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The Role of Tomato and Corn Root Exudates on *Glomus mosseae* Spores Germination and *Ralstonia solanacearum* Growth *in vitro*

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Abstract: An *in vitro* experiment was conducted to study the effect of different plant root exudates on germination of *Glomus mosseae* and the growth of bacterial wilt *Ralstonia solanacearum*. Mycorrhizal spore germination increased when the volume of Mycorrhizal Tomato Root Exudates (MTRE) increased and in contrast, a negative relationship was recorded when the volume of Non-Mycorrhizal Tomato Root Exudates (NMTRE) increased. Similarly, the Mycorrhizal Corn Root Exudates (MCRE) was able to increase the percentage of germinated spores as compared to the Non-Mycorrhizal Corn Root Exudates (NMCRE). The antagonistic effect between *Ralstonia solanacearum* and *Glomus mosseae* was also studied in this research. There was no inhibition effect of mycorrhizal and non-mycorrhizal tomato and corn root exudates on growth of *R. solanacearum*. The study indicated that *Glomus mosseae* spore germination could be influenced by the host plant or pH medium.

Key words: *In vitro*, bacteria, mycorrhizae, root exudates, antagonistic

INTRODUCTION

Plant root exudates play a key role during the presymbiotic growth phase and have been shown to stimulate hyphal branching and the catabolic metabolism of Arbuscular Mycorrhizal (AM) fungal spores (Bücking *et al*., 2008). Exudates components are thought to attract beneficial micro-organisms to the rhizosphere and also to promote their survival in this particular environment (Bayliss *et al*., 1997). Many scientific studies have been carried out using *in vitro* systems and new prospects have been opened up by using the material provided by monoxenic plates (Cano *et al*., 2008). Plant root exudates consist mainly on a complex mixture of organic acid anions, phytosiderophores, purines, sugar, vitamins, amino acids, nucleosides, inorganic ions (e.g., HCO_3^-, OH^-, H^+), gaseous molecules (CO_2, H_2), enzymes and root border cells (Dakora and Phillips, 2002). The first *in vitro* culture of AMF under aseptic conditions was accomplished by Mosse (1962). The life cycle of AMF is initiated by germination of spores (Smith and Read, 1997). The using of transformed-root organ cultures *in vitro* showed that volatile substances from roots were active (function) in the early stages of VAM formation (Linderman, 1994). Many factors can affect spore germination of AMF such as root exudates and/or volatiles, soil moisture, pH, light, temperature and CO_2 (Giovannetti, 2000).

The sporulation of the oomycete *Phytophthora fragriae* was reduced after using root exudates from a strawberry plant in *in vitro* studies (Norman and Hooker, 2000). It is well

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documented that changes in specific chemical components in plant tissues could deter pathogens. The germination of soil-borne fungus *Fusarium oxysporum* f. sp. *lycopersici* was inhibited in the presence of root exudates of tomato plants (Scheffknecht *et al*., 2006).

Treatment of tomato root exudates with insoluble polyvinylpolypyrrolidone, which binds phenolic compounds, indicated that tomato root exudates contain phenolic compounds inhibitory to *Fusarium oxysporum* f. sp. *lycopersici* microconidia germination. On the opposite, in the same study, non polyvinylpolypyrrolidone treated tomato root exudates stimulate microconidia germination of both *F. oxysporum* f. sp. *lycopersicy* and *F. oxysporum* f. sp. *radicis-lycopersici* (Steinkellner, 2005). It is well established that *in vitro* AM production is an appropriate way of getting large amounts of clean, clonal, contamination-free AM fungal material. This has opened new research opportunities for molecular biology and biochemical techniques to be applied to mycorrhizal research and the direct consequence of this is an exponential increase in our knowledge in the basic biology of this mutualistic symbiosis over the last 10 years (Cano *et al*., 2008).

The using of mycorrhizal fungi as biocontrol agents against soil-borne pathogens under the field conditions is well documented (Smith and Read, 2008). Arbuscular mycorrhizal fungi have shown a direct interaction with soil-borne pathogen through antagonism, mycoparasitism and/or antibiosis (Harrier and Watson, 2004). *Ralstonia solanacearum* is a soil-borne pathogen that causes bacterial wilt diseases in diverse plant species (Yao and Allen, 2007).

Split root experiments revealed that *R. solanacearum* was inhibited by the mycorrhizal fungus *Glomus versiforme* as a result of increased phenols content induced systemically but to a lesser extent than locally (Zhu and Yao, 2004).

Root exudates could be use for the multiplication of mycorrhizal fungi another spores *in vitro* because it serve as signals that initiate the symbiosis mechanisms of mycorrhizal fungi and the *in vitro* control ability of root exudates on the phytopathogenic bacteria *R. solanacearum* could be the first step in the management of bacterial wilt disease based on exudates compounds.

The current study consists of two experiments; the first experiment was aimed to evaluate the ability of different root exudates germination of *G. mosseae* spores. The second experiment was aimed to study the interaction between the mycorrhizal fungi *G. mosseae* germinated spores and *R. solanacearum* under laboratory controlled conditions.

MATERIALS AND METHODS

Experiment 1: Root Exudates Production

This study was done at the Laboratory of Soil Microbiology, Land Management Department, Universiti Putra Malaysia, Malaysia. Four types of root exudates were produced:

- Mycorrhizal Tomato Root Exudates (MTRE) colonized by *G. mosseae*
- Non-Mycorrhizal Tomato Root Exudates (NMTRE)
- Mycorrhizal Corn Root Exudates Colonized (MCRE) with *G. mosseae*
- Non-Mycorrhizal Corn Root Exudates (NMCRE)

Root exudates from colonized plants were produced following the method described by Shang *et al*. (2000). Seeds of red rock tomato cultivar and corn plants were rinsed for 15-20 sec with 95% ethanol for surface disinfestation. The seeds were washed several times with sterilized and distilled water. The seeds (five seeds/flask) from both plant species were placed separately in sterile glass flasks (50 mL), containing 10 mL of sterile deionized water.

The flasks were covered with cotton and sterile aluminum foil and were kept in growth chamber at 28°C in the dark for seed germination. The flasks were open after 14 days under laminar-flow and the solution of root exudates was collected using sterile Pasteur pipette. The exudates solution was immediately passed through 0.45 μm filter to remove root debris; the solution was stored at 4°C for 1 week before use. It was checked for microbial contamination. One milliliter from each exudates was taken three times and cultured on Potato Dextrose Agar (PDA) media before quantification and incubated at 28°C for 5 days. Exudates from AMF non colonized tomato roots were produced using the method described by Karlos and Safir (1987). Plants were taken from the soil (30 days old), the roots were washed carefully with distilled water and then rinsed with sterile distilled water several times. About 20-30 seedlings were placed in a flask containing 100 mL of sterile distilled water for 24 h. The solution was concentrated 1/10 of the original volume by rotary evaporation at 50°C, filtered again and stored at 4°C. The product was checked for microbial contamination by plating 1 mL on PDA and incubated at 28°C for 5 days.

Amino Acids Analysis

Amino acid concentrations were determined using HPLC by a modified method from Cohen (1994) following pre-column derivatisation with AQC reagent (6-aminoquinolyl-N-hydroxysuccinimdyl carbamate, Waters, USA). Tryptophan content was determined by alkaline hydrolyses. Cysteine and methionine were not determined.

***Glomus mosseae*, Isolation and Collection**

Mature spores of *G. mosseae* for the experiments were collected from a stock culture (Land Management Department, Universiti Putra Malaysia). The spores were transferred using a sterilized pipette to 0.45 μm diameter filter paper disc. The system was autoclaved (121°C for 15 min) before spore culture.

Culture Media

Water Agar (WA) (8%) was used as culture media. Ten milliliters of media was dispensed into glass Petri dish (9 cm diameter). The media was mixed with different volumes (2, 4, 6, 8, 10 and 12 mL) of the different root exudates separately. The media was adjusted to pH 6.5 using potassium chloride (KCl). The plates were kept in refrigerator at 4°C overnight before used.

Spore Surface Disinfestations

Before *G. mosseae* spores were cultured between both filter paper discs, the 2% chloramines T, 400 mg of streptomycin and 1 drop of Tween 80% L^{-1} distilled water was used for spore surface disinfestation. The solution passed through a 0.22 μm membrane until the first drop of the solution emerged. The contact time between solution and spores was 15-20 min. The spores were washed 5 times with sterilized and distilled water before plating onto Petri dishes. The spores were singly transferred to Petri dish (10 spores/dish) (MacDonald, 1981).

Spores Germination

Ten spores of *Glomus mosseae* numbers for each replicates were transferred to sterilized filter paper (0.45 μm) by using sterilized forceps. The spores were distributed in the filter paper and placed on 9 cm Petri-dish with WA. Petri-dishes with spores were incubated in the dark at room temperature (28±2°C). After 5 days of spore's culture in different substrate, the germ tube was clearly observed for germinated spores after filter paper staining (Phillips and

Hayman, 1970). Germinated spores were counted under binocular microscope at 100x; the color of germinated spores was yellow to brownish. The media was adjusted for all exudates to pH 6.5 using KCl before any treatment and it was measured after 5 days of spore culture. Data were analyzed by linear regression analysis to determine the relationship between percentage of spore germination and root exudate concentration using excel microsoft 2007.

Experiment 2: Root Exudates Production (As in Experiment 1)

Bacterial Suspension Preparation

According to Nesmith and Jenkins (1979), bacterial inoculums of *R. solanacearum* was prepared. Casamino acid Peptone Glucose (CPG) agar media according to Cuppelss *et al.* (1978). Inoculum was prepared by using bacterial suspension that was adjusted to 10^8 cfu mL^{-1} using a spectrophotometer at OD_{600} = 0.8. The bacterial suspension was prepared from a 24 h old culture. Ten milliliters of the suspension was inoculated into each test tube containing different volumes of different root exudates. Test tubes were incubated in at 26±2°C for 24 h.

Growth Conditions

Different volumes of different root exudates were mixed with a *R. solanacearum* suspension (10^8 cfu mL^{-1}) (5 mL/test tube). The mixture was kept in incubator for 48 h at 26-28±2°C.

RESULTS AND DISCUSSION

Experiment 1: Root Exudates Quantification

Sixteen amino acids were analyzed for the four types of root exudates (Table 1). Four replicates from each exudate were tested. All root exudates were analyzed by standard Analysis of Variance (ANOVA) and treatments means were compared using the Tukey's comparison test at $p<0.01$ using the SPSS software. ANOVA was performed for each amino acid detected.

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

	Treatments (Mmol mL^{-1})			
Amino acid	MCRE	MTRE	NMTRE	NMCRE
asp	3.8b	10.5a	1.4c	1.20d
ser	2.5a	1.4b	1.1c	0.60d
glu	4.5b	7.9a	1.6d	2.11c
gly	4.2a	1.8c	2.1b	0.58d
his	0.3a	0.5b	0.2c	0.06d
arg	0.6b	0.3d	0.5c	0.65a
thr	2.5a	0.7c	0.9b	0.28d
ala	4.8a	0.5c	2.3b	0.27d
pro	2.9a	1.0b	1.0c	0.73d
tyr	0.3a	0.1c	0.2b	0.05d
val	3.5a	0.8c	0.9b	0.20d
met	2.0b	2.8a	0.5c	0.16d
lys	1.3a	0.2c	0.5b	0.08d
ile	1.0a	0.3b	0.1c	0.08d
leu	3.14a	1.2b	0.8c	0.20d
phe	1.0a	0.8b	0.5c	0.20d

Mean values in rows followed by the same letter(s) are not significantly different, according to Tukey's HSD ($p<0.01$), were as, MTRE: Mycorrhizal tomato root exudates colonized by *G. mosseae*, NMTRE: Non-mycorrhizal tomato root exudates, MCRE: Mycorrhizal corn root exudates, NMCRE: Non-mycorrhizal corn root exudates. ANOVA was preformed for each amino acid

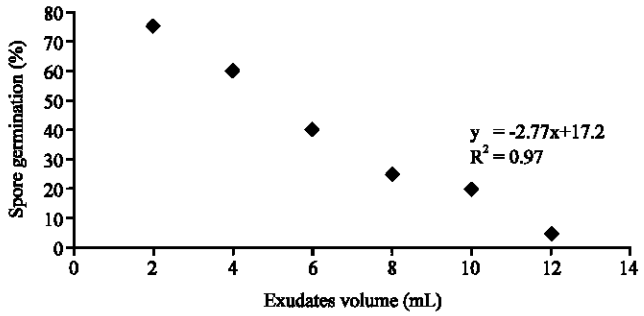

Fig. 1: Relationship between different volumes of Mycorrhizal Tomato Root Exudates (MTRE) and *Glomus mosseae* spore germination after 5 days of *in vitro* culture of the mycorrhizal fungi

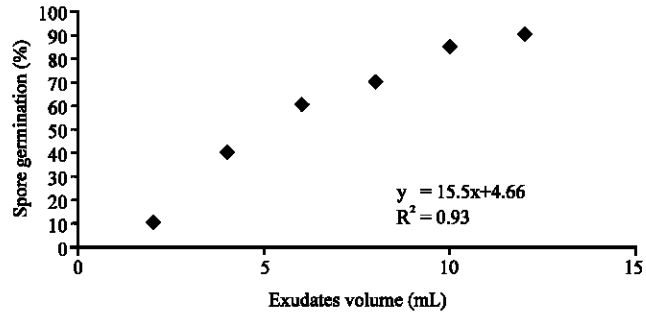

Fig. 2: Relationship between different volumes of Non-Mycorrhizal Tomato Root Exudates (NMTRE) and *Glomus mosseae* spores germination after 5 days of *in vitro* culture of mycorrhizal fungi

Spore Germination (%)

A negative linear relationship was observed between MTRE and percentage spore germination ($R^2 = 0.97$) (Fig. 1), i.e., spore germination percentage decreased with increasing the volumes of mycorrhizal tomato root exudates.

On the opposite the percentage of *G. mosseae* spore germination was increased linearly by increasing NMTRE volume ($R^2 = 0.92$) (Fig. 2).

A negative linear relationship was observed between spore germination number and exudates volume ($R^2 = 0.91$) (Fig. 3).

A positive linear relationship was recorded between NMCRE volume and the number of germinated spores ($R^2 = 0.94$) (Fig. 4).

Experiment 2: Bacterial Concentration (cfu)

The concentration of *R. solanacearum* bacterial cell in a mixture of the bacteria and serial volumes of different root exudates was measured. *Ralstonia solanacearum* concentration decreased as mycorrhizal tomato root exudates volume increased, as indicated by the negative linear relationship observed between this two variables ($R^2 = 0.97$) (Fig. 5).

Negative relationship between NMTRE and *R. solanacearum* cell concentration was observed ($R^2 = 0.93$) (Fig. 6).

MCRE was influenced on *R. solanacearum* growth negatively; the growth of *R. solanacearum* was not inhibited by increasing the MCRE volume (Fig. 7).

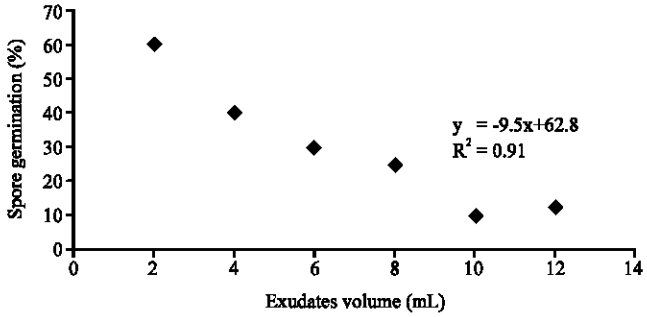

Fig. 3: Relationship between different volumes of Mycorrhizal Corn Root Exudates (MCRE) and *Glomus mosseae* spores germination after 5 days of *in vitro* culture of mycorrhizal fungi

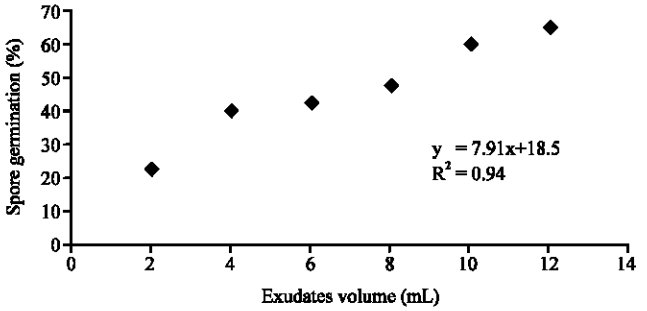

Fig. 4: Relationship between Non-Mycorrhizal Corn Root Exudates (NMCRE) and *Glomus mosseae* spores germination after 5 days of *in vitro* culture of mycorrhizal fungi

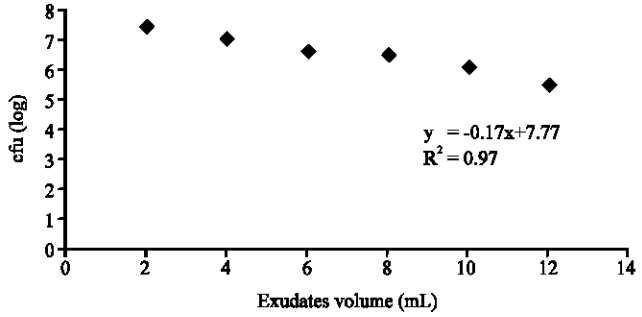

Fig. 5: Relationship between different volume of Mycorrhizal Tomato Root Exudates (MTRE) and the cfu of the *Ralstonia solanacearum* spore suspension after 48 h of incubation at 26-28± 2°C

A negative linear effect was observed between root exudates volumes of mycorrhizal corn and bacterial concentration ($R^2 = 0.93$) (Fig. 8).

pH Determination

The pH changes in a mixture of *R. solancearum* cell suspension and serial volumes of different root exudates was measured at the end of the experiment. The pH of the bacterial suspension increased linearly with the MTRE volume increase ($R^2 = 0.98$) (Fig. 9).

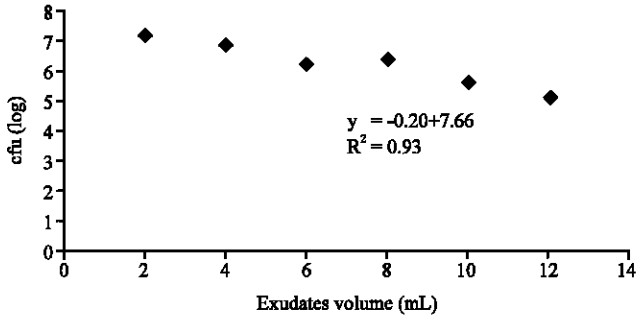

Fig. 6: Relationship between different volumes of Non-Mycorrhizal Tomato Root Exhudates (NMTRE) and the cfu of the *Ralstonia solanacearum* cell suspension after 48 h of incubation at 26-28±2°C

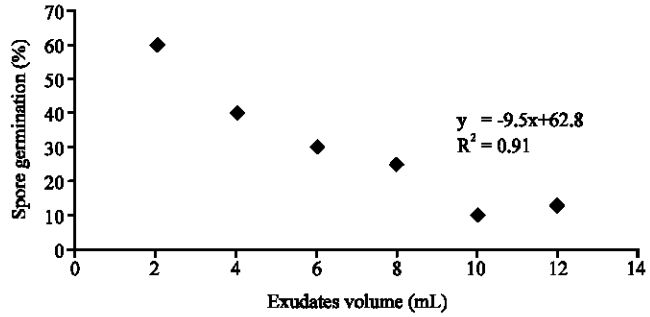

Fig. 7: Relationship between different volumes of Mycorrhizal Corn Root Exudates (MCRE) and cfu of the *Ralstonia solanacearum* cell suspension after 48 h of incubation at 26-28±2°C

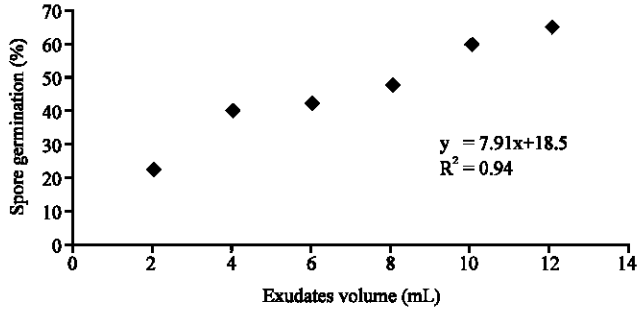

Fig. 8: Relationship between different volume of Non-Mycorrhizal Corn Root Exudates (NMCRE) and cfu of the *Ralastonia solanacearum* cell suspension after 48 h of incubation at 26-28 ±2°C

Similarly, a positive linear relationship was found (R^2 = 0.98) between NMTRE volume and pH (Fig. 10).

The pH of the mixture *R. solanacearum* and root exudates was positive and linearly correlated with exudates of NMCRE increasing volumes (R^2 = 0.90) (Fig. 11).

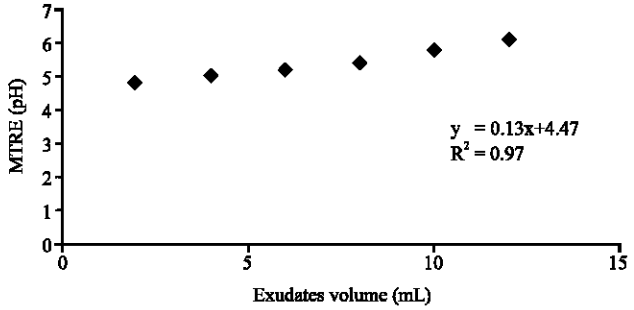

Fig. 9: Relationship between pH and volume of a Mycorrhizal Tomato Root Exudates (MTRE) mixed with a *Ralstonia solanacearum* cell suspension after 48 h of incubation at 26-28±2°C

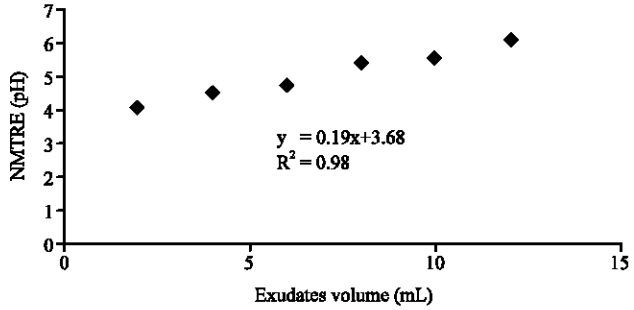

Fig. 10: Relationship between pH and volume of a Non-Mycorrhizal Tomato Root Exudates (NMTRE) mixed with a *Ralstonia solanacaerum* cell suspension after 48 h of incubation at 26-28±2°C

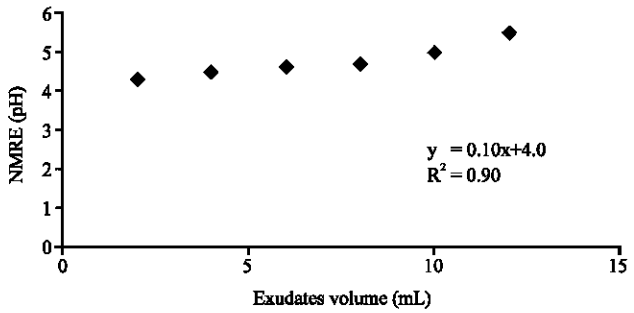

Fig. 11: Relationship between pH and volume of a Non-Mycorrhizal Corn Root Exudates (NMCRE) mixed with a *Ralstonia solanacearum* cell suspension after 48 h of incubation at 26-28±2°C

A positive relationship was also detected ($R^2 = 0.98$) between the volume of MCRE and pH value of the suspension (Fig. 12).

The present study presents a method to evaluate the effect of different root exudates in mycorrhizal fungi germination spores. Differences in spore germination of AMF due to root exudation source and quality were reported among some types of exudates, for example, root exudates of cucumber mycorrhized plants showed a reduced stimulatory effect on AM

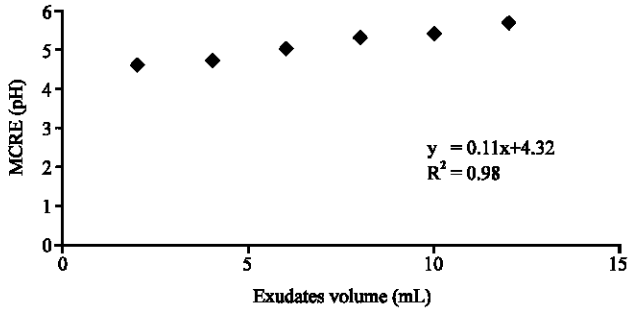

Fig. 12: Relationship between pH and volume of Mycorrhizal Corn Root Exudates (MCRE) mixed with a *Ralstonia solanacearum* cell suspension after 48 h of incubation 26-28±2°C

hyphal growth and an inhibitory effect on root colonization by AMF (Vierheilig and Piche, 2003). Similarly, Norman and Hooker (2000) found that root exudates from mycorrhized strawberry plants reduced the sporulation of *Phytophthora fragariae*. Present findings disagree with the results reported by Douds and Nagahashi (2000) as they found that the rate of spore germination can be increased by plant host root exudates. In the same way, Büching *et al.* (2008) found that crude exudation led to a slight acceleration of spore germination and increased germ tube branching. Siu and Donald (1991) stated 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 AMF.

Root exudates components such as flavonoids and volatiles (CO_2, H_2) produced due to the germination of mycorrhizal spores were the main reasons behind the germination of AMF, but the sporulation rate was reduced according to the amount of exudates used. The results obtained from this study were in the same line with that reported by Chabot *et al.* (1992), who suggested that hyphal growth of the Vesicular Arbuscular Mycorrhizal (VAM) fungus, *Gigaspora margarita* Becker and Hall, is affected by stimulatory flavonoids compounds (kaempferol, quercetin and morin).

Root exudates quantity was an important factor for spore germination number. Present data agree with that reported by Schwab *et al.* (1983), in which the quantity and composition of root extracts may not be a reliable predictor of the availability of the substrate for symbiotic VAM fungi. The data suggested that exudates quantity and quality were important stimulatory factors for spore germination. These results contradict with those obtained by Karlos and Safir (1987), they documented that the quality of exudates only an important in stimulating VAM fungus germination and hyphal elongation. The same results were confirmed by Vierheilig and Piche (2003), they observed that root exudates of non-mycorrhizal cucumber plants show a significant stimulatory effect on root colonization.

In present study, the spore germination was stimulated better by increasing the volume of NTRE and NCRE. These results can be explained by the quality of exudates used which contain more substances that stimulate the spore germination. Current results agree with the data published by Lioussanne *et al.* (2003) they suggested that root exudates collected from non-mycorrhizal tomato roots exhibited a higher attracting effect on zoospore of *Phytophthora parasitica* than root exudates from mycorrhizal tomato roots.

However, this results disagree with that published by Hepper and Smith (1976), who found that the spores of *Acaulospora lavis* maintained a few weeks at 6°C were exposed to

different pH levels and germination was higher in low pH. Another report was recorded by Bücking *et al*. (2003) they found that root exudates supplied to AM fungal spores in various concentrations significantly affect germination and hyphal branching of AMF spores in *in vitro*. *Ralstonia solanacearum* growth was negatively affected by using serial volumes of root exudates produced from corn and tomato plants. The cell concentration of *R. solanacearum* decreased when the volume of the root exudates (all types) increased. these results conflected with those published by Scheffknecht *et al*. (2006) they found that microconidia of *F. oxysporum* f. sp. *lycopersici* was enhanced in the presence of root exudates from mycorrhizal plant.

Quantification of tomato and corn root exudates colonized by AMF indicated that, amino acids were produced in a high quantity by the plant but the *R. solanacearum* was not suppressed by using different volume of MTRE. The pathogen strain (race 3 biovare 2) might explain the aggressive *in vitro* growth (Hayward, 1991).

The effect of root exudates from mycorrhizal and nonmycorrhizal tomato plants on germination of the tomato pathogen *F. oxysporum* f sp. *lycopersici* was tested by Scheffknecht *et al*. (2006). They found that germination of *F. oxysporum* f. sp. *lycopersici* microconidia was enhanced in the presence of mycorrhizal tomato root exudates. These results were in the same line with the results that establish how *R. solanacearum* growth increased by the different root exudates types used in the study.

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