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Growth responses of hydroponic green fodder system and *Fusarium oxysporum* to external supply of mycolytic enzymes applied on seaweed bagasse as carrier

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High humidity, nutritional and environmental conditions involved in hydroponic green fodder (HGF) production increases fungal contamination risk. Chemical fungicides application is limited because of their toxicity to animals. An alternative biotechnological option is the use of mycolytic enzymes such as chitinase and laminarinase. The goal of the present study was to immobilize chitinase and laminarinase on seaweed bagasse as a carrier in order to increase their action time in HGF system, and compare the effect of those enzymes on *Fusarium oxysporum* viability and HGF growth. Chitinase and laminarinase immobilization on seaweed bagasse was carried out at 4°C and stirred at 250 rpm. Immobilization was assayed at different incubation times (60, 90, 120, and 180 min). Enzymes immobilization kinetics was monitored from protein quantity balance. Enzymatic activity of chitinase and laminarinase were evaluated. Higher immobilization (65 and 84% for chitinase and laminarinase, respectively) was obtained at 120 min of incubation time. Wheat grains in HGF systems under different treatments were inoculated with *F. oxysporum* at 2×10^4 spores/ (g of seed). Kinetics of growth measured by height of green shoots, as well as wet mass, dry mass of HGF, and fungal viability after 10 days of germination were evaluated. The higher antifungal effect was observed with immobilized enzymes. It was demonstrated that applied treatments did not affect negatively the fodder growth. Moreover, the bagasse presence can improve HGF height. The obtained results demonstrated that seaweed bagasse may be effectively applied as carrier for biologically active substances in HGF system.

Key words: Antifungal effect, chitinase, laminarinase, seaweed bagasse.

INTRODUCTION

Hydroponic green fodder (HGF) consists in the germination of cereal seeds. The grain develops roots and green shoots to form a green feed for animals. This technology creates highly-nutritious, consistently reliable feed. The HGF production is especially useful for the cattle feed under semi-desert climate condition where natural pastures are not available all year around (Herrera-Torres et al., 2010). Hydroponics produces much higher crop yields in shorter time periods. This process has been proven to be beneficial economically and environmentally (Leontovich and Bobro, 2007).

However, high humidity involved in the process increases

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increases the risk of fungal contamination. Some reports described root deterioration on HGF due to fungi and yeast contamination (Capper, 1988; Saini et al., 2011). The fungi present on the root mat could cause infections or contain mycotoxins harmful to animals (Capper, 1988; Hope et al., 2005). Among the fungi species, *Fusarium* is responsible for plant diseases: some species are mycotoxigenic and some cause infections in animals and humans (Cantalejo et al*.,* 1998).

Wheat crops are susceptible to *Fusarium* head blight (FHB). This sickness is caused by *F. graminearum*, *F culmorum*, *F. avenaceum*, *F. poae*, *F. equiseti*, *F. oxysporum* among others. Control of FHB has been complicated for the plant – pathogen interaction (Saharan and Naef, 2008). Many *Fusarium* species including *F. graminearum*, and *F. oxysporum* are producers of trichothecenes and zearalenona mycotoxins. Those mycotoxins are two of the four most important *Fusarium* mycotoxins classes due to their negative effects on humans and animals. High humidity conditions allowed the production on both mycotoxins (Doohan et al. 2003). A good option for fungi control is the application of biofungicides. The hydrolytic enzymes such as exo-1, 3-β-D-glucosidase (β-1, 3-glucanase, laminarinase, EC 3.2.1.6) and β-N-acetyl-D-glucosaminidase (chitinase, EC 3.2.1.14) (Prapagdee et al. 2008) produced by *Trichoderma* spp are active against a wide variety of phytopathogenic fungi. Chitinases and laminarinases are able to lyse polysaccharides (chitin and glucans, respectively) of mature hyphae, conidia, chlamydospores, and sclerotia of *Rhizoctonia solani*, *Chaetomium globosum*, *Didymella bryoniae*, *Botrytis cinerea*, *Fusarium solani* etc. (Velusamy and Kim, 2011). *Trichoderma* spp enzymes are substantially more antifungal than other purified enzymes from any other source, when assayed under the same conditions. Furthermore, chitinases and laminarinases are nontoxic to plants and animals at high concentrations (Cano-Salazar et al., 2008).

The problems with the application of enzymes in HGF systems may be related to their elimination during plant irrigation. Thus, alternative methods for administration of these substances can be developed. The use of natural carriers could be considered an appropriate solution for prolonged release of mycolytic enzymes. In the present study seaweed bagasse obtained after alkaline hydrolysis, pressing and dehydrating of seaweed (*Sargassum* sp.), was used as support in order to increase retention and chitinase and laminarinase action time during HGF production. Seaweed has been used not only for its natural fertilizer properties (Álvarez and Gallardo, 1989), but also for its water holding properties (Cofrades et al., 2008; Dhargalkar and Verlecar, 2009). The goal of the present study was to immobilize chitinase and laminarinase on seaweed bagasse as a carrier and compare the effect of free and immobilized enzymes on *Fusarium oxysporum* viability as well as on the growth of wheat green shoots.

MATERIALS AND METHODS

Seaweed bagasse was obtained as a residue from Palaú Bioquim Co. (Saltillo, Coahuila, Mexico), which produced biofertilizers based on seaweed extract obtained after alkaline hydrolysis. *Trichoderma* chitinase, laminarinase and their substrates (*p*-nitrophenyl-β-D-N-acetyl-glucosamide and laminarin from *Laminaria digitata*, respectively) were purchased from Sigma-Aldrich Co. (Saint Louis, USA). *F. oxysporum* strain was provided from a microbial culture collection of Biotechnology Department of Coahuila Autonomous University (Saltillo, Coahuila, Mexico). This strain was used as target fungi in all *in vitro* and *in vivo* assays. All other chemicals used in this study were of an analytical grade.

Enzymes Immobilization

Before chitinase and laminarinase immobilization, seaweed residue was washed, dried, pulverized (to 150 µ) and sterilized by autoclaving (at 121°C, for 15 min). Immobilization was performed using 10 mg of dry and grinded material and 1 ml of enzyme solution (20 µg ml^{-1}) dissolved on 100 mM acetate buffer (pH 5.5). The mixtures were incubated under agitation at 250 rpm and 4°C. Immobilization was assayed for different incubation times (60, 90, 120, and 180 min), using one reaction mixture for each point. After each time, non-immobilized protein was separated from bagasse by centrifugation at 10 000 rpm for 5 min at 4°C (Heraeus Biofuge centrifuge P, 3325B, Germany). The precipitates were washed with distilled water two times. The amount of non-immobilized enzyme was calculated from measurements of protein concentration by the Bradford method (Bradford, 1976) in all recovered samples. The amount of immobilized protein was calculated from a balance of protein quantity.

Chitinase and laminarinase activities were determined spectrophotometrically on UV/Vis spectrophotometer (Varian, Cary 50, Victoria, Australia) according to methods described by Cano-Salazar et al., (2011). For chitinase activity determination, the reaction mixture contained 0.1 mL of *p*-nitrophenyl-β-D-N-acetyl-glucosamide (0.5 mM, pH 5.5), 0.45 mL of sodium acetate buffer (0.1 M, pH 5.5) and 0.1 ml of the enzyme. The reaction was carried out at 37°C during 30 min and stopped by adding 0.5 ml of 1M Na_2CO_3. Released reaction product (*p* – nitrophenol) was measured at 405 nm. One unit (IU) of chitinase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of reaction product per minute at 37°C. For laminarinase activity, laminarin (10 mg mL^{-1}) of *Laminaria digitata* was used as substrate. The enzyme reaction mixture contained 0.125 ml of laminarinase and 0.125 ml of substrate (0.1 M, pH 5.5). After incubation at 40°C for 30 min, reducing sugars were measured by Somogyi-Nelson method (Nelson 1944; Somogyi 1952). One unit (IU) of laminarinase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of glucose per minute at 40°C.

The retention of immobilized chitinase and laminarinase was determined by activity detection after repeated reaction cycles. After each catalytic cycle the enzymes immobilized on bagasse were removed from the reaction mixture and washed with acetate buffer (50 mM, pH 5.5) to eliminate any residual substrate on the bagasse. The enzyme-bagasse was reintroduced into a fresh reaction medium in order to measure its activity in successive cycles.

Antifungal activity of free and immobilized enzymes *in vitro*

The anti fungal effect of the enzymatic preparations was estimated by means of radial growth inhibition assay described previously by Prapagdee et al. (2008). The potato dextrose agar (PDA) was prepared with free chitinase and laminarinase at final concentration 10 or 20 µg ml^{-1}, as well as with 100 µg of each enzyme immobilized on

Table 1. Treatments applied to HGF system.

Abbreviation	Description
C	Uninoculated control
F	Control without treatment, inoculated with 2×10^4 spores/ (g of seeds)
BB	Control with 2.5 g of bagasse, inoculated with 2×10^4 spores/ (g of seeds)
Ch	5 ml of chitinase solution at 20 µg ml^{-1} inoculated with 2×10^4 spores/ (g of seeds)
L	5 ml of laminarinase solution at 20 µg ml^{-1} inoculated with 2×10^4 spores/ (g of seeds)
BCh	100 µg of chitinase immobilized on 2.5 g of bagasse, inoculated with 2×10^4 spores/ (g of seeds)
BL	100 µg of laminarinase immobilized on 2.5 g of bagasse, inoculated with 2×10^4 spores/ (g of seeds)

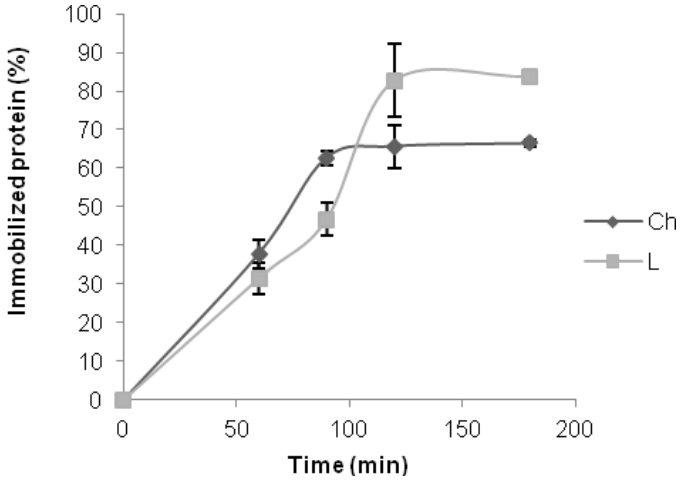

Figure 1. Kinetics of chitinase (Ch) and laminarinase (L) immobilization on the seaweed residue.

on 1 g of seaweed bagasse, and all treatments were added previously to PDA solidification. Immobilization was carried out according to the technique described above using 120 min as incubation time. The PDA without any treatment was applied as control. For inoculums, *F. oxysporum* mycelia disks (5 mm diameter) from 10-day-old culture were used. The test fungus was placed at the center of Petri dishes after PDA solidification. Plates were incubated at 27°C. Fungal growth was measured at the seventh day of assay by averaging the two diameters taken at right angles for each colony. Fungal growth inhibition was expressed as the percentage of radial growth inhibition. To calculate inhibition percentage of fungal growth, the difference in diameters between the control and treated samples was considered. All assays were performed in triplicate in at least two different experiments. A variation of less than 10% was observed when replicate assays were compared.

HGF Production under Different Treatments

HGF production was carried out in an environmental chamber (Biotronette Mark III, Melrose Park, Ill, USA) at 21±2°C. Environmental chamber was previously sanitized with phenol solution (5%). Cleaned seeds of wheat (*Triticum aestivum*) with high viability (90 to 92%) were used. Seeds were sterilized with 3% sodium hypochlorite solution and washed with water according to standard technique described by Rodríguez (2003). Five grams of seeds were placed per tray (5.5 × 5 cm). Previously, holes were made at the bottom of the trays to drain excess of solution applied for irrigation. Daily each tray was irrigated with 20 ml of nutritious solution (Vargas-Rodríguez, 2008), which contained $NH_4H_2PO_4$ at 7.08 mg L^{-1}, $Ca(NO_3)_2$ at 43 mg L^{-1}, KNO_3 at 22 mg L^{-1}, $MgSO_4$ at 61 mg L^{-1}, $CuSO_4$ at 0.06 mg L^{-1}, $MnSO_4$ at 0.31 mg L^{-1}, $ZnSO_4$ at 0.15 mg L^{-1}, boric acid at 0.75 mg L^{-1}, $(NH_4)Mo_7O_{24}$ at 0.0025 mg L^{-1}, and iron at 6.25 mg L^{-1}.

A randomized design with 3 replications was applied. Wheat seeds were inoculated with 2×10^4 macroconidium/(g of seeds) previous to enzymatic (or control) treatments addition. All treatments (Table 1) were applied on the first day of assay: 5 ml of enzyme solution at 20 µg ml^{-1}, as well as 2.5 g of bagasse with the corresponding enzyme. Treatment concentrations were selected based on the results of *F. oxysporum* radial growth inhibition *in vitro* assay.

The seedlings height was measured daily taking in account 10 stems at random. At the end of experiment (10th day after seeding), green plants with their root mats in the trays were harvested. The following data was recorded: total fresh and dry fodder mass were determined gravimetrically, as well as conversion factor (ratio of produced green fodder to the initial planted seed weight). Total fungus viability expressed as CFU/(g of HGF) was determined according AOAC method (AOAC, 1990). Briefly, consecutive dilutions were performed from suspension prepared as 1 g of HGF in 99 ml of peptone water. One milliliter of these dilutions was added to Petri dish with potato-dextrose agar. Petri dishes were incubated on microbiological incubator (Riossa E-41, México, DF, México) at 25°C for 72 h previous to quantification of colonies.

Statistical analysis

All assays were carried out twice using three repetitions. The obtained results were analyzed by the Tukey´s range test at 5% significance.

RESULTS

Enzymes immobilization

Enzyme immobilization was assayed for different incubition times (60, 90, 120, and 180 min). Higher percentage of protein immobilization on seaweed residue was 65% for chitinase (Ch), and 84% for laminarinase (L) at 120 min (Figure 1); after those times, immobilization percentages remain constant. Chitinase and laminarinase preparations presented a higher enzymatic activity (0.03±0.001 and 1.37±0.01 IU/g of bagasse, respectively) at 120 min of incubation time (Figure 2). Therefore, 120 min was selected for enzymes immobilization in further assays.

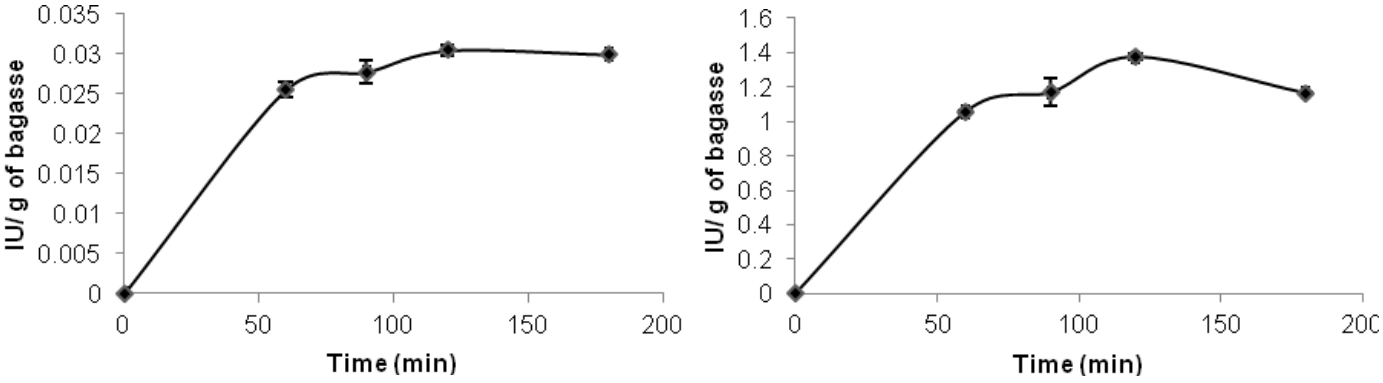

Figure 2. Enzymatic activity of chitinase (left) and laminarinase (right) during immobilization on seaweed bagasse at different incubation time.

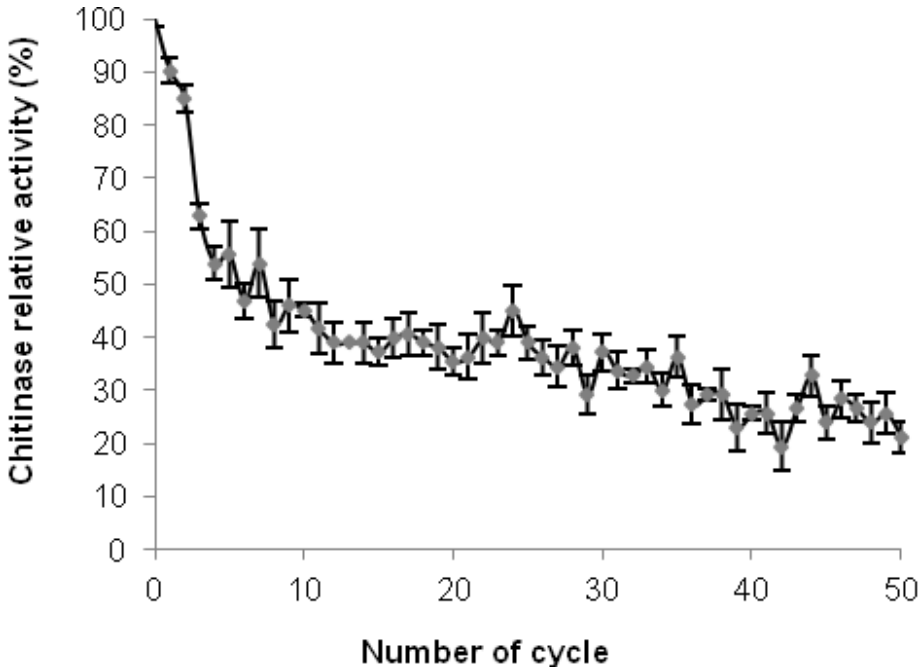

Figure 3. Remaining activity of immobilized chitinase after different cycles of enzyme application.

Specific activity of free chitinase before immobilization was 0.025 ± 0.003 IU/mg; while immobilized enzyme specific activity was 0.024± 0.002 IU/mg. Thus, there was not great variation in chitinase activity. In the case of laminarinase, initial activity of free enzyme was 0.856 ± 0.010 IU/mg. After 120 min of incubation at 4°C and centrifugation (conditions applied for immobilization), enzyme activity of free laminarinase decreased to 0.796 ± 0.008 IU/mg. Specific activity of laminarinase immobi-lized on seaweed bagasse was 0.831 ± 0.008 IU/mg. It was slightly lower than the freshly prepared enzyme, but higher than the enzyme submitted to similar conditions for immobilization.

The determination of activity in sequential cycles of reuse may be considered as a model to estimate residual activity of immobilized enzymes in HGF systems after subsequent applications of irrigated solution. In the present study, chitinase test was carried out at 50 cycles (Figure 3) and the remaining activity of immobilized chitinase decreases continuously. Enzyme lost a significant part of its activity (up to 60%) during first 15 cycles; after that, activity was lost slightly, and after 40 cycles it was maintained at 30% level. On the other hand, laminarinase totally lost its activity after four cycles of enzyme reuse

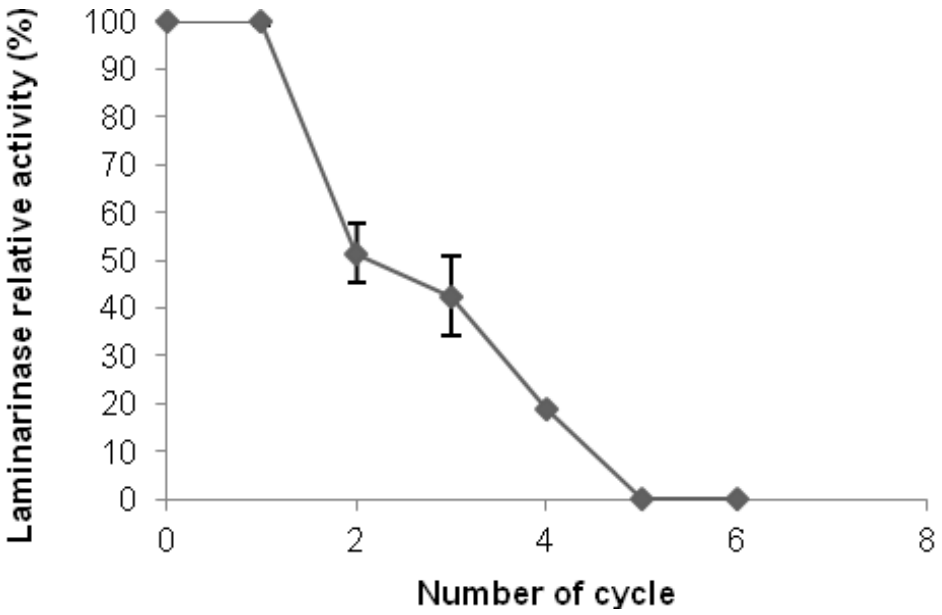

Figure 4. Remaining activity of immobilized laminarinase after different cycles of enzyme application.

Table 2. *Fusarium oxysporum* radial growth and its inhibition *in vitro* in the presence of free and immobilized chitinase and laminarinase at 7th day of assay.

Treatment	Radial growth (cm)	Inhibition (%)
Control	4.5 ± 0.3	0
Chitinase (20 µg ml^{-1})	0 ± 0	100
Chitinase (10 µg ml^{-1})	1.6 ± 0.19	64.4
Laminarinase (20 µg ml^{-1})	0 ± 0	100
Laminarinase (10 µg ml^{-1})	1.3 ± 1.6	71.1
Immobilized chitinase (100 µg in 1 g of bagasse)	0 ± 0	100
Immobilized laminarinase (100 µg in 1 g of bagasse)	0 ± 0	100

(Figure 4).

Antifungal Activity of Free and Immobilized Enzymes *in vitro*

Inhibition of *F. oxysporum* radial growth was evaluated using the solid medium in the presence of chitinase and laminarinase applied at free form or immobilized on seaweed bagasse (Table 2). Maximum inhibition (100%) in radial growth of *F. oxysporum* was detected in the presence of free chitinase and laminarinase applied at 20 µg ml^{-1}, as well as in the presence of each one of the immobilized preparations, while only partial inhibition of fungus growth was observed with free enzymes applied at 10 µg ml^{-1}.

HGF Production under Different Treatments

HGF production was carried out to define *in vivo* effect of immobilized enzymatic preparations against fungi. Statistical difference of CFU/(g of HGF) compared with control (uninoculated samples) was observed in all tests. Whereas chamber was kept open during HGF production, obtained results show total fungal count including *F. oxysporum* and other fungi present in the environment, although seeds and the environmental chamber were initially sanitized, as described above. In the control (Table 3) without *F. oxysporum* inoculation a count was 5.5 ×10^5 CFU/ (g of HGF). This indicates that conditions of humidity applied for HGF production allowed germination of different kind of fungi independently of inoculation.

Fungal counts were lower in systems treated with chitinase and laminarinase immobilized on seaweed bagasse, as well as with free chitinase (Table 3) that was considered as inhibition effect of these treatments to fungus proliferation at 89, 98 and 86%, respectively. Fungal count was greater in presence of seaweed bagasse without enzymatic treatments (Table 3). Effect of tested treatments

on properties of obtained HGF was evaluated. The detected values of HGF dry mass (DM) are similar; there is not a statistical difference between treatments ($p = 0.05$, Table 3). Furthermore DM weight was not affected by the fungal presence. During germination DM normally decreases due to the partial degradation of seed starch in order to grow. In the present study the DM gain was not very great in comparison with seeds weight (5 g) due to the fact that the process was not long enough to increase these values.

Other behavior was detected in the case of wet biomass weight (WM). This parameter was lower in control and seeds inoculated with *F. oxysporum*, which did not contain any treatment (Table 3). Despite the higher level of fungi contamination, system treated only with seaweed bagasse (BB) demonstrated the highest level of wet biomass, as well as the system treated with chitinase immobilized on bagasse (B-Ch). The treatment with free chitinase (Ch) also led to increase in this parameter (Table 3). The detected value was statistically greater than that obtained with free and immobilized laminarinase.

Similar behavior was observed for the conversion factors (CF), which demonstrate the ratio of increasing wet mass in comparison with the original seed weight, were between 7.6 and 12.1 times (Table 3). The CFs in the presence of treatments with seaweed bagasse (BB) and immobilized chitinase (BCh) are around 12, while around 10 with free chitinase. Moreover, a significant height increase ($P < 0.05$) with treatments that contained sea weed bagasse was also detected (Table 4); greater height of plants (up to 24 cm) was found in samples treated with bagasse, bagasse-laminarinase and bagasse – chitinase. The growth detected in control sample (C) without treatments was similar to other values corresponding to systems without seaweed bagasse (Table 4).

DISCUSSIONS

Seaweed bagasse obtained after alkaline hydrolysis, pressing and dehydrating of seaweed (*Sargassum* sp.) is a good carrier for chitinase and laminarinase immobilization by adsorption. Free and immobilized chitinase maintained its specific activity during immobilization. A slight difference of the average values has no statistical significance in this case. However, the immobilization protected laminarinase against slight inactivation during implemented process that was demonstrated by comparison of specific activities of free and immobilized enzyme submitted to similar conditions.

High percentages of protein immobilization as well as enzyme activity were detected (Figures 1 and 2). Similar results were reported by Cengiz et al. (2008), which demonstrated 70 to 80% of bovine serum albumin immobilized on seaweed after 60 min of incubation. Immobilization on seaweed bagasse gave considerably higher activity (6-fold) compared with data reported by Shchipunov et al. (2006) who immobilized laminarinase by entrapment on locust bean gum and reported an enzymatic activity of 0.23 IU (g of support)$^{-1}$. Cano-Salazar et al. (2011) immobilized chitinase by microencapsulation on soya lecithin liposomes quantifying the activity at 0.015 IU ml^{-1}.

Residual enzyme activity in sequential cycles of repeated applications was considerably higher in the case of immobilized chitinase than in laminarinase. The activity loss may be related to the inactivation of the enzyme or its release in the solution due to interactions detachment from carrier matrix. Jyh-Ping and Ko-Chang (1993) reported a loss of 30% of chitinase activity during the first repeated use of enzyme immobilized covalently on an insoluble polymer (hydroxypropyl methylcellulose acetate succinate), which was due to enzyme desorption. Due to the adsorption consists in physical interactions; enzymes may be removed from the support matrix during its application or during the irrigation, reducing greatly their activity. The obtained results suggest that interactions of chitinase with seaweed support may be greater than in the case of laminarinase, or that laminarinase is more susceptible to inactivation. However, the enzyme desorption does not affect the purpose for which in the present study immobilized preparations are used: released enzyme could have the possibility to interact and inactive the fungus. Thus, the obtained results demonstrated that the seaweed bagasse could be applied as carrier for chitinase and laminarinase immobilization by adsorption; although laminarinase desorption is expected in few cycles of reuse.

Both immobilized enzymes maintained the antifungal activity after their immobilization. According to Mauch et al. (1988), chitinase and laminarinase can inhibit fungal growth at concentrations of between 10 and 30 µg ml^{-1}. Instead, Cano-Salazar et al. (2011) reported that microencapsulation of chitinase and laminarinase at 10 µg ml^{-1} led to significant decrease of their antifungal activity up to 22 and 8% of radial growth inhibition, respectively. The present study demonstrates that free or immobilized enzymes can inhibit *in vitro* fungal growth (Table 2).

The obtained results (Table 3) indicate that both mycolytic enzymes immobilized on seaweed bagasse as well as free chitinase can control fungal growth on HGF, while bagasse treatment causes adverse effect. This suggests that immobilized enzymes maintain their activity during the process of HGF production. However, there is not a statistical difference between these treatments compared with chitinase applied in free form. This may be attributed to the efficient action of free enzymes on the fungus after initial spores' inoculation or to enzyme adsorption on seeds followed by its liberation during HGF growth. Moreover, the obtained results demonstrate that bagasse does not have antifungal properties by itself; in fact, bagasse increases system humidity and this condition is favorable for fungal reproduction.

Although, immobilized laminarinase is released more

Table 3. Treatments effect (Table 1) on fungal count and wheat HGF production.

Treatment	CFU/(g of HGF)	% of fungal count	Wet biomass (WM) weigh, g	Dry biomass (DM) weight, g	Conversion factor (CF)
C	$5.5 \times 10^5 \pm 6.3 \times 10^2$ [c]	100	38.23 ± 0.6 [d]	5.74 ± 0.9 [a]	7.64 ± 1.3[d]
F	$28 \times 10^5 \pm 1.4 \times 10^2$ [b]	509	39.60 ± 0.1 [d]	6.07 ± 0.5 [a]	7.92 ± 1.6[d]
BB	$45 \times 10^5 \pm 7.0 \times 10^3$ [a]	818	60.76 ± 0.2 [a]	5.38 ± 0.5 [a]	12.15 ± 1.3[a]
Ch	$0.8 \times 10^5 \pm 1.4 \times 10^3$ [d]	14	49.86 ± 0.3 [b]	5.85 ± 0.05 [a]	9.97 ± 0.5[b]
L	$15 \times 10^5 \pm 7.7 \times 10^3$ [c]	290	47.33 ± 0.3 [c]	5.70 ± 0.5 [a]	9.46 ± 1.2[c]
BCh	$0.6 \times 10^5 \pm 5.6 \times 10^2$ [d]	11	60.00 ± 0.1 [a]	5.50 ± 0.8 [a]	12.00 ± 1.0[a]
BL	$0.1 \times 10^5 \pm 1.4 \times 10^2$ [d]	2	47.56 ± 0.2 [c]	5.46 ± 0.7 [a]	9.51 ± 0.5[c]

Data in a column followed by the same letter are not significantly different according to the Tukey´s test (P=0.05). % = percentage.

Table 4. Kinetics of HGF growth under different treatments (Table 1), expressed as height increase.

Treatment	Height (cm)									
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
C	1.5±0[a]	2.0±0[a]	4.6±1[b]	10.1±1[b]	10.8±2[b]	13.8±1.4[b]	16.8±1.2	19.0±0[b]	19.0±0[b]	20.0±0.5[b]
F	1.5±0[a]	2.0±0[a]	4.1±0.2[b]	8.1±1.4[c]	11.8±0.7[b]	13.0±2[b]	15.3±1.1[c]	17.6±1.5[c]	19.0±1.5[b]	19.3±2[b]
BB	1.5±0[a]	3.0±0[a]	6.5±0.5[a]	14.3±0.5[a]	17.3±0.5[a]	20.3±0.5[a]	23.1±1[a]	24.0±0[a]	25.0±1[a]	24.8±0.2[a]
Ch	1.5±0[a]	2.5±0[a]	4.5±0[b]	9.8±1.1[b]	11.5±1.3[b]	14.1±0.7[b]	17.3±0.5[b]	18.0±0[b]	19.1±0.7[b]	20.3±0.7[b]
L	1.5±0[a]	2.5±0[a]	4.1±0.2[b]	9.6±0.2[b]	11.6±0.5[b]	14.1±0.7[b]	16.8±0.7[b]	17.6±0.5[b]	20.0±0[b]	20.1±0.2[b]
BCh	1.5±0[a]	3.5±0[a]	7.0±0.5[a]	14.6±0.2[a]	17.1±0.2[a]	19.0±0[a]	22.8±0.2[a]	24.0±1[a]	24.3±0.5[a]	24.5±0[a]
BL	1.5±0[a]	3.0±0[a]	7.0±0.8[a]	14.6±1.8[a]	17.8±2[a]	20.0±1[a]	22.8±1.2[a]	24.0±0[a]	24.0±1[a]	24.3±0.2[a]

Data in a column followed by the same letter are not significantly different according to the Tukey´s test (P=0.05).

easily in comparison with chitinase (Figure 3 and 4), its activity is higher (Figure 2). Probably the antifungal effect is associated with the early stages of fungi development from conidia (germination and initial growth) when laminarinase activity is present in the system. This enables that immobilized laminarinase treatment presents a signi-ficant antifungal effect in the HGF system (Table 3). Free laminarinase was not retained in the system enough time to develop a significant antifungal effect. Chitinase remains in the system for a longer time; this also ensures the antifungal effect (Table 3). Mycelia are considered as the source of toxic compounds and can be harmful for animal health (Cantalejo et al., 1998). The results of the present study demonstrates that their control may be achieved by means of assayed immobilized enzymes contained treatments that lead to significant decrease of CFU/(g of HGF).

Tested treatments application was not affect negatively the HGF growth. The results of DM weight obtained in the present study are similar for different treatments (Table 3), as well as to DM of rice, corn, and barley fodder hydroponic production (Vargas-Rodríguez, 2008; Fazaeli et al., 2011). This parameter was not affected by the fungal pre-sence. During germination DM normally decreases due to the partial degradation of seed starch in order to grow. Seedlings can increase DM during growth using nutrients for

example from the solution applied for irrigation (Fazaeli et al., 2011). Only a few factors can affect DM content such as seed preparation, irrigation, seedling density, and time of HGF process (Sneath and McIntosh, 2003). Those factors remained constants for all treatments; therefore a change in DM values was not expected.

In contrast, wet biomass weight was lower in control and in the HGF system inoculated with *F. oxysporum*, which did not contain any treatment. In the systems treated only with seaweed bagasse (BB) and with chitinase immobilized on bagasse (B-Ch) the highest levels of wet bio-mass were detected (Table 3). These values were higher in comparison with other treatments and with the data reported in others studies (Ajmi et al., 2009). Thus, the use of bagasse (BB), chitinase (Ch) and bagassechitinase (BCh) is beneficial in terms of yield of fresh biomass production.

The similar tendency was observed for conversion factors (CF). The CF values are greater in comparison with data reported previously for HGF of wheat (Espinoza et al., 2007), barley (Ajmi et al., 2009) and corn (López-Aguilar et al., 2009). According to Vargas-Rodríguez (2008), conversion factors commonly achieve the values up to 9. The superior values of CF obtained in the present study confirm that seaweed bagasse, and free or immobilized chitinase are beneficial in terms of biomass production, although seaweed bagasse applied separately lead to increase fungi growth.

Treatments containing seaweed bagasse showed significant height increase (Table 4) higher than growth in control sample (C) without treatments and in systems without seaweed bagasse. The detected values were higher than the one reported by Espinoza et al. (2007) who detected height of 17 cm for 10th day of HGF production. Height of fodders untreated or treated without bagasse (Table 4) was similar to hydroponic fodder reported in other studies (Espinoza et al., 2007; Ajmi et al., 2009; Al-Karaki, 2011). Difference between seedling heights is commonly attributed to the control of temperature, lightening, nutritious solution, and water quality during HGF production (López-Aguilar et al., 2009).

HGF systems tested in the present study were maintained under same conditions, except for the applied treatments. This suggests that the observed effect can be attributed to water accumulated in bagasse, as well as to the presence of some additional nutrients contained in seaweed such as carbohydrates, minerals, some essential amino acids, and vitamins (Marín et al., 2009). Moreover, the components of *Sargassum* have the potential to accumulate metals (Costa et al., 2001); this capacity allowed that nutrients added during irrigation remain available for the period of HGF production. Considering the fungi count in studied systems (Table 3), it was possible to assume that height was not the parameter that was strongly affected by fungi presence probably due to *F. oxysporum* and fungi from environment require more time to penetrate the roots of HGF and to affect its growth. However, even fungi do not have adverse effect on seedling height their growth represents a potential risk for cattle that leads to the necessity to reduce its proliferation.

Thus, seaweed bagasse is a good carrier to immobilize enzymes, such as chitinase and laminarinase, for their application for control of fungal viability in wheat HGF systems. Moreover, chitinase and laminarinase immobilized on seaweed bagasse are good alternatives for *F. oxysporum* control in HGF systems. The results indicate that the addition of seaweed bagasse on HGF systems leads to increase in plant height and wet biomass production. This finding may provide alternative means for fungi control in HGF production systems.

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