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Addition of abscisic acid increases the production of chitin deacetylase by *Colletotrichum gloeosporioides* in submerged culture

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ABSTRACT

The activity of chitin deacetylase from *Colletotrichum gloeosporioides* was studied by the addition of phytohormones (gibberellic acid, indole acetic acid, and abscisic acid) and amino sugars (glucosamine and *N*-acetyl glucosamine) in culture media. Abscisic acid exerted a positive and significant effect on enzyme production with 9.5-fold higher activity (1.05 U mg protein^{-1}) than the control (0.11 U mg protein^{-1}). Subsequently, this phytohormone was used in batch culture with higher chitin deacetylase activity being found at acidic pH (3.5) than at neutral pH (7). Furthermore, the highest activity was determined at the acceleration growth phase. The chitin deacetylase production was ascribed to the lag phase within the spore germination process and germ tube elongation instead of during the formation of appressoria, as evidenced by the scanning electronic microscopy results. Therefore, more inoculum and medium containing abscisic acid were added to the fed-batch culture, resulting in a significant increase in chitin deacetylase activity (3.64 U mg protein^{-1}). The addition of abscisic acid led to changes in the acetylation degree of chitin extracted from the cell wall of *C. gloeosporioides*, with lower degree of acetylation (DA of 75.6%) than that determined with the culture without abscisic acid (DA of 90.6%).

1. Introduction

Phytohormones are organic molecules that act as messengers with a key role in plant development. These biological compounds interact in a synergistic or antagonistic fashion within a sensitive equilibrium in response to biotic and abiotic stresses [1]. Certain pathogens successfully induce diseases through their ability to suppress or mislead plant defense responses. The susceptibility of plant organs, particularly fruits and flowers, to an invasion by phytopathogenic microorganisms increases with aging and ripening. Phytohormones, such as ethylene, accelerate senescence and increase susceptibility, whereas those that delay senescence, such as cytokinin and gibberellin, tend to increase resistance [2]. Phytohormones might cause hormonal imbalances in the early phase of infection due to plant development, and responses to environmental cues are highly regulated. Phytopathogenic microorganisms can cause disequilibrium during the disease process by producing their own phytohormones. This effect has been well documented in the

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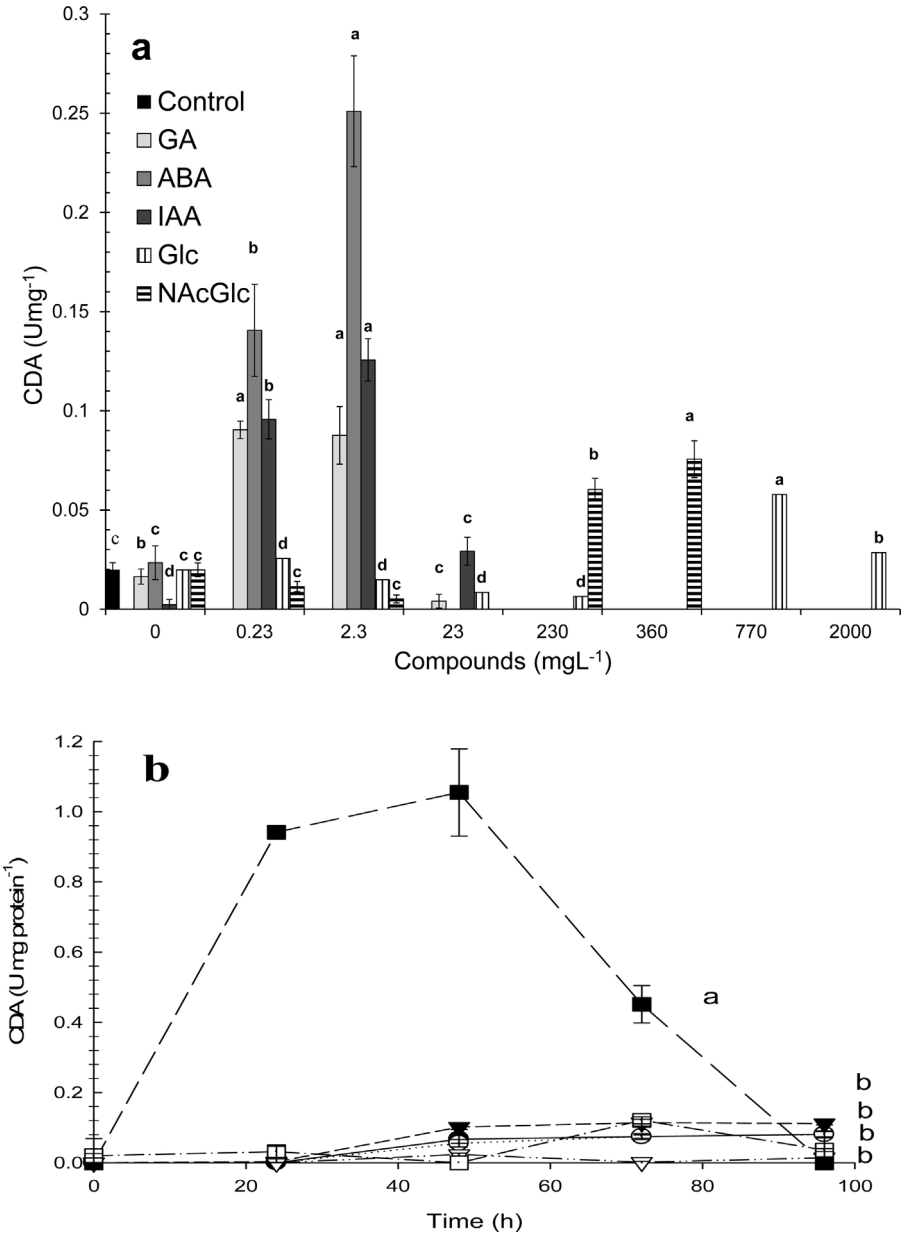

Fig. 1. SmC of *C. gloeosporioides*: a) screening of compounds at several concentrations for enhance CDA activities at 72 h and b) time course of CDA activities with added phytohormones (23 mg L^{-1}): GA (solid triangle), ABA (solid square), IAA (void triangle), and amino sugars: NAcGlc (360 mg L^{-1}) (solid circle) and Glc (770 mg L^{-1}) (void circle). Histograms with the same letter were not significantly different ($p < 0.05$) between compounds concentrations. Points with different letters were significantly different ($p < 0.05$) according to Tukeyís multiple means comparison test.

case of *Colletotrichum gloeosporioides,* which can produce indole-3-acetic acid (IAA) from tryptophan [3]. This fungus is known to produce IAA in plants, which at high concentrations inhibits the expression of plant defense molecules [4]. The application of phytohormones in plants created a suitable environment for fungal growth, as reported by Ulfers et al. [5] They applied exogenously abscisic acid (ABA) on a barley mutant with a defect in ABA biosynthesis, thus reducing its resistance against the fungus *Magnaporthe oryzae.* In another study, anthracnose produced by *Colletotrichum acutatum* was accelerated in the presence of ABA and gibberellic acid (GA) in pepper fruits. These phytohormones both accelerate or delay fruit senescence and increase the susceptibility to anthracnose [2], and also stimulate or inhibit the growth of phytopathogenic fungi [6–8]. Moreover, the production of chitin-degraded and related enzymes during the phytopathogenic process is favored as a part of the plant defense mechanisms. The phytopathogenic fungi, in turn, evade the specific plant hydrolases via the partial deacetylation of chitin in the cell wall by chitin deacetylases (CDAs), as detected in *Colletotrichum lindemuthianum* [7].

The function of phytohormones added to the culture media of fungi is unclear, and reports on the weak stimulatory or null effect of ABA on mycelial growth are limited [1]. Furthermore, increases of chitosan content in the cell walls of *Rhizopus oryzae* and *Mucor rouxii* were determined when cultivated in media supplemented with gibberellin, IAA, indole-3-butyric acid, and kinetin [9,10]. Despite these reports, to the best of our knowledge, there is no information about the effect of external phytohormones on the growth of *C. gloeosporioides* and production of CDA has not been studied yet.

In this regard, we studied the effects of phytohormones and aminosugars addition in a fed-batch culture process on

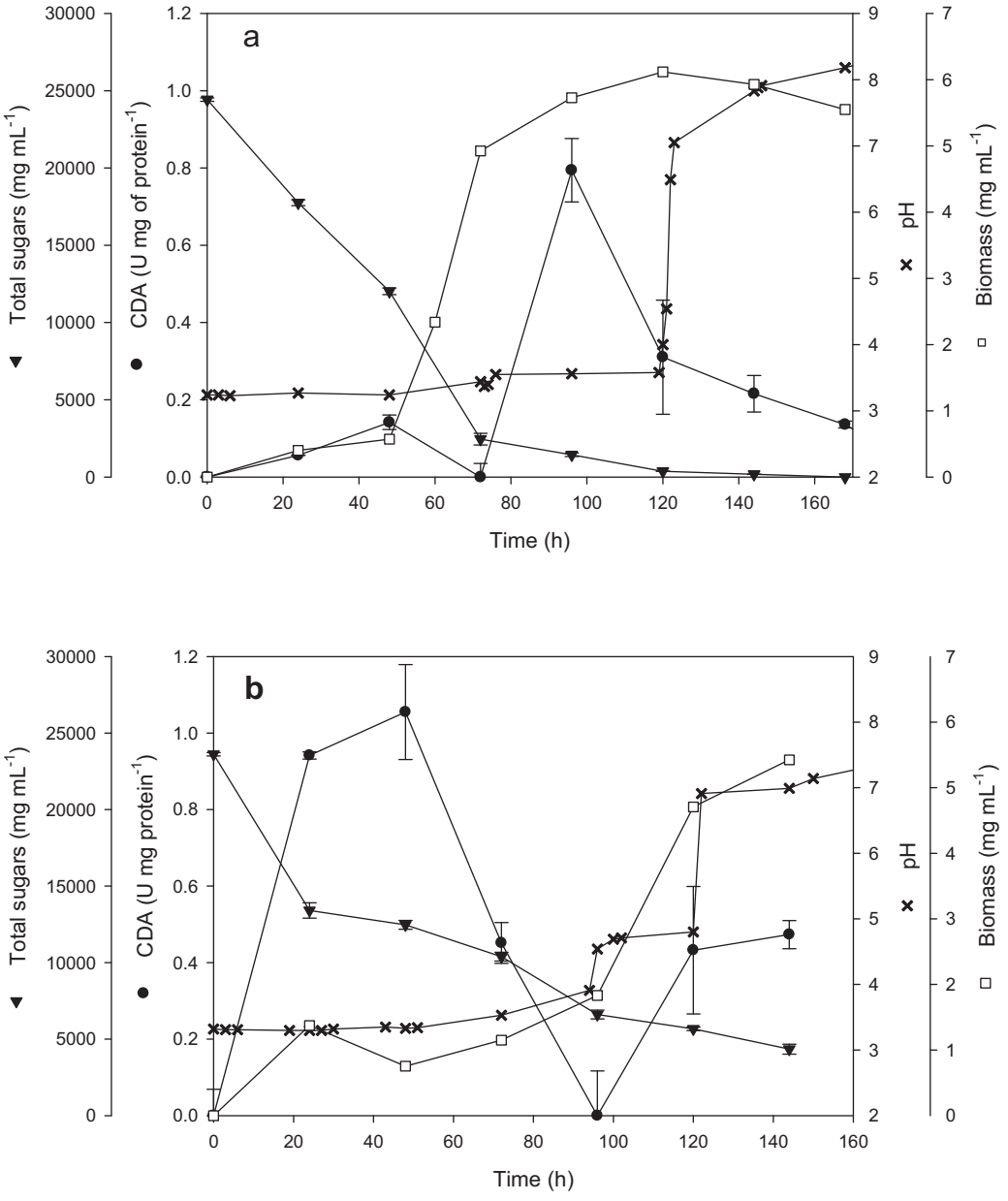

Fig. 2. Time course of biomass production (void square); CDA activities (solid triangle) and pH (cross) of *C. gloeosporioides* in SmC: a) control and b) media supplemented with ABA.

germination and biomass production as well as the production of the CDA. In addition, chitin was isolated from fungal cell walls and characterized to determine the role of CDA in determining the chemical composition of biomass when the fungus was cultured with added ABA.

2. Materials and methods

2.1. Microorganism

The *C. gloeosporioides* strain CF-6 isolated from damaged tissues of fruits, calyxes, and leaves of Italian lemon plant (*Citrus limon var* Eureka) was provided by the Culture Collection of Centro de Biotecnología Genómica of Instituto Politécnico Nacional (Mexico). The strain was cultured in slants on potato dextrose agar. Spore suspensions were obtained by mechanical stirring with a sterile solution of 0.1% (v/v) Tween 80 from 7-day-old cultures.

2.2. Media composition

The composition of glutamic acid media (GAM) for submerged culture (SmC) (g L^{-1}) was as follows: 15 g L^{-1} of glucose, 6.6 g L^{-1} of glutamic acid, 1 g of K_2HPO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 1.8 mg of $ZnSO_4{\cdot}7H_2O$, 1 mg of $FeSO_4{\cdot}7H_2O$, 0.3 mg of $MnSO_4{\cdot}H_2O$, and 0.4 mg of $CuSO_4{\cdot}5H_2O$ supplemented with 1 mg of thiamine and 1 mg of nicotinic acid [11].

2.3. Determination of effect of phytohormones and amino sugars on CDA activities of C. gloeosporioides

SmCs were carried out in flasks with 50 mL of medium for screening inductors. The medium was inoculated with 1×10^7 spores g of nutrient^{-1} and incubated at 28 °C. Samples were taken in duplicates every 24 h up to 96 h. The cultures were filtered and biomasses were determined as dry weight. The filtrates from cultures were used as crude enzyme solutions. Media were

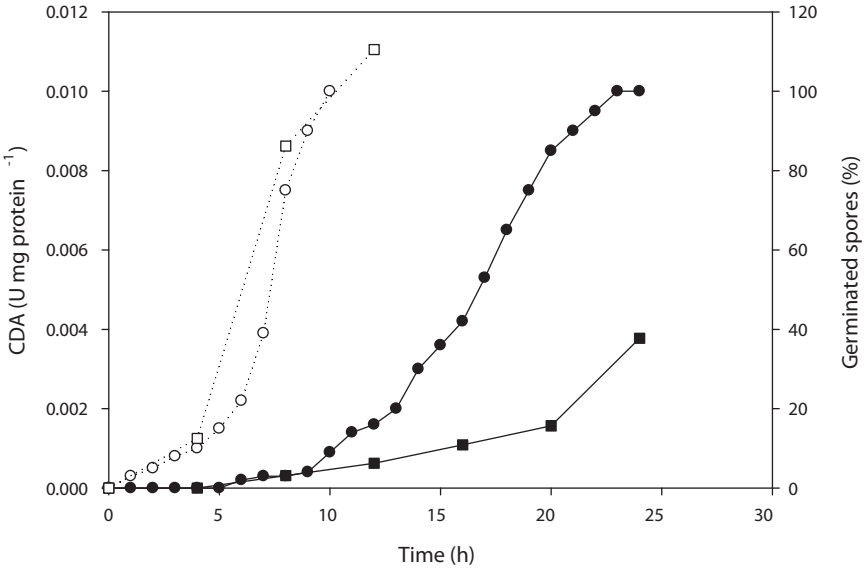

Fig. 3. Germinated spore percentages of *C. gloeosporioides* in GAM (solid circle) and GAM with added ABA (void circle). Germinated spores were counted up to 24 h after inoculation. Each data point represents the mean of two experiments with 200 observations per experiment. CDA in GAM (solid square) and GAM with added ABA (void square).

supplemented with several compounds including phytohormones, GA, ABA and IAA, at 0.23, 2.3, 23 and 230 mg L^{-1} and Glc at same range of concentrations also 770 and 2000 mg L^{-1}, samples were taken at 72 h. The time course of CDA of SmC in media with added selected concentrations of compounds experiment was carried out over 96 h.

2.4. Production of CDA of C. gloeosporioides with ABA in bioreactor

The spore suspension (1×10^7 spores g of nutrient^{-1}) was inoculated in a 3 L instrumented bioreactor (Applikon B.V. Holland) equipped with an automatic monitoring system and control facilities for agitation, pH, dissolved oxygen, aeration, temperature and medium as fed-batch. The working volume was 1.6 L of GAM without (control) or with added ABA (23 mg L^{-1}) in batch cultures. The bioreactor was operated at temperature of 28 °C, agitation at 100 rpm and the aeration rate was adjusted to a 1 vvm. For the fed-batch SmC, the composition of the medium was the same as used in the batch SmC. After 48 h of SmC into the bioreactor, it was supplemented with fresh GAM (600 mL) at volumetric feed rate of 10 mL min^{-1} with or without the addition of more inoculum (1×10^7 spores g initial dry substrate^{-1}). The pH, CDA activity, protein content, and biomass were determined every 24 h and up to 144 h.

2.5. Analyses of samples: determination of pH, biomass production, CDA activity, and total sugar and protein contents

The pH of samples was recorded using a potentiometer (pH 210HANNA, Padua, Italy). The samples were centrifuged at 12,700*g* and 4 °C for 25 min. Pellets were recovered and washed with deionized water, filtered (Whatman 40, USA), and dried overnight at 100 °C prior to biomass determination. The supernatants obtained from the fungal culture were used as the crude enzyme for further experimentation. The CDA activity was determined in the crude enzyme following the method of Kauss and Bausch [12] using ethylene glycol chitin as the substrate and D-glucosamine–HCl (0–0.035 μmol mL^{-1}) as the standard. One unit of CDA was defined as the amount of enzyme required to release 1 μmol of acetate per minute. Protein content was determined by Peterson [13] using serum albumin bovine as the standard. The amount of total sugars was determined in the supernatants following the methodology reported by Dubois et al. [14], using glucose as the standard.

2.6. Spores germination assays

To flask dishes with three coverslips, 20 mL of media was added, which was then inoculated with spore concentrations of 1×10^7 spores per g of initial dry substrate^{-1} and incubated at 27 °C and 100 rpm. A total of 200 spores (germinated and non-germinated) were counted using a light microscope (Carl Zeiss, Oberkochen, Germany), and the number of germinated spores per coverslip was determined every hour. The spore was considered germinated when the length of its germinal tube reached one-half of the spore diameters. The germinated spores were counted in triplicate, and the assay was halted at 100% of germination. The CDA activities were determined every 4 h following the procedure described in Section 2.3.

2.7. Scanning electron microscopy analysis

Scanning electron microscopy (SEM) analysis was carried out using a scanning electron microscope (JEOL JSM-5900 LV, Tokyo, Japan) with 0, 1, 4, 9, 12, 16 and 24 h samples of fungal growth. Samples were fixed in glutaraldehyde (5% vv^{-1}), treated with OsO_4 (1% wv^{-1}), dehydrated with methanol, and then covered with gold prior to analyses.

2.8. Extraction of chitin from fungal cell walls and determination of its acetylated fraction (F_A)

Biomass was washed with distilled water, and the fungal cell walls were disrupted using an Ultra-Turrax (1 min, 8000 rpm, 25 °C), filtered, and resuspended in distilled water; this procedure was repeated thrice, followed by freeze drying [15]. Subsequently, to remove lipids, proteins, and free glucans, the fungal cell walls were washed in a 1:40 biomass to solvent ratio in the following order: chloroform, chloroform-methanol (2:1 vv^{-1}), dichloromethane, dimethyl sulfoxide, and deionized water; this was followed by lyophilization [16]. Chitin purification proceeded

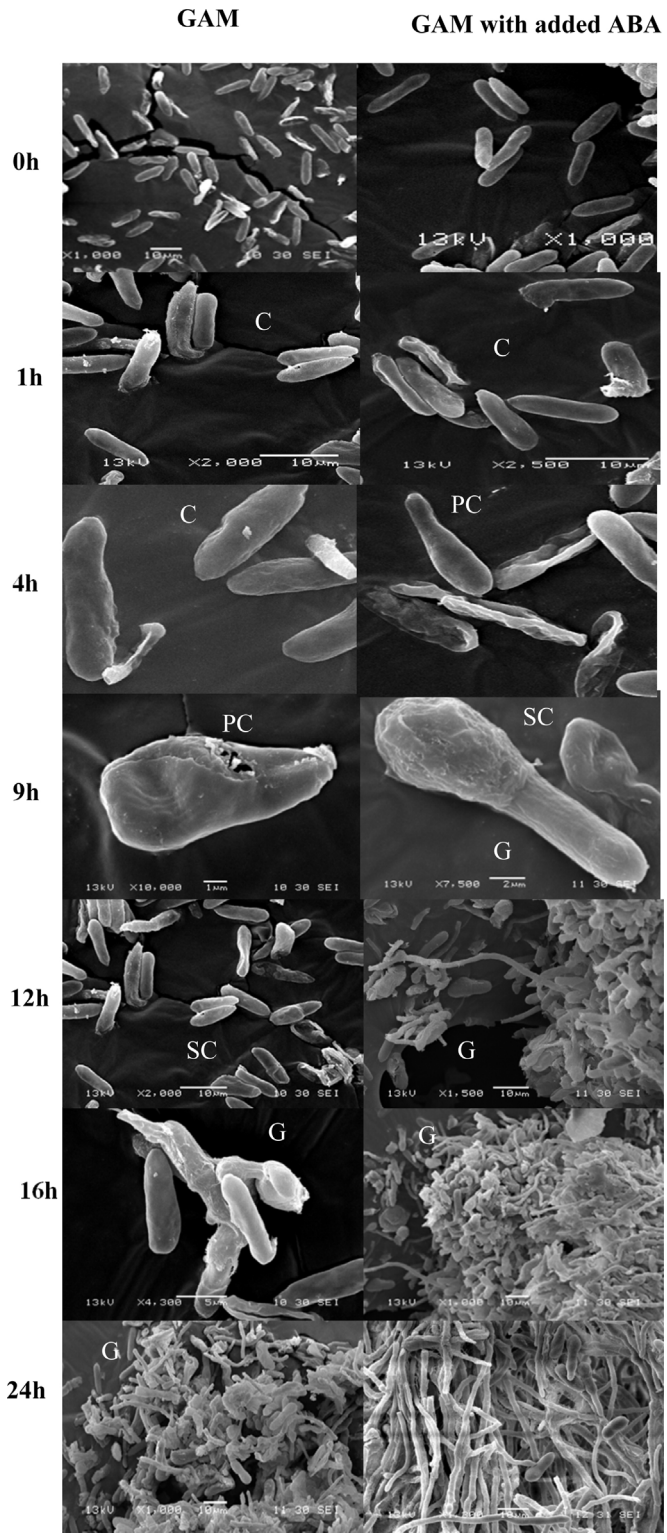

Fig. 4. Scanning electron micrographs of spores of *C. gloeosporioides* in GAM (left) and GAM with added ABA (right) for 1, 4, 9, 12, 16, and 24 h. C, SC, PC, G, and GL mean conidia, septated conidia, polarized conidia, germ tubes, and germlings, respectively.

by treatment with 1 M NaOH in a ratio of 1:40 (wv^{-1}) for 24 h at 70 °C for complete deproteinization, followed by centrifugation at 14,000 rpm at 25 °C for 15 min [17]. The pellet was washed repeatedly with hot water (70 °C) until neutralization and lyophilization [18]. The sample was resuspended in a ratio of 1:100 (wv^{-1}) in acetic acid (0.3 M) overnight at 25 °C under mechanical agitation. Then, the insoluble fraction, chitin, was separated by filtration and weighed. The yield of chitin was expressed as the percentage of chitin by fungal biomass produced (dry basis) ($Y_{Chitin/Biomass}$).

Chitins were dissolved in concentrated DCl (7.6N) for 24 h at 25 °C using mechanical agitation. Then, the samples were analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy (Bruker, Spectrospin 300, Germany) at 300 MHz with deuterated 3-(trimethylsilyl) propionic acid (TMPS) as the internal reference. The F_A values of chitins were calculated according to the method described by Einbu Aslak and Vårum [17].

2.9. Statistical analyses

Data were subjected to analyses of variance and multiple comparison of means via the Tukey–Kramer test in order to determine significant differences in CDA activities among compounds concentrations (phytohormones and amino sugars), germination, and fed-batch culture ($p < 0.05$) using the statistical program NCSS (NCSS, PASS and GESS, 2001). The specific growth rates were estimated by fitting the biomass data to the logistic model using the statistical program STATISTICA 6.0 (Stat Soft, Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Screening of phytohormones and amino sugars in CDA production

The use of phytohormones increases the amount of chitosan in the fungal cell wall [9,10], although NAcGlc and Glc also trigger the synthesis of fungal cell wall by chitin synthases, which induced dimorphism of yeast in the culture media [19]. Concomitantly, we investigated the effect of adding IAA, ABA, GA, NAcGlc, and Glc individually on CDA production. The screening for phytohormones concentration result in significantly higher CDA produced at 72 h of SmC with media with added ABA at concentration of 23 mg L^{-1} followed by GA and IAA at same concentration. High concentrations of Glc (770 mg L^{-1} and 2000 mg L^{-1}) in the media produce an increase on CDA activities (Fig. 1a). Therefore these concentrations were employed for the time course CDA experiment, the results show that ABA at addition significantly enhances enzymatic activities, as observed in Fig. 1b. However, CDA activities with GAM supplemented with NAcGlc and Glc did not differ significantly from the control, as the production was kept constant from 48 until 96 h. Similarly, SmC with phytohormones only showed a positive effect on CDA activity for ABA, with 9.5-fold higher activity (1.05 U mg protein^{-1}) than the control (0.11U mg protein^{-1}). Thus, further experimentation was carried out using GAM with added ABA.

3.2. Batch and fed-batch cultures for production of CDA

It is well documented that ABA has various biological roles during plant development, including regulation during germination, transpiration, and response to abiotic stress. Moreover, studies with anthracnose from pepper fruits have shown that ABA can suppress plant defense mechanisms, thus altering the development of *Colletotrichum* [20]. According to Vargas et al. [21], *Colletotrichum graminicola* is responsible for the production of anthracnose in maize and begins the disease cycle as a biotrophic pathogen and later becomes necrotrophic. This strain was reported to actively suppress plant defenses during the biotrophic stage after an initial microbe-associated molecular pattern-triggered activation. Despite reports that claim that ABA accelerates fungal growth in batch culture[2] and in plants [4], herein, no significant changes were noted in the biomass produced with addition

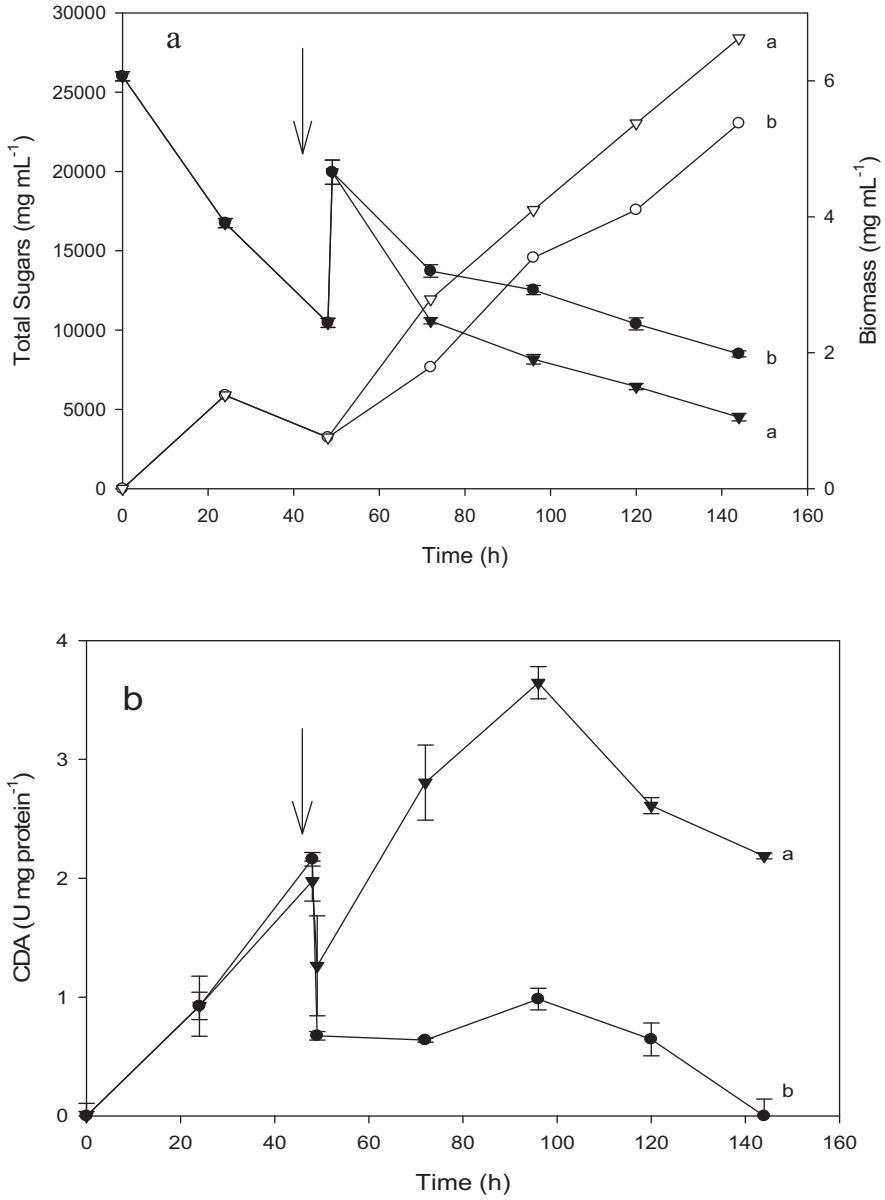

Fig. 5. Time course of biomass production and total sugars (a) and CDA activity (b) of *C. gloeosporioides* in SmC. GAM supplemented with ABA in fed batch in circles; GAM supplemented with ABA in fed batch with inoculum added (1×10^7 spores g nutrient^{-1}) in triangles. Solid symbols denote total sugars and CDA, and void circles denote biomass. Arrow shows the beginning of GAM feeding. Points with different letters were significantly different ($p < 0.05$) according to Tukeyís multiple means comparison test.

of ABA; in fact, the specific growth rate for control was significantly lesser ($\mu = 8.73 \times 10^{-3}$ h^{-1}) than that estimated with ABA ($\mu = 1.82 \times 10^{-2}$ h^{-1}) (Fig. 2). The fungal growth resulted in the depletion of glucose after 96 h of SmC in GAM, leading to the onset of stationary phase and subsequent the consumption (120 h) (Fig. 2a). By contrast, GAM with added ABA consumed sugar at a slower rate than the control; thus, *C. gloeosporioides* was at the growth phase (Fig. 2b). The CDA activities were detected for both the lag and acceleration phases. It is worth noting that GAM with added ABA displayed a significantly higher activity than that with the control, this production was detected at 24 h and 48 h. The highest specific activity of CDA in the control (0.79 U mg protein^{-1}) corresponds to the second peak at the stationary phase. This experimental result is in agreement with the findings of Pacheco et al. [11], who cultured *C. gloeosporioides* at pH 6. However, the maximum specific activity in their work was 0.018 U mg protein^{-1} at 120 h, which is considerably lower than that obtained in this work. The increase in CDA activity might be ascribed to the acidic pH from 3.2 to 4.5 during 120 h of SmC. A plausible explanation is that this strain was isolated from lemon where *C. gloeosporioides* adapted to grow, thus surpassing the natural barriers in plants including essential oils, peel complex composition, and acidic pH. In this regard, Deshmukh et al. [22], reported the growth and sporulation of *C. gloeosporioides* in a wide range of pH (4.0–8.0) in SmC.

According to the experimental evidence, CDA is influenced by ambient pH, because extracellular enzymes and permeases in fungi are produced by pH regulatory processes, and the stabilization and biological activities of enzymes and other metabolites are preserved at certain pH when they are excreted into the media [23,24]. The host modulates pH during fruit ripening, which usually begins at acidic pH and increases during maturation. For instance, this change in pH regulates the expression and secretion of pectate lyase by *C. gloeosporioides* in avocado fruits [23]. Moreover, ambient pH is

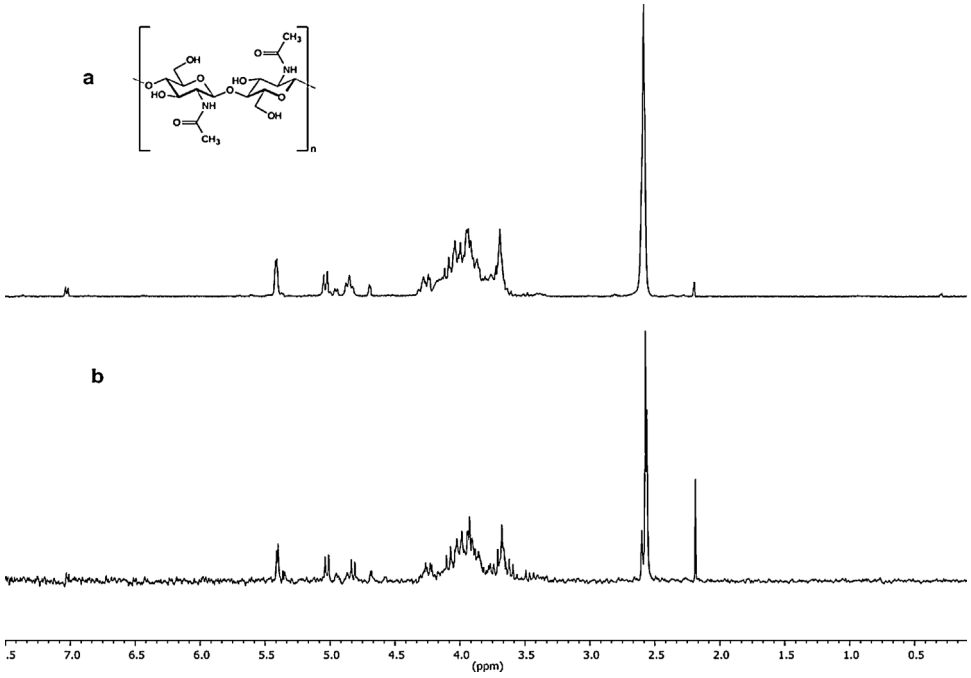

Fig. 6. ^{1}H NMR spectra of chitin purified from the cell wall of *C. gloeosporioides* of GAM (a) and GAM supplemented with ABA in fed batch with an inoculum added (1×10^{7} spores g nutrient^{-1}) (b) samples, dissolved in concentrated DCl at 25 °C (relative to the resonance of TMPS at 0.00 ppm). The resonance from H-1 of glucofuranosyl oxazoliniumion is denoted by an asterisk.

also modified by fungal growth, for example, ammonia production, as observed in Fig. 2, with gradual increase in pH.

Microscopic analyses were carried out in *C. gloeosporioides,* to determine the percentage of germinated spores, and the germ tubes, specialized hyphae, appressoria, and other related structure at the first stages of growth were observed. The CDA activities were also determined along with microscopic analyses (Fig. 3). The spores began to germinate at 7 h of incubation in the control, and 100% of germinated spores were counted at 24 h. A significant delay was noted in the germination process compared that reported by Shi et al. [25], who counted 96.4% of germinated spores of *C. gloeosporioides* in potato dextrose agar after 8 h of incubation. This delay might be due to the acidic pH in GAM (3.3), which is far from the optimum pH of 5.5–6 reported by Deshmukh et al. [22], for *C. gloeosporioides* spore germination. Nonetheless, the low pH of the medium was not a limitation for the GAM as the added ABA stimulated germination, which began at 2 h and was completed after 10 h (Fig. 3).

The conidia presented ellipsoidal morphology, as reported elsewhere, at $t = 0$ [25]. This morphology was preserved until 7 h in the control, whereas spore polarization began at 1 h in GAM with added ABA (Fig. 4). The germination process took place rapidly, germ tubes were visible at 4 h, and septate spores were seen at 9 h (Fig. 4); at the same time, however, the conidia in the control were at the polarization stage. The conidia were elongated into either long or short germ tubes, without appressoria and differentiation to new conidia. In this regard, the isolates of *C. gloeosporioides* do not form appressoria as the fungi can penetrate the host directly via the germ tubes [26,27].

3.3. CDA production in fed-batch culture

Fed-batch culture is characterized by differences in substrate consumption, and metabolic production rates. The external environment in fed-batch affected internal composition of the cells and cell morphology changes [28]. Herein, specific growth rate was controlled at 1.6×10^{-2} h^{-1} or 1.8×10^{-2} h^{-1} by feeding medium and a second strategy for CDA production improvement was experimentally conducted by adding spore inoculum during the feeding. The appropriate feeding time was selected as CDA production was proven to decrease after 48 h (Fig. 2b). The addition of spores along with fresh medium was considered an adequate fed-batch strategy due to the high level of CDA activities, as well as the positive effect of ABA on the germination process (Fig. 3). SEM studies showed that ABA stimulates germination (Fig. 4), in line with the findings of Hwang et al. [2] for anthracnose. The time course of SmC of *C. gloeosporioides* is shown in Fig. 5, where the exponential phase was extended in fed-batch SmCs (Fig. 5a). As observed, SmC presented an acidic pH range from 3.23 to 3.5 during the experiments (Fig. 5a). The highest activity was determined (3.64 U mg protein^{-1}) at 96 h of the fed-batch with an added inoculum, whereas the fed-batch with added GAM produced 2.15 U mg protein^{-1} at 48 h (Fig. 5b).

The experimental results clearly showed that the CDA activities were determined at the initial stages of fungal growth, precisely at the beginning of the acceleration phase; moreover, the activity increased significantly in fed-batch SmC with an added inoculum (Fig. 5b).

3.3. Chitin isolation from fungal cell walls from fed-batch culture with added ABA

The chitin yields were not significantly different for the control medium (GAM) and fed-batch SmC with added inoculum (3.5% and 2.7%, respectively). The F_A value determined for the chitin obtained by SmC with GAM here (0.90) is within the range found in the literature; for example, Di Mario et al. [29] reported an F_A value of 0.90–0.98 for chitin isolated from seven basidiomycete biomasses produced in SmC. This F_A value is also obtained for other chitin sources such as crustaceans, as in the study by Einbu Aslak and Vårum [17], who reported an F_A value of 0.96 for shrimp chitin. Remarkably, the F_A of chitin produced in GAM was significantly higher than the F_A determined from fed-batch SmC with added inoculum and fresh medium supplemented with ABA (F_A of 0.76) (Fig. 6). The decrease in the fraction of acetylated units on the

fungal chitin produced can be ascribed to the enhanced production of CDA in the presence of ABA in the culture medium. The successful growth and colonization of phytopathogenic fungi are related to conidia germination and evasion from plant defense hydrolases. This indicates that fungi are capable of deacetylating chitin from their cell wall via CDA activity.

4. Conclusions

In the fed-batch process, the phytohormone ABA accelerates the germination process of *C. gloeosporioides* and increases CDA production. The attained CDA activities were greater than those previously reported in batch culture processes, which is due to the improved conditions of the conducted fed-batch culture. The phytohormone ABA has an impact on the composition of the cell wall of *C. gloeosporioides*, which is reflected in the obtained chitin acetylated fractions.

Acknowledgments

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References

[1] W. Hartung, The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen, Funct. Plant Biol. 37 (2010) 806–812.
[2] S. Hwang, J. Kim, Y. Kim, H. Kim, ABA Increases susceptibility of pepper fruits to infection of anthracnose by *Colletotrichum acutatum*, Plant Pathol. J. 24 (2008) 400–406.
[3] M. Robinson, J. Riov, A. Sharon, Indole-3-acetic acid biosynthesis in *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, Appl. Environ. Microbiol. 64 (12) (1998) 5030–5032.
[4] R. Maor, S. Haskin, H. Levi-Kedmi, A. Sharon, In planta production of indole-3-acetic acid by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, Appl. Environ. Microbiol. 70 (2004) 1852–1854.
[5] S. Ulferts, R. Delventhal, R. Splivallo, P. Karlovsky, U. Schaffrath, Abscisic acid negatively interferes with basal defence of barley against *Magnaporthe oryzae*, BMC Plant Biol. 15 (2015) 7–19.
[6] C.F. Cleland, O. Tanaka, Influence of plant growth substances and salicylic acid on flowering and growth in the Lemnaceae (duckweeds), Aquat. Bot. 13 (1982) 3–20.
[7] D. Blair, O. Hekmat, A. Schuttelkopf, B. Shrestha, K. Tokuyasu, S. Withers, A.D. Van, Structure and mechanism of chitin deacetylase from the fungal pathogen *Colletotrichum lindemuthianum*, Biochemistry 45 (2006) 9416–9426.
[8] M. Iriti, F. Faoro, Abscisic acid is involved in chitosan-induced resistance to tobacco necrosis virus (TNV), Plant Physiol. Biochem. 46 (2008) 1106–1111.
[9] S. Chatterjee, S. Chatterjee, B. Chatterjee, A. Guha, Enhancement of growth and chitosan production by *Rhizopus oryzae* in whey medium by plant growth hormones, Int. J. Biol. Macromol. 42 (2008) 120–126.
[10] S. Chatterjee, S. Chatterjee, B. Chatterjee, A. Guha, Influence of plant growth hormones on the growth of *Mucor rouxii* and chitosan production, Microbiol. Res. 164 (2009) 335–347.
[11] N. Pacheco, S. Trombotto, L. David, K. Shirai, Activity of chitin deacetylase from *Colletotrichum gloesporoides* on chitinous substrates, Carbohydr. Polym. 96 (2013) 227–232.
[12] H. Kauss, B. Bauch, Chitin deacetylase from *Colletotrichum lindemuthianum*, Methods Enzymol. 161 (1988) 518–523.
[13] G.L. Peterson, Determination of total protein, Methods Enzymol. 91 (1983) 12–95.
[14] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[15] A. Niederhofer, B.W. Müller, A method for direct preparation of chitosan with low molecular weight from fungi, Eur. J. Pharm. Biopharm. 57 (2004) 101–105.
[16] L. Heux, J. Brugnerotto, J. Desbrières, M.-F. Versali, M. Rinaudo, Solid state NMR for determination of degree of acetylation of chitin and chitosan, Biomacromolecules 1 (2000) 746–751.
[17] Einbu Aslak, Kjell M. Vårum, Characterization of chitin and its hydrolysis to GlcNAc and GlcN, Biomacromolecules 9 (2008) 1870–1875.
[18] F. Tajdini, M. Amini, N. Nafissi-Varcheh, M.A. Faramarzi, Production, physicochemical and antimicrobial properties of fungal chitosan from *Rhizomucor miehei* and *Mucor racemosus*, Int. J. Biol. Macromol. 47 (2010) 180–183.
[19] N. Simonetti, V. Strippoli, A. Cassone, Yeast-mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*, Nature 250 (1974) 344–346.
[20] I. Tsigos, A. Martinou, D. Kafetzopoulos, V. Bouriotis, Chitin deacetylases: new, versatile tools in biotechnology, Trends Biotechnol. 18 (2000) 305–312.
[21] W.A. Vargas, J.M. Sanz, G.E. Rech, L.P. Rivera, E.P. Benito, J.M. Díaz, M.R. Thon, S.A. Sukno, Plant defense mechanisms are activated during biotrophic and necrotrophic development of *Colletotricum graminicola* in maize, Plant Physiol. 158 (2012) 1342–1358.
[22] A.J. Deshmukh, B.T. Mehta, A.N. Sabalpara, V.A. Patil, In vitro effect of various nitrogen, carbon sources and pH regimens on the grown and sporulation of *Colletotrichum gloeosporioides* Penz. and Sacc causing anthracnose of Indian bean, J Biopestic. 5 (2012) 46–49.
[23] D. Prusky, J. McEvoy, R. Leverentz, W. Conway, Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence, Phytopathology 91 (2001) 1105–1113.
[24] L. Ramirez-Coutiño, J. Espinosa-Márquez, M.G. Peter, K. Shirai, The effect of pH on the production of chitinolytic enzymes of *Verticillium fungicola* in submerged cultures, Bioresour. Technol. 101 (2010) 9236–9240.
[25] X.Q. Shi, B.Q. Li, G.Z. Qin, S.P. Tian, Antifungal activity and possible mode of action of borate against *Colletotrichum gloeosporioides* on mango, Plant Dis. 95 (2011) 63–69.
[26] J.J. Cascino, R.F. Harris, C.S. Smith, J.H. Andrews, Spore yield and microcycle conidiation of *Colletotrichum gloeosporioides* in liquid culture, Appl. Environ. Microbiol. 56 (1990) 2.303–2.310.
[27] K.D. Kim, B.J. Oh, J. Yang, Differential interactions of a *Colletotrichum gloeosporioides* isolate with green and red pepper fruits, Phytoparasitica 27 (1999) 1–10.
[28] J.A. Roubos, G. van Straten, A.J.B. van Boxtel, An evolutionary strategy for fed-batch bioreactor optimization; concepts and performance, J. Biotechnol. 67 (1999) 173–187.
[29] F. Di Mario, P. Rapanà, U. Tomati, E. Galli, Chitin and chitosan from basidiomycetes, Int. J. Biol. Macromol. 43 (2008) 8–12.