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Full Length Research Paper

Influence of gallic and tannic acids on enzymatic activity and growth of *Pectobacterium chrysanthemi* (*Dickeya chrysanthemi* bv. *chrysanthemi*)

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The effect of phenolic acids (gallic and tannic acids) on growth of *Pectobacterium chrysanhemi*, and its protease and pectate lyase activities was tested. The results obtained showed a significant inhibiting effect of the tannic and gallic acids on the growth of this strain. The growth rate decreases in the presence of 400 µg/ml for gallic acid and 100 µg/ml fortannic acid. The enzymatic activity retardation was observed with the two phenolic compounds as well but the rate of inhibition varied from one compound to another. The highest antimicrobial potentials and the highest effect on enzymatic activities were observed with the tannic acid at 200 µg/ml, which inhibited 91% of the tested micro-organisms, and 88% of pectate lyase activity.

Key words: Tannic and gallic acids, *Pectobacterium,* pathogenicity.

INTRODUCTION

Many plant pathogenic bacteria produce pectinolytic and macerating enzymes, and most bacteria causing soft rot on many crops belong to certain *Erwinia* species. Infection by soft rot *Erwinia* usually results in extensive maceration and rotting of parenchymatous tissue in the organs affected. In some cases maceration is directly correlated with cell death (Garibaldi and Battman, 1971). Bacterial soft rots are diseases difficult to control because of the ubiquity of the soft rot *Pectobacterium. Pectobacterium chrysanthemi* (Hauben et al., 1998), homotypic synonym of *Erwinia chrysanthemi* and recently transferred to a novel genus*, Dickeya* gen. nov., (Samson et al., 2005), is one of the main *Pectobacterium* strain known as a green house pathogens in hot climatic regions (Pérombelon and Kelman, 1980). The virulence of the *P. chrysanthemi* is mainly correlated with their ability to produce and secrete cell-wall degrading enzymes, mainly pectate lyases (Pel) (Collmer and Keen, 1986).

Determination of the role of phenolic compounds on the enzymatic activities of *P. chrysanthemi* is one of the fundamental interests for the development of new methods to combat the pathogenic bacteria. The use of the phenolic compounds (particularly tannic and gallic acids) as antimicrobial as well as enzymatic inhibitors is important for economic and environmental reasons. The antimicrobial effects of secondary metabolites and particularly those exerted by phenolic compounds were the subject of many investigations as expressed by several manners as inhibition of proteolytic activity (Waghorn and Nabb, 2003), and inhibition of the pro-duction of enzymes by the micro-organisms with bactericide or bacteriostatic action (Cheng and Costerton, 1980; Scehovic, 1998). The present article deals with the determination of the antimicrobial and antienzymatic activities of the tannic and gallic acids on *P. chrysanthemi* (Hauben et al., 1998).

MATERIALS AND METHODS

Bacteriel strain

The strain *Pch* 20.48 used in this study was provided by the Culture Stock Collection of Phytopathogenic Bacteria (CFBP, INRA Angers, France). It was maintained as deep-frozen cultures (- 80°C) in Luria-Bertani (LB) medium (10 $g.l^{-1}$ tryptone, 5 $g.l^{-1}$ yeast extract and 10 $g.l^{-1}$ NaCl, pH 7.3) supplemented with 30% glycerol (Sambrook et al., 1989). Before inoculation, a bacterial suspension of each strain was prepared in sterile distilled water (SDW) from cultures on King's B medium, and incubated at 27°C for 24 h. The bacterial inoculum was standardized from overnight stock culture in nutrient broth of 4×10^8 $cfu.ml^{-1}$.

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Bacterial growth assay

Pch 20.48 was tested against increasing doses of tannic and gallic acids (0, 25, 50, 100 200, 400, 800, 1600 and 3200 µg/ml) in Mueller-Hinton medium using the agar well diffusion method. Inhibition zones were measured 48 h after incubation and the effect was evaluated as mean of duplicate assays.

Enzymatic activity tests

Shaken cultures of *P. chrysanthemi* isolates were grown for 24 h at 27°C in nutrient broth. One ml aliquots were transferred into 50 ml of LB medium distributed in 250 ml Erlenmeyer flasks, and shaken thoroughly (250 rpm) for 48 h at 27°C to induce enzyme production. Each culture was centrifuged at 8000 g for 10 min at 4°C, and the supernatant was stored at-40°C until use. Enzymatic activities were detected in cup plate essays (Dingle et al., 1953). This enzymatic technique is based on the diffusion of the enzymatic fraction on an agarose gel containing the respective substrates. Protease activity was screened on 10 $g.l^{-1}$ powder skimmed milk (Oxoid) in Tris/HCl 0.1M buffer, pH 8.0. Pectate lyase activity was tested on 5 $g.l^{-1}$ citrus polygalacturonic acid (Sigma) in 0.05 M Tris-HCl and 10^{-3} M $CaCl_2$ at pH 8.6, and revealed by submersion in 1% CTAB (Cethyl Trimethyl Ammonium Bromide). After 30 min, activity showed a translucent blurring on white background (Wandesman et al., 1986).

For the inhibitor effect on pectate lyase and protease activities, various concentrations of tannic and gallic acids (800, 400, 200, 100 and 50 µg/ml) were added on agarose gel. Ninety µl rough enzyme preparations were placed into 6-mm diameter wells, and the Petri dishes were overnight incubated at 37°C (approx. 16 h). Enzymatic activities were screened as clear zones around the wells. Estimate of the pectate lyases and proteases activity for each replicate was made by measuring the diameter of the transparent zones surrounding the wells with and without the two phenolics compounds in concern.

RESULTS

Effect of tannic and gallic acids

Tannic acid tested with various concentrations showed a significant decrease in the growth rate of *P. chrysanthemi* up to 100 µg/l, 91% inhibition was obtained with a concentration of 200 µg/ml. The strain failed to grow when the concentration of tannic acid reached 800 µg/ml (Figure 1).

Figure 2 showed a decrease in the growth rate in response to gallic acid at concentrations equal or above 400 µg/ml. The inhibition reached 50% for the concentration ranging from 1600 to 3200 µg /ml. The results correlated with the degree of inhibition of protease and pectate lyase activities with different concentrations of the phenolic compounds (Figures 3, 4, 5 and 6). Figures 3 and 4 showed that protease activity decreased depending on the concentration of the two phenolic compounds but tannic acid was more efficient on inhibition of protease activity than gallic acid. Tannic acid caused unexpected fall of pectate lyase activity in a range 100 to 200 µg/ml (Figure 5), and a total inhibition of pectate lyase activity was observed at 400 µg/ml. A significant unexpected decrease from 100 to 13.75% of pectate lyase activity was observed as well with gallic acid when the concentration reached 800 µg/ml (Figure 6).

DISCUSSION

The aim of this study is to determine sensitivity of *P. chrysanthemi* (*E. chrysanthemi*) to both gallic and tannic acids, with regard to their effect on protease and pectate lyase activities. The results obtained showed a significant inhibitory effect of tannic and gallic acids on growth of *P. chrysanthemi*. The growth rate decreased in presence of gallic acid at concentration starting with 400 µg/ml, and 100 µg/ml for tannic acid. The highest inhibitory effect was observed for the tannic acid, which inhibited 50 and 100% of the tested strain at 200 and 400 µg/ml, respectively. The results illustrated in (Figures 1 and 2) showed a great variability from one compound to another. The importance of this retardation depends on the nature and the amount of the phenolic compound used. Our results with tannic and gallic acids show that *P. chrysanthemi* is variably sensitive to the two phenolic compound at different concentrations.

A study carried out by Chung et al. (1993) showed that tannic acid inhibits the growth of certain non-phytopathogenic bacteria as *Bacteroides fragilis*, *Clostridium perfringens* (*Clostridium welchii*) *Escherichia coli* and *Enterobacter cloacae*. Recently, Rodriguez-Vaquero et al. (2007) reported that gallic acid inhibits the growth of *E. coli* and *Klebsiella pneumoniae* at 25 and 10 µg/ml, respectively.

The more marked antibacterial effect is screened for the tannic acid at various concentrations tested ($P < 0.05$). This reduction of growth of this bacterium ranged from 28 to 47% with gallic acid ranging from 4 00 and 3200 µg/ml compared to 91 to 95% for tannic acid ranging from 200 and 400 µg/ml. The tested tannic acid was more effective on protease and pectate lyase activities than gallic acid. Protease and pectate lyase activities were variable according to the nature and the concentration of phenolic compounds of each compound. This variable sensitivity to tannic and gallic acids can be explained by the differences in size and the number of hydroxyl groups between the two acids; the tannic acids ($C_{76}H_{52}O_{46}$) are a complex mixture containing an important proportion of various ester of the gallic acid and glucose (Hodek et al., 2002).

The influence of the two phenolic compounds can be explained by the interaction with the proteases and pectate lyases, produced by *P. chrysanthemi*. Indeed, various polyphenols are known to be enzyme inhibitors and are able to settle down on some proteins and enzymes and change their enzymatic stability (Meddleton et al., 2000). Lojkowska and Holubovsca (1992) showed that the inactivation of the membrane enzymes by the phenolic compound would involve a modification of the cellular permeability, followed by a lysis of the bacterial cell.

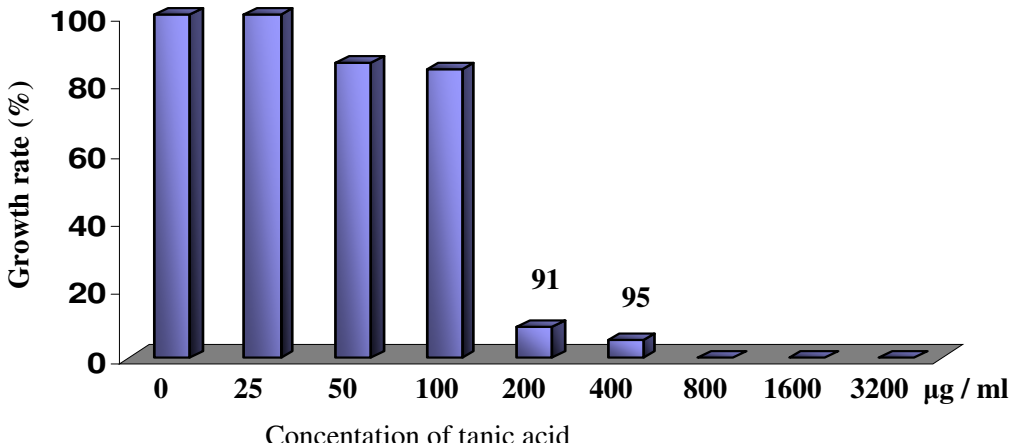

Figure 1. Effect of tannic acid on *Pectobacterium chrysanthemi* growth.

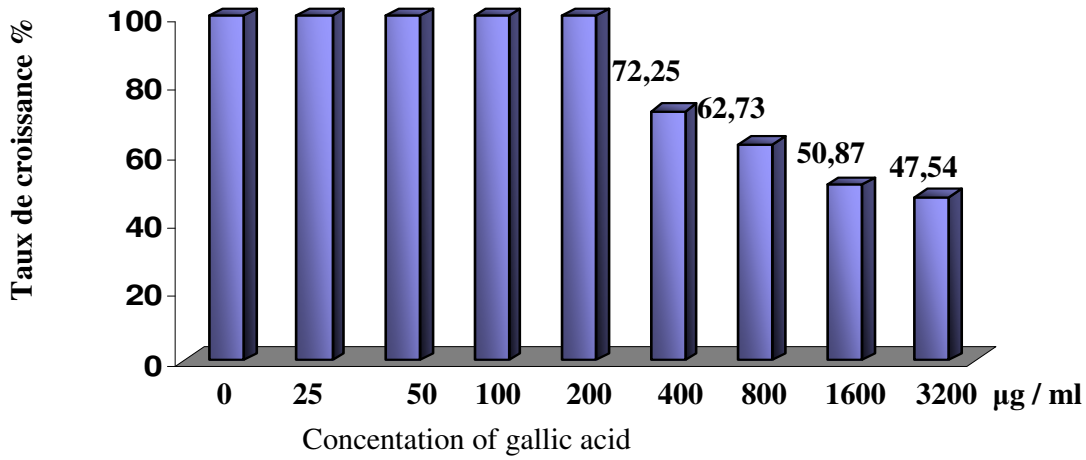

Figure 2. Effect of gallic acid on *Pectobacterium chrysanthemi* growth

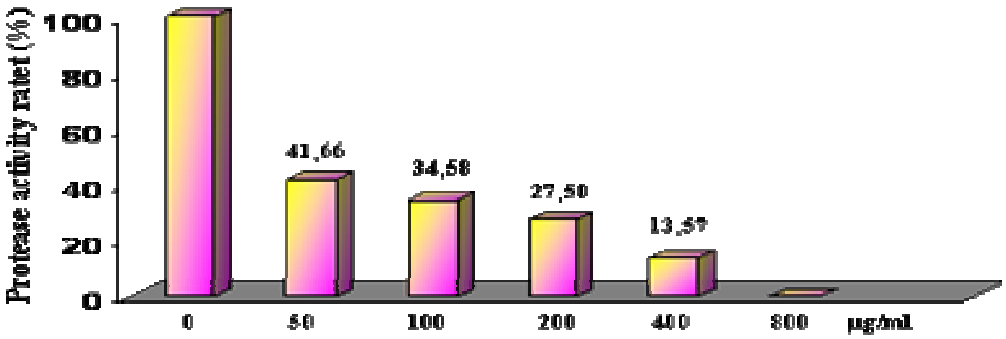

Figure 3. Inhibitory effect of tannic acid on *Pectobacterium chrysanthemi* protease activity.

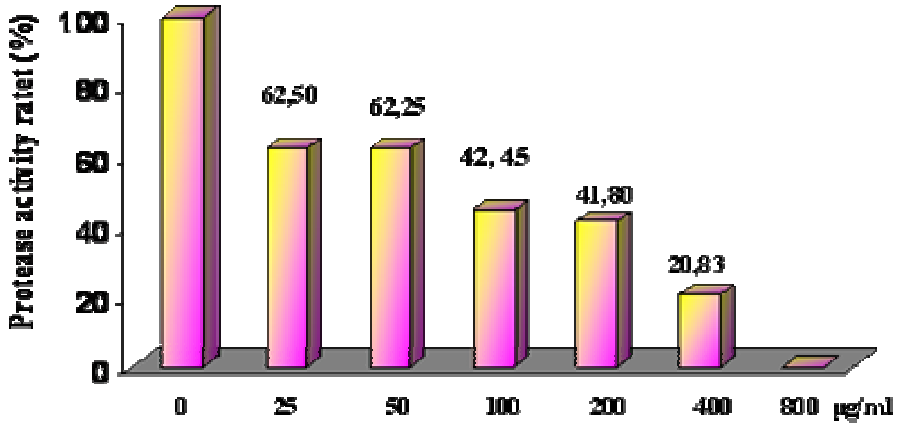

Figure 4. Inhibitory effect of gallic acid on *Pectobacterium chrysanthemi* protease activity

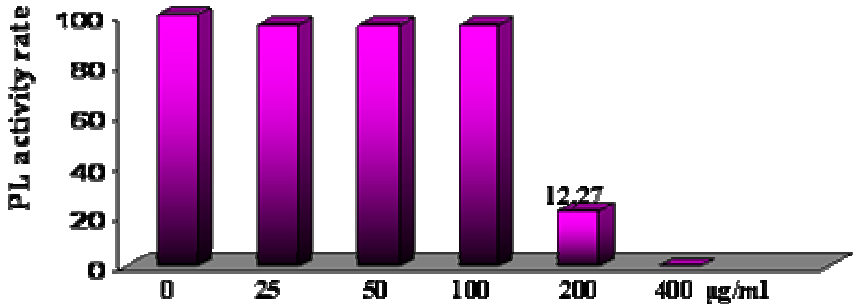

Figure 5. Inhibitory effect of tannic acid on *Pectobacterium chrysanthemi* pectate lyase activity

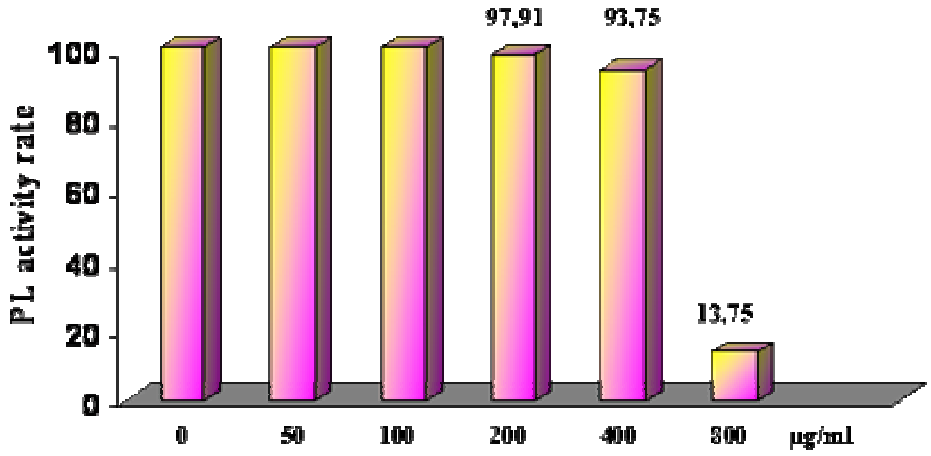

Figure 6. Inhibitory effect of gallic acid on *Pectobacterium chrysanthemi* pectate lyase activity.

The mechanism of inhibition of the bacterial enzymatic activity by the phenolic compounds is probably due to a reduction in the availability of the substrates by combination of polysaccharides and proteins (Hagerman, 1989), or by chelatation of the metal cofactor which can be partly responsible for the inhibiting enzymatic activity followed

by the inhibition of the microbial activity (Liu et al., 2003).

According to Chung et al., (1998), the inhibition of the growth of the intestinal bacteria (*Bacteroides fragilis, Clostridium perfringens, Escherichia coli* and *Enterobacter cloacae*) by the tannic acid is probably related to the high capacity of the latter to fix iron.. Iron being essential for all the micro-organisms whose acquisition is done by the synthesis of siderophores which makes it possible to transfer it inside the bacterial cell (Expert, 1999). In the case of *P. chrysanthemi,* the fall of their enzymatic activities in contact with the phenolic compounds would be related to their binding with the active sites of the enzymes which become inactive or with the precipitation of enzymatic proteins, thus preventing them to match the substrates. According to Hatano et al. (1990) and Leinmûller et al. (1991), tannins have various important binding with amylases, cellulases, β-glucosidases and proteses, and generate a complex protein-polyphenols which precipitate and become non-functional. Moreover, the phenolic compounds have generated a major interest for their biological important effects, as antibacterial, antiviral and antifungal products.

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