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Infection and necrosis of cowpea mesophyll cells by tobacco necrosis virus and two strains of tobacco mosaic virus

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Abstract

When cowpea mesophyll tissue with or without any epidermal layer was inoculated with tobacco necrosis virus (TNV), local necrotic lesions were produced. In epidermal strips isolated after inoculation of intact leaves local lesions were never observed. Homogenates of epidermal strips removed within 30 min after inoculation of the leaf with the cowpea strain of tobacco mosaic virus (Cp-TMV) or with TNV and incubated on agar for 2 or 4 days were not infectious. However, when clusters of mesophyll cells or vein pieces were still attached to the epidermal strips after stripping, the homogenates showed virus activity. When cowpea leaves were inoculated with Cp-TMV or a common strain of TMV (TMV-U) infective virus material was present in the mesophyll tissue as measured in the homogenates, at the moment of stripping, i.e. within 10 min after inculation.

It may be concluded that cowpea mesophyll cells can act as primary sites of viral ingress into the leaf and that the epidermis is not required for necrosis production after virus inoculation.

Additional keywords: Vigna unguiculata, epidermis.

Introduction

The ability of hypersensitive hosts to develop local lesions in a leaf following virus infection has been studied extensively.

Fry and Matthews (1963) reported that after TMV-U infection of tobacco, infectious material, probably RNA, moved out of the inoculated lower epidermis into the underlying mesophyll 4 h after inoculation. They also noticed occasionally that some of the mechanically inoculated leaf discs already contained infectious virus in the mesophyll when the epidermis was removed immediately after inoculation.

Couts (1980) noticed in cowpea that the transport of tobacco necrosis virus (TNV) or TNV-RNA into the underlying mesophyll tissue was fairly rapid. He did not find significant differences in lesion number and associated virus production in tissue peeled 1 h after inoculation of the leaf surface compared with unpeeled inoculated leaves. Salinas Calvete and Wieringa-Brants (1981) showed that under water-stressed conditions the passage time of TNV-strain A into the underlying mesophyll tissue in cowpea leaves was only 10 min. This passage time was measured by observing lesion formation after stripping the inoculated cowpea leaves.

Takahashi (1973) demonstrated that in tobacco infected with tobacco mosaic virus

(TMV-U) the virus replicated in the inoculated lower epidermal strips. Cowpea, however, reacts differently to virus infection. Ehara and Misawa (1967) could not find any activity of cucumber mosaic virus (CMV) in cowpea epidermal strips isolated after inoculation, although CMV replicated in tobacco epidermal strips treated similarly.

The multiplication of TNV in cowpea epidermal strips has been investigated recently (Couts, 1980; Wieringa-Brants, 1981). They suggest that the mesophyll cells adhering to the strips and the period of incubation of the strips are important for the viral activity found in the homogenates of the epidermal strips.

The involvement of the epidermis in virus replication and subsequent lesion formation has been noted by several authors (Dijkstra, 1962; Fry and Matthews, 1963; Coutts, 1980; Wieringa-Brants, 1981).

It has been suggested (Dijkstra, 1962) that in tobacco an interaction between the epidermis and mesophyll cells of 6 to 10 h duration is required for local lesion production in the mesophyll cells after a succesful virus inoculation.

It proved to be possible (Salinas Calvete and Wieringa-Brants, 1981) to induce local lesions in cowpea mesophyll tissue with TNV-A using a fine brush without carborundum. Therefore it seems unlikely that in cowpea the epidermis is involved in necrosis formation. However, when exposed mesophyll tissue is inoculated after stripping off the epidermis, the epidermis on the opposite site is still present and may influence lesion formation. Therefore experiments were carried out in which the epidermis was removed from both leaf sides before inoculation. The purpose of this study was to determine the rôle of the epidermis in the infection process of different viruses in cowpea leaves and whether the low recovery of virus from infected cowpea epidermal strips is of mesophyll origin or reflects a slow virus multiplication in the infected epidermal cells.

Materials and methods

Plants. Cowpea plants *(Vigna unguiculata* (L.) Walp., cv. Blackeye Early Ramshorn) were grown under greenhouse conditions at 25 ± 3°C and 70% r.h. Fully expanded primary leaves from 9-12-day-old cowpea seedlings were used. The seedlings had received plenty of water and had been shaded 24 h before use. *Nicotiana tabacum* L. cv. Xanthi nc was grown under the same conditions and 6-8-week-old plants were used for infectivity assays.

Virus. TNV-strain A was purified from infected 'Xanthi nc' leaves according to Kassanis (1964). The virus solution served as inoculum on naked mesophyll tissue and in the other experiments.

Tobacco mosaic virus-cowpea strain (Cp-TMV) and TMV-common strain (TMV-U) were purified from systemically infected *Nicotiana tabacum* cv. Samsun leaves using polyethyleneglycol following Gooding and Hebert (1967). A concentration of 0.14 mg ml^{-1} for Cp-TMV was used for inoculation.

Since TMV-U multiplies only in cowpea cells which become infected during mechanical inoculation without further spread of the virus (Sulzinski and Zaitlin, 1982), we used an appropriate concentration of 1.7 mg ml^{-1} as inoculum.

Virus inoculations. Primary cowpea leaves were detached from the plants and inoculated with one of the virus strains on the lower surface with carborundum 500 mesh as an abrasive. Within 5 min the leaves were washed with soap and tap water to remove the inoculum from the surface.

Naked mesophyll. The epidermis of both the upper and lower side of a leaf was removed using fine forceps. Immediately thereafter the remaining tissue was inoculated with TNV-A on the upper side using a glass spatula or a fine brush without carborundum. The leaves were incubated in closed petri dishes on filter paper and kept as dry as possible at 22 ± 3°C under continuous fluorescent light of 5.3 W m^{-2}.

Preparation and incubation of epidermal strips and epidermis-peeled leaves. The leaves were kept at room temperature on dry filter paper directly after washing and blotting. To study the multiplication of the virus in the inoculated epidermal cells the lower epidermis was removed using fine forceps. All the leaves were stripped within 30 min after inoculation. Epidermal strips from each leaf were divided into two groups before incubation: A) epidermal strips as they were pulled from the leaf, with other plant cells adhering, and B) epidermal strips without clusters of mesophyll cells or vein pieces as checked with a light microscope. Epidermal strips were incubated for 2 or 4 days on water agar as described earlier (Wieringa-Brants, 1981). The epidermis-peeled leaves from which the epidermis was used for virus multiplication studies were incubated at 22 ± 3°C and 5.3 W m^{-2}.

Infectivity assay of epidermal strips and epidermis-peeled leaves. Homogenates from the epidermal strips incubated on agar (ca 0.5 g per test) were tested as described earlier (Wieringa-Brants, 1981) by rubbing them on six half 'Xanthi nc' leaves. The opposite half-leaves served as control and were rubbed with a homogenate of 0.75 g epidermal strips frozen at zero time after stripping.

In epidermis-peeled cowpea leaves treated with TNV-A local lesions were counted 4 days after inoculation.

In cowpea no local lesions were produced by TMV-U, whereas lesions with the cowpea-Cp-TMV combination were very tiny. Therefore the cowpea leaves treated with TMV or Cp-TMV were homogenized in a mortar and pestle and these homogenates were tested on six half 'Xanthi nc' leaves each to detect virus infectivity.

Results

Infection of cowpea mesophyll tissue deprived of epidermal cells before inoculation. After TNV-A inoculation of cowpea mesophyll cells lesions developed within 40-72 h (Figs. 1 and 2). Hardly any difference in colour was noted when they were compared with lesions in inoculated leaves with one or both epidermal layers present. Virus seemed very well localized and the lesion diameter varied from 0.8 to 3 mm at 7 days after inoculation. In general these lesions were much smaller than those produced in normal leaves.

However, when the stripped area was cut out immediately after inoculation and incubated under the same conditions as the leaves, some local lesions developed within 3 days after inoculation. The lesions were homogenized and tested for virus activity.

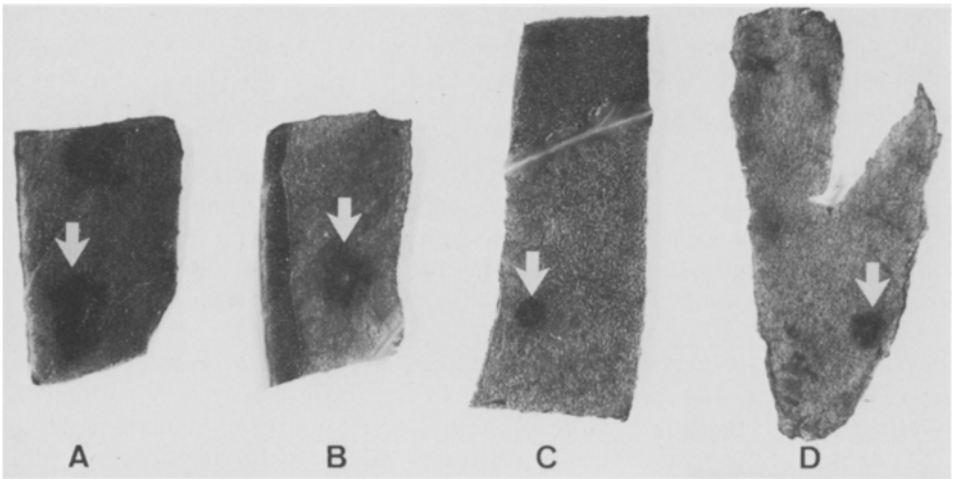

Fig. 1. Local lesions (arrow) of TNV-A in cowpea; upper leaf side 4 days after inoculation on the upper surface is shown. A) normal leaf inoculated on the upper side; B) leaf stripped at the upper side and this side inoculated immediately thereafter; C) leaf partially stripped at both sides and upper side inoculated immediately thereafter; D) leaf totally stripped at both sides and upper side inoculated immediately thereafter.

The infectivity was as of normal lesions and the homogenates induced normal lesions on 'Xanthi nc' and cowpea leaves.

When the mesophyll tissue deprived of epidermal cells was immersed in a virus suspension for 5 min, no infection occurred. Although the virus was not able to infect in this way, virus infection did occur when mesophyll cells were rubbed with a brush or a glass spatula.

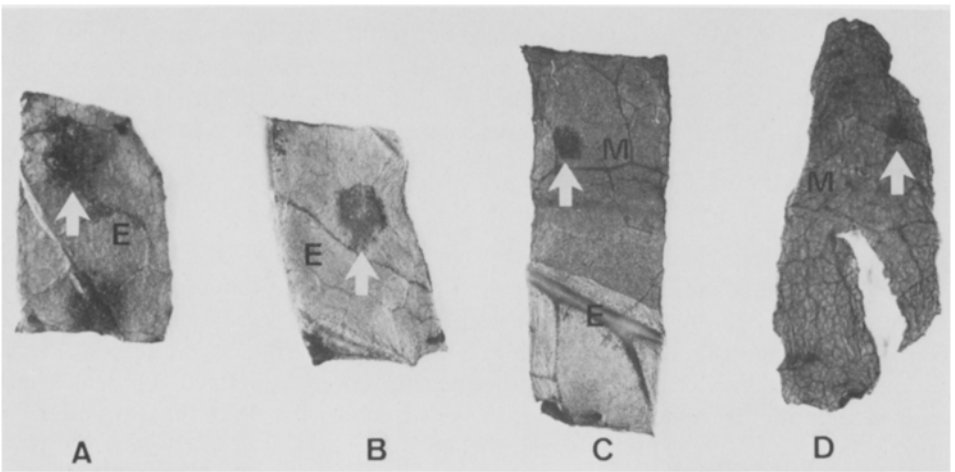

Fig. 2. Local lesions of TNV-A in cowpea; lower leaf sides are shown. The same leaf pieces as in Fig. 1. E) epidermis; M) mesophyll.

Table 1. Infectivity of homogenates of the lower cowpea leaf epidermis, stripped immediately after inoculation with TNV-A, Cp-TMV or TMV and tested on 'Xanthi nc' tobacco leaves.

Exp. no.	Incubation period of epidermal strips (days)	TNV-A group		Cp-TMV group		TMV group
		A[1]	B	A	B	B
	0[2]	9[3]	9	13	13	—[4]
I	2	15	1	0	0	0
	4	1	0	22	0	0
II	2	2	0	—	—	0
	4	54	0	—	0	—

[1] A, infected epidermal strips as they were pulled from the leaf with other plant cells adhering; B, infected epidermal strips without other plant cells.
[2] 0, control for groups A and B, i.e. epidermal strips frozen directly after stripping.
[3] Total number of lesions on six half 'Xanthi nc' leaves.
[4] —, not determined.

It can be concluded that TNV-A can provoke local lesion formation in cowpea mesophyll cells without the presence of epidermal cells.

Virus multiplication in epidermal strips. Homogenates of cowpea epidermal strips , inoculated with either virus strain, were tested on 'Xanthi nc' leaves. Results of two experiments are shown in Table 1. In none of the cowpea-virus combinations, in which epidermal strips without mesophyll cells (group B) were used, could any virus activity be detected, not even after 4 days of incubation. When green material was left with the epidermis (group A), in one experiment (Table 1, I) some virus activity could be seen in the cowpea-TNV-A combination after an incubation of 2 days but not after 4 days. In another experiment (Table 1, II) the opposite was found. In the case of Cp-TMV, virus activity was shown only after 4 days of incubation.

It is likely that in the experiments of group A the adhering mesophyll cells and vein pieces were responsible for the virus activity. Local lesion-like symptoms were never found on the stripped epidermal tissues although their homogenates were active on 'Xanthi nc' leaves (Table 1, A).

It can be concluded that not one of the three viruses tested penetrated into the epidermis cells or that the multiplication was to such a low level that it was impossible to detect virus activity with a bioassay.

Virus detection in epidermis-peeled leaves. Lesions were formed in practically all epidermis-peeled leaves which were stripped within 30 min after inoculation with TNV-A. In the cowpea-Cp-TMV combination all epidermis-stripped leaves showed lesions 7 days after peeling. The homogenates from epidermis-peeled leaves inoculated with TMV-U showed a low virus activity when tested on 'Xanthi nc' leaves. These results indicate that in each of the three cowpea-virus combinations infective virus material was present in the mesophyll at the moment of stripping, that is within

10-30 min after inoculation. So only a very short time is needed for these viruses to penetrate from the surface of an intact cowpea leaf into the mesophyll layer. Therefore it is probable that mesophyll cells can be infected during mechanical inoculation.

Discussion

It was suggested that an interaction between epidermis and mesophyll cells was necessary to induce local lesions in virusinfected hypersensitive leaves (Kasamo and Shimomura, 1977; Coutts, 1980; Wieringa-Brants, 1981). However, inoculation of cowpea mesophyll tissue without upper and lower epidermis with purified TNV-A can lead to lesion formation (Figs. 1, C, D; 2, C, D). So the epidermis does not seem to be involved in the process of normal lesion formation in cowpea. It is well known that contact of leaves with virus particles only results in infection when the leaves are injured properly. Few data are available on the changes occurring after wounding (Duafala and Nemanic, 1974); Favali et al., 1977), and the rôle of wounding the leaf in the virus infection process is still obscure.

In a normal ioculation procedure the virus has to pass the epidermis in order to infect the mesophyll cells. The time required for TNV to pass through the leaf epidermis of *Vigna unguiculata, Chenopodium quinoa* and *Phaseolus vulgaris* cv. Dubbele Witte zonder draad, subjected to waterstress, was only 10 min (Salinas Calvete and Wieringa-Brants, 1981). Similar results were found for Cp-TMV and TMV-U in cowpea (this study).

The question arises what really happens when a leaf is inoculated with a virus. There are three possibilities of viral entrance:

1) all the primary sites of viral entrance are epidermal cells,
2) all the primary sites of viral entrance are mesophyll cells, and
3) both the epidermal and mesophyll cells can act as primary sites.

In view of our results the first possibility seems the least likely for cowpea. The second and third possibility will be discussed together for some plant-virus combinations.

When tobacco is inoculated with TMV-U it seems that both mesophyll cells (Sulzinski and Zaitlin, 1982) and epidermal cells (Fry and Matthews, 1963; Takahashi, 1973) can act as primary sites of viral ingress into the leaf. Some mesophyll protoplasts prepared immediately upon mechanical inoculation appeared already infected. It has also been demonstrated that epidermal cells are infected during the process of mechanical inoculation with TMV-U (Fry and Matthews, 1963). Takahashi (1973) showed that TMV-U multiplied in tobacco epidermal strips. In our experiments with cowpea leaves the infectious virus material demonstrated in the homogenates of epidermal strips was of mesophyll origin. Coutts (1980) could not find any virus activity in epidermal strips after 50 h incubation, except in those taken just prior to lesion formation. Presumably, the epidermal cells actually contained progeny virus from the mesophyll or, more likely, were still associated with infected mesophyll cells not removed by stripping.

Although Ehara and Misawa (1967) suggested that the uncoating of cucumber mosaic virus takes place in cowpea epidermal cells this does not seem to be a general phenomenon. Lesions produced in epidermis-peeled cowpea leaves indicate that infectious Cp-TMV material was present in the mesophyll at the moment of stripping im-

mediately after mechanical inoculation. These results are in agreement with our previous results with the cowpea-TNV-A combination (Salinas Calvete and Wieringa-Brants, 1981).

It is highly unlikely that Cp-TMV or TNV-A penetrates into the epidermal cells, is uncoated and passed through to the mesophyll cells within 10 min. Direct inoculation of naked cowpea mesophyll with TNV-A using a fine brush without carborundum resulted in as many lesions as produced in control leaves (Salinas Calvete and Wieringa-Brants, 1981). Therefore, it seems likely that in cowpea the epidermis is not necessary as a primary site of entrance for Cp-TMV and TNV. It was not possible to demonstrate that in cowpea all the primary sites of viral entrance are mesophyll cells, but it is clear that they can act as such.

In the cowpea-TMV combination, homogenates of leaves stripped immediately after TMV-U inoculation and incubated for 7 days were active. This indicates that infectious TMV-U material must have penetrated into the mesophyll before removal of the epidermis. These results are in agreement with those of Sulzinski and Zaitlin (1982) who found that cowpea mesophyll cells become infected immediately upon TMV inoculation. They could not detect viral inclusion bodies in cowpea epidermis cells 7 to 10 days after inoculation. We could not find virus activity in epidermal strips either 2 or 4 days after incubation (Table 1, B).

Sulzinski and Zaitlin (1982) noted that the number of infected cowpea mesophyll protoplasts did not differ when they were prepared immediately (zero time) or 11 days after mechanical inoculation of the leaves with TMV-U. It seems likely that in the cowpea-TMV combination the primary sites of viral entrance are mesophyll cells only.

It may be concluded that in cowpea the epidermis does not play a rôle in virus lesion formation when TNV or Cp-TMV is used.

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Samenvatting

Infectie en necrose van cowpea-mesofylcellen door tabaksnecrosevirus en twee stammen van tabaksmozaïekvirus

De mogelijkheid werd onderzocht om cowpea-mesofylcellen zonder de aanwezigheid van epidermiscellen met TNV te infecteren. Kleine lokale necrotische lesies werden 40-72 uur na inoculatie zichtbaar waaruit blijkt, dat bij cowpea de epidermis niet noodzakelijk is voor de vorming van TNV-lesies. Geïsoleerd epidermisweefsel vertoonde nooit lokale lesies. Homogenaten van met TNV geïnoculeerde en daarna geïsoleerde cowpea-epidermisstukjes werden getoetst op virusactiviteit. Als de stukjes volledig vrij waren van mesofylcellen of nerfweefsel, dan vond daarin geen virusvermeerdering plaats tijdens een incubatie van 2 of 4 dagen op agar. Als na het strippen nog enkele mesofylcellen of nerfstukjes aanwezig waren, kon wel enige virusactiviteit in de homogenaten worden aangetoond.

In cowpeabladeren die geïnoculeerd werden met de cowpea-stam van TMV of de

normale stam van TMV had infectieus virusmateriaal al binnen 10 min na inoculatie het mesofyl bereikt. Blijkbaar is in cowpeabladeren de epidermis niet noodzakelijk voor de binnenkomst van virus of voor de necroseproduktie na virusinoculatie.

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