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VIRULÊNCIA, ENZIMAS LENHINOLÍTICAS E PERFIL METABÓLICO DE CRYPHONECTRIA PARASITICA EM ESTIRPES VIRULENTAS E HIPOVIRULENTAS CONVERTIDAS POR CHV1 HIPOVIRUS

VIRULENCE, LIGNINOLYTIC ENZYMES AND METABOLIC PROFILE OF CRYPHONECTRIA PARASITICA VIRULENT AND HYPOVIRULENT STRAINS CONVERTED BY CHV1 HYPOVIRUS

VIRULENCIA, ENZIMAS LIGNINOLÍTICAS Y PERFIL METABÓLICO DE CRYPHONECTRIA PARASITICA EN CEPAS VIRULENTAS E HYPOVIRULENTAS CONVERTIDAS POR CHV1 HIPOVIRUS

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RESUMO

Introdução: *Cryphonectria parasitica*, fungo responsável pelo cancro do castanheiro, causa lesões necróticas (cancros corticais) no tronco e ramos das árvores hospedeiras. O hipovírus, *Cryphonectria hypovirus* 1 (CHV1) reduz a virulência (hipovirulência) e altera a morfologia do fungo em cultura (redução da pigmentação e esporulação). As estirpes hipovirulentas CHV1 são utilizadas com sucesso na Europa como agentes de controlo biológico do Cancro do castanheiro.
Objetivos: O objetivo deste trabalho foi compreender o efeito do hipovírus na virulência e metabolismo do fungo, comparando a produção de algumas enzimas lenhinolíticas e os perfis metabólicos de estirpes virulentas e estirpes isogénicas de *C. parasitica* convertidas (com CHV1).
Métodos: A virulência de cada isolado foi avaliada por inoculação de micélio do fungo em maçãs (cv. Golden Delicious) e em ramos destacados de castanheiro com um ano de crescimento. Para a deteção da atividade de enzimas lenhinolíticas (lacases, peroxidases e celulases) foram usados vários substratos e compostos indicadores. O perfil metabólico de *C. parasitica* foi avaliado pelo sistema Biolog FF microplates pela utilização de 95 fontes diferentes de carbono.
Resultados: A utilização de MicroPlacas FF (Biolog, Inc.) indicaram que a utilização de 95 fontes de carbono pelos cinco isolados de *C. parasitica*, foram significativamente diferentes (p <0,001), quando os substratos foram agrupados em seis tipos de compostos químicos. Os maiores valores de AWCD foram obtidos para os hidratos de carbono, ácidos carboxílicos e polímeros, e os menores valores para os grupos aminas / amidas, aminoácidos e compostos diversos.
Conclusões: A avaliação da virulência de isolados de *C. parasitica* é importante para o estudo dos processos de hipovirulência mediados pelo hipovírus CHV1. Os ramos destacados de castanheiro foram, em nosso estudo, mais adequados que o teste em maçã para diferenciar as estirpes hipovirulentas das virulentas de *C. parasitica*. Os isolados virulentos evidenciaram sempre uma maior atividade de lacase induzida por ácido tânico (Lac3) e de outras enzimas lenhinolíticas (LiP, MnP e celulase) quando comparadas com os hipovirulentos. Os resultados da análise dos perfis metabólicos mostram que alguns grupos de substratos foram mais consumidos por isolados hipovirulentos. Estes estudos abrem novas perspetivas para entender o processo biológico usado pelo hipovírus, e sugerem que este é um método para discriminar estirpes hipovirulentas, e estudar a ecologia e a aptidão em campo destes isolados do fungo.

Palavras-chave: *Cryphonectria parasítica;* virulência, perfil metabólico; Biolog FF MicroPlates; enzimas lenhinolíticas; *Cryphonectria hypovirus* 1 (CHV1)

ABSTRACT

Introduction: *Cryphonectria parasitica*, the causal agent of chestnut blight, causes necrotic lesions (so-called cankers) on the bark of stems and branches of susceptible host trees. *Cryphonectria hypovirus* 1 (CHV1) infects *C. parasitica* and reduces the fungus virulence (hypovirulence) and alters the fungus morphology in culture (pigmentation and sporulation capacity). By these characteristics, the strains with hypovirus CHV1 are used in Europe as a biological control agent of chestnut blight.
Objectives: The aim of this work is to understand the effect of hypovirus on fungi virulence by comparing the production of some lignin-degrading enzymes and the metabolic profiles of some isogenic virulent and hypovirulent (converted and original) strains.
Methods: The virulence of each strain was evaluated by mycelial inoculation on apple fruits (cv. Golden Delicious) and on detached, one year old, chestnut branches. To detect the activity of ligninolytic enzymes (laccases, peroxidases and cellulases), various substrates and indicator compounds were used. The metabolic profile of *C. parasitica* was evaluated by the Biolog FF system using 95 different carbon sources.
Results: Virulent strains were found to cause more significant necrotic lesions in chestnut branches ($p<0.05$) and to produce larger lignin-degrading enzymes. The use of Biolog FF MicroPlates indicated that the use of 95 carbon sources five isolates of C. parasitica were significantly different ($p <0.001$), when the substrates were grouped into six types of chemical compounds. The highest AWCD values were obtained for carbohydrates, carboxylic acids and polymers, and the lowest values for amines/amides, amino acids and miscellaneous.
Conclusions: Virulence evaluation of *C. parasitica* strains is important to study the hypovirulence processes mediated by the hypovirus CHV1. The detached branches of chestnut were, in our study, more suitable than apple fruits test in discriminating hypovirulent from virulent strains of *C. parasitica*. Virulent strains showed higher activity of acid-tannic inducible laccase (Lac3) and other lignin-degrading enzymes (LiP, MnP, and cellulase) when compared with hypovirulent ones. The results of the metabolic profiles studies may lead to new perspectives for understanding the biological process used by the hypovirus therefore, this may suggest a method for discriminating hypovirulent strains and study ecology and field fitness in this fungal strains.

Keywords: *Cryphonectria parasitica*; virulence; metabolic profile, Biolog FF MicroPlates; ligninolytic enzymes; *Cryphonectria hypovirus* 1 (CHV1)

RESUMEN

Introducción: *Cryphonectria parasitica*, el agente causal del Chancro del castaño, causa lesiones con necrosis (chancros corticales) en la corteza de los troncos y ramas de los árboles de hospedadores susceptibles. *Cryphonectria hypovirus* 1 (CHV1) y reduce la virulencia (hipovirulencia) con alteración de la morfología del hongo en cultivo (pigmentación y capacidad de esporulación). Por estas características, el micovirus CHV1 se usa en Europa como agente de control biológico del Chancro del castaño.
Objetivos: El objetivo de este proyecto es comprender el efecto del hipovirus en la virulencia del hongo mediante la comparación de la producción de algunas enzimas ligninolíticas y de los perfiles metabólicos de algunas cepas virulentas e hipovirulentas (convertidas y originales).
Métodos: La virulencia de cada aislado se evaluó inoculando el micelio del hongo en manzanas (cv. Golden Delicious) y en ramas separadas de castaño con un año de crecimiento. Para la detección de la actividad de las enzimas ligninolíticas (lacasas, peroxidasas y celulasas) se utilizaron varios sustratos y compuestos indicadores. El perfil metabólico de *C. parasitica* fue evaluado por el sistema Biolog FF utilizando 95 fuentes de carbono distintas.
Resultados: Se constató que las cepas virulentas causan lesiones significativamente más grandes en las ramas de castaño ($p < 0,05$) y producen más enzimas ligninolíticas. El uso de Biolog FF MicroPlates indicaron que el uso de 95 fuentes de carbono por los cinco aislados de C. parasitica eran significativamente diferentes ($p <0.001$), cuando los sustratos se agruparon en seis tipos de compuestos químicos. Los valores más altos de AWCD se obtuvieron para carbohidratos, ácidos carboxílicos y polímeros, y los valores más bajos para las aminas /amidas, aminoácidos y compuestos diversos.
Conclusiones: La evaluación de la virulencia de las cepas de *C. parasitica* es importante para estudiar los procesos de hipovirulencia mediados por el hipovirus CHV1. Las ramas de castaño fueron, en nuestro estudio, más adecuadas que las manzanas para discriminar cepas hipovirulentas y virulentas de *C. parasitica*. Los aislados virulentos mostraron una mayor actividad de lacasa inducible por ácido tánico (Lac3) y otras enzimas ligninolíticas (LiP, MnP y celulasa) en comparación con los hipovirulentos. Los resultados de los perfiles metabólicos obtenidos muestran que algunos grupos químicos de sustratos fueron más consumidos por cepas hipovirulentas. Estos estudios pueden conducir a nuevas perspectivas para entender el proceso biológico utilizado por el hipovirus, por lo tanto, esto puede sugerir que este es un método para discriminar cepas hipovirlulentas y para estudiar la ecología y la aptitud de campo de estas cepas de hongos.

Palabras Clave: *Cryphonectria parasitica* virulencia; perfile metabólico; Microplacas Biolog FF; enzimas ligninolíticas; *Cryphonectria hypovirus* 1 (CHV1)

INTRODUCTION

1. THEORETICAL FRAMEWORK

The chestnut is one of the most useful trees in temperate regions with great social, ecological and economic value in mountainous regions, providing fruits of high carbohydrate food source and also timber. The chestnut fruit production is affected by the presence of lethal diseases as the Ink disease (*Phytophthora cinnamomi* Rands and *P. cambivora* (Petri) Buisman) and Chestnut blight (*Cryphonectria parasitica* (Murr.) Barr) as well as by insect pests (*Cydia splendana* HB, *Curculio elephas* Gyll and *Dryocosmus kuriphilus* Yasumatsu).

C. parasitica is a fungus of the OEPP list A2, responsible for the high current mortality of chestnut trees. It was first detected in 1904 on American chestnut (*Castanea dentata* (Marsh) Borkh) and was introduced in Italy in 1938 and progressively caused the destruction of large chestnut populations (*Castanea sativa* Mill.) in all European countries. Population biology and disease control of chestnut blight by hypovirulence have been presented in many and excellent reviews (Heiniger and Rigling 1994; Milgroom and Cortesi 1999; Robin and Heiniger 2001; Rigling and Prospero 2017). In Portugal, the disease has been present since 1989 (Abreu 1992) and is currently distributed throughout the country's chestnut regions. In terms of spatial distribution, the disease is characterized by the existence of places with a high incidence, with a high number of diseased trees, and other places with less extension and severity (Gouveia *et al*. 2001; Bragança *et al*. 2005). Characteristic symptoms of the disease are extensive necrosis (cankers) in the bark on branches and trunks that rapidly increase in size resulting in tree death (Rigling and Prospero 2017) causing severe environmental and economic costs.

This pathogen can be naturally infected by a hypovirus - *Cryphonectria hypovirus* 1 (CHV1) that induces modifications in morphological characteristics that include reduced asexual sporulation, and pigmentation capacity, it also diminishes the activity of pathogenesis–related enzymes, like oxaloacetate acethylhydrolase (OAH) (Chen *et al*. 2010) and laccase (Chung *et al*. 2008). Therefore, the introduction of hypovirulent strains has been used as a biological control method, which has been adopted and applied experimentally in Portugal since 2015 (Gouveia *et al*. 2016).

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This study provides a new combination of biological and analytical approaches to evaluate the changes related to virulence morphological characteristics, oxidative enzymes activity and metabolic profiles of virulent *C. parasitica* and its isogenic hypovirulent after the infection of the fungi with the *Cryphonectria hypovirus* 1 (CHV1).

2. METHODS

2.1 Isolates

C. parasitica isolates used in this study, listed in Table 1, were obtained from different chestnut field plantations in Trás-os-Montes region (Portugal). Isolates converted by the characterized hypovirulent strain RB111 (CHV1 donor) were also included in this study. These strains were maintained on Potato Dextrose Agar (PDA, 39g/L - Difco) agar slants at 6 - 8 °C.

Table 1 - *Cryphonectria parasitica* (virulent, hypovirulent and CHV1 converted) isolates used in this study

Date	Code	VCG	Sites	Virulence
2018	Cast13	EU11	Castrelos (Bragança)	Virulent
2018	Cast26	EU11	Castrelos (Bragança)	Virulent
2018	VBC02	EU11	Vila Boa Carção (Bragança)	Virulent
2011	RB111(CHV1 donor)	EU11	Rio Bom (Valpaços)	Hypovirulent
2018	Cast13c	EU11	Cast13 (CHV1 converted)	Hypovirulent
2018	Cast26c	EU11	Castrelos26 (CHV1 converted)	Hypovirulent
2018	VBC02c	EU11	Vila Boa Carção (CHV1 converted)	Hypovirulent
2017	Serra05	EU11	Serra (Chaves)	Hypovirulent
2013	SR442	EU11	Sergude (Felgueiras)	Hypovirulent
2018	Cast07	EU66	Castrelos (Bragança)	Virulent
2018	Cast17	EU66	Castrelos (Bragança)	Virulent
2014	VDP11	EU66	Vilar Peregrinos (Vinhais)	Virulent

2.2 Converted Isolates

Three virulent isolates (Cast13, VBC02, Cast26) were converted with a characterized hypovirulent *C. parasitica* CHV1 isolate (RB111) by hyphal anastomose as described by Rigling *et al.* (1989). Mycelium of the converted strains was transferred to fresh PDA medium and maintained at 6-8 °C at the Instituto Politécnico de Bragança culture collection.

2.3 CHV1 detection

The presence of CHV1 (*Cryphonectria hypovirus* 1) in converted isolates was confirmed through molecular methods. Total RNA was isolated from mycelium using the NorGen BioTek kit (Thorold, ON, Canada). Extraction was done using lysis buffer, precipitated with 100 % ethanol and washed in columns with wash solution as manufacturer instructions. The RNA was dissolved in elution buffer and stored at -20°C. To obtain cDNA, 3µl of total RNA was diluted in 11µl of RNase free water and incubating at 100°C for 2 minutes. The RNA dilution was mixed with 4µl of 5x Reaction Mix (Sigma) and 2µl Maxima Enzyme Mix (Sigma) and incubated under the following conditions: 10 minutes at 25°C, 30 minutes at 50°C and 5 minutes at 85°C. The ORF-A region was amplified using the primers hvep-1F (5'- TGACACGGAAGCTGAGTGTC-3´) and EP-721-4 (5´-GGAAGTCGGACATGCCCTG-3´). For the ORF-B region, the primers orfB-12aF (5´-AGACCTCAATCGGGTCTCCCT - 3´) and orfB-12aR (5´- TTCAACCACACGACGAGTTCG - 3´) were utilized. PCR amplification was performed using 1 µl of cDNA in a total of 50 µl reaction volume consisted of 10 µl of 2X Jump Start (Sigma) and 1 µl of each primer (20 pmol/µl). Thermal cycling was set up with an initial denaturation at 94°C for 2 min, followed by 33 cycles consisting of 94°C denaturation for 1 min, annealing at 55°C for 1.5 min, and elongation at 72°C for 2 min, with a final extension at 72°C for 8 min. The PCR products were visualized by agarose gel electrophoresis on 1.5 % gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, Inc) under UV illumination.

2.4 Virulence assays

2.4.1 Virulence test evaluation in apple fruits: For this assay, five isolates of *C. parasitica* (Cast13, VBC02, RB111 (CHV1 donor), Cast13c, VBC02c) were used. A lot of homogeneous apples, without defects or decay, were washed in distilled water and dried. A sterilized Pasteur glass pipette was used to obtain circular mycelial sections of *C. parasitica* for inoculation in the apple's holes made by a corky borer (5mm diameter). Each of the three replicates was identified and apples incubated at 24°C in the dark for ten days. The presence of brown lesions and their growth (length and depth) were evaluated in mm after ten days of incubation. The lesion area (cm^2) was calculated from mean radii and the rot volume (cm^3) was calculated by the mathematical formula of the cone.

2.4.2 Virulence evaluation in detached one-year growth branches of chestnuts: Chestnut branches were collected in *C. sativa* nurseries at ESAB-IPB open fields. Chestnut branches were cut into sections with approximately 20 cm long. On both cut sides of the branches, paraffin wax was applied to avoid desiccation. In the middle of each branch a cork borer (3mm diameter) was used to excise a disk of the bark tissues, and inoculations of two virulent strains of *C. parasitica* (Cast13, VBC02) and three hypovirulent strains (Cast13c, VBC02c and the CHV1 donor RB111. The name of the isolate was registered in the branch. Then, the inoculation sites were covered with cotton wool moistened with distilled water and surrounded with parafilm to avoid desiccation. Finally, chestnut branches (three branches from each isolate) were placed on a tray and incubated at 25°C. Inoculated branches were checked for fungal growth after ten days. Fungal growth was assessed as the vertical and horizontal expansion of the visible necrotic area. The area of each necrosis was calculated using the mathematical formula for elliptic surfaces.

2.5 Qualitative enzymatic analysis

Nine strains listed in Table 1 (six virulent strains, represented by three isolates of vc type EU11 and three isolates of vc type EU66; and three (wild) hypovirulent isolates were used for qualitative evaluation of enzymatic activity in Petri plate assays. A set of tests was used to detect the activity of laccases, peroxidases (lignin peroxidases - LiP, and manganese peroxidases - MnP) and cellulases, enzymes associated with plant cell-wall degradation caused by fungi.

The capacity of *C. parasitica* strains to origin brown oxidation zones around the colony (in Bavendamm test), or to decolorize the dyes inside the media, creating a halo around the colonies (in the other tests) was evaluated in four different tests, with three replicates each.

Bavendamm test (phenol oxidase test): The medium contained 1.5% malt extract, 1.5% agar and 0.5% tannic acid, pH=4.5. The solution with tannic acid was prepared, autoclaved separately and mixed with the other components before pouring into Petri plates. These plates were inoculated with plugs from fungal strains and incubated at 25°C in darkness. This test was used to detect acid-tannic inducible laccases (as *C. parasitica* Lac3).

Screening for laccases and peroxidases (LiP and MnP): It was used malt extract agar (MEA) medium supplemented with 0.04% Remazol Brilliant Blue R (RBBR) and 200µM CuSO4. The solutions of 20% of RBBR and 400mM of CuSO4 were prepared and filtered through a 0.2µm filter. As a control, two uninoculated plates were used: one with the dye (as abiotic control) and one without dye (as biotic control).

Peroxidases test: For peroxidases evaluation it was used PDA with 25mg/l Azure B dye added. After sterilization it was aseptically transferred into rectangular Petri dishes, inoculated and incubated at 25°C in darkness.

Cellulase medium: The medium was prepared with 0.5% of carboxymethyl cellulose (CMC) and 1.6% agar for the growth of nine isolates. Four days after inoculation and incubation at 25°C, the plates were flooded with a 0.1% solution of Congo Red for 45min, then the stain was poured off, and they were destained with a 1M solution of NaCl for 15min. An uninoculated plate was used as a control for media discoloration.

2.6 Metabolic profile characterization

For metabolic profile characterization two virulent isolates (Cast13 and VBC02), their converted ones (Cast13c and VBC02c) and one hypovirulent strain (RB111) were grown in 250 ml Erlenmeyer with 100 ml of PDB (Potato Dextrose Broth, 24 g/L). Erlenmeyer's were placed at 25°C in orbital shaking at 110 rpm for four days. After this the mycelium was filtered and washed with sterile water and disperser with an Ultra Turrax and transferred to glass bottles.

The global phenotypes and the utilization of 95 low molecular weight carbon sources (plus a negative control) by each of the isolates, were evaluated using the Biolog FF Microplate (Biolog Inc.), following manufacturer instructions. The obtained mycelium (viable and non-contaminated) was suspended in FF inoculating fluid supplied by Biolog in glass tubes (Cat. Nº 1006), mixed gently and adjusted to approx. 75 % transmittance at 590 nm using a Biolog Turbidimeter, previously calibrated using an FF Biolog Turbidity standard (Cat. Nº 3426). Mycelium suspensions (100 µl) was added to each well, and the FF MicroPlates were then incubated at 25°C in the dark. The optical density at 490 nm (mitochondrial activity) was determined using an ASYS UVM 340 microplate reader (Hitech GmbH) for each plate at 24 h intervals over the next seven days. Carbon sources were considered not utilized in wells in which colour development was less than, or equal to, that of negative controls. The capability of isolates to utilize different carbon sources was measured by average well-colour development (AWCD) (Garland and Mills, 1991).

2.7 Statistical analysis

Soft brown rot lesions caused by different isolates on apple fruit, and necrotic lesions on young detached chestnut branches were recorded and analysed using IBM-SPSS statistics, version 19 (SPSS Inc, 2010). Data of *C. parasitica* isolates were evaluated for normality with Kolmogorov-Smirnov and statistical analyses were performed using one-way analysis of variance (One-way ANOVA) followed by a post hoc test of LSD and Tuckey.

For the metabolic profiles analyses the average well colour development (AWCD) were calculated seven days after incubation, where AWCD equals the sum of the difference between the OD of the blank well (control) and substrate wells, divided by 95 (the number of substrate wells in the FF Microplate). AWDC of the five isolates, obtained seven days after inoculation, and the

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utilization of different groups of composts, were analysed using SPSS statistics analysis software, version 19 (SPSS Inc, 2010). Significant differences were determined by Duncan's multiple range test, and *P* values less than 0.05 were considered significant. Multivariate analyses were performed in PAST v.3.18 to reduce the number of variables resulting from metabolic profiles (Biolog-carbon source utilization), using Principal Component Analysis (PCA).

3. RESULTS

Conversion of virulent *C. parasitica* isolates and CHV1 detection

The results of conversions tests between each of the virulent strains: VBC02, Cast26 and Cast13 with the donor hypovirulent RB111 are shown in Figure 1A. Converted isolates (Cast13c, Cast26c and VBC02c) had no orange pigmentation and no sporulation when grown on PDA medium culture.

Hypovirulent strains (RB111, Cast13c and VBC02c), when compared with virulent ones showed a slow growth rate with an average of 6 mm per day, white mycelium and no spores production. On the other hand, virulent strains Cast 13 and VBC02 showed a higher growth rate with an average of 10 mm per day, orange mycelium colour, lobated colony margins and spores production.

Two converted strains were tested for the presence of hypovirus CHV1 and the donor strain RB111 was used as control. These results are shown in Figure 1B.

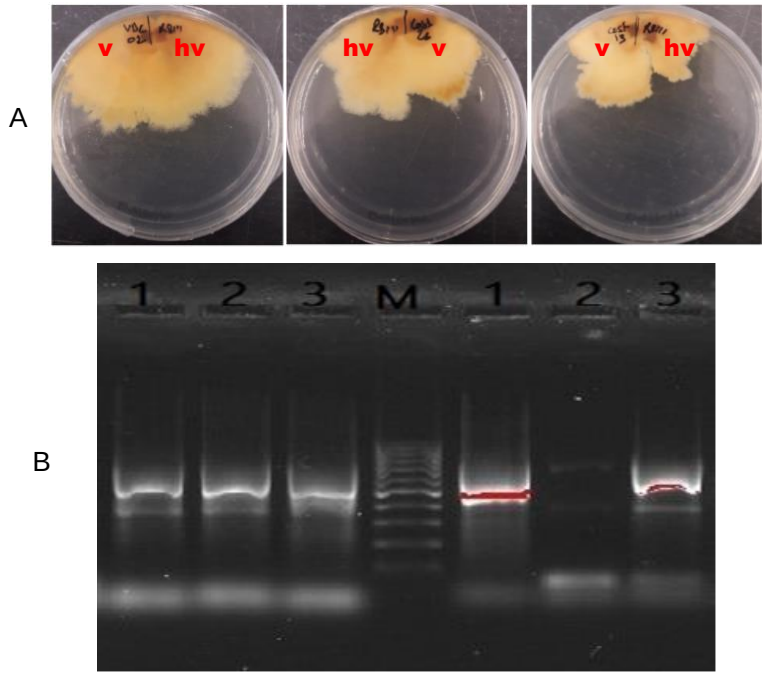

Figure 1 – A: Conversion of virulent strains of *Cryphonectria parasitica* VBC02, Cast26 and Cast13 by RB111 the CHV1 donor. B: Detection of CHV1 hypovirus in converted strains of *Cryphonectria parasitica* using two different set of primers amplifying ORFA (right) and ORFB (left). (Lanes 1: Cast13c; 2: RB111; 3: VBC02c; M: 100 bp DNA marker).

Virulence evaluation tests

All inoculated apple fruits (cv. Golden Delicious) produced soft brown rot lesions (Figure 2B) and all inoculated detached chestnut branches developed necrotic tissues (Figure 3B). Apple fruit rot lesion measurements showed that rot tissue volume caused by VBC02 is higher than caused by its converted one (VBC02c). The pattern between Cast13 and Cast13c is not significantly different as shown in Figure 2A. Rot tissue volume of the hypovirulent RB111 is higher than Cast13, a virulent isolate.

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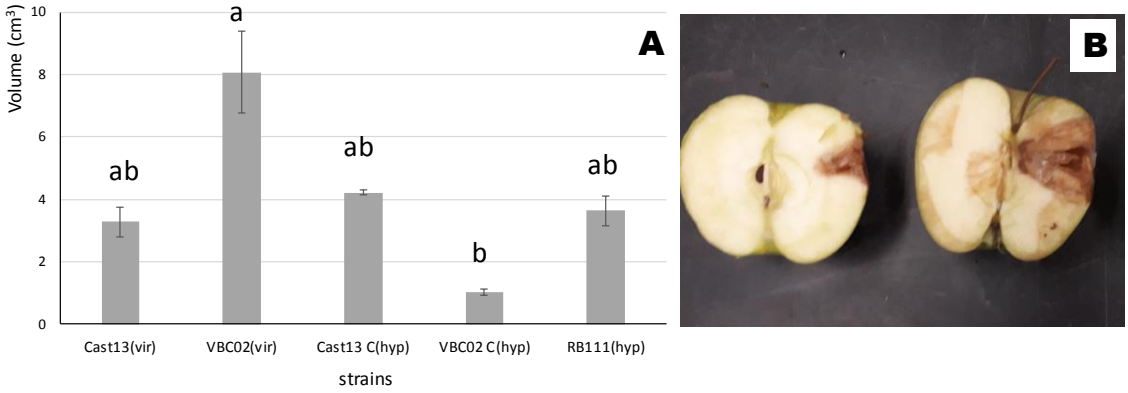

Figure 2 – A: Mean brown rot volumes (mean ± SE) on apples (cv. Golden Delicious) caused by virulent and hypovirulent isogenic strains and the hypovirulent CHV1 donor of *Cryphonectria parasitica,* 10 days after inoculation of strains (Cast13, VBC02, cast13c, VBC02c, RB111). B – Necrosis on apples. Bars with the same letter(s) are not statistically different ($p < 0.05$). (vir – virulent, hyp – hypovirulent, for easier analysis of the graph).

Virulence evaluation by the detached chestnut branches lesions revealed the two virulent *C. parasitica* strains (Cast13 and VBC02) produced similar significant large lesions (Figure 3A). Hypovirulent converted isogenic strains (Cast13c, VBC02c) produced significantly smaller lesions than virulent isogenic strains. The donor CHV1 hypovirulent RB111 produced intermediate-sized lesions but not significantly different ($p < 0.05$) from the virulent strain Cast13.

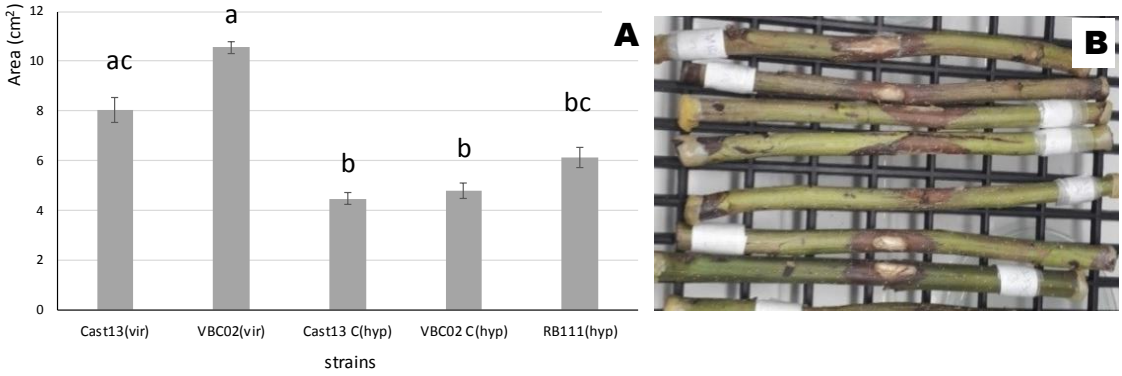

Figure 3 – A: Mean necrotic lesion area (mean ± SE) on detached chestnut branches caused by virulent and hypovirulent isogenic strains and the hypovirulent donor CHV1 strain of *Cryphonectria parasitica,* 10 days after inoculation of strains (Cast13, VBC02, cast13c, VBC02c, RB111). B – Necrosis on branches. Bars with the same letter(s) are not statistically different ($p < 0.05$). (vir – virulent, hyp – hypovirulent, for easier analysis of the graph).

Qualitative enzymatic assays

The analysis of Figure 4 reveals that virulent strains (Cast13, Cast26, VBC02, Cast07, Cast17, VDP11) caused more intense brown oxidation zones and/or larger reaction areas in Bavendamm tests, concordant with higher Lac3 activity. Virulent strains also originated bigger discoloration halos than hypovirulent strains (RB111, Serra05, SR442) did, also indicating their higher production of other lignin-degrading enzymes (LiP and MnP) and cellulase. Between virulent strains, those from EU66 vc group (Cast07, Cast17, VDP11) showed higher cellulolytic activity when compared with those from EU11 vc group.

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DOI: 10.29352/mill0213.01.00285

Strains	Bavendamm	Cellulase	Azure B	RBBR
Cast13 (EU11)	+++	2	+++	+++
Cast26 (EU11)	+++	2	+++	+++
VBC02 (EU11)	+++	2	+++	++
RB111 (donor)	+	1	+	+
Serra05	+	1	+	+
SR442	++	1	+	+
Cast07 (EU66)	+++	3	+++	+++
Cast17 (EU66)	+++	3	+++	+++
VDP11 (EU66)	+++	3	+++	+++

Figure 4 - Results obtained in the screening tests used for the detection of enzymatic activity in *Cryphonectria parasitica* strains. Bavendamm test: + to +++ refer to increasing colour reaction/area obtained in the test; Cellulase test: 1 - refers to small ø halo around colony (0-5mm), 2 - medium size ø halo (5-14mm), and 3 - big ø halo (15-25mm); Dye discoloration in Azure B and RBBR media: + to +++ refer to increasing halo diameter.

Metabolic profile characterization

The Biolog FF MicroPlate, based on the company's Phenotype Array Technology, was recently introduced for the rapid identification and characterization of filamentous fungi. We used Biolog FF Microplates to characterize the metabolic profiles of virulent and hypovirulent *C. parasitica* isolates. These microplates contain 95 different carbon sources, and 75 of them were used by all the strains (Cast13, VBC02, Cast13c, VBC02c and RB111). None of the strains was able to use all types of substrates. The set of five strains studied were unable to utilize one carbon source: 2-amino ethanol. The virulent strain VBC02 consumed less carbon sources (85) than all the other strains, and the hypovirulent strain RB111 consumed the higher number of carbon sources (93). Average well colour development (AWCD) of all carbon sources for the five *C. parasitica* strains is presented in Figure 5. After 7 days of incubation at 25°C, there were significant differences ($p < 0.001$), in metabolic capability among the five isolates, and the order was Cast13c > Cast13> VBC02c (Figure 5).

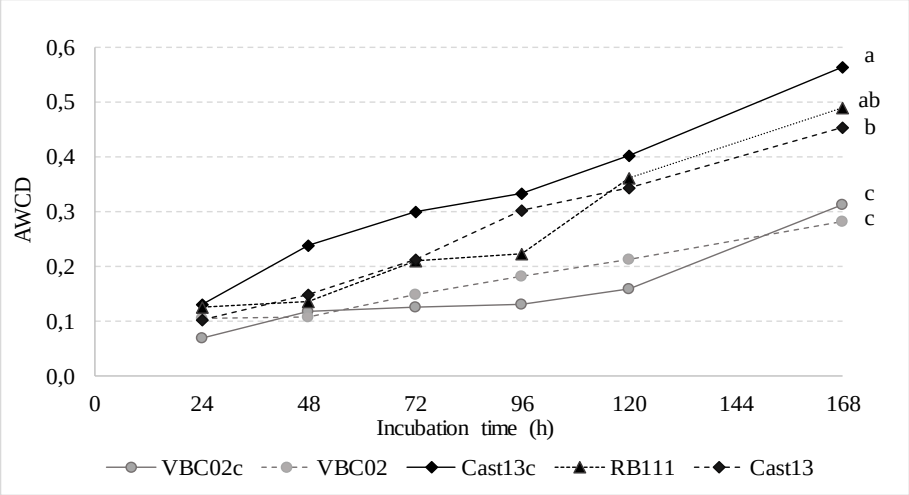

Figure 5 - Average well colour development (AWCD) of all carbon sources for the five *Cryphonectria parasitica* strains (Cast13, Cast13c, VBC02, VBC02c and RB111). Mean (AWCD) of each isolate, after seven days of incubation at 25°C followed by the same letter are not significantly different ($p < 0.05$).

According to the biochemical properties of carbon sources, the 95 substrates present in the FF Biolog Microplates (Biolog Inc.) were allocated in six chemical groups, including amines/amides, carboxylic acids, amino acids, carbohydrates, miscellaneous and polymers (Zhang *et al*. 2014). The results indicated that the utilization of six groups of carbon sources by the five *C. parasitica* isolates were significantly different ($p < 0.001$). The highest AWCD values were obtained for carbohydrates, carboxylic acids and polymers, and the lowest, for amines/amides, amino acids and miscellaneous (Figure 6).

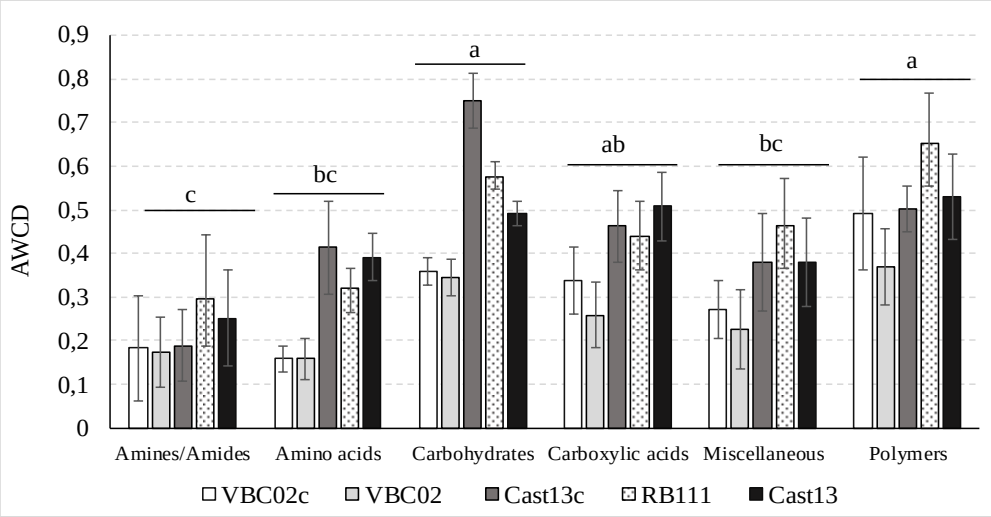

Figure 6 - Carbon sources utilization by chemical groups, for five strains of *Cryphonectria parasitica* (Cast13, Cast13c, VBC02, VBC02c and RB111) after seven days of incubation at 25°C. Mean (AWCD) of each chemical group followed by the same letter are not significantly different ($p < 0.05$).

The PCA analysis shows that the strains studied have large differences in their metabolic profiles (Figure 7), especially Cast13c and Cast13 which are in different quadrants. The axis of principal component 1 (PC1) described 57.5% of total data variability and principal component 2 (PC2) described 22.4 % of total data variability.

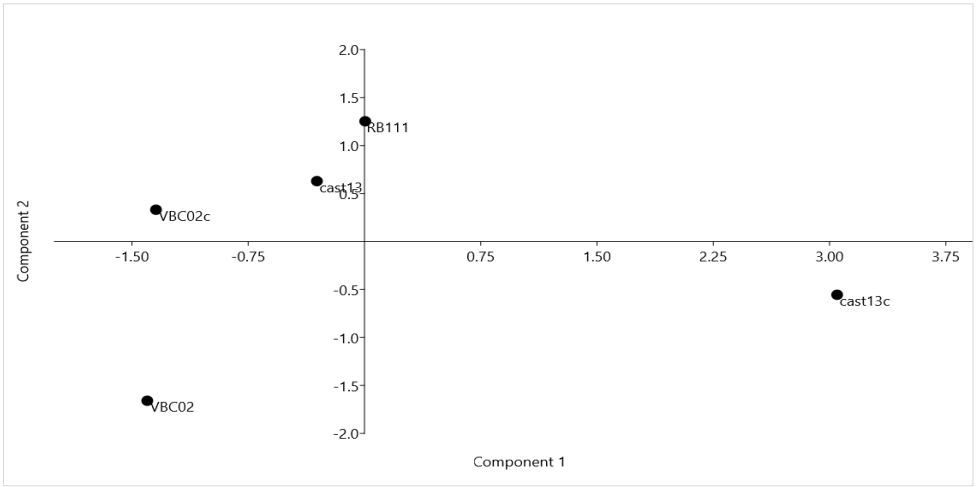

Figure 7 - Principal Component Analysis of physiological profiles obtained from *Cryphonectria parasitica* isolates (Cast13, Cast13c, VBC02, VBC02c and RB111). PC1 eigenvalue 3.2890; PC 2 eigenvalue 1.284.

4. DISCUSSION

This work aims to compare virulent strains of *C. parasitica* and hypovirulent strains (which contain CHV1 hypovirus) applied as biological control agents. For virulence evaluation tests, apple fruits and detached chestnut twigs were largely used and considered useful for detecting marked deficiencies in virulence (Elisten 1985; Lee *et al*. 2006, Faruk *et al*. 2008). In this study, as a measure of virulence, we used the necrotic tissues developed by the inoculation of mycelia of each isolate in apple fruits (cv. Golden Delicious) or on detached branches of chestnut. All isolates grew under controlled conditions and the results on detached branches of chestnut prove to be more useful as an initial screening method to quickly detect a marked difference in virulence.

Tannic acid is abundant in the bark of chestnut trees and is considered to be one of the major barriers against *C. parasitica* infections (Chung *et al*. 2008). *C. parasitica* Lac3 is the enzyme that overcomes this host defence barrier, facilitating the fitness of the pathogen. In this work, nine different *C. parasitica* strains were screened on media containing substrates and indicator compounds that enabled the visual detection of ligninolytic enzymes and cellulase activities.

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The more intense brown oxidation zones and/or larger reaction areas obtained with all virulent strains, (Cast 13, Cast26, VBC02, Cast07, Cast17, VDP11) in Bavendamm tests are concordant with a higher tannic-acid inducible Lac3 activity in these strains. These results are in agreement with Rigling *et al*. (1989) and Chung *et al.* (2008) that had previously reported that hypovirulent strains, infected with the CHV1 hypovirus dsRNA, exhibited a generalized decrease on laccases activity.

Virulent strains also originated bigger discoloration halos than hypovirulent strains (RB111, Serra05, SR442) did, also indicating their higher production of other lignin-degrading enzymes (LiP, MnP and cellulase). Between virulent strains, those from EU66 vc group (Cast07, Cast17, VDP11) showed higher cellulolytic activity when compared with those from EU11 vc group. Increased ligninolytic/cellulolytic activities seem to be concordant with higher strains virulence in *C. parasitica,* as it happens in other phytopathogenic fungi.

Five strains (two virulent, three hypovirulent - two converted and one original) were selected for further studies because they showed large differences in the qualitative assessment and they belong to the same VCG group (EU11), which resulted in a successful conversion. These strains were inoculated in different materials, namely apples and chestnut branches, to characterize their degree of virulence. Results obtained from the chestnut branches inoculation were related to the ones obtained previously with the indicators of ligninolytic enzymes production. Chestnut shoots are composed of lignin, hemicellulose and cellulose, therefore the lesions made by virulent strains, which have higher polyphenol oxidase (laccase, LiP peroxidase) and cellulase activities were more important than the damage made by strains that contain CHV1 hypovirus, which suffer from a decrease in laccase activity. Chung *et al*. (2008) have already referred that a laccase-null mutant caused a smaller lesion area on chestnut bark than did the virulent type. The reduction in the lesion area made by hypovirulent strains was also tested and proved in other studies, for *C. parasitica* (Chung *et al*. 2008) and in *Botryosphaeria dothidea* (Zhai *et al*. 2016) an infected fungus with a different type of hypovirus. Besides that, the biggest rot lesion was made by a virulent strain VBC02, and the smallest by a hypovirulent one - VBC02c. Different results were attained in the case of apple fruits inoculation in which a converted strain Cast13c caused a bigger infection area than its virulent version Cast13. This observation can be related to the reported results with the Biolog metabolic profile, that shows a high carbohydrates degradation by some hypovirulent strains, especially Cast13c. Carbohydrates represent the principal component of apples. Some of the hypovirulent strains tested showed great carbohydrates consumption but small rot lesion on apples. The reason for that can be due to the types of carbohydrates presents on apples and the different affinity of the enzymes secreted by *C. parasitica* strains for these substrates.

At the metabolic level, isolates were evaluated using Biolog FF MicroPlates, with concurrent reads of fungal utilization of 95 different carbon sources. Hypovirulent strains (Cast13c, RB111) showed great consumption of some substrates, and presented a very different metabolic profile comparing to their virulent version. Those hypovirulent strains are infected by the hypovirus CHV1, which is known to reduce the pathogenicity of the fungal but without completely cause mycelium dysfunction. On the contrary, the virus somehow looks like to keep the mycelium in a juvenile state while the debilitation is affecting pathways related to the virulence of the fungi. Another study also proved that the consumption of some amino acids, carbohydrates, lipids and nucleotides are increased in a virus-infected *C. parasitica* comparing to the wild type strain (Dawe *et al*. 2009). The fungal infection activates some metabolic pathways included in the fungal defence mechanism against the hypovirus. Some of these pathways require amino acids to activate antiviral mechanisms, they also consume different carbohydrates, which gives the mycelium the ability to take other pathways to produce energy or other compounds, and that explains the result from PCA analysis, which shows the big differences in the metabolic profiles between a virulent and its white strain.

CONCLUSIONS

In this work, all nine original strains were assayed for qualitative ligninolytic enzyme activity. At the same time, two virulent strains of *C. parasitica* were converted by hyphal anastomoses using a characterized hypovirulent strain. Then two virulent and three hypovirulent strains were chosen to test their virulence and metabolic profile. Results showed that the detached branches inoculation test is a suitable approach for the evaluation of marked virulence deficiencies. The necrotic lesion area developed by virulent strains is significantly larger than the caused by the isogenic hypovirulent strains. Besides, cellulase, laccase and lignin peroxidase activities are also reduced in hypovirulent strains. However, metabolic profiles evaluation assessed by Biolog FF microplates revealed that most of the carbon sources are more consumed by hypovirulent strains. The aforementioned achievements lead to the conclusion that the hypovirus does not cause a general debilitation of the fungus, but it partially modifies the genes related to the pathogenicity.

The metabolic analysis has revealed changes that happen in response to the hypovirus, it may also facilitate future isolate selection. As the use of specific carbon sources provides complete information on the isolates, those data can be put in a scientific database. These studies may lead to new perspectives for understanding the biological process used by the hypovirus.

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