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Effect of eosin Y on the formation of local lesions and on the accumulation of callose in 'Samsun NN' tobacco and French bean leaves inoculated with tobacco mosaic virus

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Abstract

When leaf-halves of 'Samsun NN' tobacco or bean plants were floated on a solution of 10–15 μM eosin Y after inoculation with tobacco mosaic virus (TMV) and kept at 20 °C, local lesion formation was markedly diminished. There was also a decrease in the size of the lesions.

Depending on the temperature very strong fluorescence due to callose formation was seen around the lesions in eosin Y-treated leaf-halves of 'Samsun NN' tobacco and bean plants. It lasted from 3–5 days after inoculation, whereas fluorescence around lesions in the water-treated control leaves disappeared within 2–3 days after inoculation.

When leaf discs of 'Samsun' tobacco, a systemic host for TMV, were floated on a solution of eosin Y after inoculation with TMV and kept at 20 °C for 5 days, TMV multiplication was not prevented. Callose deposition could be detected, neither in eosin Y-treated nor in water-treated control leaves.

The relation between the inhibition of local lesion formation and the accumulation of callose in eosin Y-treated leaves is discussed.

Introduction

Association of callose with local lesion formation has been reported in various virus-host combinations (Wu et al., 1969; Wu and Dimitman, 1970; Hiruki and Tu, 1972; Allison and Shalla, 1974; Shimomura and Dijkstra, 1975). The accumulation of callose on sieve plates has long been known to be induced by eosin (Schumacher, 1930). Currier (1957) and Currier and Shih (1968) demonstrated that eosin also increased the amount of wound callose in epidermal segments of *Allium* and in detached *Elodea* leaves, especially in living cells near a wound.

We now examined whether eosin Y induces the accumulation of callose and affects the formation of local lesions in leaves of 'Samsun NN' tobacco or bean plants inoculated with tobacco mosaic virus (TMV), and whether there was a relation between the two phenomena.

Materials and methods

Plants and virus. Nicotiana tabacum 'Samsun NN' and *Phaseolus vulgaris* 'Otebo' were used as local lesion hosts, and *N. tabacum* 'Samsun' as systemic host of TMV. The tobacco plants were grown under ordinary greenhouse conditions for 2.5 months and the bean plants for 10 days after sowing.

A purified preparation (10 μg/ml) of the common strain of TMV was used, and inoculations were made on the upper leaf surface previously dusted with carborundum, followed by a water rinse.

Application of eosin Y. Ten half-leaves inoculated with TMV were floated on a solution of eosin Y (5–15 μM, Wako Pure Chemical Industries, Ltd. Japan) in petridishes and kept in a controlled environment chamber under continuous illumination from fluorescent lamps. The opposite inoculated half-leaves were placed on distilled water and served as controls. After incubation for 3 days, the local lesions were counted. The lesions were measured 3–5 days after inoculation. For the detection of callose, small pieces were cut from the half-leaves at different times after inoculation.

To test the effect of eosin Y on TMV multiplication 14 leaf discs, 16 mm in diameter, were punched from 'Samsun' tobacco leaves inoculated with TMV, floated on an eosin Y solution of 5–15 μM or on distilled water in petridishes and kept at 20 °C under continuous illumination. After 5 days, the discs were ground with 20 ml of 0.1 M phosphate buffer, pH 7.0, and infectivity of the sap obtained was assayed on 'Samsun NN' leaves.

Detection of callose. Leaf pieces were decolorized with 95% ethyl alcohol after 2 min of immersion in boiling water, then stained in 0.1% aniline blue in 1/15 M K_3PO_4, pH 12.0. Observations were made with a Wild microscope or a Zeiss microscope equipped with a high pressure mercury lamp (HBO 200 W). All photographs presented here were taken with a camera mounted on the Zeiss microscope. The preparations were illuminated with reflected light passing through exciter filter BG 3/4 (transmission range approximately 270 to 480 nm with a high, flat peak from approximately 320 to 400 nm) and barrier filters 44, 47 and –65.

Table 1. Effect of eosin Y on local lesions in leaf-halves of 'Samsun NN' tobacco inoculated with TMV[1].

Incubation temperature (°C)	Concentration of eosin Y (μM)	Local lesions per half-leaf				Effect of eosin Y on leaves
		control		treated		
20	15	168[2]	(2.2)[3]	16[2]	(0.3)[3]	Browning of parts of veinlets
	10	184	(2.2)	39	(0.4)	None
	5	164		177		None
25	15	206	(2.3)	188	(2.3)	Browning of parts of veinlets
	10	218		200		None

[1] Half-leaves were incubated with eosin Y (treated) or with water (control) and the local lesions were counted on the third day after inoculation.
[2] Average number of local lesions on 10 half-leaves.
[3] Average diameter (mm) of at least 150 local lesions on 10 half-leaves at 20 °C or 25 °C, measured on the fifth and fourth day after inoculation respectively.

Tabel 1. Effect van eosine Y op de lokale lesies op bladhelften van 'Samsun NN'-tabak, geïnoculeerd met TMV.[1]

Results

Effect of eosin Y on local lesion formation. The results of experiments with 'Samsun NN' tobacco plants are shown in Table 1. Only the highest concentration of eosin Y used (15 μM) caused some leaf injury. At 20 °C, eosin Y at 10 and 15 μM decreased both lesion size and number (Fig. 1). A concentration of 5 μM eosin Y did not have any effect at this temperature. At 25 °C, 10 and 15 μM eosin Y did not decrease lesion size or number (Fig. 2).

When the leaves of very young plants (within 2 months after sowing and highly susceptible to TMV infection) were used, treatment with 10 or 15 μM eosin Y was also not so effective in decreasing lesion number and size, even at 20 °C.

The results with bean plants are shown in Table 2. Eosin Y treatment did not cause any injury at the concentrations tested. Both at 20 °C and 25 °C, eosin Y at 10 or 15 μM restricted lesion growth (Fig. 3 and 4), even at 30 °C.

Effect of eosin Y on callose accumulation. When healthy half-leaves of 'Samsun NN' plants were floated on distilled water, wound callose appeared near the cut edge (Fig. 5). This accumulation was especially pronounced in the wound region of half-leaves floated on eosin Y at concentrations of 10 and 15 μM (Fig. 6). Besides browning of veinlets in leaf-halves treated with 15 μM eosin Y, intensive callose fluorescence was visible in parts of the veinlets of these leaves. Figures 7 and 8 show such callose accumulation. Callose accumulation in the wound regions and in the veinlets does not seem to be affected by the incubation temperature.

When TMV-inoculated 'Samsun NN' leaf-halves were floated on a solution of

Fig. 1. Local lesions on half-leaves of 'Samsun NN' tobacco inoculated with TMV and incubated at 20 °C for 5 days. The left half was treated with 15 μM eosin Y and the right half with water.
Fig. 2. Same as in Fig. 1, but at 25 °C for 4 days after inoculation. Local lesions somewhat chlorotic instead of necrotic.

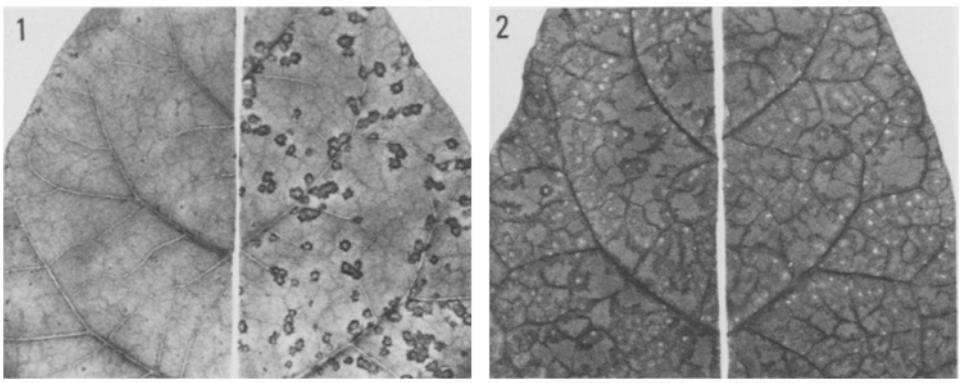

Fig. 1. Lokale lesies of bladhelften van 'Samsun NN'-tabak geïnoculeerd met TMV en gedurende 5 dagen na inoculatie geïncubeerd bij 20 °C. De linker bladhelft was behandeld met 15 μM eosine Y en de rechterhelft met water.
Fig. 2. Als bij Fig. 1, echter bij 25 °C gedurende 4 dagen na inoculatie. Lokale lesies enigszins chlorotisch in plaats van necrotisch.

Fig. 3. Local lesions on bean with TMV, at 20 °C for 5 days after inoculation. The left half was treated with 15 μM eosin Y and the right half with water.
Fig. 4. Local lesions on bean with TMV, at 25 °C for 5 days after inoculation. The left half was treated with 10 μM eosin Y and the right half with water.

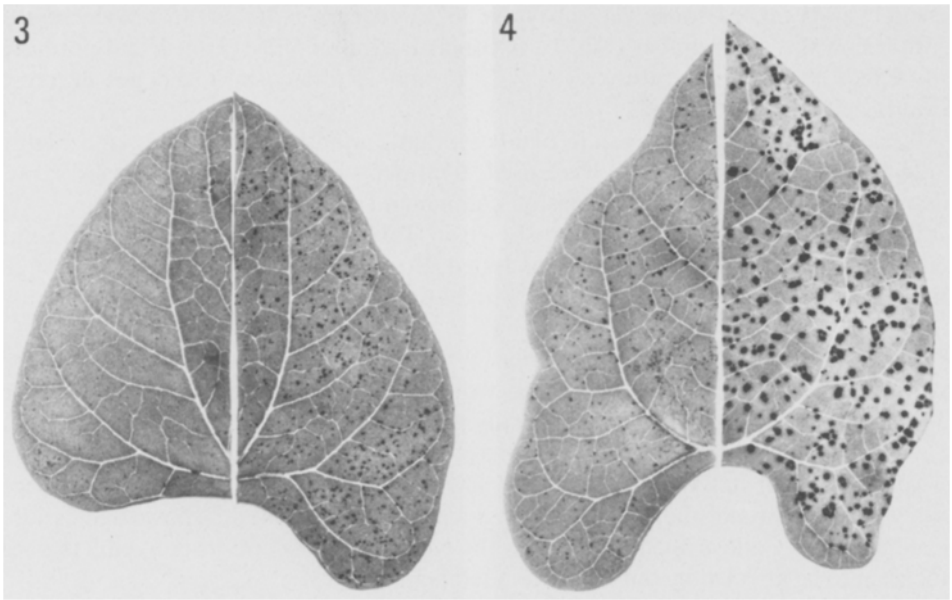

Fig 3. Lokale lesies op boon met TMV, bij 20 °C gedurende 5 dagen na inoculatie. De linkerhelft was behandeld met 15 μM eosine Y en de rechterhelft met water.
Fig. 4. Lokale lesies op boon met TMV, bij 25 °C gedurende 5 dagen na inoculatie. De linkerhelft was behandeld met 10 μM eosine Y en de rechterhelft met water.

Table 2. Effect of eosin Y on local lesions in leaf-halves of bean inoculated with TMV[1].

Incubation temperature (°C)	Concentration of eosin Y (μM)	Local lesions per half-leaf				Effect of eosin Y on leaves
		control		treated		
20	15	181[2]	(0.5)	45[2]	(0.1)[3]	None
	10	139	(0.6)	68	(0.2)	None
25	15	171	(0.9)	62	(0.2)	None
	10	177	(0.9)	102	(0.2)	None
30	15	253	(1.8)	170	(0.7)	None

[1,2] See legends to Table 1.
[3] Average diameter (mm) of 200 local lesions on 10 half-leaves at 20 °C, 25 °C, or 30 °C, measured on the fifth and third day after inoculation respectively.

Table 2. Effect van eosine Y op lokale lesies op bladhelften van boon, geïnoculeerd met TMV[1].

Fig. 5–6. Fluorescence in intact cells near the cut edge of 'Samsun NN' tobacco half-leaves. Half-leaves were either floated on distilled water (Fig. 5) or on a solution of 10 μM eosin Y (Fig. 6) and kept at 20 °C for 2 days. Bar represents 100 μm, also in the following figures.
Fig. 7–8. Fluorescence with varying intensities in the veinlets of 'Samsun NN' tobacco leaves on a solution of 15 μM eosin Y, for 2 days at 20 °C.
Fig. 9–10. Fluorescence around the lesions formed on TMV-inoculated 'Samsun NN' tobacco leaves on a solution of 15 μM eosin Y (Fig. 9) or on distilled water (Fig. 10), for 4 days at 20 °C.

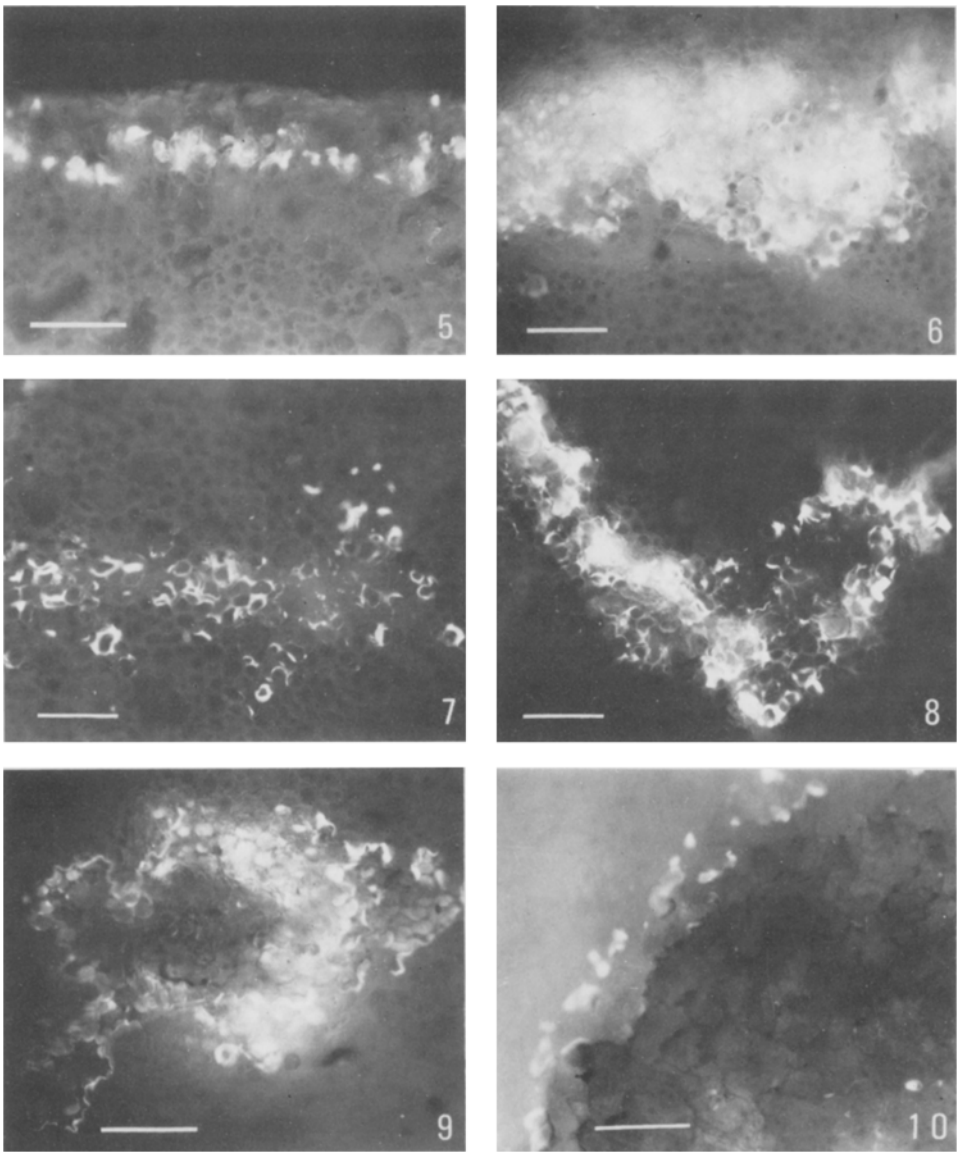

Fig. 5–6. Fluorescentie in intacte cellen in de buurt van het snijvlak van halve bladeren van 'Samsun NN'-tabak. De bladhelften waren te drijven gelegd op water (Fig. 5), of een oplossing van 10 μM eosine Y (Fig. 6) en gedurende 2 dagen bij 20 °C gehouden. Het vergrotingsstreepje geeft in alle figuren 100 μm weer.
Fig. 7–8. Fluorescentie, variërend in intensiteit, in de zijnerven van bladeren van 'Samsun NN'-tabak die gedurende 2 dagen bij 20 °C hadden gedreven op een oplossing van 15 μM eosine Y.
Fig. 9–10. Fluorescentie rondom lokale lesies op bladeren van 'Samsun NN'-tabak die gedurende 4 dagen bij 20 °C gedreven hadden op een oplossing van 15 μM eosine Y (Fig. 9), of op water (Fig. 10).

Fig. 11–12. Fluorescence in the sieve plates of veins (Fig. 11) and in parts of a veinlet (Fig. 12) of bean leaves on a solution of 10 μM eosin Y, for 2 days at 20 °C.
Fig. 13–14. Fluorescence around local lesions formed on bean leaves inoculated with TMV and floated on a solution of 10 μM eosin Y (Fig. 13) or on distilled water (Fig. 14), for 4 days at 25 °C.

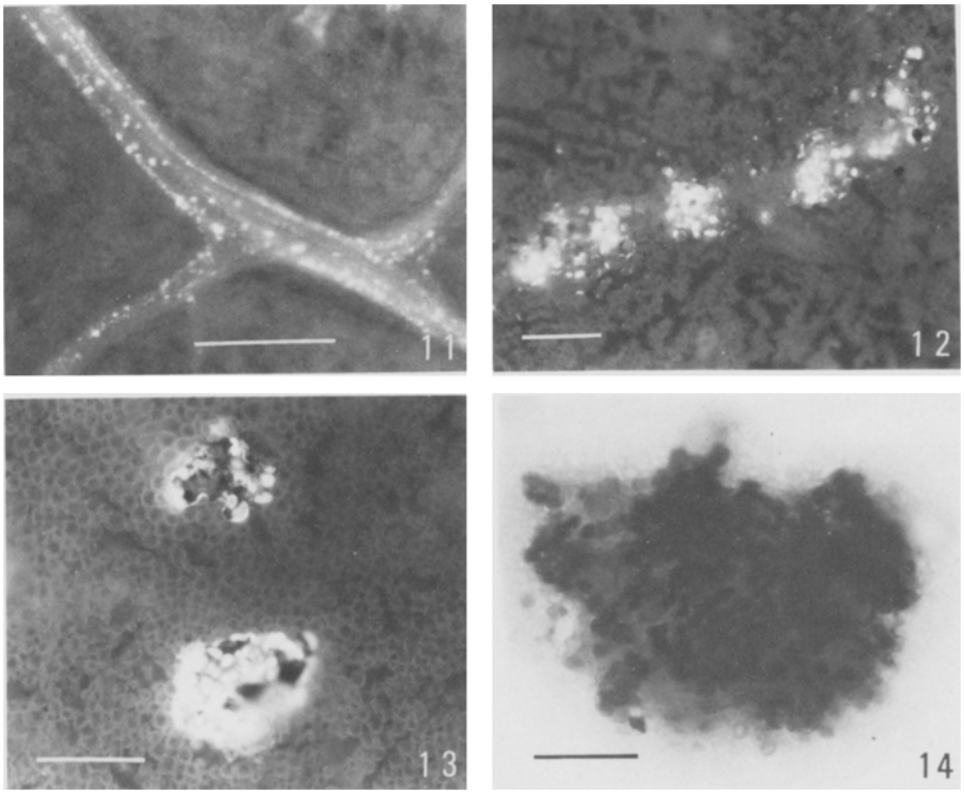

Fig. 11–12. Fluorescentie op de zeefplaten van nerven (Fig. 11) en in delen van een zijnerf (Fig. 12) van bonebladeren die gedurende 2 dagen bij 20 °C gedreven hadden op een oplossing van 10 μM eosine Y. Fig. 13–14. Fluorescentie rondom lokale lesies op bonebladeren die geïnoculeerd waren met TMV en gedurende 4 dagen bij 25 °C gedreven hadden op een oplossing van 10 μM eosine Y (Fig. 13), of op water (Fig. 14).

10 or 15 μM eosin Y at 20 °C, reduction in number and size of lesions was observed, as mentioned above. In these leaf-halves, there was a heavy deposition of callose around the tiny lesions (Fig. 9). The intensive fluorescence did not disappear, even 3–5 days after inoculation, although in half-leaves floated on distilled water it always disappeared 2–3 days after inoculation (Fig. 10). When TMV-inoculated half-leaves were floated on eosin Y at 25 °C, however, callose fluorescence around the lesions was the same as in the leaves on distilled water.

In half-leaves of healthy bean, there was not much accumulation of wound callose, and eosin Y treatment did not stimulate its formation. Treatment with eosin Y, however, induced accumulation of callose on the sieve plates of leaf veins (Fig. 11). Prominent callose fluorescence in parts of the veinlets was also observed in the treated leaves (Fig. 12).

Table 3. Effect of eosin Y on the multiplication of TMV in leaf discs of 'Samsun' tobacco[1].

Concentration of eosin Y (μM)	Number of local lesions on assay leaves[2]	Effect of eosin Y on leaves
15	162	Browning of parts of veinlets
10	170	Browning of parts of veinlets
5	199	None
0	195	None

[1] Fourteen leaf discs were treated with eosin Y and infectivity was assayed on the third day after inoculation.
[2] Average number of local lesions on 8 quarter leaves of 'Samsun NN' tobacco.

Tabel 3. Effect van eosine Y op de vermeerdering van TMV in bladschijfjes van 'Samsun'-tabak[1]

A marked deposition of callose in TMV-inoculated bean leaves was visible around the lesions which ceased to enlarge after treatment with eosin Y at 20–30 °C (Fig. 13). The amount of fluorescence was comparable to that in 'Samsun NN' at 20 °C. In the control leaf-halves of bean on distilled water, callose fluorescence around the lesions disappeared within 2 days after inoculation (Fig. 14).

Effect of eosin Y on TMV multiplication. Table 3 shows the results of biological assay of TMV in leaf discs of 'Samsun' floated on a solution of eosin Y. Eosin Y, at various concentrations, did not seem to inhibit virus multiplication. In 'Samsun' leaves treated with 10 or 15 μM eosin Y, no callose deposition could be detected except in parts of the veinlets.

Discussion

Our results show that treatment with eosin Y decreases both size and number of local lesions in 'Samsun NN' tobacco and bean leaves inoculated with TMV. Thus, the effects of eosin Y may be related to prevention of virus spread from cell to cell after establishment of infection, as well as to inhibition of virus infection. On the other hand, our work has also shown that eosin Y does not inhibit TMV multiplication in 'Samsun' tobacco leaves. Thus, prevention of virus spread from cell to cell does not seem to result from inhibition of virus multiplication, but from some factor restricting virus movement, produced during the process of local lesion formation.

Fluorescence microscopy of 'Samsun NN' tobacco and bean leaves revealed that callose reaction of wound surfaces and vein areas was increased by the presence of eosin Y, as already reported by Currier (1957) and Currier and Shih (1968) in *Allium* and *Elodea* leaves. In both 'Samsun NN' tobacco and bean leaves inoculated with TMV and incubated at 20 °C, heavy callose deposition was observed around the tiny lesions, restricted following eosin Y treatment, and this fluorescence tended to persist even after the lesions had become completely necrotic. With 'Samsun NN' at 25 °C, however, eosin Y treatment had no effect on lesion formation, and callose deposition around the lesions was the same as in the control leaves. The behaviour of tobacco cultivars with the N gene from *N. glutinosa* is known to vary with temperature,

and TMV goes systemic at high temperatures (Samuel, 1931; Kassanis, 1952; Shimomura, 1971; Otsuki et al., 1972)' It is noteworthy that at temperatures at or above 25 °C, when 'Samsun NN' plants inoculated with TMV tend to develop systemic symptoms, little callose deposition was observed.

In our experiments with 'Samsun' tobacco systemically infected with TMV, callose fluorescence characteristic of TMV infection in 'local lesion type' plants was not detected in inoculated leaves (Shimomura and Dijkstra, 1975), nor did eosin Y treatment provoke such callose deposition in these leaves. In contrast to 'Samsun NN' tobacco, bean plants inoculated with TMV and kept at high temperature continued to produce necrotic local lesions only and the plants did not become systemically infected (Kassanis, 1952). Our observations on bean leaves infected with TMV show that eosin Y enhanced callose deposition and prevented lesion formation on bean leaves incubated either at 30 °C or at lower temperatures.

We may conclude that there is a correlation between the amount of callose deposition in the leaves and the ultimate size of local lesions formed in the inoculated leaves. However, the question as to whether the presence of the callose deposition may actually prevent the spread of virus to neighbour cells has still to be answered.

Lindner et al. (1959) reported that eosin B and Y inhibit the formation of starch lesions on cucumber cotyledons inoculated with TMV, but it is not clear whether in that virus-host combination a callose reaction was also enhanced by eosin treatment.

Samenvatting

Effect van eosine Y op de vorming van lokale lesies en de ophoping van callose in bladeren van 'Samsun NN'-tabak en van boon geïnoculeerd met tabaksmozaïekvirus

Het is bekend dat er een verband bestaat tussen afzetting van callose en de vorming van lokale lesies bij verschillende virus-waardplantcombinaties. Tevens staat vast dat eosine ophoping van callose op zeefplaten veroorzaakt. Onderzocht werd nu of eosine Y van invloed is op de vorming van lokale lesies en tevens ophoping van callose veroorzaakt in bladeren van 'Samsun NN'-tabak en boon, geïnoculeerd met tabaksmozaïekvirus (TMV).

Het bleek dat bladhelften van tabak die, na inoculatie met TMV, bij 20 °C gedreven hadden op een oplossing van 10 of 15 μM eosine Y, minder en kleinere lesies hadden dan de controlehelften die op water hadden gedreven (Tabel 1; Fig. 1). Bij 25 °C werden deze effecten niet waargenomen (Fig. 2). Een concentratie van 15 μM veroorzaakte wat beschadiging van de bladeren (verbruining van een deel van de zijnerven).

Bij bladhelften van de boon was geen beschadiging te zien bij een concentratie van 15 μM. Bij bonebladeren geïnoculeerd met TMV bleek behandeling met eosine Y zowel bij 20 °C als bij 25 °C en zelfs bij 30 °C minder en kleinere lesies tot gevolg te hebben (Tabel 2; Fig. 3 en 4).

Als gezonde bladhelften van 'Samsun NN'-planten te drijven waren gelegd op water verscheen er callose in de buurt van het wondvlak (Fig. 5). Deze ophoping van callose was sterker als de bladhelften hadden gedreven op eosine Y in concentraties van 10 of 15 μM (Fig. 6). In de zijnerven, die als gevolg van de behandeling met 15 μM bruin waren geworden, was eveneens een sterke fluorescentie als gevolg van de aanwezigheid van callose te zien (Fig. 7 en 8).

Als bladhelften van 'Samsun NN'-planten na inoculatie met TMV te drijven waren gelegd op oplossingen van 10 of 15 μM bij een temperatuur van 20 °C bleek er niet alleen een reductie in aantal en afmeting van de lesies opgetreden te zijn, maar ook sterke fluorescentie als gevolg van aanzienlijke callose-afzettingen rondom de in hun uitbreiding geremde lesies (Fig. 9). De sterke fluorescentie verdween zelfs niet 3–5 dagen na inoculatie, hoewel bij bladeren die op water hadden gedreven dit al na 2–3 dagen het geval was (Fig. 10). Hadden de bladhelften na inoculatie echter bij 25 °C op eosine Y gedreven dan was de fluorescentie rondom de lesies dezelfde als in de bladhelften die op water hadden gedreven.

Bij bladhelften van gezonde bonen was niet veel ophoping van wondcallose waarneembaar en evenmin stimuleerde eosine Y de vorming ervan. Wel bracht deze stof een ophoping van callose teweeg op de zeefplaten van bladnerven (Fig. 11). Eveneens was er in de met eosine Y behandelde bladhelften fluorescentie in delen van de zijnerven (Fig. 12).

Bij bonebladeren die waren geïnoculeerd met TMV was een sterke afzetting van callose te zien rondom de lesies die zich niet meer uitbreidden als gevolg van behandeling met eosine Y bij temperaturen tussen 20 °C en 30 °C (Fig. 13). In de controlehelften die op water hadden gedreven verdween de fluorescentie binnen 2 dagen na inoculatie (Fig. 14).

Hadden bladschijfjes van 'Samsun'-tabak na inoculatie met TMV gedreven op een oplossing van eosine Y dan bleek de virusvermeerdering in deze schijfjes niet geremd te zijn (Tabel 3).

We kunnen concluderen dat er een correlatie bestaat tussen de hoeveelheid afgezette callose in de bladeren en de uiteindelijke grootte van de lokale lesies. De vraag blijft echter nog wel bestaan of callose-afzetting inderdaad de verspreiding van virus naar naburige cellen verhindert.

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