Significance and role of polygalacturonase production by Botrytis cinerea in pathogenesis

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Proefschrift

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STELLINGEN

1. The pathogenicity of *Botrytis cinerea* could be the consequence of an ecological disturbance provoked by human activities, e.g. by modern agriculture.

   This thesis.

2. As increased levels of the air pollutant ozone in ambient air can predispose plants to enhanced infection by *Botrytis cinerea*, the epidemiology of this fungus could have been consequently affected in recent years.

   Leone, G., & Tonneijck, A.E.G. 1990. Netherlands Journal of Plant Pathology 96: 65-74.

3. The claim of Schejter and Marcus that polygalacturonase of *Botrytis cinerea* does not act on pectin is wrong.

   Schejter, A., & Marcus, L. 1988. *In*: Methods in enzymology, vol 161. *Edited by*: W.A. Wood & S.T. Kellogg. 366-373.

4. Polygalacturonase activity on pectin does not depend on pectin methyl esterase. Therefore, the conclusion of Hagerman *et al.* that the degradation of pectin by *Botrytis cinerea* in their plate-assay experiments in absence of pectin methyl esterase activity was due to pectin lyase and not polygalacturonase, is incorrect.

   Hagerman, A.E., Blau, D.M., & Mc Clure, A.L. 1985. Analytical Biochemistry 151: 334-342.

5. The evidence reported by Roberts *et al.* that the concerted activities of two different pectic enzymes produced by *Erwinia carotovora* subsp. *carotovora* EC14 cause maceration of potato tuber tissues, obtained by chromosomal DNA isolation from EC14, cloning and construction of recombinant *Escherichia coli* strains, could also be reached without genetical manipulation by simply purifying the enzymes and testing them on the potato tissues.

   Roberts, D.P., Berman, P.M., Allen, C., Stromberg, V.K., Lacy, G.H., & Mount, M.S. 1986. Journal of Bacteriology 167: 279-284.

6. The evidence that the stability of the plant genome can be disrupted by stress and the possibility that this phenomenon may change the resistance of plants to diseases, imply the failure, in a relatively short time, of the introduction in the field of plants genetically engineered for resistance to a specific pathogen.

   Kuć, J. 1987. *In*: Innovative approaches to plant disease control. *Edited by*: I. Chet. 255-274.
   Marx, J. 1984. Science 224: 1415-1416.

7. The application of biotechnology for breeding purposes causes an inevitable reduction of the genetical variation of an organism and, therefore, conflicts with one of the main objectives of the Fourth Environmental Action Programme of the EC, based on the World Conservation Strategy, which aims to preserve genetic diversity in nature.

8. A forest administration based mostly on uniformly planting of non-native, genetically homogeneous conifers of the same age, strengthens the influence of air pollution in deteriorating the vitality of European forests.

9. The complaint of the New Scientist that "In Britain today, even the high fliers often cannot find decent jobs in science. Many of the best spend years on short-term contracts, with no security and little pay.", can also be easily applied to the Dutch situation.

   New Scientist of 7 April 1990, pg. 43

10. A ceasing of the rule of anonymity for referees who review a manuscript submitted for publication, would render the scientific discussion between them and the author(s) more democratic and less unilateral, and would avoid arrogant judgements, often stimulated by the feeling of protection given by secrecy.

11. The argument, often cited by scientists and specialists, that up to the present the positive effects of "alternative" therapies in medicine are not yet demonstrated, is not important for those patients who benefit using this type of therapies.

    From a letter of K. ten Holt, patient declared "incurable", to the NRC Handelsblad of 3 February 1990.

12. The etymology of the word "discovery", which means in different European languages to unveil and, therefore, to find, not only shows that Germanic and Italic languages had the same Indo-European ancestor, but also implies the pre-existence of every idea and phenomenon.

13. The Dutch authorities include Italian employees in the ethnic minorities policy, which should give some advantages in finding or maintaining a job. In view of a more united Europe, this is a discrimination against other Europeans living in Holland.

Stellingen behorende bij het proefschrift
"Significance and role of polygalacturonase production by Botrytis cinerea in pathogenesis" door Gionata Leone

Wageningen, 14 september 1990

Blind science plowes vain clods,
Insane faith lives the dream of its own cult,
A new god is only a word,
Don't believe or seek:
Everything is occult.

Fernando Pessoa: "Christmas".

Ai miei genitori,
alla mia famiglia.

PREFACE

Life is unpredictable. The fulfillment of this thesis I believe is primarily the monument to my stubborness to be a scientist. However, without having met the right persons at the rigth moment, my life would have taken a completely different course. People call it coincidence: I do not know. It went approximately as follows. First of all I met my wife Marleen who came for some time to Milan as a student in Phytopathology from the Fytopathologisch Laboratorium at Baarn. Secondly the Italian National Research Committee (CNR), a couple of months after her coming, announced fellowships to practise research in foreign laboratories. Thirdly I met Prof. Dr. Koen Verhoeff who, as the then Director of the Fytopathologisch Laboratorium, accepted me as a guest researcher and Ph.D. student although it was unsure for how long I could be financially supported. When I came to Holland, willing to take my Ph.D. here, I was only sure of a six-months financial support, which finally became three and half years spent at Baarn, covered in total by five different Italian fellowships. However, this meant to me at last to be paid for practising research, although I had no right for pension or social security (Italian rules), and allowed me to put down the backbone of this thesis.

I am grateful to many persons for their different contributions to the accomplishment of this research.

Dear Koen, you made it possible for me to join your Department and to stay in Baarn by solving all kind of bureaucratic problems concerning my fellowships and the permission to get a Ph.D. in Holland. Your constant interest in the Botrytis research did not diminish after leaving Baarn: on the contrary, you have always found time to correct and discuss this thesis. A couple of months ago, presenting a seminar at the Scottish Crop Research Institute, I was introduced with the words: "He joined the prestigious group of Koen Verhoeff". Although this group unfortunately does not exist anymore, I am proud to have been part of it for some time.

Dear Joop, you introduced me in your research line and followed all my work with great apprehension. I had to deal with unexpected, apparently insuperable difficulties which were in contrast to the published knowledge. The power of some poorly checked articles to mislead a scientist is stronger when they are published in famous reviews! Your editorial experience has been a hard but instructive school. I regret that, at the moment I am writing, your presence in the Commission is uncertain because of your serious disease, that you are facing with great courage and positivism. If you will be hindered to be present, I will place you in my mind where you have the right to be.

I acknowledge here also the great help from my friend Franco Faoro of the Istituto di Patologia Vegetale of Milan, who informed me when new fellowships were announced and took care of a lot of bureaucratic things in Italy.

Advancements in the investigations of a scientist are not only dependent on financial support and good facilities but also on:

1) students. I thank here once more Martin Kreyenbroek, Arthur Overkamp, Ed Schoffelmeer and Erik Smit for their important contribution to this research;
2) assistancy. Mrs Liem, Nelleke Schot and Lydia Waterreus have divided some of their skill and time with me and their help was always welcome. A very special thank to Mrs Liem for her patience and concern for everything in the lab.;
3) household service. I am indebted to Mr. Kanning, Mrs Dijkstra and, later, Truus van de Kemp for preparing media, sterilizing, washing and storing the glass-work. Especially Mr. Kanning, who took also care of the chemicals, I will remember his indefatigability and joviality. For every technical problem we could address ourself to Willem Liersen;
4) administration. Wil Schellingerhout and Corry Sinai always relieved me of orders, payments, etc. In particular Wil was one of the silent pillars of the Institute;
5) library. I thank Hans Schreuder for kindly requesting and sending off many reprints;
6) graphic and photographic work. I am thankful to Dennis Dutcher and to Chris van Dijk and Albert Koedam of the IPO at Wageningen. Especially Chris has been very patient and skillful with a lot of drawings.

I thank all the other colleagues of the Fytopathologisch Laboratorium at Baarn for the pleasant time we spent together.

I am also grateful to many colleagues of the IPO at Wageningen for their support in the work and understanding of my situation. In particular I thank Thijs Gerlagh and Ingrid Vos for their kind and expert advices and for providing ascospores of Sclerotinia sclerotiorum, produced by their ingenious method; and Fred Tonneijck, Chris van Dijk and Ildiko van de Linde for the excellent collaboration.

I am grateful to Prof. Dr. Ir. J. Dekker who kindly accepted to be my promotor just trusting that my research was good enough for a Ph.D.

My gratitude goes also to the LEB-Fonds for financial support of the printing of this thesis.

The skill of Felix Thiel of TFDL at Wageningen is fixed on the cover in the spectacular SEM-photo of a conidium of Botrytis cinerea. I am grateful to Mr. Geerlings of the Photo Service of the Department of Phytopathology, University of Agriculture, Wageningen, for the accomplishment of the special effects of the cover.

A special thank to my friends Arnold Brakenhoff and Fred Tonneijck for their availability to be my paranymphs.

Last but not least I thank you, Marleen, for having been the first to encourage me to undertake this enterprise. I cannot quantify how much free time it took to achieve this point. However, just during this last year, where our deepest belief was so hardly challenged and the situation of our family was also made unsure by the present (wrong) research policy, your support and optimism, together with the love and vitality of Michael, have been very precious.

Gionata Leone

Wageningen, July 1990

ABSTRACT

The saprophytic fungus *Botrytis cinerea* Pers.: Fr. causes serious problems in agriculture by being able to attack an extremely wide range of plants. During the infection process, different kind of cell wall-degrading enzymes are produced, among which the pectic enzymes polygalacturonases (PGs). These are supposed to play a major role in the penetration and colonization of the host tissues. This study deals with some fundamental aspects of the physiology of parasitism of *B. cinerea* by investigating the significance of PG production in pathogenesis.

Polygalacturonase production and PG isoenzyme composition are affected by type and concentration of a pectin-related substrate. *In vitro*, pectic enzymes are produced in a consistent sequence always starting with the constitutively synthesized isoenzyme PG2. This isoenzyme is also present in ungerminated conidia. Both the high degree of regulation of the coordinated PG production on cell wall-related polysaccharides and its association with the ability of *B. cinerea* to grow on these polymers as the only carbon source, indicate that PG production is inherent to the digestion of the pectic portion of the primary cell wall.

Polygalacturonase production is also dependent on the presence of inorganic phosphate. The involvement of phosphate is mediated by adenine nucleotides. Especially the synthesis of the inducible isoenzyme PG1, besides being controlled by a pectic substrate seems also to be regulated by the metabolic status of the fungus through the adenylate pool. The knowledge of the role of phosphate as an infection-stimulating compound has been applied to develop a rapid inoculation procedure, which can be adopted to screen bean cultivars for the resistance to *B. cinerea* and to the related weak pathogen *Sclerotinia sclerotiorum*.

The cause of the variability in PG isoenzymatic forms between different isolates has been elucidated. When variability within a given isolate, whose origin appears to reside in the factors affecting the sequential synthesis of the PG isoenzymes, can be controlled, variability in PG patterns between different isolates is correspondingly low.

The study of the biochemical and molecular properties of PG2 has shown that PG2 is an endo-enzyme whose affinity for sodium polygalacturonate is about three time higher than that for pectin, under standard conditions. The use of a new approach for the judgement of PG purity has been proposed and discussed. The physiological and biochemical properties of PG2 suggest that this enzyme could be the only PG used by *B. cinerea* to support the direct penetration of the primary cell wall.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The fungus

Botrytis cinerea Pers.: Fr. is an airborne, ubiquitous fungus which belongs to the Fungi Imperfecti (Deuteromycota), class Hyphomycetes and family Moniliaceae. The perfect state, or teleomorph, is referred to as *Botryotinia fuckeliana* (de Bary) Whetzel and the sclerotial state as *Sclerotium* Tode (Jarvis, 1980). The anamorph, conidial state *B. cinerea* is commonly known as grey mould. The name *Botrytis* is derived from the ancient Greek *botrùs*, meaning a bunch of grapes. The fungus is characterized by grey-brown clusters of oval spores, the blastoconidia, arranged in a racemose fashion; these conidia are disseminated by wind. The sequence of conidiogenesis is simultaneous and multiple. The fungal colonies are cottony and rather dry, and produce black stone-like sclerotia. Depending on isolate and cultural conditions, microconidia can be obtained *in vitro*. Although it has been suggested that they have a sexual function, like the spermatia of the rust fungi (Lorbeer, 1980), their real role remains still obscure.

Botrytis cinerea is primarily a saprophyte universally present on dead and dying plant tissue (Blakeman, 1980). As a pathogen, it usually first becomes established on dead or moribund parts of a host and then spreads into adjacent healthy tissues (Mansfield, 1980). Direct penetration of germ tubes into undamaged tissues has also been observed on different hosts, such as grape berries (Mc Clellan & Hewitt, 1973), strawberry fruit (Powelson, 1960), tomato fruit (Verhoeff, 1970) and gerbera flowers (Salinas *et al.*, 1989). *Botrytis cinerea* is also known as a secondary invader, attacking plants already infected by other pests or weakened by senescence or stress factors. The fungus is able to infect over 200 hosts (Jarvis, 1980), most of them economically important, such as field and glasshouse vegetables, ornamentals, bulb and corm-producing monocotyledons, small berry fruits including grapes and forest tree seedlings. The most common symptoms induced by *B. cinerea* are decay or rot in all kinds of fleshy organs, flecking on leaves and small necrotic lesions (spots) on different plant parts, such as tomato fruits and gerbera flowers. Its control is onerous in terms of economic costs and of the adverse impact on the environment of the chemicals applied.

Physiology of the host-fungus interaction

In the plant-*B. cinerea* interaction, cell wall-degrading enzymes secreted by the fungus play an important physiological role. The first report of a clear association between pectic and cellulolytic enzymes produced by this fungus and plant disease symptoms probably is the study of Hancock *et al.* (1964). Later, Verhoeff & Warren (1972) demonstrated the presence of different cell wall-degrading enzymes in petiole stumps of tomato plants inoculated with *B. cinerea*. More recently, various pectic enzymes have been associated with the penetration and colonization of French bean leaves by the fungus (Van den Heuvel & Waterreus, 1985).

The secretion of cell wall-degrading enzymes by microorganisms in general, is supposed to correspond with the diverse polymers present in plant cell walls (Cooper, 1983). Thus, pectic polysaccharides are degraded by pectic enzymes; hemicelluloses are degraded mainly by glucanases, arabinases and xylanases, and cellulose is broken down by a complex of enzymes mostly indicated as cellulases (Cooper, 1983). There is no doubt anymore that, among the cell wall-degrading enzymes, pectic enzymes are the major factors responsible for tissue degradation in pathogenesis, because of the structural importance of the pectic polymers, as they are largely responsible for the integrity and coherence of plant tissues (Bateman & Millar, 1966; Collmer & Keen, 1986; Verhoeff, 1980). Pectic materials are the most abundant polysaccharides present in the primary cell wall and generally predominate in the middle lamella, the region of contact between walls of contiguous cells (Cooper, 1984). Pectins and pectic substances are structured on basal chains of (1,4)-linked residues of α-D-galacturonic acid, esterified with carboxyl groups, often to a very considerable extent and modified by interruption of the chain by L-rhamnose residues or groups of other sugars (Stephen, 1983). For this reason these

polymers are often referred to as rhamnogalacturonans.

Pectic enzymes

To the group of pectic enzymes belongs a number of enzymes classified on the basis of their different mode of degrading the substrate. Thus, pectinesterases (PEs) are responsible for the hydrolysis of the esterified methyl groups from pectins. Furthermore, on the basis of the mechanism by which the α-1,4-glycosidic bond of the pectin chain is split (hydrolytic or trans-eliminative cleavage), the other pectic enzymes are classified as, respectively: polygalacturonases (PGs), polygalacturonate lyases (PGLs) and pectin lyases (PLs). Each group of enzymes can have forms cleaving internal regions of the pectic chains at random (endo-enzymes) or having a terminal point of hydrolysis (exo-enzymes). Pectic enzymes are often low-molecular-weight, stable, extracellular glycoproteins (Cooper, 1983). Pectic enzymes of fungal origin are also of industrial interest as they can be used, for instance, in the extraction, clarification and depectinization of fruit juices, in the maceration of fruits and vegetables and for the extraction of vegetable oil (Rombouts & Pilnik, 1980).

Polygalacturonases and their role in pathogenesis

Polygalacturonases are the most widely investigated pectic enzymes of fungal origin. There are several reasons for this, as: 1) PGs can account *in toto* for the phenomenon of tissue maceration (Bateman & Millar, 1966); 2) they have been involved in the cell death associated with tissue maceration (Suresh *et al.*, 1984) and; 3) they are the first pectic enzymes secreted in the sequential production of cell wall-degrading enzymes occurring on isolated cell walls and in infected tissues (Collmer & Keen, 1986).

The involvement of an enzyme leading to disintegration of the plant cell wall and to killing of protoplasts by germinating conidia of *B. cinerea* has been proposed since the beginning of this century (Brown, 1915). About sixty years later, the presence of endo-PG activity in germinating conidia of *B. cinerea* was established (Verhoeff & Liem, 1978). Notwithstanding their convincing role in pathogenesis, the role of PGs in direct penetration by germ tubes of *B. cinerea* through undamaged plant tissues is still considered speculative. Although swelling of primary cell walls of broad bean and tomato was observed by transmission electron microscopy (Mansfield & Richardson, 1981; Mc Keen, 1974; Rijkenberg *et al.*, 1980), Verhoeff (1980) stated that more investigations were necessary in order to understand whether fungal penetration implies a mechanical or a chemical process, particularly as no work was done to identify and localize the enzymes involved.

Factors affecting production of pectic enzymes in the infection process

Infection of healthy green tissues, such as leaves, by germ tubes of conidia of *B. cinerea* is usually dependent on the presence of nutrients in the inoculum. This phenomenon may be caused by the scarcity of available nutrients on leaves in comparison with the surfaces of flowers or fruits, which are much more commonly infected by the fungus (Blakeman, 1980). Since a long time, the presence of exogenous nutrients and the spore concentration in the inoculum are known to affect spore germination as well as penetration and infection by *B. cinerea* (Brooks, 1908; Blackman & Welsford, 1916; Brown & Harvey, 1927). Simple carbohydrates and/or inorganic or organic nitrogen compounds promote germination, superficial growth and formation of prepenetration structures (Blakeman, 1980). Inorganic or organic forms of phosphorus have been reported to stimulate infection (Ko *et al.*, 1981; Van den Heuvel & Waterreus, 1983). However, nutrients can also affect the regulation of the synthesis of pectic enzymes. Cooper (1983) stated that most of the cell wall-degrading enzymes are inducible and that their synthesis can be reduced by readily metabolized carbon sources. In bacteria, this repressive effect, known as catabolite repression, is mediated by decreasing intracellular 3',5'-cyclic AMP (cAMP) levels, as reported also for an endo-PGL of *Erwinia carotovora* (Hubbards *et al.*, 1978). In contrast to the inducible enzymes, constitutive enzymes are synthesized at roughly the same concentration, regardless of growth conditions and of whether a substrate is present or not (Drew & Demain, 1977). According to Van den Heuvel & Waterreus (1985) many, if not all, pectic enzymes produced by *B. cinerea* are constitutive, since they could be detected in a medium not containing a pectinaceous

substrate. However, Cooper (1983) reported that inducible enzymes are normally also synthesized to a small extent in the absence of an inducer. Therefore, detection of pectic enzyme activity in the absence of the substrate is *per se* not sufficient to classify an enzyme as constitutive. It is also not known whether pectic enzymes produced by *B. cinerea* undergo the above mentioned cAMP-mediated regulation as occurring in bacteria. For instance, the phosphate-stimulated penetration by *B. cinerea* of bean leaves reported by Van den Heuvel & Waterreus (1983), has been correlated to a concomitant phosphate-stimulated increase of activity of pectic enzymes (Van den Heuvel & Waterreus, 1985). In particular, two PGs (labelled as PG1 and PG2) were associated with the penetration stage (Van den Heuvel & Waterreus, 1985), but how phosphate could affect the PGs produced by the fungus remained an open question.

Multiple forms of pectic enzymes

Pectic enzyme activity is generally a mixture of multiple forms or isoenzymes which may differ in molecular properties (e.g. charge, size), biochemical properties (e.g. ability to degrade the substrate) and physiological properties (e.g. regulation) (Cooper, 1983). In the last decade the production of different isoenzymes has been object of more investigations also for the pectic enzymes of *B. cinerea*. The occurrence of multiple molecular forms of PGs has been reported and used to study the variability in physiological features between *B. cinerea* isolates or the effect of different cultural conditions on PG patterns (Cruickshank & Wade, 1980; Di Lenna & Fielding, 1983; Magro *et al.*, 1980; Van den Heuvel & Waterreus, 1985). However, the significance of the production of different isoenzymes is not completely understood, since very often there appears to be no correlation between isoenzymes produced *in vitro* and *in vivo* (Panopoulos *et al.*, 1984). Because of the complex regulation undergone by these enzymes, it seems justified to question whether the differences in isoenzyme profiles found so far between isolates of *B. cinerea*, are the result of artificial differences caused by the metabolic enzyme control.

Outline of the thesis

It is clear from the foregoing that, although much has been published concerning pectic enzymes produced by *B. cinerea*, our knowledge about the role and significance of PGs in pathogenesis and about their regulation in relation to the fungal development is still fragmentary. This thesis is an attempt to fill some of the gaps in our knowledge. Special emphasis has been laid upon the regulation of two pectic enzymes, viz. PG1 and PG2, following the indication of Van den Heuvel & Waterreus (1985) about their association with the infection process of bean leaves by the *B. cinerea* isolate BC1. Since this isolate has also been used throughout this investigation, the numbers assigned to the PGs are identical to those reported by Van den Heuvel & Waterreus (1985). The numbering refers to the position of bands of enzyme activity after pectin-polyacrylamide gel electrophoresis, not to the sequence of production. An *in vitro* system has been used as a basis for this study, since it has the advantage that experimental conditions can be easily controlled and manipulated. Furthermore, the plant metabolism cannot interfere with the production of the fungal enzymes, as could be expected to occur during plant-pathogen interactions. In a further investigation, also enzyme production during the host-fungus interactions has been taken into account. This approach, coupled with more intensive studies of *in vivo* enzyme production, may help to better understand the different functions of the fungus in the expression of pathogenesis.

Chapter 2 deals with the regulation of pectic enzyme production by different pectin-related polysaccharides, viz. isolated bean cell walls, citrus pectin and sodium polygalacturonate. These polymers were used as the only carbon source for the fungus. Since they are also potential inducers of pectic enzyme production, both mycelial growth and PG activity were measured daily. Furthermore, the production of different forms of pectic isoenzymes in the culture medium was followed. The influence of the conidial concentration and of the simple carbohydrates D-glucose and D-galacturonic acid on PG activity and isoenzyme pattern was also investigated.

In Chapter 3 an attempt has been made to understand whether and how phosphate regulates PG production by *B. cinerea*. Different phosphate-containing compounds were compared for their ability to stimulate PG1 and/or PG2 production. The possible

involvement of a *de novo* protein synthesis during the phosphate-dependent stimulation of PG production, was investigated with the protein synthesis inhibitors cycloheximide and actinomycin D. Determinations of the energy charge were carried out to assess whether PG production is linked to the metabolic energy status of the fungal mycelium.

Chapter 4 deals with the occurrence of a phosphate-dependent PG production in different *B. cinerea* isolates. PG isoenzyme patterns of eleven isolates were determined under cultural conditions known to stimulate only PG1 and/or PG2 production by the isolate BC1, in order to prevent variability induced by growth conditions. The specificity of the isolates as well as the enzymatic adaptation to a certain host, and the relation between PG production and pathogenicity of the different isolates, were also object of investigation by studying the plant-fungus interaction both on bean and tomato leaves.

In Chapter 5, the knowledge about the involvement of inorganic phosphate in PG production and in the infection process of *B. cinerea* has been applied to develop a rapid procedure for screening the resistance of bean cultivars to this fungus and to *Sclerotinia sclerotiorum*. Screening the resistance for these weak pathogens on bean leaves is known to be difficult as pathogenicity of both fungi often need to be stimulated by the presence of organic matter.

Chapter 6 describes the purification and characterization of PG2 and attempts to purify PG1. The major biochemical properties of PG2, such as the pH optimum, the K_m, the substrate specificity and the endo- or exo-mode of catalytic action, were determined together with some molecular characteristics such as the isoelectric point and the molecular mass. The cause of the problems encountered during the purification of the two isoenzymes was investigated by studying the electrophoretic mobilities of PG1 and PG2 as a function of pH.

This study is completed with a discussion and some general conclusions in chapter 7.

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CHAPTER 2

REGULATION BY CARBOHYDRATES OF THE SEQUENTIAL *IN VITRO* PRODUCTION OF PECTIC ENZYMES BY *BOTRYTIS CINEREA*

by

G. Leone & J. Van den Heuvel

Abstract

Cultures of *Botrytis cinerea* in a basal salt medium supplemented with different pectin-related polysaccharides (French bean cell walls; citrus pectin; sodium polygalacturonate) as the only C source, were examined daily for polygalacturonase activity, type of pectic enzymes present and mycelial growth. Total polygalacturonase activity and number of enzymes detectable were influenced by type and concentration of the substrate and by the conidial concentration at which the cultures were started. A consistent sequence in the production of pectic enzymes was found. The polygalacturonase PG2 was always the first enzyme present in the culture filtrates and was followed by a number of polygalacturonase and pectinesterase isoenzymes. PG2 was also found in ungerminated conidia. Its production is the expression of a constitutive gene as it was independent of the presence of the substrate and strictly correlated with fungal growth. D-galacturonic acid at 2 mM induced the production of some of the pectic enzymes. At 10 mM and above, however, it repressed PG2 and the subsequent production of the whole pectic isoenzyme complex, this being indicative of a feed-back repression. The results suggest that the pectic isoenzymes produced by *B. cinerea* constitute a coordinated catabolic pathway for the complete degradation of pectic polysaccharides.

Introduction

Since the beginning of this century the plant pathogen *Botrytis cinerea* Pers.: Fr. has been known to be able to produce an enzyme having macerating activity on plant cell walls, by dissolving the middle lamella (Brown, 1915). However, the report of a clear relationship between pectic enzymes produced by this fungus and plant disease symptoms was first published in 1964 (Hancock *et al.*, 1964). Very recently, two polygalacturonases (PGs) have been associated with the penetration of French bean leaves by *B. cinerea* (Van den Heuvel & Waterreus, 1985).

PGs have been repeatedly claimed as the first cell wall-degrading enzymes secreted by several fungi growing on isolated cell walls (Cooper & Wood, 1975; English *et al.*, 1971; Jones *et al.*, 1972). The sequential production of cell wall-degrading enzymes has been related to the sequence of different polymers constituting the primary cell walls that microorganisms may encounter during pathogenesis (Cooper *et al.*, 1978; Cooper & Wood, 1975). Also the production of a number of isoenzymes belonging to the PG group has been reported to occur in several fungi including *B. cinerea* (Cooper *et al.*, 1978; Cruickshank & Wade, 1980; Di Lenna & Fielding, 1983; Magro *et al.*, 1980; Marciano *et al.*, 11982; Nguyen-The *et al.*, 1984; Van den Heuvel & Waterreus, 1985), but the significance of this phenomenon is not yet clearly understood (Panopoulos *et al.*, 1984).

In view of the importance attributed to pectic enzymes in the pathogenesis of *B. cinerea*, the aim of this study was to investigate the effect of different carbohydrates used as C source on the production of PG isoenzymes by this fungus.

Materials and methods

Growth of the fungus in shake cultures

Sporulating cultures of *B. cinerea* isolate BC-1 were obtained as reported elsewhere (Van den Heuvel, 1981). Conidial suspensions were used to inoculate flasks with liquid medium (final concentration, if not stated otherwise, $2x10^4$ conidia ml^{-1}). The medium consisted of a basal salt medium (Richards' solution) containing, per 1000 ml distilled water, 10 g KNO_3, 5 g KH_2PO_4, 1.25 g $MgSO_4.7H_2O$ and 0.4 mg $FeCl_3$, supplemented with an appropriate carbon source at the desired concentration. An insoluble French bean cell wall preparation, citrus pectin, sodium polygalacturonate, D-galacturonic acid and D-glucose were employed singly or in certain combinations as carbon source. When insoluble cell walls or D-galacturonic acid were used in the experiments, it was preferable to utilize a small amount of medium in order to save substrate. Thus, three different kinds of flasks were used, depending on the experiments: 25-, 100- and 300-ml flasks containing 10, 30 and 100 ml, respectively. Under the conditions used, comparable shaking speeds were 136, 120 and 108 strokes min^{-1} for 25-, 100- and 300-ml flasks, respectively. The inoculated flasks were incubated at 19°C for the chosen growth period (never more than 20 days). At daily intervals series of cultures were harvested and the mycelium was separated from the culture liquid by filtration on preheated and preweighed filter paper (S & S no. 604, Dassel, F. R. G.). Mycelial dry weights were determined after heating at 60°C for 48 h. Fungal growth was not determined in experiments in which cell walls were used since it is not possible to establish the rate of disappearance of the insoluble cell walls in a reliable way (see also English *et al.*, 1971). The culture filtrates were assayed as described below immediately after filtration; otherwise they were stored at -20°C until use.

Isolation of cell walls

French bean plants (*Phaseolus vulgaris* L. cv. Dubbele Witte zonder draad) were grown in the glasshouse at about 25°C and were harvested 8 days after sowing. The plants were kept in total darkness during the final 40 h before harvest in order to prevent starch contamination in cell wall preparations. Hypocotyls and primary leaves were cut off and stored at -20°C in polyethylene bags until use. Cell wall preparations were obtained following the procedure of English *et al.* (1971).

Enzyme assays

PG activity of the culture filtrates was determined using a slight modification of the cup-plate assay method (Dingle *et al.*, 1953), with 0.5% sodium polygalacturonate as substrate incorporated in a 1.5% agar solution buffered at pH 5.0. Cups of 6.0 mm in diameter were cut in a 4-mm-thick agar layer and filled with 60 μl of culture filtrate. The plates were then incubated for 22 h at 30°C and developed with 5 M HCl. A clear zone around the cups was an indication of the activity of PG on the substrate. Plotting the area of the clear zones versus different concentrations of an enzyme preparation with known activity on a semilogarithmic scale, permitted to express the PG activity present in the sample assayed in units ml^{-1}. One unit of the reference enzyme mixture had been defined as the activity that releases 1 μmole of galacturonic acid h^{-1} at pH 5.0 under standard conditions (Hoffman & Turner, 1982). The assay was sensitive (limit of detection about 1.5 units ml^{-1}) and allowed the simultaneous determination of numerous samples. If not stated otherwise PG activities determined were averages from two experiments.

The presence of pectin lyases (PLs) and/or polygalacturonate lyases (PGLs) active at pH 5 in the culture filtrates was checked using the thiobarbituric acid procedure (Ayers *et al.*, 1966; Sherwood, 1966). Controls used for this assay were enzyme-substrate solutions incubated for 0 h (Sherwood, 1966).

Gel electrophoresis and pectic isoenzyme identification

Electrophoresis of culture filtrates in polyacrylamide gels containing pectin was performed using the method of Cruickshank & Wade (1980) as modified by Van den Heuvel & Waterreus (1985). Zymograms of pectic isoenzymes were developed using the

staining method of Cruickshank & Wade (1980). According to these authors, PGs appear on the gel as white bands, pectinesterases (PEs) as dark-red bands and PLs acting at low pH as yellow bands. A final rinse of the gels in 3 mM Na_2CO_3 was useful to stop enzyme reactions and enabled the storage of the gels for a long period.

The limit of detection of PG bands on the gels was comparable to the limit of detection of PG activity by means of the cup-plate assay (about 1.5 units ml^{-1}). The numbers assigned to the PG and PE bands in the present study are identical to those reported earlier (Van den Heuvel & Waterreus, 1985).

Disintegration of ungerminated conidia

Conidia of *B. cinerea* obtained as described above were suspended in the basal salt solution (final concentration $2x10^6$ conidia ml^{-1}) to which 0.11 M glucose was added, but from which KH_2PO_4 was omitted to avoid PG production and were frozen at -20°C after a microscopic check for germination. The frozen suspension was subsequently passed five times through a Biox X-press equipped with a HP-25 hydraulic press (AB Biox, Jarfalla, Sweden) to obtain complete disintegration. The disrupted material was then filtered through S & S no. 604 filter paper and subsequently through Sartorius membrane filters with a pore size of 1.2 μm. The resulting filtrate was then used for analysis.

Chemicals

All salts, D-glucose, D-galacturonic acid monohydrate and acrylamide were purchased from Merck. Citrus pectin (M_r 25.000-50.000) was purchased from Fluka; polygalacturonic acid (sodium salt, grade II) was from Sigma. N,N,N',N'-tetramethylethylenediamine and N,N'-methylenebisacrylamide were purchased from BDH Chemicals Ltd.

Results

Schematic representation of the PG and PE isoenzyme pattern in the culture filtrates

Figure 1 shows schematically all PG and PE isoenzymes found to be produced by the *B. cinerea* isolate employed in the experiments described in this paper, except PG11 and 12 which were found only in experiments (not reported here) in which yeast extract was used in the media. The pattern should not be taken as definitive, since the presence of different isoenzymes could be influenced by culture conditions and other factors related to the methods of detection. Thus, for example, some PE isoenzyme bands were often hidden if the nearest PG isoenzyme had a high activity in the gel.

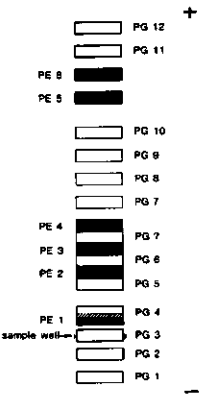

FIGURE 1. Schematic representation of the PG and PE isoenzymes produced by *B. cinerea* in cultures with different pectin-related polysaccharides as C source and detected by polyacrylamide-pectin gel electrophoresis. PGs are represented as white bands and PEs as black bands.

Assays of PL and PGL activity

Yellow bands in the electrophoresis gels, characteristic for PL activity (Cruickshank & Wade, 1980), were never detected throughout the experiments. Nevertheless, newly appearing white bands (presumably PG), not detected before in culture filtrates of *B. cinerea* (Van den Heuvel & Waterreus, 1985), were checked for PL or PGL activity. For this purpose, filtrates from cultures on leaf cell walls, producing a group of distinct white bands in the upper part of the gel, were assayed for the presence of PL and PGL activity at pH 5.0. The absence of lyase activity after all such tests confirmed that the white bands on the gels represented PG isoenzymes.

Effect of type and concentration of cell walls on pectic enzyme production

The total PG activity in cultures appeared to be influenced by the type and concentration of the insoluble cell wall preparation used in the medium as C source (Fig. 2). All treatments induced a similar increase of PG activity during the first 3 to 4 days of incubation. A further increase of PG activity was found with 0.5, but not with 1%, leaf cell walls. With cell walls from hypocotyls the reverse was found. PG2 was always the first enzyme detectable and was followed by other PG and PE isoenzymes (Fig. 3). In cultures containing 0.5% leaf cell walls, a group of PG bands was particularly distinct in the upper part of the gels from day 3 onwards (Fig. 3*a*). However, the same bands were very faint or appeared much later in experiments with another concentration or type of cell walls (Figs. 3*b*, 3*c*). Differences in number of PG isoenzymes reflected only partially those found in total PG activity. Also PE bands appeared distinct in experiments with 0.5% leaf cell walls. Unlike in host-pathogen interactions (Van den Heuvel & Waterreus, 1985), PG1 was, in general, not found in the experiments with cell walls.

Effect of pectin concentration on pectic enzyme production

With pectin as the only C source, total PG activity was higher at 0.5 than at 0.1% (Fig. 4). However, 1% pectin in the medium caused a decrease of the activity to a level similar to that obtained with 0.1%. This repressive effect resembled that found when *B. cinerea* was grown on 1% versus 0.5% leaf cell walls. If cultures were started in a medium containing 0.5% pectin and $2x10^5$ conidia ml^{-1} (results not shown) PG activity was similar to that in cultures with 0.5% pectin inoculated with $2x10^4$ conidia ml^{-1}. When the culture period was prolonged to 20 days, the activity did not change from the 10th day onwards for each pectin concentration used (results not shown).

PG2 was always the first enzyme (appearing on day 1 or 2) produced by *B. cinerea*, and mainly on the third day other PG and PE isoenzymes appeared in the culture filtrates in a sequence similar to that already seen in the experiments with insoluble cell walls (Figs. 5*a*-5*c*). Differences between the cultures were found from the second or third day and involved mainly the presence of PG1. This enzyme did not appear in cultures with 0.5% pectin inoculated with $2x10^4$ conidia ml^{-1} (Fig. 5*a*). In addition, only on 1% pectin *B. cinerea* produced clearly an isoenzyme of the PG7 to PG10 group from the 8th day onwards (Fig. 5*b*). A higher number of PG isoenzymes did not yield a corresponding higher total activity, as was already seen with cultures grown on cell walls.

Growth of the fungus in media with pectin was greater at 0.5 than at 0.1% (Fig. 6). Mycelial dry weight of cultures grown on 1% pectin were not determined since pectin stuck to the filters and hindered the filtration. This interference was not observed when media amended with 1% pectin were inoculated with higher spore concentrations. Fungal growth always reached a maximum which was then followed by a decline. Mycelial weight remained quite constant from day 9 to day 20 (results not shown). This may reflect the establishment of a balance between growth rate and rate of autolysis of the fungus. Fungal growth in the medium with 0.5% pectin inoculated with $2x10^5$ conidia ml^{-1} was intermediate between that in the medium with 0.1 and 0.5% pectin but inoculated with $2x10^4$ conidia ml^{-1}.

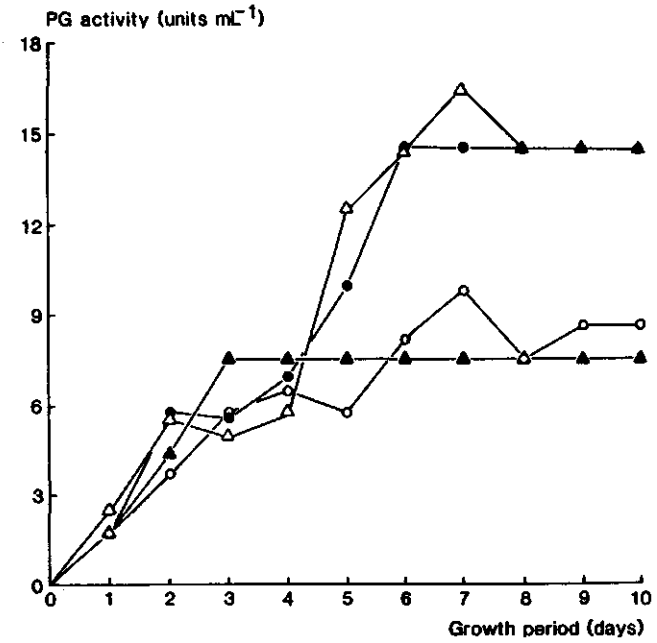

FIGURE 2. Total PG activity of *B. cinerea* grown in basal salt medium supplemented with 0.5 (closed symbols) or 1.0% (open symbols) cell walls prepared from hypocotyls (▲,△) or primary leaves (●,○) of French bean.

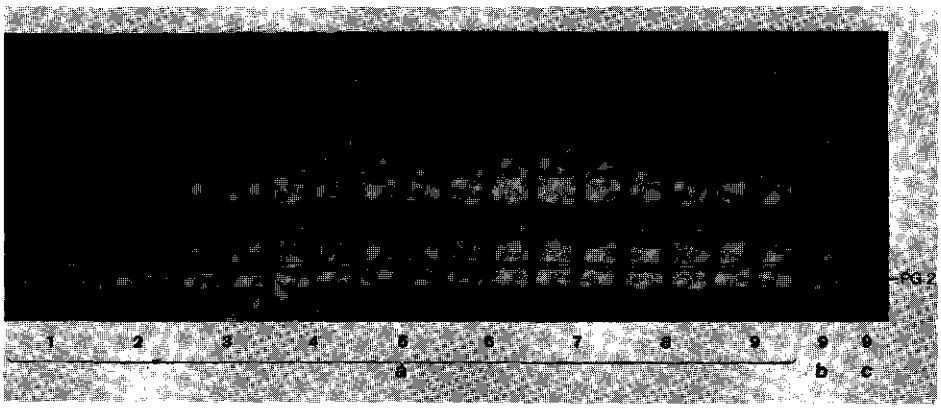

FIGURE 3. Pattern of pectic enzymes produced by *B. cinerea* in basal salt medium supplemented with cell walls prepared from primary leaves (0.5% (*a*), 1.0% (*b*)) or from hypocotyls (1.0% (*c*)). Numbers of lanes represent incubation time (days). In (*a*) the pattern of two different cultures per each day is shown.

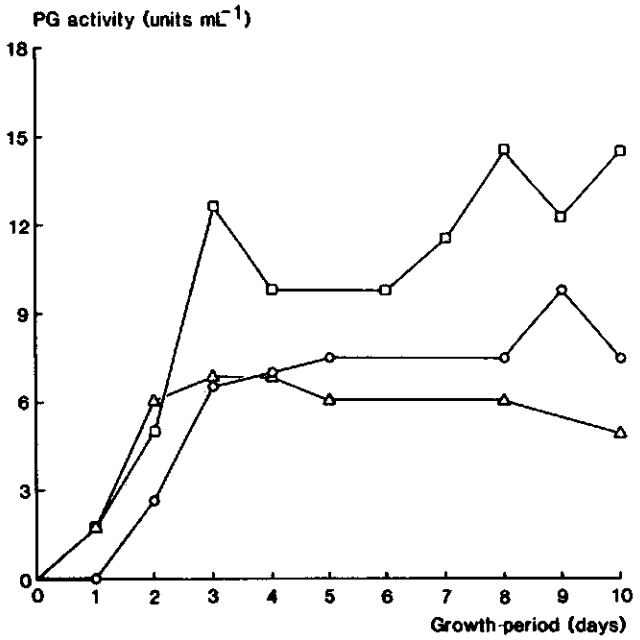

FIGURE 4. Total PG activity of *B. cinerea* grown in basal salt medium supplemented with 0.1% (△), 0.5% (□) or 1.0% (○) pectin ($2x10^4$ conidia ml^{-1}).

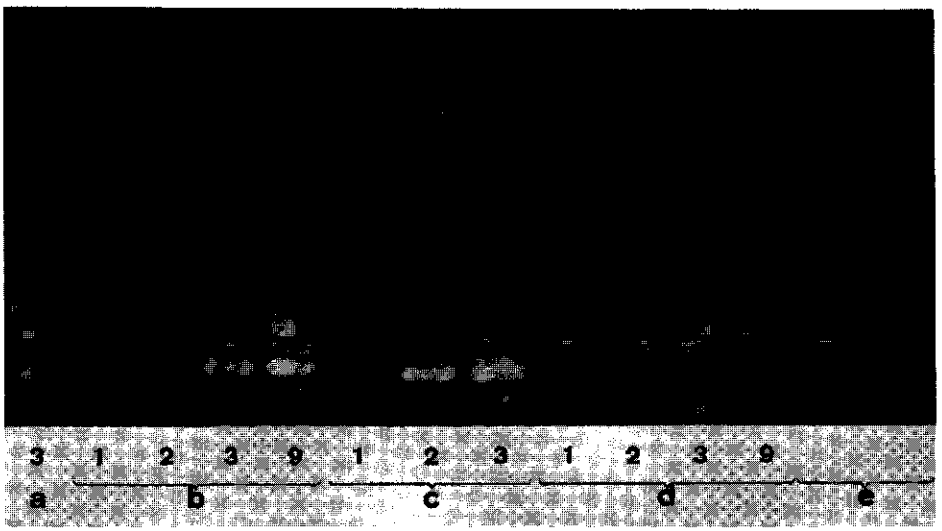

FIGURE 5. Pattern of pectic enzymes produced by *B. cinerea* in basal salt medium supplemented with 0.5% (*a*) or 1.0% (*b*) pectin ($2x10^4$ conidia ml^{-1}), with 0.5% pectin ($2x10^5$ conidia ml^{-1}, *c*) or with 0.5% sodium polygalacturonate ($2x10^4$ conidia ml^{-1}, *d*). Numbers of lanes represent incubation time (days). *e*) Identification of the PG activity present in ungerminated conidia as a single PG2 band before (right) and after (left) a 20-fold concentration step.

Effect of sodium polygalacturonate concentration on pectic enzyme production
In cultures with 0.1 and 0.5% sodium polygalacturonate PG activity remained at a low level, i. e. at about 2 and 2.5 units ml^{-1}, respectively, until day 20. At 0.5% sodium polygalacturonate PG2 was only just detectable on the first day, whereas later it was absent or barely visible (Fig. 5*d*). PG1 appeared from the second day as a vague band also, while from the third day other PG isoenzymes were just detectable in the upper part of the gel. In contrast to what was observed for the PGs, three PE isoenzymes appeared as distinct bands. In cultures on 0.1% sodium polygalacturonate isoenzyme bands were not distinctive, while in the upper part of the gel no PGs at all could be detected (results not shown).

Growth of *B. cinerea* in a medium with sodium polygalacturonate as the only carbon source was less than in a medium with pectin. The growth pattern, however, was similar during the first 10 days (Fig. 7) and thereafter. The 0.5% polygalacturonate was not completely soluble in the salt medium and this prevented the determination of the mycelial dry weight after one day of growth. The growth rates found on 0.5% sodium polygalacturonate were similar to those found on 0.1% pectin whereas the PG activity on the former substrate was much lower. This indicates that one or more products released by enzymatic degradation of polygalacturonate had a repressive and/or inhibitory effect on the fungal PGs.

PG activity in ungerminated conidia
So far, PG2 was always the first of a series of isoenzymes produced by *B. cinerea* to appear in the culture filtrates, suggesting that this enzyme may be already produced or released by germinating conidia. This prompted us to examine ungerminated conidia for the presence of PG activity. Cup-plate assays of extracts from ungerminated conidia exhibited PG activity. Gel electrophoresis of such extracts, even after a 20-fold concentration by means of ultrafiltration, yielded one single band of PG2 (Fig. 5*e*).

Effect of D-glucose on pectic enzyme production
The presence of PG2 in ungerminated conidia suggests its rapid involvement in substrate degradation, but it does not discriminate between the expression of a constitutive gene and the basal synthesis of an inducible enzyme. Therefore, the effect of D-glucose, a C source that is not a substrate for any pectic enzyme, on enzyme production was studied. Enzyme production in a medium containing 0.1% pectin plus 20 mM D-glucose was compared with that in a medium containing 0.1% pectin only but to which after two days 20 mM D-glucose was added. Pectin at 0.1% was chosen because in previous experiments with this concentration different PG and PE isoenzymes were produced at the second day. Figure 8 shows that addition of glucose to the medium with pectin, either on day 0 or day 2, strongly enhanced PG activity (see also Fig. 4). This enhanced activity was apparently due to a stimulation of the activity of, in particular, PG2, PG3 and PG4; PG1 did not seem to be stimulated (Fig. 9*a*). The enhancement of PG activity after addition of glucose on day 2 was associated with a large increase of fungal dry weight (from 0.3 mg ml^{-1} on day 2 to 1.65 mg ml^{-1} on day 3).

A rapid increase in growth on a substrate which is also a potential inducer of the enzymes may indirectly cause an increase of induced enzymes. Thus, in order to avoid a non-specific stimulation of inducible enzymes, the effect of glucose on pectic enzyme production was also studied in an experiment in which the basal salt medium was supplemented with 0.11 M glucose as the only C source, as used by Van den Heuvel & Waterreus (1985). Moreover two inoculum concentrations were compared ($2x10^4$ and $2x10^6$ conidia ml^{-1}) since it was expected that a higher conidial concentration would raise the level of constitutive enzymes. Fig. 10 shows the differences in total PG activity reached when the cultures were started with the two spore concentrations. A good correlation between PG activity and mycelial growth was observed (Figs. 10 and 11). Fig. 9*b* shows that the activity measured corresponded mainly to the band of PG2 since other PG isoenzymes were only slightly detectable. This strongly suggests that PG2 is a constitutive enzyme, whose production in vitro is dependent on rate of mycelial growth.

Effect of D-galacturonic acid concentration on pectic enzyme production
A possible effect of D-galacturonic acid, an end product of enzymatic degradation of

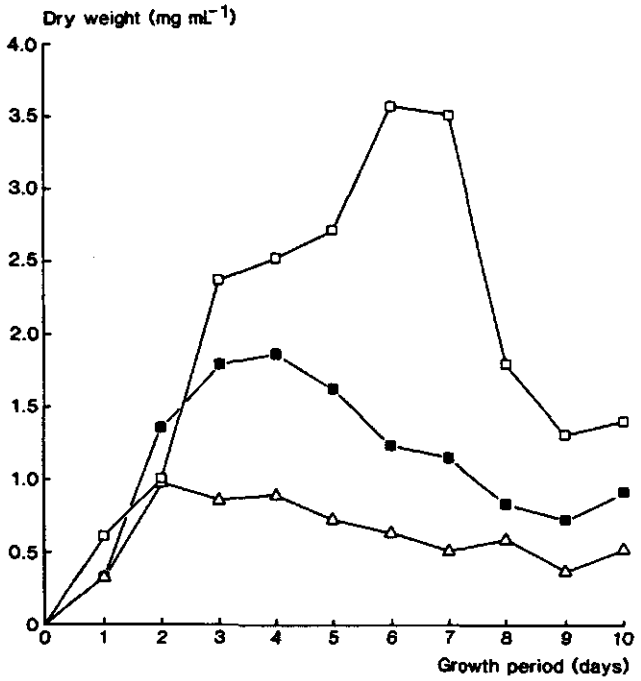

FIGURE 6. Growth of *B. cinerea* in basal salt medium supplemented with 0.1% (△) or 0.5% (□) pectin ($2x10^4$ conidia ml^{-1}) or with 0.5% pectin ($2x10^5$ conidia ml^{-1}, ■).

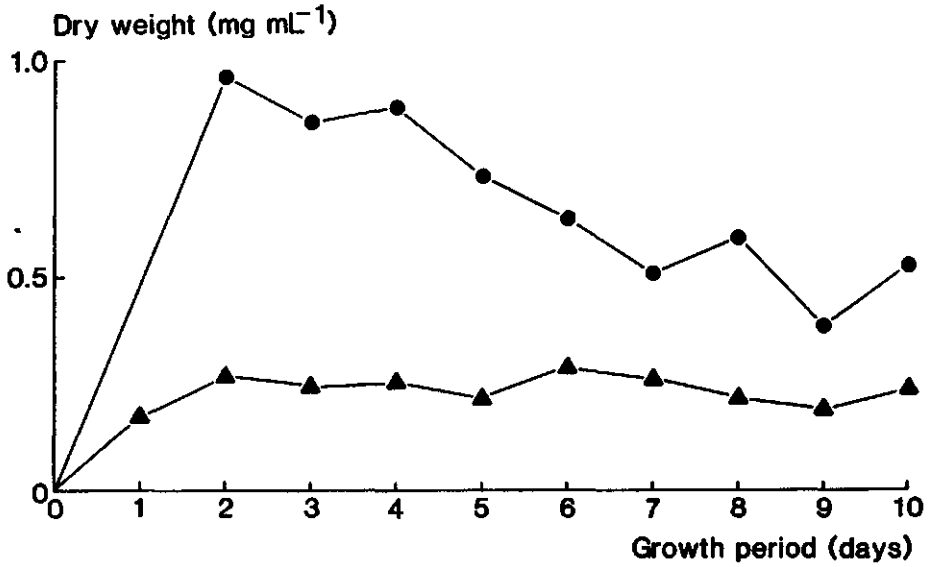

FIGURE 7. Growth of *B. cinerea* in basal salt medium supplemented with 0.1% (▲) or 0.5% (●) sodium polygalacturonate.

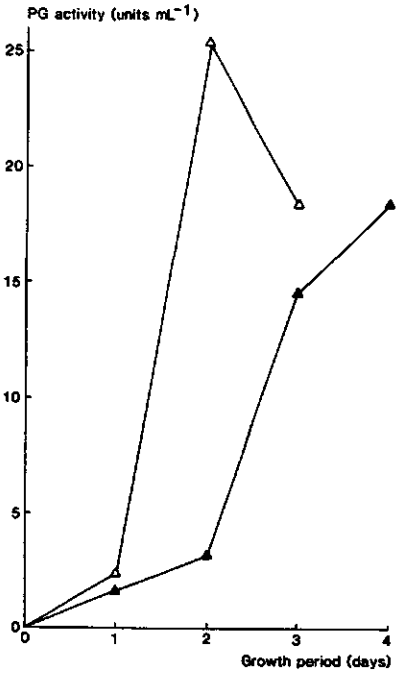

FIGURE 8. Total PG activity of *B. cinerea* grown in basal salt medium supplemented with 0.1% pectin and 20 mM D-glucose on day 0 (△) or with 0.1% pectin on day 0 and 20 mM D-glucose on day 2 (▲).

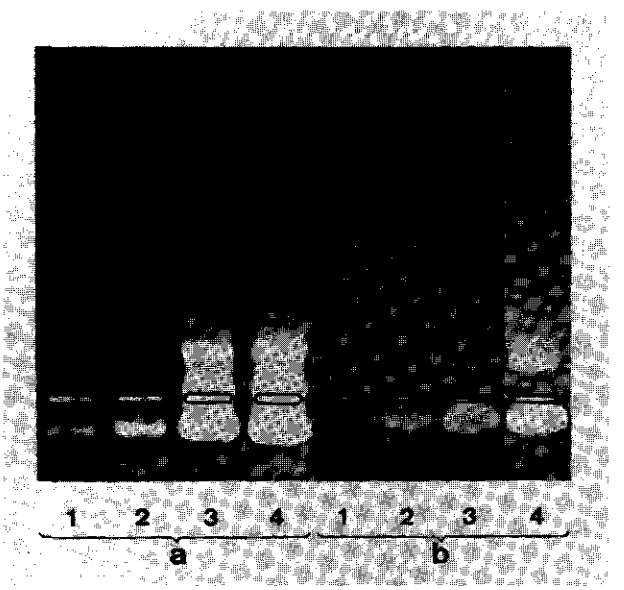

FIGURE 9. Pattern of pectic enzymes produced by *B. cinerea* in basal salt medium supplemented with 0.1% pectin on day 0 and 20 mM D-glucose on day 2 ($2x10^4$ conidia ml^{-1}, *a*) or with 0.11 M D-glucose ($2x10^6$ conidia ml^{-1}, *b*). Numbers of lanes represent incubation time (days).

pectic polysaccharides, on pectic enzyme production was studied in a system with a high production of PG2. *B. cinerea* cultures were grown on basal salt medium amended with 0.11 M D-glucose plus different concentrations of D-galacturonic acid (2, 10 and 50 mM) and inoculated with $2x10^6$ conidia ml^{-1}. Glucose was used in the media to stimulate mycelial growth and thus the constitutive synthesis of PG2 and because it had been reported (Moline & Gross, 1984; Verhoeff & Liem, 1978) that with D-galacturonic acid as the only C source, germination of *B. cinerea* conidia was weak or absent. Total PG activity and pectic isoenzyme pattern are shown in Figs. 12 and 13. The PG activity is expressed per mg mycelial dry weight since, as expected, fungal growth rate was negatively correlated with concentration of D-galacturonic acid in the medium. At 2 mM galacturonic acid PG activity increased similarly as at 0 mM but at 2 mM PG1, PG2, PG4 and PE2 were already present on day 1 (Fig. 13*a*). However, at 10 and, in particular, at 50 mM the increase in PG activity was delayed. This delay was associated with a delay in the appearance of PG2 and all other pectic isoenzymes that are normally detected (Figs. 13*b*, 13*c*). Since the negative effect of higher concentrations of galacturonic acid on PG2 gradually disappeared, it is supposed that this compound was metabolized by the fungus in the course of time.

As it was impossible to conclude from these results whether D-galacturonic acid was acting directly on the activity of PG2 (inhibition) or on its production (repression), the following experiment was carried out. *Botrytis cinerea* ($2x10^6$ conidia ml^{-1}) was grown in the basal salt medium containing 1% pectin. Preliminary experiments had shown that under these conditions a very rapid production of PG2, PG1, PE1 and PE2 took place. Furthermore, fungal growth did not appear to be influenced by D-galacturonic acid as occurred with media not supplemented with a pectic polysaccharide. After one day of growth, 10 mM D-galacturonic acid was added to the cultures and these were then sampled one day later. Controls to which D-galacturonic acid was not added were also assayed.

Table 1 shows that on the second day PG activity had increased in cultures on pectin only, but had significantly decreased if D-galacturonic acid had been added, although fungal dry weight had increased. PG2 was clearly detectable by means of gel electrophoresis in all samples assayed (results not shown). The experiment was repeated also using 20 mM D-galacturonic acid and sampling 1 and 6 hours after addition. PG activity just started to decrease at 6 hours but PG2 was always clearly detectable. These results suggest an involvement of D-galacturonic acid in the repression of the synthesis but not in the inhibition of the activity of this enzyme.

TABLE 1. Effect of addition of D-galacturonic acid to 1-day-old cultures of *Botrytis cinerea* growing in a basal salt medium supplemented with 1% pectin, on the PG activity (in units per ml culture filtrate)[a]

Carbon source	Day 1	Day 2
1% pectin	14.5 + 0[b]	21.5[c] + 0.005
1% pectin + 10 mM D-galacturonic acid	14.5 + 0	12.4[d] + 0

[a] Cultures started with $2x10^6$ conidia ml^{-1}.
[b] Averages of five replicates + standard error.
[c] Dry weight (mg ml^{-1}): 2.88 + 0.06 s.e.
[d] Dry weight (mg ml^{-1}): 3.86 + 0.09 s.e.

Discussion

A consistent sequence in the production of pectic enzymes was observed during growth of *B. cinerea* in a medium with a pectic polysaccharide as the only C source. The enzyme, originally referred to as PG2 (Van den Heuvel & Waterreus, 1985), was found to be the first secreted by the fungus in the culture media. Other PG and PE isoenzymes appeared later, their presence being mainly dependent on type and concentration of the

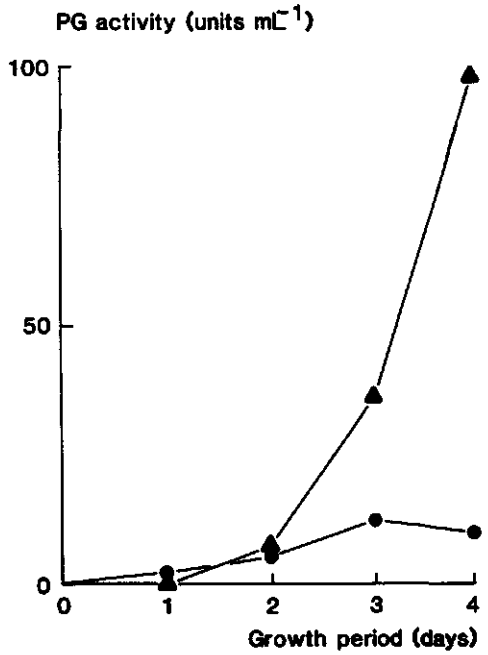

FIGURE 10. Total PG activity of *B. cinerea* grown in basal salt medium supplemented with 0.11 M D-glucose and started with $2x10^4$ (●) or $2x10^6$ (▲) conidia ml^{-1}.

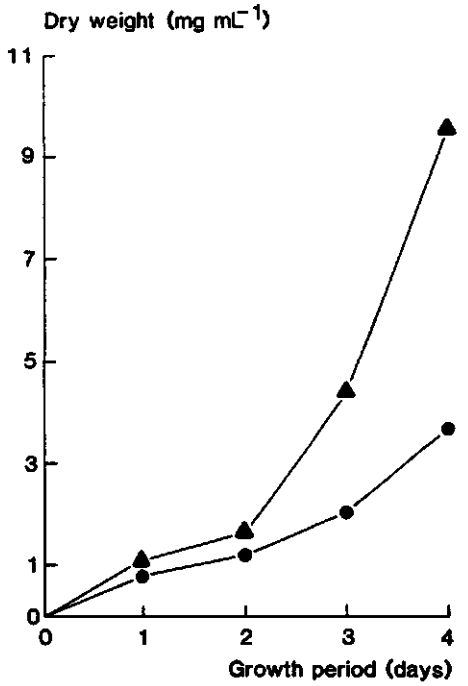

FIGURE 11. Growth of *B. cinerea* in basal salt medium supplemented with 0.11 M D-glucose and started with $2x10^4$ (●) or $2x10^6$ (▲) conidia ml^{-1}.

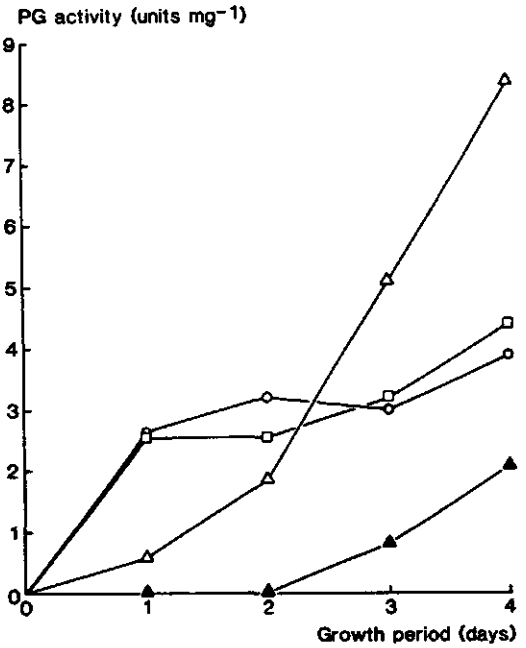

FIGURE 12. Total PG activity (expressed per mg mycelial dry weight) of *B. cinerea* grown in basal salt medium supplemented with 0.11 M D-glucose and 0 (○), 2 (□), 10 (△) or 50 (▲) mM D-galacturonic acid ($2x10^6$ conidia ml^{-1}). Values are averages of three cultures.

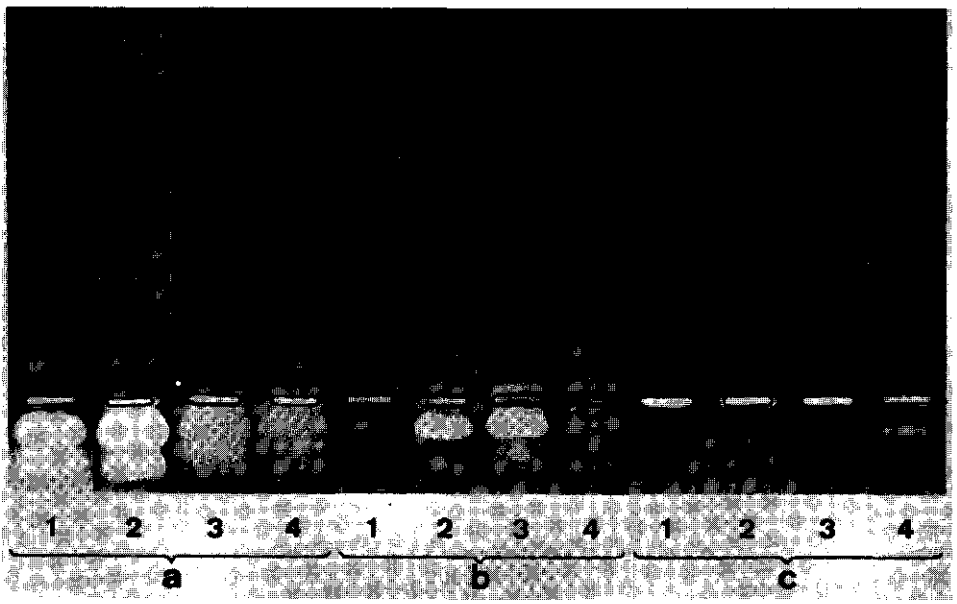

FIGURE 13. Pattern of pectic enzymes produced by *B. cinerea* in basal salt medium supplemented with 0.11 M D-glucose and 2 (*a*), 10 (*b*) or 50 (*c*) mM D-galacturonic acid ($2x10^6$ conidia ml^{-1}). Numbers of lanes represent incubation time (days).

C source used in the experiments. If PG1 was produced, it was always the second isoenzyme detectable as was already observed during the penetration stage of the infection of French bean leaves (Van den Heuvel & Waterreus, 1985).

The finding of a sequence in the secretion of pectic enzymes by *B. cinerea* helps to elucidate the role of the different isoenzymes. The enzyme complex may be interpreted as a coordinated catabolic pathway used by the fungus for a complete depolymerization of pectic polysaccharides. Thus, the reaction started by PG2 is followed by other isoenzymes that may further break down fragments released from the polymers. The fungus was able to metabolize the products formed by this enzymatic activity. The ability of *B. cinerea* to grow on cell wall-related polysaccharide fractions and/or monosaccharides, when supplied as the only energy source, is already known (Gross & Moline, 1986; Moline & Gross, 1984; Rattingan & Ayres, 1975).

A recurrent question arising from studies on microorganisms able to grow on a cell wall-related polymer, is how these microbes can sense the substrate in their environment, although such large molecules cannot enter the cell (Darvill *et al.*, 1985). It has been suggested that they may secrete a low basal level of cell wall-degrading enzymes and that the breakdown products may subsequently enter the cell, serving either as an energy source or as inducers of the synthesis of more enzyme(s) (Cooper & Wood, 1975; Lin & Kolattukudy, 1978; Stack *et al.*, 1980). The presence of PG2 in ungerminated conidia of *B. cinerea* supports this view. This mechanism may be used by the fungus to recognize a pectic polymer, thereby triggering the autocatalytic induction of the sequence of pectic and other cell wall-degrading enzymes, if necessary. It should be noted that together with endo-PG activity (Verhoeff & Liem, 1978) also cutinolytic activity (J. Salinas, personal communication) and cellulase, glucanase and cellobiase activity was found in ungerminated conidia of a *B. cinerea* isolate from tomato (Verhoeff *et al.*, 1983).

The production of PG2 appeared to be strictly correlated with fungal growth, also in the absence of a pectic polysaccharide, thus showing the characteristics of an enzyme expressed by a constitutive gene. In contrast, the other PG and PE isoenzymes could always be clearly detected after PG2 had been secreted by the fungus and had acted on the substrate. Moreover, their production was much lower in cultures growing on D-glucose than in cultures on a pectic polysaccharide. Therefore, the secretion of the PG and PE isoenzymes following PG2 may be seen as an induced production, stimulated by some breakdown product released from the substrate. D-galacturonic acid may be an inducer of the synthesis of at least the isoenzymes located in the lower part of the gel after electrophoresis, since after addition of 2 mM galacturonic acid these enzymes were already detectable after one day of growth (see Fig. 13*a*). This compound has been claimed also to be the inducer of the pectic enzyme activity of *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium albo-atrum* (5). The involvement of other breakdown products, such as short oligomers, in the induction of PGs cannot be ruled out since, for example, the isoenzymes located on the upper part of the gels were clearly induced only when the fungus was growing on cell walls.

It has been reported for several inducible PGs (e.g. Cooper, 1983; Keen & Norton, 1966; Patil & Dimond, 1968) that the addition of glucose to fungi growing on a pectic substrate can cause a strong reduction in their production. This effect, exerted by easily metabolized C sources such as glucose, has been interpreted as catabolite repression (Cooper, 1983). Our results obtained when glucose was added to cultures growing on pectin, did not provide evidence for catabolite repression as a possible regulatory mechanism for the inducible pectic enzymes of *B. cinerea* located in the lower part of the gel (Fig. 9*a*). This response may indicate that also during the colonization of host tissues the synthesis of some isoenzymes will be hardly influenced by the presence of free sugars in the surrounding environment.

Our results provide evidence for a high level of control of pectic enzyme synthesis. Under the conditions used galacturonic acid above 10 mM repressed the production and therefore the PG activity measured. Remarkably, together with PG2 the whole pectic isoenzyme complex appeared to be repressed. However, a lower concentration of galacturonic acid stimulated the secretion of some isoenzymes. Considering the sequence of isoenzymes as a system providing a coordinated degradation of galacturonan polymers, with PG2 as the starting enzyme and galacturonic acid as an end product, our results

suggest the occurrence of a feed-back repression (Drew & Demain, 1978).

Total PG activity and number of isoenzymes detectable in the course of time were influenced by type and concentration of pectic polysaccharides and/or concentration of conidia used. This effect may have been exerted by different yields of breakdown products released by enzyme action under the different experimental conditions. Particularly with sodium polygalacturonate, some of these products may have been oligogalacturonates that are known to be inhibitors of the activity of purified PGs of *B. cinerea* (Marcus & Schejter, 1983; Urbanek & Zalewska-Sobczak, 1975). The results obtained using equal concentrations of cell walls of different organs also reflect quantitative differences in the rhamnogalacturonan components present in the cell wall preparations, leaf cell walls probably containing more of them than hypocotyl cell walls. Recently, it has also been hypothesized that the complex carbohydrates of the cell walls of different organs and tissues in a single plant could be different (Darvill *et al.*, 1985).

The distinct forms of regulation mediated by the C source represent for the fungus a mechanism avoiding unnecessary pectic enzymes synthesis and/or activity. As a consequence of this metabolically dynamic process, time of sampling and conditions may be expected also to influence the number of pectic isoenzymes detectable *in vitro* and/or *in vivo* experiments. The *B. cinerea* isolate used readily produced PG1 and PG2 during early interactions on primary leaves of French bean. These enzymes were therefore postulated to be involved in the penetration of the epidermis of the leaves (Van den Heuvel & Waterreus, 1985). When this isolate was cultured *in vitro*, PG2 was quite constantly produced, whereas PG1, as other isoenzymes, appeared only under particular conditions. If cell walls were used to induce the enzymes, PG1 was unexpectedly never detected in the culture filtrates. The lack of correspondence between isoenzymes produced by microorganisms in culture and in plants, is a well known, though not yet fully understood, phenomenon (Cooper, 1983; Marciano *et al.*, 1982; Panopoulos *et al.*, 1984) and has been reported also for *B. cinerea* (Di Lenna & Fielding, 1983). An explanation for this event should take into account the potential high degree of control of each isoenzyme.

Acknowledgements

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CHAPTER 3

REGULATION BY ORTHOPHOSPHATE AND ADENINE NUCLEOTIDES OF THE BIOSYNTHESIS OF TWO POLYGALACTURONASES BY *BOTRYTIS CINEREA IN VITRO*

by

G. Leone, A. N. Overkamp, M. N. Kreyenbroek, E. Smit & J. Van den Heuvel

Abstract

The effect of orthophosphate, adenine, adenosine and a few selected adenine nucleotides on the production of two polygalacturonase (PG) isoenzymes by *Botrytis cinerea* in cultures without any pectic substrate was investigated. In the absence of P-containing compounds, PG activity was very low. Cyclic AMP, ADP and ATP stimulated the constitutive production of isoenzyme PG2 as well as fungal growth, whereas AMP stimulated the inducible production of isoenzyme PG1, but not fungal growth. The effects of orthophosphate on PG1 and PG2 production and on fungal growth were intermediate between those of AMP and the other nucleotides. The phosphate- or adenine nucleotide-dependent PG production was inhibited by cycloheximide and, to a lesser extent, by actinomycin D, implying the involvement of *de novo* enzyme synthesis. PG1 was also readily detected when fungal ATP production was inhibited by the uncoupler salicylanilide. The results provide evidence that the phosphate-dependent PG1 and PG2 synthesis by *B. cinerea* is mediated by adenine nucleotides. It is proposed that PG1 biosynthesis is not only controlled by a pectic substrate but also by the energy charge or by the proportion of AMP in the total adenylate pool.

Introduction

Conidia of the plant-pathogenic fungus *Botrytis cinerea* Pers.: Fr. are partially dependent on the presence of exogenous nutrients in the infection drop in order to germinate and infect plants (Brown, 1922; Clark & Lorbeer, 1977). In particular monosaccharides, inorganic phosphate and purine nucleotide derivatives have been reported to stimulate the ability of *B. cinerea* to cause infections (Kosuge & Hewitt, 1964; Clark & Lorbeer, 1977; Ko *et al.*, 1981; Van den Heuvel, 1981; Van den Heuvel & Waterreus, 1983). The stimulation by phosphate of the infection by *B. cinerea* on French bean leaves has been correlated with an enhancement of the activity of pectic enzymes (Van den Heuvel & Waterreus, 1985). Two polygalacturonases (PGs), viz. PG1 and PG2, were reported to be the first pectic enzymes produced a few hours after inoculation. Studying the influence of different pectin-related polysaccharides on pectic enzyme production by *B. cinerea in vitro*, Leone & Van den Heuvel (1987) found that PG2 was a constitutive enzyme, whereas PG1 was inducible. The coordinated, sequential production of both and other isoenzymes was strongly controlled by the type and concentration of the carbohydrate used as C source.

PGs have often been claimed to play an important role in host-pathogen interactions (e.g. Collmer & Keen, 1986) but how inorganic phosphate could affect their activity or production is not known. Therefore, the present study was designed to ascertain whether, and if so, how phosphate and/or adenine nucleotides regulate the biosynthesis of the PGs by the *B. cinerea* isolate BC1. To assess whether this regulative control acts independently from that found for carbohydrates (Leone & Van den Heuvel, 1987), experiments were performed in the absence of any pectic inducer. The existence of a link between pectic enzyme production and a certain P-containing compound was

investigated by adding the compound to P-free cultures of the fungus, measuring total PG activity and identifying the relative type of PG isoenzyme formed. Experiments designed to affect the intracellular concentrations of adenine nucleotides were performed in order to gain more insight into the possible importance of the energy charge or of particular nucleotides in PG synthesis regulation.

Materials and methods

Culture and growth conditions

For all experiments isolate BC1 of *B. cinerea*, from French bean, was used and was maintained on PDA slants at 4°C. For obtaining sporulating cultures, it was inoculated on slants of PDA or synthetic medium X (Last & Hamley, 1956) and incubated for 11-14 days at about 23°C under continuous fluorescent light. No differences in PG production were found between cultures derived from conidia formed on these two media. Conidial suspensions were added to 25-ml flasks, each containing 10 ml sterilized Richards' salt solution from which KH_2PO_4 was omitted, to give a final concentration of $2x10^4$ conidia ml^{-1}. The medium contained, per 1000 ml of distilled water, 10 g KNO_3, 0.25 g $MgSO_4.7H_2O$, 0.4 mg $FeCl_3$ and 20 g D-glucose as carbon source. Before use the flasks had been washed in a 2% Extran MA-03 (phosphate-free, Merck) solution, in order to avoid phosphate contamination from detergents.

A number of compounds were added to the flasks just before inoculation, to test their effect on PG production: adenine, adenosine, 3',5'-cyclic AMP (cAMP), 5'-AMP disodium salt (AMP), ATP disodium salt (ATP), KH_2PO_4, $NaH_2PO_4.2H_2O$, $Na_2HPO_4.2H_2O$ (all from Merck) and ADP sodium salt (ADP, Sigma). Controls received no P-containing compound at all. Some of them are not stable at high temperatures and were, therefore, not sterilized. The compounds were dissolved in sterile medium to give concentrated solutions and known amounts of these solutions were added to the cultures to give a final concentration of 2.5 mM, unless otherwise stated. Under those conditions, cultures of *B. cinerea* remained macroscopically and microscopically free of contamination.

For examining the response of mycelium growing under P starvation to adenosine, cAMP, AMP, ATP and KH_2PO_4, these compounds were added 48 h after the inoculation with conidia. A possible effect on PG production exerted by the protein synthesis inhibitor cycloheximide, the poly(A)-containing mRNA inhibitor actinomycin D and the uncoupler salicylanilide, was examined following addition of these compounds together with AMP, ATP or KH_2PO_4 to the cultures also 48 h after inoculation. Actinomycin D and cycloheximide were supplied to the cultures as concentrated solutions in sterile medium, giving a final concentration of 20 $\mu g\ ml^{-1}$. Because of its low solubility in water, salicylanilide was dissolved in 96% ethanol and added to sterile medium. Its final concentration was 5 $\mu g\ ml^{-1}$ and the concentration of ethanol in the culture medium was less than 0.1% (v/v). A control treatment (medium + 0.1% ethanol, without salicylanilide) was used to test a possible effect of ethanol on PG production.

After inoculation the flasks were shaken continuously at 19°C on a reciprocal shaker (136 strokes min^{-1}) for the chosen period of growth (never more than 120 h). At intervals, series of cultures were harvested and the content of each flask was filtered on preheated and preweighed filter paper (S. & S. no. 604, Dassel, F.R.G.). Mycelial dry weights were determined after heating at 60°C for 48 h. The culture filtrates were stored at -20°C until use.

Assay of intracellular adenine nucleotide levels

During the experiments designed to study the effects of addition of P-containing compounds on PG production, a very small fungal mass was generally produced (see Results). In order to gain enough mycelium to be used for the assays of the intracellular adenine nucleotide concentration, a different system needed to be developed. Three hundred-ml flasks containing 100 ml sterilized Richards' solution without KH_2PO_4 were inoculated with 7-day old mycelium grown on PDA in a Petri dish (diameter 20 cm), after scraping off one fourth of the plate surface. The cultures were incubated on a

reciprocal shaker at 120 strokes min^{-1} at 19°C. Four days after inoculation, AMP, ADP, ATP or KH_2PO_4 was added to the medium to give a final concentration of 2.5 mM. At 48 and 72 h after the addition of the P-containing compounds, the mycelium from each flask was filtered separately over a plastic sieve and quickly rinsed in phosphate-free Richards' solution. Culture filtrates were used to determine total PG activity and to identify the PG isoenzyme(s) present, in order to verify whether the fungal response to the compound was similar to that of the previous experimental systems. The sieve with mycelium was then immersed in liquid nitrogen and the frozen fungal material was ground to a fine powder using a precooled mortar and pestle. The sampling, including the freezing step, took less then 15 sec. The frozen powder was stored at -20°C for not more than one month. For the extraction, the following procedure, based on that of Bergmeyer (1974), was used. Five ml 12% $HClO_4$ (w/v) was added to 2 g of powder; the resulting slurry was homogenized for 20 sec and then centrifuged at 12000 g for 10 min. The supernatant was adjusted to pH 6.2-6.5 with 5 M K_2CO_3, centrifuged as before and assayed directly for adenine nucleotides following Bergmeyer's method (1974). The mycelial ATP concentration was determined through coupled enzyme assays using hexokinase and glucose-6-phosphate dehydrogenase (both from Merck). For each mole ATP present in the sample assayed, one mole NADPH is formed, giving a change in absorbance at 340 nm that was measured spectrophotometrically at 20°C. The ADP and AMP contents were also determined through coupled enzyme assays using myokinase, pyruvate kinase and lactate dehydrogenase (all from Merck). For each mole ADP and AMP present in the sample assayed, a proportional amount of NADH is converted to NAD^+, this giving a change in absorbance at 340 nm that was followed spectrophotometrically at 20°C. The energy charge (Atkinson, 1969) is expressed as: $\frac{[ATP]+1/2[ADP]}{[ATP]+[ADP]+[AMP]}$.

Enzyme assays

PG activity in the culture filtrates was determined quantitatively by a slight modification (Leone & Van den Heuvel, 1987) of the cup-plate assay method (Dingle *et al.*, 1953). One unit of PG activity was defined as the activity necessary to release 1 µmole of D-galacturonic acid h^{-1} at pH 5.0 under standard conditions (Leone & Van den Heuvel, 1987). PG activity was expressed in units mg^{-1} mycelial dry weight in order to compensate for differences in fungal growth resulting from the different compounds tested.

The pectic enzymes produced in the culture medium were identified by pectin-polyacrylamide gel electrophoresis (Cruickshank & Wade, 1980) as modified by Van den Heuvel & Waterreus (1985). Conditions used and numbering of the PG isoenzymes were identical to those reported earlier (Leone & Van den Heuvel, 1987). An arbitrary scale was used to give a schematic representation of the intensity of the gel bands: -, no band visible; +, vague band; ++, clear band; +++, distinct and wide band.

Statistical analysis

The experiments were performed three times; in each experiment, each treatment was done in triplicate. Values obtained per time after addition of the P-containing compounds were subjected to analysis of variance (mycelial dry weights) or of covariance (PG units mg^{-1} mycelial dry weight) (Snedecor, 1956) after transformation to log(x+1). Means were subjected to Duncan's multiple comparison test (Steel & Torrie, 1960) for significance of differences.

Results

Effect of addition of different forms of inorganic phosphate, adenine, adenosine and adenine nucleotides at the time of inoculation on mycelial growth and PG production

When KH_2PO_4, NaH_2PO_4, adenine, adenosine or adenine nucleotides were added to flasks at the time of inoculation, clear effects on mycelial growth were not easily detectable because of the scarce fungal mass produced (generally around 1 mg ml^{-1};

Table 1). Not until 72 h after inoculation were more distinct differences in mycelial dry weight noted; among the nucleotides ATP gave the strongest growth stimulation, followed by cAMP and ADP. In contrast, AMP and the other compounds had no significant effect on hyphal growth compared with the control, except for adenine which appeared to be inhibitory.

At 24 h after addition of the compounds and inoculation, PG activity was detectable only with AMP and an equimolar mixture of adenosine and Na_2HPO_4 (Table 1). At 48 and 72 h, PG activity was present in all cultures. In the presence of a P-containing compound PG activity was always higher than that of the control or than in the presence of adenine or adenosine. Among the nucleotides, cAMP, AMP and ATP gave similar PG activities and ADP gave the strongest PG stimulation. Among the inorganic forms of phosphate, the highest PG production was found with NaH_2PO_4 and with an equimolar mixture of adenosine and Na_2HPO_4.

TABLE 1. Effect of adenine, adenine nucleosides and nucleotides and different forms of inorganic phosphate (concentration used: 2.5 mM) on mycelial dry weight (in mg ml^{-1}) and on total PG production (in units mg^{-1} mycelial dry weight) by *B. cinerea*. The compounds were added to phosphate-free medium at the time of inoculation.

Compound added	hours after addition					
	24		48		72	
	Dry wt	PG activity	Dry wt	PG activity	Dry wt	PG activity
None (control)	0.96b[1]	0a	1.31bc	0.12a	1.39bc	0.54a
Adenine	0.35a	0a	0.89ab	1.88c	0.66a	0.87a
Adenosine	0.82b	0a	1.01ab	0.79b	1.22b	2.01b
cAMP	0.86b	0a	0.94ab	2.05cd	2.90ef	3.35b
AMP	1.07b	1.16b	0.75a	4.02de	1.52bcd	3.41b
ADP	1.00b	0a	1.08bc	7.61f	2.58de	7.67c
ATP	0.82b	0a	1.62cd	2.48cd	3.52f	3.22b
ATP (0.83 mM)	0.83b	0a	1.04ab	4.21de	2.09cde	3.49b
KH_2PO_4	0.76b	0a	1.32bc	3.28d	1.84cd	3.41b
NaH_2PO_4	0.80b	0a	nd[2]	nd	1.64bcd	5.92c
Adenosine + Na_2HPO_4	0.87b	1.93b	0.86ab	3.55de	1.37bc	8.25c

[1] Means within a single column followed by a different letter are significantly different (Duncan's multiple comparison test, P=0.05).
[2] nd: not determined.

The effect of the various compounds on the type of pectic enzymes produced is shown in Table 2 and Fig. 1. At 48 h after addition of these compounds, the constitutive isoenzyme PG2 was found under all conditions. In general, the PG2 band was hardly detectable in cultures without a P-containing compound, whereas in the other cultures this band was clearly visible. In cultures with AMP or a mixture of adenosine and Na_2HPO_4, a distinct band of the inducible isoenzyme PG1 was also detected after 48 and 72 h, although no pectic inducers were present in the culture medium. In all other cases, except 72 h after the addition of NaH_2PO_4, PG1 was either absent or barely visible.

Effects of addition of KH_2PO_4, adenosine and adenine nucleotides at 48 h after inoculation on mycelial growth and PG production

The phosphate-dependent total PG production and the AMP-dependent PG1 production were further investigated in experiments where only few, representative compounds, within those used above, were added to *B. cinerea* cultures growing for 48 h under P starvation. When KH_2PO_4, adenosine or adenine nucleotides were added to the medium at this time, the growth rate of the fungus was generally similar to that observed when

the compounds had been added at inoculation time. However, clear differences between treatments became already detectable 48 h after addition (Table 3). In comparison with the other P-containing compounds, AMP gave a slower growth rate throughout the experiments. At 72 h the dry weight of the cultures grown in the presence of AMP was significantly lower than that of the other nucleotides. In contrast, at the same time ATP gave the highest dry weight. The effects of KH_2PO_4 and cAMP appeared intermediate between those of AMP and ATP.

TABLE 2. Effect of adenine, adenine nucleosides and nucleotides and different forms of inorganic phosphate (concentration used: 2.5 mM) on the type of PG isoenzymes produced by *B. cinerea*. The compounds were added to phosphate-free medium at the time of inoculation.

Compound added	hours after addition					
	24		48		72	
	PG1	PG2	PG1	PG2	PG1	PG2
None (control)	-[1]	-	-	+	-	+
Adenine	-	-	-	+	-	+
Adenosine	-	-	-	+	+	+
cAMP	-	-	-	+ +	-	+ + +
AMP	+	+	+ + +	+	+ +	+ +
ADP	-	-	-	+ + +	-	+ + +
ATP	-	-	-	+ + +	-	+ + +
ATP (0.83 mM)	-	-	+	+ + +	+	+ + +
KH_2PO_4	-	-	+	+ + +	+	+ + +
NaH_2PO_4	-	-	nd[2]	nd	+ +	+ + +
Adenosine + Na_2HPO_4	+	+	+ +	+ +	+ +	+ + +

[1] -; +; + +; + + +: increasing intensity of the PG bands on pectin-polyacrylamide gels (see Materials and methods).
[2] nd: not determined.

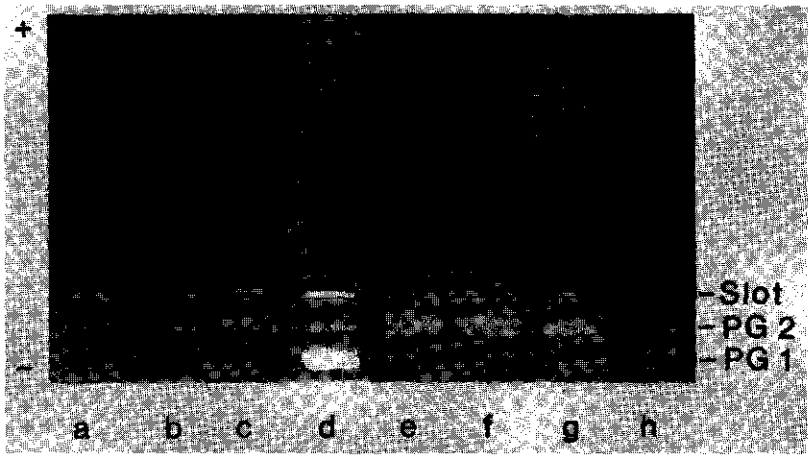

FIGURE 1. Pattern of PG isoenzymes produced by *B. cinerea* in phosphate-free Richards' solution supplemented with adenine (lane *a*), adenosine (*b*), cAMP (*c*), AMP (*d*), ADP (*e*), ATP (*f*), KH_2PO_4 (*g*), all at 2.5 mM and added at the time of inoculation; and KH_2PO_4 (2.5 mM) + salicylanilide (5 $\mu g\ ml^{-1}$) (*h*), both added at 48 h after inoculation. All the samples were taken after a 48-h-incubation period of the cultures with the compounds added.

Enhanced total PG activity was detected in the culture filtrates already at 24 h after addition of P-containing compounds (Table 3). In cultures without adenine nucleotides or phosphate, total PG activity remained significantly low. AMP and KH_2PO_4 gave the highest stimulation of PG production.

TABLE 3. Effect of adenosine, adenine nucleotides and KH_2PO_4 (concentration used: 2.5 mM) on mycelial dry weight (in mg ml^{-1}) and total PG production (in units mg^{-1} mycelial dry weight) by *B. cinerea*. The compounds were added to phosphate-free growing cultures 48 h after inoculation.

Compound added	hours after addition					
	24		48		72	
	Dry wt	PG activity	Dry wt	PG activity	Dry wt	PG activity
None (control)	1.40a[1]	0.36a	1.60a	0.50a	1.38a	1.45ab
Adenosine	1.37a	0.46a	1.83a	0.79a	1.35a	1.30a
cAMP	1.12a	1.17b	2.47bc	2.79b	3.06c	1.73ab
AMP	2.03a	2.29c	1.89ab	4.48c	1.83b	7.32c
ATP	1.22a	1.23b	2.59c	3.76b	5.06d	2.95b
KH_2PO_4	1.15a	2.32c	2.25bc	3.63b	2.47c	5.84c

[1] Means within a single column followed by different letters are significantly different (Duncan's multiple comparison test, P=0.05).

TABLE 4. Effect of adenosine, adenine nucleotides and KH_2PO_4 (concentration used: 2.5 mM) on the type of PG isoenzymes produced by *B. cinerea*. The compounds were added to phosphate-free growing cultures 48 h after inoculation.

Compound added	hours after addition					
	24		48		72	
	PG1	PG2	PG1	PG2	PG1	PG2
None (control)	-[1]	+	-	+	-	+
Adenosine	-	+	-	+	-	+
cAMP	-	+	-	+ +	-	+
AMP	+ + +	+	+ + +	+ +	+ +	+ + +
ATP	-	+	-	+ + +	-	+ +
KH_2PO_4	-	+ +	+	+ + +	+	+ + +
KH_2PO_4+salicylanilide[2]	+ +	+ +	+ + +	+	nd[3]	nd

[1] -; +; + +; + + +: increasing intensity of the PG bands on pectin-polyacrylamide gels (see Materials and methods).
[2] Final concentration: 5 μg ml^{-1}.
[3] nd: not determined.

PG2 was detectable in all treatments, but PG1 was again constantly present as a distinct band only after addition of AMP and as a vague band at 48 and 72 h after addition of KH_2PO_4 (Table 4).

Effect of addition of actinomycin D, cycloheximide and salicylanilide on the phosphate-stimulated PG production

To examine whether the observed phosphate-dependent PG production by the fungus was the result of *de novo* protein synthesis, the effect of the mRNA and protein

synthesis inhibitors actinomycin D and cycloheximide, respectively, on PG production was tested in a system where the inhibitors were added, together with AMP, ATP and KH_2PO_4, to cultures growing under P starvation for 48 h. The inhibition of PG synthesis by cycloheximide was already detectable 24 h after its addition (Table 5), whereas the effect of actinomycin D occurred generally later than that of cycloheximide. The AMP-dependent PG1 synthesis appeared to be completely inhibited by cycloheximide. When this inhibitor was administered one day after the addition of ATP instead of simultaneously, PG activity, measured 24 h later and relative to a single PG2 band, was found to be only 15% of the activity without inhibitor, whereas actinomycin D had not yet had any effect (results not shown).

Evidence for ATP having a negative effect on PG1 production came from experiments in which the ATP production during the process of electron tranport was inhibited by the uncoupler salicylanilide, in a KH_2PO_4-stimulated PG production system. Simultaneous addition of salicylanilide and KH_2PO_4 induced an approximately 50% increase of PG production 24 and 48 h later compared with the control treatment (Table 5). This increase was accompanied by the appearance of a distinct PG1 band, in addition to the usual PG2 band (Table 4; Fig. 1). The ethanol present in the medium containing salicylanylide did not affect PG production or fungal growth.

TABLE 5. Effect of actinomycin D, cycloheximide and salicylanilide on the AMP-, ATP- or KH_2PO_4-mediated (concentration used: 2.5 mM) PG production by *B. cinerea*. All compounds were added to phosphate-free growing cultures 48 h after inoculation. Values are expressed as PG units mg^{-1} mycelial dry weight relative to those of treatments without inhibitor (100%).

Compounds added	hours after addition	
	24	48
AMP + actinomycin D[1]	100	38
AMP + cycloheximide[1]	47	3
ATP + actinomycin D	100	43
ATP + cycloheximide	15	19
KH_2PO_4 + actinomycin D	65	53
KH_2PO_4 + cycloheximide	10	44
KH_2PO_4 + salicylanilide[2]	162	140

[1] Final concentration: 20 $\mu g\ ml^{-1}$.
[2] Final concentration: 5 $\mu g\ ml^{-1}$.

Effect of addition of AMP, ADP, ATP and KH_2PO_4 on intracellular adenine nucleotide levels and on energy charge

The observed effects on the biosynthesis of PG1 pointed to the possibility that PG1 production is also regulated by the intracellular concentration of adenine nucleotides and/or by the resulting energy charge. Intracellular adenine nucleotide concentrations were determined at 48 and 72 h after the addition of AMP, ADP, ATP or KH_2PO_4 to cultures of *B. cinerea* growing under P starvation for 96 h, because the most clear differences in effects on PG production were found at these times (see previous results). The different system of incubation of *B. cinerea* cultures did not change the responses in total PG activity and PG isoenzyme production already observed in the previous experiments. The adenine nucleotide concentrations of the control (continuous growth under P starvation) were determined also after 24 h (17.05 nmoles AMP, 37.9 nmoles ADP and 86.85 nmoles ATP, all expressed per g mycelial fresh weight), together with the resulting energy charge (0.75). The energy charge of the control dropped to 0.60 at 48 h and 0.49 at 72 h (Table 6). In all treatments the energy charge values found were relatively low and ranged from 0.42 (for AMP at 72 h) to 0.65 (for ADP at 48 h). Addition of AMP gave energy values always below 0.50. The lowest energy charge value at 48 h, however, was found after addition of KH_2PO_4. Among the nucleotides, ADP

and ATP constantly gave the highest energy charge values.

After AMP addition to the medium, the resulting percentage of intracellular AMP in the total adenylate pool was always higher than in all other treatments.

TABLE 6. Effect of adenine nucleotides and KH_2PO_4 (concentration used: 2.5 mM) on the intracellular adenylate concentration (in nmoles g^{-1} mycelial fresh weight), energy charge (E.C.) and % AMP in the total adenylate pool of *B. cinerea*. The compounds were added to phosphate-free growing cultures 96 h after inoculation.

Compound added	hours after addition									
	48					72				
	ATP	ADP	AMP	E.C.	%AMP	ATP	ADP	AMP	E.C.	%AMP
None (control)	78.0	56.8	41.5	0.60	23.5	50.5	77.2	55.3	0.49	30.2
AMP	66.1	87.2	72.4	0.49	32.1	51.5	114.4	95.4	0.42	36.5
ADP	124.0	73.6	49.9	0.65	20.2	86.8	66.9	38.3	0.63	19.9
ATP	215.1	387.4	158.3	0.54	20.8	81.2	61.8	37.7	0.62	20.9
KH_2PO_4	53.0	195.8	84.2	0.45	25.3	54.0	32.4	22.4	0.64	20.6

Discussion

The PG production by *B. cinerea in vitro* was found to be strongly dependent on the addition of inorganic phosphate or adenine nucleotides to the growth medium, as was reported to occur also during plant infection (Van den Heuvel & Waterreus, 1985). Without these compounds, PG production remained negligible. A similar effect of phosphate on PG production *in vitro* has been reported also for *Gaeumannomyces graminis* and *Phialophora radicicola* var. *radicicola* (Holden & Ashby, 1978). The effect of KH_2PO_4 and adenine nucleotides on the PG production by *B. cinerea* after their addition to conidia (at inoculation time) or to mycelium growing under P starvation (48 h after inoculation) was similar, but the latter responded more quickly. Not only pectic enzyme synthesis, but also fungal growth was enhanced by P-containing compounds, except for AMP, especially after addition to mycelium growing under P starvation.

The first isoenzyme appearing after addition of the P-containing compounds was PG2, known to be a constitutive enzyme whose production is strictly related to fungal growth (Leone & Van den Heuvel, 1987). This implies that an enhancement of PG production by the addition of KH_2PO_4 or a nucleotide can be interpreted primarily as an indirect effect, acting through the stimulation of mycelial growth. In contrast, the stimulatory effect of AMP on PG production occurred earlier than that of other compounds and remarkably affected the synthesis of the inducible isoenzyme PG1 in the absence of any pectic inducer. This effect was coupled to a slow fungal growth rate. Similar results as with AMP were obtained by adding to the culture an equimolar mixture of adenosine and Na_2HPO_4, but not when inorganic phosphate or adenosine were added separately. Na_2HPO_4 was chosen because the AMP preparation used in our experiments was a disodium salt. This indicates that AMP before its uptake by *B. cinerea*, may be hydrolysed by phosphatases, as occurs with phosphomonoesters in other fungi (Beever & Burns, 1980). However, it seems that, once in the fungal cell, it was only reassembled AMP that stimulated PG1 production, since adenosine and any form of inorganic phosphate added separately, failed to stimulate its synthesis. No information is at present available about the mechanisms of uptake of phosphodiesters and phosphotriesters, like ADP and ATP, by *B. cinerea*.

Addition of cAMP reverses the catabolite repression by glucose of various inducible enzymes of microorganisms growing *in vitro* on this C source (Pall, 1981). Also endopectate lyase synthesis of *Erwinia carotovora* has been claimed to undergo catabolite

repression reversed by cAMP (Hubbard *et al.*, 1978). The results of the present study, where glucose was also used as C source, did not demonstrate a similar involvement of cAMP in the inducible production of PG1 by *B. cinerea*, as PG1 synthesis never occurred in the presence of this compound. This is in agreement with the reported lack of involvement of catabolite repression in the regulation of inducible pectic enzymes of *B. cinerea* (Leone & Van den Heuvel, 1987).

The inhibition of PG production caused by actinomycin D and cycloheximide provides evidence for the involvement of *de novo* poly(A)-containing mRNA and protein synthesis during the phosphate-dependent PG synthesis. The inhibitory effect of actinomycin D was always weaker and occurred later than that of cycloheximide. This result is consistent with the mode of action of actinomycin D (Yoshikawa *et al.*, 1978). As this inhibitor is supposed to reach its site of action later than cycloheximide, PG2 mRNA synthesis might remain at a normal level for a fairly long period, thus giving rise to a still normal level of PG2.

The effect on PG synthesis shown by salicylanilide when supplied in combination with KH_2PO_4, is remarkable. The supposed inhibition of ATP synthesis caused by uncoupling of the electron transport after addition of salicylanilide was accompanied by a stimulation of PG synthesis and the appearance of PG1 24 h after the additions, as was observed also in the experiments with AMP. At that time, PG1 was never detected after addition of KH_2PO_4 alone. Furthermore, total PG activities produced under cultural conditions of low average number of bound phosphate groups per adenine moiety (e.g. 2.5 mM AMP) or of relatively low total bound phosphate concentration (2.5 mM ADP, or 0.83 mM ATP), were always higher than those produced under conditions of high average number of bound phosphate groups per adenine moiety (2.5 mM ATP). All these results point to the involvement of the adenylate pool in the regulation of the synthesis of PG production, and in particular of PG1, by the isolate BC1 of *B. cinerea*.

The determinations of the intracellular concentrations of the adenine nucleotides yielded energy charge values that were generally low. The energy charge of *B. cinerea* under P starvation (control) dropped from 0.75 at 24 h to 0.49 two days later. This could be the result of the exhaustion by the fungus of its own phosphate supply in the course of the experiments, as was reported to occur also for other fungi (Beever & Burns, 1980). Although little is known about growth under P limitation in microorganisms, similar drops in the energy charge, accompanied by a clear reduction of the ATP concentration, were reported for bacteria and yeasts undergoing different kinds of nutrient limitation (Chapman & Atkinson, 1977). Surprisingly, also after addition of a P-containing compound the adenylate energy charge of *B. cinerea* remained below 0.65. Similar values were obtained also during the phosphate-mediated inhibition of ethylene biosynthesis in *Penicillium digitatum* (Mattoo *et al.*, 1983). This effect can be partially explained by uptake of P from inorganic or organic sources being an energy-requiring process for the fungus (Beever & Burns, 1980).

Two opposite effects on *B. cinerea* were observed following addition of a P-containing compound to the growth medium: an ATP-mediated effect (high growth rate; constitutive PG2 production; little or no PG1 production; tendency for the energy charge to increase) and an AMP-mediated effect (low growth rate; PG1 production; little PG2 production; tendency for the energy charge to decrease). The effect of ADP on growth and PG isoenzyme production, was very similar to that of ATP; the effect of KH_2PO_4 was intermediate between that of ATP and AMP. Our results suggest that a low energy charge in the fungal cell is one of the regulatory factors of PG1 synthesis. However, the observed energy charges showed often only small differences, and an underestimation caused by disturbances of the cellular compartmentalization, which can take place during the extraction of the adenine nucleotides, cannot be ruled out (Delmer & Brody, 1975; Thomas & Dawson, 1977). Following addition of AMP, the percentage of AMP in the total adenylate pool in the mycelium was higher than after any other addition. This suggests that, if not the energy charge, the proportion of AMP in the adenylate pool plays an important role in the metabolic control of PG1 production. Thus, besides the regulation exerted by the type and concentration of the substrate (Leone & Van den Heuvel, 1987), PG1 synthesis may be controlled, at a different level, by the metabolic status of the fungus through the adenylate pool. This strongly supports the view of the coordinated involvement of the sequentially produced pectic enzymes of *B. cinerea* in the

digestion of pectic polymers (Leone & Van den Heuvel, 1987).

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CHAPTER 4

IN VIVO AND *IN VITRO* PHOSPHATE-DEPENDENT POLYGALACTURONASE PRODUCTION BY DIFFERENT ISOLATES OF *BOTRYTIS CINEREA*

by

G. Leone

Abstract

Eleven isolates of *Botrytis cinerea*, coming from different host plants of various geographic locations and isolated in different years, were compared with isolate BC1 for: a) their *in vitro* and *in vivo* production of total polygalacturonase (PG): b) pattern of PG isoenzymes formed in the presence of inorganic phosphate (KH_2PO_4) or AMP and: c) for their ability to cause lesions on leaves of French bean and tomato. All isolates were strongly phosphate-dependent for *in vitro* PG production, showing a complete lack of activity when phosphate was absent from the growing medium. In the presence of AMP four isolates had *in vitro* a PG production significantly higher than with KH_2PO_4 and five isolates showed also the specific induction of the synthesis of a PG1-like isoenzyme, as occurring with BC1. Under the same conditions, three isolates showed no PG synthesis. All isolates were characterized by the production of cathodic forms of PGs comprising at least a PG1- or a PG2-like isoenzyme. Seven isolates produced two isoenzymes with an electrophoretic mobility equal to that of PG1 and PG2 of isolate BC1. The results point to a low level of intraspecific variation for the isoenzyme pattern of the different *B. cinerea* isolates. All isolates gave rise to spreading lesions on French bean and tomato leaves when glucose and KH_2PO_4 were present in the inoculum drops, but not when both compounds were absent. Differences in total PG production did not affect the ability to cause spreading lesions or to infect the two host plants used.

Introduction

The occurrence and participation of pectic enzymes in tissue maceration by *Botrytis cinerea* Pers.: Fr. have been documented for over 70 years (Brown, 1915), but only recently their isoenzymatic forms came into focus (Cruickshank & Wade, 1980; Di Lenna & Fielding, 1983; Magro *et al.*, 1980; Leone & Van den Heuvel, 1987; Van den Heuvel & Waterreus, 1985). *B. cinerea* is also well-known for its phenotypic variability possibly caused by heterocaryosis (Di Lenna *et al.*, 1981; Grindle, 1979; Hansen & Smith, 1932). Three *B. cinerea* isolates were reported to differ markedly in their polygalacturonase (PG) and protein patterns (Magro *et al.*, 1980). In contrast, Cruickshank & Wade (1980) found different PG isoenzyme patterns only between different *Botrytis* species. Working with a *B. cinerea* isolate from bean (BC1), Leone & Van den Heuvel (1987) found that PG isoenzyme production occurred sequentially and that each isoenzyme could undergo a high level of regulation. All these reports mentioned that the fungus was grown on a pectic polysaccharide as C source. Since the PGs are involved in its metabolism (Leone & Van den Heuvel, 1987), isoenzyme patterns of different isolates could be influenced by differences in the degree of metabolic enzyme control. In that case, the isoenzyme pattern of isolates of the same fungal species may show artificial differences. In a study on the involvement of phosphate in the *in vitro* PG production by *B. cinerea*, it was found that when the fungus was grown on glucose as the only C source, only the PG isoenzyme PG2 or a combination of PG1 and PG2 was produced up to 72 h after inoculation (Leone *et al.*, 1990). This depended on the addition to the growth medium of several inorganic or organic phosphorus-containing compounds or of AMP, respectively. Since these cultural conditions specifically stimulated the production of only

two PGs and prevented the production of other PG forms, they could be used for comparing different isolates, paying attention only to PG1, PG2 and possible novel PGs.

The aim of the present study was to ascertain whether: a) the phosphate-dependent PG production by BC1 found *in vitro* (Leone *et al.*, 1990) occurs with other *B. cinerea* isolates; b) differences exist in PG isoenzyme production *in vitro* and/or *in vivo* between different isolates; and c) a relation exists between the quantitative and/or qualitative PG production and the pathogenicity of *B. cinerea* (expressed as the ability to produce spreading lesions). An answer to these points might give a better insight in the significance of PGs for the pathogenicity of *B. cinerea*. Studies of the complex regulation of these enzymes suggested more a digestive role than a role restricted to penetration (Leone & Van den Heuvel, 1987; Leone *et al.*, 1990).

Materials and methods

Plant material

Plants of French bean (*Phaseolus vulgaris* L. cv. Dubbele Witte zonder draad) and tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) were grown in a glasshouse at 20-25°C. Primary leaves of two-week-old French bean plants and leaflets of the sixth true leaf of 9-10-week-old tomato plants were excised from the plants and used for inoculation.

Source and growth condition of B. cinerea isolates

Designations and sources of the isolates to be compared are given in Table 1. All isolates were maintained on PDA slants at 4°C. Sporulating cultures were obtained after transferring the isolates on slants of synthetic medium X (Last & Hamley, 1956) and incubating them for 11-14 days under continuous fluorescent light at about 23°C.

TABLE 1. Origin of isolates of *Botrytis cinerea* used in the experiments.

Isolate code	Source	Country, year of isolation
BC1[a]	Bean	the Netherlands, 1970
BC4[a]	Tomato	the Netherlands, 1970
BC10[a]	Cyclamen	the Netherlands, 1983
BC12[a]	Gerbera	the Netherlands, 1986
BC19[a]	Pepper	Spain, 1987
CBS 126.58[b]	Pear	the Netherlands, 1958
CBS 156.71[b]	Cyclamen	the Netherlands, 1971
CBS 179.71[b]	Chicory	the Netherlands, 1971
CBS 261.71[b]	Barley	South Africa, 1971
Gr 4/85S[c]	Grape	Italy, 1985
- [c]	Pea	Italy, 1972
St 18/85S[c]	Strawberry	Italy, 1985

[a] From the collection of the Willie Commelin Scholten Phytopathological Laboratory, Baarn, the Netherlands.
[b] Supplied by the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.
[c] Supplied by the Istituto di Patologia Vegetale, Torino, Italy.

For *in vitro* experiments, conidial suspensions at a final concentration of 2×10^4 conidia ml^{-1} were prepared to inoculate 25-ml flasks containing 10 ml sterilized Richards' solution from which KH_2PO_4 had been omitted (Leone *et al.*, 1990). Just before inoculation KH_2PO_4 or 5'-AMP disodium salt (adenosine-5'-monophosphate; AMP, Merck) was added to the medium to give a final concentration of the compound of 2.5 mM, as AMP is not stable at high temperature (Leone *et al.*, 1990). After 4 days of incubation at 19°C on a reciprocal shaker at a continuous shaking rate of 136 strokes.min^{-1}, the mycelium formed was separated from the culture liquid by filtration

on preheated and preweighed filter papers (S&S no. 604, Dassel, F.G.R.). Culture filtrates were directly used for enzyme assay and electrophoresis.

For *in vivo* experiments, conidial suspensions at a final concentration of $2x10^4$ or $2x10^6$ conidia ml^{-1} were prepared in a 0.11 M glucose + 0.067 M KH_2PO_4 solution, as reported by Van den Heuvel (1981). Drops (20 μl) of conidial suspensions were placed on the adaxial surface of detached primary French bean leaves and tomato leaves (up to 30 drops per leaf), lying in transparent plastic trays. Incubation was as reported by Van den Heuvel (1981). After 12, 24 and 48 h, drops belonging to one leaf were removed by aspiration and pooled, thus forming a single sample. The collected samples were stored at -20°C or used directly for enzyme assay and electrophoresis.

Enzyme assays

PG activity in culture filtrates or in inoculum drops was measured quantitatively by the cup-plate assay method of Dingle *et al.* (1953) as reported by Leone & Van den Heuvel (1987). One unit of enzyme activity was expressed as the activity necessary to release 1 μmole of D-galacturonic acid h^{-1} at pH 5.0 under standard conditions (Leone & Van den Heuvel, 1987). Qualitative determination of PG isoenzyme production was performed by pectin-polyacrylamide gel electrophoresis as reported by Van den Heuvel & Waterreus (1985). Zymograms of PG isoenzymes were developed using the staining procedure of Cruickshank & Wade (1980). Numbers assigned to PG bands for isolate BC1 were identical to those reported by Leone & Van den Heuvel (1987). The electrophoretic mobility of PG isoenzymes of isolate BC1 was used as a basis for the comparison between the different isolates. Electrophoretic mobility was calculated by measuring the distance from the upper end of the sample well to the middle of the activity bands.

Statistics

All experiments were repeated at least once. In the *in vitro* experiments, each treatment was replicated three times. On the data of the PG units mg^{-1} mycelium dry weight, analysis of variance was performed with the statistical package GENSTAT V after transformation of the data to log(x+1). Significance of differences within means relative to the interaction "isolates x compounds" was tested by the Duncan's multiple range test (Duncan, 1955). The same test was also applied separately to the means calculated for the factors "isolates" and "compounds", using their relative standard errors. Therefore, the significance obtained from one set of means cannot be compared with that obtained from another set of means.

Results

Effect of addition of KH_2PO_4 or AMP on the production of PG by different isolates of B. cinerea

In Table 2 the results are shown of the *in vitro* experiments on total PG production; the PG activities are expressed in units mg^{-1} mycelial dry weight in order to correct enzyme activity for possible differences in mycelial growth between the different isolates. Omission of phosphorus in either inorganic or organic form from the culture medium (control), resulted in a complete lack of PG activity. In contrast, all isolates showed PG activity when KH_2PO_4 had been added to the medium. The response of the isolates to the stimulation by KH_2PO_4 was quantitatively different and ranged from 2.4 units mg^{-1} mycelial dry weight (isolate CBS 156.71) to a ca twelve-fold higher PG activity of BC12 (28.4 units mg^{-1} mycelial dry weight). The activities produced were generally low, as nine isolates showed an activity of less than 10 units mg^{-1}, while seven isolates had an activity of even less than 5 units mg^{-1}.

When AMP had been added to the culture medium, three isolates (BC12, BC19 and CBS 156.71) did not produce any PG activity, notwithstanding their positive response to the addition of KH_2PO_4. For all other isolates, PG production was clearly detectable. Four isolates (CBS 126.58, CBS 179.71, CBS 261.71 and pea) showed a significantly greater stimulation of PG activity than after KH_2PO_4 addition. The isolates BC1 and Gr

4/85S also showed this stimulatory effect, but PG activity did not differ significantly.

Table 2 also shows that, independently from the isolate tested, the average stimulation of PG activity by KH_2PO_4 or AMP differed significantly from the control but not from each other. Furthermore, the average PG production, regardless of the compound added to the culture medium, showed marked differences between isolates. Thus, BC19 and CBS 156.71 were weak PG producers, whereas BC12, Gr 4/85S and St 18/85S, were strong PG producers. The two isolates from cyclamen (BC10 and CBS 156.71) also differed significantly from each other with regard to PG production.

TABLE 2. PG activity (in units mg^{-1} mycelial dry weight) produced by different *B. cinerea* isolates after addition of KH_2PO_4 or AMP (2.5 mM) to a phosphate-free medium (control) at inoculation time.

Isolate	Compound added			Average
	None (control)	KH_2PO_4	AMP	
BC1	0.0a[1]	4.9cde	5.7def	3.5b[2]
BC4	0.0a	8.4fgh	7.1efg	5.2bc
BC10	0.0a	9.8ghi	6.7defg	5.5bc
BC12	0.0a	28.4k	0.0a	9.5de
BC19	0.0a	3.8bcd	0.0a	1.3a
CBS 126.58	0.0a	2.9bc	5.3def	2.7b
CBS 156.71	0.0a	2.4b	0.0a	0.8a
CBS 179.71	0.0a	4.8cde	19.6jk	8.1cd
CBS 261.71	0.0a	4.8cde	16.1ij	7.0c
Gr 4/85S	0.0a	13.6hij	15.3ij	9.6de
Pea	0.0a	5.0cdef	13.2hij	6.0c
St 18/85S	0.0a	19.0jk	15.7ij	11.6e
Average	0.0a[3]	9.0b	8.7b	

[1] Means (relative to the interaction "isolates x compounds") followed by the same letter are not significantly different (Duncan's multiple range test, P=0.05).

[2] Means (relative only to isolates) followed by the same letter are not significantly different (Duncan's multiple range test, P=0.05).

[3] Means (relative only to compounds) followed by the same letter are not significantly different (Duncan's multiple range test, P=0.05).

The types of PG isoenzymes secreted in the culture medium by the different isolates are shown in Fig. 1A-C. All isolates, except CBS 156.71, produced an isoenzyme comparable to PG1 of BC1. Slight differences in the relative electrophoretic mobility of this isoenzyme were noted with the isolates BC10 and BC19 (Table 3). The specific stimulation of the induction of PG1 synthesis by AMP addition, already known for BC1 (Leone *et al.*, 1990), was observed also with the isolates CBS 126.58, CBS 179.71, CBS 261.71, Gr 4/85S and pea. The PG1- like isoenzyme of the isolate CBS 179.71 seemed subdivided into two bands migrating very closely to each other (Fig. 1A). The same effect was observed on some gels with the PG1-like isoenzyme of the isolates BC12 and BC4 (Fig. 1C; Table 3).

A PG isoenzyme with a relative electrophoretic mobility equal to that of PG2 of BC1 was found to occur distinctly with the isolate CBS 126.58 and, as a vague band, with isolates BC4, CBS 179.71, Gr 4/85S and St 18/85S (Fig. 1A-C; Table 3). Isolates CBS 156.71 and CBS 261.71 produced a PG2-like isoenzyme with a REM_{PG2} of 0.62 and 0.50, respectively (Table 3). The isolates Gr 4/85S, pea and St 18/85S showed in addition a distinct band of activity localized at the sample well and one at a slightly higher position. These bands were similar to those of the isoenzymes PG3 and PG4 already described for isolate BC1 (Leone & Van den Heuvel, 1987).

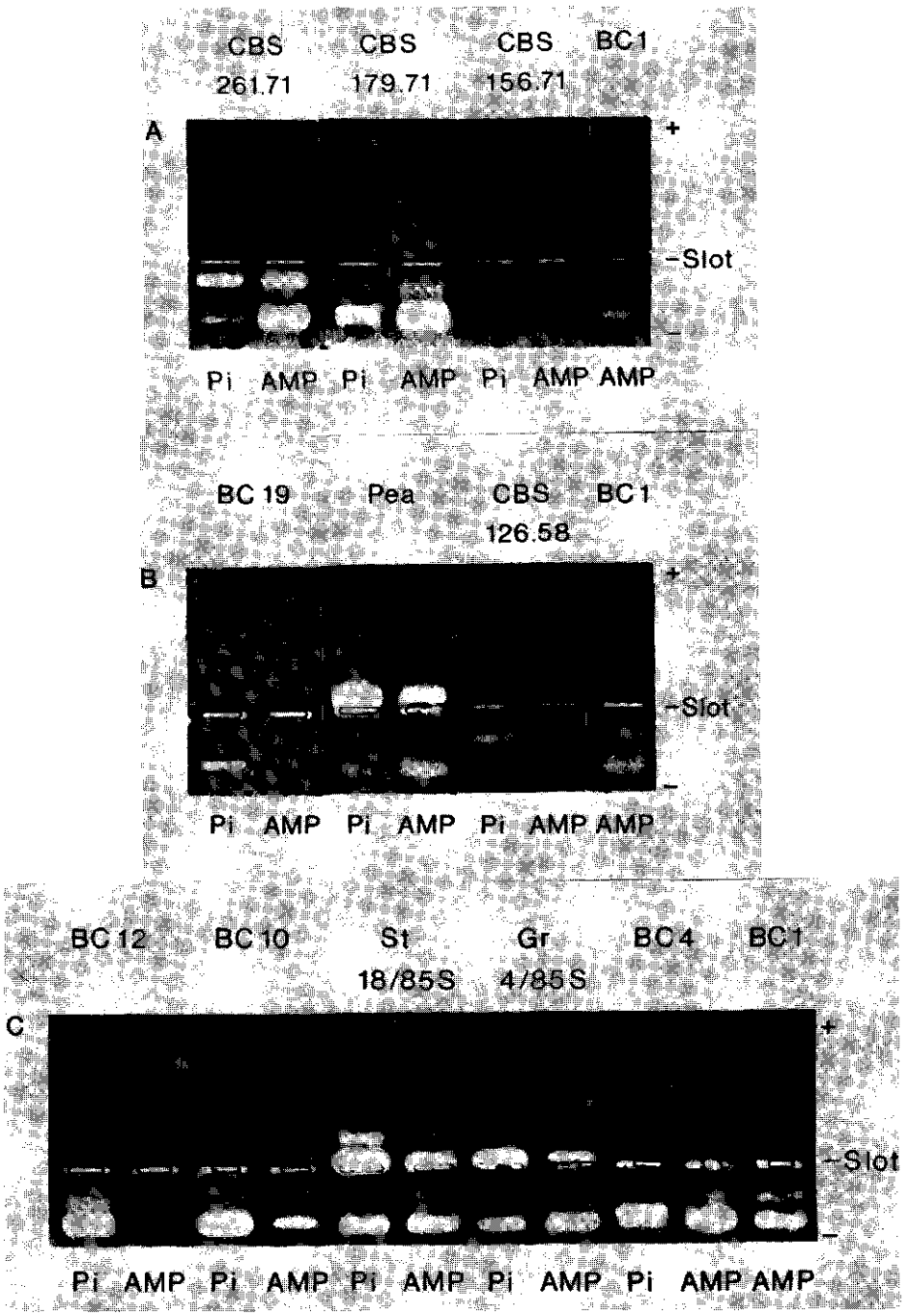

FIGURE 1. Comparison of the polygalacturonase isoenzyme patterns produced by *Botrytis cinerea* isolates after addition to the medium of KH_2PO_4 or AMP. A: isolates CBS 261.71, CBS 179.71, CBS 156.71 and BC1. B: isolates BC19, pea, CBS 126.58 and BC1. C: isolates BC12, BC10, St 18/85S, Gr 4/85S, BC4 and BC1. PG1 is the isoenzyme of BC1 migrating closest to the cathode; PG2 is the adjacent one.

TABLE 3. Relative electrophoretic mobility (REM) of PG1- and PG2-like isoenzymes produced by different *B. cinerea* isolates *in vitro* and/or *in vivo*.

Isolate	REM_{PG1}[a]	REM_{PG2}[b]
BC1	1.00	1.00
BC4	0.86; 1.00	1.00
BC10	0.86	1.00
BC12	0.86; 1.00	1.00
BC19	0.82	1.00
CBS 126.58	1.00	1.00
CBS 156.71	1.00	0.62
CBS 179.71	0.86; 1.00	1.00
CBS 261.71	1.00	0.50
Gr 4/85S	1.00	1.00
Pea	1.00	-
St 18/85S	1.00	1.00

[a] REM_{PG1} = electrophoretic mobility of PG1-like isoenzyme (produced by *B. cinerea* isolate)/electrophoretic mobility of PG1 from BC1.
[b] REM_{PG2} = electrophoretic mobility of PG2-like isoenzyme (produced by *B. cinerea* isolate)/electrophoretic mobility of PG2 from BC1.

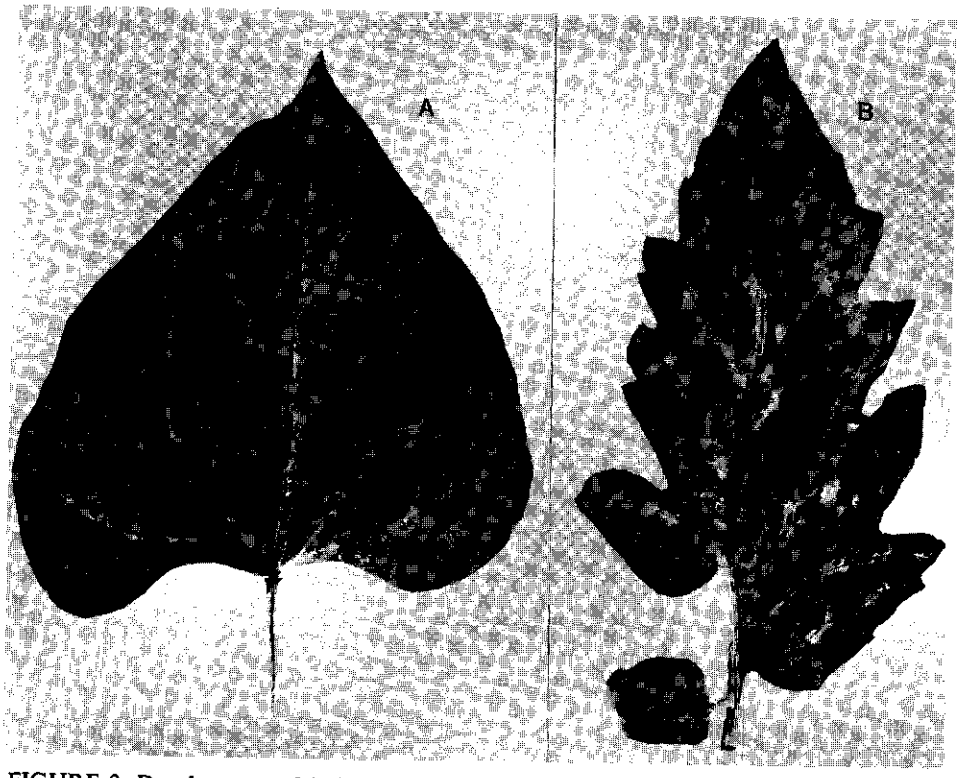

FIGURE 2. Development of lesions by *Botrytis cinerea* (BC1) on bean leaves at 72 h (A) and on tomato leaves (B) at 48 h after inoculation with drops of a glucose (0.11 M) + KH_2PO_4 (0.067 M) solution containing $2x10^4$ conidia ml^{-1}.

Disease development on French bean and tomato leaves

The development of disease on inoculated French bean and tomato leaves was followed for seven days. Two days after inoculation with $2x10^4$ conidia ml^{-1}, only few isolates had produced clear lesions, whereas three days after inoculation lesions were present on all inoculated leaves (Fig. 2). The symptoms observed on the host were generally the same for all isolates. Tomato leaves appeared much more quickly affected and rotted earlier than bean leaves and often showed a chlorotic area around the necrosis (Fig. 2B). Seven days after inoculation at least 50 to 100% of the leaf area was infected and showed profuse sporulation of the fungus.

PG production by different isolates on French bean and tomato leaves

In inoculation drops collected from French bean and tomato leaves inoculated with $2x10^4$ conidia ml^{-1}, no PG activity was detected at 12 h after inoculation. After 24 h on bean or tomato and after 48 h on bean leaves, PG production did not differ markedly between the isolates (Table 4). After 48 h on tomato leaves the activity had clearly increased and differences between isolates became more distinct. This appeared related to the quicker disease development observed on tomato leaves (Fig. 2B).

TABLE 4. PG activity of inoculum drops of a glucose (0.11 M) + KH_2PO_4 (0.067 M) solution containing $2x10^4$ conidia ml^{-1} of different *B. cinerea* isolates collected from French bean and tomato leaves at different times after inoculation.

	hours after inoculation			
Isolate	24		48	
	bean	tomato	bean	tomato
BC1	2.5	2.8	2.5	9.0
BC4	2.2	2.0	1.6	4.3
BC10	2.2	2.5	1.6	7.4
BC12	3.2	2.2	2.5	5.1
BC19	2.2	2.8	1.6	9.0
CBS 126.58	2.2	2.5	2.8	9.0
CBS 156.71	2.0	2.0	2.0	5.1
CBS 179.71	2.5	2.8	3.2	5.1
CBS 261.71	2.5	3.2	1.6	9.0
Gr 4/85S	2.2	2.8	1.6	9.0
Pea	2.8	2.5	2.0	13.8
St 18/85S	2.8	4.3	2.8	17.7

Identification of the type of PG isoenzymes formed during the plant- pathogen interaction showed that all *B. cinerea* isolates produced PGs migrating to the cathode or remaining in the vicinity of the sample well (Fig. 3A-C). PG forms secreted during the *in vivo* interactions corresponded to those already observed during the *in vitro* experiments, but some isolates showed an additional band, mostly present in the samples collected on tomato leaves 48 h after inoculation. Thus, on that host and at that time, isolate CBS 261.71 showed a PG3-like isoenzyme; isolates BC10, BC19 and Gr 4/85S a PG2-like isoenzyme; isolate CBS 156.71 a PG1-like isoenzyme; isolate pea a PG2- and a PG4-like isoenzyme and another one localized below the PG1-like isoenzyme. The latter new isoenzyme was further observed, less distinctively, with isolates BC1, BC10, BC12, BC19, CBS 126.58, CBS 179.71 and CBS 261.71.

When bean leaves were inoculated with isolate BC1, BC4, BC10, BC12, Gr 4/85S or St 18/85S at $2x10^6$ conidia ml^{-1} and inoculum drops were collected 24 h after inoculation, PG isoenzyme production was much more stimulated than in inoculum drops with $2x10^4$ conidia ml^{-1} (Fig. 4). In the former case, there were more and wider PG bands on the gels for each isolate tested. All these isolates had in common at least a PG1-, a PG2- and a PG3-like isoenzyme band. The isolates Gr 4/85S and St 18/85S showed a PG3-like

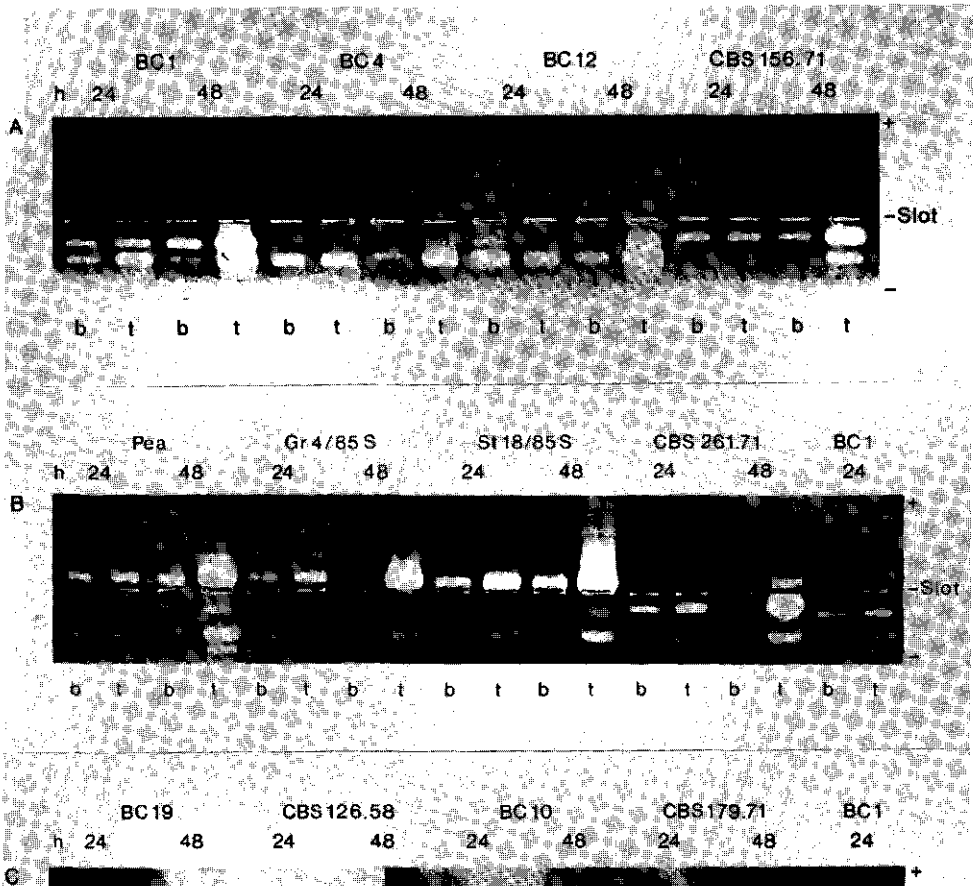

FIGURE 3. Comparison of the polygalacturonase isoenzyme patterns produced by different *Botrytis cinerea* isolates 24 and 48 h after inoculation of bean (b) and tomato (t) leaves with drops of a glucose (0.11 M) + KH_2PO_4 (0.067 M) solution containing $2x10^4$ conidia ml^{-1}. A: isolates BC1, BC4, BC12, and CBS 156.71. B: isolates pea, Gr 4/85S, St 18/85S, CBS 261.71 and BC1. C: isolates BC19, CBS 126.58, BC10, CBS 179.71 and BC1.

isoenzyme activity band stronger than the other isolates and only isolate Gr 4/85S showed a PG localized below the PG1-like isoenzyme, as was observed with other isolates on tomato leaves 48 h after inoculation with $2x10^4$ conidia ml^{-1}.

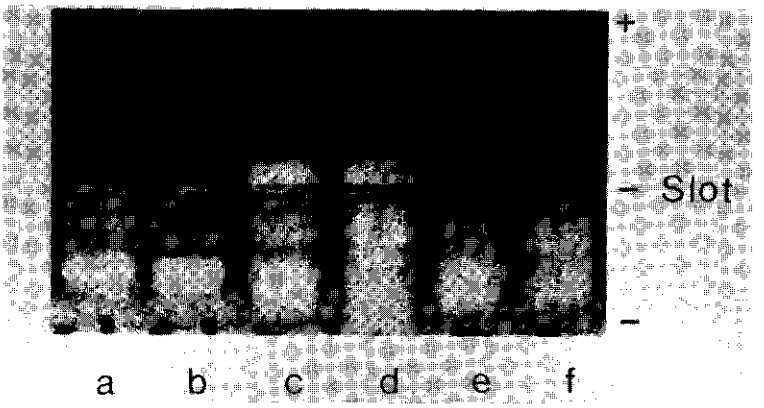

FIGURE 4. Comparison of the polygalacturonase isoenzyme patterns produced by different *Botrytis cinerea* isolates 24 h after inoculation of bean leaves with drops of a glucose (0.11 M) + KH_2PO_4 (0.067 M) solution containing $2x10^6$ conidia ml^{-1}. Lane *a* = BC10; *b* = BC12; *c* = St 18/85S; *d* = Gr 4/85S; *e* = BC4 and *f* = BC1.

Discussion

With regard to PG production, the *B. cinerea* isolates used in this study were all strongly dependent on phosphate, supplied either as KH_2PO_4 or as AMP. In the absence of phosphate, no PG activity could be detected in the growth medium. The phosphate-dependent PG production found for isolate BC1 (Leone *et al.*, 1990), therefore, appears to be a more general characteristic of *B. cinerea*, since some of the isolates studied originated from distant geographical regions or had been isolated in different years. Only five isolates responded to addition of AMP with a PG1 stimulation and four of them with a PG production significantly higher than with KH_2PO_4, as reported for BC1 (Leone *et al.*, 1990). This indicates that the ability of PG1 synthesis following stimulation by AMP is an unstable character of *B. cinerea*. Three isolates showed no PG production after AMP addition, suggesting that they are mutants for AMP transport or utilization. This result strongly supports the hypothesis that the intracellular effect of AMP on PG production is very specific and is not caused by its degradation into inorganic phosphate as a result of phosphatase activity (Leone *et al.*, 1990).

All isolates were able to infect French bean or tomato leaves when conidia were suspended in a 0.11 M glucose + 0.067 M KH_2PO_4 solution, but not when conidia were suspended in water only. Thus, no host specificity was shown by the different isolates. Tomato leaves appeared more rapidly attacked by any isolate than bean leaves and this fungal activity corresponded with the higher total PG activity detected 48 h after inoculation.

Isolate St 18/85S gave the highest total PG production *in vitro* and on tomato leaves 48 h after inoculation. On the other hand, isolates BC19 and CBS 156.71 gave the lowest total PG production *in vitro*. Nevertheless, they were able to infect bean and tomato leaves as quickly as the other isolates and also showed enhanced PG activity 48 h after inoculation on tomato leaves. BC1 and BC4 showed low PG activity *in vitro* and also produced low PG activity on their respective host-plants (bean and tomato). All these results indicate a lack of enzymic host-adaptation and of a clear-cut relationship between the rate of PG production and the level of pathogenicity, as was also reported for other *B. cinerea* isolates (Di Lenna *et al.*, 1981; Di Lenna & Fielding 1983; Lorenz & Pommer, 1985). PG activity and, sometimes, number of PG isoenzymes increased more

quickly on the more susceptible reaction of tomato leaves and, on bean leaves, upon inoculation with $2x10^6$ compared to $2x10^4$ conidia ml^{-1}. Enhanced pectic enzyme activities and/or multiple isoenzymatic forms produced by *B. cinerea* are therefore more the result of the infection process than the cause of it and appear connected with catabolic or digestive activities of the fungus as was reported from *in vitro* studies by Leone & Van den Heuvel (1987) and Leone *et al.*, (1990).

Isolate BC1 is known to produce the isoenzymes PG1 and PG2, together or singly, depending on the experimental conditions (Van den Heuvel & Waterreus, 1985; Leone & Van den Heuvel, 1987; Leone *et al.*, 1990). These two isoenzymes have been used as a basis for a comparison between the different *B. cinerea* isolates used in this study. On the ground of the electrophoretic mobility, rather great similarities between the isolates became evident. All isolates were characterized by the production of cathodic forms of PGs, comprising at least a PG1- or a PG2-like isoenzyme, within 48 h after inoculation both *in vitro*, under KH_2PO_4 or AMP stimulation, or *in vivo* during lesion development. *In vivo*, a PG isoenzyme localized below the PG1-like band was observed with different isolates, including isolate BC1. This isoenzyme had not yet been described in the previous studies carried out with isolate BC1 (Van den Heuvel & Waterreus, 1985; Leone & Van den Heuvel, 1987; Leone *et al.*, 1990). Considering that the isolates differed from each other as to their origin (host plant) and time and place of isolation, the results presented here show a low level of variability in the PG pattern of *B. cinerea*. This implies also that intraspecific variation of PGs may be too low for their possible use as molecular markers for a genetic analysis of *B. cinerea* (Michelmore & Hulbert, 1987). The slight differences in electrophoretic mobility found when comparing the PG1- and PG2-like isoenzymes suggest the presence of small differences in their molecular structure, therefore indicating the occurrence of mutations or of modifications at translational or post-translational level (Verkleij, 1980).

The finding of similarities in the PG isoenzymes within the *B. cinerea* isolates studied are in good agreement with the results of Cruickshank & Wade (1980) but are in contrast with those reported by Magro *et al.* (1980). The fact that the PG isoenzymes could be differentially produced by a single isolate *in vitro* or *in vivo* (compare for example the pattern found *in vivo* after inoculation with $2x10^4$ or $2x10^6$ conidia ml^{-1}), and the knowledge that growth conditions *in vitro* can alter the isoenzyme pattern of isolate BC1 (Leone & Van den Heuvel, 1987), indicate that the metabolic control exerted on the isoenzymes can be an unexpected source of variation in the comparison of different isolates. The occurrence of shifts of isoenzyme patterns in organisms may also be related to the possible differential activation during development of the genes involved in the synthesis of these enzymes (Michelmore & Hulbert, 1987; Scandalios, 1969).

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CHAPTER 5

A RAPID PROCEDURE FOR SCREENING THE RESISTANCE OF BEAN CULTIVARS (*PHASEOLUS VULGARIS* L.) TO *BOTRYTIS CINEREA* AND *SCLEROTINIA SCLEROTIORUM*

by

G. Leone & A. E. G. Tonneijck

Abstract

A method to inoculate detached bean leaves with *Botrytis cinerea* or *Sclerotinia sclerotiorum* without using a carbon-nitrogen source is described. Leaves were incubated in plastic trays that were covered with transparent plastic bags to maintain air humidity above 95%. Spores of either fungus inoculated in water alone were generally unable to cause infection. Additions of KH_2PO_4 (62.5 mM) or of mixtures of inorganic phosphate and glucose (1.1 or 5.5 mM) to the inoculum stimulated fungal pathogenicity. Also spore concentration ($2x10^5$ or $2x10^6$ spores ml^{-1}) influenced the ability to produce lesions. At $2x10^6$ spores ml^{-1} both fungi were able to infect primary bean leaves when inoculated in an inorganic phosphate solution. These conditions were used for a preliminary screening for resistance of 15 bean cultivars to both fumgi. Only partial resistance to the pathogens was found. Since the procedure is simple and does not require blooming plants, it can be rapidly adopted in screening and breeding programs.

Introduction

Studying the interaction between the air pollutant ozone and the weak pathogens *Botrytis cinerea* Pers.: Fr. and *Sclerotionia sclerotiorum* (Lib.) de Bary in causing foliar injury to bean (*Phaseolus vulgaris* L.) (Leone *et al.*, 1989), we had to develop an inoculation procedure that gave quantifiable results in a short time. While information on the ozone sensitivity of different Dutch bean cultivars is available (Tonneijck, 1983), information is lacking on both the susceptibility of the cultivars to *B. cinerea* or *S. sclerotiorum* and on the possible relationship between ozone sensitivity and susceptibility to the fungi. Therefore, it was considered necessary to gather information about the level of resistance of different bean cultivars to the pathogens before taking into account the interaction with ozone.

The screening of bean cultivars to *B. cinerea* and/or *S. sclerotiorum* is difficult since the fungi need a nutrient source and free moisture in order to invade healthy leaf tissue (Blakeman, 1980; Hunter *et al.*, 1981). Mature blossoms are a common nutrient support for weak pathogens used in screening programs (Bailey, 1987; Dickson *et al.*, 1988; Hunter *et al.*, 1981).

This article describes a simple and rapid procedure to inoculate young bean leaves with *B. cinerea* or *S. sclerotiorum* spore suspensions without the need of a carbon-nitrogen supply. This method was applied for screening the resistance of bean cultivars of known ozone sensitivity to the fungi.

Material and methods

Seeds of 15 bean cultivars (Table 3) were planted in 12-cm diameter plastic pots filled with a high fertility peat-clay potting mixture (Triomf no. 17, modified, Trio BV, Westerhaar, the Netherlands) and grown in a glasshouse with charcoal-filtered air at a

16-h photoperiod, a 20/18°C day/night temperature regime and 75% relative air humidity. Pots were watered daily with tap water. Fourteen days after planting, beans were thinned to two plants per pot. All plants were 21 days old (primary leaves fully expanded) when used for cultivar screening.

The fungi were isolated from naturally infected bean plants. *Botrytis cinerea* was maintained on potato dextrose agar (PDA) slants at 4°C. In order to obtain sporulating cultures, the fungus was inoculated on slants of synthetic medium X (Last & Hamley, 1956) and incubated for 11 days at 20°C in the dark. *Sclerotinia sclerotiorum* ascospores collected on filter papers were from our Institute. Free ascospores were obtained by immersing the filter papers in distilled water. Suspensions for inoculation were adjusted to $2x10^5$ or $2x10^6$ spores ml^{-1} in distilled water, in a 62.5 mM KH_2PO_4 (Pi) solution or in a Pi-glucose solution (62.5 mM Pi + 1.1 mM glucose or 62.5 mM Pi + 5.5 mM glucose) for both fungi.

Detached primary leaves were placed on plastic grids on a layer of wet filter paper in plastic trays. The grids avoided a direct contact between the leaves and the filter paper. The cut end of each petiole was inserted in wet floram foam. Trays containing eight leaves were enclosed in transparent plastic bags and placed in an environmentally controlled chamber under a 16-h photoperiod (fluorescent light, ca 1,200 lux) and a 20/18°C day/night regime. Under these conditions, non-inoculated leaves remained vital for at least one week. In preliminary experiments with primary leaves of cultivar Lit, spray inoculations with $2x10^5$ or $2x10^6$ spores ml^{-1} did not give satisfactory results as lesions were only observed if drops coalesced. Drop inoculations gave more reproducible results and were further used. Ten-20-μl drops of a spore suspension prepared as described above, were placed on the adaxial side of each of four leaves. In screening for the resistance of different cultivars to both fungi, only drop inoculation in Pi solution containing $2x10^6$ spores ml^{-1} was used. Two days after inoculation, the number of successful lesions was scored.

Data of successful lesions, based on 3 replicate experiments, were subjected to ANOVA after angular transformation using the statistical package Genstat V. Where the F-test showed significant differences, means were compared in a LSD-test.

Results and discussion

Inoculum composition and spore concentration were found to influence the ability of the fungi to produce lesions (Table 1). Water inoculated spores of *B. cinerea* were generally unable to cause infections on bean leaves and infection was observed at a very low level only with *S. sclerotiorum* at $2x10^6$ spores ml^{-1}. To obtain infections by *B. cinerea* and *S. sclerotiorum* on healthy leaves, spores need to be stimulated by nutrients such as dead or dying plant material and monosaccharides without or in combination with inorganic phosphate (Pi) and purine nucleotide derivatives (Clark & Lorbeer, 1977; Ko *et al.*, 1981; Hunter *et al.*, 1981; Van den Heuvel & Waterreus, 1983). The most commonly used methods to screen bean plants for resistance are also based on this condition of nutrient stimulation, mostly with mature blossoms as nutrient support for the fungi (Hunter *et al.*, 1981; Dickson *et al.*, 1988). However, such a procedure can give variable results because it is difficult to standardize.

In our method, addition of a combination of Pi and glucose to the inoculum stimulated fungal pathogenicity at $2x10^5$ spores ml^{-1}. Simple carbohydrates like glucose are known to promote germination, superficial growth and formation of prepenetration structures (Blakeman, 1980). However, at $2x10^6$ spores ml^{-1} Pi alone was sufficient to reproduce this effect that was stronger for *S. sclerotiorum* than for *B. cinerea*. This suggests that Pi stimulates a direct penetration of both fungi into the leaf, as Pi involvement in the regulation of the production of the cell wall-degrading enzyme polygalacturonase by *B. cinerea* has been reported (Leone *et al.*, 1987). Since addition of Pi alone was sufficient in stimulating the fungal infectivity, only inoculations in a 62.5 mM Pi solution with $2x10^6$ spores ml^{-1} were employed.

Another factor strongly influencing the infection process by *B. cinerea* and *S. sclerotiorum* is the need for a relative air humidity above 93% (Blakeman, 1980; Hunter

TABLE 1. Successful lesions (in percentage of the total number of inoculation drops) on primary bean leaves (cv. Lit) 48 h after inoculation with *Botrytis cinerea* or *Sclerotinia sclerotiorum* spores (concentration: $2x10^5$ or $2x10^6$ spores ml^{-1}) in droplets of different composition.

	Pathogens and spore concentration			
	B. cinerea		S. sclerotiorum	
Inoculum composition	$2x10^5$	$2x10^6$	$2x10^5$	$2x10^6$
Water	0.0	0.0	0.0	2.1
62.5 mM Pi	0.0	2.5	0.0	36.7
62.5 mM Pi + 1.1 mM glucose	5.0	35.0	6.2	41.2
62.5 mM Pi + 5.5 mM glucose	65.0	90.0	6.2	63.3

et al., 1981). By incubating the detached bean leaves in plastic trays with wet filter paper and covered with transparent plastic bags, relative humidity could easily be maintained above 95%.

The ANOVA results of the resistance of 15 bean cultivars to *B. cinerea* and *S. sclerotiorum* are summarized in Table 2. Significant effects were found for the main factors fungi and cultivars but not for their interaction.

TABLE 2. ANOVA results of the screening of 15 bean cultivars for resistance to *Botrytis cinerea* and *Sclerotinia sclerotiorum*.

Source of variation	D.f.	S.s.	M.s.	V.r.	F prob.
Replicate	2	4586.4	2293.2		
Fungus	1	3912.5	3912.5	16.4	<0.001
Cultivar	14	7260.3	518.6	2.2	0.020
Fungus x Cultivar	14	2797.6	199.8	0.8	0.625
Residual	58	13803.1	238.0		
Total	89	32359.9			

In drop inoculation in Pi solution containing $2x10^6$ spores ml^{-1}, *S. sclerotiorum* caused significantly more lesions than *B. cinerea* regardless of the bean cultivar (Table 3). The cultivars Centrum and Comprise appeared to be relatively resistant towards *B. cinerea* whereas Groffy and Stratego were relatively susceptible. The cultivars Gamin and Centrum showed the lowest level of infection by *S. sclerotiorum* and cultivar Tuf the highest. Significant differences in susceptibility between the cultivars could be detected regardless of the fungus. None of the cultivars was completely resistant to both fungi: cultivar Centrum was the most resistant whereas Tuf was the most susceptible. A relationship between the reported ozone sensitivity/tolerance of the cultivars (Tonneijck, 1983) and the observed susceptibility/resistance to the fungi could not be found.

The primary aim of the procedure developed was not the screening of cultivars for resistance to *B. cinerea* and/or *S. sclerotiorum*, but to have a reliable inoculation and incubation method for bean leaves in order to study quantitatively the interaction between ozone and weak pathogens in causing foliar injury (Leone *et al.*, 1989). Nevertheless, since the major factors determining the infection on leaves by both fungi (spore concentrations, infection-stimulating compounds and relative humidity) can be controlled and easily manipulated, we believe this procedure can be successfully applied also to large-scale screening and breeding programs.

TABLE 3. Successful lesions (in percentage of the total number of inoculation drops) on primary leaves of 15 bean cultivars 48 h after inoculation with *Botrytis cinerea* or *Sclerotinia sclerotiorum* spores (concentration: $2x10^6$ spores ml^{-1}) in a 62.5 mM Pi solution. Bean cultivars are ranked in decreasing order of ozone sensitivity (Tonneijck, 1983).

Cultivar	B. cinerea	S. sclerotiorum	Average[a]
Pros	40.0	67.5	53.7
Stratego	47.5	55.0	51.2
Tuf	41.7	75.8	58.7
Prelude	38.3	38.3	38.3
Centrum	3.3	30.0	16.6
Comprise	3.3	43.3	23.3
Fran/Toccata	40.8	48.3	44.5
Lit	22.5	49.2	35.8
Prevato	21.7	43.3	32.5
Precores	24.2	46.7	35.4
Belami	24.2	42.5	33.3
Gamin	25.0	21.7	23.3
Groffy	48.3	39.2	43.7
Narda	37.5	64.2	50.8
Berna	30.8	67.5	49.1
Average[b]	29.9	48.8	

[a] Least significant difference between cultivars (LSD; P=0.05): 31.3.
[b] Least significant difference between fungi (LSD; P=0.05): 6.5.

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CHAPTER 6

PURIFICATION AND CHARACTERIZATION OF A CONSTITUTIVE POLYGALACTURONASE ASSOCIATED WITH THE INFECTION PROCESS OF FRENCH BEAN LEAVES BY *BOTRYTIS CINEREA*

by

G. Leone, E. A. M. Schoffelmeer & J. Van den Heuvel

Abstract

The constitutively produced polygalacturonase isoenzyme PG2, was isolated from culture filtrates of *Botrytis cinerea*, purified and characterized. The purification procedure involved gel filtration, ion exchange chromatography and chromatofocusing. Homogeneity of purified PG2 was checked by staining for PG activity after gel electrophoresis and by preparation of titration curves. The shape of titration curves of PG2 and two other PG isoenzymes explained the difficulties found in separating superimposed pectic enzyme activities during the purification procedure. PG2 hydrolyzed sodium polygalacturonate more quickly than pectin. Under standard assay conditions, at pH 4.4, the K_m value for the hydrolysis of polygalacturonate was 0.46 mg substrate ml^{-1}; for pectin, the K_m was provisionally estimated to be 1.18 mg substrate ml^{-1}. The optimal pH for PG2 activity with polygalacturonate was 4.5 and with pectin 4.0. PG2 activity was also influenced by the presence of NaCl or $CaCl_2$ in the reaction mixture. Analysis of the breakdown products by paper chromatography and a comparison of the reaction rate by viscosimetry and reducing group assay, revealed that PG2 has an endo catalytic mode of action on polygalacturonate. The isoelectric point and the molecular mass of PG2 were estimated to be 9.1 and 23.0 kDa, respectively.

Introduction

Botrytis cinerea Pers.: Fr. is a widespread fungus able to infect the aerial parts of many plant species, causing flecking, tissue maceration and soft rots (Verhoeff, 1980). Cell wall-degrading enzymes play an important role in the expression of these symptoms (Hancock *et al.*, 1964; Verhoeff, 1980). Pectic enzymes, and polygalacturonases (PGs) in particular, are the first cell wall-degrading enzymes secreted by many microorganisms growing on isolated cell walls (Cooper & Wood, 1975; English *et al.*, 1971; Jones *et al.*, 1972). In the phosphate-stimulated infection of French bean leaves by *B. cinerea*, two PGs, viz. PG1 and PG2, were reported to accumulate in inoculum drops, 6-12 h after inoculation, during fungal penetration of the outer epidermal cell wall (Van den Heuvel & Waterreus, 1985). Under conditions without penetration, activities of both PGs remained negligible. The two PGs were also associated, together with other PGs as well as pectin esterases (PEs), with fungal colonization of the leaf tissues. Research on the regulation of the synthesis of pectic enzymes by *B. cinerea* showed the sequential nature of their production, that is started by the constitutively produced PG2, and indicated that PG production is inherent to the digestion of pectic polymers by the fungus (Leone & Van den Heuvel, 1987; Leone *et al.*, 1990).

The supposed important role played in the infection process by PG1 and PG2 justified a study of their mode of action. This report deals with the purification and the characterization of the principal enzymatic properties of PG2, and with attempts to purify PG1.

Materials and methods

Growth of the fungus and production of PGs

A culture of isolate BC1 of *B. cinerea* maintained on a PDA slant at 4°C was used as a stock culture throughout all experiments. For obtaining profusely sporulating cultures, the fungus was passed on slants of medium X (Last & Hamley, 1956) and incubated for 11 days at about 23°C under continuous fluorescent light (Van den Heuvel, 1981).

In order to find the best medium and conditions for a large-scale enzyme production, 300-ml Erlenmeyer flasks containing 100 ml of one of several variations of Richards' solution (Leone & Van den Heuvel, 1987) were inoculated with spore suspensions of different concentrations. The inoculated flasks were incubated at 19°C for four days on a reciprocal shaker at 108-120 strokes min^{-1}. In some cases, two days after inoculation, AMP was added to the medium at a final concentration of 2.5 mM (Leone *et al.*, 1990) and the cultures were incubated for three days more.

At the end of the incubation period the mycelium was separated from the culture liquid by filtering the liquid in successive steps through filter paper (S & S no. 604, Dassel, Federal Republic of Germany) and Sartorius membrane filters of 0.65, 0.45 and 0.2 μm pore size. Later, the last step was omitted after finding a considerable loss of PG activity. The resulting filtrates were quickly frozen in a cold ethanol bath and lyophilized to dryness.

After selection of the best conditions for production of PG1 and PG2 (see Results) several batches of 30 flasks were inoculated, incubated and processed, giving aliquots of ca 3 l of culture filtrate for further purification.

Preparation of cross-linked polyuronides for affinity chromatography

Sodium polygalacturonate was prepared by alkaline de-esterification of citrus pectin (Fluka, M_r 25,000-50,000) following the method reported by Rombouts *et al.* (1979). Cross-linked polygalacturonate and alginate with a cross-linking degree of 0.57 were prepared using epichlorohydrin as described by Rombouts *et al.* (1982). Swelling of the resulting material and chromatographic procedures were as reported by Marcus & Schejter (1983).

Enzyme purification

Culture filtrates were lyophilized as described above. The residues were redissolved in a minimal quantity of 10 mM barbital buffer, pH 8.0, and 30-ml samples were passed through a 2.2x48 cm column of Sephadex G-25 Medium equilibrated and eluted with the same buffer, at a flow rate of 50 ml h^{-1}. Previous experiments (Van den Heuvel & Waterreus, 1985) had indicated that the pI of the predominant PG isoenzymes was between 8.5 and 9.5. This characteristic was used as a basis for the following purification step. PG-containing fractions obtained from different Sephadex G-25 Medium runs were pooled and applied to a CM-Sepharose CL-6B column (2.3x16 cm) also equilibrated with 10 mM barbital buffer, pH 8.0. PGs were eluted with a linear gradient of 0-0.3 M NaCl in the same buffer, at a flow rate of 20 ml h^{-1}. Fractions containing PG1 or PG2 were pooled and separately processed for chromatofocusing. Both fractions were first desalted via a Sephadex G-25 Medium column (2.2x48 cm) equilibrated with 25 mM triethylamine-HCl buffer, pH 11.0. The PG-containing eluate was pooled and 35-ml samples were then applied to a 1.1x27 cm column of Polybuffer exchanger PBE 118 (Pharmacia), equilibrated with the same buffer. The column was eluted with 250 ml 2.2% (v/v) Pharmalyte pH 8-10.5, adjusted to pH 8.0 with 5 M HCl, at a flow rate of 10 ml h^{-1}.

All chromatographic steps were done in a cold room at 2°C. Absorbance of the eluates from the different columns was monitored at 280 nm.

Assays for PG activity

PG activity was determined quantitatively by two methods. The first was the 2-cyanoacetamide assay as reported by Gross (1982), based on the spectrophotometric determination of reducing groups released from sodium polygalacturonate or pectin. D-galacturonic acid was used as the standard. This method is applicable only if the

concentrations of D-glucose and L-cysteine in the samples are negligible (Honda *et al.*, 1982). The second method was a slight modification (Leone & Van den Heuvel, 1987) of the cup-plate assay (Dingle *et al.*, 1953). Details of the assay procedure and of the determination of PG units were as previously reported (Leone & Van den Heuvel, 1987).

Analysis of pectic enzymes

Protein concentrations were determined using the method of Bradford (1976) with BSA as the standard.

Fractions of culture filtrates or eluted from chromatographic columns were subjected to electrophoresis in polyacrylamide gels supplemented with pectin, as described by Van den Heuvel & Waterreus (1985). Pectic enzymes (PGs and PEs) were detected by the zymogram technique using the procedure of Cruickshank & Wade (1980). This method was routinely used to follow the course of the purification procedure. The numbering of PG isoenzymes was as already reported (Leone & Van den Heuvel, 1987).

For the study of the electrophoretic mobility of purified PGs as a function of pH, titration curves were prepared. Ultrathin gels (thickness 0.7 mm) with relative concentrations of acrylamide + cross-linker (T) and cross-linker (C) of 4.7% and 3.8%, respectively, containing Pharmalyte pH 3-10 and/or pH 8-10.5, were cast as described by Righetti (1983). A pH gradient was created in the gels in one direction by running the unloaded gels at 15 W constant for 45 min. After application of 60 μl enzyme sample along the pH gradient, the gels were electrophoresed in a direction perpendicular to the pH gradient for 15 min at 1000 V constant. The uniformity of the pH gradient was checked after the electrophoresis step using a small surface pH electrode. Gels with a pH gradient created by Pharmalyte 8-10.5 only were also used for determining the isoelectric point (pI) of PGs. PGs were located in the gels after incubating the gels at 30°C in a 0.1 M citric acid/0.2 M Na_2HPO_4 buffer (pH 4.5) to which 0.5% (w/v) sodium polygalacturonate had been added just before incubation. The incubation time depended on the isoenzyme. As a rule, the best incubation time for PG1 was 2 h, for PG2 0.5 h, and for PG3 2.5 h. After carefully washing out the remaining sodium polygalacturonate with distilled water, the gels were immersed in a fresh 0.05% (w/v) ruthenium red solution for 1 h and subsequently rinsed with distilled water until a distinct white area was visible.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by using 0.8 mm-thick gels with T=11.3% and C=2.6%. The SDS concentration was 0.1% (w/v) in the gels and in the running buffer (0.05 M Tris/0.1 M glycine buffer, pH 8.7) and 2% in the samples. The samples were prepared as described by Ried & Collmer (1985) and contained 0.001% bromophenol blue as tracking dye. A Bio-Rad SDS preparation was used throughout the experiments since it allowed enzyme renaturation necessary for activity staining (Lacks *et al.*, 1979). As molecular mass markers BSA, ovalbumin, chymotrypsinogen A and ribonuclease A (Pharmacia) and cytochrome C (Merck) were employed. Two gels, one of which contained 0.09% (w/v) pectin, were run simultaneously. After pre-electrophoresis at 280 V constant for 0.5 h, 10-μl samples were applied to the gels and proteins were concentrated at 50 V constant for another 0.5 h. Electrophoresis was finally carried out at 500 V constant for 2 h. Protein staining was performed by the urea-silver stain method of Chauduri & Green (1987) or by the periodic acid-silver stain method of Dubray & Bezard (1982) for glycoproteins. PG activity in the pectin-amended gels was detected with the method of Cruickshank & Wade (1980). The molecular mass of PG2 was determined from a plot of the relative mobility of the marker proteins against the log of their molecular mass.

Viscosimetry

The viscosity of solutions of sodium polygalacturonate after various periods of incubation (0-48 h) with PG2 was measured with an Ubbelohde no. 4 viscosimeter. The reaction mixtures, held at 30°C, consisted of 1 ml of an enzyme solution (9.9 units ml^{-1}) and 14 ml of a solution containing 2.4% (w/v) sodium polygalacturonate and 0.14 M ammonium oxalate in 0.1 M acetate buffer, pH 4.5. Ammonium oxalate was added to facilitate complete solubility of the substrate.

Analysis of enzymatic breakdown products
Paper chromatography was used for the qualitative analysis of breakdown products released from sodium polygalacturonate by the action of PG2. Enzymatic reaction mixtures, incubated at 30°C, consisted of 800 µl 0.24% (w/v) sodium polygalacturonate (previously washed with 70% ethanol) and 0.14 M ammonium oxalate in 0.1 M acetate buffer, pH 4.5, and 160 µl of a PG2 solution (9.9 units ml^{-1}). At intervals (0-48 h), a portion of the incubation mixture was removed and the reaction was stopped by immersing the samples in a boiling water bath for 5 min. Twentyfive-µl samples were spotted on Whatman no. 3MM chromatography paper. The chromatograms were subsequently eluted with 1-butanol:96% acetic acid:H_2O (4:2:3; v/v/v). Uronic acid spots were visualized as reported by Marcovic & Slezarik (1984).

Results

Effect of the culture medium on PG production
Attempts were made to optimize PG production in culture. Two principal aims were pursued: 1) to reach the best conditions for a high production of PG1 and PG2, and 2) to have the lowest possible production of other PG isoenzymes. The highest activities were obtained with a modified Richards' solution (containing per 1000 ml distilled water 5 g KNO_3, 2.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_2O$, 0.4 mg $FeCl_3$ and 10 g glucose) supplemented with 0.01% yeast extract and inoculated with $2x10^6$ conidia ml^{-1}. However, the resulting culture liquid contained various other PG isoenzymes and, more importantly, had an extremely high viscosity which hindered filtration and concentration. The latter characteristic was often found when the fungal growth rate was highly stimulated by yeast extract or high phosphate concentrations. Therefore, phosphate-limited growth followed by AMP-stimulated PG synthesis was selected (Leone *et al.*, 1990). Thus, for large-scale batch cultures the following conditions were used. The medium contained per 1000 ml distilled water 10 g KNO_3, 0.25 g $MgSO_4.7H_2O$, 0.4 mg $FeCl_3$ and 20 g glucose, and was inoculated with $2x10^4$ conidia ml^{-1}. After incubation for 2 days, AMP (final concentration 2.5 mM) was added to the medium. Although using these cultural conditions PG production was reduced of about 3.5 times, the low viscosity of the culture liquids permitted an easier and quicker start of the purification.

Affinity chromatography of pectic enzymes on cross-linked polyuronides
The powder obtained after lyophilization of the culture filtrates was dissolved in distilled water and extensively dialysed against 0.01 M acetate buffer, pH 5.2, for 48 h. Ten ml of the dialysed sample was then applied to a column (1.6x27 cm) of cross-linked polygalacturonate and eluted with 10 mM acetate buffer, pH 5.2. Two peaks of PG activity, as assessed by cup-plate assay, were obtained after application of a gradient of 0-0.5 M NaCl in the same buffer. The peaks contained several PG isoenzymes among which PG2 was predominant, as assessed by zymograms after electrophoresis. The PE isoenzymes PE1 and PE2 (Leone & Van den Heuvel, 1987) were also detected in the first activity peak. No protein absorbance was associated with the pectic enzyme peaks, indicating that the activity was caused by a very low enzyme concentration.

Attempts were made to improve the separation of PG1 and PG2 from the other pectic isoenzymes. Changes in the pH of the elution buffer (pH 4.2) or in its molarity (0.1 M), in the steepness of the salt gradient, or in the cross-linking degree of the polygalacturonates (0.46) were all unsuccessful. A preparation of alginate (undegradable by PGs) with a cross-linking degree of 0.57 showed too strong swelling properties, perhaps caused by a too low molecular mass, and could not further be used as chromatographic substrate. Therefore, we decided to apply a different purification procedure.

PG purification
The lyophilized culture filtrate of *B. cinerea* was chromatographed on Sephadex G-25 Medium and eluted with 10 mM barbital buffer, pH 8.0. PG activity, measured by the

cup-plate assay, eluted as one peak in the void volume. Zymograms after electrophoresis showed that this activity was mainly caused by PG1 to PG4. Ion exchange chromatography of the pooled PG-containing fractions on a column of CM-Sepharose CL-6B at pH 8.0 showed that PGs were eluted by ca 0.1 M NaCl as two distinct peaks, if measured by the 2-cyanoacetamide method (Fig. 1). If PG activity was determined with the cup-plate assay, only the second peak was found. The first peak contained mainly PG1, PG2 and PG3, while the second peak contained almost exclusively PG2 and PG3 (Fig. 2). Pooled fractions from either of both peaks were desalted using a Sephadex G-25 Medium column at pH 11.0. However, after the desalting step PG1 activity was completely lost, this making impossible a further purification and characterization of this isoenzyme. Chromatofocusing of the desalted, pooled fractions from the second peak on a column of PBE 118 using Pharmalyte 8-10.5 as the eluent yielded again two PG peaks if measured with the 2-cyanoacetamide method and only one peak if measured with the cup-plate assay (Fig. 3). The first peak contained solely PG2, whereas the second peak contained mainly PG3 and some PG2, as assessed by zymograms after electrophoresis. Since the pooled PG2 fractions from the first peak were apparently free of any other pectic enzyme, this PG2 preparation was judged as pure. No protein peaks were associated with the PG peaks themselves. In Table 1 the results of the enzyme purification are summarized.

Electrophoretic analysis

The PGs did not elute from the CM-Sepharose CL-6B column in the sequence expected on the basis of their increasing pIs, i.e. PG4 → PG3 → PG2 → PG1, but instead PG4 → PG1 → PG3 → PG2 (Fig. 2). This prompted us to investigate the electrophoretic mobility of PG1, PG2 and PG3 as a function of pH by means of their titration curves. These are schematically illustrated in Fig. 4 for the pH range 3-10. The curves of PG1 and PG3 were flat over a wide pH range, but this was not the case for PG2. Therefore, the charges and the binding forces of PG1 and PG3 at the pH used for the cation exchanger must have been weaker than those of PG2, causing the displacement effect observed. The charge of PG3 seemed to be even weaker, as its activity had been spread over more fractions than PG1 (Fig. 2). The pI of PG2 and PG3 was 9.1 and 8.9, respectively, as determined from the titration curves in the pH range 8-10.5 of samples purified by chromatofocusing and containing solely PG2 or PG3 (Figs 5 A and B). The pI of PG1 could not be determined accurately from the titration curves obtained from a mixture of PGs; it was higher than that of PG2 because after gel electrophoresis the PG1 band was always closest to the cathode.

The molecular mass of PG2 was estimated to be 23.0 kDa by SDS electrophoresis.

Substrate specificity of PG2

The capability of PG2 in hydrolyzing pectin was studied, as pectic polysaccharides are important components of the rhamnogalacturonan portion of the primary plant cell wall. PG2 could hydrolyze pectin but sodium polygalacturonate appeared to be a better substrate. At equivalent substrate concentration (2%), PG2 catalyzed the release of reducing groups from sodium polygalacturonate about six times more quickly than from pectin (Fig. 6). It has to be stressed that sometimes, during the determination of activity by the 2-cyanoacetamide method in the course of pectin degradation, we observed strongly fluctuating values for the released reducing groups. We were not able to ascertain the cause of these disturbances. However, the differences found between sodium polygalacturonate and pectin in the degree of degradation by PG2 were not affected by these disturbances.

The difference between the rates of hydrolysis of sodium polygalac- turonate and pectin by PG2 was quantified by determining the K_m values for both substrates at pH 4.4. Also in this case, sometimes fluctuating values were found for the rate of pectin degradation. The results were therefore plotted as a substrate-saturation curve (Fig. 7 A), whereas the results from sodium polygalacturonate could be used for a Lineweaver-Burk plot (Fig. 7 B). Using the computer program of Page (1987), the K_m for sodium polygalacturonate was calculated to be 0.46 mg substrate ml^{-1}. For pectin, the K_m was provisionally estimated to be 1.18 mg substrate ml^{-1}. Therefore, the affinity of PG2 for sodium polygalacturonate is about three times higher than for pectin.

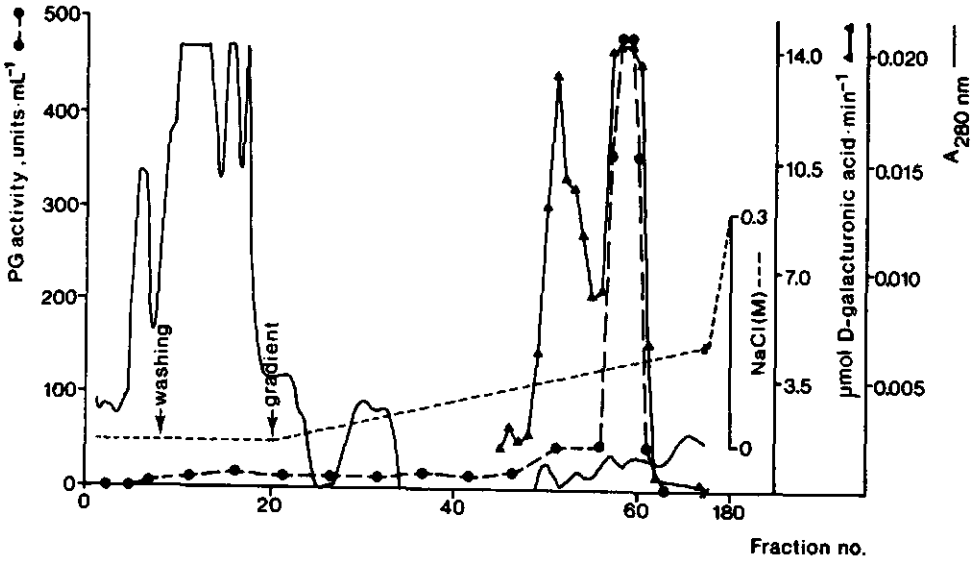

FIGURE 1. CM-Sepharose CL-6B ion exchange chromatography of the pooled PG-containing fractions resulting from the gel filtration on Sephadex G-25 Medium. The column was eluted with 60 ml 10 mM barbital buffer (pH 8.0). Then, 500 ml of a linear gradient of 0 to 0.3 M NaCl in the same buffer was applied. The flow rate was 20 ml h^{-1} and 5-ml fractions were collected. PG activity was measured by the cup-plate assay (●) and by the 2-cyanoacetamide method (▲). Fractions 50 to 53 and 57 to 60 were pooled.

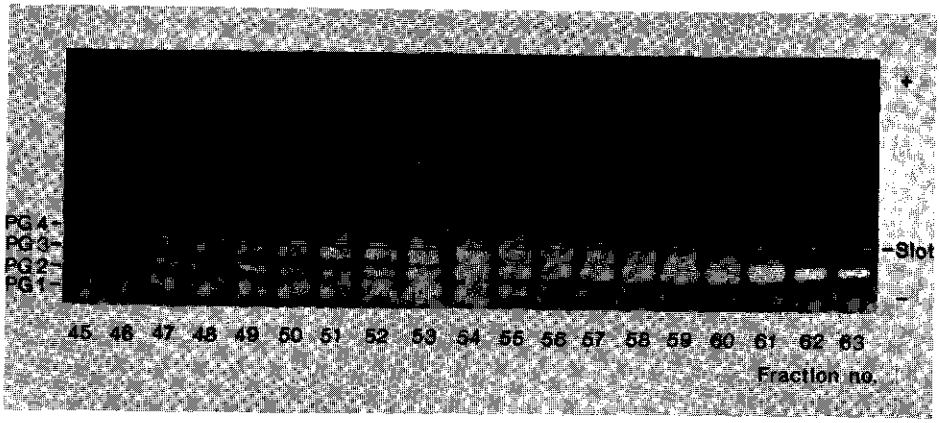

FIGURE 2. PG isoenzymes eluted by ion exchange chromatography on CM-Sepharose CL-6B (Fig.1). Numbers indicate the assayed fractions. Pectic enzymes were detected by the zymogram technique after pectin-polyacrylamide gel electrophoresis as described in Materials and methods.

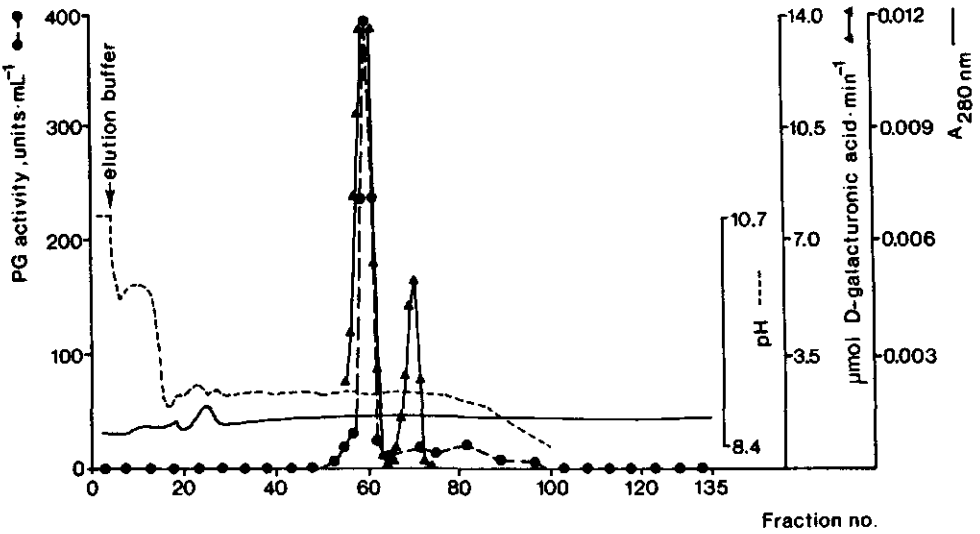

FIGURE 3. Chromatofocusing on PBE 118 of the pooled PG2 and PG3-containing fractions (57-60) resulting from CM-Sepharose CL-6B chromatography (see Fig. 1). The column was eluted with 250 ml 2.2% (v/v) Pharmalyte pH 8-10.5. The flow rate was 10 ml h^{-1} and 2-ml fractions were collected. PG activity was measured by the cup-plate assay (●) and by the 2-cyanoacetamide method (▲).

TABLE 1. Scheme of purification of PGs of *Botrytis cinerea*

Purification step	Volume (ml)	Protein concn. (µg/ml)	PG activity (units/ml)	Specific activity (units/µg protein)	Degree of purif.	Yield (%)
Crude culture liquid	2620	1	4.96	4.96	1.0	100.0
Lyophilized culture filtrate	30	10	145.50	14.55	2.9	33.6
Sephadex G-25 Medium (1)	40	nd[a]	79.05	>79.05	>15.09	24.3
Sepharose CL-6B						
PG2 + PG3	20	nd	104.05	-	-	16.0
PG1 + PG3	20	nd	10.20	-	-	1.6
Sephadex G-25 Medium (2)						
PG2 + PG3	35	nd	42.85	-	-	11.5
PBE 118						
PG2	21.5	nd	53.49	-	-	8.8
PG3	8.5	nd	12.58	-	-	0.8

[a] nd = not detectable, i.e. concentration below detection limit of 1 µg protein ml^{-1}.
[b] Based on detection limit of 1 µg protein ml^{-1}.

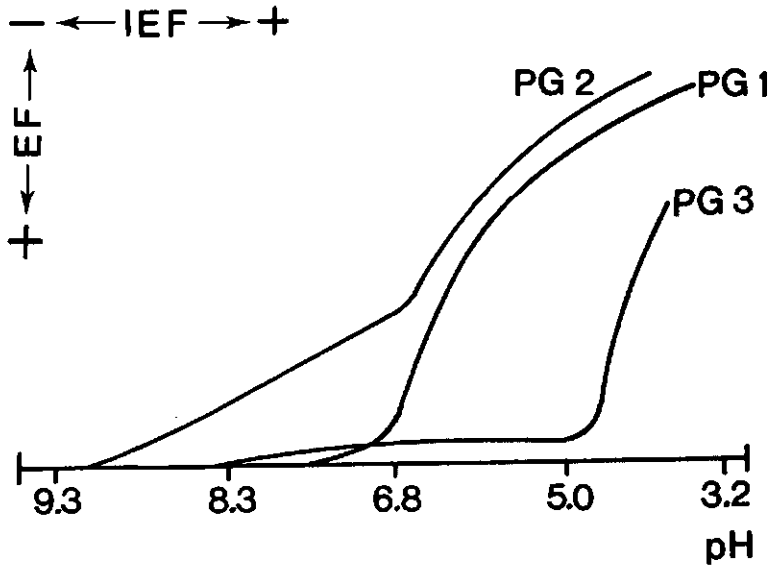

FIGURE 4. Schematic representation of the titration curves of PG1, PG2 and PG3 in the pH range 3-10. Titration curves of PG2 and PG3 were separately obtained from fractions containing solely PG2 or PG3 (see legend of Fig. 5). Subsequently, the titration curve of PG1 was recognized evaluating the electrophoretic mobilities of the enzymes from a fraction containing a mixture of PG1, PG2 and PG3.

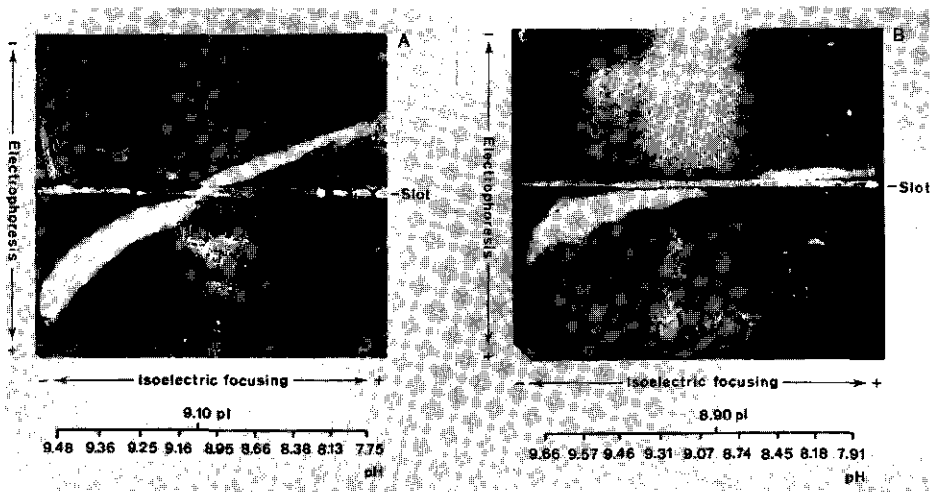

FIGURE 5. Titration curves in the pH range 8-10.5 of PG2 (A) and PG3 (B) purified by chromatofocusing. PG2 (8.7 cup-plate units ml^{-1}) was from the pooled fractions (56-61) eluted from the PBE 118 column (see Fig. 3): PG3 (26 cup-plate units ml^{-1}) was from fraction 72 eluted from the same column. Focusing, electrophoresis and enzyme visualization were as described in Materials and methods.

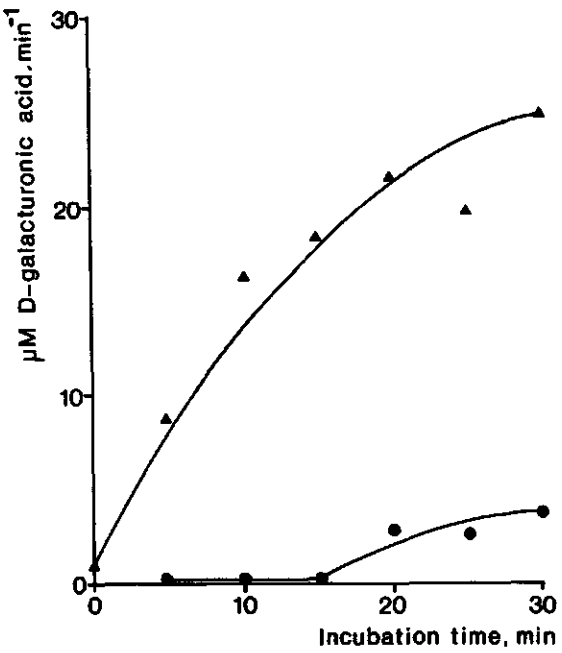

FIGURE 6. The hydrolysis of sodium polygalacturonate (▲) and pectin (●) by PG2. The reaction mixtures consisted of 155 μl 37.5 mM acetate buffer (pH 4.4), 20 μl 2% (w/v) sodium polygalacturonate or pectin and 25 μl PG2 (145.5 cup-plate units ml^{-1}). Activity was measured by the 2-cyanoacetamide method.

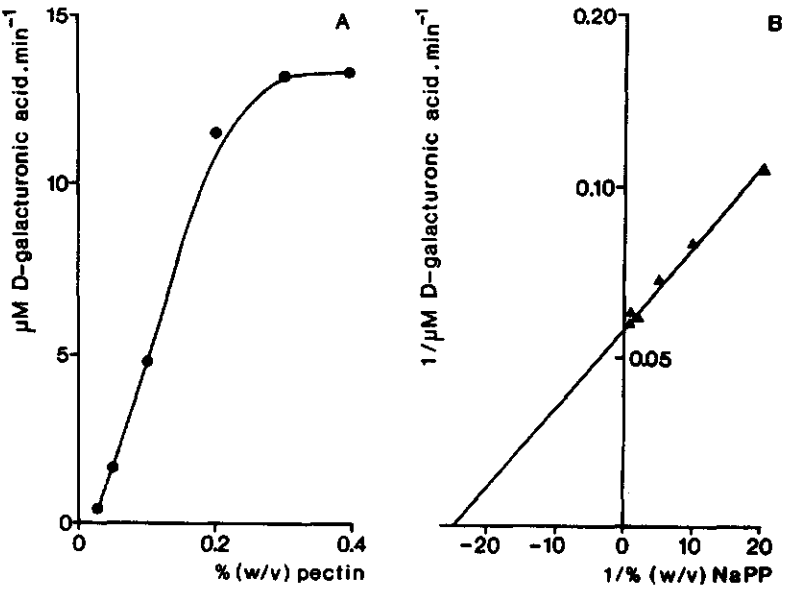

FIGURE 7. Substrate-saturation curve of the initial activity of PG2 against pectin concentration (A) and Lineweaver-Burk plot of the initial activity of PG2 against sodium polygalacturonate concentration (NaPP) (B). The reaction mixtures consisted of 155 μl 37.5 mM acetate buffer (pH 4.4), 20 μl pectin or sodium polygalacturonate (at 0.025, 0.05, 0.1, 0.2, 0.3 and 0.4%, w/v) and 25 μl PG2 (18.25 cup-plate units ml^{-1}). Activity was measured by the 2-cyanoacetamide method.

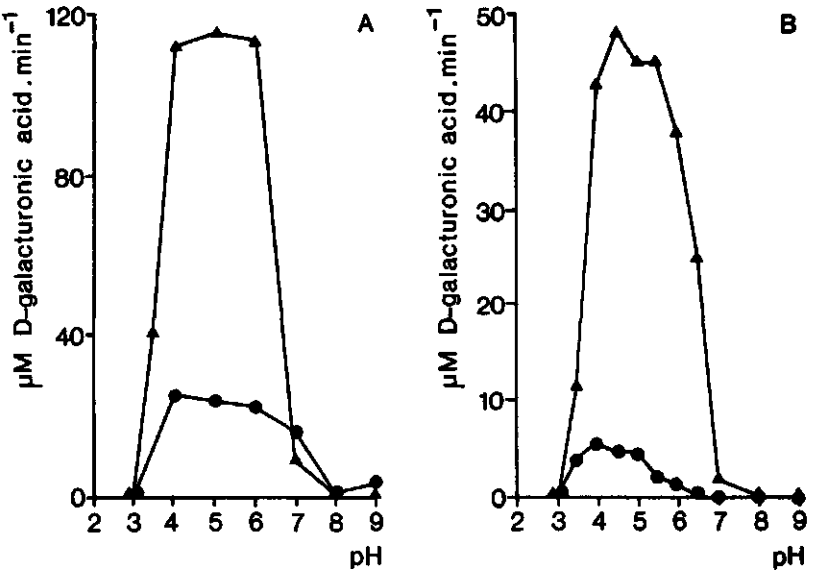

FIGURE 8. Hydrolysis of sodium polygalacturonate (▲) or pectin (●) by a mixture of PGs (A) or by purified PG2 (B) at different pHs. The following buffer solutions were used: 50 mM acetate buffer (pH 3.0-5.0), 50 mM phosphate buffer (pH 6.0-7.0) and 50 mM barbital buffer (pH 8.0-9.0). The reaction mixtures consisted of 155 µl buffer solution, 20 µl 2% (w/v) sodium polygalacturonate or pectin and 25 µl enzyme (25.6 cup-plate units of a mixture of PG1, PG2 and PG3 ml^{-1} or 145.5 cup-plate units ml^{-1} of PG2). Activity was measured by the 2-cyanoacetamide method.

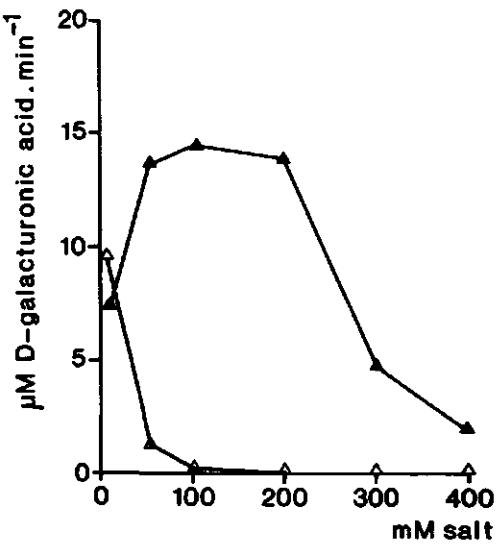

FIGURE 9. Effect of different NaCl (▲) and $CaCl_2$ (△) concentrations on PG2 activity. The reaction mixture consisted of 135 µl 37.5 mM acetate buffer (pH 4.4) containing various amounts of NaCl or $CaCl_2$, 40 µl 2% (w/v) sodium polygalacturonate and 25 µl PG2 (18 cup-plate units ml^{-1}). Activity was measured by the 2-cyanoacetamide method.

Optimal pH

A mixture of PG1, PG2 and PG3 was able to hydrolyze sodium polygalacturonate in the pH range 3.5-7.0 and maximally between pH 4 and 6 (Fig. 8 A). The hydrolysis of this substrate by a pure PG2 preparation was optimal at pH 4.5 (Fig. 8 B). Since the breakdown of sodium polygalacturonate by PG2, alone or in combination with PG1 and PG3, occurred in the same pH range (3.5-7.0), this implies that the pH optima of PG1 and PG3 are close to that of PG2.

Pectin was broken down by a mixture of PG1, PG2 and PG3 in the pH range 4-7 (Fig. 8 A). The pH optimum for hydrolysis of pectin by PG2 was pH 4 (Fig. 8 B). Unlike sodium polygalacturonate, pectin was hydrolysed by the mixture of PG1, PG2 and PG3 at a wider pH range than by PG2 alone.

It should be noted that with both substrates, the mixture of PG1, PG2 and PG3, which contained about one/sixth of the activity of the PG2 preparation, released 2 to 3 times more reducing groups than pure PG2. This indicates that PG1 and PG3 took part in the degradation of the substrates performed by PG2.

Effect of NaCl and $CaCl_2$ on PG2 activity

The effects of various concentrations of NaCl and $CaCl_2$ on PG2 activity are shown in Fig. 9. PG2 activity was stimulated by 50-200 mM NaCl, whereas 300 mM and higher concentrations had an inhibitory effect. $CaCl_2$ had a highly negative effect on PG2 activity. Increasing the concentration of $CaCl_2$ caused an increased precipitation of insoluble substrate, possibly calcium polygalacturonate. Therefore, the negative effect of $CaCl_2$ on PG2 may not be the result of a direct enzyme inhibition but, rather, the result of a decreased availability of degradable substrate.

Mode of catalytic action

In order to determine whether PG2 has an endo or exo mode of catalytic action, two approaches were used. In the first, the viscosity of an enzyme-substrate mixture was measured as a function of the time of incubation of the mixture. The time necessary to reduce the viscosity of sodium polygalacturonate to 50% was 60 min, whereas at the same time the release of reducing groups, as measured by the 2-cyanoacetamide method, was only about 2% (Fig. 10). A sharp decrease in viscosity associated with a relatively slow increase in the number of reducing groups is a strong indication that PG2 has an endo catalytic mode of action (Nasuno & Starr, 1966).

In the second approach, the breakdown products formed during the hydrolysis of sodium polygalacturonate with PG2 were analysed by paper chromatography. In this case, the products were found to be a range of di-, tri-, tetra-, and penta-galacturonic acids as well as D-galacturonic acid, using mono- and tri-galacturonic acid as references (results not shown). The release by an enzyme of a mixture of monomer and oligomers from a polymeric substrate is also a clear indication of an endo catalytic mode of action.

Discussion

The supposed involvement of PG1 and PG2 in the penetration process of bean leaves by *B. cinerea* (Van den Heuvel & Waterreus, 1985) incited the study of some of their biochemical properties. The purification of these enzymes was found to be difficult mainly because of their low production *in vitro* and the superimposition of different pectic enzyme activities throughout the whole separation procedure. However, it was possible to purify PG2, but PG1 activity was completely lost just before the last chromatographic step.

A large-scale enzyme production was complicated mostly because of the intrinsic nature of PG synthesis by *B. cinerea*. It was already known that this fungus produces coordinately and sequentially a number of PG isoenzymes, their synthesis being regulated by the type and concentration of the carbohydrate used as C source (Leone & Van den Heuvel, 1987). A further difficulty resided in the fact that PG2 is constitutively produced, while PG1 is inducible and its synthesis seems to be regulated also by the energy status of the fungal cell (Leone *et al.*, 1990). Moreover, the best

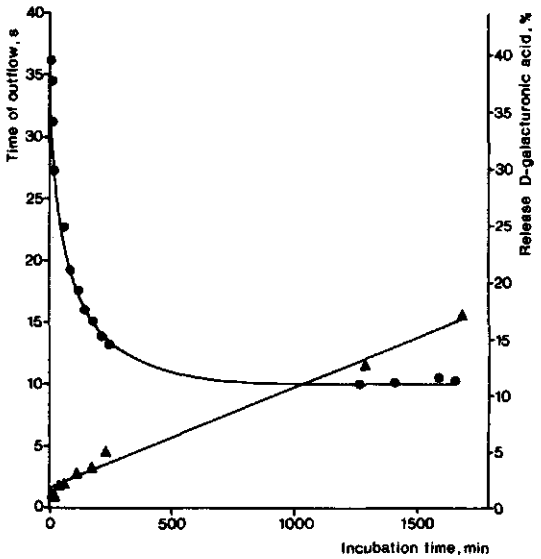

FIGURE 10. Changes in viscosity (●) and release of reducing groups (▲) from sodium polygalacturonate as a function of the time of incubation with PG2. Viscosimetry and measurements of the release of reducing groups were as described in Materials and methods. The release of reducing groups is expressed as % of the amount of sodium polygalacturonate present at the start of the experiment.

conditions for PG2 production (rapid and extensive growth stimulation) led to a highly viscous culture filtrate that hindered its isolation. The high viscosity may have been caused by the known abundant production of polysaccharides or mucilage by *B. cinerea* (Kamoen *et al.*, 1978; Mc Keen, 1974). Therefore, conditions of phosphate-limited growth, followed by AMP-stimulated PG1 production, as used in the study of phosphate involvement in PG synthesis (Leone *et al.*, 1990), were chosen for the production of PGs for purification.

Purification of pectic enzymes by means of a single affinity chromatography step has been reported (Marcus & Schejter, 1983; Rexova-Benkova & Tibensky, 1972). The application of this technique for the purification of PG1 and PG2 seemed attractive, but in our hands it was unsuccessful because of: a) the inability to separate the superimposed enzyme activities eluted and; b) the known degradation of the cross-linked polyuronides caused by polygalacturonase activity (Rombouts *et al.*, 1982). However, it has to be stressed that in the reports cited, the enzyme activities purified were not checked for the presence of different pectic isoenzymes by the zymogram technique as we did.

In the course of the purification procedure, we found that the eluted peaks of activity contained constantly two to four superimposed pectic isoenzymes. Furthermore, with ion exchange chromatography PG1, PG2 and PG3 were not eluted in the sequence expected on the basis of their pI. We could explain this displacement effect from the titration curves of the three enzymes. The presence of a flat area in the curves of PG1 and PG3 including their pI, reflects a weak net charge on their molecules over a quite large pH range. This causes these enzymes to elute over a wide pH range on either side of their pI. The difficulty of separating and purifying cell wall-degrading enzymes is a known problem (Cooper, 1977). It has often been reported that supposedly homogeneous pectic enzyme preparations showed, during their characterization, activities of different type (Cooper *et al.*, 1978; Förster & Rasched, 1985; Miller & Macmillan, 1970; Urbanek & Zalewska-Sobczak, 1975; Wang & Keen, 1970). As an explanation of this phenomenon, the existence of unseparable multi-enzyme particles was hypothesized (Cooper, 1977). Our results indicate that the small differences in charge at pH 8.0 and/or the closeness

of pIs of *B. cinerea* PGs are the cause of the superimposition of their activities during the purification procedure. These peculiar characteristics, reported also for the polygalacturonate lyase isoenzymes of *Erwinia chrysanthemi* (Bertheau *et al.*, 1984), should therefore be taken into account as a possible explanation of the above-mentioned failures in obtaining homogeneous pectic enzyme preparations.

In our study, the protein concentration of purified PG solutions could not be determined using the method of Bradford (1976). Likewise, PG activity was never associated either with protein bands that could be revealed by applying a sensitive staining after electrophoresis, e.g. the urea-silver stain (Chauduri & Green, 1987) or the glycoprotein periodic acid-silver stain (Dubray & Bezard, 1982), or with absorbance peaks obtained during chromatographic separations. This implies that the enzyme activity measured was below the level of protein detectability of commonly used methods. No other protein bands were detected in the purified PG2 preparations. Nevertheless, sometimes such a PG2 fraction appeared to be contaminated by PG3. Therefore, we always used activity staining in pectin-polyacrylamide gels as the main parameter for judging the purity of a PG2 preparation. A PG isoenzyme preparation was considered to be pure if it was completely free of any other pectic isoenzyme.

The complete loss of PG1 activity before the chromatofocusing step was unexpected. The activity of a purified tomato PG has been reported to be lost in a buffer at pH 11 (Pressey, 1984). During the desalting of PG1 by means of gel filtration, the column was equilibrated with triethylamine buffer at pH 11. It is possible that this condition was too harsh also for PG1, but not for the other PGs, or that the presence of salts was essential for its activity.

Pure PG2 could be obtained after extensive purification of *B. cinerea* culture filtrates. This enzyme is constitutively produced, is present in ungerminated conidia and is the first pectic enzyme secreted by the fungus (Leone & Van den Heuvel, 1987). Therefore, we studied some of its biochemical and catalytic properties in order to understand better its mode of action. PG2 has a high affinity for sodium polygalacturonate with a K_m of 0.46 mg substrate ml^{-1}. The K_m value found is similar to those known for the PGs of other microorganisms (Rombouts & Pilnik, 1980), but is about three times smaller than that of a *B. cinerea* PG reported by Urbanek & Zalewska-Sobczak (1975). With the 2-cyanoacetamide method, incubation for 3 h at 30°C was necessary to detect the activity of a tomato PG (Gross, 1982), whereas in our case incubation for 10 min at 30°C was sufficient to detect activity of PG2. Thus, PG2 shows the characteristics of a very active enzyme. This property could be the reason for the low but highly regulated PG production by the fungus. Metabolically, it is unnecessary for the fungus to synthesize large amounts of an active enzyme.

Although the hydrolysis of sodium polygalacturonate appeared to be most efficient, PG2 was also able to degrade pectin under the same conditions, with a provisionally estimated K_m of 1.18 mg substrate ml^{-1}. Therefore, PG2 is able to degrade the rhamnogalacturonan portion of the plant cell wall. Our finding is in contrast to the claim of Schejter & Marcus (1988) that PG of *B. cinerea* do not act on pectin. The origin of the disturbances observed during the determination of PG activity by the 2-cyanoacetamide method in the course of pectin hydrolysis, could not be ascertained. However, since a number of different cell wall-related sugars are known to react with 2-cyanoacetamide (Honda *et al.*, 1982), the disturbances in the course of pectin degradation by PG2 could have been caused by a not uniform release of reducing carbohydrates other than D-galacturonic acid from the pectin used.

PG2 catalysed the hydrolysis of sodium polygalacturonate optimally at pH 4.5 and of pectin at pH 4.0. These values are comparable with the pH optimum ranges found for PGs of other fungi (Lee & West, 1981; Rombouts & Pilnik, 1980). In particular during the hydrolysis of pectin by a mixture of PG1, PG2 and PG3, PG1 and PG3 seemed to be responsible for the substrate degradation above pH 6.0.

Extracellular enzymes such as PGs come across various cations in or around their substrate. The ions are often present in a free form but Ca^{2+} may be also bound to the pectic fractions of the plant cell walls. We found that both the type and the concentration of the salt used had an effect on PG2 activity. The hydrolysis of sodium polygalacturonate was stimulated by up to 200 mM NaCl, whereas higher concentrations were inhibitory. This finding is in agreement with the effect of NaCl on

polymethylgalacturonases of *Alternaria alternata* (Vàzquez *et al.*, 1986). $CaCl_2$ was strongly inhibitory to PG2 activity. This effect was not exerted directly on the enzyme, but seemed rather the result of the formation of insoluble cross-connections between pectic chains, probably through calcium bridges between free carboxyl groups. It should be noted that the acquisition of resistance of cell walls to hydrolysis by cell wall-degrading enzymes, after incorporation of Ca^{2+} ions, has often been hypothesized (Bateman & Beer, 1965; Förster & Rasched, 1985; Vàzquez *et al.*, 1986).

Many PGs of different microorganisms have an endo catalytic mode of action (Ayers *et al.*, 1969; English *et al.*, 1972; Lee & West, 1981; Rombouts & Pilnik, 1980). Also PG2 behaves as an endo-enzyme as found with sodium polygalacturonate as substrate. While PG2 activity could be measured easily both by the cup-plate assay and the 2-cyanoacetamide method, the activities of PG1 and PG3 could be detected by the latter method only (Fig. 1 and 3). The cup-plate assay measures the initial destruction of the colloidal properties of sodium polygalacturonate, caused by the action of the enzyme(s) (Dingle *et al.*, 1953). Our results showed clearly that the activities of PG1 and PG3 are strongly underestimated when using the cup-plate assay. A mixture of PG1, PG2 and PG3 always degraded both sodium polygalacturonate and pectin more than a pure PG2 preparation, in the whole pH range 3-7. This suggests that either one or both PG1 and PG3 are exo-enzymes and/or have a much lower affinity for the polymeric substrate than has PG2. The sequential production of pectic enzymes by *B. cinerea* (Leone & Van den Heuvel, 1987) indicates that their true substrates may be oligogalacturonides released by the action of PG2.

Acknowledgements

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Studies in the physiology of parasitism of *B. cinerea* are the major object of this thesis, following the indication that two polygalacturonases, viz. PG1 and PG2, were associated with the penetration by this fungus of bean leaves (Van den Heuvel & Waterreus, 1985). Knowledge about the types and the role of the enzymes involved in the degradation of the plant cell wall is of fundamental importance for the understanding of plant-pathogen interactions. At the host cell wall level, degradation of the rhamnogalacturonan component is important not only to allow the penetration of the pathogens, but also to elicit defence mechanisms of plants (Amin *et. al.*, 1986). An elucidation of the physiological aspects involved in the penetration process by *B. cinerea* provides a better understanding of the phytopathological processes and, eventually, may stimulate the formulation of control measures which are less harmful for the environment than the traditional chemical control. More than two decades ago, Bateman & Millar (1966) stated that the area of regulation of pectic enzyme synthesis and activity of plant pathogens was in need of critical investigation. Up till now, however, this field of research has been largely neglected for *B. cinerea*. The research performed and reported in this thesis has been devoted primarily to the study of these fundamental aspects of the parasitism of *B. cinerea*.

Significance of polygalacturonase production by Botrytis cinerea

Several studies have shown that *B. cinerea* produces a number of pectic isoenzymes belonging to the PG group (Cruickshank & Wade, 1980; Di Lenna & Fielding, 1983; Drawert & Kreft, 1978; Magro *et al.*, 1980). In all these investigations, *B. cinerea* was grown on media containing only a pectinaceous substrate as carbon source. In the studies presented here, when the fungus was grown in a basal salt medium containing a cell wall-related polysaccharide (isolated bean cell walls, citrus pectin or sodium polygalacturonate) as the only carbon source, growth and PG production appeared to be related (Chapter 2). Type and concentration of the substrate influenced growth and total PG activity similarly before the start of mycelial autolysis. Pectic isoenzymes were found to be produced in a consistent sequence. The *B. cinerea* isolate BC1, mostly used in this study, firstly produces the constitutive enzyme PG2 and secondly, if produced anyhow, the inducible PG1 (Chapter 2). PG2 breaks down sodium polygalacturonate with an endo-mode of action (Chapter 6). Subsequently, also other pectic enzymes are, in general, synthesized by the fungus. The numbering of the pectic isoenzymes used in this study is the same as that given by Van den Heuvel & Waterreus (1985); this numbering is based on the location of bands of PG activity in gels after electrophoresis, and not on the sequence of production of PG isoenzymes by *B. cinerea*.

Botrytis cinerea is known to be able to grow on cell wall-related polysaccharide fractions and/or monosaccharides as the only carbon source (Gross & Moline, 1986; Rattigan & Ayres, 1975). However, this appears to be the first study that demonstrates that the ability to digest pectic polymers is directly associated with the production of pectic enzymes by this fungus. The regulation of PG1 and PG2 has been found to be mediated both by the carbon source, most likely through enzyme inhibition by pectic oligomers and/or feedback repression by D-galacturonic acid (Chapter 2), and by the adenylate pool, most likely through the metabolic energy status of the fungal cell (Chapter 3). This indicates that the significance of PG production by *B. cinerea* lies in the provision of nutrients from rhamnogalacturonan-related polymers of the plant cell walls.

Another characteristic of the regulation of PG production by *B. cinerea*, fitting in the view of PG involvement in digestive processes, is that PG2, PG3 and PG4, after their synthesis, are hardly affected by the presence of simple carbohydrates, such as D-glucose (Chapter 2). This implies that catabolite repression is not involved in the regulation of some pectic enzymes of *B. cinerea*. This characteristic is relevant as far as fungal colonization of host tissues is concerned. The production of certain pectic

isoenzymes will be barely affected by the increasing release of free sugars (other than D-galacturonic acid!) caused by the coordinated action of the whole array of cell wall-degrading enzymes of *B. cinerea*. Also PG1 does not undergo catabolite repression (Chapter 3). Its synthesis could never be derepressed by addition of cAMP to a medium containing D-glucose as the only C source, as should be expected when an inducible enzyme is governed by catabolite repression (Cooper, 1983; Pall, 1981). However, PG1 production is highly controlled not only by a pectic substrate but also by the energy charge or by the proportion of AMP in the adenylate pool (Chapter 3). Thus, PG1 induction is dependent on the combined presence of the pectic inducer and of a low metabolic energy status of the fungal cells. Such a situation is likely to occur when the infection process starts from germinating conidia. After its induction, however, as long as the energy charge will increase as a result of nutrient acquisition, PG1 synthesis will be repressed. The occurrence of this event can be pictured as a signal that tissue disintegration is already providing an adequate energy source for fungal growth. Hence, further PG1 production is, at that moment, unnecessary. Some factors and their interaction involved in the regulation of the synthesis and activity of PG1 and PG2 are schematically illustrated in Fig. 1.

All *B. cinerea* isolates used in this study were strongly dependent on inorganic phosphate for PG production (Chapters 3, 4 and 5). Although phosphate importance for PG biosynthesis has been established (Chapter 3), the results presented in this thesis represent only a theoretical basis to be further developed. The significance of a phosphate source for the fungus in order to become infectious is still not clear. This phenomenon can be manipulated and used in inoculation procedures which can be applied in screening and breeding programs (Chapter 5). However, a practical application without further investigating the importance of phosphate for the physiology of parasitism of *B. cinerea*, eventually would be shortsighted. *Botrytis cinerea* is primarily a saprophytic organism (Blakeman, 1980). Inorganic phosphate may mimic somehow the effect of dead plant material, which is the primary substrate of a saprophyte, and may trigger the metabolic activities of the fungus necessary for tissue degradation and transformation. The involvement of PG production in the digestion of cell wall-related pectic polymers, fits in the characteristic of a saprophyte whose ecological task originally could have been that of recycling dead plant material. In this context, the pathogenicity of *B. cinerea* could be the consequence of an ecological disturbance provoked by human activities, e.g. by modern agriculture. Practices in modern agriculture may stimulate the fungus through the provision of plenty of plant substrates in the form of dead, weakened or succulent material.

Role of PGs produced by Botrytis cinerea in pathogenesis

The above described involvement of pectic enzymes produced by *B. cinerea* in digestive processes, might seem to be in contradiction to a possible role of PGs in the penetration of healthy host tissues by the fungus. The hypothesis of an involvement of PGs of *B. cinerea* in direct penetration has been mainly supported by: 1) electron microscopical observations of the swelling of primary cell walls in *B. cinerea*-host interactions (e.g. Mansfield & Richardson, 1981; Mc Keen, 1974; Rijkenberg *et al.*, 1980) and; 2) the electrophoretic identification of PG1 and PG2 in inoculation drops and their association with the penetration of French bean leaves by the isolate BC1 (Van den Heuvel & Waterreus, 1985). However, whether direct penetration of germ tubes of *B. cinerea* through healthy tissues involves a mechanical or a chemical process has been questioned from the end of the last century (Elad, 1989; Verhoeff, 1980).

If direct penetration is supported by enzymatic processes, then the enzymes produced must be secreted by the fungus in order to act in the environment surrounding the penetrating germ tube or hypha. In order to understand which pectic enzyme is secreted by *B. cinerea*, the following experiment was performed. Using an experimental design as described for the study of the effect of D-glucose on pectic enzyme production (Chapter 2), the type of enzymes, present 7 days after inoculation in the culture liquid before and after disruption of the mycelium by ultrasonication (applying a 50 W power for 1 min), was determined by means of electrophoresis. In the first case, only PG2, PE1 and PE2 (Fig. 1, Chapter 2) were detected. In the second one, in addition to PG2, PE1 and PE2 also traces of PG1, PG3, PG4 and PG5 were detected. This finding gives rise

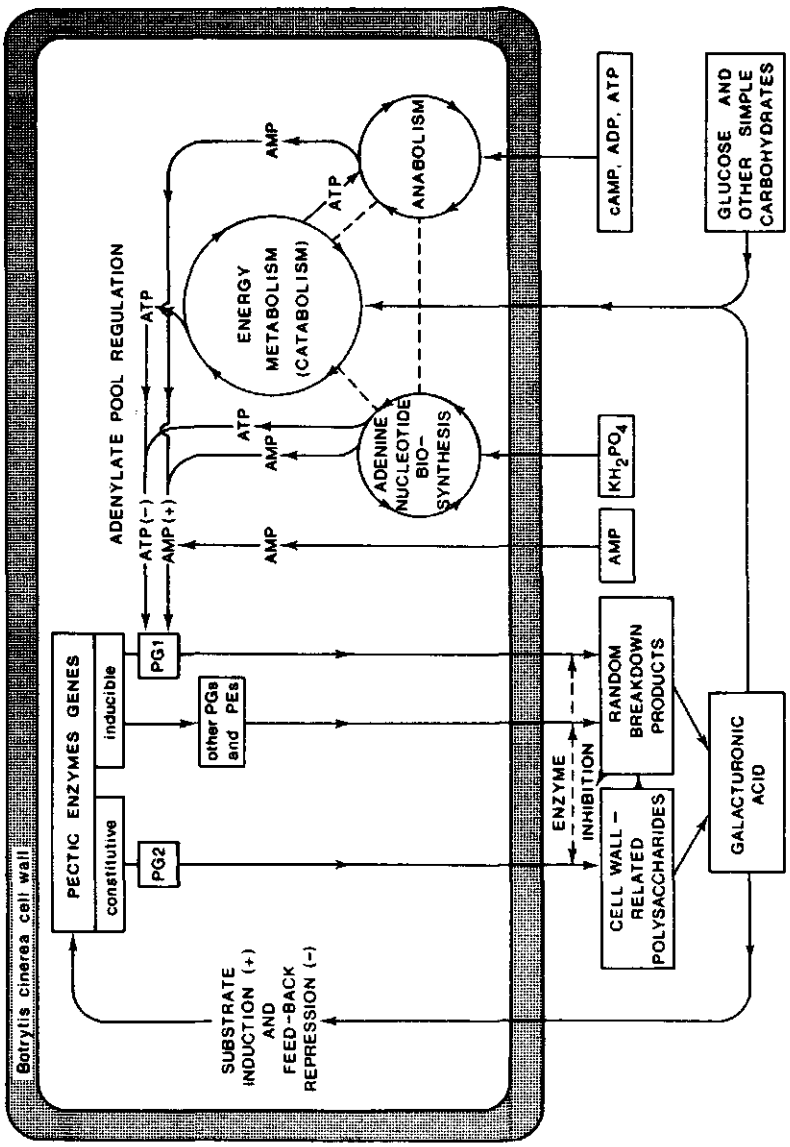

FIGURE 1. Schematic representation of the regulation by carbohydrates and adenine nucleotides of the synthesis and activity of PG1 and PG2 of isolate BC1 of *Botrytis cinerea*.

to at least two considerations. The first one is that PG2, which is constitutively produced and already present in ungerminated conidia (Chapter 2), could be the only PG used by BC1 during the direct penetration of the primary cell wall of the host. However, the velocity of the penetration process plays a decisive role as to whether other pectic enzymes than PG2, produced by *B. cinerea*, are involved at this stage of the infection. The secretion of PEs would make the degradative action of PG2 even more effective, as the affinity of PG2 for a demethylated pectic substrate is about three times higher than for pectin (Chapter 6). A quick softening of the primary cell wall caused by the endo-mode of action of PG2 (Chapter 6), will help the mechanical pressure of the growing hyphal tip in succeeding in the penetration process, if the plant cell has no possibility to check it. However, any enzymatic action during penetration must be very localized, as scanning electron microscopic observations of bean tissues, bearing spores with germ tubes attempting to penetrate, show no evident morphological changes on the tissue around the penetrating fungal structure (Garcia-Arenal & Sagasta, 1980; Leone, unpublished).

A second consideration, derived from the experiment described above, is related to the detection of PGs *in vitro* and *in vivo*. Methods for the isolation of PGs from culture liquids or infected tissues, including also fungal structures, could cause mycelial disruption. Thus, activities subsequently detected, might actually be the result of a mixture of intracellular and extracellular fungal enzymes. In that case, incorrect conclusions could be drawn about the role of an enzyme in the infection process. Based on the results reported here, only a few of the many pectic enzymes produced by *B. cinerea* under different conditions may have an extracellular function. One of these extracellular enzymes is PG2, which, on the basis of its regulation and biochemical properties, may display a double role both in direct penetration of the host tissues (Van den Heuvel & Waterreus, 1985), and in the induction of the chain production of other isoenzymes for catabolic activities (Chapter 2). The latter function could be mediated by the release of monomers and oligomers from the rhamnogalacturonan component of the cell wall, caused by its mode of action (Chapter 6). As to the role of PG1 and other PGs, more investigations are needed in order to establish their exact role in the pathogenesis of *B. cinerea*. The regulation of the inducible PG1 (Chapter 3) and its apparent lower affinity for sodium polygalacturonate compared with PG2 (Chapter 6), indicate so far that PG1 has another role than PG2 in the breakdown of rhamnogalacturonan-related polymers.

In Chapter 4 it was concluded that the differences in PG production of various *B. cinerea* isolates were not related to the ability of the isolates to infect the host challenged. This implies that, in the presence of nutrients, the level of the PG production and its isoenzymatic composition are not related to the level of pathogenicity. Therefore, whatever the specific role played by any isoenzyme, PG production must be regarded as only one of a complex of factors contributing to the expression of pathogenicity of *B. cinerea*. An enumeration of factors participating in the pathogenesis of *B. cinerea* may include cutinolytic enzymes, other cell wall-degrading enzymes, proteases, toxins, oxygen radicals and/or other fungal metabolites (Edlich *et al.*, 1989; Mohavedi & Heale, 1990; Salinas *et al.*, 1986). A complete understanding of the role of PGs can be obtained only by taking into account the contribution of the individual factors involved in pathogenesis, and of their interaction, by studying extensively the plant-fungus interaction in only one host-*B. cinerea* model system.

Source of variability of polygalacturonase production and its significance

Pectic enzymes produced by *B. cinerea* can be separated in a number of electrophoretic variants of the enzymes. These multiple forms are often referred to as isoenzymes (or isozymes) (e.g. Cooper, 1983). Such a classification has been used throughout this thesis. The phenomenon of multiplicity of PGs is also common in other microorganisms producing pectic enzymes. However, the relation between these isoenzymes within a microorganism is not clearly understood (Panopoulos *et al.*, 1984). One of the major puzzles is that certain isoenzymatic forms are often produced *in vitro* but not *in vivo* or *vice versa* (Panopoulos *et al.*, 1984). This shows that knowledge of the biochemical or physiological factors conditioning the enzyme production *in vitro* and/or *in vivo* is still missing. Variability in PG isoenzyme patterns has been reported to occur within a given

isolate and between different isolates of *B. cinerea* (Magro *et al.*, 1980). An explanation of this phenomenon has tentatively been proposed by Magro *et al.* (1980), viz. enzyme multiplicity provides *B. cinerea* with a greater ability for infecting a wide host range. However, the investigations presented here show a low level of variability in the PG pattern between eleven *B. cinerea* isolates, differing from each other as to their origin (host plant) and time and place of isolation (Chapter 4).

Two sources of variability can be distinguished: variability within a given isolate and variability between isolates. The former one finds its origin in the nature of PG production, being inherent to the catabolic activities of the fungus (Chapter 2). As it has been reported for higher organisms during their development (Scandalios, 1969), this variability may be due to a shift in isoenzyme profiles following a differential activation of the genes responsible for their synthesis during substrate degradation. When dealing with dynamic processes, such as those linked to physiological functions, time of sampling and cultural conditions affect isoenzyme patterns detectable *in vitro* and/or *in vivo* (Chapters 2 and 4). Hence, the PG variability occurring within a given isolate can be primarily affected by even small variations in the experimental conditions.

As to the variability between different isolates, it has been shown that, when using cultural conditions known to prevent variability within an isolate, a low level of intraspecific variation is found (Chapter 4). Therefore, it seems justified to suppose that in previous studies at least part of this intraspecific variability could have been based on artificial differences due to the metabolic control of the enzymes. The primary cause of the remaining variation, then, may be ascribed to genetical instability for specific characters, such as AMP involvement in PG1-like isoenzyme synthesis (Chapter 4), to mutations and/or to modifications of proteins at translational or post-translational level (Verkleij, 1980).

When conidia of *B. cinerea* were used as inoculum suspended in a solution containing nutrients, no host specificity and no isoenzymatic host adaptation was shown by the different isolates during the infection process. These results, coupled to a lack of association between the rate of PG production and the level of pathogenicity, indicate that multiple PG isoenzymatic forms are not the primary cause of the infection process. Therefore, once more, the view of the connection of PG production with fungal digestive activities is supported.

Aspects of methodologies used in studying PGs

When dealing with investigations on enzymes, one of the most critical points, in order to obtain correct information and to interpret results, are the methods used. This paragraph has no provocative purpose: rather, its aim is to show the possible origin of disagreement between studies on PGs produced by *B. cinerea*. During this study, I encountered three major points of controversy between the results of my experiments and other work published on *B. cinerea*. 1) The presence of multiple forms of PGs produced by the isolate chosen for this study was in agreement with some studies dealing with PG patterns (Cruickshank & Wade, 1980; Di Lenna & Fielding, 1983; Magro *et al.*, 1980; Van den Heuvel & Waterreus, 1985), but it was not reported in other studies on purification and characterization of pectic enzymes of the fungus (Marcus & Schejter, 1983; Schejter & Marcus, 1988; Urbanek & Zalewska-Sobczak, 1975). 2) The isolate used in this study produced PGs easily detectable by enzyme assays but the concentration of the enzymes, once isolated, were far below the level of detectability of proteins, as compared with the literature (Marcus & Schejter, 1983; Schejter & Marcus, 1988; Urbanek & Zalewska-Sobczak, 1975). 3) The results obtained by detecting the enzyme activity with different assays during PG purification were not always in agreement with each other.

As to the first point, the lack of observing multiplicity of *B. cinerea* PGs in the studies cited (Marcus & Schejter, 1983; Schejter & Marcus, 1988; Urbanek & Zalewska-Sobczak, 1975), may be based mainly on three possibilities, either separate or in combination. 1) The authors were not aware of the phenomenon of multiplicity of PGs of *B. cinerea* during their study. 2) The methods used to detect enzyme activity were not sensitive enough to show all possible different isoenzymes (see also comment to the third point). 3) The isolates used in those studies produced only the two PG forms which were purified by the authors.

As to the second point, the following may help to explain this. 1) Isolate BC1 generally produces small amounts of extracellular proteins, as measured in culture filtrates (Chapter 6), and PGs represent only a small percentage of the total proteins produced. 2) The cultural conditions used in order to obtain a good PG production, could have adversely affected the total protein production by the fungus. In this study, fungal growth was supported batch-wise, whereas Marcus & Schejter (1983) and Schejter & Marcus (1988), who reported a much higher protein concentration in their culture filtrates, used a fermentor. 3) Methods to determine protein concentrations could have influenced final measurements. In the course of my investigations, the method of Bradford (1976) was applied, whereas in the investigations cited, the method of Lowry *et al.* (1951) was used. The former method does not interfere with, among others, carbohydrates, while the latter one does (Bradford, 1976). Hence, when measuring protein concentrations in an environment where also high carbohydrate concentrations are supposed to be present, such as in fungal culture filtrates, the method of Lowry might give unrealistic results.

As to the third point, fractions eluting from chromatography colums were tested for PG activity using three different assays (Chapter 6): cup-plate assay (Dingle *et al.*, 1953), 2-cyanoacetamide assay (Gross, 1982) and electrophoretic zymograms (Cruickshank & Wade, 1980). Only the last method allows the separation and identification of pectic isoenzymes, without giving quantitative information. Therefore, a combination of a quantitative assay for activity (cup-plate assay and/or 2-cyanoacetamide assay) and electrophoretic zymograms is necessary, in order to obtain a complete picture of the purification procedure. This has to be done, as it appeared that single peaks of activity eluted from the colums, and measured by a quantitative assay, were still composed of different isoenzymes when tested by the zymogram technique (Chapter 6). Furthermore, even if working at levels below the detectability of sensitive protein-staining methods, the zymogram technique could reveal whether a single purified band of PG activity was free from other forms of the enzyme (Chapter 6). This finding implies that in previous works, where no zymogram technique was applied and the concept of PG purity after extensive purification was based only on the presence of a single protein band after electrophoresis (Marcus & Schejter, 1983; Schejter & Marcus, 1988; Urbanek & Zalewska-Sobczak, 1975), it is possible that this single protein band could hide other pectic isoenzymes below the level of protein detectability. The study of the biochemical properties of such an enzyme could then lead to uncorrect conclusions.

As a concluding remark, it can be postulated that a scientist, through the method applied, affects the study of natural phenomena and, hence, can draw conclusions apparently in contrast with those of another scientist. One of the major pronouncements from Einstein's special relativity theory (Capra, 1983) is that different observers will order events differently in time, if they move with different velocities relative to the observed events. All this should make scientists less frightened of so called "unexpected results" and they should be aware that moderation and relativism are necessary when they judge the work of their colleagues.

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SUMMARY

The fungus *Botrytis cinerea* Pers.: Fr. is a saprophyte universally present on weakened or dead plant material. However, *B. cinerea* can cause also serious problems in agriculture not only by attacking over 200 different hosts, but also because its control is onerous in terms of economic costs and of the adverse impact on the environment of the fungicides applied. The most common symptom induced by *B. cinerea* is the decay of the plant tissues infected. Substances secreted by the fungus, such as the cell wall-degrading pectic enzymes, polygalacturonases (PGs), support this action. Furthermore, PGs are supposed to play a major role in the penetration and the colonization of the plant tissues. The awareness of the importance of PGs for the physiology of parasitism of *B. cinerea*, led to the study of some fundamental aspects of the significance of PG production by this fungus in pathogenesis.

By studying the effects of different cell wall-related polysaccharides and of two monosaccharides on total PG production, isoenzymatic composition and fungal growth *in vitro*, basic information on the regulation of PG production by *B. cinerea* has been gained. Type and concentration of the carbohydrates and the conidial concentration in the inoculum affect fungal growth and total PG activity in a similar way, before the beginning of mycelial autolysis. Pectic enzymes are produced in a consistent sequence always starting with PG2. This enzyme is already present in ungerminated conidia. Its synthesis is the expression of a constitutive gene as it is independent of the presence of the substrate and strictly associated with fungal growth. D-galacturonic acid at 2 mM induces the production of some of the pectic enzymes. At 10 mM and above, however, it represses PG2 and the subsequent production of the whole pectic isoenzyme complex. As D-galacturonic acid is the end-product of the action of PGs on the cell wall, this implies the involvement of a feedback repression. The results indicate that the pectic isoenzymes produced by *B. cinerea* constitute a coordinated catabolic pathway for the complete degradation of pectic polysaccharides. Since *B. cinerea* is able to grow on cell wall-related polysaccharides as the only carbon source, it is assumed that pectic enzyme production supports their digestion.

Inorganic phosphate (Pi) and purine nucleotide derivatives are known to stimulate the ability of germ tubes of conidia of *B. cinerea* to invade healthy leaf tissue. In a previous study, this stimulation was associated with a rapid production of pectic enzymes, in particular PG1 and PG2, a few hours after the inoculation of bean leaves. However, how Pi could affect PG production by *B. cinerea* was not known. To assess whether and how Pi and/or adenine nucleotides regulate the biosynthesis of the PGs of isolate BC1, experiments were carried out with cultures without any cell wall-related substrate. In the absence of P-containing compounds, PG production is negligible. Cyclic AMP, ADP and ATP stimulate the constitutive production of PG2 as well as fungal growth, whereas AMP stimulated the inducible production of PG1, but not fungal growth. The effects of Pi on PG1 and PG2 and on fungal growth are intermediate between those of AMP and of the other nucleotides. The Pi- or adenine nucleotide-dependent PG production involves *de novo* enzyme synthesis, as it can be inhibited by the protein synthesis inhibitors cycloheximide and, to a lesser extent, actinomycin D. When ATP production in the fungal oxidative phosphorylation is inhibited by the uncoupler salicylanilide, PG1 is detected in the culture liquid. Experiments designed to affect the intracellular concentrations of adenine nucleotides also provide evidence that the Pi-dependent PG1 and PG2 biosynthesis by BC1 is mediated by adenine nucleotides. It is proposed that, besides being regulated by a pectic substrate, PG1 synthesis is also controlled, at a different level, by the metabolic status of the fungus through the adenylate pool.

Botrytis cinerea is known for its phenotypic variability between isolates, supposed to be based on heterocaryosis. Intraspecific variability has been reported to occur also for a physiological character such as PG isoenzymatic composition. However, because of the complex metabolic regulation undergone by these enzymes, it seems justified to question whether the differences in isoenzyme profiles found so far between isolates, are the result of artificial differences induced by cultural conditions. Therefore, the cultural conditions used to study the specific regulation by Pi of PG production by isolate BC1, which specifically stimulate the synthesis of only PG1 and PG2, were applied to

compare the PG production and the isoenzymatic composition of eleven *B. cinerea* isolates. Furthermore, the specificity of the isolates and the isoenzymatic adaptation to the host-plant as well as the relation between PG production and the ability of the various isolates to cause spreading lesions have been investigated both on bean and on tomato leaves. PG production of all isolates *in vitro* is strongly dependent on Pi. All isolates produce cathodic forms of PGs, which comprise at least a PG1- or a PG2-like isoenzyme. This indicates a low level of intraspecific variability for the PG isoenzymatic patterns of the different *B. cinerea* isolates. When conidia are suspended in a solution containing nutrients, no host-specificity and no isoenzymatic host-adaptation can be found between the isolates during the plant-fungus interactions. Differences in total PG production did not affect the ability to cause spreading lesions or to infect the two host plants used.

The knowledge obtained on the involvement of Pi in PG production and in the infection process of *B. cinerea*, has been applied to develop a simple inoculation procedure to screen the resistance of bean cultivars to this fungus and to the related weak pathogen *Sclerotinia sclerotiorum*. Screening for resistance of bean leaves to these fungi is known to be difficult, as variable results are often obtained because the pathogenicity of both fungi need to be stimulated by nutrients. In the procedure developed, when bean leaves are inoculated with spores of *B. cinerea* or *S. sclerotiorum* suspended in a solution of Pi, without any carbon-nitrogen source, the level of infection of both fungi is enhanced compared to inoculations with spores suspended in water. Since the inoculation with Pi is rapid and does not require blooming plants, the method can be easily adopted in screening and breeding programs.

On account of the possible importance of PG2 in the infection process by *B. cinerea*, the major biochemical and molecular properties of this enzyme were studied. In this study the purification steps and the homogeneity of the purified PG2 preparation were constantly checked by means of electrophoretic zymograms, as the specific PG concentration was too low to be measured by a protein assay. The cause of the difficulties found to separate superimposed pectic enzymes activities during the purification procedure, was investigated by studying the electrophoretic mobilities of PG2, PG1 and of another PG isoenzyme. PG2 breaks down sodium polygalacturonate more quickly than pectin. Under standard assay conditions, the K_m value for the hydrolysis of sodium polygalacturonate is 0.46 mg substrate ml^{-1}; for pectin, the K_m is estimated to be 1.18 mg substrate ml^{-1}. The pH optimum for the degradation of polygalacturonate by PG2 is 4.5 and of pectin 4.0. PG2 activity is also influenced by the presence of NaCl or $CaCl_2$ in the reaction mixture. Analysis of the breakdown products by paper chromatography and a comparison of the reaction rate by viscosimetry and reducing groups assay, indicates that PG2 has an endo catalytic mode of action. The isoelectric point and the molecular mass of PG2 are estimated to be 9.1 and 23.0 kDa, respectively.

From a general evaluation of the results it is proposed that the primary significance of the sequential, coordinated PG production by *B. cinerea* is inherent to the digestion of the pectic portion of the plant cell wall. This fits in the characteristics of a saprophyte ecologically adapted to live on dying or dead plant material. However, both the physiological and biochemical properties of PG2 convince also of its direct involvement in a quick softening of the primary cell wall, in order to facilitate fungal penetration.

SAMENVATTING

De saprofytische schimmel *Botrytis cinerea* Pers.: Fr. komt universeel voor op verzwakt of dood plantaardig materiaal. *B. cinerea* kan echter ernstige problemen in de landbouw veroorzaken, niet alleen omdat deze schimmel meer dan 200 verschillende gewassen kan infecteren, maar ook vanwege de kostbare bestrijding ervan en het ongunstige effect van de gebruikte fungiciden op het milieu. Het meest voorkomende symptoom van *B. cinerea* is het verrotten van de geinfecteerde planteweefsels. Door de schimmel geproduceerde stoffen zoals de celwandsplitsende pectische enzymen, polygalacturonasen (PG's), ondersteunen dit proces. Bovendien worden PG's geacht een belangrijke rol te spelen bij het proces van penetratie en kolonisatie van planteweefsels. Daarom werd onderzoek uitgevoerd naar de betekenis van de PG-produktie door deze schimmel tijdens de pathogenese.

Fundamentele informatie over de regulatie van de PG-produktie werd verkregen door het bestuderen *in vitro* van de effekten van verschillende aan de celwand gerelateerde polysacchariden en van twee monosacchariden op de totale produktie van PG, op de isoenzym samenstelling en op de schimmelgroei. Afhankelijk van de concentratie van de verschillende koolhydraten en van de conidiën concentratie van het inoculum werd de totale PG-activiteit tot aan het moment van autolyse op dezelfde wijze beinvloed als de schimmelgroei. Pectische isoenzymen worden in een vaste volgorde geproduceerd, waarbij PG2 altijd als eerste verschijnt. Dit enzym is reeds aanwezig in ongekiemde conidiën. PG2 is een constitutief enzym omdat de produktie ervan onafhankelijk is van de aanwezigheid van het substraat en strikt verbonden is met de schimmelgroei. D-galacturonzuur induceert in een concentratie van 2 m*M* de produktie van enkele pectische enzymen. Bij 10 m*M* en hogere concentraties remt het echter de vorming van PG2 en de daaropvolgende produktie van het hele pectische isoenzym complex. Aangezien D-galacturonzuur het eindprodukt is van de PG-werking op de celwand, duidt dit op een "feedback" repressie. De resultaten wijzen uit dat de door *B. cinerea* geproduceerde pectische isoenzymen een gecoördineerde katabolische weg vormen voor de volledige afbraak van pectische polysacchariden. Omdat *B. cinerea* in staat is te groeien in cultures met als enige koolstofbron polysacchariden die verwant zijn aan de celwand, wordt geconcludeerd dat de produktie van pectische enzymen de vertering van deze polysacchariden mogelijk maakt.

Het is bekend dat anorganisch fosfaat (Pi) en derivaten van purine nucleotiden de binnendringing van kiembuizen van *B. cinerea* conidiën in gezond planteweefsel stimuleren. In voorafgaand onderzoek werd verband gelegd tussen deze stimulatie en de snelle produktie van pectische enzymen, vooral van PG1 en PG2, binnen enkele uren na inoculatie van bonebladeren. Het was echter niet bekend hoe Pi de PG-produktie door *B. cinerea* kon beinvloeden. Teneinde vast te stellen of en op welke wijze Pi en/of adenine nucleotiden de biosynthese van PG's reguleren, zijn experimenten uitgevoerd met cultures van isolaat BC1 welke geen aan de celwand verwant substraat bevatten. De PG-produktie is te verwaarlozen in de afwezigheid van P-bevattende componenten. Cyclisch AMP, ADP en ATP stimuleren zowel de constitutieve produktie van PG2 als de groei van de schimmel terwijl AMP de induceerbare produktie van PG1 stimuleert maar niet de groei. De effecten van Pi op PG1, PG2 en op de schimmelgroei liggen tussen die van AMP en de andere nucleotiden in. De Pi- of adenine nucleotide-afhankelijke PG-produktie impliceert een *de novo* enzymsynthese, aangezien deze produktie kan worden geremd door de eiwitsynthese remmers cycloheximide en actinomycine D. Wanneer de ATP produktie in de oxydatieve fosforylatie van de schimmel wordt geremd door de "uncoupler" salicylanilide, kan PG1 in het cultuurfiltraat worden gemeten. Experimenten waarin de intracellulaire concentratie van adenine nucleotiden wordt beinvloed, tonen aan dat adenine nucleotiden betrokken zijn bij de Pi-afhankelijke biosynthese van PG1 en PG2 door het isolaat BC1. De synthese van PG1 wordt dus niet alleen gereguleerd door een pectineachtig substraat maar ook door de metabolische toestand van de schimmel welke bepaald wordt door de verhouding van de verschillende adenine nucleotiden.

Botrytis cinerea is bekend om de fenotypische variabiliteit tussen isolaten, die vermoedelijk berust op heterocaryose. Intraspecifieke variabiliteit werd ook beschreven

voor een fysiologische eigenschap zoals de samenstelling van PG isoenzymen. Vanwege de complexe metabolische regulatie van deze enzymen is de vraag gerechtvaardigd of deze verschillen in isoenzymprofielen tussen isolaten niet het resultaat zijn van kunstmatige verschillen ten gevolge van de gebruikte groeiomstandigheden. Daarom werden de groeicondities, die gebruikt zijn om de specifieke regulering van de PG-produktie door Pi van isolaat BC1 te bestuderen, toegepast om de PG-produktie en de samenstelling van isoenzymen van elf *B. cinerea* isolaten met elkaar te vergelijken. Tevens werden de specificiteit van de isolaten en de adaptatie van isoenzymen aan de waardplant alsmede de relatie tussen PG-produktie en het vermogen van de verschillende isolaten om spreidende lesies te veroorzaken onderzocht op bone- en tomatebladeren. In alle isolaten is de PG-produktie *in vitro* sterk afhankelijk van Pi. Alle isolaten produceren kathodische vormen van PG's met minimaal een PG1- of een PG2-achtig isoenzym. Dit duidt op weinig intraspecifieke variabiliteit van de PG isoenzympatronen tussen de verschillende *B. cinerea* isolaten. Verschillen in specificiteit tussen de verschillende isolaten worden niet gevonden wanneer de twee plantesoorten worden geïnoculeerd met conidiën gesuspendeerd in een voedingsoplossing. Ook produceert elk isolaat op beide plantesoorten dezelfde isoenzymen. De gevonden verschillen in de totale PG-produktie hebben geen invloed op het vermogen om spreidende lesies te veroorzaken of om de twee gebruikte waardplanten te infecteren.

De verkregen kennis over de betrokkenheid van Pi bij de produktie van PG's en bij het infectieproces van *B. cinerea* werd gebruikt om een simpele inoculatie methode te ontwikkelen ten einde de resistentie van bonecultivars tegen deze schimmel en tegen het verwante zwakte pathogeen *Sclerotinia sclerotiorum* te toetsen. Toetsing op resistentie tegen deze schimmels levert dikwijls variabele resultaten op omdat de pathogeniteit van beide schimmels gestimuleerd moet worden door nutrienten. Vaak worden hiervoor bloemblaadjes gebruikt. Inoculatie van bonebladeren met sporen van *B. cinerea* of *S. sclerotiorum* die gesuspendeerd zijn in een oplossing met Pi zonder enige koolstof of stikstofbron, verhoogt het niveau van infektie van beide schimmels vergeleken met inoculaties met sporen die alleen in water zijn gesuspendeerd. Omdat de inoculatie met Pi snel en reproduceerbaar is en geen bloeiende planten vereist, kan de methode gemakkelijk worden toegepast in toetsings- en veredelingsprogramma's.

Vanwege het mogelijke belang van PG2 in het infectieproces van *Botrytis cinerea* werden de belangrijkste biochemische en moleculaire eigenschappen van dit enzym nader onderzocht. Hierbij zijn de zuiveringsstappen en de homogeniteit van het gezuiverde PG2-preparaat gekontroleerd door middel van electroforetische zymogrammen, omdat de specifieke PG-concentratie te laag was om te worden gemeten met een eiwitbepaling. Tijdens de zuiveringsprocedure konden sommige PG's moeilijk van elkaar gescheiden worden. De reden ervan werden onderzocht met behulp van de titratie curven van PG1, PG2 en van een ander isoenzym. PG2 breekt natrium polygalacturonaat sneller af dan pectine. Onder standaard condities is de K_m waarde voor de afbraak van natrium polygalacturonaat 0,46 mg substraat ml^{-1}; de K_m waarde van de afbraak van pectine is ongeveer 1,18 mg substraat ml^{-1}. Het pH optimum voor de afbraak van natrium polygalacturonaat door PG2 ligt bij 4,5 en voor die van pectine bij 4,0. De aktiviteit van PG2 wordt ook beinvloed door de concentratie van NaCl of $CaCl_2$ in het reaktiemengsel. Analyse van de afbraakprodukten met behulp van papierchromatografie en vergelijking van de reaktiesnelheid door viscosimetrie en door bepaling van de reducerende groepen tonen aan dat PG2 een endo-enzym is. Het isoelectrisch punt van PG2 ligt bij 9,1 en zijn molecuulmassa is 23,0 kDa.

Concluderend kan worden gesteld dat de afbraak van het pectische deel van de celwand van de plant door verschillende PG isoenzymen die na elkaar worden geproduceerd een onderdeel is van de vertering van dit substraat door *B. cinerea*. Dit is in overeenstemming met de karakteristieken van een saprofyt die ecologisch is aangepast om te leven op stervend of dood plantaardig materiaal. De resultaten van de fysiologische en biochemische eigenschappen van PG2 maken bovendien duidelijk dat dit isoenzym direkt betrokken is bij een snelle verzachting van de primaire celwand teneinde de binnendringing van de schimmel te vergemakkelijken.

RIASSUNTO

Il fungo *Botrytis cinerea* Pers.: Fr. è un saprofita che cresce ovunque sia presente tessuto vegetale indebolito o morto. Tuttavia *B. cinerea* può anche causare seri problemi in agricoltura. Infatti, non solo è in grado di attaccare più di 200 tipi di piante ospiti, ma il suo controllo è anche oneroso economicamente e i trattamenti fungicidi hanno conseguenze negative per l'ambiente. Il sintomo più comune indotto da *B. cinerea* è la disgregazione del tessuto vegetale infettato. Sostanze secrete dal fungo, quale gli enzimi pectici poligalatturonasi (PG) che degradano la parete cellulare dell'ospite, sostengono quest'azione. Inoltre si suppone che le PG svolgano un ruolo essenziale durante la penetrazione e la colonizzazione dei tessuti. La consapevolezza dell'importanza delle PG per la fisiologia del parassitismo di *B. cinerea*, ha stimolato lo studio di alcuni aspetti alla base del significato della produzione PGca da parte di tale fungo nella patogenesi.

Si sono ottenute informazioni fondamentali sulla regolazione della produzione PGca di *B. cinerea* studiando *in vitro* gli effetti di diversi polisaccaridi affini alla parete cellulare vegetale e di due monosaccaridi sulla produzione totale, composizione isoenzimatica delle PG e sulla crescita fungina. Il tipo e la concentrazione dei carboidrati, nonchè la concentrazione conidica nell'inoculo influiscono similarmente sulla crescita fungina e sulla produzione PGca prima dell'inizio dell'autolisi miceliare. Gli enzimi pectici sono prodotti in sequenza, sempre iniziata dall'isoenzima PG2. Questo enzima è già presente nei conidi non germinati. La sua sintesi è l'espressione di un gene costitutivo poichè è indipendente dalla presenza del substrato ed è strettamente associata alla crescita fungina. L'acido D-galatturonico a una concentrazione di 2 mM induce la produzione di alcuni enzimi pectici. A una concentrazione superiore a 10 mM, invece, reprime PG2 e la conseguente produzione dell'intero complesso di enzimi pectici. Giacchè l'acido D-galatturonico è il prodotto finale dell'azione delle PG sulla parete cellulare, ciò implica il coinvolgimento di una repressione da retroazione. I risultati indicano che gli isoenzimi pectici prodotti da *B. cinerea* costituiscono una via catabolica coordinata per la completa decomposizione dei polisaccaridi di origine pectica. Poichè *B. cinerea* è in grado di crescere su polisaccaridi affini alla parete cellulare quale unica fonte di carbonio, si assume che la produzione di enzimi pectici sostenga la loro digestione.

Il fosfato inorganico (Pi) e alcuni nucleotidi purinici stimolano la capacità dei conidi di *B. cinerea* di infettare tessuto fogliare sano. In uno studio precedente, questa stimolazione venne associata a una rapida produzione di PG1 e PG2, poche ore dopo l'inoculazione di foglie di fagiolo. Tuttavia, in quale modo il Pi potesse influire sulla produzione PGca di *B. cinerea* era, finora, ignoto. Per determinare se e come il Pi e/o i nucleotidi adenilici regolino la biosintesi delle PG dell'isolato BC1, si sono eseguiti esperimenti con colture prive di substrati affini alla parete cellulare. In assenza di composti contenenti fosforo, la produzione PGca è trascurabile. AMP-ciclico, ADP e ATP stimolano sia la produzione costitutiva di PG2 che la crescita miceliare, mentre AMP stimola la produzione inducibile di PG1 ma non la crescita fungina. Gli effetti del Pi su PG1 e PG2 e sulla crescita fungina sono intermedi tra quelli dell'AMP e degli altri nucleotidi. La produzione delle PG dipendente da Pi o da nucleotidi adenilici implica una sintesi enzimatica *de novo* in quanto essa può essere bloccata dagli inibitori della sintesi proteica, cicloesimide, e, in minor misura, attinomicina D. Quando la produzione di ATP nella fosforilazione ossidativa fungina inibita dall'agente disaccoppiante salicilanilide, PG1 viene rilevata nel liquido di coltura. Esperimenti pianificati per influire sulla concentrazione intracellulare dei nucleotidi adenilici, danno anch'essi prova che la biosintesi di PG1 e PG2 dipendente da Pi nell'isolato BC1 è mediata da nucleotidi adenilici. Si propone che la sintesi di PG1, oltre che regolata da un substrato pectico, sia anche controllata, a un livello differente, dallo stato metabolico del fungo per mezzo del complesso adenilico.

Il fungo è conosciuto per la variabilità fenotipica tra isolati, che si suppone sia dovuta a eterocariosi. Tale variabilità intraspecifica è stata anche descritta per un carattere fisiologico quale la composizione isoenzimatica delle PG. Tuttavia, a causa della complessa regolazione metabolica a cui sono sottoposti questi enzimi, è apparso giustificato chiedersi se le differenze in profili isoenzimatici descritte finora tra diversi isolati non fossero il risultato di differenze artificiali indotte dalle condizioni di coltura.

Perciò le condizioni colturali utilizzate per studiare la specifica regolazione di PG1 e PG2 da parte del Pi per l'isolato BC1, sono state applicate per confrontare la produzione PGca e la composizione isoenzimatica di undici isolati di *B. cinerea*. Inoltre, la specificità degli isolati e l'adattamento isoenzimatico alla pianta ospite, nonchè la relazione tra la produzione PGca e la capacità dei vari isolati di produrre lesioni espanse, sono state esaminate su foglie di fagiolo e di pomodoro. Per tutti gli isolati la produzione PGca *in vitro* è dipendente da Pi. Tutti gli isolati producono forme catodiche delle PG, le quali comprendono almeno un isoenzima PG1- o PG2-simile. Ciò indica un basso livello di variabilità intraspecifica per i profili isoenzimatici delle PG tra i differenti isolati di *B. cinerea*. Quando si prepara una sospensione conidica in una soluzione contenente glucosio e Pi, durante l'interazione fagiolo- o pomodoro-fungo gli isolati non mostrano nè specificità nè adattamento isoenzimatico all'ospite. Differenze nella produzione PGca totale non influiscono sulla capacità di causare lesioni espanse o di infettare le due piante ospiti usate.

La conoscenza ottenuta sul coinvolgimento del Pi nella sintesi PGca e nel processo d'infezione di *B. cinerea*, è stata applicata per sviluppare un metodo d'inoculazione onde saggiare la resistenza di cultivars di fagiolo a questo fungo e all'affine patogeno debole *Sclerotinia sclerotiorum*. E' risaputo che saggiare la resistenza di foglie di fagiolo a questi funghi è difficile in quanto si ottengono spesso risultati variabili. Ciò perchè la patogenicità di entrambi i funghi deve essere stimolata da sostanze nutritive. Nel metodo sviluppato, quando si inoculano foglie di fagiolo con spore di *B. cinerea* o *S. sclerotiorum* sospese in una soluzione di Pi priva di fonti di carbonio e azoto, il livello di infezione di entrambe i funghi aumenta rispetto a inoculazioni con spore sospese in sola acqua. Giacchè il metodo di inoculazione con Pi è rapido e non richiede piante in fiore, lo si può facilmente adottare per programmi di selezione e di miglioramento genetico.

In seguito alla possibile importanza di PG2 nel processo d'infezione di *B. cinerea*, si sono studiate le principali caratteristiche biochimiche e molecolari di questo enzima. Nel presente studio le fasi della purificazione e l'omogeneità del preparato purificato di PG2 sono state costantemente controllate con zimogrammi elettroforetici, poichè la concentrazione specifica delle PG era troppo bassa per essere misurata per mezzo di un'analisi proteica. La causa delle difficoltà incontrate nel separare attività sovrapposte di enzimi pectici durante il procedimento di purificazione si è investigata studiando la mobilità elettroforetica di PG1, PG2 e di un altro isoenzima. PG2 degrada più velocemente il poligalatturonato sodico della pectina. In condizioni standard, il valore della K_m per l'idrolisi del poligalatturonato sodico è di 0,46 mg di substrato per ml; la K_m relativa alla pectina è stata stimata essere di 1,18 mg di substrato per ml. Il pH ottimale per la degradazione da parte di PG2 del poligalatturonato è di 4,5 e della pectina di 4,0. L'attività di PG2 è anche influenzata dalla presenza di NaCl o di $CaCl_2$ nella miscela di reazione. L'analisi dei prodotti di degradazione mediante cromatografia su carta e la comparazione della velocità di reazione mediante viscosimetria e saggio dei gruppi riducenti, indicano che PG2 è un endo-enzima. Il punto isoelettrico di PG2 è di 9,1 e la sua massa molecolare è di 23,0 kDa.

Valutando globalmente i risultati di questo studio, si propone che il significato primario della produzione sequenziale e coordinata delle PG da parte di *B. cinerea* è inerente alla digestione della porzione pectica della parete cellulare vegetale. Ciò è in accordo con le caratteristiche di un saprofita ecologicamente adattato a vivere su tessuto vegetale morente o morto. Tuttavia, le proprietà fisiologiche e biochimiche di PG2 convincono anche di un suo ruolo diretto nel rapido dissolvimento della parete cellulare primaria, onde facilitare la penetrazione fungina.

CURRICULUM VITAE

Gionata Orazio Maria Leone is born on 4 January 1956 at Milan, Italy. He took his diploma of High School in classical studies in 1975 and obtained his degree in Agriculture (cum laude: Plant Protection as main subject) from the University of Milan, in 1980. He collaborated with the Istituto di Patologia Vegetale of the University of Milan until 1984 working at first on analysis of residues of fungicides against Botrytis cinerea in grapes and strawberries, and, later, on the effect of the light quality on the hypersensitive reaction of Datura stramonium and Phaseolus vulgaris plants to Tobacco Mosaic Virus. From 1982 to 1984 he teached Biology in Evening Technical Schools for adults of the municipality of Milan. From 1984 to 1987 he joined the Department of Physiology of the Fytopathologisch Laboratorium "Willie Commelin Scholten" of the University of Amsterdam and the Royal University of Utrecht, Baarn, Holland. There he performed research on the physiological and biochemical aspects of the enzymes, polygalacturonases, produced by Botrytis cinerea during pathogenesis. From 1987 to 1989 he joined the Department of Physiology and Resistance of the Research Institute for Plant Protection (IPO) at Wageningen, where he investigated the effects of the air pollutant ozone on the predisposition of Phaseolus vulgaris beans to the weak pathogens Botrytis cinerea and Sclerotinia sclerotiorum. Since July 1990 he is joining the Department of Detection and Biotechnology of the IPO. His research interests concern the development of techniques to detect serologically plant viruses and fungi. He published various articles in each field of study.