INTERSPECIFIC HYBRIDIZATION WITHIN THE DIPLOID SPECIES OF LOTUS (LEGUMINOSAE).

by

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INTRODUCTION

Species of the genus Lotus (Leguminosae) are principally located in two geographic areas: the Pacific coast of North America and the Mediterranean region. In addition, endemic species are found in such widely scattered places as South Africa, Australia and Japan. Although it could justifiably be reported in 1957 that there was a lack of precise knowledge concerning the cytology and taxonomy of Lotus (Bubar, 1957), there has been a considerable improvement in just a few years (Larsen, 1958a,b; Callen, 1959; Callen, Bubar and Grant, 1959; Gillett, 1959). One of the recurrent problems in the classification of Lotus has been whether or not the North American species, which are best known as members of the genus Hosakia, should be included with those of the Old World species. Brand (1898) and other European authors have excluded the North American species from the genus Lotus. However, the more recent view as expressed by Callen (1959) is that the New World species should be incorporated into a single genus, Lotus.

The principle species of agronomic interest is L. corniculatus L. ($2n = 24$). Birdsfoot trefoil, as it is commonly called, is a leguminous forage crop. Until recently this species has been relatively little used in North American agriculture. However it is now considered to have distinct possibilities for future pasture and for

even hay production on secondary soils where alfalfa and red clover are not successful (MacDonald, 1946). Part of the role of interspecific hybridization will be to provide new germ plasm for breeding with the present cultivars and eventually to provide a means to transfer desirable characteristics from one species to another.

From the theoretical point of view, Lotus is of considerable interest. There are two basic chromosome numbers in the genus, namely, $\underline{x} = 6$ and $\underline{x} = 7$. These numbers are represented by both the Old World and the New World species. This information lends support for the establishment of a single genus for both the New and Old World species. In the Old World species, tetraploids with somatic chromosome numbers of 24 and 28 are found, however, no tetraploids have as yet been discovered in the North American species. Of particular interest are the evolutionary events that have occurred to produce these two basic chromosome numbers. Bubar (1957) has given evidence that $\underline{x} = 7$ is the basic number for the genus. The origin of L. corniculatus itself, is another problem which calls for resolution. By means of interspecific hybridization it may be possible to establish hybrids and to then create amphidiploids. The latter could not only aid in establishing the past history of L. corniculatus, but could also give potentially valuable genotypes for further forage improvement.

A further fundamental role of interspecific hybridization would be to examine the pairing relationships of the chromosomes at meiosis in interspecific hybrids in order to elucidate the natural relationships of the species.

An example of the contribution that can be made to theoretical knowledge by means of interspecific hybridization is the hybridization studies carried out by Clausen, Keck and Hiesey (1945) between species of the tribe Madiinae in California. In brief they postulated a certain evolutionary pathway, and then by means of interspecific hybridization were able to prove their hypothesis by experimentally synthesizing plants already in existence.

It should be remembered that artificial hybridization may bring together two genotypes which would not normally meet in nature, as a result of some ecological or geographic isolation barrier (Heiser, 1949). Thus hybridization under artificial conditions may not give the true relationships of the species to one another as found under natural conditions. However, this is only of secondary consideration in the production of desirable genotypes for plant breeding purposes.

The occurrence of interspecific hybridization in plants under natural conditions is well recognized. Stebbins (1959) has recently reviewed the role of natural interspecific hybridization in the evolution of plants, and no attempt will be made to review the subject here. It should be pointed out that differences have been detected in the frequency of some species to hybridize under natural conditions. Stebbins (1959) states "In Lotus and Trifolium the species native to California often grow side by side in mixed colonies, and even in the case of closely related species which have the same chromosome number, hybrids have never been seen, in spite of frequent searches for them".

The use of statistics as developed by Anderson (1949) primarily for the detection of natural hybrid populations, can possibly be used under the appropriate conditions to establish the nature of gene-flow in back cross progenies and in F_2 progenies from artificial hybridizations.

The role of interspecific hybridization has had a considerable impact both on theory as in revealing the degree of homology of the chromosomes in hybrids and in practical applications, such as the transfer of genes for resistance to disease. That interspecific hybridization has played an important role in the

development of economic plants is well known. Hybridization has been most important in garden and greenhouse ornamentals, such as roses, tulips, hyacinths, narcissus, pansies, and orchids. To a lesser extent hybridization has been important in the production of such useful fruits as apples, cherries, plums, peaches and grapes. In the cereal grains a different phase of hybridization may be seen in that the main interest is to transfer particular characters to one species through hybridization, backcrossing and finally selection (Stebbins, 1950).

We have seen that experimental interspecific hybridization has already had important theoretical and practical applications. It is hoped that the interspecific hybridization studies of Lotus will have equally significant consequences.

LITERATURE REVIEW

Interspecific hybridization in the genus Lotus

The literature concerned with interspecific hybridization in Lotus is not extensive. The reason for this is that the genus as a whole has received little attention in the past from either taxonomy, agronomy or from the point of view of genetics or cytotaxonomy.

Certain closely related species of Lotus have morphological attributes in common which makes positive identification sometimes difficult. This condition has been brought about partly through polyploidy in which the polyploids have been given specific rank. Three such closely related species are the diploid species L. tenuis and L. uliginosus and the tetraploid, L. corniculatus. Larsen (1954) has stated that these three species are not difficult to distinguish in northern Europe. However, in southern Europe he has found that these species are extremely polymorphic so that it is difficult to tell them apart by employing floral manuals. He suggests that the variation may be due to interspecific hybridization. Ottley (1944), who has monographed the American species of Lotus, believes that some of the American specimens she has examined are interspecific hybrids.

In contrast to these reports of suspected hybridization between species of Lotus, Stebbins (1959) has stated that he has never observed hybrids between closely related species of Lotus which grow side by side in mixed colonies in California despite frequent searches for them. Stebbins has also pointed out that hybrid swarms are unknown throughout the Leguminosae. Thus we have conflicting views in regards to the extent of hybridization between species of Lotus in nature.

With the increasing importance of L. corniculatus as a forage crop, greater interest has been aroused in the artificial production of hybrids for higher yielding genotypes. Dawson (1941) attempted the interspecific cross between L. corniculatus (2n = 24) X L. tenuis (2n = 12). However, he was unsuccessful. Tome and Johnson (1945) carried out crosses between these species but used an autotetraploid form of L. tenuis. Although they were successful in producing pods as a result of crosses between these species, they failed to obtain mature seed. They repeated reciprocal crosses between L. corniculatus X L. tenuis as earlier attempted by Dawson and also L. tenuis (2x) X L. tenuis (4x). None of these crosses were successful.

On the basis of seed set, McKee (1949) reported what was believed to be successful hybridizations between several

species of Lotus as follows:

L. corniculatus var. arvensis X L. corniculatus var tenuifolius
L. corniculatus var. tenuifolius X L. corniculatus var arvensis
L. corniculatus var. arvensis X L. divaricatus
L. corniculatus var. tenuifolius X L. divaricatus
L. corniculatus var. arvensis X L. corniculatus var. hirsutus
L. corniculatus var. tenuifolius X L. corniculatus var. hirsutus

Emasculation was not practiced. At the time of publication of McKee's paper it had not been determined whether these were true interspecific crosses.

When Keim (1952) performed his interspecific crosses in Lotus he removed the keel of the flower to lessen the chances of self-pollination. Furthermore in some of the crosses he employed the dominant genetic marker of pubescence carried by the pollen parent. In reciprocal crosses of L. corniculatus X L. tenuis (4x) he obtained hybrids both with and without embryo culture. The data of the cross G4N2 (L. tenuis, 4x) X E1 (L. corniculatus) are an example of his series of hybridizations: flowers pollinated 26, pods formed 17, seeds produced 148, and shriveled seed 92. In the cases where the pubescent marker was employed as the male most of the progeny exhibited this character indicating successful hybridization. Keim also carried out some miscellaneous crosses involving L. corniculatus, L. tenuis, L. uliginosus, L. uliginosus (4x), and L. tenuis (4x). In

the cross between the diploid species L. uliginosus x L. tenuis, 83 seeds were obtained while in the cross between the tetraploid forms of these species, only 4 seeds were produced. No further information was supplied on the putative hybrids from these miscellaneous crosses.

Mears (1955) also attempted reciprocal crosses between L. corniculatus X L. tenuis (4x) and was successful in obtaining them. Mears obtained a higher seed set when the tetraploid form of L. tenuis was used as the female in crosses with L. corniculatus, rather than as the male parent. From these crosses 33 hybrids were obtained. They were vigorous and expressed an intermediate phenotype in regards to the parental characteristics. In tests for fertility she obtained an average of 17 seeds per pod in plants grown in the greenhouse whereas an average of 20.7 seeds per pod were derived from plants grown in the field. In the following crosses:
L. corniculatus X L. uliginosus (4x) and the reciprocal cross;
L. tenuis (4x) X L. uliginosus (4x) and reciprocal;
L. uliginosus X L. tenuis; and L. tenuis X L. corniculatus;
no mature living hybrids were obtained. However in the cross L. uliginosus X L. uliginosus (4x) she was successful in obtaining 6 hybrids, one of which was a triploid while the others were tetraploids. These hybrids were obtained by employing embryo culture techniques.

One interspecific hybrid in Lotus in which there can be no reasonable doubt as to its true hybridity is a successful cross obtained between L. cambriensis (2n = 12) X L. ornithopodioides (2n = 14) by Seaney (1957). The correct taxonomic spelling for the former species is L. coimbriensis. Seaney reported the diploid chromosome number for this hybrid to be 13. Attempts to double the chromosome number of the hybrid were reported to have been unsuccessful. Furthermore, Seaney found the hybrid to be extremely difficult to propagate by cuttings. Seaney has also reported successful hybridization of the diploid species L. filicaulis X L. tenuis and obtained seed.

Bent (1958) reported the pollination of 7,742 flowers in carrying out interspecific hybridization employing the following species of Lotus: L. corniculatus var. japonicus, L. corniculatus, L. tenuis (2x), L. tenuis (4x), L. uliginosus (4x), and L. uliginosus (2x). Emasculation was practiced in all cases. From these pollinations he observed a pod set of 1,461. He was able to culture 1,218 embryos and successfully raised 17 embryos to maturity. Three new interspecific hybrids were obtained with the aid of embryo culture, namely, L. uliginosus X L. tenuis, L. uliginosus (4x) X L. corniculatus, and L. uliginosus X L. corniculatus. Furthermore he also obtained some viable seed from his initial pollination of 7,742 flowers.

Self-sterility in Lotus

The term 'self-sterile' is used when self-seeds are not produced by undisturbed isolated flowers. The sterility may be caused by genetic, environmental or mechanical effects which may act before, during or after fertilization (Bubar, 1957).

That self-sterility exists in Lotus has been known since 1878 when Darwin reported that several plants of L. corniculatus which had been individually covered produced no seed. In more recent times Silow (1931) has reported that L. corniculatus is not completely self-sterile, and occasionally plants set seed after self-fertilization. In regards to L. uliginosus, Silow stated that he considered this species to be incapable of spontaneous self-pollination. However, he found that after artificial self-pollination nearly all the plants set seed. Dawson (1941) in his experiments on self-fertilization with L. corniculatus and L. uliginosus obtained results similar to those of Silow. These results were further confirmed by MacDonald (1946) for L. corniculatus, and by McKee (1949) for L. corniculatus and L. uliginosus. In addition, McKee noted that individual plants varied widely with respect to self-sterility.

Giles (1949) studied specifically different aspects of self-sterility in L. corniculatus. Besides confirming observations from previous studies which indicated that

plants of L. corniculatus are highly self-sterile, he found that the stigmatic membrane had to be ruptured before the stigma became receptive for pollen germination. Giles also noted that even the most highly self-sterile plants produced some self-seeds.

Bubar (1957) obtained the following degrees of self-fertility by manipulation of the flowers (in which the stigmatic membrane was artificially ruptured): high self-fertility in L. corniculatus var. japonicus, L. filicaulis, and L. weilleri; some in L. uliginosus; slight in L. corniculatus and L. tenuis; and none in L. jacobaeus. The following taxa were completely self-sterile without manipulation: L. corniculatus, L. tenuis, L. uliginosus, L. weilleri, L. suaveolens and L. jacobaeus. A different situation was observed in the following species which showed that in the absence of manipulation there was a high degree of self-fertility. These species were: L. corniculatus var. japonicus, L. angustissmus, L. hispidus, L. palustris, L. filicaulis, L. parviflorus, L. divaricatus, L. peregrinus and L. tetragonolobus.

Bent (1958) like the previous investigators found variation to exist between plants within a given species in respect of self-sterility. In eight clones of L. corniculatus he found four that would self, although the values he obtained

were much lower than in crosses between clones. Likewise, in L. uliginosus he found considerable interclonal variability, with only one clone failing to set seed on selfing.

Emasculation of Lotus

When MacDonald (1946) carried out a study of the effect of pollination within and between some Lotus species (L. corniculatus var. vulgaris, L. corniculatus var. tenuifolius, L. uliginosus, and L. hispidus), he found that emasculation was impractical since injury and severe drying of the flower parts resulted. Similarly Donovan (1957) believed that emasculation was not practical in L. corniculatus. When Bent (1958) attempted emasculation of L. corniculatus and L. tenuis he reported nearly 100 per cent flower-drop. However, Bent reported successful emasculation of L. japonicus and L. uliginosus. He emasculated the flowers about two days before their normal anthesis. His method was as follows: the fused petals of the keel were clasped with forceps from above and gently pulled to remove the keel. With care and practice it was possible to remove the keel and the entire ten anthers in one operation. Following emasculation, flowers were allowed to mature for two days before pollination.

Mears (1955) emasculated Lotus flowers in a similar manner to that of Bent. On completion of emasculation, Mears placed a cylinder of nylon tulle over the inflorescence.

The nylon cloth had a density of 22 threads to the inch and the cylinder was fitted with a draw-string opening.

Erbe (1955) employed similar methods of emasculation as those of Mears and Bent. He found that the use of a Beebe binocular loupe facilitated emasculation. H e observed that emasculation of very immature flowers inevitably resulted in blossom-drop.

Erbe (1955) made a study of the fertility relationships of L. corniculatus and L. tenuis (4x). From his observations he concluded that there was sufficient seed set as a result of self-pollination in these two species of Lotus to make emasculation essential when controlled pollinations are to be made.

Pollination and fertilization in Lotus

From a study of the reports of earlier investigators and from his own observations MacDonald (1946) concluded that the only natural pollinating agents in Lotus were the large-bodied Hymenoptera. In actual tests MacDonald found that only bumble bees and honey bees were effecting pollination. The role of bees in pollination of Lotus has been more recently supported by Morse (1955).

According to Erbe (1955) part of the function of the bee in pollination is to rupture the stigmatic membrane

which is forced against the abdomen of the bee while it is seeking nectar. That it is necessary for the stigmatic membrane in Lotus to be broken before fertilization can be effected is a hypothesis that has been supported by most investigators with Lotus who have considered this problem. Mears (1955) has reported that Knuth, McKee, Keim, and Hansen have all come to this conclusion.

In artificial pollinations, a small wooden spatula to which a small piece of sandpaper or similar material is frequently attached, is used for brushing against the stigma to break the stigmatic membrane and for transferring the pollen to the female parent (Bubar, 1957; Mears, 1955; Keim, 1952). Erbe (1955) initially used a very fine abrasive material namely the striking surface to be found on a safety match box. However according to Erbe there was an increase in the percentage pod set on pollination when the practice of intentional light rubbing to break the stigmatic membrane was discontinued and was replaced by the limited irritation produced by the application of the pollen.

Bubar (1957) showed that after the stigmatic membrane had been ruptured a fluid medium was released onto the surface of the stigma. He reported that this medium was used up by the germinating pollen. When this medium was exhausted repeated pollinations did not lead to fertilization.

Giles (1949) made a study of the germination of pollen tubes in L. corniculatus both between compatible and incompatible crosses. He found that two hours after pollination, pollen tubes were well established in the upper portion of the style and that the germinated pollen grains had emptied their contents in both compatible and incompatible matings. There appeared to be no difference in the rate of pollen tube growth nor in the number of the tubes penetrating the style between compatible and incompatible crosses. However after 24 hours, even though the potentially incompatible tubes were not inhibited at the distal end of the ovary, the potentially compatible pollen tubes were more abundant in the proximal half of the ovary.

By a series of trials Keim (1952) demonstrated that there was no relationship between time-of-day of pollination and success in fertilization. Bent (1958) found that the pollination of the flowers two days after emasculation was consistently more favorable to successful fertilization than immediate pollination.

Another problem in the correct timing of pollination has been raised by some observations of Bubar (1957). By examining serial sections of ovules he has discovered that the self-fertile species which he investigated (L. corniculatus japonicus, L. palustris, and L. divaricatus)

all had their ovules at the same stage of development within the ovary. Self-pollination appears to occur only at a suitable stage of maturity of the ovules for fertilization to take place. In contrast, Bubar found that in the self-sterile species he examined (L. corniculatus L. tenuis, L. suaveolens, and L. weilleri) there was a variation in the stage of ovule development within each ovary.

Embryo culture in Lotus

Rappaport (1954) has made an excellent review of in vitro culture of plant embryos. He also has cited a 1949 unpublished thesis of Solomon which contains a synoptic review of the literature regarding embryo culture. Rappaport considers that the two principle achievements of plant embryo culture are:

(1) overcoming the dormancy of certain seeds, and

(2) the provision of cultural methods which permits the growth of viable hybrids that were formerly unobtainable.

Before proceeding into the details of specific techniques adapted by investigators for the culture of Lotus embryos, a brief review will be given of the developments of embryo culture and its importance for obtaining desired hybrids.

The first investigator to excise plant embryos and bring them successfully to maturity was Hannig in 1904

(cited by Keim, 1952). Laibach (1929) became well known for obtaining hybrid plants between Linum austriacum X L. perenne by successfully growing embryos to maturity on cotton soaked in sugar in his 'test-glasses'.

The first worker in North America was Tukey (1933) who was able to grow embryos of sweet cherry in vitro. Later La Rue (1936) showed that it was possible to culture embryos of several species of gymnosperms, dicotyledons, and monocotyledons on a single medium and therefore demonstrated that the nutrition of the embryos was not species specific. The contemporary work of Randolph and Cox (1943) in embryo culture has had widespread influence in recent years including the embryo culture of Lotus.

Keim (1952) after making a number of experiments in the culture of embryos of Trifolium and Lotus decided in favor of the method that will now be briefly described. A small room without windows was chosen as a sterile dissection room. A table in this room was rinsed with a 50 per cent solution of 'Chlorox'. A binocular dissecting microscope and a suitable lamp were placed in a working position. After the door of the sterile room had been closed the interior of the room was sprayed with a 1 per cent phenol solution. The legume or pod to be dissected was immersed in a 1 per cent aqueous solution of 'S.T.37' antiseptic for one minute. After the removal of the ovule

it was placed on the stage of the binocular dissecting microscope and the embryo removed. The dissecting needles were kept free from microorganisms by periodic flaming while the hands were rinsed occasionally with 50 per cent ethanol. On one occasion, with this technique Keim was able to establish 81 embryos in culture without a single case of microbial contamination.

Some differences of opinion have been expressed as to the most suitable culture medium to employ for culturing Lotus embryos. Keim employed the culture medium devised by Randolph and Cox (1943). However, Mears (1955) preferred White's embryo culture medium, whereas, Bent considered that Knudsen's culture medium was superior to Randolph and Cox's for culturing Lotus embryos.

What might be considered as minor details are sometimes of great importance and this aptly applies to observations on embryo culture procedures. Bent (1958) observed the death of embryos in embryo culture that he considered resulted from the leaching out of some deleterious substance from the plastic caps used on the embryo culture bottles at the time of sterilization.

As to the role of embryo culture for interspecific hybridization in Lotus, Mears (1955) observed that the normal environment for the developing embryo namely the

endosperm ceases to develop and thus leaves the hybrid embryo to perish. She also found occasionally that embryos appear to degenerate when the endosperm development appears to be normal.

It may be seen from this literature review that interspecific hybridization in the genus Lotus is as yet only in the early stages of development. The principles underlying the techniques are largely unknown. The methods of embryo culture although promising, are wasteful of many living embryos which may contain important genotypes. With the increased agronomic interest in species of Lotus it may be expected that a knowledge of factors concerned with interspecific hybridization will be of immense importance for future breeding experiments in this genus.

MATERIALS AND METHODS

One of the major problems which has faced those individuals who have attempted to carry out interspecific hybridization in the genus Lotus has been the need for the development of a satisfactory technique for making crosses. A number of variations in technique have been attempted to-date, but the success of these techniques has not been as great as desired. Seaney (1958) has recently indicated the urgent need for developing still better techniques in making interspecific crosses in Lotus. Accordingly, particular emphasis was given to this aspect of the problem. Considerable time has also been devoted to the development of an embryo culture technique, and details of the methods used will follow.

The Species

The plants used in this study are all diploid European species. Seed was obtained from the considerable collection of Lotus species maintained at Macdonald College by Drs. Bubar, Callen, and Grant. The seed stems from diverse origins, both from natural and cultivated stands. The sources of these collections are given in the Appendix Table I.

The species employed in this study have been divided into two groups. Division I contains those species closely related to L. corniculatus. These species are commonly referred to as

belonging to the L. corniculatus group. Division II contains other diploid European species used in this study.

Division I

L. corniculatus var. alpinus	2n = 12
L. corniculatus var. eremanthus	2n = 12
L. corniculatus var. heterophyllarius	2n = 12
L. filicaulis	2n = 12
L. japonicus	2n = 12
L. tenuis	2n = 12
L. uliginosus	2n = 12

Division II

L. arabicus	2n = 14
L. arenarius	2n = 14
L. campylocladus	2n = 14
L. coimbriensis	2n = 12
L. drepanocarpus	2n = 14
L. jacobaeus	2n = 14
L. judaicus	2n = 14
L. maroccanus	2n = 14
L. ornithopodioides	2n = 14
L. strictus	2n = 14
L. weilleri	2n = 14

Not every species has been used equally frequently in crossing attempts. L. japonicus, L. tenuis, and L. uliginosus have been used in crosses most frequently, followed by L. corniculatus var. alpinus and L. arabicus. Crosses have been carried out reciprocally when abundant flowers were available. Reciprocal crosses have been carried out most extensively between L. japonicus, L. tenuis and L. uliginosus.

The chromosome number was determined for each species used in this study. A standard cytological procedure was used

for the determination of chromosome numbers and for karyological analyses. Root tips from potted plants were pretreated with 0.002 M 8-hydroxyquinoline (Tjio and Levan, 1950) for 1 hour, after which they were stained by the Feulgen method (Darlington and La Cour, 1947) and squashed for the examination of metaphase chromosomes. Slides were made permanent by McClintock's method (McClintock, 1929).

Culture

For the summer months the species have been maintained in two groups. One group consisting of a minimum of three plants for each species was planted in nursery rows in the field. The second group was raised in pots set in sand in cold frames. Approximately, 170 individual plants could be maintained in this manner. The latter plants were used principally for crossing experiments while the field plants were used for provision of pollen and observation of morphology.

During the winter months eighty plants were maintained in the greenhouse. Artificial illumination was provided by 150 watt reflector floodlights and controlled so that the plants received approximately sixteen hours total light daily. The plants were maintained at a temperature of 18°C.

Emasculation

The usual forceps method of emasculation was considered to be injurious to the flower, and therefore, responsible for

the severe flower-drop which amounted to 100 per cent loss in some trials. Consequently, an alternative emasculation technique was sought. After some trials another method was employed with greater success and is carried out in the following manner. A small incision is first made in the keel of the flower; a thin glass tube is then inserted; and the anthers are subsequently sucked out by means of a partial vacuum leaving the rest of the flower intact.

A typical pencil-like glass tube for emasculation measures 16 cm. in length and has a diameter of 1.5 cm. The working tip is drawn out to an external diameter of approximately 1.00 mm. and with a bore of 0.8 mm. At about 6.0 cm. from the tip, there is a round hole in the wall of tube which acts as a valve, when the hole is closed and opened, by the first digit finger. This valve permits a much reduced suction while the tip of the glass tube is being manoeuvred into the flower. Under operating conditions with the electric air pump providing the partial vacuum, the suction at the working tip of the tube was 25.5 cm. of mercury with the finger valve closed, and 6.5 cm. with the valve open.

When a plant was to be emasculated, it was brought into the laboratory. The infloresence to be emasculated was pinned down to a Petri dish that had been previously filled with paraffin and was covered with a moist filter paper. The filter

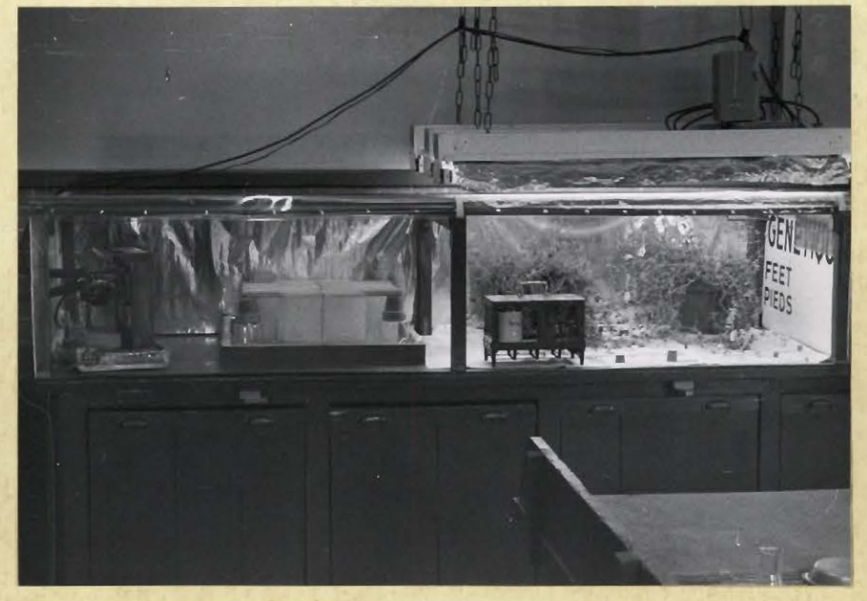
paper was replaced if an anther was ruptured during emasculation and after emasculation of each plant so as to reduce the possibility of contamination. Emasculation was carried out with fine dissecting needles, the pencil-like glass tube and employing a partial vacuum as already described. Sixfold magnification provided by a binocular dissecting microscope was suitable for the purpose. When this technique was properly applied it was difficult to distinguish emasculated flowers from those of the controls.

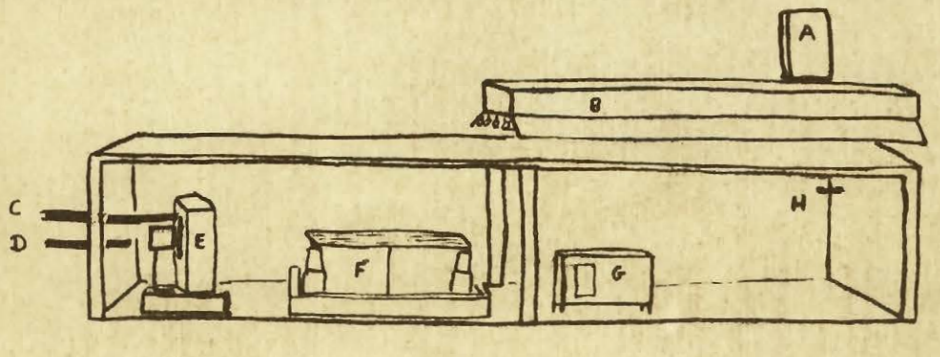
Although this method for the removal of the anthers left the flowers free of overt damage, experience showed that during the high temperatures of summer, many of the flowers still lost their color and died before they were mature enough to be pollinated. Therefore, additional procedures were developed and were carried out as follows. As soon as the plant has been emasculated it is sprayed with 10 parts per million 2-4-5 trichlorophenoxypropionic acid. It is then placed in a growth chamber until the emasculated flowers have matured.

The growth chamber is illustrated in Figures 1 and 2. The chamber measures 2.33 meters in length, 0.77 m. in width and stands 0.46 m. high. Clear plastic forms the top and sides. An arrangement for lowering the temperature was devised by employing the honeycomb portion of an automobile heater, connected to a cold water source. An eletric fan circulated

Figure I

GROWTH CHAMBER





A Light timer
B Fluorescent lights
C Water out
D Cold water in
E Cooler and fan
F Humidifier
G Thermo hygrograph
H Thermostat

Figure 2

GROWTH CHAMBER DETAIL

Left half of growth chamber

Right half of growth chamber

View towards left end wall

Lotus plant in far right corner

air through the cooler when a thermostat closed the electric circuit. The main purpose of the cooler was to remove the heat radiating from the fluorescent lights and to maintain the temperature below a set level. A humidifier was made by suspending fifty sheets of paper towelling into water in a tray measuring 60 cm. square. Light for the growth chamber was supplied by six fluorescent tubes 1.20 meter in length, arranged alternately, cool white and daylight type. At 18 cm. from the tubes 600 foot candles were registered by a General Electric foot candle meter model number 80W40Y16. The light was kept on for fourteen hours a day. The temperature maintained was 24°C $\pm$ 2°C. The relative humidity was kept between 80-90 per cent. Up to fifteen Lotus plants could be accommodated at one time.

The technique for pollination

A small wooden flat stick (a tongue depressor), 11.5 cm. in length and 1.0 cm. in width was used for pollinating. At one end of the stick was glued a small rectangle of fine sandpaper. This 'pollinating stick' or 'crossing stick' was similar to those used by other workers with Lotus who have found it is necessary to break the stigmatic membrane in order to make an effective pollination (Knuth, 1908; Keim, 1952; Hansen, 1953).

The flower to be pollinated is manipulated so that the

stigma protrudes visibly. The pollen was transferred by means of the sandpaper on the 'crossing stick' to the stigma of the plant to be pollinated. The contact of the stigma with the sandpaper was judged sufficient to rupture the stimatic membrane and no attempt was made to rub the stigma vigorously.

The determination of the proper stage of embryo development from the growing legume

With present methods the protoembryo is too young to be successfully cultured. Since it is wasteful to excise the legume while the developing embryo is at the protoembryo stage or at later stages when embryo abortion has already begun, a method was therefore sought to predict the right time for excision of the embryo. Somewhat to my surprise the following method was successful and was carried out with little difficulty. The legume to be diagnosed was placed on a sterilized Petri dish top and a portion of the pod which generally contained four or five ovules was cut off with a scalpel that had been sterilized by passing through an alcohol flame. The open end of the legume was then sealed with vaseline to prevent possible contamination and dehydration. The asceptic equipment which was used may possibly be unnecessary. If on dissection of the excised portion of the legume only immature embryos were found, the remainder of the legume is left to develop until it is estimated that the embryos would be mature enough to be successfully cultured *in vitro*.

Preparation of the embryo culture medium

The medium used for embryo culture was essentially that of Randolph and Cox (1943). As the solution B of this medium was found to be unstable, various attempts were made to keep the metallic salts in solution. Only when elaborate precautions were taken to exclude free oxygen was the solution stable enough to prevent the formation of a precipitate for a limited period. Since this precipitate gave a positive test for iron it was decided to substitute the ferrous sulfate of the culture medium with various iron containing chelates. The solution finally used has been called D. Solution A is unchanged. The constituents of these solutions are as follows:

Solution A

Calcium nitrate (hydrated)	23.6 g.
Potassium nitrate	8.5 g.
Potassium chloride	6.5 g.
Distilled water	500.0 ml.

Solution D

'Sequestrene 330 Fe'	4.7 g.
'Calgon'	1.0 g.
Magnesium sulfate (hydrated)	3.6 g.
Distilled water	500.0 ml.

'Sesquestrene 330 Fe' is the trade name for technical sodium ferric diethylenetriamine pentaacetate obtained from the Geigy Company Incorporated, Bayonne, New Jersey, U.S.A. Calgon is a commonly used water softener which contains sodium

hexametaphosphate. Glass distilled water was used both for the nutrient solutions and for the embryo culture medium. After the ingredients were dissolved in their respective half-litre volumes the solutions were kept in a refrigerator at 4°C.

A standard one ounce clear glass bottle with a screw cap was used as a container for culturing the embryos. This bottle stands about 7.5 cm. high with an overall diameter of approximately 3.2 cm. The neck opening is 2.0 cm. A plastic cap composed of hard black phenol formaldehyde plastic was usually used. The cap was boiled in a strong soap solution and then autoclaved to leach out any compounds that were in the past suspected of hindering growth of the embryo (Bent, 1958). Caps of linear or low pressure polyethylene have also been used. Besides being translucent, resistant to autoclaving and freedom from leaching they have the advantage that they will maintain a set position on the neck of the bottle to allow the exchange of gasses between the developing embryo and the outside air. The polyethylene caps were not completely ideal because their thread did not quite match the thread on the neck of the bottles.

After the initial batches of embryo culture medium had been autoclaved, it was found that there had been a shift in the pH beyond the optimum of 6.5 to levels that were too acidic. To overcome this condition the pH of the medium, while it was in its liquid state and before sterilization, was adjusted to a pH of 7.3 with the addition of one-hundredth Normal NaOH.

In addition, the sugar was autoclaved separately as a concentrated solution (20 per cent sugar), and then added to the medium under asceptic conditions.

One hundred culture bottles made a convenient number to prepare at one time. The procedure for the preparation of the embryo culture medium was commenced by dissolving 5.6 g. of 'Bacto-Agar' in 720 ml. of hot water. To this agar solution was added 4.0 ml. each of solution A and solution B. The entire solution was then adjusted to a pH of 7.3. The solution was added to the culture bottles by means of a funnel equipped with a short length of rubber tubing and a pinchcock clamp. The required 7.2 ml. of solution was measured approximately by bringing up the level in the culture bottle to a predetermined mark of a glass sheet behind the bottle. The bottles were capped tightly and then the caps given a quarter turn to allow the escape of air on sterilization. The necessary 0.8 ml. of 20 per cent sucrose solution was placed in individual small shell vials and the tops plugged with cotton. Vials and bottles were then autoclaved at 20 pounds pressure for 15 minutes. While the vials and bottles were still quite hot the measured sugar solutions were poured into their respective culture bottles. The combining of the two solutions was quickly carried out, and so farhas resulted in no contamination. The cooling of the bottles was slowed with an insulation of towels to lessen the formation of condensate on the inside walls. There

was a tendency for the culture medium to become dried on storage. However, if the culture bottles together with an open beaker of water were placed in a small carton within a plastic bag, and placed in the refrigerator the medium did not become dehydrated as quickly, and this reduced the frequency of preparation of the culture medium.

The technique for transferring the embryo into culture medium

The excision of the embryos and their transfer into culture bottles was carried out in a small sterile room. The room was kept free from organisms by ordinary cleanliness, ultra violet light, freedom from draughts, and by means of an air supply which was filtered. The following sterile items were required: a Petri dish containing moist filter paper discs, and a number of Petri dishes lined on the bottom half with moist filter paper on which had been placed a micro culture slide or well slide with two concavities.

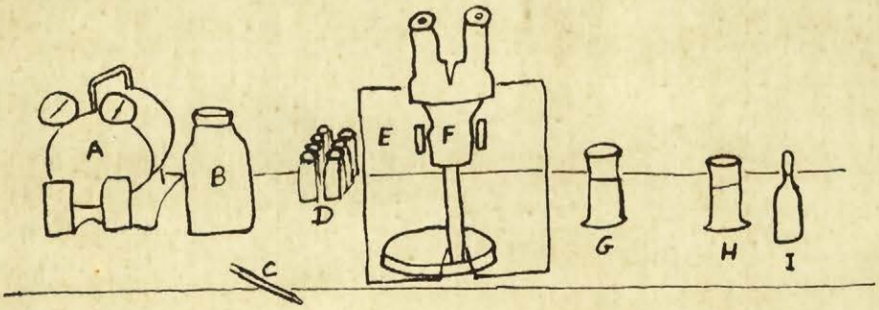
The procedure for excision of the embryos has been evolved from the technique of Randolph (1955) that was used for Iris embryos. The technique for the excision of Lotus embryos was carried out in the following manner. After the completion of each step in the procedure of excision the needles employed were flamed in a Bunsen burner and placed in a jar of antiseptic consisting of a 50 per cent aqueous hexylresorcinol solution ('S.T.' 37, Sharpe and Dohme, Philadelphia). At the start of each excision the hands and the microscope stage were wiped with 50 per cent

ethanol. The legume for dissection was immersed with a pair of forceps in 'S.T. 37' for one minute. It was then placed on a 9 cm. disc of sterile filter paper on the stage of the dissecting microscope where the ovules were removed. The filter paper was then slid aside and the ovules were transferred into one of the Petri dishes equipped with a culture slide. One of the wells of the slide was filled with the antiseptic and a single ovule submerged in the solution and the embryo dissected from the ovule. The embryo was transferred by means of a dissecting needle to the surface of the culture medium. The antiseptic in the well of the slide was replaced after each ovule was dissected. The once-used antiseptic and ovule debris were removed from the well slide through a glass tube by means of suction supplied by an air pump. For dissection it was found convenient to have one sharp and one flattened dissecting needle. The plastic screen surrounding the dissecting microscope was designed to deflect the breath of the operator. Figures 3, 4, and 5 illustrate the main steps in this embryo excision and transfer technique.

The bottles containing the newly excised embryos were placed in a dark section of the incubator which maintains a temperature of 25°C $\pm$ 2°C. The incubator is illustrated by means of a diagram in Figure 6. An incubator was needed primarily because the temperature in the laboratory sometimes exceeds 30°C during the summer months.

Figure 3

EQUIPMENT FOR EXCISION OF THE EMBRYOS



A Air pump
B Safety bottle
C Glass suction tube
D Culture bottles
E Plastic screen
F Dissecting microscope
G 50% 'S.T. 37'
H 50% ethanol
I 50% 'S.T. 37'

Figure 4

CONSECUTIVE STEPS IN EXCISION OF EMBRYOS

Needles flamed

Sterile filter paper about to be placed on stage of microscope

Pod immersed in antiseptic

Dissection of pod to obtain ovules

Figure 5

CONSECUTIVE STEPS IN EXCISION OF EMBRYOS
CONCLUDED

Transfer of ovules to Petri dish containing well slide.

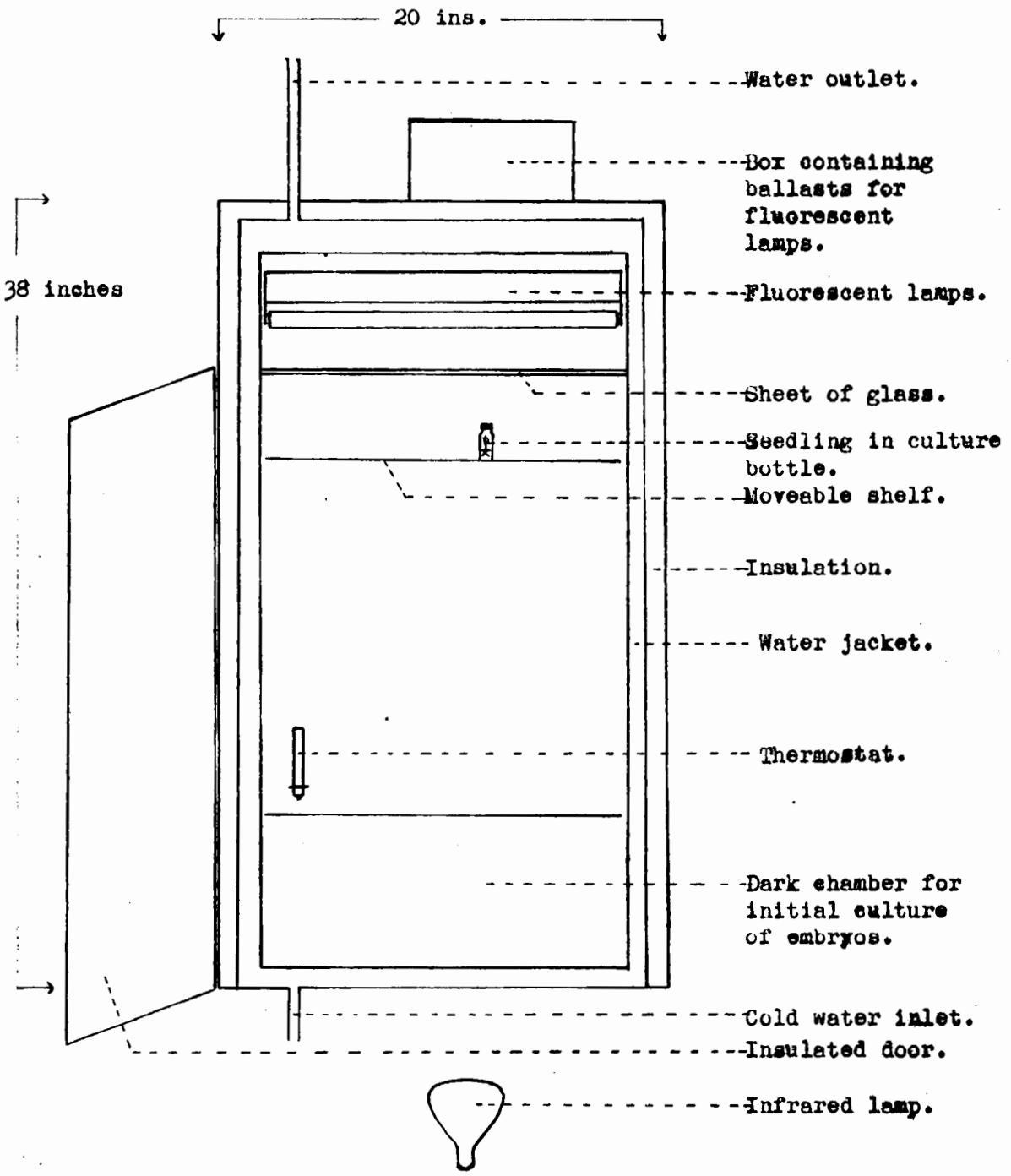
Excision of embryos

Embryo placed on culture medium.

Suction removal of used antiseptic and debris from well slide.

Figure 6

DIAGRAM OF INCUBATOR



The incubator was made from an old seed germinating chamber. From my experience with attempts to dispose of the heat from the fluorescent lamps the most important design features were: (1) placing of the lamp ballast outside the incubator and, (2) providing a free circulation of air around the fluorescent lamps. Four 20 watt lamps were used, arranged alternately daylight and cool white type. The lights were kept on continuously. At 8 cm. from the lights the culture bottles received about 1000 foot candles as measured by a General Electric foot candle meter model number 80W40Y16. Cold water was continuously circulated through the water jacket. Fortunately the cold water supply was never warmer than 25°C. During the winter months when the air in the laboratory becomes dry the loss of water from the culture medium was lessened by placing a tray of water in the incubator into which hung a number of sheets of absorbent paper.

After three days in darkness in the incubator, about three-quarters of the embryos normally have opened their cotyledons and added some length to their radicles. At this stage of growth the bottles were moved to a position in the incubator with the lowest light intensity and were then moved successively to higher intensities. During the first attempts at <u>in vitro</u> culture the writer lost 15-20 per cent of the embryos because the radicles did not penetrate the surface of the medium. This difficulty was resolved simply by tipping over the minute seedling with a needle under sterile conditions.

Figure 7 shows some of the seedlings in culture. The span of time between excision and placement of the seedlings in soil is not a critical one. Any of the seedlings in the group of three in Figure 7 could be successfully transferred to soil. Usually five weeks passed before the transfer was undertaken.

The transfer of the seedling to soil

The following method was evolved for the transfer of the seedlings from the sterile moist climate of the culture bottle to soil after losses were experienced which involved up to half of the seedlings. Clay pots with a mouth diameter of 9 cm., filled within 5 cm. of the top with soil, were autoclaved at 20 pounds pressure for 20 minutes. The cap of the culture bottle was removed and the medium broken up with a glass rod. Using a pair of forceps the young plant was gently pulled from the bottle and the large lumps of medium washed off the roots. The seedling was then planted in the sterile soil in a pot. The pot was placed in a shallow wooden box filled with moist vermiculite and the entire box with pots was placed in the growth chamber for a week with the temperature about 24°C and the relative humidity 80-90 per cent. The growth chamber was illuminated for fourteen hours a day. At the end of the week the seedlings were moved to the greenhouse and handled in the ordinary manner except that they were still kept in the box containing the vermiculite. So far there have been no losses

Figure 7

SEEDLINGS GROWN FROM EXCISED EMBRYOS

L. japonicus seedlings grown from embryos excised twenty six days previously. All are from the same pod.

Putative hybrid from the cross L. japonicus X L. tenuis. The seedling was twelve days old

when seedlings of the same size, or older than those shown at the top of Figure 7 were transferred.

Auxiliary techniques

Attempts have been made to culture excised stems bearing flowers, since at certain times it was not desirable to move a particular plant for emasculation purposes. Depending to some extent on the original vigor of the plant it has been found feasible to grow Lotus stems in Hoagland's solution. The formula for this solution was used as given by Meyer (1939) with a minor variation, namely, the substitution of 'Sequestrene 330 Fe' as the source of iron. Polyethylene containers were used for culturing the excised stems. Holes were drilled in the lids of the containers, and the stems inserted through these holes into the solution which almost filled the container. Once a day the containers were aerated.

Both in the excised stem method and in the more common method of growing the plants in soil in pots many of the interspecific pollinations failed to produce a pod or legume. Therefore, it was considered that the pollen tube might not grow down the style quickly enough to effect fertilization while the ovum was still receptive. Since it is well known that certain plant hormones will stimulate plant growth, the following method was tried in order to stimulate pollen tube growth. After the flower had been pollinated a fine shower

of a hormone powder made from 50 parts per million 2-4-5 trichlorophenoxypropionic acid in talcum powder was allowed to fall on the stigma from a camel hair brush.

The method used by Giles (1949) for storing L. corniculatus pollen has been tried for the diploid species of Lotus. In this technique pollen of each species is placed in a small gelatine capsule; the capsule is placed in a shell vial and corked; and the vial placed under refrigeration at 2-3°C. The method of testing pollen germination was to place the pollen in a 20 per cent sucrose solution containing 30 parts per million boric acid. The use of the boric acid for this purpose was proposed by Chiscon (1958). On the suggestion of Dr. E.R. Boothroyd of McGill University, a well for germinating the pollen was conveniently obtained by making a ring of vaseline on an ordinary microscopic slide. This ring can easily be made by using an eyedropper and heating the vaseline to a liquid state.

A simple and most useful auxiliary technique was the test for hydrogen cyanide in Lotus. The technique followed has been that as given by Dawson (1941).

OBSERVATIONS

Culture

The diploid species of Lotus employed in this project required no special techniques in their culture. They were found to respond favorably to ordinary greenhouse cultural methods and can be maintained as individual plants in field plots. It has been found that the percentage seed germination can be increased by scarification of the seed between two pieces of sand paper or even more effectively, by means of a dissecting needle while holding the seed with forceps. With the exception of L. jacobaeus, it has been found that flower production can be considerably increased by staking the plant and if possible by training the plant up the stake.

The response of flowering to day-length is marked in most of the species. For example, L. tenuis produced only aborted flower buds when exposed to a 15 hour day while with 16 hours total light (natural and artificial) it flowered readily. Furthermore it was found that flower production could also be increased by having two 150 watt reflector floodlights per square meter of bench space as compared to the former one light per square meter. The following species flowered in the winter in the greenhouse with a total minimum day-length of artificial and natural

light of 16 hours: (a) Division I (species closely related to L. corniculatus), L. corniculatus var. alpinus, L. corniculatus var. heterophyllarius, L. filicaulis, L. japonicus, L. tenuis, and L. uliginosus. (b) Division II (other diploid species), L. arabicus, L. arenarius, L. coimbriensis, L. jacobaeus, L. judaicus, L. maroccanus, L. ornithopodiodes, and L. weilleri. L. campylocladus and L. drepanocarpus never came into flower in the winter even though they were mature healthy plants, and were subjected to the same environmental conditions as the other species.

Species characteristics of interest for interspecific hybridization.

The term 'self-sterility' applied to a particular species of Lotus needs to be interpreted with some caution. Not only was it observed that there was intraspecific variation in the sterility exhibited by different plants, but it was also observed that the reaction of a plant of the same genotype appeared to vary with the season. As an example of the latter observation L. tenuis, L. jacobaeus, and certain lines of L. maroccanus which were all reported to have a high degree of self-sterility (Bubar, 1957) were discovered in the late summer and fall of 1958 to produce selfed seed.

The following species data have proved useful in interspecific hybridization. The term 'rate of growth'

refers to the development of the plant as observed in the growth of the stems and the proliferation of the leaves. L. japonicus which requires 15 weeks under greenhouse conditions to attain anthesis is taken as representing the normal rate of growth for the diploid species studied. The term 'average size of flower" refers to the average flower-size of a diploid species of Lotus. The flower of L. japonicus which measures approximately 11 mm. in length has been selected as representative of the average flower-size for the diploid species.

L. arabicus L., 2n = 14

All plants of this species which have been tested give a strongly positive reaction in the test for the presence of HCN. This species is an annual with a medium rate of growth. The flower is pink and of average size. The flowers recover very poorly after emasculation. The species will self on artificial self-pollination.

L. arenarius Brot., 2n = 14

Plants of this species have a positive reaction to the H CN test. L. arenarius is a perennial with a medium rate of growth.

L. campylocladus Webb., 2n = 14

Plants of this species have a strong positive reaction to the HCN test. This species is a perennial with a very

slow rate of growth. This plant has never produced flowers while grown by the author so that at present it has not been possible to use this species in crossing experiments.

L. coimbriensis Willd. 2n = 12

Different individuals of this species have a positive or a negative reaction to the HCN test. This species is an annual with a slow rate of growth. The flowers are quite small and do not lend themselves well to emasculation.

L. corniculatus L. var. alpinus Ser., 2n = 12

Plants of this species have a positive reaction to the HCN test. This is a perennial species with a slow and somewhat intermittant growth. The flower is of average size with only one or two flowers per infloresence. Although the flower color is like L. corniculatus, the keel tip, in the plants observed were a very dark brown. This latter characteristic might be useful in inheritance studies. Flowering was observed to occur at irregular intervals. Emasculation has not been successful with this species as the flower dies and falls off.

L. corniculatus L. var. heterophyllarius Pet.-Stib., 2n = 12

Plants of this species have a negative reaction to the HCN test. This species is an annual with a medium rate of growth. The flower size is average and has a good recovery after emasculation. Plants of this species have been found to be highly self-fertile.

L. drepanocarpus Durieu, 2n = 14

Plants of this species have a strong positive reaction to the HCN test. They are perennial with a rapid rate of growth. It has not been possible to bring this species into flower in the winter.

L. filicaulis Durieu, 2n = 12

Plants of this species have a negative reaction to the HCN test. It appears to be an annual with a rapid rate of growth. Flower size is slightly smaller than the average for the genus. Recovery after emasculation is only fair, and there are few flowers on the plant when it is fully in flower.

L. jacobaeus L., 2n = 14

Plants of this species have a strong positive reaction to the HCN test. This species which is native to the Cape Verde islands, has a very different appearance to most of the species of Lotus. It is a perennial with a bush-like habit supported by a woody stem. The large flower is a dark red wine in color. The flowers recover very well from the effects of emasculation, and the plant remains in flower almost continuously.

L. japonicus (Regel) Larsen, 2n = 12

Plants of this species have a positive reaction to the HCN test. Although this species appears to be an annual its life has been extended as much as five months by growth

in the greenhouse. Its rate of growth is moderate. The flower size is average and the flowers recover quite well after emasculation. L. japonicus flowers relatively profusely and thus provides a suitable source of flowers for hybridization. The species is self-fertile.

L. judaicus Boiss. 2n = 14

Plants of this species have a strong positive reaction to the HCN test. It is a perennial with a somewhat slow rate of growth.

L. maroccanus Ball. 2n = 14

Plants of this species have a positive reaction to the HCN test. However as one plant gave a negative reaction there may be some further negative plants to be found in a large population of this species. The flower is of average size. Flower production is plentiful with several flowers being carried on the same inflorescence.

L. ornithopodiodes L., 2n = 14

Plants of this species have a positive reaction to the HCN test. The species is an annual with a very rapid rate of growth. Although the flowers are small, recovery after emasculation has been excellent. It is a self-fertile species.

L. tenuis Waldst. and Kit., 2n = 12

Different plants of this species have given a positive

or a negative reaction to the HCN test. It is a perennial species with a rapid rate of growth. The flower-size is slightly below average for the genus and has a recovery rate after emasculation that is relatively low.

L. uliginosus Schkuhr. 2n = 12

Plants of this species have a negative reaction to the HCN test. They are perennial with an extremely rapid rate of growth. The flower is slightly larger than average and recovers very well after emasculation. The species will self-fertilize itself readily on artificial manipulation. With its negative test for HCN and its easily emasculated flowers, plants of L. uliginosus are suitable to use as females in interspecific hybridization crosses.

When plants of a particular species are said to have positive or negative reaction to the HCN, the presence of the opposite reaction or even an intermediate reaction can not be discounted until a large population has been examined. The following results that were obtained from a sample of seed taken at random from a large population of L. tenuis, may be used as an illustration. The plants were grown in the greenhouse and the HCN test was made when the plants were one month of age. The results are shown in Table 1.

Table 1

Reaction to the HCN test of a sample of plants of *L. tenuis*

Reaction to the HCN test	Plant Numbers	Percentage
Strong positive	8	10.4
Positive	49	63.6
Weak positive	16	20.8
Negative	4	5.2

Cytology

All the species used in this project were examined cytologically to confirm the presence of the chromosome numbers previously reported in the literature. The species listed in 'Materials and Methods' all conformed in chromosome number to those reported in the literature. However other species which are reported in the literature as being diploid or possessing diploid races (*L. angustissimus* *L. parviflorus*, *L. villosus*, *L. suaveolens* and *L. coccineus*) were examined cytologically for possible use in this study, but were found to be tetraploid rather than diploid.

Emasculation and flower recovery

In *Lotus* the anthers are enclosed in a small chamber formed from fused petals known as the keel or carina. The

opening to the keel is a small pore that enables the stigma to protrude when the flower is manipulated by a bee. The standard method of emasculation involves the removal of the keel along with the 10 anthers, which tends to result in the mutilation of the flower. When the writer applied this method to L. japonicus and L. tenuis in the summer of 1959 there was virtual complete loss of the flowers that had been emasculated. Employing a modified technique as described in the 'Materials and Methods', it was found that large Lotus flowers such as those of L. uliginosus could be emasculated at the rate of 30 flowers an hour, while small flowers such as those of L. ornithopodioides, could be emasculated at the rate of 15-20 flowers an hour. This method of emasculation has been found to be very effective in the prevention of self-fertilization. For example, in recent hybridizations a group of 38 legumes were produced on plants of L. uliginosus after emasculation and pollination with pollen from another species. However there was not a single fully developed fertilized ovule found in any of the 38 legumes. Each legume usually contains about 17 fertilized ovules on selfing. Some of the limitations of this emasculation technique have been found to be the necessity to be close to a supply of electricity to operate the air pump, and the desirability of having the plants in pots so that they may be brought into the laboratory for emasculation.

Although the removal of the anthers by this method of emasculation leaves the flower apparently free from overt damage, it was found that in some of the species that the flower-drop of emasculated flowers was still high especially so during the high temperatures of summer.

Several small experiments were carried out in an attempt to find a way to reduce the percentage flower-drop of Lotus flowers after emasculation. In one of these experiments performed in June 1959, two plants of L. tenuis were emasculated by the procedure as described in the 'Materials and Methods'. The plants were maintained in the laboratory where it was hoped that the lower temperatures prevailing might allow the flowers to come to maturity so that interspecific pollinations could be made. The 46 emasculated flowers slowly died without any of them showing any signs of coming to maturity while the plants themselves and the control flowers appeared to be normal. It was observed in the emasculated flowers that one of the first signs of moribundity was the change in color of the flower pedicel from that of the normal green-yellow color to a light yellow. As this color change was considered to possibly be the visible signs of some mechanism of abscission it was decided to attempt to alter the normal course of events by the application of a hormone.

To test this hypothesis plants were maintained under the same environmental conditions except that control flowers were not emasculated and had a small application of pure lanolin placed on the flower pedicels, while the emasculated flowers to be tested had a small application of lanolin containing a 1 per cent concentration of indole acetic acid placed on the flower pedicels. The result of this experiment was that 17 of the total of 22 treated flowers remained attached to the plant of L. tenuis while all the control flowers fell off. However none of the hormone treated flowers came to maturity or showed any signs of doing so.

Since the hormone conferred some advantage in that the emasculated flowers would now stay attached to the plant, a series of small experiments were undertaken to attempt to discover a way to bring the flower to maturity. These experiments involved various concentrations of indole acetic acid and napthalene acetic acid in lanolin as a base and applied to the pedicel and the ovary of the emasculated flower. The results of these experiments were essentially the same: the treated flowers remained attached while the control flowers fell off. However in no case did the treated flower mature sufficiently to be pollinated.

With the knowledge that a hormone could have some useful effect on the emasculated flower a series of small experiments were instituted to try the effects of likely hormones in a fine water spray. When an aqueous spray containing 20 p.p.m. indole acetic acid was applied to a L. tenuis plant after 44 flowers had been emasculated, these flowers soon fell off as well as all the other flowers the plant possessed. The plant, however, recovered. The plant above as well as the next two plants to be mentioned were kept in pots of soil placed in sand in cold frames. Ordinary summer temperatures prevailed. When a plant of L. tenuis received an aqueous spray of 10 p.p.m. napthalene acetic acid after 25 flowers had been emasculated, all 25 flowers remained attached to the plant for a period of two days before the first flower dropped. None of the flowers showed signs of coming to maturity. Likewise another plant treated in a similar fashion had 13 of a total 22 emasculated flowers still on the plant after 4 days.

These small scale trials were continued employing different concentrations of hormones and modifying the temperature and humidity by means of a growth chamber in order to try to bring the emasculated flowers to maturity. Finally, a technique was worked out which involved spraying the entire plant bearing the emasculated flowers with 10 p.p.m. 2-4-5 trichlorophenoxypropionic acid and

maintaining the plant at a temperature of approximately 24°C and a relative humidity of 80-90 per cent by means of a growth chamber.

Of the total of 696 emasculated flowers, in which the plants bearing them were sprayed with hormone and the plants placed in the growth chamber, 296 or 42 per cent of the flowers matured sufficiently so that it would have been possible to pollinate them. If only the flowers termed large (a stage of maturity of the flower immediately before normal dehisence of the anthers) are considered, the percentage of emasculated flowers coming to maturity rises to 66 per cent.

A different response to the hormone was obtained with different species tested. For example plants of L. uliginosus in which a total of 121 flowers were emasculated, were sprayed with the hormone and maintained in the growth chamber. It was found that 43 per cent of the flowers matured sufficiently to be pollinated, while with plants of L. japonicus the equivalent data were 124 flowers emasculated with a 45 per cent recovery and for L. tenuis, 153 flowers emasculated, with 20 per cent of the flowers being recovered. It will be recalled that virtually all the flowers of L. tenuis fell off under summer conditions when the plant remained untreated. Excised stems of

L. uliginosus cultured in Hoagland's solution in a container in the growth chamber on which a total of 288 flowers were emasculated and sprayed with the hormone, had a flower recovery rate of 44 per cent.

During the summer emasculated flowers on plants in the cold frames were subjected to temperatures as high as 30°C. In contrast plants in the greenhouse in the winter were maintained at an average temperature of approximately 18°C and a relative humidity of 50-60 per cent. Under the latter conditions a group of L. uliginosus plants in which 653 flowers were emasculated and then the plants sprayed with the hormone, had a flower recovery rate of 56 per cent. A group of L. tenuis under similar conditions with 71 flowers emasculated had a flower recovery rate of only 12 per cent.

The data from the emasculated flowers on plants that were sprayed with the hormone and placed in the growth chamber also demonstrates the respective fates of flowers judged to be 'large', 'medium' and 'small' at the time of emasculation. This is a flower-maturity classification in which the term large was applied to flowers of any species that are judged to be at the stage of maturity immediately before dehiscence of the anthers. 'Medium' and 'small' flowers represent those which are respectively less mature.

A recovered flower is one that is considered sufficiently mature so that it could be pollinated for interspecific hybridization. The 25 per cent of the flowers which were classified as 'large' had a recovery rate of 66 per cent. The 48 per cent of the flowers classified as 'medium' had a recovery rate of 37 per cent while the 27 per cent of the emasculated flowers classified as 'small' had a recovery rate of 30 per cent.

Interspecific hybridization

Attempted interspecific crosses were initially carried out almost entirely with L. tenuis, and to a lesser extent with L. maroccanus and L. jacobaeus, all being used as females. The emphasis on these species was because they were reported to be highly self-sterile (Bubar, 1957), and emasculation was not considered to be absolutely essential. Of the 194 crosses made, 26 produced either seed or embryos for embryo culture or both. Ten small lots of putative hybrid seed were obtained. Twenty-three of the crosses gave a total of 296 embryos. With the technique of embryo culture and transfer of seedlings to soil still being perfected only 34 of the putative hybrids were brought to maturity. However all of these putative hybrids turned out to be selfs.

During the second phase of this project in which the

newly developed techniques of embryo culture, emasculation and emasculated flower recovery have been applied, 1,960 flowers have been emasculated, of these, 1,004 emasculated flowers have recovered to the level of maturity where they could have been pollinated, 976 have been pollinated interspecifically, and 364 legumes were produced as a result of these interspecific hybridizations.

From these developing legumes four living putative hybrid embryos have been excised which were of sufficient size to potentially grow in the embryo culture medium. Their parentage and progress of development at the time of writing is as follows: (1) this first embryo was removed from a legume which was initiated by the interspecific hybridization of L. japonicus X L. tenuis. It is a young seedling growing in a pot in soil and looks like L. japonicus and is believed to be a self of L. japonicus; (2) the second was excised from a legume initiated by the cross L. uliginosus X L. tenuis. It is still alive in culture but growth has apparently ceased; (3) the third embryo was excised from a legume that resulted from the interspecific hybridization of L. uliginosus X L. japonicus. This putative hybrid appears to be dying at the time of writing; (4) this last embryo was excised from a legume initiated by the cross L. ornithopodioides X L. jacobaeus and is growing well in culture.

The following data demonstrates the difficulty of interspecific hybridization in the diploid species of Lotus

with an account of the results gained from the dissection of a recent group of legumes which were produced by interspecific pollinations. The term 'collapsed ovule' is given to an ovule that has enlarged almost to the size of a fully mature ovule and then has degenerated as the endosperm has disappeared and left the surrounding seed coat in a flattened condition. If a diligent search is made in the collapsed ovule a minute 'proto-embryo' is usually found. An 'empty legume' is one that is judged to contain unfertilized ovules or ones that show no visible signs of development. A 'degenerate' legume is one in which pollination produced only a very slight increase in length of the ovary which subsequently degenerated. The legumes were dissected approximately 21 days after pollination. Of the total of 141 legumes examined 122 of them contained collapsed ovules with an average of 3.7 ovules per pod. There were 12 'empty legumes'. The degenerate legumes totalled seven. One viable putative hybrid was obtained from these legumes.

Besides the usual collapsed ovules found in this group an ovule was found that appeared to be completely normal. When the ovule was opened the entire apparently normal endosperm was removed from the seed coat. No embryo or proto-embryo could be observed. This was the only observation of normal endosperm failing to contain an embryo.

In an attempt to discover the course of events leading to the collapsed ovules in interspecific hybridizations in Lotus, a single legume that resulted from the interspecific hybridization of L. heterophyllarius X L. tenuis was examined as to the condition of the ovules and the hybrid embryos at different intervals. Eleven days after pollination (of the emasculated flower) a section of the legume was removed (as described in 'Materials and Methods'). Five ovules were found in this section of the legume and they appeared to be normal in every respect. The endosperm was normal and contained a proto-embryo of the size to be expected at eleven days after pollination. Twenty-one days after pollination, the remaining portion of the same legume was examined to discover the condition of the ovules. Inside this portion of the legume nine fertilized ovules were found. Three of these ovules were in a partially collapsed condition and showed no trace of the embryos. Two more ovules were also partially collapsed but each contained a moribund heart-shaped embryo. The fifth partially collapsed ovule contained a heart-shaped embryo that appeared to be alive. Another heart-shaped embryo larger than the others, was found in a slightly collapsed ovule, but the embryo appeared dead. Finally two severely collapsed ovules were found that each contained minute moribund pre-heart shaped embryos. These abnormal ovules and embryos

appear to be a direct consequence of the interspecific hybridization rather than the technique of sampling ovules at different stages of development, since previous experiments in this study employing legumes produced by selfing a self-fertile species have shown that normal growth would continue in the legume after the initial section of the legume had been removed.

Embryo culture

Before the present technique of embryo culture as described in 'Materials and Methods' was developed, the contamination of cultures due to microorganisms involved 50-70 per cent of the cultures, In practically every case the foreign growth, was considered to be the result of bacterial colonies that appeared to grow out from the embryo in culture. In only two or three cases did a colony of fungus grow on the surface of the culture medium independently of the embryo. The new methods developed for the excision of embryos and their transfer to culture bottles produced cultures routinely free from microorganisms so that contamination was a very rare event. For example, in a group of 61 non-hybrid embryos grown as controls, there was not a single case of contamination.

Many proto-embryos were found in the dissection of legumes that were a product of interspecific hybridization. These ranged in size and stage of development from a minute

'ball' of cells, complete with the suspensor, to torpedo-shaped embryos. None of these proto-embryos visibly proliferated on the culture medium as used.

As it was not possible to culture the proto-embryos after they had been excised, an attempt was made to culture the complete ovules known to contain proto-embryos. The ovules were placed on and in some cases partly in the embryo culture medium employed. Although the trials were made on a very small scale it was interesting to note that the two ovules that had been partially submerged in the medium remained green and apparently alive for several days after the ovules placed on the surface of the medium had died. When the two ovules that had been partially submerged had also died the proto-embryos were examined to see if any growth had occurred and no growth could be detected.

Auxiliary techniques

On occasion it was not desirable to move a particular plant for emasculation purposes. A method is described in the section on 'Materials and Methods' which was used to culture excised stems of Lotus. Flowers on the excised stems of L. uliginosus were emasculated and subsequently matured quite successfully. Likewise, pollination produced legumes that compared favorably with legumes produced by plants grown in soil in pots. However, excision of developing

legumes following interspecific hybridization has not yielded any viable hybrid embryos to date.

In the section 'Materials and Methods' a method is described for the storage of pollen and for germination tests on an artificial medium. Although the control pollen germinates extremely well on the application of these germination techniques, the stored pollen gave results that are at the moment inconclusive. The writer observed that the percentage of germination depended to some extent on the concentration of the pollen in the sugar solution. This is in agreement with observations of Giles (1949) on pollen of L. corniculatus. Since at present it is difficult to obtain a precise ratio of pollen to sugar solution and no method has been found to distribute the pollen evenly throughout the solution, detailed percentages of pollen germination probably are not very meaningful.

A method to stimulate pollen tube growth has been described in the section 'Materials and Methods'. However, the evaluation of this method has not been completed.

DISCUSSION

Preliminaries to interspecific hybridization in Lotus

It need hardly be stated that one of the first requirements in order to carry out interspecific hybridization is to be able to bring the species into flower. The fact that L. drepanocarpus and L. campylocladus did not come into flower is strong evidence that a difference in photoperiodism exists between species of Lotus. Since species of Lotus have such a wide geographic range, from temperate to tropical regions, a difference in photoperiodism between species might be expected. However, photoperiodism is also influenced by the age of the plant and by differences in temperature and these factors must be considered (Curtis and Clark, 1950). The minimum day-length of 16 hours appears to be satisfactory for most diploid species used in this study. This day-length period was selected partly because Bubar (1957) reported observing abnormalities in flowers of L. corniculatus, L. tenuis, and L. uliginosus when a day-length considerably exceeding 16 hours was employed.

An observation of some possible significance was the discovery that potentially viable putative hybrids resulted only from interspecific crosses in which the female parent was normally self-fertile, or produced selfed-progeny after

manipulation of the flower in which the stigmatic membrane was ruptured.

From a theoretical point of view it would seem that the choice of a self-fertile plant to be employed as a female in an interspecific cross might be advantageous, as any possible genetic incompatibility system carried in the pollen of the other species would be less likely to encounter a similar system in the female. The writer considers it probable that if a large population of supposedly self-sterile species were examined for self-fertile individuals some would be found.

A useful preliminary preparation to interspecific hybridization in Lotus was the determination of the reaction of plants of a particular species to the test for hydrogen cyanide (HCN). The advantage of this principle from the point of view of interspecific hybridization has been that HCN provides a genetic marker for indicating whether or not hybridization has really taken place. In the case where the female plant tests negative for HCN and the male positive, a positive test in the offspring would indicate that the offspring was a hybrid, since Dawson (1941) has shown the capacity for the cyanogenesis is a simple dominant characteristic.

As was stated in the 'Observations', chromosome number determinations have revealed a number of tetraploid races for species previously known only as diploids. There is the possibility of error in the identification of these tetraploid races, however, since other such cytodemes are known (L. angustissimus, L. creticus; Larsen, 1955, 1958a) and others unpublished (Grant, 1960), it might be expected that tetraploid races for other species will be found. In addition, artifically induced tetraploids of Lotus species are being produced by different individuals, and therefore, it is absolutely essential for the ploidy to be determined in the material with which hybridization studies are to be carried out.

The emasculation procedure and the treatments instituded in this study to enable the emasculated flowers to recover and be pollinated has greatly increased the probability of successful flower recovery. The effect of the increased humidity and the controlled temperatures of the growth chamber can be assumed to lessen the dessication of those parts of the flower that have been injured in the course of emasculation. The method of emasculation used in this study produces the minimum of injury to the flower. The effect of the hormone is more difficult to explain. However it is known

that many plant auxins are polar in their movement. That is, the auxin is produced in the apical parts of the plants and tends to move to the more basal portions. It is also known that flowers can be the source of these auxins (Fuller and Tippo, 1950). In the case of Lotus, it is possible that emasculation of the flower interferes with the production of a certain flower hormone that normally moves through the pedicel of the flower and would normally prevent the operation of a mechanism of abscission. The role of the 2-4-5 trichlorophenoxypropionic acid would be a possible substitute for the action of the natural hormone and thus prevent or delay the abscission of the flower.

In the opinion of the writer there should be some doubt as to the wisdom of using sandpaper to rupture the stigmatic membrane in place of the method which occurs in nature, namely, the rupture of the stigmatic membrane by the abdomen of the bee. Although it is appreciated that the membrane must be ruptured and the flow of stigmatic fluid initiated, the writer considers that the use of relatively course abrasives is likely to cause sufficient injury to the stigma, to interfere with pollination and possibly to be a contributing factor in flower-drop. Erbe (1955) believed that he obtained a greater pod set when he discontinued the practice of rubbing

the stigma with an abrasive. An improved technique for this problem is probably required.

Interspecific hybridization in Lotus and the role of embryo culture

During the first phase of this study a number of interspecific pollinations were made in which the female parent was reputedly self-sterile and thus emasculation was not considered absolutely essential. As reported in the section under 'Observations' a number of the crosses resulted in progeny that were the result of selfing of the female parent. The suggestion is therefore made that; (1) similar to Silow (1931), some self-sterile Lotus species give a small proportion of selfed progeny and, (2) the presence of 'foreign' pollen may have influenced self-fertilization.

Mears (1955) has raised the question whether embryo culture for interspecific hybridization in Lotus is really necessary. She suggests that healthy embryos that can grow in vitro may be classified as potentially viable seeds. The present writer supports this view with the reservation that the embryo must be at least the size equal to one-half the total volume of the ovule if a normal seed is to be produced. Furthermore the investigator must be prepared to make a very large number of crosses, perhaps

in the neighborhood of 10,000 before such a hybrid seed is likely to be formed (refer to the pollination data of Bent cited in this thesis).

Does this mean that the technique of embryo culture that is designed to culture a relatively large embryo as described in this thesis and in other studies on Lotus is not required? The answer to this question the writer believes is a cautious 'yes', which thus places the emphasis on a need for proto-embryo culture. Although the previous investigators of interspecific hybridization in Lotus correctly concluded that a barrier to hybridization existed in the form of hybrid inviability that had its effect within the ovule, an error may have been made as to the precise stage in the development of the embryo in which the disharmony between the hybrid embryo and the endosperm occurred. The writer believes that the observations made in the course of this study support the idea that the crisis in development of the embryo occurs in the proto-embryo stage rather than at the mature or nearly mature embryo stage.

What is the cause of the embryo abortion in these interspecific crosses? One of the causes most often cited for embryo abortion in plants in general is somatoplastic sterility originally suggested by Brink and

Cooper (1947). This involves essentially a developmental disturbance which produces an overgrowth of the integument which in turn reacts to depress the endosperm development. Since the present writer has never observed such an overgrowth of the integument in hybridization in Lotus somatoplastic sterility does not appear to be the right hypothesis for the species studied. Mears (1955) has come to a similar conclusion. Brock (1954,1955) has found that chromosome breakage in the hybrid endosperm of certain Lilium crosses plays a role in the breakdown of the endosperm and the consequent death of the embryo. It is difficult to see how such a cause could be at work in Lotus for as described in the account of the one legume that was initiated by the cross of L. heterophyllarius X L. tenuis the endosperm initially achieves a volume that is comparable to that observed in an intraspecific cross. By this stage many mitotic divisions have occurred without visible ill effects. However an embryological study will be necessary to determine if such a factor exists.

The writer considers that the disappearance of the endosperm of a fertilized ovule in an interspecific cross of Lotus may possibly be due to the following condition. The hybrid zygote begins to secrete some substance,

perhaps an enzyme, that is potentially capable of lysing or otherwise interfering with the metabolism of the endosperm. However while the embryo is yet small and the endosperm is relatively large, the concentration of the hypothetical substance is unable to produce its lysing effect, yet when a certain threshold of concentration is reached as a consequence of the increased number of the cells in the embryo, the endosperm, possibly quite suddenly, is lysed. The resistance of the embryo to its own lytic agent and the sensitivity of the endosperm might be related to the different genetic constitution of the two tissues in that the embryo would have the genotype AB while the endosperm would have the genotype AAB.

Under 'Observations' mention was made of a single ovule that was taken from a legume that had been initiated by an interspecific cross in which the ovule contained normal endosperm but the embryo could not be located. Although this may represent another category of hybrid-endosperm disharmony, to be added to the more common observation of the collapsed ovule with the contained proto-embryo, the writer considers that the death and the disappearance of the embryo may mean no more than that the embryo contained a lethal combination of genes which resulted in the premature death of the embryo.

The author believes that it might be possible to

culture the hybrid proto-embryos. Although White (1954) states that attempts at such culture have not been successful, the work of Blakeslee and his associates (Blakeslee, 1945; Blakeslee and Satina, 1944) in which they were able to bring embryos as small as 0.1 mm. in length successfully to maturity indicates the promise in this field.

Embryo culture in Lotus

Some of the modifications of the embryo culture technique instituted by the writer to suit the peculiarities of Lotus are not of a major character, however, they have aided in the successful culture of embryos to mature plants. Although plant embryos have been cultured at 30°C and over (Rappaport, 1954), the writer considers 25°C a more suitable temperature for culturing Lotus embryos and Gist (1957) holds a similar view. The important innovation that has been made is the dissection of the ovules while they are submerged in an antiseptic. It is believed that this procedure has resulted in the reduction of contamination by microorganisms in cultures of embryos to the stage where contamination can now be considered a very rare event. More important perhaps is the fact that it is now possible to rapidly dissect embryos with only the rare occurrence of injury to the embryo as the embryo can now be virtually

'floated' free from the rest of the ovule. The writer considers that a proportion of the abnormal embryos reported by Keim (1952) and by Bent (1958) were possibly caused by mutilation when the ovules were dissected.

The improvements made by the author for techniques of emasculation and for emasculated flower recovery have made it possible to carry out interspecific hybridizations in large numbers and in combinations of species which were formerly not amenable to hybridization because of the heavy flower-drop. Also the improvements made in the present embryo culture methods have made it possible to bring more hybrids to maturity. The author has indicated that future success in the production of hybrid Lotus species may be greatly accelerated through a successful proto-embryo culture technique.

SUMMARY

Interspecific hybridization has been carried out between diploid species of Lotus with emphasis on those species closely related to L. corniculatus with the intention of analysing the degree of homology of the chromosomes between species and in so doing elucidate the relationships of the species and to select hybrids of potential value for forage crop improvement.

1. The somatic chromosome numbers of the species used in this study have been determined and have been found to conform to those numbers reported in the literature. Some possible tetraploid races have been found for species previously only reported as diploids.

2. The majority of the Lotus species used in this study flowered with a minimum day-length of 16 hours.

3. As the result of obtaining selfed-progeny from so-called self-sterile plants, the writer considers that some self-fertile plants would probably be found in a population of reputedly self-sterile plants of a species of Lotus. However, there appears to be intraspecific variation in plants for this character, and the response of the same individual may not be constant.

4. Various techniques have been tried in order to successfully emasculate a plant. An emasculation procedure has been developed that produces no overt damage to the flower.

5. An emasculated flower recovery technique has been developed that permits on the average 42 per cent recovery of the flowers. The technique involves the use of a hormone (2-4-5 trichlorophenoxypripionic acid) and a growth chamber in order to provide a temperature of approximately 25°C and a relatively high humidity of 80-90 per cent.

6. Vigorous rubbing of the stigma in the pollination technique, as has been practiced in the past, has been discontinued, as this procedure was considered to be a partial cause of early flower drop.

7. A method has been developed to establish the correct time for excission of the embryo in any given legume. The method involves taking a sample of 4 or 5 ovules from a portion of the legume removed from the plant at various times and sealing the cut portion of the legume with vaseline.

8. The embryo culture medium preparation has been modified to prevent iron in solution from precipitating by the use of a chelated iron. Also a technique has been devised for maintaining the stability of the p.H. of the medium on sterilization.

9. The embryo culture technique has been modified so that the former 50-70 per cent contamination has been reduced to a very low level. The main feature of the new

method involved the dissecting of the ovule while submerged in an antiseptic. An incubator for embryo culture has been designed and constructed.

10. A method has been evolved to transfer the embryo culture seedling to soil without losses.

11. The following auxiliary techniques were employed for aid in developing interspecific hybrids: the culture of flower-bearing excised stems, pollen storage, stimulation of pollen tube growth and the use of the hydrogen cyanide test. In the latter test a population of L. tenuis contained individuals that were strongly positive, positive, weakly positive, and negative in the reaction to the test.

12. During the latter phase of this project 1,960 flowers have been emasculated, 976 have been pollinated interspecifically, and 364 legumes (pods) were produced as result of these crosses. From these legumes 4 living putative hybrids were excised.

13. The putative hybrids were only obtained from females that were self-fertile or were self-fertile on having the stigmatic membrane broken.

14. The most common product of an interspecific hybridization was a collapsed ovule containing a proto-embryo. A hypothesis was put forward to account for this circumstance.

15. A discussion was undertaken as to whether embryo culture is really necessary for the embryos that have obtained the size equal to half the ovular volume. The need for a proto-embryo culture technique was discussed.

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APPENDIX

TABLE I

Species	Accession Number	Source and Origin of Seed*
L. arabicus L.	B-36	Seed introduced from Spain
L. arenarius Brot	B-34	Seed obtained from Rabat, Morocco
L. campylocladus Webb	B-127	Seed obtained from Coimbra, Portugal
L. coimbriensis willd	B-126	Seed obtained from Commonwealth Scientific and Industrial Research Organization, Canberra, Australia. Introduced from Portugal.
L. corniculatus var. alpinus Ser.	B-77	Seed obtained from C.Favarger, Institut de Botanique de l'Universite de Neuchatel
L. corniculatus var. ermanthus Chiov.	B-87	Seed obtained from U.S.D.A. Ames, Iowa. Introduced from Kenya.
L. corniculatus var. heterophyllarius Pet-Stib	B-86	Seed obtained from Uppsala, Sweden
L. drepanocarpus Durieu	B-125	Seed obtained from Botanic Garden, Adelaide, South Australia.
L. filicaulis Durieu	B-37	Seed obtained from U.S.D.A. P.I. 51864
L. jacobaeus L.	B-128	Seed obtained from Lisbon, Portugal.
L. japonicus (Regel)Larsen	B-129	Seed collected by Prof. Isawo Hirayoshi, Japan.
L. judaicus Boiss	B-85	Seed obtained from Central Experimental Farm. Introduced from Mount Carmel, Israel.
L. maroccanus Ball	B-35	Seed obtained from Rabat, Morocco
L. ornithopodioides L.	B-130	Seed obtained from Commonwealth Scientific and Industrial Research Organization, Canberra, Australia. Introduced from Tunisia.
L. strictus (Janka) Brand	B-88	Seed obtained from Central Experimental Farm. Introduced from Botanic Garden, Copenhagen.

Species	Accession Number	Source and Origin of Seed*
L. tenuis Waldst and Kit	B-131	Seed obtained from U.S.D.A. Ames, Iowa. Introduced from Turkey.
L. uliginosus Schkukr	B-132	Ceskoslovenska Akademie Zemedelskychved, Vyshumny Ustav Krmivareky Brno 1, Czechoslovakia.
L. Weilleri Maire	B-133	Seed obtained from Paris, France.

* A number of these seed samples were obtained through the kindness of Dr. J.S. Bubar, Department of Agronomy, Macdonald College.