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TRANSMISSION OF BEAN COMMON MOSAIC VIRUS BY SEED OF PHASEOLUS VULGARIS L. CULTIVAR BEKA

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CONTENTS

CHAPTER 1

INTRODUCTION

Transmission of viruses to the progeny of their hosts through seeds has proved to be of great importance in the spread of some virus diseases of different character. It already has been known for many years that several viruses transmitted by sap or by aphids can be seed-borne. Recent findings of LISTER (1960) emphasize the importance of seed infection. He detected that some soil-borne viruses transmitted by nematodes to roots of their host plants, can also infect the seed of these plants.

The way transmission occurs has been studied frequently from a practical point of view, since a high degree of cleanliness of seed is often required. Moreover the occurrence of embryo infection has been studied in order to obtain a better insight into the relation between host-plant and virus. An extensive literature deals with problems in this special field.

A striking aspect of the phenomenon of transmission of viruses by seed is its rather limited occurrence. Not only is the number of viruses known to be transmitted by seed relatively small, but the phenomenon may also be limited to definite strains of a given virus. Besides it may be restricted to certain host-plants, even to certain host varieties. Another limitation consists of the fact that in cases of virus transmission by seed only a part of the seed harvested from virus-diseased plants shows infection. The percentage of infected seeds may depend, among other factors, on the developmental stage of the host-plant at the time of its infection. In this respect some authors report that with a number of viruses, only infection before flowering can result in virus trans-

mission by seed (FAJARDO, 1930; COUCH, 1955; CROWLEY, 1957b). This suggests that infection of micro- and megaspores might take place, but that infection of a developing embryo is impossible. It was, however, observed that some viruses can infect embryos during an early developmental stage (CROWLEY, 1959).

These discrepancies in results obtained by different authors may be due to the difference in host-plants and viruses they used for their experiments.

From the many factors involved in the occurrence of seed infection by virus, the way a host-plant is influenced by a virus is highly important. In this respect a systemic reaction may result in one effect and a hypersensitive one in another. In the former case, virus-transport to developing flower buds may be possible; in the latter it may be limited or abortion may occur of infected tissues such as pollen, ovules, young seeds and flower buds. It need not be argued that the anatomical and physiological characteristics of these sexual organs, and the way they behave when reached by infectious material, play an important rôle in transmission of virus by seed.

Environmental conditions influencing developmental processes of infected host-plants are important also and may be responsible for the contradictory results obtained by authors in experiments concerning transmission of virus by seed.

Many data obtained from different combinations of host-plants and viruses have already afforded an insight into the way seed infection may occur, but in each case studied there are still gaps in our knowledge, giving rise to hypotheses. This can even be asserted for the way in which bean common mosaic virus [1]) is transmitted by seed of *Phaseolus vulgaris* L., a virus-host combination often studied, e.g. by FAJARDO (1930), VAN DER WANT (1954) and CROWLEY (1957b).

The purpose of the present study is to detect more of the rules which affect the occurrence of the transmission of bean common mosaic virus to the progeny of *Phaseolus vulgaris* L. The cultivar Beka, also used by VAN DER WANT (1954), was chosen as experimental plant, because after inoculation it may react with systemic symptoms as well as with necrosis. Though van der Want observed acro-necrosis and flower bud necrosis, the significance of these symptoms for virus transmission by the seeds is still not clear. Moreover, inoculation of plants in different growth stages leads to dissimilar syndromes. The relation of this behaviour of the bean plant to the possibility of virus transmission by seed is still unknown. Other questions are still open also, such as why only a part of the seed becomes infected and why plant infection after flowering does not result in seed infection at all. CALDWELL (1934 and 1962) and BENNETT & ESAU (1936) proposed that the reason developing embryos cannot be infected is because of the absence of plasmodesmal connections between the embryo and the parent plant. This supposition needs verification.

A detailed study was planned of the processes occurring after ino-

[1]) According to Rev. appl. Myc. 35, 1957, suppl. iss. August 1957.

culation in different developmental stages of the plant, in hopes that this study would lead to a better understanding of the way in which virus material reaches the micro- and megaspores and also the embryo. First and foremost, the development of the healthy 'Beka' plant had to be studied, especially the developmental processes occurring in the healthy ovules and seeds, for comparison with equivalent events occurring in those of plants infected with virus. Special attention had to be given to the transport of virus material to flower buds, ovaries and ovules, because their developmental stage at the time they are infected might influence their reaction. In particular, the behaviour of buds that become infected shortly before or after flowering might be important in relation to embryo infection.

The reduction in seed production in the case of plants infected in an early stage of their development had to be studied, because it might be connected with a partial infection of the seed of these plants. Moreover, the presence of virus in seeds during the stage of ripening had to be studied, just as embryo infection in mature and dried seeds, because a decrease of the percentage of virus-infected seeds might be possible during maturation, desiccation, storage and germination.

It is hoped that this detailed study of different aspects of the phenomenon of virus transmission by seeds of *Phaseolus vulgaris* L. cv. Beka will also contribute to a better understanding of transmission of viruses by seed in general.

CHAPTER 2

LITERATURE

2.1. Transmission of viruses by seed

The transmission of viruses through seed to the progeny of an infected plant, as far as is known, can take place in two different ways:

Viruses which stand desiccation, and which can stay infective in the seed-coat or in the pulp residues adjacent to the seed-coat, can infect seedlings during germination. This is known of tobacco mosaic virus (TMV) in tomato (Ainsworth, 1934; Crowley, 1957b and 1958; Broadbent, 1961; Taylor *et al.*, 1961) and in *Capsicum frutescens* L. (Crowley, 1957b) and also of cucumis-virus 2 in cucumber (van Koot & van Dorst, 1959). Most seed-borne viruses, however, are transmitted by infection of the embryo.

A list of viruses which are transmitted by the seed of their hosts is given by Crowley (1957b) and Carter (1962). Several authors have occupied themselves with the question of why many viruses, among which are the most infective ones such as TMV, are not transmitted by the embryos of their hosts.

In some cases, embryo infection seemed to be prevented by abortion of the infected micro- and macrospores following a disturbance of

meiosis, e.g. aspermy virus in tomato (CALDWELL, 1952). A disturbance of the formation of pollen of *Nicotiana glutinosa* infected with aspermy virus is reported by WILKINSON (1953).

For some other non-seed-transmitted viruses, e.g. yellow bean mosaic virus in bean and cucumber mosaic virus in pungent pepper, both of which reduce the fertility of their hosts, it was assumed that the virus did not directly interfere with micro- and megaspore formation, but that the reduction of fertility was due to a disturbance of the normal hormonal control of plant growth.

DUGGAR (1930) supposed that inactivation by "some specific protein or other specific material in the seed" might prevent virus transmission by seed. Evidence of inactivation of southern bean mosaic virus in nearly all seeds of bean plants during maturation and storage of the seeds was given by CHEO (1955) and by ZAUMEYER & HARTER (1943). A decrease in the percentage of barley seeds infected with barley mosaic virus was also explained by inhibition or inactivation of the virus during storage of the seed (GOLD *et al.*, 1954). The lack of embryo infection of tomato seed with TMV and of cucumber seed with cucumber mosaic virus, however, could not be explained by inactivation of these viruses, as no virus-inactivating substances were detected in these seeds, nor could any TMV-inactivating activity be observed with tomato embryos growing in vitro (CROWLEY, 1955 and 1957a).

In his most recent publication CALDWELL (1962) suggested that the absence of transmission of virus by embryos might be due to the absence of a surplus of high-energy phosphate compounds in tissues where rapid synthesis of cellular components occurs.

In most cases seed-borne viruses are transmitted by only a relatively low, but variable percentage of the seeds of their hosts. COUCH (1955), GROGAN *et al.* (1950) and VON MERKEL (1929) reported that the host plant variety may play a rôle in determining the percentage seed infection. The importance of the strain of the virus has been demonstrated by CATION (1952). Also the age of the plant at the time of inoculation and the environmental conditions seem to be of importance (FAJARDO, 1930; SINGH *et al.*, 1960; ATHOW & BANCROFT, 1959; CROWLEY, 1957b). Summarizing the facts which might be responsible for the great variation in the percentage of seed infection, CROWLEY (1959) supposed that "it would seem likely that any factor physiological or genetical that can influence the concentration or survival of infectious virus particles in the floral meristem of a plant will have an effect on the percentage of seed transmission".

Several authors observed that seed infection did not occur when the plants were infected towards or after the time of flowering (FAJARDO, 1930; COUCH, 1955). CALDWELL (1934 and 1962) suggested that "the markedly different rates of growth respectively of the endospermic and of the embryonic tissues would result in the breakdown of the plasmodesmata between these tissues and between them and those of the nucellus". This lack of plasmodesmal connections between embryonic and parent tissue would prevent virus infection

of the embryo. This hypothesis, which was also suggested by BENNETT & ESAU (1936), supposes that virus-transport from cell to cell takes only place through plasmodesmal strands. The necessity of plasmodesmal connections for virus-transport, however, was doubted by KASSANIS *et al.* (1958), who calculated that the velocity of virus-transport in callus tissue in which with staining and with electron-microscopical examination no plasmodesmal structures could be detected, is the same as the velocity of virus-transport through leaf tissue.

CROWLEY (1959), who stated that southern bean mosaic virus, tobacco ringspot virus and barley stripe mosaic virus could infect developing embryos of their hosts during the early stage of their development, therefore supposed that not the lack of plasmodesmal connections but "the structure of the epidermal cells of the embryo or some difference in their metabolism prevents infection of the developing embryos".

2.2. TRANSMISSION OF BEAN COMMON MOSAIC VIRUS BY SEED

Evidence of the transmission of bean common mosaic virus by seed of *Phaseolus vulgaris* L. was first given by REDDICK & STEWART (1919). Thereafter several authors working with different bean cultivars observed transmission of bean common mosaic virus by seed in different percentages:

	%
REDDICK & STEWART (1919)	50
ARCHIBALD (1921)	23 and 43
KENDRICK & GARDNER (1924)	10 and 25
BURKHOLDER & MÜLLER (1926)	50
VAN DER MEULEN (1928)	32
VON MERKEL (1929)	21 to 51
PIERCE & HUNGERFORD (1929)	33 and 48
FAJARDO (1930)	36 and 57
NELSON (1932)	18, 50 and 29
HARRISON (1935b)	20 to 59 and 55
SMITH & HEWITT (1938)	66 and 56
CROWLEY (1957b)	83
CRISPIN MEDINA & GROGAN (1961)	42 to 45
QUANTZ (1962)	93

Differences in percentage of seed infection between different cultivars of bean were noted by REDDICK & STEWART (1919), KENDRICK & GARDNER (1924) and VON MERKEL (1929). PIERCE & HUNGERFORD (1929) and FAJARDO (1930) observed that seeds harvested from infected plants grown from infected seeds showed a higher percentage of infection than seeds harvested from plants infected during their development. FAJARDO (1930) reported that the stage of the plant at the moment of infection influences the percentage of

infected seeds. He also observed that "the appearance of flowers marks the turning point in this respect, since plants infected after the flowers were set, produced almost no infected seeds". Germinal force of the seeds was not influenced by the virus.

NELSON (1932) was the first who tried to explain why it never happens that all seeds are infected with virus. He observed by anatomical studies that the placental tissue of the carpel of the bean plant is fed by two separate vascular bundles situated in the ventral wall of the ovary. The ovules are alternately connected with one of these two vascular bundles and moreover every seed seems to have its own vascular supply. Nelson supposed that the infection of a bean seed depends on the vascular connection with an infected or a non-infected part of the plant. However the results of his study on the position of infected seeds within the pod did not agree with his supposition. His negative results will be partly due to the fact that bean common mosaic virus is also transmitted by pollen, which was first stated by NELSON & DOWN (1933). A definite distribution of pods containing infected seeds at the plants, or of infected seeds within the pods, also could not be detected with soybean plants infected with tobacco ringspot virus by ATHOW & LAVIOLETTE (1962).

CROWLEY (1957b) found that a high temperature before flowering of infected plants increased the percentage infected seeds; a high temperature after flowering did not have such an effect. He concluded that developing embryos can no more become infected. CRISPIN MEDINA & GROGAN (1961), however, failed to detect a clear influence of temperature on the percentage of seed infection.

2.3. VIRUS-TRANSPORT IN PLANTS

The way virus is transported from the site of infection to the flower buds is of importance in relation to transmission of virus by seeds. A review of literature on transport of viruses through plants is given by BEEMSTER (1958) and BRANTS (1961). Many contradictory results as to the direction and the velocity of the transport have been obtained. Several authors agree that virus-transport is correlated with food-transport (ROBERTS, 1950; BENNETT, 1956; BRANTS, 1961). SAMUEL (1934) and BENNETT (1940) found that acropetal transport of virus was accelerated when the plant was developing flowers and fruits. Little seems to be known concerning the virus-transport to the flowers and fruits themselves. Highly varying data are obtained about the rate of virus-transport through plants as well as about the time which has to pass after inoculation before the virus has been transported from the inoculated leaf into the stem. BEEMSTER (1958) concludes that the moment at which virus is transported out of the inoculated leaf depends on different factors, such as the character of the virus and that of the host, the age of the leaf and that of the plant, and the environmental conditions, all of which help to explain the varying results obtained by different authors on this subject.

CHAPTER 3

MATERIAL AND METHODS

3.1. Materials

Phaseolus vulgaris L. cultivar Beka was used as experimental plant in all cases. Plants were grown in an aphid-free glasshouse at temperatures from 20° to 25° C. Large pots, each containing one experimental plant, were kept in peat.

A strain of bean common mosaic virus provided by Ir. N. Hubbeling, Instituut voor Plantenziektenkundig Onderzoek, Wageningen, was used in all experiments. Bean common mosaic virus in this paper is indicated by the abbreviation BCMV.

3.2. Inoculation methods

Sap, pressed out of leaves of bean plants that were infected with BCMV and showed distinct mosaic symptoms, was used as inoculation material. A leaf of a 'Beka' plant was dusted with carborundum powder and then inoculated with sap containing BCMV. After about one minute this leaf was rinsed with tap-water. A simple leaf, or the middle leaflet of a compound leaf at the main stem, was chosen as the place of inoculation.

Healthy plants of the cultivar Beka, 10 to 14 days old, were used as test plants. If BCMV had been present in the inoculum, the test plant showed symptoms 10 to 14 days after inoculation.

3.3. Testing of seeds

The presence of virus in seeds can be checked by three methods.

a. *The seedling assay*

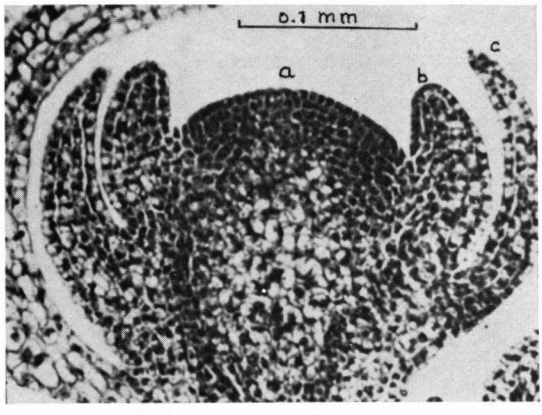
Each seed to be tested is sown in a small pot. The percentage of seed infection can be estimated after the appearance of symptoms, which may occur before the seedlings have developed a 2nd compound leaf at the main stem.

b. *The test plant assay*

Sap samples pressed from cotyledons and from the rest of the embryo are tested separately on two test plants. It is obvious that this method is too time- and space-consuming.

c. *The local lesion assay*

This method is based on the hypersensitivity to BCMV infection of certain bean cultivars, e.g. 'Topcrop' (Quantz, 1957). Young, excised, simple leaves, inoculated with sap and incubated on moist filter paper in a closed Petri-dish under artificial light at 32° C, develop dark-brown lesions after 2 to 3 days if BCMV is present. This method was tried during two successive seasons. A comparison with the seedling assay revealed that the seed infection determined with the local lesion assay ranged from 60 to 80 % of that found with



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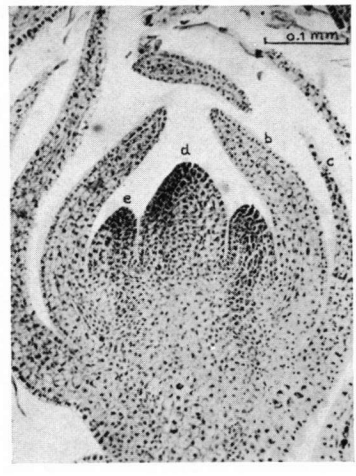

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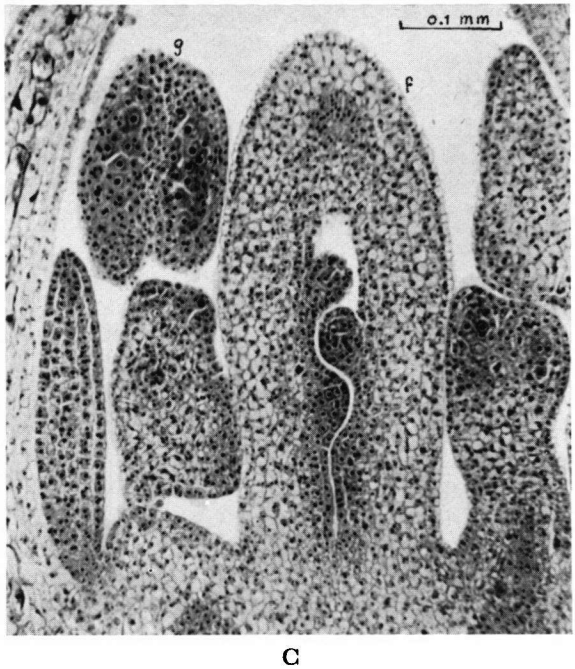
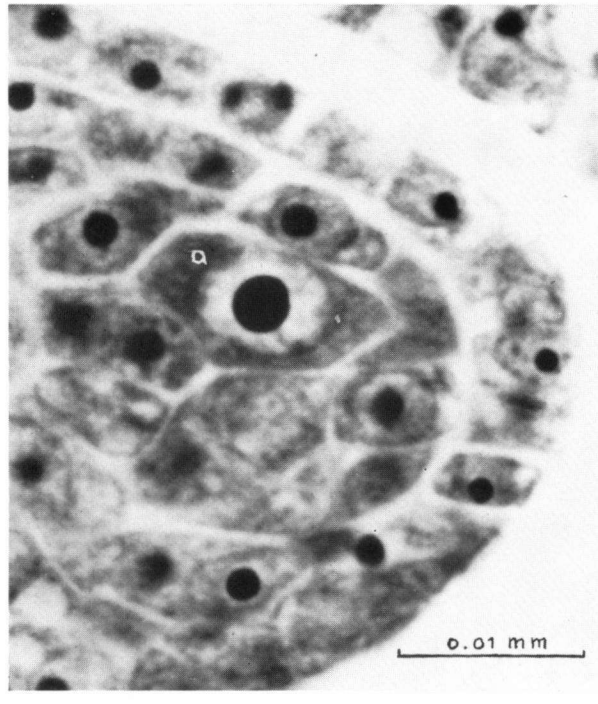


C

Plate 1. A, B and C. Flower buds in different stages of development. a: apical meristem; b: petal-primordium; c: sepal-primordium; d: ovary-primordium; e: anther-primordium; f: ovary with ovule-primordia; g: developing anther.



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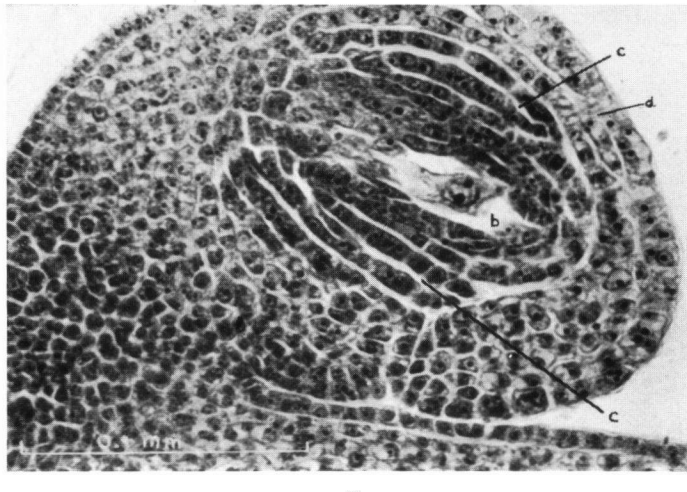
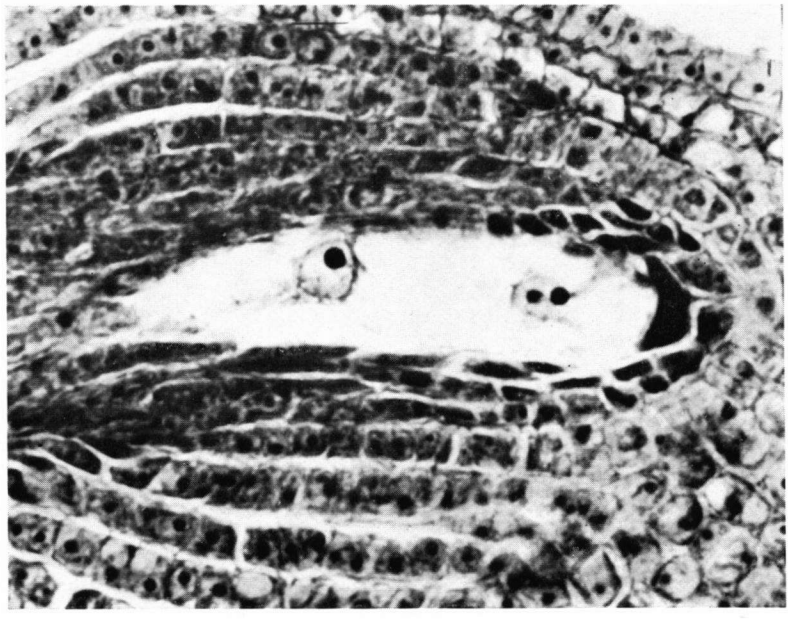


B

Plate 2. A: Ovule-primordium with megaspore mother cell. B: Ovule-primordium with mono-nucleate embryo-sac. a: megaspore mother cell; b: mono-nucleate embryo-sac; c: inner integument; d: outer integument.



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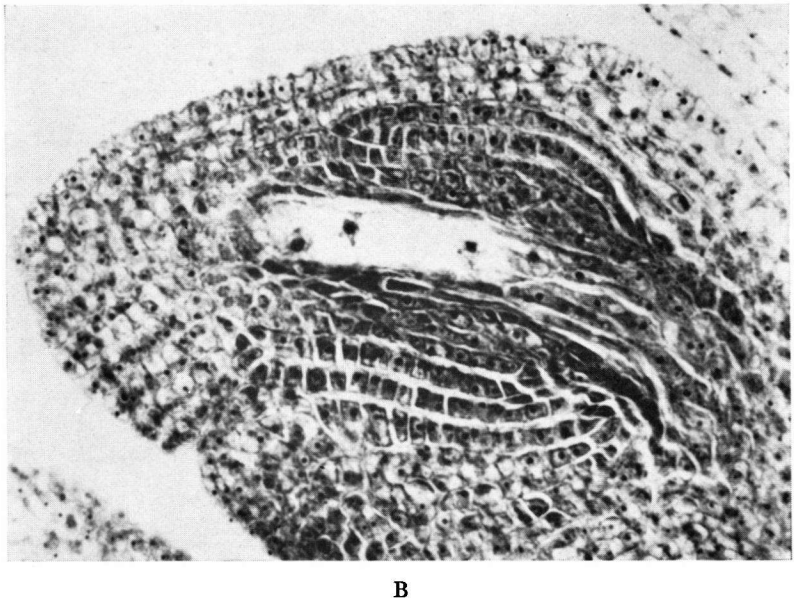


B

Plate 3. A: Ovule-primordium with a two-nucleate embryo-sac. B: Ovule-primordium with a four-nucleate embryo-sac.

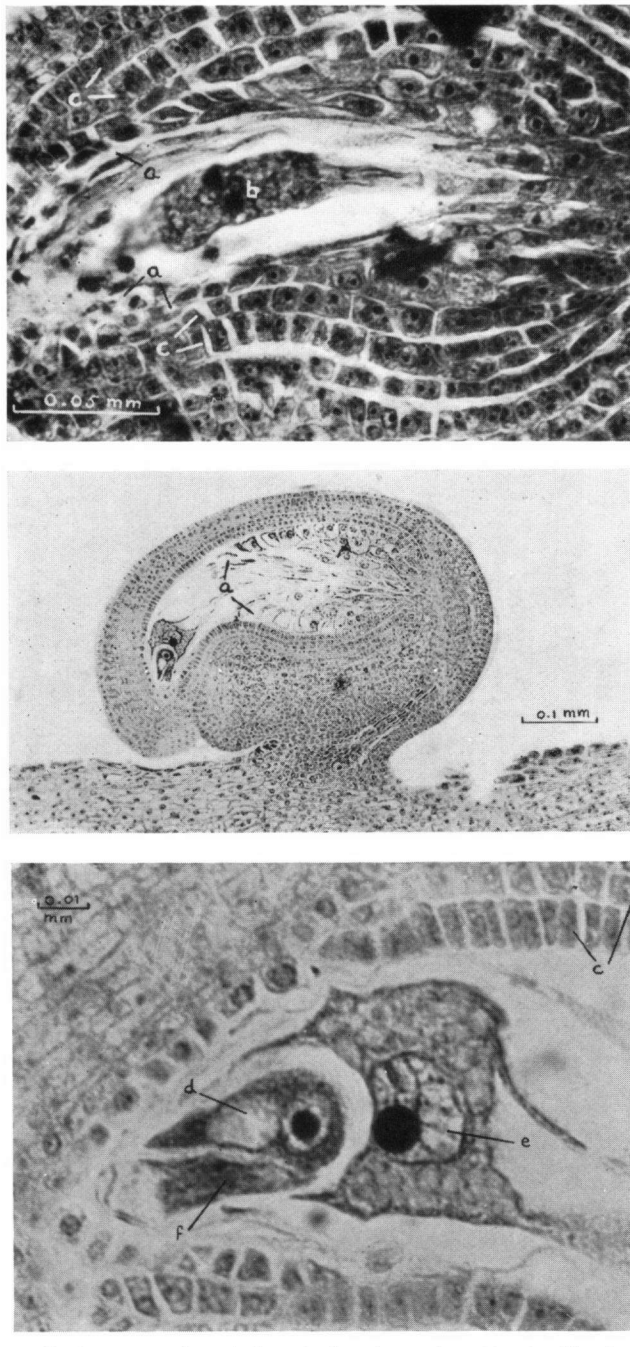
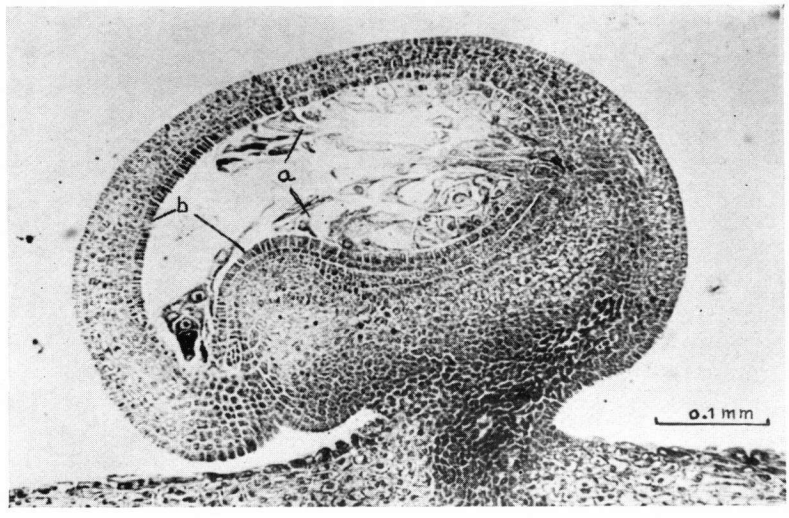


Plate 4. A: Embryo-sac, 2 to 3 days before flowering. B: Fertilized ovule at day of flowering. C: Detail of B. a: disintegrating nucellar tissue; b: polar nuclei; c: inner integument; d: zygote; e: fused polar nuclei; f: pollen tube.



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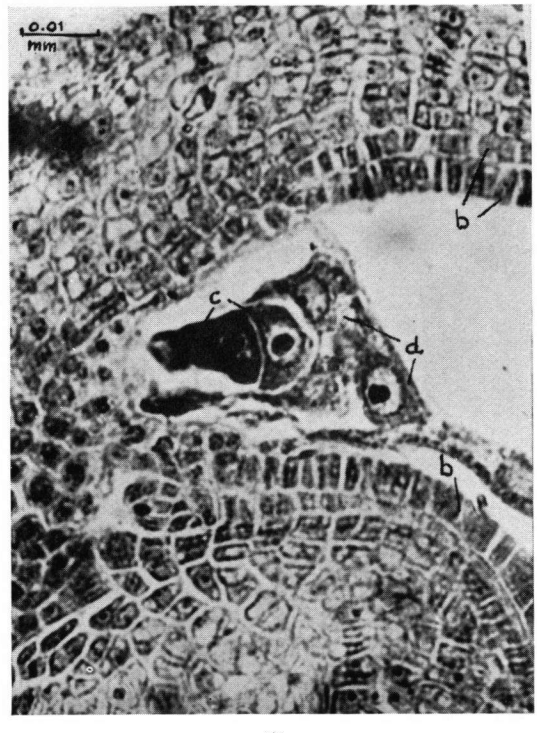
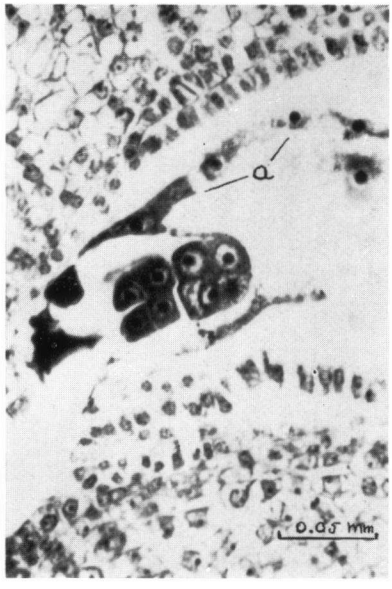


B

Plate 5. A: Ovule with a two-celled pro-embryo. B: Detail of A. a: disintegrating nucellar tissue; b: inner integument; c: pro-embryo; d: endosperm.



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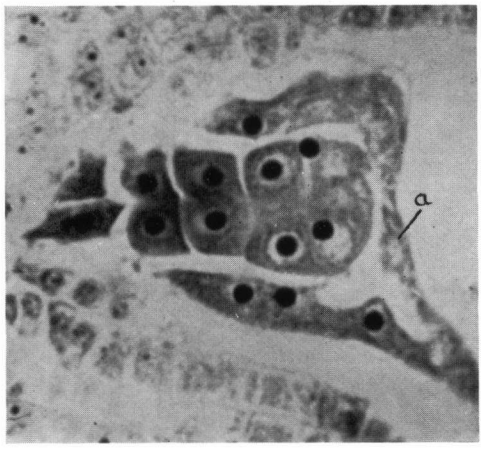

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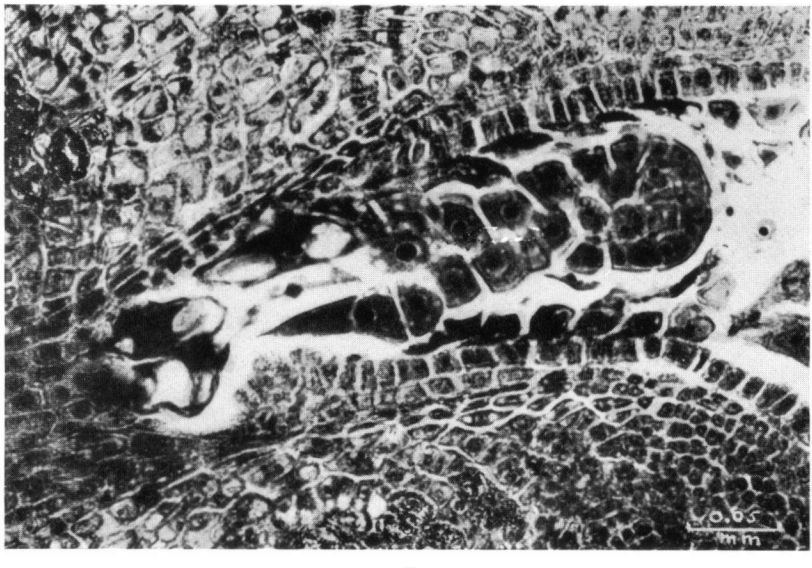
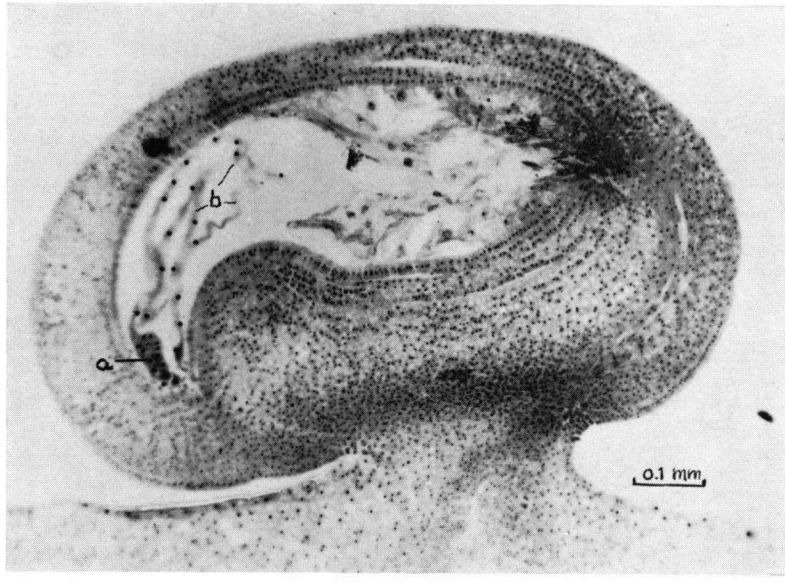


C

Plate 6. Developing embryos, 2 days (A), 4 days (B), and 6 days (C) after flowering. a: endosperm.



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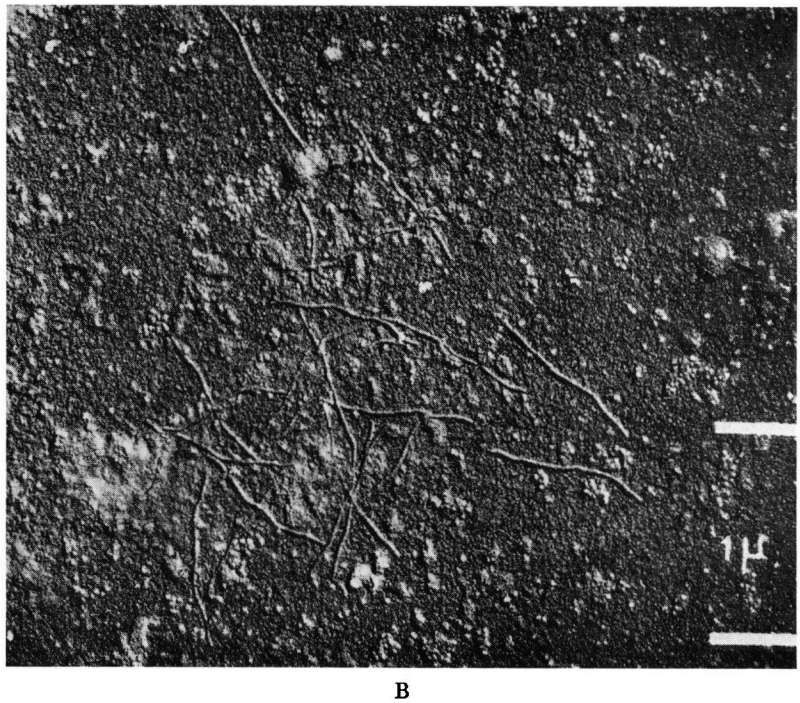

B

Plate 7. A: Ovule, 6 days after flowering. a: embryo; b: endosperm. B: Electron-micrograph of a dip-preparation of an ovule 3 days before flowering showing particles of bean common mosaic virus.

the seedling assay. In an extensive study QUANTZ (1962) stated that in most cases only 80 % of the number of seeds that appeared to be contaminated when using the seedling assay could be detected by the local lesion method. Therefore the latter is not reliable enough for our purpose.

In our experience the seedling assay gives the most trustworthy picture of virus transmission from parent plants to their progeny. Moreover this method is the most convenient and so it was used in all experiments.

3.4. ANATOMICAL RESEARCH

For studying the anatomy of the flower buds, ovaries and ovules, the paraffin microtome and the ultra-microtome were used. For sectioning with the paraffin microtome the objects were fixed in the fixatives of Bouin, Carnoy or Craf. After 24 hours' fixation the objects were upgraded through alcohol and benzene and embedded in paraffin of m.p. 58° C. Longitudinal sections, 7 to 12 μ thick, were deparaffinized in xylene and graded down via ethanol and water and finally stained in a solution of ferrous-haematoxylin.

To prepare ovule-tissues for electron-microscopical research ovules were fixed and embedded according to WALTER (1957) with some modifications:

after 30 minutes fixation in a 1 % buffered osmium tetra-oxide solution (pH 7.2) two times, for 10 minutes each, in Tyrode's solution. The ovules were upgraded through ethanol:

Two times, for 10 minutes each, in each of 50 %, 60 %, 70 %, 80 %, 90 % and 96 % ethanol and two times, for 15 minutes each, and one time for 20 minutes in 100 % ethanol. After dehydration the ovules were infiltrated with a monomer-methacrylate mixture of 2 parts methyl-methacrylate and 8 parts butyl-methacrylate as follows:

Two times, for 20 minutes each, in a mixture of 100 % ethanol and uncatalyzed monomer-methacrylate;

Two times, for 20 minutes each, in uncatalyzed monomer-methacrylate mixture;

One time for 20 minutes in monomer-methacrylate mixture with 2 % polymerization catalyzer (2,4-dichlorobenzoylperoxyde). The fixation, dehydration and infiltration were carried out at about 5° C. After infiltration the material was brought to room temperature.

Gelatin capsules were filled with a thick liquid pre-polymerized methacrylate mixture. In the top of each capsule was orientated one ovule infiltrated with catalyzed monomer-methacrylate mixture. The capsules were kept for 48 hours at 48° C for polymerization of the methacrylate.

To prepare these ovule-containing sticks for sectioning with the ultra-microtome, pyramidal structures (one per stick) were carved out at different places of the embryo-sac-surrounding tissues (PEASE, 1960).

Ultrathin sections were cut from the pyramidal structures with the aid of a glassknife microtome, built according to the principle

of Philips–Haanstra. The sections mounted on formvar covered grids were stained with 2 % $KMnO_4$ at room temperature for 10 minutes, differentiated in 5 % citric acid and dried at room temperature according to LAWN (1960). They were examined with a Philips E.M. 100 electron microscope.

CHAPTER 4

THE DEVELOPMENT OF THE HEALTHY PLANT

For a clear comprehension of the experiments concerning transmission of virus by seed, a thorough knowledge of the vegetative and the generative development of the healthy 'Beka' plant is indispensable. A review of these developmental processes observed in potted plants under glasshouse conditions is given below. Fig. 1 is a diagram of a fully developed plant.

4.1. THE VEGETATIVE DEVELOPMENT; GROWTH STAGES AND NUMBERING

About 8 days after sowing, the hypocotyledon emerges with its two

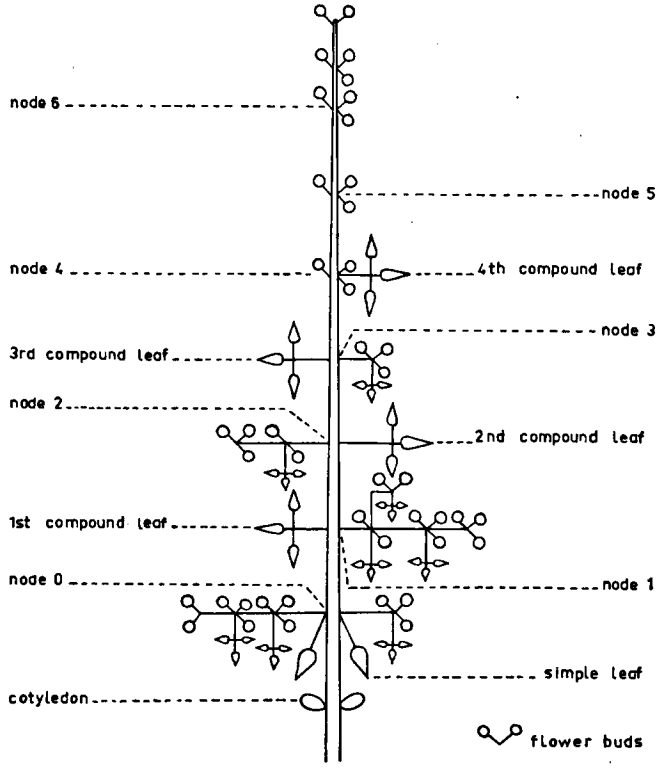

Fig. 1. Diagram of a healthy 'Beka' plant.

cotyledons enclosing the growing point, which later gives rise to the main stem with its leaves: two opposite simple ones followed by the 1st, 2nd, 3rd and 4th compound leaves successively. In some cases a 5th compound leaf is formed. The nodes, at which these leaves are placed, will be indicated with the numbers 0, 1, 2, 3, 4, and 5. From one of the simple leaves at node 0 an axillary shoot develops with two to three compound leaves, each bearing one to three flower buds at its base. The axillary shoot from the other simple leaf usually does not grow out very far. As a rule an axillary shoot also develops from node 1. It may bear two compound leaves each with one to three axillary flower buds, and one to three apical ones. Axillary shoots arising from the nodes 2 and 3 bear each only one compound leaf with two to three flower buds and apically a whorl of two to three flower buds. From the axillary shoots at the nodes 1 and 2, shoots of the second order may arise bearing themselves a compound leaf and flowers. A shoot at node 4 is exceptional; it only develops in case a 5th compound leaf is present. Generally only two to three flower buds develop at node 4. Apically from node 4, three to five whorls of two to three flower buds each develop at the main stem. In most cases the highest whorls do not reach maturity.

The developmental stages of the plants used in our experiments are indicated with the numbers 0 to 4. By stage 0 that stage is meant at which the simple leaves are just unfolded; and by stages 1 to 4 those stages, at which respectively the 1st, 2nd, 3rd and 4th compound leaf is fully unfolded but still immature. In the stages 0, 1, 2 and 3 the leaflets of the leaf placed directly above the fully unfolded one are just starting to unfold.

The period between the opening of the oldest and of the youngest flower bud is the flowering-stage. Generally the buds at node 4 or those at node 5 are first to flower, followed by the basal buds of the axillary shoots at the nodes 1, 2 or 3. This order of succession is important in relation to the abortion of flower buds of diseased plants to be discussed later.

None of the flower buds of healthy plants dies before flowering, with the occasional exception of those belonging to the topmost whorls of the main stem and shoots. During the first two weeks after flowering, however, a great number of young pods abscise. The total number of pods containing ripened seeds varied from two to seven per plant in our experiments.

4.2. The generative development

Studies about the development of the embryo-sac and embryo of *Phaseolus vulgaris* L. were carried out by Brown (1917) and Weinstein (1926). These authors used other bean cultivars than 'Beka' for their experiments, just as Takeuchi (1956) studied morphologically and cyto-chemically the developmental process of the embryo from its very early to its fully matured stage in seed.

For study of the generative development of *Phaseolus vulgaris* L. cv. Beka, flower buds pistils and young developing pods were gathered

from nodes 4 and 5 from healthy plants, from the moment the buds had just differentiated until 6 days after the day of flowering. Flower buds from nodes 4 and 5 were used because in the experiments about the transmission of the virus the infection of these buds was studied in particular. Intact young buds were immersed in the fixing fluid (p. 441). From the larger buds the floral envelopes were first removed to insure penetration of the fluid.

Microscopical examination of the oldest flower buds from nodes 4 and 5, which buds are the farthest developed ones of the plant, revealed the following:

Primordia of sepals and petals, enclosing an apical meristem, can already be distinguished in the oldest buds present in stage 0 of plants 12 to 14 days old (plate 1 A). About 5 days later, these primordia have elongated and the anther-primordia are split off from the apical meristem, which will differentiate further into the pistil (plate 1 B). The ovary becomes hollow. Soon after, the ovule-primordia start developing (plate 1 C). This occurs at the time the 1st compound leaf unfolds. During the growth of the plants between stage 0 and stage 1, microspore mother cells are formed within the anthers and within each ovule there appears a megaspore mother cell which is separated from the epidermis by a single layer of cells (plate 2 A). This cell gives rise to the megaspores. The functional megaspore, which forms the embryo-sac, enlarges (plate 2 B). Its surrounding cells form the nucellar tissue, which now becomes surrounded by the outer integument. During the development of the embryo-sac, which takes place between the developmental stages 1 and 3 of the plants, the inner integument shoves between the nucellar tissue and outer integument, separating the outer one from the nucellus (plates 2 B and 3 B). The first nuclear division of the functional megaspore gives rise to two daughter nuclei, which pass to the respective ends of the sac (plate 3 A). After a second nuclear division two nuclei (plate 3 B), and after a third division four nuclei are seen at each end of the embryo-sac. The polar nuclei start migrating and become situated not far from the egg-apparatus 2 to 3 days before flowering (fig. 2 and plate 4 A). The inner integument then borders the nucellar tissue except at the micropilar end. At this time the nucellar tissue starts to disintegrate bringing the lateral sides of the embryo-sac in immediate contact with the inner cell-layer of the inner integument before fertilization (plates 4 A, 4 B and 4 C). The cells of the inner cell-layer of the inner integument become rectangular and elongated in a direction perpendicular to the embryo-sac, forming an integumentary tapetum. During the day of flowering the egg-cell fuses with one of the male nuclei, the polar nuclei fuse with the other male nucleus (plates 4 B and 4 C). After fertilization, the nucellar tissue almost disappears, except at the chalazal end of the sac where its cells grow larger (plates 4 B and 5 A). One day after the day of flowering a 2-celled, and one day later already a 16-celled embryo can be observed in the embryo-sac (plates 5 A, 5 B and 6 A). About 6 days after flowering a suspensor with swollen elongated basal cells

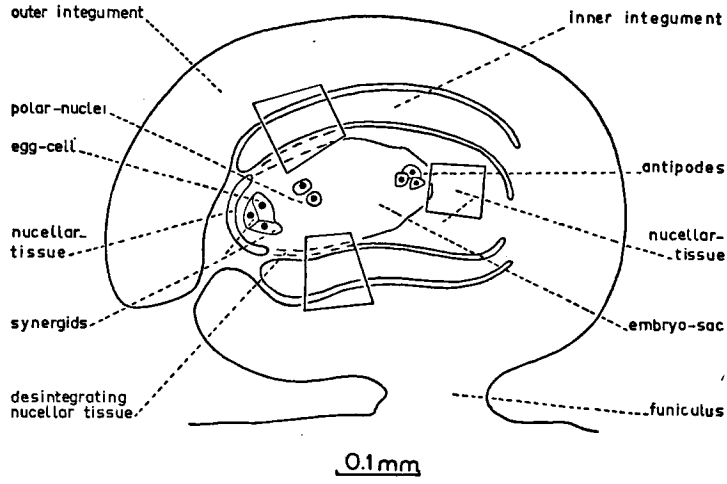

Fig. 2. Diagram of an ovule, 2 to 3 days before flowering. The framed parts in the figure mark the tissues from which ultrathin sections were cut (p. 000).

is formed, which pushes the globular embryo towards the chalazal end of the sac (plates 6 B and 6 C). The endosperm which is formed by the fusion of two polar nuclei and one of the male gametes forms a peripheral coenocytic layer in the embryo-sac (plate 7 A). Takeuchi states that, according to the classification of MAHESHWARI (1950), the endosperm formation belongs to the "nuclear type", which means that cell-walls are only formed at a later stage. At the day of flowering and also before, no pro-embryo or endosperm was detected within the embryo-sac. Besides, dissection of flower buds of one day before flowering revealed that no microspores left the loculi of the anthers. Consequently fertilization before flowering is improbable.

The present observations about the development of the embryo-sac and the pro-embryo agree with those of Brown, Weinstein and Takeuchi.

CHAPTER 5

THE DISEASE SYMPTOMS

5.1. INTRODUCTION AND LITERATURE

Bean common mosaic was first recognized by IWANOWSKI in Russia in 1899. A summary of the literature has been given by VAN DER WANT (1954). A description of the symptoms shown by several cultivars of *Phaseolus vulgaris* L. infected with BCMV was given by HUBBELING (1955). VAN DER WANT (1954) carried out some experiments on the relation between the development of plants and leaves at the time of infection with BCMV, and the nature of the symptoms. If he inoculated young bean plants of the cultivar Beka at the time the

simple leaves were unfolded but still immature, the 1st compound leaf showed curling of the leafblade and mosaic ("rolmozaiek"). If at the time of inoculation the simple leaves were full-grown and the 1st compound leaf was already visible the latter developed veinal necrosis ("steengrauw") after some days. Bean plants inoculated 17 days after sowing soon had growth-retardation. The apical parts of the main stems of many plants became necrotic and axillary buds of the simple leaves started sprouting. Necrosis of stem tips and abnormal ramification also occurred in plants inoculated 20 to 37 days after sowing. If plants were inoculated 41 days after sowing no axillary shoots were produced though in some cases stem tips became necrotic. Flower buds, if present at the time of inoculation, showed a brown discoloration and dropped. Some infected plants had a chlorotic streak along the full length of the pod. This typical symptom is called "aalstreep" by van der Want.

These observations reveal a correlation between the character of the disease symptoms of leaves, shoots and pods on the one hand and the growth stage of the plants on the other hand. It seemed desirable to look further into symptoms shown by diseased plants, paying attention not only to apical necrosis and necrosis of flower buds, but also to the behaviour of those buds which flowered and produced seeds without being aborted and which might be contaminated with virus. Therefore experiments were carried out to study the behaviour of plants after inoculation in different developmental stages. The influence of the site of inoculation on the behaviour of plants was also studied.

5.2. The influence of the developmental stage of the plant at the moment of inoculation, and of the site of inoculation, on the habitus of the plant

Plants were inoculated in growth stages 0, 1, 2, 3 and 4 and also in two older stages: at the height of flowering and just after the flowering period. To observe a possible influence of the site of inoculation on the habitus of the plant in each growth stage, inoculations were carried out on different leaves (Table 1).

In all experiments only those plants were used which fitted into the description of the growth stage. Therefore they were selected out of a great number of plants raised beforehand. As a control the 1st compound leaves of two plants in each of the stages were treated with water. Five plants were used for the study of each growth stage. The total number of plants used was 139.

A first experiment was carried out with plants sown in June; a second, with plants sown in August. The development of each inoculated plant was studied.

In both experiments it appeared that in a certain stage of development the leaf chosen for inoculation was of but little influence on the future habitus of the plant. All inoculated leaves showed epinasty after about 5 days and local reactions 10 to 14 days after treatment. Inoculated leaves with the petiole in a normal position in the morning

TABLE 1

Scheme of the combinations (+) of developmental stages of the plants and sites of inoculation.

stage of development of the plant	nodes of the main stem at which the inoculated leaf was placed:				
	0	1	2	3	4
0	+				
1	+	+			
2	+	+	+		
3	+	+	+	+	
4	+	+	+	+	+
flowering	+	+	+	+	+
after flowering	+	+	+	+	+

became epinastic during day-time long before the leaves of healthy plants. It may be concluded that the virus infection induced this metabolic disturbance.

The symptoms occurring after inoculation of plants in different developmental stages are given in Table 2. Different symptoms appeared on the leaves according to the growth stage in which the plants had been inoculated. They will be discussed in detail in § 5.3.

After inoculation of the plants in stage 0, initial retardation of the growth of the main stem was followed by a vigorous development of sprouts at nodes 0, 1 and 2. These shoots, especially those in the axils of the simple leaves, formed a greater number of laterals than similar sprouts of healthy plants. Also, secondary and tertiary axillary buds present in the axils of the leaves at the main stem developed, conferring upon the plants the feature of a witches' broom a few months after inoculation. By continuous development of new sprouts the flowering period was increased compared to that of healthy plants. A great many flowers were misshapen. Their sepals were sometimes split and filiform and might protrude above the petals. The petals were sometimes partly green and remained small. The vexillum was usually bent backward, partly split or consisted of two small leaves separately inserted on the receptaculum. The alae were narrowed, sometimes also partly split and widely divergent from each other. In a healthy flower the lower petals were united to the spirally wound carina containing the ten stamina and the pistil, which situation promotes self-pollination. In the malformed flowers the lower petals were not united, the stamina and the pistil were diverted from each other, a situation which seems to be unfavourable for self-pollination.

Abnormal flowers were not strictly confined to plants infected with virus; they also occurred exceptionally with healthy plants. In infected plants, however, the malformed flowers were more frequent.

The pods developed on plants inoculated in the simple leaf stage were often short, broad, crooked and locally narrowed where young seeds stopped developing. The average number of seeds per pod was

TABLE 2

Symptoms occurring after inoculation of plants in different developmental stages.

symptoms shown by:	stage of development at the moment of inoculation					
	0	1	2	3	4	during and after flowering
main stem	growth retarded	acro-necrosis above nodes 3 or 4	acro-necrosis above node 5	normal	normal	normal
axillary shoots	ramified	necrosis of axillary shoots; new laterals ramificated	acro-necrosis; new laterals ramificated	acro-necrosis or normal	normal	normal
compound leaves: 1st	fine or coarse mosaic	epinasty	normal	normal	normal	normal
2nd	coarse mosaic	necrosis	epinasty	normal	normal	normal
3rd [1]	coarse mosaic	transition symptoms	necrosis	epinasty	normal	normal
4th [1]	coarse mosaic	transition symptoms	transition symptoms or necrosis	necrosis	epinasty	normal
flower buds	often misshapen	necrosis	necrosis safe the oldest ones at nodes 4 and 5	youngest buds aborted; all others flower	normal	normal
pods	often malformed with non-developed seeds	often malformed with non-developed seeds	"aalstreep" symptom	"mosaic"	normal	normal
average number of seeds per pod	1.8	1.7	2.9	3.0	2.9	3.0

[1]) Symptoms are often weak and difficult to distinguish.

much lower in diseased than in healthy plants and plants inoculated in stage 2 or later (Table 2).

Plants inoculated in stage 1 were initially retarded in growth. All axillary buds abscised with the exception sometimes of the buds at node 0, if the latter were little developed. The main stem often died off above node 3 or 4. Later on, secondary and tertiary shoots developed at nodes 0, 1, 2 and 3. These newly formed shoots grew out vigorously into ramified stems, often giving the plants a witches'-broom-like appearance, not to be distinguished from a plant inoculated in stage 0. All of the flower buds that developed later, which flowered and produced seeds in spite of the infection, were born on shoots showing coarse mosaic.

Plants inoculated in stage 2 lost nearly all flower buds, because parts of axillary shoots and the tip of the main stem at which they were situated became necrotic or because they became necrotic themselves. Some of the oldest flower buds, which were nearly always situated at nodes 4 and 5, and occasionally also at the basal clusters of sprouts at nodes 1 and 2, stayed healthy.

Only pods originating from these flowers showed the "aalstreep" symptom. Soon after flower bud abortion the axillary shoots showed acro-necrosis. New sprouts showing coarse mosaic were formed at the time the pods with "aalstreep" symptoms were nearly mature.

If the plants were inoculated in stage 3 acro-necrosis of axillary shoots occurred occasionally, while only the youngest flower buds at these shoots abscised; all others flowered. The pods often showed a mosaic. Inoculation of the plants in stage 4, during or after flowering, was of no influence on flower bud formation, flowering, pod- and seed production in comparison with the controls. With diseased as well as with healthy plants many young pods abscised.

In field experiments the reactions of bean plants to BCMV infection were studied in the field after inoculation at different developmental stages.

At the end of May, six groups each of five plants were grown, covered by an insect cage to prevent the plants from natural infection by aphids. The plants of the six groups were inoculated mechanically on the 1st compound leaf, respectively in the stages 0, 1, 2, 3, 4 and after flowering. Some days after inoculation the insect cages were removed from all plants.

The reactions of the plants in the field agreed with the reactions of plants inoculated in a similar way in the glasshouse, except that plants in the field became more firm, and that field plants inoculated in stage 3 gave rise to new sprouts with leaves showing mosaic symptoms a long time after inoculation. Similar glasshouse plants never did so.

The results of these experiments on the habitus of the plants after inoculation in different stages of development confirmed the observations of van der Want. The behaviour of flower buds, axillary buds, shoot tips, main stems and leaves points to a growth period of these organs during which they are hypersensitive to virus infection. The

character of this hypersensitivity, perhaps of importance for the way virus might or might not become seed-borne, was studied in more detail. Observation of the behaviour of leaves becoming infected in different stages of development might contribute to clarify the occurrence of flower bud necrosis.

5.3. The relation between the developmental stages of compound leaves at the time of their infection and the character of the consequent disease symptoms

The behaviour was studied of the 1st, 2nd, 3rd and 4th compound leaves of the main stem after inoculation of plants at different developmental stages. In every case one of the simple leaves was chosen for inoculation with the virus. Because the development of the three leaflets of one compound leaf proceeds in a similar way, measurements and observations were restricted to the middle one. The length of the midrib from the base to the tip of the blade of this leaflet was measured and the stage of development was determined at the time of its infection. Leaflets shorter than 3 mm were measured with the aid of a dissecting microscope. The time of infection of the leaflets, assumed, to be the time at which the virus reached an infectious level in the leaflets in hours after inoculation, had first to be determined.

The following experiment concerned the 1st compound leaves. Similar experiments not described here, were performed with the higher ones. Thirty-six 11-day-old plants were inoculated. The lengths of the middle leaflets of the 1st compound leaves of these plants varied from 3 to 6 mm. At 12 hour intervals, starting 12 hours after inoculation, the 1st compound leaf was excised from three plants. Test plants were inoculated with sap obtained from each of these leaves separately. These latter plants were observed after three weeks. Simultaneously, a similar experiment was carried out with 19-day-old plants, in order to compare the rate of virus transport in older plants with that in younger ones. In older plants the lengths of the middle leaflets varied from 50 to 60 mm at the moment of inoculation. Table 3 shows the results of both experiments. In the younger plants the virus could be demonstrated in the sap of the middle leaflets of the 1st compound leaves between 84 and 96 hours; in the older plants, between 96 and 108 hours after inoculation.

The experiment about the relation between developmental stages of leaflets at the time of their infection and the character of the consequent disease symptoms was carried out with 100 plants from which about five per day were inoculated beginning at the 7th day and continuing until the 22nd day after sowing. In the 7-day-old plants the leaflets would probably be invaded by the virus within 96 hours; in the 22-day-old plants the virus would probably invade the leaflets shortly after 96 hours. Therefore the time of infection for all 1st compound leaves was assumed to be 96 hours after inoculation. The lengths of the middle leaflets of the 1st compound leaves, measured 96 hours after inoculation, varied from 2 to 140 mm in the 16 groups of plants.

TABLE 3

Time after inoculation at which the virus was demonstrated in the 1st compound leaf of plants inoculated on a simple leaf, at two ages.

hours after inoculation	number of leaflets in which virus could be demonstrated in plants inoculated at the age of:	
	11 days [1]	19 days [2]
12	0 [3]	0 [3]
24	0	0
36	0	0
48	0	0
60	0	0
72	0	0
84	0	0
96	2	0
108	3	2
120	3	2
132	3	3
144	3	3

[1] length of leaflets at the moment of inoculation varied from 6 to 10 mm.
[2] length of leaflets at the moment of inoculation varied from 50 to 60 mm.
[3] there were 3 leaflets in each treatment.

The following disease symptoms developed in four weeks after inoculation:

a. curling with a coarse mosaic.

The leaves show a clear variegated mosaic. Dark-green patches of varying shape, size and number are irregularly scattered over the leaf blade. These patches are often oblong and placed symmetrically in relation to the midrib or the lateral veins, thus giving rise to typical regular patterns. The bases of all leaflets are narrowed; the blades are misshapen, sometimes folded along the axis. The tips of the leaflets may be abnormally long and thin.

b. a fine mosaic.

The leaves show a mosaic consisting of small dark-green patches scattered in lighter green fields. There is less narrowing of the leaf base than with the coarse mosaic. The leaf blade is only slightly misshapen. It is neither folded nor curled. The leaf surface is even.

The symptoms described under a. and b. are considered as "chronic" by van der Want.

c. transition symptoms.

The leaves show a normal green; they are usually brittle and their surface is irregularly vaulted. The leaf blades are somewhat curved with the tips pointing downwards.

d. chlorosis and necrosis.

Here, too, the leaflets are more or less curved with their tips pointed

downwards. The interveinal tissue shows slight chlorosis, which may pass over into a strong yellowing. The leaves feel tougher and they are more brittle than healthy leaves.

The veins are sunken and show a red to dark-brown discoloration over long distances. This discoloration seems to be restricted to the walls of the epidermal cells, though it may extent to the cell-walls of one or two under-lying cell-layers. The contents of these cells may also show a light-brown discoloration. Staining of the brown cell-walls of the veins with ammoniacal gentian-violet solution differentiated in hydrochloric acid indicated the presence of suberin and corky substances (SCHÖMMER, 1952).

The symptoms described under c. and d. are considered as "acute" by van der Want.

e. epinasty of the leaf petiole.

Though the leaflets are normal in shape and colour, the virus induces a characteristic epinastic response, perhaps due to a disturbance in growth-substance metabolism (GRIEVE, 1943). After some weeks the abnormal epinastic reactions may disappear.

A correlation was found between the developmental stage of a leaflet at the time of its infection and the symptom which developed thereafter. Table 4 shows six classes of leaflets of different lengths, those of one class all showing the same symptom.

TABLE 4

The relation between the lenghts of the leaflets of the 1st compound leaves at the time of infection (assumed to be 96 hours after inoculation of the plant), and the symptoms they showed.

lengths of leaflets in mm	disease symptoms
2 – 5	coarse mosaic
4.7– 6	fine mosaic
5.8– 19.5	transition symptom
15 –112	chlorosis and necrosis
110 –132	epinasty
129 –140	symptomless

It seemed desirable to learn which developmental stages of the leaflets correlate with the classes of leaf-sizes and their corresponding disease symptoms. Therefore growth curves were made of the middle leaflets of the 1st, 2nd, 3rd and 4th compound leaves at the main stems of ten healthy plants sown at the same date as the plants of the former experiment. The growth rates of these leaflets had been measured from the 7th to the 40th day after sowing. The growth curves of the middle leaflets of the compound leaves of one of the ten plants is given in Fig. 3a. The growth curves of the leaves of the other plants were similar. The six classes of leaf-sizes given in Table 4 are projected in the growth curve of the middle leaflet of the 1st compound leaf of one of the ten plants (Fig. 3b).

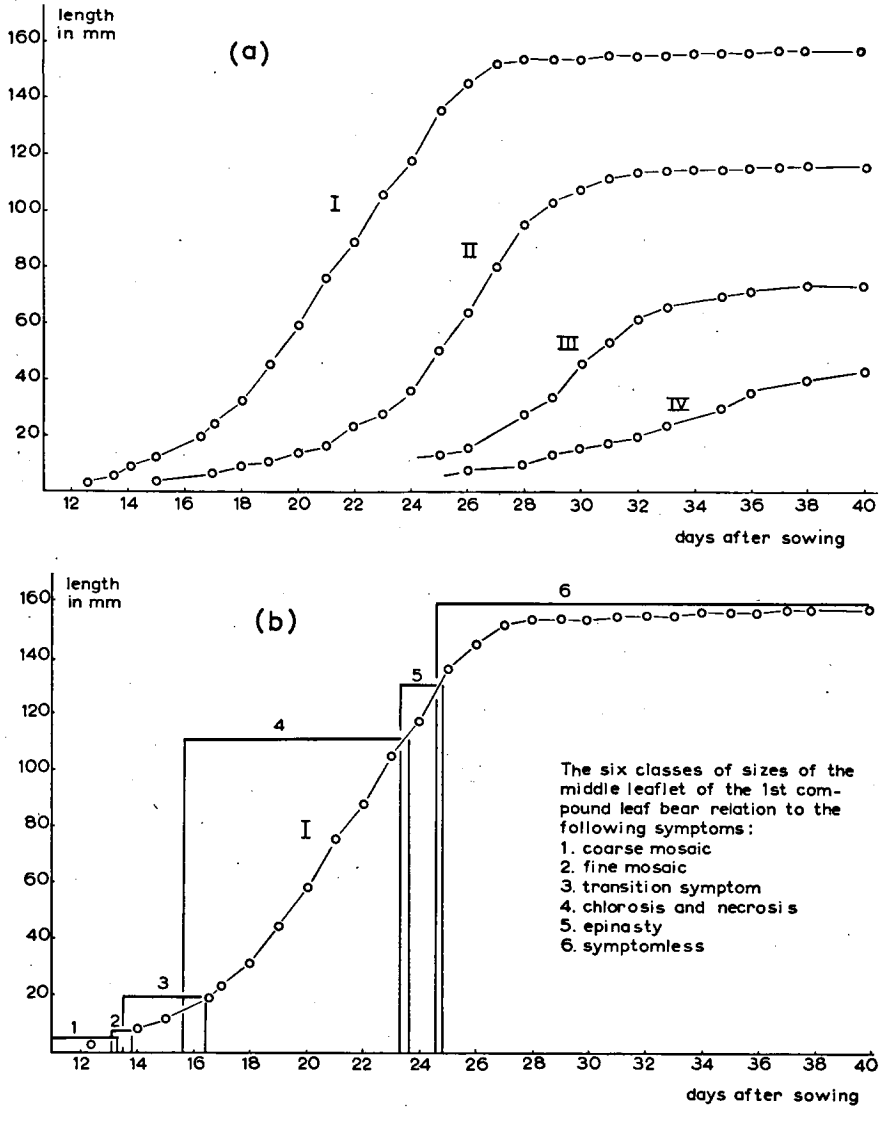

Fig. 3. a. Growth curves of the middle leaflets of the compound leaves at the main stem.

b. The relation between the growth stages at the time of infection of the 1st compound leaf and the different symptoms this leaf develops.

Growth curves of the middle leaflets of the:

I 1st compound leaf
II 2nd compound leaf
III 3rd compound leaf
IV 4th compound leaf.

The following conclusions may be drawn:

1. A leaf will show a coarse mosaic with curling if it becomes infected with BCMV during an early stage of development, during which mainly cell-divisions occur.
2. A leaf will show a fine mosaic if it becomes infected somewhat later, but still in an early stage of development. In this case, leaf deformations are only slight.
3. Transition symptoms appear if the leaf becomes infected at the time when cell-elongation begins to predominate over cell-division.
4. Chlorotic and necrotic symptoms will appear if a leaf becomes infected during the grand period of growth, the period in which mainly cell-elongation occurs.
5. Epinasty without any discolorations or transformations appears if a leaf becomes infected at the end of the grand period of growth.
6. If the leaf has matured or nearly matured before infection, the virus does not induce any symptoms, but virus is present in the leaf.

It must be emphasized that the sizes of the leaflets leading to different disease symptoms overlap, which may be due to individual dissimilarities between the plants. Therefore the numbers given in Table 4 have to be considered as relative. Moreover under other environmental conditions other leaf sizes might be found leading to comparable results.

The same relation was found between the developmental stages of the 2nd compound leaves and the appearance of symptoms. To avoid too many details these dates are not introduced in Fig. 3b.

The relation was not extensively studied for the 3rd and 4th compound leaves, as the different symptoms could not always be distinguished clearly. This may be connected with the lack of clearly different developmental stages during the development of these leaves; the growth curves were flattened (Fig. 3a). Similar results were obtained from experiments performed a year later.

In all cases described, the virus became systemic in the leaves. Leaves which were in the grand period of growth at the time of their infection showed necrotic symptoms. Leaves which had not yet reached that stage showed mosaic symptoms without necrosis. When infection happened, while leaves had reached maturity or nearly so, they did not show disease symptoms. From these observations it was deduced that necrosis of other tissues than that of leaves e.g. shoots and flower buds, might also be connected with infection during their grand period of growth. Therefore, in this respect the behaviour was studied of internodes of main stem and shoots, and also of peduncles of flower buds.

5.4. The relation between the developmental stage of shoots and flower buds at the time of their infection, and the occurrence of necrosis

Plants were inoculated in stages between 0 and 4. In all, 100 plants were inoculated. The occurrence of flower bud- and acro-necrosis was noted. Of five healthy plants the growth was measured of some of those parts that were similar to the ones that became necrotic with infected plants. The following parts were measured: the basal internodes of the axillary shoots at nodes 1 and 3; the internode between nodes 4 and 5 of the main stem; the peduncles of the oldest flower buds at node 4. Growth curves were made.

To determine approximately the time after inoculation at which virus reached an infectious level in the plants of which the occurrence of necrosis was studied, 36 plants were inoculated in stage 0 and 36 plants were inoculated in stage 4. Every 12 hours during 6 days, starting 12 hours after inoculation, from three plants of each group tips were excised above node 0. Test plants were inoculated with sap of these tips separately.

Virus could not be demonstrated in tips excised 108 hours after inoculation or earlier. It was, however, detected in those picked 120 hours after inoculation, both of plants inoculated in stage 0 and in stage 4. In plants inoculated in stages between stage 0 and stage 4, necrosis occurred as follows (Fig. 4):

The main stem of plants inoculated in stage 0 did not become necrotic. It developed laterals with leaves all showing mosaic symptoms.

Plants, inoculated soon after stage 0 showed necrosis of the main stem above node 0. Inoculation in later sub-stages between stages 0 and 1 caused necrosis of the main stem, respectively, above nodes 1, 2 or 3. Plants, inoculated in sub-stages just before stage 1 showed no acro-necrosis. The internodes beneath node 4 of these plants seemed to have passed the hypersensitive period of growth. The part of the main stem above node 4, however, had not yet reached this period. The 4th compound leaf, lost after inoculation in an earlier stage by necrosis of the main stem above nodes 0, 1, 2 or 3, now developed mosaic symptoms, while flower buds at node 4 and at higher nodes did not drop, and later produced seeds.

Inoculation of plants in stage 1 and in sub-stages between stages 1 and 2, however, caused acro-necrosis above node 4. Plants, inoculated in stage 2 showed necrosis above node 5, while inoculation of plants in sub-stages between stages 2 and 3 resulted in acro-necrosis of the main stem above node 6 or higher. Inoculations in later stages no longer caused acro-necrosis.

A similar course of events happened with axillary shoots at node 0, 1, 2 and 3.

It was concluded that the later the inoculation happened, the more the necrotic nodes were situated near the tip of the main stem or shoots.

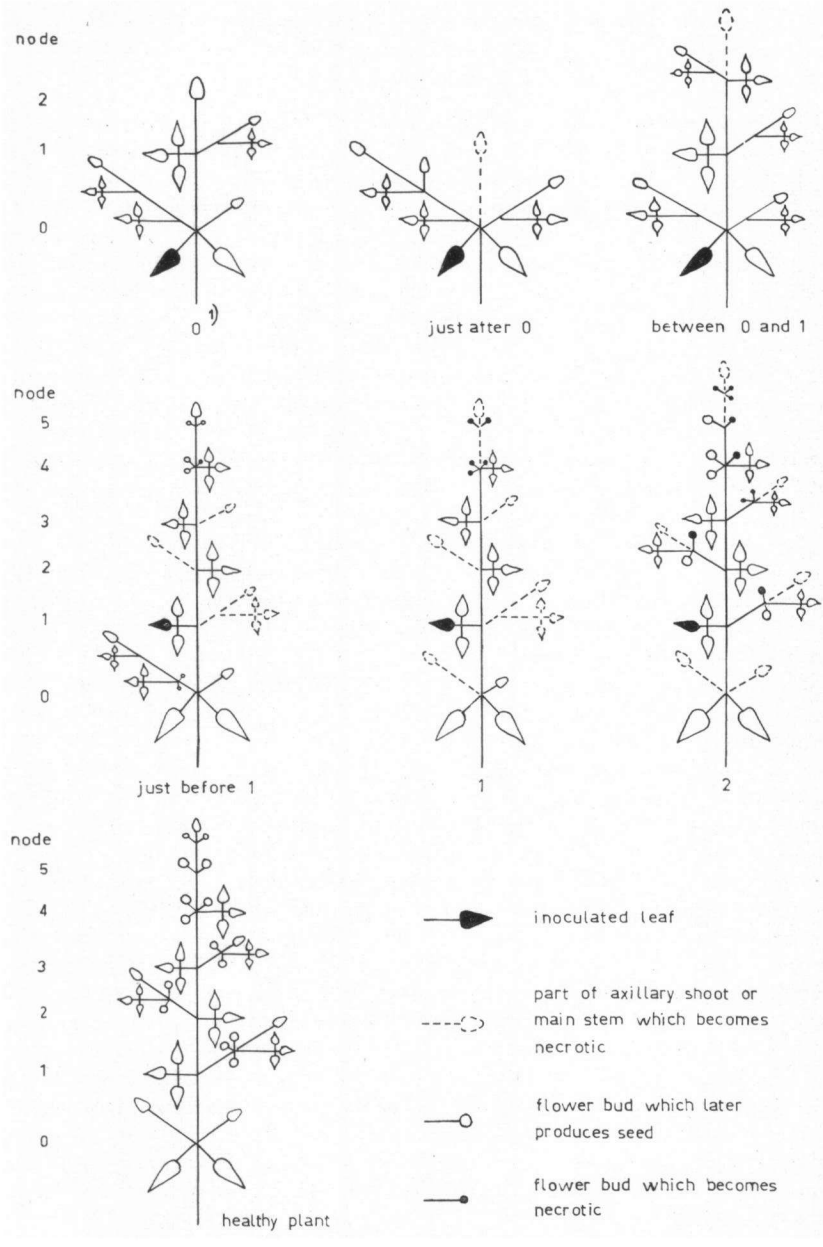

Fig. 4. Necrosis of flower buds and parts of main stem and axillary shoots after inoculation of plants in different stages of their development. The diagrams represent plants about 5 weeks after sowing.

1) growth stage of the plant at the time of inoculation.

As for the flower buds it may be mentioned that inoculation before or in stage 0 did not result in flower bud necrosis. Inoculation in sub-stages after stage 0 resulted in a loss of all flower buds carried by those apical parts of stem and shoots which became necrotic.

After inoculation of plants in a sub-stage just before stage 1, flower buds at nodes 4 and higher nodes did not drop and later produced seeds (p. 455). However, inoculation of plants in stage 1 or in sub-stages between stage 1 and 2, which resulted in acro-necrosis above node 4, caused also necrosis of the flower buds at node 4 and at the basal nodes of axillary shoots above which acro-necrosis occurred.

In stage 2, the oldest flower buds at these nodes had passed the critical stage and did not become necrotic any more after inoculation of the plant in this stage or later stages. The later the inoculation was carried out after stage 2, the more flower buds had passed the critical stage and stayed apparently healthy. Inoculation in stage 4 and later stages did not cause flower bud necrosis any more.

It appeared that with the infected plants, necrosis of internodes of stems and shoots coincided with their grand period of growth, if virus material infected those parts during that period (Fig. 5). This is in accordance with the finding that necrotic symptoms of leaves appear if infection takes place during their grand period of growth. If this also applies to the peduncles could not be stated.

It will be clear that the production of infected seeds can be expected of only those flowers whose buds are not lost by flower bud necrosis or necrosis of main stem and shoots. Therefore it was necessary to study which ones were apt to produce seeds contaminated with virus after the plants were inoculated in different developmental stages.

CHAPTER 6

THE OCCURRENCE OF SEED INFECTION

It had already become clear that abortion of flower buds could be expected after inoculation between the stages 0 and 4. A picture had to be acquired of the possibility of infection of seeds produced by all non-aborted buds after inoculation of plants in the stage 0 to 4, during flowering and after flowering.

6.1. THE OCCURRENCE OF INFECTION OF SEEDS OF PLANTS INOCULATED IN DIFFERENT STAGES OF THEIR DEVELOPMENT

The occurrence of seed infection was studied in six different experiments. In each of these experiments, plants were inoculated in one of the following stages: 0, 1, 2, 3, 4, during flowering and after flowering. In the experiments 1 and 2, moreover, the influence of the site of inoculation on the occurrence of seed infection was studied. Table 1, shows a scheme of the combinations of the stages of the plants and sites of inoculation used. In experiments 3, 4, 5 and 6, only the relation between the stages of the plants at the moment of inoculation and seed infection was studied. All plants of these ex-

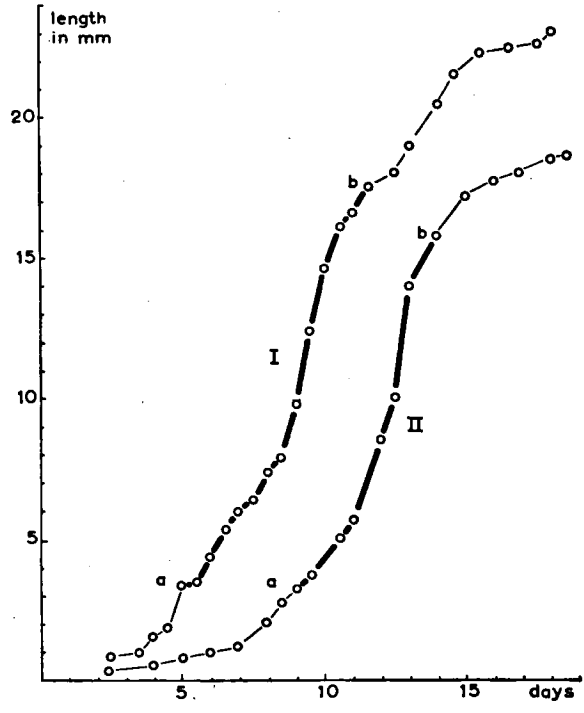

Fig. 5. The relation between the growth stages of internodes at the time of infection and the occurrence of necrosis. I growth curve of the internode of the main stem between nodes 4 and 5. II basal internode of the axillary shoot at node 3 of the main stem. The thickened part of the curves marks the critical period of the growth of the internodes. If the virus reaches an infectious level in the plant during this period, the internode becomes necrotic.

periments were inoculated on the middle leaflet of the 1st compound leaf. Simultaneously with experiments 1 and 2, the infection of seeds of diseased plants grown from infected seeds (i.e., secondarily infected plants) was examined.

The development of branches, flower buds, flowers, pods and seeds of all plants was noted. The seeds harvested were marked with a code number indicating their place within the pods and the place of the pods on the plants. It was asked whether the percentage of infection of seed of infected plants grown in the field would be comparable with that of seed of infected plants grown in the glasshouse. Therefore seeds from plants grown and inoculated in similar growth stages in the field were also tested. All seeds were tested by the seedling assay. The experiments were carried out in two successive summers.

In experiments 1 and 2, the site of inoculation appeared not to influence the percentage of infection, the locations of pods containing infected seeds, or the distribution of infected seeds within the pods. Therefore the results of all six experiments can be discussed together (Table 5).

TABLE 5

Percentage of seed infection in relation to the developmental stages of the plants at the moment of inoculation.[1])

stages of development of the plants at the moment of inoculation	number of glasshouse experiment:														field experiments	
	1		2		3		4		5		6					
	a	b	a	b	a	b	a	b	a	b	a	b	c	d	a	b
0	54	16	40	5	195	29	78	12	209	19	78	10	654	14	45	5
1	30	0	30	0	97	6	41	2	516	103	45	4	759	15	31	5
2	114	0	127	0	76	0	92	0	201	0	195	0	840	0	82	0
3	134	0	192	0	101	0	135	0					562	0	50	0
4	249	0	256	0	84	0	139	0					728	0	49	0
during flowering	201	0	248	0	229	0	230	0					908	0	50	0
after flowering	168	0	249	0	211	0	199	0					827	0	48	0

a: number of seeds tested; b: number of seeds infected; c: total number of seeds tested; d: average percentage of seeds infected.

[1]) All seeds originated from the first flowering period.

Infection of seeds harvested from plants inoculated in stage 0 came to an average of 14 %. No flower buds were aborted.

All axillary buds and flower buds of plants inoculated in stage 1 became necrotic. New laterals arose showing mosaic symptoms. Seeds originating from these sprouts were infected to an average of 15 %.

Plants inoculated in stage 2 lost all flower buds, except the most developed ones, which were situated mainly in clusters at nodes 4 and 5, less frequently in clusters on lateral shoots at the axils nearest to the main stem at nodes 1, 2 and 3 (Fig. 4). Eight hundred forty seeds originating from these flowers were checked; not one appeared to be infected. After these seeds had ripened the plants showed new vegetative growth: sprouts developed, which showed mosaic symptoms and on which flowers, and later pods, developed. Infection of 270 seeds harvested from these pods averaged 13 %.

Of plants inoculated in stage 3 more flower buds had passed the critical stage of abortion. None of their seeds proved to be infected. Plants inoculated in stage 3 in the glasshouse later did not produce mosaic-showing laterals. Plants inoculated in stage 3 in the field sometimes did. The infection of 150 seeds harvested from the mosaic-showing laterals of these plants averaged 15 %.

Plants inoculated in stage 4 or later stages both in the glasshouse and in the field produced healthy seeds. No mosaic-showing laterals were formed by these plants.

The infection of seeds of plants inoculated in the field was comparable to that of seeds from plants inoculated in the glasshouse. The percentage infection of 453 seeds produced by secondary infected plants averaged 11 %. The experiments revealed that seeds which transmitted virus were formed in pods only on those shoots which showed mosaic symptoms.

CHAPTER 7

ANATOMY AT THE TIME OF INFECTION OF THOSE FLOWER BUDS WHICH MAY PRODUCE INFECTED SEEDS AND OF FLOWER BUDS WHICH BECOME NECROTIC

Flower buds may be divided into three groups:

1. Those which have not yet reached the critical stage at the time of their infection and which may produce infected seeds;
2. Those which become infected during the critical stage and are aborted, or are lost by acro-necrosis of main stem and shoots;
3. Those which have passed the critical stage at the time of their infection and which later, in spite of infection of the plant, never produce infected seeds.

It would be interesting to determine the border-line between the stages of development of these three groups in anatomical respect. Therefore experiments were carried out to determine:

a. the anatomy, at the time of their infection, of buds equivalent to the farthest developed flower buds of group 1 which later produced infected seeds.
b. the anatomy at the time of their infection, of buds equivalent to the least developed buds of group 3 which later never produced infected seeds.

7.1. The anatomy, at the time of their infection of buds equivalent to the farthest developed flower buds which later produced infected seeds

Experiments already described revealed that only those buds produced infected seeds which were present on plants inoculated in early stages or at newly formed shoots of plants inoculated in later stages (p. 460). They were the ones which had not yet reached the critical stage of bud necrosis and also were not lost by shoot necrosis. Those among these buds which were farthest developed at the time of their infection are the ones situated at nodes 4 of plants inoculated in a sub-stage of the plant shortly before stage 1 (p. 457, Fig. 4). These buds become necrotic if plants are inoculated in stage 1. Therefore the buds at the nodes 4 of plants inoculated in a sub-stage just before stage 1 were considered in the following experiments, because these buds must be the oldest ones capable of producing virus infected seeds.

Ninety plants, inoculated in a sub-stage shortly before stage 1, were divided at random into three groups. With the first group of plants, the time (expressed in days after inoculation) was determined after which virus had probably reached an infectious level in the flower buds at node 4. Because the flower buds themselves were too small to be tested, the time was determined after which virus reached an infectious level in the tip of the main stem above node 3. Every 12 hours, starting 12 hours after inoculation, from three plants of the first group tips, 5 to 7 mm in length, were excised above node 3. Test plants were inoculated with sap of these three tips separately. From plants of the second group, tips were excised and fixed at similar intervals. The anatomy was studied of the oldest flower buds at node 4 of those tips which were likely to have been infected comparable to those of similar plants of group 1 which appeared to have been invaded by virus. From plants of the third group, seeds formed in pods at node 4 were harvested and tested with the seedling assay.

Virus could not be demonstrated in tips excised 108 hours after inoculation or earlier. It was, however, detected in all three of those picked 120 hours after inoculation. Microscopical examination of flower buds at node 4 of tips excised 120 hours after inoculation showed that within the ovules of the oldest buds the functional megaspore was strongly enlarged. This cell was still mono-nucleate or had started to divide (plate 2 B). Within the anthers, microspore mother cells were present. Five out of 59 seeds harvested from nodes 4 of plants of the third group turned out to be infected. With a similar experiment, carried out shortly after the former, similar results were

obtained: seven of 67 seeds turned out to be infected. With both experiments no exact answer is given to the question which is precisely the stage of development at the time of infection of the oldest flower buds which later produce infected seeds, nor can it be said excactly with which stage of development of the flower buds the period of flower bud necrosis starts. The results of the experiments suggest strongly, however, that the period of necrosis of the flower buds starts approximately with the development of the embryo-sac within the ovules. So, if virus has been built up to an infectious level in the plant before the flower buds have reached this period of development, their seeds formed later may be infected.

7.2. The anatomy, at the time of their infection, of buds equivalent to the youngest flower buds which later, in spite of their infection, did not produce infected seeds

The youngest flower buds which produced healthy seeds in our experiments were those which were not aborted after inoculation of the plants in stage 2. It was stated that these non-dropping flower buds are mainly situated at nodes 4 and 5, less frequently in the clusters on lateral shoots at the axils nearest to the main stem at nodes 1, 2 and 3. To determine approximately the stage of development of these buds at the time of their infection, an attempt was made to answer the following questions:

a. At what rate is virus transported to the flower buds?

b. Which anatomical stage of development is reached by the youngest flower buds that do not drop, at the time virus has just been built up to an infectious quantity within them?

The following experiments were performed:

Of 30 plants inoculated in October in stage 2 at the middle leaflet of leaf 1, from each of three plants two flower buds were picked every 24 hours during 10 days starting with the day of inoculation. These buds were situated at nodes 4 and 5 and at the axillary shoots situated at nodes 0, 1, 2 and 3 of the main stem. The two largest buds of the basal clusters at the axillary shoots were chosen. The buds of each pair were pressed out together and their sap was assayed with a test plant.

Six days after inoculation, the virus was present in an infectious quantity in flower buds picked from axillary shoots situated at the nodes 1, 3, 4 and 5 of the main stem. After 7 days it was also present in those picked from axillary shoots situated at node 2; but only after 9 days, in those picked from axillary shoots at node 0 (Table 6). If it is assumed that the site where virus can be first demonstrated is also the site to which virus material is first transported, the results indicated that virus material was first transported upwards to the flower buds at nodes 1, 3, 4 and 5. The flower buds at the axillary shoots at node 2 seemed to be passed over, as in these buds virus could first be demonstrated one day later. Thereafter virus material was

TABLE 6

Time after inoculation at which virus was first demonstrated in flower buds.

days after inoculation	nodes from which the buds were picked:					
	0	1	2	3	4	5
1	0 [1])	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	3	0	3	2	3
7	0	3	3	3	3	3
8	0	3	3	3	3	3
9	1	3	3	3	3	3
10	3	1	3	3	2	3

[1]) Each figure is based upon a test of one bud pair from each of three different plants inoculated in stage 2.

transported downwards to flower buds at the axillary shoots placed at node 0. The experiment was repeated in October and November. Similar results were obtained.

Now that it had appeared possible to demonstrate virus in flower buds, an attempt was made to determine which anatomical stage the ovules of the youngest, non-excised and non-dropped flower buds had reached at the time virus had just been built up to an infectious quantity within their ovaries.

This experiment was carried out in May. It could be expected that the time after inoculation necessary for virus material to be transported to the buds would be shorter than in the experiment carried out in October, since temperature and light conditions were more favourable (POUND & BANCROFT, 1956; BANCROFT & POUND, 1956). Sixty plants inoculated in stage 2 at the middle leaflet of the 1st compound leaf were divided into three groups. Every 24 hours, from 48 hours until 168 hours after inoculation, pairs of the oldest flower buds were picked from three plants of the first group, as in the former experiment. The ovaries were excised. The lengths of the ovaries varied between 1 and 3 mm. The inocula prepared from the pairs of ovaries, and also from the pairs of flower buds from which these ovaries had been excised, were separately rubbed on the simple leaves of test plants.

The data in Table 7 show that 5 days after inoculation virus was demonstrated for the first time both in flower buds and ovaries at nodes 1, 3, 4 and 5. Six days after inoculation the virus was demonstrated for the first time in flower buds and ovaries at the sprouts at nodes 0 and 2 (Fig. 6). As it was expected, the rate of transport of the virus to the flower buds and ovaries turned out to be higher than in the former experiments carried out in October and November.

Simultaneously with the picking of flower buds from the first group, for testing for the presence of virus, the oldest flower buds

from similar places were gathered from three plants of the second group. They all were immediately fixed in order to select later, for an anatomical examination, those buds in which virus material had probably just been built up to an infectious level. The fixed material

TABLE 7

Time after inoculation at which virus was first demonstrated in flower buds or ovaries from different nodes.

days after inoculation	nodes from which the buds were picked:											
	0		1		2		3		4		5	
	fl.b.[1]	ov.[1]	fl.b.	ov.	fl.b.	ov.	fl.b.	ov.	fl.b.	ov.	fl.b.	ov.
2	0 [2]	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	**3**	**2**	0	0	**2**	**2**	**2**	**2**	**3**	**2**
6	**3**	**3**	**2**	**3**	**2**	**2**	**3**	**3**	**3**	**2**	**3**	**3**
7	**2**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**

[1] fl.b. = flower bud; ov. = ovaries.
[2] Each figure is based upon a test of one bud pair or ovary pair from each of three different plants inoculated in stage 2.

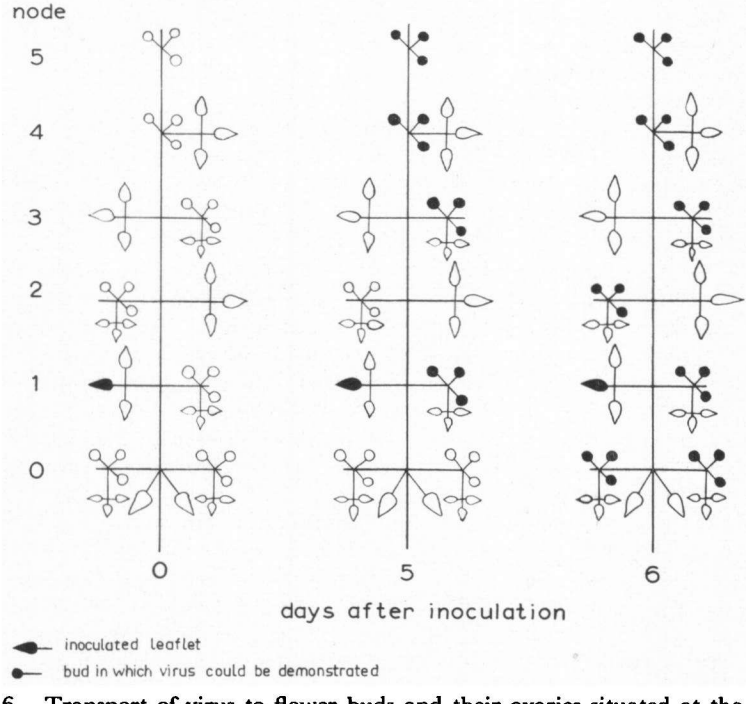

Fig. 6. Transport of virus to flower buds and their ovaries situated at the basal nodes of axillary shoots and at nodes 4 and 5 of the main stem, after inoculation of the plant in stage 2.

was stored because the results of the experiment with similar buds of the plants of the first group had to be awaited. Then, only buds were examined which had been picked from nodes 4 and 5 and from axillary shoots at nodes 1 and 3, 5 days after inoculation and at nodes 0 and 2, 6 days after inoculation. In these flower buds, according to the results obtained with similar flower buds of plants of the first group, virus material had probably also just been built up to an infectious quantity.

Microscopical examination revealed that some of the oldest ovules of flower buds from nodes 2, 4 and 5 contained an eight-nucleate embryo-sac. Microspores were present in the anthers at this time. All other flower buds contained ovules that were less developed. From the 24 inoculated plants of the third group nearly all flower buds dropped by necrosis of parts of the axillary shoots at which they were situated, or by necrosis of themselves. Only some of the oldest flower buds at nodes 4 and 5 and at the basal clusters of axillary shoots at node 2 stayed on.

If flower buds of the plants of the three groups were comparable, it may be assumed that each ovule of flower buds that did not drop, contained already an eight-nucleate embryo-sac or a farther developed one at the time virus had been built up to an infectious level within their ovaries. From former experiments (p. 460) it was evident, that these buds would not produce virus infected seeds.

Results of a similar experiment carried out in May and June led to similar conclusions.

The results obtained from experiments which are discussed in this chapter lead to the following conclusions:

Infection of seed of the bean cv. Beka depends on the stage of development of the flower buds at the time of infection of the plant. The flower buds farthest developed which produced infected seeds contained ovules, at the time of their infection, in which the embryo-sac started to develop: the functional megaspore was strongly enlarged, but still mono-nucleate. The anthers contained microspore mother cells at that time. Also the lesser developed buds could produce virus infected seeds.

If the embryo-sac within the ovules had approximately passed the mono-nucleate stage, but had not yet reached the eight-nucleate stage at the time virus was built up to an infectious quantity within the ovaries, the flower buds became necrotic.

It may be asked, why not all seeds, but only about 15 % of them, became infected when buds are infected in a young stage.

CHAPTER 8

FACTORS, WHICH MAY BE RESPONSIBLE FOR THE LOW PERCENTAGE OF VIRUS TRANSMISSION BY SEED

The percentage of infected seeds of plants showing mosaic symptoms is rather low in our experiments, especially in relation to findings of

authors who used other bean cultivars or other virus strains for their experiments. Some factors, which may be responsible for the fact that only a small number of seeds of plants infected in an early stage of development transmitted virus, will be discussed.

8.1. The influence of maturation, drying, storage and germination on the percentage of virus transmission by the seed

Zaumeyer & Harter (1943) reported that southern bean mosaic virus, which is not transmitted by seeds, was present in high concentration in seed of systemically infected plants of *Phaseolus vulgaris* L. in early stages of development, but was apparently inactivated in later stages of ripening. Cheo (1955), who studied this phenomenon in more detail, found that as seed matured, the virus content of the embryo increased while that of the seed-coats and the pod decreased. Virus in the embryo was, however, inhibited very rapidly when dehydration of the seeds occurred, but not so in seed-coats and leaves. Completely ripened or dried seeds did not transmit the virus; however, fresh immature seeds from systemically infected plants did. Cheo suggested that the inhibition of the virus in the embryos might be due to certain chemical changes during the final stages of ripening.

Gold *et al.* (1954) stated that barley false stripe virus was transmitted through only 50 to 90 % of the seed, though all developing embryos examined contained the rod-shaped particles in a concentration approximately as high as in leaf tissue. They explained this by inactivation of the virus during storage.

In view of these findings, an attempt was made to discover whether the low percentage of seed which transmitted BCMV in our experiments was due to inactivation of the virus during maturation, desiccation, storage or germination.

About 25 to 30 days after the end of flowering, 25 green immature pods were chosen at random from 20 out of 75 bean plants which had been inoculated in stage 0. From each of these pods two green immature seeds were picked at random. After removal of the seed-coat the cotyledons of each seed were separated from the rest of the embryo and held together by a small piece of wire. The embryo without cotyledons is called the "incomplete embryo". To inactivate superficial virus material the pairs of cotyledons and the incomplete embryos were kept submerged in a concentrated soap solution for 2 minutes after which they were rinsed in running tap-water for 30 minutes. Control experiments revealed that in this way infectious material disappeared from healthy cotyledons and incomplete embryos, which had been kept submerged for 30 minutes in undiluted inoculum. With a glass spatula each pair of cotyledons and each incomplete embryo were separately crushed on a ground-glass slide after the addition of some droplets of tap-water. The inocula thus acquired were rubbed on both simple leaves of separate test plants. About three weeks later, 45 to 50 days after flowering, 75 of the oldest, now mature and dry pods were picked at random from the remaining

50 plants. Out of each pod two dry brown seeds were picked at random. Fifty of these seeds were dried for 10 days at 24° C. Then they were soaked in Petri-dishes between moist filter paper at 24° C for 48 hours and tested for the presence of virus in cotyledons and embryos by the use of test plants. Fifty dry seeds were sown out directly after harvesting; another 50 seeds were sown out after a year's storage. The experiment was repeated during the same season.

TABLE 8

The percentage of seed infection as influenced by maturation, drying, germination and storage.[1])

A	the percentage of seeds in which virus could be demonstrated with the test plant assay in:							
	only cotyledons		only incomplete embryos		both cotyledons and incomplete embryos		total percentage of infected seeds	
	exp. I	exp. II	exp. I	exp. II	exp. I	exp. II	exp. I	exp. II
immature seeds	4	2	4	4	4	10	12	16
dry, mature seeds	2	4	6	8	0	2	8	14
B	the percentage of seeds in which virus could be demonstrated with the seedling assay							
directly after harvest							11	15
after a year's storage							12	12

[1]) Each percentage is based on testing of 50 objects.

Table 8 shows for both experiments (I and II) that:

1. In infected seeds virus could be demonstrated in the cotyledons, in the incomplete embryos or in both. These observations agree with those of CROWLEY (1957b), CRISPIN MEDINA & GROGAN (1961) and QUANTZ (1962).
2. The percentage of infection of immature and mature seeds determined with the test plant assay and the percentage of seed infection determined with the seedling assay, directly after harvesting and after a year's storage of the seeds, were all about equal. Thus, the low percentage of seed infection cannot be due to inhibition or inactivation of virus during maturation, drying, storage or germination of the seeds.

It might be explained by a low percentage of infection of mega- and microspores. In all experiments in which the occurrence of seed infection was studied, the seeds originated from self-pollination. Therefore it had to be examined if embryo infection originated from an infected megaspore, infected microspores, or both.

8.2. Crossings with mega- and microspores of healthy and diseased plants

Transmission of virus by pollen of bean plants infected with bean common mosaic virus was first suggested by Reddick & Stewart (1918).

Nelson & Down (1933) first demonstrated the transmission of BCMV through pollen as well as through egg-cells to the progeny of the plant.

Frandsen (1952) confirmed the transmission of BCMV by pollen.

Crispin Medina & Grogan (1961) also demonstrated transmission of BCMV by either pollen or ovules of infected plants in high percentages by crossing diseased and healthy plants. They found that pollen usually transmitted virus to a larger percentage of progeny than ovules did.

It was asked if our strain of BCMV would be transmitted through the pollen as well as through the eggs to the progeny of the bean cultivar Beka and to what extent.

Two methods of pollination developed by Buishand (1956) were used:

a. The rubbing method with emasculation of the bud.

The standard is bent backwards with a pair of forceps. Then the keel is carefully pulled to pieces and stamens can be removed. Next the stigma is pollinated by carefully rubbing with a thickly pollinated stigma originating from the male parent.

b. The clamping method without emasculation of the bud.

If the left-hand wing is pressed downward, the stigma with the style-brush emerges from the keel. The stamina remain in the closed keel. Next a stigma with pollen is pulled out from the male parent and hooked behind the stigma to be pollinated.

Crossings were accomplished between *Phaseolus vulgaris* cv. Beka and *Phaseolus vulgaris* cv. Dubbele Witte zonder draad, because the cotyledons and the hypocotyledons of seedlings brought about by hybridization show a purple discoloration, which distinguishes them from the seedlings from seeds produced by selfing.

The transmission of BCMV by pollen was studied by pollinating flowers of healthy bean plants cv. Dubbele Witte zonder draad with pollen of bean plants cv. Beka which had been inoculated in stage 0. For this pollination the clamping method without emasculation of the bud was used.

The transmission of BCMV by eggs was studied by pollinating flowers of bean plants cv. Beka, inoculated in stage 0, with pollen of healthy bean plants cv. Dubbele Witte zonder draad. For this pollination the rubbing method with emasculation of the bud was applied, as the clamping method could not be applied to the malformed flowers of the diseased 'Beka' plants. It was impossible to make the stigma with the style-brush emerge from the keel by pressing the left-hand wing downward.

The results of the crossings indicate that BCMV can be transmitted by pollen and ovules of infected bean plants cv. Beka (Table 9). The percentages of ovule- and pollen-transmission are about equal, respectively 27 and 31 %. Also 50 seeds brought about by selfing were harvested from the same diseased 'Beka' plants, which were used for the crossing: infected Beka ♀ × healthy Dubbele Witte ♂.

TABLE 9

Percentage infection of seed produced by hybrids after crosses involving healthy 'Dubbele Witte' and diseased 'Beka' bean plants.

crossings	number of pollinations	percentage of successful pollinations	number of hybrid seeds	percentage of seeds infected
Dubb. Witte ♀ × [1]) Beka ♂	156	40	213	31
[1]) Beka ♀ × Dubb. Witte ♂	153	19	36	27
[1]) Beka ♀ × [1]) Beka ♂	(self pollination)		50	15

[1]) Inoculated in stage 0.

The infection of these seeds amounted to 15 %. So the percentage of infected seeds from self-pollinated flowers of infected plants is about half the percentage of infected seeds from artificial crossings, in which only one of the parents was infected. The same effect was found by CRISPIN MEDINA and GROGAN (1961) with *P. vulgaris* cv. Sutter Pink.

It may, however, be assumed that infection of an embryo with the strain of BCMV used in all the present experiments with bean plants cv. Beka originated from an infected pollen grain or an infected egg-cell, may be from both.

It may be concluded that the percentage infected embryos, whose infection is caused by entrance of virus via the egg-cells or contaminated pollen, appeared to be maintained at the original level after maturation, storage and germination of seeds. Therefore, the phenomenon that not all seeds of plants infected in a young stage transmitted virus, can only be due to the fact that not all mega- and microspores became invaded by it.

It was tried to get an answer to the question why not all megaspores of early infected plants became invaded with the virus. The infection of microspores was kept out of consideration.

8.3. INFECTION OF OVARIES AND OVULES OF PLANTS INOCULATED IN A YOUNG GROWTH STAGE

To get an answer to the question why not all egg-cells of early infected plants become invaded by virus it first was asked if perhaps not all flower buds, ovaries or even ovules became infected with the virus.

To obtain a clear picture of the presence of virus in ovaries and pods, ovules and seeds, these organs were tested in different stages of their development.

Ovaries and pods in ten different stages of development were picked at random from a group of 25 plants, inoculated in growth stage 0 (Table 10). If not too small, ovules and seeds were removed from their respective ovaries and pods. To inactivate superficial virus material, the ovaries and pods, ovules and seeds were kept submerged in a concentrated soap solution for 2 minutes and rinsed in tap-water for 30 minutes. Control experiments revealed that in this way infectious material disappeared from healthy ovaries and pods, ovules and seeds, which had been kept submerged for 30 minutes in undiluted inoculum. Ovaries and pods, ovules and seeds were squeezed separately on a rough slide with a glass spatula after some droplets of a buffer-solution at pH 7 had been added. The slide, with the small quantity of inoculum thus obtained, was rubbed on one of the simple leaves of a test plant.

TABLE 10

Stages of development of ovaries and pods.

numbers of the stages	developmental stages
I	ovaries without cavity or with a small cavity, but still without ovule-primordia.
II	ovaries with a cavity, in which ovules start developing.
III	ovaries containing ovules with a 1- to 2-nucleate embryo-sac
IV	,, ,, ,, ,, a 4-nucleate embryo-sac.
V	,, ,, ,, ,, a 8-nucleate embryo-sac.
VI	pods containing very young seeds varying from 0.5 to 1 mm.
VII	,, ,, ,, ,, ,, ,, ,, 1.5 to 3.5 mm.
VIII	green, full-grown pods.
IX	yellowing pods.
X	dry pods.

Sap, for inoculum, was pressed out of the larger pods. The dry pods (stage X, Table 10) were soaked in water for 24 hours before testing.

Ovary-primordia without a cavity, or with a cavity but not yet containing ovule-primordia (stage I) measuring less than 0.3 mm, were too small to test in this way. From them and also from those in stage II preparations were made, by "the dipping method" according to BRANDES (1957) with some modifications, as follows:

After a rinse in tap-water, the ovaries were cut lengthwise into two halves under the binocular microscope. The cut surfaces of both halves of each ovary were kept in contact with a droplet of twice distilled water for 30 seconds, placed on a formvar film covering a grid. The droplet just overlapped the aperture of the grid. The grids were shadow-casted with gold-palladium.

Ovules, excised from six different ovaries 2 days before flowering

(stage V), were tested by means of the dipping method as well as by the test plant assay. Ovules and seeds, from ovaries and pods respectively, in the stages VI, VII, VIII and IX were tested only with the test plant assay. The inoculum of seeds of stage VIII and IX was prepared with virus-tongs.

Electron-microscopical examination did not reveal any virus particles in the ovary-primordia of stage I, picked 16 days after inoculation of the plant (Table 11). In only one out of ten ovary-primordia in stage I, picked 20 days after inoculation, were some virus particles observed, though they could be observed easily in eight out of ten ovaries in stage II, picked from the same plants 20 days after inoculation. In the latter, virus could also be demonstrated with the test plant assay.

TABLE 11

Presence of bean common mosaic virus in ovaries and pods at different developmental stages. The bean plants were inoculated in stage 0.

development of ovaries and pods:				presence of virus in ovaries and pods, tested with:			
				test plant assay		dipping method	
stages (see table 10)	days after inoculation	days before (—) or after (+) flowering	lengths of ovaries and pods, in mm	numbers tested	numbers infected	numbers tested	numbers infected
ovaries							
I	16	— 40 to — 30	± 0.5	—[1]	—[1]	10	0
I	20	— 40 to — 30	± 0.5	—	—	10	1
II	20	— 40 to — 30	0.8 to 1	10	8	10	8
III	35	— 25 to — 15	2.5 to 3.5	10	10	—[1]	—[1]
IV	40	— 15 to — 10	4 to 6	10	10	—	—
V	50	— 2	5 to 8	10	8	—	—
pods							
VI	50	+ 5	8 to 12	9	7	—	—
VII	80	+ 10	23 to 32	10	10	—	—
VIII	90	+ 35	70 to 110	10	10	—	—
IX	100	+ 60	70 to 110	10	4	—	—
X	100	+ 60	70 to 110	10	0	—	—

[1]) Where no data are given, no tests were made.

From the moment that ovules started developing (stage II) until 2 days before flowering (stage V) virus could be demonstrated in at least 80 % of the ovaries.

Virus appeared to be present in at least 78 % of the pods until they turned yellow. In that stage (X) virus could be demonstrated in only four of ten pods. In dry pods soaked in water no virus was detected at all. Virus was easily observed electron-microscopically in nine out of ten ovules from ovaries in stage V excised 2 to 3 days before flowering (Table 12, and plate 7 B).

In similar ovules virus could be demonstrated with the test plant assay: in two experiments respectively five out of ten and eleven out

TABLE 12

Presence of bean common mosaic virus in ovules and seeds at different developmental stages. The bean plants were inoculated in stage 0.

development of ovules and seeds:				presence of virus in ovules and seeds, tested with:			
				test plant assay		dipping method	
stages (see table 10)	days after inoculation	days before (—) or after (+) flowering	lengths of the ovules and seeds, in mm	numbers tested	% of infection	numbers tested	numbers infected
V	50	— 2	0.3 to 0.7	10	50	10	9
V	50	— 2	0.3 to 0.7	15	73	—[1]	—[1]
VI	50	+ 5	0.5 to 1	34	85	—	—
VII	80	+ 10	1.0 to 3.6	20	75	—	—
VIII	90	+ 35	9 to 20	37	12	—	—
IX	100	+ 60	10 to 18	24	4	—	—

[1]) Where no data are given, no tests were made.

of fifteen ovules appeared to be infected. So it turned out that the percentage of infected ovules 2 days before fertilization was much higher than the percentage of seeds that transmitted virus in our experiments.

Young seeds from pods up to and including stage VII also appeared to be infected to a high percentage. It may be supposed that during these early developmental stages the virus is present in the still green integuments, which constitute a great part of the volume of the young seed.

The more the integuments lose their original character and acquire that of a dry seed-coat, the lower the percentage of seed infection, until at last, in stage IX, the percentage of infection of the seed is defined only by that of the embryo. With inoculum prepared from dry testae soaked in water no test plants could be infected.

From the experiments it became evident that no virus could be detected in ovary-primordia in stage I. This may be due to their meristematic character, which disappears when differentiation into ovary-wall and ovules occurs.

Absence of virus from meristematic tissues of virus-infected plants has been reported by several authors. No virus was detected in growing-points of diseased plants belonging to many different species (LIMASSET & CORNUET, 1949). MOREL & MARTIN (1955) succeeded in growing healthy potatoes from excised apical meristems originating from plants infected with virus Y. Unpublished experiments by A. F. Schippers–Lammertse have shown that the top-meristem and frequently the first two leaf-primordia of a tobacco plant inoculated with TMV remain virus-free, while somewhat older leaf-primordia underneath contain virus in a high concentration. Several authors (WHITE, 1943; MELCHERS & BERGMANN, 1959; BRANTS *et al.*, 1962) have stated that

growing-points of tomato roots infected with TMV, cultivated in White's liquid, also are virus-free.

From the moment ovules started to develop, virus could be demonstrated in nearly all ovaries of plants inoculated in stage 0 and so the low percentage of seed infection of BCMV cannot be due to a small number of ovaries infected with virus.

It is also improbable that the low percentage of seed infection is caused by a lack of infection of a number of ovules before their fertilization, because a few days before flowering they are infected to a high percentage. In these ovules probably a barrier was present which prevented the entrance of virus into the egg-cell and later into the embryo. In younger ovules virus might have entered the egg-forming cells or the egg-cells. It may be that if examination of younger ovules had been possible, a lower percentage of ovule infection had been found. In this way it may be explained why the percentage of infected egg-cells could not reach the same height as that finally reached in the ovules.

8.4. The cause of reduction in number of seeds per pod on plants infected in a young growth stage

Caldwell (1952) observed that the infection of tomato plants with aspermy virus disturbed the prophase of meiosis, and accordingly stopped the division of the spore mother cells of both micro- and megaspores. The production of seed and pollen of the infected plants was highly reduced.

It was stated that the number of seeds per pod of bean plants infected with BCMV in stage 0 or 1 was also greatly reduced (Table 2).

Since leaves, flower buds and axillary shoots infected at a critical stage of their development become necrotic, it was supposed that the development of ovules or ovule-primordia, mega- or microspores, invaded by virus in some definite stage, might also be disturbed by their infection. If so, this would reduce the number of seeds per pod but also reduce the percentage of infected seeds.

It might, however, also be possible that the reduction of fertility of plants showing mosaic symptoms is due to an impairment of vigour of the whole plant or to an impairment of vigour of mega- and microspores.

To clarify these questions, the development of ovule-primordia, ovules and seeds was compared in plants showing mosaic symptoms, in healthy plants in starved condition and in normal healthy ones.

Ninety plants, sown simultaneously in the autumn of 1961, were divided into three groups. The plants of the first group, grown in large pots, were inoculated in stage 0. The plants of the second group, also kept in large pots, were not inoculated. The plants of the third group were cultivated in small pots and shadowed with a jute screen to impair the normal growth. They, too, were not inoculated.

At the time the plants were about in stage 1 the number of normal ovule-primordia per ovary was checked. For this purpose 50 flower

buds were picked at random from 15 plants per group. The ovaries were excised and brought into a drop of lactophenol under a coverglass for microscopical examination.

In ovaries of the diseased plants, as well as in those of the healthy plants in starved condition, ovule-primordia with retarded growth and thickenings of the placenta marked the places where evidently ovule-primordia had failed to develop (Fig. 7). Only normally developing ovule-primordia were counted.

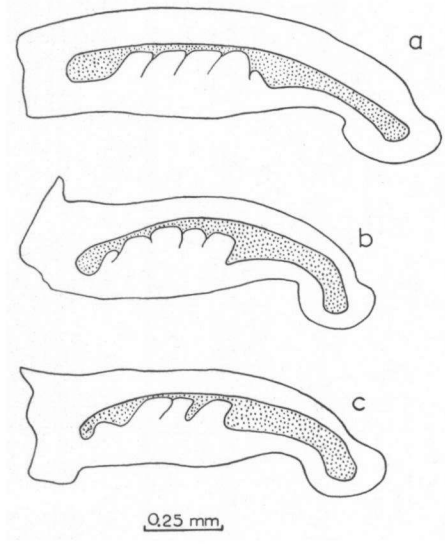

Fig. 7. Ovule-primordia with retarded development in ovaries of: a) a healthy plant grown under light-deficient conditions b) and c) plants inoculated in stage 0.

The number of ovaries containing, respectively, 2, 3, 4, 5 and 6 ovule-primordia were counted separately for each group (Table 13A, experiment I). The total number of ovule-primordia formed in 50 ovaries of plants with mosaic symptoms and that of ovule-primordia formed in 50 ovaries of healthy plants in starved condition differed from the number of ovule-primordia formed in normal healthy plants.

Ovaries of healthy plants contained mostly four or five ovule-primordia; those of plants with mosaic symptoms, three or four; those of the badly developed plants still less. The lack of vigour of the last group of plants appeared to be the cause of the reduced number of ovule-primordia per ovary. This was probably also the case with the reduced number of ovule-primordia in pods of the mosaic-diseased plants, which also showed reduced growth.

To determine the occurrence of ovule abortion in infected plants in a later stage, a second counting was made just before flowering. Fifty buds, nearly opened, were picked at random from 15 plants of each of the same three groups. From each of these flowers the ovary

was cut and its number of ovules was determined under the binocular microscope (Table 13B, experiment I).

No ovule-primordia with retarded growth or abnormally developed ovules were detected with the dissecting microscope.

A comparison of the numbers in Table 13A I and 13B I shows that the number of normal ovules present in the ovaries just before flowering did not differ from that in younger ones.

TABLE 13

A. The number of ovule-primordia several weeks before fertilization in ovaries of healthy plants, infected plants and plants grown under light-deficient conditions.

state of the plants	number of ovaries, in which the number of ovule-primordia amounted, respectively to: 2	3	4	5	6	total number of ovule-primordia in 50 ovaries	number of ovule-primordia in 50 ovaries [1]
Experiment I							
healthy	0	1	35	14	0	213	100
infected	0	23	27	0	0	177	83
light-deficient	14	26	9	1	0	147	69
Experiment II							
healthy	0	2	39	9	0	207	100
infected	3	19	28	0	0	175	84,5
light-deficient	12	26	12	0	0	150	72,4

B. The number of ovules just before fertilization in ovaries of healthy plants, infected plants and plants grown under light-deficient conditions.

state of the plants	number of ovaries, in which the number of ovules amounted, respectively, to: 2	3	4	5	6	total number of ovules in 50 ovaries	ovules in 50 ovaries [1]
Experiment I							
healthy	0	1	34	14	1	215	100
infected	1	23	24	2	0	177	82,3
light-deficient	10	28	12	0	0	152	70,6
Experiment II							
healthy	0	1	40	8	1	209	100
infected	1	18	31	0	0	180	86,1
light-deficient	15	23	12	0	0	147	70,3

[1]) Expressed as % of the number of ovule-primordia (ovules) in ovaries of healthy plants.

It was concluded that ovule-primordia with retarded growth observed in ovaries of plants in stage 1 had not developed any further and that abortion of ovules did not occur before flowering.

So the decrease in seed production of diseased plants is partly due to the inhibition of ovule development. This can be considered as the result of the impairment of vigour of the diseased plants, since exactly the same phenomenon could be noticed in healthy plants grown under adverse conditions.

The results of a similar experiment, also carried out with 90 plants in the autumn of 1961, were in agreement (Tables 13A II and 13B II).

The influence of infection of the plant with BCMV on the fertilization of the ovules and the development of the seeds was studied in spring and summer of 1962 with 75 plants divided into three groups of 25. The plants of the three groups were treated in a way similar to the plants of the three groups of the former experiment. In contrast to the preceding experiment carried out in autumn (p. 473), the mosaic-diseased plants grew vigorously, and produced large leaves. The plants of both other groups were comparable to those of the former experiment. Two months after sowing, 50 nearly full-grown pods of every group were picked at random. The contents of these pods were divided into three classes:

1. Ovules which stopped developing after flowering. Within these ovules no embryo could be distinguished by microscopical examination. This failure of the ovules to grow may be the result of: lack of pollination, inadequate pollination or infertility of the ovules.
2. Seeds with inhibited development, varying in size from somewhat larger than mature unfertilized ovules, to half as large as normally developed seeds. Within these seeds an embryo could always be distinguished.
3. Normally developed seeds.

The experiment was repeated with another 75 plants sown at the same date. Table 14 shows that the total number of unfertilized ovules and more or less developed seeds was comparable in pods of healthy plants and in plants showing mosaic symptoms, respectively 222 and 216 in the first experiment and 179 and 182 in the second experiment. This indicates that the infection of the plants did not influence the number of ovule-primordia present per ovary, contrary to our former results (Tables 13A and 13B).

This lack of influence of virus infection may be ascribed to the luxurious vegetative growth of the plants showing mosaic symptoms grown in spring and summer in contrast to the growth retardation of the plants showing mosaic symptoms grown in autumn.

The total number of ovules and seeds in the pods of the plants grown under reduced light-intensity was much less than that of the other two groups, which agrees with our former results (Table 13A and 13B).

TABLE 14

The development of seeds in the pods of healthy plants, infected plants and plants grown under light-deficient conditions.

state of the plants	non- or inadequately fertilized ovules		badly developed "seeds"		normally developed seeds		total number of ovules and seeds	ovules [1] and seeds
		%		%		%		
Experiment I								
healthy	8[2]	3,6[4]	22[2]	9,9[4]	192[2]	86,4[4]	222	100
infected	63	29,1	18	8,3	135	62,4	216	97,2
light-deficient	13	8,3	64	41,2	78	50,3	155	69,8
Experiment II								
healthy	4[3]	2,2[4]	24[3]	13,3[4]	151[3]	80,4[4]	179	100
infected	49	26,9	10	5,4	123	67,5	182	101,6
light-deficient	4	3,3	48	39,3	70	56,5	122	68,1

[1]) Expressed as % of the total number of ovules and seeds in pods of healthy plants.
[2]) Number of ovules or seeds in 50 pods (Exp. I).
[3]) Number of ovules or seeds in 40 pods (Exp. II).
[4]) The percentage in relation to the total number present in all the pods of each group.

The present results confirm our supposition that reduction in number of ovules or ovule-primordia per ovary of infected plants is due to impairment of vigour of the mother plant induced by virus but not due to the presence of the virus itself in these special tissues.

The data in Table 14 also show that only a small number of ovules stopped developing after flowering in pods of normal healthy plants and in those of plants grown under reduced-light conditions. The percentage of ovules which stopped developing in pods of plants showing mosaic symptoms, however, was several times higher.

The percentage of badly developed seeds in pods of healthy plants was about the same as that in pods of mosaic-diseased plants, while that in pods of healthy plants grown under reduced-light conditions was several times higher. Thus it appeared that plants with virus showed a high number of non-fertilized ovules and those without virus but grown under unfavourable conditions showed a high number of seeds inhibited in development. Concerning the site of ovules and seeds in the pods it was observed (Fig. 8):

a. ovules which stopped developing after flowering were situated at random.

b. seeds with inhibited development were nearly always situated at the proximal end of the pod. If more of the badly developed seeds were present, then the less developed they were, the nearer they were situated to the proximal end of the pod.

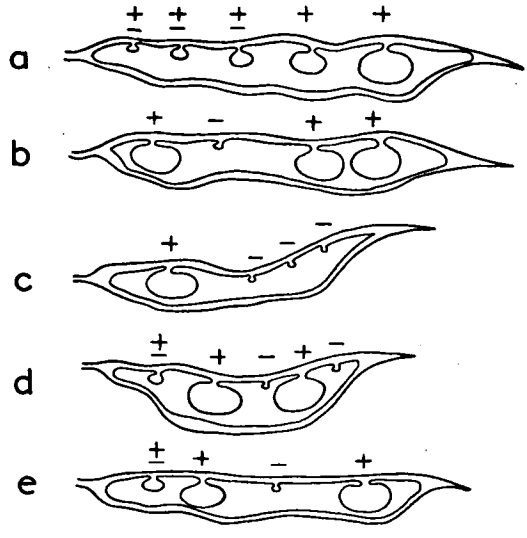

Fig. 8. The site of non- or inadequately fertilized ovules (—), seeds with inhibited development (±), and normally developed seeds (+) in pods of plants inoculated in stage 0 (c, d and e) and in a pod of a healthy plant grown under deficient light (a and b).

These observations also suggest that the inhibition of development of seeds is connected with an insufficient supply of nutrients, while the high number of ovules which stopped developing after flowering, situated at random in pods of plants showing mosaic symptoms, has to be ascribed to another factor connected with the presence of virus.

CRISPIN MEDINA & GROGAN (1961) observed that the percentage of germination of pollen and the length of pollen tubes of mosaic-diseased bean plants were much less than those of healthy plants. Such an impairment of vigour of pollen and perhaps of egg-apparatus may be responsible for the high number of ovules which stopped developing, of mosaic-diseased plants in our experiments.

If so, this impairment of vigour of micro- or megaspores is due to the presence of the virus and not to the impairment of vigour of the whole mother-plant.

In summary, the decrease in production of normal seeds by healthy plants in starved condition may be caused by:

1. a decrease in the number of ovule-primordia initiated per pod.
2. an inhibition of seed development.

The decrease in production of normally developed seeds of plants showing mosaic symptoms may be caused by the same factors which

can be ascribed to a lack of nutrients and moreover to a non- or inadequate fertilization of ovules.

In what way the presence of the virus may be responsible for the non- or inadequate fertilization of ovules is unknown. Anyhow, the phenomenon can only be of minor importance in determining the percentage of infected seeds and cannot explain why so many seeds are non-infected.

CHAPTER 9

ABSENCE OF SEED INFECTION WITH PLANTS INFECTED SHORTLY BEFORE OR AFTER FLOWERING

9.1. INTRODUCTION

In our experiments seed infection occurred only if ovules became infected before the embryo-sac was formed. Thereafter, infection of flower buds resulted in abortion, which hypersensitive period stopped when the embryo-sac had nearly matured(p.465). Flower buds invaded by virus after the hypersensitive period, before or after flowering, always produced healthy seeds. These observations are in agreement with those of FAJARDO (1930), who stated that bean plants infected with bean common mosaic after the flowers were set, produced almost no infected seeds. CROWLEY (1957b) reported that the temperature prevailing before flowering of bean plants infected with BCMV influenced the percentage of infected seeds, while the temperature after flowering was not. These observations led to the supposition that fertilization stops the possibility of embryo infection; this cessation might be due to the lack of plasmodesmal connections between the embryo and its surrounding tissues (CALDWELL, 1934, 1962; BENNETT & ESAU, 1936).

No literature is available concerning the possibility of infection of these virus-free-seed-producing ovules of plants which are inoculated shortly before or after flowering, nor concerning the anatomical proceedings of the embryo-sac and the tissues that surround the embryo-sac, during this period.

To verify the hypothesis that fertilization, which occurs at the day of flowering, stops the possibility of virus transmission by seed, a number of experiments was carried out. The moment of infection of buds producing virus-free seed had to be determined. For that purpose the infection of ovaries and ovules of the buds that had just passed the critical period of abortion, and their behaviour during infection, was studied. These buds were chosen because, of the virus-free-seed-producing buds, they were the ones whose time of flowering was most remote from the moment of inoculation of the plant. They seemed to offer the best opportunity to study the question why their egg-cells and embryos do not become infected. These youngest flower buds which were not aborted after they became infected were the oldest buds out of the clusters situated at nodes 4 and 5, less frequently

out of the basal clusters on lateral shoots at nodes 1, 2 and 3, of plants inoculated in stage 2 (Fig. 9).

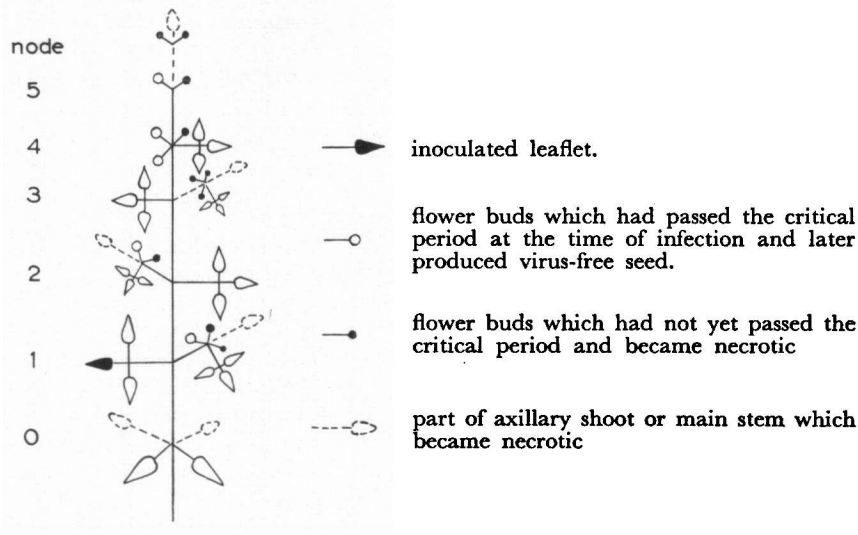

Fig. 9. Diagram of a plant inoculated in stage 2, showing flower bud necrosis and necrosis of a part of the main stem and axillary shoots.

It was asked how many days before fertilization virus material might enter these flower buds, their ovaries and ovules. Next it was asked whether before fertilization this virus material would be built up to an infectious level high enough to be demonstrable with the test plant assay or with the dipping method, especially in the ovules. Therefore the rate of transport of virus material from the inoculated leaf to the buds, their ovaries and ovules had to be determined. The developmental stage of the ovules at the time of infection and at the time virus had been built up to an infectious level had to be studied, just as their further development until their fertilization and thereafter.

Anatomical changes during the period of virus multiplication might elucidate why no embryo infection occurred with the flower buds studied. It was impossible to study all phenomena with the same plants. Therefore, in our experiments, out of a great number of plants sown at the same date a number of comparable plants in stage 2 were selected and inoculated simultaneously. These plants were divided at random into four groups, with which were studied, respectively:

a. the time of infection of flower buds and ovaries by virus material with respect to the moment of inoculation;
b. the time at which virus had been built up to an infectious level within flower buds, ovaries and ovules;
c. the anatomy of the ovules of flower buds at the time they were infected with virus and at the time virus had been built up to an infectious level;

d. the time of flowering of buds comparable to those of the other groups. The last mentioned group was also used for checking which flowers similar to those studied in the other groups would drop and which would produce virus-free seeds.

Healthy plants sown simultaneously with the inoculated ones were used to compare the development and flowering of the buds similar to those which dropped from the diseased plants.

9.2. Experiments

Experiment 1

One hundred thirty-nine plants, inoculated in stage 2, were divided into five groups. Twelve plants of the same age were not inoculated. Every 24 hours, from 24 hours till 144 hours after inoculation, two flower buds were picked from nodes 4 and 5, from five plants of the first group. The ovules were excised from the ovaries after the latter had been excised from the buds. The inocula, made from the pairs of flower buds without ovaries, and from the pairs of ovaries without ovules, and from the ovules from each pair of ovaries together, were separately tested for the presence of virus. Virus could be demonstrated for the first time in ovaries and buds gathered 5 days after inoculation (Table 15).

Simultaneously, two flower buds were picked from nodes 4 and 5, from five plants of the second group. After the ovaries from these flower buds had been excised, the pairs of buds without ovaries, and the pairs of ovaries, were incubated separately for 3 days partly submerged in White's fluid (White, 1943) in small tubes. It was

Table 15

Time after inoculation at which buds at the nodes 4 and 5 and their ovaries had been invaded by virus material, and time at which virus had just been built up to an infectious level.

days after inoculation	number of the node from which the buds were picked:							
	4				5			
	tested immediately		tested after an incubation period of 3 days		tested immediately		tested after an incubation period of 3 days	
	buds without ovary	ovaries	buds without ovary	ovaries	buds without ovary	ovaries	buds without ovary	ovaries
1	0 [1]	0	0	0	0	0	0	0
2	0	0	3	3	0	0	2	2
3	0	0	3	2	0	0	3	2
4	0	0	2	2	0	0	4	2
5	5	3	5	3	5	5	4	1
6	5	5	4	2	5	5	3	3

[1]) Each figure is based upon a test of one bud pair from each of five different plants inoculated in stage 2.

supposed that the virus material that had entered the flower buds or ovaries before they were excised would be transformed into a demonstrable virus quantity during incubation. After that, the inocula made from the pairs of these flower buds, and from the ovaries, were tested for the presence of virus.

Table 15 shows that after 3 days of incubation, virus could be demonstrated in three of five pairs of flower buds and ovaries picked from nodes 4 and 5 two days after the plants were inoculated, but in neither flower buds nor ovaries picked from similar sites one day after inoculation. This means that virus material entered the flower buds and ovaries approximately 2 days after inoculation. In comparison with the findings of the experiment with group 1 showing that virus had reached an infectious level in buds and ovaries gathered 5 days after inoculation, it was concluded, that virus material needed 2 days to reach the buds and ovaries and thereafter 3 days to be built up to an infectious level within them.

That not all flower buds and ovaries picked 2 days after inoculation or afterwards showed infection after 3 days of incubation may partly be due to the fact that some of them disintegrated by bacterial infection. The presence of virus could not be verified in any of the inocula made from ovules from pairs of ovaries, even 6 days after inoculation.

Simultaneously with the picking of flower buds from plants of the first and the second group for virus detection, all buds were gathered from the clusters at the nodes 4 and 5, from five plants of the fourth group. These buds were immediately fixed for an anatomical study. Later those buds had to be selected which might just have been invaded by virus material and also those in which virus might have been built up to an infectious level. From the foregoing experiments it will be clear that only those flower buds picked 2 days, and 5 days after inoculation had to be embedded in paraffin, because only these might respectively just have been invaded by virus material or contain virus which just had been built up to an infectious level.

Microscopical examination revealed that the oldest flower buds of the clusters from nodes 4 and 5, picked 2 days after inoculation, contained ovules with a four-nucleate embryo-sac (plate 3 B). The other buds were less developed. The oldest flower buds of the clusters at nodes 4 and 5, picked 5 days after inoculation, contained ovules with an eight-nucleate embryo-sac. The other buds were less developed. These observations confirm results obtained in former experiments (p. 465), from which it was concluded, that flower buds which contained ovules with an eight-nucleate embryo-sac or ovules in a more advanced stage of development at the time that virus reached an infectious level within them had passed the period of flower bud necrosis. These flower buds never produced infected seeds. The fifth group of plants was used to study the days of flowering. Of 12 inoculated plants of the fifth group and 12 non-inoculated plants of the same age, the day of flowering of all buds was noted. The time of flowering turned out not to be influenced by virus infection of the plants (Table 16). The oldest buds of healthy and virus infected plants flowered on the 9th

TABLE 16

Number of days after inoculation at which the buds flowered which originated from plants inoculated in stage 2, and from comparable healthy plants.

days after inoculation	inoculated plants: number of flowers at the nodes:						healthy plants: number of flowers at the nodes:					
	0	1	2	3	4	5 and higher	0	1	2	3	4	5 and higher
9					8	2				2	6	2
10			4		10	4			5		16	1
11			2				2	7	1	2	11	7
12							7	14	8	7	2	14
13							7	12	15	9		10
14							10	3	9	6		4
15							11	2	10	2		1
16							10	1				7
17							11	1	1	1		5
18							8		2			1
19							4	1				1
20							1		1	1		
21							1					

and 10th days after inoculation. The buds which were first to flower were situated at nodes 4 and 5 and less frequently on the axillary shoots at nodes 2 and 3. All younger buds became necrotic. The number of flower buds in the clusters at nodes 4 and 5 of plants of the fourth group with ovules that contained an eight-nucleate embryo-sac, 5 days after inoculation, was of the same order as the number of flower buds that did not became necrotic in the clusters at nodes 4 and 5 of plants of the fifth group. This is in agreement with the supposition mentioned above that flower buds from the clusters at nodes 4 and 5 which were not aborted were those ones which contained ovules with an eight-nucleate embryo-sac at the time virus had reached an infectious level in these flower buds and their ovaries. All seeds originating from these flower buds turned out to be virus-free.

As it must be assumed that the flower buds from the nodes 4 and 5 of the plants of the five groups were comparable, the experiment proved that:

a. virus material entered the ovaries of the buds that produced virus-free seed, at the nodes 4 and 5, two days after inoculation of the plants in stage 2, that is about 7 or 8 days before they flowered. At that time the buds contained ovules with a four-nucleate embryo-sac.

b. virus was built up to an infectious quantity in these buds and their ovaries 5 days after inoculation, that is 4 or 5 days before they flowered. At this time their ovules contained an eight-nucleate embryo-sac.

This means that infection of ovaries 7 to 8 days and 4 to 5 days before flowering, the times at which respectively virus material had

just reached the ovaries and at which virus had just been built up to an infectious quantity within the ovaries, does not result in infection of the seeds they will produce later. Although virus multiplication starts already within the ovaries about 7 days before fertilization, the infection of the megaspores seems to be difficult or even impossible.

Experiment 2

Still, ovule infection had to be studied more thoroughly. The fact that in the former experiments virus could not be detected with the test plant assay in ovules of flower buds at nodes 4 and 5 of plants inoculated in stage 2, within 6 days after inoculation might be explained by the following hypotheses:

a. the ovules were not or not yet invaded by the virus.

b. the time was too short for virus material in ovules to be built up to an infectious level.

c. the amount of inoculum obtained from all ovules together from one ovary was too small to demonstrate infectious virus.

Therefore ovules were tested for the presence of virus at a later stage with both the test plant assay and the dipping method. Fifty-seven plants, inoculated in stage 2, were divided in three groups of 15 plants and a fourth group of 12 plants. Twelve plants of the same age were not inoculated. Every 24 hours, from the 5th until the 9th day after inoculation, ovules from three flower buds at each of the nodes 4 and 5 from three plants were tested for infectivity. From all ovules of a single ovary an inoculum was prepared. The inocula thus obtained were rubbed out separately on test plants. Thus every 24 hours nine different inocula were tested. Simultaneously ovaries were picked from buds at node 4 of each of three plants of the second group. Two ovules in juxtaposition were taken out of each of them. A dip-preparation made of each ovule (p. 470) was examined electron-microscopically. Table 17 shows that 5 and 6 days after inoculation no virus could be demonstrated in the ovules by either of the two test methods. Seven days after inoculation, however, virus could be demonstrated with both methods. The rather low number of positive results obtained with the test plant method may be due to the extremely small amount of inoculum, the diameter of the largest ovule being only about 0.5 mm.

The number of dip-preparations in which virus particles could easily be distinguished would perhaps have been higher, had not some of them been too thick for examination.

Simultaneously with the picking of ovules for virus detection, flower buds from nodes 4 and 5 from three plants of group 3 were fixed to study the anatomy of the ovules at the moment virus could be demonstrated for the first time in comparable ovules of groups 1 and 2. Only those flower buds picked 7 days after inoculation were embedded in paraffin. Microscopical examination revealed that these flower buds contained ovules with an eight-nucleate embryo-sac. In

TABLE 17

Number of days after inoculation at which virus could be demonstrated for the first time in ovules from buds originating from nodes 4 and 5 of plants inoculated in stage 2.

days after inoculation	number of preparations in which virus could be demonstrated by means of:			
	the test plant assay		the dipping method	
	experiment I	experiment II	experiment I	experiment II
5	0/9 [1]	0/9	0/6 [2]	0/10
6	0/9	0/9	0/6	0/10
7	2/9	6/9	4/6	9/10
8	5/9	6/9	4/6	7/10
9	4/9	2/9	3/6	—

[1] numerator: the numbers of inocula with virus; each inoculum originated from all ovules of one ovary; the nine ovaries originated from the nodes 4 and 5 of three different plants.
denominator: the numbers of inocula tested.

[2] numerator: the numbers of dip-preparations from ovules excised from buds at the nodes 4 in which virus particles could be observed.
denominator: the numbers of dip-preparations examined.

most of them the polar nuclei had already migrated to the centre (Fig. 2). The dates of flowering of the flower buds at nodes 4 and 5 of 12 plants of group 4 and of 12 non-inoculated plants were noted (Table 18).

The youngest flower buds at nodes 4 and 5 of inoculated plants, which would have flowered on the 11th, 12th, 13th or 14th day after inoculation, dropped. Probably virus material invaded these buds in a critical stage, at the time the ovules were between the mono- and eight-nucleate embryo-sac stage. Two buds flowered on the 11th day after inoculation. Their petals however did not open. They had probably just passed the critical stage when they were invaded by virus material. This was also the case with the older buds which flowered on the 9th or the 10th day after inoculation.

TABLE 18

Flowering of buds from nodes 4 and 5 of bean plants inoculated in stage 2 with BCMV, and of comparable buds from healthy plants.

days after inoculation	number of flowers of:	
	healthy plants	infected plants
9	8	12
10	15	14
11	8	2
12	3	
13	0	
14	1	

If it is supposed that the oldest flower buds at nodes 4 and 5 of the plants of groups 1 and 2 would have flowered simultaneously with those of groups 3 and 4, it may be concluded that infective virus present in the ovules 7 days after inoculation can be built up in the ovules 2 or 3 days before flowering. The seeds produced later by similar flower buds of the 12 inoculated plants turned out to be virus-free. The results obtained with a similar experiment carried out in the same season led to similar conclusions (Table 17, experiment II).

TABLE 19

Time of infection of ovary and ovules of buds having passed the critical period at the time of infection, in relation to the time of inoculation and flowering. These buds are the youngest ones that always produced virus-free seeds.

days after inoculation		days before flowering
10	flowering	0
9	flowering	0 or 1
8	. . . . .	
7	virus was built up to an infectious quantity in the ovules. embryo-sac eight-nucleate. polar nuclei had migrated to the middle.	2 or 3
6	. . . . .	
5	virus was built up to an infectious quantity in the ovaries. embryo-sac eight-nucleate. . . .	4 or 5
4	virus material probably had entered the ovules.	5 or 6
3	. . . . .	
2	virus material entered the ovaries. embryo-sac four-nucleate.	7 or 8
1	. . . . .	
0	inoculation	10 or 11

The data may be summarized as follows (Table 19):

1. The experiments discussed in this chapter revealed that after inoculation of the plant in stage 2, only those flower buds produced seeds whose ovules contained an eight-nucleate embryo-sac at the moment virus could be demonstrated in them for the first time. Less developed ones abscised. These seed-producing flower buds were mainly the older ones at nodes 4 and 5; less frequently they were the older ones on the axillary shoots at nodes 1, 2 and 3.

2. Virus material entered the ovaries of the oldest buds at nodes 4 and 5 as soon as 2 days after inoculation, i.e. 7 or 8 days before flowering, at the time they contained ovules with a four-nucleate embryo-sac.

3. Infectious virus could be demonstrated for the first time within these buds and their ovaries 5 days after inoculation, i.e. 4 or 5 days before flowering. At that time the ovules contained an eight-nucleate embryo-sac.

4. Infectious virus could be demonstrated within the ovules of these buds for the first time 7 days after inoculation, or 2 to 3 days before they flowered. At that time the ovules contained an eight-nucleate

embryo-sac in which the polar nuclei had already migrated to the middle.

5. Flower buds similar to those mentioned under 1, 2 and 3 only produced healthy seeds.

The apparent inability of the virus to infect the egg-cell, although virus reached an infectious quantity in the ovaries and ovules before fertilization, suggests a barrier between the egg and the virus infected tissues of the ovules.

As it would be of interest to obtain more knowledge about the character of this barrier, the embryo-sac with its surrounding cell-layers was studied electron-microscopically.

9.3. Electron-microscopical examination of the cell-layers bordering the embryo-sac

Ovules were chosen from buds at nodes 4 and 5, 2 to 3 days before flowering, both from healthy plants and plants inoculated in stage 2. Longitudinal sections through the cell-layers bordering the sac (Fig. 2), and through the nucellar tissue were examined electron-microscopically. In most cases the nucellar tissue had completely disappeared at places lateral to the sac, where in some cases some loose cell-wall fractions were left. At the chalazal end bordering the embryo-sac, also, cell fractions and loose cells were observed. By disappearance of the nucellar tissue, the lateral sides of the embryo-sac had come into immediate contact with the inner cell-layer of the inner integument, which bears the character of an epidermis with a cuticle. In the latter, plasmodesmal connections with the embryo-sac were never observed, though plasmodesmata could be found frequently between mutual cells of the inner and also between those of the outer integument (plates 8 and 9). The absence of plasmodesmal connections between inner integument and embryo-sac may be connected with the fact that during the development of the embryo-sac the extending inner integument separates the nucellar tissue from the outer integument. The latter was observed by light-microscopical examination of developmental stages of the ovule (p. 444). No attempt was made to detect ectodesmata in the cuticular layer of the inner integument. It may, however, be difficult or even impossible for virus material to pass from cells of the inner integument to the egg-cell. Entering of virus into the sac via its chalazal end would be a possibility.

It may be, however, that the disintegration of the nucellus-cells at this site, which process proceeds also after fertilization, as light microscopical examination revealed (plates 4 B and 5 A), makes transport of virus material of BCMV to the egg-cell difficult or impossible.

The data concerning the possibility of transmission of the virus through seed may be summarized as follows:

Virus which invades ovaries before the embryo-sac starts to develop within the ovules may invade cells of the ovules, the future egg-cell included, and be transmitted through about 15 % of the seeds. If

virus invades ovaries in the period between the mono-nucleate and the eight-nucleate stage of the embryo-sac of their ovules, no virus is transmitted by seeds because all these flower buds abscise.

Disintegration of the nucellar tissue starts early, at the time the embryo-sac has reached the eight-nucleate stage. It may be that virus entering the nucellus after that stage cannot infect the nuclei of the sac, the egg-cell included, and so the embryo, which develops after fertilization, also remains free from virus.

CHAPTER 10

DISCUSSION

Different aspects of the occurrence of seed transmission of bean common mosaic virus (BCMV) by seeds of *Phaseolus vulgaris* L. cultivar Beka were studied. The experiments were preceded by an analysis of the development of the healthy 'Beka' plant and by a study of the anatomy of its flower buds in different developmental stages. Next, the different ways in which 'Beka' plants may react after having become infected with BCMV in different developmental stages were studied in relation to seed infection.

It appeared that plants inoculated at a very young stage developed only shoots with leaves that showed mosaic symptoms and with pods that contained less seeds than those of healthy plants. Plants which were somewhat older when inoculated showed necrotic symptoms in leaves, acro-necrosis in shoots and main stem, and necrosis of several flower buds. Later, these plants developed new sprouts which showed mosaic symptoms. Plants inoculated in the 1st compound leaf stage lost all flower buds by acro-necrosis and flower bud necrosis. The later the plants were inoculated after the 1st compound leaf stage the less necrotic symptoms appeared.

Special attention had to be given to the occurrence of flower bud necrosis, as this phenomenon restricts the transmission of virus by seed. It was asked if necrosis of flower buds might be connected with a definite period of their development in a way similar to that in which necrosis and a developmental stage of leaves and shoots are correlated. As the latter are less difficult to study than flower buds which contain so many heterogeneous organs, the relation was examined first between the stages of leaves and shoots during which they became infected and the disease symptoms they would show. Necrotic leaf symptoms appeared when virus reached an infectious level in leaves during their grand period of growth, i.e. during the period in which cell-elongation predominates over cell-division. Leaves infected before this period developed mosaic symptoms, while leaves infected after this period became epinastic or did not show any symptoms (Fig. 3b). Necrosis of shoots and main stem appeared to be related to a strong enlargement of the internodes at the time virus reached an infectious level within the plants (Fig. 5). These internodes

Plate 8. Electron-micrograph of an ultra-thin section from the cell-layers which border the lateral sides of the embryo-sac of an ovule, 3 days before flowering. a: embryo-sac; b: inner integument; c: outer integument; d: disintegrating nucellar tissue. The white darts point at plasmodesmal-like structures in the cell-walls.

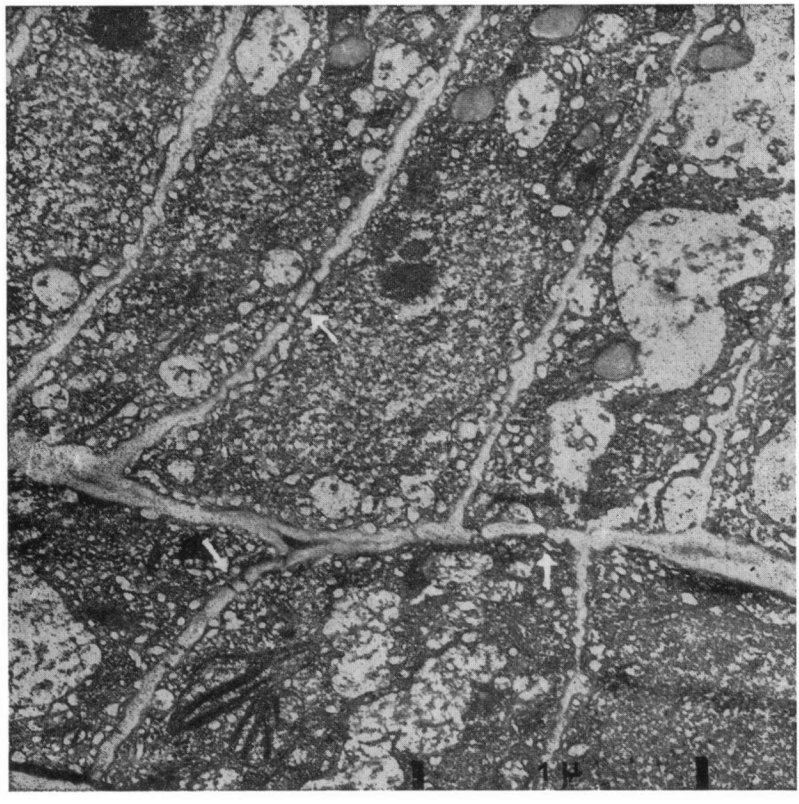

Plate 9. Electron-micrograph of an ultra-thin section from the inner integument which borders the lateral sides of the embryo-sac of an ovule 3 days before flowering. The white darts point at plasmodesmal-like structures in the cell-walls.

and the parts of the shoots or main stem above these internodes died. The basal internodes first pass through this period of strong enlargement, followed successively by the internodes more acropetally situated. Therefore the older the plants were at the time of inoculation, the nearer to their tips were the necrotic parts of the main stem and the shoots, until at last all internodes had passed the critical period and acro-necrosis occurred no more.

It was evident that the behaviour of a tissue seemed to depend on its stage of growth at the time it became invaded by infectious material. During the stage of cell-division a tissue seemed to support virus in so far that infection resulted in a mosaic pattern, whereas tissue succumbed, if infected in a stage wherein cell-elongation predominates over cell-division. Mature or nearly mature tissue though invaded, showed hardly any macroscopic symptoms.

It became obvious that flower bud necrosis is due to a course of events similar to that causing leaf- and shoot necrosis. Those flower buds dropped in which the ovules were strongly enlarging and in which the embryo-sac was within range from the mono-nucleate to the eight-nucleate stage at the time virus reached an infectious level in them. Sometimes, also, the peduncles were enlarging rapidly at that time. It was impossible to decide whether in some cases necrosis of the peduncles was the cause of abscision; all buds that dropped however, were in the critical period in which they contained ovules between the mono- and eight-nucleate stage. It will be clear that all buds that abscised can be left out of consideration as to the production of infected seeds. Only those flower buds which had not yet reached this critical period, or those which had just passed it, produced seeds and therefore could be studied with regard to the production of infected seeds. Only the former when invaded by virus produced infected seeds. Among these, the infection averaged 15 %. Flower buds invaded by virus after the critical period produced only healthy seeds.

With regard to the flower buds which became infected before they reached the critical period of bud necrosis, it was asked why only about 15 % of their seed was infected.

Because experiments revealed that BCMV is inactivated in pod-walls and seed-coats, it was supposed that the low percentage of embryo infection might also be caused by inactivation of virus within the embryos during maturation, desiccation, during storage or germination of the seeds. Inactivation of virus in embryos during maturation and storage is reported by GOLD *et al.* (1954) and CHEO (1955). It appeared, however, that of embryos excised from immature green seeds, from fully grown matured ones, from yellowing pods and dried seeds stored during one year, all tested with the test plant assay, about 15 % were infected. Seeds from the same plants transmitted the virus to about the same percentage of the seedlings. It was concluded that virus was inactivated in the walls of pods and in seed-coats, but not in the embryo. The percentage of infected embryos appeared to be maintained at the original level after maturation,

storage and germination of seeds. As inactivation could not explain the low percentage of embryo infection it was supposed that this low percentage was due to the fact that not all egg-cells and pollen became infected.

Because in all experiments seed originated from self-pollination of the flowers, it first had to be determined whether embryo infection originated from infected megaspores, or infected microspores, or both. Artificial crossings revealed that embryo infection may originate from an infected megaspore and an infected microspore, both of which appeared to be infected to a low percentage. That not all seeds of plants infected in a young stage transmitted the virus, therefore must be due to the fact that not all mega- and microspores became invaded with virus.

An attempt was made to elucidate the question why not all egg-cells of early infected plants became infected with virus. The microspores were kept out of consideration.

With the test plant assay and the dipping method (p. 470) virus could be demonstrated in at least 80 % of the ovaries of plants infected in a young stage, from the moment that ovule-primordia started to develop within them. The low percentage of egg-cell infection therefore cannot be ascribed to the non-infection of ovaries. However, no virus could be demonstrated with the dipping method in ovary-primordia before ovule-primordia started to develop within them. This may be connected with the meristematic character of the ovary-primordia before they differentiate into ovary-wall and ovules. Failure to demonstrate virus in meristematic tissues has been recorded frequently.

Experiments were designed to determine whether perhaps not all ovules became invaded with virus. For technical reasons—younger ovules were too small—the presence of virus in ovules of plants inoculated in a young stage could only be studied some days before fertilization. Some days before flowering, virus could be demonstrated in about 80 % of the ovules. Thus, the percentage of ovules in which virus had reached a demonstrable quantity was much higher than the percentage of seeds which transmitted virus. Also 80 % of immature seeds appeared to be contaminated with virus, if all parts of the seeds were tested together. In ovules some days before flowering, and also later, probably a barrier was present which prevented the entrance of virus into the egg-cell and later into the embryo. The character of this barrier will be discussed later. In younger ovules, in which no such a barrier was present, virus might have entered the egg-forming cells or the egg-cell. It may be that if examination of younger ovules had been possible a lower percentage of ovule infection had been found. In this way it may be explained why the percentage of infected egg-cells could not reach the same height as that finally reached in the ovules.

It was also studied whether the reduced number of seeds in pods of plants infected in a young stage might be partly responsible for the low percentage of infected seeds. It might be that infection of ovules

or young seeds at some critical stage of development would result in abortion, just as infection of leaves, shoots and flower buds at critical periods of their growth resulted in necrosis.

Ovule- and seed development of three groups of plants were compared: plants infected in a young developmental stage, healthy plants, and healthy plants grown under deficient light.

The reduction in seed production of both virus-infected plants and healthy plants grown under deficient light appeared to be due to two factors: a decrease in the number of ovule-primordia initiated per ovary and an inhibition of the development of young seeds. Both were probably due to a lack of nutrients. Moreover the reduction in seed production of infected plants seemed to be due to a non- or inadequate fertilization of ovules, which phenomenon was seldom observed with healthy plants grown under normal conditions or under deficient light. If all non- or inadequately fertilized ovules had developed into virus-transmitting seeds, their frequency would still only be partly responsible for the low percentage of seed infection.

It may be concluded that this low percentage correlates with a barrier inhibiting transport of virus material from the walls of the young ovules to the embryo-sac or the egg-cell. A similar barrier has to be supposed, preventing microspores to become infected to a high percentage after they have reached a certain stage of development. Non- or inadequate fertilization of the egg-cells of diseased plants perhaps may be also partly responsible for the low percentage of transmission of the virus by seed.

An attempt was made to elucidate also why flower buds having passed the critical stage with ovules in which virus reached an infectious level, never produced infected seeds. That fertilization stops the possibility of seed infection with bean common mosaic virus was already proposed by FAJARDO (1932) and CROWLEY (1957). To verify this hypothesis, experiments were carried out to study the behaviour of those buds which had passed the critical stage and which were farthest remote from flowering. Virus material entered the ovules of these buds 5 to 6 days before flowering, i.e. 5 to 6 days before fertilization, while virus was built up to an infectious level 2 to 3 days before flowering. That no egg-cell- or embryo infection occurred, however, could be deduced from the lack of contaminated seeds produced later.

It was suggested that in these ovules there was also already present, some days before fertilization a barrier which might prevent virus-transport. Light-microscopical examination revealed that during the development of the embryo-sac the inner integument grows out between nucellus and outer integument. Electron-microscopical and light-microscopical examination indicated that the nucellar tissue has already started to disintegrate some days before fertilization, leaving the lateral sides of the embryo-sac in immediate contact with the inner integument. No plasmodesmal-like structures were observed in cell-walls of the inner integument bordering the embryo-sac. If virus material can be transported from cell to cell by plasmodesmata only, it probably cannot enter the embryo-sac from the inner integument.

It may, however, enter the embryo-sac from the chalazal end. At this side, cells of the nucellar tissue strongly enlarge and seem to loosen from each other before fertilization; some seem even to disintegrate. That no infection of the egg-cell or, later, of the embryo occurred in our experiments after the embryo-sac reached the eight-nucleate stage may therefore be ascribed to the impossibility of transport of virus material through disintegrating nucellar tissue and through the egg-cell- or embryo-surrounding medium, respectively to the egg-cell or the embryo. Nor will multiplication of virus at these sites be possible.

CALDWELL (1934, 1962) and also BENNETT & ESAU (1936) supposed that lack of plasmodesmal connections between embryo and parent tissue would prevent the embryo from becoming infected. CROWLEY (1959) supposed that not the lack of plasmodesmal connections but "the structure of the epidermal cells of the embryo or some difference in their metabolism prevents infection of the developing embryos". About the presence of plasmodesmal- or ectodesmal structures in the outer cell-walls of developing embryos nothing is yet known. Independent of the presence or absence of plasmodesmal connections a barrier may be found in the disintegrating nucellar tissue and the embryo-surrounding medium, preventing virus multiplation and transport. Data about the localization of a virus in different parts of an ovule at different times of its development are needed for a better understanding of virus transmission by seed.

Possibly that localization of virus with fluorescent antiserum, which technic at its present state of development is not feasible in our experiments, may become useful in the future.

SUMMARY

1. In this study an attempt was made to elucidate why transmission of bean common mosaic virus (BCMV) by seed of *Phaseolus vulgaris* L. cultivar Beka is always restricted to a certain percentage of seeds harvested from diseased plants.

2. The development of the healthy 'Beka' plant, especially the formation of flower buds, pods and seeds, was analyzed. Special attention was given to the development of the ovaries and ovules from their primordial stage until some days after fertilization.

3. The influence of infection upon the development of shoots, flower buds and seeds was studied. Inoculation of the plants before the 1st compound leaf at the main stem had fully developed resulted in initial retardation of growth followed by a vigorous development of sprouts. All compound leaves showed mosaic symptoms. Pods formed on these plants often were malformed by non-development of ovules and seeds. Plants which became infected in later stages showed necrosis of flower buds and of definite parts of the main stem, shoots and leaves. The developmental stage of the plants determined the sites at which necrosis would occur.

Plants which became infected after all compound leaves at the main stem were developed never showed symptoms.

The relation between the developmental stages of leaves, internodes and flower buds at the time they became infected, and the character of the symptoms they later developed was studied in detail.

4. It became apparent that the first two compound leaves of the main stem each could show the following symptoms:

a. a coarse mosaic with curling if it became infected during an early stage of development, during which mainly cell-divisions occur.

b. a fine mosaic with only slight deformations if a leaf became infected somewhat later, but still in an early stage of development.

c. chlorotic and necrotic symptoms if a leaf became infected during the grand period of growth, in which mainly cell-elongations occur.

d. epinasty without any discoloration or malformation if a leaf became infected at the end of the grand period of growth.

e. no symptoms if a leaf had matured or nearly matured before infection took place; virus was, however, present in the leaf.

5. Necrosis of shoots and main stem turned out to be related to the grand period of growth of internodes at the time virus reached an infectious level in the plant. Basal internodes are the first to pass through this period of strong enlargement, successively followed by internodes situated more acropetally. Therefore, the older the plants were at the time of infection, the more the necrotizing parts of the main stem and shoots moved in an apical direction, until at last all internodes had passed the critical period and acro-necrosis occurred no more.

6. The relation between the stages of development at the time of infection and the character of the subsequent symptoms is far more difficult to study with flower buds, which contain so many heterogenous organs, than with stems, shoots and leaves. Flower buds became necrotic when infection occurred during the period at which the embryo-sac within the ovules developed from a mono-nucleate into an eight-nucleate state.

It could not be determined whether this necrosis and dropping of buds must be considered as a result of virus infection of the strongly enlarging peduncles being in the grand period of growth or of the simultaneously enlarging ovaries, ovules and embryo-sac. However, it became clear that buds with ovules between the mono- and eight-nucleate stage when invaded by virus, could be disregarded as to the production of infected seeds.

7. All those flower buds which had passed the critical period of necrosis at the time they became infected produced healthy seeds. Infected seeds were produced only by those flower buds which had not yet reached the critical period of necrosis at the time virus reached an infectious level in the plant. Therefore to obtain infected seeds, inoculation had to be performed in an early stage of development.

The infection of, and transmission by seeds of even these buds, however, averaged only 15 %. Factors which might be responsible for this low percentage were studied.

8. Although virus turned out to be inactivated in pod-walls and seed-coats during maturation and drying, it did not seem to be inactivated in the embryos during maturation and drying, nor during storage and germination of the seed. Therefore, the low percentage of seed infection probably is not due to inactivation of the virus.

9. Cross-pollination experiments revealed that embryo infection might originate from an infected egg-cell or an infected pollen grain. Because the percentage of infected seeds did not appear to change during maturation and drying of the seed, it had to be supposed that the low percentage of seed infection is due to the fact that only a low percentage of mega- and microspores becomes invaded with virus.

The percentage of infected seeds which originated from artificial cross-pollinations between a healthy and a diseased plant turned out to be twice as high as the percentage of infected seeds which originated from self-pollination.

10. A study was made of why so low a percentage of the egg-cells of plants which were infected in an early stage of development, became invaded with virus.

No virus particles were detected in dip-preparations of ovaries which had not

yet differentiated into ovary-walls and ovule-primordia. Their still-meristematic character may be related to the absence of infectious virus.

Virus particles could be detected in dip-preparations of about 80 % of the ovaries in which ovules just started to develop, prepared 20 days after inoculation of the plant. So the low percentage of infected egg-cells cannot be due to the fact that a large proportion of the ovaries does not become infected.

Virus particles could be observed in dip-preparations of about 80 % of the ovules 2 to 3 days before fertilization. In these ovules, and also in a high percentage of young seeds, virus reached an infectious level. As infection of the embryos averaged about 15 %, however, a barrier was supposed to be present in nearly mature ovules some days before flowering and after fertilization, preventing the entrance of virus into the egg-cell and later into the embryo. It may be that only a low percentage of young ovules becomes infected before this barrier is formed. This might explain the low percentage of egg-cell infection. The character of this barrier was not studied with these buds but with those which never produced infected seeds, described under 12, 13 and 14.

11. The cause of the reduction in seed production of plants infected in an early stage of development might also be partly responsible for the low percentage of seed infection. Therefore this phenomenon was examined. The reduction of seed production appeared to be due to:

a. a decrease in the number of ovule-primordia initiated per ovary.

b. an inhibition of the development of young seeds.

c. a non- or inadequate fertilization of ovules.

As the phenomena mentioned under a. and b. could also be observed with healthy plants grown under deficient light, they may be due to a lack of nutrients. A high percentage of non- or inadequately fertilized ovules was observed only with plants infected with BCMV. In what way the presence of the virus may be responsible for this phenomenon is unknown. Anyhow, the phenomenon can only be of secondary importance in determining the percentage of infected seeds.

12. Experiments were carried out to examine why flower buds never produced infected seeds if they had passed the critical period of necrosis at the time they became infected with virus. The time of infection of these flower buds, their ovaries and ovules was determined in relation to the moment of fertilization. The anatomical development of the ovules was studied from the moment virus material entered the ovules until virus reached an infectious level within them.

13. Virus material entered the ovaries 7 or 8 days before flowering, at the time they contained ovules with a four-nucleate embryo-sac. Infectious virus could be demonstrated for the first time within these ovaries 4 or 5 days before flowering. At that time their ovules contained an eight-nucleate embryo-sac.

Infectious virus could be demonstrated within the ovules themselves for the first time 2 or 3 days before flowering. At that time they contained an eight-nucleate embryo-sac in which the polar nuclei had already migrated to the middle.

Because flower buds comparable to those of which the infection of ovaries and ovules was studied produced only healthy seeds, the hypothesis was proposed that a barrier might be within the ovules which might prevent infection of the egg-cell and later of the embryo.

14. Light-microscopical and electron-microscopical examination of the embryo-sac and the embryo-sac-surrounding tissues of ovules 2 to 3 days before flowering, revealed that the nucellar tissue had started to disintegrate already some days before fertilization, leaving the lateral sides of the embryo-sac in immediate contact with the inner integument. No plasmodesmal-like structures were observed in cell-walls of the inner integument bordering the embryo-sac.

If virus material can be transported from cell to cell only by plasmodesmata, it probably cannot enter the embryo-sac through the inner integument. Entrance through the chalazal end of the embryo-sac may be difficult or impossible as at this site the nucellar tissue also has already started to disintegrate before fertilization.

15. The disintegrating nucellar tissue, and the medium surrounding the egg-cell and later the embryo, may prevent virus multiplication and virus-transport. This fact, together with the impossibility of virus material to enter the embryo-sac through the integuments, may be considered as a barrier which prevents the infection of egg-cell and later the embryo. In this way it can be explained why flower buds which become infected just before or after fertilization never produce seeds infected with BCMV.

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