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INHERITANCE OF FATTY ACID COMPOSITION OF SEED OIL IN THE BUFFALO GOURD, CUCURBITA FOETIDISSIMA HBK

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INHERITANCE OF FATTY ACID COMPOSITION OF SEED OIL IN THE BUFFALO GOURD, *CUCURBITA FOETIDISSIMA* HBK

by

Allen Craig Gathman

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Allen Craig Gathman

entitled Inheritance of Fatty Acid Composition of Seed Oil in the Buffalo Gourd, Cucurbita foetidissima HBK.

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

W P Bemis	Sept. 23, 1983
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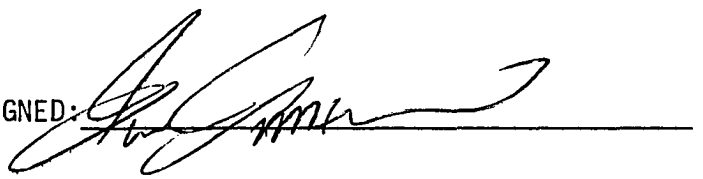
SIGNED: 

To my father

Craig Gathman

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While it is admittedly a cliche, the people who have helped in my progress as a student are in truth too numerous to mention here. To a smaller group, however, I owe so much that it would be improper for me to put my name on this work without inviting them to share such credit as it may deserve. First, my major professor, Dr. W. P. Bemis, throughout my career in the College of Agriculture, has unfailingly guided me with quiet confidence in my ability. By giving me responsibility and the freedom to fulfill it in my own way, he has helped me to grow over the years I have worked with him.

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ABSTRACT

The buffalo gourd, Cucurbita foetidissima HBK, is a xerophytic perennial native to the southwestern United States and northern Mexico. A vigorous spreading vine, it produces edible oil and protein in the seeds, and edible starch in its fleshy storage root. This study concerns the inheritance of content of each fatty acid in the seed oil of the buffalo gourd, including heritability studies, physiological relationships between the fatty acids, and environmental influence on the oil composition. The mechanisms of fatty acid biosynthesis and desaturation and environmental effects on them are reviewed, as is the inheritance of fatty acid composition in commercial oilseed crops.

In this study, crosses were made between plants selected for low or high linoleate content and the progeny analyzed by gas chromatography, using a non-destructive half-seed method. Analyzed seed of extremely high and low linoleate content were planted, and crosses made among the resulting plants. Their progeny were analyzed and half-seeds planted again, to be selfed or sib crossed.

The fatty acid composition of buffalo gourd seed was found to be determined by the embryonic genotype. Linoleate and oleate content were negatively correlated, as has been previously shown in this and other species.

Heritability of oleate and linoleate content was determined by regression of progeny values on midparent values and found to be approximately 0.86 in the first year, while palmitate gave no significant regression. In the second year, palmitate exhibited a heritability of 0.39, but oleate and linoleate had heritabilities near 0.4. The notable decrease in their heritability was examined by multiple regression analysis of progeny values with environmental parameters and midparent values. A significant regression was obtained with day length for oleate and linoleate content; however, correlations also were found with high and low temperatures during the seed maturation period. The various environmental parameters tested were too strongly correlated to distinguish the causative factor with certainty.

CHAPTER ONE

INTRODUCTION

The buffalo gourd, Cucurbita foetidissima HBK 1817, is a xerophytic cucurbit native to the southwestern United States and northern Mexico. It is a low, spreading vine with gray-green, pubescent leaves, which vary from entire to deeply lobed, the latter form predominating in Mexico (Bailey, 1943). The species is drought-resistant and perennial by virtue of its large, fleshy storage root (Dittmer and Talley, 1964); during adverse weather conditions, such as drought or cold winter weather, the vines senesce, but the roots survive to produce new vines when conditions are favorable. It reproduces both asexually and sexually, the former by adventitious rooting at the nodes, and the latter by means of seeds produced in hard, fibrous gourds.

Although the species has been described as monoecious (Bailey, 1943), an antherless mutant was discovered by Curtis and Rebeiz (1974) which produces male sterile plants. Further investigation of this mutant by Dossey, Bemis, and Scheerens (1981) showed it to be conditioned by a single dominant allele, symbolized by G for gynoecy (Cucurbit Gene List Committee, 1982). This allele is widespread in natural populations, prompting the description of the species as "monogynodioecious" (Bemis et al., 1978). Gynoecious lines have been shown to outyield monoecious lines (Wilkins, 1980), and have been used in the production of line hybrids (Bemis et al., 1978; Bemis, Berry, and Weber, 1979a, b; Gathman

and Bemis, in press).

The buffalo gourd has been found in association with American Indian artifacts dated as early as 7,000 B.C. (Whitaker, Cutler, and MacNeish, 1957), and has been used by certain tribes for food, medicinal and ritual purposes (Gilmore, 1919; Niethammer, 1974). Until the Second World War, however, this species remained an uncultivated weed of restricted value. The outbreak of hostilities caused, among other things, an interruption of vegetable oil supplies which stimulated the consideration of other sources of oil. Curtis (1946) outlined the domestication potential of some xerophytic cucurbits, particularly C. foetidissima. He emphasized its value as a source of oil and protein, noting that "it seems ironic that the answer to some of the problems of diseased, undernourished people may be growing in wide areas around them, a neglected weed." Other early reports on this species continued to emphasize its value as an oilseed (Bolley, McCormack and Curtis, 1950; Shahani et al., 1951; Paur, 1952).

Only relatively recently has an interest developed in the buffalo gourd as a source of starch. Berry et al (1975) reported a starch content of 55% dry weight in C. Foetidissima roots. The excellent rheological properties of this starch (Dreher et al., 1983), combined with the high yields currently attainable (Nelson et al., 1983), make it likely to be the first salable product of the buffalo gourd. Commercial development of C. foetidissima as a starch source is now being conducted by the University of Arizona under contract to a private investment company.

The buffalo gourd still shows potential for use as an oilseed crop, notably in the high plains area of Texas, where a recent economic analysis suggests it could compete favorably with sunflower (Young, Morgan, and Shultz, 1982). In order to realize this potential, it will be necessary to breed for improved and more consistent oil yield and composition.

Yields of buffalo gourd seed to date have been highly variable. In a test at the Marana Agricultural Experiment Station, seven line hybrids and three open-pollinated selections were tested in replicated plots. The first year yields varied from 20 to 900 kg/ha. Normally second year yields are substantially higher; however, in this test residual herbicide damage adversely affected the second year results. The data obtained were too variable to be readily interpreted; although the best plot yielded the equivalent of over 3,000 kg/ha of seed, another replication of the same variety yielded less than 500 kg/ha. Projected yields of 2,000 kg/ha have yet to be attained on any sizable acreage (Gathman and Bemis, in press). Substantial plant breeding work will be required to obtain such yields on a regular basis.

Oil content and composition of buffalo gourd seed also display considerable variation. An analysis of seed from 85 accessions gave a crude fat content of 32.9 $\pm$ 4.7% (Scheerens et al., 1978). Fatty acid composition of this oil has been determined by a number of investigators. The predominant fatty acids are palmitate (6.1 - 24.4%), stearate (1.0 - 10.2%), oleate (10.0 - 36.2%), and linoleate (39.3 - 77.2%), while traces of myristate and linolenate also occur (Wood and Jones, 1943; Shahani

et al., 1951; Earle et al., 1959; Bemis et al., 1967; Scheerens et al., 1978; Vasconcellos et al., 1980; Khoury et al., 1982). Small amounts of conjugated dienoic (ca. 2%) and trienoic (ca. 0.1%) fatty acids have been found in oil from this plant, but are not sufficient to detract from its food value (Vasconcellos et al., 1980).

Of particular interest is the variability in linoleate content of this oil. Although causation is difficult to establish, a negative correlation has been established between dietary intake of polyunsaturated fatty acids and incidence of coronary heart disease (Stamler, 1979); for this and related reasons, high polyunsaturated fatty acid content is desirable in commercial vegetable oils in this country. On the other hand, oils with low polyunsaturated fatty acid content have better stability and keeping qualities than more unsaturated ones, and thus may be desirable for some applications (Robertson, Thomas, and Burdick, 1971). It is therefore apparent that a knowledge of the mode of inheritance of fatty acid composition of buffalo gourd oil would be valuable in the breeding of oilseed lines with divergent fatty acid compositions.

This study represents an effort to elucidate the inheritance of seed oil composition in the buffalo gourd, with special attention to oleate and linoleate content. The heritability of fatty acid composition was studied; selections were made for high and low linoleate content; and the influence of invironment on oil composition was investigated.

CHAPTER TWO

LITERATURE REVIEW

While little previous work has been done on the inheritance of fatty acid composition in the buffalo gourd, a sizable literature exists on this topic in other crops. As it appears likely that many oilseeds have biochemical and genetic similarities, a knowledge of the physiology of oil composition and its inheritance in commercial oil producing crops is necessary as a background to the present study.

Fatty Acid Biosynthesis and Desaturation

The understanding of the genetics of a biochemical system is dependent to some extent on understanding the physiology of the system. Fatty acid biosynthesis is a complex process, offering many possible points of genetic control.

It is well established that de novo fatty acid synthesis occurs in chloroplasts in photosynthetic tissue, and in plastids in other tissues such as developing seeds. Studies using leaf tissue (Wharfe and Harwood, 1978), developing seeds (Stumpf, 1975a), and isolated chloroplasts (Stumpf and James, 1963) have all shown 1-^{14}C acetate to be incorporated readily into palmitate, stearate, and oleate. The pathway of synthesis of these compounds begins with the formation of acetyl-CoA acetyl thiokinase. Malonyl-CoA is then formed from acetyl-CoA by carboxylation, and longer chain fatty-acyl CoA is formed through successive additions of acetate groups from acetyl-CoA by a soluble enzyme complex

known as fatty acid synthetase. The end product of de novo fatty acid synthesis is palmitate (16:0). Stearate (18:0) is then formed by elongation of palmitate, and desaturated to oleate (18:1) (Harwood, 1975; Stumpf et al., 1980).

The metabolic pathways involved in further desaturation of fatty acids are less clear. In the case of the step most pertinent to this study, the desaturation of oleate to linoleate, at least two controversies exist.

Oleate Desaturation: Substrate

First, the actual substrate for the reaction has been questioned. Originally, it was assumed to be oleyl-CoA, as exogenous 1-^{14}C oleyl-CoA is readily metabolized by microsomal preparations of developing safflower seeds (Vijay and Stumpf, 1971, 1972), "aged" potato tubers (Ben Abdelkader et al., 1973), and pea leaves (Dubacq, Mazliak, and Tremolieres, 1976), yielding labelled linoleate. In particular, Vijay and Stumpf (1971) found that, while most of the newly synthesized linoleate in such reactions was esterified to phosphatidylcholine (PC), their microsomal preparation would not desaturate exogenous 1-^{14}C oleyl-PC. Furthermore, they found significant amounts of radioactivity in linoleyl-CoA at the end of the reaction. Having found that sn-glycero- 3- phosphocholine (GPC) acted as a scavenger to remove residual oleyl-CoA, they added it to the reaction mixture to test the suitability of the remaining oleyl-PC as a substrate for desaturation. No desaturation occurred, and they concluded that the actual substrate for oleate desaturase is oleyl-CoA.

However, when the same system was examined by Stymne and Appelqvist (1978), the results were different. They observed that almost all of the exogenous labelled oleate was incorporated into oleyl-PC within the first 10 minutes, revealing the presence of a high level of oleate phoshatidylcholine acylase activity. Desaturation, however, continued throughout the first hour, suggesting that oleyl-PC rather than oleyl-CoA was the substrate for the desaturase. Experiments by Slack, Roughan, and Browse (1979) on the safflower microsomal desaturase system also indicated that oleyl-PC was the substrate. When they used 1-^{14}C oleyl-CoA as a substrate, no label was found in linoleyl-CoA, while over 60% of the exogenous oleate was converted to linoleyl-PC in one hour. Exogenous oleyl-PC still functioned poorly as a substrate, implying that endogenous PC may be more accessible to the desaturase. They suggested that the technique used in the experiments of Vijay and Stumpf (1971) to distinguish oxygen esters and thioesters may have overestimated the latter, causing erroneously high estimates of acyl-CoA amounts in the reaction products. They also found that GPC acted as an inhibitor of oleate desaturation regardless of substrate, rather than merely as an oleyl-CoA scavenger.

Experiments with Chlorella vulgaris (Gurr, Robinson, and James, 1969), Torulopsis utilis (Talamo, Chang, and Bloch, 1973), pea leaves (Slack, Roughan, and Terpstra, 1976), soybean, linseed, and safflower cotyledons (Slack, Roughan, and Balasingham, 1978), and Fusarium oxysporum (Wilson, Adams, and Miller, 1980) have all implicated oleyl-phospholiped as the substrate for oleate desaturation. It thus seems likely

that this is the main pathway for linoleate biosynthesis in most organisms. An important implication of this finding is that oleate is desaturated while bound in a membrane, as phospholipids are found almost exclusively in membranes in the cell (Lehninger, 1975; p. 287). This allows for regulation of oleate desaturase function by a myriad of factors affecting membrane fluidity and structure.

Oleate Desaturation: Site

The subcellular location of the oleate desaturase system has also been disputed. Numerous authors (see Oleate desaturation: substrate) have found oleate desaturase activity in "microsomal" fractions from plant tissues, typically 100,000 g centrifugation pellets from homogenized material, containing primarily fragments of endoplasmic reticulum (ER). Weaire and Kekwick (1975), however, found fatty acid synthetase activity in avocado mesocarp and cauliflower buds to be strongly dependent on the method of disintegration of the tissue. Material homogenized with an Atom-Mix laboratory blender showed fatty acid synthetase activity in a soluble fraction, with malonyl-CoA as the preferred substrate and stearate as the main product. When a vegetable grater was used instead to gently grind the tissues, the fatty acid synthetase was found in a 2,000 g particulate fraction composed largely of chloroplasts. This enzyme system used acetate as a substrate and produced a wide spectrum of fatty acids, in which oleate predominated. Significant amounts of linoleate and linolenate were also found, indicating the presence of desaturase activity. The authors suggested that vigorous grinding disrupted chloroplasts, releasing the soluble

components of the fatty acid synthetase system into the supernant while destroying the activity of the membrane-bound components such as stearate and oleate desaturases.

In a study using various centrifugation fractions of pea leaves, Dubacq et al. (1976) found only the microsomal fraction to be capable of desaturating exogenous oleyl-CoA. Plastids showed no desaturase activity, although the grinding process was described as "gentle." Roughan, Grattan, and McManus (1979) found isolated spinach chloroplasts to incorporate 1-^{14}C acetate into linoleate and linolenate only under conditions allowing active synthesis of diacylgalactosylglycerol (DGG). They concluded that acyl-DGG was the substrate for a relatively non-specific desaturase enzyme whose *in vivo* function was mainly linolenate production while leaving the site of *in vivo* linoleate synthesis unclear.

In a more recent paper, Tremolieres et al. (1980) again found the oleate desaturase activity in pea leaves to be primarily associated with the microsomal fraction. Low levels of activity were associated with a plastid fraction, but were always found in conjunction with antimycin-insensitive NADH-cytochrome c reductase activity, which is a marker for ER. Interestingly, this ER contamination persisted despite sucrose density gradient purification of the chloroplast fraction, a fact interpreted by the authors as an indication of a membrane fusion between the ER and the outer chloroplast membrane. Such an interpretation agrees well with the data of Weaire and Kekwick (1975), as gently ground tissue would yield chloroplasts with more of this ER attached. Roughan and Slack (1980) have presented a model of plant lipid metabolism

incorporating the roles of enzymes in the chloroplast stroma, lamellae, and envelope, and in the ER, into an interdependent system. If oleate is desaturated exclusively in the ER, the process would be expected to be wholly under the control of the nuclear genotype, as opposed to that of the plastid; while this is the case in many plants, rape and soybean are prominent exceptions (see Fatty acid inheritance in commercial crops) possibly implying a more complex interaction of plastid and nuclear genomes.

Fatty Acid Inheritance in Commercial Crops

A number of studies have been made of the inheritance of oil composition in commercial oil-producing crops, revealing varying modes of inheritance in different species.

Safflower

One of the earliest studies of inheritance of oil quality was performed on safflower (Carthamus tinctorius L.) (Knowles and Mutwakil, 1963). The study made use of iodine number of oil samples, a measure of unsaturation level. Most safflower varieties at the time had a relatively constant oil composition, with 76% linoleate, 16% oleate, 1% stearate, and 7% palmitate, giving an iodine value of 138-145. Two introductions from India had been found to have iodine values of 85 - 95, with the percentages of oleate and linoleate approximately reversed. The lower iodine value was shown to be determined by a single partially cominant gene, symbolized Ol. The heterozygotes had iodine values of 111 - 130.

An Iranian safflower introduction with an intermediate iodine value (121) was later studied in crosses with commercial and low iodine value Indian lines (Knowles and Hill, 1964). The intermediate value was found to be the result of a third allele at the Ol locus, named ol^1. Lines homozygous for this allele have approximately equal amounts of oleate and linoleate. Another locus in safflower regulates the amount of stearic acid in the oil. Homozygous st/st lines contain 5 - 10% stearic acid, as opposed to 1% in St/St lines.

Corn (Maize)

The fatty acid composition of corn (Zea mays L.) oil is also under genetic control. One of the first studies of inheritance of fatty acid composition to rely solely on gas chromatographic analysis of seed was performed on lines of corn with high and low linoleate content (Poneleit and Alexander, 1965). The 12.5% difference in linoleate content between the lines Illinois High Oil (IHO) and R84 was found to be determined by a single gene, with high linoleate content recessive to low. The gene was subsequently symbolized ln (de la Roche, Alexander, and Weber, 1971).

The above authors also noted a strong negative correlation (-.97) between oleate and linoleate content of the oil, a result which is not surprising if the two fatty acids are viewed as successive stages in a pathway. Similar correlations for oleate and linoleate have been noted in soy (Smith, 1981), rape (Kondra and Thomas, 1975; Kondra and Wilson, 1976), sunflower (Putt, Craig, and Carson, 1969), peanut (Brown et al., 1975), and buffalo gourd (Scheerens et al., 1978) oils.

In a later study (de la Roche et al., 1971), crosses were made between IHO, R84, and a third line, C103, which has a low (43%) linoleate content. The monogenic difference between IHO and R84 was confirmed, and C103 was also found to differ by a single gene from R84. The lesser (10.8%) difference in linoleate content between C103 and IHO, however, showed more complex inheritance, with no discrete classes and transgressive segregates in the F_2 and F_3 generations. The authors concluded that at least two genes were involved in the inheritance of this fatty acid difference.

The existence of at least two loci affecting oleate and linoleate content of corn oil has been confirmed cytogenetically. Monosomic (Plewa and Weber, 1976) and translocational (Shadley and Weber, 1979) analyses have located such loci on chromosomes 2 and 5, respectively, of the corn genome. Tests of isogenic lines with different cytoplasms have ruled out any large effect of cytoplasmic genotype on the fatty acid composition of corn (Gregory and Grogan, 1976).

Diallel analyses (Poneleit and Bauman, 1970; Widstrom and Jellum, 1975) have shown oleate, linoleate, and palmitate in corn to be subject mainly to additive gene effects, while both additive and dominance effects are important in the inheritance of stearate content. Significant but small environment and reciprocal effects were observed in the inheritance of fatty acid composition. As implied by these observations, oleate and linoleate levels in corn are highly heritable (Weber, 1978).

Rape

The inheritance of fatty acid composition of rapeseed (Brassica napus L.) has been examined, particularly for erucic and eicosenoic acid content, as these unusual fatty acids limit the use of oils containing them. Both fatty acids are controlled by the same two loci, acting in the embryo (Harvey and Downey, 1964; Kondra and Stefansson, 1965). Of greater importance to this study is the mode of inheritance of the common fatty acids in rape, in particular oleate and linoleate. It has been estimated that from 2 to 6 genes control the amount of these fatty acids in various lines (Kondra and Thomas, 1975; Kondra and Wilson, 1976.) Evidence from reciprocal crosses indicate that content of these compounds is primarily controlled by the maternal genotype in rape (Thomas and Kondra, 1973).

The heritability of oleate and linoleate content in rape varies widely depending on the lines involved; in most cases it is from .6 to .8, but in one cross the heritability of oleate content was as low as .06. (Kondra and Thomas, 1975; Kondra and Wilson, 1976; Kondra and Thomas, 1978). It was predicted that the desirable high linoleate character could be transferred to commercial low-erucic-acid lines.

Soybean

Most of the work on the inheritance of fatty acid composition of soybean (Glycine max (L.) Merrill) seeds has focused on linolenic acid. The high level of this trienoic acid in soybean oil (5 - 9%) is generally thought to contribute to the poor flavor stability of the oil,

and the progress of plant breeding efforts to reduce it has been very slow (Smith, 1981).

An early survey of linolenate and linoleate content of oil from 251 soybean accessions (White, Quackenbush, and Probst, 1961) revealed the paucity of variation of linolenate percentage that has hampered breeding efforts to date. Linoleate content, however, varied from 35.8 to 53.4%. Crosses revealed no clear mode of inheritance for either fatty acid, and it was suggested that both were under polygenic control. By the use of mutation breeding, crossing low-linolenate lines, and selection for high oleate content, the level of linolenate has been gradually reduced to a low of 4.2% (Howell, Brim, and Rinne, 1972; Hammond and Fehr, 1975; Wilson, Rinne, and Brim, 1976; Wilson, Burton, and Brim, 1981).

The fatty acid composition of soybean oil is primarily determined by maternal genotype, although linolenate content is somewhat influenced by pollen parent genotype. The content of oleate and linoleate is independent of pollen parent (Brim, Schultz, and Collins, 1968).

Sunflower

Investigations of the inheritance of sunflower (Helianthus annus L.) oil composition have been hampered by the fact that it is strongly influenced by temperature during seed maturation (see Environmental effects on lipid composition: Sunflower). By comparing different lines with similar flowering times in similar environments, however, Putt et al., (1969) were able to identify genetic effects on oil composition of

this crop. The heritability of sunflower seed fatty acid composition was later determined under controlled environmental conditions to be "moderately high," and it was predicted that plant breeders would be able to modify the oil quality of this species (Kinman, 1972).

Other Crops

Existing genetic variation in flax (Linum usitatissimum L.) fatty acid composition was investigated recently (Green and Marshall, 1981), in an effort to determine if its linolenate content could be reduced to a low level by plant breeding. While the variation in this fatty acid was found insufficient for production of edible linseed oil lines, considerable variation was found in overall fatty acid composition. Within variety parent-offspring correlation coefficients revealed significant heritable variation for each of the fatty acids examined, including palmitate, stearate, oleate, linoleate, and linolenate. As an earlier study has shown fatty acid composition of flax to be controlled by embryo genotype (Yermanos and Knowles, 1962), selections could be made on single F_2 seed.

Fatty acid composition of peanut (Arachis hypogea L.) oil has been shown to be significantly affected by genotype, year, and location (Worthington, Hammons, and Allison, 1972; Young et al., 1974; Brown et al., 1975). F_2 segregation studies performed by Tai and Young (1975) showed normal distributions of each constituent fatty acid, a result which was interpreted to mean that fatty acid inheritance in peanuts is polygenic. The seed oil of the pumpkin (Cucurbita pepo L.) has been

analyzed for fatty acid composition (Power and Salway, 1910; Riebsomer and Nesty, 1934; Markovic and Bastic, 1976) and found to contain palmitate (6 - 13%), stearate (4 - 7%), oleate (24 - 37%), and linoleate (45-57%). No information is available on the inheritance of fatty acids in this plant.

Environmental Effects on Lipid Composition

The fatty acid composition of storage and membrane lipids in many organisms is affected by the environment. While photoperiod and humidity have been implicated in some cases, by far the most important influence is that of temperature. Most germane to this study is the effect of environment on the composition of seed storage lipids in oil-producing crops.

Sunflower

The first oilseed crop in which temperature was discovered to have a major effect on oil composition was sunflower. Barker and Hilditch (1950a, b) studied oil from varieties of sunflower grown in different parts of Africa and in England and found that all varieties tended to have similar oil composition in the same location, while a given variety had very different oil composition when grown in different locations. In all cases, lower linoleate content (and lower total unsaturation) was associated with higher temperature. They concluded that oil composition in the seed was determined primarily by temperature, while admitting the possible influence of incident light and rate of seed development. Studies of sunflowers grown at different locations and

with different planting dates have confirmed the inverse correlation of temperature with linoleate content, and its direct correlation with oleate content (Robertson, Thomas, and Burdick, 1971; Johnson and Jellum, 1972; Keefer et al., 1976).

In an attempt to exclude the possible influence of photoperiod, Grindley (1952) grew sunflowers in the summer and winter in tropical Khartoum and analyzed their seed oil. The summer crop averaged 33% linoleate, while the winter-grown seed averaged 55% linoleate. While the difference in average daily photoperiod between the two seasons was less than two hours, the average daily minimum temperature was over 10 degrees centigrade lower in the winter. It was concluded that temperature was the main cause of differences in seed oil composition. This conclusion was supported by the more rigidly controlled experiments of Canvin (1965), who grew sunflower and other oilseeds in growth chambers with constant photoperiod and light intensity. Analysis of sunflower seeds which matured at four controlled temperatures (10, 16, 21, and 26.5 C.) showed linoleate percentage to be inversely correlated with temperature, ranging from ca. 75% at the lowest to ca. 30% at the highest temperature. A recent growth chamber study by Tremolieres, Dubacq, and Drapier (1982) showed light intensity to have a strong influence on sunflower seed oil composition, with high linoleate content favored by low light intensity at the flower head. Although linoleate content at the lowest light intensity studied was reduced from 55% to 35% of total fatty acids, significant effects were observed only when light was reduced to 10% of full sunlight intensity or less.

Rape

The growth chamber experiments of Canvin (1965) also revealed an increase in unsaturated fatty acids of rape (Brassica napus) at lower temperatures, including erucic acid as well as linoleate and linolenate. Similar, although lesser, effects were noted in field experiments with rape, turnip rape (B. campestris L.), and white mustard (Sinapsis alba L.), grown in different locations and in different seasons (Craig, 1961; Appelqvist, 1968), although humidity, photoperiod, and light intensity effects were not controlled in these studies.

In a growth chamber study with controlled light intensity, photoperiod, and humidity, opposite results occurred (Appelqvist, 1971). Total unsaturation of rapeseed, mainly due to linoleate content, was found to increase with increasing temperature under these conditions, suggesting that the field effects were due to lower humidity in the hotter climatic conditions, rather than temperature per se. The author concluded that longer maturation time under higher humidity favored the later-developing (i.e., unsaturated) fatty acids. Tremolieres et al. (1982) observed increases in oleate and linoleate, and a decrease in linolenate, with increasing temperature during maturation of rape seeds in growth chambers. Changes in light intensity caused no alteration of fatty acid composition of the seed oil. Humidity was not controlled in this experiment, and thus cannot be ruled out as a factor in the results.

Other Oilseeds

The oil of flax (Linum usitatissimum) grown in North Dakota in two successive years showed a great difference in unsaturation (Painter, Nesbitt, and Stoa, 1944). In the year with higher average temperature during the seed maturation period, the average iodine value of the oil produced was 162, while in the cooler year it was 187. In growth chamber experiments, higher temperature and shorter photoperiod caused decreased linolenate content in linseed oil (Sosulski and Gore, 1964; Canvin, 1965; Yermanos and Goodin, 1965; Dybing and Zimmerman, 1966).

Nine corn hybrids showed no change in oil fatty acid composition in response to different planting dates (Jellum and Marion, 1966), but in controlled "phytotron" environments, two of three inbreds and one open-pollinated variety showed decreased linoleate content at higher temperatures (Thompson, Jellum, and Young, 1973). Soybeans grown in controlled environments in various locations showed decreased linoleate and linoleate content with higher temperature, while photoperiod and light intensity had no effect on oil composition (Howell and Collins, 1957). Interestingly, a growth chamber study of "high linoleic acid" sesame (Sesamum indicum L.) seed showed an increase in unsaturation (due to higher linoleate content) under higher temperatures (Brar, 1980).

Other Tissues and Organisms

Temperature effects on lipid fatty acid composition have been observed in most higher plant tissues. In general, as with most oilseeds, temperature and lipid unsaturation show an inverse relationship.

In leaves this results in higher linolenate content at lower temperatures; this is the case in leaves of wheat (de la Roche et al., 1972), alfalfa (Grenier et al., 1972), rape (Smolenska and Kuiper, 1977), Vicia faba (Lem et al., 1980), maize (Diepenbrock and Stamp, 1982), and cucumber (Horvath et al., 1983). A similar relationship holds for the roots of cotton (St. John and Christiansen, 1976), wheat (Willemot, 1977), soybean (Rivera and Penner, 1978), and rye (Clarkson, Hall, and Roberts, 1980). In general, tissue cultures of higher plants also show increased desaturation at lower temperatures (Radwan and Mangold, 1976).

Unicellular organisms follow a similar pattern except at very low temperatures, with an inverse relationship between temperature and unsaturation. Examples include yeast (Kates and Baxter, 1962), algae (Patterson, 1970), bacteria (Fulco, 1970; Cronan and Vagelos, 1972), and protozoa (Skriver and Thompson, 1979). In the animal kingdom, fish show increased lipid unsaturation when acclimated to lower temperatures (Cossins and Prosser, 1978).

In general, most organisms exhibit an increase in lipid desaturation with decreasing temperature. In certain oilseeds, particularly those which are exposed directly to the environment, low humidity may cause hastened maturation with a concomitant decrease in unsaturation. Light intensity and photoperiod may affect oil unsaturation, but they are generally compounded with other factors. In the case of sunflower, light intensity has an effect on unsaturation, but is important only at levels not often found in the field.

Mechanism and Consequences of Temperature Effects on Lipid Composition

Mechanism

The phenomenon of increased desaturation in tissues at reduced temperatures goes counter to expectation in a sense; enzymes such as oleate or linoleate desaturase should be more active at higher temperatures, so that desaturation should increase with increasing temperature. In fact, this was observed to occur in cell-free extracts of the yeast Torulopsis utilis (Meyer and Bloch, 1963). Although extracts from cells grown at lower temperatures did show increased in vitro desaturase activity, a given extract displayed reduced activity at lower assay temperatures, suggesting that in this organism, low temperature causes increased synthesis of desaturase.

On the other hand, the fatty acid composition of the protozoan Tetrahymena pyriformis has been found to respond to chilling despite cycloheximide treatment, suggesting control after the stage of desaturase synthesis (Skriver and Thompson, 1979). Such conflicting results illustrate the need to consider different organisms separately.

A study with detached developing soybean and flax cotyledons (Slack and Roughan, 1978) showed greatly increased unsaturation within six hours of transfer from 22.5 to 13 degrees.

Furthermore, unsaturation showed a significant decrease in the same time after transfer from 22.5 to 32 degrees. Unless the turnover rates of both fatty acids and desaturase are unusually fast, decreased

synthesis of the enzyme could not cause such a rapid decrease in unsaturated fatty acid content.

The most likely mechanism for this phenomenon in oilseeds is the one proposed by Harris and James (1969a) after studies on cut seeds of flax, sunflower, and castor bean (Ricinus communis). They found that decreasing temperature caused an increase in unsaturated fatty acids in the sunflower and castor bean seeds, though not in the flax seeds. Oxygen is required for the desaturation reaction (cf. Harwood, 1975) and the concentration of oxygen in solution in equilibrium with air decreases with increasing temperature. Thus, when the sunflower or castor bean seeds were incubated under elevated oxygen pressures, their desaturation was found to increase with increasing temperature. Chloroplasts were found to be present in the developing flax seeds used, providing an endogenous oxygen source. Similar results were obtained on experiments on daffodil bulbs, leaf tissue, and the alga Chlorella sorokiniana (Harris and James, 1969b); the photosynthetic tissues showed a temperature response in fatty acid composition only when grown in the dark. A more recent experiment with cultured sycamore (Acer pseudoplatanus) cells revealed a similar dependence of desaturation on oxygen tension (Rebeille, Bligny, and Douce, 1980). It seems probable that this is the major mechanism for increased desaturation under low temperature in higher plants.

Consequences

That increased desaturation at low temperatures confers benefits on plants seems clear. Cold hardiness or frost hardening of wheat seedlings (de la Roche et al., 1972) and spruce chloroplasts (Senser and Beck, 1982) is associated with increased desaturation. It has been suggested that cold hardiness is a result of increased membrane flexibility off-setting the effect of low temperature. In one test of this theory, Lyons, Wheaton, and Pratt (1964) tested the mitochondria from a number of chilling-resistant and chilling-sensitive plant species for their ability to swell in hypotonic solutions. The chilling-resistant species had more flexible mitochondria, and more unsaturated fatty acids. In another experiment, cells of Rauwolfia serpentina cultured at 10 degrees C. produced protoplasts which fused more readily than those from cells cultured at 25 degrees, again demonstrating greater flexibility (Yamada et al., 1980). Furthermore, tests have shown that mixtures of fatty acids in proportions commonly found in plant lipids show a distinct lowering of freezing point as unsaturation increases (Lyons and Asmundson, 1965).

Increased unsaturation of membrane lipids at low temperatures thus causes increased membrane fluidity, which is apparently related to enhanced functioning of membrane-bound enzymes (Quinn and Williams, 1978). The value to the plant of increased unsaturation of storage lipids in oilseeds, if any, is unclear. If this is merely a pheiotropic effect of the oxygen dependence of desaturases, no such value need be sought; however, it is possible that fluidity of storage lipids is a factor in their mobilization during seed germination.

CHAPTER THREE

MATERIALS AND METHODS

Crossing Scheme

Seeds were produced by controlled crosses on buffalo gourd plants at the University of Arizona Tucson Experiment Station. In summer 1979, crosses were performed on selected plants whose siblings had been determined to have notably high or low linoleate content. High x high and low x low crosses were made between several stocks. One-gram aliquots (approximately 25 seeds) of seed from these crosses were analyzed for fatty acid composition. Seeds from crosses showing extremely high or low linoleate content were then analyzed individually by a non-destructive half-seed method (see sample preparation). Seeds extremely high or low in linoleate content were then planted in pots in the greenhouse and subsequently transplanted to the field in June, 1980. Few flowers were obtained in the first season; crosses were not obtained until the following year.

In the summer of 1981, controlled high linoleate x high linoleate, low linoleate x low linoleate, high linoleate x low linoleate, and low linoleate x high linoleate crosses were made between the 1979 progeny plants. Ten seeds from each of 30 crosses were analyzed individually for fatty acid composition; the plants produced from these seeds were grown in the greenhouse and then transplanted to the field in April, May, and June, 1982. In this season, all plants were self-pollinated or

crossed to siblings. Ten individual seeds from each of 28 crosses were analyzed. To test the influence of environmental conditions, a number of crosses were repeated on dates at least ten days apart. Analyses were performed on duplicate 25 to 50 seed bulk samples from each date for 17 of these crosses.

Seed Harvesting

Fruit were allowed to ripen in the field for a minimum of 40 days after pollination, then hand-picked. Seeds were extracted by a modification of the method of Scheerens et al. (1978): Fruit were cleaved and submerged individually in beakers of water until the placental tissue was disintegrated by fermentation. The seeds from each gourd were washed, air-dried, and stored at 25 degrees until used in analyses, a period of one to four months.

Sample Preparation

Bulk seed aliquots were ground to 10 mesh in a laboratory Wiley mill and extracted with hexane. Individual seeds were prepared for analysis by a half-seed method similar to that previously described (Gathman and Bemis, 1981): Seeds were soaked in water for 4 - 12 hours. The seed coat was dissected away, the nucellar membrane was removed, and the embryo was cut in half across the cotyledons. The distal halves of the cotyledons were placed in a 1 ml microflex tube (Kontes K-749000-0001) and ground with a nylon pestle. The proximal half of the embryo, containing the undamaged shoot and root tips, was supported on glass wool in a plastic vial containing an inorganic embryo culture solution (Randolph

and Cox, 1943) and cultured in an incubator at 30 degrees. After one week the half-seedling was transplanted into soil in a 4-inch pot and placed in the greenhouse. Transplants were moved to the field in late spring.

Esterification of Fatty Acids

Bulk seed hexane extracts were dried under a stream of nitrogen, then redissolved in a mixture of 4.5 ml of methanol/sulfuric acid (0.5% sulfuric acid by volume) and 2.5 ml of toluene. This mixture was refluxed at 100 degrees for 3 hours in a sealed 10 x 100 mm screw-cap tube.

Ground half-seeds were suspended in 0.45 ml of methanol/sulfuric acid and 0.25 ml of toluene, then refluxed at 100 degrees for 3 hours in sealed 1 ml microflex tubes. In both bulk and single seed analyses, water and hexane were added to the resulting mixture to form a bilayer. The layers were separated by centrifugation at 1,000 RPM for 10 minutes, and the upper layer, containing esterified fatty acids, was removed by pipetting, dried under a stream of nitrogen, and redissolved in hexane.

Chromatography

Fatty acid methyl esters dissolved in hexane were analyzed with a Perkin-Elmer model 880 gas chromatograph with a 1.8m by 3.175 mm stainless steel column, packed with acid-washed Chromosorb W. The liquid phase was either 10% Silar 7CP or 10% Silar 10C, in columns prepared by Applied Science Laboratories. The injector temperature was 250 degrees; the carrier gas was nitrogen at a flow rate of 50 ml/min; and the column temperature was 200 degrees. The chromatograph used was equipped with a

flame ionization detector. Elution times of fatty acid methyl esters were compared with those of standards obtained from Sigma Chemical Company.

Proportions of fatty acid methyl esters were determined by measurement of peak areas, either with a mechanical disk integrator or by triangulation. Standard mixtures of methyl oleate and methyl linoleate were prepared gravimetrically to test the resolution of these compounds. All fatty acid data are expressed as percentage of total fatty acids.

Determination of Crude Fat Content

One-gram aliquots of seed were ground to 10 mesh, weighed, and refluxed in hexane for six hours in a Goldfisch apparatus. The hexane was driven off by heating and the remaining oil determined gravimetrically. Crude fat contents are expressed as percent of total seed weight.

Environmental Data

High and low temperature and rainfall data were obtained from the official records of the Tucson Experiment Station. Day lengths were approximated by the period from sunrise to sunset, which was calculated by the formula

$$\cos(H) = -\tan(L)\tan(D)$$

where H is the half-day length in hours, L is the latitude (0.562 radians for Tucson), and D is the solar declination (Sellers, 1965; eq. 3-3). The solar declination was approximated by the formula

$$D = 23.45 \left(\cos\ 0.986\ (N + 250.5)\right)$$

where N is the day number, given that March 1 is day 1 (Sellers, personal communication.

CHAPTER FOUR

RESULTS AND DISCUSSION

Over 900 single seed and bulk analyses were conducted on seeds from the three years in which crosses were performed. This large data base provides a number of insights into the inheritance of fatty acid composition of buffalo gourd seed oil.

Precision and Accuracy of Measurements

All analyses were performed in triplicate in order to minimize the inherent imprecision of measuring peak areas, whether by triangulation or with a mechanical integrator. These triplicate analyses were averaged to give the percentage composition of the samples used. In cases where they varied excessively, samples were reanalyzed or deleted from the study. By examining the standard deviation of the triplicate analyses, an estimate of the precision of fatty acid measurements may be obtained.

Two crosses, designated 401 and 402, from the 1981 progeny were chosen as a representative sample of the precision of fatty acid measurement in this study. The coefficient of variation for each fatty acid was determined for each of these seeds. These coefficients were then averaged for each of the two crosses, giving two estimates of the precision of measurement of each fatty acid. In Table 1 these average coefficients of variation are compared with the coefficient of variation of each fatty acid throughout the 1981 population. It can be seen that the within-seed

Table 1. Precision of measurements: comparison of coefficients of variation between analyses and throughout population.

	MEAN %			COEFFICIENT OF VARIATION		
FATTY ACID	401	402	POPULATION	401	402	POPULATION
Linoleate	54.3	53.3	52.2	1.66	1.57	17.4
Oleate	35.4	33.8	35.7	2.26	1.98	27.6
Palmitate	7.8	9.6	8.8	9.55	5.61	18.9
Stearate	2.4	3.2	3.4	27.76	12.34	40.4

401 and 402 are 10-seed progenies chosen to represent the 1981 population. The coefficients of variation given for these progenies are averages of the coefficients for each of the 10 seed. The coefficients given for the entire population reflect both within-seed and between-seed variation.

coefficient of variation for oleate and linoleate is small compared to the variation of the population, indicating good precision.

The coefficients of variation within seeds for palmitate are higher; while the actual standard deviation of triplicate analyses was about the same for all fatty acids, the lower mean percentage of palmitate in the oil accentuates the effect of its standard deviation. Stearate, the smallest measured component, suffers still more from this effect; its coefficients of variation are so high that little significance can be attached to the determinations of stearate content made in this study. It was difficult in any case to measure the area of the stearate peak in chromatograms, as it always overlapped the oleate peak. Due to the imprecision of determination of stearate, as well as its status as a minor component of buffalo gourd oil, it will generally be omitted from further statistical consideration here.

In order to perform the large number of analyses needed for this study, it was necessary to shorten the time of analysis to a minimum. This was achieved by raising the temperature of the column and the flow rate of nitrogen above commonly used levels. While the goal of speed was achieved, the separation of oleate and linoleate was adversely affected. The two peaks were clearly resolved for most of their area, but showed some overlap at their bases (Figure 1). In order to determine the effect of this overlap on the accuracy of measurement of these compounds, standard mixtures of the pure fatty acid methyl esters were prepared gravimetrically. The results of analysis of these standards are shown in Table 2.

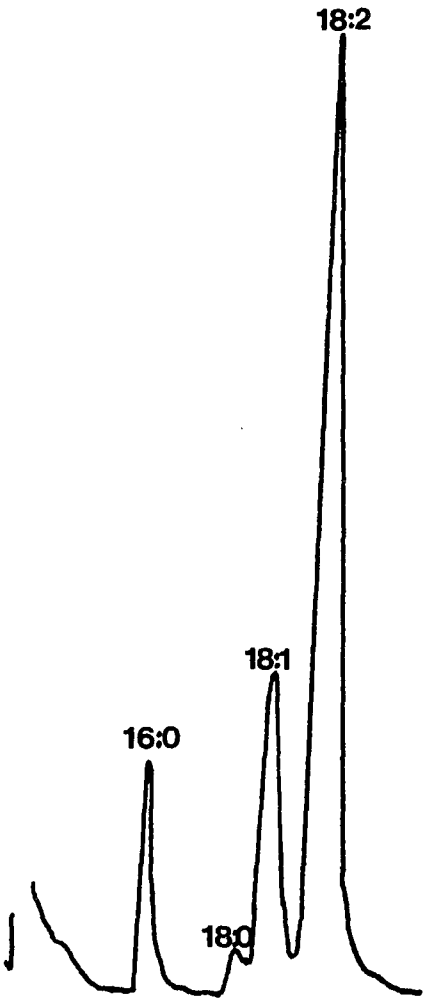

Figure 1. Typical Chromatogram of Fatty Acid Methyl Esters from Buffalo Gourd Seed Oil.

16:0-Palmitate; 18:0-Stearate; 18:1-Oleate; 18:2-Linoleate.

Table 2. Standard mixtures: comparison of observed data and data corrected for peak overlap.

	UNCORRECTED %				CORRECTED %			
	Linoleate		Oleate		Linoleate		Oleate	
COLUMN:	1	2	1	2	1	2	1	2
Linoleate: Oleate Standard % %								
90:10	86.2	87.3	13.8	12.7	89.2	90.3	10.8	9.7
80:20	74.3	74.8	25.7	25.2	77.3	77.8	22.7	22.2
70:30	67.5	68.4	32.5	31.6	70.5	71.4	29.5	28.6
60:40	57.3	58.4	42.7	41.6	60.2	61.4	39.8	38.6
50:50	50.1	49.7	49.9	50.3	51.9	47.0	48.1	53.0
40:60	41.3	40.9	58.7	59.1	38.3	38.0	61.7	62.0
30:70	35.6	35.1	64.4	64.9	32.6	32.2	67.4	67.8
20:80	25.8	25.0	74.1	75.0	22.8	22.0	77.1	78.0
10:90	15.0	16.2	85.0	83.8	12.0	13.2	88.0	86.8

SIGNIFICANCE OF F BETWEEN COLUMNS:

Uncorrected	Corrected
0.110	0.458

Column 1: Silar 10C

Column 2: Silar 7CP

Values are averages of triplicate analyses.

It was found that the overlap of the two peaks produced the most pronounced effects when they were most unequal in size. The 10% linoleate: 90% oleate mixture notably gave inaccurate results on both column types used, with linoleate overestimated by as much as 60% of the amount actually present. Large peaks were consistently underestimated and small peaks overestimated.

The following formula was devised to correct for the deviations:

$$C = \left[\left(\frac{y\ |O-H|}{H}\right)^{\frac{1}{x}}\right] (0.1\ H)$$

H is half the sum of the observed values for oleate and linoleate percentage. C is the correction factor, which is subtracted from the observed value O when D<H and added when D>H. The constants x and y were added to allow for adjustment of the correction factor, and were empirically found to give good results when $x = 33.3$ and $y = 0.0000001$.

The same correction was used for both oleate and linoleate, so that the total of the two fatty acids remained unchanged. As can be seen from Table 2, this correction brought all standard analyses to no further than 3.2% from the actual values. An analysis of variance performed on the two columns used showed them not significantly different; the F values for this analysis are given in the same table.

All statistical analyses were performed both with and without correction; while values obtained differed somewhat, the same variables were significant with either method. Therefore, the statistical tests reported here are all based on corrected percentages of oleate and linoleate.

Table 3. Correlation coefficients of fatty acids.

	LINOLEATE	OLEATE	PALMITATE
1981:			
Oleate	-0.9815 (.001)		
Palmitate	0.3751 (.021)	-0.5190 (.002)	
Stearate	0.1475 (.218)	-0.2823 (.065)	0.2927 (.058)
1982:			
Oleate	-0.9785 (.001)		
Palmitate	0.1122 (.205)	-0.2906 (.015)	
Stearate	-0.2909 (.015)	0.1557 (.126)	0.1821 (.090)

Each coefficient is followed by its significance in parenthesis.

General Observations on the Data

The high variability of fatty acid content in buffalo gourd seed oil noted previously (see Chapter One) was evident in the stocks used in this study. Linoleate content ranged from 25 to 85 percent; oleate, 4 to 65%; palmitate, from 6 to 13%; and stearate ranged from 1 to 6%. It is noteworthy that the two fatty acids used as criteria for selection had a wider range of variation than has previously been found in this species.

Correlation coefficients for the fatty acids measured are given in Table 3. As has been previously reported for this and other plants (see Chapter Two: Fatty acid inheritance in commercial crops: Corn), oleate and linoleate were found to be highly negatively correlated. For 921 single seed analyses from 1979, 1981, and 1982, the correlation coefficient was -0.977. This is further evidence that oleate and linoleate represent successive steps in a pathway in the buffalo gourd as in other higher plants (Stumpf et al., 1980).

A small but significant negative correlation has been observed between linoleate content and total crude fat content of corn (Poneleit and Bauman, 1970). Such a correlation, if large enough, could represent an obstacle in breeding simultaneously for high yield and high unsaturation in a seed oil. In order to determine if this problem exists in the buffalo gourd, 58 bulk seed samples were analyzed for crude fat content as well as oil composition. The results of these analyses (Figure 2) show that no significant correlation exists between linoleate content and total crude fat content in the stocks tested.

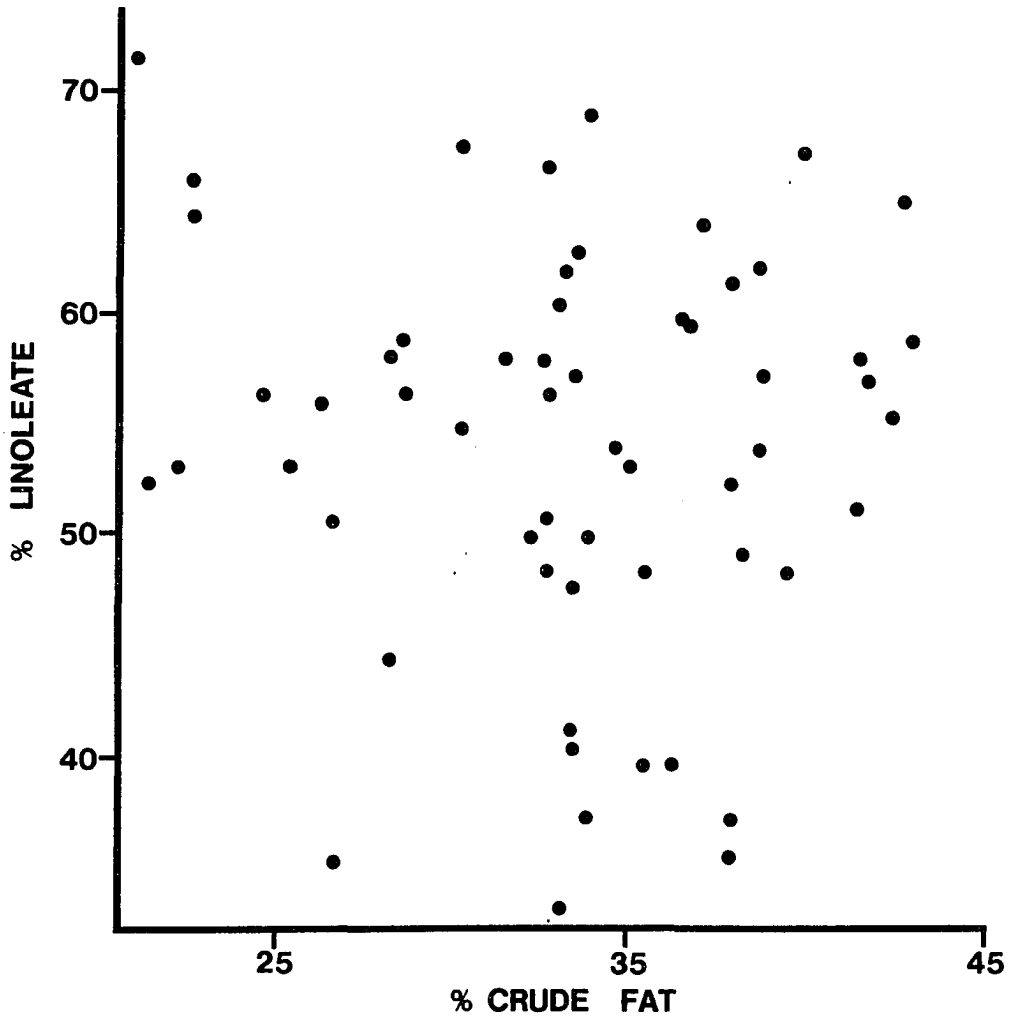

Figure 2. Linoleate Content Versus Crude Fat Content of Buffalo Gourd Seeds

$r = -0.08$

In rapeseed and soybeans, the fatty acid composition of the seed oil is largely determined by the genotype of the maternal parent, while in other crops such as corn and safflower the embryo genotype determines oil composition (see Chapter Two: Fatty acid inheritance in oilseed crops). The latter situation allows the breeder to make selections on single seed of a segregating generation, greatly reducing the land required for a breeding program. An analysis of variance of fatty acid composition of single buffalo gourd seeds of the 1981 progeny (Table 4) shows that maternal and paternal parents are equally significant in influencing this character; it may thus be concluded that neither maternal effect nor cytoplasmic inheritance has an important effect on oil composition in this species. The phenotype of a buffalo gourd plant for oil composition can therefore be defined as the oil composition of the seed which produced it.

One of the original objectives of this research was to determine the number of loci involved in the control of oleate desaturation in this species. Unfortunately, due to the very different environmental variance of the seasons in which the F_1 and F_2 seeds developed (see Environmental Effects), the method of Wright (Castle, 1921) could not be used. In order to determine if discrete F_2 classes occurred, 100 single seed from an F_2 cross were analyzed. The resulting data approximated a normal distribution with no discrete classes apparent, indicating that oleate desaturation in the buffalo gourd is under polygenic control.

Table 4. Analysis of variance of fatty acid content: progeny by parents

	MATERNAL PARENT		PATERNAL PARENT	
FATTY ACID	F	SIG.	F	SIG.
LINOLEATE	44.3	.001	68.4	.001
OLEATE	45.9	.001	77.8	.001
PALMITATE	34.1	.001	21.1	.001
STEARATE	8.2	.001	17.0	.001

SIG.: Significance of F for the variable indicated.

Heritability of Oil Composition

An important step in a breeding program is the determination of the heritability of the characteristics to be altered. This determination was possible in 1981, as progeny were obtained from parents of known oil composition as seeds, and thus of known phenotype.

Heritability of fatty acid composition of buffalo gourd seed oil was determined by the method of Falconer (1981, p. 151). The average percentage of each fatty acid in each 10-seed progeny was used as the dependent variable in a regression analysis, with the midparent value (the average of the values of the two parents) as the independent variable. The slope of the regression line (B) in such an analysis is an estimate of narrow sense heritability. The results of these regressions are given in Tables 5, 6, and 7; linoleate and oleate had high, and approximately equal, heritabilities. It should be noted that the values in Tables 5 and 6 differ slightly from those previously published (Gathman and Bemis, 1983); this is due to the application of the correction factor (see Precision and Accuracy of Measurements). The regression for palmitate was not significant.

It was observed that the variance of progeny families differs greatly between families; for linoleate percentage, the within-family variance ranged from 8.4 to 160.1. In order to determine if this difference in variance would affect the estimation of heritability, all regressions were repeated using single seed data. As can be seen from Tables 5, 6, and 7, the resulting heritability estimates do not differ significantly from those based on progeny averages. The single seed regression

for palmitate did give a significant heritability; however, the explained variability (r^2) for this regression is so small that little importance can be attached to this estimate on the basis of this year's data alone.

Simple regressions of progeny averages on midparent fatty acid percentage were again performed for the 1982 crosses (Table 8). The heritability of palmitate is significant for these data, and somewhat higher than in the previous year, although within one standard error of the former value. This confirming result implies that palmitate content does in fact have a heritability in the vicinity of 0.4, and could conceivably be altered by a breeding program if desired.

Linoleate and oleate, however, show markedly lower heritabilities than before. The crosses performed in 1982 were exclusively selfs and sib crosses; did this assortative mating scheme cause a decrease in measured heritability? The effect of assortative mating on heritability measurements has been investigated (Falconer, 1981: p. 161 - 164), and it has been shown that measured heritability is increased by the factor

$$\frac{1+\frac{1}{2}h^2r}{1+\frac{1}{2}h^4r}$$

where r is the correlation of parents and h is the original heritability. That is, assortative mating causes an apparent increase in heritability, rather than a decrease, and cannot be responsible for the discrepancy.

A genuine decline in heritability of this magnitude must reflect either a decrease in additive genetic variance, which is unlikely, as

Table 5. 1981 Simple regressions: linoleate

INDEPENDENT VARIABLE	r	r^2	B	S.E.B	SIG.
Average Progeny:					
Midparent Linoleate %	0.8589	0.7377	0.8499	0.096	<.001
(constant)			5.1757	5.332	.340
Single Seed:					
Midparent Linoleate %	0.7352	0.5406	0.8576	0.045	<.001
(constant)			4.4887	2.550	.079

r: Correlation coefficient.
B: Slope of regression line.
S.E.B: Standard error of B.
SIG.: Significance of F for the variable indicated

Table 6. 1981 Simple regressions: oleate

INDEPENDENT VARIABLE	r	r^2	B	S.E.B	SIG.
Average Progeny:					
Midparent Oleate %	0.8622	0.7434	0.8658	0.096	<.001
(constant)			7.3287	3.256	.032
Single Seed:					
Midparent Oleate %	0.7402	0.5479	0.8705	0.046	<.001
(constant)			7.4398	1.552	<.001

r: Correlation coefficient.
B: Slope of regression line.
S.E.B: Standard error of B.
SIG.: Significance of F for the variable indicated.

Table 7. 1981 Simple regressions: palmitate

INDEPENDENT VARIABLE	r	r^2	B	S.E.B	SIG.
Average Progeny:					
Midparent Palmitate %	0.2965	0.0879	0.2578	0.157	.112
(constant)			6.5782	1.401	<.001
Single Seed:					
Midparent Palmitate %	0.2471	0.0614	0.2559	0.058	<.001
(constant)			6.5941	0.519	<.001

r: Correlation coefficient.
B: Slope of regression line.
S.E.B: Standard error of B.
SIG.: Significance of F for the variable indicated.

Table 8. 1982 Simple regressions: progeny mean with midparent

FATTY ACID	r	r^2	B	S.E.B.	SIG.
LINOLEATE	0.4234	0.1797	0.2660	0.077	.001
(constant)			52.889	3.730	<.001
OLEATE	0.4466	0.1994	0.2484	0.068	.001
(constant)			13.510	2.874	<.001
PALMITATE	0.5008	0.2508	0.3884	0.091	<.001
(constant)			4.7019	0.776	<.001
STEARATE	0.5915	0.3499	0.4388	0.081	<.001
(constant			1.3402	0.290	<.001

r: Correlation coefficient

B: Slope of regression line.

S.E.B: Standard error of B.

SIG.: Significance of F for the variable indicated.

both high and low linoleate selections were included in the 1982 crossing scheme; or an increase in environmental variance. The latter possibility seems very likely, as the 1979 and 1981 crosses were made on two-year-old plants from May to June, while the first-season plants used in 1982 flowered later, and crosses were made on them from July to September.

Environmental Effects

During the 1982 season a number of crosses were repeated on different dates. In order to determine if date of pollination could affect fatty acid composition of buffalo gourd seed, 17 such pairs of crosses made on dates at least 10 days apart were analyzed. The results of these analyses for the 8 most widely separated pairs of crosses are shown in Table 9. It can readily be seen that most crosses repeated on different dates differ in oleate and linoleate content. For the majority of the crosses, the later replication shows higher linoleate content, although in very late crosses, this effect was reversed. It was therefore hypothesized that some environmental parameter loosely correlated with date of pollination was influencing the polyunsaturation of buffalo gourd seed oil.

The environmental parameters tested for possible correlation with linoleate content were photoperiod (approximated as day length), daily high temperature, daily low temperature, and rainfall. As the seed of buffalo gourd reach 90% viability in 38 days after pollination (Alves-Costa and Bemis, 1972), these parameters were averaged over the period from 1 to 40 days after pollination (DAP), with the exception of rainfall, which was summed over that period. The equivalent averages

and sum were also calculated for three subdivisions of the maturation period; 1 - 20, 21 - 40, and 11 - 30 DAP. Figures 3 - 12 show the data for these variables during the crossing periods.

Multiple regressions were performed for each time period and each fatty acid, giving the results in Table 10. The regressions were performed in a stepwise manner, incorporating variables into the equation in order of their power to increase the explained variation. The first variable incorporated in all cases was the midparent fatty acid percentage. For linoleate and oleate, the only other significant variable was day length. The period 21 - 40 DAP gave the highest value for r^2, indicating that this is the critical period in seed development for environmental influence on linoleate and oleate content. This agrees with previous observations on fatty acid changes during the maturation of buffalo gourd seeds, in which linoleate was found to increase markedly during the same period (J. W. Berry, unpublished data), indicating that oleate desaturation was proceeding actively.

Palmitate showed a significant regression for 1 - 20 DAP with total rainfall, indicating a different environmental influence from that affecting unsaturated fatty acids. The results of the multiple regression for palmitate (Table 13) cast some doubt on the importance of this influence, as rainfall accounts for an increase in explained variation of only .0555. While rainfall or a related factor such as humidity may influence palmitate production during the first 20 DAP, the effect is apparently not great. MacCarthy and Stumpf (1980) observed increased palmitate/C18 fatty acid ratios with increasing temperature in cell cultures

Table 9. Effect of pollination date on oil unsaturation.

CROSS NUMBER	DATE OF POLLINATION	LINOLEATE	OLEATE %	MIDPARENT LINOLEATE %
538	07/22/82	53.7	33.0	53.6
	08/11/82	70.1	16.7	
555	07/26/82	63.9	25.5	51.8
	08/09/82	69.7	20.3	
591	07/27/82	54.8	35.1	45.9
	08/15/82	60.8	29.3	
592	07/29/82	60.4	28.1	46.5
	08/13/82	66.7	22.7	
600	07/13/82	64.0	24.2	51.2
	07/27/82	63.6	26.0	
625	07/23/82	57.8	29.5	44.8
	08/07/82	62.4	26.0	
655	08/31/82	80.7	8.4	54.5
	09/14/82	77.9	9.9	
695	08/30/82	63.3	26.4	31.2
	09/13/82	59.8	29.4	

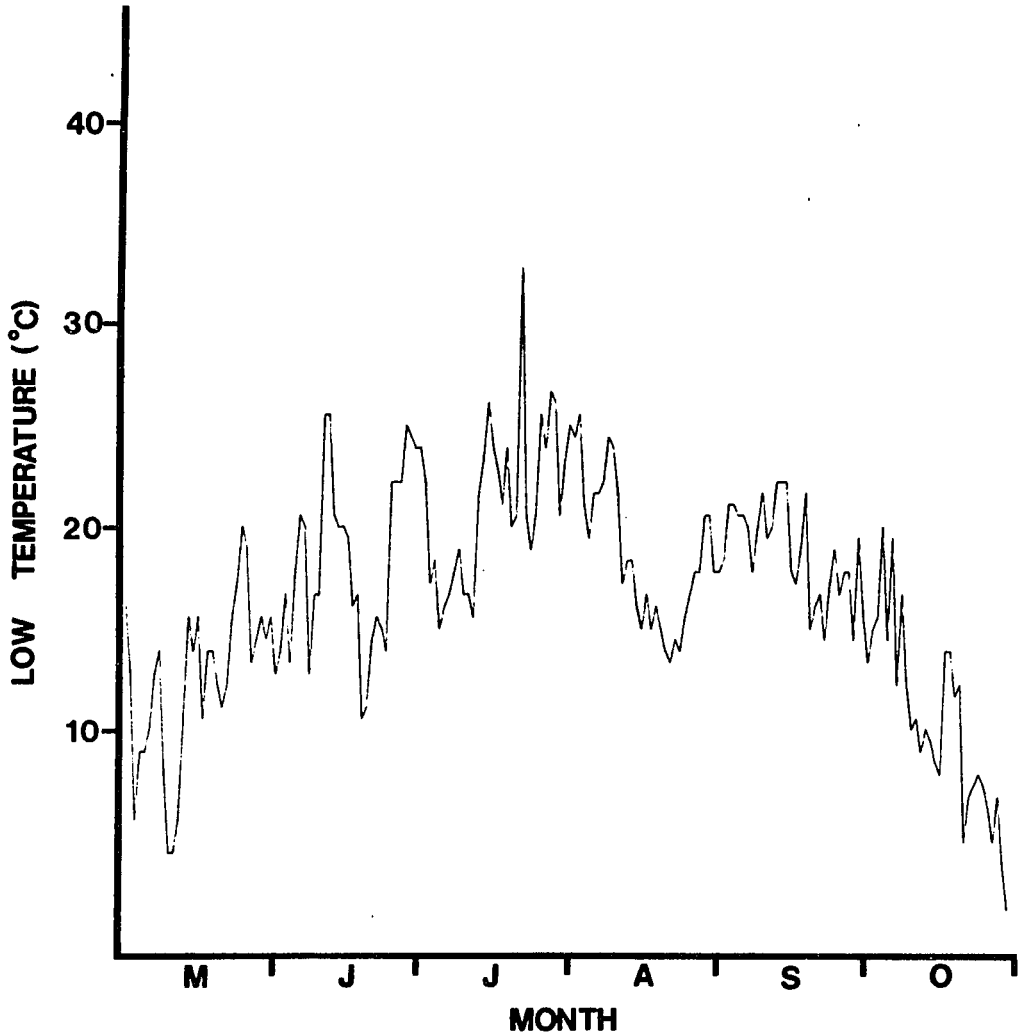

Figure 3. Daily Low Temperatures, Tucson Experiment Station: 1979.

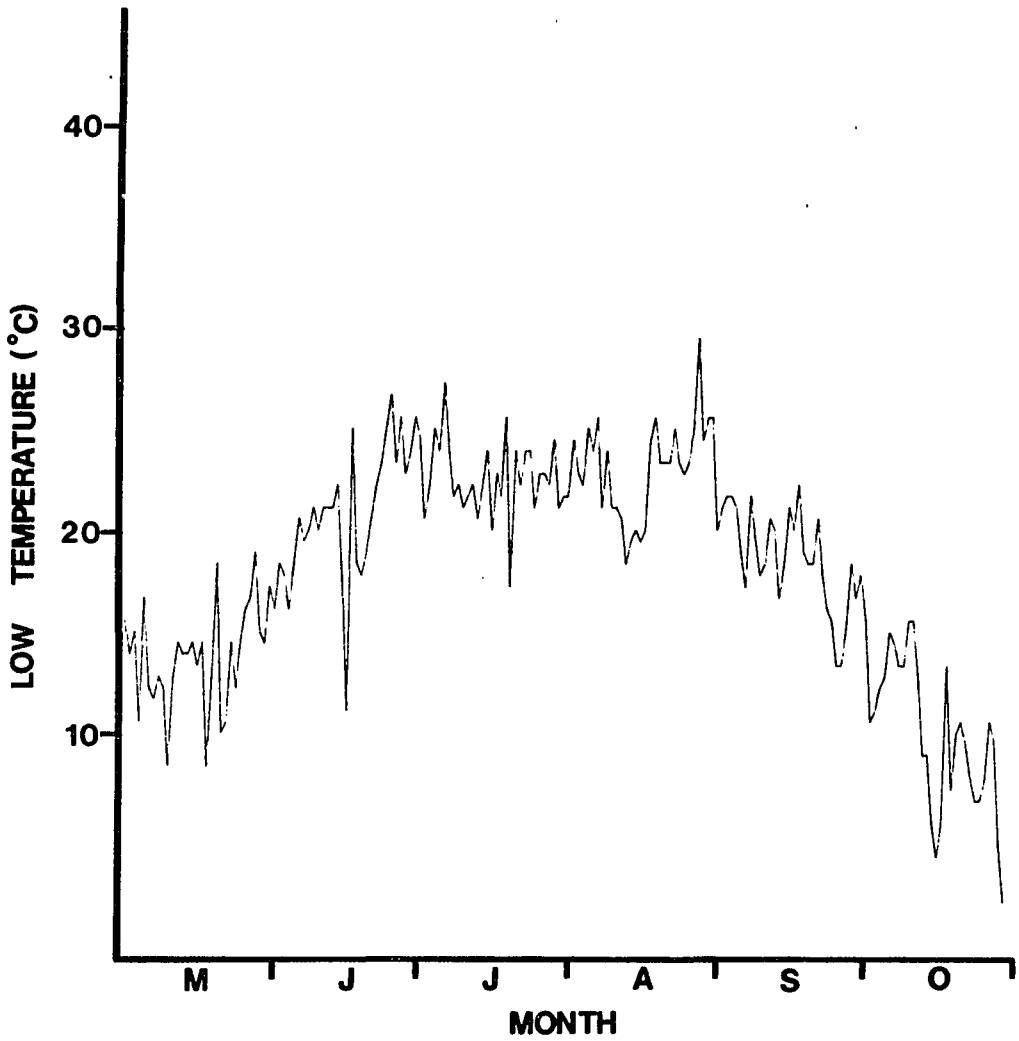

Figure 4. Daily Low Temperatures, Tucson Experiment Station: 1981.

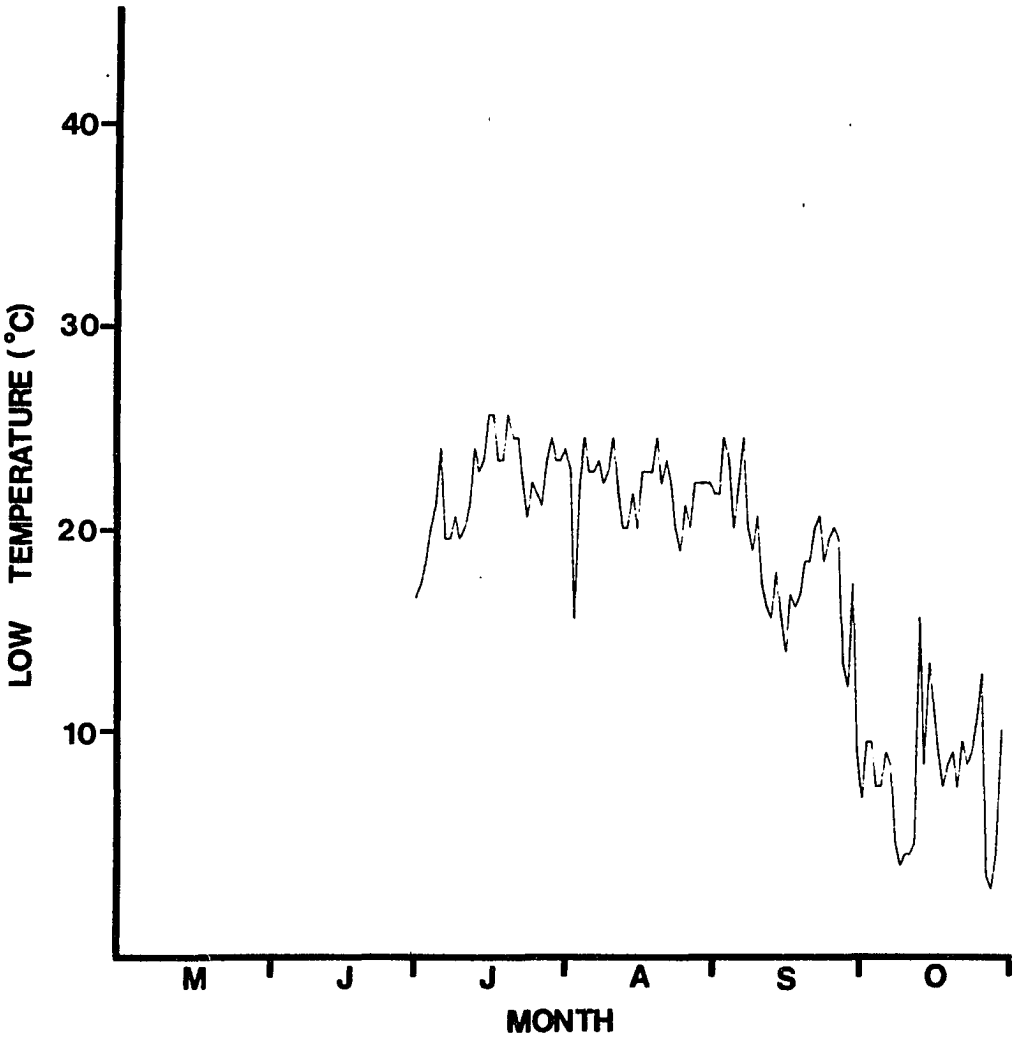

Figure 5. Daily Low Temperatures, Tucson Experiment Station: 1982.

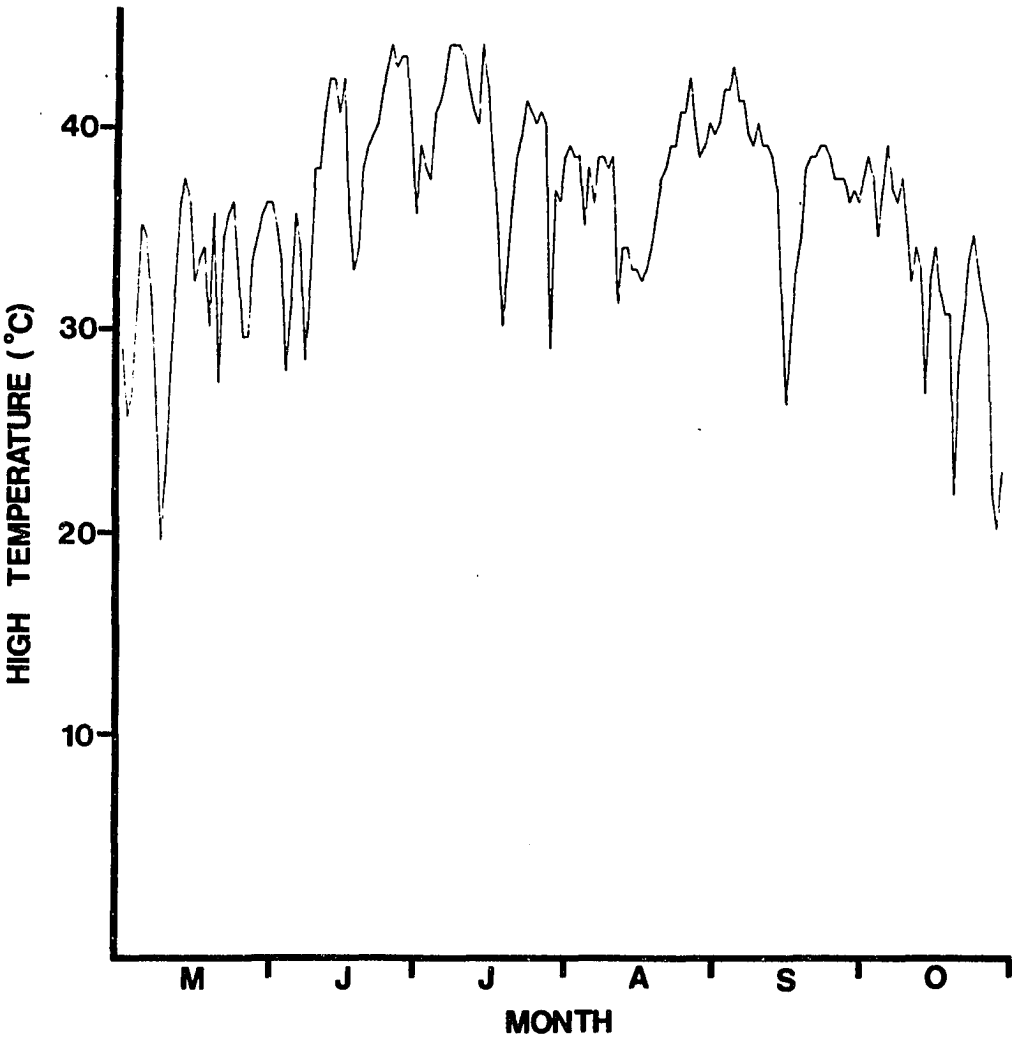

Figure 6. Daily High Temperatures, Tucson Experiment Station: 1979.

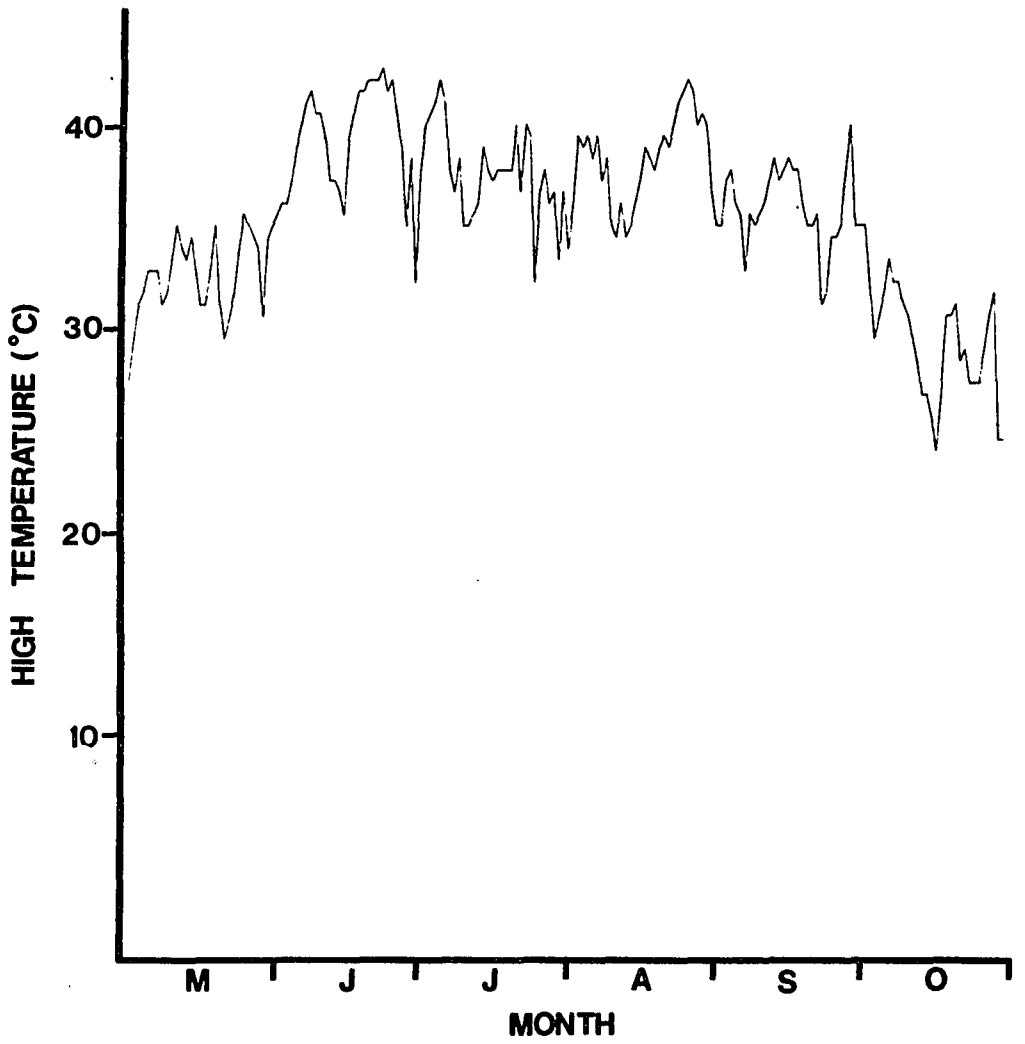

Figure 7. Daily High Temperatures, Tucson Experiment Station: 1981.

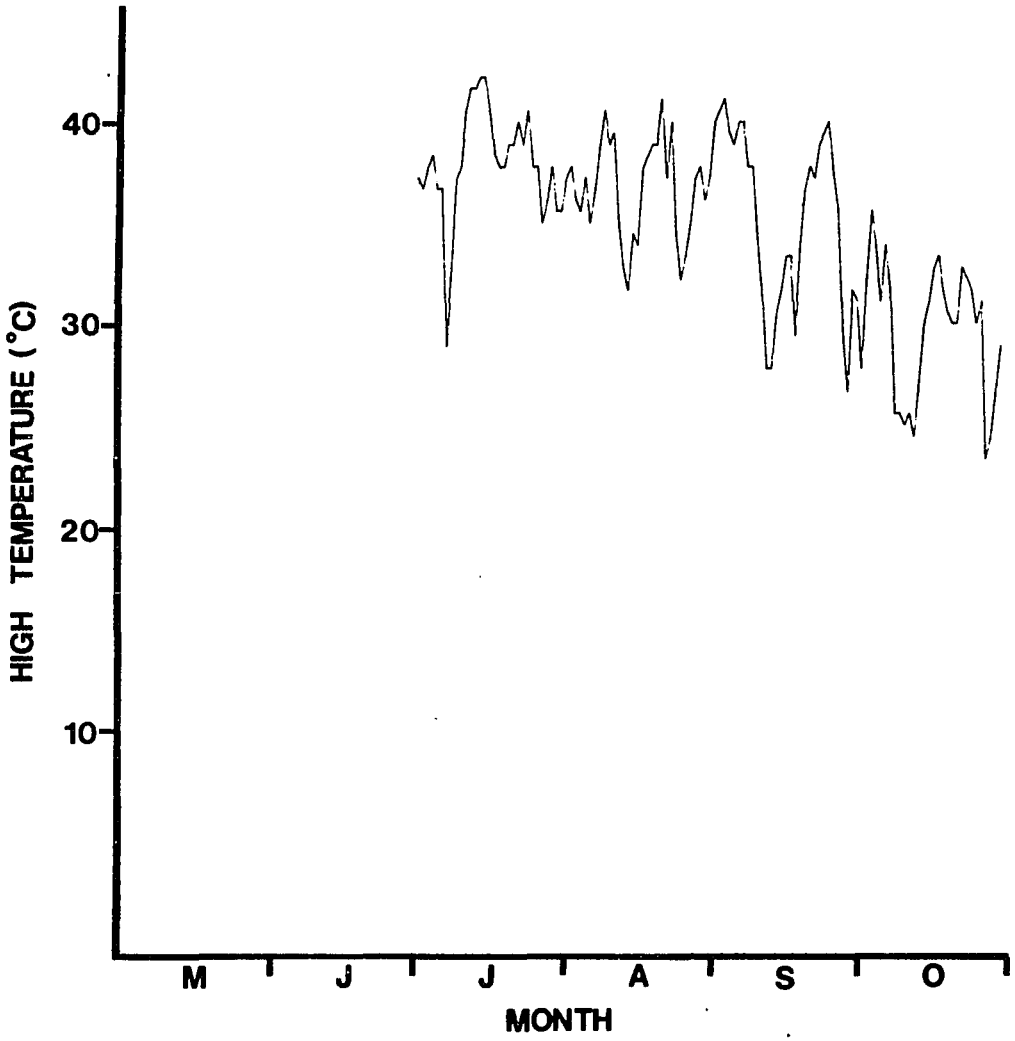

Figure 8. Daily High Temperatures, Tucson Experiment Station: 1982.

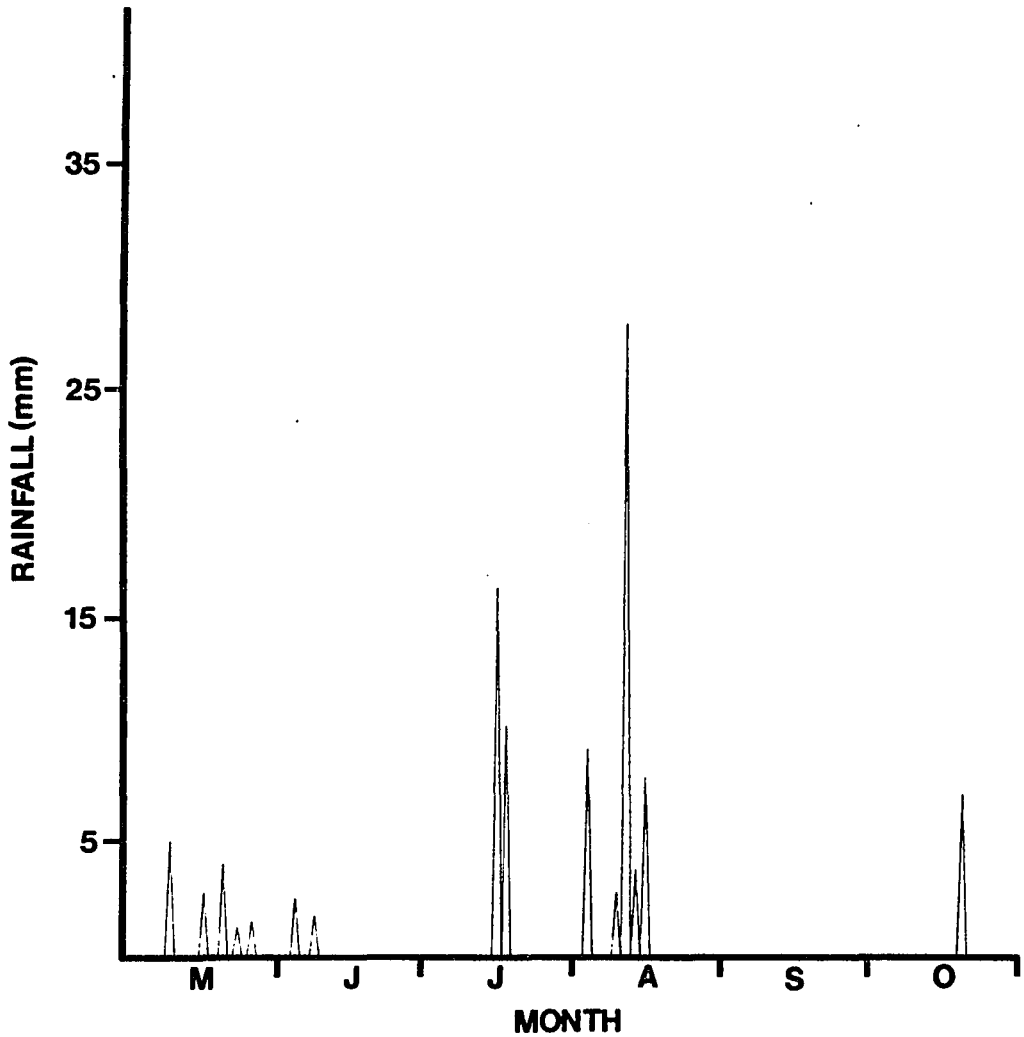

Figure 9. Daily Rainfall, Tucson Experiment Station: 1979.

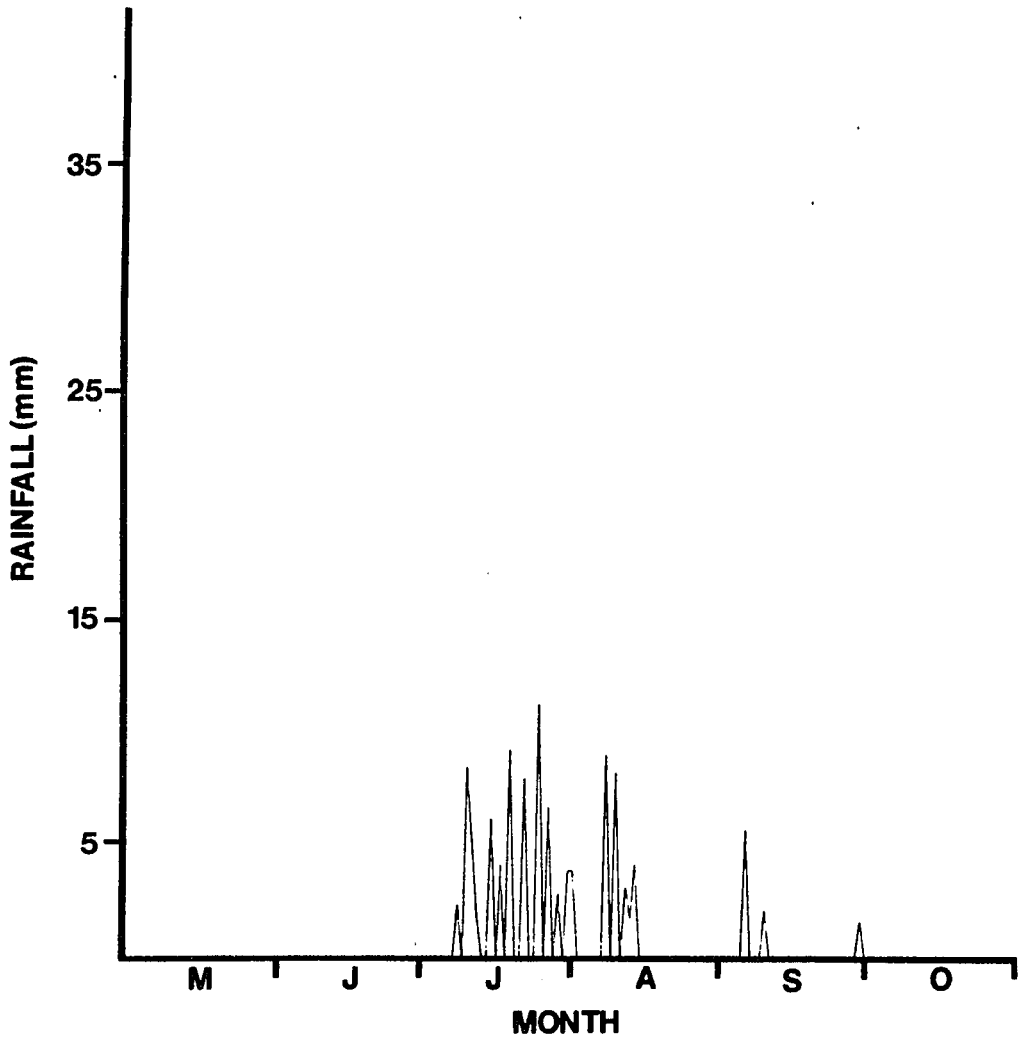

Figure 10. Daily Rainfall, Tucson Experiment Station: 1981

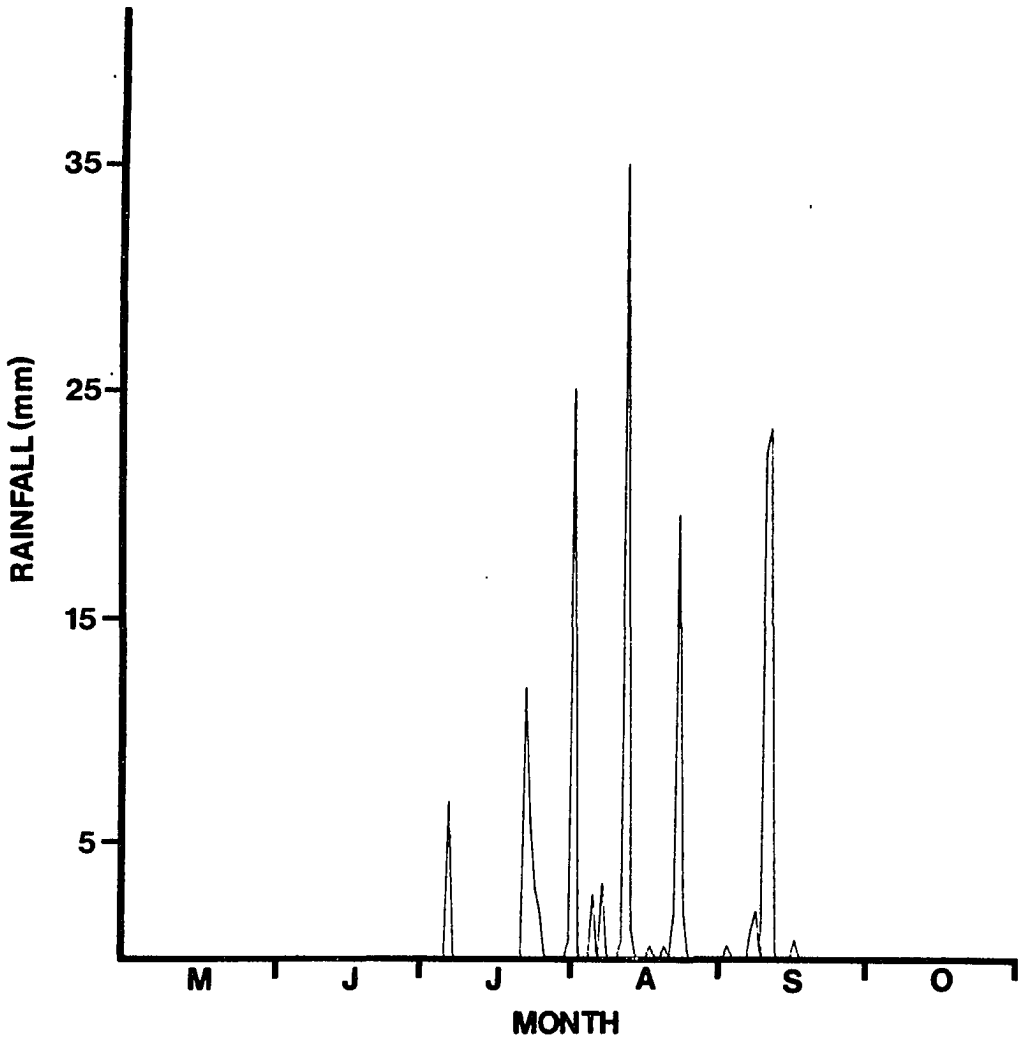

Figure 11. Daily Rainfall, Tucson Experiment Station: 1982.

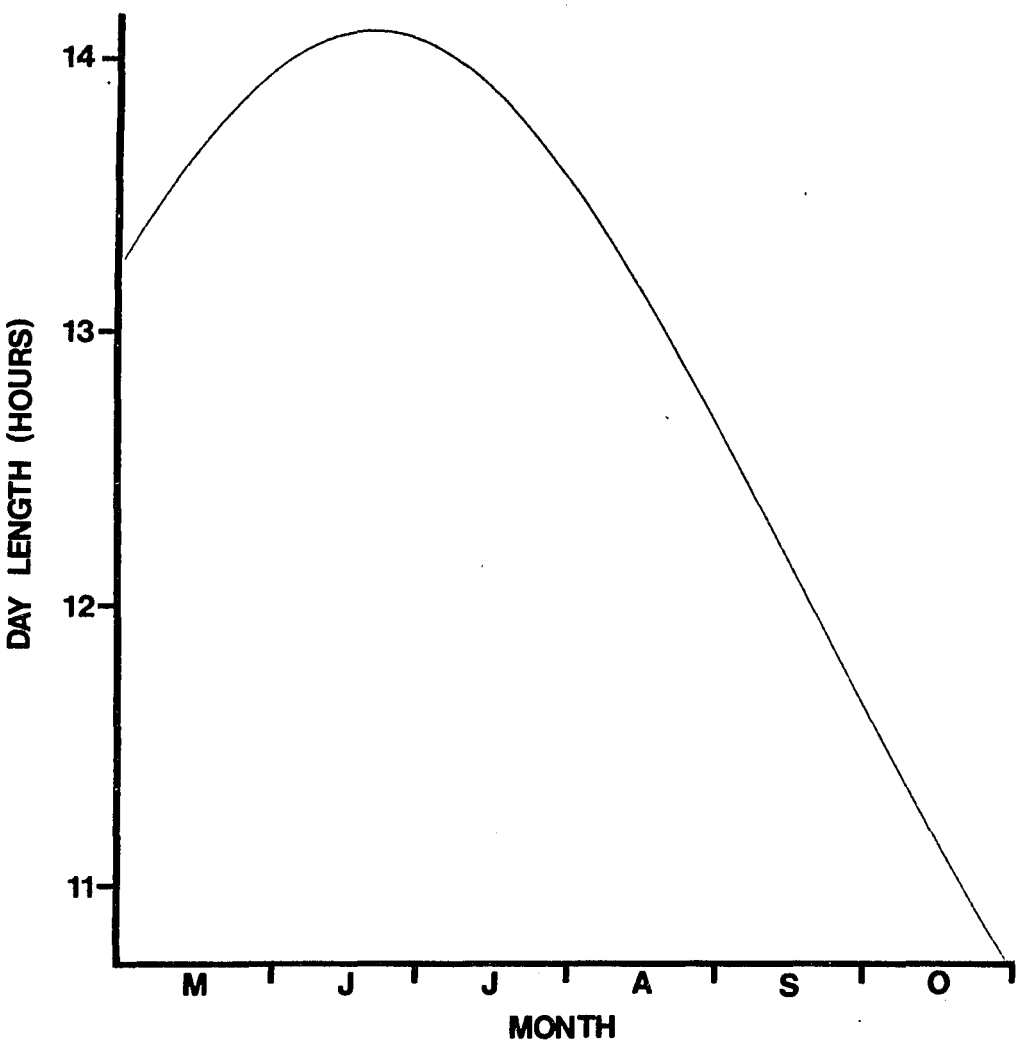

Figure 12. Day Length, Tucson Experiment Station.

Table 10. Determination of critical period for environmental influence.

FATTY ACID	CRITICAL PERIOD (DAP)	HIGHEST r^2	VARIABLES	SIG.
LINOLEATE	1 - 40	0.7252	Midparent Linoleate %	<.001
			Day Length	<.001
	1 - 20	0.7222	Midparent Linoleate %	<.001
			Day Length	<.001
	21 - 40	0.7260	Midparent Linoleate %	<.001
			Day Length	<.001
	11 - 30	0.7243	Midparent Linoleate %	<.001
			Day Length	<.001
OLEATE	1 - 40	0.7007	Midparent Oleate %	<.001
			Day Length	<.001
	1 - 20	0.6978	Midparent Oleate %	<.001
			Day Length	<.001
	21 - 40	0.7015	Midparent Oleate %	<.001
			Day Length	<.001
	11 - 30	0.6998	Midparent Oleate %	<.001
			Day Length	<.001
STEARATE	(no significant regressions for environmental parameters)			
PALMITATE	1 - 40	(no significant regressions for environment)		
	1 - 20	0.5539	Midparent Palmitate %	<.001
			Rain	.044
	21 - 40	(no significant regressions for environment)		
	11 - 30	(no significant regressions for environment)		

DAP: Days after pollination

r: Correlation coefficient

SIG.: Significance of F for the variable indicated.

of Catharanthus roseus G. Don, Glycine max (L.) Merr., and Nicotiana tabacum L., and Jaworski et al. (1974) found that the palmitate elongase system of safflower seed was inhibited in vitro by high temperature. No evidence for a corresponding in vivo temperature effect was found in this study of buffalo gourd seed oil.

The multiple regressions for linoleate and oleate content are shown in Tables 11 and 12. In each case, the addition of day length to the regression equation more than doubles the explained variation. Furthermore, the narrow sense heritability, as the regression coefficient B for midparent value, is for each fatty acid greatly increased over the value in the simple regressions (Table 8). It follows from this that environment had a significant effect on unsaturation of buffalo gourd seed oil in 1982.

It is interesting to note that photoperiod was the environmental factor most strongly correlated with linoleate and oleate content. No such influence has been found for soybean, and sunflower shows a marked temperature response in unsaturation under constant photoperiod (see Chapter Two: Environmental effects on lipid composition). Both fatty acids gave significant correlations with average high and low temperatures in multiple regressions, but the correlations were not as strong as with photoperiod. The difficulty of separating these factors, however, is shown by their correlation coefficients, given in Table 14. Day length has a correlation coefficient of over 0.9 with both high and low temperature. As can be seen from these correlations and from graphs of these factors (Figures 3 - 8, 12), in Tucson in the summer and early

fall, the longest days and highest temperatures roughly coincide.

Thus it is possible that the correlation of linoleate and oleate content with day length reflects a temperature effect. It should be noted (Tables 11 and 12) that the regression coefficients for day length with oleate and linoleate content are roughly equal and opposite in sign; oleate content increases and linoleate content decreases as the days become longer. As the longer days are generally hotter, seeds developing during long days are exposed to a greater number of hours of hotter temperature. This suggests that linoleate content may show a stronger correlation with a combination of these factors than with either factor alone. To test this, an index of degree-hours was calculated by the formula

$$D = (H-26.7)L$$

where D is degree-hours, H is average high temperature, and L is average day length. While this index gave a significant multiple regression with linoleate content, the explained variation (r^2) was .5029, slightly lower than the value of .5271 found in the multiple regression with day length (Table 11). This factor only approximates the actual course of temperature change during the day, however, and temperature cannot be ruled out as the causative factor of unsaturation changes in buffalo gourd oil on the basis of these data.

Table 11. 1982 Multiple regressions: linoleate

INDEPENDENT VARIABLE	MULTIPLE r	r^2	r^2 CHANGE	B	S.E.B.	SIG.
Midparent Linoleate %	0.4239	0.1797	0.1797	0.4420	0.066	<.001
Day Length	0.7260	0.5271	0.3474	-7.3917	1.185	<.001
(constant)				135.01	13.47	<.001

r: Correlation coefficient

B: Slope of regression

S.E.B: Standard error of B.

SIG.: Significance of F for the variable indicated.

Table 12. 1982 Multiple regressions: oleate

INDEPENDENT VARIABLE	MULTIPLE r	r^2	r^2 CHANGE	B	S.E.B.	SIG.
Midparent Oleate %	0.4466	0.1994	0.1994	0.3946	0.061	<.001
Day Length	0.7015	0.4921	0.2926	6.8611	1.242	<.001
(constant)				-76.449	16.44	<.001

r: Correlation coefficient.

B: Slope of regression line.

S.E.B: Standard error of B.

SIG.: Significance of F for the variable indicated.

Table 13. 1982 Multiple regressions: palmitate

INDEPENDENT VARIABLE	MULTIPLE r	r^2	r^2 CHANGE	B	S.E.B.	SIG.
Midparent Palmitate %	0.5008	0.2508	0.2508	0.3445	0.091	<.001
Rainfall	0.5535	0.3064	0.0555	0.2695	0.131	.044
(constant)				4.5979	0.755	<.001

r: Correlation coefficient.

B. Slope of regression line.

S.E.B: Standard error of B.

SIG.: Significance of F for the variable indicated.

Table 14. Correlation coefficients of environmental parameters

	HIGH TEMPERATURE	LOW TEMPERATURE	DAY LENGTH
LOW TEMPERATURE	0.9695		
DAY LENGTH	0.9262	0.9592	
RAINFALL	0.5767	0.7385	0.6742

All coefficients are significant at the .001 level.

a/ All parameters are averages for 21 to 40 days after pollination, except rainfall, which is the total rain during that period. Data were collected from May 1, 1982 to October 31, 1982.

CHAPTER FIVE

CONCLUSIONS

The results of the work reported here have important ramifications for the possible alteration of buffalo gourd seed oil quality through plant breeding, as well as for the understanding of fatty acid synthesis in this species. While a great deal more research remains to be done, it is possible now to suggest the possible directions such work should take.

The techniques used for determination of fatty acid composition in this study, while generally satisfactory, should be modified somewhat. The relatively high precision of measurement found for the major fatty acids obviates the need for triplicate analyses of oil samples for the most part. The time saved by omitting these replications would then allow each individual chromotographic analysis to be performed more slowly, through the use of lower column temperatures and slower carrier gas flow rates. The increased accuracy thus obtained would be very worthwhile.

The process of oleate desaturation in buffalo gourd seeds is apparently similar to that observed in various other plants. Linoleate and oleate are strongly negatively correlated, and the conversion of oleate to linoleate is under polygenic control. Like a number of oilseeds, buffalo gourd exhibits an environmental effect on lipid unsaturation; the controlling factor is quite possible temperature.

In order to ascertain the number of loci involved in oleate desaturation, and to distinguish their physiological effects, the development of inbred lines will be necessary. Such development is in progress, and will undoubtedly contribute to understanding of the physiology of fatty acid metabolism in this species in the future.

While this study clearly demonstrated that an environmental parameter affects oleate desaturation in buffalo gourd seeds, the high correlation of the various environmental factors made it impossible to clearly distinguish them. Photoperiod correlated most strongly with linoleate content in these data, but the causative factor may well be temperature, as it seems to be in some other oilseeds (see Chapter Two: Environmental effects on lipid composition). This possibility should be tested. The most defniitive method of distinguishing between the various environmental factors is to raise buffalo gourd plants in controlled environment chambers where day length and temperature may be varied independently, making it possible to determine whether temperature, photoperiod, both, or even another factor is responsible for the observed environmental effect. Unfortunately, the size of buffalo gourd plants makes this difficult except when very large growth chambers are available.

Another method of distinguishing between photoperiod and temperature effects is to grow plants in different locations and different times. As research on buffalo gcurd is presently being conducted in numerous countries, this method might be feasible through a cooperative effort.

The results of this study indicate that breeding for altered palmitate content in buffalo gourd oil should be possible. Breeding for altered linoleate and oleate content should also be feasible, but will require a special program. One of two breeding methods could be employed. First, a program such as that for sunflower oil composition breeding could be established (see Chapter Two: Fatty acid inheritance in commercial crops - Sunflower), in which the breeding stocks could be grown under a constant environment, and selections made for oil composition. The heritability data for 1981 (Tables 5 and 6) demonstrate that such a program could work; the parental and progeny seeds both developed at the same time of year, and the weather was comparable in the two years (Figures 3, 4, 6 and 7). Under these circumstances the heritability of linoleate content is extremely high, and progress in a breeding program should be rapid.

Second, a research program could be designed to identify genotypes relatively insensitive to temperature. The 1982 multiple regressions (Tables 11 and 12) indicate that long days, or their concomitant high temperatures, increase linoleate content and decrease oleate content of buffalo gourd seed oil. Compared to the 1981 simple regressions (Tables 5 and 6), however, the 1982 regressions explain considerably less of the observed variation; approximately 50% as compared to 75% in 1981. Furthermore, even when environment is taken into account, the heritability of linoleate and oleate content is considerably lower in 1982. Both of these observations indicate that an additional source of variation is still unaccounted for. Quite possibly this source is

variability in the temperature response of different genotypes, in which case it may be possible to select for lines which have a desirable fatty acid composition which is independent of environment.

The existence of such genotypic differences in temperature response is also suggested by the data in Table 9; crosses 655 and 695 show only a small increase or even a decrease in linoleate content over a period of approximately two weeks, although photoperiod and temperature were both declining during that period (see Figures 5, 8 and 12). This may represent an inherent limit to the environmental response, as both members of each pair of crosses have rather high linoleate content, especially considering their midparent values; or it may reflect a true difference in the environmental response of these lines. One method of distinguishing these possibilities would be to repeat a number of self-pollinations on several different dates, so that separate regressions could be performed for different genotypes. If variation were found, selections could then be made for plants with a low value of B for day length (or related parameter). While a similar program using a growth chamber to vary the environmental parameters would give more specific information on the actual causative factors, the field study outlined would have nearly equal pragmatic value in breeding for altered oil composition.

In the laboratory, buffalo gourd seeds offer a potentially useful system for studying the physioloty of oleate desaturation. It is relatively easy to obtain quantities of developing seeds during the time when desaturase activity appears to be high (21 - 40 DAP). Preliminary

studies have shown that a microsomal preparation from such seeds is capable of *in vitro* oleate desaturation (unpublished data). Furthermore, environmental influence aside, there is obviously a strong genetic effect on the linoleate content of oil in mature buffalo gourd seeds. Buffalo gourd seeds should therefore be a useful system for studying the means of genetic control of desaturation. Numerous factors could help to determine the final composition of the oil, including the rates of incorporation of oleate and linoleate into triglycerides, the activity of transacylases which presumably insert oleate into PC to be desaturated, the supply of O_2 to the desaturase, and the structure of the ER membrane, as well as the actual quantity and activity of the oleate desaturase present.

In vitro studies could detect differential incorporation into triglycerides for oleate and linoleate by using labelled oleate, if such incorporation takes place *in vitro*; transacylase activity can be measured similarly, as shown by Stymne and Appelqvist (1978; see Chapter Two: Fatty acid biosynthesis and desaturation, Oleate desaturation: substrate) and the supply of O_2 can be regulated (Rebeille et al., 1980). Membrane structure and oleate desaturase quantity and activity variations between genotypes differing in oil composition would remain confounded, but the field of possible points of genetic control could be narrowed somewhat in such studies. If genetic control of linoleate content in *C. foetidissima* seed oil is polygenic, several of these factors may be responsible for producing different linoleate levels in different lines.

The physiology of temperature effects on lipid desaturation is still imperfectly understood. It is established that O_2 concentration has an influence on desaturation rates at different temperatures, but it seems unlikely that it is the only factor involved, considering that some oilseed species show little response to temperature change (Canvin, 1965). Several experiments have shown that oilseed cotyledons will change their rate of desaturation *in vitro* in reaction to temperature or oxygen tension changes (see Chapter Two: Mechanism and consequences of temperature effect on lipid composition - Mechanism); however, genetic regulation of desaturase production in response to temperature cannot be ruled out in higher plants. If buffalo gourd does show genotypic differences in temperature response, such genetic regulation would seem the likely cause. It would be worthwhile to test microsomal preparations from seeds developing under different temperatures for divergent *in vitro* desaturase activity.

The buffalo gourd is notable not only for its economic potential, but also for the unique opportunity it presents for genetic studies. While it has many characteristics similar to those of commercial oilseeds, the variability of germplasm available in collections of this species from the wild far exceeds that found in plants long domesticated. The value of such diversity in elucidating physiological mechanisms is clear.

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