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SECRETARÍA DE EDUCACIÓN PÚBLICA

Instituto Politécnico Nacional
"La Técnica al Servicio de la Patria"

# XXXVI CONGRESO NACIONAL DE CONTROL BIOLÓGICO

## 7 Y 8 DE NOVIEMBRE 2013

## OAXACA DE JUÁREZ, OAXACA, MÉXICO.

Editores:
Alfonso Vásquez López,
Rafael Pérez Pacheco.

# In vitro culture of arbuscular mycorrhizal fungi from *Agave* soils

Adrien Gallou[1,2], Evangelina Esmeralda Quiñones-Aguilar[1], Gabriel Rincón-Enríquez[1]*

[1] Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. Av. Normalistas No. 800, Colinas de la Normal. CP 44270, Guadalajara, Jalisco México.
[2] Present adress: Laboratorio de Biología Molecular, Centro Nacional de Referencia de Control Biológico, Km 1.5 Carretera Tecomán-Estación FFCC, Col. Tepeyac. CP 28110, Tecomán, Colima, México.
*For correspondence. E-mail grincone@gmail.com. Tel. +52 (33) 33455200 Ext. 1703

## Introduction

The *Agave* plant is an important economic crop and had been exploited and broadly utilized in different fields (Simpson *et al.,* 2011). The best know application of *Agave* is the production of tequila. In 2011 and 2012, the "Consejo Regulador del Tequila (CRT)" has calculated the production of tequila in Mexico at 261 and 253 millions de liters, respectively. Unfortunately, this culture is susceptible to numerous pathogens. In the last years, the CRT has observed the presence of diseases on 20-30% of tequila plants. Conventional agriculture mostly relies on the application of chemical pesticides, which are often only partially effective against soil-borne diseases. In addition, repeated applications of pesticides may be detrimental to human health and to the environment. Alternative or complementary approaches to fight agave diseases, including biological control strategies, are necessary for modern agriculture.
One of the most common mutualistic association in nature is the Arbuscular Mycorrhizal (AM) symbiosis which is formed between soil fungi belonging to the Phylum Glomeromycota (Schüβler *et al.,* 2001) and more than 80% of higher plants including the vast majority of agricultural crops (Smith and Read, 2008). The AM fungus, an obligate biotroph, receives carbohydrates from the host plant required to complete its life cycle, in exchange of which it provides the plant with nutrients (e.g. phosphate, nitrogen and sulphur) and water. The establishment of this symbiosis involves major change in the physiology of the host plant causing a global reprogramming of plant functions and modulating its responses to biotic and abiotic stresses (Jung *et al.,* 2012). Numerous studies have reported the reduction in incidence and/or severity caused by soil-borne pathogens in presence of AM fungi (Jung *et al.,* 2012). The protective effects conferred to plants by AM fungi may be localized, i.e. at the site of the host cell containing arbuscules (Cordier et al. 1998) or systemic (Pozo *et al.,* 2002) via a mycorrhiza induced resistance (MIR) mechanism (Jung *et al.,* 2012).
Only a few studies have reported mycorrhiza occurrence in species of *Agave* and fewer studies, the effects of the AM symbiosis on the physiology of *Agave* plants. The more efficient strategy, in order to evaluate the *Agave*-AM fungal interaction, is the use of *in vitro* AM fungal cultivation (Declerck *et al.,* 2005). Consequently, the main objective of this research is to develop *in vitro* culture of AM fungi originated from different *Agave* soils, in order to evaluate the impact of the AM symbiosis on the *Agave* diseases.

## Materials and methods

*Establishment of trap cultures*

Soil samples were collected from eight sites of the state of Michoacán (México) in the "Denomination of Origen of Mezcal (DOM)" (Table 1). Soil samples were collected from each site from the rhizosphere of *Agave cupreata* plants. Trap cultures were established in the greenhouse to stimulate the production of spores in order to allow the establishment of the *in vitro* AM fungal culture.

**Table 1.** Sites of the soil samples (DOM- Michoacán).

| City | Locality | Site of sampling | Type of plantation | Geographic location | Altitude (msnm) |
|---|---|---|---|---|---|
| Madero | Etúcuaro | El Huizachal | *A. cupreata* cultivated | N 19°25' 31.4'' O 101°12' 51.4'' | 1624 |
| | | Las Campesinas | *A. cupreata* cultivated | N 19°24' 20.1'' O 101°11' 57'' | 1537 |
| | | Rancho Carlos Rojas | *A. cupreata* wild-type | N 19°23' 17'' O 101° 13' 34.2'' | 1853 |
| Morelia-Madero | Tumbisca | El limón | *A. cupreata* cultivated | N 19°32' 21.4'' O 101°05' 41.8'' | 1866 |
| | | Agua Dulce | *A. cupreata, A. tequilana* cultivated | N 19°32' 4.4'' O 101°04' 39.4'' | 2132 |
| Tzitzio | Tzitzio | Paso Ancho | *A. cupreata* cultivated | N 19°30' 1.7'' O 100°54' 51.1'' | 1006 |
| | | Barranca de las Nueces | *A. cupreata* cultivated | N 19°33' 55.1'' O 100°55' 39'' | 1471 |
| | | Cerro del Metate | *A. cupreata* wild-type | N 19°34' 22.3'' O 100°56' 27.5'' | 1915 |

*Spore extraction and disinfection procedure*

Spores were extracted from a 50-100 $cm^3$ of each trap culture sample by wet sieving without sucrose density gradient centrifugation in order to not damage the viability of the spore. Spores were surface-sterilized following a procedure adapted from Cranenbrouck *et al.* (2005). Disinfected spores were subsequently transfer on Petri plates (90 mm in diameter) containing the Modified Strullu-Romand (MSR) medium (Declerck et al. 1998), solidified with 3 g $L^{-1}$ Phytagel (Sigma-Aldrich) and incubated in the dark at 25℃.

*Establishment of the* Root Organs Culture (ROC)

Following germination, a plug of medium gel supporting a germinated spore was transferred with a scalpel into a bi-compartmented Petri plate (90 mm in diameter) containing the MSR medium (Cranenbrouck et al. 2005). An actively growing transformed carrot root (70 mm long) was previously placed and the gel plug containing the spore placed at the near vicinity of the roots. The Petri plates were incubated at 25℃ in the dark.

## Results

After the disinfection and association procedures of the different sampling soils, we have allowed to establish the *in vitro* culture of two AM fungi originated from two different

locations. The first one is from the cultivated site “El Huizachal” and the second one is from the wild-type site “Cerro del Metate”.
These two AM fungal cultures showed the same patterns of AM fungal symbiotic establishment under *in vitro* ROC conditions. The first stage of the AM fungal association is rapidly established between the modified carrot roots and the AM fungi (i.e. 2-5 days) (Fig. 1A). During the first month, the AM fungi develop an important extra-radical mycelium (Fig. 1B) and after two months, we observed an important production of young/small spores and mycelium (Fig. 1C). The first production of newly viable spore (generation; G1) is only obtained after three to four months of cultivation in ROC systems. After six months of cultivation, the numbers of spores (G1) obtained is low (10 ± 5 spores) (Fig. 1D).
Only few spores (i.e. limited number of material after the first generation) of the G1 generation have been used in order to determine this taxonomic classification (Fig. 1E and F). Actually, the species of the two AM fungi are unknown.
The second generation of the *in vitro* ROC system has been established and as the first one, has allowed to obtain the production of newly viable spore (G2). The third generation of *in vitro* ROC system is in progress.
In order to produce more viable spores, we have performed an experiment with the in *vitro* Arbuscular Mycorrhizal Plant (AMP) culture systems (Dupré de Boulois *et al.,* 2006) and associated the spore G1 with *Medicago sativa* plants in these systems (Fig. 1G). These *in vitro* AMP culture systems allowed the production of numerous newly viable spores (100 to 1000 spores; personal communication) (Fig. 1H).

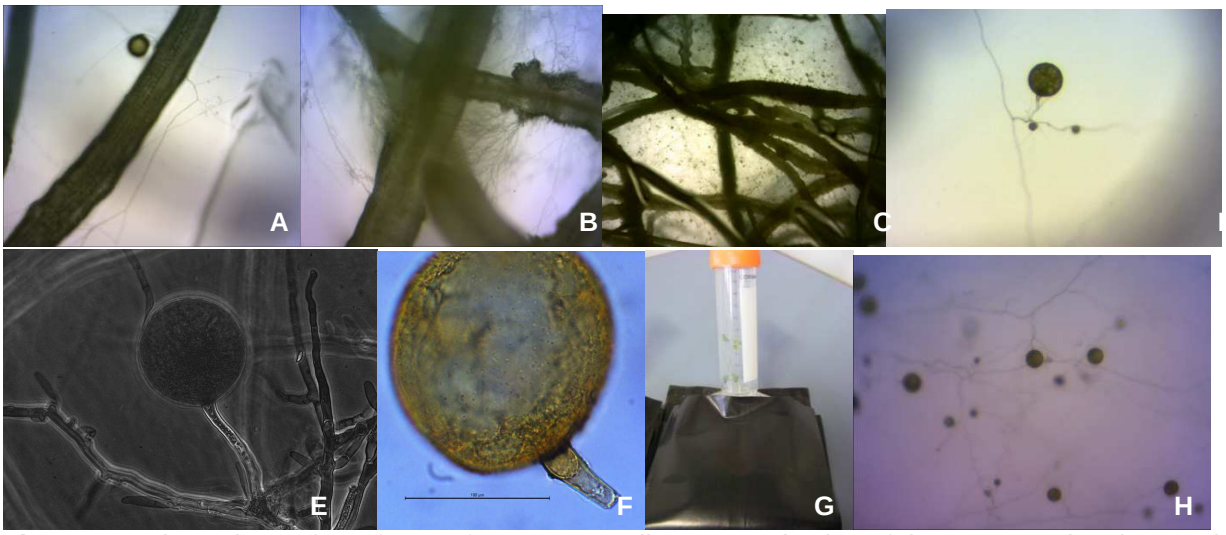


**Figure1.** *In vitro* culture of AM fungus from *Agave* soils. A: Germination of the spore mother (generation; G0), three days after transfer of the plug at the near vicinity of the roots. B: Hyphal production, one month after transfer. C: Hyphal and spore productions, two months after the transfer. D: New viable spore (G1), three months after transfer. E: Young spore in formation. F: New spore (G1) with Melzer’s coloration. G: *In vitro* Arbuscular Mycorrizal Plant (AMP) culture system of three weeks with a *Medicago sativa* plant. H: Production of a new generation of spore (G2) with the *in vitro* AMP culture system.

## Discussion

These results showed for the first time the establishment of two AM fungi originated of *Agave* soils under *in vitro* condition. It should be note that the AM fungal *in vitro* culture

has really start at the beginning of the 1980. On the +/- 228 AM fungal species from the 11 families described (Schüßler and Walker, 2010), during the last 30 years, only 17 AM fungal species from 3 families were successfully cultivated under *in vitro* culture.
The production of an important number of spores with the *in vitro* AMP culture systems has allow to start the morphological description of the AM fungi. In addition, the molecular identification is in progress following the protocol of Krüger *et al.* (2012) and the establishment of *in vivo* monosporal culture is also in progress.
In conclusion, the production of *in vitro* cultivation of AM fungi originated from *Agave* soils is the first step in order to understand/decipher the effects of the AM symbiosis on the physiology of *Agave* plants and particularly, to study the protective effects conferred to *Agave* plants by AM fungi against the agave diseases.

## Acknowledgements

This research was sponsored by the “Fondo Mixto CONACYT-Gobierno del Estado de Michoacán” under contract “Utilización de recursos microbianos para el control biológico de la pudrición del cogollo de agave tequilero en la DOT-Michoacán” (MICH-2010-03-148208).

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