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EFFECTS OF CONCANAVALIN A ON THE GERMINATION OF SMUT TELIOSPORES AND ON THE HYPHAL GROWTH

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ABSTRACT

Sporisorium scitamineum cells, that causes smut disease in sugar cane, respond to Concanavalin A (ConA). This lectin stimulates the cytoagglutination of teliospores, an excessive elongation of germinative tubes and induces morphological changes in hyphae, in the same way that it occurs in other cellular types. These events seem to play a defensive role in plants against pathogenic infections. In fact, ConA prevents *S. scitamineum* germination. Binding assays reveal that the distribution of ConA specific receptors is heterogeneous in both location and stage of cycle. Generally, it seems that the lectin activates mitotic events during the life cycle of cells that have been previously able to germinate. For example, increased cell budding is observed in released sporidia after contact with high concentration of the lectin. Moreover, desorption assays indicate that bound ConA is partially desorbed by methyl-mannose and by sugar cane glycoproteins, suggesting that important polysaccharide ligands involved in the defensive response against smut disease could be contained in sugar cane juice produced by resistant varieties.

KEYWORDS: ***Sugar cane, glycoproteins, defence, lectin, desorption***

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INTRODUCTION

Concanavalin A (ConA) is a lectin, produced by *Canavalia ensiformis*, that requires Ca^{2+} to produce cytoagglutination[1] and able to develop arginase activity in the presence of Mn^{2+}. [2] ConA interacts with different cell types, not only with plant cells. For example, ConA not only binds to lichenized Chlorophyceae[2] and cyanobacteria[3] but also binds to the surface of mammalian cells and acts as a stimulator of mitosis by binding to insulin receptors in lymphocytes.[4] Both binding action and stimulation of mitosis are reversed by incubation of the cells with α-D-methyl-mannopiranose.[5] The effect of the addition of ConA to a culture of *Ustilago maydis* has been studied by Saavedra et al.,[6] as a contribution to the understanding of the mechanisms of recognition and infection of plants by fungi. They demonstrated that the binding of ConA to the basiodiospores causes several morphological changes, such as spore aggregation, multiple branch formation in hyphae and an increased budding activity of cells. The basidiospores, which usually are thin and elongated structures, are transformed into more long cells showing thickening in some areas that causes cell deformations. The binding of ConA to the *U. maydis* basiodiospores was evidenced by using a fluorescent conjugate of the lectin. A higher fluorescence intensity at the tips and areas of budding of the basiodiospores have been observed, suggesting a heterogeneous distribution of saccharidic structures (ligand) on the surface of the fungal cell wall in relation to the different stages of its growth.Lectins from *Triticum vulgaris*, *Phaseolus lunatus*, *Dolichos biflorus* and maize coleoptyles produce inhibition of both teliospore germination and fungal growth of *U. maydis*[7] in a similar way to that found for some sugarcane glycoproteins on teliospores of *Sporisorium scitamineum*,[8,9] phylogenetically close to *U. maydis*. Nevertheless, ConA behaves as an in vitro activator of the germination of teliospores and basidiospores of *U. maydis* rather than as an inhibitor of the process. Thus, it has been proposed that this activation can be due to the binding of the lectin to polysaccharide-containing ligands in the surface of the cell wall of the basiodiospores. Morphological changes, and, probably, a previous activation of the parasitic abilities of the pathogen derive from this binding. The growth kinetics of the *U. maydis* mycelium cultured on ConA is discretely activated by the lectin from the beginning of the incubation time compared to that shown in cultures in the absence of ConA. The binding of the lectin, the subsequent agglutination of these cells, the location of the ligands in the germinative top of basidiospores, and the evident morphological modifications that ConA induces in the basidiospores of *U. maydis* bring sufficient evidences to support the idea that the lectin substantially modifies the normal pathway of in vitro development of the basidiospores.[7] ConA modifies the cell surface of the basidiospores in such a way that it allows the biological cycle of *U. maydis* to continue??? The distribution of ligands containing mannose and/or glucose, specific for the binding of ConA, should vary during the life cycle of *U. maydis*, appearing at the budding zone during the yeast stage as well as at the tips and septate areas of fungal mycelium. These sugars of the cell surface of *U. maydis*, specific for ConA, can also participate as elicitors in the recognition of the plant. [7] On the other hand, results reported by Saavedra et al. [6] allow to postulate the existence of a recognition mechanism activated during the first contact of *U. maydis* spores (and/or basidiospores) with the surface of stems, leaves or seeds of different species from the genus *Zea* (maize and teocintles, *Z. perennis*). The plant would develop fixed-surface molecular structures, the nature and specificity of which must be similar to those that bind ConA, since polysaccharides and glycoproteins containing mannose–glucose units abound in the cell walls of the fungus and at the germinative tips of the same organism. These molecular interactions lectin-ligand would be the first signal of the fungal infection of the plant.Since *S. scitamineum* is phylogenetically very close to *U. maydis*, as has been said above, the effects of ConA on smut teliospores can provide valuable information about the recognition systems between the host and the pathogen.

ABBREVIATIONS

ConA, concanavalin A; FITC, fluorescein isothiocyanate; HMMG, high molecular mass glycoproteins; Tris, Tris (hydroxymethyl) aminomethane.

MATERIALS AND METHODS

Teliospores of the pathogen *Sporisorium scitamineum* (Syd.) and six months-old plants of *Saccharum officinarum* (L.), Mayarí 55-14 cv. resistant to smut, field grown in the Botanic Garden of Complutense University of Madrid (Madrid, Spain), were used throughout this work.

Teliospore germination and inoculation procedure

Teliospores of *S. scitamineum* were isolated from whips collected from diseased Barbados 42231 plants (susceptible to smut) in experimental crops of the National Institute for Sugarcane Investigation (INCA) in Matanzas, Cuba. Teliospores were sterilized in surface and incubated in sterile Lilly and Barnett[10] (1951) medium at 38 °C for 5 days, as previously described.[11] Single sporidial colonies were isolated and re-incubated in the same medium. In order to determine the mating type of each isolate, random mating experiments were performed. Mating reaction was evidenced by the appearance of aerial mycelium and isolates were arbitrarily designed as either plus or minus.[12] Five six months-old plants of Mayarí 5514 variety were inoculated as previously described with compatible + and – sporidia.[13] Five non-inoculated plants were used as controls. Inoculated and non-inoculated plants were sampled at 6 hours post-inoculation. Stems were maintained at -20 °C.

Purification of sugarcane HMMG (high molecular mass glycoproteins)

HMMG sugarcane glycoproteins from inoculated and non-inoculated plants of Mayarí variety were obtained from stalks as previously described [13] and used in desorption assays.

Germination assay

Percentage of teliospore germination in the presence of increasing concentrations of ConA (0–0.25 mg.mL^{-1}) was evaluated according to the protocol described by Sánchez-Elordi et al.,[13] by using a light Olympus BX51 microscope fitted with an Olympus DP72 camera for capturing images. Images were analyzed with the Cell A Image Acquisition Software. Each analysis was performed in triplicate.

Labeling ConA with fluorescein isothiocyanate (FITC) and desorption assays

For competition assays, Sánchez-Elordi protocol was followed with modifications. [13] ConA (1.0 mL from 1.0 mg mL^{-1} lectin solution), prepared in 10 mM Tris-HCl, pH 9 buffer, was mixed with 0.1 mL of 1mg mL^{-1} fluorescein isothiocyanate (FITC) dissolved in the same buffer. Mixture was maintained at 30°C for 2 h with vigorous shaking in the dark. Then, the mixture was dialyzed against 2L of Tris-HCl buffer, pH 9.0, at 4°C for 24 h in the dark to remove the excess of unbound FITC. The dialysate, containing labeled fluorescent ConA, was used for binding and competition assays.[14] Firstly, initial fluorescence level of labeled ConA solution was quantified by using a Kontron SF25 spectrofluorometer. Excitation and emission wavelengths used were 490 and 517 nm, respectively. Then, pre-hydrated teliospores (1.5 mg dry weight) were incubated with 300 µL of ConA-FITC at 30 °C for 2 h with vigorous shaking in the dark. Samples were centrifuged at 9200 × g for 10 min at 4 °C and the fluorescence emission of the supernatants was measured. After extensive washing to remove all unbound fluorescence, labeled teliospores were incubated for 1 hour in the presence of 300 µL of 100 mM methyl-mannose (a) or HMMG from non-inoculated (b) and inoculated (c) My plants, according to Legaz et al.[15] Samples were then centrifuged and fluorescence in supernatants was measured in order to evaluate the desorption action that each agent exerts on the bound ConA. Each analysis was performed in duplicate. Light and fluorescent microscope images of labeled spores were obtained before and after desorption by using Olympus BX51 microscope fitted with an Olympus DP72 camera and analyzed by using the Cell A Image Acquisition Software. Fluorescence images were taken with an excitation filter of 465 nm.

RESULTS AND DISCUSSION

The incubation of smut teliospores in buffered solutions of ConA at variable concentration increased the number of agglutinated cells, with a concomitant decline of free-living spores. In parallel, the increase of the lectin concentration added to the incubation media markedly increased the release of sporidia, from 226 ± 20 in the absence of ConA to 754 ± 69 for a concentration of 0.25 mg mL^{-1} lectin (Figure 1). In apparent disagreement with this last result, the number of germinated teliospores decreased for this concentration of lectin, as observed in Figure 2.Moreover, numerous morphological changes could be observed in cells after contact of these with increasing concentrations of the lectin, in relation to the control (3A, B).Firstly, the teliospores appeared strongly agglutinated (Figure 3C). Germination produced elongated (Figure 3C) and branched (Figure 3D, E) hyphae, some of those expanded at their ends (Figure 3D). By increasing the dose of ConA in the incubation medium, the aggregates (Figure 3G) and the excessive hypha elongation (Figure 3I) become more evident, and the mitotic activity giving rise to a large amount of sporidia (Figure 3F) which are produced by budding. Some of these sporidia can be seen at the budding (Figure 3H) or conjugation (Figure 3I) phases. All of these alterations were observed by light microscopy (Figure 3).

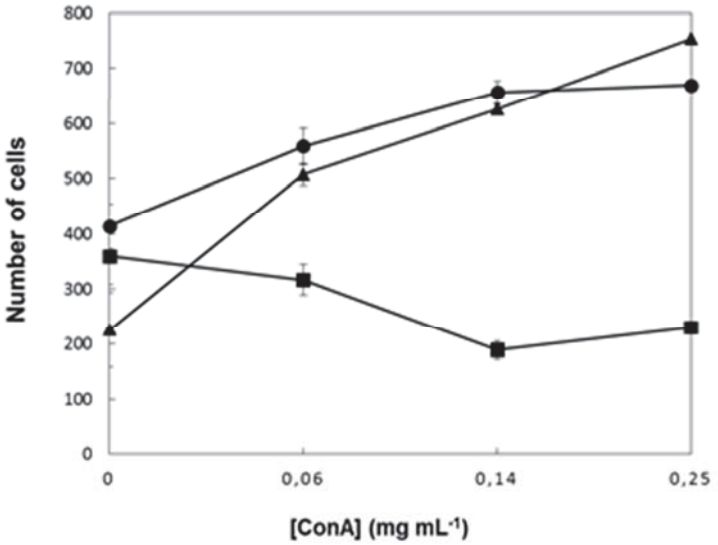

Figure 1

Number of aggregated (●) and released teliospores (■) or liberated sporidia (▲) in presence of variable concentrations (0- 0.25 mg mL^{-1}) ConA.

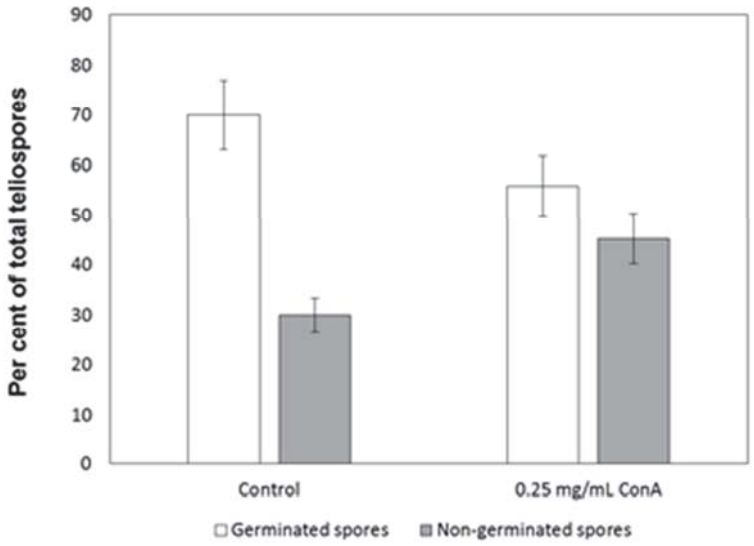

Figure 2
Percentage of germinated and non-germinated teliospores in presence or absence of ConA in medium.

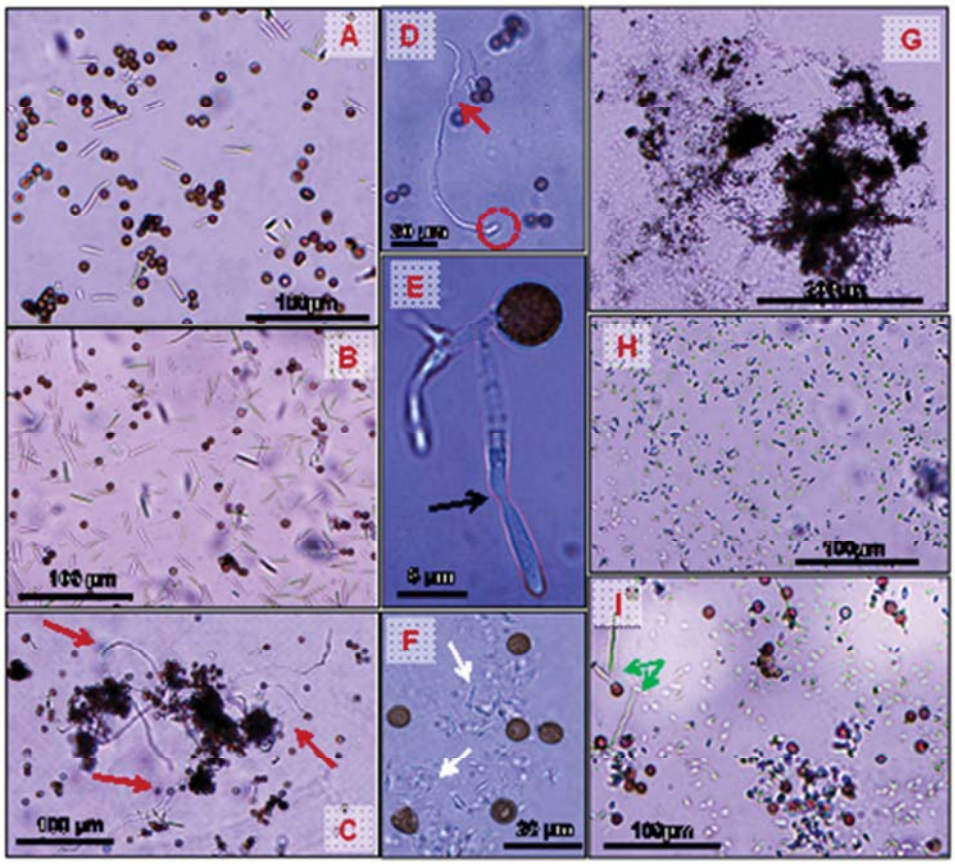

Figure 3
Light microscopy of smut teliospores, treated or not with ConA. A and B) Control, untreated spores; C) Teliospores incubated in 0.06 mg mL^{-1} ConA; D and E) Teliospores incubated in 0.14 mg mL^{-1} ConA; F-I) Teliospores incubated in 0.25 mg mL^{-1} ConA. White arrows indicate abundance of sporidia produced after budding; black arrow notes the point of formation of a new hypha, red arrows point to elongated and branched hyphae and yellow arrows show conjugation. Red circle indicates a thickening visualized at the end of many hyphae treated with ConA (0.14 mg mL^{-1}).

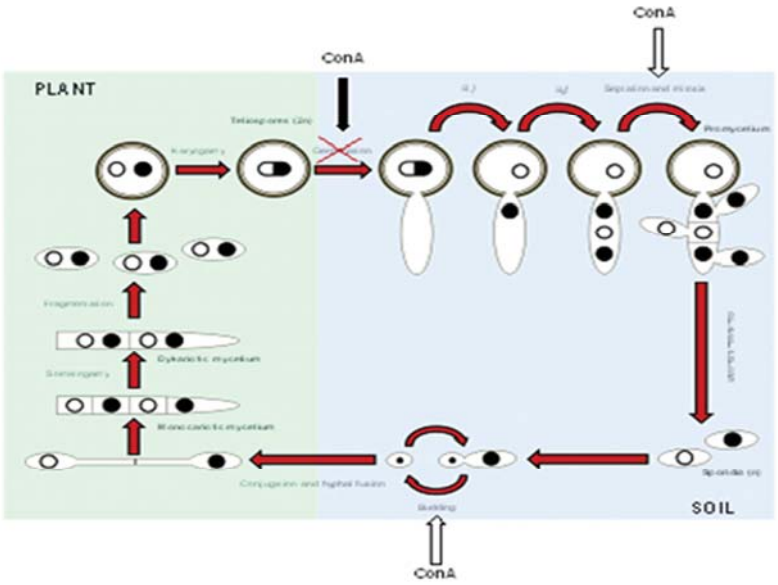

Figure 4
Life cycle of Sporisorium scitamineum. Maximum activity points of ConA are indicated.

In relation to that described in the literature,[6] ConA seemed to slightly inhibit germination but, after this first phase, it acted by favoring these stages of the cycle of the pathogen which could be associated to the mitotic division of the cells (Figure 4) after the first two reductive divisions (meiosis). In addition, ConA seemed to stimulate the budding of sporidia before sexual conjugation. For this reason, the relationship dose-response between sporidia production and ConA concentration revealed a large number of sporidia (Figure 3H) although teliospore germination rate significantly declined (Figure 2). The highest concentration of ConA seemed to stimulate excessively the budding, and might be hindering the normal feed cycle. The sporidia of *S. scitamineum* responded to ConA, so that they should possess in their cell wall a ligand containing superficial residues of glucose and mannose.Figure 5A shows a non-homogeneous distribution of ConA-FITC in *S. scitamineum* cells.

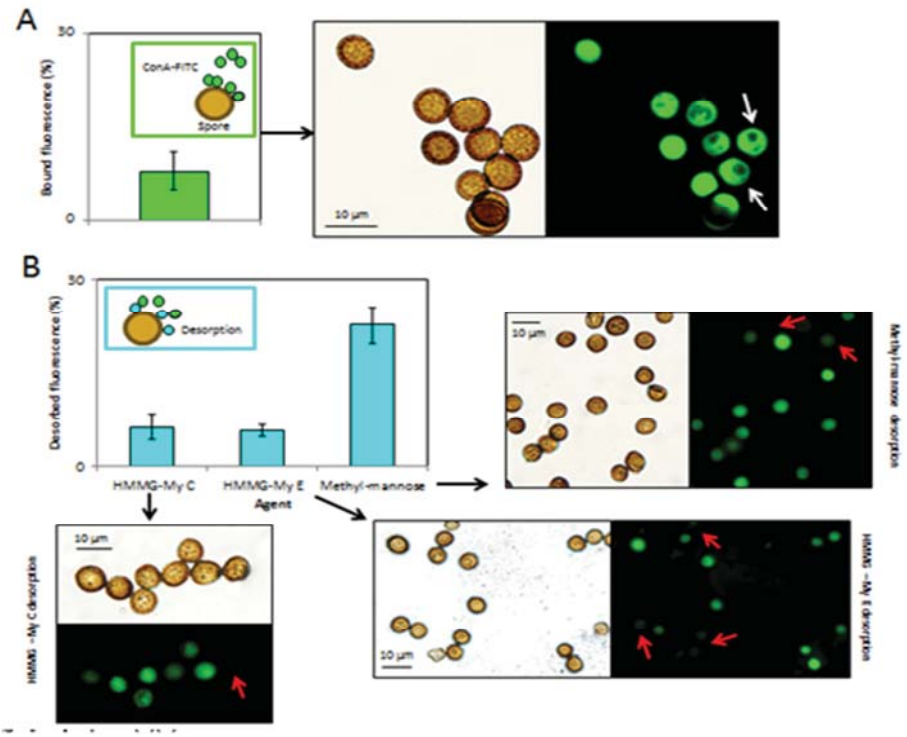

Figure 5
A) Percentage of bound ConA-FITC to teliospore cell wall. White arrows indicate non-fluorescent areas in cells. B) Percentage of desorbed fluorescence in labeled cells after incubation with HMMG from inoculated plants, non-inoculated plants or methyl-mannose. Red arrows point to cells that have lost the fluorescence, probably after contact with the desorption agent.

In fact, non-fluorescent areas seemed to correspond to the germinative spore. It suggested that ConA receptors initially occurred on teliospores wall, which were responsible for excessive agglutination of cells, but they could disappear before the emergency of the germinative tube. However, although the germinative spore was delimited by ConA distribution, cells are not able to germinate. Probably the lectin could prevent appearance of other signals.On the other hand, the expression of ConA receptors seemed to increase in already formed germinative tubes, that turned into elongated cells, and in already released sporidia to stimulate cell budding. Then, it might be considered that ConA caused effects on cells only if they have been previously able to germinate.The invasive ability of the pathogen depends not only on the hyphal growth but also on its potential to form appressoria. [16] Teliospores of *S. scitamineum* germinate and undergo meiosis to form four haploid basidiospores. However, haploid cells are not infective and only the dikaryotic hyphae formed by fusion of compatible sporidia can infect the host [17] (Fernández-Álvarez et al. 2009). Germination of smut spores occur on the internodal surface, which is followed by the formation of appressoria on the inner scales of the young buds and on the base of the emerging leaves.[16] Appressoria formation and plant penetration requires the fungal adhesion to hydrophobic surfaces, represented in nature by cell wall glycoproteins [18] and the occurrence of specific signals.[19] Thus, the adhesive ability of fungal cells can be nullified by blocking the access of fungal hyphae to cell wall glycoproteins, role that is played by secreted sugar cane glycoproteins in resistant cultivars.[20] by simulating a false quorum signal. This is a mechanism designed to replace the genuine quorum signal, which is triggered by the own teliospores in order to increase the size of the inoculum potentially infective.[13] Possibly, ConA might play the same blocking role, as it has been described for *Rhodosporidium toruloides*.[21] However, in this case, ConA does not inhibit the growth of fungal mycelium although the morphological alteration of hyphae could be interpreted as an additional inconvenience to form appressoria (Figure 3D).The fact that ConA was able to use ligands with either glucose or mannose in their polysaccharidic moiety could be the basis for a similarity of mechanisms of action with the linkage of glycoproteins of defense produced by the sugar cane as a response to mechanical injuries,[22] infections by fungi,[8] bacteria[23] or association with endosymbionts,[24] such as *Gluconacetobacter diazotrophicus*. In fact, the desorption of bound ConA after incubation of the teliospores with methyl-mannose showed similarities with the desorption of bound HMMG induced by sucrose or α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside[22] but still more indicative with the partial desorption of linked ConA by the action of the cane-produced HMMG (Figure 5). Moreover, it would be important to consider differences between percentage of teliospore germination described herein and those concerning teliospore aggregation and germination according to the changing chemical composition of sugar cane glycoproteins composition before and after smut invasion, as found for arginase activity.[25] These results did not show differences in desorption capacity between HMMG from inoculated or non- inoculated plants. It would indicate a similar content-by-cell of polysaccharide ligand for HMMG that competes with ConA in the assays.

CONCLUSION

We know that ConA causes morphological and biological changes in *S. scitamineum* growth from teliospores. This indicates that smut cells possess specific receptors on their cell wall for the lectin during specific life stages of *S. scitamineum* development. ConA prevents spore germination although stimulates mitotic division in tubes and sporidia, if they are formed. Since sugar cane glycoproteins partially desorb ConA, these results suggest that teliospore cell wall contain an important number of different polysaccharide ligands so that said proteins act as a defense factor against the pathogen invasion.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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