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Ascophyllum nodosum seaweed extract effect on morphology and cellulolytic ability of the fungus *Fusarium oxysporum* f. sp. *vasinfectum*

Efeito do extrato da alga marinha *Ascophyllum nodosum* na morfologia e habilidade celulolítica do fungo *Fusarium oxysporum* f. sp. *vasinfectum*

Efecto del extracto de algas *Ascophyllum nodosum* sobre la morfología y capacidad celulolítica del hongo *Fusarium oxysporum* f. sp. *vasinfectum*

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Abstract

This work aimed to verify the effect *in vitro*, of *Ascophyllum nodosum* (AN) seaweed extract on the morphology and cellulolytic capacity of the fungus *Fusarium oxysporum* f. sp. *vasinfectum* (FOV). Thus, the fungus was placed in contact with different doses of the extract, being these: 0, 0.5, 1.0, 2.0, 4.0 and 8.0%. It was verified that the product, with increasing doses, progressively induced mycelial growth of the fungus, as measured by the diameter of the colonies and fresh mass of mycelium grown in PD (potato-dextrose) culture medium. This result was also corroborated by the progressive increase in the activity of the β-1,3-glucanase and chitinase enzymes required during the hypha elongation process. However, the AN extract progressively reduced FOV sporulation with increasing doses. Furthermore, the cellulolytic capacity of the phytopathogen was significantly reduced in the presence of the

algae extract, which was measured by the activity of the enzymes endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase. Thus, these facts constitute important information for the management of fusariosis, since the inhibition of sporulation and decreasing degradation capacity of the cellulose by the pathogen, can translate into declined disease in compatible host-pathogen interactions.

Keywords: Fusariosis; Cotton wilt; Fungi physiology; Mode of action; Biomolecules.

Resumo

Este trabalho objetivou verificar o efeito, *in vitro*, do extrato da alga marinha *Ascophyllum nodosum* (AN) sobre a morfologia e capacidade celulolítica do fungo *Fusarium oxysporum* f. sp. *vasinfectum* (FOV). Assim, o fungo foi colocado em contato com diferentes doses do extrato, sendo estas: 0, 0.1, 0.2, 0.4 e 0.8%. Foi verificado que o produto, com o aumento das doses, induziu progressivamente o crescimento micelial do fungo, medido pelo diâmetro das colônias e massa fresca do micélio crescido em meio de cultivo BD. Tal resultado foi corroborado pelo aumento, também progressivo, da atividade das enzimas β-1,3-glucanase e quitinase, requeridas durante o processo de elongação da hifa. No entanto, o extrato de AN reduziu progressivamente a esporulação de FOV com o aumento das doses. Ainda, a capacidade celulolítica do fitopatógeno foi significativamente reduzida na presença do extrato da alga, sendo esta variante medida por meio da atividade das enzimas endo-β-1,4-glucanase, exo-β-1,4-glucanase e β-glicosidase. Assim, tais fatos se configuram em importantes informações para o manejo da fusariose, uma vez que a inibição da esporulação e, também, da capacidade de degradação da celulose pelo patógeno, pode se traduzir em diminuição doença, em interações patógeno-hospedeiro compatíveis.

Palavras-chave: Fusariose; Murcha do algodoeiro; Fisiologia de fungos; Modo de ação; Biomoléculas.

Resumen

Este trabajo tuvo como objetivo verificar el efecto, in vitro, del extracto del alga Ascophyllum nodosum (AN) sobre la morfología y capacidad celulolítica del hongo *Fusarium oxysporum* f. sp. *vasinfectum* (FOV). Por tanto, el hongo se puso en contacto con distintas dosis del extracto, que son: 0, 0,1, 0,2, 0,4 y 0,8%. Se encontró que el producto, con dosis crecientes, inducía progresivamente el crecimiento micelial del hongo, medido por el diámetro de las colonias y la masa fresca del micelio crecido en medio de cultivo BD. Este resultado fue corroborado por el aumento, también progresivo, de la actividad de las enzimas β-1,3-

glucanasa y quitinasa, requeridas durante el proceso de elongación de hifas. Sin embargo, el extracto de AN reduce progresivamente la esporulación del FOV con dosis crecientes. Asimismo, la capacidad celulolítica del fitopatógeno se redujo significativamente en presencia del extracto de algas, siendo esta variante medida por la actividad de las enzimas endo-β-1,4-glucanasa, exo-β-1,4-glucanasa y β-glucosidasa. Así, estos hechos son información importante para el manejo de la fusariosis, ya que la inhibición de la esporulación y, también, de la capacidad de degradación de la celulosa por parte del patógeno, puede traducirse en una disminución de la enfermedad, en interacciones patógeno-huésped compatibles.

Palabras clave: Fusariosis; Algodón marchito; Fisiología de los hongos; Modo de acción; Biomoléculas.

1. Introduction

The genus *Fusarium* contains some of the most important phytopathogenic fungi species for world agriculture (Dean et al., 2012). Other species of this genus are, admittedly, important producers of mycotoxins and, still others, are becoming worrisome pathogens for humans (van Diepeningen & Hoog, 2016). The species *Fusarium oxysporum* f. sp. *vasinfectum* is responsible for the cotton wilt that is one of the most serious problems of the crop. A few years ago, this fungus was responsible for the decay of the cotton crop in the Brazilian conditions of cultivation, however, thanks to the availability of resistant varieties, the productivity of the crops is assured (Bedendo, 2011).

The control of fusariosis is mainly hampered by the fungus's ability to produce resistance structures, the chlamydospores, which remain dormant in the soil for long periods. Thus, when conditions are favorable and host is available, the structure germinates on the roots of the plant and the hyphae penetrates directly or through wounds. Hypha is the fungal mycelium-forming unit and has, in its constitution, chitin and β-1,3-glucan polymers. Chitin, the most abundant constituent, is a polymer formed by β-1,4-N-acetylglucosamine units and its metabolism is divided into two processes: synthesis and degradation. Assuming that hyphae growth occurs at the apex of the structure and that it is constantly rigid (Alexopoulos et al., 1996), the fungus must be able to produce enzymes that degrade the structural components of the cell wall of the hypha to grow. Thus, chitinases and β-1,3-glucanases are the enzymes responsible for catabolism of the cell wall. The latter, in turn, being less rigid can, this way, grow. After this process, the activity of cell wall degradation enzymes

decreases and enzymes of the type chitin-synthase and glucan-synthase are produced for the reconstitution of the polymers of chitin and β-glucan, respectively (Adams, 2004; Bauermeister et al., 2010). The process is cyclical and as soon as the fungus needs to grow again, the cell wall degradation enzymes are produced again.

Enzymes also mediate the advancement of the fungus inside the plant. In a logical metabolic sequence, so that the fungus *F. oxysposum* f. sp. *vasinfectum* reaches the cell interior, initially, it should be able to degrade the suberin, which covers the outer organs of the plant, then the middle lamella, aided by pectinolytic enzymes and finally the cell wall. The latter, due to its complex constitution, demands the high ability of the fungus to produce extracellular enzymes.

Cellulases, hemicellulases and ligninases are the enzymes produced at the moment of cell wall degradation and are related to cellulose, hemicellulose and lignin degradation, respectively, being molecules that, when present, confer a high degree of pathogenicity to the microorganisms (Pascholati, 2011). Highlighting the cellulases, these enzymes are produced in greater quantity due, mainly, to the high cellulosic content of the vegetables in general. Thus, endo-β-1,4-glucanases or β-1,4-D-glucan-glucohydrolases, exo-β-1,4-glucanases or β-1,4-D-glucan-cellobiohydrolases and β-glucosidases are enzymes of integrated action, but responsible for distinct steps within the degradation process of the cellulose polymer. The first, at random, breaks down β-1,4 glycan bonds of the glucan chain. Then exoglucanase acts on the non-reducing ends of the molecule, releasing cellobiose molecules (glucose disaccharides). Finally, the hydrolysis of cellobiose is catalyzed by β-glucosidase (Schwan-Estrada et al., 2003; Pascholati, 2011).

Several natural products of biological origin have already been tested and presented promising results in the interference of the metabolism of the fungus *F. oxysporum* f. sp. *vasinfectum* (Lima et al., 2014; Silva et al., 2014; Freddo et al., 2016). Among these products, the extracts obtained from marine algae are widely used (Jayaraman & Ali, 2015). The algae *Ascophyllum nodosum* (L.) Le Jolis is the most studied and commercially exploited (Ugarte et al., 2006). Its effect on plant health has already been proven by the treatment of cucumbers with two doses of the product (0.5 and 1.0%) and reduction of diseases caused by the fungi *Alternaria cucumerinum*, *Didymella applanata*, *Fusarium oxysporum* and *Botrytis cinerea* (Jayaraman et al., 2011). Extracts from other seaweed, such as *Alaria esculenta*, *Fucus vesiculosus*, *Spirulina plantensis* and *Eklonia maxima*, have already been tested *in vitro* and have shown a fungicidal effect against a number of pathogens, including *Fusarium roseum*

and *Fusarium oxysporum*, but at very high doses, 50 and 90%, impossible to be practiced in extensive crops (Coşoveanu et al., 2010).

Understanding the growing need for new technologies for the management of plant diseases, which meet the current demand for sustainable production, the use of natural products in agriculture, among them seaweed extracts, is increasingly significant. However, it is necessary to understand the effect of these products on the entire agroecosystem, especially on the pathogen. Thus, this work aimed to verify the *in vitro* effect of *Ascophyllum nodosum* seaweed extract on the morphology and cellulolytic capacity of the fungus *Fusarium oxysporum* f. sp. *vasinfectum*.

2. Material and Methods

All the tests described below were carried out at the Laboratory of Microbiology and Biotechnology, at Biology Department, State University of Maranhão, São Luís, MA.

2.1 *In vitro* effect of *A. nodosum* extract on mycelial growth, mycelium fresh mass and sporulation of the fungus *F. oxysporum* f. sp. *vasinfectum*

The fungus *F. oxysporum* f. sp. *vasinfectum* (FOV) was obtained by the isolation of fragments from the vascular system of cotton plants with typical symptoms of fusariosis. After obtaining pure culture, inoculation in healthy seedlings, re-isolation, and identification of the species by morphological characteristics of the colony and conidium, the fungus was preserved in test tubes containing PDA (potato-dextrose-agar) culture medium.

The assay to evaluate mycelial growth of FOV consisted of Petri dishes (80 mm in diameter), containing PDA culture medium, plus five doses of *A. nodosum* extract (AN), obtained from the commercial product Acadian Seaplants®, being: 0.5, 1.0, 2.0, 4.0 and 8.0%. The 0 (zero) dose served as test control. After solidification of the medium, mycelial discs (5 mm) containing fungus structures were transferred to the center of the plates. These were than incubated in BOD type chambers at 25 °C and 12 h photoperiod, generated by fluorescent lamps of 15 W. Daily, the colonies diameter was measured, in opposite directions, until, after 7 days of evaluation, one of the plates reached the border. The values of the 6th day of measurement were used for the analysis of the mean mycelial diameter of the pathogen, expressed in mm.

The plates from the previous experiment were used to determine the sporulation of the fungus at different doses of AN. In this way, each one received 20 mL of distilled and autoclaved water, and then the mycelium was scraped with a soft bristle brush. The suspension formed was filtered in lint and the volume adjusted to 100 mL. Using a Neubauer type hemacytometer, the number of conidia formed was calculated and the values adjusted in the order of 10^6 conidia mL^{-1}.

The fresh mass of the mycelium was evaluated by growth of the fungus in liquid PD (potato-dextrose) culture medium, plus the same doses of the extract used previously. Thus, erlenmeyers containing 100 mL of the preparations after receive 5 disks of mycelium containing pathogen structures, were then incubated for 7 days on an orbital shaker and kept in continuous dark at 25 °C with stirring speed set at 150 rpm. After the period, the mycelium produced was separated from the filtrate and the fresh mass of the structure was determined on a precision scale, being the values expressed in g.

All the assays described so far have been performed in duplicate. These were grouped and their averages calculated. The tests contained six treatments with six replicates each. After completing the tests, the means of each treatment were obtained and compared by analysis of variance using the Tukey's test ($p = 0.05$). The correlation of the applied treatments (doses of the extract) with the results was verified by linear regression analysis, being considered a high correlation for the values of $R^2 \geq 0.7$ (Toledo and Ovalle, 2008). All data were analyzed using R software, version 4.0.0.

2.2 Effect of *A. nodosum* extract on the total protein content and activity of the β-1,3-glucanase and chitinase enzymes produced by the mycelium of *F. oxysporum* f. sp. *vasinfectum*

Samples of 1 g of pathogen mycelium obtained from the FOV cultivated in PD plus the same doses of AN used in the previous tests were macerated in the presence of liquid nitrogen. Following the described procedures, the preparation received 4 mL of 100 mM sodium phosphate buffer, pH 6.0 and was centrifuged at 20,000 *g* for 25 min at 4 °C. The obtained supernatants (protein extract) were used to determine the total protein content of the sample and activities of the enzymes β-1,3-glucanase and chitinase.

The total protein content of the samples was quantified according to Bradford (1976). To that end, 800 µL of the protein extract received 200 µL of Bradford's reagent (*Bio-Rad Protein Assay*). After 5 min, the absorbance was determined at 595 nm, the protein

concentration was expressed as mg equivalents of bovine serum albumin (BSA) g^{-1} sample, based on BSA standard curve.

The activity of the enzyme β-1,3-glucanase was determined for the mycelium of FOV by the colorimetric quantification of the glucose released in the laminarin substrate, according to Kombrick & Hahlbrock (1986). To do this, 150 µL of the protein extract received 150 µL of laminarin already dissolved in 100 mM sodium phosphate buffer before (1) or after (2) heating at 37 °C for 3 h. Thus, the concentration of reducing sugars was determined according to Lever (1972). We determined the concentration of reducing sugars from the standard glucose curve and the amount of glucose released by laminarin was obtained based on the subtraction: (1) - (2). The results were expressed as µg glucose min^{-1} protein mg^{-1}.

To verify the activity of the chitinase at the time of FOV mycelial growth, the colloidal chitin substrate was used, according to a protocol adapted by Moerschbacher et al. (1988). Thus, to 100 mg of the substrate, 400 µl of 0.2 M sodium phosphate buffer plus 500 µL of the protein extract produced in sodium borate buffer (0.1 M) were added. The preparation was then placed in a water bath at 50 °C for 1 h and then boiled at 100 °C for 5 min. After this time, the samples were centrifuged for 10 min at 7,000 rpm and the supernatant measured in a spectrophotometer at 595 nm. Values were expressed as units of mg^{-1} chitinase protein, according to standard protein curve (Bradford, 1976).

2.3 Determination of the cellulolytic capacity of the fungus *F. oxysporum* f. sp. *vasinfectum* under the action of *A. nodosum* seaweed extract

Initially, mycelial discs containing FOV structures were transferred to 100 mL of the Modified Melin Nokrans culture medium (MMN) (Marx, 1969), plus the same doses of the *A. nodosum* extract used in the previous trials (Melo, 2017). For the induction of enzymes, the glucose of the medium was replaced by microcrystalline cellulose (1.0%) for the determination of exo-β-1,4-glucanase activity and, by carboxymethylcellulose (CMC) (1%), for the determination of the activity of endo-β-1,4-glucanase and β-glucosidase enzymes. The activity of the enzymes was determined by indirect spectrophotometry, based on the release of glucose molecules in the medium (Schwan-Estrada et al., 2003). Thus, the exo-β-1,4-glucanase dosage was based on the reaction of 50 µL of the enzyme source obtained by collecting aliquots of the medium every 6 days over 30 days and 450 µL of microcrystalline cellulose (1.0%) in 0.05 M sodium acetate buffer, pH = 5.0. The mixture was incubated for 30 min at 50 °C and the reaction quenched with the addition of 1.5 mL of 1.0% *p*-

hydroxybenzoic acid hydrazide. After, the mixture boiled at 100 °C for 5 min and then was cooled on ice. The absorbance was determined at 410 nm (Schwan-Estrada et al., 2003).

Endo-β-1,4-glucanase activity was measured by the reaction of 50 µL of the enzyme source plus 450 µL of 1.0% CMC in 0.05 M sodium acetate buffer, pH = 5.0. After 30 min of incubation at 50 °C, the reaction was quenched by the addition of 1.5 ml of 1.0% *p*-hydroxybenzoic acid hydrazide, the preparation heated at 100 °C for 5 min and then cooled in ice. The absorbance of the reaction was determined at 410 nm (Schwan-Estrada et al., 2003).

Finally, β-glycosidase had its enzymatic activity determined with ρ-nitrophenyl-β-D-glucopyranoside as the substrate. The reaction mixture, consisting of 300 µL of ρ-nitrophenyl-β-D-glucopyranoside, 0.02 M, in 0.05 M sodium acetate buffer and 200 µL of the enzyme source, was incubated at 50 °C for 30 min. Then, 500 µL of 1.0 M Na_2CO_3 solution was added to the mixture to paralyze the reaction and the released ρ-nitrophenol was determined in a spectrophotometer at 405 nm (Deshpande & Erikson, 1988).

2.4 Experimental design and data analysis of enzymatic assays

The bioassays for the enzymatic analyzes were performed in triplicate, as well as the absorbances for each replicate that were determined three times in a row. After finalization, the triplicate was grouped in averages for each experimental unit and these were used to calculate the mean of the treatments.

All enzymatic assays were performed in a completely randomized design. The treatments consisted of the different doses of AN (0.5, 1.0, 2.0, 4.0, 8.0%), plus the control (dose zero), being these formed of six repetitions each.

The obtained means were submitted to analysis of variance and the data compared by the Tukey test, p = 0.05. The correlation of the applied treatments (doses of the extract) with the results was verified by linear regression analysis, being considered a high correlation for the values of $R^2 \geq 0.7$ (Toledo and Ovalle, 2008). All data were analyzed using R software, version 4.0.0.

3. Results

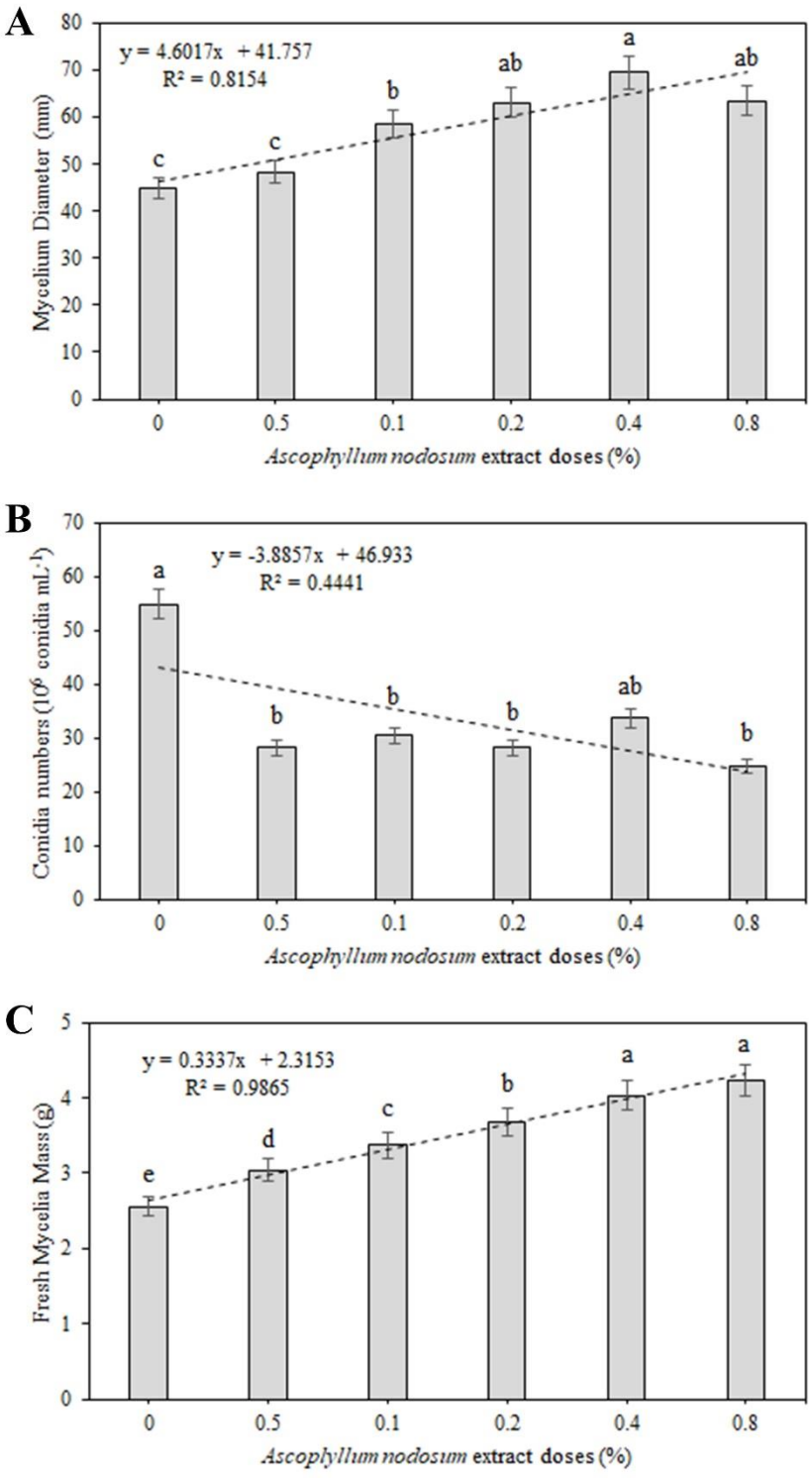
3.1 *In vitro* effect of *A. nodosum* extract on mycelial growth, mycelium fresh mass and sporulation of the fungus *F. oxysporum* f. sp. *vasinfectum*

The results obtained for the mean diameter and fresh mass of the FOV mycelium showed significant differences between the treatments, in which higher values were observed for fungus growth under the action of AN doses than in the control. The increase of the doses resulted in a progressive increase in the diameter of the colonies and fresh mass of the structure (Figures 1A and 1B).

The effect of the product on the induction of FOV growth could also be observed from the images related to the mycelial growth of the fungus in the culture media with the addition of the different doses of AN. There was a clear increase in the diameter of the colonies from the use of the algae extract (Figure 2).

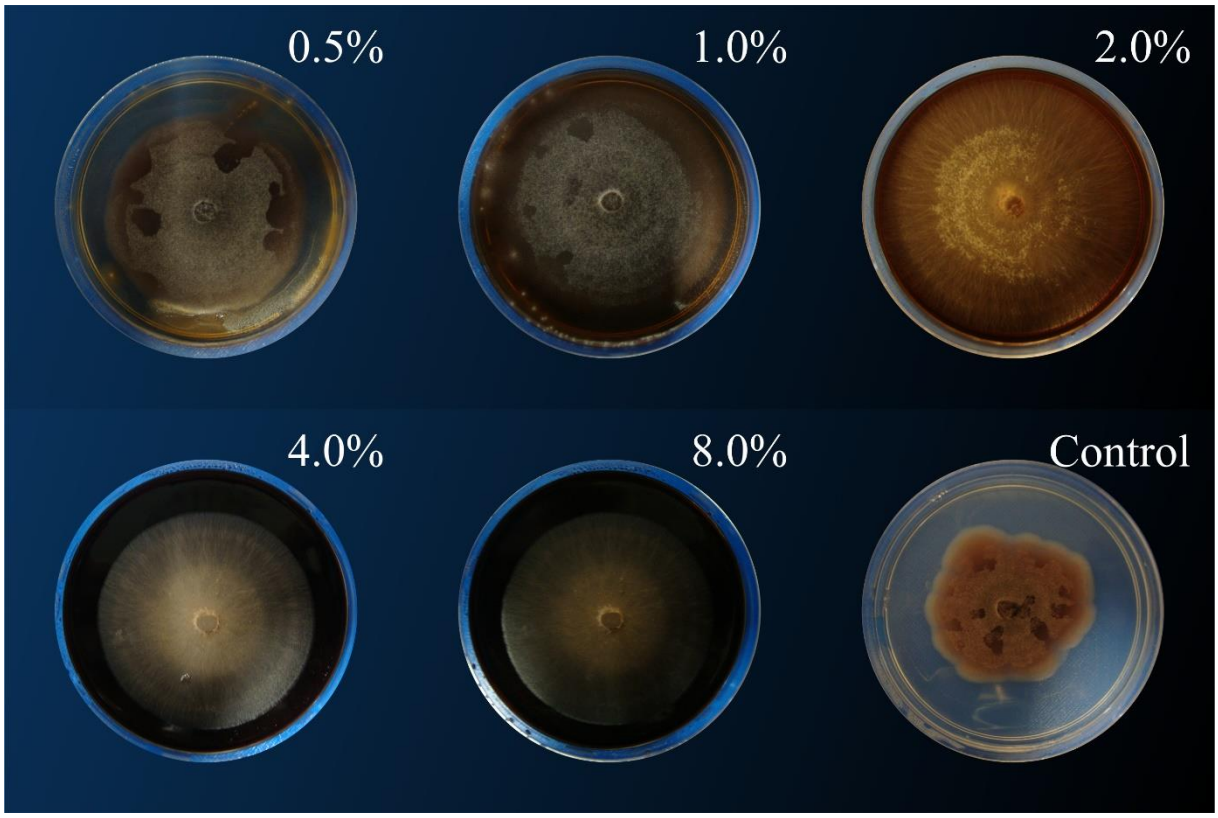
In contrast, sporulation of the fungus suffered a marked interference of AN doses (Figure 1C), where it was verified that all the colonies grown in the presence of the extract showed significantly lower sporulation than the control. In addition, there was no significant difference between the concentrations used, and it was demonstrated that the product directly interferes with the production of infective propagules of the pathogen (Figure 1C). With the data obtained from the three tests described, it was possible to observe that the high mycelial production of the pathogen did not imply high sporulation.

Figure 1. Effect of *A. nodosum* extract on the mean diameter of mycelium (A), fresh mass of mycelium (B) and sporulation (C) of fungus *F. oxysporum* f. sp. *vasinfectum*. Columns of the same group, followed by the same letter, do not differ by Tukey test, p = 0.05. The bars above the columns represent the standard error of the mean.



Source: Research data.

Figure 2. Morphological aspect of *F. oxysporum* f. sp. *vasinfectum* colonies showing the effect of *A. nodosum* extract doses on mycelial production after 7 days of incubation.



Source: Research data.

3.2 Effect of *A. nodosum* extract on the total protein content and activity of the β-1,3-glucanase and chitinase enzymes produced by the mycelium of *F. oxysporum* f. sp. *vasinfectum*

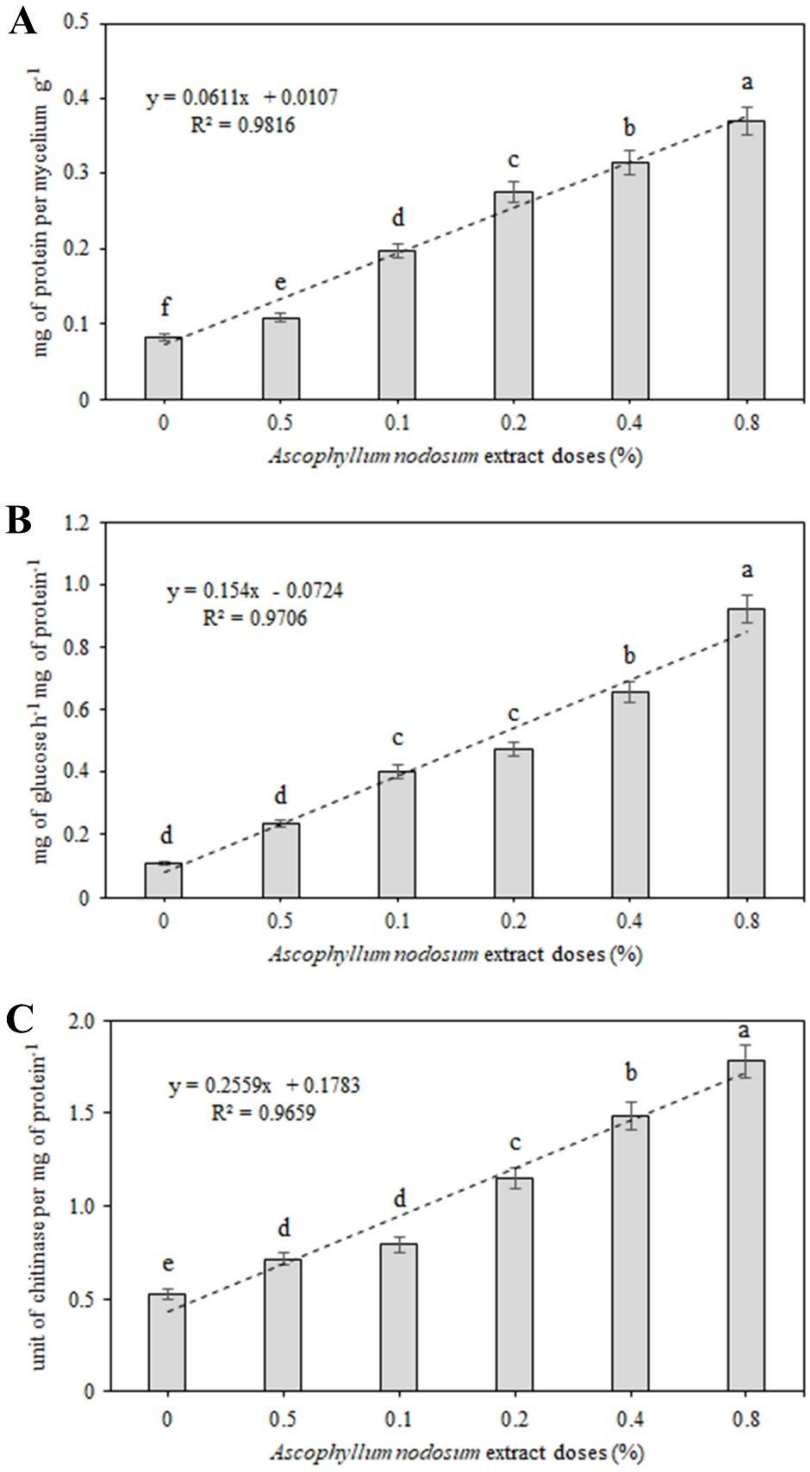
The total protein content of the FOV mycelium was strongly influenced by AN dose. Thus, it was found that all dosages used increased the peptides concentration of fungal mycelium grown in the marine algae extract, and these treatments differed significantly from the control treatment. In addition, exposure of the mycelium of the fungus to increasing doses of the extract implied a progressive increase in the total content of these molecules per gram of fresh mycelium (Figure 3A).

In a manner like that observed for the total protein content, AN significantly increased the activity of the enzyme β-1,3-glucanase. With the exception of the 0.5% dose, all the others had a higher amount of monosaccharides released than the control and increase of the doses resulted in progressive increases in the glucose content of the samples (Figure 3B). The same pattern was observed in the evaluation of chitinase activity. All doses of the product differed

statistically from the control, registering higher activity of the enzyme in chitinase units per mg of protein in treatments with the product in relation to the zero dose (Figure 3C). Furthermore, successive increases in chitinase activity were observed as the extract doses were increased.

The enzymatic activity observed in the mycelium of *F. oxysporum* f. sp. *vasinfectum* under the action of different doses of the extract of *A. nodosum*, is in total agreement with the bioassays performed in this work for the verification of fungal mycelial growth. Considering that the hyphae grow in a scheme of breaking and reconstruction of the polymer's constituent of the cell wall, greater mycelial growth implies in greater enzymatic activity, as verified.

Figure 3. Total protein content (A) and activity of the enzymes β-1,3-glucanase (B) and chitinase (C) in the mycelium of the fungus *F. oxysporum* f. sp. *vasinfectum* under the action of different doses of *A. nodosum* seaweed extract. Columns of the same group, followed by the same letter, do not differ by Tukey test, p = 0.05. The bars above the columns represent the standard error of the mean.



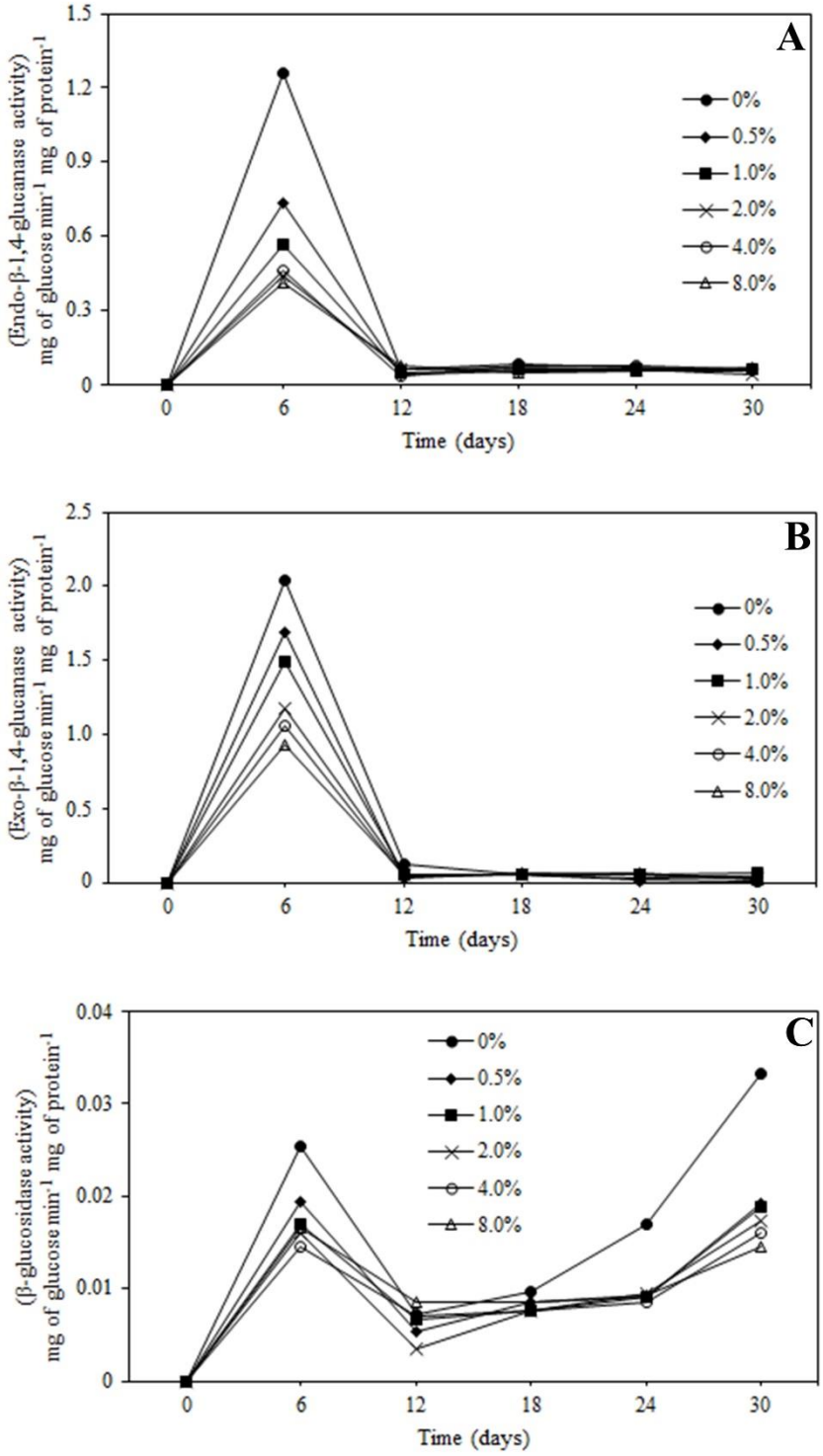
Source: Research data.

3.3 Cellulolytic capacity of fungus *F. oxysporum* f. sp. *vasinfectum* under the action of *A. nodosum* seaweed extract

The cellulolytic capacity of FOV, expressed in mg of glucose mg min^{-1} mg of $protein^{-1}$, was numerically higher on the sixth day of fungus growth in MMN culture medium for the three cellulases. From the twelfth day, there was a reduction in the activity of the enzyme's endo-β-1,4-glucanase and exo-β-1,4-glucanase, remaining thus until the end of the analysis. The β-glucosidase enzyme also showed a decrease in activity at 12 days of analysis, which was not maintained on subsequent days with progressive increases in enzymatic action. For the three cellulases, it was observed the reduction of the cellulolytic capacity of the pathogen from the culture of the fungus in MMN medium plus the AN dose (Figure 4).

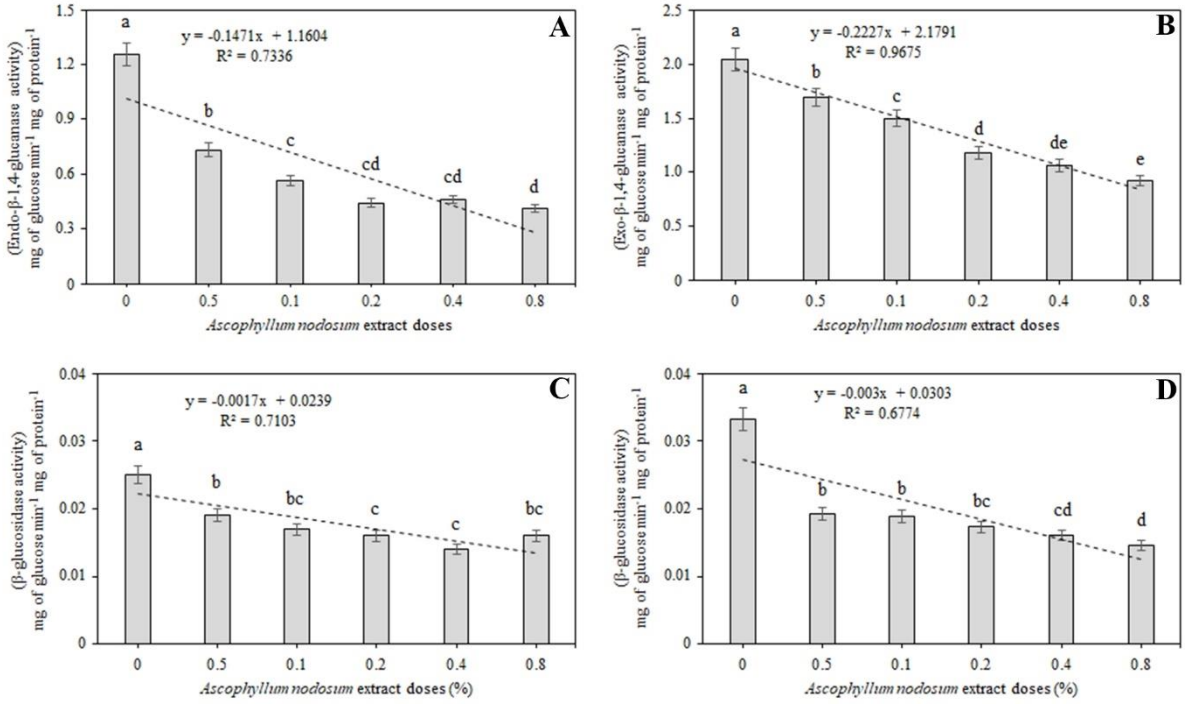
The analysis of variance in enzyme action peaks, on the 6th day of analysis for endo and exoglucanase (Figures 5A and 5B, respectively) and on the 6th and 30th days for glucosidase (Figures 5C and 5D, respectively), showed the effect of the AN doses in reducing the cellulolytic capacity of the fungus. Such parameter was significantly lower in all the treatments with the product, when compared to the control treatment. In addition, progressive increases in the doses of the extract resulted in a progressive reduction in the activity of the three enzymes (Figure 5).

Figure 4. Cellulolytic activity of fungus *F. oxysporum* f. sp. *vasinfectum* under the action of different doses of *A. nososum* seaweed extract. (A) endo-β-1,4-glucanase; (B) exo-β-1,4-glucanase; (C) β-glucosidase. The percentages shown in the graphs correspond to the doses used in the bioassay.



Source: Research data.

Figure 5. Peaks of action of the cellulolytic enzymes produced by the fungus *F. oxysporum* f. sp. *vasinfectum* under the action of different doses of the *A. nodosum* extract. Activity of the enzymes endo-β-1,4-glucanase (A) and exo-β-1,4-glucanase (B) on the 6th day of analysis and activity of the β-glucosidase enzyme in 6th (C) and 30th (D) days of analysis. Columns of the same group, followed by the same letter, do not differ by Tukey test, p = 0.05. The bars above the columns represent the standard error of the mean.



Source: Research data.

4. Discussion

Seaweed extracts have shown an effect on the growth of a wide range of phytopathogenic fungi (Coşoveanu et al., 2010; Jiménez et al., 2011; Ambika & Sujatha, 2014, 2015). Coşoveanu et al. (2010), showed reductions in the mean diameter of the colonies of the fungi *Fusarium roseum*, *F. oxysporum*, *Alternaria alternata*, *A. dauci*, *A. longipes*, *Trichoderma viride*, *Botrytis cinerea*, *Aspergillus niger* and *Penicillium expansum*, ranging from 50% to 90%, when in the presence of *Alaria esculenta*, *Fucus veiculosos*, *Fucus* sp. and *Eklonia maxima* macroalgae extracts, all commercially available. Peres et al. (2012), obtained results that contrast with those presented in this study, since the authors observed a suppressive effect of the alcoholic extract of *A. nodosum* on the growth of the fungus *Colletotrichum lagenarium*. However, the extract had no significant effect on growth

inhibition of *Aspergillus flavus* fungus. Against *Monilinia fructicola*, *A. nodosum* extract presented responses like those observed in these tests. According to the results presented by the authors, the product induced mycelial growth of the fungus and this increment was proportional to the increase of the applied doses of the extract (Oliari et al., 2014).

Studies carried out by Colapietra & Alexander (2006) prove the efficiency of A. nodosum extracts as biostimulants. Additionally, according to the manufacturer's specifications, the seaweed is recognized for being excellent in complementing growth hormones (auxins, gibberellins, cytokinins, abscisic acid), amino acids (alanine, aspartic and glutamic acid, glycine, isoleucine, leucine, lysine, proline, tyrosine, tryptophan and valine), important nutrients (N, P, K, Ca, Mg, S, B, Fe, Mn, Cu and Zn) and has a pH ranging from 7.8 to 8.2 (Norrie & Keathley , 2005). Such characteristics were also pointed out by Limberger & Gheller (2012) and explain the non-interference of the product in the mycelial development of the colonies and the stimulus to produce fresh mycelial mass, observed in the tests.

Knowing the effects of products applied to crops, on the sporulation of filamentous fungi is of great importance for phytopathologists and producers since the spore, when disseminated and deposited on the surface of a susceptible host, is a propagule with the potential to cause disease in the sound tissue. In normal biological development, filamentous fungi produce conidia after stopping vegetative growth. Under axenic conditions, the sporulation of these individuals is also modulated by the nutritional depletion of the culture media or by different stresses such as, for example, the high concentration of organic and inorganic molecules (Wulandari et al., 2009; Conrad et al., 2014). One of the hypotheses raised to explain the beginning of the process is the probable existence of sensors that signal, to the conidiogenic cell, the nutritional status of the environment (Roncal & Ugalde, 2003; Su et al., 2012).

Oliari et al. (2014) did not verify the interference of *A. nodosum* extract on the sporulation of *M. fructicola*, under the conditions in which they developed the tests. On the other hand, Mattner et al. (2014) highlight that the extract of the referred algae induces the sporulation of fungi due to the high pH that the product presents, which causes stresses on the microorganism, leading to the formation of infectious propagules. However, what was observed here was the inhibition of the sporulation of the fungus, probably generated by the high nutritional availability of the medium, also promoted by the addition of *A. nodosum* doses. Works such as de Barros et al. (2015) confirm the observed effect. The authors verified

an inhibitory effect of algae extract on the formation and germination of *Alternaria brassicicola*, an agent of alternariosis in cauliflower seedlings.

Another probable cause of the interference of *A. nodosum* on the sporulation of FOV, is in the molecular constitution of the alga. Fucanas, algal polysaccharides present in the species, are identified as agents that inhibit the germination of the infectious propagules of several phytopathogens (Khan et al., 2011; Stadnik & Freitas, 2012) and, since these molecules are the probable agents for inhibiting the germination of these structures, they can be pointed out as the possible cause of the decrease in the germination of *F. oxysporum* f. sp. *vasinfectum* in the presence of the extract, observed in the results described here.

Proteins are demanded by fungi, because in addition to their physiological functions, they can also be sources of nitrogen, carbon, and sulphur (Marzluf, 1997). Many of these molecules are involved in the plant defence process (Schwan-Estrada et al., 2008), however, in microorganisms, active forms of proteins, enzymes, are important for several processes, among them, the maintenance of the plasticity of the hypha cell wall (Bartnick-Garcia, 1970). Specific enzymes participate in the biosynthesis of the chitin and glucan molecules that will make up the cell wall (Adams, 2004). These enzymes form long chains of N-acetylglucosamine, linked by β-1,4 bonds, and glucans linked by β-1,3 bonds, respectively (Odds et al., 2003; Adams, 2004). Such an organization gives the hypha rigidity, and the growth of the structure is conditioned by the fungus' ability to partially break the cell wall to grow. The results observed for the activity of β-1,3-glucanase and chitinase, enzymes responsible for the partial breakdown of hypha at the time of growth of the structure, complement the results obtained in the growth and production of fresh mycelial mass of the fungus *F. oxysporum* f. sp. *vasinfectum*, in different concentrations of algae extract. The increase in the concentration of the extract induced increases in the vegetative structure of the fungus, so it is natural that an increase in the activity of the enzymes has also been observed, since, to grow, the microorganism demands these molecules.

From the results obtained, it can be said that the algae extract *A. nodosum*, in the concentrations and conditions applied in these tests, increases the vegetative growth of FOV, also resulting in an increase in the activity of β-1,3-glucanase enzymes and chitinase, responsible for the plasticity of the hyphae.

The activity of cellulases on a substrate occurs synergistically between endo and exoglucanases, where it is observed that the activity of one enzyme component is favored by the hydrolytic action of the other (Wood, 1985; Ryu & Mandels, 1980). The β-glycosidase is common in microorganisms and other living beings such as plants, bacteria, and animal

tissues, being the last to act in the cellulose degradation process (Hirayama et al., 1978). Endo and exoglucanases from a typical enzyme-cellulase system are inhibited by the presence of cellobiosis (Guedes et al., 2017), as observed for the three enzymes produced by FOV in the assays. Suppressive effects of the activity of these three enzyme components have already been verified, in different bioassays, when in the presence of *A. nodosum* kelp.

Wang et al. (2008) verified, *in vitro*, that florotanines extracted from *A. nodosum*, reduced rumen fermentation, cellulolytic and protein degradation of the tested forage. In a similar study, Belanche et al. (2016) also found that the presence of florotanins in the seaweed offered to the animals as a supplementary part of the diet, reduced the digestibility of forage by 24% and, because of that, there was a change in the structure of the bacterial community of the animals' rumen. As in these cases, it was observed in this work that the seaweed, applied as an extract, reduced the cellulolytic activity of the fungus *F. oxysporum* f. sp. *vasinfectum*. In addition, the cultivation of the fungus in increasing concentrations of the extract implied also progressive reductions in the cellulolytic activity of the microorganism. There is no work in the literature that has verified the interference of *A. nodosum* extract on the cellulolytic capacity of phytopathogenic fungi, so this potential needs to be further investigated. Thus, these data are unprecedented in this context.

5. Conclusion

The seaweed extract *A. nodosum* induces the vegetative growth of the fungus *F. oxysporum* f. sp. *vasinfectum*, probably due to its high availability of soluble sugars. This fact is confirmed by the increase in the total protein content and activity of the enzymes β-1,3-glucanase and chitinase, responsible for the breakdown of the hypha cell wall, when it is in the elongation phase. However, the product inhibits the sporulation of the pathogen, this inhibition being directly proportional to the increase in doses, according to what was observed in the tests performed. Thus, under the conditions in which these tests were performed, it can be said that there is a progressive inhibition of the cellulolytic activity of the fungus with increasing doses of the extract. This fact constitutes important information for the management of fusariosis, since the inhibition of the cellulose degradation capacity by the pathogen, can translate into decreased disease, compatible pathogen-host interactions. However, it is important that similar tests are carried out under field conditions to confirm the responses observed here. These discoveries are unprecedented in the context of the control of phytopathogens from seaweed extracts.

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