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Biostimulation of inoculation with *Glomus proliferum* and application of humic acid in the *in vitro* growth of *Lunularia cruciata*[1]

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ABSTRACT

In this study, we evaluated the growth of the liverwort *Lunularia cruciata*, inoculated or not with the arbuscular mycorrhizal fungi (AMF) *Glomus proliferum* (15 spores per Petri dish), in Strullu-Romand Variant (SRV) medium modified and enriched with humic acid (HA) at different concentrations (0, 20, 40 and 80 mg C L^{-1}), as well as the sporulation of the AMF. We assessed the absolute growth rate (AGR) and relative growth rate (RGR) at inoculation, as well as at 7, 14, 21, 28, 43, 52 and 60 days after inoculation (DAI), whereas we assessed sporulation at 25, 43, 60 and 70 DAI. The main determinant of *L. cruciata* growth was the presence of AMF. With and without *G. proliferum* inoculation, respectively, the AGR peaked at 39 and 42 DAI, and the RGR was 0.0474 and 0.0387 $cm^2\ cm^{-2}\ d^{-1}$. Doses of 20 and 80 mg C L^{-1} of HA had a positive influence on the growth of *L. cruciata*. With and without HA, respectively, the AGR peaked at 38 and 39 DAI, and the RGR was 0.0484 and 0.0422 $cm^2\ cm^{-2}\ d^{-1}$. The sporulation of *G. proliferum*, which was as high as 199 spores $plate^{-1}$, was influenced by HA, especially at 20 and 80 mg C L^{-1}.

Key words: arbuscular mycorrhizal fungi, liverworts, monoxenic cultures, humic substances

Introduction

Arbuscular mycorrhizal fungi (AMF) are organisms of the phylum Glomeromycota (Schüssler *et al.* 2001), considered one of the most important groups on the planet (Read 1992), because it is responsible for the mutualistic symbiotic relationship known as a mycorrhiza. Most plant families are able to form this type of relationship (Brundrett 2009; Wang & Qiu 2006), including nonvascular plants such as liverworts (Fonseca *et al.* 2006; Ligrone *et al.* 2007; Fonseca *et al.* 2009). In the case of such plants, the relationships is referred to as arbuscular mycorrhiza-like (AML) symbiosis (Berbara *et al.* 2006; Moreira & Siqueira 2006; Souza *et al.* 2010; Kottke & Nebel 2005, Humphreys *et al.* 2010).

One of the main features of AMF is their obligate biotrophy, which means that they need a plant to complete their life cycle (Siqueira *et al.* 1985). Cultures are generally produced with a plant in soil (Fonseca *et al.* 2006) or through *in vitro* monoxenic culture with transformed roots (Bécard & Fortin 1988; Souza & Declerck, 2003). AMF culture techniques in axenic environments have been increasing with the optimization of *in vitro* studies (Declerck *et al.* 2005).

Alternatives have been sought for *in vitro* production of AMF, especially in relation to the plant symbiont. More recent work attempting to accomplish *in vitro* culture of AMF with stalks of *Anthoceros punctatus* L. reports the establishment of AML growth of external mycelium and the production of new glomerospores (Schüssler 2000). Since then, several studies with hornworts and liverworts collected from natural habitats have recorded the presence of mycorrhizal fungi in the stalk (Duckett *et al.* 2004; Russell & Bulman 2005; Fonseca *et al.* 2006), indicating the possibility of using these plants as hosts for the *in vitro* culture of AMF. Fonseca *et al.* (2006, 2009) found that *Glomus proliferum* Dalpe & Declerck managed to complete the life cycle and establish AML symbiosis when in the presence of *Lunularia cruciata* (L.) Dumortier ex. Lindberg in a Strullu-Romand Variant (SRV) culture medium modified from Strullu-Romand medium (Declerck *et al.* 1998; Fonseca *et al.* 2006) with 29.2 mM sucrose, a 10/14-h light/dark cycle and a temperature of 25°C. The attempt to use an avascular plant such as *L. cruciata* for *in vitro* culture creates the need to adapt the culture medium for the growth of AMF. Vitamins, amino acids and other growth-promoting substances should be tested to determine their efficiency in

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developing both the symbiont and the fungus in the *in vitro* culture. However, humic substances are macromolecules of high molecular weight, with no set formulation, typically composed of aromatic and aliphatic chains, as well as ketones, phenols, enols, etc. Humic substances are present in vermicompost and soil, and are subdivided into three fractions: humin, humic acid (HA) and fulvic acid (Santos & Camargo 1999). The humic fractions influence soil fertility through the general improvement of physical and chemical conditions for biological activity and, moreover, because they contain physiologically active substances (Stevenson 1994; Façanha *et al.* 2002; Canellas *et al.* 2001, 2002; Nardi *et al.* 2007; Zandonadi *et al.* 2007). Thus, HA may influence rhizospheric biota, particularly AMF (Lima 2008). However, the effects of HA on mycorrhizae have been little studied. Gryndler *et al.* (2009) found that small amounts of organic soil matter can influence development and Gryndler *et al.* (2005) found that the addition of humic substance promoted the development of intra-and peri-apical AMF.

In the present study, we sought to determine the growth of *Lunularia cruciata*, inoculated or not with the AMF *Glomus proliferum* in a modified SRV medium enriched with HA. We also evaluated the sporulation of inoculated AMF.

Materials and methods

Experiment setup

Lunularia cruciata propagules were obtained from the growth of stalks in axenic cultures maintained in SRV medium at the Laboratory of Soil Biology at the Federal Rural University of Rio de Janeiro (Fonseca *et al.* 2006). The HA was extracted from cattle manure vermicompost. The extraction followed the methodology proposed by the International Humic Substances Society (Swift 1996) with adaptations (Benites *et al.* 2003). The HA had the following composition (Garcia *et al.* 2012a): C = 56.7%; H = 4.84%; O = 34.6%; N = 3.07%; S = 0.72%; H/C = 0.08; O/C = 0.61; C/N = 18.4; carboxylic, phenolic and total acid = 9.2, 2.03, and 11.27 mol kg^{-1} (C), respectively; and E_4/E_6 ratio = 4.22.

Liverwort segments of approximately 3.0 cm were transferred from the original Petri dishes to plates containing a medium enriched with varying levels of HA and cultured for one week at a temperature of 24°C and a 10/14-h light/dark cycle. After this period, we performed the inoculation with the AMF, *Glomus proliferum* Dalpe & Declerck (MUCL 41827) from the *In vitro* Glomeromycota Collection of the Mycology Department of the l'Université Catholique in Louvain, Belgium. The fungus multiplied and was maintained in monoxenic cultures of *Lunularia cruciata*. Fifteen glomerospores of *G. proliferum* were placed on each plate, as near as possible to the rhizoids.

The experiment consisted of the growth of the liverwort, inoculated or not with *Glomus proliferum*, in an SRV medium (20 g L^{-1} sucrose) with varying doses of HA (20, 40 and 80 mg C L^{-1}) and a control (without HA). The experimental design was completely randomized, with a 2×4 factorial arrangement in 6 repetitions (each plate constituted a repetition). The liverworts were transplanted, and, after one week, uncontaminated plates were either inoculated or not with the AMF. The growth of the liverwort was evaluated at inoculation, as well as at 7, 14, 21, 28, 43, 52 and 60 days after inoculation (DAI). The number of glomerospores on the plates was evaluated at 25, 43, 60 and 70 DAI through direct counting of glomerospores with an inverted microscope.

Statistical Analyses

In relation to all variables studied, Lilliefors and Bartlett tests were performed for levels of HA, the presence or absence of AMF and seasons after transplanting. The data were subjected to ANOVA ($p<0001$), followed by regression analyses performed using the program SAEG 8.0 (UFV 2000).

Quantitative growth analysis - To monitor the growth of *Lunularia cruciata*, we used the technique proposed by Lima *et al.* (2006): photos of each experimental unit (plate) were taken and worked with in an image editor (Adobe Photoshop) and SIARCS® software (Embrapa, Brasília, Brazil; http://www.catalogosnt.cnptia.embrapa.br/catalogo20/catalogo_de_produtos_e_servicos/arvore/CONTAG01_411_911200615045.html). The editing of images consisted only of cleaning, keeping the plant material in black and the rest of the image in white. The software used allowed us to determine the coverage area of the plate (in cm^2) and the length (in cm) of the organism under study.

We adopted the functional method for the coverage area of the plate and the data were fitted by regression, thus deriving the growth rates. Thus, among the various models proposed by Hunt (1981), we chose to work with the Richards model for area, based on iterative processes. Data analysis was guided by the ANOVA. The primary data showed strong heterogeneity among samples, and the functions were therefore fitted after the processing of the data by means of the natural logarithm in order to minimize the effect of heteroscedasticity (Neter & Wasserman 1974; Araujo 2003). The model selection was based on the significance of the coefficients and the value of the coefficient of determination (R^2), in conjunction with the global trend of temporal variation of the variable measured. The Richards function, the absolute growth rate (AGR) and relative growth rate (RGR), respectively, were calculated according to the following expressions (Hunt 1981):

$$C = a\left(1 \pm e^{(b-cT)}\right)^{-1/d} \quad (cm^2) \quad \text{(eq. 1)}$$

$$AGR = \frac{ace^{b-cT}}{d} \cdot \left(1 \pm e^{b-cT}\right)^{-\left(1/d+1\right)} \quad (cm^2 \cdot day^{-1}) \quad \text{(eq. 2)}$$

$$RGR = \frac{ce^{b-cT}}{d\left(1 \pm e^{b-cT}\right)} \quad (cm^2 \cdot cm^{-2} \cdot day^{-1}) \quad \text{(eq. 3)}$$

where T is time; e is the exponent; and a, b, c, and d are the coefficients of the Richards equation. The AGR expresses the rate of biomass production per unit of time. The RGR expresses the rate of biomass production per unit of preexisting material (Hunt 1981).

Results and discussion

As can be seen in Fig. 1, the ANOVA showed that, within the coverage area of the liverwort, there was a significant effect for the interaction HA*AMF ($p<0.05$). In the mycorrhizal liverworts, growth was unaffected by the lower doses of HA, showing an increase only at the highest dose. In the liverworts without AMF, the effect of the HA was apparent only at concentration of 80 mg C L^{-1} of HA. Intermediate doses of HA (20 and 40 mg C L^{-1}) had an effect opposite of that expected, with the values of the area of liverwort being lower than those obtained with the control treatment. This may indicate a direct effect of the HA present in the medium, which could have altered the absorption of nutrients, probably in a dose-dependent manner.

Fonseca & Berbara (2008) reported that in the presence of *Glomus proliferum* liverworts showed a mean reduction of 30% of dry weight as compared with non-inoculated specimens. The authors inferred that the fungus is a photosynthetic burden for the liverwort and characterized the association as parasitic and non-mutual, as expected. However, in the present study it was demonstrated that the association was mutual, since plant growth was favored by the colonization of the AMF, irrespective of the presence of humic substances.

The growth curve for the coverage area of the plates with the liverwort was sigmoidal. In the curve (Fig. 2a and 2b), we identified three phases, as described by Pereira and Machado (1987): in the initial phase (0-14 DAI), the area gradually increases or there occurs the establishment of the liverwort and symbiosis; the intermediate stage (15-42 DAI) corresponds to exponential or logarithmic growth—a continuous period of rapid plant growth and consolidation—resulting in liverworts twice their original size; and the final phase (from 43 DAI) was one of maturation, in which growth is once again slow or nonexistent.

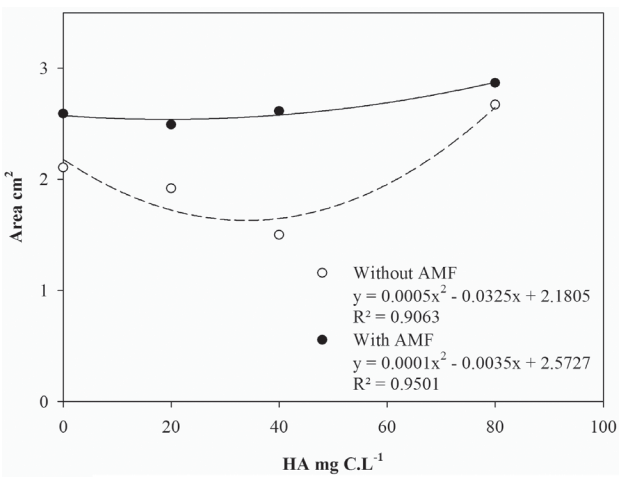

Figure 1. Area of the plate covered by the liverwort *Lunularia cruciata*, inoculated or not with the arbuscular mycorrhizal fungi (AMF) *Glomus proliferum*, by humic acid (HA) dose.

Comparing the liverworts inoculated with *Glomus proliferum* and those not inoculated, the inoculated specimens showed a higher coverage area of the plate. The increase of the area over time indicates that from 7 DAI to 60 DAI the best results were found in plants inoculated with AMF (Fig. 2a), with the highest AGR occurring at 39 DAI with a value 0.107 cm^2 d^{-1}, whereas in the treatment with no AMF, the maximum AMF was observed at 42 DAI with 0.084 cm^2 d^{-1}. Benincasa (2003) showed that the AGR can be used in order to estimate the average speed of growth over the period of evaluation. Because the AGR was reached sooner and with a greater value for the treatment with AMF than for the treatment without, these results indicate that the AML symbiosis promoted an increase in the speed of growth of the liverwort studied.

Treatment with HA at a concentration of 80 mg C L^{-1} also promoted a greater increase in the liverwort area between 7 and 60 DAI compared with the HA-free medium (control) (Fig. 2b). The maximum AGR in the treatment with HA occurred at 38 DAI with 0.1076 cm^2 d^{-1}, and in the treatment without HA at 39 DAI with 0.0932 cm^2 d^{-1}. Therefore, 80 mg C L^{-1} of HA also increased the speed of growth compared with the medium without HA (control).

The RGR is an important parameter in the evaluation of dry plant matter, being used in performance comparisons between different plants or to compare the effects of different treatments (Beadle 1987; Barcelos *et al.* 2007). The increases in RGR were 0.0474 cm^2 cm^{-2} d^{-1} and 0.0387 cm^2 cm^{-2} d^{-1} of growth, respectively, in liverworts with and without AMF. Thus, the RGR of the liverworts inoculated with the AMF showed higher values. However, over the course of the experiment, that rate began to decrease sharply (Fig. 2c). The RGR of the treatment without AMF remained almost constant until approximately 25 DAI, when it dropped, and the two curves intersected at 37 DAI with an RGR of 0.032 cm^2 cm^{-2} d^{-1}. This behavior in the RGR demonstrates that inoculation favors the early development of the liverwort when compared with treatment without AMF. This shows the advantage provided by the AMF in terms of rapidity with which the plant establishes itself in a new environment, similar to what occurs in mycorrhizal plants transplanted to the field, which grow more vigorously and have an early start of production when compared with non-inoculated plants (Moreira & Siqueira 2006).

The RGR of the liverworts treated with and without HA corresponded to 0.0484 cm^2 cm^{-2} d^{-1} and 0.0422 cm^2 cm^{-2} d^{-1}, respectively. Therefore, the RGR of the liverworts treated with HA was higher. However, at approximately 28 DAI this rate decreased sharply, and the two curves intersected at 30 DAI with 0.036 cm^2 cm^{-2} d^{-1} (Fig. 2d). Therefore, it is

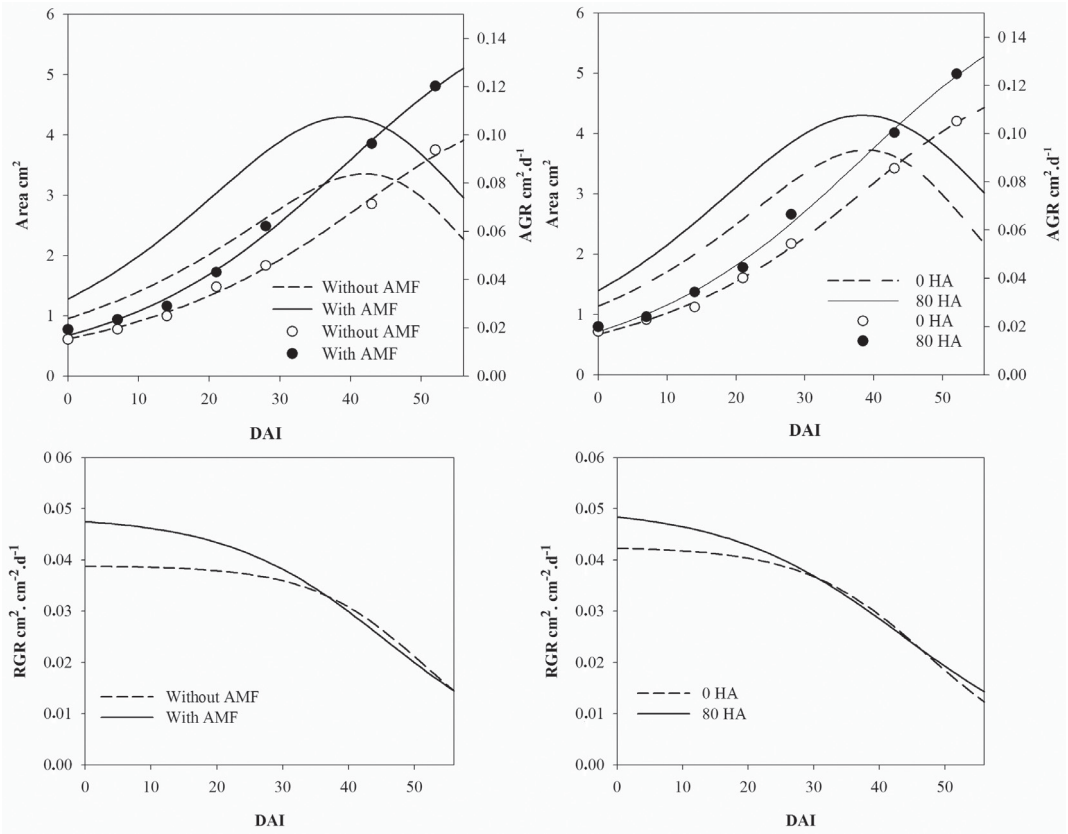

Figure 2. Results of cultures of the liverwort *Lunularia cruciata**: (a) area of the plate covered by *L. cruciata* and absolute growth rate (AGR; ● and ○ for "with" and "without" conditions, respectively) with and without inoculation with the arbuscular mycorrhizal fungi (AMF) *Glomus proliferum*; (b) area of the plate covered by *L. cruciata* and AGR with 0 and 80 mg C L^{-1} of humic acid (HA; ● and ○, respectively) in the SRV culture medium; (c) relative growth rate (RGR) with and without AMF inoculation; (d) RGR with 0 and 80 mg C L^{-1} of HA in the SRV culture medium.
DAI – days after inoculation.
*Coefficients obtained via the Richards mathematical model and its coefficient of determination (R^2) of the treatments: with AMF—*a*=6.2817, *b*=3.7975, *c*=0.0831, *d*=1.7119 and R^2=0.99; without AMF—*a*=4.5734, *b*=5.9914, *c*=0.1163, *d*=2.9935 and R^2=0.99; with HA—*a*=6.624, *b*=3.2504, *c*=0.0745, *d*=1.4821 and R^2=0.99; and without HA—*a*=5.086, *b*=5.0048, *c*=0.1055, *d*=2.478 and R^2=0.99.

clear that inoculation with AMF and the addition of HA, independently, stimulate the growth of liverworts. Considering that the life cycle of a liverwort in a Petri dish is limited to 100 days (unpublished data), it is likely that the rate of reduction in the RGR of *Lunularia cruciata* slowed over time. A reduction in RGR paralleling an advance in the physiological cycle has also been observed in other plants (Urchei *et al.* 2000; Zabot *et al.* 2004).

In evaluating the influence of the addition of different concentrations of HA to the culture medium, we observed that initial growth was similar at all doses, the effects of the addition of HA were evident only from 52 DAI and a dose of 80 C mg L^{-1} promoted greater increases in growth of the liverwort (Fig. 3). The other doses (20 and 40 mg C L^{-1}) were not sufficient to increase plant growth when compared with the control treatment. The increase in liverwort area obtained with the highest dose of HA (80 mg C L^{-1}) is probably attributable to the increased availability of C in the culture medium.

Working with *in vitro* clover and basil roots and different concentrations of HA added to the culture medium, Lima (2008) noted that the doses of 20 and 160 mg C L^{-1} promoted root growth while 40 and 80 mg C L^{-1} led to a reduction in growth rate. By monitoring the rhizosphere pH, the author also found that decreases in the concentration of HA induced corresponding increases in acidification during root growth, acidification being greatest at a dose of 20 mg C L^{-1}. Beginning at concentrations of 40 mg C L^{-1}, acidification begins to decrease, and at a dose of 160 mg C L^{-1} the rhizosphere actually becomes alkali. Along with this phenomenon, we observed an adaptive response to the root growth medium with a high concentration of HA. In addition, alkalization of the pH in the rhizosphere may cause a reduction in the absorption of ions from the solution, resulting in impaired root development. Although similar behavior may have occurred in the liverwort plates, variations in pH were not analyzed during that experiment.

In addition to the growth of the liverwort, we assessed the sporulation of *Glomus proliferum*. The number of glomerospores recorded in the present study was lower than that observed by Fonseca *et al.* (2008). That difference might

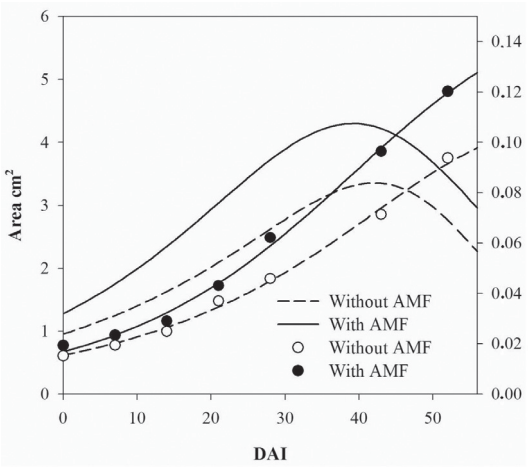

Figure 3. Average growth, in area, of the liverwort *Lunularia cruciata* cultured in SRV culture medium, with 0 and 80 mg C L^{-1} of humic acid humic acid (HA), together with the absolute growth rate (AGR; ● and ○, respectively), at inoculation (0), as well as at 7, 14, 21, 28, 35, 42, 49 and 60 days after inoculation (DAI).

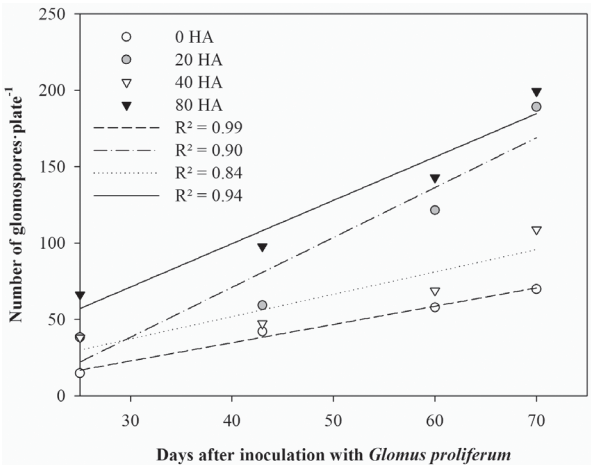

Figure 4. *In vitro* sporulation of *Glomus proliferum* associated with *Lunularia cruciata* in SRV culture medium with increasing doses of humic acid (HA; 0, 20, 40 and 80 mg C L^{-1}) at 25, 43, 60 and 70 days after inoculation (DAI).

be attributable to the fact that those authors used a medium enriched with sucrose and P. In the present study, HA in SRV medium had a positive effect on the sporulation of *G. proliferum* at the lowest dose (20 mg C L^{-1}) and at the highest dose (80 mg C L^{-1}), the fungus reaching a production rate of 199 glomerospores $plate^{-1}$ over the 70-day experimental period (Fig. 4).

The effects of adding HA to plants are still poorly understood. Garcia *et al.* (2012b) studied the variation in HA exposure time and dose and found differences in the production of enzymes related to oxidative stress. Roots supplemented with humic substances present varying responses, including: positive influence on transportation and facilitation of the uptake of ions; increased respiration and rates of reactions that occur in the Krebs cycle, ensuring an increase in adenosine triphosphate (ATP) production; increased synthesis of nucleic acids and chlorophyll content and also an increase or inhibition of enzymes (Nannipieri *et al.* 1993; Façanha *et al.* 2002). In addition, biochemical mechanisms, such as ATPase activity, are stimulated by certain levels of HA (Varanini *et al.* 1993; Berbara *et al.* 1995; Façanha *et al.* 2002; Canellas *et al.* 2002).

The addition of HA to the culture medium can influence processes such as sporulation, production of external mycelium (Gryndler *et al.* 2005) and mycorrhizal colonization. Further studies are necessary to clarify this issue.

Liverworts inoculated with *Glomus proliferum* presented growth rates higher than those of non-inoculated liverworts, indicating the mutualistic nature of the relationship. Similarly, the enrichment of an SRV culture medium with HA at 80 mg C L^{-1} promotes the development of *Lunularia cruciata*. Interaction between AMF inoculation and medium supplementation with AH can be observed in the area of plant cover, suggesting that this type of association may favor an increased photosynthetic rate, which is directly related to plant cover area. The production of glomerospores was most pronounced at HA doses of 20 and 80 mg C L^{-1}, indicating that HA affects the process of multiplication of these glomerospores. Further studies are needed in order to elucidate the mechanisms responsible for this phenomenon.

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