

THE EFFECT OF DEVELOPING EMBRYOS ON PLANT VIRUSES

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Summary

The theory that inactivation of tobacco mosaic virus by developing embryos prevents its seed transmission was tested by growing tomato embryos in media containing the virus. In many trials, using a variety of techniques, no evidence of any virus-inactivating activity was detected.

I. INTRODUCTION

Crowley (1955) demonstrated the presence of virus inhibitors in the seed of several plants, but found no virus inactivators. Hence the theory that inactivators in seeds prevent the seed transmission of plant viruses must be abandoned, unless the inactivator is some transitory product of the metabolism of embryos. The investigations described here were made to determine whether such transitory virus inactivators do occur. The relevant literature has been reviewed recently by Bennett (1956) and Crowley (1955).

Evidence will be presented in the next paper (Crowley 1957) that several of the highly infectious plant viruses commonly infect the testa, and sometimes the endosperm. Despite this the embryo develops without becoming infected. Ignoring the reasons for the lack of microspore or macropore infection, some mechanism must exist to prevent infection of the embryo during its development. Two mechanisms have been postulated: (1) that the lack of plasmodesmatal connections prevents infection; and (2) that viruses are inactivated in the endosperm, or at the interface of the embryo and endosperm. There is general agreement among embryologists and anatomists that there are no plasmodesmatal connections to embryos, and among virologists that the likely path for movement of viruses between cells is by way of the plasmodesmata. However, as all other tissues of plants are connected by plasmodesmata it is impossible to detect experimentally any other means of between-cell movement of virus particles.

The second suggestion that viruses are inactivated in the endosperm seems a reasonable alternative. If a virus is able to multiply actively in the endosperm and if the endosperm is largely (and in many seeds totally) absorbed by the developing embryo some explanation for the lack of embryo infection is necessary. All other metabolites are absorbed by the embryo as small molecules, presumably following the breakdown of larger molecules by enzymes excreted by the embryo. It would therefore seem possible for viruses in the endosperm to be broken down similarly. This theory can be investigated even if only indirectly. If embryos do in fact prevent their own infection by breaking down viruses present in the surrounding medium, then embryos growing in a culture medium containing virus should break down at least a portion of this virus. It also follows that if such a mechanism does operate

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it must do so throughout the entire period of the development of the embryo, and that the stage of development of the embryo used should be unimportant in experimental work.

The assumption that the technique could detect such a virus-inactivating effect seems likely for two reasons: (1) the volume of culture medium used per embryo was, in many of the experiments detailed below, far less than that surrounding a naturally developing embryo, and (2) the virus concentration in the medium was, nearly always, less than that of naturally infected tissues. The embryos thus had less virus to inactivate when cultured in this way than under natural conditions.

II. MATERIALS AND METHODS

Tomato embryos were grown on White's medium containing tobacco mosaic virus. After periods of 1-12 days, the infectivity of the medium was compared with the infectivity of similar samples containing the same number of dead embryos

TABLE 1
EFFECT OF TOMATO EMBRYOS ON INFECTIVITY OF TOBACCO MOSAIC
VIRUS IN THE MEDIUM IN WHICH THEY ARE GROWING

Treatment	Mean Number of Lesions per Leaf			
	Expt. 1	Expt. 2	Expt. 3	Mean
Live embryos	53	37	76	55
Dead embryos	55	35	63	51
Without embryos	57	28	51	45

(killed by boiling for 5 min) and with similarly treated samples of medium containing no embryos. All infectivity assays were carried out in a randomized block design on *Nicotiana glutinosa*. The tobacco mosaic virus used in all experiments was diluted from a purified preparation kindly provided by Dr. R. J. Best. It was sterilized by filtration through a Lloyd's sintered-glass filter (Lloyd 1945). On several occasions the infectivity of the embryos cultured in the virus solution was tested by washing them in water, rinsing in "Teepol", again washing, and inoculating (in lots of 20 or more) to *N. glutinosa*. None was found to be infected. In this respect the cultured embryos behave as they do *in vivo*. Apparently the damage sustained by the embryos in dissection is not adequate for their infection under embryo-culture conditions.

III. RESULTS AND DISCUSSION

In the first experiments, tomato embryos were placed, 10 per tube, in 0.5 ml of a culture medium containing a 1/1000 dilution of a preparation of tobacco mosaic virus (containing 6 mg of virus protein/ml). They were incubated for 14 days at 25°C, and assayed. The results (Table 1) of three trials provided no evidence of any reduction by the growing embryos of the infectivity of the medium. In two experiments there was an insignificant increase in the infectivity of the medium. This

is attributed to the fact that the embryos used in these experiments were almost mature and in the course of their growth absorbed water from the medium, and so concentrated the virus.

(a) *Aerobic Embryo-culture*

Some workers have suggested that, under the relatively anaerobic conditions of complete immersion, embryos do not develop normally but tend to germinate immediately. This change, if it does occur, could significantly alter the metabolism of the embryos, including their effect on virus particles present in the surrounding medium. Two techniques were used to overcome this difficulty. The first was to shake the culture tubes either constantly or intermittently throughout the experiments. The results of such experiments were in every way similar to those in which the culture tubes were unshaken. The second technique was to place the embryos on

TABLE 2
EFFECT OF TOMATO EMBRYOS ON THE INFECTIVITY OF
TOBACCO MOSAIC VIRUS

Values are the mean number of lesions per half leaf
(20 replicates)

Treatment	Dilution of Virus Preparation in Medium		
	1/100	1/1000	1/10,000
Live embryos	15.6	18.8	2.4
Without embryos	17.2	13.5	1.0

strips of filter paper extending down into the medium. Using this technique the embryos developed normally, and germination in culture occurred only when mature embryos were used. The results in Table 2 are of one experiment in which this technique was used. Three dilutions of the virus in the medium were used, to enable the more certain detection of any inactivation caused by the growing embryos, for, if the virus is being inactivated, the lower the concentration of the virus the greater the proportion of the virus that must be inactivated. The results of this and several similar experiments gave no indication of virus inactivation.

(b) *Variations in the Composition of the Medium*

The physical and chemical properties of the culture medium also influence, to some extent, the metabolism of the embryos, and might affect the results of these experiments. The following variations were tried:

- (i) The sugar concentration of the medium was varied from 2 to 8 per cent. because this had been reported by Rappaport (1954) to have a pronounced effect on the development of embryos in culture.

- (ii) The medium was used at pH 4, 5, 6, 7, and 8, because acidity could greatly affect the development of the embryo, and the infectivity of the virus.
- (iii) Coconut milk, both sterile and unsterile, was added to the medium. This was found by van Overbeek, Conklin, and Blakerslee (1942) to contain excellent growth stimulators.

In none of these experiments was evidence of virus inactivation detected, nor did any of the modifications significantly effect the development of the embryo. The medium used finally contained 2 per cent. sucrose, and was adjusted to pH 7.

TABLE 3
EFFECT OF DEVELOPING TOMATO EMBRYOS ON TOBACCO MOSAIC VIRUS AT
DIFFERENT INCUBATION TEMPERATURES

Values are the mean number of lesions per half leaf

Virus Concentration in Medium	Incubation Temperature (°C)	Incubation Time		
		1 day	3 days	6 days
1/100	3	176	201	181
	25	179	138	154
1/1000	3	37	27	31
	25	35	29	21
1/10,000	3	4.7	5.2	5.0
	25	4.5	3.5	3.5

A further modification was to use embryos of different ages. Some experiments were made with the youngest embryos that could be dissected, others with half mature or mature embryos. No differences were detected and the age of the embryo is considered unimportant; any mechanism preventing embryo infection must operate throughout its development. For most of the experiments described the embryos used were between one-half and two-thirds mature, because at this stage they are growing rapidly and are easily dissected and handled.

(c) Combined Effect of Embryo and Endosperm

A further possibility worth investigating was that the inactivating mechanism could be of such a nature as to require the presence of the endosperm for its operation. To test this, both embryo and endosperm were cultured as a whole by using very immature tomato seeds in which the endosperm was still almost gelatinous and could be removed from the seed intact. These endosperms, together with the embryo developing within them, were then cultured in the manner described above. No evidence of virus inactivation was obtained.

The addition of dead embryos to the medium sometimes appeared to increase its infectivity. This difference could not be attributed to the absorption of water from

the medium by the embryos because the embryos, having been boiled in sterile distilled water, are far from dry when cultured. As they do not grow, they would not take up more water. Both treatments contain pieces of filter paper of the same size; thus absorption of virus particles on to the filter paper is not the cause. The most probable explanation is that some substances are lost into the medium from the embryos, which through being boiled are presumably "leaky". This could also account for the fact that the live embryos did not increase the infectivity of the medium as much as the dead embryos. It is possible that the live embryos do equally increase the infectivity of the virus, but the increase is masked by a simultaneous virus-inactivating process, with the result that neither process is detectable. Experiments were therefore carried out in which this difference between leaky (boiled) and

TABLE 4
EFFECT OF REPEATED ADDITIONS OF TOMATO EMBRYOS ON THE INFECTIVITY
OF TOBACCO MOSAIC VIRUS

Values are the number of lesions per half leaf. Differences between temperature treatments not significant at $P = 0.05$

Virus Concentration in Medium	Incubation Temperature (°C)	Incubation Time		
		1 day	2 days	4 days
1/50	3	37	45	41
	25	28	36	36
1/500	3	3.2	5.5	5.5
	25	2.1	3.0	4.7
1/5000	3	0.6	0.8	1.0
	25	0.3	0.8	0.4

"non-leaky" (live) embryos was eliminated by using only live embryos cultured at two temperatures, 3 and 25°C. The effects of a virus-inactivating mechanism should be detectable by a comparison of the infectivity of the media, because the virus inactivation, being a physiological reaction, would be accelerated by a factor of four times for a 20°C rise in temperature. The results of one such experiment are given in Table 3.

From these results it is clear that at 3°C the infectivity of the medium remained relatively constant for the duration of the experiment at all three virus concentrations. At 25°C, there appears to be a reduction in the infectivity of the virus at the 1/1000 dilution, but such a conclusion is inconsistent with the results at the lowest dilution, where the same number of embryos did not significantly reduce the infectivity of one-tenth of the amount of virus. Because of this, and the fact that at the lowest virus concentration the change in infectivity did not continue throughout the experiment, the apparent drop in infectivity is thought not to be real.

(d) *Effect of Repeated Additions of Embryos to the Medium*

One further possibility, by which the technique used could fail to demonstrate the presence of a virus-inactivating mechanism, is that such a mechanism might not continue to operate in artificial embryo-culture. Although no reason is known why this should be so, an attempt was made to obtain evidence that would eliminate even this possibility. In several experiments 10 embryos were added to each tube daily. Previously, they had been added only at the beginning of the experiment. In this way any decline in the virus-inactivating activity of the cultured embryos would be counteracted by the repeated additions of more embryos. The results of one experiment carried out in this manner are shown in Table 4.

The experimental error is high because the technique involves unavoidable sampling errors, diluting errors, and slight variations in the treatments. All the inocula in which embryos were growing at 25°C were less infective than inocula in which they had been growing at 3°C. This is attributed to the secretion, by the growing embryo, of some substances that inhibit the infectivity of the virus. It is not thought to be due to inactivation of the virus by the embryos, because the reduction in infectivity is as great, or greater, in the most concentrated inocula as in the most dilute, and the reduction in infectivity does not increase with increasing incubation time. For these reasons it is concluded that these results give no indication of any virus-inactivating activity of the developing embryos.

Some other explanation must therefore be sought to explain the means by which embryos can remain virus-free while developing within infected tissues, and, of the explanations that have been advanced, the most adequate is Bennett's (1956) suggestion that viruses are unable to infect the developing embryo because it lacks plasmodesmatal connection with other tissues.

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V. REFERENCES

- BENNETT, C. W. (1956).—Biological relations of plant viruses. *Annu. Rev. Pl. Physiol.* **7**: 143–70.
CROWLEY, N. C. (1955).—The effect of seed extracts on the infectivity of plant viruses and its bearing on seed transmission. *Aust. J. Biol. Sci.* **8**: 56–67.
CROWLEY, N. C. (1957).—Studies on the seed transmission of plant virus diseases. *Aust. J. Biol. Sci.* **10**: 449–64.
LLOYD, J. B. (1945).—A versatile pressure filter. *Pharm. J.* (4S) **54**: 169.
VAN OVERBEEK, J., CONKLIN, M. E., and BLAKERSLEE, A. F. (1942).—Factors in coconut milk essential for growth and development of small *Datura* embryos. *Amer. J. Bot.* **29**: 472–7.
RAPPAFORT, J. (1954).—In vitro culture of plant embryos and factors controlling their growth. *Bot. Rev.* **20**: 201–25.