Effect of Validamycin A on the Production of Cellulase, Xylanase and Polygalacturonase by *Rhizoctonia solani*

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Validamycin A decreased the biomass and extracellular protein produced by cultures of *Rhizoctonia solani* A79 grown on medium containing cellulose as the carbon source. In the presence of the antibiotic, the production of inducible enzymes active on filter paper (FPase), carboxymethylcellulose (CMCase) and (cellobiase) was decreased, but not the production of constitutive enzymes involved in hemicellulose (xylanase) and pectin (polygalacturonase) degradation. When incorporated in the assay mixtures, validamycin A did not affect the activity of the above enzymes, but the antibiotic did act as a substrate for cellobiase. Validamycin A did not affect the distribution of cellulase enzymes in stationary phase cultures of *R. solani*, and incorporation of inositol in the culture medium did not reverse the effect of the antibiotic on cellulase production. Addition of inositol to the medium decreased the activities of FPase and CMCase in the culture supernatant but had no effect on cellobiase activity. The results are discussed in relation to the way validamycin A controls rice sheath blight.

INTRODUCTION

Validamycin A is an antibiotic used in Japan and elsewhere to control *Rhizoctonia solani*, the causal agent of rice sheath blight. The antibiotic acts as a paramorphogen, reducing hyphal growth unit length (i.e. increasing branch frequency) without affecting the organism's specific growth rate (Robson *et al.*, 1988, 1989). The stimulatory effect of validamycin A on branching leads, *in vitro*, to the formation of dense colonies which expand in radius more slowly than untreated colonies (Nioh & Mizushima, 1974; Trinci, 1985; Robson *et al.*, 1988, 1989). The effect of validamycin on the morphology of mycelia of *R. solani* has been correlated with a decrease in the phosphatidylinositol content of the biomass (Robson *et al.*, 1989). Although neither fungicidal nor fungistatic, validamycin is able to control rice sheath blight by reducing the rate of spread of the pathogen from the lower to the upper regions of rice plants. In addition, the antibiotic reduces 'cushion' development and the rate of formation and development of fungal lesions (Wakae & Matsuura, 1975). Anatomical studies of plant tissue at the boundaries of fungal lesions revealed that, compared with lesions developed on untreated plants, validamycin A reduced the disintegration of parenchyma tissue (Endo & Wakae, 1983; Endo *et al.*, 1983), suggesting that the antibiotic might affect the production of extracellular enzymes (cellulase and pectinase) involved in tissue maceration. Although a previous *in vitro* study failed to demonstrate an effect of validamycin A on the production or activity of cellulase (Endo *et al.*, 1983), the antibiotic is known to inhibit the production by *R. solani* of β -1,3-glucan synthase, β -glucosidase and laminarinase (Uyeda *et al.*, 1985, 1986). Another paramorphogen, L-sorbose,

Abbreviations: CMCase, carboxymethylcellulase; FPase, enzyme with activity against filter paper; PGase, polygalacturonase.

has been shown to affect the production of β -glucosidase and cellulase by *Trichoderma reesei* (Bisaria *et al.*, 1986; Sahoo *et al.*, 1986; Nanda *et al.*, 1986). The purpose of the present study was to investigate the effects of validamycin A on the production of extracellular enzymes by *R. solani*.

METHODS

Organism and media. *Rhizoctonia solani* A79 was obtained from Dr A. Goulds, University of Manchester, and was maintained at 4 °C on malt extract agar (Lab M) slopes under paraffin oil. *R. solani* was grown at 25 °C in shake flask [250 ml conical flasks containing 50 ml volumes of Vogel's (1956) medium shaken at 200 r.p.m.] liquid culture with sixteen 1 × 1 cm squares of cellulose filter paper (Whatman no. 1) as the sole carbon source in each flask. Flasks were inoculated with 2 ml volumes of a mycelial macerate prepared by homogenizing *R. solani* biomass in distilled water. The resultant macerate was filtered through two layers of muslin and the suspension adjusted with water to give an optical density of 1.0 at 540 nm.

Enzyme preparation. Mycelia and undegraded cellulose were collected from cultures using a single layer of muslin and washed with 50 ml distilled water. The washed biomass was suspended in 20 ml ice cold buffer (0.1 M-citrate/phosphate buffer, pH 5) and homogenized for 30 s at high speed (MSE homogenizer). The resulting homogenate was sonicated (100 W, MSE M284 ultrasonic disintegrator) in an ice bath for periods of 30 s with 60 s gaps between successive sonications to avoid overheating the sample. The amount of protein released after each sonication was measured using the Bradford reagent (Bradford, 1976) and six successive cycles of sonication was found to give maximal protein release from the biomass (results not shown). The homogenate was centrifuged at 4 °C for 40 min at 13000 *g* and the supernatant (the soluble fraction) collected. This fraction was examined at a magnification of × 400 by light microscopy to verify that it was free of cell debris. The pellet was washed twice by suspending it in 20 ml ice cold buffer followed by centrifugation. Finally the pellet was suspended in 20 ml cold buffer (the insoluble fraction).

Biomass determinations. Due to the presence of undegraded substrate (cellulose), chitin was used as an indicator of fungal biomass (Ride & Drysdale, 1972).

Enzyme assays. Filter paper (FPase), carboxymethylcellulase (CMCase), xylanase and cellobiase activities were determined as described by Lowe *et al.* (1987). The reaction mixture was incubated at 40 °C and pH 5 to measure FPase activity, 50 °C and pH 5 to measure xylanase activity, 55 °C and pH 5 to measure cellobiase activity and 55 °C and pH 4 to measure CMCase activity. Polygalacturonase (PGase) activity was determined by incubating a 0.25 ml sample with 0.5 ml 0.1 M-citrate/phosphate buffer (pH 5) and 0.25 ml of a suspension (20 mg ml⁻¹) of polygalacturonic acid (Sigma) at 50 °C for 60 min in a reciprocal shaking water bath (150 strokes min⁻¹). The reaction was terminated by the addition of 2 ml modified dinitrosalicylic acid reagent and the reducing sugar was determined (Lowe *et al.*, 1987) after removal of undegraded polygalacturonic acid by centrifugation (MSE Microcentaur, high speed, 2 min). Enzyme activities are expressed as nkat (ml culture supernatant)⁻¹, nkat (mg culture supernatant)⁻¹, nkat (mg dry weight of biomass)⁻¹ or total nkat (katal = amount of enzyme activity that transforms 1 mol substrate s⁻¹).

Effect of validamycin and cellulose on enzyme activity. To determine the effects of validamycin A and cellobiose on enzyme activity, enzyme assays were performed in the presence of either 100 µM-validamycin A or cellobiose.

Temperature and pH optima of the enzymes. Enzyme activities were measured in the culture supernatant at pH 5 as a function of temperature over the range 20–60 °C for incubation times of 20–120 min. Enzyme activities were also measured at the optimum temperature over the pH range 2–9 in increments of 0.5 pH unit (in 0.1 M-citrate/phosphate buffer).

RESULTS

Temperature and pH optima of enzyme activities in the culture supernatant

The pH and temperature optima of the enzyme activities in the supernatant of stationary phase cultures of *R. solani* grown on medium containing filter paper as the carbon source were: FPase, pH 4.5–5.5, 40 °C; CMCase, pH 4, 55 °C; cellobiase, pH 5, 50 °C; xylanase, pH 5, 50 °C; PGase, pH 5–5.5, 50 °C.

Effect of validamycin A on biomass, extracellular protein production and enzyme production

Validamycin A at a concentration of 1 µM caused a significant ($P < 0.05$) reduction in the biomass of cultures of *R. solani* grown on filter paper (Table 1), but no further decrease in biomass was observed when the concentration of validamycin A in the medium was increased to 100 µM. Validamycin A also caused a significant ($P < 0.05$) decrease in the amount of

Table 1. *Effect of validamycin A on biomass concentration, extracellular protein concentration and pH of cultures of R. solani A79*

R. solani A79 was grown at 25 °C in shake flask culture in Vogel's medium containing filter paper as sole carbon source and in the presence of 0, 1, 25 or 100 µM-validamycin A. After 6 d, the biomass, extracellular protein and pH of the cultures were determined. Results represent the means of five replicate flasks ± SE.

Validamycin A concn (µM)	Estimated biomass per flask (mg)*	Extracellular protein (µg mg ⁻¹)†	Final pH of culture supernatant
0	61.7 ± 5.8	16.5 ± 1.3	6.30 ± 0.02
1	40.0 ± 2.5‡	14.9 ± 2.3‡	6.24 ± 0.03
25	38.4 ± 6.2‡	11.2 ± 2.0‡	6.43 ± 0.11
100	39.1 ± 4.0‡	2.9 ± 1.4‡	6.10 ± 0.04

* Chitin was used to estimate biomass and the results are expressed as mg mycelial dry weight.

† Results are expressed as µg protein (mg dry weight mycelium)⁻¹.

‡ Significantly (using the *t*-test, *P* < 0.05) different from the control lacking validamycin A.

Table 2. *Effect of validamycin A on the production of some extracellular enzymes of R. solani A79*

R. solani was grown at 25 °C in shake flask culture in Vogel's medium containing filter paper as sole carbon source and in the presence of 1, 25 or 100 µM-validamycin A. After 6 d, enzyme activities in the culture supernatants were determined. Each enzyme assay was performed in triplicate and the results represent the means of five replicate flasks ± SE. Dry weight was estimated from measurements of chitin content (see Methods).

Enzyme	Enzyme activity [nkat (mg dry wt) ⁻¹]			
	0	Validamycin A concn in medium (µM):		
		1	25	100
FPase	0.13 ± 0.01	0.03 ± <0.01*	0.03 ± <0.01*	0.02 ± <0.01*
CMCase	1.13 ± 0.12	0.03 ± <0.01*	0.11 ± <0.05*	0.02 ± <0.01*
Cellobiase	0.83 ± 0.04	0.22 ± 0.02*	0.17 ± <0.08*	0.12 ± 0.02*
Xylanase	6.35 ± 0.12	6.69 ± 0.39	6.57 ± 0.37	6.97 ± 0.37
PGase	0.39 ± 0.03	0.30 ± 0.03	0.27 ± 0.04	0.29 ± 0.03

* Significantly (using the *t*-test, *P* < 0.05) different from control lacking validamycin A.

extracellular protein produced per mg of biomass; reductions in extracellular protein of 10%, 32% and 82% were observed in the presence of 1, 25 and 100 µM-validamycin A respectively. However, no significant difference (*P* > 0.05) was found in the pH of the supernatants of cultures grown in the presence and absence of validamycin A.

Table 2 shows the effect of validamycin A on the activity of several extracellular enzymes in the supernatant of stationary phase cultures of *R. solani*; 1 µM-validamycin A caused significant (*P* < 0.05) reductions in the extracellular activities of FPase (a 77% reduction compared with the control), CMCase (97% reduction) and cellobiase (73% reduction), but had no significant (*P* > 0.05) effect on the xylanase or PGase activities of the culture supernatants. No further reduction in FPase or CMCase activity was observed when the validamycin A concentration was increased to 100 µM, but a further significant (*P* < 0.05) decrease in cellobiase activity occurred at a validamycin concentration of 100 µM.

In order to investigate the effect of the antibiotic on the distribution of enzymes in cultures of *R. solani*, mycelia were fractionated into insoluble and soluble fractions and the activities of each enzyme in the various fractions were determined. Table 3 shows the enzyme activities in the three fractions after 6 d growth of *R. solani* (stationary phase) in the presence and absence of

Table 3. *Effect of validamycin A on the total activity and distribution of some enzymes produced by R. solani A79*

R. solani was grown at 25 °C in shake flask culture in Vogel's medium containing filter paper as sole carbon source and in the presence and absence of 25 µM-validamycin A. After 6 d, mycelium and culture filtrate were harvested and the mycelium fractionated into soluble and insoluble fractions (see Methods). The enzyme activities in each of the three fractions were determined. Results are expressed as nkat (estimated mg dry weight mycelium)⁻¹. Each enzyme assay was performed in triplicate and the results represent the means of three replicate flasks. Dry weight was estimated from measurements of chitin content (see Methods).

Enzyme	Validamycin A concn (µM)	Enzyme activity [nkat (mg dry wt) ⁻¹]			
		Total*	In culture supernatant	In soluble fraction of biomass	In insoluble fraction of biomass
FPase	0	0.27	0.13	0.02	0.12
	25	0.08†	0.03†	0.00†	0.05†
CMCase	0	2.04	1.13	0.18	0.73
	25	0.33†	0.11†	0.00†	0.22†
Cellobiase	0	3.27	0.83	0.07	2.37
	25	1.36†	0.17†	0.01†	1.18†
Xylanase	0	7.38	6.35	0.16	0.87
	25	7.32	6.57	0.00	0.75
PGase	0	0.40	0.39	0.01	0.00
	25	0.37	0.37	0.00	0.00

* Sum total of the three fractions.

† Significantly (using the *t*-test, *P* < 0.05) different from control lacking validamycin A.

25 µM-validamycin A. In both the control and validamycin A treated cultures, approximately 50% of the FPase activity was found in the culture supernatant and 50% in the insoluble fraction of the biomass. The FPase activity in each fraction was reduced by the antibiotic and the total FPase activity was decreased by about 70% (from 0.27 to 0.08 nkat mg⁻¹). Approximately half (53%) of the CMCase activity was found in the culture supernatant with the remaining activity occurring in the insoluble (36%) and soluble (9%) fractions. Validamycin A reduced the CMCase activity in each fraction but had a greater effect on the extracellular activity than on activities in the soluble and insoluble fractions. Overall, CMCase activity was reduced by 84% (from 2.04 to 0.33 nkat mg⁻¹).

Most of the cellobiase activity, unlike that of the other enzymes, was found in the insoluble fraction (72%) with the remainder in the culture supernatant (28%). Again, the activity in both fractions was reduced in the presence of the antibiotic, but, as with CMCase, the activity of the culture supernatant was affected more than that of the insoluble fraction. Overall, cellobiase activity was reduced by 58% (from 3.27 to 1.36 nkat mg⁻¹).

Xylanase activity was restricted largely to the culture supernatant (86%) with the remainder being present in the insoluble fraction (14%). PGase activity was found only in the culture supernatant. Validamycin A had no significant (*P* > 0.05) effect on either xylanase or PGase activity, and had no appreciable effect on the distribution of either enzyme.

Validamycin A (up to 100 µM) had no effect on the activity of any of the enzymes when added to the assay systems, although the antibiotic was found to be a substrate for cellobiase (results not shown). Validamycin A contains a β-1,4-linked glucose residue which appears to be important for antibiotic activity (Wakae & Matsuura, 1975). In order to investigate the possible role of this linkage group in the effect of the antibiotic on enzyme production, the effect of cellobiose (a β-1,4-disaccharide) on enzyme production was studied. The presence of 100 µM-cellobiose in the medium had no significant (*P* > 0.05) effect on the production of extracellular activity of any of the enzymes tested (results not shown).

Table 4. Effect of inositol on the production of extracellular FPase, CMCase and cellobiase by *R. solani* A79 grown in the presence and absence of validamycin A

R. solani A79 was grown at 25 °C in shake flask culture in Vogel's medium containing filter paper as carbon source and in the presence and absence of validamycin A and inositol. Culture supernatants were harvested after 6 d and the enzyme activities determined. Each enzyme assay was performed in triplicate and results represent the means of five replicate flasks $\pm$ SE.

Enzyme	Validamycin A concn (μ M)	Enzyme activity in culture supernatant (nkat ml ⁻¹)			
		Inositol concn in medium (mM)			
		0	0.1	1.0	5.0
FPase	0	0.12 $\pm$ 0.01†	0.07 $\pm$ <0.01*	0.05 $\pm$ <0.01*	0.04 $\pm$ <0.01*
	1	0.03 $\pm$ <0.01*	0.04 $\pm$ <0.01*	0.02 $\pm$ <0.01*	0.01 $\pm$ <0.01*
CMCase	0	1.35 $\pm$ 0.08†	0.95 $\pm$ 0.04*†	0.48 $\pm$ 0.04*†	0.16 $\pm$ 0.02*†
	1	0.39 $\pm$ 0.05*	0.28 $\pm$ 0.01*†	0.26 $\pm$ 0.01*†	0.23 $\pm$ 0.01*†
Cellobiase	0	0.67 $\pm$ 0.03†	0.52 $\pm$ 0.06†	0.51 $\pm$ 0.01†	0.59 $\pm$ 0.04*
	1	0.30 $\pm$ 0.02*	0.31 $\pm$ 0.01	0.28 $\pm$ 0.03	0.34 $\pm$ 0.06

* Significantly (using the *t*-test, $P < 0.05$) different from control lacking validamycin A and inositol.

† Significantly ($P < 0.05$) different from control containing validamycin A but lacking inositol.

Interaction of inositol and validamycin A

Since it has been shown (Robson *et al.*, 1989) that the effect of validamycin A on the morphology of *R. cerealis* is correlated with a reduction in the phosphatidylinositol content of the biomass, experiments were made to determine if addition of inositol to the medium would reverse the effect of the antibiotic on enzyme production. A further reason for doing this experiment was that it has been shown that inositol antagonizes the effect of validamycin A in controlling rice sheath blight (Wakae & Matsuura, 1975).

Table 4 shows the interaction between inositol and validamycin A on enzyme activities in the supernatant cultures of *R. solani*. The presence of 0.1, 1 or 5 mM-inositol caused a significant ($P < 0.05$) decrease in FPase activity in the absence of validamycin A, but had no significant effect in its presence. Inositol caused a significant ($P < 0.05$) decrease in CMCase activity in both the presence and absence of validamycin A. The CMCase activity in the absence of the antibiotic was significantly ($P < 0.05$) reduced when the inositol concentration was increased from 0.1 to 5 mM. However, no further reduction in activity was found in the presence of validamycin A when the inositol concentration in the medium was increased above 0.1 mM (Table 4). The presence of inositol, even up to a concentration of 5 mM, had no significant ($P > 0.05$) effect on cellobiase activity in either the presence or absence of validamycin A.

DISCUSSION

The production of xylanase by *R. solani* grown on cellulose (Tables 2 and 3) may be due either to the inducers present in filter paper (Lynch *et al.*, 1981) or, as seems more likely, to the constitutive nature of the enzyme. PGase is also a constitutive enzyme and was produced when *R. solani* was grown on glucose (results not shown), as well as when it was grown on cellulose (Tables 2 and 3). Thus, the results in Tables 2 and 3 show that validamycin A reduces the production of the inducible enzymes examined (FPase, CMCase and cellobiase) but not of the constitutive enzymes (xylanase and PGase) involved in the maceration of plant tissue. Further, Table 3 shows that the reduced activities of FPase, CMCase and cellobiase in the culture supernatant were not simply the result of a change in the distribution of these enzymes in the biomass. Thus, as the antibiotic had no direct effect on enzyme activity, the results suggest that validamycin A inhibits the production of the cellulase complex. By contrast, L-sorbose (another paramorphogen) has been shown to increase the extracellular activity of β -glucosidase (Bisaria *et al.*, 1986; Sahoo *et al.*, 1986) and endoglucanase (Sahoo *et al.*, 1986; Nanda *et al.*, 1986) in *Trichoderma reesei*. The overall β -glucosidase activity was unchanged by L-sorbose, the increase

in extracellular activity being correlated with a decreased activity of wall bound enzyme. The increased activity of endoglucanase in *T. reesei* has been correlated with the increased number of hyphal tips present in L-sorbose treated cultures (it has been suggested that cellulase is secreted from hyphal tips; Bisaria *et al.*, 1986).

The decreased production of the cellulase complex caused by validamycin A does not appear to be due to the β -1,4-glycosidic linkage between the glucose and validoxylamine A residue nor to its utilization as a carbon source, as addition of up to 100 μ M of the disaccharide (β -1,4 linked glucose residues) cellobiose to the medium had no significant effect on the production of the extracellular enzymes by cultures grown on filter paper.

The inhibitory effect of 1 μ M-validamycin A on FPase, CMCase and cellobiase production, the restriction of the effect of the antibiotic to the production of the cellulase complex, and the reported inability of validamycin A to reduce the overall production of constitutive β -glucosidase (Uyeda *et al.*, 1986), suggest that the antibiotic may interfere with the induction of the cellulase complex. Cellulase enzymes are repressed during growth on readily utilized carbon sources (e.g. glucose) and are induced during growth on cellulose, cellobiose (Mandels & Reese, 1960) or derivatives of cellulose such as carboxymethylcellulose (Willick *et al.*, 1984). The induction of cellulase enzymes by insoluble cellulose has led to the hypothesis that low constitutive levels of cellulase may release small molecular mass products which either act as, or become converted to, an inducer (Mandels & Reese, 1960; Mandels *et al.*, 1962). Cellobiose can induce cellulase production in many fungi and the disaccharide sophorose has been shown to act as a powerful inducer of cellulase production, although only in *T. reesei* (Steinberg & Mandels, 1979). Sophorose is generated by the action of β -glucosidase on the products of cellulose breakdown by a transglycosylation reaction (Vaheri *et al.*, 1979). Inactivation of the surface bound β -glucosidase by the inhibitors nojirimycin or glucono- δ -lactone has been shown to prevent cellulase induction by cellulose, but not by sophorose (Kubicek, 1987). Cellulase induction in *T. reesei* is considered to involve the initial action of low levels of constitutive endoglucanase with the subsequent formation of an inducer (sophorose) from the products of cellulose hydrolysis by β -glucosidase via a transglycosylation reaction. A similar system of inducer formation may well operate in other cellulolytic fungi. The discovery that various transglycosylation products, including both di- and trisaccharides containing β -1,6-, β -1,4- and β -1,2-glycosidic linkages, are formed by endoglucanase and β -glucosidase (Okada & Nisizawa, 1979; Gritzali & Brown, 1979), has led to speculation that, like sophorose in *T. reesei*, other cellulase inducers may be formed by transglycosylation reactions. As validamycin A is a substrate for β -glucosidase (Asano *et al.*, 1987), the presence of the antibiotic could compete with the natural substrate, reducing the formation of inducer, and hence cellulase production. Alternatively, validamycin A may compete with the inducer, but not cause induction of cellulase.

If validamycin A causes a decrease in the production of the cellulase complex *in vivo*, it would explain both the decreased ability of the pathogen to penetrate host tissue (Wakae & Matsuura, 1975) and the observed reduction in tissue degeneration (Endo *et al.*, 1983) when infected plants are treated with the antibiotic. However, antagonism of the protective effects of validamycin A by inositol (Wakae & Matsuura, 1975) does not appear to result from a reversal of cellulase inhibition (Table 4). The decreased production of cellulase caused by inositol was presumably due to its utilization as a carbon source for growth (Merivouri *et al.*, 1984).

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