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Bio-compartmental *in vitro* System for *Glomus mosseae* and *Ralstonia solanacearum* Interaction

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Abstract: The life cycle of arbuscular mycorrhizal fungi (AMF) is initiated by spore germination. The interaction between *Glomus mosseae* and *Ralstonia solanacearum* was achieved by following the bio-compartmental *in vitro* system. The system was modified to be useful for different microbes with different types of medium. Mycorrhizal fungi spores were germinated using water agar, nutrient agar and soil media, while casamino acid-peptone-glucose (CPG) media was used for *R. solanacearum*. All medium were mixed with different volumes of tomato and corn root exudates. The hyphal length of *G. mosseae* greatly affected by the exudates particularly, mycorrhizal tomato root exudates (MTRE) and mycorrhizal corn root exudates (MCRE). The growth of *R. solanacearum* was suppressed due to *G. mosseae* spores germination which can produce different volatile and non volatiles substances. The aim of this experiment was to investigate the influence of root exudates volatiles on *R. solanacearum* and the hyphal of *G. mosseae* growth under laboratory conditions using a new modified technique.

Key words: Mycorrhizal fungi, root exudates, bacterial wilt, corn, tomato

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) form symbioses with 80% of vascular plant species roots (Smith and Read, 2008). The first *in vitro* culture of AMF under aseptic conditions was accomplished by Mosse (1962). Transformed-root organ cultures *in vitro* showed that volatile substances from roots were active function in the early stages of AMF formation (Linderman, 1994). The effects of root exudates on *in vitro* spore germination and/or hyphal growth of endomycorrhiza have been studied by numerous works (Sood, 2003). Some references documented that the role of exudates has little influence on the stimulation of AMF spore germination but they may have a role in other phases of the AMF development. Root exudates produced from clover (Karlos and Safir, 1987), onion (Graham, 1982), carrot (Nagahashi *et al.*, 1996), tomato and corn (Tahat *et al.*, 2010), have been shown to increase hyphal growth and development of AMF. Many factors can affect spore germination of AMF such as root exudates and/or volatiles, soil moisture, pH, light, temperature and CO₂ (Becard and Pfeffer, 1993). The study done by Becard and Piche (1989) shows that the hyphal development and spore germination of *Gigaspora margarita* is due to the effects of root metabolites. Those metabolite volatile substances and exudates factors are produced by roots.

Root exudation includes actively secreted polysaccharides, enzymes, volatile compounds including CO₂ and ethylene, low molecular weight metabolites such as organic acids, amino acids, sugars and phenols (Marschner, 1995).

The germination of *Fusarium oxysporum* f.sp *lycopersici* was inhibited in the presence of root exudates from tomato plant (Scheffknecht *et al.*, 2006). Differential growth of *F. oxysporum* f.sp *chrysanthemi*, *Trichoderma harzianum*, *Clavibacter michiganensis* and *Pseudomonas chlororaphis* was explained by substances released from *Glomus intraradices* under *in vitro* culture conditions (Filion *et al.*, 1999). Root exudates of non-mycorrhizal tomato exhibit a higher attracting effect on zoospores of *Phytophthora parastica* more than exudates collected from mycorrhizal tomato roots (Lioussanne *et al.*, 2009). Siu and Donald (1991) suggested that *in vitro* production of flavonoids from alfalfa roots (*Medicago sativa* L.) may regulate or facilitate the development of AMF symbioses and offer new hope for developing pure plant-free culture of mycorrhiza.

The goal of this study was to evaluate the biological indirect interactions between mycorrhizal fungi *G. mosseae* volatiles compounds and *R. solanacearum* growth under conditions laboratory following new technique.

MATERIALS AND METHODS

Root exudates production: Four types of root exudates were produced:

- Mycorrhizal tomato root exudates colonized by *G. mosseae* (MTRE)
- Non-mycorrhizal tomato root exudates (NMTRE)
- Mycorrhizal corn root exudates (MCRE)
- Non-mycorrhizal corn root exudates (NMCRE)

Exudates from colonized plant were produced following the method of Shang *et al.* (2000). Seeds of tomato plants were rinsed for 15-20 sec with 95% ethanol for surface sterilization. The seeds were washed several times with sterilized and distilled water and placed in sterile glass flasks (50 mL), containing 10 mL of sterile deionized water. The seeds (five seeds/flask) plants were covered with cotton and sterile aluminum foil and were kept in growth chamber (28°C) in dark conditions for seeds germination. The flasks were open after 14 days under laminar-flow and the solution (root exudates) was collected using sterile Pasteur pipette. The exudates solution was immediately passed through 0.45 µm filter to remove root debris or any microorganisms; the solution was stored at 4°C until use. It was checked for pathogen availability using PDA media before quantification. Exudates from non colonized tomato and corn roots were produced using the method of Karlos and Safir (1987). Plants were taken from the soil (30 days old), the roots were washed carefully by distilled water rinsed with sterile distilled water several times. About 20-30 seedlings were placed in a flask containing 100 mL sterile distilled water for 24 h. The solution was concentrated 1/10 the original volume by rotary evaporation at 50°C and filter sterilized again and stored at 4°C. The product was checked for saprophyte contamination using PDA media.

Root exudates quantification: Amino acid contents were determined in the root exudates in previous study by Tahat *et al.* (2010).

Water agar medium preparation: Water agar was prepared by taking eight gm of water agar powder and dissolved in one liter distilled water. The media was autoclaved (121°C for 15 min). pH media was adjusted to pH 5.5 using KCl. Ten milliliter of medium were poured in each Petri-dishes. The media was mixed with (3,6,9, mL) for each treatment (MTRE), (NMTRE), (MCRE) and (NMCRE) (1:1 v/v) prepared before poured in Petri dishes.

Nutrient agar medium preparation: Ten grams of nutrient agar was mixed with 1 L distilled water. Shacked well then pH value was adjusted to pH 6.0. The mixture was autoclaved (121°C for 15 min). Five gram streptomycin was added to the media for saprophytic microbe's inhibition. Ten milliliter of media were poured in each plate. The media was mixed with (MTRE), (NMTRE), (MCRE) and (NMCRE) (1:1 v/v) prepared.

Soil medium preparation: Ten of *G. mosseae* spores were sandwiched between two millipore membrane (0.45 µm) pore diameters. Fine sterilized and sieved soil (20 g) was used to cover the upper millipore membrane and another layer of soil was used under the second millipore membrane. The system was watered with 3, 6, 9 mL of (MTRE), (NMTRE), (MCRE) and (NMCRE).

Bio-compartmental in vitro cultural system: To study the role of germinated spores volatile on *R. solanacearum* growth we did a slight modification on bio-compartmental system was done. Two glass Petri dishes were used with the same size (9 cm diameter). Different medium were poured in each dish, the upper dish contains CPG media for *R. solanacearum* while the bottom dish contain water agar, nutrient agar and soil media separately (Fig. 1a-c).

***R. solanacearum* isolation and growth:** The colonies of *R. solanacearum* were monitored after 3 days of incubation. The growth was seen in NMTRE and NCTRE while the treatments TMRE and CMRE were free *R. solanacearum* colonies.

Hyphal length measurement: The hyphal length was measured under the microscope using an eyepiece grid.

Data analysis: Completely randomized design was used (4 treatments with 4 replications). Excel program was used for the means separation.

RESULTS

Hyphal length: Hyphal length was varied among treatments related to the different volumes used. At 3 mL volume, the TMRE was the highest and it was significantly different when compared with NMTRE and CMRE. When the volume of exudates increase, the growth of hyphae was increased significantly in the same treatment. At 6 mL exudates volume, the hyphal length was significantly different among TMRE, NMTRE and CMRE. TMRE and NMTRE were not different statistically at 6 mL volume. When the volume of exudates was 9 mL from TMRE used, the hyphal length was

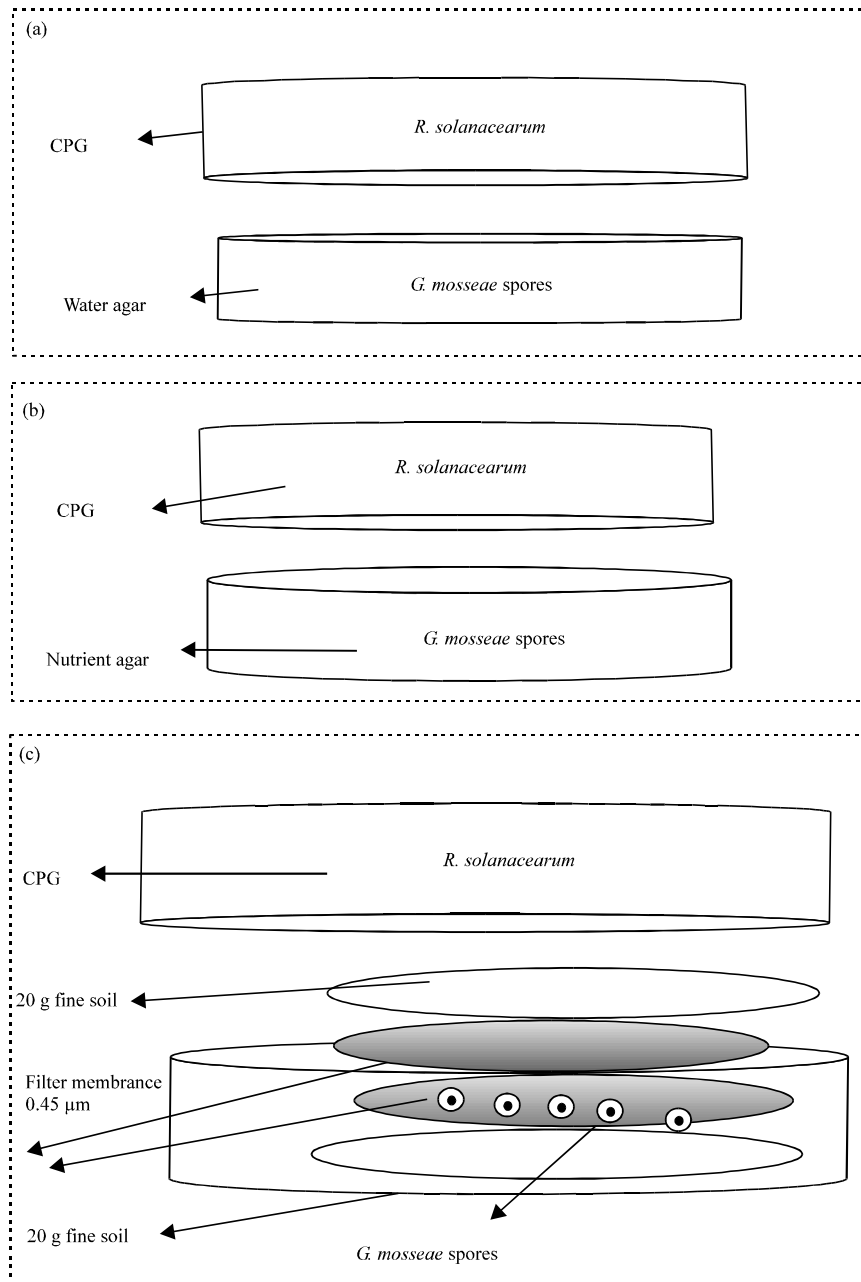


Fig. 1(a-c): Modified bio-compartmental system of *G. mosseae* spore germination using three types of medium (water agar (a), nutrient agar (b) and soil (c), CPG medium used for *R. solanacearum* growth

statistically increased compared to NMTER and CMRE. TMRE treatment was not different from CMRE, when the volume of root exudates was equal (Fig. 2).

***R. solanacearum* growth:** *R. solanacearum* growth and development was estimated visually. The growth was not observed in TMRE and CMRE. In NMTRE and NMCRE

the colonies growth were very weak and small size. The colonies were recognized by the pinky color.

DISCUSSION

Bio compartmental *in vitro* system can be use broadly in mycorrhizal research and other microbes

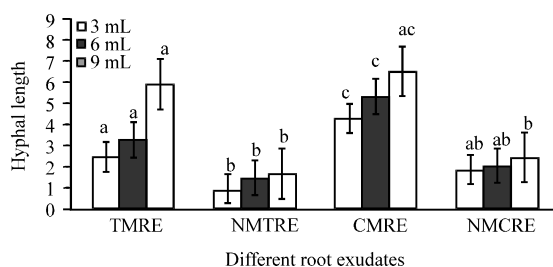


Fig. 2: The effect of different volume of root exudates from different host on the hyphal length of *G. mosseae*. Whereas, Mycorrhizal tomato root exudates colonized by *G. mosseae* (MTRE). Non-mycorrhizal tomato root exudates (NMTRE). Mycorrhizal corn root exudates (MCRE). Non-mycorrhizal corn root exudates (NMCRE). Bars followed by the same letter are not significantly different Tukeys using HSD ($p = 0.5$)

Table 1: Amino acid contents in different types of root exudates

Amino acid	MCRE (Mmole/ML)	MTRE (Mmole/ML)	NMTRE (Mmole/ML)	NMCRE (Mmole/ML)
asp	3.8±0.06a	10.5±0.05b	1.4±0.05c	1.20±0.07d
ser	2.5±0.05a	1.4±0.02b	1.1±0.08c	0.60±0.06d
glu	4.5±0.09a	7.9±0.08b	1.6±0.09c	2.11±0.08d
gly	4.2±0.01a	1.8±0.01b	2.1±0.08c	0.58±0.01d
his	0.3±0.01a	0.5±0.01b	0.2±0.08c	0.06±0.03d
arg	0.6±0.01a	0.3±0.02b	0.5±0.01c	0.65±0.07d
thr	2.5±0.01a	0.7±0.05b	0.9±0.01c	0.28±0.08d
ala	4.8±0.04a	0.5±0.04b	2.3±0.06c	0.27±0.03d
pro	2.9±0.04a	1.0±0.08b	1.0±0.08c	0.73±0.21d
tyr	0.3±0.07a	0.1±0.03b	0.2±0.01c	0.05±0.01d
val	3.5±0.05a	0.8±0.04b	0.9±0.01c	0.20±0.05d
met	2.0±0.04a	2.8±0.06b	0.5±0.03c	0.16±0.03d
lys	1.3±0.02a	0.2±0.06b	0.5±0.04c	0.08±0.03d
ile	1.0±0.07a	0.3±0.07b	0.1±0.04c	0.08±0.02d
leu	3.1±0.07a	1.2±0.03b	0.8±0.06c	0.20±0.05d
phe	1.0±0.04a	0.8±0.04b	0.5±0.01c	0.20±0.04d

Means in rows followed by the same letter are not significantly different, according to Tukeys HSD ($p \leq 0.01$). MTRE: Mycorrhizal tomato root exudates colonized by *G. mosseae*. NMTRE: Non-mycorrhizal tomato root exudates. MCRE: Mycorrhizal corn root exudates and NMRC: Non-Mycorrhizal Corn Root Exudates

(St-Arnaud *et al.*, 1996). In the current experiment, slight modification was done in this system to allow the indirect interaction between two microbes using different medium. The initial germination for *G. mosseae* spores was observed in all medium used. The present results matches with that reported by Linderman (1994), Graham *et al.* (1982), they succeeded to develop and germinate *G. versiforme* and *G. intraradices* on water agar media. *G. mosseae* successfully germinated in nutrient agar media (Srinath *et al.*, 2003). The quantity and the quality of root exudates are involved in spore germination and hyphal length (Karlos and Safir, 1987). This work illustrated that when the volume of root exudates increased the hyphal length was increase due to the spore's life span. The *G. mosseae* hyphal growth was

affected by the volatiles produced from the germinated spores in the medium used. The amino acid contents were varied related to the difference between the hosts qualitatively and quantitatively (Table 1). The results presented were closed to that reported by Tawaraya *et al.* (1996), they observed that hyphal growth of *Gigaspora margarita* was strongly affected by the exudates of onion (*Allium cepa*). Srinath *et al.* (2003) were successes to develop a new in vitro experimental system to study the morphogenesis of a single extra-radical mycelium of the *Glomus intraradices*. In their study they used six types' agar-based media. They found that the mycelium of *G. intraradices* showed differences between-treatment in architecture, morphogenesis and formation of branched absorbing structures.

In the current report, the volume and the source of exudates used were significantly playing a role in hyphal growth and elongation using different medium. Hyphal length was significantly different in (MCRE) when 3 and 6 mL volume used. NMCRE and NMCRE were not different statistically in all volumes used indicated that mycorrhizas fungi colonization may play a great role in volatiles produced qualitatively and quantitatively. The documented results by Napierala-Filipiak *et al.* (2002) were different from the current our results, that they found that volatile compounds (primarily terpenes, sesquiterpenes) extraction were qualitatively the same in roots of mycorrhizal and nonmycorrhizal plants. They also observed that plant hosts inoculated with the fungi resulted in non-significant increases in the total amount of the volatiles. The mycorrhizal fungi spores showed diversified effect on the concentrations of several terpenoids.

The volities produced by the germinated spores and the hyphal growth were able to suppress the growth *R. solanacearum* colonies in MTRE and MCRE and the obesit was in the treatments of NMTER and NMCRE that is the colonies were developed slowly. results reported in this study very close to that reported by Norman and Hooker (2000), they illustrated that the sporulation of *Phytophthora fragariae* was reduced after using root exudates from a strawberry plant in *in vitro* studies.

CONCLUSION

The bio-compartmental system was useful tool to a chief the interaction between different microbes which need different medium to grow. The root exudates was an important and great factor affecting hyphal growth and development. More studies are needed to investigate the role of anther root exudates in mycorrhizal fungi development and management.

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