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Short communication
THE KINETIN RIBOSIDE AS *In Vitro* STIMULATOR OF *Glomus clarum* SPORES GERMINATION

Comunicación corta
La kinetina ribósido como estimulador de la germinación *In Vitro* de esporas de *Glomus clarum*

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ABSTRACT. Nowadays the *in vitro* mycorrhization of plants is a challenge in agricultural biotechnology and it depends of the germination potential of arbuscular mycorrhizal fungal (AMF) propagules, specially the spores, their colonization abilities and the media and systems of culture. The aim of this work was to evaluate the effect of two concentrations (0,05 mg L^{-1} y 0,07 mg L^{-1}) of the auxin AIA and the cytochinin kinetin riboside on *in vitro* germination and germinative tube length of *G. clarum* spores in E medium (modified MS). E and MSR media were used as controls. All culture media had influence on both variables performance. The concentration of kinetin riboside of 0,07 mg L^{-1} had a positive effect on germination percentage, reaching values of 100 % in that medium after 10 days of incubation. Those values were statistically similar to those founded in MSR medium. However, the highest values of germinative tube length were obtained in MSR medium and they were 45 % higher to those measured in E medium combined with kinetin riboside (0,07 mg L^{-1}). The AIA concentrations used had an inhibitory effect on spore germination and also on the germinative tubes growth.

Key words: mycorrhizae, plant growth substances, spores germination, mycelium

RESUMEN. La micorrización de plantas *in vitro* constituye hoy en día un reto de la biotecnología agrícola y depende en gran medida del potencial germinativo de los propágulos de hongos micorrízicos arbusculares (HMA) utilizados, en especial las esporas, sus habilidades colonizativas y de los sistemas y medios de cultivo. El objetivo de este trabajo fue evaluar el efecto de dos concentraciones (0,05 mg L^{-1} y 0,07 mg L^{-1}) de la auxina AIA y la citoquinina kinetina ribósido en la germinación y el crecimiento del tubo germinativo *in vitro* de esporas de *G. clarum* en medio de cultivo E (MS modificado). Se utilizó además el medio E y el SRM como controles. Todos los medios de cultivo influyeron sobre el comportamiento de ambas variables. La concentración de kinetina ribósido de 0,07 mg L^{-1} tuvo un efecto positivo sobre el porcentaje de germinación, alcanzándose valores en este medio cercanos al 100 %, transcurridos 10 días de incubación. Estos valores fueron estadísticamente similares a los encontrados en el medio SRM. Sin embargo, los mayores valores de crecimiento del tubo germinativo se obtuvieron en el medio SRM y superaron en un 45 % aproximadamente a los alcanzados en medio E combinado con la concentración de kinetina ribósido de 0,07 mg L^{-1}. Las concentraciones de AIA utilizadas tuvieron un efecto inhibitorio sobre la germinación e igualmente sobre el crecimiento de los tubos germinativos.

Palabras clave: mycorrhizae, sustancias de crecimiento vegetal, germinación de esporas, micelio

INTRODUCTION

The capability of Mycorrhizal Arbuscular Fungi (AMF) of increasing growth and survival of plants has elecited the interest of scientists all over the world. At present, the use of these microorganisms as substitutes of chemical fertilizers and pesticides has been promoted in harmony with the development of sustainable production practices (1).

The mycorrhization of *in vitro* plants is today a challenge of agricultural biotechnology and greatly depends on the germination potential of arbuscular

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mycorrhizal fungi (AMF), specially the spores, their colonization abilities and the culture media and systems. The methodological improvement of culture systems (composition of culture media and environmental growth conditions) will contribute to the development of systems that guarantee the massive production of *in vitro* plants (2).

The E culture medium (3) was designed to replace the SRM medium (Strullu and Romand modified), commonly used for *in vitro* culture of AMF, since it does not guarantee the nutritional requirements of potato *in vitro* plants. This medium was produced from modifications practiced to the MS medium (Murashige & Skoog), to assure the mycorrhization of potato plants (*Solanum tuberosum* L. cv. Desiré) with the AMF *Glomus clarum* (Nicolson & Schenck), in partially *in vitro* culture systems (3). However, in totally *in vitro* systems, also studied by the same author, in which plants under these conditions are usually produced, the germination of the spores in the E[A] medium was not achieved or did not occur with the necessary speed. According to Fernández (2), the probable cause of the absence or delay of germination was the presence of ethylene in the containers, ethylene is known as a plant growth regulator that can negatively influence the germination of AMF spores (4, 5) and it is produced by potato plants when grown in closed containers (6).

The germination of spores is one of the most important process during the life cycle of these fungi, from which, the success of symbiosis establishment (7), both under natural or *in vitro* conditions, will greatly depend on. Though spores in general, do not need external factors to germinate (8), this is a complex process influenced by: dormancy and storage, pH, temperature, humidity, light, aereation (oxygen, CO_2), inorganic ions, microorganisms, oxidizing agents, antibiotics and pesticides. Other recent investigations recognize radical factors released by the host known as proteins, CO_2, extracell protons flow and strigolactones (9) as stimulators of spore germination (10), growth and pre-symbiotic hyphal ramification (11, 12).

Other growth regulators like auxins could have a certain influence on the AMF spore germination, since it is known that mycorrhized plants vary the levels of these hormones as compared to non-mycorrhized plants (13), modify their colonizing potential and the possibility that these fungi produce some of them is discussed (13).

[A] Fernández, K. Establecimiento de un sistema eficiente de micorrización in vitro de plántulas de Solanum tuberosum L. y Medicago truncatula Gaertn [Tesis de Doctorado], Universidad de La Habana, La Habana, Cuba, 2013, p. 100.

For all the above, the purpose of this paper has been to evaluate the effect of the AIA auxin and cytokinin riboside on the germination and growth of the germinative tube of de *G. clarum* spores in E culture medium.

MATERIALS AND METHODS

In order to meet the objective an experiment with two repetitions in time was done under lab controlled conditions. Two growth regulators were tested on the germination and length of the germinative tube *in vitro* of *G. clarum* - MUCL 46238 spores, isolated from a Cuban ecosystem.

G. clarum spores of 4 months culture, were acquired in GINCO (*in vitro* collection of Glomeromycota, BCCM/MUCL, Microbiology Unit, Catholic University of Lovaina, Lovaina la Nueva, Belgium, http://www.mbla.ucl.ac.be/ginco-bel) and were supplied in Petri dishes of 90 mm of diameter, in association with transformed roots (Ri T-DNA) of carrot (*Daucus carota* L.) in SRM medium (Strullu and Romand modified) (14), solidified with 3 g L^{-1} of Gel Gro®.

Spores were extracted from the culture after solubilizing the SRM medium with a citric acid solution (1,92 g 100 mL^{-1}) and sodium citrate (2,94 g 100 m L^{-1}) (15), then they were kept in deionized sterile water till use. Later on, they were inoculated in E medium (MS modified) (3) without sugar and vitamins combined with two concentrations (0,05 mg L^{-1} and 0,07 mg L^{-1}) of kinetin riboside (Duchefa Biochemie) and indol acetic acid (AIA - Sigma); E and SRM media were used as controls.

Five spores per Petri dish of 90 mm of diameter were inoculated with the help of a micropipette Eppendorf (20 µL) and 10 plates of each culture medium were used. Later on, plates were sealed with Parafilm (Pechiney, PlasticPackaging, Chicago, IL 60631) and were incubated at 27°C till finishing the studies.

Evaluations

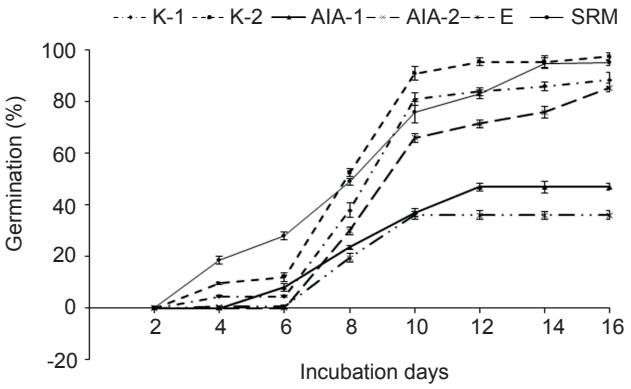
The germination percentage (%) and the length of the germinated tube of the spores were calculated (mm). Germination was monitored every two days after incubation and till day 16. A germinated spore was considered as such when the growth of the germinated tube was seen from the sporofore or from the tip of the supporting hypha, if the spore had it. The germinative tube length was evaluated at the end of the experiment (16 days) using a micrometer. Evaluations were done using a dissecting microscope (10-40X, Olympus SZ40, Olympus Optical GmbH, Germany).

Statistical analysis

Upon confirming the normalcy and homogeneity of variance (Test de Brown-Forsythe), using the statistical package Statistical, data were subjected to the Analysis of Variance of simple classification and then to the Tuckey's Test, in order to identify significant differences among treatments at p< 0,05. The values of germination percentages were transformed as per the expression arcsenÖx/100 and its normal distribution confirmed. With the values of both variables confidence intervals of the mean 95% probability were calculated, according to the number of repetitions and the reproducibility of data. The Statistica software package was used.

RESULTS AND DISCUSSION

When evaluating the effect of the two kinetin riboside and AIA concentrations in E medium on the *G. clarum* spore germination percentage (Figure 1), a differentiated observable effect of the culture media over the variable was recorded since the first four days of incubation.

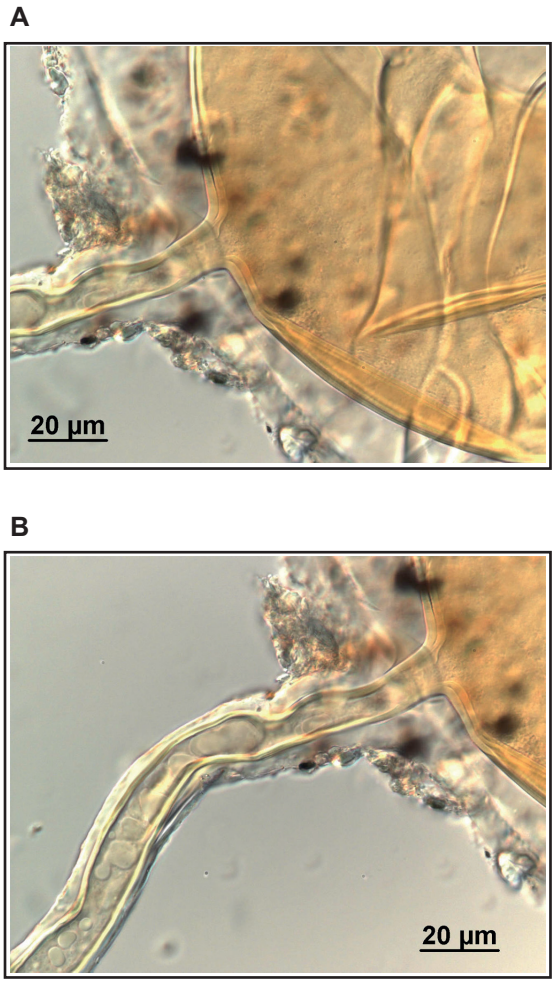


K (kinetin riboside) and AIA (indol acetico acid), (1)-0,05 mg L^{-1} and (2)-0,07 mg L^{-1}. Bars represent mean of 50 replicates ± confidence intervals for p<0,05

Figure 1. Germination percentage of *G. clarum* spores after 16 days of incubation in culture medium E combined with two AIA concentrations and kinetine riboside and media E and SRM as controls

The germination percentages of spores started increasing gradually as days passed, reaching a maximum value for each culture medium at day 10. From this moment on, the values of the variable remained constant till day 16 when the experiment finished, being higher in the spores found in E-kinetin 2 (0,07 mg L^{-1}) and SRM media, without significant differences among them. After practically 16 days, 100 % of the spores had germinated in these two media.

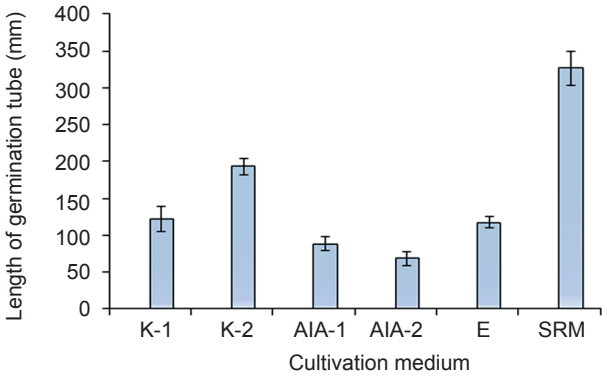
The germinative tubes observed showed a typical growth of *G. clarum* with a great quantity of cytoplasmatic content (Figure 2) and extended upright within culture media, without appreciating effects of the media composition over their behavior, though it did on their length.



Pictures taken with the Microscope made up of brillian field (250-500X, Olympus BH-2, Olympus Optical GmbH, Germany)

Figure 2. A and B: *G. clarum* spores MUCL 46238. B: Germinative hypha of *G. clarum* showing the cytoplasmatic content (CC)

Figure 3 shows the effect of the composition of the media on the growth of germinative tubes of *G. clarum* spores. Unlike the germination percentage, the highest values of this variable were attained in the SRM medium, commonly used for AMF *in vitro* culture in transformed roots by the E medium combined with 0,07 mg L^{-1} of kinetin riboside, that in this case showed values 45 % lower than those found in the SRM medium.



K (kinetine riboside) and AIA (Indol acetic acid), (1)-0,05 mg L^{-1} and (2)-0,07 mg L^{-1}.
Bars represent the mean of 50 replictes ± confidence intervals for p<0,05

Figure 3. Influence of two AIA concentrations and Kinetine riboside on the length of the germinative tube (mm) of *G. clarum* spores germinated 16 days after incubation on the E medium and SRM as controls

In both media, growth values of germinative tubes statistically exceeded those observed for the rest of culture media. The lowest values of this variable were recorded in the media containing AIA.

Mycorrhized plants increase the accumulation of cytokinins both in roots and stems, so it is obvious the close relationship between mycorrhizal establishment and the evolution of the plant hormone in the different parts of the plant (12).

Though there are no reports on the consulted literature that highlight a direct effect of cytokinins on the events previous to the colonizing process of mycorrhizal fungi, no doubt that in this experiement, there are direct evidences that the kinetin riboside –at the concentration of 0,07 mg L^{-1} in medium E- significantly stimulate spore germination of *G. clarum* and the germinative tube growth.

Nevertheless, in the case of ectomycorrhizal fungi, there are evidences that cytokinins produced by plants stimulates mycelium ramification (13). If this would be confirmed, it would be evident that hyphae from mycorrhizal fungi have receptors, at least, for some plant hormones and these could play a role in the asymbiotic stage of their life cycle.

Certain radical secondary metabolytes as flavonoids or terpenoids, depending on the plant species, though they are not essential in the arbuscular mycorrhizal symbiosis, can affect spore germination, the production of hyphal ramification and radical colonization (10, 11, 12). Isoflavonoids have similar structures to strogens and in *R. intraradices* it has been shown the existence of joining sites similar to those used by strogen, that seemingly play a role in growth regulation of the hypha (16).

On the other hand, a cDNA of *R. intraradices* (Ginmyc1) has been cloned with similar sequence of receiving protein from a steroid hormone and gen products have been detected only in the external hyphae, which suggests that they can play a role in the pre-colonization stage (17).

In medium E combined with any of the two AIA concentrations, germination values significantly lower than in spores incubated on other culture media, were recorded. Such values did not surpass 50 % of the germination, expressing an inhibitory effect of this hormone to the concentration used on the behavior of the variable.

It is probable that AIA concentrations used have had a negative effect on germination and growth of germinative tubes, taking into account studies made by Hidayat *et al*. (7), by inoculating, together with spores, isolated bacteria from the external part of *G.* sp., under *in vitro* conditions. According to these authors, effects found in the behavior of both variables were related to the AIA production by the bacterial isolates used. If the AIA concentration were in nanomolar values that stimulated spore germination, and on the contrary, if AIA values were in the order of micromolar, as those used in this experiment, germination was inhibited.

In a similar study, Kaneko and Tanimoto (17) also referred that plant hormones as AIA can stimulate spore germination and that hyphal growth of *Gigaspora margarita* and *G. fistulosum* at nanomolar concentrations can ibhibit them at high values (micromolar) (17)

CONCLUSIONS

- The application of kinetin riboside of 0,07 mg L^{-1} to medium E stimulates spore germination of *G. clarum* reaching germination values of 100 %, similar to those attained in a SRM medium. However, the AIA concentrations used (0,05 mg L^{-1} and 0,07 mg L^{-1}) in the same medium (E), had an inhibitory effect on germination and on the growth of spore germinative tubes. The highest values of germinative tube length were reached in the SRM medium, commonly used for the AMF *in vitro* culture.

- Although trials related to the effect of the cytokinins on the germination of spores are not abundant in literature, it is necessary to take into account the results of this study it is precise, not only to complement the advances attained in the mycorrization of *in vitro* plants in our country, but also because they are valuable results to consider in the attempts to culture *in vitro* AMF species considered recalcitrant that are resistant to the cullture under these conditions.
- The absence of descriptions on the role of plant hormones in the biology of fungi in general, could simply be a reflect that the action of the hormones *in vivo* is regulated by concentration gradients that are difficult to reproduce experimentally.

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