MONTANA STATE UNIVERSITY

A study of gene action on erucic acid inheritance in selected geographical strains of oriental mustard
(Brassica juncea, L. Cosson.)
by Kailash Prasad Agrawal

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:
Erucic acid is a single unsaturated fatty acid with 22 carbon atoms. This has many potential commercial uses. It is found in varying quantities in different strains of Brassica juncea (L.) Cosson. This study was directed towards studying gene action relative to the inheritance of erucic acid content in the oil of B. juncea. General and specific combining abilities were to be estimated with a view to determining the future breeding program for increasing erucic acid content in commercial oriental mustard.

A modified method of micro-quantitative analysis was employed for oil extraction and esterification of seed oil. The gas liquid phase chromatograph provided the tool for the separation and quantitative study of fatty acid composition.

The effect of date of maturity on erucic acid content of seed oil was estimated from two years data. Results indicated that seed maturity was not a major factor in determining the erucic acid content of seed oil.

The effect of different climatic conditions on erucic acid content of seed oil was studied by analyzing one strain of B. juncea (oriental yellow mustard) grown at nine locations in 1960 and three Brassica accessions grown under seven different climatic conditions in 1962.

Results of analysis indicated that climatic differences influenced erucic acid content of the seed oil and that the effect was not uniform for different strains at different locations. The magnitude of the effect was not sufficiently great to interfere with the determination of major genetic differences.

Seventeen strains of Brassica differing in erucic acid content were selected for a study of erucic acid inheritance. Selfed and crossed seed from ten parental lines in three replications were analyzed for fatty acid composition. Erucic acid values were analyzed statistically for a study of gene action by correlation and regression and also by a method of analysis of variance suggested by Hayman (25). Results of statistical analysis indicated the presence of significant additive variance in these lines. Variance due to interaction was non-significant but there was evidence of the presence of dominance gene effects due to certain lines. Crosses involving one of ten lines exhibited large reciprocal differences indicating the presence of maternal effects. Further analysis by a method suggested by Sprague and Tatum (64) indicated that certain lines had high or low general combining ability and other lines combined high general and specific combining ability. Further testing would be necessary to determine the most effective parents and methods for a breeding program.

A STUDY OF GENE ACTION ON ERUCIC ACID INHERITANCE IN SELECTED
GEOGRAPHICAL STRAINS OF ORIENTAL MUSTARD
(Brassica juncea, / L./ Cosson.)

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A thesis submitted to the Graduate Faculty in partial
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INTRODUCTION

Search for a new crop which could utilize acres not needed for food and fiber production is occupying the time of many researchers. This new crop must possess some unique quality with respect to either protein, starch or fat in order that it may be used as a source of raw material by industry. Chemical analysis in the past has indicated the presence of a typical fatty acid, erucic acid, in varying quantities, in the oils of seed of plants belonging to the family Cruciferae.

Erucic acid is a monoene of 22 carbons. The long unsaturated carbon chain makes it a potential raw material for conversion into different short chain carbon substances. At present it would seem that a fat source must contain more than 50 percent erucic acid for economic commercial extraction and utilization. Seed oil from crambe, which belongs to the same plant family Cruciferae, has been found to contain 45 to 55 percent erucic acid.

Strains of oriental mustard (Brassica juncea L.), which are commercially grown in the United States and are well adapted for high seed yields and high fat percentages, contain fats with low erucic acid content (from 20 to 25 percent). Oil from some of the related strains of B. juncea from the world collection have been found to contain erucic acid up to 50 percent. This source could possibly be used to transfer the gene or genes for erucic acid content to the commercial varieties of B. juncea.

Commercial requirements of the presence of at least 50 percent erucic acid presently favor the selection of crambe as a potential commercial crop even though it is relatively low yielding, both in respect to oil content and seed, and is not well adapted to dryland production.

At this point there would seem to be two alternatives, (1) accept crambe and work to improve yield, drouth resistance, and oil percentage and discard commercial varieties of B. juncea, which are known to possess these characters to a greater degree, or (2) locate additive genes for erucic acid in available strains of B. juncea and transfer them to adapted commercial types of B. juncea.

The present series of experiments were designed to study the mode of gene action for erucic acid inheritance in strains of B. juncea of differing geographical origin.

A minimum of information on procedure was available at the time of initiation of this undertaking in 1960. It was necessary to first determine the effects of such factors as environment and maturity on erucic acid content and to develop a method for rapidly extracting and analyzing very small samples of oil for erucic acid content. These necessary experiments of a preliminary nature are made a part of this thesis.

The technique of diallel cross analysis was selected as the method to be employed in evaluating the mode of gene action.

General and specific combining ability of the different lines was

to be investigated in an effort to determine the feasibility and method

of a future improvement program.

LITERATURE REVIEW

I. Fats, Fatty Acid Composition and Chemical Determination.

Fats obtained from seeds are in the form of triglyceride esters
of fatty acids. Three molecules of the fatty acids are associated with
one molecule of glycerol with ester bonds. These three fatty acids may
be alike or different, as reported by Eckey (9), Markley (43), Jamieson
(34) and Kirchenbauer (42). In general, fatty acids found in plants have
an even number of carbon atoms and may be saturated or unsaturated.

The mechanism of fat synthesis in the plant is not known but it
is thought that triglycerides, being insoluble, are actually synthesized
in the parts where they are found.

Garner and Allard (15) noted that in peanut, soybean, cotton and
safflower seed fat synthesis does not set in during the early stages of
seed development but occurs about midway between blooming and final matur-
ity of the seeds.

Mohamad and Sultan (47), working with B. campestris L., noted that
rapid oil formation started 20 days after fertilization and continued for
the next 20 days.

Jones (37) and Jones and Shaw (38) noted a similar phenomenon in
Macadamia integrifolia, Maiden et. Betche. Very little oil formation in
the beginning was followed by a period of rapid oil synthesis, followed
again by a slower period of oil synthesis. A decrease in reducing sugars
was associated with the increase in oil content.

Eckey (9) quoted several workers who studied oil formation in

walnuts, almonds and linseed. These workers found that a decrease in carbohydrate content occurred concomitant with a rapid increase in oil content.

Based on these findings, as well as those of Thor and Smith (quoted by Eckey) with pecan, it was concluded that the amount of carbo- hydrate involved was too small to account for the appreciable amount of fat formed. Eckey (9) suggested that much of the oil formed in the seed is from materials translocated from other parts of the plant. Eckey (9) and Kirchenbauer (42) suggested that probably the carbohydrates synthesized by the plant as a result of photosynthesis are translocated to the area of synthesis where the carbohydrates are converted to fat.

Esau (10) mentions development of a thick integument layer in the Cruciferae which consists of outer and inner integument each consisting of several cell layers. The outer integument serves as a protective covering whereas the inner integument serves as an area of starch storage during seed development. Hokansson (20, 21) traced the development of seed in B. oleracea and B. rapa. From his studies he concluded that embryo development takes place in eight stages. During the beginning stage, the endosperm starts growth immediately after triple-fusion and continues growth for 14 days. The endosperm surrounds the young embryo for a short period. Fourteen days after fertilization the endosperm starts diminishing and by 21 days the sporophyte tissues (integuments) as well as the endosperm disappears. The embryo grows and by 28 days the cotyledons are fully de- veloped. The cotyledons are the fat storage tissues of the Brassica seed.

He further suggested the indication of three time periods in post fertilization development:

(1) The first period of about ten days during which an increase of sporophyte tissue, especially the inner integument, takes place. This is the location of starch storage.

(2) The second period of vigorous development of endosperm, accompanied by a diminishing of the inner integument.

(3) The third period of rapid growth of the embryo and development of cotyledons and formation of oil.

This suggests that starch is first stored in the integuments followed by its use in the manufacture of endosperm tissues. The endosperm supplies the nutrients for the growing embryo and development of the cotyledons.

Yermanos (72) suggested that fatty acid composition in oils from flax is controlled by the genotype of the seed. This would be expected on the basis of the evidence that fats are synthesized after fertilization and in tissues formed as a result of fertilization and subsequent embryonic development.

Erucic acid is a characteristic fatty acid component of Cruciferae oil and constitutes a major portion of the fatty acids in the oils of the genus <u>Brassica</u>.

It is monoethenoid acid, also termed as monoene, and has 22 carbons. A monoethenoid acid contains two less hydrogen atoms than the corresponding saturated acid. The empirical formula of erucic acid is $C_{22}H_{42}O_2$ $\left[CH_3(CH_2)_7CH=CH(CH_2)_{11}-COOH\right]$. According to Markley (43) its molecular

weight is 338.5, neutralizing value 165.7, and iodine value 75.0. To date it has been found in only two plant families, the Cruciferae and Tropacolaceae.

Seeds from plants of Brassica species, viz. B. nigra (L.) Koch, B. campestris, B. juncea and B. napus L., have been analyzed for fatty acid composition at various times by different workers. Eckey (9) reported analysis of several samples of B. napus with an observed erucic acid content of 45 to 55 percent.

Hilditch (29, 30) reports 50 percent erucic acid in samples of rape seed and 50 percent in black mustard. Mehlenbacher (45) reports 45 to 54 percent erucic acid content in oils of B. napus, 40.6 percent in B. nigra and 38.7 percent in B. campestris. Mikolajizak et al. (46) have reported erucic acid content in oils from different sources of Brassica as follows: 31 percent in B. campestris, 43 percent in B. nigra, 36 percent in B. oleracea, 37 to 42 percent in B. carinata Braun., 22 percent in B. juncea and 44 to 50 percent in B. napus. Steffanson (66) has isolated one variety of rape (B. napus), completely devoid of erucic acid in the seed oil. Chemical analysis of seed oils from different strains of B. nigra, B. campestris and B. juncea performed by the Montana Agricultural Experiment Station indicated differences in erucic acid content among these species and among strains within species.[1]

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[1] Quarterly Report Nos. 1-12: Investigation on fatty acid composition of the oil from Brassica and related genera of mustard seed. Prepared by Dr. K. Goering.

Craig and Wetter (3), and Craig (4) studied the influence of environment on fatty acid composition of rape seed. Their studies indicated that soil and moisture differences significantly influence erucic acid content.

Harvey (23) studied the inheritance of erucic acid content in B. napus L. In his studies he used one variety of rape which was free of erucic acid to cross with another having a high percentage of erucic acid. He determined gene action by studying F_2 and F_3 progenies. From his studies he concluded that erucic acid content was controlled by two additive genes and that each gene had an equal effect. He found no evidence of dominance or maternal effects.

Soxlet's method of oil extraction is commonly used for quantitative determination of total oil percentage. This method is quite accurate for quantitative determinations but it takes approximately 18 hours for complete extraction, and a minimum of ten grams of seed. This method is not readily adapted to the extraction of oils from smaller quantities of seed for qualitative purposes. Mehlenbacher (45) suggested grinding seed in a Waring blender and extraction of oil from the crushed seed by ethyl ether or other suitable solvent. This method requires a relatively large sample for grinding in the blender. The "Swedish method" suggested by Tröeng (69) consists of grinding five g. of seed in a metallic tube by metallic balls in the presence of Skelly F solvent. A large number of samples can be handled by this method in a comparatively short time. Harvey (22) used a Carver laboratory press equipped with a plunger and

cylinder and pressure gauge to crush the seed at a constant pressure. The maximum pressure should not exceed 2500 pounds per square inch. Traces of oil present on the top of the press are washed with an excess of Skelly F into a beaker, filtered, and evaporated on a steam bath under vacuum. This method is rapid and makes possible extraction of oil from small samples.

The general process of trans-esterification for determination of fatty acid composition consists of refluxing one g. of oil with 100 ml. of methanol and 10 ml. of sodium methoxide for about six hours. This is followed by a second process of trans-esterification by treatment with diazomethane. The complete process requires eight to ten hours. The amount of methyl esters obtained is usually sufficient for several injections.

Harvey (22) used two ml. of two percent acetyl-chloride in methanol for trans-esterification of oils from single seeds. He found refluxing for 30 minutes to an hour was sufficient for complete esterification. Acetyl-chloride serves as a catalyst in this method as contrasted to sodium methoxide in the method described above. The amount of methanol is dependent on the amount of oil. If proper care is taken, sufficient esters are obtained from a few seed for injection into the gas chromatograph.

Gas liquid phase chromatography is similar to other systems of chromatography. Gas liquid phase chromatography also utilizes a system of two phases, one stationary phase which serves as the adsorbing or

partitioning agent and another, moving phase, which serves as a means of transport of material for separation. In gas liquid phase chromatography, the stationary phase is a liquid which is generally distributed over an inert solid support in order to give it a large surface area for exchange, (41). The moving phase is a gas.

Under difinitely set operational conditions, the retention value or retention time which is characteristic of a certain component is recorded on a graph.

Gas liquid phase chromatography has been successfully used for the analysis of fatty acid composition of seed oils from Brassica by Harvey (22, 23), Mikolajizak, et al. (46) and is also used in the analytical work at the Montana Agricultural Experiment Station.

II. Brassica juncea and its Relation to Other Species of the Genus Brassica.

The nomenclature of forms within the genus Brassica has been quite confusing in the past owing to the morphological similarities within the species and also due to some cross compatability among some species. Sinsakaia (63) tried to classify the species of Brassica according to chromosome number but still there remained some confusion in the B. campestris group as well as the B. napus group. Pearson (57) stated that all members of the ten chromosome group were closely related and should be classified as one species. His classification was based on chromosome number. Due to previous incorrect cytological investigations, he grouped B. juncea and B. napus as one species although he recognized

morphological differences in these two forms and noted that they did not intercross easily.

Moringa (48, 49, 50), after a series of cytological, morphological, and interhybridization studies, subdivided the genus into six main groupings. He indicated that the three forms with the lowest number of chromosomes possess the basic genomes, i.e. B. campestris (n = 10) - genome A; B. nigra (n = 8) - genome B; and B. oleracea L. (n = 9) - genome C; whereas, the other three forms B. juncea (n = 18); B. napus (n = 19); and B. carinata (n = 17) are amphidiploids having the genome constitution AB, AC, and BC, respectively. Work of U (70), Sikka (61, 62), Sun (67, 68) and Crane (5) gave further evidence to Moringa's groupings.

A wide variety of forms differing in general morphology are present in almost all the species of the genus Brassica. Given on the following page are certain distinguishing characteristics which have been suggested by Moringa (50), Sabnis (60), Sun (67, 68) and Musil (52) as useful key characters for differentiating species.

	Brassica species					
	campestris	nigra	juncea	carinata	napus	oleracea
Leaf characters:						
clasping	yes	no	no	no	no	no
leathery	no	no	no	yes	yes	yes
leaf shape	wide at base	short ovate	long	-	-	-
hairs	few marginal	sparingly and stiff	few marginal	glaucus	few marginal	glaucus
color	bright green	light green	bright green	blue green	blue green	blue green
Pod characters:						
length, mm.	30	11	29	-	48	-
beak length	long	short	medium	short	-	-
shape	flattened	angular	slightly flattened	flattened	-	-
position relative to raceme	-	appressed	spreading	appressed	appressed	-

Moringa (50) and U (70) proposed a possible explanation for the amphidiploid origin of B. juncea, B. napus and B. carinata. They suggested that interspecies hybridization in nature followed by doubling resulted in the formation of the amphidiploid. In crosses between B. campestris and B. oleracea, U (70) obtained a very low seed set but among hybrids there was one amphidiploid (2n = 38) which was phenotypically similar to B. napus.

Subsequently, the statement of Moringa (50) and U (70) regarding the amphidiploid origin of B. napus, B. juncea and B. carinata has been verified by many workers.

Howard (32) obtained napus forms as a result of crossing auto-tetraploid forms of B. chinensis L. and B. oleracea.

Frandsen (14) also obtained B. pseudo-napus by crossing colchicine induced tetraploid forms of B. campestris and B. oleracea. This pseudo-napus had fairly regular meiosis and crossed freely with natural occurring forms. Similarly, B. pseudo-napus has been obtained by Olsson (54, 56) using different forms from the campestris and oleracea group. Synthesis of winter-hardy rape by crossing tetraploid forms of B. campestris and B. oleracea has been accomplished.

Synthesis of pseudo-carinata has been achieved by Frandsen (14). The fertility of this amphidiploid was not very high but it had fairly regular meiosis and crossed freely with natural B. carinata.

B. juncea has been artificially synthesized by intercrossing different forms of B. nigra with different forms of B. campestris. Frandsen (13) synthesized B. juncea by crossing tetraploid forms of the two species, B. nigra and B. campestris. Howard (31, 32), Ramanujam and Srinivasachar (59), and Olsson (55) successfully isolated artificial B. juncea from hybrids between tetraploid forms of B. nigra and B. campestris. The pseudo-juncea resembled the natural B. juncea in morphological characters and intercrossed freely with the natural forms.

Dobzhansky (7) points out that different geographical races or

strains of allopatric organisms have originated as a result of environmental diversities and as a result of natural selection. These allopatric populations respond to environmental differences by genotypic differentiation. He defines geographical races as genetically distinct allopatric races. Diversities among allopatric organisms are much greater than among sympatric organisms. There is always the probability of finding diverse gene complexes in different geographical races or strains, which may be true in case of the strains under consideration.

Olsson (53), based on the result of the hybridization experiments between morphologically different forms of B. napus of diverse origin and on the result of cytological examinations, suggested that this species originated in different regions by intercrossing of B. campestris and B. oleracea on different occasions. B. juncea and B. carinata types may have originated in a similar manner. Gates (16) suggested that many new types have evolved in the genus Brassica at different times and places.

Allard (1) has discussed different types of incompatability mechanisms operating in self and cross fertilized plants which does not fit in the present situation. Pearson (57), Hokansson (20, 21) and Downey (8) found reciprocal differences in incompatability mechanisms. They report instances of interspecies hybridization of crosses involving diploid and tetraploid individuals. In general, they found better seed set when the individual strains with higher chromosome numbers were used as the female parent than in the reciprocal crosses. Müntzing (51)

suggests that disturbances in the hybrid seed development may be due to a change in the relationship between maternal tissues, endosperm tissues, and embryo tissues which normally have the respective chromosomal relationship of 2n : 3n : 2n. Hokansson (20) actually observed that quantities of endosperm and starch formed in reciprocal crosses of 2n x 4n types in barley were different. This could, in turn, have an influence on embryo development.

III. Estimation of Components of Variation in Quantitative Inheritance Studies.

Quantitative characters which exhibit continuous variations are, in most cases, controlled by a number of genes acting as a gene block. These genes may have an additive or complementary effect or the genes may be interacting to produce a phenotypic variation. It is not possible to separate the effects of individual genes in such cases, yet, measurement of the combined effect in appropriate materials may give a general idea of the genetic situation involved.

Another type of continuous variation may be found to be controlled by single genes. This type of continuous variation is due to different degrees of gene expression in different environments. Allard (1) believes that if environment is held constant for all the progenies of such monogenic hybrids, segregation into discrete classes should occur.

A study of continuous variation requires a statistical approach and no single method has been devised which will apply to all genetic situations. Different genetic models have been devised and methods

suggested to analyze the genetic situations characterized by a particular model. In all these situations, the model is usually much simpler than what may be the actual situation.

Fisher is considered to be the forerunner of modern biometrics. In his paper of 1918 (11) he presented a gene model which included dominance effects at a single locus. He stated that there may be a deviation from the simple additive effects between loci, similar to dominance at one locus, if more than one locus affected the genetic character. He called this deviation epistacy, and introduced the term dual epistacy to indicate the interaction of all possible pairs of loci affecting the gene character. In this paper he discussed the possibility of the study of the accumulated effect of additive and interacting factors. He suggested that the heritable variances observable among any group of organisms may be regarded as a sum total of the variance due to individual factors. The proportion of heritable variance may be easily estimated from covariance or the mean products of the measurements of related individuals so that without being able to recognize any single factor, we have a direct means of estimating their total contribution to heritable variance. This concept provided the basis for the development of techniques for measurements of heritability in animal breeding.

In a subsequent paper Fisher et al. (12) discuss the effect of dominance and epistasis and describe what they call third degree statistics to measure the degree of dominance. F_2 and F_3 population data are required.

Fisher's suggestion was quite useful. Hull's (33) paper may be thought to be an extension of the work of Fisher. Hull (33), Griffing (17) and Kempthorne, et al. (40) used techniques of regression analysis for estimating heritability and to study inheritance of quantitative characters.

Similarly, Powers (58) studied the inheritance of quantitative characters by means of correlation and regression in tomatoes. Sprague and Tatum (64) evolved a method for evaluating general and specific combining ability for a set of single crosses in corn. Specific combining ability was used to measure heterosis effects.

Mather (44) developed statistical methods or formulas for partitioning components of variation under a given set of conditions. His genetic model is similar to that of Fisher (11). He proportioned the observed variations into three main components which he describes as:

E - Nonheritable - environmental

H - Genetic - unfixable (dominance or epistatic)

D - Genetic - fixable (additive)

Yates (71), about the same time, suggested a method of analysis of data from diallel crosses and also developed techniques of analysis of variance for testing reciprocal differences in diallel crosses.

Techniques developed by the above workers provided a foundation for the development of a much stronger analytical method for separation of components of variation in a given genetic environment. This analytical method is generally referred to as "Diallel Cross Analysis".

A diallel cross is taken to be a set of homozygous lines with a complete set of F_1 progeny including a total of n^2 individuals or families (depending on whether F_1 or later generations are employed).

The method of analysis and interpretation of results is dependent on the following conditions:

(1) Use of only one set of F_1 progenies or both reciprocal.

(2) Use of F_1 progenies (both reciprocals or single) plus the inbred parents.

(3) Nature of inbred parents, that is: (a) If they are random inbred parents from a random mating population, then genetic interpretation can be safely drawn with respect to the population as a whole. (b) If the inbred lines selected are non-random from a population, then the inferences drawn can be valid for only the particular population of inbreds.

(4) If F_2 and F_3 and backcross generations can be included in the study, extra information may be obtained, linkage may be determined and the number of alleles estimated, etc. Some loss in the precision of the experiment may occur due to inadequate population size.

Methods of analysis suggested by different workers from time to time may be categorized into three main approaches:

(1) Kempthorne (39) and Anderson et al. (2) defined a genetic environment characterized by a random mating population at equilibrium. If inbred lines of a diallel cross represent random samples from this population, then the genetic mechanism as studied from the analysis of

the diallel cross may be used to define the population as a whole. This approach is quite rigid. Kempthorne developed methods for estimating components of variation for inbreeding populations and also extended it to populations with partial inbreeding.

(2) Hayman (24, 25, 26, 27) and Jinks (35, 36) have proposed methods of analyses that are not as rigid regarding random selection of inbred lines. The inbred population may be selected for certain characters and accordingly they suggest methods to estimate components of variation under a given set of conditions. These components are descriptive of a population from which the selected lines might have been sampled. The components give an approximate description of the genetic situation in the given set of inbred lines.

In this kind of situation any particular line may be added or removed from the general diallel table without any effect on the final interpretation of the result, as stated by Dickinson and Jinks (6).

(3) The third approach to diallel cross analysis is that of Griffing (18, 19), who estimates the general and specific combining ability of the inbred lines and interprets the combining ability in terms of gene action. Griffing has suggested methods for determining the components of variance after calculating the combining ability.

These three approaches to diallel analysis are based on certain assumptions which characterize particular situations, discussed by each author. Methods have been extended further to apply to other situations. Studies of F_2 and F_3 or backcross generations have been used in the

diallel analysis to estimate parental homozygosity by Dickinson and

Jinks (6) and for the study of linkage by Hayman (28).

MATERIALS AND METHODS

I. Collection of Plant Materials.

Seventeen geographical strains of B. juncea originally from ten different geographical regions were selected and obtained from different sources for the present study. Their place of origin, description of the type, and source of seed for this study are shown below:

	Identification	Place of origin	Seed source
1.	Fordhook Fancy	United States	W. Atlee Burpee & Co.
2.	Florida Broad Leaf	"	"
3.	Large Smooth Leaf	"	"
4.	Chinese Broad Leaf	"	R. H. Shumway, Seedman
5.	P.I. 173847	India	Regional Plant Introduction Station, Pullman, Washington
6.	P.I. 183920	"	"
7.	P.I. 175067	Nepal	"
8.	P.I. 207465	Afganistan	"
9.	P.I. 169077	Turkey	"
10.	P.I. 169085	"	"
11.	P.I. 179192	"	"
12.	P.I. 195552	Ethiopia	"
13.	Japan No. 91	China	Dr. Roebbelen, Goettingen, Germany, through Dr. J. R. Schaeffer
14.	Tibet No. 96	Tibet	"

Identification	Place of origin	Seed source
15. Montana Brown	Europe	Prof. R. F. Eslick, Original seed.
16. Oriental Yellow	Orient	" (60-9220-12)
17. (P.I. 173847 Sel.) B. juncea	India	" (60-8870)

These 17 strains were originally identified as B. juncea by the source from which the seeds were obtained. Some discrepancies have been noted. Percentage of oil, iodine number, and fatty acid composition in the seed of the 17 strains of Brassica selected for study of erucic acid content of the seed oil are presented in Table I.

II. Hybridization.

In the spring of 1961, nine seeds from each of the 17 strains were seeded in three replications in hills three feet apart. On emergence they were thinned to one plant per hill.

On initiation of flower buds, one inflorescence from each plant was bagged with kraft paper bags to insure self pollination. Care was taken to clip off the flowers which had opened prior to bagging.

In addition to selfing, every plant in the replicate was crossed to each other plant, i.e., all possible combinations, so that each plant served as a pollen donor for the rest and also received pollen from each of the other plants in the replicate. Summarily, a diallel series with reciprocals was established in triplicate.

Emasculations and pollinations were preferably done in the morning

Table I. Oil percentage in the seed and iodine number and fatty acid composition of the oil from 17 strains of **Brassica** selected for study of erucic acid content of the seed oil.

Identification	No. of samples averaged	Oil percent- age	Iodine No.	Percentage of some of the fatty acids in oils of different strains of **Brassica**.					
				C_{16}	C_{18}	C_{20}	C_{22} Saturated	C_{22} Unsaturated	C_{24}
P.I. 169077	2	33.6	118.5	4.2	61.2	14.0	0.8	19.7	0.4
P.I. 169085	2	34.3	119.1	4.0	60.4	14.0	0.9	20.6	0.6
P.I. 179192	3	34.8	117.2	4.1	55.5	16.8	0.8	21.9	1.3
Brown Seeded B. juncea	2	35.8	106.6	3.2	50.6	11.8	0.8	23.5	0.4
Oriental Yellow	13	34.8	116.3	4.6	56.8	14.7	0.2	23.7	0.4
Montana Brown	2	34.1	121.2	4.0	56.7	15.1	0.4	24.0	0.5
Tibet No. 96	1	32.3	123.0	3.2	51.4	9.5	1.0	33.5	1.5
Japan No. 91	1	34.1	115.3	3.3	46.4	12.2	1.0	35.1	1.7
P.I. 183920	1	32.8	108.0	6.9	36.9	16.8	1.9	36.5	1.9
Chinese Broad Leaf	3	32.2	113.4	3.8	44.5	12.7	1.8	36.7	1.3
Florida Broad Leaf	2	32.1	112.7	3.6	46.8	11.2	0.6	36.8	0.7
Fordhook Fancy	2	32.6	111.5	3.2	42.9	11.6	0.6	40.8	0.8
Large Smooth Leaf	2	33.0	115.2	3.6	42.7	11.4	0.6	40.8	1.3
P.I. 195552	3	30.3	114.0	4.7	42.8	8.8	0.6	42.7	0.8
P.I. 207465	2	30.1	102.1	2.6	41.5	11.4	0.2	43.7	1.1
P.I. 173847	1	36.3	97.8	2.9	36.9	10.9	1.5	46.6	1.7
P.I. 175067	1	35.3	98.5	2.7	39.9	8.6	1.1	47.1	1.3

23

hours as usually it was difficult to find pollen in the afternoon.
Occasionally extra flowers were bagged in the morning for pollen collec-
tion, if the pollination was to be done in the afternoon.

In general, four buds were used for emasculation. They were
emasculated about two days prior to opening and pollinated immediately.
The pollinated flowers were bagged in kraft paper bags. Bags were
secured by means of a support beside the plant. This helped prevent
losses from wind. Plants were checked regularly for pod set. Bags
were removed as soon as pods developed to minimize wind injury. Selfed
and crossed seed was collected from each plant as the seed matured. At
the onset of fall, all plants were harvested irrespective of maturity.

III. Effect of Immaturity on Erucic Acid Content.

Immaturity was anticipated as a possible complicating factor as
some strains were quite late in flowering. Random eight-foot rows of
oriental yellow mustard were harvested starting about a month before
maturity. Subsequent harvests were made every four days until the final
harvest. Seeds obtained after threshing were analyzed for fatty acid
composition to determine the effect of premature harvest on erucic acid
content.

On the various dates of harvest, pod samples were collected for
moisture determinations. The percentage moisture in the seed is a
relative measure of the degree of maturity. This experiment was conducted
in the years 1961 and 1962. The correlation coefficient between percent-
age moisture and percentage erucic acid using the two years combined

data was calculated.

IV. Effect of Environment on Erucic Acid Content.

In trials conducted in 1960 and 1962, the effect of environment on percentage of erucic acid in B. juncea was studied. Four replication composited samples of oriental yellow mustard from each of the nine locations where mustard trials were conducted in 1960 were analyzed for erucic acid content. In the 1962 study, seeds of three Brassica ascessions grown at seven locations in four replications were analyzed for erucic acid content of the seed fat. Seed of replications one and two, and three and four were combined to provide two replications for analysis. A wider choice of species or strains within the genus Brassica permitted more reliable conclusions to be drawn with respect to the effect of environment on erucic acid content.

V. Chemical Analysis.

Based on a review of the literature, the fatty acid composition of the seed was considered to be controlled by the genotype of the seed rather than by the maternal plant. Seed obtained from the diallel cross constituted the F_1 population as far as the oil composition determinations were concerned. This seed and selfed seed from the parent plants were used for the determination of the oil composition.

The method of chemical analysis as suggested by Harvey (22) was followed with slight modifications. Initially, esters of a few samples were made using the method suggested by Harvey and also by the official method used for larger samples. Similarly, esters were injected in two-

foot and five-foot columns of the chromatograph to note any differences due to column length. The complete procedure for analysis, as used, is described below.

(1) Fat Extraction: For the chemical analysis, four seeds from each sample of selfed and crossed progenies were used for the determination. Seeds were cold pressed in a closed cylinder operated by hydraulic pressure. The instrument was provided with a pressure gauge so that care could be taken to maintain a pressure of less than 2500 psi. hydraulic pressure. At higher pressures the oil may undergo chemical changes due to the heat developed. The equipment used for this hydraulic press is a modification of the Carver Laboratory Press and was constructed for this study by using a hydraulic jack, a pressure gauge, a Carver cylinder and plunger, some channel iron and four long bolts. The instrument construction is illustrated in Figure 1. This apparatus cost only a fraction of the Carver Laboratory Press and proved satisfactory for these tests.

Oil obtained from the four seeds was not usually sufficient to overflow and remained on the plunger top with the meal. This meal and oil was washed from the cylinder and plunger with an excess of Skelly F into a 50 ml. beaker. Meal was separated from the oil-solvent solution by vacuum filtration using a porcelain vacuum filter. The oil-solvent solution was then transferred to a 25 ml. pear-shaped flask with ground glass neck and the solvent was removed by evaporation under vacuum using a rotary evaporator and hot water bath as illustrated in Figure 2.
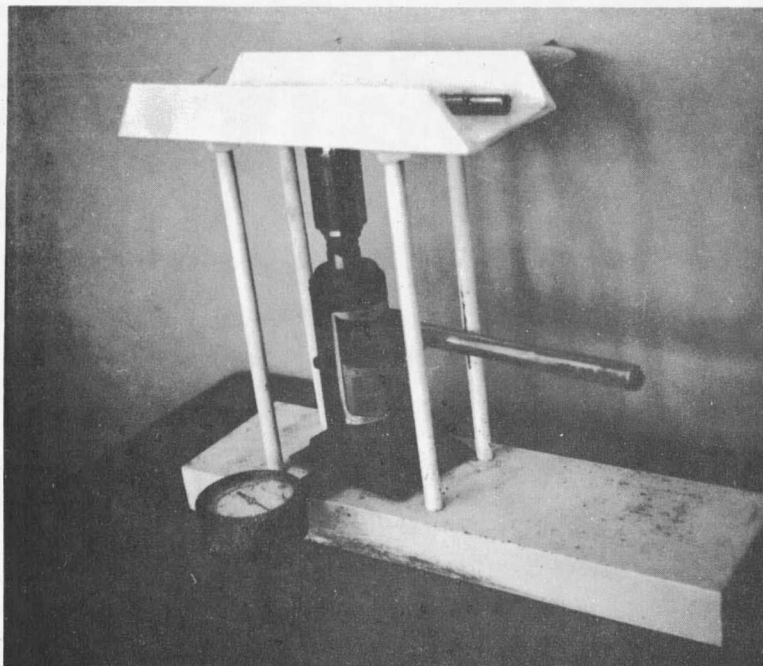
Figure 1. Laboratory press used for oil extraction.
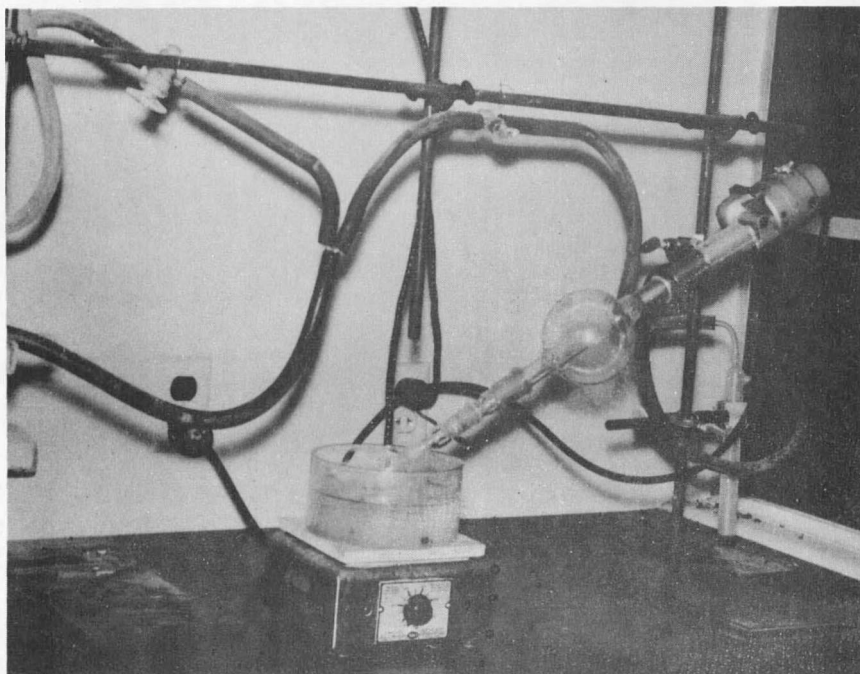
Figure 2. Rotary vacuum evaporator with hot water bath.

(2) <u>Trans-esterification</u>: After evaporation of the solvent,
5 ml. of two percent acetyl chloride in methanol was added to the oil
in the pear-shaped flask. Subsequently, the flask was attached to a
condenser and refluxed for approximately one hour. Cold water was cir-
culated through the water jacket of the condenser in order to check
evaporation of methanol. Constant heat was applied to the flask by
means of an electric heating mantle. The apparatus for the refluxing
process is shown in Figure 3.

Acetyl chloride used in the process of trans-esterification
serves as a catalyst. This is highly reactive. The solution of acetyl
chloride and methanol should be prepared fresh each day for use, as
chlorine escapes during storage. Preparation of this solution requires
special care. Acetyl chloride should be added slowly, drop by drop, to
methanol to avoid a laboratory explosion.

After refluxing the oil-methanol mixture for one hour to insure
complete esterification, the excess of methanol and acetyl chloride was
evaporated under vacuum. Esters left in the flask were picked up with
an excess of Skelly F and transferred to four-dram sample vials for
storage. The Skelly F was again evaporated off by slow heating on a hot
plate.

Methyl esters were then ready for injection into the columns of
the Aerograph (gas liquid phase chromatograph).

(3) <u>Composition Determination</u>: One to two μl. samples of esters
were drawn in a 10 μl. fixed-needle syringe and injected into the column
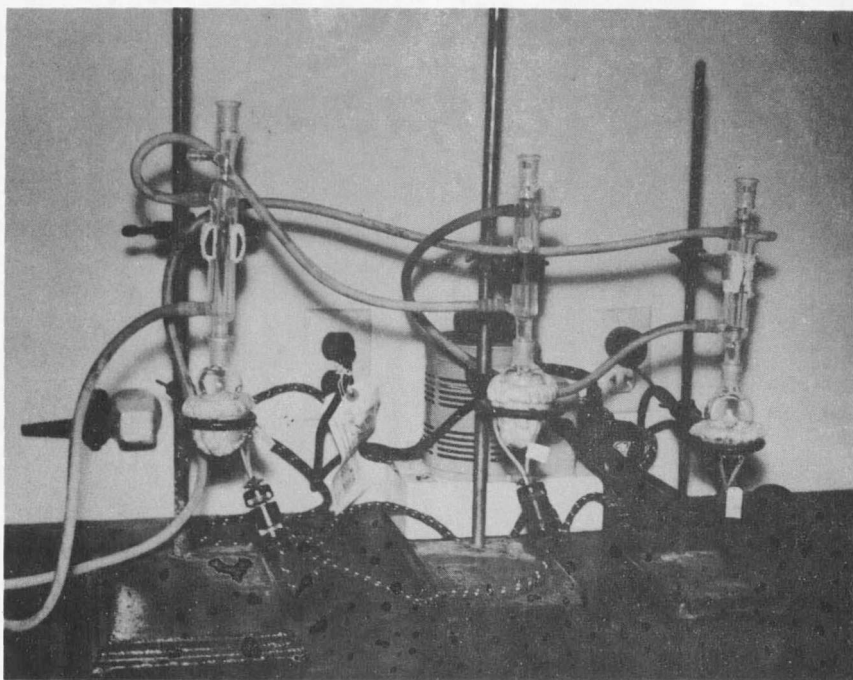
Figure 3. Apparatus for trans-esterification including condensor and heating mantle.

of the gas liquid phase chromatograph unit. The G.L.P.C. unit used for
this study consisted of a Model A - 110 C - Aerograph equipped with a
Minneapolis Honeywell Recorder illustrated in Figure 4. A disc inte-
grator was also attached to the recorder to integrate the peak area into
linear distance which is shown at the base of the recorder graph. A
24-inch column was used. Butane 1-4-diallyl succinate polyester spread
over fire-brick was used as the liquid phase. This was tightly packed
in the two-foot column. Helium gas, flowing at 17 psi. pressure through
the column served as the moving gaseous phase. The column was operated
at 230°C. The recorder chart operated at a speed of 1 cm. per minute.
Elution time at this pressure was approximately 15 minutes. The column
had to be repacked after about every 50 injections.

Elution time for different fatty acids, under a given set of condi-
tions was determined by injecting known samples. The area under each
peak was measured by the linear distance travelled by the pen of the
disc integrator in the particular time taken by each ester. Adjustments
had to be made in the reading of the integrator pen for correction of the
base line for those peaks, which may not have touched the base line. The
base line is set at electrical zero. The integrator pen is adjusted on
the basis that when the recorder pen is at zero, the integrator pen moves
parallel to the base line. Before each injection, the recorder pen is
brought to zero.

After proper adjustments for the base line parallax, areas under
different peaks were determined in terms of relative distance travelled
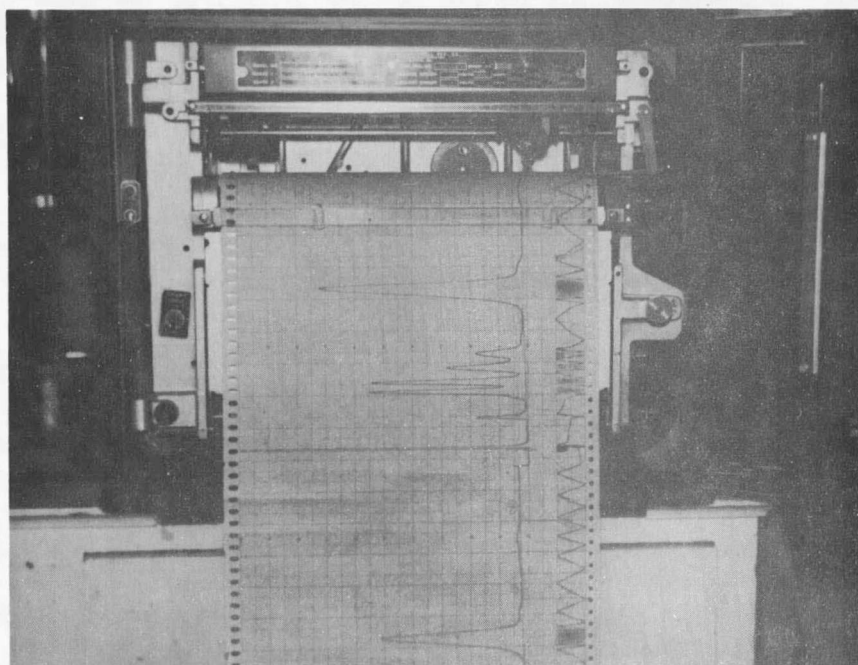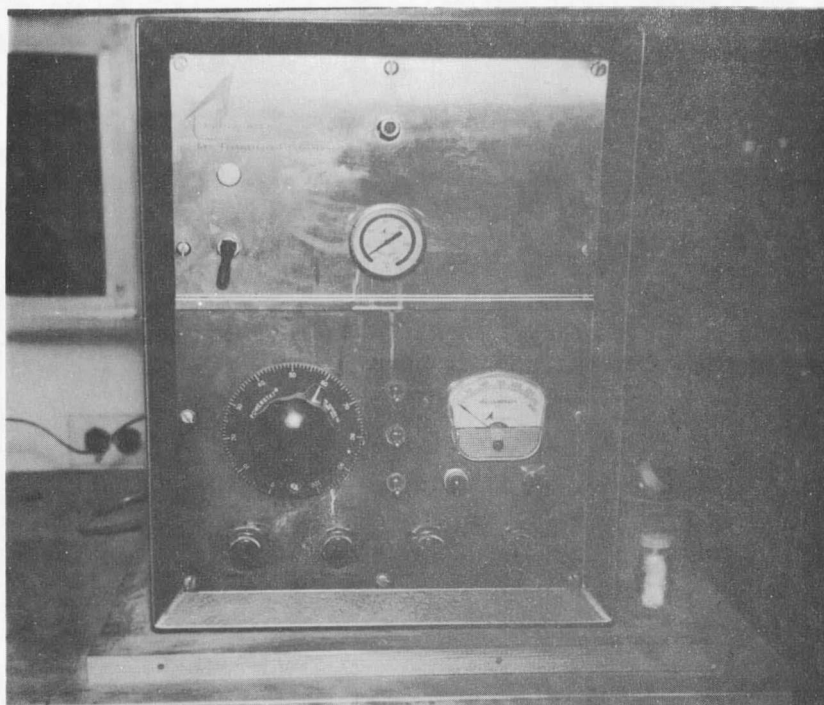
Figure 4. Gas-liquid phase Chromatograph equipment.

Top - Model A - 110 C - Aerograph
Bottom - Minneapolis Honeywell Recorder

by the integrator pen. Subsequently, erucic acid percentage in the sample was calculated. Percentage of erucic acid was determined for 450 samples which included 300 samples which represented a complete set of data for 10 parents, and their F_1 progenies, including reciprocals, for three replications. A complete set of crosses for the remaining seven lines could not be obtained due to failure of germination, probable incompatibility and late maturity.

VI. Statistical Analysis.

Statistical analysis was performed by steps. The first step was to determine if there were any significant differences among parents, among crosses, and between reciprocals within crosses. A further test of variation due to regression was conducted. These tests were performed by the methods of analysis of variance as suggested by Steele and Torrie (65). A parent-progeny regression analysis was done using the model $Y_{ij} = B_0 + B_1 X_1 + B_2 X_2 + B_3 X_3 + E_{ij}$; where, X_1 and X_2 represent the mean values of the male and female parents (obtained by averaging the three values from the three replications) and X_3 is the cross product of X_1 and X_2. The latter may be a measure of gene interaction. The extent to which the progeny may be influenced will be determined by the significance and magnitude of the regression coefficient B_3.

The partial regression coefficients were determined and tested for significance by the T- test.

At this point, an evaluation of total and averages indicated that one parental line, Florida Broad Leaf, was contributing large differ-

ences associated with reciprocals. It was suspected that perhaps this line was the principal contributor to calculated average significant differences between reciprocals.

Subsequently, a new diallel table, omitting Florida Broad Leaf, was established which included only nine parental lines. Tests for significance were performed by means of analysis of variance, as before, for differences among crosses and between reciprocals.

Data obtained from crosses, including Florida Broad Leaf, were analyzed separately for the presence of maternal effects. Regression analysis was performed as before, using only the nine parents and their F_1 generation, including reciprocals, to test whether the regression of parents on F_1 due to interaction was significant.

A further rigorous test for significance of additive and dominance variance in the diallel set was performed by the methods of analysis suggested by Hayman (25). This test is based on the method suggested by Yates (71) for measuring the significance of additive variance and for testing the reciprocal differences in diallel crosses. The appropriate statistical model is given by Yates as follows:

$y_{rs} = m + j_r + j_s + j_{rs} + k_r - k_s + k_{rs}$ where,

$y_{rs} =$ the entry in the rth row and s^{th} column of the diallel table,

m = grand mean,

j_r = mean deviation from the grand mean due to the rth parent,

j_{rs} = remaining discrepancy in the rs^{th} reciprocal sum,

$2 k_r$ = differences between the effects of the rth parental line

used as male parent and as female parent,

2 k_{rs} = remaining discrepancy in the rs^{th} reciprocal differences.

Yates suggested the method of calculating the mean sum of squares for these four constants. According to Hayman (25) they measure the following:

j_r, (a) = variation between the mean effects of each parental line which is a measure of additive variance.

j_{rs}, (b) = variations in the reciprocal sums not attributable to j_r. j_{rs} is used to measure the significance of j_r and is also a measure of dominance variance.

k_r, (c) = average maternal effect of each parental line.

k_{rs}, (d) = variations in the reciprocal differences not ascribable to k_r. This is used as error to test the reciprocal differences, k_r.

Hayman (25) in his analysis of variance used the letters a, b, c, and d to represent j_r, j_{rs}, k_r, k_{rs}, respectively, as indicated above in parenthesis. He extended this analysis by subdividing the sum of squares for j_{rs}, (b) into three components 1, 1_r, and 1_{rs}, which he denoted as b_1, b_2, and b_3, respectively. These components measure mean dominance deviation, further dominance deviation due to the rth parent, and the remaining discrepancy in the rs^{th} reciprocal sum, respectively. The extended model of Hayman is as follows:

$$y_{rs} = m + j_r + j_s + 1 + 1_r + 1_s + 1_{rs} + k_r - k_s + k_{rs}$$

In the analysis of variance 1 and l_r are tested with l_{rs} as an estimate of error. He further suggests that in the absence of reciprocal effects, the components k_r and k_{rs} will measure the environmental variance. The sum of squares for the components j_r, j_s, 1, l_r, l_{rs}, k_r, k_{rs}, are calculated by using parents and the F_1 progeny including both reciprocals. The method of analysis is one of first obtaining sums and differences of the reciprocals both for individual crosses as well as for the parental lines. This is followed by the calculation of sum of squares for the different variables. For this analysis, data from all three sets of diallel crosses are pooled together by summation, and the total values are employed for the necessary calculations. The detailed steps of calculation are presented in Appendix Tables V-VIII.

General and specific combining ability of the different lines were determined by a method suggested by Sprague and Tatum (64). A table of averages was assembled, excluding parents. The two reciprocal values were averaged. This was possible because of a lack of significant reciprocal differences. Values of totals and means for the different lines were computed. Then σ_G^2, general combining ability, was calculated by the formula given below:

$$\sigma_G^2 = \frac{n-1}{n(n-2)} \cdot \left[\left[\frac{\frac{(n\,T_i - T)^2}{2}}{\frac{n\,(n-1)(n-2)}{4}} \right] - \frac{E}{r} \right]$$, where,

n, is the number of lines, T_i is the totals of individual lines, T is the grand total, E is the standard error, and r is the number of repli-

cations. $\frac{E}{r}$ in the present study was calculated to be 0.62, and n = 9.

Specific combining ability was calculated in three steps:

(1) Actual erucic acid content multiplied by (n - 2).

(2) These above values adjusted by the appropriate rows and column totals using the following formula:

$$(n - 2)(ab) - T_a - T_b + \frac{2}{n - 1} T, \text{ where,}$$

a, b, c, ... i are the nine lines and T_a and T_b are the totals for the lines a and b respectively.

(3) This gave the specific values for determination of specific combining ability, which is given by the formula:

$$\sigma \frac{2}{S} = (X_k - \frac{E}{r}) \frac{(n - 2)}{(n - 3)}, \text{ where, k goes from a to i.}$$

$$X_a = \frac{(ab^2 + ac^2 + \ldots + ai^2)}{(n - 2)(n - 3)(n - 2)}$$

RESULTS

Of the seventeen strains which were seeded, two, Plant Introductions 173847 and 183920, did not germinate. P.I. 175067 was very late and did not start flowering by the end of the season. Fourteen strains remained for crossing and selfing. Plant Introductions 207465 and 195552 formed no seed on hybridization with other strains when these strains were used as the female parent. In reciprocal crosses, however, there was evidence of occasional seed set. Selfing of these plants was successful. These two strains were eliminated from the general analysis.

Two foliosa strains - Large Smooth Leaf and Chinese Broad Leaf, were comparatively late in flowering, so that in certain crosses well-developed seeds were not obtained. This was attributed to early frost. To verify this, strains with which these two failed to form seed under field conditions were planted in the greenhouse and crosses were made with good success.

The seed obtained was not included in the general analysis to avoid the possible effect of different environments on erucic acid content.

After this series of failures due to germination, probable cross incompatability, and immaturity, seeds from selfing and crossing were recovered from ten source lines in three replications, i.e., from three maternal plants.

Concerning the preliminary comparison of the methods of chemical analysis, the following observations were made:

(1) Variations in the percentage of erucic acid up to 3.8 per-
cent were noted, in one case, when two different methods of esterifica-
tion were used. In general, however, the differences between the two
methods were slight, as can be noted in Table II.

(2) The method of esterification had a more pronounced effect
on determining the percentage composition of lower-chain fatty acids
than erucic acid.

(3) It was observed that alcohol had to be present in excess for
complete esterification. Two ml. of methanol as suggested by Harvey
(22) for esterification of fats from single seed was found insufficient
for esterification of oil from four seeds. Better samples of esters
were obtained when 5 ml. of methanol were used for oil from four seeds.
Incomplete esterification is evidenced by inconsistency of the different
peaks in reaching the base line of the chromatograph. Differences due
to complete and incomplete esterifications as evidenced by the base line
of the chromatograph are illustrated in Figure 5.

(4) Refluxing for a period of 30 to 40 minutes was found to be
adequate if alcohol was present in excess.

(5) An excess of alcohol and acetyl chloride remaining after
esterification, if left with esterified samples during storage, resulted
in changes in fatty-acid composition.

(6) A comparison of the two-foot column with the five-foot
column indicated that the five-foot column was more efficient in separ-
ating smaller peaks which emerged at close intervals. Separation of the

Table II. Comparison of two methods of determination of erucic acid percentage in seed oil of Oriental Yellow mustard.

Sample No.	Four seed technique using 2-ft. column Total C_{22}	Official method of analysis using 5-ft. column			Difference from official method
		C_{22_I}	$C_{22_{II}}$	Total C_{22}	
	%	%	%	%	%
1.	23.3	20.0	3.1	23.1	0.2
2.	22.4	18.3	0.8	19.1	3.3
3.	23.7	19.0	0.9	19.9	3.8
4.	24.9	18.8	2.5	22.3	2.6
5.	20.9	18.9	4.1	23.0	-2.1
6.	24.3	20.0	4.8	24.8	-0.5
7.	23.2	19.3	1.8	21.1	2.1
8.	22.6	20.6	2.3	22.9	-0.3
9.	24.0	22.5	1.3	23.8	0.2
10.	24.1	22.9	2.0	24.9	-0.8
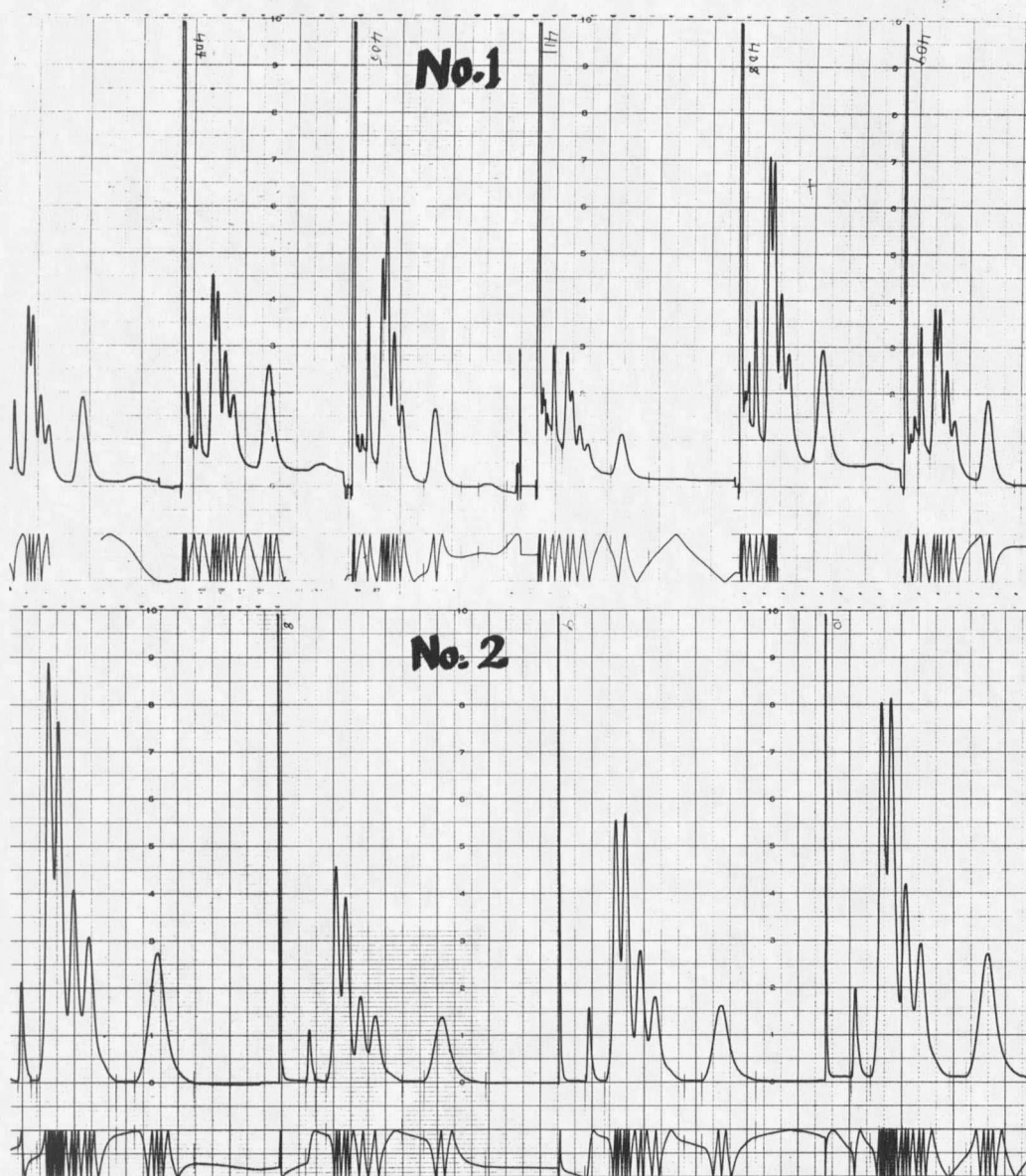
Figure 5. Two sets of graphs showing difference in base line due to
correct and faulty esterification of oil. No. 1 is faulty,
No. 2 is correct.

major fatty acids was done efficiently by the two foot column, and comparatively faster.

Effect of Environment on Erucic Acid Percentages.

The effect of growing conditions at nine different locations in 1960 on the erucic acid content of the oil of oriental yellow mustard is reported in Table III. Environment did not cause an appreciable variation in erucic acid percentage. A total variation of 3.6 percent was noted, whereas, in the present study, erucic acid content of different strains ranges from 20.2 to 45.7 percent.

Data obtained from the chemical determination of erucic acid percentage in the oil of three Brassica accessions grown at seven locations in 1962 are reported in Table IV. The analysis of variance of the data, Table V, indicated significant differences between accessions for erucic acid content at the five-percent level. The location influence on percentage erucic acid was more pronounced than the variation due to varieties. Variation in erucic acid content due to location was significant at the one-percent level. The variety x location interaction was significant at the five-percent level. The maximum range within a variety was 5.2 percent.

Effect of Maturity on Percentage of Erucic Acid.

Results of moisture and erucic acid composition determinations for ten samples from 1961 and twelve samples from 1962 are presented in Table VI. The relationship between percentage of moisture and per-

Table III. Effect of growing conditions at nine locations on the erucic
acid content in the oil of Oriental Yellow mustard grown
during the year 1960.

Location	Condition	Percentage erucic acid in seed oil
Bozeman	Irrigated	22.5
Huntley	Irrigated	21.5
Sidney	Irrigated	20.6
Creston	Irrigated	23.9
Huntley	Dryland	21.5
Sidney	Dryland	21.0
Creston	Dryland	22.6
Moccasin	Dryland	22.5
Havre	Dryland	20.3
	Average	21.8

Table IV. Effects of growing conditions at seven locations on the
erucic acid content in the seed fat of three __Brassica__ acces-
sions grown during the year 1962.

Locations	__Brassica juncea__ Oriental Yellow	__Brassica juncea__ Selection	__Brassica pervidis__ Tender green	Average
	%	%	%	%
Creston, irrigated	22.0	19.9	18.2	20.0
Sidney, irrigated	23.6	24.2	21.5	23.1
Bozeman, irrigated	19.6	20.0	18.8	19.5
Creston, dryland	21.3	19.8	18.3	19.8
Sidney, dryland	20.7	24.8	22.4	22.6
Havre, dryland	22.3	25.0	20.6	22.6
Moccasin, dryland	23.8	22.0	18.9	21.6
Average	21.9	22.2	19.8	21.3
Maximum range	4.2	5.2	4.2	3.6

Table V. Analysis of variance of effect of growing conditions at seven locations on erucic acid content in the oil of three accessions of _Brassica_ grown during the year 1962.

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F - test
Replication	1	0.14	0.140	
Varieties	2	48.61	24.305	17.06*
Error (a)	2	3.85	1.425	
Sub-total	5	52.60		
Locations	6	83.03	13.838	8.81**
Variety x Location	12	45.55	3.796	2.42*
Error (b)	18	28.27	1.570	
Total	41	209.45		

*Significant at the five percent level.
**Significant at the one percent level.

Table VI. Percentage erucic acid in oil from seed samples of oriental yellow mustard, harvested on successive dates in the years 1961 and 1962.

	1961		1962	
Date of harvest	Percent moisture in seed at harvest	Percent erucic acid in oil	Percent moisture in seed at harvest	Percent erucic acid in oil
	x	y	x	y
August 16	65	23.3	72	21.3
August 20	64	22.4	68	22.4
August 24	60	23.7	61	21.9
August 28	55	24.9	66	23.9
Sept. 1	51	20.9	64	22.8
Sept. 5	40	24.3	62	20.3
Sept. 9	35	23.2	53	23.1
Sept. 13	35	22.6	44	21.1
Sept. 18	20	24.0	23	23.6
Sept. 24	-	-	13	22.8
Sept. 29	-	-	10	23.7
October 4	-	-	9	24.1
October 6	19	24.1	-	-

centage of erucic acid was found to be negative as evidenced by a corre-
lation coefficient of -0.41, which is not significant at the five percent
level. The percentage of erucic acid in the 22 determinations was with-
in a range of 20.3 to 24.1, i.e., a difference of 3.8 percent, which was
not consistently associated with the date of harvest.

Erucic Acid Inheritance.

Chemical analysis indicated differences in percentage of erucic
acid in selfed and hybrid seeds obtained from the same plant. Differences
in erucic acid composition between selfed and crossed seeds from a single
plant of oriental yellow mustard as evidenced by the relative area under
the peak in the chromatograph are illustrated in Figure 6.

Results of chemical analysis of selfed and crossed seed of 10
geographical lines (average of three sets) are presented in Table VII.
The data for the three sets of diallel crosses are given in Appendix
Tables I, II, and III.

Results of the statistical analysis of erucic acid percentage of
selfed and F_1 hybrids of a diallel cross involving 10 parental lines are
presented in Table VIII. Differences among crosses were found signi-
ficant at the one percent level. Differences between reciprocals were
found significant at the five percent level.

In the regression analysis reported in Table IX, variation between
crosses due to regression was found to be significant at the one percent
level. The regression equation was $Y_{ij} = B_o + B_1 X_1 + B_2 X_2 + B_3 X_3 + E_{ij}$.
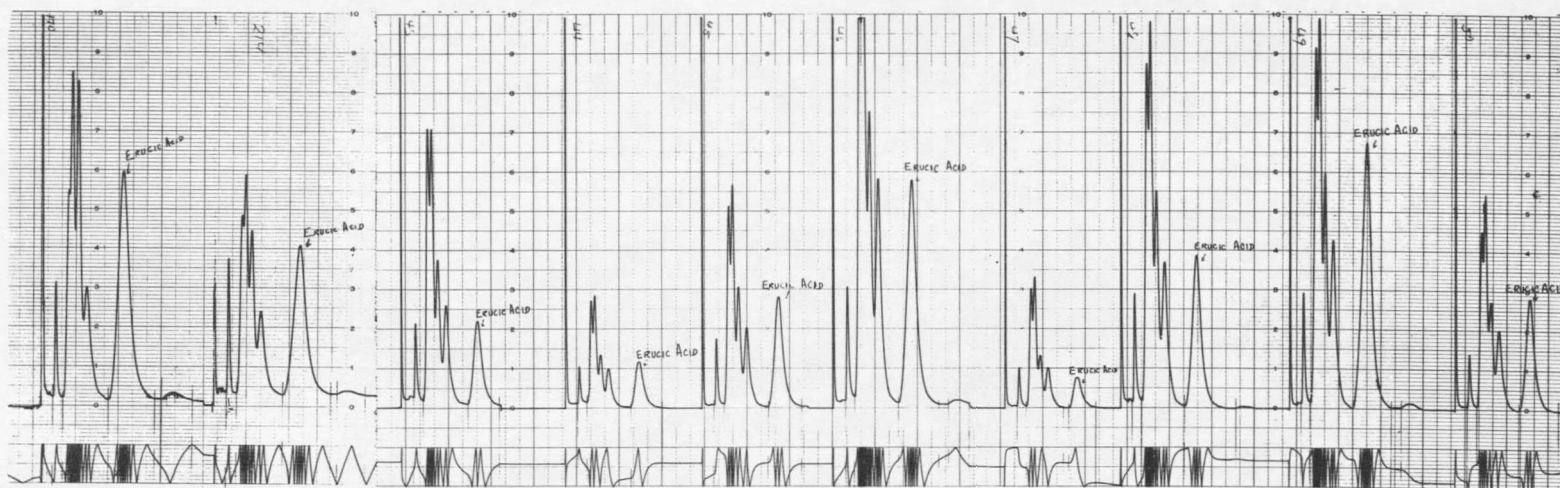
Figure 6. Relative size of different peaks obtained when oil from seed of the same plant of oriental yellow mustard was used. The area under the erucic acid peak for any sample is measured in relation to the area under all peaks for the sample. Chromatograph 47 is a self. The others are from the oil from crossed seed.

Table VII. Erucic acid percentage in the oil from selfed parents (underscored) and their F_1 progenies in a diallel cross of ten strains of B. juncea of different geographical origins (average of three replications).

Parents Female → Male ↓	Percentage erucic acid in										Total
	Montana Brown	P.I. 179192	Oriental Yellow	P.I. 169085	Fordhook Fancy	P.I. 169077	Brown Seeded B. juncea	Tibet No. 96	Florida Broad Leaf	Japan No. 91	
Montana Brown	<u>21.6</u>	21.7	27.7	22.8	32.6	22.7	25.8	41.2	38.1	34.8	289.0
P.I. 179192	26.0	<u>22.8</u>	24.8	25.5	36.0	27.3	21.7	30.3	39.2	35.6	289.2
Oriental Yellow	24.7	28.5	<u>23.2</u>	24.3	33.9	21.9	22.0	34.2	38.0	37.5	288.0
P.I. 169085	26.7	25.5	26.8	<u>25.2</u>	33.5	25.2	23.1	33.0	42.3	41.3	302.6
Fordhook Fancy	38.6	31.2	38.7	39.1	<u>28.6</u>	32.8	30.4	35.5	44.3	39.9	359.1
P.I. 169077	25.0	26.6	24.2	24.8	34.2	<u>28.7</u>	21.6	33.2	35.4	34.2	287.9
Brown Seeded B. juncea	20.5	26.6	28.0	27.8	36.1	24.9	<u>32.9</u>	30.9	42.7	26.0	296.4
Tibet No. 96	33.3	31.8	32.9	24.8	42.1	30.2	29.5	<u>36.4</u>	38.3	40.5	339.8
Florida Broad Leaf	34.2	36.4	25.8	21.2	33.6	33.1	35.4	38.7	<u>40.7</u>	41.9	351.0
Japan No. 91	38.1	35.7	38.2	37.8	40.6	31.1	35.2	40.6	40.9	<u>41.3</u>	379.5
Total	288.7	286.8	290.3	283.3	351.2	277.7	277.6	354.0	399.9	373.0	<u>3182.5</u>

Table VIII. Analysis of variance to determine source of variation, in erucic acid content in seed oil from a diallel cross with ten parental lines of Brassica juncea.

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F - test
Crosses	89	10756.44	120.86	59.0**
Reciprocals	1	13.06	13.06	6.37*
Within crosses (Error)	180	368.71	2.05	
Total	269	11125.15		

**Significant at the one percent level.

*Significant at the five percent level.

Table IX. Determination of the significance of multiple regression due to males, females, and cross product, males x females, on erucic acid content of the oil from the F_1 progeny in a diallel cross of ten parental lines of *Brassica juncea*.

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F - test
Regression	3	4506.74	1502.25	20.67**
Deviation from regression (Error)	86	6249.70	72.67	
Total	89	10756.44		

**Significant at the one percent level.

Values of regression coefficients obtained for the regression equation are given below.

Symbols	Regression coefficient	Calculated t - value of the partial correlation coefficient	Measures of
B_0	-10.2402	--	
B_1	1.0431	3.04**	effect of female parent
B_2	0.9198	2.68**	effect of male parent
B_3	- 0.0187	1.67	effect of male x female interaction

The interaction effect was small and non-significant when tested at the five percent level.

Similar analysis for nine parental lines are reported in Tables X and XI. Differences between crosses were found to be significant at the probability level of one percent. The basic table for calculations is appended as Appendix Table IV.

Variation between crosses due to regression was also found to be significant at the one percent level. Values of the regression coefficients obtained from the regression analysis of the diallel set of nine parental lines are presented below.

Table X. Analysis of variance to determine sources of variation, in erucic acid content in seed oil, from a diallel cross with nine parental lines of <u>Brassica juncea</u>.

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F - test
Crosses	71	7848.16	110.54	59.43**
Reciprocals	1	0.95	0.95	<1
Within crosses (Error)	144	287.62	1.86	
Total	215	8115.78		

**Significant at the one percent level.

Table XI. Determination of the significance of multiple regression due to males, females, and cross product, males x females, on erucic acid content of oil from the F_1 progeny in a diallel cross of nine parental lines of <u>Brassica</u> <u>juncea</u>.

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F - test
Regression	3	2349.74	783.25	9.69**
Deviation from regression (Error)	68	5498.42	80.85	
Total	71	7848.00		

**Significant at the one percent level.

Symbols	Regression coefficient	Calculated t - value of the partial correlation coefficient	Measures of
B_0	-14.4839	--	
B_1	1.1600	-2.44*	effect of female parent
B_2	1.1983	2.52*	effect of male parent
B_3	- 0.0277	1.69	female x male interaction

The B_3 value was determined to be non-significant, as in the previous analysis.

The analysis of variance of the diallel table of nine parents indicated that differences due to reciprocal effects were non-significant when the Florida Broad Leaf strain was not used in the study. The analysis of variance for testing crosses involving Florida Broad Leaf is presented in Table XII. The F - test indicated the presence of significant variation due to crosses, due to reciprocals and due to crosses x reciprocals.

Results obtained thus far may be summarized as follows:

(1) Wide variations in erucic acid percentage were observed to be significant in different crosses.

(2) These variations between F_1 crosses were associated with erucic acid level of both male and female parents.

(3) Additive gene effects for erucic acid percentage were evident in the ten strains under study.

(4) Dominance influences were indicated as low and non-significant and not symetrically distributed.

Table XII. Analysis of variance for testing significance of variation
in erucic acid content between crosses and between recipro-
cals in a set of crosses involving Florida Broad Leaf as
male and female parent with the nine strains of _Brassica_
juncea.

Sources of variation	Degrees of freedom	Sum of squares	Mean square	F - test
Crosses	8	386.25	48.28	18.78**
Reciprocals	1	398.75	398.75	155.15**
Crosses x reciprocals	8	309.21	38.65	15.08**
Error	36	92.44	2.56	
Total	53	1186.65		

**Significant at the one percent level.

(5) There was evidence of symetrical reciprocal differences in crosses involving Florida Broad Leaf whereas such symetrical reciprocal effects were not evident in crosses involving the other nine parental lines.

Data for the nine parental diallel were analyzed by the method suggested by Hayman (25). Results obtained confirmed the results obtained by the regression analysis discussed previously. Complete results of the analysis are presented in Table XIII. From this analysis it may be concluded that:

(1) Variance attributed to additive gene effect is highly significant.

(2) Variance attributed to the average maternal effect was nonsignificant. This indicated asymetrical distribution of any maternal effects in the nine parental lines.

(3) Variance attributed to mean dominance deviation was nonsignificant. Dominance deviations attributed to individual parents were significant. This indicates asymetrical distribution of dominance deviations in certain parents. For those interested, the details of calculations are included as Appendix Tables VI, VII, and VIII.

Results of analysis to determine the general and specific combining ability for the nine parental strains are presented in terms of σ^2_G and σ^2_S in Tables XIV and XV.

Examination of the means of different parental lines and values of σ^2_G associated with each parental line indicate that lines five and ten have good general combining ability for high erucic acid content. Line

Table XIII. Analysis of variance of erucic acid content for testing
significance for different components of genetic variance
and reciprocal differences in a set of diallel crosses
involving nine geographical strains of Brassica juncea.

Sources of variation 1/	Degrees of freedom	Sum of squares	Mean square	F - test
a	$(n-1) = 8$	6352.38	794.05	17.29**
b_1	1	68.49	68.49	2.64
b_2	$(n-1) = 8$	884.18	110.52	4.26**
b_3	$\frac{1}{2}n(n-3) = 27$	700.93	25.96	
b	$\frac{1}{2}n(n-1) = 36$	1653.60	45.93	1.50
c	$(n-1) = 8$	124.67	15.58	0.50
d	$\frac{1}{2}(n-1)(n-2)=28$	856.03	30.57	
Total	$(n^2-1) = 80$	8986.68		

**Significant at the one percent level.
1/
a = Measure of additive variance.
b_1 = Mean dominance deviation.
b_2 = Dominance deviation due to individual parents.
b_3 = Error variance for testing dominance.
b = Error variance for testing additive variance, and also a
       measure of dominance variance.
c = Average maternal variation
d = Error for testing maternal effect and also for testing
       dominance variance (b).

Table XIV. Average values of erucic acid percentage in the oil from the selfed parents of nine geographical strains and their F_1 progenies (average of both reciprocals from three replications).

Parents	Percentage erucic acid in indicated cross									Total
	Montana Brown	P.I. 179192	Oriental Yellow	P.I. 169085	Fordhook Fancy	P.I. 169077	Brown Seeded B. juncea	Tibet No. 96	Japan No. 91	
Montana Brown	21.6	23.8	26.2	24.7	35.6	23.8	23.1	37.2	36.4	252.4
P.I. 179192	23.8	22.8	26.6	25.5	33.6	26.9	24.1	31.0	35.6	249.9
Oriental Yellow	26.2	26.6	23.2	25.5	36.3	22.9	25.0	33.5	37.8	257.0
P.I. 169085	24.7	25.5	25.5	25.2	36.3	25.0	25.4	28.9	39.5	256.0
Fordhook Fancy	35.6	33.6	36.3	36.3	28.6	33.5	33.2	38.8	40.2	316.1
P.I. 169077	23.8	26.9	22.9	25.0	33.5	28.7	23.2	31.7	32.6	248.3
Brown Seeded B. juncea	23.1	24.1	25.0	25.4	33.2	23.2	32.9	30.2	30.6	247.7
Tibet No. 96	37.2	31.0	33.5	28.9	38.8	31.7	30.2	36.4	40.5	308.2
Japan No. 91	36.4	35.6	37.8	39.5	40.2	32.6	30.6	40.5	41.3	334.5

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Table XV. Average percentage erucic acid in oil of the F_1 progenies
of each of the nine geographical strains and the calculated
values of general and specific combining ability as deter-
mined from a diallel cross.

Parental lines	Mean F_1's	Sigma Square G	Sigma Square S
1. Montana Brown	28.89	4.29	6.36
2. P.I. 179192	28.42	6.78	1.81
3. Oriental Yellow	29.26	2.71	2.10
4. P.I. 169085	28.88	4.35	6.78
5. Fordhook Fancy	35.95	35.61	3.23
6. P.I. 169077	27.48	13.59	2.46
7. Brown Seeded B. juncea	26.88	19.44	1.38
8. Tibet juncea	34.00	16.66	7.27
10. Japan No. 91	36.69	46.58	7.36

eight has medium general combining ability and lines one, two, three and four possess average general combining ability. Lines six and seven though associated with higher values of σ^2_G are poor combiners.

Comparatively high values of σ^2_S are associated with parental lines eight and ten which also have high values of σ^2_S. The two lines, Fordhook Fancy and Japan No. 91, are the most likely parents for obtaining crosses producing advanced generations with higher erucic acid content whereas Tibet juncea needs further testing.

Results of incomplete F_1 hybrids and parents not included in the analysis are recorded in Appendix Table IX for those who may have an interest in this incomplete data.

DISCUSSION

That three of the new Plant Introductions, from India and Nepal, did not establish plants in the field is characteristic of new introductions when macro-climatic changes are involved.

The two strains P.I. 207465 and P.I. 195552 did germinate, grow, and form seed on selfing. No hybrid seed was formed on the plants when pollen from other strains of B. juncea was used for cross pollination. This indicated some type of cross incompatability was involved. Occasional seed set was obtained in crosses of reciprocal types, i.e., when the pollen of these two strains was used to pollinate plants of the other strains of B. juncea.

Plants of these two strains were different morphologically from the remaining strains. Morphology of leaves, pods and beaks indicated that P.I. 207465 was of the species B. campestris rather than B. juncea. Similar studies of P.I. 195552 indicated that this was quite dissimilar to other strains in morhphological characters and has since been identified as B. carinata. Distinguishing morphology of these two strains in comparison to the other ten strains (under study) can be noted in Figures 7 and 8.

Results of crosses involving these two strains could be explained on the basis that inter-species hybridization was being attempted. Moringa (48, 50), Howard (32), Frandsen (13, 14), and Olsson (54, 55, 56) have reported cases of inter-species hybridization in Brassica. Better success was achieved when the maternal plant was the one having the higher number of chromosomes. The failure of seed set when P.I. 207465

ORIENTAL-YELLOW | FLORIDA-BROAD LEAF | P.I. 169077 (TURKEY) | FORD-HOOK-FANCY | P.I. 169085 (TURKEY) | TIBET.No:96 TIBET

P.I. 179192 (TURKEY) | JAPAN.No.91 CHINA | MONTANA BROWN | BROWN SEEDED JUNCEA | P.I. 207465 AFGANISTAN | P.I.195552 ETHIOPIA

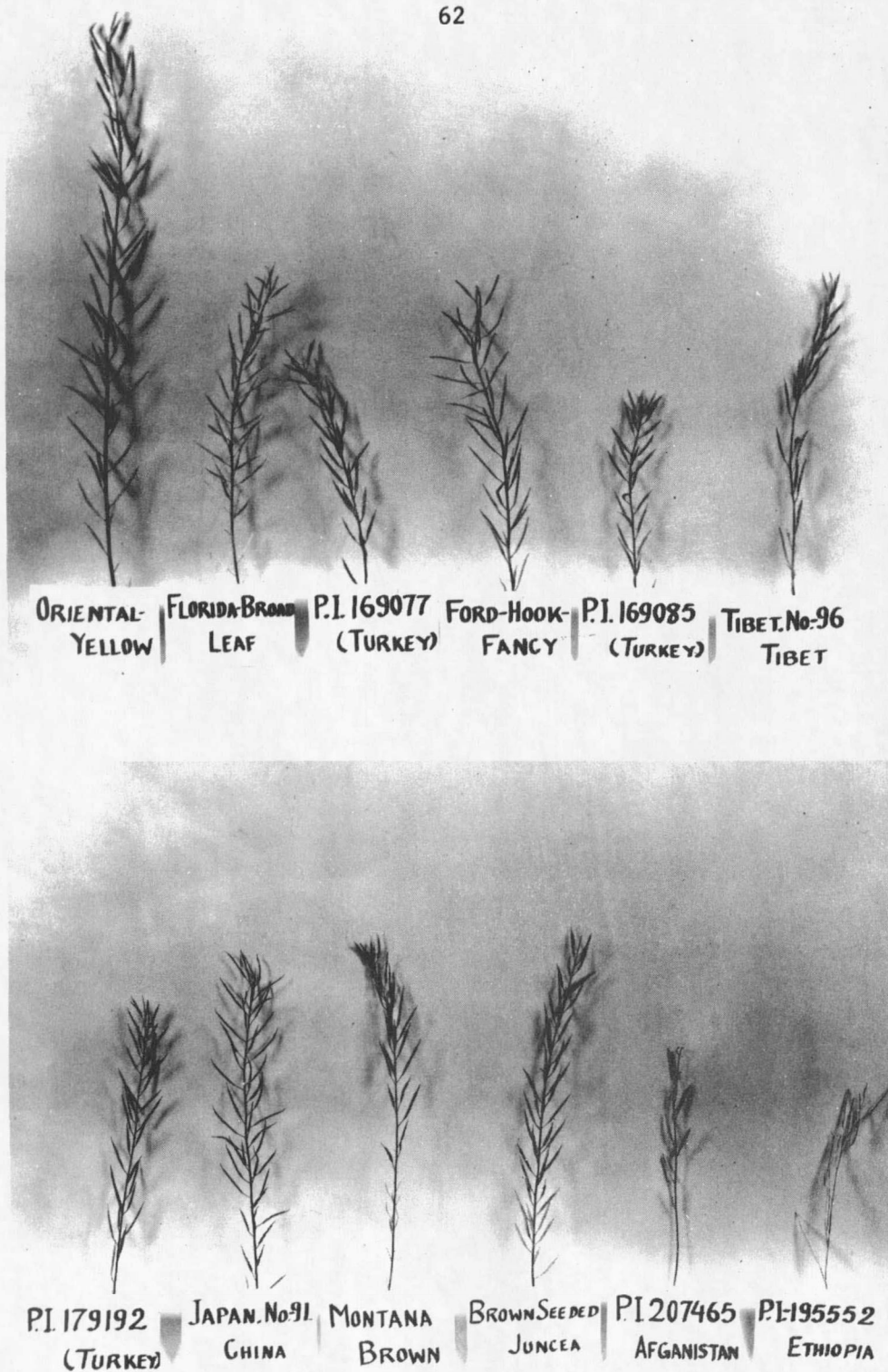Figure 7. The arrangement of pods in the racemes of twelve
Brassica strains.
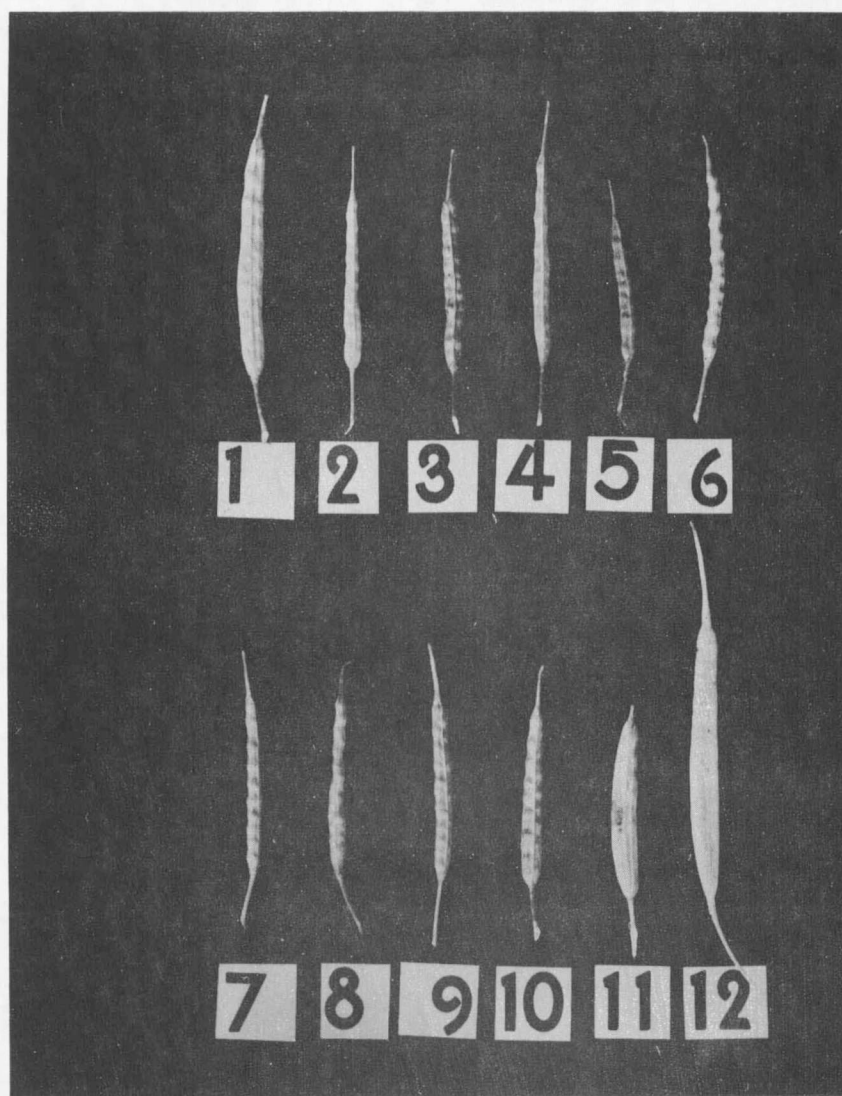
Figure 8. The shape of pod and length of beak of twelve
strains of *Brassica*.

1. Oriental Yellow
2. Florida Broad Leaf
3. P.I. 169077
4. Fordhook Fancy
5. P.I. 169085
6. Tibet No. 96
7. P.I. 179192 (Turkey)
8. Japan No. 91 (China)
9. Montana Brown
10. Brown Seeded *B. juncea*
11. P.I. 195552 (Ethiopia)
12. P.I. 207465 (Afganistan)

(n = 10) and P.I. 195552 (n = 17) were used as female parents in a cross
with B. juncea (n = 18) could be due to this same phenomenon.

The other strains appeared to be quite uniform in their morphologi-
cal characters for the types and also conformed to the species character-
istics. Each strain represented a type of its own quite distinct from
the others in some respect.

In order to determine inheritance of a quantitative character, it
is desirable to know as much as possible about the influence of environ-
ment on the character under study. If the character is not markedly in-
fluenced by environment and a genotype x environment, interaction does not
exist, then the results may be given broader interpretation and the physical
conduct of a study may be considerably simplified. The environmental
studies conducted in conjunction with this study resulted in no significant
differences for one genotype grown at nine locations in 1960 but did in-
dicate significant location effects for three genotypes grown at seven
locations in 1962. Considering the range of erucic acid content of the
parental lines to be studied, only small environmental effects on erucic
acid content were noted. These two trials indicated that environment is
a minor consideration in studying inheritance of major gene differences for
erucic acid content. Craig (4) reported similar results from his study of
environmental effects on erucic acid percentage in eight varieties of
B. napus.

The parents for the present study varied considerably in maturity.
To determine if the maturity of the seed at harvest had an influence on
erucic acid content, date of harvest studies were conducted in 1961 and

1962. Moisture content of the seed was used as an indication of maturity. The mositure content of the seed declines as seed matures. Percentage moisture in the seed harvested at the last date of harvest was down to about 10 percent in the year 1962, whereas moisture content of the seed on the initial harvest date was much higher. Varying percentages of erucic acid were obtained on different dates and it was hoped to establish a relationship, if any, between these two measures. A negative correlation coefficient was obtained which was not significant. This indicated that erucic acid percentage was not influenced by maturity at harvest.

An analysis of the results of erucic acid determination of the oil from selfed and crossed seed obtained from the same plant gave evidence of significant differences due to crosses. This supported the theory that erucic acid content is controlled to some degree by the genotype of the seed rather than by the sporophyte mother plant. Work by Harvey (23) with B. napus and Yermanos, et al. (72) with flax support these findings. Carbohydrates needed for fat synthesis or for the synthesis of different fatty acids are synthesized by the mother plant. This is influenced by the genotype of the mother plant as well as the environment in which the mother plant is grown. Thus, quality and quantity of starch synthesized by the mother plant and the translocation to the areas of fat synthesis may be the cause of the reciprocal differences noted in one strain.

In the presence of large maternal effects, correct genetic estimates could not be made with reliability. A line exhibiting reciprocal differences was removed from the diallel before determination of the components of genetic variation.

The genetic study was approached in two ways:

(1) By means of regression analysis the following information was provided:

(a) Some of the differences between crosses were due to parent-progeny regression, i.e., genetic.

(b) There was significant additive gene effects for erucic acid content of the ten strains under study.

(c) Interaction effects were statistically non-significant in these lines, on the average.

(2) The method of analysis of variance for diallel crosses, as suggested by Hayman(25), provided the second method of analysis. This method also tested for the significance of additive and dominance gene effects but the method further separated the dominance effect into two components: (a) average dominance deviations, and (b) dominance deviation contributed by lines.

By applying this method, the significance of the dominance effect of the lines was tested and the results obtained by the previous method were varified. This test indicated the presence of dominance deviation due to certain lines.

Hayman's method is based on the following main assumptions:

(1) no maternal effect

(2) diploid segregation

(3) homozygosity for the factor or factors under study.

The first assumption was tested and verified in the course of the analysis. The assumption of diploid segregation in B. juncea is supported

by Moringa (50). The assumption of homozygosity for the loci is not as easily verified. As discussed earlier, there would seem little doubt that B. juncea is an amphidiploid species which originated as a result of inter-specific hybridization and subsequent doubling. This suggests homozygosity at each locus except for instances of mutations subsequent to the formation of the amphidiploid. Results of our study of erucic acid in three sets indicates a constancy of erucic acid percentage in each line and F_1 hybrid. This may be interpreted to mean that loci controlling erucic acid formation are probably homozygous.

After the determination of the presence of additive gene effects and/or random dominace effects as shown by the analysis of variance, the next step was to locate which line or lines were contributing the major additive and/or dominance gene effects. This was achieved by calculating the general and specific combining ability of the different lines.

There was evidence of good general combining ability in lines five and ten. Line five, however, had a much lower specific combining ability than line ten, which indicated that this line was uniform in transmitting its erucic acid character to its progeny. The higher value of specific combining ability associated with line ten is an indication that certain specific combinations of this line are better in erucic acid than others and might be contributing to the higher value. Line eight exhibited a medium value for general combining ability associated with a higher value for specific combining ability. Lines eight and ten need further evaluation.

The three parental lines - Japan No. 91, Fordhook Fancy and Tibet No. 96 appear to be promising for future use in evolving lines with higher erucic acid content.

SUMMARY

Erucic acid is a monoethenoid acid with 22 carbon atoms. This has many potential commercial uses and its presence in considerable quantities in seed oil from species of the family Cruciferae could make this family important commercially. Commercial varieties of mustard suited to this climate are high yielding, both with respect to seed and oil, but are low in erucic acid content. Chemical examination of oils from some of the geographical strains indicated the presence of high erucic acid content. This gave an indication of potential sources from which genes for erucic acid could be transferred to the commercial strain. To achieve this, the type of gene action for erucic acid and also the general and specific combining ability of these strains needed to be known. This formed the main objective of this study.

Chemical analysis of seed for fatty acid composition was done by a method of micro-quantitative analysis. The method was appropriately modified and checked for use in this particular study. Four seeds were pressed in a laboratory press and oil extracted by an excess of Skelly F. Trans-esterification of oil was achieved by refluxing oil with 5 ml. of 2 percent acetyl chloride in methanol for approximately one hour. The excess of methanol and acetyl chloride left was evaporated. The methyl esters obtained were injected into a gas liquid phase chromatograph for composition determination. A two-foot column was used.

Effect of date of maturity on erucic acid content of seed oil was estimated by harvesting plants at four-day intervals starting about a month before maturity in the years 1961 and 1962. Moisture percentage

and erucic acid percentage of these samples were determined and the cor-
relation between these two factors was determined to be negative and non-
significant. It was concluded that seed maturity was not a major factor
in determining erucic acid content of the seed oil.

The effect of different environmental conditions on erucic acid
content of seed oil was determined by analyzing one entry grown at nine
locations in 1960 and three Brassica accessions grown under seven different
climatic conditions in four replications in 1962. Location had an effect
on erucic acid content of seed oil in 1962 but not in 1960 which indicated
that certain environments did significantly influence the erucic acid per-
centage of the seed oil and that the effect was not uniform for different
strains at all locations. The magnitude of the effects was not suffic-
iently great to interfere with the determination of major genetic
differences.

Seventeen strains of Brassica differing in geographical origin
and erucic acid content of the seed oil were selected for a study of
erucic acid inheritance. Seed from each of the seventeen strains were
planted in hills three feet apart, in three replications, at Bozeman, in
the year 1961. These plants were selfed and crossed to each other in
all possible combinations. Selfed and crossed seed obtained were analyzed
for erucic acid content. Chemical analysis was followed by statistical
analysis to determine the mode of gene action and the general and specific
combining ability of the different strains.

Seven strains were not included in the final analysis for reasons
of failure to germinate, late maturity, or incompatability. Ten parental

lines were finally examined for gene action. Statistical analysis was done by two different methods suggested by different workers. Analysis by methods of correlation and regression resulted in the finding of significant additive gene effects, non-significant dominance effects, and a maternal effect, when the ten parental lines were examined. When they were re-examined after exclusion of crosses involving Florida Broad Leaf, the maternal effect was found to be non-significant. Analysis of crosses involving Florida Broad Leaf separately, indicated significant differences among crosses, and the presence of a significant maternal effect.

The data obtained from the nine parental lines were further analyzed by means of analysis of variance as suggested by Hayman (25). Results of analysis indicated presence of significant additive effects, non-significant average dominance effects, and an indication of random dominance effects due to certain lines. The maternal effect was found to be non-significant.

A further analysis was conducted utilizing the method suggested by Sprague and Tatum (64) to determine which lines were contributing to the major genetic variance. In this analysis, average calculated values from both the reciprocals were used to calculate the general and specific combining ability of each line.

The results indicated that three strains - Japan No. 91, Fordhook Fancy, and Tibet No. 96 appear to be better combiners and could be used to the best advantage in a future breeding program for the improvement of erucic acid content in Montana commercial oriental yellow mustard.

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APPENDIX

Appendix Table I. Erucic acid percentage in the oil from selfed parents and their F_1 progenies in the diallel cross of ten strains of B. juncea of different geographical origin - Set I.

Parents Female Male → ↓	Percentage erucic acid in										Total
	Montana Brown	P.I. 179192	Oriental Yellow	P.I. 169085	Fordhook Fancy	P.I. 169077	Brown Seeded B. juncea	Tibet No. 96	Florida Broad Leaf	Japan No. 91	
Montana Brown	21.1	22.0	27.8	24.8	31.2	22.4	26.9	41.5	37.7	33.1	288.5
P.I. 179192	29.2	23.5	25.0	25.2	35.4	27.8	20.8	31.4	38.7	35.6	292.6
Oriental Yellow	24.5	27.3	25.1	24.3	35.0	20.7	22.8	33.0	37.4	38.4	288.5
P.I. 169085	28.3	25.0	28.8	26.3	32.3	26.0	23.1	32.5	39.8	40.6	302.7
Fordhook Fancy	37.7	30.4	39.1	40.4	28.9	32.0	29.8	37.0	45.1	38.8	359.2
P.I. 169077	25.7	27.1	23.4	26.2	34.1	30.3	20.5	33.4	35.1	35.0	290.8
Brown Seeded B. juncea	21.5	27.8	26.9	28.3	37.1	25.5	33.8	29.2	42.3	26.4	298.8
Tibet No. 96	30.7	32.8	34.5	26.2	42.4	30.3	27.8	35.7	38.7	40.9	340.0
Florida Broad Leaf	35.3	34.9	26.9	32.3	33.6	30.2	34.3	40.6	39.2	40.7	348.0
Japan No. 91	38.4	36.7	38.2	39.4	39.5	32.7	33.5	40.1	42.4	38.7	379.6
Total	292.4	287.5	295.7	293.4	349.5	277.9	273.3	354.4	396.4	368.2	3188.7

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Appendix Table II. Erucic acid percentage in the oil from selfed parents and their F_1 progenies in the diallel cross of ten strains of <u>B</u>. <u>juncea</u> of different geographical origin - Set II.

Parents Female → Male	Percentage erucic acid in										Total
	Montana Brown	P.I. 179192	Oriental Yellow	P.I. 169085	Fordhook Fancy	P.I. 169077	Brown Seeded <u>B</u>. <u>juncea</u>	Tibet No. 96	Florida Broad Leaf	Japan No. 91	
Montana Brown	20.8	21.9	27.7	21.7	33.5	23.2	25.6	41.8	37.6	36.3	290.1
P.I. 179192	24.5	23.4	24.7	25.5	36.4	25.1	20.9	30.7	37.5	36.7	285.4
Oriental Yellow	24.8	29.7	24.4	26.0	35.2	21.2	21.3	34.6	38.6	37.7	293.5
P.I. 169085	26.2	25.2	25.6	25.0	32.6	26.5	22.2	33.9	42.0	40.3	299.5
Fordhook Fancy	38.0	31.4	39.3	38.9	28.2	35.6	29.0	33.5	42.1	40.2	356.2
P.I. 169077	25.9	26.2	24.0	25.6	34.9	28.9	21.6	32.4	37.1	35.2	291.8
Brown Seeded B. juncea	20.2	26.9	28.8	26.9	37.7	24.9	32.8	32.2	40.5	27.7	298.6
Tibet No. 96	34.6	30.9	32.3	23.4	41.5	30.8	30.7	36.8	38.0	42.0	341.0
Florida Broad Leaf	34.2	37.1	24.3	32.9	33.4	35.3	36.5	36.4	39.2	41.6	350.9
Japan No. 91	38.1	33.7	39.2	36.7	41.1	31.2	34.4	41.9	40.1	42.2	378.6
Total	287.3	286.4	290.3	282.6	354.5	282.7	275.0	354.2	392.7	379.9	3185.6