ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. XXXIV — nr 3
1965

The impact of virus diseases on the viability of pollen and the development of embryo sac in *Lupinus luteus* L.

T. KAZIMIERSKI and E. M. KAZIMIERSKA

INTRODUCTION

Virus diseases are the cause of serious losses in a number of species of cultivated plants. Yellow lupin grown in Poland on vast areas in the Great Valleys region also shows symptoms of virus infection resulting in what had until recently been a slight reduction in yields but has now become a very serious problem.

The leaves, racemes, and pods of yellow lupin plants infected by virus diseases are deformed and the general appearance of such specimens differs from that of non infected plants. The sick plants yield a low crop seeds (Kazimierski 1961), often mis-shaped and insufficiently filled. Low yields in infected plants may be due, among other things, to low pollen viability, slow rate of growth and shortness of pollen tubes, and to the withering of embryo sacs.

Only few studies treat of *L. luteus* embryology. The problem is mentioned by Gollmick (1937) who investigated the proembryo development; references to the microsporogenesis and structure of the funiculus can be found in Maheshwari's (1950) and Schnarf's (1931) works. To our knowledge, however, no studies on the impact of pathogenic agents on the *L. luteus* embryo sac are available. The cause and effect sequence between crop yield and macrosporogenesis in representatives of other species, namely, in *Trifolium pratense* (Poljakowa 1954) and *Medicago sativa* (Maleewa 1960) was the subject of investigations.

The present paper proposes to study the effect of virus diseases on generative cells.

MATERIAL AND METHODS

Pollen was collected from the lateral racemes in the first row of plants showing strong symptoms of virus infection. The main inflorescence in such plants had either formed no pods or only very small

ones. Samples of pollen from lateral flowers of healthy plants were also collected for comparison.

A mixture of acetocarmine and glycerine was used to stain the pollen in order to determine the degree of its viability. This reagent stains selectively viable pollen cells red leaving the dead ones unchanged. Coloured and non-coloured pollen grains were counted on ten microscope slides under 400 × enlargement.

Pollen germination was tested in humidors at 20—23°C on the following five media

1. agar-agar 1%, sucrose 1%, citric acid 0.001%
2. „ 1%, „ 5%, „ „ 0.001%
3. „ 1%, „ 10%, „ „ 0.001%
4. „ 1%, „ 15%, „ „ 0.001%
5. „ 1%, „ 20%, „ „ 0.001%

Germinating and non germinating pollen grains were counted on ten microscope slides after 6 and 24 hours. The length of the pollen tubes was measured in not less than 30 germinating pollen after 24 hours. When germination gave poor results, the length of pollen tubes was measured in all germinating pollen grains. Part of the pollens inseminated in a medium containing 20% sucrose was treated with strong Navashin's fixing fluid after 6 and 24 hours, in order to establish the behaviour of nuclei in the germination process. Then the material was stained with crystal violet and orange G, differentiated in Lugol's fluid and, after treatment with xylene, enclosed in Canada balsam.

Material for study of the development of embryo sac was fixed in FAA fixing agent (Formaldehyde 40%, ethyl — alcohol 70%, glacial acetic — acid, ratio 9:0.5:0.5), was preserved in 70% alcohol and embedded in paraffin according to the method used in microtechnical preparation. It was later cut into section 10 μ thick, stained in Heidenhain's hematoxyline and basic fuchsin according to Feulgen's method, counterstained with fast green and embedded in Canada balsam.

The material for investigation was taken from 20 infected plants and 14 healthy ones. The drawings were made with PZO apparatus build on the principle of Abbe's apparatus; the photographs were taken with an Exa camera and the microscope used was a PZO MB-10.

RESULTS

Lupine pollen germinates on agar-agar medium with sugar added (Aleksiejenko 1951). The first pollen germination tests were carried out on the following medium: agar-agar, 1%, sucrose 5% and citric acid, 0.001% (Mackiewicz 1958). The number of germinating pollen grains was determined after 6 and 24 hours. The following results were obtained (Table 1).

Table 1

Germination of *L. luteus* pollen grains in healthy and virus infected plants, on medium containing 5% sucrose, after 6 and 24 hours

Germinating pollen grains were counted after hours	Plants: healthy — number of pollen grains				Plants: infected — number of pollen grains			
	non-germinating	germinating	total	% of germinating pollen grains	non-germinating	germinating	total	% of germinating pollen grains
6	1021	88	1109	7.93	722	61	783	7.79
24	576	103	679	15.18	454	54	508	10.63

After 6 hours the percentage of germinating pollen grains was low and nearly the same for healthy plants and for those with symptoms of virus diseases. After 24 hours the percentage slightly increased.

It seemed rather unlikely for *L. luteus* to have a low percentage of germinating pollen grains. It may have been that the pollen of infected plants required a different concentration of sucrose. A second series of tests was therefore made in which the quantities of agar-agar and citric acid were left unchanged while sugar was added to the medium in quantities of 1, 5, 10, 15 and 20%.

Table 2

Germination of *L. luteus* pollen grains in healthy and virus infected plants after 6 hours

Medium components	Plants: healthy — number of pollen grains				Plants: infected — number of pollen grains			
	non-germinating	germinating	total	% of germinating pollen grains	non-germinating	germinating	total	% of germinating pollen grains
1. 1% of sucrose	816	10	826	1.2	3315	46	3361	1.3
2. 5% of sucrose	703	123	826	14.8	1844	106	1950	5.4
3. 10% of sucrose	490	329	819	40.1	2091	306	2397	12.7
4. 15% of sucrose	377	298	675	44.1	1605	329	1934	17.0
5. 20% of sucrose	180	542	722	70.9	1684	398	2082	19.1

The results are shown in Table 2. On the medium containing 1% of sucrose there is practically no difference in the germination of pollen grains from healthy and infected plants. The percentage of germinating

pollen grew with the increase of sucrose content in the medium. The highest values were obtained on the medium containing 20% of saccharose. It is interesting to note that both the pollen of healthy and infected plants show a rising tendency of germination corresponding to increasing sucrose content. On the other hand, with various culture media there were differences in the percentual increase of germinating pollen grains in healthy and infected plants. The percentage of germinating pollen in healthy plants grew much more rapidly than that in sick ones. The slight increase in the percentage of germinating pollens when a medium containing 10% of sucrose was replaced by one containing 15% is not easily explained. The increase in the percentage of germinating pollen grains of healthy and infected plants was so slight after 24 hours that it was hardly worth noting.

Differences in the intensity of germination in pollen of healthy and sick plants were noticeable already when the medium containing 5% of sucrose was used and they became increasingly more apparent as the sugar was increased in the medium. With 5% sucrose, the percentage of germinating pollen grains in healthy plants was by 9.4% higher than in sick ones. On a medium containing 20% of sucrose the difference in germination between pollen grains of healthy and infected plants was 51.8% to the adventage of the former.

Table 3

Length in μ of *L. luteus* pollen tubes in healthy and virus infected plants (after 24 hours)

Medium components	Plants:					
	healthy			infected		
	average	minimum	maximum	average	minimum	maximum
1. 1% of sucrose	66.1	36.0	138.3	67.2	36.0	152.7
2. 5% of sucrose	109.7	36.0	262.2	99.8	36.0	384.4
3. 10% of sucrose	136.7	43.2	384.4	114.3	36.0	327.0
4. 15% of sucrose	142.3	43.2	348.6	118.2	36.0	298.2
5. 20% of sucrose	190.7	86.4	403.2	110.2	36.0	298.2

The length of pollen tubes was measured after 24 hours and the average results obtained are listed in Table 3. They show that on a medium containing 1% of sucrose, there was no difference in the length of pollen tubes between pollen grains of healthy and sick plants. Differences began to be noticeable when 5% sucrose was used and they increased with the rise of sucrose content in the medium. At 5% sucrose concentration, the pollen tubes in pollen grains of healthy plants were by 9.9 μ longer than those of sick plants, and for 20% sucrose the

difference reached 80.5 μ. The minimum length of pollen tubes in infected plants remained at the 36 μ level, whereas in healthy plants pollen it increased beginning with the 5% content in the medium. Maximum length of pollen tubes occurred on media containing 20 and 5% sucrose for healthy and infected plants, respectively.

The germability of healthy plants pollen grains was uniform and the length of their pollen tubes was much more standarized than in pollen grains of sick plants in the case of which germination lacked

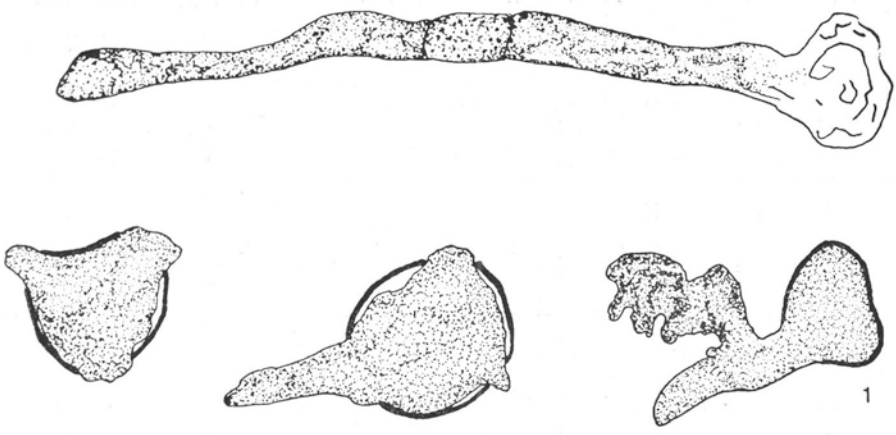


Fig. 1. Germinating pollen grains of healthy and virus infected yellow lupine. Long pollen tube in healthy and short pollen tubes in infected plants. Magnific.: 400 ×.

uniformity. In some of the infected plants no more than 2—3 germs sprouted in the pollen grains and their pollen tubes were extremely short (Fig. 1). This phenomenon was observed at all sucrose concentrations used.

Among the non-germinating pollen grains of infected plants a number were deformed. Some others swelled quite normally on the medium and their pores were distinctly visible, but they did not develop tubes.

The results of testing pollen viability during germination were compared with data obtained after the pollen grains had been treated with an acetocarmine and glycerine mixture (Photo *a* and *b*). As shown in Table 4, the percentage of pollen grains which absorbed stain was high in healthy plants. In infected plants, on the other hand, the percentage of pollen grains which absorbed red stain showed considerable fluctuation, oscillating from 48 to 95%. A similar reaction to the acetocarmine glycerine mixture was shown by the pollen germinating on the media i.e. where the percentage of pollen stained by the mixture was

low the percentage of pollen grains germinating on media with various sucrose contents was low as well, and vice versa. Differences existed in the value of viability percentages. Probably some of the pollen grains which had absorbed the acetocarmine, did not germinate on the media and hence the higher percentage of viable pollens under the aceto-carmine test.

Table 4

Results of analysis of *L. luteus* pollen grains in acetocarmine and glycerine mixture

No. of specimen	Healthy plants			
	Number of pollen grains			
	coloured	non-coloured	total	% of coloured pollen grains
1	385	3	388	99.2
2	666	29	695	95.8
3	413	9	422	97.8
4	285	2	287	99.3
Total	1749	43	1792	97.6
Plants showing symptoms of virus infection				
1	277	301	578	47.9
2	787	130	917	85.8
3	100	81	181	55.2
4	895	110	1005	89.0
5	385	104	489	70.5
6	634	34	668	94.9
7	821	252	1073	76.5
8	737	112	849	86.8
9	509	47	556	93.3
10	325	39	364	89.2
Total	5470	1210	6680	81.8

The generative cell in pollen grains germinating on the medium divide into male nuclei both in the pollen grain and in the pollen tube. Generally speaking the process of generative cell division in healthy and in virus infected plants developed along similar lines. None the less, among the pollen grains of sick plants a certain number were noted to posses long pollen tubes containing non-divided generative cells.

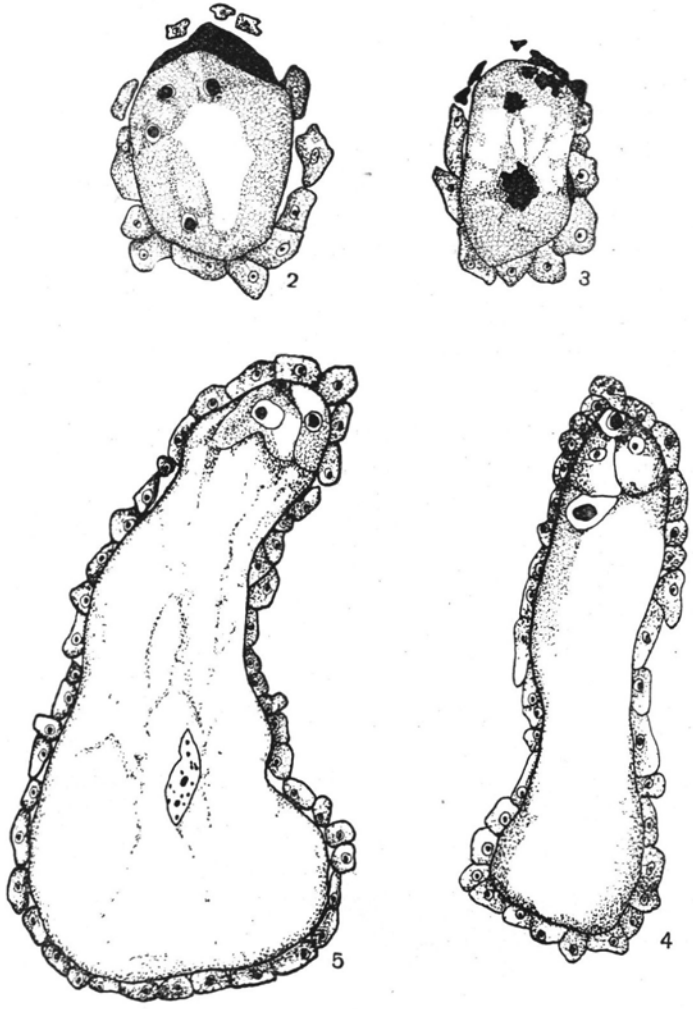
More than three hundred ovules of infected and healthy plants were analysed and development of the embryo sac was examined from the four nucleate phase on.

The yellow lupine embryo sac is monosporic, eight nucleate and it

develops according to the Polygonum type. The changes observed in the ovule structure fall into two cathegories:

1. deformation in the development of placenta,
2. degeneration of ovules.

Deformation in the development of placenta consists in its excessive elongation which transforms the placenta to a slightly flattened cylinder



Figs. 2—5. Embryo sacs of healthy and virus infected yellow lupine. *2* — normal four nucleate embryo sac; *3* — four nucleate embryo sac with degenerating nuclei; *4* — embryo sac with small quantity of cytoplasm and second nucleus of embryo sac situated under the egg apparatus; *5* — egg apparatus at initial stage of degeneration with a second nucleus of embryo sac split into a dozen or so basic bodies. Magnific.: Fig. *2*, *3* — 500 ×, Fig. *4*, *5* — 300 ×.

almost filling the interior of the ovary. The cells of the deformed placenta were large, strongly elongated, both wider and longer than the surrounding cells of the ovary (Photo *c*).

The lupine ovary is unilocular. Its interior is covered by one layer of epidermis, with two placenta situated along the ventral stitch line, which alternate in producing ovules. The ovule is campylotropous (Photo *d*). Degenerated ovules differ in shape from normal ones. In some ovules the integuments were also degenerated (Photos *e*, *f* and *g*). These integument cells were compressed and possessed no nuclei or cytoplasm. They formed a shapeless compact mass around the nucellus, and easily absorbed the fast green stain. In such ovules the cells of the nucellus were large, the vacuoles were filled and often possessed no nuclei or cytoplasm (Photo *f*). In the interior of such degenerate ovules sometimes walls of the embryo sac, without cytoplasm, egg apparatus or polar nuclei (Photo *e*) and often remnants of desintegrating egg apparatus and polar nuclei could be seen (Photos *e*, *h*, and *i*). In one of the earlier described deformed embryo sacs, possessing no egg apparatus, polar nuclei or cytoplasm, three cells were discovered strongly elongated and expanded at the micropylar end, growing from the nucellus into the embryo sac (Photo *j*).

There were noticeable disturbances in the development of the embryo sac at the four nuclei and eight nuclei stages. In the four nuclear phase of the embryo sac the disturbance consisted in desintegration of the nuclei (Figs. 2 and 3). In the eight nuclei embryo sac, the nuclei degenerated either directly after dividing or at a later period. Most disturbances occurred during ripening of the embryo sac. The alterations which occurred in the embryo sac at this period were:

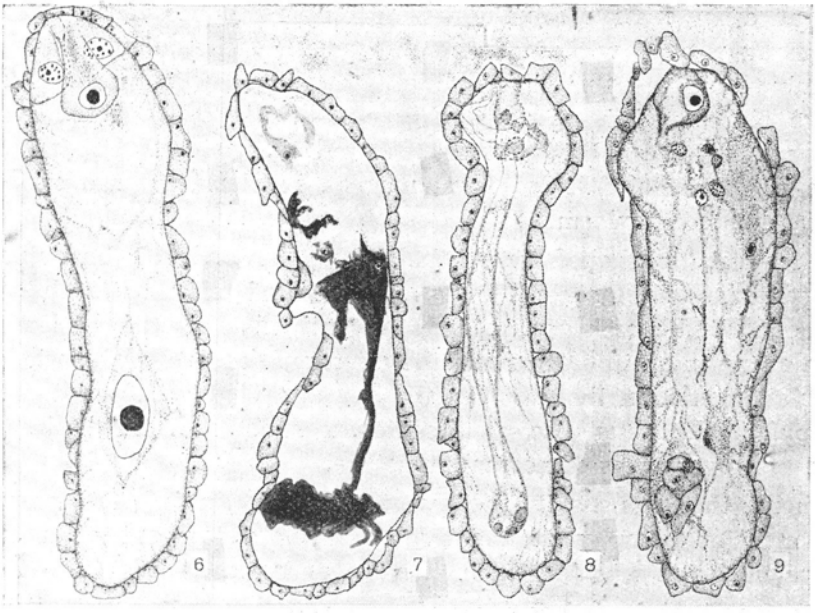
1. Cytoplasm degeneration until its total disappearance and the polar nuclei of the embryo sac were shifted to the micropylar end of the embryo sac (Fig. 4);

2. synergid degeneration and disappearance before fertilization;

3. splitting of the second nucleus of the embryo sac into a dozen or so basic bodies (Fig. 5);

4. total disintegration of the egg apparatus and polar nuclei of the embryo sac. In this case large stained bodies of indefinite shape were visible in the embryo sac (Fig. 6 and 7). Examination of the cross-section of such ovules revealed that hematoxyline stained substances in the embryo sac merged with the colouring agent in the adjoining cells of the nucellus. It is possible that these embryo sacs weakened by virus infection were invaded by fungus hyphae which caused total disintegration of the embryo sac.

Among other interesting observations the abnormal growth of the pollen tube towards the interior of the embryo sac should be mentioned.



Figs. 6—9. Embryo sacs of healthy and virus infected yellow lupine. *6* — Ripe embryo sac of healthy plant. *7* — Totally degenerated embryo sac. *8* — Embryo sac with pollen tube in its interior; degenerating synergids whose only remnants are nuclei hardly affected by colouring. *9* — Embryo sac with zygote, endosperm nuclei and four cellular additional embryos
Magnific.: 330 ×.

In several instances where fertilization had taken place, traces of pollen tubes were found only in the micropylar end of the embryo sac. In one specific case the pollen tube had probably been introduced into the interior of the embryo sac not through the micropyle since no trace of its passage was found in it. In this particular embryo sac degeneration of the synergids was far advanced and there were initial symptoms of disintegration in the egg cell (hardly visible nucleus, no normal vacuoles and nontypical cytoplasm). Under the degenerate egg apparatus the pollen tube was twisted spirally probably in order to avoid lifeless synergids. The whole pollen tube, with sperms and vegetative nucleus visible in it, was inside the embryo sac (Fig. 8).

One of the ovules contained a zygote and endosperm nuclei and also four-cellular adventitious proembryo in the chalazal end of the embryo sac (Fig. 9). The proembryo was in contact with the nucellus cells. It is hard to tell which cells it had originated; was it the antipodal which had failed to pass into the chalazal end of the embryo sac and produced the additional embryo, or perhaps the cells of the nucellus. The latter supposition seems more probable since in yellow lupine the antipodals are known to disappear at an early stage, namely when the egg apparatus is formed.

In an other embryo sac there were only remnants of the egg apparatus namely cells without nuclei, however in the micropylar end of the embryo sac there was an additional cell rich in cytoplasm and with a large nucleus. This was probably one of the cells of the nucellus which might have substituted the degenerate egg cell. Also the shape of the polar nuclei differed from normal. In these embryo sacs there was not much cytoplasm.

In a normally shaped ovary the micropylar ends of the ovules are directed towards the style. The egg apparatus is situated in the micropylar end of the embryo sac and the antipodals in the chalazal end. In one ovary in an infected plant the micropyle of two ovules was at the flower stalk end. In these ovules the egg apparatus was situated at the micropylar end, thus also at the flower stalk end. The third ovule in the said ovary was of normal structure.

Among the changes in the ovary structure in infected plants we must mention atrophy of the ovules and development of a suberic diaphragm which was formed in the flower stalk.

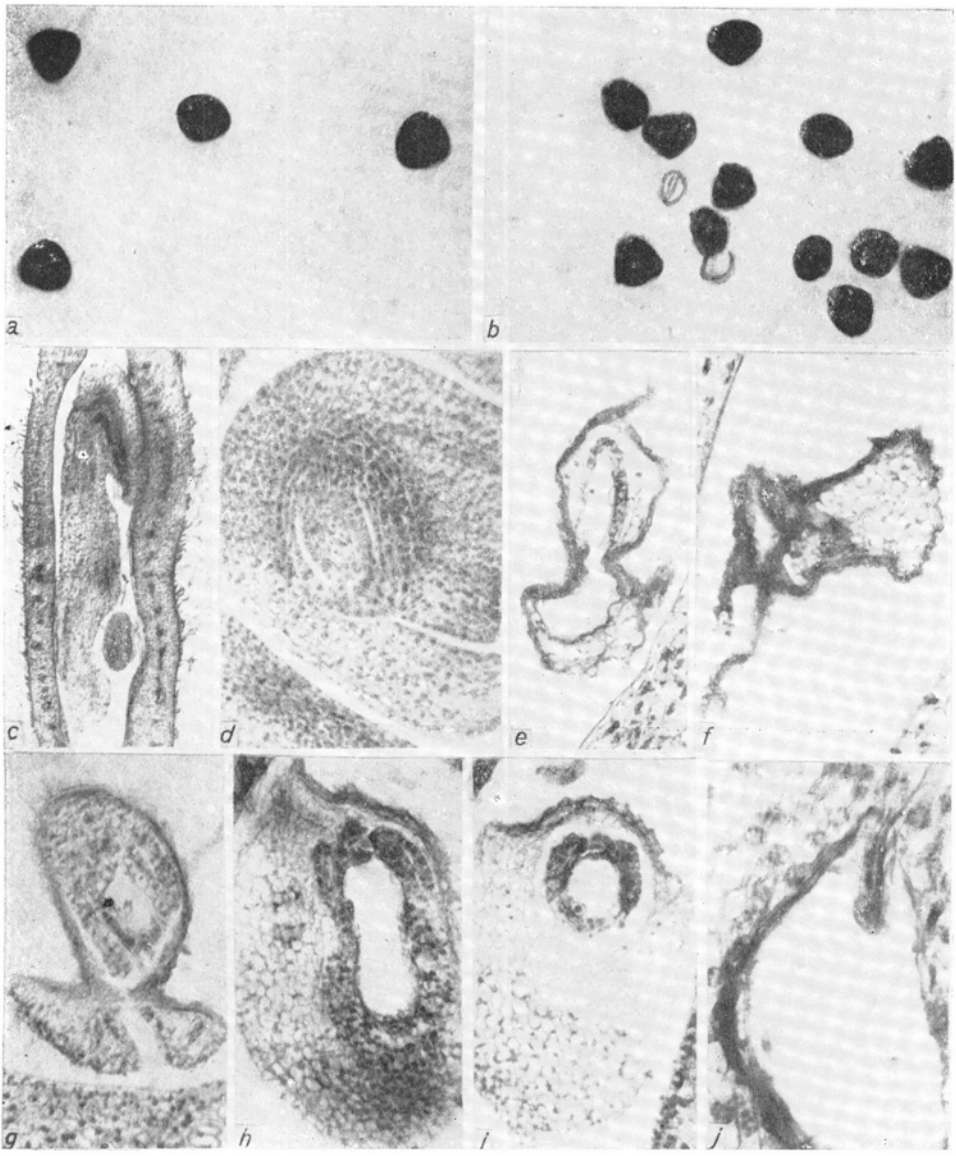
The fact that in infected plants the pods were insufficiently yield suggested that perhaps the cross-section of their vessels differed from that of healthy plants. The cross-section of inflorescence stalks cut at 0.5 cm from the lowest flower whorl showed that the average surface of vessels in a bunch of healthy plants was 177.7 μ^2 while in infected ones it was 315.1 μ^2. Water flows through these vessels as in any pipe, i.e. according to the general principles of hydrodynamics. The abundance of leaves in infected plants and the cross section of their vessels which had to supply a surface area larger than in healthy plants, suggest the conclusion that in the transpiration process more water was drawn by the former plants than by the latter.

DISCUSSION

Embryological, cytological and anatomical comparative investigation of the pollen grains, ovules and inflorescence stalks of healthy and virus infected yellow lupine plants showed that virus diseases caused serious disturbances in the development of embryo sacs, in pollen germination and in vessel structure.

Pollen germination on media containing low sucrose concentration (1% and 5%) was poor both in healthy and in infected plants. On media containing more sucrose the percentage of germinating pollen grains was much higher in the former than in latter plants. Pollen tubes growing from pollen grains of healthy plants at any concentration of sucrose in the medium were comparatively longer than those produced by infected pollen grains.

Plate I



Pollen grains and ovules of healthy and virus infected yellow lupine plants. *a* — Pollen grains of healthy specimen; *b* — pollen grains of a plant with symptoms of virus infection; *c* — deformation in the development of placenta; *d* — normally shaped ovule with integuments and nucellus; *e*, *f* and *g* — ovules deformed at various development stages with degenerated integument and nucellus cells; *h* and *i* — various degrees of ovules deformation with visibly deformed embryo sacs; *j* — embryo sac without cytoplasm, egg apparatus and polar nuclei; three nucellus cells are growing from the micropylar end towards the interior of the embryo sac.

Pollen viability tests results obtained by the method of staining pollen grains with a mixture of acetocarmine and glycerine as compared with the results of pollen germination on agar-agar media show that the first method yields higher results (Kazimierski 1961) than the second. This could perhaps be explained by the fact that carmine also stains pollen grains containing cytoplasm which neverthless are not always capable of germinating. The actual viability of pollen can be determined in cultures on adequatly prepared media.

In healthy as well as in sick plants the generative cells in pollen grains divide into male nuclei both in the pollen grain and in the pollen tube. Non-divided generative cells were sometimes found in the pollen tubes of infected plants pollen grains. It is noteworthy that the generative cell in infected plants adsorbed the crystal violet stain much more intensinely than that of pollen grains in healthy plants.

The ovules in infected plants had degenerate integuments and nucelli. There were also irregularities in the position of the pollen tube inside the embryo sac.

CONCLUSIONS

1. Comparison of the germination of pollen grains in healthy and virus infected yellow lupine plants has shown the percentage of germinating pollen grains in healthy plants to be much higher than in sick ones. Also the pollen tubes grown by healthy plants are much longer.
2. In virus infected plants the components of the embryo sac degenerate at various stages of its development. Some embryo sacs were found to be in a state of complete degeneration. Ovules in infected plants were often deformed, lacking integuments, some cells were without nuclei and sometimes without cytoplasm.
3. In cross-section the surface of vessels in infected plants is larger than in healthy ones.
4. Defective yielding of seeds in virus infected plants is caused by the low viability of pollen grains and by degeneration of the embryo sac.
5. In order to obtain high yields of vigorous yellow lupine seeds measures should be taken to destroy pests, particulary virus bearing insects (with sucktorial and sting organs in their mouths). Also virus resistant varieties should be developed and given priority in cultivation.

The authors are indebted to Dr. Stefan Krupko, Professor of Botany, Adam Mickiewicz University, Poznań for advice and information he most kindly offered.

(Entered 9.V.1963)

REFERENCES

Aleksiejenko A. I., 1951, Żizniesposobnost' pylcy i rylca u liupina, Sieliekcja i siemienowodstwo 11.

Gollmick F., 1937, Über Artkreuzungen bei Lupinen, Züchter. 9:65—68.

Kazimierski T., 1961, Anomalie w budowie kwiatów i strąków u *Lupinus luteus* L., Rocz. Nauk Roln. 84-A:811—820.

Mackiewicz H., 1958, Żywotność pyłku u trzech gatunków łubinu, Rocz. Nauk Roln. 79-A:103—138.

Maheshwari P., 1950, An introduction to the embryology of angiosperms (Russian translation, I.I.L., Moskwa, 1954).

Maleewa Z. P., 1960, Żizniesposobnost' zarodyszewogo mieszka i pylcy u lucerny raznych srokow posiewa, Trudy po Prikład. Bot. Genet. i Sielek. 33:265—272.

Poljakowa T. F., 1954, Sostojanie matierinskich wosproizwoditielnych elementow u krasnogo kliewiera piered opłodotworeniem, Woprosy siemienowodstwa krasnogo kliewiera, 115—124, Leningrad.

Schnarf K., 1931, Vergleichende Embryologie der Angiospermen, Berlin.

Wpływ chorób wirusowych na rozwój woreczka zalążkowego łubinu żółtego

Streszczenie

Porównując kiełkowanie ziarn pyłku z roślin zdrowych i wirusowo chorych łubinu żółtego na sztucznych pożywkach wykazano, że rośliny zdrowe mają dużo większy odsetek pyłków kiełkujących niż rośliny chore. Także łagiewki pyłkowe pyłków z roślin zdrowych są dłuższe niż u pyłków z roślin chorych.

U roślin wirusowo chorych elementy składowe woreczka zalążkowego degenerują w różnych fazach jego rozwoju. Znajdowano woreczki zalążkowe kompletnie zdegenerowane. Zalążki z roślin chorych często były zdeformowane, nie miały osłonki, w komórkach brak było jąder i niekiedy cytoplazmy. Rośliny chore mają większą powierzchnię przekroju poprzecznego naczyń.

Słabe wiązanie nasion przez rośliny wirusowo chore jest powodowane słabą żywotnością pyłku, degeneracją woreczka zalążkowego. Otrzymywanie wysokich zbiorów zdrowych nasion łubinu żółtego wymaga zwalczania na plantacjach szkodników przenoszących choroby wirusowe (owadów o narządach pyszczkowych ssąco-kłujących) oraz prowadzenia hodowli w kierunku otrzymania form odpornych na choroby wirusowe.