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Methylxanthines as inducers of pectin lyase in *Penicillium griseoroseum* cultured on sucrose

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R. C. MINUSSI, M. C. BARACAT-PEREIRA, J. L. C. COELHO AND D. O. SILVA. 1997. Pectin lyase (PL) from *Penicillium griseoroseum* can be induced by xanthine, theobromine, theophylline and especially by caffeine and hypoxanthine (5 mmol l^{-1} with 0·01% yeast extract (YE)). For caffeine and hypoxanthine, PL activity was, respectively, 5·2 and 3·7 times higher than with YE alone. The simultaneous addition of caffeine or hypoxanthine (5 mmol l^{-1}) and YE (0·1%) had a synergistic effect on PL activity as compared to the addition of these substances alone (0·2% YE; 10 mmol l^{-1} caffeine; 10 mmol l^{-1} hypoxanthine). Increasing caffeine concentrations (0–10 mmol l^{-1}) for a constant YE content of 0·01%, resulted in an increase in PL activity and a decrease in mycelial mass. For a constant caffeine concentration (5 mmol l^{-1}) and increasing YE contents (0–0·2%), a higher PL activity and mycelial mass were detected. The addition of caffeine (10 mmol l^{-1}) at the beginning of incubation increased PL activity and decreased mycelial mass, while caffeine added after 12 and 24 h resulted in decreases in PL activity and increases in mycelial mass. The results presented here indicate that methylxanthines, especially caffeine, can induce PL in *P. griseoroseum*.

INTRODUCTION

Pectinolytic enzymes produced by non-pathogenic micro-organisms have a wide range of applications in the industrial clarification of juices and wines and in the manufacture of pectin-free starch hydrolysates (Ghildyal *et al.* 1981). The use of pectinolytic complexes to degum natural fibres, such as flax, has been the subject of some study because of the potential applications in the textile industry (Sharma 1988).

Pectin lyase (PL) is the only pectinase known to hydrolyse highly esterified pectin without the previous action of other enzymes (Alaña *et al.* 1989). Reports on the ability of pectin lyase to macerate diverse plant tissues and clarify juices can be found in the literature (Ishii and Yokotsuka 1971, 1972; Szajer and Szajer 1982).

Most commercial enzymatic complexes used today contain cellulases that attack cellulose fibres making them less resistant to industrial processing. *Penicillium griseoroseum* synthesizes a pectinolytic complex with lower cellulase activity. In early studies, this micro-organism was found to produce PL using sucrose as a carbon source only when yeast extract was added to the medium (Baracat-Pereira *et al.* 1994). However, in a later study the addition of tea extract to the sucrose medium was also found to induce PL (Baracat-Pereira *et al.* 1997).

Since methylxanthines are present in high concentrations in tea, the objective of this study was to investigate their effects on the growth and PL production of *P. griseoroseum*.

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MATERIALS AND METHODS

Micro-organism

Penicillium griseoroseum was originally obtained from seeds of forest trees at the Departamento de Fitopatologia, Universidade Federal de Viçosa, Minas Gerais, Brazil.

Cultivation conditions

Inoculum was produced by culturing *P. griseoroseum* on oatmeal agar for 9 d at 25°C. The basal medium used for experiments contained (in g l^{-1}): sucrose, 4·0; KH_2PO_4, 8·0;

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K_2HPO_4, 2·48; $MgSO_4.7H_2O$, 1·1; $(NH_4)_2SO_4$, 1·0; with the pH adjusted to 6·3. Fifty ml of this medium were added to Erlenmeyer flasks (125 ml) and treatments consisted of the addition to the flasks of yeast extract (YE) and methylxanthines at different concentrations. The media were inoculated with 5×10^4 spores ml^{-1} and incubated for 48 h at 25°C on a rotary shaker (150 rev min^{-1}) (Brumano *et al.* 1993).

Growth determination and enzyme assay

Cultures were harvested by filtering through a 400-mesh sieve (37-μm pore size) and growth was determined according to Calam (1969) as mycelial dry weight (MDW). PL activity was determined in the culture filtrate spectrophotometrically (A_{235}) according to Albersheim (1966). The reaction mixture consisted of 1·0 ml of 2·5% citric pectin (Sigma®) in 100 mmol l^{-1} phosphate buffer (pH 6·8) and 1·5 ml of culture filtrate. Samples of 0·5 ml of the reaction mixture were added to 4·5 ml of 0·01 mol l^{-1} HCl at 0 and 30 min of incubation at 40°C. PL activity unit (U) was defined as nanomoles of unsaturated uronides produced ml^{-1} of culture filtrate min^{-1}.

RESULTS

The effects of nitrogen bases (xanthine and hypoxanthine) and methylxanthines (caffeine, theophylline and theobromine) on the production of PL by *P. griseoroseum* were studied by culturing the fungus on sucrose supplemented or not with 0·01% yeast extract and containing 5 mmol l^{-1} of the different compounds (Fig. 1). Maximum PL activities were obtained when cultures were grown on the mineral medium containing caffeine or hypoxanthine and yeast extract. Increases in enzyme activity in the treatments with caffeine and hypoxanthine were 5·2 and 3·7 times, respectively, the activity measured in the treatment with YE alone. Similarly, the addition of both theophylline and YE resulted in high values of PL activity. Caffeine and hypoxanthine were capable of inducing PL even in the absence of YE. The addition of xanthine, theophylline and theobromine resulted in PL activity levels similar to those recorded for the control (YE alone). Mycelial dry weight was lower when caffeine was present in the culture medium giving higher PL/MDW ratios.

A synergistic effect of caffeine and YE on PL activity was observed when both compounds were added simultaneously at a final concentration of 5 mmol l^{-1} and 0·1%, respectively. PL activity corresponded to that obtained with the addition of equivalent amounts of each substance alone, i.e. either 0·2% YE or 10 mmol l^{-1} caffeine (Fig. 2). Similar effects were observed for hypoxanthine, although the levels of PL activity recorded were lower than for caffeine (Fig. 2).

The higher the caffeine concentrations (0–10 mmol l^{-1}), for a constant YE content of 0·01%, the higher the PL activity obtained and the lower the mycelial growth, leading consequently to increasing PL/MDW ratios. This demonstrates the effect of caffeine on PL induction (Fig. 3a). However, a constant caffeine concentration of 5 mmol l^{-1} but increasing levels of YE (0–0·2%), produced increases in PL activity as well as in mycelial dry weight which led to lower PL/MDW ratios (Fig. 3b). When different proportions of YE and caffeine were combined so that the sum of both was kept constant at 0·03%, high PL activities could only be obtained when YE was added at concentrations above 0·01% (Fig. 4). PL activity and mycelial dry weight values obtained

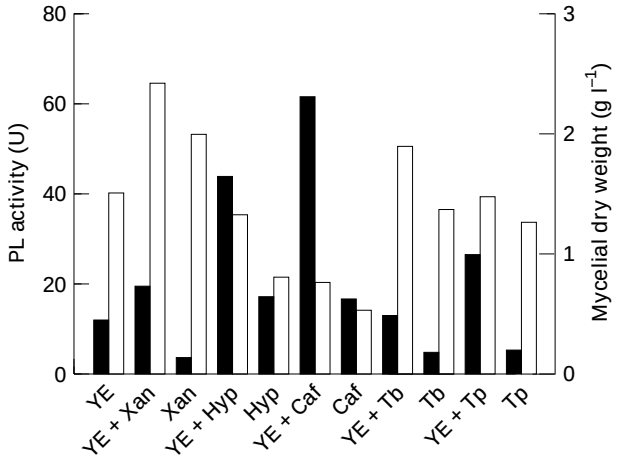

Fig. 1 Pectin lyase (PL) activity (■) and mycelial dry weight (□) of *Penicillium griseoroseum* cultured on 0·4% sucrose supplemented or not with 0·01% yeast extract (YE) in the presence or absence of 5 mmol l^{-1} methylxanthines (xanthine (Xan), hypoxanthine (Hyp), caffeine (Caf), theobromine (Tb), theophylline (Tp))

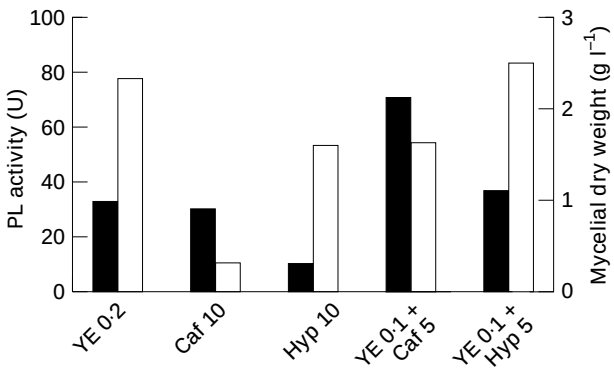

Fig. 2 Pectin lyase (PL) activity (■) and mycelial dry weight (□) of *Penicillium griseoroseum* cultured on 0·4% sucrose added with 0·2% yeast extract (YE 0·2), 10 mmol l^{-1} caffeine (Caf 10), 10 mmol l^{-1} hypoxanthine (Hyp 10), 0·1% yeast extract and 5 mmol l^{-1} caffeine (YE 0·1 + Caf 5), 0·1% yeast extract and 5 mmol l^{-1} hypoxanthine (YE 0·1 + Hyp 5)

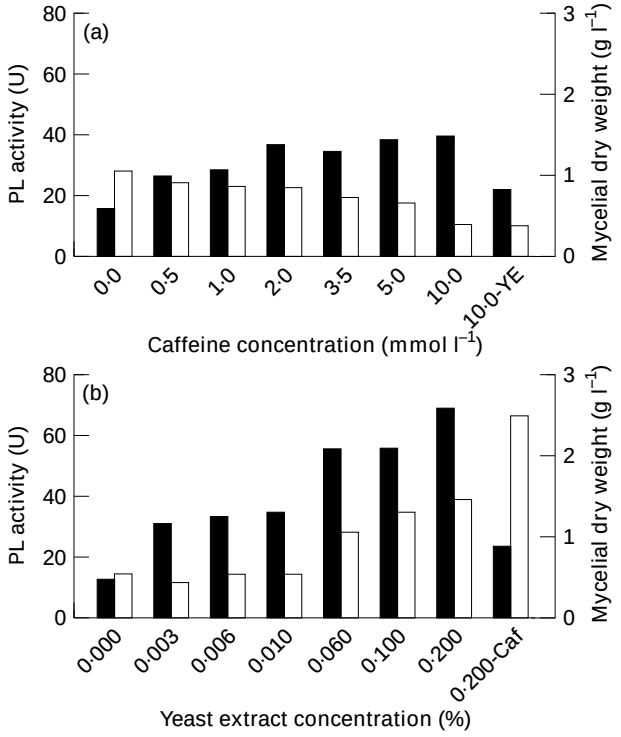

Fig. 3 Pectin lyase (PL) activity (■) and mycelial dry weight (□) of *Penicillium griseoroseum* cultured on 0·4% sucrose and (a) 0·01% yeast extract and crescent concentrations of caffeine (0–10 mmol l^{-1}) and 10 mmol l^{-1} caffeine without yeast extract (10·0-YE) or (b) 5 mmol l^{-1} caffeine and crescent concentrations of yeast extract (0–0·2%) and 0·2% yeast extract without caffeine (0·200-Caf)

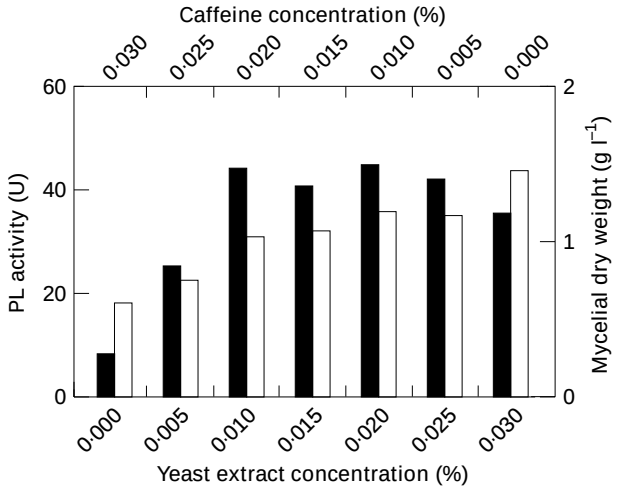

Fig. 4 Pectin lyase (PL) activity (■) and mycelial dry weight (□) of *Penicillium griseoroseum* cultured on 0·4% sucrose supplemented with yeast extract (0–0·03%) and caffeine (0·03–0%). Different proportions of yeast extract and caffeine were combined so that the sum of both was kept constant at 0·03%

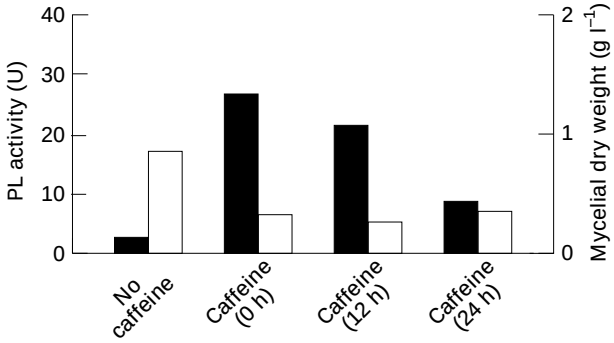

Fig. 5 Pectin lyase (PL) activity (■) and mycelial dry weight (□) of *Penicillium griseoroseum* cultured for 48 h on 0·4% sucrose, in the presence or absence of 10 mmol l^{-1} caffeine added at times zero, 12 and 24 h of cultivation

by cultivating *P. griseoroseum* in 0·03% caffeine corresponded to 25 and 40%, respectively, of the values obtained when the fungus was grown in 0·03% YE (Fig. 4), resulting in lower PL/MDW ratios.

The effects of adding caffeine at 10 mmol l^{-1} at 0, 12 and 24 h after inoculation is shown in Fig. 5. Adding caffeine at the time of inoculation promoted the highest PL activity and the lowest mycelial dry weight and adding it at later times decreased PL activity and increased mycelial dry weight. These results demonstrate that when caffeine is used as an inducer, PL induction in *P. griseoroseum* occurs within the first hours of culture.

DISCUSSION

Penicillium griseoroseum does not produce PL in the presence of sucrose unless YE is added to the medium (Baracat-Pereira *et al.* 1994). However, it did produce PL in the presence of tea extract, with or without sucrose (Baracat-Pereira *et al.* 1997). Results obtained in this study indicate that caffeine, hypoxanthine and theophylline induced PL synthesis in *P. griseoroseum* in media which contained sucrose as the carbon source either in the presence or absence of YE. Methylxanthines are present in high concentrations in tea extract. The average contents reported in the literature are 1·5–4·0% for caffeine, 0·2–0·4% for theobromine and 0·02% for theophylline (Finger *et al.* 1992). Methylxanthines act as phosphodiesterase inhibitors in animals but are relatively inefficient inhibitors of fungal phosphodiesterases (Pall 1981). In fungi, inhibition by these substances generally occurs only when they are present at high concentrations (5–10 mmol l^{-1}). Methylxanthines, such as theophylline and caffeine, affect other enzymes by bonding to purines, nucleosides and nucleotides (Pall 1981). This bonding is mainly a result of the chemical nature of these compounds since all of them are purine bases. Some authors have reported that cAMP may

be involved in enzyme synthesis regulation in bacteria (Botsford and Harman 1992). Tröger and Meyer (1995) have presented a scheme for the pharmacological action of theophylline on the cellular levels of cAMP. According to this model, high doses of theophylline would lead to phosphodiesterase inhibition and, consequently, to increases in cAMP levels. We believe that such increase may be involved in the regulation of PL synthesis by *P. griseoroseum* (Baracat-Pereira *et al.* 1997).

YE and methylxanthines seem to have different effects on PL induction. When caffeine or hypoxanthine were added singly, PL activity was lower than when added together with YE. This indicates the existence of different induction mechanisms in *P. griseoroseum*. PL activity was superior to that recorded when these compounds were used alone at concentrations equivalent to the sum of individual concentrations. A synergistic effect of these compounds on PL activity could be observed when they were added simultaneously to the culture medium.

Caffeine caused a reduction in *P. griseoroseum* dry weight. Mycelial mass was reduced when compared to the treatment with YE. Mittag (1994) studied the effects of caffeine and caffeine salts on *Candida albicans* growth and structure and reported that these compounds were antifungal when present within the range of 2–4 mg ml^{-1}. Mittag (1994) suggested that sub-inhibitory concentrations of caffeine and caffeine salts probably cause increases in the levels of cAMP inducing the formation of multiple buds with reduced separation rates. These events result in further inhibition of the multiplication of daughter cells. Caffeine apparently had inhibitory effects on *P. griseoroseum* growth. According to Roussos *et al.* (1994), *P. verrucosum* uses caffeine as a nitrogen source when no other nitrogen compound is available. This probably results from the ability of *P. verrucosum* to synthesize xanthine dehydrogenase and uricase. YE-containing media are much richer and therefore are more suitable for higher mycelial mass production.

The growth, and PL synthesis by *P. griseoroseum*, are dependent on the time at which caffeine is added to the culture medium after inoculation. The later the caffeine is added, the higher the mycelial mass produced and the lower the PL activity measured.

Our results indicate that methylxanthines, especially caffeine, are PL inducers in *P. griseoroseum* grown on a sucrose medium. This is especially important for the industrial production of this enzyme. Sucrose is available at relatively low costs in Brazil and caffeine can be extracted from leaves of several plants which are commercially abundant and cheap.

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