AMBIENT pH-REGULATED ENZYME SECRETION IN ENDOPHYTIC AND PATHOGENIC ISOLATES OF THE FUNGAL GENUS *Colletotrichum*

Walter Maccheroni Jr.[1]*; Welington Luiz Araújo[2]; João Lúcio Azevedo[2,3]

[1]*Allelyx Applied Genomics / Techno Park, km. 104, Rod. Anhangüera - 13067-850 - Campinas, SP, Brasil.*
[2]*USP/ESALQ - Depto. de Genética, C.P. 83 - 13400-970 - Piracicaba, SP - Brasil.*
[3]*UMC - Núcleo Integrado de Biotecnologia - Av. Dr. Cândido Xavier de Almeida e Souza, 200 - 08780-911 - Mogi das Cruzes, SP - Brasil.*
**Corresponding author <walter.maccheroni@alellyx.com.br>*

ABSTRACT: In fungi a genetic system ensures that enzymes are secreted mainly at ambient pH values corresponding to their optima of activity. Although a great deal of information has been obtained concerning this environmental response, there is a lack of studies involving phytopathogenic, endophytic and entomopathogenic fungi as well as different aspects of fungus-host interactions. This study compares in a plate-clearing assays, the effect of ambient pH in the secretion of amylase, cellulase, lipase, pectinase and protease by endophytic, phytopathogenic, and entomopathogenic isolates belonging to several species of *Colletotrichum*. All enzymes were secreted in a pH-dependent manner by all isolates. Endophytes and pathogens showed distinct patterns of protease secretion, with optima at alkaline and acid growth conditions, respectively. In liquid medium, a Pi-repressible acid phosphatase of an endophytic isolate responded to ambient pH, having a 14-fold increase in secreted specific activity at acid pH, as compared to alkaline pH. Furthermore, part of a *Colletotrichum pac*C homologue gene, coding for a transcriptional factor responsible for pH-regulated gene expression, was cloned. Ambient pH seems to be a general factor controlling enzyme secretion in fungus-host interactions through a conserved genetic circuit.
Key words: *Glomerella*, enzyme secretion, endophytes, pathogenic fungi, *pac*C

SECREÇÃO DE ENZIMAS MEDIADA PELO pH DO AMBIENTE EM ISOLADOS PATOGÊNICOS E ENDOFÍTICOS DO FUNGO *Colletotrichum*

RESUMO: Em fungos, um sistema de regulação gênica garante que enzimas sejam secretadas predominantemente em valores de pH do ambiente próximos aos pH ótimos de atividade correspondentes. Embora muita informação tenha sido acumulada sobre essa resposta adaptativa, não existem estudos envolvendo fungos fitopatogênicos, endofíticos e entomopatogênicos, bem como sobre outros aspectos relacionados às interações fungo-hospedeiro. No presente trabalho foi comparado, em meio sólido, o efeito do pH do ambiente na secreção das enzimas amilase, celulase, lipase, pectinase e protease por isolados endofíticos, fitopatogênico e entomopatogênicos pertencentes a diferentes espécies de *Colletotrichum*. Para todas as enzimas e em todos os isolados, observou-se um padrão de secreção dependente dos valores do pH do ambiente. Isolados endofíticos e patogênicos apresentaram padrões distintos de secreção de protease, com ótimos em pH de crescimento alcalino e ácido, respectivamente. Em meio líquido, uma fosfatase ácida Pi-repressível, secretada por um isolado endofítico, respondeu ao pH do ambiente, apresentando um aumento de 14 vezes na sua atividade específica durante o crescimento do fungo em meio ácido, quando comparado a meio alcalino. Além disso, foi clonada parte do gene *pac*C de *Colletotrichum*, o qual codifica um fator de transcrição responsável pela regulação dependente do pH do ambiente. É plausível a hipótese de que o pH ambiente é um fator de amplo espectro controlando a secreção enzimática durante as interações fungo-hospedeiro por meio de um circuito genético conservado.
Palavras-chave: *Glomerella*, secreção enzimática, endófitos, fungo patogênico, *pac*C

INTRODUCTION

During fungal infection a range of hydrolytic enzymes is secreted to help promoting host colonization. Depending of the ecological niche occupied by each fungus, a particular set of enzymes, mainly composed of proteases and carbohydrases, are displayed to degrade specific tissues and scavenge for nutrient resources (Yakoby et al., 2000). Because these enzymes work outside the fungal cell, activity as well as the mechanisms that control synthesis and secretion are under the influence of several environmental factors such as ambient pH (Caddick et al., 1986; Maccheroni Jr. et al., 1995).

In the saprophyte *Aspergillus nidulans*, pH-regulated gene expression is under the control of at least seven genes (Maccheroni Jr. et al., 1997; Negrete-Urtasun et al., 1999; Tilburn et al., 1995). Gene *pac*C codes for a transcriptional factor activated by proteolysis at alkaline growth pH, and is responsible for inducing alkaline-expressed genes (e.g. alkaline protease) and repressing acid-expressed genes (e.g. acid protease). Genes *pal*A, B, C, F, H, and I are components of a transduction pathway that senses ambient pH and activates the PacC protein. This genetic system seems to be well conserved among fungi (Penãlva & Arst, 2002), but only very recently a *pac*C homologue has been identified in a phytopathogen, the filamentous fungus *Sclerotinia sclerotiorum* (Rollins & Dickman, 2001).

In phytopathogenic fungi, pectinases are involved in plant cell wall-degradation and are thought to be important determinants of pathogenicity and virulence (Annis & Goodwin, 1997). Early reports (Hancock, 1966; Sherwood, 1966) demonstrated that secretion of specific pectinolytic activities by phytopathogenic fungi was dependent on the pH of culture medium. Thus, polygalacturonases (PGs) usually have acid optimum pH for activity and are produced and secreted preferentially at acid growth conditions, whereas for pectin/pectate lyases (PLs) the opposite occurs. It has been then proposed that the concomitant rise in plant tissue pH and increase in PL activity and synthesis would account for the switch between a transient biotrophic phase to a highly destructive necrotrophic phase in *Colletotrichum lindemuthianum* causing bean anthracnose (Bailey et al., 1992). More recent works with *Colletotrichum gloeosporioides* have shown that ambient pH regulates PL transcription *in vitro* (Shih et al., 2000; Yakoby et al., 2000); *in planta* PL expression in the necrotrophic phase of infection has also been demonstrated (Shih et al., 2000).

Colletotrichum comprises a variety of phytopathogenic species used as models for studies concerning pathogenicity and fungal-plant interaction (Perfect et al., 1999). A few members have also been described as endophytes, establishing asymptomatic plant infections (Araújo et al., 2001; Azevedo et al., 2000; Freeman & Rodriguez, 1993) and as opportunistic pathogens of insect (Teixeira, 2000) and even man (Ritterband et al., 1997). The aim of this study was to examine the extension of ambient pH regulation on the *in vitro* secretion of several enzyme activities by endophytic, phytopathogenic and entomopathogenic isolates of *Colletotrichum*, and to identify a *pac*C homologue in the genus.

MATERIAL AND METHODS

Isolates and growth conditions

Colletotrichum isolates and respective hosts are listed in Table 1. Endophytes were isolated from surface-sterilized parts of healthy plants and pathogens directly from diseased plant or insect tissues using standard methodologies [all references in Table 1]. Isolates were grown on Potato Dextrose Agar medium and conidia were obtained after 7-14 d. Enzyme assays were carried on solid (1.5%agar) and liquid minimal medium(containing, L^{-1} : KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 1.5 g; $Na_2B_4O_7.10H_2O$, 0.5 mg; $CuSO_4.5H_2O$, 0.5 mg; $FeSO_4.7H_2O$, 0.5 mg; $MnSO_4.H_2O$, 0.3 mg; $NaMoO_4.2H_2O$, 1 mg; $ZnSO_4.7H_2O$, 0.1 g; glucose, 10g; amonium tartrate, 1 g) buffered at pH 5.0, 6.8, and 8.0 with 50 mmol L^{-1} of citrate (Na^+), MOPS, and Tris-HCl, respectively, and with modifications as specified for each experiment. Growth in liquid media was carried out in a rotary shaker at 200 rpm. All growth experiments were performed at 28°C.

Enzyme secretion assays on solid media

Enzyme secretion was evaluated in minimal glucose-free medium, supplemented with 0.1% yeast extract and one of the following carbon sources: 0.5% dry skim milk Nestlé (protease); 0.5% CM-cellulose Sigma (cellulase); 0.5% citrus pectin Sigma (pectinase); 0.2% soluble corn starch Sigma (amylase); 1% Tween 20

Table 1 - Wild-type isolates of *Colletotrichum* and their respective hosts.

Isolate/designation[a]	Host[b]	Reference
Endophyte		
C. musae 7 (E1)	Banana leaves	Maccheroni & Azevedo, 1998
C. gloeosporioides CAR1 (E2)	Sweet orange leaves	This work
C. gloeosporioides CCC (E3)	Sweet orange petals	Teixeira, 2000
Plant pathogen		
C. gloeosporioides CCE (PP1)	Sweet orange petals	Teixeira, 2000
C. acutatum 3KLCC (PP2)	Key lime flower buds	Kuramaeizioka et al., 1997
C. sublineolum (PP3)	*Sorghum* sp. leaves	Kamida, 1998
Insect pathogen		
C. gloeosporioides $CTAA_2$ (IP1)	*Orthezia praelonga*	Teixeira, 2000

[a] Designations used in text and Table 2 are in parentheses. [b] Banana (*Musa cavendish*); Sweet orange (*Citrus sinensis* (L.) Osbeck, var. Pera); Key lime (*Citrus aurantifolia* (L.) Swingle); *Orthezia praelonga* (Homoptera).

Amersham (lipase) or 2.4% purified crab chitin Sigma (chitinase). Chitin and plates for chitinase detection were prepared according to Hankin & Anagnostakis (1975). Degraded substrate zones were visualized as previously described for protease, amylase, lipase, pectinase, and chitinase (Hankin & Anagnostakis, 1975) and cellulase (St. Leger et al., 1997). Isolates were point-inoculated onto solid media and allowed to grow for 3-4 d (endophytes) or 5-6 d (pathogens) or until the colony reached at least 2.5 cm in diameter.

Phosphatase analysis

Conidia (10^8) were inoculated into 500 mL Erlenmeyer flasks containing 200 mL of liquid minimal medium, buffered at pH 6.8 followed by growth for 36 h. The mycelium was harvested, washed with sterile water and further grown in 100 mL of minimal medium, buffered at different pH values, for 24 h under the same described conditions. For phosphatase derepression, further growth was carried out in Pi-free minimal medium (KH_2PO_4 was replaced with an equimolar concentration of KCl). Thereafter, preparation of cell-free extract and determination of phosphatase activities were performed as described previously (Maccheroni & Azevedo, 1998).

Cloning procedures

Genomic DNA was extracted from mycelium grown for 48 h in liquid Potato Dextrose medium according to Andrade-Monteiro et al. (1994). Approximately 200 ng of total DNA were used as template in PCR reactions with degenerated oligonucleotides PAC1 (forward primer; 5'-GTNTGCGARCGNCACGTNGG; consensus amino acid sequence VCERHVG) and PAC2 (reverse primer; 5'-RTCRTCVGCRTGVGTYTTNACRTG; consensus amino acid sequence HVKTHADD). Degeneracy codes are: R= A or G; Y= C or T; V= A, C or G, and N= A, C, G or T. Amplification was performed in a final volume of 25 µL containing: assay buffer (Tris-HCl, 70 mmol L^{-1}, pH 9.0); $MgCl_2$, 1.5 mmol L^{-1}; 2.5 U of Taq DNA polimerase; 1 mg of each primer and 200 mmol L^{-1} of each dNTP. PCR was carried out using the following profile: 94°C for 4 min; 5 cycles of 94°C for 1 min, 38°C for 1 min, and 72°C for 2 min; 30 cycles of 94°C for 1 min, 46°C for 1 min, and 72°C for 2 min; final extension of 72°C for 5 min. PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide. A fragment of expected size was cut off from the gel, eluted, cloned into pGEM-T vector (Promega), and the recombinant plasmids were electroporated into *Escherichia coli* DH5a competent cells. Plasmid DNA from three clones was prepared by routine, alkaline lysis and sequenced on both strands with universal primers (M13 forward and reverse primers) on an ABI 377 automated sequencer using standard ABI protocols.

RESULTS AND DISCUSSION

Ambient pH-regulated enzyme secretion by endophytic and pathogenic isolates of *Colletotrichum* grown on solid media

Plate clearing assay has proven to be a method of great simplicity and value in measuring enzyme secretion in fungi (St. Leger et al., 1997) and therefore, was used in the present study. All carbon sources were degraded by all isolates in a pH-dependent manner (Table 2), except for chitin that was not degraded at all (results not shown). Amylase activity was not observed at alkaline pH and although starch had been degraded at neutral and acid pH, enzyme secretion was discrete and occurred only under the fungal colonies. Pectinase was produced in all growth conditions, but secretion occurred consistently only at acid pH indicating that pectin degradation is due probably to a polygalacturonase activity. In general, lipase was absent at acid pH and secreted at neutral and alkaline pH, whereas cellulase was observed in most growth conditions and secreted preferentially at neutral and alkaline pH.

Degradation of protein was observed in all growth conditions with optimal levels at the extremes of the assayed pH range. In this case, a more distinct pattern was observed among isolates, with endophytes se-

Table 2 - Influence of solid medium pH on the secretion of five enzyme activities by endophytic and pathogenic isolates of *Colletotrichum*[a].

Isolate	Amylase			Cellulase			Lipase			Pectinase			Protease		
pH	5.0	6.8	8.0	5.0	6.8	8.0	5.0	6.8	8.0	5.0	6.8	8.0	5.0	6.8	8.0
E1	0.7	0.6	n.d.	0.8	0.9	1.0	n.d.	1.3	2.2	1.3	1.0	1.0	1.0	1.0	1.2
E2	0.8	0.8	n.d.	n.d.	1.0	1.3	n.d.	1.2	1.7	1.3	1.0	1.0	1.0	1.0	1.9
E3	0.8	0.8	n.d.	n.d.	1.0	1.5	n.d.	1.2	1.9	1.4	1.0	1.0	1.0	1.0	1.3
PP1	1.0	1.0	n.d.	1.0	1.1	1.3	n.d.	1.2	1.7	1.4	1.0	1.0	1.3	1.0	1.0
PP2	1.0	1.0	n.d.	n.d.	1.4	1.5	n.d.	1.7	1.7	1.7	1.0	1.0	1.4	1.0	1.0
PP3	1.0	1.0	n.d.	1.0	1.0	1.4	n.d.	n.d.	1.5	1.3	0.7	n.d.	1.8	0.7	1.0
IP1	1.0	1.0	n.d.	1.0	1.2	1.3	n.d.	1.3	2.1	1.4	1.0	1.0	1.3	1.0	1.0

[a] Results are the mean of three independent experiments and represent the enzyme activities expressed as the ratio between the diameters of the degraded zone and the colony. Values >1 indicate enzyme secretion into the medium resulting in a degraded zone around the colony. Values =1 and <1 indicate degraded zones only under the colony. n.d. (not determined) indicates no degraded zone or that it could not be estimated.

creting more protease activity at alkaline pH and pathogens at acid pH. Distinct optimum pH-types of protease activities seem to be differentially secreted by the two groups of isolates. Indeed, in isolates of the fungal genus *Verticillium*, secretion of different profiles of protease activities was also observed and seems to correlate with a specific ecological niche, discriminating between insect and plant pathogens (St. Leger et al., 1997). The fact that the opportunistic entomopathogen IP1 showed similar patterns to those of plant pathogens, regardless of the enzyme activity tested, may lay on the observation that this isolate may be also pathogenic to plants (Teixeira, 2000). Ambient pH-regulated enzyme secretion has already been shown to occur in other fungi occupying different niches and be involved in different aspects of the corresponding interactions. In the ericoid endophytic fungi *Hymenoscyphus ericae* and *Rhodothamus chamaecistus*, acid proteases probably involved in nitrogen acquisition from the environment are secreted only at low growth pH and some of the enzymatic properties seem to correlate with the pH of the soils of their host plants (Leake & Read, 1990). In the insect pathogenic fungus *Metarhizium anisopliae*, alkaline pH is a physiological signal that triggers the synthesis and secretion of several proteases and a chitinase proposed to be virulence factors in host cuticle penetration (St. Leger et al., 1998; 1999).

Effect of ambient pH on phosphatase production by C. musae grown in liquid medium

Four distinct phosphatase activities produced in vitro by endophytic isolates of C. musae have been previously identified by Maccheroni & Azevedo (1998). Constitutive acid phosphatase, alkaline phosphatase, and phosphodiesterase activities were observed only inside the cells and thus, are not expected to be deeply influenced by ambient pH (Caddick et al., 1986). The only extracellular activity observed was a Pi-repressible acid phosphatase that may respond to variations in ambient pH. To assess those expectations, the effect of ambient pH on the production of these four enzyme activities was carried out under nutritional sufficiency and Pi-starvation conditions. Only the Pi-repressible acid phosphatase markedly responds to ambient pH, having a 14-fold increase in secreted activity at acid pH as compared to alkaline pH (Table 3).

Identification of a *pac*C homologue in *C. sublineolum*

A PCR strategy was used to identify a *pac*C homologue in *Colletotrichum*. The PacC sequences from *A. nidulans*, *A. niger*, and *P. chrysogenum* (GeneBank accession numbers S54308, S63587, and AAC36492, respectively) were aligned and a highly conserved amino acid block, comprising the DNA binding motif (Denison, 2000), was chosen to designate a pair of degenerated primers. These primers were then used to amplify genomic DNA from all *Colletotrichum* isolates in Table 1. Except for isolates E1 and E3, amplification from all others yielded a DNA fragment of expected size. Isolate PP3 showed the most distinct band against the amplified background and therefore, it was further cloned and sequenced. A 213 bp nucleotide sequence (GeneBank accession number AF260325) coding for a putative polypeptide 100% similar to the PacC proteins of *Aspergillus* was revealed (Figure 1) and strongly suggests the existence of a *pac*C homologue in *Colletotrichum*. In conclusion ambient pH may be a general factor controlling enzyme secretion in *Colletotrichum*. Furthermore, the identification of a *pac*C homologue in *C. sublineolum* reinforces the idea of a conserved genetic circuit widespread in nature and being responsible, at least in part, for pH-regulated gene expression that takes place in fungus-host interactions (Rollins & Dickman, 2001).

Table 3 - Influence of liquid medium pH on the synthesis and secretion of phosphatases by the E1 endophyte of *C. musae*.

Enzyme activity[b]	Growth condition[a]								
	pH 5.0			pH 6.8			pH 8.0		
	+Pi	Pi		+Pi	Pi		+Pi	Pi	
	CE	CE	CM	CE	CE	CM	CE	CE	CM
Acid phosphatase	4.6 ± 0.6[c]	8.2 ± 0.4	n.d.	7.9 ± 0.2	7.1 ± 0.6	n.d.	6.1 ± 0.7	4.4 ± 0.3	n.d.
Alkaline phosphatase	1.1 ± 0.3	0.8 ± 0.2	n.d.	1.4 ± 0.1	1.3 ± 0.1	n.d.	1.6 ± 0.3	2.0 ± 0.1	n.d.
Phosphodiesterase	1.8 ± 0.2	6.5 ± 0.8	n.d.	3.6 ± 0.5	8.0 ± 0.7	n.d.	4.9 ± 0.2	8.9 ± 0.3	n.d.
Pi-repressible acid phos.	2.4 ± 0.2	6.0 ± 0.3	28.4 ± 5.2	3.6 ± 0.1	4.4 ± 0.2	6.2 ± 0.5	2.9 ± 0.4	2.8 ± 0.2	2.0 ± 0.3

[a] Minimal medium (+Pi) and Pi-free minimal medium (-Pi) buffered at different pH values. Cell-free extract (CE) and culture medium (CM). [b] Acid phosphatase (100 mmol L^{-1} sodium citrate, pH 5.0, 2 mmol L^{-1} EDTA), alkaline phosphatase (100 mmol L^{-1} CHES, pH 10, 10 mmol L^{-1} $MgCl_2$) and Pi-repressible acid phosphatase (100 mmol L^{-1} sodium citrate, pH 6.0, 2 mmol L^{-1} EDTA), and phosphodiesterase (100 mmol L^{-1} Tris-HCl, pH 7.5, 10 mmol L^{-1} $MgCl_2$) were assayed over respectively, 1 mmol L^{-1} r-nitrophenylphosphate and bis-r-nitrophenylphosphate as substrates. [c] Mycelial and secreted specific activities expressed as U (mg dry weight mycelium $^{-1}$± standard error). One unit of phosphatase activity (U) was defined as one nmol substrate hydrolyzed per min at 37ºC. Three independent sets of experiments were performed and enzyme assays were carried out in duplicate. n.d., not determined.

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An    104   RKSTNNLNLTCQWGSCRTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQDLKK 159
Ag     98   RKSTNNLNLTCQWGSCRTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQDLKK 153
Cs      1   RKSTNNLNLTCQWGSCRTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQDLKK 56
Pc     86   RKSTNNLNLTCQWGTCNTTTVKRDHITSHIRVHVPLKPHKCDFCGKAFKRPQDLKK 141
Ss     73   RKSTNNLNLTCGWNSCRTTTVKRDHITSHIRVHVPLKPHKCEFCGKAFKRPQDLKK 128
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Figure 1 - Alignment of the partial amino acid sequence of PacC from *C. sublineolum* (Cs) and the corresponding protein regions of *A. nidulans* (An), *A. niger* (Ag), *P. chrysogenum* (Pc) and *S. sclerotiorum* (Ss). Primer sequences were not used for translation of the *S. sclerotiorum* protein. Numbers on the sides of the alignment refer to the original position of amino acids in the respective proteins. Identical and similar residues are shown on black and grey backgrounds, respectively. Sequence alignment was performed using the Clustal W program (http://www.hgsc.bcm.tmc.edu/SearchLauncher).

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