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Chitosan-Elicited Callose Synthesis in Soybean Cells as a Ca^{2+}-Dependent Process[1]

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ABSTRACT

A new method for the rapid and quantitative fluorometric determination of callose is described. In suspension-cultured cells of *Glycine max*, synthesis of callose starts within 20 minutes of treatment with chitosan and parallels over hours the accumulation of 1,3-linked glucose in the wall. Poly-L-lysine also elicits callose synthesis. The effect of chitosan is enhanced by Polymyxin B at low concentrations; this antibiotic alone at higher concentrations can also induce callose synthesis. Callose synthesis is immediately stopped when external Ca^{2+} is bound by ethylene glycol-bis-(2-aminoethyl ether)-*N,N'*-tetraacetate or cation exchange beads, and partly recovers upon restoration of 15 micromolar Ca^{2+}.

Callose synthesis is observed only when membrane perturbation causing electrolyte leakage from the cells is induced by one of the above treatments. It does not appear to be due to *de novo* synthesis or proteolytic activation of 1,3-β-D-glucan synthase. It is concluded that this Ca^{2+}-dependent enzyme is directly activated by the influx of Ca^{2+} occurring concomitantly with the leakage of cell constituents. This suggestion is also discussed in conjunction with the chitosan-induced synthesis of phytoalexin in the same cells.

Callose is a polysaccharide containing a high proportion of 1,3-β-linked glucose and is easy to detect cytochemically, for instance using aniline blue fluorescence or staining with resorcin blue (10). It is deposited in response to physical or chemical stress adjacent to the plasma membrane and often at the connections that unite the protoplasts of contiguous cells (*e.g.* plasmodesmata, sieve pores), presumably to isolate the injured area (10). The localized deposition and speed of callose formation may be important in the first line of defense against pathogens as callose is also a major component of papillae or wall appositions which are formed at sites of attempted penetration by invading fungal hyphae (1, 4). Callose is similarly found around lesions in certain virus-infected plants where it may help to prevent spreading of the virus (4, 20).

In addition, callose is often formed as a transient wall material at sites which are altered during special developmental steps (*e.g.* pollen maturation, sieve pores formation; 12). It is also deposited during *in vivo* growth of pollen tubes. In compatible tubes it seals the spent pollen grain from the cytoplasm containing the nuclei near the tube tip. In incompatible matings there is often a heavy callose deposit just behind the arrested tube tip (2). More recent reports imply callose also in gravitropism (15).

All the above processes require that callose synthesis is a well regulated process. Plasmolysis-induced callose formation in epidermal cells of onion is prevented when oxalic acid or EDTA are present; these substances presumably act by trapping Ca^{2+} (9). The biochemical basis of these observations was not established.

We have recently shown that addition of chitosan to suspension-cultured soybean cells results in increased leakage of electrolytes, UV-absorbing material, and proteins (29). This event was counteracted by polyanions such as polygalacturonate or polyaspartic acid and also effected by poly-L-lysine, indicating that it is due to the polycationic nature of chitosan. Although Ca^{2+} is displaced by the above polycations from the wall and, therefore, most likely also from the plasma membrane surface, the major injury of the plasma membrane appears to be caused by disturbing the membrane fluidity, as indicated by the observation that higher mol wt polycations are more effective than lower mol wt polycations (30).

The chitosan-induced leakage of cell constituents is followed by a change in various physiological parameters (18). Chitosan was added to cells in nutrient medium in amounts just sufficient to ensure that no further increase in fresh weight occurred for 43 h. Under these conditions we observed a production of the phytoalexin glyceollin, alterations in the wall phenolics, and increased resistance against the fungal wall degrading enzyme mixture 'Driselase' (18). The walls also became highly fluorescent with decolorized aniline blue and this staining property disappeared on preincubation with a mixture of endo- and exo-1,3-β-glucanase, proving that chitosan, in addition to the above effects, also induced formation of callose.

In the present report we present some quantitative and regulatory aspects of callose formation which might be understood taking into account the recently observed Ca^{2+}-requirement of the 1,3-β-glucan synthase (17). The results also allow some hypothetical suggestions toward a possible role of Ca^{2+} in the induction of other chitosan-induced physiological changes regarded to be of importance in resistance phenomena.

MATERIALS AND METHODS

Cell Treatment. The suspension culture of *Glycine max* cv Harosoy 63 and the growth conditions were as described previously (18). The cells were subcultured in modified B_5 medium at 5-d intervals. Some preliminary experiments were performed with cultures of *Phaseolus vulgaris* cv 'Grandessa' (subculture: 7 d), *Nicotiana tabacum* cv 'Samsun', and *Petroselium hortense*. The latter two cultures were kindly provided by L. Willmitzer

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and K. Hahlbrock (Köln, FRG), respectively.

For experiments with incubation times up to 4 h, the cells were collected by vacuum filtration on filter paper and 6 g were resuspended in 100 ml of 10 mM Tes/NaOH (pH 7.0) with 2% (w/v) sucrose. Aliquots of 5 ml (300 mg of cells) were pipetted into 20-ml plastic vials closed with caps and gently mixed for the indicated time on a roller mixer (Denley Technology, Sussex, England). Substances were added from concentrated stock solutions to give 5.5 ml final volume. On addition of chitosan and poly-L-lysine, the cells become partly and transiently agglutinated and thus difficult to pipette. Each point in the experiments reported represents the data from one individual 5-ml sample, prepared as described above.

In some experiments, the concentration of free Ca^{2+} in the cell suspension was altered by means of AG 50 W-X8 cation exchange resin (Biorad). The beads (200–400 mesh) were equilibrated with 1 M KCl or $CaCl_2$, washed with Tes buffer, and added to the cell suspension (1 g wet weight/g cell fresh weight).

In experiments requiring more than 4 h incubation time (Fig. 1), all manipulations were performed under sterile conditions as described (18), using B_5 medium. The cells were harvested as above and stored in ethanol (3 ml/g) for at least 12 h at 4°C. The cell mass was then suction-dried and disintegrated in 3 volumes of water in a glass Potter homogenizer. Cell walls were collected by centrifugation (5 min, 380*g*) and washed three times with water and once with acetone. The resulting wall pellet was air dried and amounted to 2.8 to 3% of the initial cell fresh weight.

Cell Permeability. Electrolyte leakage was followed by measuring the change in conductivity of the suspension buffer. The conductivity at the start of incubation was taken as 0% and the conductivity of a sample in which the cells had been completely destroyed by heating until boiling in a microwave oven represents 100%.

Callose Extraction. The cells from the above sample (300 mg) were collected by vacuum filtration on glass fiber discs (Whatman type GF/A, 2.5 cm) and washed with about 10 ml of water. To remove autofluorescent soluble material the cells were soaked for at least 2 min in 10 ml of ethanol. The suction-dried cells were transferred into a glass Potter homogenizer and disintegrated in 3 ml of 1 N NaOH. The resulting suspension was incubated at 80°C for 15 min to solubilize the callose and centrifuged (5 min, 380*g*). Aliquots (50–200 μl) of the supernatant were used for the callose assay.

For determination of the callose formed in the 1,3-β-D-glucan synthase assay (see below) the pellet was solubilized by heating to 80°C for 5 min in 250 μl of 1 N NaOH. Aliquots of 50 to 200 μl of this solution were used for callose determination.

Callose Determination. Samples and/or 1 N NaOH (total volume 200 μl) were mixed with 400 μl of 0.1% (w/v) aniline blue WS in water (Merck, Darmstadt, FRG), resulting in a violet-red color. After addition of 210 μl of 1 N HCl the color changes to deep blue, indicating neutral to acidic pH values. The final pH value was adjusted by addition of 590 μl 1 M glycine/NaOH buffer (pH 9.5) and the tubes were mixed vigorously. During the following incubation for 20 min at 50°C and further 30 min at room temperature, the aniline blue becomes almost completely decolorized. Fluorescence of the assay was read in a Jobin-Yvon JY3 D spectrofluorometer (excitation 400 nm, emission 510 nm, slit 10 nm, energy 6, gain 50). Calibration curves were established using a freshly prepared solution of the 1,3-β-glucan pachyman in 1 N NaOH. The standard curve was linear between 0.1 and 2 μg pachyman/assay, the upper amount reflecting about full scale fluorescence. Using this calibration curve, amounts of callose were expressed as pachyman-equivalents.

Enzyme Assay. For determination of 1,3-β-D-glucan synthase activity the cells were washed on the filter with about 10 ml of 100 mM Tes/NaOH (pH 7.0), and 2 g were homogenized in a glass Potter homogenizer at 0°C in 4 ml of the above buffer, but containing 1 mM DTT and 1 mg/ml soybean trypsin inhibitor. The homogenate was centrifuged for 3 min at 480*g* and the supernatant used in the enzyme assay. Alternatively, a microsomal fraction was prepared from this supernatant as described (17), employing 50 mM Tes/NaOH (pH 7.0) containing 1 mM DTT to wash and suspend the microsomes.

Aliquots of 50 μl enzyme preparation were mixed with 50 μl assay buffer containing 50 mM Tes/NaOH (pH 7.0), 20 mM cellobiose, 16% (w/v) glycerol, 10 mM $MgCl_2$, 0.04% (w/v) digitonin. The relatively high final Mg^{2+} concentration (5 mM) was used to allow a better control of $[Ca^{2+}]$. For this purpose the assay buffer contained, in addition, either 4 mM EGTA for assays without Ca^{2+}, or 4 mM EDTA plus 3.6 mM $CaCl_2$ for assays with Ca^{2+} (27). The reaction was started with 5 μl of 20 mM UDP-glucose containing 58,000 cpm of UDP-[^{14}C]glucose. After incubation at 25°C for 10 min the reaction was terminated by immersion in a boiling water bath for about 5 min and the [^{14}C]glucan formed measured as described (17).

To determine the callose formed in the same enzyme assay with the fluorometric method described above, the UDP-[^{14}C]-glucose was omitted and zero time controls without substrate were run to correct for the slightly elevated endogenous callose content of extracts from chitosan-treated cells. The reactions were stopped by addition of 1.5 ml of 65°C ethanol. The tubes were heated at 65°C for 5 min and centrifuged for 10 min at 380*g*. The pellet was drained by inversion of the tubes on filter paper and used for callose determination.

Ca^{2+} Determination. The $[Ca^{2+}]$ in the above enzyme assays was calculated (16) to be <10 nM (termed 'no Ca^{2+}') when EGTA was added. When the EDTA/$CaCl_2$/$MgCl_2$ mixture (27) was used at the above concentrations, $[Ca^{2+}]$was measured in scaled-up assay mixtures to range from 90 to 130 μM free Ca^{2+} when the 480*g*-supernatant was used as an enzyme source and from 130 to 190 μM when the microsomal fraction was used. These values were determined using a Ca^{2+}-sensitive electrode (17) and a Ca^{2+}-standard diluted in quartz-distilled H_2O containing 5 mM $MgCl_2$. A concentration of free Ca^{2+} around 100 μM is saturating when the 1,3-β-glucan synthase is assayed under the above conditions (17).

The cell suspension used for the *in vivo* callose induction experiments was prepared without further additions of Ca^{2+}. Free Ca^{2+} in this suspension was 8 to 20 μM, determined using the Ca^{2+}-sensitive electrode with a Ca^{2+}-standard diluted in quartz-distilled H_2O.

Polysaccharide Analysis. Uronic acids were determined with the biphenylol test (7). The noncellulosic wall polysaccharides were hydrolyzed with 2 N TFA (1 h, 120°C). The sugar composition of this and of the nonhydrolyzed cellulosic fraction were further analyzed by GC as described (5).

Alternatively, the total cell wall fraction was twice methylated with potassium dimsyl, hydrolyzed and acetylated (6). The products were analyzed on a 20-m OV-225 WCOT glass capillary column (160–200°C, linear program with 1°/min). The elution times of the especially interesting 1,4-5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol were obtained by methylation of cellulose and laminarin, respectively. The identity of the sugar derivatives was verified on a Hewlett Packard gas chromatograph/mass spectrometer model 5995 with a 25-m SP-1000 fused silica capillary column (170–210°C, linear 1°/min).

Materials. Chitosan preparation from crude crab shell chitosan (29) and staining of callose with aniline blue and fluorescence microscopy were as described before (18). Pachyman was prepared by B. A. Stone (Bundoora, Australia) and kindly provided by W. Eschrich (Göttingen, FRG). So-called 'glucan-elicitor' was prepared by NaOH-extraction of mycelium from *Phytophthora*

megasperma f.sp. glycinea (3). All other materials were as described (17, 18).

RESULTS

Induction of Callose Formation. The sugar composition of walls from untreated cells was compared to that of cells treated for 43 h with chitosan. The content of noncellulosic glucose as determined by hydrolysis with 2 N TFA increased from 2.4 to 6.9% on treatment with chitosan (noncellulosic plus cellulosic sugars plus uronic acids = 100%). The cellulose content of both samples was comparable (32%).

A second method was used to confirm this and to investigate at the same time the linkage type of the wall monomers. Walls of cells which were incubated with chitosan for various time periods were subjected to methylation analysis. The 1,3-linked glucose content was already significantly increased after 1 h and reached about 8% after 7 h (Fig. 1). Walls obtained from cells incubated for 43 h with chitosan contained 8.7% of 1,3-linked glucose and 28% of 1,4-linked glucose (total sugars = 100%). For the chitosan-treated cells the peak in the gas chromatogram with a retention time characteristic for 1,3-glucose gave very strong signals for the respective ions in the mass spectrometer, whereas for control cells only traces of these ions were found. This indicates that in the gas chromatographic analysis minor amounts of an unknown impurity eluted with the same retention time as 1,3-glucose and that the values of about 1% given in Figure 1 for the control cells are overestimated to some extent. No significant change in the amount or linkage type of other cell wall monomers was observed (data not shown). In parallel with the increase of 1,3-linked glucose, also the amount of cell wall material extracted with hot alkali and fluorescent with aniline blue increased (Fig. 1).

The fluorometric callose determination could also be used with whole cells and enabled us to perform short-time studies without sterile handling. This has the additional advantage that the complex nutrient solution could be replaced by buffer, conditions under which less chitosan is needed to induce electrolyte leakage (18, 29) and the $[Ca^{2+}]$ can be measured and controlled.

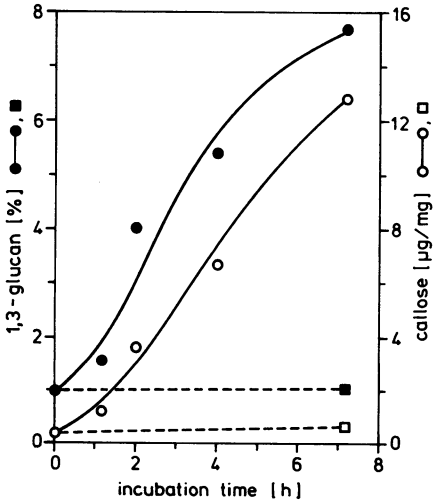

FIG. 1. Influence of incubation time on 1,3-glucan (●, ■) and callose (○, □) content of cell walls of chitosan-treated (●, ○) and control (■, □) cells. Chitosan (1 mg/g fresh weight) was added under sterile conditions at zero time to cells which were, in contrast to all other experiments reported, suspended in B_5 medium. The glucan content is expressed as 1,3-glucose as percentage of the total sugars recovered from the gas-chromatographic analysis and callose in µg pachyman-equivalents per mg dry cell wall, as determined fluorometrically. For a note on the significance of the 1% of 1,3-glucan in control cells see "Results".

On addition of chitosan detectable callose formation starts after about 20 min, is constant after about 1 h, and becomes higher proportionally to the amount of chitosan added (Fig. 2B). Leakage of electrolytes precedes callose formation (Fig. 2A).

In preliminary analogous experiments with suspension-cultured cells of *Phaseolus vulgaris*, *Nicotiana tabacum*, and *Petroselinum hortense*, chitosan similarly induced membrane permeability, which was also followed by enhanced formation of callose (data not shown). When UDP-glucose at a final concentration of 0.5 mM was added to soybean cell suspensions, together with 0.5 mg/g chitosan, the initial time course was the same as shown in Figure 2B and only after about 1 h or more a 20 to 30% higher callose content was observed. Similarly, also, the addition of 0.25 mM $MgCl_2$ instead of UDP-glucose did not alter the initial time course but after 2 or 3 h the amount of callose formed was about 30% higher (data not shown).

When the chitosan preparation used was autoclaved, it became more active (Table I), presumably as some longer chains of the

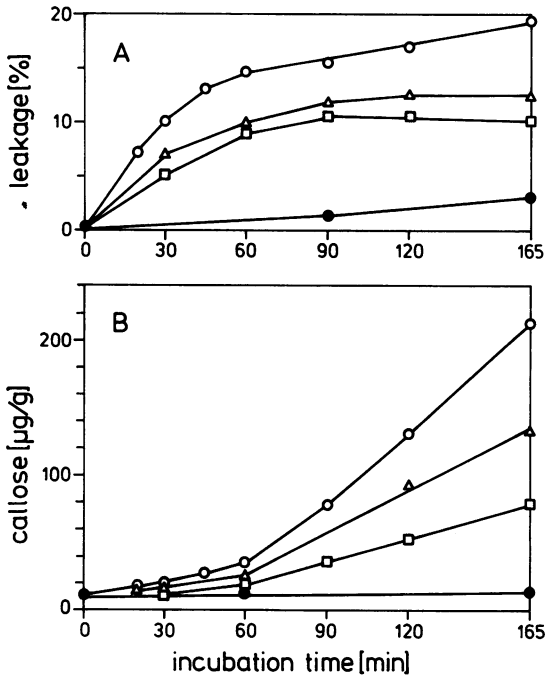

FIG. 2. Electrolyte leakage (A) and callose content (B) of soybean cells as a function of chitosan concentration and incubation time. Cells were suspended in buffer and chitosan added at zero time at 0 (●), 0.25 (□), 0.5 (△), or 2.5 (○) mg/g fresh weight, respectively, Leakage is given as percentage of the conductivity attained with totally destroyed cells and callose content in µg pachyman-equivalents per g fresh weight.

Table I. *Influence of Various Polycations on the Increase in Membrane Permeability and Callose Content of Soybean Cells*

Incubation time was 2 h and the other conditions were as in Figure 2.

Additions	Leakage	Callose[a]
0.5 mg/g fresh wt	%	µg/g
None (control)	4	3
Chitosan (autoclaved)[b]	23	129
Chitosan	13	102
Poly-L-lysine (120,000)[c]	38	32
Poly-L-lysine (60,000)	41	27
Poly-L-lysine (11,000)	7	17
Poly-L-lysine (4,000)	3	1

[a] Expressed as µg pachyman/g fresh weight. [b] Chitosan was autoclaved at 121°C for 20 min. [c] Mol wt.

polymer became degraded to molecules which have a better access through the cell wall to the membrane. The importance, however, of a relatively high mol wt of the polycations employed is evident from the results with poly-L-lysine (Table I). Smaller molecules are less effective in causing both electrolyte leakage and callose synthesis. The respective monomers L-lysine and D-glucosamine are inactive in inducing these effects. Addition of polygalacturonate to chitosan reduced its ability to induce callose formation (data not shown). A similar effect has been shown previously for the induction of electrolyte leakage (29) and glyceollin production (18).

The effect of chitosan both on cell leakage and callose synthesis is enhanced by Polymyxin B at low concentrations. Under these conditions, Polymyxin B alone was ineffective (Table II). At higher concentrations it could also act alone as an elicitor of callose synthesis, accompanied by electrolyte leakage. No significant increase in callose formation was found 2 h after addition of a crude preparation of 'glucan-elicitor' from *P. megasperma* f.sp. glycinea in concentrations (2.5 mg/g cell fresh weight = 0.2 mg/ml) sufficient to raise the glyceollin content of similar soybean cell cultures 10- to 20-fold when they were incubated in B_5 medium for 43 h (18).

Ca^{2+} Requirement of Callose Synthesis. As determined with an ion-sensitive electrode, the suspensions of soybean cells in Tes-buffer contained 8 to 20 μM free Ca^{2+}. At this concentration the chitosan-induced callose synthesis was maximal; at moderately higher $[Ca^{2+}]$ there was no further increase in callose synthesis. On the other hand, at concentrations of $Ca^{2+} \geqq 1$ mM, higher quantities of chitosan were necessary to obtain the same rates of electrolyte leakage (29) and callose formation as in the Tes-buffer used (data not shown). This presumably is due to secondary effects of Ca^{2+}, namely stabilizing of membranes.

On addition of EGTA at a final concentration of 0.5 mM, the signal from the Ca^{2+} electrode was below the detection limit which is at about 1 μM. Under these conditions, no (Fig. 3B) or almost no (Fig. 4) callose is formed in chitosan-treated cells. EGTA is equally effective when it is added either together with chitosan or 1 h later (Fig. 3B). As far as can be determined with the callose assay employed, EGTA also immediately stopped callose synthesis at a time when it proceeds with a constant rate (Fig. 4). Addition of EGTA also renders the cells more leaky (Fig. 3A). This effect is small without chitosan but dramatic when chitosan is present.

When cell suspensions in which callose synthesis was arrested by addition of EGTA were brought back to the initial $[Ca^{2+}]$, callose synthesis starts again, although at diminished rates (Fig. 4). The latter appears understandable if one considers that even within the 10 min during which EGTA was present, considerable amounts of cell constituents must have been lost (compare Figs. 4 and 3A).

As EGTA caused a great increase in electrolyte leakage we have also manipulated $[Ca^{2+}]$ by means of cation-exchange beads

Table II. *Influence of Polymyxin B and Chitosan on the Increase in Electrolyte Leakage and Callose Synthesis*

Cells were incubated for 2 h as in Figure 2 without or with chitosan (0.5 mg/g).

Polymyxin B Added	No Chitosan		Chitosan Added	
	Leakage	Callose	Leakage	Callose
μM	%	$\mu g/g$	%	$\mu g/g$
0	3	3	11	108
1	4	3	35	134
5	7	12	38	130
10	21	35	49	99

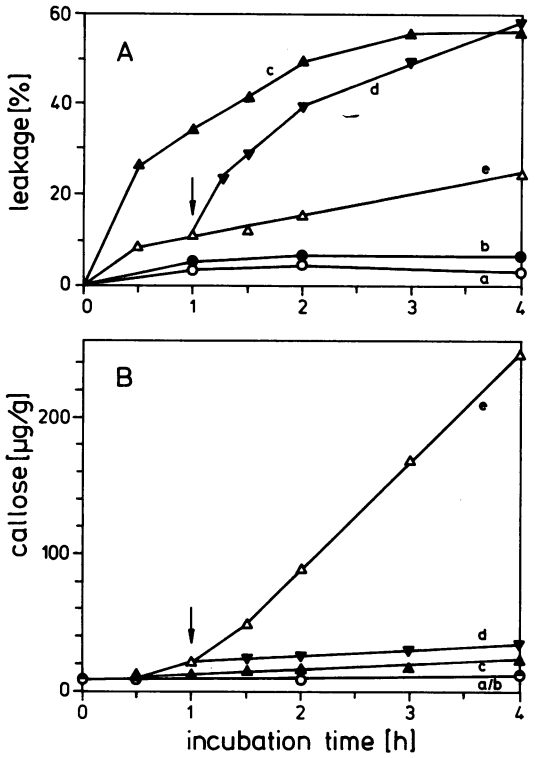

FIG. 3. Influence of Ca^{2+} on electrolyte leakage (A) and callose content (B) of chitosan-treated cells. The cells were suspended in buffer at about 15 μM free Ca^{2+}. If present, chitosan (0.5 mg/g fresh weight) was added at 0 h, followed in some samples and at various times by 2 mM EGTA to result in a $[Ca^{2+}] < 0.1$ μM. Other conditions as in Fig. 2. a (○), control (no chitosan, no EGTA); b (●), control (no chitosan, EGTA at 0 h); c (▲), + chitosan (+ EGTA at 0 h); d (▼), + chitosan (+ EGTA at 1 h, arrows); e (△), + chitosan (no EGTA).

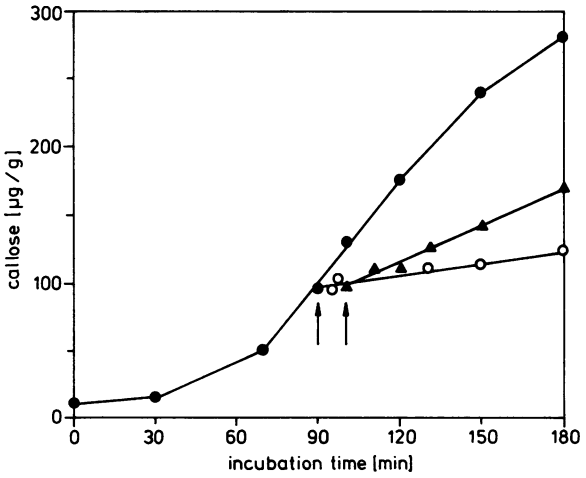

FIG. 4. Partial reversion by Ca^{2+} of the decrease in callose synthesis caused by EGTA. Same conditions as in Figure 3. Callose synthesis was induced at zero time by chitosan (●) at about 15 μM free Ca^{2+}. At the first arrow $[Ca^{2+}]$ was brought to < 0.1 μM by addition of EGTA (○) and at the second arrow back to about 15 μM (▲) by the addition of a concentrated solution of $CaCl_2$ in amounts sufficient to result in the same voltage at the Ca^{2+}-selective electrode as before addition of EGTA.

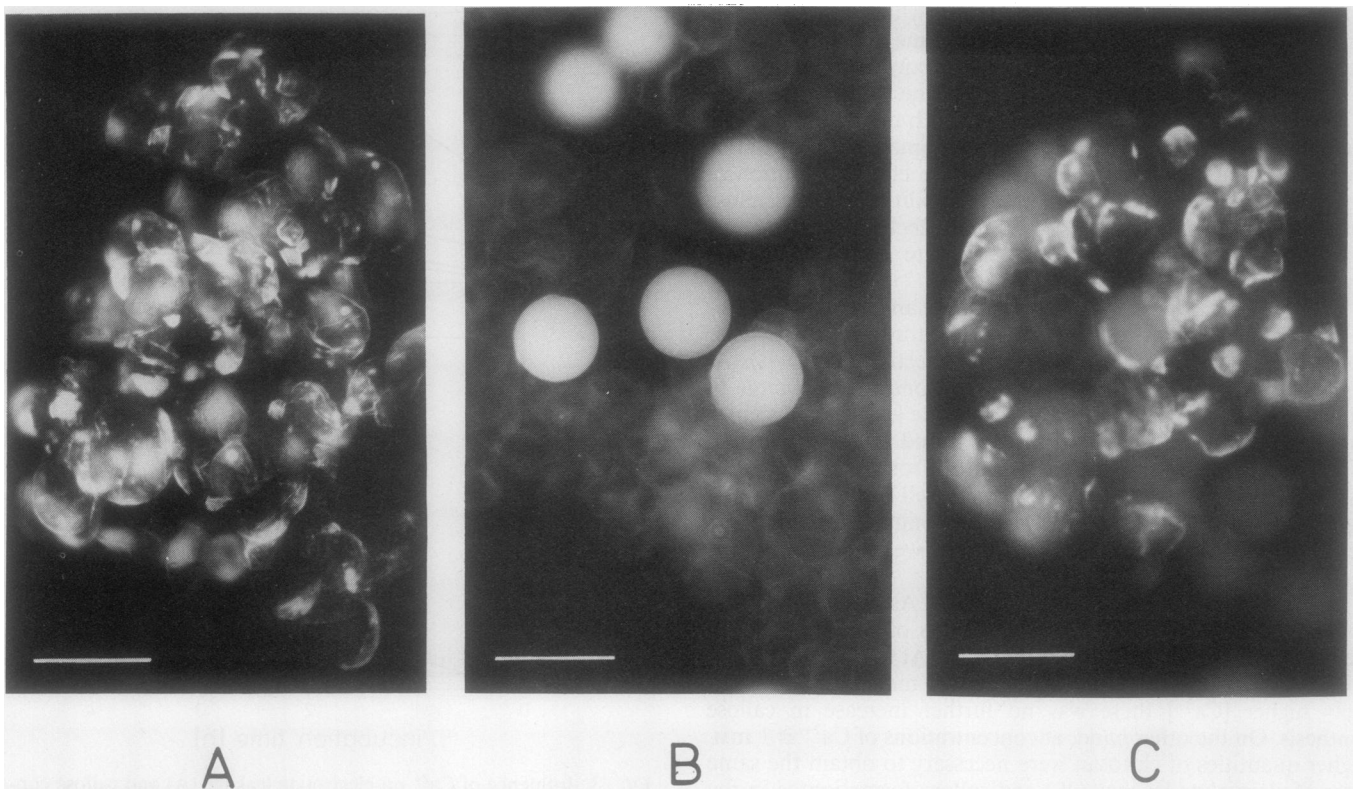

FIG. 5. Dependence of chitosan-induced callose synthesis on the presence of external Ca^{2+}, as effected by ion-exchange beads. A, Control (0.5 mg chitosan/g, incubation time 2 h, [Ca^{2+}] about 15 μM); B, same but with ion-exchange beads in the K^+-form, [Ca^{2+}] < 1 μM; C, mixture of the ion-exchange beads in K^+- and Ca^{2+}-form, [Ca^{2+}] about 15 μM. Cells were stained with aniline blue and photographed in the fluorescence microscope as described in Köhle *et al.* (18). The magnification bar represents 0.1 mm.

Table III. *Activity of the 1,3-β-Glucan Synthase in Chitosan-Treated Cells and Its Stimulation by Ca^{2+}*

Polymerization of glucose from UDP-glucose was determined for comparison either with ^{14}C-labeled substrate or with the fluorometric callose assay. For remarks on [Ca^{2+}] in the enzyme assay mixture see "Materials and Methods". The mean values of four replicate assays from the same cell extract (480 g supernatant) are given.

Source of Enzyme	[^{14}C]Glucan Formed		Callose Formed	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
	μg/10 min/50 μl			
Control cells	0.2	4.8	0.2	4.6
Cells treated with chitosan[a]	0.3	5.0	0.2	4.4

[a] 0.5 mg chitosan/g cell fresh weight for 2 h.

which have no direct access to the plasma membrane surface. When the K^+ form of the beads was used, only a slight increase in leakage was observed, when compared to a suspension which received chitosan alone (data not shown). Nevertheless, the chitosan-induced callose synthesis was greatly prevented (Fig. 5B). That this is not due to possible toxic effects exerted by the resin is evident from Figure 5C which shows that callose synthesis becomes possible when exchange beads in the Ca^{2+} form are added in amounts (three parts Ca^{2+}-form per two parts of K^+-form) sufficient to ensure a [Ca^{2+}] of about 15 μM. Regardless of which form of cation exchange resin was added, the cell suspensions did not form any callose when chitosan was omitted. Due to difficulties in separation of cells from resin beads, the quantitative fluorometric callose determinations could not yet be performed when the cells are mixed with the ion-exchange beads; the results are therefore only presented as photographs. The control without resin beads is presented (Fig. 5A) to demonstrate that the callose formed is sometimes present at very localized dots but more often in extended caps which suggest that considerable parts of the cell surface are involved in callose synthesis.

Glucan Synthase Activity. Activity of 1,3-β-glucan synthase was measured by two methods. The incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose into polymers and the values obtained by fluorometric callose determination are almost consistent if the incubation time was 10 min (Table III). With incubation times of 0.5 to 1 min, we observed an apparent lag-phase when the fluorometric assay was used, whereas the assay with UDP-[^{14}C]glucose gave incorporation rates fully proportional to time (data not shown). Thus, for short incubation times where presumably the proportion of short-chain molecules is higher, it is advisable to use the classical ^{14}C-assay.

No significant differences in enzyme activity were found between extracts from chitosan-treated and control cells (Table III). In both preparations the activity of the enzyme was about 15- to 25-fold, stimulated by the presence of Ca^{2+}. It is of special interest that the 1,3-β-glucan synthase activity measured without Ca^{2+} was not elevated in chitosan-treated cells.

Results corresponding to those of Table III were also obtained using chitosan treatment for 30 min. When crude cell homogenates, resuspended 480*g*-pellet (= membranes adhering to cell walls) or washed microsomal particles (17) were used for experiments similar to those reported in Table III, no significant differences between control and chitosan-treated cells were found.

DISCUSSION

Callose formation was measured using a new quantitative fluorometric assay based on the histochemical aniline blue stain-

ing technique which is widely assumed to indicate the presence of 1,3-β-glucans (10). This specificity, however, has also been questioned (21). The contradictory views seem to result from the presence in commercial aniline blue of several fluorochromes at varying abundance, and from the influence of the pH and environment on the fluorescence properties (21, 28). The fluorochrome specific for 1,3-β-glucans (11) is not yet commercially available. We have, therefore, pragmatically overcome these problems in the assay described by making use of the alkali solubility of callose in combination with a buffer of sufficient capacity to control the pH value. In addition, use of a spectrofluorometer allows, in contrast to a fluorescence microscope, selection of the exact wavelength of excitation and emission.

A serious shortcoming of the assay, in regard to quantitative aspects, is the fact that fluorescence intensity depends on the DP[3] of the 1,3-β-glucan (11). This was also indicated by the observation that in the assay described, about 100-fold more laminarin (DP ≈20) than pachyman (DP 250–690) is needed to give the same fluorescence. The data in Table III show that the callose produced *in vitro* under optimal assay conditions and with 10 min of incubation time gives about the same fluorescence as pachyman. In contrast, the callose produced in chitosan-treated cells appears to contain some shorter chains: If one assumes that the 1,3-glucan is quantitatively recovered by the methylation analysis, one can calculate, for instance from the values reported in Figure 1 for 7 h incubation time, that 75 μg of 1,3-glucan were contained per mg of wall material compared with 13 μg/mg measured fluorometrically. Thus, the latter technique underestimates callose formed *in vivo* by a factor of about 6, with a tendency toward even higher factors at shorter incubation times. This might, in part, explain the findings during the initial phase of about 1 h, where the rate of callose synthesis measured fluorometrically steadily increases (Figs. 2B, 3B, 4).

Other factors may additionally contribute to the initial phase of low callose production. Binding of chitosan to the wall and the resulting Ca^{2+} displacement proceeds over about 30 min (30), although the appearance of electrolytes, presumably resulting mainly from inside the cell, is far more rapid and shows already diminished rates when callose formation apparently begins (Figs. 2 and 3). A major ion contributing to the overall increase in conductivity of the suspension buffer is K^+. Other cell constituents leaking out might lag behind; this was observed to some extent in chitosan-treated soybean cells for UV-absorbing material and proteins (29). Similar observations were made with tobacco cells treated with Polymyxin (25). Thus, although convenient to measure, the increase in external electric conductivity is only a rough indicator of the alteration of membrane properties and, most likely, does not properly reflect the change in a critical cell parameter which has to fall below or rise above a certain threshold value to effect metabolic changes, as discussed below for Ca^{2+}.

The first callose formation is detected about 20 min after addition of chitosan and, thus, can be classified as a process presumably too rapid to involve transcriptional *de novo* synthesis of enzyme proteins (for a time course of such processes compare Hille *et al.* [14]). Consistent with this assumption, no increase in total 1,3-β-glucan synthase activity was detected in chitosan-treated cells (Table III). One could also consider a process of covalent modification of this enzyme as the possible cause of increased callose production. It was previously shown that proteolytic activation can render this enzyme even more active than the presence of Ca^{2+} at saturating concentrations. This stimulation was effected either by trypsin or, to some extent, endogenous proteinases. The latter were presumed, on the basis of the observed inhibition by soybean trypsin inhibitor, from an enzymic

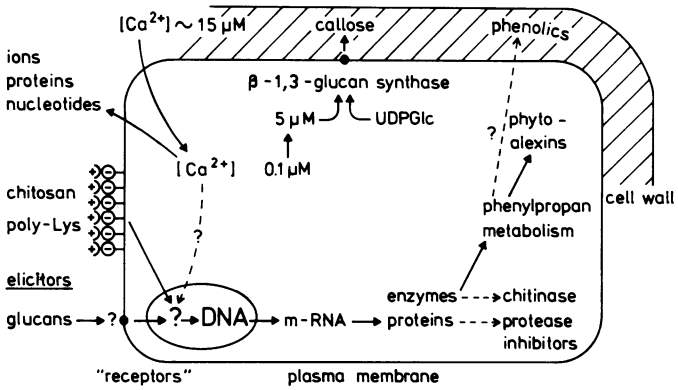

FIG. 6. Proposed events leading to chitosan-elicited callose synthesis followed by induction of some other metabolic changes regarded to be of importance in resistance against pathogens. For more details see "Discussion".

activation taking place in crude homogenates (17). However, the present observation that extracts of chitosan-treated cells do not show an increase in Ca^{2+}-independent 1,3-β-glucan synthase activity (Table III) shows that this proteolytic step has no physiological significance during chitosan-induced callose synthesis. The same evidence also argues against other types of covalent modification of 1,3-β-glucan synthase, such as phosphorylation/dephosphorylation. In addition, we have found that with microsomal particles which have been washed, the Ca^{2+}-induced activation is readily reversible when Ca^{2+} is complexed, and does not require nucleotides (17). These experiments have more recently been extended (unpublished results) to show that reversion also occurs at low temperatures and does not require any significant time. Thus, as long as positive evidence for a mechanism involving covalent modification of 1,3-β-glucan synthase is lacking, we propose that the activation of this enzyme by Ca^{2+} (Table III) results from a direct and reversible interaction of this ion with the enzyme. It has been suggested before on the basis of inhibitor studies that the activation of 1,3-β-glucan synthase by Ca^{2+} is not mediated by calmodulin but possibly by other calciproteins or phospholipids (17).

Chitosan-induced callose formation is not possible without the presence of external Ca^{2+} (Figs. 3B, 4, 5) and leakage of cell constituents appears to be an additional prerequisite. It can be induced by various agents such as chitosan, poly-L-lysine (Table I) and Polymyxin B (Table II), all of which seem to be at least partly nonspecific as they allow for the release of various substances from the cell, following their gradients of concentration (Fig. 6). It is generally assumed that the concentration of free Ca^{2+} in the cytoplasm of eucaryotic cells is held in the range of 0.1 μM. Although difficult to measure directly, this has been shown for many animal cells and also among plants for *Chara* and *Nitella* (26). The 1,3-β-glucan synthase activity *in vitro* is strongly increased when the concentration of free Ca^{2+} is above 0.5 μM, with half saturation of the effect at about 5 μM and saturation at 50 to 100 μM (17). The stimulation by Ca^{2+} at saturating concentrations is 15- to 25-fold when the enzyme is assayed in the presence of 5 mM Mg^{2+} (17; Table III) and becomes 30- to 40-fold when Mg^{2+} is omitted (unpublished results). This enzyme, termed 1,3-β-glucan synthase II when it is measured at about 1 mM UDP-glucose, a condition also used by us to study callose synthesis (Table III), is regarded to be a marker enzyme for the plasma membrane (for literature see [19]). It appears logical to assume that the enzyme is vectorially arranged in the membrane as its substrate UDP-glucose presumably comes from the cytoplasmic side (8, 23) and its product callose is deposited at the wall surface. Thus, a local increase in Ca^{2+} ions at the cytoplasmic side due to the membrane perturbation could di-

[3] Abbreviations: DP, degree of polymerization.

rectly activate the enzyme and initiate callose formation (Fig. 6).

The above working hypothesis is formulated assuming a mainly nonspecific opening of Ca^{2+} channels by the externally applied agents. If the concentration of chitosan (Fig. 2) or Polymyxin B (Table II) or the mol wt of poly-L-lysine from 11,000 to 60,000 are increased, then the increase in electrolyte leakiness correlates to some extent with an increase in callose formation. Such a correlation is not observed, however, when the various substances are compared. Chitosan appears to be more effective for callose induction than Polymyxin B or poly-L-lysine, whereas the latter two substances effect lower callose formation at a greater electrolyte leakage. Obviously, there is, to some extent, in addition to nonspecific leakage induction of a superimposed effect which possibly consists of opening more specific ion channels. This appears possible if one considers the mode of interaction of the substances with the membrane. The polycations appear to combine by charge-charge interaction; major targets are the polar heads of membrane phospholipids, as indicated *e.g.* by the use of poly-L-lysine for the fusion of pure liposomes (13). Poly-L-lysine is more effective with increasing mol wt (Table I; [30]) and this suggests that it mainly acts like a fixed block which disarranges membrane fluidity. The effect appears to be enhanced when surface-associated Ca^{2+} is displaced from the membrane, for instance by EGTA (Fig. 3A). This agrees with the observation that divalent ions partly prevent the effect of polycations on leakage (29). Both polycations used might, however, additionally interact with negatively charged exposed parts of membrane proteins and thereby change their catalytic properties. To what extent the two polycations interact with the various potential anionic membrane sites may depend on the distances between their charged groups and on the rigidity and spatial arrangement of the molecule backbone. The antibiotic Polymyxin B is a cyclic polypeptide with hydrophobic side chains which can intercalate into membranes and interact with phospholipids (22, 25) and thereby also may open both nonspecific and ion-specific channels. The concentrations of the three agents used as callose elicitors were chosen to just slightly damage cell membranes and cause moderate leakage (Figs. 2A, 3A) presumably leaving most of the cells alive. It appears trivial that at higher concentrations the above substances more severely damage and eventually fully destroy the cells. The beginning of such a negative effect is, for instance, evident for the combination of 10 μM Polymyxin B and chitosan (Table II). At concentrations of 100 μM and higher, Polymyxin B fully destroyed the cells without callose formation (data not shown). At such high concentrations the antibiotic also directly inhibits 1,3-β-glucan synthase *in vitro* (17). Callose formation appears to be possible only when the loss of cell constituents, such as substrates and cofactors, is in some balance with the presumed inflow of Ca^{2+}.

It has been observed before that chitosan, in addition to callose formation, can also elicit other metabolic changes in soybean cells; a few of them which appear to be of importance for resistance against pathogens have been studied to some extent (18). On a time scale, callose formation appears to be an early event which is followed by the others mentioned in Figure 6. For instance, the first effects on glyceollin synthesis can be seen after about 10 h and are prominent after 1 to 2 d (18). For the example of phytoalexin synthesis in soy bean suspension cultures induced by glucan elicitors it has been suggested that a major aspect of their regulation is *de novo* synthesis of respective enzymes (14). In other systems, such a mechanism has also been suggested for proteins which are of direct potential use against pathogens, such as chitinase or proteinase inhibitors (24). It is still uncertain whether or not specific membrane receptors for the above mentioned glucan elicitors exist. The effect of polycations on gene expression has been suggested to possibly result from their direct interaction with nuclear DNA (for literature see [24]). We propose another hypothesis. Any realization of genetic information requires a yet unknown mechanism for signal transduction (? in Fig. 6) which in soybean cell cultures obviously can be triggered by such diverse substances as glucans, chitosan, or other polycations. The above discussed Ca^{2+}-dependent callose formation, on addition of chitosan or poly-L-lysine, could be taken as an indicator that a rise in the concentration of cytoplasmic Ca^{2+} had occurred. It is tempting to speculate that this also might function as a second messenger to trigger gene expression. The observation that glucan-elicitors of glyceollin synthesis did not induce significant callose formation within the 2 h of application studied does not rule out this hypothesis. One could consider that a hypothetical opening of receptor-specific Ca^{2+} channels may lead to long-time alterations of $[Ca^{2+}]$ which are just sufficient to induce enzyme synthesis but remain below a threshold necessary for rapid callose formation.

LITERATURE CITED

1. AIST JR 1976 Papillae and related wound plugs of plant cells. Annu Rev Phytopathol 141: 145–163
2. ANDERSON MA, RD HOGGART, AE CLARKE 1983 The possible role of lectins in mediating plant cell-cell interactions. Prog clin Biol Res 138: 143–161
3. AYERS AR, J EBEL, B VALENT, P ALBERSHEIM 1976 Host pathogen interactions X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. Plant Physiol 57: 760–765
4. BELL AA 1981 Biochemical mechanisms of disease resistance. Annu Rev Plant Physiol 32: 21–81
5. BLASCHEK W, D HAASS, H KÖHLER, G FRANZ 1981 Cell wall regeneration by *Nicotiana tabacum* protoplasts: Chemical and biochemical aspects. Plant Sci Lett 22: 47–57
6. BLASCHEK W, D HAASS, H KÖHLER, U SEMLER, G FRANZ 1983 Demonstration of a β-1,4-primer glucan in cellulose-like glucan synthesized *in vitro*. Z Pflanzenphysiol 111: 357–364
7. BLUMENKRANTZ N, G ASBEE-HANSEN 1973 New method for quantitative determination of uronic acids. Anal Biochem 54: 484–489
8. BRETT CT 1978 Synthesis of β-(1→3)-glucan from extracellular uridine diphosphate glucose as a wound response in suspension-cultured soy bean cells. Plant Physiol 62: 377–382
9. ESCHRICH W 1965 Physiologie der Siebröhrencallose. Planta 65: 280–300
10. ESCHRICH W 1975 Sealing systems in phloem. *In* MH Zimmermann, JA Milburn, eds, Encyclopedia of Plant Physiology, Vol 1. Springer-Verlag, Berlin, pp 39–56
11. EVANS NA, PA HOYNE, BA STONE 1984 Characteristics and specificity of the interaction of a fluorochrome from aniline blue (sirofluor) with polysaccharides. Carbohydrate Polymers 4:215–230
12. FINCHER GB, BA STONE 1981 Metabolism of noncellulosic polysaccharides. *In* W Tanner, FA Loewus, eds, Encyclopedia of Plant Physiology, Vol 13B. Springer-Verlag, Berlin, pp 68–132
13. GAD AE 1983 Cationic polypeptide-induced fusion of acidic liposomes. Biochim Biophys Acta 728: 377–382
14. HILLE A, C PURWIN, J EBEL 1982 Induction of enzymes of phytoalexin synthesis in cultured soy bean cells by an elicitor from *Phytophthora megasperma* f.sp. *glycinea*. Plant Cell Rep 1: 123–127
15. JAFFE MJ, AC LEOPOLD 1984 Callose deposition during gravitropism of *Zea mays* and *Pisum sativum* and its inhibition by 2-deoxy-D-glucose. Planta 161: 20–26
16. JEWELL BR, JC RÜEGG 1965 Oscillatory contraction of insect fibrillar muscle after glycerol extraction. Proc R Soc Lond B Biol Sci 164: 428–459
17. KAUSS H, H KÖHLE, W JEBLICK 1983 Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from soybean cells. FEBS Lett 158: 84–88
18. KÖHLE H, DH YOUNG, H KAUSS 1983 Physiological changes in suspension-cultured soy bean cells elicited by treatment with chitosan. Plant Sci Lett 33: 221–230
19. ROBINSON DG, R GLAS 1983 The glucan synthases from suspension-cultured sugar cane cells. J Exp Bot 34: 668–675
20. SHIMOMURA T, J DIJKSTRA 1975 The occurrence of callose during the process of local lesion formation. Neth J Plant Pathol 81: 107–121
21. SMITH MM, ME MCCULLY 1977 A critical evaluation of the specificity of aniline blue induced fluorescence. Protoplasma 95: 229–254
22. STORM DR, KS ROSENTHAL, PE SWANSON 1977 Polymyxin B and related peptide antibiotics. Annu Rev Biochem 46: 723–763
23. TIGHE DM, MC HEATH 1982 Callose induction in cowpea by uridine diphosphate glucose and calcium phosphate-boric acid treatments. Plant Physiol 69: 366–370
24. WALKER-SIMMONS M, L HADWIGER, CA RYAN 1983 Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. Biochem Biophys Res Commun 110: 194–199

25. WEIMBERG R, HR LERNER, A POLJAKOFF-MAYBER 1983 Induction of solute release from *Nicotiana tabacum* tissue cell suspensions by Polymyxin and EDTA. J Exp Bot 34: 1333–1346
26. WILLIAMSON RE, CC ASHLEY 1982 Free Ca^{2+} and cytoplasmic streaming in the alga *Chara*. Nature 296: 647–651
27. WOLF HU 1971 Divalent metal ion buffers with low pH-sensitivity. Experientia 29: 241–249
28. WOOD PJ, RG FULCHER 1982 Dye interactions: a basis for specific detection and histochemistry of polysaccharides. J Histochem Cytochem 31: 823–826
29. YOUNG DH, H KÖHLE, H KAUSS 1982 Effect of chitosan on membrane permeability of suspension-cultured *Glycine max* and *Phaseolus vulgaris* cells. Plant Physiol 70: 1449–1454
30. YOUNG DH, H KAUSS 1983 Release of calcium from suspension-cultured *Glycine max* cells by chitosan, other polycations, and polyamines in relation to effects on membrane permeability. Plant Physiol 73: 698–702