CHAPTER 1. INTRODUCTION

Originating in Central America, the sunflower was taken to Eastern Europe where, mainly in Russia, it was developed as an oilseed crop. In Australia oil seed sunflower production commenced in the late 1960's, using Russian material. Current cultivars, derived from Russian high oil varieties, have oil contents of 35% to 60% with protein contents of 15% to 20% (Matheson, 1976).

The crop has found a niche as an extra option for summer cropping, particularly in eastern Australia where it is grown as a rainfed or irrigated crop from the central highlands of Queensland to southern Victoria. The production of sunflower oil in Australia has increased markedly over the last decade. In 1973/74 154,820 hectares of sunflower were sown yielding 100,400 tonnes of seed while in 1983/84 254,400 hectares were sown yielding 188,300 tonnes of seed.

Approximately 90% of sunflower seed produced is crushed leading to the production of an edible oil containing high levels of the polyunsaturated fatty acid linoleic acid. Oil of such high quality (linoleic acid content) is not obtained from other more common oils such as cotton, peanut and soybean. Sunflower oil is used in the manufacture of polyunsaturated margarine, salad dressing and oils, cosmetics, soap and paints (Lovett, Harris and McWilliam, 1976).

Much of the sunflower oil produced in Australia is low in linoleic acid and does not meet the statutory level of 62% required for use in polyunsaturated products. The cause of this reduced level appears to be high temperature during seed production which depresses linoleic acid synthesis. One approach to overcome this problem is to select areas and sowing times that favour the production of oil with a high linoleic acid content. Harris, McWilliam and Bofinger (1980) have examined this approach and have predicted that with existing cultivars, whilst some areas are

suited to high quality oil production, adequate supplies of polyunsaturated oil would not be reliably obtained from the Australian sunflower crop. An alternative approach would be to select sunflower genotypes that have the capacity to produce high levels of linoleic acid over the range of temperature conditions experienced during seed maturation.

This thesis attempts to examine the occurrence of sunflower genotypes with the capacity to produce high levels of linoleic acid over a wide range of temperatures and also to examine methods that can be used to facilitate the selection of such genotypes.

CHAPTER 2. LITERATURE REVIEW

2.1 INTRODUCTION

The sunflower *Helianthus annuus* L is grown widely throughout eastern Australia. The cypsellae (or seeds) are up to 18mm long and consist of a husk or pericarp lined with testa and endosperm which enclose the embryo. The embryo consists of two large cotyledons and a small growing point, in which the plumule and radicle are visible (Vaughan, 1970). The seeds are rich in oil which is predominantly triacylglycerol. The major fatty acids of sunflower oil are the di-unsaturated linoleic acid, the mono-unsaturated oleic acid and the saturated fatty acids, palmitic and stearic acid (Earle, Vanettan, Clark and Wolff, 1968) (Diagram 2.1).

DIAGRAM 2.1 Major fatty acids of sunflower oil

$CH_3\ (CH_2)_{14}\ COOH$	palmitic acid (16:0)
$CH_3\ (CH_2)_{16}\ COOH$	stearic acid (18:0)
$CH_3\ (CH_2)_7\ CH{=}CH\ (CH_2)_7\ COOH$	oleic acid (18:1)
$CH_3\ (CH_2)_4\ CH{=}CH\ CH_2\ CH{=}CH\ (CH_2)_7\ COOH$	linoleic acid (18:2)

2.2 FACTORS INFLUENCING THE OIL CONTENT OF SUNFLOWER SEED

2.2.1 The effect of Temperature on the Oil Content of Sunflower Seed

Genotype is important in determining oil content in sunflowers as seen by the successful selection for high-oil cultivars (Putt, Craig and Carson, 1969). However, within these cultivars, differences in oil content due to genotype are relatively small (Fick and Zimmerman, 1973). The major control of oil content appears to be exerted by the environmental conditions experienced during crop growth.

Several researchers have investigated the effect of temperature on the oil content of sunflower seed under controlled temperature conditions. Canvin (1965) reported that, when plants were grown in constant temperatures, oil

content was highest in sunflower seed grown at 21°C while seed grown at 10°C, 16°C and 26.5°C had lower contents. Over a range of fluctuating temperatures from 18/13°C to 33/28°C, Downes (1974) found that oil percentage decreased with increasing temperature. Harris, McWilliam and Mason (1978) reported a similar trend in oil percentage with increasing temperature but noted that variability in their data was high.

Although high temperature has been shown to depress oil content under controlled conditions, in the field no consistent temperature effect has been demonstrated. Some studies report a higher oil content of sunflower seeds grown at higher temperatures. Robertson and Russell (1972) reported a 5.5% increase in oil content when the mean daily temperature from pollination to maturity was 27.5°C compared to oil content in seed matured with a mean daily temperature of 25.5°C. Similarly, Johnson and Jellum (1972) found higher oil percentage in one season when mean temperatures of 24-27°C occurred during a 31 day period after flowering than in another season when mean temperatures of 20°C were experienced during the same growth stage.

In Australia, data from field trials conducted by Keefer, MacAllister, Uridge and Simpson (1976) in which the crop was sown at ten intervals over two growing seasons in Central Queensland showed no consistent relationship between oil percentage and mean temperature during flowering and seed development. Although studies showed some suggestion of a similar trend to that recorded in their controlled environment studies, Harris *et al* (1978) could find no significant effect of temperature on oil yield in field sites across an altitudinal gradient from the Northern Tablelands to the North West plains of N.S.W. Robertson, Morrison and Wilson (1978a) found that sunflowers grown at latitudes above 39°N which experienced average temperatures of 18-20°C had a slightly higher oil content than those grown below 39°N latitude which experienced average temperatures of 25-26°C, but also could not show a significant effect of temperature or latitude on total oil content. Goyne, Simpson, Woodruff and Churchett (1979) have reported oil content to be linearly related to mean maximum temperature during the period of oil accumu-

lation for the cultivars Sunfola 68-2 and Hysun 30, but oil percentage increased with increasing temperature in Hysun 30 while the opposite trend occurred in Sunfola 68-2.

From the varied responses obtained under field conditions, it appears that oil content of sunflowers is influenced not by temperature alone but also by other environmental factors (Harris *et al.*, 1978; Goyne *et al.*, 1979) (see Section 2.2.2).

2.2.2 Other Factors Influencing Oil Content of Sunflower Seed

A range of factors other than temperature has been shown to influence the oil content of sunflower seed. These factors include water stress, disease, salinity, the application of mineral fertilizers and the position of seed within the capitulum (head).

The effect of water stress on the oil content of sunflower seed has been extensively studied. Talha and Osman (1974) applied water stress at four stages of plant growth - slow elongation, rapid elongation, flowering and ripening. Significantly lower oil percentages were obtained for all stress treatments. Muriel and Downes (1974), however, have reported that water stress had a consistent effect on oil percentage only when it occurred after flowering, when it resulted in a reduced oil content. The effect of water stress appears to be more complex since other researchers (Unger, 1978; Jessop and Binns, 1980; Rawson and Turner, 1982) have found no consistent or significant reduction in oil content over a range of water levels experienced under field conditions.

Salinity also has been reported to affect oil content in sunflower seeds. Gharsalli, Djemal-Daoudi and Cherif (1982) incorporated varying levels of sodium chloride in the potting mixture of sunflowers. Oil percentage of the seeds fell from 55.4% at zero NaCl to 51.6% at 0.10M NaCl and to 43.5% at 0.15M NaCl. Increased sodium chloride also reduced plant height, the number of seeds per head and the seed weight. These effects are similar to those observed under water stress and therefore the effect

of sodium chloride on oil content in sunflower seed may be due to an osmotic effect on water uptake from the soil.

The oil content of sunflower seed has been reported to decrease in the presence of several diseases. Some of the diseases studied include downey mildew (*Plasmospera halsedii*), rust (*Puccinia helianthi*), verticillium wilt (*Verticillium alboatrum*) and alternaria (*Alternaria helianthi*) (Zimmer and Zimmerman, 1972; Brown, Kajornchaiykul, Siddiqui and Allen, 1974; Siddiqui and Brown, 1977; Allen, Kochman and Brown; 1981). These are all diseases that affect the leaf of the sunflower and their effect on oil content logically could be expected to be due to reduced assimilate availability during seed filling. The reduction in oil yield from these diseases is dependent on the time of infection of the plant - those occurring early in sunflower growth causing the greatest reduction in oil yield. Other diseases also causing a reduction in oil content of sunflower include head rot (*Rhizopus spp* and *Botrytis spp*) and sclerotinia (*Sclerotinia sclerotiorum*) (Zimmer and Zimmerman, 1972; Dorrell and Huang, 1978).

There is an extensive literature on the effect of mineral fertilizer application on the oil content of sunflower seed. The response of sunflower to nitrogen application has been varied. Some researchers have found a decrease in oil percentage of the seed and an increase in oil yield per unit area over a range of nitrogen applications (Zubriski and Zimmerman, 1972; Cheng and Zubriski, 1978). Other workers have reported a decrease in both oil percentage and oil yield with decreasing nitrogen (Pacucci and Scarascia-Mugnozza, 1972; Mathers and Stewart, 1982). In still other experiments, while the oil percentage decreased with increasing nitrogen levels, oil yield per unit area was greatest at the low nitrogen levels (Muirhead, Low and White, 1982). Saric, Jocic and Veresbaranji (1972) found that low levels of nitrogen fertilizer increased the oil percentage of sunflower seed but that high levels depressed oil percentage with oil yields following the same trend. The varied response of sunflower oil content to

nitrogen fertilizer is probably due to the different soil types that were used in each study, since the results reported come from several different countries. Further, the initial level of nitrogen prior to fertilizer application is rarely reported, and added nitrogen therefore does not necessarily reflect the actual amounts available to the plant.

Phosphorus and potassium are the other two minerals that have been extensively examined for their effect on sunflower. In general oil content is increased by phosphorus, phosphorus plus potassium or nitrogen plus phosphorus (Appelqvist, 1977; Kalra and Tripathi, 1980; Chaudary and Paturde, 1982). Again, the responses can be expected to be dependent on soil type and cropping history and therefore are site specific.

The position of seed within the head of the sunflower also influences the oil percentage of that seed. Fick and Zimmerman (1973) examined the oil content of seeds from three zones of the head. Zone 1 was the peripheral third of the head; zone 3 was the centre third of the head, while zone 2 was the third of the head between zones 1 and 3. Seeds in zone 3 had the lowest oil percentages with seeds in zone 2 having the highest. These results were not due to differences in hull percentages as dehulled kernels showed the same trend. It is not clear why this trend occurs.

This section has discussed reductions in oil content of the seed. In a crop situation oil yield per unit area is the most important consideration. An increase in oil percentage (i.e. oil content) in the seed does not necessarily result in an increased oil yield. Other parameters such as the number of seeds per head and seed weight also effect the oil yield of a crop.

Since so many factors appear to influence the oil content of the developing sunflower seed it is very difficult to successfully predict the oil percentage of a sunflower crop (Harris *et al.*, 1978; Goyne *et al.*, 1979).

2.3 FACTORS INFLUENCING THE FATTY ACID COMPOSITION OF SUNFLOWER OIL

2.3.1 The Effect of Temperature on the Fatty Acid Composition of Sunflower Oil

The effect of temperature on the fatty acid composition of sunflower oil has been studied under controlled environment conditions. Canvin (1965) found that the proportions of linoleic and oleic acid varied greatly with the temperature experienced during seed development while palmitic and stearic acids were unaffected. The proportions of linoleic acid fell from around 75% at 10°C to about 30% at 26.5°C with a corresponding rise in oleic acid content from 20% to 65%. A phytotron study by Harris *et al* (1978) has provided similar results over a range of alternating 'day/night' temperatures from 15/5°C to 35/25°C. Tremolieres, Dubacq and Drapier (1982) have reported a slightly different response of sunflower acids to temperature. Linoleic and oleic acid percentages were found to plateau at temperatures above 22°C unlike the previous two studies where linoleic acid continued to decrease and oleic acid to increase to the highest temperature - 26.5°C (Canvin, 1965) and 30°C (mean) (Harris *et al.*, 1978).

In contrast to oil content, the effect of temperature on the fatty acid composition of sunflower oil has been widely recorded under field conditions. As early as the 1950's the effect of temperature on the fatty acid of sunflower oil had been noted. Bridge, Crossley and Hilditch (1951) grew sunflowers in different regions of Australia and reported that oil from sunflowers grown in the Northern Territory had a lower linoleic acid content than oil from sunflowers grown at more southerly latitudes. Similar results were reported by Grindley (1952) who grew sunflowers in Khartoum, Sudan, during both summer and winter, and found that oil from winter-grown plants contained the higher proportion of linoleic acid. In a date-of-planting study, Johnson and Jellum (1972) reported no apparent relationship between oil composition and temperature during seed development, but many studies since have confirmed that there is a linear decrease in linoleic acid

and a corresponding linear increase in oleic acid in response to increasing temperature (Robertson, Thomas and Burdick, 1971; Keefer *et al.*, 1976; Harris *et al.*, 1978; Robertson *et al.*, 1978a; Unger, 1980; Robertson and Green, 1981). Good correlations have been obtained between linoleic (and oleic) acid content and temperatures during seed formation (Keefer *et al.*, 1976; Harris *et al.*, 1978; Robertson *et al.*, 1978a). Keefer *et al* (1976) found oleic and linoleic acids were linearly related to the mean temperature over a period 21-35 days after flowering ($y = 2.27x + 110.67$ for linoleic acid) while Harris *et al* (1978) and Robertson *et al* (1978a) found that the percentage contents of these acids were more closely related to the mean minimum temperature during the seed filling phase of growth. Robertson *et al* (1978a) obtained the equation $y = -1.95x + 89.09$ for linoleic acid (where x = average minimum daily temperature during seed maturation) while Harris *et al* (1978) obtained $y = -1.41x + 86.4$ for linoleic acid (where x = mean minimum temperature from mid-flowering to harvest). In all these studies more than 80% of the variation in the percentage of either acid in the oil was accounted for by the temperature terms used in the regression equations.

2.3.2 Other Factors Influencing Fatty Acid Composition of Sunflower Oil

Although temperature exerts the major influence on the fatty acid composition of sunflower oil, a range of other factors has been investigated to determine their effect. These include salinity, disease, mineral fertilizers, the position of the seed within the head, light and water stress.

Gharsalli *et al* (1982) incorporated increasing amounts of sodium chloride into the potting mixture used for sunflowers. The increased sodium chloride resulted in an increase in linoleic acid content with a corresponding decrease in oleic acid. Linoleic acid increased from 45% at zero NaCl to 52% at 0.15M NaCl while oleic acid decreased from 40% to 30%.

In contrast to the effect on oil content, disease has been found to have very little effect on the fatty acid composition of sunflower oil. The diseases studied included downy mildew (*Plasmopera halstedii*), rust (*Puccinia helanthi*), verticillium wilt (*Verticillium alboatrum*), head rot (*Rhizopus spp* and *Botrytis spp*), sclerotinia wilt (*Sclerotinia sclerotiorum*) and charcoal stem rot (*Macrophomena phaseoli*) (Zimmer and Zimmerman, 1972; Harris *et al.*, 1978; Dorrell and Huang, 1980).

Not much information on the effect of mineral fertilizers on seed fatty acid composition is available in the literature. Nitrogen has been reported to increase the relative percentages of saturated fatty acids while phosphorus and potassium have tended to increase the content of unsaturated fatty acids (Appelqvist, 1977). However, the absence of documentation of any effect on the proportions of each unsaturated acid would seem to imply that any effect which may occur is of little importance.

The position of the seed within the head has been found to influence the fatty acid composition of the oil. Zimmerman and Fick (1973) examined the fatty acid composition of the oil from seeds in three different zones of the head. Linoleic acid content increased from the periphery to the centre of the head, with a corresponding decrease in oleic acid. Plamitic acid also increased slightly from the periphery to the centre of the head. However, differences in fatty acid composition of seeds within a zone of the head were not significant. Contrasting results were reported by Sukhija, Borthakur and Bhatia (1980) who showed that the level of linoleic acid decreased from the periphery to the centre of the head. Oleic acid levels also followed this trend leading to a decrease in the level of total unsaturated fatty acids in the oil, the difference being accounted for by the palmitic acid content which increased from the periphery to the centre (from 4.2% to 14.8%). The different results obtained for linoleic and oleic acid from these two studies could be due, in part, to the effect of temperature on the fatty acid composition of the oil. Both studies

used field-grown sunflowers so it is possible that seeds in different zones of the head experienced slightly different temperatures during the phase of rapid oil synthesis, since, in sunflowers, flowering starts at the periphery and moves across the head over a period of several days.

Whatever the reason for the differences, it seems clear that head position influences fatty acid composition of the oil and therefore any study on oil composition needs to be consistent in sampling from a particular zone of the head.

Tremolieres *et al* (1982) have studied the effect of light intensity on the fatty acid composition of sunflower oil. At the onset of oil accumulation, the light intensity irradiating the head of the sunflower was reduced by a filter applied around the head, resulting in only a 10-20% reduction of light intensity at the leaves. Linoleic acid content increased as light was reduced - from 30% linoleic acid at full light intensity to 58% linoleic acid at 3% light intensity. An adequate description of the filter used was not given and it is possible that some of the response could be due to a shading causing a lower temperature inside the filter. However, such effects are not likely to cause changes in composition of this order, therefore the mechanism of the reported changes is unclear.

Literature on the effect of water stress on the fatty acid composition of sunflower oil is sparse. In the study of Talha and Osman (1974) differences in oil composition were observed between the water stressed and the control plants. Oleic acid levels increased by up to 15% while linoleic acid levels decreased by as much as 8% when water stress was applied at the stage of slow elongation. Kharchenko (1979) has reported that the synthesis of oleic acid at high temperatures is more intensive under water stress. Stressed plants had 71.2% oleic acid in their oil compared to 55.8% in the control. Since one of the effects of water

stress is to cause an increase in the temperature of the plant, part of this effect again could be due to changes in the temperature in the seed tissue. However, the size of the change would appear to be too great for this alone to account for the reported differences.

Under field conditions oil from fully watered crops contained 3-4% more linoleic acid than crops which suffered water stress from before anthesis to maturity (Dubbelde, pers. comm.). The crops matured under high temperatures (Harris, Dubbelde and McWilliam, 1982) and a difference of this order may have been due to differences in the internal temperature of the plant. These results, however, suggest that if water stress does influence oil composition the effect is minimal compared with that of ambient temperature during seed growth.

2.4 THE DEVELOPMENT OF THE SUNFLOWER SEED

2.4.1 Oil and Dry Matter Accumulation in the Developing Sunflower Seed

The accumulation of oil and dry matter in sunflower seeds has been studied by several researchers. Although slight differences exist, the overall pattern of oil and dry matter accumulation reported is very similar between individual studies.

In the developing seed, dry matter appears to increase almost linearly from pollination to physiological maturity (Harris *et al.*, 1978; Robertson *et al.*, 1978a). In contrast, a lag period has been reported before oil accumulation commences. Several different durations have been reported for the lag phase - 200 day-degrees after pollination (≅ 10 days) (Harris *et al.*, 1978), 13 days after flowering (DAF) (Dorrell, 1978), 14 days after flowering (Robertson *et al.*, 1978a) and 38 days after flowering (Gunstone and Padley, 1967). A period of rapid oil accumulation is reported to follow the lag phase. A range of values also has been reported for the length of the rapid oil accumulation phase - 12 days (Dorrell, 1978; Harris *et al.*, 1978), 14 days (Robertson *et al.*, 1978a) and 18 days (Gunstone and Padley, 1967).

The accumulation of triacylglycerols (the major storage lipid) in the developing seed followed the pattern of total oil accumulation. Rapid synthesis of triacylglycerols occurred between 14 DAF and 28 DAF, reaching a maximum at physiological maturity (Robertson, Chapman and Wilson, 1978b). Physiological maturity, or maximum seed dry weight, has been reported to occur at 650 day-degrees (Harris *et al.*, 1978), 902 day-degrees (Anderson, 1975) and 28 DAF (Dorrell, 1978).

Individual plants of sunflowers, especially open pollinated but also hybrid cultivars, commence flowering at different times. Also, since flowering in sunflowers commences at the periphery and moves across the head to the centre over a period of several days, the age of the seeds would decline from the periphery to the centre of the head. In studies which report on whole crop oil development (Chisholm and Hopkins, 1961; Anderson, 1975) both of these effects would be expected to be incorporated and extend the apparent length of the development period. While ensuring that flowering commenced on the same day, most studies cited in this section did not take into account the range of seed ages in the head, and, therefore, the seed samples used probably contained seed of several different physiological ages. An attempt to avoid this problem was reported in the study of Harris *et al* (1978). Heads were bagged prior to anthesis and cross-pollinated only once when the majority of the stigma were receptive. Since Peredovic is high self-incompatible (Vithange and Knox, 1977), this approach endeavoured to ensure that all embryos in the head started development at the same time.

The use of samples containing seeds of different ages could explain some of the differences reported in the duration of both the lag phase and the rapid phase of oil accumulation. Some differences could also be due to the use of different cultivars - Peredovic (Anderson, 1975; Dorrell, 1978; Harris *et al.*, 1978), Sun Gro 380 (Robertson *et al.*, 1978b) and an unknown cultivar (Gunstone and Padley, 1967). Since different rates of oil accumulation occur at different temperatures (Pereira, 1978), differences between

studies could also be due to different temperatures during seed maturation.

One exception to the general pattern of oil accumulation was reported by Hopkins and Chisholm (1961). Oil formation commenced 10 DAF and continued steadily for seven weeks. This long, slow accumulation of oil could be due to the cultivar used, Advance, which is a low oil, confectionery sunflower compared to the high oil sunflowers used in the other studies, but could also be partly due to low temperatures during oil accumulation.

In the study of Pereira (1978), oil and dry matter accumulation during development of the embryo and the pericarp of the seed were investigated in three temperature regimes (30/22°C, 25/17°C and 20/12°C). The embryo was the major site of oil accumulation. After a lag period of 12 days from flowering, oil synthesis occurred rapidly in the embryo until 28 DAF (728 day-degrees, 588 day-degrees) at the two higher temperatures and until 36 DAF (612 day-degrees) at the lowest temperature. Different rates of oil accumulation in the embryo were reported for the three temperatures.

At the two higher temperatures, pericarp oil content reached about 30% while, at the lowest temperature, pericarp oil content reached only 15%. Triacylglycerol accumulation in the embryo followed the pattern of total oil accumulation except that maximum triacylglycerol content occurred slightly later than maximum oil content. Unfortunately, the results in this study by Pereira (1978) were presented as curves with no actual data points given, and therefore, no indication is given of the variation in the data.

2.4.2 Changes in Fatty Acid Composition of Oil during Seed Development

The fatty acid composition of sunflower seeds has been found to change considerably during seed development. The oil of immature seeds has been found to have a much higher percentage of palmitic acid (16:0) than that of mature seeds. A range of values for palmitic acid content in immature seed oil has been reported - 10% (Hopkins and Chisholm, 1961); 17% (Gunstone and Padley, 1967); 19.6% (Harris *et al.*, 1978); 20.8%

(Robertson *et al.*, 1978b) and 26.5% (Dorrell, 1978). In all studies, the proportion of palmitic acid decreased during seed development to a final content of about 5-7%. Stearic acid (18:0) content has been found to follow two slightly different patterns. Hopkins and Chisholm (1961) and Gunstone and Padley (1967) reported that stearic acid was highest in oil from immature seeds and declined during seed development to around 1-2%. Other workers (Harris *et al.*, 1978; Robertson *et al.*, 1978b; Dorrell, 1978) have found that stearic acid levels are low in immature seed oil (1.8 - 3.7%) but increase to a maximum content (7.7 - 8.1%) at about 14-17 DAF before decreasing to a final content of about 3-5%.

Oil from immature seeds has also been found to contain several fatty acids which are normally absent or found only in trace amounts in the oil of mature seeds. Gunstone and Padley (1967) found that about 45% of the oil from immature seed consisted of two unidentified fatty acids corresponding to a carbon number of 19.8 and 20.2. Robertson *et al* (1978b) reported 0.5% hexadecenoic acid (16:2), 10.7% linolenic acid (18:3), 1.5% arachnidic acid (22:0) and 2.6% unknown fatty acid as components of the oil of immature sunflower seed. Similarly, Dorrell (1978) found the oil of immature seeds to contain 11.6% of other fatty acids consisting of linolenic, arachnidic and behenic acids.

The changes in oleic (18:1) and linoleic (18:2) acids during seed development are more complex, due to the influence of temperature on the synthesis of these acids (Section 2.3.1). Although different studies have found actual values to differ, the change in linoleic acid during development appears to generally follow a similar pattern (Hopkins and Chisholm, 1961; Harris *et al.*, 1978; Robertson *et al.*, 1978b; and Dorrell, 1978). Initially, linoleic acid percentage decreases, reaching a minimum level at about 13-15 DAF and then increases until about 25-36 DAF. Changes in oleic acid levels show an inverse pattern. Both the drop in linoleic acid and the increase in stearic and oleic acid percentages occur at the start of the

period of rapid oil accumulation.

Pereira (1978) carried out a detailed study of the accumulation of fatty acids in both the embryo and the pericarp of the sunflower seed over three temperature regimes. The changes in palmitic and stearic acids in the embryo were similar to those discussed above, in all three temperature regimes. Different patterns of linoleic and oleic acid changes in the embryo were found for each temperature treatment. Both the higher temperature treatments exhibited a decrease in linoleic acid which was then followed by an increase in percentage as described above for whole seeds. The inverse pattern was found for oleic acid in the embryo. In the highest temperature regime, the percentage of oleic acid was higher than that of linoleic acid for the whole period of embryo development, but in the middle temperature regime, although oleic acid percentage was higher at early stages of development, linoleic acid became the dominant fatty acid at later stages. In the lowest temperature treatment, linoleic acid was the dominant fatty acid for the whole period of embryo development.

In the pericarp, linoleic and oleic acids were found to be not as strictly complementary as they were in the embryo. Although the fatty acids in the pericarp were found to be influenced by temperature, the extent of the influence was less than in the embryo, especially with linoleic acid. The unsaturated fatty acids were found to make up about 87% of the oil in the embryo at all temperatures, but in the pericarp the unsaturated fatty acid content decreased with temperature, especially at the lowest temperature where saturated fatty acids comprised 36% of the lipid of the pericarp.

It appears that palmitic acid content is high in immature seeds but that this is rapidly altered once oil accumulation commences. The proportions of linoleic and oleic acid in the oil appear to undergo slight changes during development until they achieve their final ratio which is dependent on the temperature conditons during development. The data on fatty acid composition would have been more clearly interpreted if they

had been reported as the weight of each fatty acid during development. A drop in the percentage of an acid could be caused by increased synthesis of another fatty acid, not a decrease in the content of that acid itself. If the data had been reported on a weight basis it would have been possible to determine whether the quantity of fatty acid was in fact increasing or decreasing.

2.5 THE BIOSYNTHESIS OF SUNFLOWER OIL

2.5.1 Biosynthesis of Saturated Fatty Acids

The synthesis of palmitic and stearic acids in plants has been reviewed in detail by Stumpf (1977, 1980).

Palmitic acid synthesis in plant tissues involves a *de novo* system that utilizes acetyl-CoA, malonyl-CoA, acyl carrier protein (ACP) and palmitoyl-ACP synthetase to form palmitoyl-ACP. Another set of enzymes, palmitoyl- ACP elongase, then converts palmitoyl-ACP to stearoly-ACP (Stumpf, 1980).

In developing seeds acetyl-CoA is generated in the proplastid by the pyruvate dehydrogenase complex (PDC). This enzyme complex oxidatively decarboxylates pyruvate to form acetyl-CoA and CO_2 (Stumpf, 1977). Thompson, Reid, Lyttle and Dennis (1977) have examined this enzyme in proplastids from developing castor bean (*Ricinus communis* L). PDC is a high molecular weight complex which requires NAD^+, CoA and thiamine pyrophosphate for full activity. Acetyl-CoA is converted to malonyl-CoA by the action of acetyl-CoA carboxylase. A number of plant acetyl-CoA carboxylases have now been studied (Stumpf, 1977, 1980) and Stumpf (1980) has concluded that there are probably two types of carboxylases present in plant cells, one of which would be located in proplastids (or chloroplasts) to be used for palmitoyl-ACP synthesis and elongation.

Palmitoyl-ACP synthetase occurs widely in plants where it appears to synthesise palmitoyl-ACP by a series of reactions identical to those of the

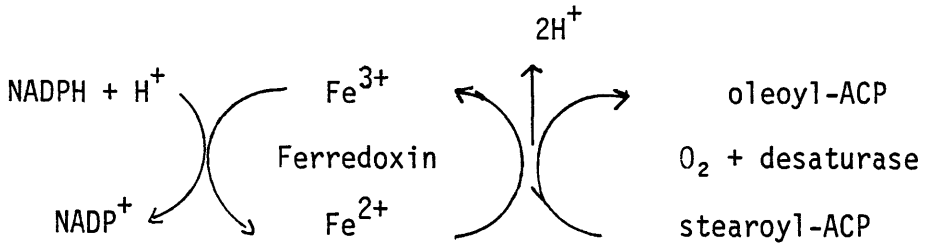
bacterial system (Harwood, 1975). Acetyl-CoA acts as a starter unit and successive 2-carbon units are added from malonyl-CoA, generating CO_2 with each addition. The acyl intermediates are carried by ACP and NADPH is required. The overall reaction is (Harwood, 1975) -

$$\text{acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ \rightarrow \text{palmitic acid} + 7\ CO_2 + 14\ NADP^+ + 8\ \text{CoA} + 6\ H_2O$$

Stearic acid is formed by the elongation of palmitic acid. The enzyme system involved, palmitoyl-ACP elongase, has been examined in a number of tissues (Jaworski, Goldschmidt and Stumpf, 1974). The enzyme complex requires NADPH and, using palmitoyl-ACP as the substrate, adds a 2-carbon unit from malonyl-CoA forming stearoyl-CoA.

2.5.2 Biosynthesis of Unsaturated Fatty Acids

The desaturation of stearate to linoleate involves two desaturases (Stumpf, 1975). The enzyme catalysing the first desaturation step, stearoyl-ACP desaturase, is a soluble enzyme located in plant proplastids and chloroplasts (Stumpf, 1980). Stearoyl-ACP is the substrate and molecular oxygen and a suitable reductant, usually NADPH, are required. Ferredoxin acts as an intermediate electron carrier between the reductant and the desaturase (Nagai and Bloch, 1968; Jaworski and Stumpf, 1974; Stumpf and Porra, 1976). Oleoyl-ACP has been identified as the product of the reaction (Stumpf and Porra, 1976; Ohlrogge, Shine and Stumpf, 1978). The reaction occurs as follows (Stumpf, 1975):



The nature of the substrate for the second desaturation step has been a controversial topic over the last decade. Both oleoyl-CoA and

oleate esterified to phosphatidylcholine (PC) have been proposed as the real substrate for oleate desaturation. Evidence supporting oleoyl-CoA as the substrate comes from investigations of Vijay and Stumpf (1971) and Dubacq, Mazliak and Tremolieres (1976). Using microsomes from safflower (*Carthamus tinctorious*) seeds and pea (*Pisium sativum*) leaves, respectively, these workers reported that oleoyl-CoA was rapidly desaturated to linoleoyl-CoA in the presence of molecular oxygen, and that both oleoyl-CoA and linoleoyl-CoA were rapidly esterified to the 2 position of PC.

In support of the other substrate, Slack, Roughan and Tempstra (1976) found that when [^{14}C] oleoyl-CoA was incubated with pea leaf microsomes the label was rapidly incorporated into PC, and released as free fatty acid. The loss of label from oleoyl-PC and the concommittant increase in label in linoleoyl-CoA proceeded at linear rates over a 60 minute period. These workers concluded that pea leaf microsomes possess an oleate desaturase for which 3-sn phosphatidylcholine oleate (oleoyl-PC) is either the substrate or an immediate precursor of the substrate. Stymne and Appelqvist (1978), using microsomes from developing safflower seeds reported that label from [^{14}C] oleoyl-CoA appears in PC before it is seen in any other lipid class investigated. They found that considerable desaturation continued after the disappearance of free [^{14}C] oleoyl-CoA and that no free [^{14}C] linoleoyl-CoA could be detected. Further, Stymne and Appelqvist (1978) propose that the presence of considerable amounts of label in thioesters (oleoyl-CoA and linoleoyl-CoA) in the study of Vijay and Stumpf (1971) was an artefact caused by the method of assay for thioesters which has been shown to assay not only thioesters but also phospholipids such as PC. Slack, Roughan and Balasingham (1978) identified PC as the major lipid in developing seeds of soybean, linseed and safflower. These workers found that [^{14}C] acetate supplied to detached cotyledons was incorporated mainly into the acyl groups of PC, diacylglycerol (DAG) and triacylglycerol (TAG). Initially, the label was predominantly in oleate

but, subsequently, entered the linoleoyl and linolenoyl groups of these lipids. Slack, Roughan and Browse (1979) found that safflower microsomes would desaturate labelled oleate esterified at both positions 1 and 2 of PC. Using sunflower seed microsomes incubated with [^{14}C] oleoyl-CoA, Rochester and Bishop (1982) found that the linoleate formed was largely esterified to PC, or occurred as a free fatty acid. Their results suggested that in the sunflower, oleate esterified at the 2 position of PC is the preferred substrate.

Attempts to demonstrate the presence of an oleoyl-CoA desaturase in the microsomal fraction of developing safflower seeds by the appearance of linoleoyl-CoA were inconclusive (Slack *et al.*, 1978). Although their results strongly suggest that the major portion of labelled linoleate was formed from [^{14}C] oleoyl-CoA via [^{14}C] oleoyl-PC, Stymne and Appelqvist (1978) could not rule out a minor contribution from an oleoyl-CoA desaturase. Further investigation (Stymne and Glad, 1981) has shown that the desaturation of oleate occurs solely while it is esterified to PC and that any label present in acyl-CoA could be completely accounted for by acyl exchange. These workers reported the presence of two different mechanisms of acyl exchange to transfer oleoyl-CoA to PC. One involves the transfer of oleate with the release of free CoA while the other involves the exchange of oleate from oleoyl-CoA for unsaturated 18-carbon fatty acids of PC which would lead to the release of labelled linoleoyl-CoA.

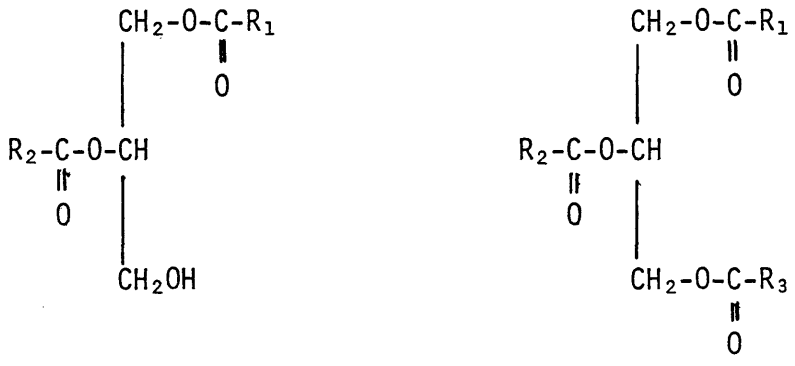
In view of recent evidence it appears that oleate bound to PC is the real substrate for oleate desaturate. In plants this enzyme is membrane-bound and restricted to the endoplasmic reticulum (Slack *et al.*, 1976; Dubacq *et al.*, 1976), and NADPH and molecular oxygen are required for full activity (Stymne and Aqqelqvist, 1978).

2.5.3. Synthesis of Triacylglycerols in Seeds

Triacylglycerol is the major storage lipid in sunflower seeds (Gunstone and Padley, 1967). The most widely occurring pathway for the

de novo synthesis of triacylglycerols is the Kennedy pathway (Gurr, 1980). Briefly, this pathway involves the esterification of the 1 and 2 positions of sn-glycerol-3-phosphate with fatty acids to produce 1,2-sn-diacylglycerol phosphate (phosphatidic acid). The phosphate group is then removed to give rise to a diacylglycerol (DAG) and a third fatty acid is esterified at the vacated sn 3 position to give a triacylglycerol (TAG) (Diagram 2.2).

DIAGRAM 2.2 The structure of diacylglycerol and triacylglycerol



Diacylglycerol Triacylglycerol

R_1 R_2 R_3 represent acyl chains derived from fatty acids.

Evidence for the occurrence of this pathway in plants has come from several sources. Barron and Stumpf (1962) demonstrated that a particulate fraction of the mesocarp of avocardo (*Persea americana*) could synthesise labelled TAG from [^{14}C] glycerol. The order of labelling of intermediates was glycerol phosphate, phosphatidic acid, DAG and, finally, TAG, supporting the occurrence of the Kennedy pathway. A similar labelling sequence was reported for flax (*Linum* spp) seed embryos (Dybing and Craig, 1970). Using either [^{14}C] acetate, [^{14}C] malonate or [^{14}C] carbon dioxide, label was rapidly incorporated into phospholipids (PL) and DAGs but more slowly into TAGs. After transferring the embryos to a non-radioactive incubation medium, the label in the TAG continued to increase while the label in the PL and DAG decreased. The observations indicate a turnover of DAG and PL, consistent with their role as intermediates in TAG synthesis.

A similar labelling sequence was again demonstrated in Crambe (*Crambe abyssinica*) seed slices incubed with [^{14}C] glycerol, but phosphatidic acid was not highly labelled *in vivo* (Gurr *et al.*, 1974). Phosphatidylcholine was found to be the most rapidly labelled phospholipid in developing seeds of soybean (*Glycine max*), linseed (*Linum usitatissium*) and safflower (Slack *et al.*, 1978). Wilson and Rinne (1976) prepared synthetic phosphoglycerides labelled with ^{3}H in the glycerol backbone and ^{14}C in the acyl groups, and studied the metabolism of these phosphoglycerides in developing soybean cotyledons. Their study revealed that PL could be metabolized to DAG and that the DAG formed could be either recycled to PL or converted to TAG, indicating that PL's play a key role in TAG formation. Slack *et al* (1978), using pulse labelling, showed that the label from acetate was first incorporated into oleate and then entered linoleate and linolenate, first esterified to PC, subsequently to DAG and finally to TAG.

In view of the evidence for PL involvement and especially the role of PC in the synthesis of unsaturated TAG, Roughan and Slack (1982) have suggested a modified Kennedy pathway (Diagram 2.3). Evidence now suggests that linoleate is formed from oleate whilst bound to PC (see Section 2.5.1). For the modified Kennedy pathway to operate, the linoleate must be capable of being released from the PC molecule. Stymne and Glad (1981) have demonstrated the presence of an acyl-CoA:PC acyltransferase in the microsomes of developing soybean cotyledons. The enzyme is ATP-independent and catalyses the exchange of acyl groups between oleoyl-CoA and polyunsaturated fatty acids at the 2 position of PC. This enzyme could provide polyunsaturated CoA esters for the formation of DAGs from glycerol-3-phosphate (Stymne and Glad, 1981) or for incorporation into the 3 position of TAGs (Roughan and Slack, 1982).

For the modified Kennedy pathway to operate DAGs must be released from PC. The mechanism of this release has not been demonstrated experimentally but the enzyme, cholinephosphotransferase, is thought to be involved (Slack *et al.*, 1978). In animal microsomes, this enzyme has

been found to catalyze the exchange of phosphorylcholine between cytidine monophosphate and DAG (Kanoh and Ohno, 1975; Francescangeli, Horrocks and Porcellati, 1981). The enzyme appears to have no specificity for either DAG or phosphatidylcholine species. Roughan and Slack (1982) propose that on thermodynamic grounds, this enzyme should operate in plants as it does in animals.

DAG acyltransferase catalyzes the addition of the third acyl group to the DAG to form TAG. In avacardo mesocarp this enzyme appears to be fairly non-specific for the acyl donor since both acyl-ACPs of varying chain lengths and acyl-CoAs were suitable substrates (Shine, Mancha and Stumpf, 1976). Little is known about the DAG specificity of this enzyme in oilseeds but, in pig liver, the enzyme has no specificity for DAG as it has been shown to randomly acylate DAGs of varying degrees of unsaturation (Akesson, 1969). Roughan and Slack (1982) propose that the fatty acid composition of the 1 and 2 positions of TAG molecules would be dependent on the relative pool size of the different DAG species and, therefore, ultimately on the pool sizes of the different PC species since cholinephosphotransferase is non-specific for the DAG substrate.

Most of the evidence for the involvement of phosphatidylcholine in the biosynthesis of TAGs comes from labelling studies and further research, especially into the enzymology of some of the reactions of the modified Kennedy pathway (Roughan and Slack, 1982), needs to be carried out to confirm the role of PC.

2.5.4 Control of the Biosynthesis of Unsaturated Fatty Acids

The fatty acid composition of the oil of several oilseeds (eg sunflower, rape (*Brassica rapus*)) varies markedly with the temperature experienced during the synthesis of this oil (Canvin, 1965). In sunflower oil total unsaturation remains unchanged but the ratio of oleic to linoleic acid changes with temperature. The mechanisms responsible for this

DIAGRAM 2.3

Modified Kennedy pathway of triacylglycerol synthesis in sunflower seeds (adapted from Roughan and Slack, 1982)

16:0 = either palmitic or stearic acid

18:1 = oleic acid

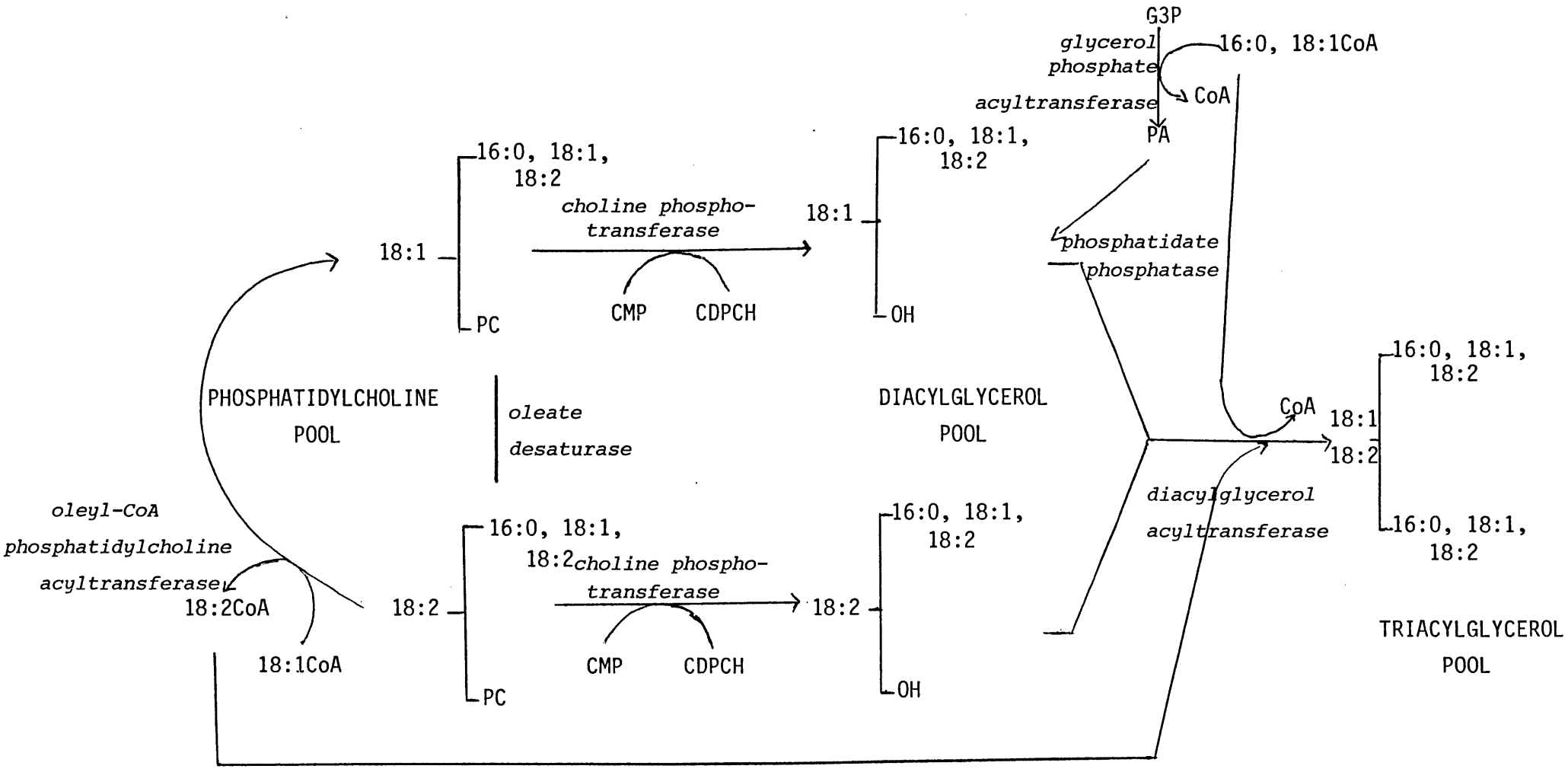
18:2 = linoleic acid

CMP = cytidine 5'-monophosphate

CDPCH = cytidine 5'-diphosphatidylcholine

G-3-P = sn-glycerol 3-phosphate

PA = sn - 3 phosphatidic acid


G3P
glycerol phosphate acyltransferase
16:0, 18:1CoA
CoA
PA
16:0, 18:1, 18:2
18:1
choline phospho-transferase
CMP
CDPCH
PC
OH
phosphatidate phosphatase
PHOSPHATIDYLCHOLINE POOL
oleate desaturase
DIACYLGLYCEROL POOL
CoA
18:1
18:2
diacylglycerol acyltransferase
TRIACYLGLYCEROL POOL
oleyl-CoA phosphatidylcholine acyltransferase
18:2CoA
18:1CoA
18:2

change are not fully understood although several possible mechanisms have been reported.

Harris and James (1969) proposed that the major effect controlling an increase in the amount of unsaturated acids at low temperatures was the increase in available O_2 (due to the greater solubility of O_2) which was rate-limiting for desaturation. Dompert and Beringer (1976) reported only a slight increase in linoleic acid levels by flushing sunflower heads growing at high temperature with gas mixtures containing oxygen. These authors concluded that temperature dependent rates of O_2-diffusion were therefore unlikely to determine fatty acid composition. Mazliak (1979) also found that although oxygen is a limiting factor of desaturation in oilseeds it did not appear to be the sole or the main controlling factor of the desaturation activity.

It has been suggested (Mazliak, 1979) that the membrane fluidity itself could be the regulating factor controlling the activity of the membrane-bound fatty acid desaturases. This mechanism implies that temperature changes that cause alterations of membrane fluidity would trigger the onset of fatty acid desaturation in cell membranes. This theory has been proposed for controlling fatty acid composition in the protozoan, *Tetrahymena* (Nozawa and Kasai, 1978), but no evidence has been presented yet for plant systems. However, in leaf tissue, Williams, Khan and Mitchell (1983) found the rate of synthesis of linoleic acid not to be determined by substrates such as oxygen or by the physical properties such as low membrane fluidity.

Browse and Slack (1983) working with safflower cotyledons found that oleate desaturase was not affected by reduced oxygen levels, but could not rule out the fact that in the developing seed the diffusion barrier created by the seed coat or pericarp was sufficient to reduce the concentration of oxygen reaching the desaturase. The work of these authors studying the effect of temperature on the rates of oleoylphosphatidylcholine desaturation and on fatty acid synthesis has led them to propose that low temperature does not cause increased activity of desaturases but that fatty acid synthesis

declines more under low temperatures than desaturase activity. Therefore the different activities of these enzymes determines the fatty acid composition of oil depending on the temperature conditions.

The incorporation and conversion of radioactive oleoyl-CoA in sunflower microsomes was studied by Rochester and Bishop (1982). They reported no effect of incubation temperature on the formation of linoleic acid from oleoyl-CoA between 15°C and 30°C. If fatty acid synthesis in sunflower declines at low temperatures while desaturase activity is unaffected (as proposed by Browse and Slack, 1978) then the fatty acid composition of oil produced at low temperatures would be expected to be very different to that produced at high temperatures.

2.6 THE PROBLEM IN AUSTRALIA - A POSSIBLE SOLUTION

Australian processors require a minimum linoleic acid content of 62% in sunflower oil used for the manufacture of polyunsaturated margarines. Sunflower oil produced in Australia is often low in linoleic acid and fails to meet this requirement. Harris, McWilliam and Bofinger (1980) examined the probability of the occurrence of temperatures suited to the production of sunflower oil with high linoleic acid content in eastern Australia. It was concluded that, with existing cultivars, adequate supplies of oil with the required linoleic acid content will not be reliably obtained from the Australian sunflower crop. The development of cultivars which can maintain a high level of linoleic acid over the range of temperatures experienced in sunflower growing areas is a necessity if the requirement for polyunsaturated oil for margarine manufacture is to be met.

Using inbred lines with a wide range of flowering dates, Putt, Craig and Carson (1969) have shown that genetic control of fatty acid composition in sunflower occurs, independent of environment. Therefore, breeding for different levels of linoleic and oleic acids in sunflower oil seems a feasible objective.

Considerable variation exists in the fatty acid composition of oil from sunflower seeds. Putt *et al* (1969) reported a wide range of fatty acid contents in 56 lines grown in one location in one season, and in single seeds of the cultivar Peredovic (Table 2.1). Under controlled environment conditions Kinman (1972) reported similar ranges for fatty acid content in sunflower oil (Table 2.1).

TABLE 2.1 Range in the fatty acid composition of the oil of sunflowers grown in common conditions

	PERCENTAGE OF TOTAL FATTY ACIDS		
FATTY ACID	Putt *et al* (1969)		Kinman (1972)
	56 lines	Individual seeds	
Palmitic	4.7- 8.2	4.5- 9.4	3-40
Stearic	1.7- 9.1	2.5-12.4	
Oleic	13.9-40.3	14.8-46.4	12-66
Linoleic	47.9-76.4	34.3-75.4	25-80

Selections have been made for high linoleic acid content in sunflowers but these have been found to be just as temperature sensitive as other cultivars (Harris pers. comm.). The fatty acid compositions of some oilseeds are relatively insensitive to temperature. Safflower and castor oil, for example, show very little change in the composition of seed oil with temperature (Canvin, 1965). The fatty acid compositions of some oilseeds have been successfully altered by breeding. Commercial rapeseed oil normally contains about 25-50% erucic acid (22:1) which can have adverse physiological effects in animals when ingested. Inbreeding and selection for low erucic acid has resulted in the isolation of strains of rapeseed with less than 1% erucic acid in their seed oils (Downey and McGregor, 1975). Kharchenko (1979) has reported the

establishment of the high oleic "Pervents" cultivar of sunflower. This cultivar has an oleic acid content of about 80% and shows very little change in fatty acid composition with temperature.

Since some oilseeds can maintain a stable fatty acid composition with changing temperatures and since breeding has led to the successful alternation of the fatty acid composition of oilseeds, it therefore could be possible to select a sunflower genotype that has high linoleic acid levels independent of the temperature experienced during seed development.

One approach to this problem using early germination at cold temperatures has been made by Downes and Tonnet (1982). A relationship between the rate of germination of a seed at 4°C and the level of linoleic acid in the seed of the mature plant was established. Using this relationship Downes and Tonnet (1982) have made selections for stable linoleic acid content. Although these selections do not maintain the same linoleic acid content at all temperatures, the depression of linoleic acid synthesis at high temperatures seems to have been reduced.

Screening techniques are needed that will facilitate the search for temperature-stable genotypes. In particular, techniques are needed that could screen the limited amounts of seed available in the early stages of breeding programmes.

It is hypothesized that one possible technique could be the use of embryo culture. In agriculture today, embryo culture has a diverse range of applications such as overcoming non-viability of hybrids, overcoming seed dormancy and related problems, and for the study of experimental embryogenesis (Young, Thorpe and Jensen, 1981). Embryo culture can be divided into two main types. One involves the removal of fairly mature embryos and their subsequent development into seedlings in culture, while the other involves the development of immature embryos in culture (Raghavan, 1980). This second type of embryo culture may be useful in the search for sunflower genotypes that have stable linoleic acid contents. If embryos

from a single sunflower head could be cultured in a range of temperatures the stability, or otherwise, of the fatty acid composition of the oil could be known by the time some remaining seed had matured on the head. Such a technique would be advantageous since it would be suitable for the small seed samples that are often found in breeding programmes and because it does not require large scale controlled environment facilities to gain the response of fatty acid composition to temperature.

CHAPTER 3. THE DEVELOPMENT OF SUNFLOWER EMBRYOS AND THE EFFECT OF HIGH TEMPERATURE DURING DEVELOPMENT ON THE FATTY ACID COMPOSITION OF THE EMBRYO OIL

3.1 INTRODUCTION

The use of embryo culture has been suggested as a means to facilitate the search for sunflower genotypes with stable linoleic acid contents (Section 2.6). However, before the technique was explored it was necessary to determine the stage of growth at which embryos might be most sensitive to the effects of external influences (especially temperature) on fatty acid synthesis.

It was hypothesised that the ideal time to take embryos for culture would be at the onset of rapid oil synthesis. At this stage any effect of temperature would be expected to be most readily seen, since there would not be a large amount of oil already present to mask changes, and the rapid synthesis of oil would quickly express new trends in fatty acid composition. The accumulation of oil in developing sunflower seeds has been studied by several researchers (Harris *et al.*, 1978; Robertson *et al.*, 1978a; Dorrell, 1978). Although a rapid oil accumulation phase occurred in all the studies, a range of values has been reported for the time of onset of this phase (Section 2.4.1). Therefore it was deemed necessary to define the time of onset of rapid oil accumulation in developing sunflower seeds in order to select appropriate material for the evaluation of temperature effects.

It was also considered necessary to establish at which stage of development the oil composition of the sunflower embryo was most sensitive to temperature. It could perhaps be expected that differences in temperature during rapid oil accumulation would have the most effect simply because that is when the oil is being synthesised, but the temperature conditions experienced before or after this phase could also influence the fatty acid composition of the oil. In zero-erucic acid rape plants subjected to high

temperatures at different stages of seed development, high temperature during rapid oil accumulation influenced the linoleic and linolenic acid percentages of the oil, while high temperature before or after this phase had little or no effect (Appelqvist, 1980). In sunflowers the phase of oil accumulation most sensitive to temperature needs to be defined.

Experiments were therefore undertaken with the objectives of:

a) determining precisely the period of rapid oil synthesis; and

b) examining the effect of exposure to high temperature at differing growth stages on the final oil composition of seeds at maturity.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Boron trifluoride-methanol complex (14%) was obtained from Merck (Munich, West Germany); butylated hydroxytoluene from Sigma Chemical Company (St Louis, M.O., U.S.A.) and the GLC column packing GP 10% SP-222-PS was obtained from Supelco Inc. (Bellefonte, Penn., U.S.A.). All other solvents and chemicals used, unless otherwise stated, were analytical grade.

3.2.2 Plant Materials

An open-pollinated cultivar was chosen for all experiments to provide the potential for detection of greater genetic variability than might be expected in hybirds. Selections of the most successful open-pollinated cultivar for Australian conditions, Peredovik (Sunfola 68-2 or 68-3), were chosen and have been used throughout the experiments reported in this thesis except where otherwise stated.

All heads were bagged prior to anthesis. When approximately half the stigma on a head were receptive, pollen was collected from several heads, mixed, and then brushed over the stigma with a camel-hair brush. This brush pollination was carried out only once on each head to endeavour to have all

embryos commence development at the same time. The bags were replaced over the heads at least until the remaining stigma had scenesced.

3.2.3 Determination of Dry Weight

Samples were oven dried to constant weight at 65°C.

3.2.4 Extraction of Lipids

The method of lipid extraction used in this and all subsequent experiments was a modification of that of Folsh, Lees and Stanley (1957). Embryos were removed from seeds, weighed and homogenized with 10 ml of chloroform:methanol (2:1) in Ten Broeck homogenizer. Lipids were partitioned into the chloroform phase by the addition of 0.73% NaCl (2.0 ml) and butylated hydroxytoluene (0.5 mg) was added. After centrifugation the chloroform layer was removed and placed in a preweighed flask and the sample was evaporated to dryness on a rotary evaporator. Ethanol (1.0 ml) was added to the flask and the sample was re-evaporated. The weight of the lipid was recorded and the sample was then re-dissolved in chloroform containing butylated hydroxytoluene (0.5 mg/ml) to a final concentration of 5 mg/ml. The samples were then stored at -15°C.

3.2.5 Preparation of Methyl Ester Derivatives

In all experiments the following procedure was carried out. A 2 to 3 mg sample of lipid was evaporated to dryness under a stream of nitrogen and then re-dissolved in benzene (0.5 ml). Methyl esters were prepared using 2.0 ml of boron trifluoride in methanol (Morrison and Smith, 1964). After a one hour incubation at 65°C the methyl esters were extracted twice with petroleum spirit (B.R. 40-69°C; 5.0 ml) and dried over anhydrous sodium sulphate. The extracted methyl esters were evaporated to dryness under a stream of nitrogen and re-dissolved in 200 µl of carbon disulphide.

3.2.6 Gas-Liquid Chromatography

GLC analyses of methyl esters were performed at 175°C on 6mm x 1.82m

column packed with GP 10% SP-222-PS using a Packard series 7800 gas chromatograph with flame ionization detector (see Appendix 3:1 for full operational settings of the GLC). Peaks were identified by comparison with standard methyl esters and peak areas were calculated by the method of Carroll (1961). The procedure for the conversion of fatty acid percentages to weight of fatty acids is given in Appendix 3:2.

3.2.7 Thin-Layer Chromatography

Lipids were fractionated by thin layer chromatography on Kieselgel G (Merck) using the solvent mixture hexane:diethyl ether:acetic acid (70:30:1) (Nichols, 1963). Lipid bands were visualized with iodine vapour and identified by co-chromatography with standards. The triacylglycerol bands were scraped from the plates and assayed.

3.2.8 Assay for Triacylglycerols

Triacylglycerols were assayed by the hydroxamic acid method (Renkonen 1961). Reagents were prepared as described by Snyder and Stephens (1959) except for the ferric perchlorate, which was prepared according to Goddu, LeBlanc and Wright (1955).

3.2.9 Experimental Procedures

Experiment 1. Development of Sunflower Embryos

Sunflower plants were grown in the field at Armidale, N.S.W., (latitude 30.31 S, altitude 981 m). Twenty heads were pollinated at the same time and then covered with gauze bags to minimize bird damage. Seeds were sampled from heads during development and the embryos removed. Dry weight, oil and triacylglycerol content of the embryo were recorded and the fatty acid composition of the oil was determined. Air temperatures during the experiment were recorded by a continuous reading thermohygrograph in a Stephenson's screen placed at the edge of the plot about one metre from the ground.

Experiment 2. The Effect of High Temperature at Different Stages of Embryo Development on the Final Fatty Acid Composition of the Oil

Sunflower plants were grown under natural conditions in 30cm pots until anthesis when the plants were brush pollinated and transferred to a glasshouse (mean day/night temperatures 25/15°C) until the treatments were imposed. Groups of plants were transferred to a Shearer growth cabinet (30/22°C day/night temperatures) and exposed to high temperatures at different stages of seed development and for varying periods (Table 3.1).

Both the growth and rate of physiological development of sunflower seeds have been found to be related to temperature (Anderson, 1977; Harris *et al.*, 1978; Pereira, 1978). It was therefore necessary to use a scale other than time to record development in an endeavour to ensure that the results were not affected by the physiological status of the seeds. Heat units were therefore chosen as they are widely used in the estimation of plant development rates. The heat sum was calculated as day-degrees $= \sum_{i=1}^{n} (0.5\ (T_{max_i} + T_{min_i}) - T_{base})$ where T_{max_i} = daily maximum temperature, T_{min_i} = daily minimum temperature, T_{base} = temperature below which development does not occur and n = number of days. A base temperature of 0°C was used (Anderson, 1977). From Harris *et al* (1978) rapid oil accumulation was found to occur between approximately 250 to 500 day-degrees after pollination with physiological maturity occurring at approximately 800 day-degrees. Using these values as a guide, the treatments as described in Table 3.1 were imposed. Seeds were harvested from all treatments when a heat sum of 800 day-degrees had been reached. Embryos were removed from the pericarp and the lipid was extracted, methylated and analysed for fatty acid composition.

3.2.10 Statistical Analysis

Regression analysis was carried out to describe the pattern of dry matter accumulation, and oil and fatty acid accumulation during seed development. Data from experiment 2 were subjected to an analysis of

TABLE 3.1. High temperature treatments used in experiment 2.

Treatment	Duration of different temperature exposures during seed development (expressed as Heat Sum (day-degrees) after pollination).	
	Low temperature 25/15°C.	High temperature 30/22°C.
1	0-800	-
2	100-800	0-100
3	0-100, 200-800	100-200
4	0-200, 300-800	200-300
5	0-300, 400-800	300-400
6	0-400, 500-800	400-500
7	0-500, 600-800	500-600
8	0-600, 700-800	600-700
9	200-800	0-200
10	300-800	0-300
11	400-800	0-400
12	500-800	0-500
13	-	0-800

variance and Duncan's Mutiple Range Tests were used to determine the significance of differences between means.

3.3 RESULTS

3.3.1 Experiment 1. Development of Sunflower Embryos

Embryo dry weight increased in a linear fashion from about 150 day-degrees to about 500 day-degrees, while oil accumulation commenced at about 200 day-degrees and continued for 300 day-degrees (Figures 3.1a and 3.1b). The accumulation of triacylglycerols followed the pattern of total oil accumulation (Figure 3.1c). The fatty acid percentages were converted to weight of fatty acids by the method set out in Appendix 3.2. The pattern of accumulation of individual fatty acids (linoleic, oleic, stearic and palmitic) is shown in Figures 3.2 and 3.3. The accumulation of linoleic acid followed the same pattern as total oil and triacylglycerols. The weight of oleic acid increased rapidly from 200 day-degrees until about 380 day-degrees, remained fairly constant until about 500 day-degrees and then decreased. The amount of saturated fatty acids (stearic and palmitic) increased linearly from about 200 to 400 day-degrees and then remained constant.

3.3.2 Experiment 2. High Temperature Effects during Development

The results of this experiment are presented in Table 3.2. The high temperature treatments during seed development had no significant effect on the percentages of saturated fatty acids in the oil of mature seed. Prolonged exposure to high temperature for 400, 500 or 800 day-degrees following pollination (treatments 11, 12 and 13) led to a significant ($p \leq 0.05$) decrease in linoleic acid percentages, with corresponding increases in oleic acid. Although the length of the high temperature exposure increased over the three treatments, the means were not significantly different from each other. Treatments 9 and 10 were exposed to

FIGURE 3.1

The development of embryos from field-grown sunflower seeds.

a) The increase in the dry weight of sunflower embryos

The fitted line represents the equation

$y = 363.8 - 5.0594099x + 0.0214389x^2 - 0.0000223x^3$

$r^2 = 0.98$

where y = dry weight and x = heat sum

b) The increase in oil content of sunflower embryo

The fitted line represents the equation

$y = 288.3 - 3.6767685x + 0.0140150x^2 - 0.0000137x^3$

$r^2 = 0.98$

where y = oil content and x = heat sum

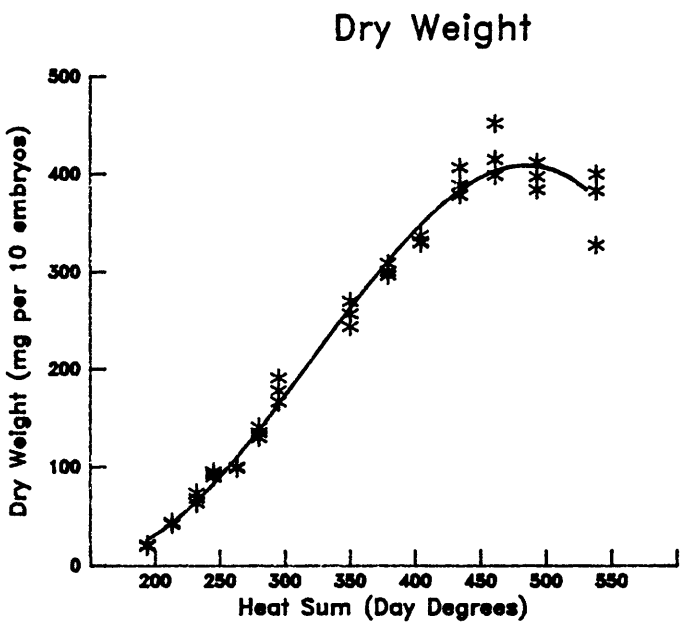
c) The increase of triacylglycerol content in developing sunflower embryos

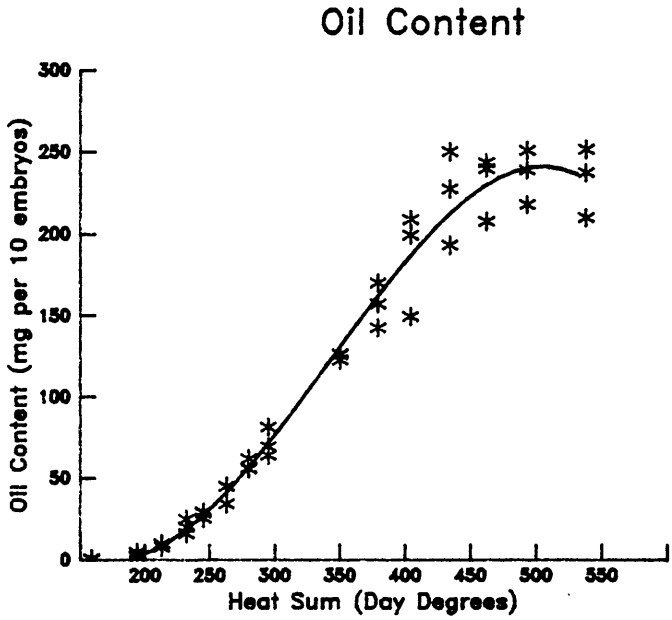
The fitted line represents the equation

$y = 339.6 - 4.2618210x + 0.0159791x^2 - 0.0000154x^3$

$r^2 = 0.97$

where y = triacylglycerol and x = heat sum


Dry Weight
Dry Weight (mg per 10 embryos)
0
100
200
300
400
500
200
250
300
350
400
450
500
550
Heat Sum (Day Degrees)


Oil Content
Oil Content (mg per 10 embryos)
0
50
100
150
200
250
300
200
250
300
350
400
450
500
550
Heat Sum (Day Degrees)

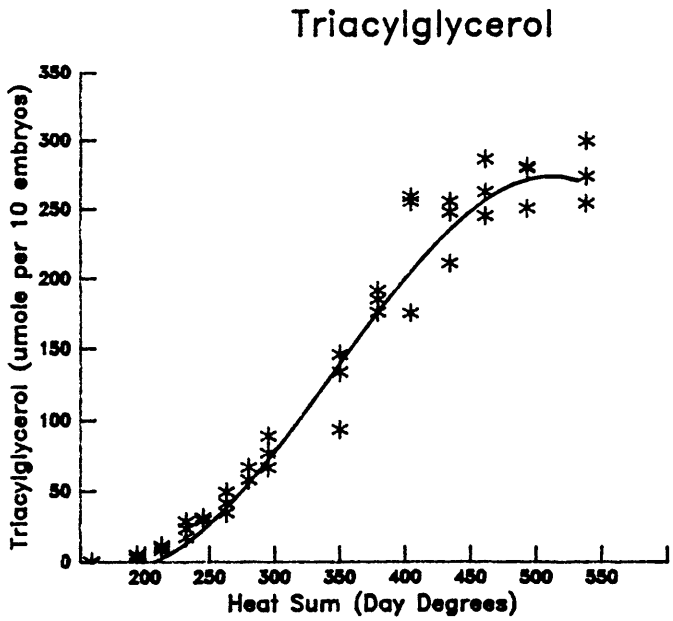

Triacylglycerol
Triacylglycerol (umole per 10 embryos)
0
50
100
150
200
250
300
350
200
250
300
350
400
450
500
550
Heat Sum (Day Degrees)

FIGURE 3.2

The accumulation of saturated fatty acids in developing sunflower embryos.

a) Palmitic acid

The fitted line represents the equation

$y = -8.5 + 0.551222x - 0.0000372x^2$

$r^2 = 0.91$

where y = plamitic acid and x = heat sum

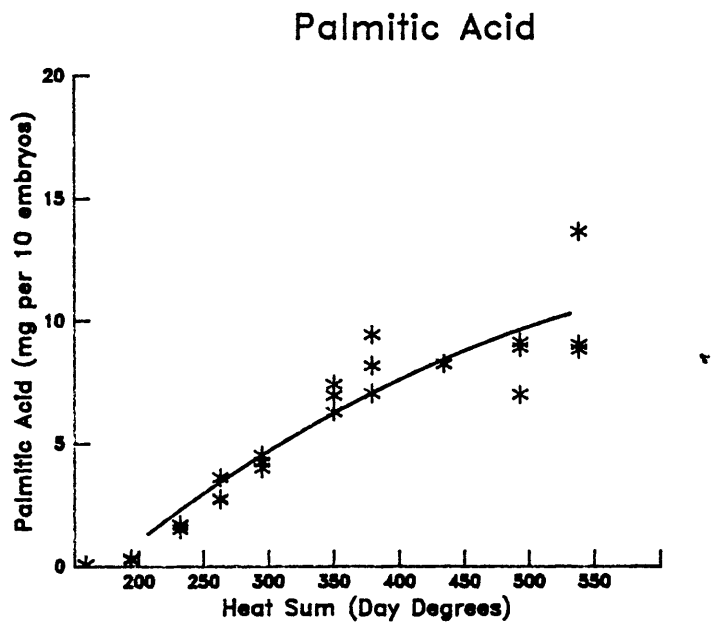
b) Stearic acid

The fitted line represents the equation

$y = -9.6 + 0.593711x - 0.0000376x^2$

$r^2 = 0.89$

where y = stearic acid and x = heat sum


Palmitic Acid
Palmitic Acid (mg per 10 embryos)
0
5
10
15
20
200
250
300
350
400
450
500
550
Heat Sum (Day Degrees)

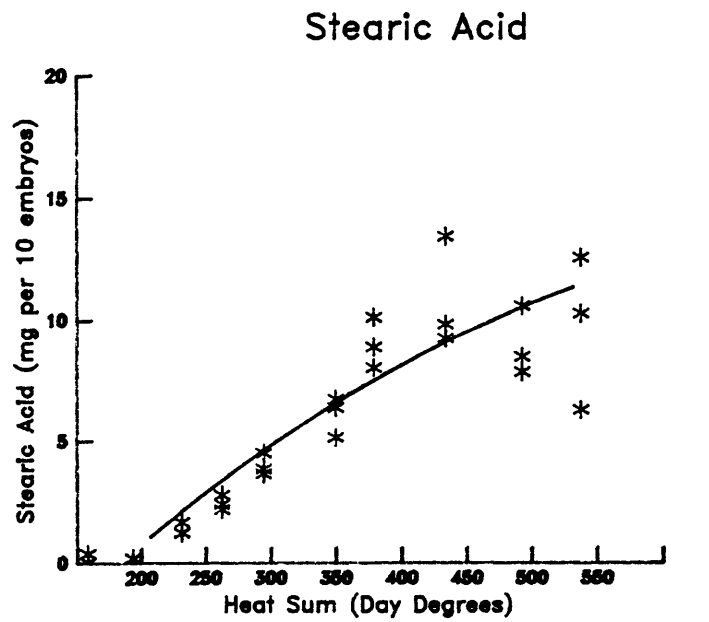

Stearic Acid
Stearic Acid (mg per 10 embryos)
0
5
10
15
20
200
250
300
350
400
450
500
550
Heat Sum (Day Degrees)

FIGURE 3.3

The accumulation of unsaturated fatty acids in developing sunflower embryos.

a) Oleic acid

The fitted line represents the equation

$y = 65.7 - 0.9436527x + 0.0039819x^2 - 0.0000043x^3$

$r^2 = 0.96$

where y = oleic acid and x = heat sum

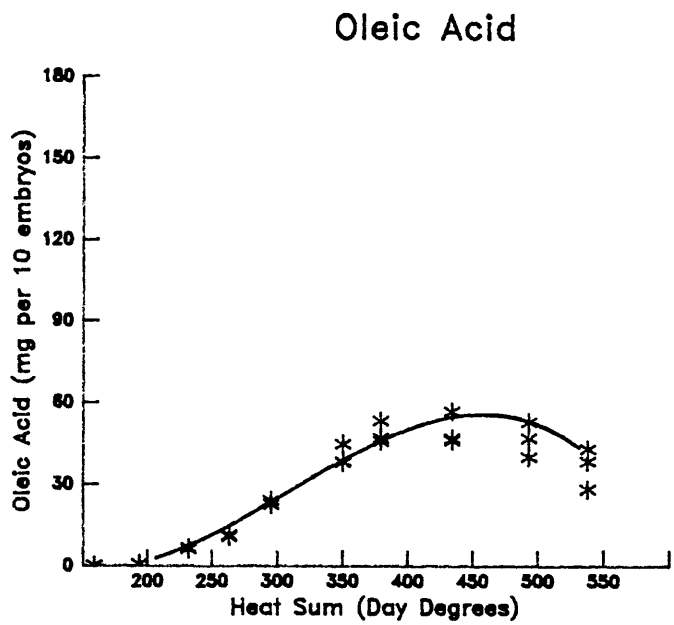
b) Linoleic acid

The fitted line represents the equation

$y = 192.7 - 2.2874079x + 0.0081013x^2 - 0.0000074x^3$

$r^2 = 0.98$

where y = linoleic acid and x = heat sum


Oleic Acid
Oleic Acid (mg per 10 embryos)
180
150
120
90
60
30
0
200
250
300
350
400
450
500
550
Heat Sum (Day Degrees)

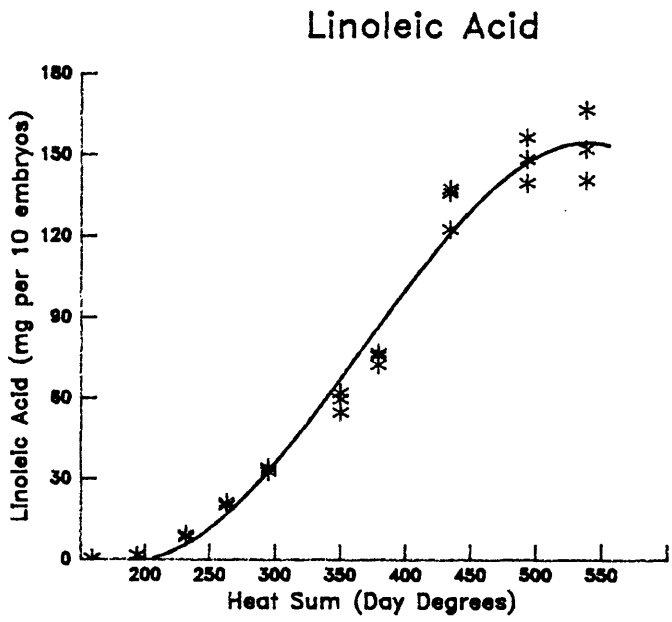

Linoleic Acid
Linoleic Acid (mg per 10 embryos)
180
150
120
90
60
30
0
200
250
300
350
400
450
500
550
Heat Sum (Day Degrees)

TABLE 3.2. The effect of high temperature at different stages of growth on the final fatty acid composition of the oil. (Values represent the mean of four heads).

Treatment	High Temperature Exposure (day-degrees after pollination)	Fatty Acid Composition (% total fatty acid)			
		Palmitic	Stearic	Oleic	Linoleic
1	0	4.7	2.9	14.9[a]	77.2[c]
2	0-100	5.2	3.3	14.4[a]	76.5[c]
3	100-200	4.7	3.3	16.0[a]	75.4[bc]
4	200-300	5.2	3.8	13.1[a]	77.2[c]
5	300-400	4.4	4.6	21.6[ab]	68.5[ab]
6	400-500	4.9	3.6	20.5[ab]	70.7[abc]
7	500-600	4.8	2.8	15.6[a]	75.8[c]
8	600-700	4.9	3.1	15.5[a]	76.5[c]
9	0-200	5.8	3.6	13.6[a]	76.5[c]
10	0-300	5.3	2.8	14.6[a]	76.8[c]
11	0-400	5.2	2.8	24.8[b]	66.9[a]
12	0-500	4.9	3.5	24.5[b]	66.3[a]
13	0-800	4.8	2.5	26.8[b]	65.5[a]

[1] Values in the same column followed by the same letter did not differ significantly ($p \leq 0.05$).

relatively long periods of high temperatures (200 and 300 day-degrees respectively) early in embryo development, but the fatty acid composition of oil from these plants was not significantly different from the low temperature control (treatment 1). There is a suggestion, however, that the 300-400 and 400-500 day-degree phase of embryo development (treatments 5 and 6) were sensitive to temperature. The fatty acid composition of oils from heat treatments 2, 3 and 4, which experienced high temperatures during some of the 0-300 day-degree period, were not significantly different to that of the control. Similarly heads that experienced high temperatures after 500 day-degrees (treatments 7 and 8) had fatty acid composition that was not significantly different to that of the low temperature control.

3.4 DISCUSSION

The pattern of dry weight and oil accumulation in the embryo was very similar to that for whole seeds (Harris *et al.*, 1978; Robertson *et al.*, 1978a; Dorrell, 1978). The period of rapid oil synthesis was the same as that reported by Harris *et al* (1978) (200-500 day-degrees), but physiological maturity (or maximum dry weight) occured about 150 day-degrees earlier. This difference could be due to the use of embryos (or kernels) compared to whole seeds, or to the fact that the results presented by Harris *et al* (1978) are based on three populations of sunflowers, while the results presented here are from one population alone.

The content of saturated fatty acids in the developing embryo remained constant from about 400 day-degrees after pollination and that of linoleic acid from about 500 day-degrees, but the content of oleic acid fell from about 450 day-degrees until 700 day-degrees. It seems more than a coincidence that the apparent loss in total oil content over this period (approx. 20 mg per embryo) was the same as the amount of oleic acid lost. In the study by Pereira (1978) in which oil accumulation in the kernel and pericarp of the sunflower seed was studied, it was reported

that the oil content of the pericarp increased from about 35 days after pollination, while oil content of the kernel decreased. It could therefore be possible that oil lost from the kernel was transferred to the pericarp and from the results of this experiment it appears that it could be oleic acid that is involved in this transfer.

In experiment two, only treatments 11, 12 and 13 were significantly affected by high temperature exposure, while there was the suggestion of an effect on treatments 5 and 6. From the results it therefore appears as if the influence of temperature on fatty acid composition was not due to prolonged exposure to high temperature, but to selective exposure to high temperature during the 300-500 day-degrees phase of embryo development.

Rapid oil synthesis in the embryo occured between 200 and 500 day-degrees after pollination (experiment 1). The hypothesis that this phase of embryo development would be most sensitive to temperature has been partially substantiated. The three treatments causing a significant alteration in fatty acid composition were exposed to high temperature for all or most of this phase. In treatments 5 and 6, where there is suggestion of an alteration in fatty acid composition, high temperature exposure was experienced for part of the rapid phase of oil synthesis. The early part of this phase, however, appears to be unaffected by high temperature, since treatment 4 (200-300 day-degrees exposure) and treatment 10 (0-300 day-degrees exposure) showed no significant change in fatty acid composition. In rapeseed individual fatty acids on triacylglycerol molecules have been found to undergo exchange reactions in the early stages of oil accumulation (Gurr, 1980; Appelqvist, 1980). The occurrence of this type of fatty acid exchange in the early phase of rapid oil accumulation could explain the results obtained for treatments 4 and 10. Triacylglycerol molecules which were synthesised early in seed development (ie between 0 and 300 day-degrees) may undergo acyl exchange reactions so that the fatty acid composition of this oil no longer reflects the temperature conditions under which it was synthesised, but reflects the

temperature conditions of the period following (ie 300-500 day-degree period).

From an examination of the results of the two experiments, it appears that the ideal time to culture sunflower embryos would be between 300 and 500 day-degrees after pollination. At this stage of development, rapid oil synthesis would be occuring and the embryo would be most sensitive to changes in temperature.

CHAPTER 4. THE DEVELOPMENT AND ASSESSMENT OF A TECHNIQUE FOR THE CULTURING OF ISOLATED SUNFLOWER SEED

4.1 INTRODUCTION

In the previous chapter the stage of embryo development at which it would be most suitable to culture sunflower embryos was defined, but it still remained to find a culture medium that would support embryo growth. This is one of the most important aspects of embryo culture work. Since there is a vast literature on the culture of embryos involving many different species and conditions only a brief general outline of the components of embryo culture media and their effect on embryo growth will be given in this chapter. Comprehensive reviews have been published by Sanders and Ziebur (1963), Narayanswami and Norstog (1964), and Raghavan (1980).

The main constituents of the medium are agar, a carbon source, minerals, vitamins, amino acids and hormones. The agar is simply used to solidify the culture medium and the concentration used ranges from 0.25% to 2.0% (Sanders and Ziebur, 1963; Narayanswami and Norstog, 1964).

Since embryos in culture have been removed from their energy supply from the plant and normally are not fully autotropic, the medium must be supplemented with an organic carbon source. Sucrose is the most common carbohydrate used (DeFossard, 1976; Raghavan, 1980) probably because it is the sugar that is translocated in the plant. Sucrose concentrations used in culture media range from 0.5% to 18% with the optimum concentration varying with age and species (Sanders and Ziebur, 1963).

Mineral salts have been added to culture media since it was realised that they were essential for plant growth. Elements such as potassium, calcium, magnesium, nitrogen, phosphorus, sulfur and iron are commonly included (Sanders and Ziebur, 1963). Trace elements such as boron, copper, manganese and zinc are also added. Their inclusion is necessary because they are constituents of complex organic molecules such as enzymes and a deficiency of these elements can results in slow growth or death (DeFossard,

1976).

When the importance of vitamins in plant metabolism was recognised, these also were included in culture media. Commonly used vitamins include biotin, pantothenic acid, niacin, inositol, pyridoxine, thiamine and ascorbic acid (Sanders and Ziebur, 1963; Raghavan, 1980). However, their presence is not always essential because embryos, being partially autotropic, can probably synthesise vitamins for their own requirements providing that the substrates are present in adequate quantities (Raghavan, 1980). Amino acids have also been added to culture media to stimulate the growth of embryos (Sanders and Ziebur, 1963; Narayanswami and Norstog, 1964; Raghavan, 1980). Hormones such as auxins, gibberellic acid and cytokinens have also been added to media but the response of embryos to these hormones has varied depending on the age and species of the embryo (Sanders and Ziebur, 1963; Raghavan, 1980).

Embryo culture can have one of two aims - either to convert an immature or dormant embryo into a seedling, or to allow the embryo to develop but not germinate. The composition of a medium would be dependent on the purpose for which the embryos are cultured. A less complex medium is required when an embryo is germinated and quickly becomes autotropic than when an embryo is required to follow normal development, since in the latter case nearly all the nutritional requirements must be provided in the culture medium.

Determining the correct concentrations of the constituents of the culture medium is one of the most important aspects of embryo culture work. This chapter describes a series of experiments that were undertaken to develop an embryo culturing system, especially the determination of a suitable culture medium that would allow the normal development of embryo components, including the fatty acids, in the developing sunflower embryo. Once the culturing system is defined it will be necessary to compare embryos grown in this system with those grown normally in a head. The second part

of this chapter describes a series of experiments that were carried out to compare growth and development of 'cultured' and head-grown embryos.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

All chemicals used in the preparation of culture media were analytical grade except where otherwise stated. Refined white sugar (CSR) was used as a sucrose source in media.

4.2.2 Preparation of Culture Media

Media were prepared after the method of DeFossard (1976). Stock solutions of 10X or 100X concentrations were prepared separately for the components of the medium - minerals, growth factors and amino acids, and hormones. The components were mixed together in the required amounts on the day of preparation. Sucrose and agar were added and the solution was heated to dissolve the agar. After dispensing 10ml of solution per culture tube, the medium was autoclaved. Details of the concentrations of media constituents are given in Appendix 4.1.

4.2.3 Sterilisation and Culturing Techniques

Seeds or segments of the capitulum (head) were removed from plants 10 to 13 days (approx. 300 day-degrees) after hand pollination. The material was disinfested in a solution of sodium hypochlorite (1% w/v available chlorine) and 0.1% detergent (7x, Biotech) and then rinsed three times in sterile distilled water. For experiments with excised embryos, embryos were aseptically removed from the pericarp after the seeds were sterilized. In some experiments individual seeds still attached to a core of spongy head tissue were aseptically removed from sterile segments of head. All tissue (excised embryo, intact seed or seed plus head tissue) was placed in 10ml of sterile culture medium (see Plates 4.1 and 4.2).

PLATE 4.1

Photograph of excised embryos (at left) and isolated seeds plus head tissue as prepared for tissue culture experiments.

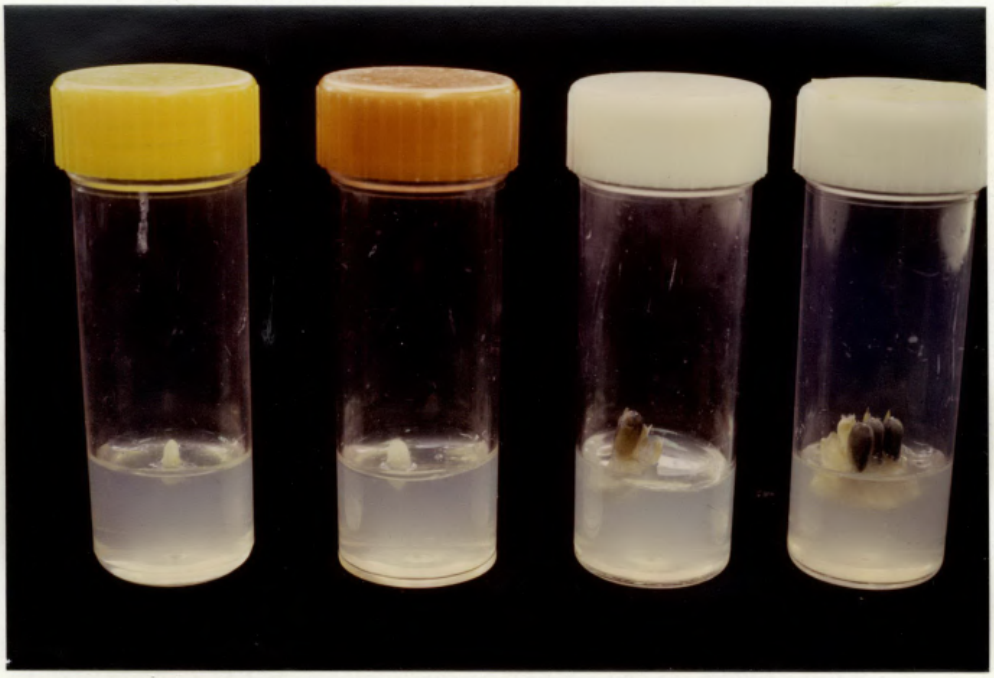


PLATE 4.2

Photograph of a typical culture experiment.

4.2.4 Electron Microscopy

Pieces of embryo cotyledon (1mm^3) were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 6.8) for 3 hours at 4°C. After 4 washes in phosphate buffer (30 minutes each) the samples were left overnight in phosphate buffer. The tissue was post-fixed in buffered 1% osmium tetroxide for 3 hours at 4°C. After one wash in phosphate buffer the tissue was dehydrated through a series of ethanols (50%, 70%, 95% and 100% ethanol) and finally infiltrated with Spurr's low viscosity resin A (Spurr, 1969). Ultrathin sections were stained with uranyl acetate and lead nitrate (Reynolds, 1963) and examined under the transmission electron microscope.

4.2.5 Experimental Procedures

Part A: Development of the Technique of Isolated Seed Culture

A series of preliminary experiments was carried out to define the most suitable conditions for culturing sunflower embryos. The parameters studied were:

a) The type of tissue to be cultured - excised embryo, intact seed or seed still attached to some of the spongy head tissue.

b) The effect of different concentrations of the media components on embryo growth. Those studied were -

i) mineral concentration (low/high).

ii) sucrose concentration (4-16%).

iii) the presence of growth factors and amino acids.

iv) the effects of indole butyric acid and other auxins.

v) concentration of agar (0-1.5%).

A single embryo, seed or seed plus head tissue was placed in each culture tube containing 10ml of the appropriate medium. The method of preparation of culture media is presented in Appendix 4.1. For the first few experiments tissue was grown in the light at 25/15°C but in later

experiments groups of tubes were placed in boxes or put in wire baskets and covered in foil.

Embryo growth was recorded by measuring the length of the embryo or the wet weight of the embryo, and also by the visual appearance of the embryo, i.e. colour and firmness of cotyledons, presence of necrosis, etc.

When several media were found to give reasonable embryo growth, an experiment was carried out to examine the oil content and the fatty acid composition of the oil from cultured embryos. Oil analyses for this experiment were performed by C.P. Rochester, C.S.I.R.O Plant Physiology Unit, Macquarie University, North Ryde.

Part B: Assessment of the Technique of Isolated Seed Culture

An experiment was carried out to compare the growth of embryos from cultured seeds with that of embryos grown in the head. The composition of the medium used is given in Table 4.7. At 300 day-degrees after pollination seeds from half a sunflower head were placed in culture. At this stage the sunflower plant with half the head still intact and the cultured seeds were placed in a Shearer growth cabinet at 28/20°C day/night temperatures. After seven days embryos from both treatments were removed from seeds and analysed for oil content and the fatty acid composition of the oil.

Tissue from embryos grown in both sets of conditions was taken for examination under the transmission electron microscope. A segment of the cotyledon of the embryos was removed, cut into 1mm cubes and then prepared for electron microscopy (Section 4.2.4).

A further experiment was set up to examine the effect of incubation temperature on fatty acid composition of oil from embryos of cultured seeds. At approximately 300 day-degrees after pollination five sunflower heads were removed and seeds with head tissue attached were cultured.

Fifteen seeds from each head were placed in incubators set at temperatures of 15, 18, 21, 24 and 27°C. When the heat sum at each incubation temperature had reached 200 day-degrees, (i.e. 500 day-degrees after pollination) the embryos were removed and analysed for dry weight, oil content and fatty acid composition. Data were recorded for each head in each treatment.

4.2.6 Statistical Analyses

In culture experiments, standard deviation or standard errors were calculated for all mean values. Regression analysis was used to fit lines of best fit to the response of embryo fatty acid composition to temperature, and a test of non-parallelism was used to compare the fitted lines.

4.3 RESULTS

4.3.1 The Development of the Technique of Isolated Seed Culture

A series of experiments was carried out to define the culture medium that would support embyro growth. Preliminary experiments revealed that excised sunflower embryos exhibited precocious germination when cultured (Table 4.1). Increasing the sucrose concentration of the medium reduced the germination rate but embyro growth was still poor (Table 4.2). It was therefore apparent that excised embryos could not be grown readily in culture medium and alternative tissue was tested.

Whole seeds were detached from the head and placed in media. However, growth of embryos in these seeds was poor and it was reasoned that this was probably due to inadequate uptake of nutrients from the media through the small pore through which vascular connections normally traverse the pericarp. Methods were tested whereby the pericarp was punctured in an attempt to achieve greater uptake (Diagram 4.1). However, the growth of embryos in these seeds remained unsatisfactory (Table 4.3).

It was therefore reasoned that it may be possible to achieve greater uptake if the vessels contained in the spongy tissue of the head

TABLE 4.1

The effect of mineral and sucrose concentration on the growth of excised embryos and embryos from intact cultured seeds. Values represent a mean of 10 embryos ± standard deviation.

Mineral Concentration	Tissue Type	Increase in Embryo Length (mm)	
		4% sucrose	8% sucrose
LOW	excised embryo	*	*
	intact seed	0.6 ± 0.3	0.4 ± 0.3
HIGH	excised embryo	*	*
	intact seed	1.3 ± 0.3	1.0 ± 0.3

* precocious germination of embryos.

TABLE 4.2

The effect of increasing sucrose concentration on the development of excised embryos. Medium-high minerals, 0.9% agar plus appropriate sucrose concentrations. Values represent the mean of 10 embryos ± standard deviation.

Sucrose concentration (%)	Increase in embryo length (mm)	Precocious germination (%)
4	0	50
8	0.2 ± 0.3	50
12	0.5 ± 0.3	20
16	0.5 ± 0.5	20

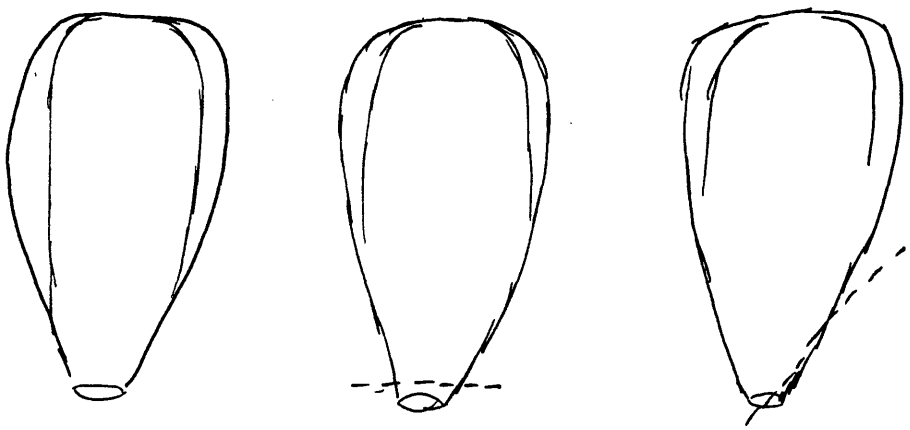
DIAGRAM 4.1

Schematic representation of seeds with punctured pericarps -

(a) intact seed

(b) seed cut at base

(c) seed cut diagonally at the side



(a) (b) (c)

TABLE 4.3

The effect of the tissue type cultured on the growth of the embryo. Medium used 4% sucrose, high minerals (see appendix 4.1) and 0.9% agar. Values represent the mean of ten embryos ± standard deviation

Tissue Cultured	Increase in Embryo Length (mm)
excised embryo	0.3 ± 0.3*
intact seed	0.9 ± 0.2
seed cut at base	0.7 ± 0.3
seed cut diagonally at side	1.3 ± 0.4
seed with head tissue attached	2.8 ± 0.3

* some precocious germination.

(see Plate 4.1) were to be exposed in the medium. Slices of head, comprising a segment of the spongy tissue and a seed with vascular connections to this tissue kept intact, were therefore tested. Embryos from this tissue grew more than excised embryos or embryos from intact or punctured seeds (Table 4.3). This system was therefore used in all subsequent experiments.

The effect of different agar concentrations in the medium on embryo growth was examined and the results are presented in Table 4.4. The highest concentration of agar appeared to adversely affect embryo growth. Although the increase in embryo weight was comparable at 0, 0.6% and 0.9% agar, the embryos grown on 0.9% agar showed less necrosis than embryos grown at the other concentrations.

While the inclusion of auxins, indole butyric acid or 6 auxin mix in the culture medium increased the growth of embryos in cultured seeds, the inclusion of growth factors and amino acids seemed to adversely affect growth (Table 4.5). However, when the culture media were assessed for their capacity to support oil synthesis, the presence of indole butyric acid, growth factors and amino acids gave the most oil accumulation in embryos from cultured seeds (Table 4.6). Fatty acid synthesis appeared to be normal in embryos from the cultured seeds since the fatty acid composition of the oil fell within the expected limits (Table 4.6). Therefore the medium containing high minerals, growth factors and amino acids and indole butyric acid was chosen to be used in all future experiments. The constituents of this medium are presented in Table 4.7.

4.3.2 Assessment of the Technique of Isolated Seed Culture

The development of embryos from cultured seeds was compared to that of embryos remaining in the head. The results are presented in Table 4.8. The increase in dry weight of cultured embryos was 73% of

TABLE 4.4

The effect of agar concentration on the growth of embryos from cultured seeds. Values represent mean of 10 embryos ± standard deviation.

Medium: 4% sucrose plus high minerals.

Agar concentration %	Increase in embryo weight mg/embryo
0	15.0 ± 3.8
0.6	17.1 ± 3.4
0.9	18.6 ± 1.6
1.5	11.0 ± 3.2

TABLE 4.5

The effect of indole butyric acid, 6 auxin mix, growth factors and amino acids on the growth of embryos in cultured seeds (seeds + spongy tissue used). Basic medium high minerals, 4% sucrose plus 0.9% agar. Values represent the mean of 10 embryos ± standard deviation.

Treatment	Increase in embryo length (mm)		Increase in embryo wet weight	
	0	plus growth factors and amino acids	0	plus growth factors and amino acids
0	3.5 ±0.6	2.4 ±0.4	12.9 ±2.4	11.6 ±2.7
IBA 1.0μM	3.9 ±0.6	3.2 ±0.4	21.1 ±2.2	17.5 ±4.3
6 Auxin mix	3.7 ±0.4	3.2 ±0.5	18.8 ±4.5	12.9 ±3.4

TABLE 4.6

The assessment of 6 culture media for their capacity to support oil synthesis in the developing embryo of cultured seeds (with head tissue attached).

Medium 1 Basic medium high minerals, 4% sucrose + 0.9% agar.

Medium 2 Basic plus growth factors and amino acids.

Medium 3 Basic plus 1.0μM IBA.

Medium 4 Basic plus 1.0μM IBA and growth factors and amino acids.

Medium 5 Basic plus 6 auxin mix.

Medium 6 Basic plus 6 auxin mix and growth factors and amino aicds.

Medium	Wet Weight (mg/10 embryos)	Oil Content (mg/10 embryos)	Fatty Acid Composition (% total fatty acids)			
			palmitic	stearic	oleic	linoleic
1	246	5.1	3.1	0.8	22.9	72.1
2	238	4.8	8.5	2.2	17.7	71.0
3	261	8.1	7.3	2.1	23.8	66.3
4	303	11.8	4.3	1.1	22.8	71.5
5	301	3.6	6.3	1.5	19.9	71.8
6	306	4.6	6.6	2.8	29.1	60.6

TABLE 4.7

Constituents of final culture medium.

Macronutrients (mM)	NH_4NO_3(10); KNO_3(10); NaH_2PO(1); $CaCL_2$(2); $MgSO_4$(1.5).
Micronutrients (μM)	H_3BO_3(50); $MnSO_4$(50); $ZnSO_4$(20); $CuSO_4$(0.1); Na_2MoO_4(0.1); $CoCl_2$(0.5); KI(2.5); $FeSO_4$(50); Na_2EDTA(50); Na_2SO_4(450).
Growth Factors (μM)	Inositol (600); nicotinic acid (40); Pyridoxine HCl(6); thiamine HCl(40); biotin (1); D-Ca-pantothenate(5); ascorbic acid(10); choline chloride(10).
Amino Acids (μM)	L-cysteine HCl(120); glycine(50).
Hormone (μM)	Indole butyric acid (1.0).
Carbon Source	Sucrose (4%)
Agar	Difco-Bacto (0.8%). pH adjusted to 5.5.

TABLE 4.8

Comparison of the development of embryos from 'cultured' seeds and seeds grown in the head. Values represent the mean ± standard deviations for 3 replicates. Heat sum = 168 day-degrees.

Parameter	Head-grown (control) embryos	Embryos from Cultured Seeds
Increase in dry weight (mg/10 embryos)	163.2 ± 10.7	118.4 ± 1.0
Increase in oil content (mg/10 embryos)	66.5 ± 1.8	56.2 ± 0.7
Fatty acid composition (% of total fatty acids)		
palmitic	5.4 ± 0.3	5.7 ± 0.4
stearic	10.1 ± 0.9	10.0 ± 0.4
oleic	57.2 ± 1.3	56.9 ± 1.8
linoleic	27.3 ± 0.9	27.4 ± 1.9

that of head-grown embryos. Oil from the two types of embryos was almost identical in fatty acid composition. TLC analysis of oil extracted from both cultured and head-grown embryos was very similar, consisting of predominantly triacylglycerol, with some diacylglycerol and polar lipid present.

Transmission electron microscopy was used to examine the ultrastructural details of cells from both groups of embryos (Plates 4.3 and 4.4). No ultrastructural differences were detected between the tissue of embryos allowed to develop on the head and those from culture. Cells from both tissue types could be seen to contain nuclei, oil bodies and protein bodies. Oil bodies were abundant in the cytoplasm of both cell types but the cells from the cultured tissue appeared to contain slightly more protein bodies.

The change in fatty acid composition of oil from cultured embryos in response to increasing incubation temperature is shown in Figures 4.1a to 4.1d. The saturated fatty acids were relatively unaffected by temperature while the unsaturated fatty acids varied markedly across the range of temperatures used. The percentage of linoleic acid in the extracted oil of all heads fell with increasing temperature with a corresponding increase in oleic acid percentage. Regression analysis was used to fit a line to the results for each head. Linoleic acid (and oleic acid) was linearly related to temperature for all heads. A test for non-parallelism revealed that the lines for four heads formed a family of parallel lines with the equation $LA = 104.1 - 3.04T$ ($r = 0.975$) while one head's response was non-parallel ($LA = 128.96 - 4.12T$ ($r = 0.991$)). The same situation occurred with the regression lines relating oleic acid to temperature.

After 200 day-degrees in culture there were no significant differences ($p \leq 0.05$) in the increase of dry weight and oil content of embryos at each temperature, indicating the effectiveness of using heat sums to relate the development of embryos at different temperatures.

PLATE 4.3

Electronmicrograph of a cell from an embryo developed on the head (x5880).

o = oil body; p = protein body; n = nucleus.

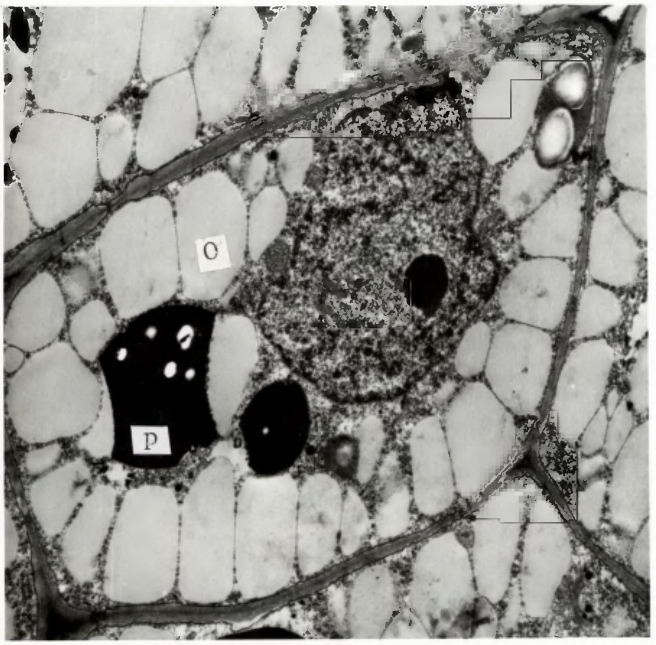


PLATE 4.4

Electronmicrograph of a cell from an embryo from a cultured seed (x5880).

o = oil body; p = protein body; n = nucleus.

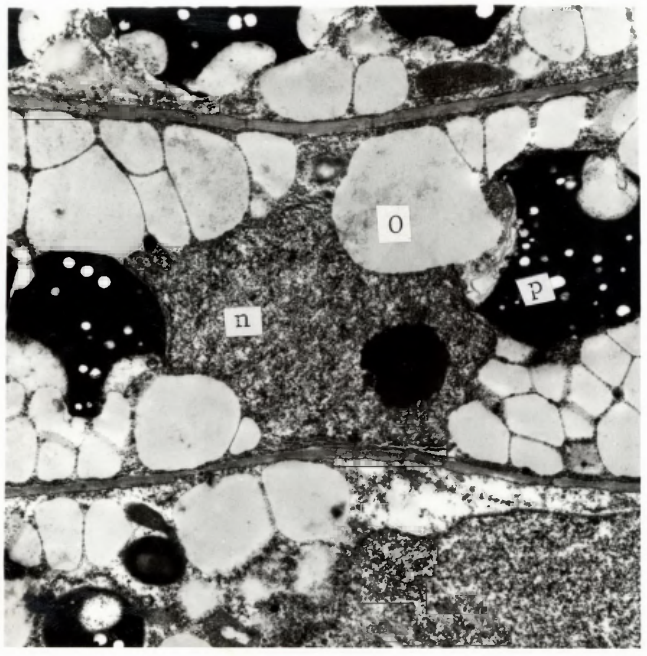


FIGURE 4.1

The change in fatty acid composition of oil from cultured embryos in response to increasing incubation temperature

a) Palmitic Acid

line represents

$y = 0.0183x + 5.619$ $r^2 = 0.114$

b) Stearic Acid

line represents

$y = 0.218x + 2.930$ $r^2 = 0.556$

c) Oleic Acid

the dark line represents heads A, B, D, E and the equation is

$y = 2.725x - 11.700$ $r^2 = 0.984$

the broken line represents head C and the equation is

$y = 3.986x - 37.980$ $r^2 = 0.996$

d) Linoleic Acid

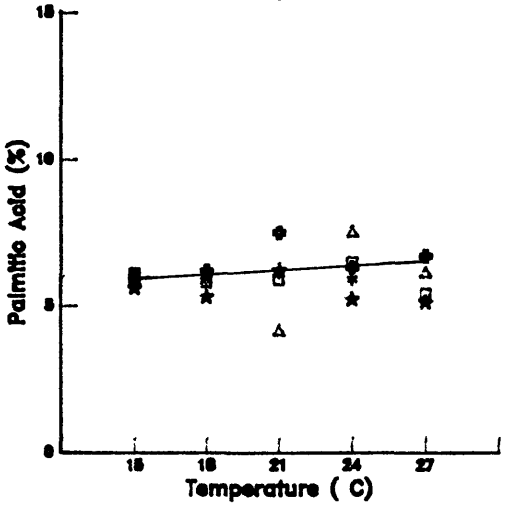
the dark line represents head A, B, D, E and the equation is

$y = -3.040x - 104.10$ $r^2 = 0.975$

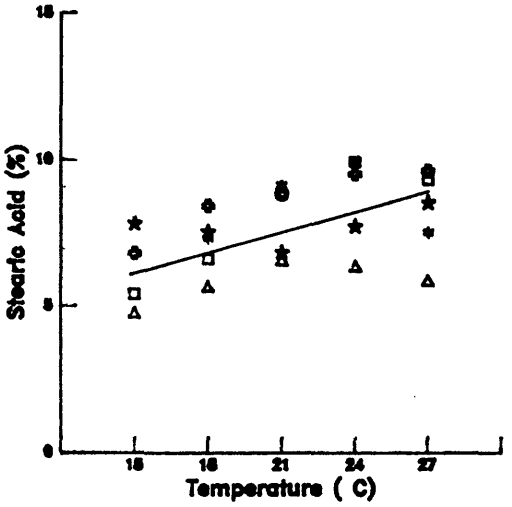
the broken line represents head C and the equation is

$y = -4.12x + 128.96$ $r^2 = 0.991$

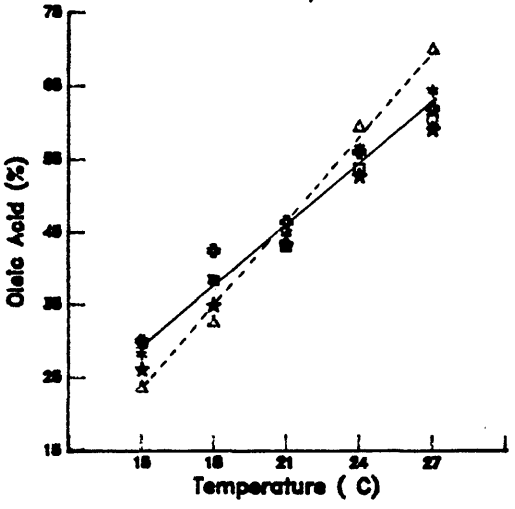
a) Palmitic Acid



b) Stearic Acid



c) Oleic Acid



d) Linoleic Acid

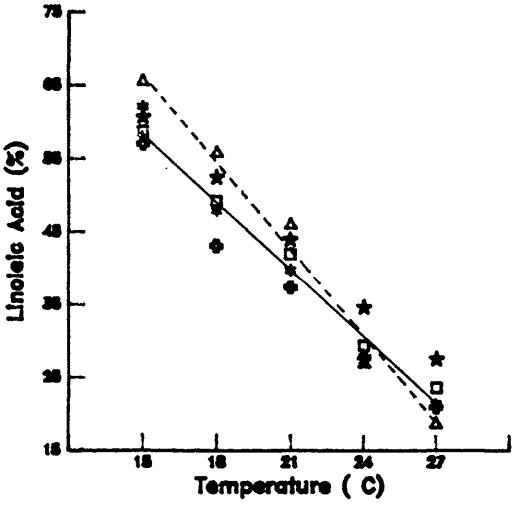


TABLE 4.9

Increase in dry weight and oil content of embryos from isolated seeds cultured in a range of constant temperatures. Values represent the mean of five replicates.

Incubation Temperature °C	Increase in Dry Weight mg/10 embryos	Increase in oil content mg/10 embryos
15	125.1	62.0
18	124.6	61.9
21	129.2	63.8
24	129.1	63.7
27	115.5	58.2
	SE = 12.04	SE = 4.95

4.4 DISCUSSION

Although increasing sucrose concentration had previously been found to totally inhibit precocious germination of cultured embryos (Sanders and Ziebur, 1963), this was not the case with sunflower embryos. Lack of embryo growth recorded could have been due to the medium not providing the full range of needed vitamins, growth factors and nutrients, but since alternative material was found to be suitable for culture the search for a medium that would support the growth of excised embryos was not pursued.

Embryos within intact seeds placed directly on the culture medium grew poorly while good embryo growth was obtained from seeds cultured with spongy head tissue attached. While the reason for this difference has not been closely examined, it would appear that sufficient uptake of nutrients could not occur when seeds were placed directly on the medium. Normally the seed is connected to the head by a vascular connection which traverses the otherwise impermeable pericarp. When the seed is removed from the head and placed in culture it is possible that this vascular connection may become damaged or blocked so preventing continued uptake. Alternatively, the small area available for transfer of medium components may simply be inadequate to allow enough uptake of nutrients and growth factors from the medium. The tissue of the sunflower head has a rich vascular network, particularly around the periphery of the head where material was taken for culturing (Morozov, 1958). The use of seeds still attached to spongy head tissue probably increased nutrient uptake by exposing a larger surface area of vascular tissue across which uptake could occur.

The medium that was found to give satisfactory growth contained sucrose (4%) as an energy source and a range of minerals necessary to plant growth. Although their initial inclusion seemed to suppress embryo growth, the presence of growth factors such as biotin, thiamine

and pyridoxine were found to be necessary for maximum oil synthesis, as was the presence of indole butyric acid.

The transmission electron microscopy study of cultured and head grown tissues revealed that the cells from both types of tissue were ultrastructurally similar. There was a suggestion that the 'cultured' cells contained a larger number of protein bodies than the head-grown tissue. This could be a real difference since the 'cultured' tissue had an increased supply of nitrogen and amino acids to the cells from the culture medium, but it could also be an artefact produced by the method of sectioning and cutting the tissue.

The five heads used to examine the fatty acid composition with increasing temperatures each gave a slightly different response. For the relationship between linoleic acid percentage and temperature (Figure 4.1d), the response of four heads formed a family of parallel lines but one head gave a significantly different response. A similar result was obtained for the oleic acid response to temperature (Figure 4.1c). The variation is not altogether unexpected as an open-pollinated cultivar Sunfola 68 has been used throughout the experiments to provide genetic variability. One head appears to be slightly more sensitive to increasing temperature than the other four heads used in the experiment.

The overall changes in fatty acid compositions of embryos from cultured seeds with increasing incubation temperature is very similar to the response obtained by Canvin (1965) and Harris *et al* (1978) using whole plants under controlled environmental conditions. The results of the culture experiment also compare favourably with results from field studies (Harris *et al.*, 1978; Robertson *et al.*, 1978a; Keefer *et al.*, 1976).

Although the growth of embryos from cultured isolated seeds was slightly slower than growth of embryos remaining in the head the composition of the oil produced by both types of embryos was identical.

Ultrastructural development also appeared normal. The response of the fatty acid composition of oil from cultured embryos to increasing temperature was also as expected. Therefore the technique of isolated seed culture appears to be a useful system in which to study the effect of external influences, especially temperature, on the fatty acid composition of sunflower oil.