SEED PRODUCTION IN *LILIUM REGALE* E. H. WILS.

By

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INTRODUCTION

From 1878 to the present there have been repeated references in the literature to apomictic reproduction in the genus Lilium, varying from the popular, by and for amateurs, to the detailed morphological and cytological observations of Beal (2,3) and Stewart and Emsweller (24). Although an analysis of all species has not been made, the following have been reported as apomictic: L. superbum Linn. by Parkman (16), Pfeiffer (17), and Woodcock and Coutts (30); L. pumilum DC. by Preston (19) and Slate (21); L. longiflorum Thunb. by Slate (21) and Woodcock and Coutts (30); L. speciosum Thunb. by Woodcock and Coutts (30); and L. Leichtlinii var. Maximowiczii (Regel) Baker by Stewart and Emsweller (24).

Of all species of Lilium, L. regale has been reported most often as apomictic due to the unsuccessful attempts of many breeders to incorporate its desirable characters into hybrids [Beal (2,3), Pfeiffer (18), Preston (19), Slate (21), and Woodcock and Coutts (30)].

Despite this mass of breeding evidence there are many indications that apomixis did not occur in all instances when L. regale was used as the female parent. Wilson (29) described the origin of L. x sulphurgale Perry as L. regale x L. myriophyllum var. superbum (Baker) E. H. Wils. and L. x princeps E. H. Wils. as L. regale x L. Sargentiae E. H. Wils. Woodcock and Coutts (30) reported L. x Pride of Charlotte as a hybrid developed from L. regale x L. Sargentiae. Stewart (26) has indicated the existence of the following unnamed hybrids; L. regale x L. candidum Linn. (two instances) and L. regale x L. Henryi (Baker).

Morphological and cytological evidence also indicates that L. regale is not entirely apomictic in its reproductive behavior. Cooper (6) has

demonstrated the regular and consistent formation of a haploid egg and synergids in L. regale. His observations have shown that the upper polar and antipodal nuclei of the embryo sac are not developed in the manner indicated previously by Coulter, Chamberlain and Schaffner (8). However both reports agree on the regular formation of a haploid egg and synergids. Further O'Mara (15) and Cooper (7) have proved the regular formation of haploid male nuclei in L. regale. Since microgametogenesis is frequently aberrant in apomictic species, the regular formation of the male gametes in Lilium is additional, although not conclusive, evidence of sexual reproduction.

Even more striking evidence in support of the hypothesis, that apomixis is not as prevalent as previously supposed, results from the work of Beal (2,3) who investigated the formation of the zygote and embryo in L. regale. His results indicate that the embryo does not arise as a bud from the nucellus or integument, that the male gametes are discharged in the embryo sac, and there is no difference in rate or manner of embryo and endosperm development between L. regale selfed and interspecific pollinations. His only evidence that apomixis occurred was the similarity of the F_1 and F_2 populations to the female parent.

Perhaps the most logical explanation of these contradictory reports may be found in the results of observations of the behavior of other species of Lilium, where the occurrence of both sexual and asexual seed in the same species has been reported. Pfeiffer (17) obtained two true hybrids of L. superbum x L. canadense Linn. in an otherwise apomictic population. This contention is further born out by the recent observations on chromosome morphology by Stewart and Emsweller (24) in the cross L. Leichtlinii var. Maximowiczii x L. tigrinum Ker-Gawl. They observed both sexual and

apomictic seedlings in the F_1 progeny. It has also been shown that the same species when used as the female parent may produce sexual seeds when crossed with a second species [Stewart (25) and apomictic seeds when crossed with a third Pfeiffer (17)].

Obviously, a single typical mode of reproduction, whether sexual or apomictic, has not yet been established in L. regale, and may not exist. The present investigation was undertaken in an attempt to determine whether or not apomixis occurs in L. regale.

MATERIALS AND METHODS

Bulbs of L. regale were obtained from reliable commercial sources and grown in the greenhouse following the usual cultural procedures. Necessary precautions were taken to prevent self pollination and, since flowering occurred from January to March, no insects were present. The flowers were emasculated two days before anthesis. Two days after anthesis pollination or other treatment was effected. At planned intervals ovaries were collected, cut into approximately five-millimeter sections, and killed in Nawaschin's solution. The standard tertiary butyl alcohol method was followed, material embedded, and serial sections were cut and stained with Heidenhain's iron-alum hematoxylin, using fast green as a counter stain. Styles were collected, if they had not abscissed, and longitudinal sections were prepared and stained with safranin and fast green. Observations were made using 2.6x and 10x dry objectives and 43x and 90x apochromatic oil-immersion objectives with 15x compensating oculars.

Two methods were employed in an attempt to induce apomixis without penetration of the ovary by the pollen tubes. Emasculated but unpollinated flowers were used as checks. First, pollen extracts were prepared substantially in accordance with the chloroform method of Gustafson (12). In one case entire anthers were extracted but in the other only the pollen itself was used. The extracted material was finally mixed with lanolin and applied in that form. Either of the extracts in lanolin or lanolin alone was applied either to the stigma or to the cut made on the surface of the ovary when removing the style. Second, sixty hours after intraspecific pollination the style was removed and the lower centimeter embedded. In each method ovaries were embedded or allowed to mature.

In view of the failure during the first two years to obtain mature seed in the case of all but one interspecific cross, several treatments, using various concentrations of boron, sucrose, calcium chloride, and malic acid, were devised in an attempt to induce seed set. To permit the pollen tubes of the short-styled species, L. callosum, to penetrate the embryo sac of L. regale without traversing the long style of the female parent, the style was cut to one cm. The pollen was then applied to the cut surface or the anther was inserted in the stylar canal.

Since the ultimate aim was to observe cytologically the method of embryo formation in L. regale, various interspecific and intraspecific pollinations were made and both the styles and ovaries were collected at planned intervals. Additional pollinations were made to provide material for genetic studies of F_1 and F_2 populations.

Since distinct genetic markers are lacking in intraspecific crosses of L. regale, X-ray induced chromosomal aberrations were employed to determine whether seed production was sexual or asexual in this type of cross. L. regale pollen was removed by screening from dehisced anthers, placed in containers, and given X-ray irradiation of 500, 1000, or 2000 Roentgen units, through the courtesy of Dr. S. H. Wollman of the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland, at the same intensity, the dosage being determined by varying the exposure. In addition, anthers which were partially dehisced were exposed to 2000 Roentgen units (Table 1). All of these pollens were then used in excess in crosses on L. regale and the ovaries allowed to mature.

Table 1

Containers, dosage and time of exposure of L. regale pollen to X-ray irradiation.

Pollen Contained in	Roentgen units	Exposure (minutes)
Gelatin capsules	500	1.47
Glass vials	1000	2.94
Glass vials	2000	5.88
Anthers	2000	5.88

RESULTS

ATTEMPTS TO INDUCE APOMIXIS

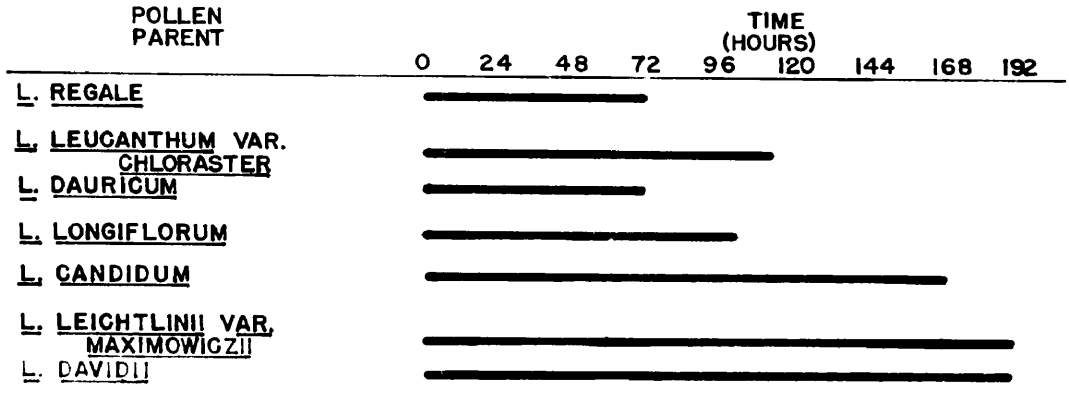
Pollen and pollen tube studies.

The experiments undertaken to determine whether pollination is necessary to induce apomixis or the presence of some chloroform-soluble growth regulator in the pollen which will initiate apomixis, since some types of apomixis are autonomous, produced negative results. This suggested that apomictic development did not occur without pollination and subsequent experiments were undertaken to determine the relationship of the progressive growth of the pollen tube to its effect on the induction of apomixis. The removal of the style sixty hours after pollination effectively blocked the male gametophyte from the ovary. This was deemed sufficient time for the hormones or growth substances from the pollen tubes to be effective without fertilization. No seeds were ever found, even though the pollen tubes were occassionally observed to have penetrated the centimeter of the stylar canal adjacent to the ovary.

Since excision of the style might have initiated some type of necrotic reaction which would hinder apomictic development, a detailed study was made of the growth of the pollen tubes of L. Davidii Duch., L. Leichtlinii var. Maximowiczii, L. leucanthum var. chloraster (Baker) E. H. Wils., L. longiflorum, and L. regale down the style of L. regale and the results correlated with observations of the initiation of embryo formation. These observations clearly indicated that embryo development occurred only in those embryo sacs which had been penetrated by the pollen tube. An analysis of growth rates showed that the pollen tubes of L. regale traversed the entire style in 72 hours, L. longiflorum in 102 hours, L. leucanthum var. chloraster in 114 hours, L. candidum in 168 hours, and L. Leichtlinii var. Maximowiczii and L. Davidii in 192 hours (Text Figure 1).

TEXT FIGURE 1

TIME REQUIRED FOR POLLEN TUBES TO TRAVERSE THE STYLE OF L. REGALE



Breeding behavior.

Since the question of whether interspecific or intraspecific pollination is necessary for successful seed production is still debatable, 463 crosses were made with the object of obtaining mature seed. A total of 19 species, including L. regale, were used as pollen parents (Table 2).

Table 2.

Number of pollinations and capsules formed following interspecific and intraspecific pollinations on L. regale.

Pollen Parent	Pollinations	Capsules Formed
L. amabile Palibin	19	0
L. auratum Lind.	19	0
L. Bolanderi S. Wats.	2	0
L. callosum Sieb. & Zucc.	45	0
L. candidum	7	0
L. dauricum Ker.-Gawl.	27	0
L. Davidii	95	0
L. Leichtlinii var. Maximowiczii	58	0
L. leucanthum var. chloraster	124	96
L. longiflorum	138	0
L. michiganense Farwell	4	0
L. pumilum	3	0
L. regale*	113	48
L. Roezlii Regel.	3	0
L. speciosum	17	0
L. superbum	11	0
L. tigrinum	47	0
L. tsingtauense Gilg.	3	0
L. Washingtonianum Kellogg	26	0
Total	463	144

*This includes the pollinations using X-rayed pollen.

Only 96 crosses using L. leucanthum var. chloraster and 44 using L. regale as pollen parents set seed. The other 17 species, involving 524 crosses, yielded no viable seed. Although these values are the totals for a four-year period this trend was discernable at the end of the second season. This was at variance with previously reported results and, since

there was evidence of embryo sac breakdown as early as 300 hours after pollination, it was suggested that the slow growth rate of some species was the cause of the failure to set seed.

In an attempt to increase the amount of pollen germination and to speed the rate of growth of the pollen tubes of certain species, a variety of methods was employed. The use of boron and other substances, known to regulate pollen germination and pollen tube growth, apparently failed to increase the rate of growth as evidenced by the lack of capsule and mature seed formation in any case. The use of a shortened style also failed to increase seed set. However, in this case there was considerable enlargement of the ovary when compared with that of a non-shortened style.

STUDIES OF THE EMBRYO SAC FOLLOWING INTERSPECIFIC AND INTRASPECIFIC POLLINATIONS.

The preliminary results demonstrated that at least the actual penetration of the embryo sac by the male gametophyte was necessary for seed set. Consequently the cellular development, following penetration, was studied in an attempt to determine the reproductive pattern of L. regale.

Normal development - fertilization.

The structure and development of the embryo sac in Lilium has been carefully investigated over a period of years. A study of embryo sacs in this material showed them to have developed in the normal manner, that is, the embryo sac contained three equal-sized micropylar nuclei, two polar nuclei centrally located, and two or three chalazal nuclei, one or two of which often showed early stages of degeneration.

After the pollen tubes had penetrated the embryo sac, male nuclei were observed possessing various configurations. They appeared at first

to be rod shaped (fig. 1), then horseshoe-like or spiral (fig. 2), and finally vermiform (fig. 3) or somewhat rounded (fig. 4). The male gamete assumed a position next to a micropylar nucleus (fig. 4) which was assumed to be the egg nucleus and the two nuclei became somewhat flattened at the surface of contact. A slight indentation of the egg appeared, in which the male nucleus partially rested. The next stage was the development of a large nucleus which was interpreted as the zygote, at least it was larger than the surrounding synergids. These stages of fertilization were observed following pollination with L. regale and L. leucanthum var. chloraster.

Abnormal development - fertilization

In the vast majority of the embryo sacs observed the sequence of events was as described above. However, certain aberrant behavior was seen in three crosses, L. regale x L. leucanthum var. chloraster. In a few instances, in these three crosses, the clear distinction between male and female nuclei was not in evidence. The two synergids also seemed to be in various stages of fusion or at least so closely associated with each other that they could easily be confused with the male and female nuclei. In one embryo sac the evidence of synergid fusion was further emphasized because there was a dividing nucleus, which appeared to be in an early daughter-cell stage as evidenced by the presence of a cell plate and spindle fibers (fig. 5), and a large nucleus (fig. 6). This large nucleus was definitely not of endosperm origin and a faint wall indicated a previous fusion. Regardless of whether the dividing nucleus or the large nucleus is interpreted as being the zygote, there is evidence of either fusion or cell division in the remaining one. Other observations showed this large nucleus in early prophase (fig. 8, 9) early anaphase (fig. 10), or telephase (fig. 11) while the two smaller nuclei showed no further fusion. At

first these observations seemed promising as indicating an explanation of apomixis, but it must be emphasized that they were extremely rare.

Normal development - embryo.

The first division of the zygote occurred approximately 75 hours after fertilization and the plane of division was perpendicular to the long axis of the embryo sac, while the plane of the second division was parallel to the long axis. Subsequent divisions initiated the formation of the embryo proper as an ellipsoid of tissue. Further elongation of the suspensor to a length of six to ten cells was observed. Approximately 600 hours after pollination the suspensor and embryo were clearly defined. Progressive growth of the embryo by cell division occurred until finally a linear embryo extending nearly the entire length of the mature seed was formed, 1100 hours after pollination. All of these observations were made on intraspecific pollinations and in contrast, even though the initial development was similar, after 600 hours the interspecific crosses proceeded to develop at a somewhat slower rate. Thus embryos resulting from pollination by L. leucanthum var. chloraster at 1480 hours were only three quarters as long as the mature L. regale embryos. One embryo of the cross L. regale x L. Leichtlinii var. Maximowiczii observed at 1176 hours was only one third as long as the corresponding embryo from an intraspecific L. regale cross (fig. 12, 13). This same ratio held with L. longiflorum at 816 hours (fig. 14, 15). Thus the initial slow growth of the embryo of these last two crosses is reflected in a failure to set any viable seed.

Abnormal development - embryo.

In the latter stages of embryo development (fig. 16) from 600 hours to 1100 hours, various types of abnormal embryos were present. Again, these observations were only made on material produced by interspecific

pollination. The abnormal embryos were located at the micropylar end of the embryo sac and surrounded by cells which were apparently nucellar in origin. Endosperm was never observed. The shape of these abnormal embryos varied considerably (fig. 17, 18, 19, 20).

In a cross where the pollen parent was L. Leichtlinii var. Maximowiczii only two embryos were observed in hundreds of sections from five ovaries. One of these was normal in appearance and the other was composed of only a spherical group of cells.

When L. longiflorum was used as the pollen parent a great diversity was observed in embryo formation. In one ovary abnormal embryos only were observed. These varied from irregularly shaped groups of cells with suspensor, approaching normal embryos, to mere spherical masses of cells. In the second ovary 11 embryos were observed of which five were apparently normal [redacted] three abnormal, and three intermediate (fig. 22, 23, 24). The intermediate type was irregularly shaped but differed from the abnormal embryo described above, in that they were located in a large embryo sac which had some endosperm around its periphery. A third ovary, in which only four embryos were seen, had two normal and two abnormal embryos. Still another ovary showed only one abnormal embryo, while the fifth showed no embryos at all.

The only interspecific cross in which any embryo reached viable maturity was L. regale x L. leucanthum var. chloraster. In this cross only occasional abnormal fertilization stages were observed.

Pollinations with X-rayed pollen.

A study of the chromosome morphology of progeny to determine sexual or apomictic development of the cross L. regale x L. leucanthum var. chloraster, which was the only interspecific cross setting viable seed, would be impossible, since the two species have identical chromosomes. Likewise, in

an intraspecific cross this could not be done. Therefore, in order to determine whether the reproductive mechanism was sexual or asexual in either case, the use of X-ray induced chromosomal aberrations as markers was suggested. The number of pollinations, capsules formed, and observations on seeds produced, when L. regale was pollinated with irradiated L. regale pollen at different X-ray dosages, are summarized in Table 3. There was a marked reduction in the frequency of successful crosses with increased dosage. The differences between the two 2000 R treatments is understandable if the shielding effect of the glass container of the pollen is considered. As indicated, the shielding resulted in less reduction in the number of successful crosses than when the pollen was exposed directly to the irradiation. At the lower dosage of 1000 R, the pollen was also exposed in glass vials and this probably accounts for the insignificant reduction in successful crosses.

As would be expected, the total number of seeds present in any one capsule is about the same. However, when they were divided into three classes, that is, seeds with no embryos, seeds with normal embryos (embryos more than half the length of the seed), and seeds with small embryos (embryos one half the length of the seed or less) (fig. 25), it becomes apparent that there was a tremendous effect of the X-rayed pollen in individual ovules as well as on the ovary as a whole. There is a negative correlation between dosage and the number of normal seeds and a positive correlation between seeds with small embryos and seeds with no embryos.

Table 3

Effect of X-irradiation on Pollen and Seed Formation of L. regale.

Dosage (in R)	Container	No. of Pollinations	Capsules Set	% Set	Number of Capsules Analyzed	Average No. seed/ Capsules	Seed Description: No embryos No.	No embryos % of Total Seeds	Normal embryos No.	Normal embryos % of Seeds with Embryos	Small Embryos No.	Small Embryos % of Seeds with Embryos
0	Anther	1	1	100	1	521	71	13.6	446	99.1	4	.9
500	Gelatin	8	8	100	4	506	515	25.4	1469	97.0	45	3.0
1000	Glass	23	22	96	8	471	1387	36.8	2298	96.4	85	3.6
2000	Glass	13	9	69	9	486	2574	58.8	1620	89.9	183	10.1
2000	Anthers	69	2	3	*							

*Not ripe at time of writing.

DISCUSSION

Maternal inheritance has been recognized in Lilium for some seventy years and has been reported as occurring in six species of the genus. Most of these reports are based on field observation of the progeny, and they must be acknowledged to be open to question. But one cannot dismiss the recent observations of Beal (2, 3) and Stewart and Emsweller (24) which confirm the existence of apomixis. The main problem is not whether apomixis occurs, although that is important, but the conditions causing and the developmental stages leading to the production of apomictic seed. It must be stated at the outset that not all reports are unanimous as to the occurrence of apomixis in Lilium. Both cytological investigations and breeding experiments have been reported which are at a variance with the entirely apomictic development reported by some workers. Beal (2, 3) has failed to find any indications of apomictic development during fertilization stages or embryo development. There are several named and unnamed hybrids using some of these same six apomictic species as the female parent. These facts indicate that a re-evaluation of the literature and experimental evidence is in order for the purpose of weighing the evidence for and against apomictic reproduction in the genus Lilium.

The possible mechanisms of apomixis in Lilium are the formation of diploid female gametes by non-reduction, fusion of the micropylar nuclei, or sporophytic budding.

This discussion considers; first, the time of initiation of apomixis and mechanisms which could induce it; second, the cellular details indicating sexual or asexual seed formation; and third, a survey of the evidence which would indicate that Lilium is sexual or apomictic.

THE INDUCTION OF APOMIXIS

Various progressive stages were isolated in order to determine the developmental stages and the nature of the initiating mechanism of apomixis. The formation of viable apomictic seeds is not autonomous, since mature flowers which were emasculated but not pollinated never formed seed. Stout (28), in 1933, suggested some type of pollen tube reaction as the causative mechanism of apomixis.

That apomixis is not initiated in Lilium by several known growth regulators has been shown recently by Beal (1). In his experiments indole-acetic, napthalenacetic, and napthoxyacetic acids in lanolin when applied to the top of the ovary, caused occasional enlargement of the ovaries to almost mature size, but such treatment never resulted in apomictic seed development. In the present study it was found that chloroform extracts of pollen likewise did not initiate apomictic development or significant and consistent ovary enlargement. It is suggested that the explanation for the increase in the size of the ovaries, as reported by Beal (1) and also in this investigation, may be found in Gustafson's (13) observations on the relation of pollen and auxin. He reported that some plants contain enough auxin in the ovary to induce parthenocarpic growth, others require additional auxin through pollination to initiate growth, while still a third group requires not only pollination but fertilization before initiation of ovary growth. The results of this study indicate that L. regale may be classified primarily in Gustafson's (13) third category.

In this investigation, the whole series of pollinations, in which the style was removed just before the pollen tubes reached the ovary, ruled out the possibility of a substance diffusing ahead of the male gametes and stimulating the apomictic reactions, since no seeds were ever obtained

following such treatment. Correlation of pollen-tube growth down the style with examination of the ovary of the same pistil corroborated this evidence, since embryos were never formed unless the pollen tubes had actually penetrated the embryo sac. Additional evidence was obtained from L. regale x L. Davidii in which the pollen tubes grew all the way down the style but never initiated embryo development, even after 1000 hours. Therefore, if L. regale is apomictic the stimulus is supplied only after the pollen tube penetrates the embryo sac.

It is conceivable that non-diffusable substances contained in the pollen tube could be effective in inducing apomixis. However, it was observed that viable seeds were not formed in the crosses L. regale x L. longiflorum and L. regale x L. Leichtlinii var. Maximowiczii, even though sectioned material did show occasional penetration of the embryo sacs by the pollen tubes. Apparently, the mere discharge of the male gametes into the embryo sac is in itself not sufficient to induce apomictic development.

This was confirmed by the series of experiments which were undertaken to determine the cause of the constant failure of L. regale to form seed in any interspecific cross (Table 2) other than that with L. leucanthum var. chloraster. Since the micropylar nuclei showed visible indications of disintegration 300 hours after pollination if fertilization had not occurred, it was considered likely that this disintegration had been initiated before the actual observation. This led to a study of pollen tube growth rate and an evaluation of the number of pollen tubes present in the stylar canal of L. regale. This study clearly indicated that in many cases the rate of growth and the number of pollen tubes present were significantly lower when interspecific pollinations on L. regale were compared with intraspecific L. regale pollinations.

A combination of these two factors (slow pollen-tube growth and early disintegration of the micropylar nuclei) suggested a possible cause for the failure to set seed in some instances. A survey of the literature indicated the existence of many chemicals which exert some type of control over pollen germination and pollen-tube growth. It was anticipated that treatment of L. regale with some of these regulatory substances might cause more rapid growth of and an increase in the number of the pollen tubes, thus allowing the male nuclei to enter the embryo sac prior to initiation of disintegration. Since pollinations with the various supplemental treatments failed to form any viable seed, it may be assumed that the material was not absorbed by the plants; that the treatments were at the wrong concentration; or that the substances tested were not the limiting factors.

The styles of L. regale were shortened prior to the application of pollen from the short-styled L. callosum in an attempt to determine if, in some instances, the length of the style was the limiting factor in pollen-tube growth and subsequent seed set. The results indicated that factors other than the length of the style were involved, since viable seed did not develop. However, the extreme enlargement of the ovaries as a result of this treatment, when compared with non-shortened styles, indicated that the pollen tubes had at least grown partially into the ovary in order to deliver the auxin required for enlargement.

All of the above observations indicated that, at least, active penetration of the embryo sac was necessary before viable seeds were formed. Thus, the cellular mechanism of embryo development offered the only remaining clue to potential apomixis in this species.

CYTOLOGICAL OBSERVATIONS OF THE EMBRYO SAC AND EMBRYO

Gustafson (11) and Stebbins (22) have both recently reviewed the extensive literature concerning apomixis and attempted to bring some semblance of order to the terminology. In the course of their discussions, no mention is made of the genus Lilium. However, in other plants the formation of a diploid female gamete, the fusion of two micropylar nuclei, or sporophytic budding have been reported and any one of the three would explain the apomictic phenomenon attributed to L. regale. Stout (27) has suggested some type of apogamy while Hall et al (14) mentioned "budding" as the possible cellular mechanism of apomixis in Lilium. However, neither authors presented any evidence on which to base their statements.

There is adequate evidence in the literature to eliminate the formation of diploid gametes as the natural mechanism of apomixis in L. regale, despite the recent experimental observations of Rosenberg (20) indicating that diploid female gametes might be induced in L. longiflorum. The short exposure to a very low temperature, which he reported as inducing the formation of diploid female gametes, was not involved in the other numerous reports of apomixis and it was certainly not a factor in this study. Cooper (6) has shown that the embryo sac formation in L. regale conforms to normal development of the embryo sac in Lilium and results in the formation of haploid micropylar nuclei. Further, O'Mara (15) and Cooper (7) have shown that the male gametes are haploid and typical in their development. Beal (2, 3) has recently shown that the male gametes are discharged into the embryo sac and that normal fertilization occurs in intraspecific crosses. The present investigation extends these observations to interspecific crosses in the majority of cases observed. In both the observations of Beal (2, 3) and the present investigation there was no indication that

the embryo developed from any nucleus other than one of the complex of micropylar nuclei. Thus, present observations and those cited in the literature seem to eliminate not only diploid gametes, but also sporophytic budding as the origin of apomictic seed.

The observation of abnormal embryos is of considerable interest, since they occurred only in interspecific crosses which failed to set viable seed. Beal (4) did not believe them to be of importance in an explanation of the supposed apomictic behavior, due to their low frequency of occurrence. However, it may well be that these represented occasional hybrid embryos in which the genomes were sufficiently compatable to allow some abnormal development. Probably many abnormal embryos abort at early stages of their development and evidently none are sufficiently balanced to allow complete differentiation. If these embryos were apomictic, they would necessarily have the balanced genome of L. regale and there would be no reason for them to develop in an abnormal manner.

To summarize the discussion up to this point: pollination, pollen-tube growth, penetration of the embryo sac by the pollen tube, and discharge of the male gametes must occur before embryo development begins. Both the male and the female gametes are apparently normal and, in all but an occasional embryo sac, a normal embryo develops from a zygote which resulted from the normal fusion of the male and the female gametes. Further, sporophytic budding does not occur. The only exceptions observed to this normal sequence of events in fertilization and embryo development were the occasional fusion of synergids and the formation of abnormal embryos after interspecific hybridization. Neither observation, individually or collectively, is sufficient to account for the high frequency of apomictic development believed by previous investigators to occur in L. regale.

EVIDENCE FOR THE OCCURRENCE OF APOMIXIS IN *LILIUM*

Breeding.

The following is an analysis of breeding experiments by which the apomictic behavior of *L. regale* has supposedly been established. Pfeiffer (18) and Preston (19) crossed 30 species, varieties, and hybrids on *L. regale* obtaining no seed with 14 of these and 38% successful crosses with the other 16 pollen parents. Various other authors have reported successful crosses when nine of the pollen parents employed by Pfeiffer (18) and Preston (19) were used and when using nine additional ones. These investigators, however, present no numerical data. The F_2 populations have been studied by Beal (2, 3), Parkman (16), Pfeiffer (18), and Preston (19) and they have never found any indications of a male parent. These data indicate that the pollen parent has some effect on the induction of apomixis.

Cytological.

Cytological evidence of the occurrence of apomixis in *Lilium* is rather limited. Stewart and Emsweller (24) observed the chromosome morphology of root tips resulting from the cross *L. Leichtlinii* var. *Maximowiczii* x *L. tigrinum* and found only 1% sexual plants. Beal (2, 3) has observed meiosis in the progeny of apomictic populations of *L. regale* and found no peculiarities which would serve to distinguish them from progeny of selfed *L. regale*. The fusion, or partial fusion in some cases, of two sets of micropylar nuclei occasionally observed in three ovaries in this investigation may be regarded as a means of apomictic development. This is the only cytological observation of the embryo sac known to the author which offers even a reasonable clue to the cellular mechanism of apomixis in *L. regale*.

EVIDENCE FOR SEXUALITY IN LILIUM.

Breeding.

While apparently apomictic behavior in Lilium has been unusual enough to be reported frequently, especially in the popular literature, the occurrence of hybrids has remained unpublished or included only in general reports. L. x Golden Gleam was reported as a hybrid between L. pumilum and L. martegon var. album F.C.C. [Slate (21), Wilson (29)], while a hybrid of L. pumilum x. L. aurantiacum Weston has been described [Woodcock and Coutts (30)]. L. x Horsfordii was recorded as the hybrid L. Leichtlinii var. Maximowiczii x L. dauricum var. venustum f. Batemanniae E. H. Wils. [Woodcock and Coutts (30)] and the same female parent gave a sexual hybrid when crossed with L. tigrinum [Stewart and Emsweller (24)], L. superbum has yielded sexual hybrids when crossed with L. canadense [Pfeiffer (17)] and L. carolinianum Michx. [Stewart (25)]. Sexual hybrids of L. regale pollinated with L. candidum, L. Henryi, L. myriophyllum var. superbum, and L. Sargentiae have been cited in the introduction.

The formation of viable seed in L. regale following certain interspecific pollinations, as cited in the literature, is at variance with the results of this investigation, under conditions where contamination by L. regale pollen was impossible, in the majority of instances (Table 4).

Table 4.

A Comparison of the Seed Set Following Interspecific Pollinations as Reported in the Literature and as Observed in this Investigation.

Male Parent	Literature		Rappleye	
	Seed Set	No Seed	Seed Set	No Seed
L. pumilum	0	4	0	3
L. Leichtlinii var. Maximowiczii	0	1	0	58
L. auratum	1	2	0	19
L. candidum	5	25	0	7
L. longiflorum	1	23	0	138
L. speciosum	2	4	0	17
L. tigrinum	3	6	0	47
L. leucanthum var. chloraster	4	0	96	28

Two species formed no viable seed in either case. L. leucanthum var. chloraster as a pollen parent was previously reported 100% effective in causing seed set, while in this investigation this pollen initiated seed formation in 96 out of 124 attempts. Of particular importance, however, are the five species which were reported in the literature to be 20% effective in inducing seed set in 72 attempts. In this investigation, when the same species were used as pollen parents, there was no seed set in 228 attempts. The differences in the results obtained could be understood on the basis of genetic differences between the L. regale stocks used if the results of this investigation had been all negative. However, it is difficult to accept this as an explanation when only some of the crosses behaved differently. A second possibility is the effects of different environments, since all other crosses were evidently made on outdoor plantings while those reported here were made on forced greenhouse plants. A third possible explanation is the failure of adequate protection against L. regale pollen in the crosses reported in the literature.

Emsweller (10) has reported much the same experience with L. longiflorum. Although this species is recorded as forming apomictic seed following many interspecific pollinations, he has never obtained a single mature seed from an interspecific cross, having tried over a period of 13 years, using 10 different pollen parents.

Cytological.

Observations of Beal (2, 3) and those reported here show that, in the great majority of observed embryo sacs, normal sexual fusion occurs and that an embryo develops to maturity from the resulting zygote. These observations of fertilization are in close agreement with those of Coulter et al (8) in

L. philadelphicum Linn., Cooper (6) in L. Henryi, and Blackman and Welsford (5) in L. martagon Linn. and L. auratum. It is to be noted that the crosses which employed L. leucanthum var. chloraster as the male parent were very successful in the production of viable seed. However, this species is very closely related to L. regale and possibly even the same species [Stewart (23)] . In crosses with more distant species as the pollen parent there was a high rate of incidence of the abnormal type of embryo. Since gradations between normal and abnormal embryo types have been observed, this strongly suggests sexual reproduction and that even the normal embryos are sexual.

It may be well to point out that the information available at the onset of this investigation indicated only apomictic seed formation in L. regale. Thus, the preliminary work and interpretations were carried out presuming its occurrence. However, the failure to observe any morphological behavior which would substantiate this supposition led to an intensive search for crosses which could be proven, by examination of the progeny's chromosomes, to be either sexual or apomictic. Thus, normal or apomictic behavior in the embryo sac could be definitely established in a given cross and compared with the other. The first series of experiments, the attempt to induce wide interspecific crosses with supplemental chemical treatments, failed completely. A second series, using X-rayed pollen, showed positively that seed formation in intraspecific crosses was sexual. This established the intraspecific crosses with L. regale as sexual and furnished a normal for comparison with interspecific crosses.

The fact that the use of X-rayed pollen resulted in an approximately linear negative correlation between dosage and the number of successful crosses and number of seeds with mature embryos and a positive correlation

between dosage and the number of seeds with incomplete embryos and seeds with no embryos, means that the pollen has functioned in the formation of these embryos. Since there was a reduction in number of successful crosses, it was conceivable that the dosage of 2000 Roentgen units may have rendered the pollen incapable of secreting some hypothetical substance which stimulated the formation of apomictic seed. However, the differences in percentages of seeds with mature embryos, seeds with immature embryos, and seeds with no embryos, at different dosages, can not be explained on such a basis. The small or partially formed embryos would seem to be the result of X-ray induced lethals functioning at different stages in the development of the embryo which had been stimulated into development by fertilization with the X-rayed pollen. Dobzhansky (9) has described a similar situation in Drosophila.

EVIDENCE FOR THE OCCURRENCE OF BOTH APOMICTIC AND SEXUAL SEED IN LILIUM

The occurrence of both sexual and apomictic seed in several species of Lilium has already been cited. Perhaps the most striking evidence of this behavior is to be found in a series of crosses made independently by Pfeiffer (17) and correlated with those made by Stewart (25) while studying the relationships of the eastern North American lilies, using L. superbum, a reported apomictic species, as the female parent. Stewart (25) observed that selfing and intraspecific crosses produced a sexual F_1, as did crossing with L. carolinianum. Using L. michiganense as a male parent, seeds were obtained which possessed embryos but which never germinated. L. philadelphicum as the pollen parent produced neither seed nor capsule enlargement. Pfeiffer (17) in crossing L. superbum x L. canadense, found two sexual seedlings among some 2000 apomictic seedlings. Thus, again there is very strong evidence that a wide range of reactions may be induced when different pollen parents are used on a single apomictic species.

A correlation of the observations of previous investigators indicating apomixis in L. regale with the results of this investigation, which shows the reproductive system to be sexual, indicates that the same variety of reactions noted above for L. superbum occurs in L. regale.

SUMMARY

Although Lilium regale has been frequently reported as an apomictic species, morphological and cytological observations indicated that the formation of an embryo occurred only after the actual fusion of the male and female gametes following either interspecific or intraspecific pollinations. No true interspecific cross formed viable seeds. Viable embryos developed from the resulting zygote after intraspecific pollinations and this was confirmed when X-rayed pollen at various dosages gave a positive correlation of dosage with both the number of seeds with no embryos and number of seeds with small embryos. A negative correlation was observed between dosage and both the number of seeds with normal embryos and the number of capsules set. This indicated that the embryos were the result of sexual fusion.

In the cross L. regale x L. leucanthum var. chloraster, a few embryo sacs were observed in which not only the male and female gametes were observed in various stages of fusion, but also the two synergids were observed to be fusing. It is suggested that fusion of synergids might explain the apomictic behavior of L. regale which has been reported in the literature.

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PLATE I

Figure 1. *L. regale* x *L. regale*, 120 hours after pollination. Uncoiled male nucleus (♂). (X2100).

Figure 2. *L. regale* x *L. leucanthum* var. *chloraster*, 132 hours after pollination. Spiral male nucleus (♂). (X2100).

Figure 3. *L. regale* x *L. leucanthum* var. *chloraster*, 108 hours after pollination. Coiled male nucleus (♂). (X2100).

Figure 4. *L. reglae* x *L. regale*, 114 hours after pollination. Male nucleus (♂) laying beside (♀). (X2100).

Figure 5. *L. regale* x *L. leucanthum* var. *chloraster*, 180 hours after pollination. Note 2 small nuclei (S) separated by a cell plate (CP) and spindle fibers (SF). See figure 6. (X2100).

Figure 6. *L. regale* x *L. leucanthum* var. *chloraster*, 180 hours after pollination. This photomicrograph was taken in exactly the same position as Figure 5 only at a different focus. Note cell plate (CP) shown in Figure 5. (X2100).

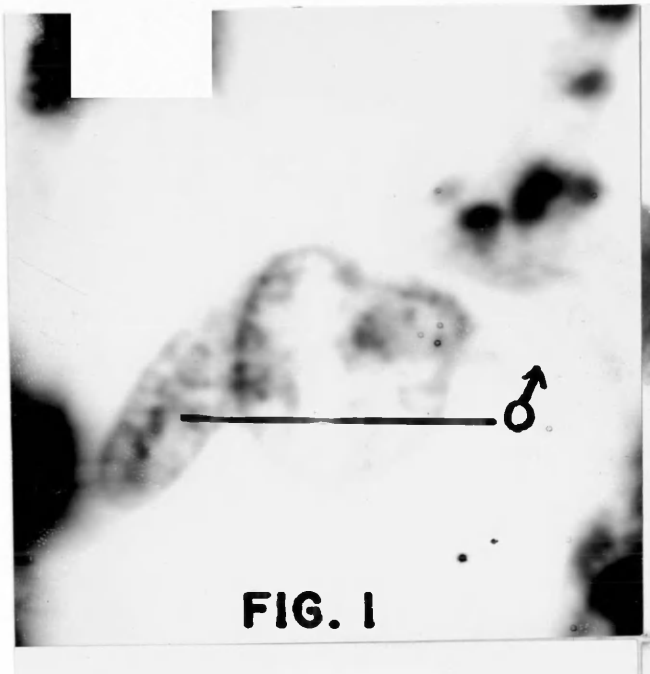

FIG. 1

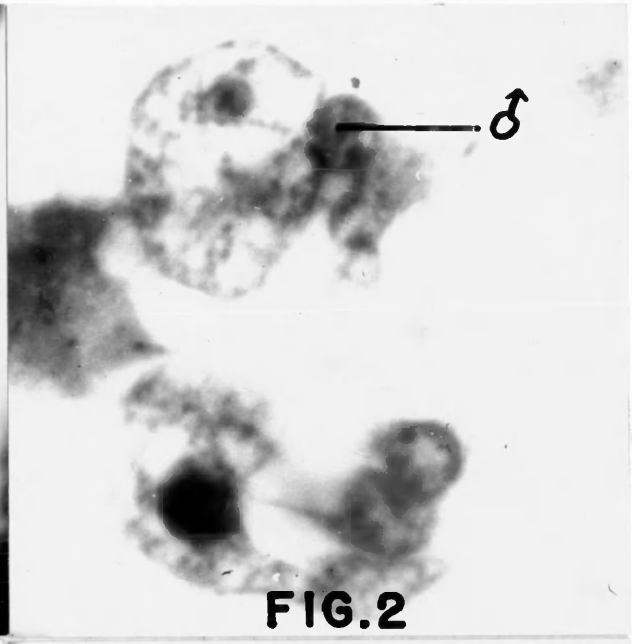

FIG. 2

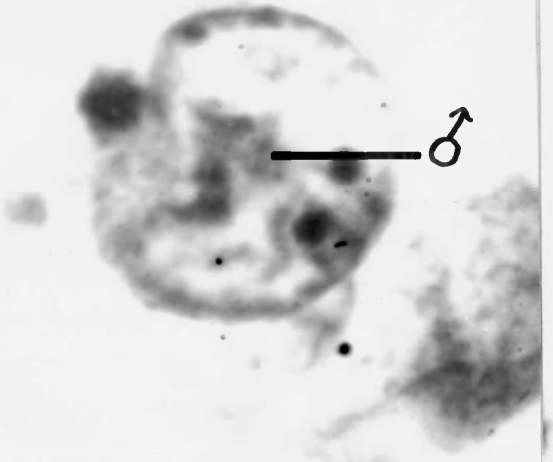

FIG. 3

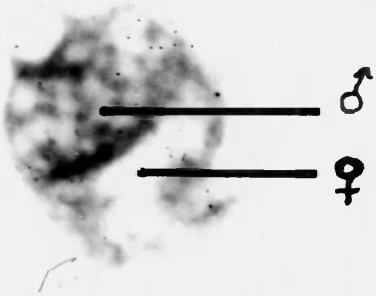

FIG. 4

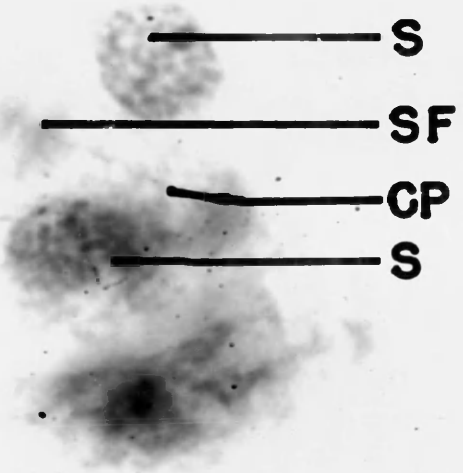

FIG. 5

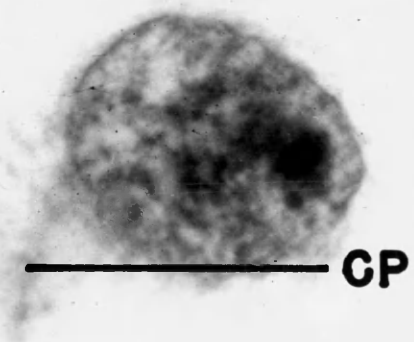

FIG. 6

PLATE I

PLATE II

Figure 7. L. regale x L. leucanthum var. chloraster, 216 hours after pollination. Two small nuclei (S) and one large nucleus (L). (X2100).

Figure 8. L. regale x L. leucanthum var. chloraster, 216 hours after pollination. Two nuclei partially fused. See Figure 9. (X2100).

Figure 9. L. regale x L. leucanthum var. chloraster, 216 hours after pollination. This photomicrograph shows the next section of the same ovule illustrated in Figure 8. Note large nucleus in prophase stage of division. (X2100).

Figure 10. L. regale x L. leucanthum var. chloraster, 216 hours after pollination. Two small nuclei (S) and anaphase stage of division of adjacent nucleus. (X2100).

Figure 11. L. regale x L. leucanthum var. chloraster, 216 hours after pollination. Two small nuclei (S) and telophase stage of division of adjacent nucleus. (X2100).

Figure 12. L. regale x L. Leichtlinii var. Maximowiczii, 1176 hours after pollination. This is the largest embryo observed in any true interspecific cross. Compare with Figure 13. (X200).

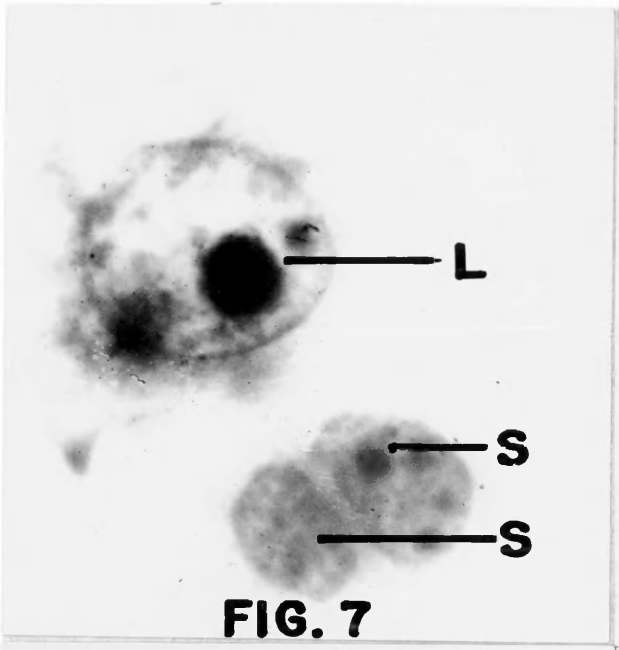

FIG. 7

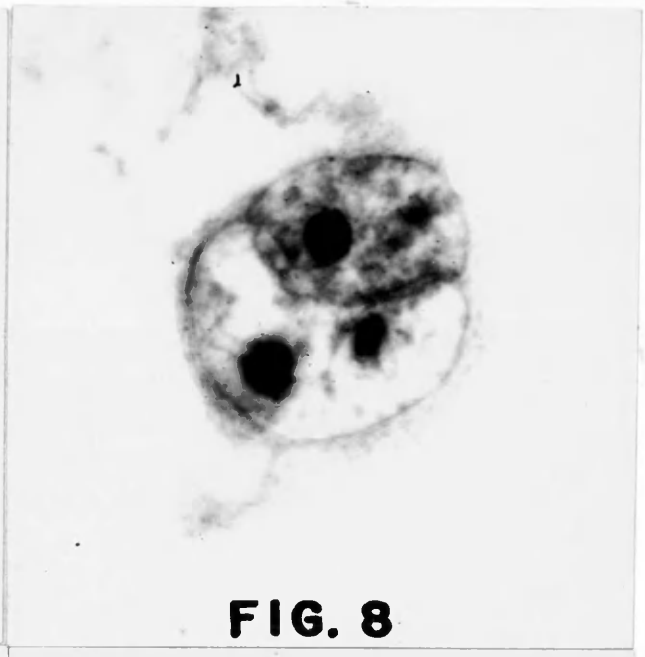

FIG. 8

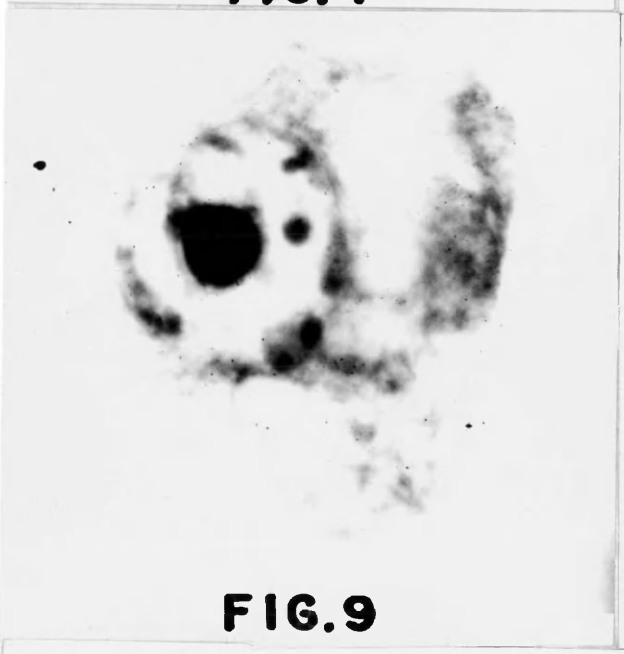

FIG. 9

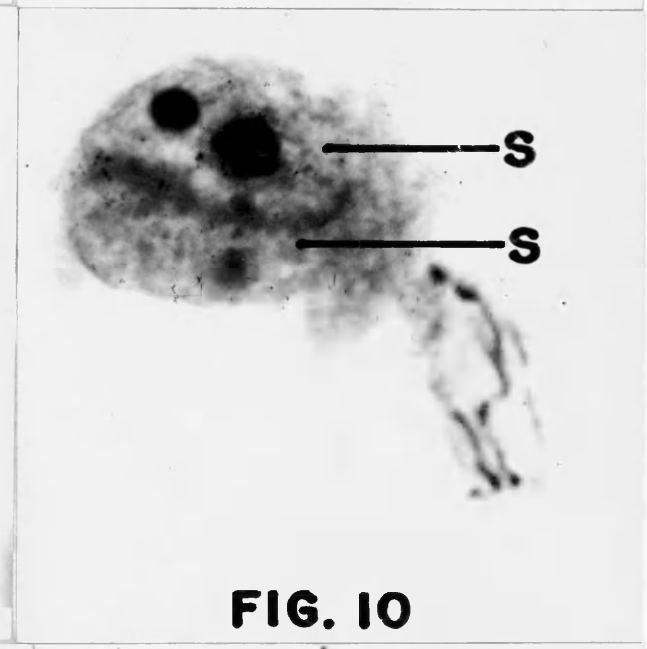

FIG. 10

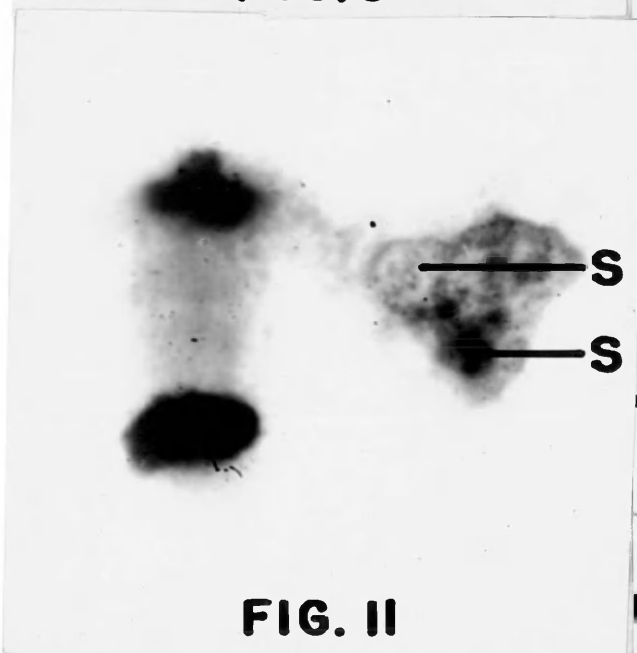

FIG. 11

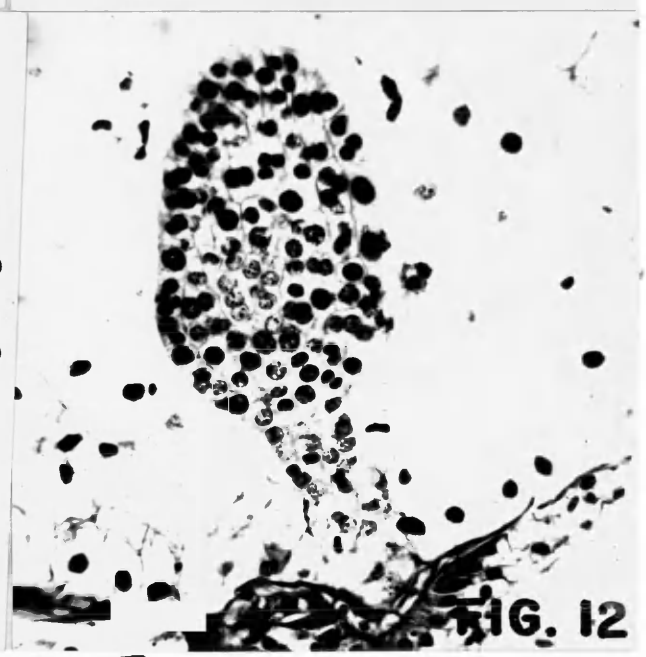

FIG. 12

PLATE II

PLATE III

Figure 13. L. regale x L. regale, 1176 hours after pollination. Embryo nearly full sized. Compare with Figure 12. (X50).

Figure 14. L. regale x L. longiflorum, 816 hours after pollination. Embryo, suspensor, and endosperm in embryo sac. Compare with Figure 15. (X300).

Figure 15. L. regale x L. regale, 744 hours after pollination. (X120).

Figure 16. L. regale x L. longiflorum, 816 hours after pollination. This embryo is normal and is comparable to intraspecific L. regale embryos at 600 hours after pollination. Endosperm is present although not shown in this photomicrograph. (X300).

Figure 17. L. regale x L. longiflorum, 816 hours after pollination. Abnormal embryo with no endosperm. This is comparable to the lower portion of the embryo shown in Figure 16. (X300).

Figure 18. L. regale x L. longiflorum, 816 hours after pollination. Abnormal embryo. See Figure 16. (X300).

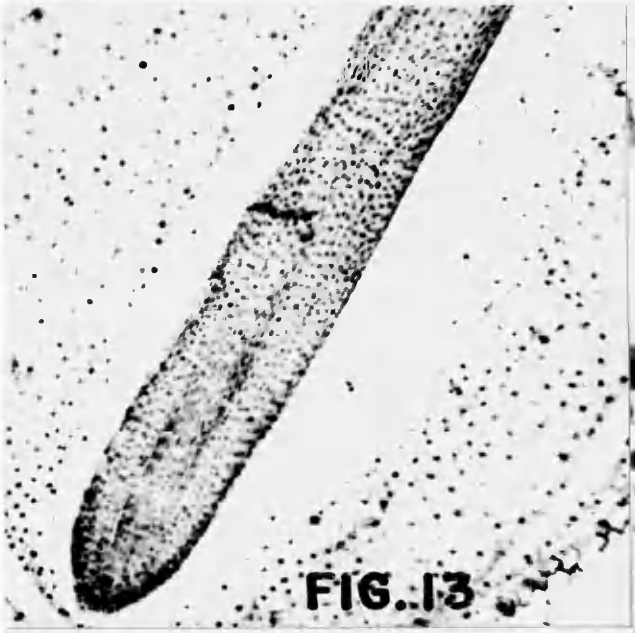

FIG. 13

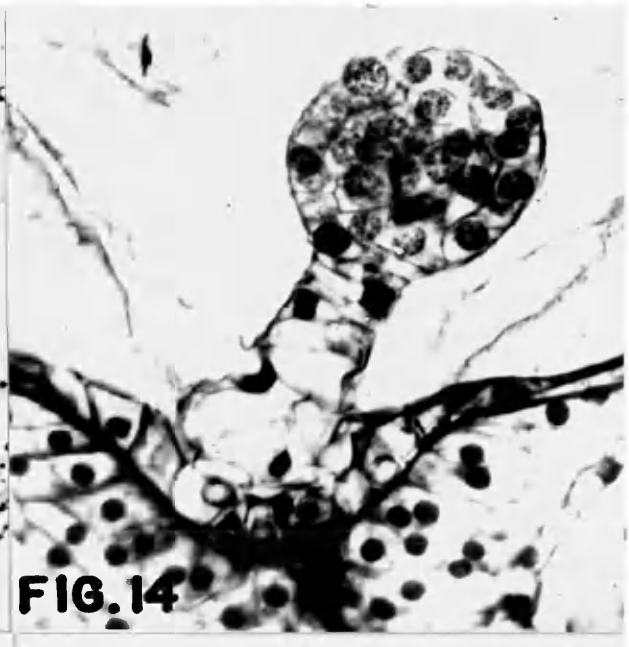

FIG. 14

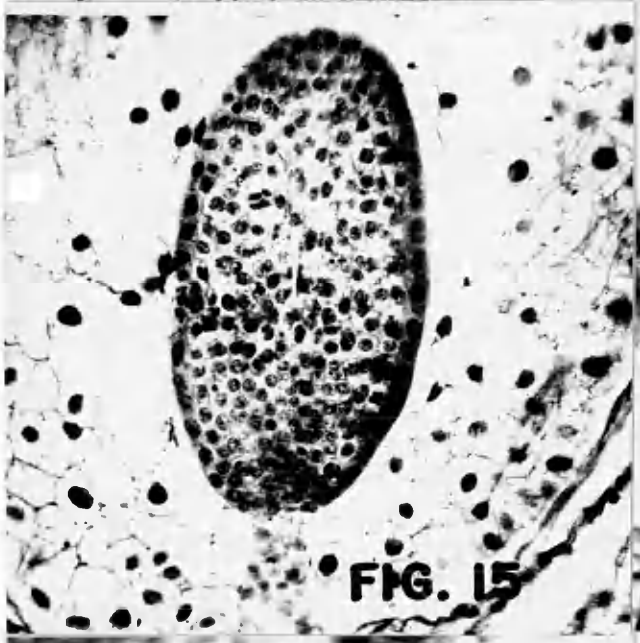

FIG. 15

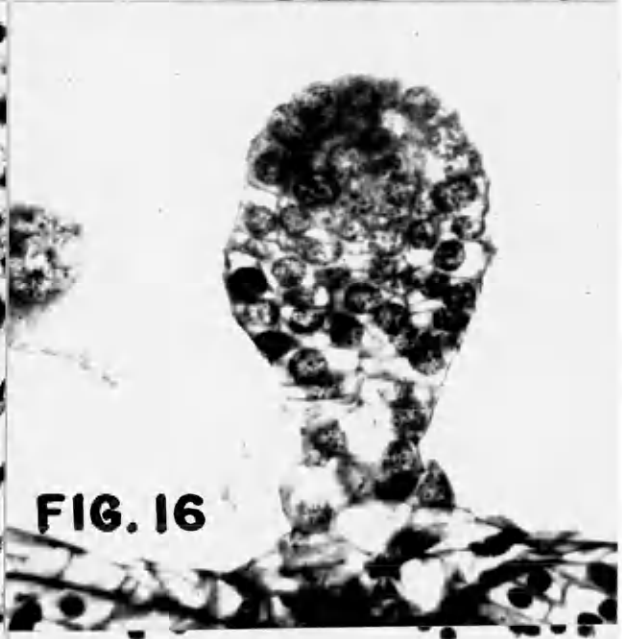

FIG. 16

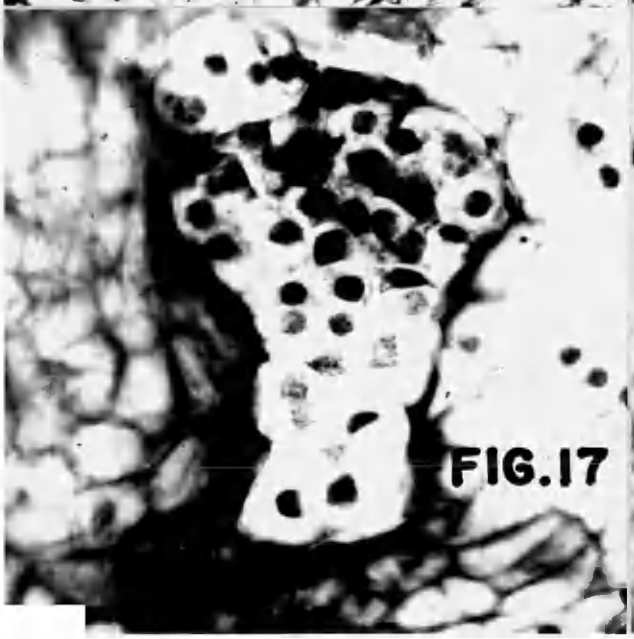

FIG. 17

FIG. 18

PLATE III

PLATE IV

Figure 19. L. regale x L. longiflorum, 816 hours after pollination. Abnormal embryo. See Figure 16. (X300).

Figure 20. L. regale x L. longiflorum, 816 hours after pollination. Abnormal embryo. See Figure 16. (X300).

Figure 21. L. regale x L. longiflorum, 960 hours after pollination. Abnormal embryo. See Figure 16. (X300).

Figure 22. L. regale x L. longiflorum, 816 hours after pollination. Intermediate type of abnormal embryo. This type of embryo is misshapen but, in contrast to the abnormal type, there is some endosperm present. (X300).

Figure 23. L. regale x L. longiflorum, 816 hours after pollination. Intermediate type of abnormal embryo. (X300).

Figure 24. L. regale x L. longiflorum, 816 hours after pollination. Intermediate type of embryo (E) with endosperm (EN). (X300).

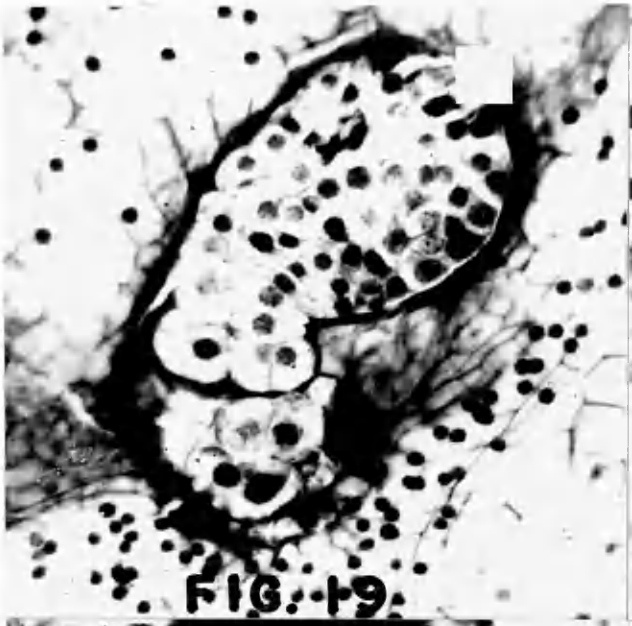

FIG. 19.

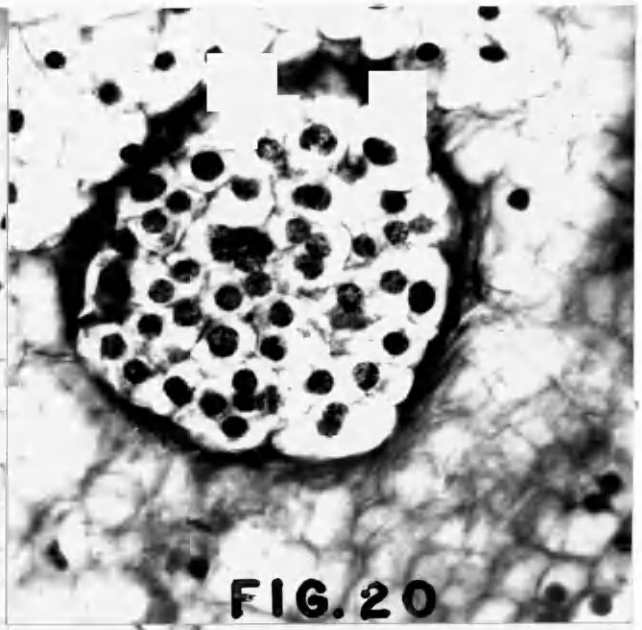

FIG. 20

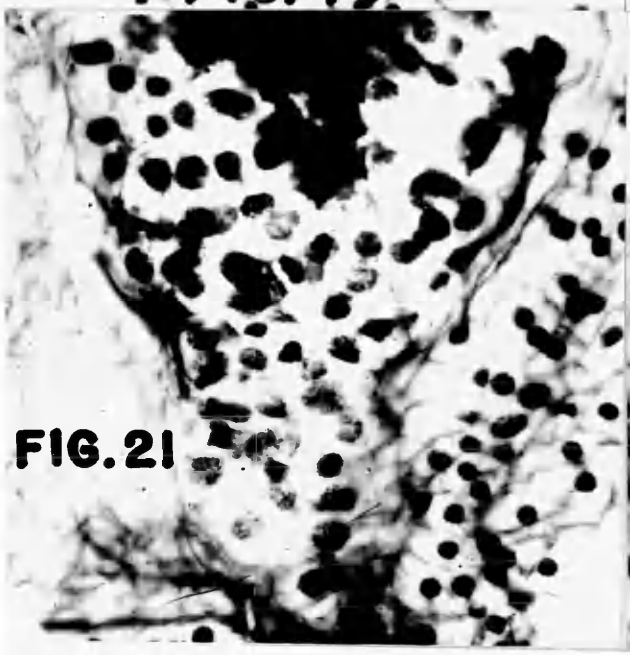

FIG. 21

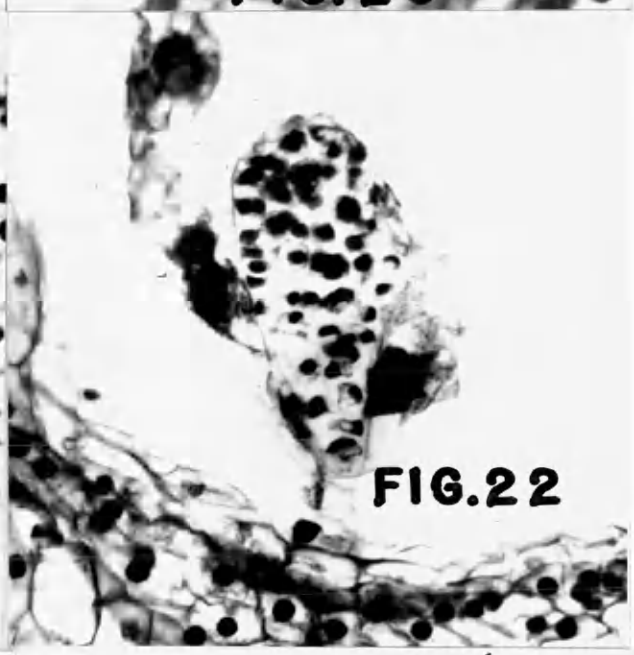

FIG. 22

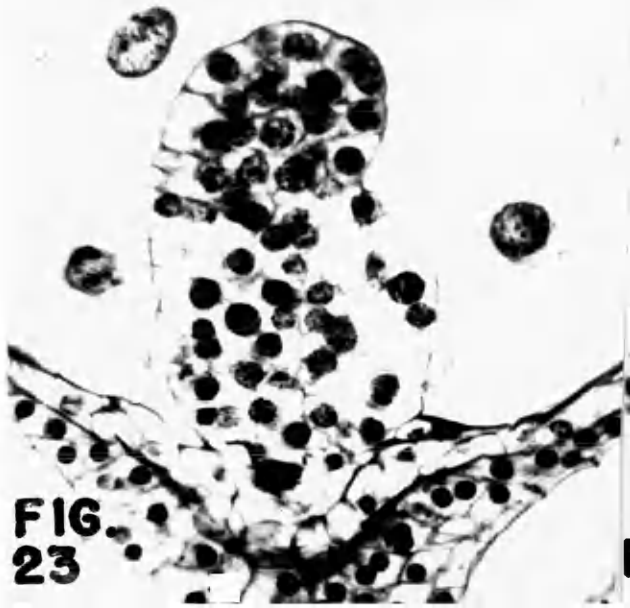

FIG. 23

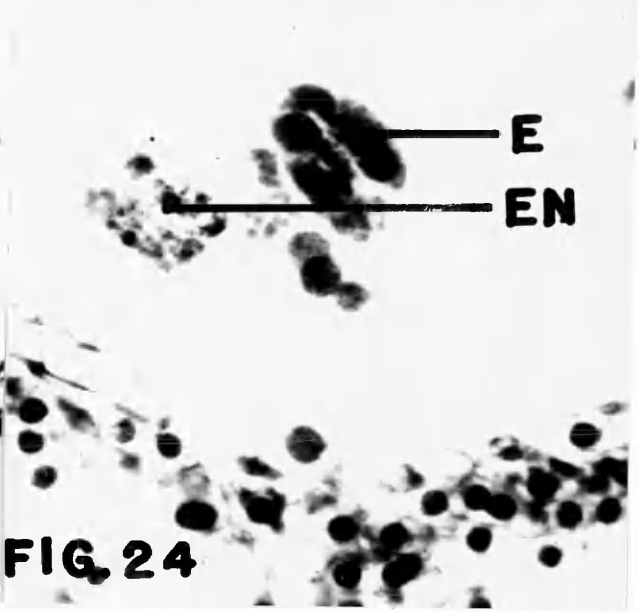

FIG. 24

PLATE IV

PLATE V

Figure 25. *L. regale* x *L. regale*. (Pollen x-rayed at 1000 R). (x2.2)

Top row: Seeds with no embryos or endosperm.

Second row: Seeds with no embryos and probably endosperm present.

Third row: Seeds with mature embryos and endosperm.

Fourth row: Seeds with embryos less than half the length of the mature embryos and endosperm.

Bottom row: Twin embryos. This is the only twin observed in over 20,000 seeds examined.

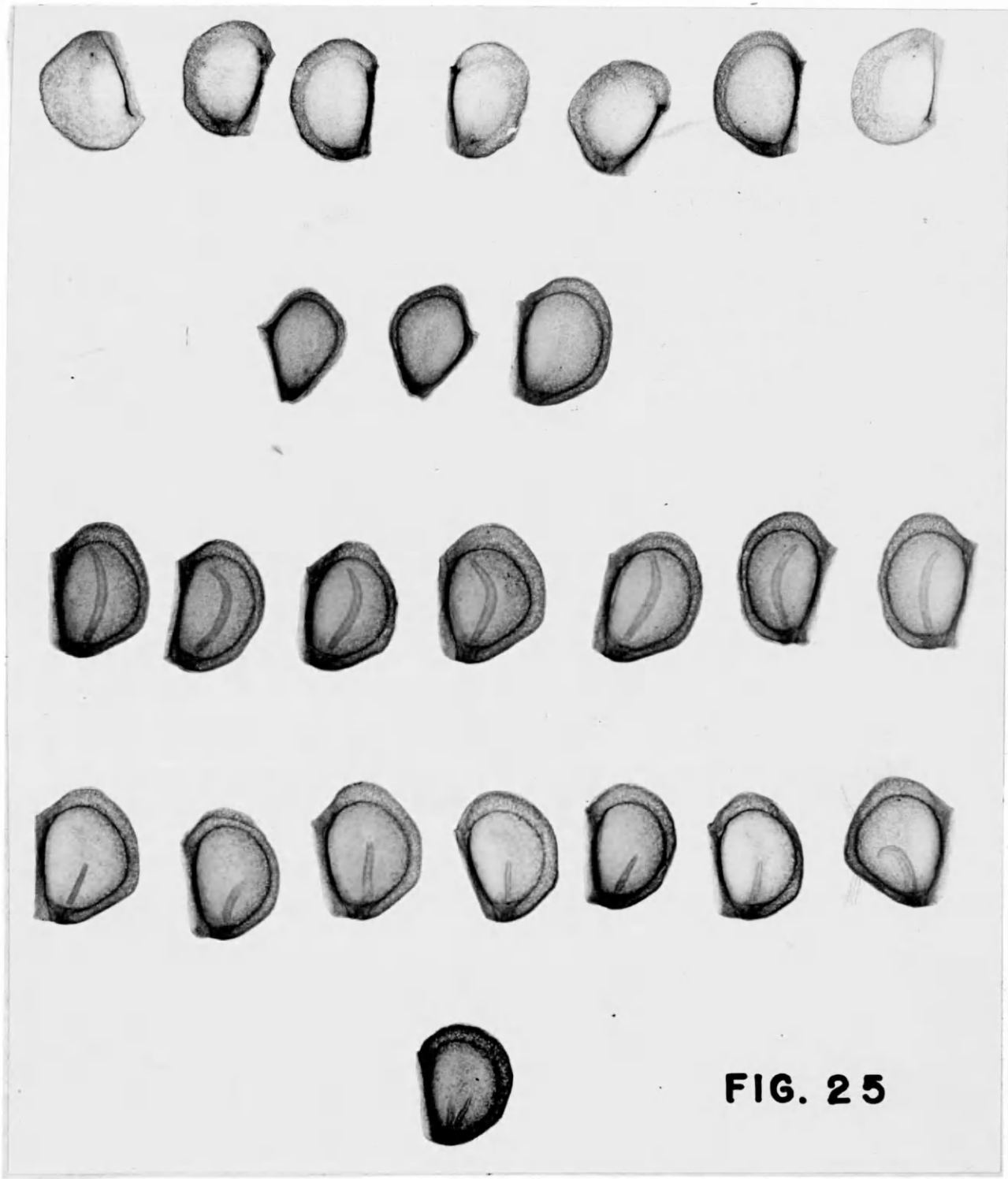

FIG. 25

PLATE V