Revista Latinoamericana de MICROBIOLOGÍA
ALAM

Vol. 51, Nos. 1-2
January - March 2009
April - June 2009
pp. 23 - 31

RESEARCH CONTRIBUTION

Colletotrichum lindemuthianum (Sacc. & Magn.) Scrib. is a potential cellulases producer microorganism

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Colletotrichum lindemuthianum (Sacc. and Magn.) Scrib. is a potential cellulases producer microorganism

ABSTRACT. *Colletotrichum lindemuthianum* a hemibiotrophic fungus is the causal agent of anthracnose in *Phaseolus vulgaris* L. This a complex and peculiar vegetative life cycle with unique cell differentiation and growth process, and a high degree of genetic and metabolic plasticity which contribute with its adaptation under adverse environmental conditions. Therefore, the fungus is able to propagate and to survive easily in its specific host and plant detritus, where extracellular enzymes play an important role in the biopolymers degradation. A source of carbon and other nutrients for this fungus is the large quantity of lignocellulose available in the tequila industry waste, this motivates the thought in the production of sugars from enzymatic degradation of cellulosic waste from the agave as an interesting and inexpensive alternative to obtain products from this solid waste. *C. lindemuthianum* was subject to screening of cellulases, we founded that this fungus secretes a high level of cellulases in medium containing agave lignocellulosic waste when it was compared with other cellulose fungi producer. The enzymatic activity of these lytic enzymes showed saccharification percentage value of 34.3 at optimal pH and temperature. This fungus is an ideal model to study aspects as development, cell differentiation and production of extracellular enzymes with biotechnological application.

Key words: *Colletotrichum lindemuthianum*, hemibiotroph, anthracnose, extracellular enzymes, saccharification, agave waste.

RESUMEN. *Colletotrichum lindemuthianum* un hongo hemibiotrofo el agente causal de la antracnosis en *Phaseolus vulgaris* L. Este muestra un complejo y peculiar ciclo de vida con un singular proceso de crecimiento y diferenciación celular, así como una gran plasticidad genética y metabólica, la cual contribuye a su adaptación a diversas condiciones ambientales. Con ello, el hongo es capaz de propagarse y sobrevivir fácilmente en su hospedante o en detritus vegetal, donde las enzimas extracelulares tienen una función importante en la degradación de biopolímeros. Una fuente de carbono y otros nutrientes para este hongo es la gran cantidad de lignocelulosa disponible en el desecho de la industria del tequila, lo que motiva a pensar en la producción de azúcares a partir de la degradación enzimática de los residuos celulósicos provenientes del agave como una alternativa barata e interesante para obtener productos de desechos sólidos. *C. lindemuthianum* fue sujeto a un escrutinio de celulasas; encontramos que este hongo secreta una gran cantidad de celulasas en medio que contiene residuo lignocelulósico de agave, cuando fue comparado con otros hongos productores de celulasas. La actividad enzimática de estas enzimas líticas mostró porcentajes de sacarificación de 34.3 a temperatura y pH óptimos. Este hongo es un modelo ideal para estudiar aspectos como el desarrollo, diferenciación celular y la producción de enzimas extracelulares con aplicación biotecnológica.

Palabras clave: *Colletotrichum lindemuthianum*, hemibiotrofo, antracnosa, enzimas extracelulares, sacarificación, residuo de agave.

Footnote: JSL, AFG and REMM had an equitable research contribution

1. INTRODUCTION

It is well known that fungi posses an amazing secretion system that allow them to produce an extraordinary essential metabolism necessary to obtain compounds from the surrounding to support defense, growth and development processes. A very wide range of waste polymers must be degrading to monomers outside of fungal cell by hydrolytic extracellular enzymes, then, the simpler and smaller molecules will be taken up by the fungi and used as nutrients involved in the energy generation, building blocks in the cell structure and other physiological functions. Extracellular enzymes are normally produced and secreted by the fungi, they are constitutive enzymes and always are present in span life cycle, and others are produced in response

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First version received: October 27, 2009; first version revised: February 02 and February 10, 2010; second version received in revised form: April 08, 2010; second version revised: April 30, 2010. Accepted: May 07, 2010.

Rev Latinoam Microbiol 2009; 51 (1-2): 23-31

to presence of particular substrates, known as inducible enzymes. The fungal extracellular enzymes are stables to environmental conditions (*e.g.* large array of substrates and physicochemical conditions), such as, growth medium, pH, heavy metal, salinity, temperature and final product tolerance. These characteristics of fungal extracellular enzymes encourage researching the best enzyme producer fungal strain, the cost-effective enzymatic hydrolysis process and enzymes tolerant to the extreme environmental conditions. Currently industrial use of extracellular enzymes from microorganisms is an integral part of a wide variety of commercial processes and applications.

The early scientific investigations on fungi were concerned with their devastating and destructive effects on plant crops more than with ancestral beneficial effects in manufacture of beer, bread, wine, chess and other foods. However, a small number of fungal species are pathogens or harmful and some of them could to have a beneficial effect. Also, it is possible to unite in a specific topic the classical approach to the study of fungi and the current view of biotechnology concerning the use of fungi. An example is the deuteromycete *C. lindemuthianum* the causal agent of common bean anthracnose disease, in which, both the secretion system as extra cellular enzymes are an important key in its life cycle. This fungus has two feeding behavior to obtain carbon and nitrogen by using the lytic enzymes to break the plant cell wall polymers, in the hemibiotrophic behavior it is feeding of plant living cell, whereas in saprophytic behavior it is feeding of plant detritus. To know the molecular factors that support the cell plasticity to adapt in the environment and to be able to survive and to propagate the fungal specie, will be very useful to form a general sketch of the potential biotechnological use of *C. lindemuthianum* as extracellular enzymes producer such as cellulases. *C. lindemuthianum* cells were used in the hydrolysis of polysaccharides obtained from the cellulosic waste of the agave (*Agave tequilana* Weber). This knowledge will define a better way of handling the causal agent of bean common anthracnose disease. We focusing this topic with a general review of *C. lindemuthianum* biology and then we show the enzymatic work of its cellulases on lignocellulosic waste produced from agave head which is used for Tequila manufacture, an emblematic Mexican and international alcoholic beverage corresponding for Mexico as the first origin domination, awarded sence 1974.

2. *P. VULGARIS* ANTHRACNOSE

The anthracnose is a common and widespread bean (*P. vulgaris*) disease is caused by the fungus *C. lindemuthianum*. The disease was first registered on bean (*P. vulgaris*) in France in 1843 year. The causal agent was designated with several names such as, *Gleosporium lindemuthianum* (1878), *Septoria legunimun* (1882), *Septoria leguminum* var *phaseolorum*, *C. lindemuthianum* (1889), *C. lagenarium* (1893), *Gleosporium lindemuthianum* (1894) and *Glomerella lindemuthiana* (1993). In 1875, in Germany in other bean species were detected the disease symptoms; the pathogenic fungus was denominated as *S. legunimun* pro *Lindemuth.* Recently, the causal agent of the common bean anthracnose disease has been clearly identified as a fungus that presents imperfect and perfect forms, which have been denominated *Colletotrichum lindemuthianum* and *Glomerella cyngulata f. sp phaseoli*, respectively (Rodriguez and Yoder 1987).

When the fungus is established in a susceptible plant, the first noticeable symptoms are easily detected. They are yellowish spots that later will develop into ulcerous necrotic wounds affecting all plant structures (leaves, stems, flowers and fruits). Later, a generalized infection is seen in the plant; the mycelia growth and the fungus fruiting structures may be detected over the surface of the plant which soon will cause its death. In the infested bean crop areas, the fungus prevails annually and in the bean detritus for more than twelve years. The remarkable resistance of *C. lindemuthianum* and its capacity for survivability in any environmental condition renders its presence capable of losses in bean crops. In fact, the damage caused by this fungus in bean crops is so great that it had produced an economical loss in productive and consumer countries of the American continent (Dillard *et al.* 1993). Furthermore, *C. lindemuthianum* is a fungus that has a reduced number of plant hosts, mainly *P. vulgaris.* In less extent and severity, this fungus can also colonize *P. acutifolius* var *lactifolius, P. coccineus, P. aureus, P. lunatus, P. limensis, Medicago sativa* and *Vicia faba* (Delphine *et al.* 1988). Also, in bean resistant plants *C. lindemuthianum* cause «the hypersensitive response» - groups of red-brownish wounds of different sizes that are produced by the plant to delimit the spread of the pathogenic fungus and to kill it (Elliston *et al.*1976).

3. TAXONOMY

Colletotrichum lindemuthianum is considered as hemibiotrophic fungus, its taxonomical classification was a difficult, confused task. This fungus had been named with different synonymous throughout the years. Then, it could be hardly identified through classical taxonomy, because it produces acervuli with or without fruiting body depending of the quality and amount of substrate (Sicard *et al.*1997). Now, the fungal names are given according to principles and rules of the International Code of Botanical Nomenclature, although, there is still some controversy in the designated names to some fungus. Some authors have even proposed particular, systematic, fungal classification which is still in

Rev Latinoam Microbiol 2009; 51 (1-2): 23-31

discussion because certain fungal species are difficult to be classified than others. The *C. lindemuthianum* classification was made following the Alexopoulos and Mim (1979) proposal. In this case, most authors agreed that *C. lindemuthianum* belongs to: Family, Melanconiaceae; Order, Melanconiales; Sub Class, Coelomycetidae; Class, Deuteromycetes; Sub Division, Deuteromycotina; Division Amastigomycota; Kindom Myceteae; Super Kindom, Eucariota. They are considered taxonomic units below the species such as the ***form specialis*** (*f. sp*) - a group of individuals in the same species that have the ability to infect several plant species; while the **physiological race** is pathogenic only for certain varieties of plant species.

4. VEGETATIVE CYCLE

Colletotrichum lindemuthianum deploys a complex life cycle which has various development phases and two ways to take food. In every phase may be seen as unique, differentiated stages that let the fungus survive. Independently of the fungus development phase, the spore germination occurs in a similar manner (Figure 1). In the imperfect form of *C. lindemuthianum* the reproduction is asexual, the spores are produced inside acervulus and immerse in water soluble pre-formed mucilage (O'Connell 1996). The development of fungal spore shows a biphasic behavior which means two life styles, as a saprophyte and biotroph; therefore, the fungus has been classified as hemibiotroph. In life style saprophytic the fungus growth in any carbon source including crystalline cellulose which may be easily converted into molecules fuel by extracellular lytic enzymes. On the other hand, as a biotroph fungus has the ability to feeding of nutriments outright of living plants.

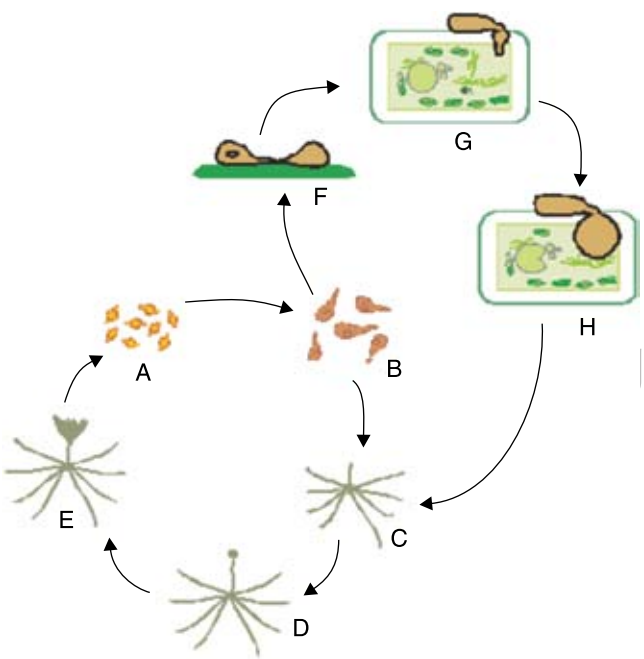

Figure 1. C. lindemuthianum *biological cycle. A. Spores, B. Germination, C. Vegetative Growth, D. Aerial structures, E. Sporogenesis, Spore adhesion Penetration, H. Primary hyphae Formation.*

As a saprophyte fungus, the spore germination process begins with the spore adhesion to the plant surface under adequate humidity conditions; specifically, correct aqueous content in the spore envelope (mucilage). At this level, the oval spores of the fungus round off by water absorption and active growth. Later, the germinating tube is formed (germinule phase), and the hyphae elongates to colonize the substrate. The aerial mycelia appear; then the fungal reproductive structures are formed where the spores are storage. Finally, their life cycle is completed and it starts all over again.

During the spore adhesion, the hydrophobicity of vegetal surface, the physical-chemistry bidirectional signalization and the mucilage play a major role (Young and Kaus 1984; Sela-Buurlage *et al.* 1991; Mercure *et al.* 1994). The mucilage is formed by heavy molecular weight glycoproteins, a variety of enzymes and germination inhibitors; but it does not contain chitin (O'Connell *et al.* 1996; Hughes *et al.* 1999). Besides, it acts as a structure that protects the spore of dehydration and even as a protective barrier against environmental toxic and defense plant metabolites. The 6 μm length fimbriae structures which are part of the germinule and the appresorium also participate in the adhesion process (O'Connell 1996). The *C. lindemuthianum* hyphae grow constantly reaching a size of 2 or 3 times bigger than their original size spore. It is believed that critical nutrient conditions and chemical bidirectional communication induce the formation of the dome in the hyphae tip or appresorium. At same time, it initiates the melanin synthesis and the formation of the septum that separates cytoplasm from appresorium and germinule. Also, the plasmatic membrane presents a biochemical differentiation process which separates into two domains, one in the domo or appresorium and the other in the infection peg (Pain *et al.* 1996). The appresorium produces and accumulates melanin an important factor involved in the turgency pressure which is required for the *C. lindemuthianum* to penetrate into the plant cell (Kubo and Furusawa 1991).

A new distribution and localization of plasmatic membrane proteins occur in the base of appresorial dome. It is necessary for the formation of a penetration pore, the synthesis of new cell walls layers, and the secretion of some other materials (O'Connell 1996). The melanin is storage in a cell wall layer which lies very close to the plasmatic membrane (Bailey *et al.* 1992). The penetration pore is rounded by an

Rev Latinoam Microbiol 2009; 51 (1-2): 23-31

internal wall layer with funnel shape called, «appresorial cone». This structure is contiguous with the peg penetration wall and does not have chitin or melanin. The apical growth starts again with the infection peg emission and the injection of the plasmatic membrane and the citosol through the penetration pore then grows down to the intramural space between plasmatic membrane and cell wall. This will become the infection hyphae which is present in the biotrophic phase (Pain *et al.* 1996).

Knowledge about the biotrophical phase between *C. lindemuthinanum* and bean cells interaction recently has started. However, during this phase, the primary hyphae growing between the vegetal plasmatic membrane and the cell wall is know. This fungus does not form specialized structures such as haustorium, like the ones seen in some strict biotrophs (O'Connell *et al.* 1987; Green *et al.* 1995).

One characteristic of biotrophic intracellular interaction between plant cell and fungus, it is that, pathogen avoids or suppresses the initiation event of the plant defense responses, those that switch on the «hypersensibility reaction» or the synthesis and deposition of callose on cell wall (Heath and Skalamera 1997).

5. GENOTYPIC AND PHENOTYPIC PLASTICITY

Colletotrichum lindemuthianum shows plasticity against various environmental stimuli such as substratum in quality and quantity, thermal and aqueous stress resistance, and the invasion capacity of the specific host in relation with the live nutritional source. The variability of the *C. lindemuthianum* colonies is are the result of the fungus adaptation growth to the nutrient support. It can be explained as the fungal morphological colonies changes by the environmental pressure (Van Kan *et al.* 1990). The biological manifestation that the fungus shows is the great capacity to survive in different environmental conditions such as extreme temperatures between 4 – 50 ºC, dramatic variations of soil pH and the alterations in substrate quality (Dillard *et al.* 1993). Eventually, the fungus is induced into a co-evolution process due to the emergence of resistant bean varieties which allow the easy establishment of the infection. As a result, there is a formation of stable variants of the same fungal specie that can be detected comparing virulent strains of the same specie against different type of host. The race pathogenic variability in the same species of *C. lindemuthianum* is another form of plasticity shown by this organism. Considering the genetically heterogenic population in spite of the morphological uniformity, the phytopathogenic species express differences in their pathogenic potential (virulence) to different varieties of the same species. Many races and clones have been described for *C. lindemuthianum (Table 1)*. Barrus in 1911 made the first report of α y β races, Burkholder in 1923 described γ race; in 1942 Andrus and Wade reported delta race; In a French thesis of Blondet, he gave evidences of race, cited by Charrier and Bennerot in 1970; Hubbeling in 1974 reported δ race; Schnock in 1974 indicated κ race presence; and Tu *et al.* in 1984 reported ε race in Canada. Recently, three biodiversity centers have been reported to have the biggest quantity of *C. lindemuthianum* races*:* The first is located in Mexico and the southern part of the United States, the second in Colombia, Peru and Brazil, and the third in the Central America countries (Balardin *et al.* 1997; Sicard *et al.* 1997). Numerous studies have been made to identify new races of *C. lindemuthianum* in countries where bean (*Phaseolus* spp) is cultivated; therefore, different groups of bean varieties denominated, «differential set of common bean varieties» were used in those researches. The new races have been classified using different nomenclatures such as, assignation Greek letter or with binary number that making it difficult to compare the races

Table 1. Set of *P. vulgaris* differential varieties to differentiate races of *C. lindemuthianum* and its assignation with Greek letter proposal by Drijfhout and Davis (1989). (*) = Michigan Dark Red Kidney. (-) = cultivar resistant; (+) = cultivar sensible.

Cultivar	Fungal races Alpha	Beta	Delta	Epsilon	Gamma	Iota	Kappa	Lambda
Coco a la Crème	-	+	-	-	+	+	-	+
Cornell 49242	-	-	-	-	-	+	+	-
Evolutie	-	-	-	-	-	+	-	-
Kaboon	-	-	-	-	+	+	-	+
MDRK*	-	+	+	-	+	+	+	+
Mexique 222	-	-	-	-	-	-	-	-
PI 165422	+	+	-	-	-	-	-	+
PI 207262	-	+	-	-	-	-	-	-

Rev Latinoam Microbiol 2009; 51 (1-2): 23-31

from one to another (Drijfhout and Davis 1989; Pastor Corrales *et al*. 1995). In Mexico for example, Yerkes and Telis in 1952 used the next differential set of common bean varieties; Amarillo 155, Bayo 164, Canario 1001, MDRK, Michelite, Negro 150, Negro 152 and Parry Marrow, three of them are American varieties and other three are Mexican varieties. Then, they could identify the MA1 to MA10 races and formed three groups. The group I is formed by the MA1 to MA6 races; the group II is formed by the MA7 and MA17 races; the group III is formed by the A8 to MA10 races. Later, the group IV was classified and it is formed by MA19 and MA20 races. Lastly, the alpha group is formed by the MA11 to MA15, and MA21 and MA22 races. Consequently, the correct comparison could be possible between all the races reported by participating countries following the proposals of *Tables 1 or 2*. Therefore it is necessary to have a single agreement on the criteria for naming the different races of *C. lindemuthianum*.

Table 2. Set of *P. vulgaris* differential varieties and assignation number to the binary assignation to races of *C. lindemuthianum.* Binary system: *2n*, *n* is equivalent to number place of the cultivar in the set. The sum of each bean varieties values with susceptible reaction give a binary number of a specific race. Example, the race 17 of *C. lindemuthianum,* is virulent to Michelite (1) + Widusa (16) varieties. (*) = Michigan Dark Red Kidney. Pastor Corrales *et al.* 1995.

Varieties	Assignation number	Bean resistance gene
Michelite	1	
MDRK*	2	*Co-1*
Perry Marrow	4	Co-1^3
Cornell 49242	8	*Co-2*
Widusa	16	*Co-1^5,Co-9^3*
Kaboon	32	Co-1^2
México 222	64	*Co-3*
PI 207262	128	*Co-4^3, Co-9*
TO	256	*Co-4*
TU	512	*Co-5*
AB 136	1024	*Co-6, co-8*
G2333	2048	*C0-4^2, Co-5, Co7*

6. GENOME

The *C. lindemuthianum* genome of various isolates was analyzed using flow cytometry to measure the genome size. It revealed that the strains presented a meaningful variation in the genome size, up to 40 %. Later, the Southern analysis of fungal, cloned telomeres revealed that the chromosome number varies from nine to twelve. While the chromosomes separation using electrophoresis in gel of pulse field (PFGE) showed that there are two different sizes: the polymorphic and small chromosomes of 2.5 Mbp, and the big chromosomes of 7 Mbp that were not resolved by the electrophoretic method. Furthermore, two repeated grouped elements were found in different polymorphic chromosomes, they have a single copy of a conserved region sequence which was used as a probe of small chromosomes. However, this probe could not be detected in some stocks (O'Sullivan *et al*. 1998).

Evidences indicate that part of the variations observed in *C. lindemuthianum* genome organization is due to genome and/or chromosomal essential regions which are necessary for the fungus vegetative cycle (O'Sullivan *et al*. 1998). Moreover, it can be speculated that the biological variations detected in the pathogenic virulence of the fungus against different bean varieties is just due to the differences in their genome size. Besides, *C. lindemuthianum* is an organism that only a reduced number of genes have been cloned, but none of them is related to its pathogenicity or virulence (Table 3). Whereas the host (*P. vulgaris*), several different resistance genes have been identified and named *Co-1*, *Co-1^2*, *Co-1^3*, *Co-1^5*, *Co-2*, *Co-3*, *Co-4*, *Co-4^2*, *Co-4^3*, *Co-5*, *Co-6*, *Co-7*, *co-8*, *Co-9*, and *Co-9^3*, all of them have been correlated with the plant resistance (Table 2). In *C. lindemuthianum* has been reported twelve virulence genes assigned whit the letters *a, b, c, d, e, f, g, h, I, j, k, and l* compatibles with the common bean differential varieties show in Table 3, respectively (Tamayo *et al.* 1995; Basset 1996). Every day into

Table 3. Colletotrichum lindemuthianum cloned genes.

Genes	Protein	Function	References
Clpg1	Endopolygalacturonase	Necrotrophic development	Centis *et al.* 1996
Clpg2	Endopolygalacturonase	Necrotrophic development	Centis *et al.* 1997
Clk1	Ser-Tre protein kinase	Signalization	Dufresne *et al.*1998
CIH1	Biotrophy-related protein	Biotrophy	Perfect *et al.* 1998
gpd	Glyceraldheide-3P- dehydrogenase	Houskeeping genes	Templeton *et al.* 1992
Clpt1	*C. lindemuthianum* protein transport 1	Rab/GTPase protein transport	Dumas *et al.* 2001

Rev Latinoam Microbiol 2009; 51 (1-2): 23-31

biodiversity centers, it is reporting new *C. lindemuthianum* pathotypes indicating a large pathogenic variability of this phytopathogen fungus, as it was recently demonstrated by Sanchez Garcia *et al*. (2009).

7. SACCHARIFICATION OF AGAVE (*A. TEQUILANA*) LIGNOCELLULOSIC WASTE BY CELLULASES FROM *C. LINDEMUTHIANUM*

Recently has started a study of the secretion system and the extracellular enzymes in *C. lindemuthianum*, two important factors for the survival of specie both for hemibiotrophic as saprophytic lifestyles. A few *C. lindemuthianum* extracellular enzymes that hydrolyze complex biopolymers have been reported, such as; proteases, pectin and pectate lyases (Wijesundera *et al*. 1989), polygalacturonases and chitinases (Anderson 1978; Keon 1990), and recently cellulases (Acosta Rodriguez *et al*. 2005). Other enzyme reported is the chitin deacetylases that modified the chitin, (Tsigos and Bouriotis 1995). Some of these enzymes have been proposed to hydrolysis of cellulose from agroindustry and municipal wastes and recycling paper industry. Also is true that agave lignocellulosic waste containing a large amount of Carbon that could be recycling for several chemical and biological processes, *e.g.* to produce cellulose fiber, molasses, unicellular protein, organic acid, sugar alcohol, syrup, and ethanol. The key for to obtaining this cellulose products derivates involves the enzymatic hydrolysis of plant hemicelluloses and cellulose to oligo- and mono-saccharides and other molecules. With this information in mind we screened a stock of wild isolates of *C. lindemuthianum* from infected bean plants collected of crops field situated in the common bean producer area of the north central Mexican plain, to select the best wild strain cellulases

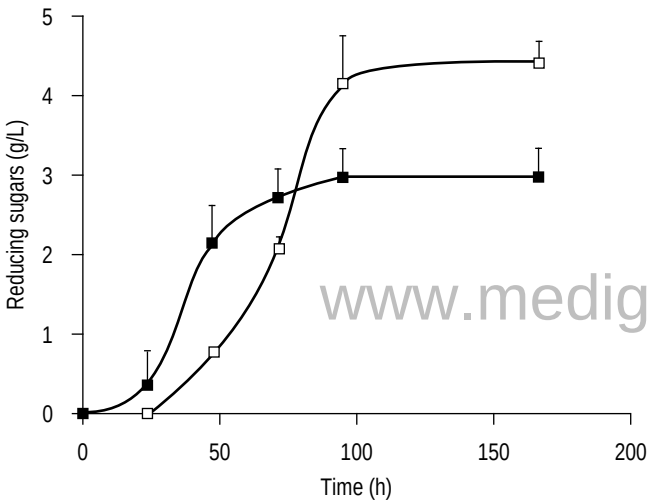

Figure 2. *Production of reducing sugars from lignocellulosic agave waste. Agave lignocelluosic waste without (-■-) and with (-□-) sulfuric acid treatment were hydrolyzed with a total crude enzyme coktail from* C. lindemuthianum. *The enzymatic activity was assay in accord to Habu et al., method (1997) and the reducing sugars produced were assay by DNS method. The agave bagasse was pre-washed with water, and then was hydrolyzed with 2 % H_2SO_4 at 150 °C per 10 min. N = 3, size simple = 3. Tukey ($\alpha < 0.05$).*

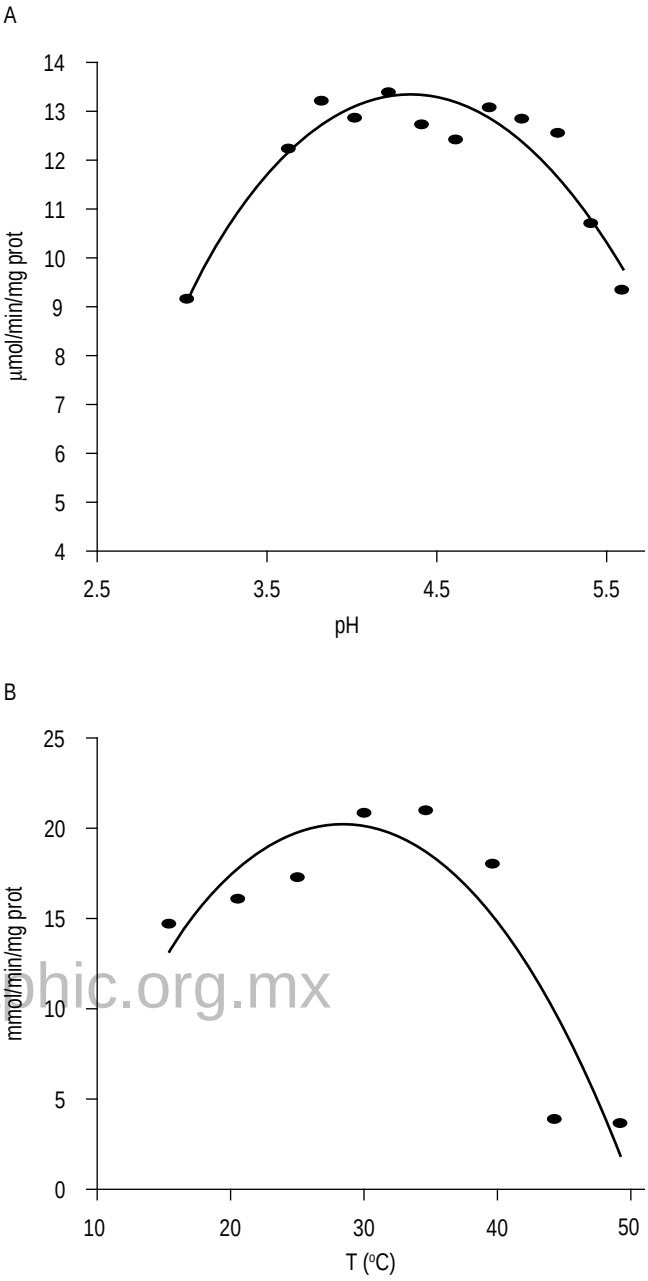

Figure 3. *Effect of pH and temperature on cellulases obtained of* C. lindemuthinaum. *The cellulase specific activity was measured with 1% agave cellulose waste acid treated as substrate. A) pH, B) Temperature. N = 3, size sample = 3. Tukey ($\alpha < 0.05$). Points represent means of each treatment.*

Rev Latinoam Microbiol 2009; 51 (1-2): 23-31

producers, the result was a selection of three fungal isolates named AFG 1-3, all of them produce β-glycosidases, endo- and exo-cellulases, assayed with the substrates; *p*-nitrophenyl glycoside, carboxymethyl cellulose and avicel, respectively (Flores Garcia e*t al*. 2005).

The solid lignocellulosic waste a major by-product of tequila industry was assayed with cellulases obtained from fungal AFG-3 isolate. The fungal cellulases hydrolyzed the agave lignocellulosic waste to mono- and oligosaccharides, recorded by reducing sugars (Figure 2), these enzymes have a broad range of optimal pH into acid zone and they are moderately resistant at high temperature for its enzyme work (Figure 3). The best result of the hydrolysis enzymatic was obtained with agave lignocellulosic waste pre-hydrolyzed with sulfuric acid. An explication is that there are not any residual monosaccharides that inhibiting the fungal cellulases or cellulose microcrystalline form is perturbed by acid treatment. After acid treatment it is observed that cellulose fibers are too far and broken in short fibers, doing that the cellulose fibers may be better exposed to enzymatic attack (Figure 4). When cellulases from C. *lindemuthianum* were compared to cellulases from another organism, based on the hydrolysis of the lignocellulosic residue pre-hydrolyzed with H_2SO_4, it was observed that the enzymatic activity of C. *lindemuthianum* cellulases was similar to commercial cellulases obtained from *Penicillium funiculatum*. Enzymatic activities values and saccharification index values were similar between them (Table 4). Also the enzymatic hydrolysis of crystalline cellulose101 (Sigma Co.) was less efficient with both the *C. lindemuthianum* cellulases as the commercial cellulases. Two explications are possible: the cellulases are inhibited by monosaccharides and these have scarcity tolerance to end products; or a poor substrate affinity of the cellulases by the cellulose in its natural microcrystalline form could be shown.

8. CONCLUSIONS

The study of *C. lindemuthianum* fungus represents a constant challenge for any scientific research. That is because this pathogenic fungus still held a lot of questions regarding to its biology. However, they may be related to the genetic, biochemical and molecular behavior of the fungus that may be considered as fungal ubiquitous process in the extracellular enzyme production during the phytopathogenesis event and cycle life. The extraordinary feeding behavioral pattern and the unique, biological development of *C. lindemuthianum* make it an interesting biological model of study. Furthermore, the economical importance of fungus when it grows as a biotroph in common bean plants (*P. vulgaris*) highly increases its relevance and the interest of the researchers toward its biology fields.

This organism -as a simple, unique, single cell- presents various differentiation processes and morphologies; the hy-

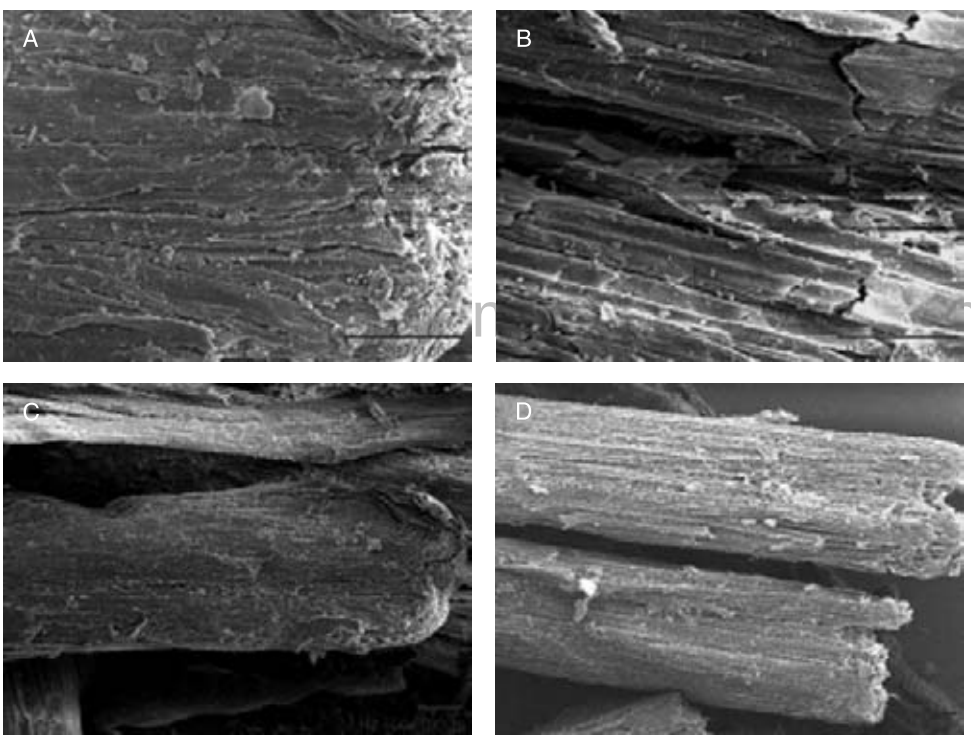

Figure 4. *Transmission micrographs of cellulosic fibers from agave waste. A. Fiber of agave waste without (A and C) and with (B and D) acid treatment. Cellulosic agave waste was treated with 2% H_2SO_4 at 150 °C by 10 min. Dry up fibers were coated with thin layer of cooper by 20 min/15 mA in Edwards S150A Sputter coater. A and B at 300 X; C and D at 100x.*

Rev Latinoam Microbiol 2009; 51 (1-2): 23-31

Table 4. Comparisson of enzymatic saccharification of lignocellulosic agave waste with two fungal cellulases source. 10 U/ml of fungal cellulases were added to 2 g of with or without acid hydrolysis lignocellosic agave waste suspended in 100 ml of 50 mM acetate buffer pH 4.8, then incubated at 40 °C in shaking. Saccharification percentage was determined at 96 h using DNS method.

Cellulases source	Acid hydrolysis	Saccharification (%)
C. lindemuthianum	+	34.3
	-	23.8
P. foniculatum™	+	29.73
	-	19.40

phae, the appresorium, the infection peg and the primary hyphae which have their own and independent cell functions. Therefore, the different basic mechanisms that govern the cell growth and differentiation may be approached in the same organism. At the same time, is very important to know the biochemical and genetic -mainly the DNA content- mechanisms that regulate the extraordinary morphological and colonial variability and how all this is related to the specific virulence effect against a certain common bean varieties. Is also of great importance the understanding of the pathology of new and virulent *C. lindemuthianum* wild isolates, and to know about the fungal adaptation to new and resistant bean varieties.

At industrial level the use of large amount of agave lignocellulosic waste to obtain any by-products right now is not possible, because there are not available cellulase enzyme preparations. However, *C. lindemuthianum* secretes an important level of cellulases in medium containing crystalline cellulose or agave lignocelluosic waste prehydrolyzed with sulfuric acid. From a different point of view, this fungus is an ideal model to study different aspects of its development such as growth, biochemical, molecular, cell differentiation and the production of extracellular enzyme with biotechnological applications.

9. ACKNOWLEDGMENTS

The research was support partially by grants from Consejo Estatal de Ciencia y Tecnología del Estado de Michoacan and Universidad Michoacana de San Nicolas de Hidalgo. AFG and JSL were postgraduate fellowship of CONACYT-Mexico.

10. REFERENCES

1. Acosta Rodriguez, I., M.G. Pinon Escobed, M.G. Zavala Paramo, E. López Romero, & H. Cano Camacho. Degradation of cellulose by the bean-pathogenic fungus *Colletotrichum lindemuthianum*. Production of extracellular cellulolytic enzymes by cellulose induction. Antonie van Leeuwenhoek, 2005; 87: 301-310.
2. Alexopoulos, C.J. & C.W. Mims. 1979. Introductory Mycology. Third Edition. Ed. Wiley.
3. Alzate Marin, A.L., G. Soares Baia, T.J. de Paula Junior, G.A. de Carvalho, E. Gonzalves de Barros & M. Alves Moreira. Inheritance of anthracnose resistance in common bean differential cultivars AB 136. Plant Disease, 1995; 81: 996-998.
4. Anderson, A.J. Extracellular enzymes produced by *Colletotrichum lindemuthianum* and *Helmintosporium maydis* during growth on isolated bean and corn walls. Phytopathology, 1978; 68: 1585-1589.
5. Anderson, D.W. & R.L. Nicholson. Characterization of a laccase in the conidial mucilage of *Colletotrichum graminicola*. Mycologia, 1996; 88: 996-1002.
6. Bailey, J.A., R.J. O´Connell, R.J. Pring & C. Nash. 1992. Infection strategies of *Colletotrichum* species. pp. 88-120. In: J.A. Bailey & M.J. Jeger (Eds). *Colletotrichum*: Biology, Pathology and control CAB International, Wallingford.
7. Balardin, R.S., A.M. Jarosz & J.D. Kelly. . Virulence and molecular diversity in *Colletotrichum lindemuthianum* from South Central, and North America. Phytopathology, 1997; 87: 1184-1191.
8. Basset, M.J. List of genes of *Phaseolus vulgaris*. Annual Report of the Bean Improvement Cooperative, 1996; 39: 1-19.
9. Bonnen, A.M. & R. Hammerschmidt. Role of cutinolytic enzymes in infection of cucumber by *Colletotrichum lagenarun*. Physiology and Molecular Plant Pathology, 1989; 35: 475-481.
10. Centis, S., B. Dumas, J. Fournier, M. Marolda & M-T. Esquerré-Tugayé. Isolation and sequence analysis of CLPG1, a gene coding for an endopolygalacturonase of the phytopathogenic *Colletotrichum lindemuthianum*. Gene, 1996; 170: 125-129.
11. Centis, S., I. Guillas, N. Sejalon, M-T. Esquerre-Tugaye & B. Dumas. Endopolygalacturonase genes from *Colletotrichum lindemuthianum*. Cloning of CLPG2 and comparison of its expression to that of *CLPG1* during saprophytic and parasitic growth of the fungus. Molecular Plant Microbe Interactions, 1997; 10: 769-775.
12. Charrier, A. & H. Bennerot. Contribution al etude des races physiologiques de l´ anthracnose du haricot. Annals of Phytopathology, 1970; 2: 489-506.
13. Dickman, M.B., S.S. Patil & P.E. Kolattukudy. Effect of organophosphorus pesticides on cutinase activity and infection of papaya by *Colletotrichum gloesporoides*. Physiological Biochemistry 1983; 73: 1209-1214.
14. Dickman, M.B. & S.S. Patil. Cutinase deficient mutants of *Colletotrichum gloesporoides* are nonpathogenic to papaya fruit. Physiology and Molecular Plant Pathology 1986; 28: 235-243.
15. Dillard, H.R. & A.C. Cobb.. Survival of *Colletotrichum lindemuthianum* in bean debris in New York State. Plant Disease 1993; 77: 1233-1238.
16. Drijfhout, E. & H.J.C. Davis. Selection of a new set of homogeneously reacting bean (*Phaseolus vulgaris*) differential to differentiate races of *Colletotrichum lindemuthianum*. Plant Pathology 1989; 38: 391-396.
17. Dufresne, M., J.A. Bailey, M. Dron & T. Langin. clk1, a serin/threonine protein kinase encoding gene, is involved in pathogenicity of *C. lindemuthianum* on common bean. Molecular Plant Microbe Interactions 1998; 11: 99-108.
18. Dumas, B., C. Borel, C. Hebert, J. Maury, C. Jaquet, R. Balsse & M.T. Esquerre-Tugaye. Molecular characterization of *CLPT1*, a SEC-4 like Rab/GTPase of the phytopathogenic fungus *Colletotrichum lindemuthianum* which is regulated by the carbon source. Gene 2001; 272: 219-225.
19. Flores Garcia, A. & M.M. Martinez Pacheco. Capacidad celulolítica de *Colletotrichum lindemuthianum* en la fase saprofitica. Ciencia Nicolaita 2005; 42: 60-72.
20. Green, J.R., N.A. Pain, M.E. Cannell, G.L. Jones, C.P. Leckie, S. McCready, K. Mendgen, A.J. Mitchell, J.A. Callow and R.J. O'Connell. Analysis of differentiation and development of the specialized infection

structures formed by biotrophic fungal plant pathogens using monoclonal antibodies. Canadian Journal of Botany 1995; 73: s408-s417.
21. Habu, N., K. Igarashi, M. Samejima, B. Petersson & K-E. Eriksson. Enhanced production of cellobiose dehydrogenase in cultured of *Phanerochaete crysosporium* supplemented with bovine calf serum. Biotechnology and Applied Biochemistry 1997; 20:97-102.
22. Heath, M.C. & D. Skalamera. Cellular interaction between plants and biotrophic fungal parasites. In: Tommerup IC, Andrews JH, ed. Academic Press. Advances in Botany Research 1997; 24: 196-225.
23. Hughes, H.B., R. Carzaniga, S.L. Rawling, J.R. Green & R.J. O'Connell. Spore surface glycoproteins of *Colletotrichum lindemuthianum* are recognized by a monoclonal antibody, which inhibits adhesion to polystyrene. Microbiology 1999; 147: 1927-1936.
24. Hubbeling, N. Resistance in beans to the lambda race of *Colletotrichum lindemuthianum*. Proc. 19th International Horticultural Congress 1A 1974: 293.
25. Keon, J.P.R., G. Waksman & J.A. Bailey. A comparison of the biochemical and physiological properties of a polygalacturonase from two races of *Colletotrichum lindemuthianum*. Physiological and Molecular Plant Pathology 1990; 37: 193-206.
26. Kubo, Y., & I. Furusawa. Melanin biosyntheses: Pre-requisite for successful invasion of the plant host by appressoria of *Colletotrichum* and *Pyricularia*. In: G.T. Cole & H.C. Hoch (Plenum, Eds). The fungal spore and disease initiation in plants and animals. New York. 1991: 205-218.
27. Mercure, E.W., B. Leite & R.L. Nicholson. Adhesion of ungerminated conidia of *Colletotrichum graminicola* to artificial hydrophobic surfaces. Physiology and Molecular Plant Pathology 1994; 45: 421- 440.
28. O'Connell, R.J. Absence of a specialized interface between intracellular hyphae of *Colletotrichum lindemuthianum* and cells of *Phaseolus vulgaris*. New Phytopatologist 1987; 107: 725-734.
29. O'Connell, R.J., N.A. Pain, K.A. Hutchinson, G.L. Jones & J.R. Green. Ultrastructure and composition of the cells surfaces of infection structures formed by the fungal plant pathogen *Colletotrichum lindemuthianum*. Journal of Microscopy 1996; 181: 204-212.
30. O'Sullivan, D., P. Tosi, F. Creusot, B.M. Cooke, T.H. Phan, M. Dron & T. Langin. Variation in genome organization of the plant pathogenic fungus *Colletotrichum lindemuthianum*. Current Genetic 1998; 33: 291-298.
31. Pain, N.A., J.R. Green, G.L. Jones & R.J. O'Connell. Composition and organization of extracellular matrices around germ tubes and appressoria of *Colletotrichum lindemuthianum*. Protoplasma 1996; 190: 119-130.
32. Pain, N.A., R.J. O'Connell & J.T. Green. A plasma membrane associated protein is a marker for differentiation and polarization of *Colletotrichum lindemuthianum* appresoria. Protoplasma 1995; 188: 1-11.
33. Pastor Corrales, M.A., M.M. Otoya, A. Molina & S.P. Singh. Resistance to *Colletotrichum lindemuthianum* isolates from Middle America and Andean South America in different common bean races. Plant Disease 1995; 79: 63-67.
34. Perfect, S.E., R.J. O'Connell, E.F. Green, C. Doering-Saad & J.R. Green. Expression cloning of a fungal proline-rich glycoprotein specific to the biotrophic interface formed in the *Colletotrichum*-bean interaction. Plant Journal 1998; 15: 273-279.
35. Rodriguez, R.J. & O.C. Yoder. Selectable genes for transformation of the fungal plant pathogen *Glomerella cingulata* fsp *phaseoli* (*Colletotrichum lindemuthianum*). Gene 1987; 54: 73-81.
36. Sánchez García, B.M., A. Flores Oliva, A. Sánchez Arizpe, S. Pineda Rodríguez, G. López Jiménez, S. Fraire Velasquez, E.R. Garrido Ramírez, J.A. Acosta Gallegos, J. Simpson Williamson & R. Rodriguez Gerra. *Colletotrichum lindemuthianum* pathotypes in Oaxaca and San Luis Potosi, Mexico and resistance in common bean. Agricultura Tecnica de Mexico 2009; 35: 46-57.
37. Schnock, M.G., G.M. Hoffman & J. Kruger. A new physiological strain of *Colletotrichum lindemuthianum* infecting *Phaseolus vulgaris* L. Horticultural Science 1975; 10: 40.
38. Sela-Buurlage, M.B., L. Epstein & R.J. Rodriguez. Adhesion of ungerminates *Colletotrichum musae* conidia. Physiology and Molecular Plant Pathology, 1991; 39: 345-352.
39. Sicard, D., Y. Michelakis, M. Dron & C. Neema. Variability of resistance to *Colletotrichum lindemuthianum* in the three centers of diversity of common bean of its host *Phaseolus vulgaris*. Phytopatology 1997; 87: 807-813
40. Tamayo, P.J., M. Otoya & M.A. Pastor-Corrales. Diversidad de los patotipos de *Colletotrichum lindemuthianum*, el patógeno de la antracnosis, en Río Negro, Antioquia. Fitopatología Colombiana, 1995; 19: 1-6.
41. Templeton, M.D., E.H.A. Rikkerink, S.L. Solon & R.N. Crowhurst. The cloning and molecular characterization of the glyceraldheyde 3-phosphate dehydrogenase encoding gene and cDNA from the plant pathogenic fungus *Glomerella cingulata*. Gene 1992; 122: 225-230.
42. Tu, J.C., J.W. Sheppard & D.M. Laidlaw. Occurrence and characterization of the epsilon race of bean anthracnose in Ontario. Plant Disease 1984; 68: 69-70.
43. Tsigos, T.Y. & V. Bouriotis. Purification and characterization of chitin deacetylase from *Colletotrichum lindemuthianum*. Journal of Biological Chemistry 1995; 270: 26286-26291.
44. Van Dyke, C.G. & C.W. Mims. Ultrastructure of conidia, conidum germination and appressorium development in the plant pathogenic fungus *Colletotrichum truncatum*. Canadian Journal of Botany, 1991; 69: 2455-2467.
45. Wattard, C., D. Kobiler, A. Dinoor & D. Prusky. Pectate lyase of *Colletotrichum gloesporoides* attacking avocado fruit: cDNA cloning and involvement in pathogenicity. Physiology and Molecular Plant Pathology 1997; 50: 197-212.
46. Wijesundera, R.L.C., J.A. Bailey, R.J.W. Byrde & A.H. Fielding. Cell wall degrading enzymes of *Colletotrichum lindemuthianum*: their role in the development of bean anthracnose. Physiology and Molecular Plant Pathology 1989; 34: 403-413.
47. Yerkes, W.D. & O.M. Telis. New races of *Colletotrichum lindemuthianum* in Mexico. Phytopathology 1956; 46: 564-567.
48. Young, D.H. & H. Kauss. Adhesion of *Colletotrichum lindemuthianum* spores to *Phaseolus vulgaris* hypocotyls and polystyrene. Applied and Environmental Microbiology 1984; 47: 616-619.

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