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Ethylene regulation of β-1,3-glucanase in tobacco

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Abstract. Ethylene treatment (approx. 20 $\mu l \cdot l^{-1}$ in air for 2 d) of tobacco (*Nicotiana tabacum* L. cv. Havana 425) plants markedly increases the endo-β-1,3-glucanase (EC 3.2.1.39) content of leaves. The antigenic form of the enzyme induced is the same one whose production is blocked by treating cultured cells with combinations of auxin ($1.1 \cdot 10^{-5}$ M α-naphthaleneacetic acid) and cytokinin ($1.4 \cdot 10^{-6}$ M kinetin). Evidence is presented that cultured tobacco cells require ethylene for β-1,3-glucanase accumulation: i) ethylene treatment increased the accumulation of β-1,3-glucanase in callus tissues >10 d after subculturing and in cell-suspension cultures; ii) callus tissues can produce ethylene; iii) conditions known to inhibit ethylene production (1 mM $CoCl_2$; 33° C treatment) or ethylene action (approx. 1.6 $mmol \cdot l^{-1}$ norbornadiene in air) inhibited β-1,3-glucanase accumulation by callus tissues treated for 4 d following subculturing; and, these inhibitory effects were prevented by exogenous ethylene. Combinations of auxin and cytokinin blocked ethylene-induced accumulation of β-1,3-glucanase by cell-suspension cultures. The results favor a model in which ethylene induces β-1,3-glucanase accumulation, and auxin and cytokinin inhibit this induction process.

Key words: Auxin and ethylene action – Cell culture (ethylene, β-1,3-glucanase accumulation) – Cytokinin and ethylene action – Ethylene (glucanohydrolase-regulated) – β-1,3-Glucanase – *Nicotiana*.

Introduction

Enzymes with endo-β-1,3-glucanase (EC 3.2.1.39) activity are widely distributed in species of higher plants (reviewed by Ballance and Manners 1978). Treatment of tomato and bean plants with the "stress" hormone ethylene increases the β-1,3-glucanase activity in leaves (Abeles and Forrence 1970; Pegg 1976; Boller et al. 1983). Activity of this enzyme is also induced in parts of plants infected by viral, bacterial, and fungal pathogens (reviewed by Pegg 1977). This observation and the fact β-1,3-glucans are components of fungal cell walls (Bartnicki-Garcia 1968) has led to the hypothesis that β-1,3-glucanase is important in the defense reaction of plants (reviewed by Boller 1985).

Earlier, we purified a specific endo-β-1,3-glucanase which is a major component (approx. 5%) of the soluble-protein fraction prepared from cultured tobacco cells and from the lower leaves and roots of tobacco plants (Felix and Meins 1985, 1986). Production of this enzyme by cultured tobacco tissues is regulated by auxin and cytokinin. The contents of β-1,3-glucanase and its mRNA increase by up to 10- and 20-fold, respectively, over a 7-d period when callus tissues are transferred from a medium containing the auxin α-naphthaleneacetic acid (NAA) and the cytokinin kinetin to a medium with either or both growth regulators omitted (Mohnen et al. 1986; Felix and Meins 1986). Mixtures of auxin and cytokinin added to the culture medium completely block this induction.

The question arises as to whether or not the β-1,3-glucanase regulated in culture is induced as part of the response of the intact plant to ethylene and pathogens. Here we show that the specific β-1,3-glucanase purified from cultured tissues is induced in plants treated with ethylene and provide evidence that ethylene is also required for induction of this enzyme in cultured cells.

Materials and methods

Plant materials and tissue culture. All tissues and organs used in this study were from *Nicotiana tabacum* L. cv. Havana 425

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Abbreviations: NAA = α-naphthaleneacetic acid; NDE = norbornadiene

plants grown from seed in a greenhouse. The cloned tissue-culture line 275N was isolated from pith-parenchyma tissue (Eichholz et al. 1983). Stock tissues of 275N were cultured as described earlier (Meins and Lutz 1980) on a basal medium consisting of the concentrations of salts, thiamine, and *myo*-inositol recommended by Linsmaier and Skoog (1965), 10 $g \cdot l^{-1}$ agar ("hochrein" grade; Merck, Darmstadt, FRG) and 5.0 $mg \cdot l^{-1}$ chlorophenol red (Eastman Kodak, Rochester, N.Y., USA) supplemented with $1.1 \cdot 10^{-5}$ M NAA and $1.4 \cdot 10^{-6}$ M kinetin. Suspension cultures were shaken at 125 $rev \cdot min^{-1}$ in 125 ml screw-capped Delong flasks (Bellco, Vinesland, N.J., USA) containing 40 ml of liquid medium. The liquid medium used was the standard cytokinin + auxin medium with agar omitted and 4% (w/v) sucrose (Binns and Meins 1973). The cultures were transferred at 14-d intervals using 4.0 ml of an inoculum filtered through a 150-μm pore-size sieve. The cell-suspension line S275N was obtained by serially transferring shake cultures of 275N tissue and filtering the inoculum through a 150-μm pore-size sieve at each transfer.

For experimental treatments of 275N, tissues were precultured for 7 d on the complete auxin + cytokinin medium and then subcultured for various lengths of time on basal medium supplemented as indicated with NAA and kinetin. Tissues were incubated either in shell vials covered with Kaput closures containing 10 ml of medium (Meins and Lutz 1980) or in 94- or 145-mm-diameter, plastic Petri dishes (Greiner, Nuertingen, FRG) sealed with Parafilm (American Can Co., Greenwich, Conn., USA) containing 10 ml of medium per piece of tissue. For experimental treatments of S275N, stock cultures were washed four times by centrifugation at 700 · *g* and resuspension in liquid basal medium. Delong flasks (125 ml) containing 50 ml of test medium were inoculated with approx. 3 ml packed-volume of cells and shaken at 125 rev. min^{-1}.

Preparation of protein extracts. Frozen tissues were homogenized with a Polytron homogenizer (Kinematica, Kriens, Switzerland) in 2 to 3 volumes of extraction buffer containing 0.1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 200 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl, pH 8.0. Suspension cultures were harvested by centrifugation (700 · *g*), frozen at −80° C, and then shaken at room temperature for 45 min in 2 to 3 volumes of the homogenization medium. Soluble protein fractions were obtained by centrifuging the tissue extracts for 15 min at 10000 · *g*. Protein was measured by a modification of the Bradford (1976) method for small samples (Felix and Meins 1985) using bovine gamma globulin (Bio-Rad, Glattbrugg, Switzerland) as a standard.

Treatment with ethylene and norbornadiene. Ethylene was added to shell vials and Delong flasks sealed with rubber closures (Werthemann, Basel, Switzerland) to give a concentration in the air space of 10 to 20 $\mu l \cdot l^{-1}$. Norbornadiene (NDE) was added to chambers containing callus cultures in plastic Petri dishes to give concentrations in the air space of approx. 0.4 to 3.0 $mmol \cdot l^{-1}$.

Ethylene measurements. Ethylene was measured in 1-ml samples of gas with a GC-RIA gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an aluminum oxide column and a flame-ionization detector connected to a GC-Processor (RRP-G1; Shimadzu). The column was calibrated using the standard gas mix ("Can mix 3"; Supelco Inc., Bellefonte, Pa., USA).

Assays for β-1,3-glucanase. β-1,3-Glucanase was measured by "rocket" immunoelectrophoresis (Laurell and McKay 1981) using the authentic protein purified from 275N tissue as standard (Felix and Meins 1985). The activity of β-1,3-glucanase was estimated reductiometrically as described earlier (Felix and Meins 1985).

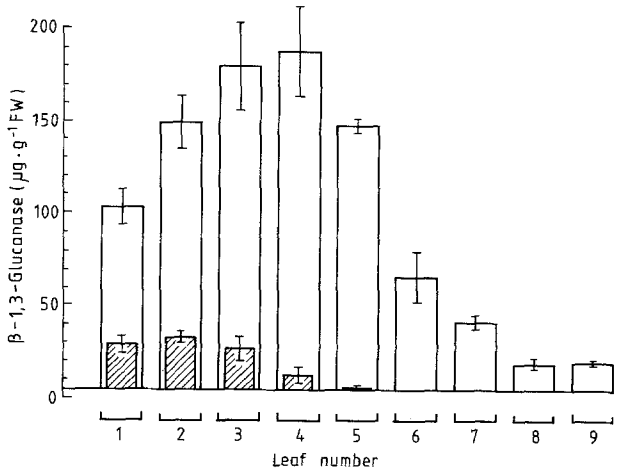

Fig. 1. Ethylene induction of β-1,3-glucanase in Havana 425 tobacco leaves. Plants incubated for 2-d in air (*cross-hatched bars*, <0.05 $\mu l \cdot l^{-1}$ ethylene) or in air supplemented with 20 $\mu l \cdot l^{-1}$ ethylene at the start of the experiment (*open bars*). *Horizontal line*, limit of detection, 4 μg β-1,3-glucanase $\cdot g^{-1}$ FW; *error bars*, ±SE for leaves from three replicate plants. Leaves are counted from the bottom of the plant

Results

Induction of β-1,3-glucanase in tobacco leaves by ethylene. Two-month-old tobacco plants were divided into three groups. The first group served as a zero-time control. The second group was incubated for 2 d in chambers containing approx. 20 $\mu l \cdot l^{-1}$ ethylene in air. An additional control group was incubated for 2 d in chambers without added ethylene. No ethylene (<0.05 $\mu l \cdot l^{-1}$) was detected in the control chambers either initially or after 2 d incubation. The β-1,3-glucanase content of protein extracts prepared from leaves was measured by "rocket" electroimmunoassay and expressed on a fresh weight (FW) basis. Plants in the zero-time and 2-d control groups exhibited the distribution of β-1,3-glucanase described earlier (Felix and Meins 1986). The enzyme content was below the limit of detection (<4 $\mu g \cdot g^{-1}$ FW) in young leaves near the top of the plant, increased with leaf age, and was high in leaves near the bottom of the plant (Fig. 1). Ethylene treatment markedly increased the β-1,3-glucanase content of all the leaves assayed. The greatest induction, approx. 150 $\mu g \cdot g^{-1}$ FW, was found in leaves 3 and 4 counting from the bottom of the plant. These results show that the antigenic form of β-1,3-glucanase regulated by auxin and cytokinin in cultured

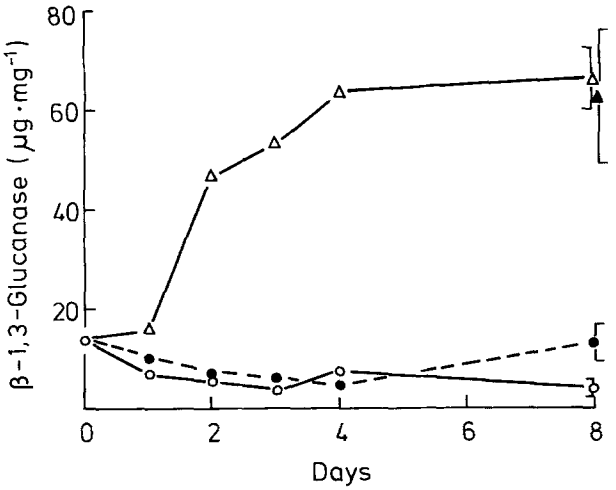

Fig. 2. The effect of exogenous ethylene on the accumulation of β-1,3-glucanase by early callus cultures of 275N tobacco tissue incubated on hormone-free medium (△, ▲) or auxin + cytokinin medium (○, ●). Cultures were incubated in air (△, ○) or with ethylene at an initial concentration of approx. 10 μl·l^{-1} (▲, ●). β-1,3-glucanase is expressed on a soluble-protein basis. Values after 8-d incubation are the means of four replicate cultures; *error bars*, ±SE

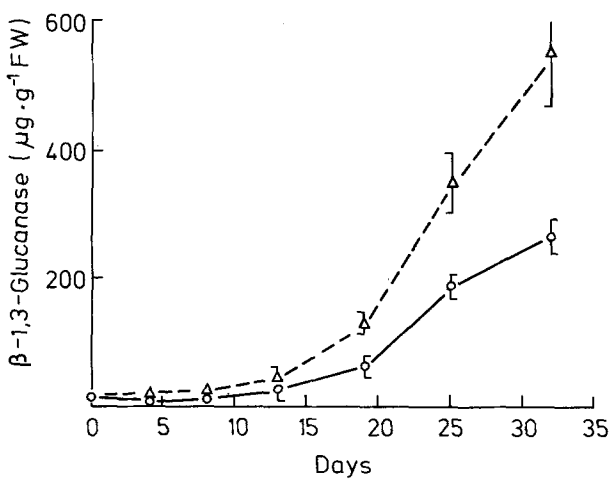

Fig. 3. Effect of exogenous ethylene on the β-1,3-glucanase content of 275N tobacco tissue in long-term culture. Tissues were incubated on auxin + cytokinin medium with (△—△) and without (○—○) approx. 10 μl·l^{-1} ethylene added. *Error bars*, ±SEM for three replicate cultures

tissues can also be induced in leaves by ethylene treatment.

The effect of ethylene on cultured tissues. The first series of tissue-culture experiments was performed with "early" cultures, i.e. 275N tissues 8 d after subculturing, in which auxin + cytokinin completely blocks β-1,3-glucanase accumulation (Felix and Meins 1985). Tissues were incubated under inductive conditions (hormone-free medium) and non-inductive conditions (auxin + cytokinin medium). One set of cultures was treated with approx. 10 μl·l^{-1} ethylene in sealed vials. A control set of cultures was incubated in loosely-capped vials. The ethylene content in the air space above the control tissues remained below the limit of detection, <0.05 μl·l^{-1}. Expressed on a per mg protein basis, the β-1,3-glucanase content of induced tissues increased approx. fivefold over an 8-d period. During the same period the β-1,3-glucanase content of non-induced tissues remained at a constant, low level (Fig. 2). Ethylene treatment did not significantly affect enzyme accumulation, although a small increase was observed in tissues incubated on non-inductive medium for 8 d with ethylene. Similar results were obtained when β-1,3-glucanase activity was measured, indicating that ethylene did not alter the specific activity of the enzyme or induce other, immunologically distinct forms of the enzyme. Tissues in the early phase of culture do not appear to exhibit the ethylene response observed with leaves in the intact plant.

The effectiveness of auxin and cytokinin in blocking β-1,3-glucanase accumulation depends on the length of the culture period (Eichholz et al. 1983; Felix 1985). In the period from approx. 10 d to 32 d after subculturing, tissues accumulate very high concentrations of β-1,3-glucanase, up to 15–20% of the soluble protein, even when incubated on auxin + cytokinin medium. To find out whether tissues in this "late" phase respond to ethylene, we compared the β-1,3-glucanase content of tissues incubated for up to 32 d on auxin + cytokinin medium with and without ethylene added. Ethylene enhanced the β-1,3-glucanase accumulation of the aged tissues giving concentrations up to twofold higher than in control cultures after 32 d (Fig. 3). This represented a net accumulation since treated and control tissues showed the same increase in FW, 391 ± 34-fold and 365 ± 11-fold, respectively, after 32 d of incubation. Therefore, callus tissues can respond to ethylene by accumulating β-1,3-glucanase and this inductive effect depends on the age of the tissue.

We found that β-1,3-glucanase accumulation by short-term cell-suspension cultures could be regulated by combinations of ethylene, auxin and cytokinin. Suspension cultures of 275N tobacco cells containing different combinations of auxin and cytokinin were incubated with and without approx. 15 μl·l^{-1} ethylene added to the air phase at the start of the experiment. The suspension cultures with ethylene added exhibited the auxin and

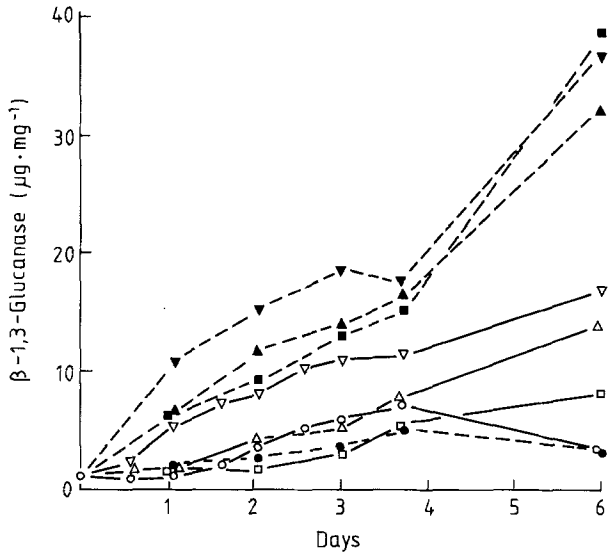

Fig. 4. Effect of exogenous ethylene and combinations of auxin and cytokinin in the culture medium on the accumulation of β-1,3-glucanase by suspensions of S275N tobacco cells. Cells were incubated in sealed flasks containing hormone-free medium (▽), auxin medium (△), cytokinin medium (□), or auxin + cytokinin medium (○). *Filled symbols* connected by broken lines indicate cultures treated with 10–20 $\mu l \cdot l^{-1}$ ethylene. β-1,3-Glucanase content is expressed on a soluble-protein basis as the mean of two replicate cultures

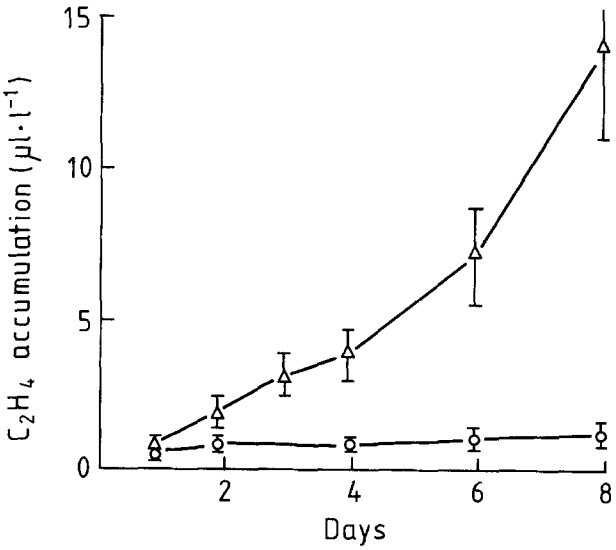

Fig. 5. Ethylene accumulation in sealed vials containing 275N tobacco tissue. Incubation on hormone-free medium (○–○) and auxin + cytokinin medium (△–△)–. *Error bars*, ±SE for four replicate cultures

cytokinin response typical of early callus cultures (Felix and Meins 1985). When cells were incubated in media with auxin, cytokinin, or both hormones deleted, the β-1,3-glucanase content per mg protein increased by up to 40-fold over 6 d (Fig. 4). This induction was markedly inhibited by auxin + cytokinin added to the medium. The suspension cultures without ethylene added also accumulated β-1,3-glucanase in the inductive media, but accumulation was reduced by 60–80%. These results show that ethylene can induce β-1,3-glucanase accumulation in early suspension cultures and that this induction is blocked by mixtures of auxin and cytokinin added to the medium.

Ethylene production by early callus cultures. It is possible that early callus cultures fail to respond to ethylene because they produce sufficient endogenous ethylene to permit β-1,3-glucanase accumulation. We measured the ethylene content of the air phase of cultures incubated on basal medium and on auxin + cytokinin medium. One set of cultures used for the β-1,3-glucanase measurements shown in Fig. 1, was maintained in shell vials with loosely-fitting caps. A second set of cultures was maintained in shell vials sealed with rubber caps or Parafilm. No ethylene (< 0.05 $\mu l \cdot l^{-1}$) was detected in the air phase of the loosely capped cultures. Accumulation of ethylene in the air phase of the sealed cultures depended on the hormonal constitution of the culture medium (Fig. 5). The ethylene content of cultures on basal medium was approx. 1 $\mu l \cdot l^{-1}$ and increased slightly during the incubation period. The ethylene content of cultures on hormone-containing medium, on the other hand, increased markedly throughout the culture period and was 16-fold higher after 8 d than that of the cultures maintained on basal medium. This large effect could not be accounted for by the slight differences in growth, approx. 20% lower FW after 8 d on basal medium than on auxin + cytokinin medium. In other experiments (Felix 1985) we confirmed that the effect on ethylene accumulation was primarily due to auxin, as reported by others (e.g., Huxter et al. 1979; Miller and Pengelly 1984).

Studies with ethylene inhibitors. The experiments reported so far show that cultured 275N tobacco tissues can produce ethylene and that exogenous ethylene can increase β-1,3-glucanase in early cell-suspension cultures and late callus cultures. To find out whether or not ethylene is required for β-1,3-glucanase accumulation, early callus cultures were treated with known inhibitors of ethylene production and ethylene action. Callus tissues were precultured for 7 d on auxin + cytokinin medium, incubated for 4 d on basal medium with and without inhibitor, and then assayed for β-1,3-glucanase content. The data were expressed both as μg enzyme per mg protein and as total enzyme accumu-

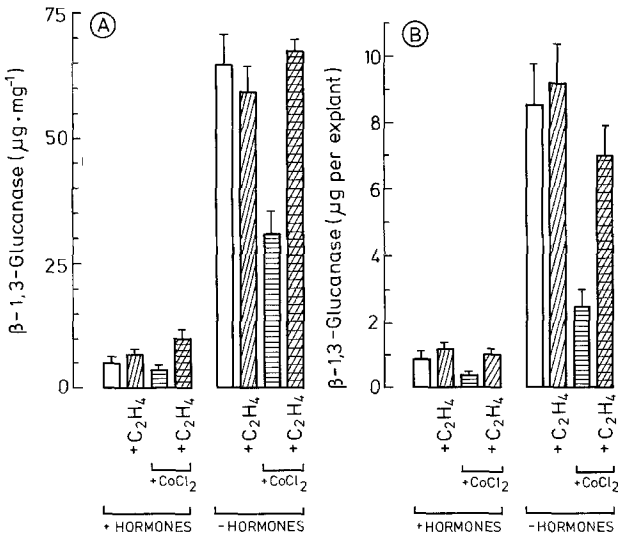

Fig. 6A, B. Effect of $CoCl_2$ on β-1,3-glucanase accumulation by 275N tobacco tissue expressed on a soluble-protein (**A**) and per-explant (**B**) basis. Tissues were incubated for 4-d as indicated on hormone-free medium (−Hormones), on auxin+cytokinin medium (+Hormones), with ($+C_2H_4$) and without ($-C_2H_4$) ethylene, and with ($+CoCl_2$) and without ($-CoCl_2$) 1 mM $CoCl_2$ in the medium. *Error bars*, ±SE for three replicate samples

Table 1. Effect of norbornadiene and 33° C treatment on β-1,3-glucanase accumulation by callus cultures of 275N tobacco tissue

Medium	Treatment[a]	β-1,3-Glucanase content (μg) per:	
		mg protein	explant
Auxin +cytokinin	Zero time	8.5±0.6[b]	0.57±0.12
	26° C	5.1±1.0	0.87±0.21
	26° C+C_2H_4	6.8±0.8	1.19±0.19
	26° C+NDE	2.9±0.5	0.40±0.04
	26° C+NDE+C_2H_4	3.6±0.5	0.74±0.15
	33° C	4.2±0.9	0.35±0.12
	33° C+C_2H_4	3.4±0.1	0.41±0.05
Basal	26° C	64.7±5.9	8.54±1.20
	26° C+C_2H_4	59.3±5.0	9.20±1.15
	26° C+NDE	27.8±1.2	3.74±0.42
	26° C+NDE+C_2H_4	47.6±3.4	5.90±0.70
	33° C	14.8±3.0	1.45±0.36
	33° C+C_2H_4	56.7±14.2	3.52±0.94

[a] Tissues incubated in sealed Petri dishes for 4-d supplemented as indicated with 10 $\mu l \cdot l^{-1}$ ethylene (+C_2H_4) and 1.6 $mmol \cdot l^{-1}$ norbornadiene (+NDE)

[b] Mean±SE for tissues from three replicate Petri dishes

lation per callus explant. Parallel experiments were performed with and without approx. 10 $\mu l \cdot l^{-1}$ ethylene added, to distinguish between general toxic effects and ethylene-related effects.

Cobaltous ion is known to inhibit ethylene biosynthesis (Lau and Yang 1976). We verified that 1 mM $CoCl_2$ blocked accumulation of ethylene by at least 96% in cultures of 275N tissue incubated with and without auxin+cytokinin added. $CoCl_2$ treatment also inhibited β-1,3-glucanase accumulation by 60% in tissues incubated on hormone-free medium (Fig. 6). This inhibition was completely reversed by exogenous ethylene. Similar results were obtained when β-1,3-glucanase content was expressed on a per-explant basis indicating that $CoCl_2$ did not have nonspecific effects on growth over the short period of the experiment. Other experiments showed that $CoCl_2$ inhibition and reversal by ethylene could also be obtained when tissues were incubated on media containing auxin or cytokinin added separately.

High-temperature treatment is also known to inhibit ethylene biosynthesis (Yu et al. 1980). We verified that raising the culture temperature from 26° C to 33° C inhibited ethylene accumulation by 88–93% and inhibited the accumulation of β-1,3-glucanase by tissues incubated on hormone-free medium (Table 1). Reversal of this effect by ethylene was variable. The reversal was nearly complete when the data were expressed on a protein basis; however, 33° C treatment also had marked nonspecific effects. Growth of Havana 425 tobacco tissues is greatly inhibited at 33° C (Meins 1975) and there was only slight ethylene reversal when the data were expressed as total enzyme per explant.

Norbornadiene (NDE) has been reported to have "anti-ethylene" effects (Sisler and Yang 1984). We found NDE to have a narrow effective range. An initial concentration of approx. 0.45 $mmol \cdot l^{-1}$ in the air space did not block β-1,3-glucanase accumulation and a concentration of approx. 2.3 $mmol \cdot l^{-1}$ was very toxic. At a concentration of approx. 1.6 $mmol \cdot l^{-1}$, NDE inhibited β-1,3-glucanase accumulation by approx. 60% (Table 1). This effect was partially reversed by ethylene, approx. 80% and approx. 60%, when β-1,3-glucanase content was expressed on a protein and explant basis, respectively.

Discussion

Treatment of bean and tomato plants with ethylene increases the β-1,3-glucanase activity in leaves (Abeles and Forrence 1970; Pegg 1976; Boller et al. 1983). The present experiments show that in tobacco ethylene increases the β-1,3-glucanase content of leaves. Moreover, the antigenic form induced is the same one regulated by auxin and cytokinin in cultured cells.

Following ethylene treatment the β-1,3-glucanase content of older leaves increased to higher concentrations than in younger leaves near the top of the plant. This indicates that factors other than ethylene determine the basic distribution of the enzyme in leaves. One possibility is that auxin and cytokinin, which block production of β-1,3-glucanase in cultured cells, act in the same way in the intact plant. These hormones are thought to be present at higher concentrations in young leaves than in leaves near the bottom of the plant (Pengelly and Meins 1983; Letham 1978), i.e. the gradients of hormones and β-1,3-glucanase are inversely correlated as predicted by our working hypothesis. Reports of direct effects of these hormones on β-1,3-glucanase in intact plants and detached leaves are conflicting. The increase in enzyme activity during abscission of bean petioles was delayed by treatment with auxin and cytokinin (Abeles and Forrence 1970). Cytokinin inhibited enzyme activity in senescing leaves of *Nicotiana glutinosa*, whereas auxin was not effective (Moore and Stone 1972). Auxin treatment of pea epicotyls slightly decreased β-1,3-glucanase activity (Davies and Maclachlan 1968). High concentrations of the auxin indole-3-acetic acid increased enzyme activity in pea pods (Mauch et al. 1984).

There are three lines of evidence that ethylene is required for β-1,3-glucanase accumulation by cultured tobacco cells. First, cultured tissues can produce ethylene. Second, ethylene treatment can promote β-1,3-glucanase accumulation by cultured tissues. Finally, known inhibitors of ethylene production ($CoCl_2$ and 33° C treatment) and ethylene action (NDE) also inhibit β-1,3-glucanase accumulation by early callus cultures. These treatments had general toxic effects that were most conspicuous when the data were expressed on a per-explant basis, and interpretation of the 33° C experiments is complicated by the effect of temperature on the relative solubility of ethylene in air and in tissues. Nevertheless, in each case, ethylene treatment partially or completely reversed the inhibition. Ethylene appears to be necessary for the induction of β-1,3-glucanase in cultured cells.

The inhibitory effect of auxin and cytokinin on β-1,3-glucanase accumulation is not mediated by ethylene. Incubation of tissues on auxin + cytokinin medium had opposite effects on β-1,3-glucanase and on ethylene accumulation. Moreover, the ethylene inhibitors tested did not prevent the inhibitory effects of auxin and cytokinin.

The experiments with cell-suspension cultures provide direct evidence for an interaction of ethylene, auxin, and cytokinin in regulating β-1,3-glucanase accumulation. They show that ethylene is effective in promoting accumulation provided auxin, cytokinin, or both hormones are omitted from the culture medium. These results favor a two-step model in which ethylene induces β-1,3-glucanase, and auxin and cytokinin prevent the induction.

The molecular mechanism for ethylene induction of β-1,3-glucanase in tobacco is not known. There is indirect evidence to suggest ethylene acts at the mRNA level. First, ethylene induces the appearance of β-1,3-glucanase in upper leaves of the plant, which, in control plants, do not contain detectable β-1,3-glucanase mRNA (Shinshi et al. 1987). Second, auxin and cytokinin block both the ethylene effect and accumulation of β-1,3-glucanase mRNA in cultured cells (Mohnen et al. 1985). Finally, ethylene induces chitinase mRNA accumulation in bean and tomato plants (Broglie et al. 1985, 1986). The activities of β-1,3-glucanase and chitinase are coordinately induced by ethylene in these plants (reviewed by Boller 1985), and the mRNAs for the two glucanohydrolases are coordinately regulated in cultured tobacco cells (Shinshi et al. 1987). The cell-suspension system described here provides an experimental system well suited for testing the mRNA hypothesis and for investigating the interaction of auxin, cytokinin and ethylene in regulating β-1,3-glucanase.

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