

# Effect of Glucose and Adenosine Phosphates on Production of Extracellular Carbohydrases of *Alternaria solani*<sup>1</sup>

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## ABSTRACT

Production of carbohydrases by *Alternaria solani* is inhibited by glucose under low growth conditions. In an enriched medium, glucose has little effect on the production of polygalacturonase and cellulase while it still suppresses production of  $\beta$ -glucosidase. Low levels of all three enzymes were produced in the absence of their respective substrates. Such regulation has been found with many organisms. However, far greater production of these carbohydrases occurred with additions of adenosine phosphates to the growth media. Highest stimulation of enzyme production was by adenosine 5'-phosphate. Adenosine 5'-triphosphate and cyclic 3',5'-adenosine monophosphate gave lesser amounts. Starvation appears to induce production of extracellular carbohydrases and adenosine 5'-phosphate may have a role in the starvation process.

Extracellular carbohydrases which degrade pectin and wall material of host tissue have been invoked as a mechanism by which pathogenic fungi invade plants (1-3, 13). Substrate induction of enzyme production and its repression by simple sugars have been reported (9, 11, 14). The effect of cAMP on negating enzyme repression by simple sugars, although well established in bacteria (10), has yet to be explored fully in fungi.

We examined the influence of substrate, glucose, and adenosine phosphate compounds on the production of PG,<sup>2</sup> C<sub>x</sub>, and BG by the fungus *Alternaria solani* (Ell. and G. Martin) L. R. Jones and Growt., a foliar pathogen of solanaceous crops.

## METHODS

The isolate of *A. solani* was obtained originally from tomato by J. B. Skaptason of Cornell University in the early 1940s and we have maintained it since 1960 by weekly transfers on potato dextrose agar with no observed loss in pathogenicity. Inoculum for the experimental cultures was an agar plug cut with a No. 2 cork borer from a 3-day-old colony growing on a plate of

0.1% glycerol, 1% Ionagar No. 2 (oxid) mineral salts (14) which had been inoculated 3 days before with a suspension of mycelial fragments from a 1 to 2-week-old slant. For the experiments the fungus was grown on liquid-shake culture in either minimal or enriched media at 25 C. The minimal medium consisting of glucose (20 g/l) and mineral salt was that of Goatley (6). The enriched medium (5) consisted of 2 g of casein amino acids (Difco), 1 g of yeast extract (Difco), 1.5 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of MgSO<sub>4</sub>, 10 ml of trace element stock (7) and water to make 750 or 900 ml, and later brought to 1 liter with the addition of various experimental compounds. The media were autoclaved at 15 p.s.i. for 20 min. Experimental compounds were dissolved in water, passed through a 0.22  $\mu$ m Millipore filter and added to the basal medium. Experimental solutions (7.5 ml) were added to basal medium to bring the culture to a volume of 25 or 75 ml in 125-ml flasks that were capped with plastic foam plugs. Plastic plugs were chosen rather than cotton to remove sources of cellulose contamination. Following growth, the fungus was separated from the broth by centrifugation, washed once with water, and dried at 70 C to constant dry weight.

The supernatant broth was analyzed for activities of PG, C<sub>x</sub>, BG. Samples held 1 to 3 days were frozen after harvest with little loss in enzyme activity. PG and C<sub>x</sub> were assayed viscometrically employing 1 ml of broth and 9 ml of 1.2% sodium polypectate or 0.25% carboxymethylcellulose in 0.1 N sodium citrate buffer (pH 5.2). Lesser volumes of broth (0.2 or 0.5 ml) were used to assay cultures of high activity. The polypectate and carboxymethylcellulose were solubilized by blending with buffer at 80 C. Specific activities of PG and C<sub>x</sub> were calculated by the formula:

$$\text{Units of activity} = \frac{1000}{T_{50} \times \text{dry weight}} \text{ per ml of culture}$$

where  $T_{50}$  is the time required to reduce viscosity by half. Activity of BG was measured as absorbance at 420 nm in 1 hr at 50 C of a solution containing equal volumes of culture filtrate and 4 mg per ml of *o*-nitrophenyl- $\beta$ -D glucoside (Sigma Chemical Co.) in "Z" buffer (10) without mercaptoethanol. The reaction was stopped by the addition of one-fourth volume 1 M sodium carbonate. The absorbance of a test solution was corrected for the absorbance of a blank containing reagents.

$$\text{Unit of activity} = \frac{A_{420} \times 1000}{\text{dry weight}} \text{ per ml of culture}$$

## RESULTS

**Endopolygalacturonase Activity.** The production of PG in a growing culture of *A. solani* was followed by measuring activ-

<sup>1</sup> We dedicate this paper in memory of Milton Zucker, our friend and former colleague. Milton often stressed the dual importance of understanding one's field of study and having competence to study. His success as an outstanding investigator is based in part on his thorough grasp of physiology and his high degree of competence to study biochemistry.

<sup>2</sup> PG: endo-polygalacturonase; C<sub>x</sub>: cellulase (EC. 3.2.1.4); BG:  $\beta$ -glucosidase (EC. 3.2.1.21).

ity of media during 4 to 15 days. Because maximum activity occurred in 8 to 12 days, a growth period of 10 to 11 days was chosen. Mycelial growth and PG activity varied with nutrients in the medium (Table I). The fungus can produce PG in the absence of pectin. When glucose is present with pectin, PG appearance is depressed 40-fold in minimal medium and increased slightly in enriched medium.

The effect of adenosine phosphates on PG production by cultures growing in glucose plus pectin in the enriched medium are presented in Figure 1. Highest levels of PG were found at low dosages of AMP and ATP, whereas cAMP caused appreciable increase in PG production only at the highest concentration. The increases in PG production were not due to the presence of phosphate because enzyme activity was not affected by a 10-fold increase in inorganic phosphate of the control medium. The effect of the adenosine phosphate compounds is on enzyme production because no stimulation of enzyme activity was observed when the compounds were added to enzyme solution at the time of assay. Mycelial dry weights in the experiments represented in Figure 1 varied between 6 to 8 mg/ml, and were not affected appreciably by treatment.

The effects of cAMP and glucose on PG appearance in the medium were studied. The fungus produced no PG on the enriched media and only a trace when 6.1 mM cAMP was present. Glucose (20 g/l), caused 6.2 units of PG per mg dry

Table I. Effects of Nutrients on Growth and Polygalacturonase Production of *Alternaria solani*

| Carbon Source                     | Minimal | Medium                     | Enriched | Medium                     |
|-----------------------------------|---------|----------------------------|----------|----------------------------|
|                                   | Dry wt  | Polygalacturonase activity | Dry wt   | Polygalacturonase activity |
|                                   | mg/ml   | units/mg dry wt            | mg/ml    | units/mg dry wt            |
| Glucose (20 g/l)                  | 1.10    | 0.8                        | 4.9      | 3.3                        |
| Glucose (20 g/l) pectin (0.4 g/l) | 1.53    | 10.5                       | 7.0      | 9.3                        |
| Pectin (0.4 g/l)                  | 0.12    | 416.3                      | 2.3      | 2.3                        |

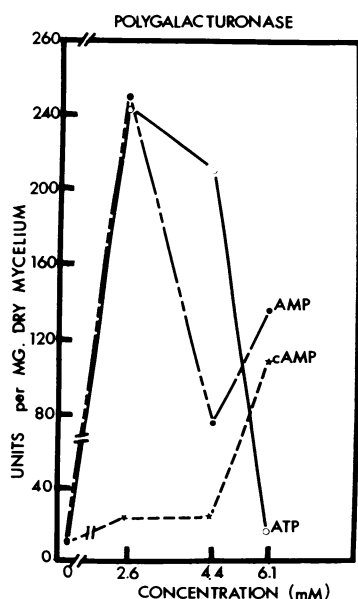


FIG. 1. Effect of AMP, ATP, and cAMP on the production of PG by *Alternaria solani* in an enriched medium.

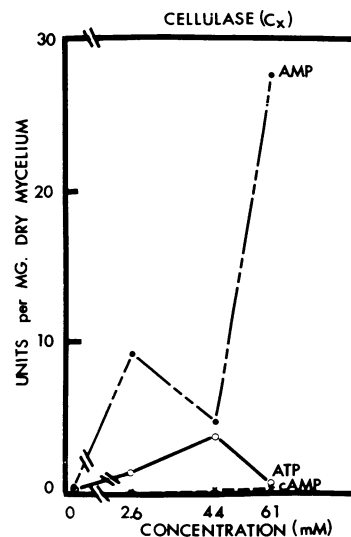


FIG. 2. Effect of AMP, ATP, and cAMP on the production of  $C_x$  by *Alternaria solani* in an enriched medium.

Table II. Effect of Glucose and AMP on Cellulase Production by *Alternaria solani* Growing in an Enriched Medium

| Substrate                          | Growth       | Cellulase Activity |
|------------------------------------|--------------|--------------------|
|                                    | mg dry wt/ml | units/mg dry wt    |
| Glucose (20 g/l)                   | 5.4          | 0.1                |
| AMP (6.1 mM)                       | 1.1          | 0.0                |
| Glucose (20 g/l) plus AMP (6.1 mM) | 6.7          | 28.7               |

wt. of mycelium to be formed, but glucose plus 6.1 mM cAMP caused the formation of 16.1 units. Apparently, stimulation of PG production by cAMP requires the presence of glucose. Production of PG was not stimulated by cAMP in the presence of pectin.

**Cellulase Activity.** Low levels of  $C_x$  were formed by *A. solani* in minimal and enriched media containing carboxymethylcellulose or cellobiose. Because of low enzyme activity, effects of glucose on  $C_x$  production could not be detected. However, high  $C_x$  activity was found when the fungus was grown on enriched media containing AMP (Fig. 2). Production of  $C_x$  was less with ATP than with AMP, and cAMP had little effect. The effects of AMP and ATP were not due to an increase in phosphate content in the medium because a 10X increase in inorganic phosphate caused only a slight stimulation of  $C_x$  production. High production of  $C_x$  by AMP required glucose (Table II), thus the relationship appears to be synergistic.

**$\beta$ -Glucosidase.** Unlike that of PG and  $C_x$ , production of BG in enriched medium was reduced to one-half or less in the presence of glucose (Table III). No specific inducer is required; however, production per mg dry weight of fungus was greater in the presence of carboxymethylcellulose and cellobiose than without. We obtain the same results with pectin as those of carboxymethylcellulose and cellobiose. Cyclic AMP has mixed effects, it caused an increase in enzyme production in the presence or absence of glucose, in the presence of carboxymethylcellulose and cellobiose. These are poor substrates for growth of the fungus, and increased production of BG may result from prolonged growth under starvation conditions rather than from specific induction. However cAMP caused a slight decrease in enzyme formation in the presence of glucose

Table III. Effect of Carbon Source and cAMP on Beta-glucosidase Production by *Alternaria solani* Growing in an Enriched Medium

| Carbon Source                               | Growth        |      | Beta-glucosidase Activity |      |
|---|---------------|------|---------------------------|------|
|   | 6.1 mM cAMP   | None | 6.1 mM cAMP               | None |
|   | mg dry wt, ml |      | units/mg dry wt           |      |
| Glucose (20 g/l)                            | 6.6           | 6.2  | 45                        | 6    |
| None  | 1.1           | 0.8  | 482                       | 13   |
| Glucose + carboxymethyl-cellulose (2.4 g/l) | 6.8           | 9.2  | 26                        | 35   |
| Carboxymethylcellulose                      | 1.0           | 1.2  | 489                       | 103  |
| Glucose + cellobiose                        | 6.4           | 5.3  | 9                         | 35   |
| Cellobiose (2 g/l)                          | 1.9           | 1.9  | 300                       | 174  |

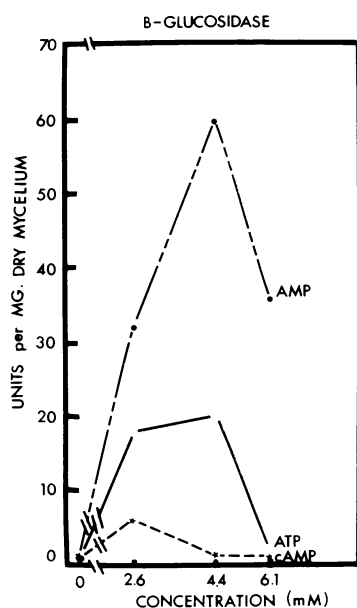


FIG. 3. Effect of AMP, ATP, and cAMP on the production of BG by *Alternaria solani* in an enriched medium.

plus carboxymethyl cellulose and glucose plus cellobiose. Activity of BG in enriched medium together with glucose plus pectin was increased greatly by AMP, moderately by ATP, and mildly by cAMP (Fig. 3). The increase was not due to an increase in phosphate *per se*.

### DISCUSSION

Production of PG,  $C_x$ , and BG by *A. solani* is highly influenced by cultural additives. This fact known for some time (1, 11, 13) is not our major finding, but it is reiterated briefly for this pathogen. The enzyme activities can increase logarithmically with arithmetical increases in components in the media. Moreover, the ratio of enzyme activity, *i.e.*, PG- $C_x$ -BG, varied independently with treatment. This lack of proportionalities between enzyme yields suggests that the appearances of the enzymes in the media may be independently controlled. Production of all three enzymes did not require the presence of any of their respective substrates nor glucose. Enzyme production per milligram of growth appears to increase in a minimal medium. Effects of glucose varied with enrichment of the media. Glucose suppressed production of PG in minimal media and caused a slight increase in its production in enriched me-

dia. In contrast to the other enzymes, production of BG in enriched media was suppressed by the presence of glucose.

Various adenosine phosphates appeared to affect production of these carbohydrases in the media. AMP was most effective and no specific carbon source was required for the increases in enzyme production by AMP. ATP was effective at low concentrations but lost its effect at high levels. Cyclic AMP, even at very high concentrations was the least effective adenosine phosphate on enzyme production. The absence of large increases in carbohydrases in high cAMP treatments suggests that cAMP activity is common to that of AMP and is not necessarily due to a role as a messenger for enzyme induction (10).

Of special interest to us is the extent to which enzyme production of extracellular carbohydrases of *A. solani* are affected by adenosine phosphates more so than by carbon source. This may not be surprising if one considers that *A. solani* is a weak saprophyte at best and most growth probably occurs in or on plants. Hence the pathogen usually would need no specific inducer-repressor systems to indicate when pectin, cellulose, and related products are present since they are always present in the plant. However, this fungus apparently still needs regulating systems that control appearance of extracellular carbohydrases, depending on conditions of starvation or growth. A contrast between starvation and growth would be the pectin treatment of minimal and enriched media of Table I. In the life of the fungus, starvation prevails during competition with microbes for plant refuse in soil and during spread through host tissue, whereas a nonlimiting supply of substrates may prevail during initial penetration of host cells.

Although all three enzymes are increased by AMP, a certain priority of production is apparent under a given set of circumstances. For instance, as in Figure 4, maximum production of PG occurs at 2.6 mM AMP, whereas maximum production of BG was at 4.4 mM AMP, and maximum production of  $C_x$  was at 6.1 mM AMP. If high AMP is a starvation signal, then enzyme production is scheduled differently as the fungus proceeds to undergo increasing degrees of starvation, *i.e.*, PG before BG before  $C_x$ . The sequence BG before  $C_x$  is interesting because  $C_x$  end-products are substrates for BG. If  $C_x$  were produced before BG, then  $C_x$  would not alleviate starvation since cellobiose, the end-product, would not be metabolized. From Figure 4, the PG production was bimodal in relation to AMP concentration, suggesting either two different PG enzymes or two different regulations, or both.

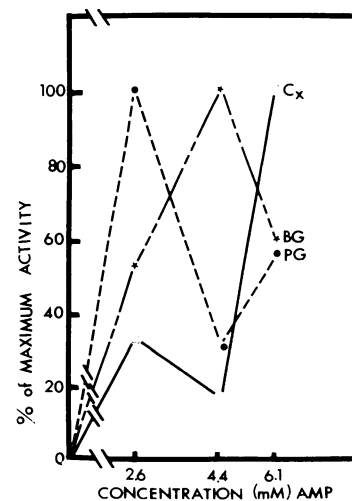


FIG. 4. Differential effect of AMP on the production of PG,  $C_x$ , and BG by *Alternaria solani* in an enriched medium.

We cannot tell from our experimental design whether the actions of adenosine phosphates are on enzyme synthesis or on enzyme excretion from the fungal cell. However, the sequential effect of AMP on the appearance of the extracellular carbohydrases (Fig. 4) is more suggestive of a signal controlling synthesis than of one controlling release.

Hulme and Stranks (8) reported that a starving mycelium appears to produce the highest amounts of enzymes. Our data show this starvation effect (Table 1) and in nonstarved mycelia AMP mimics this starvation effect (Fig. 4). We have no idea whether extracellular AMP or ATP has any effect on intracellular levels of these compounds, *i.e.*, energy charge (4). Further studies integrating extracellular levels of adenosine phosphates with energy charge data may explain these effects.

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