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STRUCTURAL FEATURES OF FUNGAL β-D-GLUCANS FOR THE EFFICIENT INHIBITION OF THE INITIATION OF VIRUS INFECTION ON *NICOTIANA TABACUM*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco-mosaic virus; inhibitors of viral infection; β-D-glucans; *Phytophthora parasitica*, *P. megasperma*; *Fusarium oxysporum*; laminaran; β-(1 → 3)-linked D-glucopyranosyl residues with (1 → 6) linked branched saccharides.

Abstract—Glucans of fungal origin have been shown to inhibit the early stages of infection of *Nicotiana* by numerous viruses of different taxonomic groups. Several glucans were isolated from the cell walls of *Phytophthora parasitica*, *Phytophthora megasperma* f. sp. glycinea (*Pmg*) and *Fusarium oxysporum*, and their antiviral activity compared on tobacco leaves inoculated with tobacco mosaic virus. These polysaccharides consist of a mixture of (1 → 3)(1 → 6)-β-D-glucans with M_r varying from 1.1×10^3 to 2×10^6. Requirements for a prominent antiviral activity of the fungal polysaccharides are a β-(1 → 3)(1 → 6)-D-glucan structure with mono-, di-, tri- or tetra-glucosidic side branches attached to a linear main chain of β-(1 → 3)-linked-D-glucose residues. Very high activity is correlated with a high degree of branching at position 6 and with the size and glycosidic nature of the side chains. The molecular masses and the organized structure of fungal β-D-glucans are not essential for their antiviral activity. The structural motif for antiviral activity in *Nicotiana* is distinct from that required for elicitation of phytoalexins in soybean cotyledons.

INTRODUCTION

Inhibitors of plant viruses can be divided into two major categories [1, 2]; inhibitors of virus multiplication and inhibitors of virus infection. The former are substances that, when applied to leaves already infected, decrease the rate at which the virus replicates and spreads. The latter are substances that, when applied to leaves before or simultaneously with virus inoculation, prevent infection from occurring. Inhibitors of infection have been the most extensively studied antiviral substances. Some have been found in extracts of plants and appeared to be proteinaceous in nature [1, 2]. Others have been obtained from microorganisms [for reviews, see 1, 2]. Even though some of them have been suspected for a long time to be polysaccharides, there are only a few cases where such polysaccharides have been partially purified and characterized.

For example, it was reported that cell walls of *Phytophthora infestans* contain a polysaccharide that, when mixed with potato virus X, prevented infection from occurring in tobacco [3–5]. The characteristics of the polysaccharide from *P. infestans* resemble those of mycolaminaran, a water-soluble and branched β-(1 → 3)-D-glucan with an average degree of polymerization of approximately 36 glucose units [6]. Recently, soluble mycolaminaran from the cytoplasm of *P. megasperma* was shown to protect several *Nicotiana* species from infection when used at a concentration range similar (*ca* 100–1000 $\mu g\,ml^{-1}$) to that of the antiviral *P. infestans* glucan [7]. The same range of concentrations of lichenan, a linear β-(1 → 3,4)-D-glucan from *Cetraria islandica*, exhibited antiviral activity against numerous viruses of different taxonomic groups tested on several *Nicotiana* species [8]. Furthermore, we have also shown previously [9] that a glucan preparation from the cell wall of *P. megasperma* f.sp. *glycinea* (*Pmg*) which contained highly branched β-D-glucans, protected several Nicotianae from infection by several single-stranded RNA viruses of different taxonomic groups. The host plants successfully protected included *N. tabacum* (nine different cultivars), *N. sylvestris*, *N. glutinosa* and *N. clevelandii*. By recombining the data of these different reports, it appears that a number of parameters and features of the glucan-induced antiviral activity are common [3–5, 7–9]: (i) the antiviral activity was demonstrated by the inhibition of symptom development and virus accumulation in the various Nicotianae; (ii) the glucans were effective when added directly to the inoculum or applied at various times before or very shortly after mechanical virus inoculation, indic-

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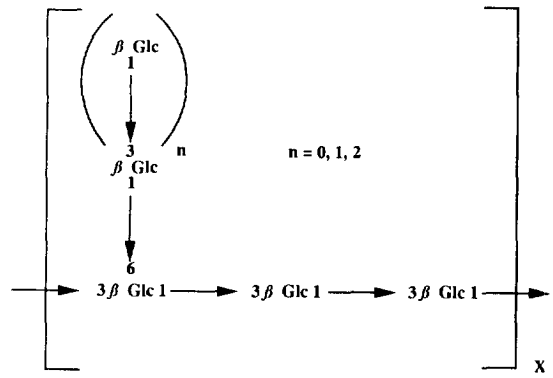
ating that early stages of infection were inhibited and (iii) the glucans did not act directly on the virus, but rendered the host less susceptible to infection. However, one major difference was the much lower concentration, by three orders of magnitude, of *Pmg* cell wall glucan required for these effects [9] when compared to lichenan, soluble mycolaminaran and the *P. infestans* glucan. While all these glucans possess a main chain of (1 → 3)-linked β-D glucopyranosyl residues with some branching, the *Pmg* cell wall glucan preparation, also known as a very active elicitor of phytoalexin synthesis in soybean cotyledons [10], was characterized as highly branched β-D-glucans with terminal-, 3-,6-, and 3,6-linked glucosyl residues. In the present study, the structural requirements for very high antiviral activity were investigated by assaying (1 → 3)-linked-β-D glucans with various degrees of (1 → 6) branching and characterized from *P. parasitica* and *Fusarium oxysporum*. This analysis also aimed to determine whether the same or different motifs were responsible for antiviral activity in Nicotianae and elicitor activity in soybean [11, 12].

RESULTS AND DISCUSSION

Structure of native glucans and derivatives assayed for antiviral activity

Neutral polysaccharides were isolated from the cell walls of *P. parasitica* grown on Huguenin and on Hall media and of *F. oxysporum* grown on synthetic liquid medium. Their structures were determined by methylation, Smith degradation, acetolysis, and ^{13}C NMR spectrometry. These polysaccharides have a main chain of (1 → 3)-linked-β-D-glucopyranosyl residues with (1 → 6) linked branched saccharide residues [13–15]. These glucans exhibited various lengths of side chains and various degrees of branching. The glucans produced by *P. parasitica* grown on Huguenin medium [13, 14] and on Hall medium [15] were designated **1** and **2**, respectively (Fig. 1). Those isolated from *F. oxysporum* correspond to structure **3** (Fig. 1). In order to obtain further informations on the relationships between antiviral activity and structural features, the conformations of these fungal polysaccharides were studied and different derivatives of these compounds were prepared.

For the determination of the ordered structures, the formation of complexes with Congo Red was investigated. The λ_{max} of Congo Red shifted to higher wavelength in the presence of glucans **1**, **2** and **3** (Fig. 2). Such complexes are known to be due to the presence of a single helical conformation [16, 17]. The λ_{max} of complexes of glucans **1** and **2** with Congo Red decreased on the addition of sodium hydroxide (Fig. 2), indicating the presence of a low degree of organized conformation whereas the glucans **3**–Congo Red complexes were stable in sodium hydroxide up to 0.1 M; these findings indicate the presence of a single stable helical conformation for glucans **3**. Like Kogan *et al.* [18], we observed that the stability of the ordered structure of glucans depends on the length of the side chains.



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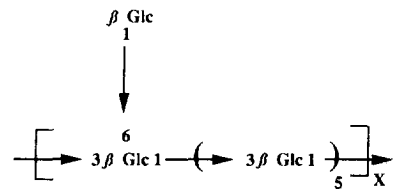

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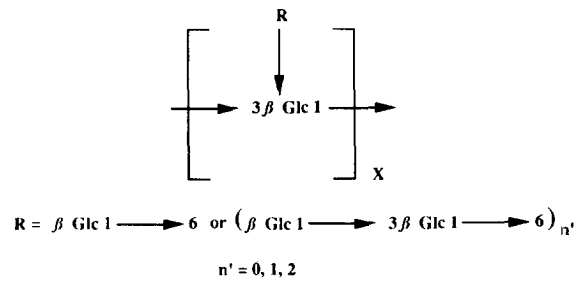

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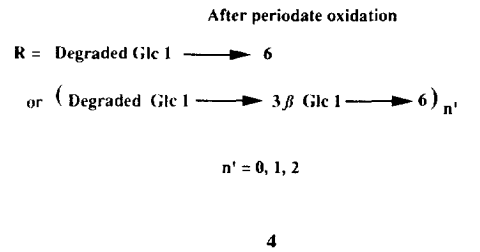

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Fig. 1. Structures of glucans assayed for antiviral activity. Structures **1** and **2** correspond to native glucans purified from *P. parasitica* grown in Huguenin medium and in Hall medium, respectively. Native glucans of structure **3** have been purified from *F. oxysporum*. Glucans of structure **4** were obtained by oxidation of glucans of structure **2**.

The glucans **2** were submitted to periodate oxidation followed by borohydride reduction. The oxidized glucosaccharides correspond to the structure **4** (Fig. 1). It was previously reported that glucans **1** and **2** produced by *P.*

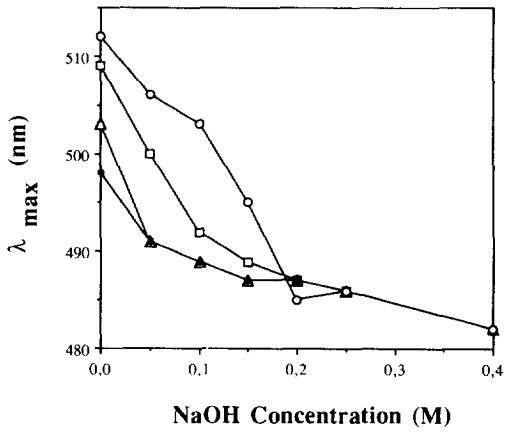

Fig. 2. Plot of maximum absorption of Congo Red complexed with fungal glucans vs the concentration of sodium hydroxide. Complexation was carried out in the presence of: native glucans **1** (—□—) and **2** (—△—) from *P. parasitica*; native glucans **3** from *F. oxysporum* (—○—). Control without polysaccharide (—●—).

parasitica contain three molecular weight fractions A, B, and C [15]. These fractions were prepared by gel permeation chromatography on a Sepharose CL-4B column (Table 1) and several of them were tested separately for their antiviral activity (Table 2). The results were compared with those obtained using a *Pmg* glucan preparation which contained a complex mixture of highly branched glucan fragments ranging in size from hexasaccharides up to polysaccharides with M_r exceeding 100 [10]. Commercial laminaran (Sigma) was also assayed as a reference glucan with a main chain of (1 → 3)-linked-β-D-glucopyranosyl residues with rare (1 → 6)-linked branched saccharides.

Estimation of antiviral activity

Some of the earlier reports on antiviral activities of glucans [7–9] have demonstrated that they induced a host-mediated, but as yet unknown, mechanism of inhibiting early stages of virus infection. In systemic hosts, this resulted in a delay or suppression of both symptoms and virus production, depending on the concentration and the nature of the glucan assayed. In such hosts, differential quantitative effects are rather difficult to measure and interpret. In contrast, local lesion hosts are very useful and sensitive test hosts since the number and location of the infection sites are easily visualized as local necrotic lesions and the extent of virus multiplication at each infection site is reflected by the size of the lesion. As only the number, not the size, of these lesions was changed by treatment with the glucans [7–9], it was concluded that the mechanism triggered by the glucans involved inhibition of establishment of infection and not inhibition of virus replication and spread from the first infected cells.

Table 1. Relative amounts and molecular weights of *P. parasitica* glucans. Native glucans **1** and **2** were purified from *P. parasitica* grown in Huguenin medium and in Hall medium, respectively, and were subsequently separated by chromatography on Sepharose Cl-4B

Glucan fractions (relative amounts in %)		M_r
1A (31)	**2**A (6)	2000
1B (26)	**2**B (12)	30
1C (43)	**2**C (82)	7.5

Other parameters of the protection efficiency were the timing between application of the glucans and virus inoculation, and the relative concentrations of glucans and virus [7–9]. Glucans were protective if applied before, at the same time as or very shortly after virus inoculation [7–9]. Treatments with glucans were efficient by spray, injection or mechanical inoculation [9]. In the experiments described in Table 2, we used the optimized experimental system, in which the glucan to be assayed was co-inoculated with tobacco mosaic virus (TMV) on the local lesion host *N. tabacum* var. Samsun NN. This procedure enabled a very accurate determination of the concentrations of both the virus and the glucan applied to leaves.

The results in Table 2 are expressed in two different ways: the relative lesion number, which is direct reflection of the measurements, and the degree of protection, which is obtained by calculation (see legend to Table 2). It should be emphasized that high degrees of protection (corresponding to a low relative lesion number) are much more accurately determined than low degrees of protection (corresponding to high relative lesion number). This is a consequence of the variability in the number of lesions induced after a range of high degrees of protection, a difference of a few per cent (e.g. 95–98%) is easier to measure than a larger difference in a range of low degrees of protection (e.g. 0–20%).

Structure-activity relationships in antiviral glucans

Since a common feature of the antiviral glucans described so far is the presence of long chains of (1 → 3)-linked-β-D-glucopyranosyl residues, we assayed laminaran as a reference compound. The results (Table 2) merely confirm those of Zinnen *et al.* [7] and demonstrate a low but significant protecting activity. We found dose-response effects similar to those previously reported for the *Pmg* glucans, but requiring glucan concentration higher by 2–3 orders of magnitude. When laminaran was pretreated with purified tobacco 1,3-β-glucanases, the antiviral activity was completely lost (data not shown), showing the requirement for the main chain of (1 → 3)-linked-glucosyl residues in the β configuration.

A stronger inhibition of TMV-induced local lesions on *N. nicotiana* leaves was observed when virus and native glucans **1** and **2** from *P. parasitica* of **3** from *F. oxysporum* were mixed and inoculated together.

Table 2. Antiviral activities of glucans of various sources and structures

Source	Glucan*	Concentrations ($\mu g\,ml^{-1}$)	Relative lesion number† (% of control)	Protection‡ (%)
P. parasitica (Huguenin medium)	Native **1**	1	12.8 ± 0.8	87.2
		10	0.7 ± 0.04	99.3
	(**1A**)	1	7.9 ± 0.6	92.1
		10	2.3 ± 0.1	97.7
	(**1C**)	1	11.3 ± 0.9	88.7
		10	4.5 ± 0.3	95.5
P. parasitica (Hall medium)	Native **2**	1	5.4 ± 0.8	94.6
		10	0.1 ± 0.1	99.9
	(**2A**)	1	8.1 ± 1.3	91.9
		10	1.1 ± 0.1	98.9
	(**2B**)	1	2.0 ± 0.3	98.0
		10	0.2 ± 0.03	99.8
	(**2C**)	1	7.7 ± 0.6	92.7
		10	0.8 + 0.1	99.2
	4 = Oxidized **2**	1	26.4 ± 5.8	73.6
		10	19.6 ± 3.7	80.4
P. megasperma	*Pmg*	1	6.4 ± 0.7	93.6
		10	0 ± 0	100.0
F. oxysporum	(**3**)	1	100 ± 15.2	0
		10	77.9 ± 12.7	22.1
L. digita	Laminaran	20	64.8 ± 19.5	35.2
		500	12.5 ± 1.2	87.5
		1000	4.0 ± 0.3	96.0

*Glucans assayed were: native glucans **1** and **2** from *P. parasitica* (Fig. 1), native glucans **3** from *F. oxysporum* (Fig. 1) and glucans **4** obtained by oxidation of glucans **2** from *P. parasitica* (Fig. 1); frs (**1A**), (**1C**), (**2A**), (**2B**) and (**2C**) obtained as described in Table 1; *Pmg* = cell wall glucan preparation from *P. megasperma* obtained as described in ref. [10]; commercial laminaran.

†Relative lesion number corresponds to 100 (nt/nc) where nt and nc are lesion numbers on treated and control leaves, respectively. Lesion numbers are the mean of lesions counted on 12 discs (20 mm in diameter) punched on each leaf. For each test (treatment and control) two leaves from three plants were inoculated (TMV plus glucan or TMV alone). The relative error was calculated by using the *t* test of Student, at *P* 95%; in this case, it can be assumed that the distribution is normal.

‡Values of degree of protection in % were calculated from nt/nc as 100 (1 − nt/nc).

To examine the effect of the length of the side chains and of the degree of branching, the antiviral activities of polysaccharides **1**, **2** and **3** were compared. We observed different degrees of protection. Table 2 shows that 1 $\mu g\,ml^{-1}$ of native glucans **1** and **2** induced significant protection of about 87–95% and that 10 $\mu g\,ml^{-1}$ completely prevented infection; at the same dose, glucans **3** were inactive at 1 $\mu g\,ml^{-1}$ and at 10 $\mu g\,ml^{-1}$ the protection was about 20%. These results, compared to those obtained with laminaran, showed a correlation between the length of β-linked branches and the antiviral activity since glucans **3** contain side chains composed of a single D-glucosyl group (Fig. 1) and glucans **1** and **2** contain di-, tri- or tetra-saccharides branches (Fig. 1).

In addition, the protection conferred by the highly branched glucans **2**, which has about one branch at each main-chain glucosyl unit (Fig. 1) was higher than that observed with less branched glucans **1**, which possess one branch for three main-chain glucosyl units. A much lower protection was observed with glucans **3** with a low degree of branching of one out of five D-glucose residues. The minimum protection was observed for laminaran with the lowest degree of branching of one out of about 30 D-glucose residues. Thus, the degree of branching of glucans also appears important for their antiviral activity. This is supported by the results obtained with the polysaccharide preparation from *P. megasperma* (*Pmg*) which is a mixture of highly branched β-D-glucans [10].

The low activity of glucan **3**, however, shows that ordered structures of branched (1 → 3)-β-D-glucans are not by themselves sufficient for the effective protection of *N. tabacum* var. Samsun NN against TMV.

To further examine the relationship between the antiviral activity and the side chain structure, we examined

the protection effect of oxidized glucans **2** (structure **4**, Fig. 1) against viral infection induced by TMV in tobacco leaves. In the side chains of native glucans **2** the non-reducing terminal glucosyl residues and the glucosyl residues monosubstituted at position 6 (Fig. 1) are periodate sensitive. Thus periodate oxidation of native glucans **2** led to a decrease in the number of non-reducing terminal residues which were degraded from cleavage of both the C-2–C-3 and C-3–C-4 bonds in the D-glucosyl groups. At the same dose (10 $\mu g\,ml^{-1}$) the protection of oxidized glucans **2** (80%) was weaker than that of the corresponding native glucans **2** (100%). This decrease confirms the positive contribution of side chains of β-D-(1 → 3) glucans and the importance of terminal glycosyl residues in side chains for the protective effect.

The effect of the size of the most active glucans was also investigated. The antiviral activity of three molecular weight fractions A, B and C, present in native glucans from *P. parasitica*, listed in Table 1, was examined. All these compounds showed similar activities (Table 2). Thus, it appears that the molecular weight of glucans had no influence on the antiviral activity. These results are in good agreement with those obtained with the *Pmg* glucan fraction after molecular sieving on Ultrogel AcA 34 [9]: there was high activity in all fractions over the whole range of molecular masses.

In conclusion, our results demonstrated that several structural parameters could affect the antiviral activity in tobacco. Requirements for a prominent activity of fungal polysaccharides are a β-(1 → 3)(1 → 6)-D-glucan structure with mono-, di-, tri- or tetra-glucosidic side branches attached to a linear main chain of β-(1 → 3)-linked-D-glucose residues. Furthermore, very high activity is correlated with a high degree of branching at position 6 and with the size and the glycosidic nature of side chains. The molecular weights and the organized structure of fungal β-D-glucans are not essential for their antiviral activity in tobacco. It is noteworthy that native glucans **2**, which have an antiviral protection efficiency close to that of the *Pmg* glucan fraction (Table 2), are also the ones that show the closest structural features to *Pmg* glucan fragments [10, 19]. The latter have another biological activity, as elicitors of phytoalexin synthesis in soybean. A structural analysis very elegantly showed the structural requirements for elicitor activity, namely chains of (1 → 6)-linked-glucose residues with (1 → 3) branching [11, 12, 19]. The minimal structure required for elicitor activity was shown to be a heptaglucoside with a chain of five (1 → 6)-linked-glucose residues and two (1 → 3) branches of a single glucose residue linked to glucose residues 2 and 4 of the main chain. Several assays performed with this heptaglucan indicated no antiviral activity in tobacco (data not shown). This is not surprising, in view of the structural requirements described above for antiviral activity. It can be concluded, that even though the *Pmg* glucan preparation has a dual biological activity (elicitor of phytoalexins in soybean cotyledons and inhibitor of virus infection in Nicotianae), the structural features required for the two activities are distinct.

EXPERIMENTAL

Cultures. Isolate 26 of *P. parasitica* from the fungal culture collection of INRA Antibes was grown either on Huguenin liquid medium [20] for 12 days at 24° without shaking or on modified Hall liquid medium [21] containing (per litre) 2 g asparagine and 30 g glucose for 3 days at 24° and 125 rpm in a rotary shaker. The isolate of *F. oxysporum* from the fungal culture collection of INRA Montfavet was grown without shaking on synthetic liquid medium for 3 weeks at 24° under a 16 hr light day regime. The mycelium was separated from the medium by centrifugation.

Extraction and purification of glucans from mycelial cell walls. Glucans were isolated from mycelial walls. The *Pmg* glucan preparation was a generous gift of P. Albersheim and A. Darvill (Complex Carbohydrate Research Center, Athens, Georgia, U.S.A.) and was obtained as indicated in ref. [10]. The cell walls were prepared as previously reported [13]. They were extracted with water at 100°. The insoluble residue was suspended in 1 M NaOH for 2 hr at 60° and centrifuged [22]. The supernatant soln was neutralized with HOAc and then dialysed against distilled water overnight. The non-dialysable fraction was lyophilized, dissolved in 0.01 M NaP_i buffer, pH7, and fractionated by CC on D52 DEAE-cellulose, equilibrated with the same buffer. The column was first eluted with the same buffer, and then with a 0–1 M NaCl linear gradient in the same buffer. Each fr. was dialysed and purified by chromatography on a column of Sephadex G-25 eluted with water.

Determination of molecular mass. A soln of glucans (3 mg) in distilled H_2O (0.5 ml) was applied to a column (1.6 × 80 cm) of Sepharose CL-4B. The column was equilibrated and eluted with distilled H_2O at a flow rate of 10 $ml\,hr^{-1}$ and the eluent was collected in 4 ml frs. The carbohydrate content of each fr. was determined with anthrone reagent [23]. The column was calibrated with standard dextrans from Pharmacia (France).

Methylation analysis. Glucans were methylated twice by the Hakomori method [24] as previously described [13].

Periodate oxidation. Glucans were oxidized as described in the literature [13].

Acetolysis. Acetolysis of glucans was performed according to ref. [25] in a mixture of Ac_2O–HOAc–H_2SO_4 (10:10:1) as previously described [13].

Complex formation with Congo Red. The change of absorption maximum of Congo Red in the presence of glucans was measured as previously described [26]. Glucans in NaOH soln (1 $mg\,ml^{-1}$) and Congo Red in NaOH soln (38 μM) were mixed in equal vols and the λ_{max} values measured using a Beckman DU-68 spectrophotometer.

Plants. The host plants used in these experiments were *Nicotiana tabacum* var. Samsun NN. The plants were grown in a greenhouse under controlled conditions. The plants were placed in a growth chamber at 22° ± 1° (16 hr photoperiod, 2 × 10 Wm^{-2} total irradiance), sev-

eral days before and during the incubation period following inoculation with virus and treatment with glucans.

Virus. TMV strain U1 was purified from infected tobacco leaves as previously described [27].

Inoculation of leaves with virus. Virus infection was usually accomplished by inoculation of the two or three first fully expanded leaves by rubbing the upper surface with a glass pad and abrasive Celite in the presence of a suspension containing purified virus. A virus concentration of 0.1 $\mu g\,ml^{-1}$ was used for inoculation in order to induce about 200 local lesions per control leaf (not treated with glucan).

Treatment of the leaves with glucans. In all experiments, the glucans were mixed with virus and applied by mechanical inoculation. For each treatment (mock or glucan solns) two leaves from three different plants were treated.

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REFERENCES

1. Bawden, F. C. (1954) *Adv. Virus Res.* **2**, 31.
2. Hirai, T. (1977) in *Plant Disease: An Advance Treatise* (Horsfall, J. G. and Cowling, E. B., eds), Vol. 1, pp. 285–305. Academic Press, New York.
3. Hodgson, W. A., Munro, J., Singh, R. P. and Wood, F. A. (1969) *Phytopathology* **59**, 1334.
4. Singh, R. P., Wood, F. A. and Hodgson, W. A. (1970) *Phytopathology* **60**, 1566.
5. Wood, F. A., Singh, R. P. and Hodgson, W. A. (1971) *Phytopathology* **61**, 1006.
6. Wang, M. C. and Bartnicki-Garcia, S. (1974) *Carbohyd. Res.* **37**, 331.
7. Zinnen, T. M., Heinkel, C. M., Hudspeth, M. E. S. and Meganathan, R. (1991) *Phytopathology* **81**, 426.
8. Stubler, D. and Buchenauer, H. (1991) in *Mechanisms of Plant Defense Responses* (Fritig, B. and Legrand, M., eds), p. 455. Kluwer Academic Publishers, The Netherlands.
9. Kopp, M., Rouster, J., Fritig, B., Darvill, A. and Albersheim, P. (1989) *Plant Physiol.* **90**, 208.
10. Sharp, J. K., Valent, B. and Albersheim, P. (1984) *J. Biol. Chem.* **259**, 11 312.
11. Sharp, J. K., Valent, B. and Albersheim, P. (1984) *J. Biol. Chem.* **259**, 11 321.
12. Cheong, J. J., Birberg, W., Fügedi, P., Pilotti, A., Caregg, P. J., Hong, N., Ogawa, T. and Hahn, M. G. (1991) *Plant Cell* **3**, 127.
13. Fabre, I., Bruneteau, M., Ricci, P. and Michel, G. (1984) *Eur. J. Biochem.* **142**, 99.
14. Perret, J., Bruneteau, M., Michel, G., Marais, M. F., Joseleau, J. P. and Ricci, P. (1992) *Carbohydr. Polymers* **17**, 231.
15. Bruneteau, M., Perret, J., Rouhier, Ph. and Michel, G. (1992) *Carbohydr. Res.* **286**, 345.
16. Ohno, N. and Yadomae, T. (1987) *Carbohydr. Res.* **58**, 293.
17. Gomaa, K., Kraus, J., Franz, G. and Roper, H. (1991) *Carbohydr. Res.* **217**, 153.
18. Kogan, G., Alfold, J. and Malser, L. (1988) *Biopolymers* **27**, 316.
19. Albersheim, P. and Darvil, A. G. (1985) *Sci. Amer.* **253**, 44.
20. Huguenin, B. (1974) *Ann. Phytopathol.* **4**, 425.
21. Hall, R., Zentmyer, G. A. and Ervin, D. C. (1960) *Phytopathology* **59**, 770.
22. Iino, K., Ohno, N., Suzuki, I., Miyazaki, T., Yadomae, T., Oikawa, S. and Sato, K. (1985) *Carbohydr. Res.* **141**, 111.
23. Shields, R. and Burnett, W. (1960) *Anal. Chem.* **32**, 885.
24. Hakomori, J. (1964) *J. Biochem.* **55**, 205.
25. Bayard, B. and Montreuil, J. (1972) *Carbohydr. Res.* **24**, 427.
26. Ogawa, K., Tsurugi, J. and Watanabe, T. (1972) *Chem. Letters* 689.
27. Von Wechmar, M. B. and Van Regenmortel, M. H. V. (1970) *S. Afr. Med. J.* **44**, 151.