Effectiveness of Plant-Derived and Microbial Polysaccharides as Elicitors for Anthraquinone Synthesis in *Morinda citrifolia* Cultures

Heike Dörnenburg and Dietrich Knorr*

Department of Food Technology, Berlin University of Technology, Königin-Luise-Strasse 22, 14195 Berlin, Germany

Various plant-derived and microbial polysaccharides were applied to cultured cells of *Morinda citrifolia*. Application of polysaccharides elicited secondary metabolism of these cultures and induced anthraquinone production. Polysaccharides involved in interactions between plants and microorganisms (chitosan/chitin and pectin) were most effective in inducing anthraquinone synthesis. Seven and 14 days of incubation with Chitin 50, a water-soluble chitin derivative with a degree of acetylation of 53%, resulted in an anthraquinone synthesis approximately 2.5 times higher than the control. For pectins the active components were found to be galacturonic acids with a degree of polymerization higher than five units. Pectins isolated from *M. citrifolia* cell walls have been shown to elicit anthraquinone synthesis over longer periods than other pectins tested and induced a 5.6-fold increase of anthraquinone production without inhibitory effects on the growth of the cell culture.

INTRODUCTION

It is now widely accepted that microbial invasion of intact plants will result in synthesis of antimicrobial secondary metabolites. Polysaccharides derived from cell walls of plants (e.g., pectin or cellulose) and microorganisms (e.g., chitin, chitosan, or glucans) are known to be endogenous and exogenous elicitors in plant–microorganism interactions (Mauch et al., 1988). Chitosan had been shown to be a very efficient elicitor (Brodelius et al., 1989; Kauss et al., 1989; Sauerwein et al., 1991). Other polysaccharides produced and excreted by plant pathogen microorganisms, such as xanthan and curdlan (Johnson et al., 1991) or gellan (Morimoto and Murai, 1989), have also been identified as elicitors in plant cell culture systems. Fukui et al. (1983) observed induction of shikonin formation by agar and found that polysaccharides containing acidic functional groups (agaropectin and pectic acid) were active for elicitation. However, these exogenous polysaccharides were less active than the endogenous polysaccharides of cell wall origin (Fukui et al., 1990). Exogenously applied alginate was also found to be one of the inducing agents of echinatin biosynthesis (Ayabe et al., 1986).

Results of studies on the elicitor effects of various acidic, basic, and neutral polysaccharides on *Morinda citrifolia* suspension cultures are reported here. This cell line was selected as a model because it had been shown to produce phytoalexins including anthraquinones (Wijnsma et al., 1986) and because their production in suspension cultures could be induced by biotic elicitors such as homogenized preparations of *Aspergillus niger* (van der Heijden et al., 1988).

MATERIALS AND METHODS

Cell Culture. The cell suspension culture of *M. citrifolia* was cultivated in 200-mL flasks with 60 mL of a modified B5 medium (Gamborg et al., 1968) supplemented with 2 g/L casein hydrolysate. 2,4-Diphenoxyacetic acid was replaced by 10^{-5} M naphthylacetic acid. The pH of the medium was adjusted to 5.5 with 1 N NaOH. Cells used in the experiments were in the exponential phase (14 days), incubated at 23.5 ± 0.2 °C and 100 ± 1 rpm under continuous light.

Elicitor Treatment. These experiments were carried out in 100-mL flasks containing 25 mL of medium at various concentrations (0, 25, 50, 100, and 250 μg/mL) of polysaccharides including sodium alginate, chitosans from crab shells (Sea Cure 143, Sea Cure+, BLV, and Chitin 50; Protan Biopolymers, Drammen, Norway), cellulose (Sigma), β-cyclodextrin (Roquette, Frankfurt, Germany), locust bean gum and guar gum (Wolff, Hamburg, Germany), curdlan and konjac (FMC Bioproducts, Philadelphia, PA), apple pectin and pectic acid (Herbstreith & Fox, Neuenbürg, Germany), citrus pectin (Pomosin, Grossenbrode, Germany), poly(galacturonic acid) (Sigma), alginate from *Pseudomonas syringae* (Dr. Schmauder, Halle, Germany), gellan, rhamsan, welan, and xanthan (Kelco, Hamburg, Germany), levan (Dr. Groß, Göttingen, Germany), and oligogalacturonic acid (Dr. Endreß, Neuenbürg, Germany). These commercial grade polysaccharides were used without further purification. Two milliliters of *M. citrifolia* suspension were used as inoculum for 25 mL of medium. Samples were taken after 2, 4, 7, and 14 days of incubation at 100 ± 1 rpm under continuous light.

Chitosan Purification. Chitosan isolated from *Mucor rouxii* cell walls (Dunkel, unpublished data) was dissolved in 1% acetic acid. The chitosan solution was dialyzed for 24 h against distilled water in tubes with a molecular weight cutoff of 2000 followed by freeze-drying. The degree of acetylation was determined according to the method of Muzzarelli and Rocchetti (1986).

Extraction and Isolation of Cell Wall Pectins from *M. citrifolia* Cultures. One hundred grams fresh weight of *M. citrifolia* cultures at several growth phases (12-, 18-, and 24-day-old cultures) were extracted with 50 mL of distilled water at 80 °C for 5 h. After centrifugation at 15 000*g* for 15 min, the pectin solution (supernatant) was mixed with 450 mL of 96% ethanol to precipitate insoluble pectin fragments. Following centrifugation the pectin pellets were redissolved in 25 mL of distilled water and then dialyzed for 24 h in tubes with a molecular weight cutoff of 2000 against distilled water. Pectin isolation was concluded by freeze-drying.

Analyses. Cell growth was monitored by obtaining fresh weights by filtration through a Büchner funnel after 7 and 14 days of incubation. Analysis of anthraquinones was performed by repeated extraction with 80% ethanol according to the method of Zenk et al. (1975) followed by centrifugation at 4000*g* and spectrophotometric determination at 434 nm. All experiments were carried out in four replications.

RESULTS AND DISCUSSION

Screening of Microbial and Plant Polysaccharides. Several polysaccharides isolated from plants or microorganisms or produced extracellularly by microorganisms have been selected and screened for their elicitor effects

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Table 1. Polysaccharides, Their Molecular Structure and Elicitor Effect (Percent of Control) on Anthraquinone Synthesis (AQ) in *M. citrifolia* Cultures after 14 Days of Elicitation [Concentrations Were 250 μg/mL Medium with the Exception of Chitosan (50 μg/mL)]

polysaccharide	molecular structure[a]	elicitor	AQ (%)
	Plants		
guar gum	β-1,4-D-Man$_2$-α-1,6-D-Gal (1,8)*	active	135 ± 9
CM-cellulose	β-1,4-D-Glc-CH$_2$COOH	active	167 ± 9
pectin	α-1,4-D-Gal-UA	active	202 ± 9
konjac	β-1,4-D-Man-β-1,4-D-Glc (1,6)*	(inactive)	123 ± 1
cellulose	β-1,4-D-Glc	inactive	104 ± 10
β-cyclodextrin	α-1,4-D-Glc	inactive	104 ± 10
alginate	β-1,4-D-Man-UA-α-L-Gul-UA	inactive	88 ± 6
locust bean gum	β-1,4-D-Man$_4$-α-1,6-D-Gal (4)*	inactive	98 ± 3
	Microorganisms		
alginate (*Pseudomonas*)	Ac-(β-1,4-D-Man-UA-α-L-Gul-UA)	active	132 ± 16
rhamsan (*Alcaligenes*)	⟨ ⟩-α-Ac-D-Glc-β-Ac-D-Glc	active	131 ± 10
xanthan (*Xanthomonas*)	β-D-Glc$_2$-β/α-D-Man$_2$-β-Glc-UA	active	152 ± 9
chitosan (*Mucor*)	β-1,4-N-Ac-D-Glc-amine	active	130 ± 4
curdlan (*Agrobacterium*)	β-1,3-D-Glc	(inactive)	120 ± 9
levan (*Zymomonas*)	β-2,6-Frc-1,6-Rha$_{12\%}$	(inactive)	122 ± 9
gellan (*Pseudomonas*)	⟨ ⟩	inactive	96 ± 8
welan (*Alcaligenes*)	⟨ ⟩-α-L-Rha oder -α-L-Man	inactive	90 ± 4

[a] Abbreviations and symbols: ⟨ ⟩, β-1,3-D-Glc-β-1,4-D-Glc-UA-β-1,4-D-Glc-α-1,4-L-Rha; Ac, acetyl; Frc, fructose; Gal, galactose; Glc, glucose; Gul, gulose; Man, mannose; Rha, rhamnose; UA, uronic acid; ( )*, (Man:Gal) or (Man:Glc); underlined, active molecules and glycosidic bonds.

(Table 1). Compounds inducing anthraquinone concentrations >120% (control = 100%) were considered as active elicitors.

The data indicate that the elicitor effects of polysaccharides derived from microorganisms can be correlated with the presence of acetyl groups in their molecules. Uronic acid containing polysaccharides such as pectin and xanthan proved to be effective inducers of anthraquinone synthesis. We could not observe an elictor effect of plant-derived alginate. This was surprising because the structure of polyguluronates in alginate is closely related to that of polygalacturonates in pectin. These results indicate an increased secondary metabolite production induced by positive or negative charges of polysaccharides or based on their linear or helical structure. These findings are confirmed by comparison of the effects of cellulose and (carboxymethyl)cellulose (Table 1). On the other hand, the degree of branching (number of side chains of the polymer) or the high number of galactose units in the case of guar gum (Table 1) resulted in metabolite-inducing effects. This was in contrast to findings with locust bean gum (low degree of branching), which did not stimulate anthraquinone synthesis. *M. citrifolia* derived pectins combine properties which seem to be signaling factors for an efficient elicitor. We observed elicitor effects as a result of α-glycosidically bound polysaccharide chains as found in pectin molecules. In alginates, linear and helical forms alternate within the chain and, in fact, the elicitor effect on anthraquinone production was missing. Konjac, which has been shown to have limited elicitor effects, is a linear polysaccharide without side chains. Since plant cell cultures of *M. citrifolia* did not develop into large cell aggregates, they most likely produced only a primary cell wall. This might be one reason for stimulating effects on secondary metabolism by guar gum, which possesses one side chain per two mannose units, in contrast to locust bean gum with only one side chain per four mannose units (Table 1). Differences in elicitor activity of guar gum and locust bean gum could be due to effects on different pectin structures, i.e., short side chains and highly esterified galacturonic acids in the middle lamella of plant cell walls and highly branched rhamnogalacturonans in the primary cell wall (Hwang et al., 1993).

The role polysaccharides, e.g., alginate from *Pseudomonas*, chitosan, rhamsan, xanthan, or pectin, play in plant–microorganism interaction must also be considered. Pectin

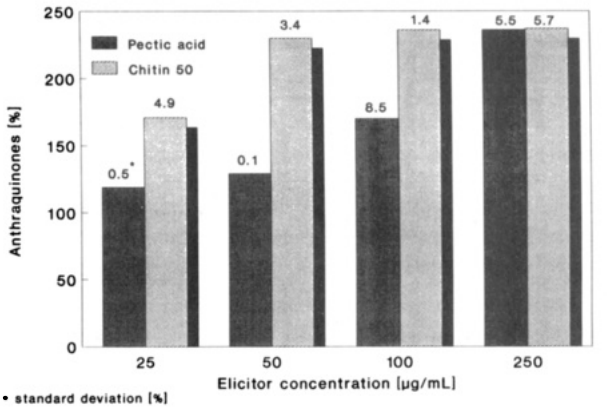

Figure 1. Effect of elicitor concentration on anthraquinone production in *M. citrifolia* cell cultures after 7 days of incubation (control = 100%).

and chitosan gave the best results by activating secondary metabolism in *M. citrifolia* cultures and were selected for further investigations.

Effect of Elicitor Concentration on Anthraquinone Production. A comparison of pectic acid and Chitin 50 showed an effect of Chitin 50 on anthraquinone biosynthesis at concentrations lower than that of pectic acid. Concentrations higher than 50 μg/mL Chitin 50 did not result in a further increase of anthraquinone production, while raising the concentration of pectic acid was correlated with increased anthraquinone production (Figure 1).

The different responses of *M. citrifolia* cultures to pectins and Chitin 50 might be the result of specific plant defense strategies as exemplified in Figure 2. The initial reaction of plant cells to elicitor treatment seems to be an increase in the synthesis of hydrolytic enzymes, such as chitinases and lysozymes, to counterattack pathogens. In such cases the products of enzymatic hydrolysis of fungal cell walls are suspended chitin fragments, which can act as exogenous elicitors initiating plant-derived synthesis of phytoalexins (such as anthraquinones), antibiotics able to suppress microbial growth (Dörnenburg and Knorr, 1994).

Chitosans as Elicitors. The degree of acetylation of chitosan or chitin was found to be important in inducing defense metabolisms in plant cell cultures (Table 2). Preliminary studies indicated a lesser effect of origin of chitosan than of its similarity to chitin molecules, which

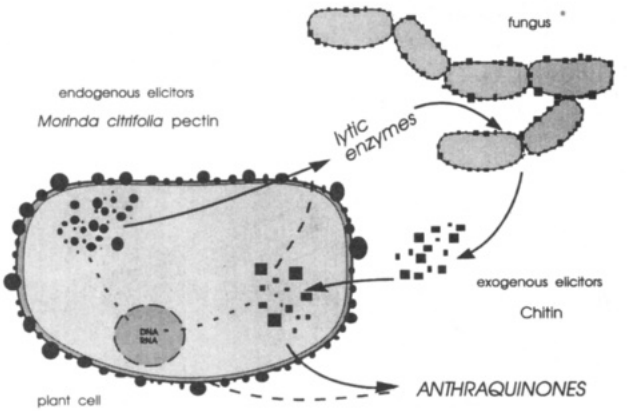

Figure 2. Suggested model for effects of endogenous and exogenous elicitors on plant cell cultures.

Table 2. Elicitor Effect of Various Chitosans and Chitin on Anthraquinone Synthesis (AQ) in *M. citrifolia* Cultures after 14 Days of Incubation

elicitor[a]	degree of acetylation (%)	AQ (%)
control		100 ± 2
chitosan		
Sea Cure 143 from *Mucor rouxii*	14	137 ± 4
	21	130 ± 4
Sea Cure+	26	144 ± 4
BLV	28	154 ± 9
chitin		
Chitin 50	53	254 ± 7

[a] Concentrations from 25 to 100 μg/mL with highest elicitor activity (higher concentrations resulted in inhibition of anthraquinone synthesis or cell death).

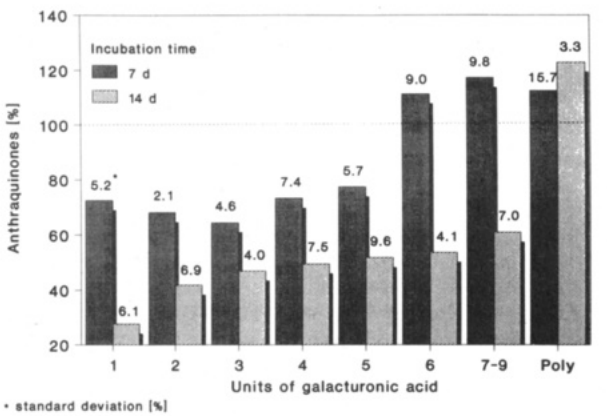

Figure 3. Effect of degree of polymerization of galacturonic acid (250 μg/mL) on anthraquinone production in *M. citrifolia* cell cultures after 7 and 14 days of incubation (control = 100%).

are the key cell wall polysaccharides for signal recognition in fungi. In contrast to data given by Kauss et al. (1989), our results led to the supposition that positively charged glucosamine molecules of chitosans were not solely responsible for elicitor effects.

Pectins as Elictors. Examinations of different galacturonic acid oligomers regarding their role in inducing anthraquinone synthesis in *M. citrifolia* showed a decrease in production when galacturonic acid with a short chain length of one to five units was applied. As shown in Figure 3, only oligomers with more than five units caused elicitor activities.

After 14 days of elicitation oligogalacturonic acids with a degree of polymerization of one to nine units did not result in stimulating effects on anthraquinone synthesis. They inhibited secondary metabolite synthesis but did not affect growth of the cell culture. However, application

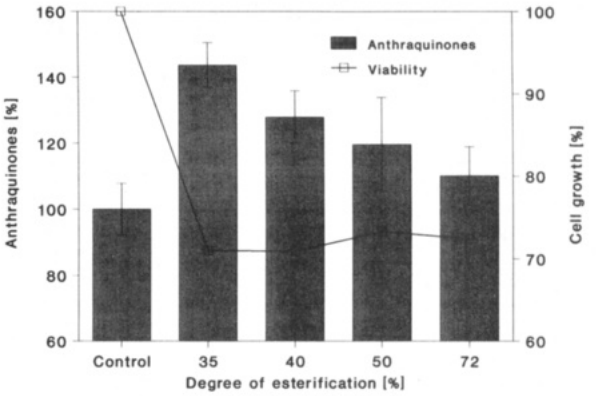

Figure 4. Effect of degree of esterification of citrus pectin (250 μg/mL) on anthraquinone production in *M. citrifolia* cell cultures after 7 days of incubation.

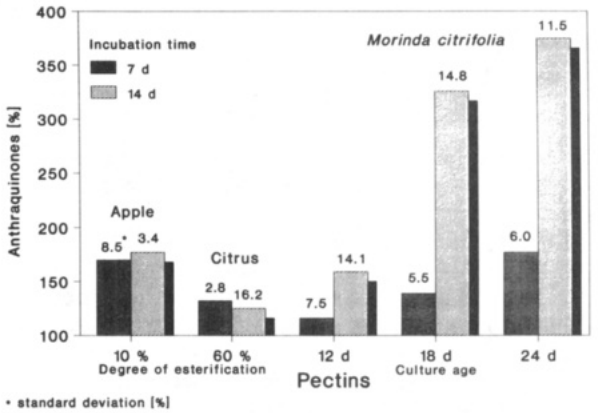

Figure 5. Stimulation of anthraquinone synthesis in *M. citrifolia* cultures by different pectins (100 μg/mL) after 7 and 14 days of incubation (control = 100%).

of poly(galacturonic acid) to the cell culture caused a continuous increase of anthraquinone synthesis during incubation time (Figure 3). This indicates a degradation of the oligomers and polymers to active elicitors or to inactive ones such as monomers or oligomers by polygalacturonase activities in the cell culture (Dörnenburg, 1993). These effects are in agreement with data provided by Bruce and West (1982) and by Nothnagel et al. (1983). These authors also observed a degradation of elicitor active cell wall fragments from plants into shorter but inactive oligomers and other degradation products by pectinolytic enzymes.

Elicitation of anthraquinone synthesis was dependent on the degree of esterification of citrus pectins (Figure 4) and apple pectins (data not shown). Concentrations of 250 μg of citrus pectin/mL of medium increased anthraquinone synthesis in *M. citrifolia* and inhibited cell growth after 7 days of incubation (Figure 4). Cell growth as well as anthraquinone production was not affected during an incubation time of 2 weeks (data not shown). However, we observed an increase in secondary metabolite production during the first week of incubation after elicitation with pectin.

A very important aspect for increasing anthraquinone production has been the kind of pectin used as elicitor. A comparison of elicitor effects of apple pectin, citrus pectin, and pectins derived from *M. citrifolia* cell cultures on anthraquinone synthesis is presented in Figure 5. Elicitation was dependent on the source, degree of esterification, or the age of the cultures from which cell wall pectins had been isolated. Pectins isolated in the stationary phase (from 24-day-old cultures) seemed to have a lower degree of esterification than pectins from the exponential phases.

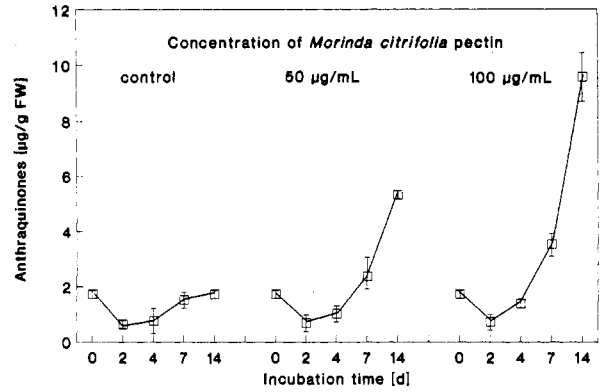

Figure 6. Elicitation of anthraquinone synthesis by cell wall pectin isolated from *M. citrifolia* cultures.

This might be the result of esterase activities which are also involved in fruit ripening. After 14 days of incubation, a significant increase in anthraquinone concentration was observed within the cells. This might also have been the result of induction of polygalacturonase activity (Dörnenburg, 1993) followed by a degradation of the pectins to elicitor active fragments.

Application of cell wall pectins isolated from a 24-day-old suspension culture (late exponential growth phase) led to a maximum of anthraquinone production (Figures 5 and 6). Anthraquinone concentration within the cells decreased during the initial 4 days of incubation primarily due to enlargement of cell size. After this lag phase, the concentration of anthraquinones in *M. citrifolia* cultures increased. Pectin concentrations of 100 µg/mL of medium caused a stimulated anthraquinone production in *M. citrifolia* cultures after 4 days of incubation. After an incubation time of 2 weeks, anthraquinone concentrations 5.6 times higher than the control could be observed (Figure 6).

Conclusions. The effectiveness of polysaccharides as active elicitors was dependent on their molecular structures. Negative charges exerted by acetyl groups in microbial polysaccharides (alginate, chitosan, and rhamsan) or by carboxyl groups within microbial as well as plant polysaccharides (xanthan and pectin) were able to elicit plant cell response to a higher degree than positive charges of amino groups in chitosan. A comparison of elicitor effects by alginate and pectin revealed the importance of glycosidic bonds within polysaccharides. Linear molecules with β-glycosidically bound saccharides were less active than helical structures which are the result of α-glycosidically bound polymers. Polysaccharides with a high degree of branching were more active than those with a lower number of side chains.

The elicitor effect of pectin depended on the degree of esterification and the degree of polymerization of galacturonic acid. Low degrees of esterification led to negative charges in the polymer, resulting in increased anthraquinone synthesis. Chain lengths between 10 and 13 of α-1,4-linked oligogalacturonides have been shown to induce accumulation of phytoalexins. Oligogalacturonides of shorter chain length did not possess this effect (Cervone et al., 1989). We observed a signal recognition when the oligogalacturonic acid chain was larger than five units. Shorter molecules are known to be elicitor-inactive (Nothnagel et al., 1983; Jin and West, 1984). An increase of anthraquinone production over time was observed by elicitation with poly(galacturonic acid) and pectins derived from *M. citrifolia* cell walls. Our hypothesis is based on degradation of these elicitors by polygalacturonases from the cell cultures into more active poly(galacturonic acid) fragments. However, the degradation of pectin fragments also led to a decrease of anthraquinone synthesis due to the presence of oligogalacturonic acids with chain lengths shorter than six units.

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