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Insulation of basiodiomicet fungies phosphorus solubilizers and nitrogen fixers for application as mycorrhizas in lechuga (*Lactuca sativa*)

Aislamiento de hongos basiodiomicetos solubilizadores de fósforo y fijadores de nitrógeno para su aplicación como micorrizas en lechuga (*Lactuca sativa*)

GÓMEZ-VALADEZ, Alejandrina†*, JIMÉNEZ-GONZÁLEZ, Angélica, CONDE-BAEZ, Laura and GAYOSSO-MEXIA, Ana Rosa

Universidad Politécnica de Pachuca, Carretera Pachuca-Cd. Sahagún, Km 20, Ex-Hacienda de Santa Bárbara, municipio de Zempoala, Hidalgo, México. C. P. 43830

ID 1st Author: *Alejandrina, Gómez-Valadez* / **ORC ID:** 0000-0003-3588-7142, **CVU CONACYT ID:** 423508

ID 1st Coauthor: *Angélica, Jiménez-González* / **ORC ID:** 0000-0002-5653-1172, **CVU CONACYT ID:** 84352

ID 2nd Coauthor: *Laura, Conde-Baez* / **ORC ID:** 0000-0003-0250-7916, **CVU CONACYT ID:** 414113

ID 3th Coauthor: *Ana Rosa, Gayosso-Mexia* / **ORC ID:** 0000-0002-5194-1666, **CVU CONACYT ID:** 872221

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Abstract

Poor agricultural practices have caused eroded soils, therefore alternatives are sought for the solubilization and fixation of nutrients by mycorrhizal fungi, that grow on the plants roots forming a mutualist symbiosis. Therefore, in this work, *Basidiomycetes* fungi were isolated and those that are able to solubilizing phosphorus and nitrogen were selected for their inoculation in lettuce (*Lactuca sativa*), this is high consumption and fast growth vegetable. Five substrates (fertile soil, eroded soil, humus and horse manure) were studied with a not inoculated control, where the response variable was hypocotyl growth and the *L. sativa* radicle, measured every 24 hours for 30 days. It was observed that the HM3 fungi stimulated the highest growth of the plant in eroded soil, generating an alternative to agriculture and contributing to bioremediation and exploitation of damaged soils.

Phosphorus, Nitrogen fixers, Mycorrhizae

Resumen

Las malas prácticas de agricultura han ocasionado suelos erosionados, por lo tanto se buscan alternativas para la solubilización y fijación de nutrientes mediante hongos mycorrhizal, los cuales crecen en las raíces de las plantas formando una simbiosis mutualista, por lo tanto en este trabajo se aislaron hongos basidiomicetos y se seleccionaron aquellos capaces de solubilizar fósforo y nitrógeno, para su inoculación en Lechuga (*Lactuca sativa*), este vegetal es de alto consumo y rápido crecimiento. Se estudiaron cinco sustratos (suelo fértil, suelo erosionado, humus y estiércol de caballo) con un testigo sin inocular, donde la variable de respuesta fue el crecimiento del hipocótilo y la radícula de *L. sativa*, medidos cada 24 h por 30 días. Se observó que el hongo que estimulo el mayor crecimiento de la planta fue el HM3 en suelo erosionado, generando una alternativa a la agricultura y contribuyendo a la biorremediación y aprovechamiento de suelos dañados.

Fosforo, Fijadores nitrógeno, Mycorrhizas

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* Correspondence to Author (email: alegomez@upp.edu.mx)
† Researcher contributing as first author.

Introduction

Mycorrhizas are non-pathogenic mutual symbiotic associations between fungi and plant roots (Turk et al., 2006). They are divided into endomycorrhizas (vesicle-arbuscular), these penetrate the root cells (Frey-Klett et al., 2007); and in ectomycorrhizas, which are characterized by forming outside the cortical cells of the root producing a structure called “Hartig's network” (Galindo-Flores et al., 2015). Among the most studied mycorrhizal fungi are arbuscular belonging to the genus Glomeromicota, which are characterized by participating in crops (Andrade and Silveira, 2008; Khaosaad et al., 2006).

On the other hand, ectomycorrhizas play a fundamental role in the development of woody trees such as pinus sp. (pines), Quercus sp. (oaks), etc. (Rúa et al., 2015). In this family are the fungi basidiomycetes and ascomycetes (Pierre-Emmanuel et al., 2010), on which few studies have been carried out to investigate the influence of these fungi on agricultural crops. In both cases (endo and ectomycorrhizas), the symbiosis is carried out because the fungi have a heterotrophic character and can obtain their carbon source from the metabolites provided by organisms such as plants, and in reciprocity they solubilize and fix the mineral nutrients and provide the soil water that their hosts need to grow (Honrubia, 2009).

Among the most important nutrients for plant development are nitrogen (N) and phosphorus (P), which limit growth. Different factors involved for nutrient availability are known, including pH, the amount of organic matter, humic and fulvic acids, citrates and oxalates (Rosatto-Moda, et al., 2014).

However, the assimilation of nutrients is carried out with the help of mycorrhizal fungi, since, by producing acidic substances such as lactic, citric, malic and gluconic acid, an ionization process of nutrients that are not directly available is initiated in the soil for vegetables (Katiyar et al., 2013). Due to these organic acids, NH4 is oxidized to assimilable nitrogen (Stavros et al., 2012), as is phosphorus, which is found as volcanic ashes or limestones and oxides of Fe and Al (Raij, 2011).

It has been determined that an appropriate interaction between soil-plant-fungus can provide farmers with a saving of 25 to 50% of fertilizers, since the nutrients existing in the soil are also used, in addition the crops have a greater development in terms of height, vigor and leaf area (Cruz-Hernández et al., 2014). Consequently, a 15 and 50% increase in fruit yields has been observed, leading to an alternative to sustainable agriculture (Noda, 2009).

Another benefit of this symbiosis is the protection against pathogens and a greater resistance to environmental stress (Nadeem et al., 2014), on the other hand, Vos et al., (2013) mention that mycorrhizas help fight some parasites such as nematodes, which cause rot of plant tissues.

The cultivation of vegetables for consumption is important, as they are a food that provide vitamins, minerals and fiber. Lettuce (Lactuca sativa) is one of the most consumed vegetables. In 2010, 340,383 tons were grown in Mexico, and its rapid growth facilitates laboratory study (SAGARPA, 2011).

However, due to poor agricultural practices and the abuse of chemical products such as fertilizers, herbicides and insecticides, a soil that is scarce in available nutrients has been generated, and their deficiency causes cultivated species to slow their growth or die. Therefore, in this work it is proposed to isolate phosphorus and nitrogen fixing basidiomycetes fungi, for mycorrhizal application in L. sativa using as substrate eroded and fertile soils.

Materials and methods

A random sampling of basidiomycete fungi was carried out in the town of Santa Monica, municipality of Epazoyucan in the state of Hidalgo, Mexico (19 ° -97 'North; 98 ° -61' West), during the rainy season in September. 2015. The fungi collected were subjected to an astringent treatment as indicated by Hine-Gómez and Abdelnour-Esquivel (2013). The stem was sectioned and 1 cm^3 segments were cut which were inoculated in papa dextrose agar (PDA) for seven days at 28°C.

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To select the fungi the samples were reseeded in culture medium yeast extract-mannitol-agar-blue bromothymol (ELMARC) for fungi with the ability to solubilize nitrogen (Angeles-Nuñez and Cruz-Acosta, 2015), and in Sundara medium and Sniha (SS) for phosphorus solubilizers. The selected fungi were reseeded in PDA medium and their spores were collected with 0.01% Tween 20.

To study the influence of fungi on the development of L. sativa, an experimental design of a factor with four levels and five replicates was used, using four substrates classified based on their type using pH and electrical conductivity (Salgado-Transit et al. al., 2011), which were designated as follows: fertile soil (SF), eroded soil (SE), humus (H) and horse manure (EC). To each substrate, five seeds of L. sativa were placed as indicated by Kim et al., (2010), 100 µL of a solution with 10^6 mL^{-1} spores of each of the selected fungi was inoculated (variable) Independent).

For each treatment a control was used without inoculation. As response variables, hypocotyl and radicle growth were measured every 24 hours for 30 days. To observe the effect of the microorganisms native to the soils used, the growth of L. sativa in Murashige and Skoog (MS) medium was also tested using the same response variables for 15 days. A statistical analysis of the results was carried out by means of an analysis of variance (ANOVA) and Tukey test, with the SPSS ™ 17.0 program.

At the end of this period, staining of the roots was performed on all treatments following the methodology described by Muñoz et al., (2009), in order to know if the isolated fungi generated a mycorrhizal symbiosis.

Results and discussion

25 different species of basidiomycete fungi were collected, of which three presented halos of solubility of both N and P, and were named as HM1, HM2 and HM3. The species with the highest solubility halo for nitrogen was HM3 and for phosphorus HM2.

This solubilization phenomenon occurs because the fungus secretes organic acids that modify the pH, for the ELMARC medium, the ammoniacal nitrogen is converted into assimilable nitrogen (NO_2, NO_3, and N_2), and in the SS medium the calcium phosphate present is ionized to PO_4^{3-}, which is a compound assimilable by the microorganism, depending on the amount of acid secreted is the size of the halo (Cordero et al., 2008; Cerón-Rincón and Arístízabal-Gutiérrez, 2012).

When performing the experimental design on the substrates selected with the fungi HM1, HM2 and HM3, it was observed that there was growth with the exception of horse manure, where both in the control group and in the experimental group there was no development of lettuce seeds during the 30 days of experimentation. This is due to the fact that there is a difference in pH and electrical conductivity (Table 1) far from the appropriate values for the growth of both the seeds and the microorganisms used (pH 6-7 CE 1.0-1.4 dS m^{-1}) (Zarazúa-Villaseñor et al., 2007, Carranza et al., 2009). In addition, horse manure was the substrate that had the highest salinity value, which indicates that water absorption is affected since an alteration in osmotic pressure is generated (Achilli and Childress, 2010).

Substratum	Initial pH	Initial CE (dS m^{-1})	Final pH	Final CE (dS m^{-1})
MS medium	6.8	1.16	5.6	0.54
Humus	8.36	0.78	7.09	0.34
Fertile soil	7.23	1.09	7.02	0.25
Eroded soil	10.19	2.21	8.43	0.79
Horse manure	10.55	3.36	8.65	0.86

Table 1 pH and EC of the substrates used in the inoculation of the selected fungi and L. sativa

As for the other substrates that were studied, Fig. 1 shows the hypocotyl measurements of the plants at 720 h. It was observed that in all cases (SE, SF and H) there is greater growth in the groups treated with the selected fungi compared to the control groups (P <0.05). In addition, the greatest growth occurred in the soil eroded with the HM3 fungus, followed by fertile soil and humus. Checking that the characteristics of the substrates directly influence the promotion of the interaction between fungi and L. sativa in their growth and potentialization.

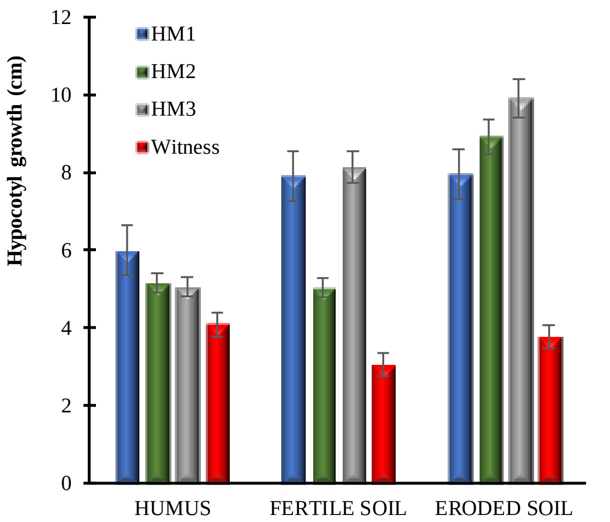

Figure 1 Growth of the hypocotyl of L. sativa at 720 h, with the different substrates and fungi selected

In addition to the above, in the group with the HM3 fungus, in addition to having the highest growth compared to the control group and the other experimental groups, it was observed that the appearance of the seedlings was of greater vigor (Fig. 2): the hypocotyl was thicker, larger leaves and the amount of seedlings obtained exceeded the number of inoculated seeds.

Therefore, the eroded soil is considered as the best substrate for the growth of L. sativa with isolated fungi, this symbiosis reflected an increase in the development of seedlings since it usually has a growth of 6.73 cm at 30 days (Terri-Alfonso et al., 2014), and with the HM3 fungus a length of 10.03 cm was reached in 30 days.

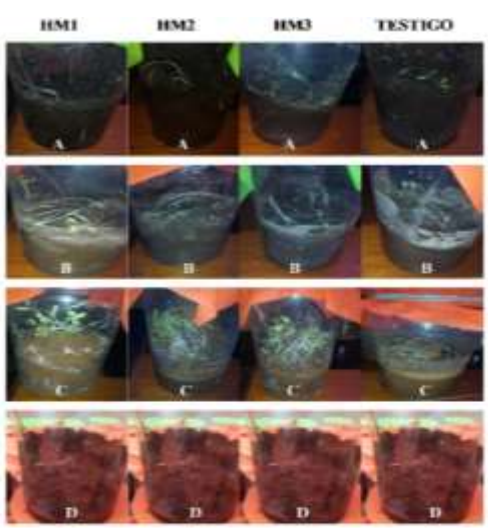

Figure 2 Sprouts of L. sativa at 720 h, on different substrates, with inoculated fungi.

Regarding the radicle, in Fig. 3 the development in the different substrates is shown at 720 h, in the analysis of variance a P <0.05 was obtained, and Tukey's analysis showed that the greatest growth in the investigated groups have HM2 in eroded Earth (3.0 cm). However, in general, there is no relationship between the addition of the isolated fungi and the length of the radicle, this with respect to the control groups.

However, it is recommended to make other types of measurements on the radicle, such as the root area and dry weight of this part of the plant since some differences were observed between the type of radicle developed in the control and experimental groups, suggesting that Fungi can help spread the root for better nutrient utilization (Noda, 2009).

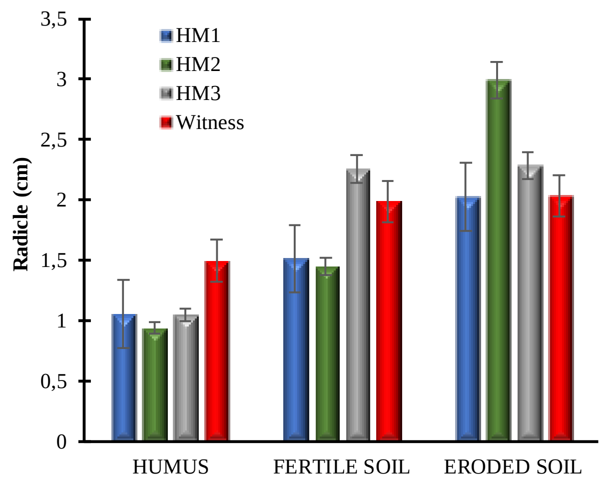

Figure 3 Growth of the L. sativa radicle at 720 h with the different substrates

In order to monitor the effect of the isolated fungi on L. sativa in a controlled environment and environments, the MS medium was used as a substrate and it was observed that the seeds with the fungi germinated at 48 hours and the control until the 120 hours.

This indicates that there is an effect when adding these microorganisms since they stimulate the growth of the embryo and this leads to the growth of the hypocotyl (Terry-Alfonso, 2014)

In addition the fungi adhered to the radicle of the plant observing the development of a symbiosis.

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During the treatment in the MS medium, which lasted 360 hours, a distribution and increase of the seedlings in the experimental groups was observed with the three fungi isolated as with the previous substrates, this phenomenon is known as potentialization because the When forming the association, they help plant species distribute the growth to obtain a greater amount of nutrients (Larkan and Smith, 2007), growing between 10 to 15 seedlings with the inoculated fungus and 5 seedlings in the control group (Fig. 4).

Figure 4 Test in MS medium with Lactuca sativa, at 360 hours of inoculation with the selected fungi (HM1, HM2 and HM3).

On the other hand, the three fungi studied generated a greater growth of the hypocotyl compared to the control at 360 hours (Fig. 5). ANOVA analysis showed that there is a significant difference in treatments compared to the control group ($P < 0.05$), however, there is no difference between the fungus MH1 and MH2 according to the Tukey test with a $P = 0.954$, with HM3 being the that stimulates greater growth.

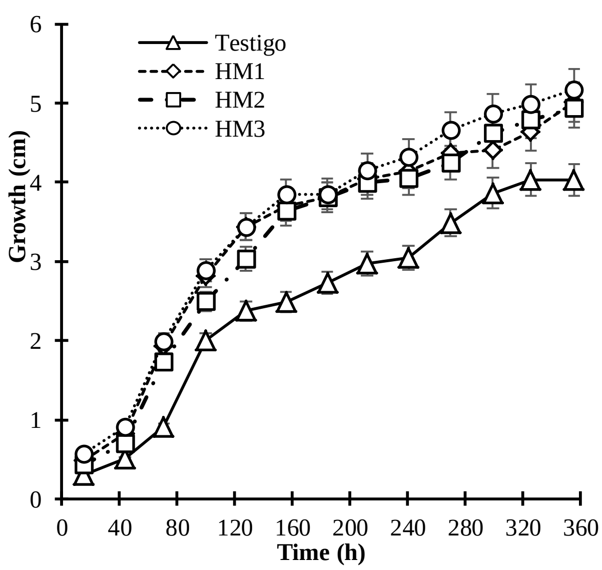

Figure 5 Growth of the hypocotyl of L. sativa inoculated with the selected fungi (HM1, HM2 and HM3)

Regarding the development of the radicle, it was determined that the control groups had a higher growth compared to the experimental groups (Fig. 6), this phenomenon of lower root development and higher hypocotyl growth with the fungus demonstrates that they produce substances bioactives capable of helping to increase nutrient fixation and thus stimulate the growth of L. sativa in the MS medium (González-Perigó et al., 2015), without having to develop a larger root surface.

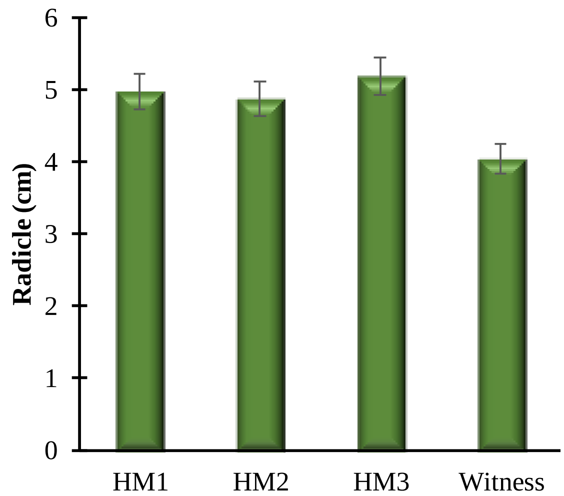

Figure 6 Growth of the L. sativa radicle, at 360 hours of inoculation with the selected fungi

Finally, the technique of clearing and staining on the roots was carried out (Muñoz et al., 2009) to check the presence of mycorrhizas, and it was observed that the isolated basidiomycete fungi formed a mycorrhizal symbiosis in the root cells of the plant unlike the control groups (Fig. 7).

This symbiotic behaviour has been observed in fungi of the Zygomycetes type such as Glomus sp., Which is one of the most studied fungi in this type of behaviour in order to increase the growth of some plants such as Zea mayz (maize) (Martín-Alonso et al., 2012), Solanum lycopersicum (tomato) (Mujica et al., 2014) Carica papaya (papaya) (Quiñonez-Aguilar et al., 2014), among others.

However, the promotion of a fungus of basidiomycete to mycorrhiza type in crops has so far been little studied.

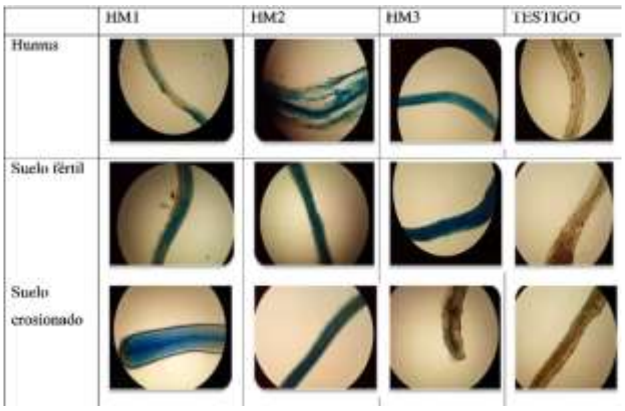

Figure 7 Root staining in L. sativa at 720 h growth with inoculated fungi, observed in the 400 X optical microscope

Finally, the positive effect of the promotion of mycorrhizal symbiosis of isolated fungi, especially of HM3 in L. sativa, suggests a potential use for its application in eroded soils, and with that in addition to increasing the production of this vegetable, it also I would be doing bioremediation indirectly.

This is possible because in this interrelation of species (fungus-plant), the plants select, attract and stimulate the microbial communities through exudates secreted by the roots (Cerón-Rincón and Aristízabal-Gutiérrez, 2012), and the Chemotaxis, in reciprocity, microorganisms stimulate growth through nutrient solubilization and production of bioactive substances (siderophores, antibiotics, hydrolytic enzymes and cyanidic acid) (Peña and Reyes, 2007).

Conclusions

Asylated basidiomycete fungi and selected phosphorus and nitrogen fixers are capable of forming mycorrhizal associations in L. sativa, they also provide a positive effect on the growth of said crop, observing that with the HM3 fungus the highest growth of hypocotyl was observed in soil eroded, this provides an alternative for sustainable agriculture and a contribution to the bioremediation of damaged soils.

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