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Research Paper

PHYTOBACTERIA BIOTIZATION OF FOUQUIERIA SPLENDENS CALLUS AGAINST CADMIUM

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Abstract

"Biotization" has been defined as a metabolic response of in vitro-grown plant material to a microbial inoculant(s), leading to the developmental and physiological changes enhancing biotic and abiotic stress resistance. Also, it has been reported that in vitro culture of tissue explants with beneficial microbes may enhance protection against like the heavy metals contamination. This study analyzed the effect on growth of a desert plant *Fouquieria splendens* bacterized callus with an endophyte bacterium against cadmium. In this study, even all the endophytes employed have been characterized as medium indole acetic acid producers; the obtained fresh biomass does not shown a notably effect on bacterized callus as a promotion response by them, but against the heavy metal there was a promotion response of cells growth with *Staphylococcus pasteuri* strain Fs3. These results suggest a positive biotization response of callus biomass of *Fouquieria splendens*; where even the endophytes do not increase at all the gain of biomass; they were able to protect the cells against cadmium under *in vitro* conditions. Thus these strains could be recommended as adequate endophytes for the establishment of *in vitro* plant-bacterium interactions.

Key words: *biotization, callus culture, endophytes, co-cultures*.

INTRODUCTION

In vitro co-culture of tissue explants with beneficial microbes may enhance protection and tolerance against a variety of abiotic stresses like the heavy metals contamination; this experimental process is known as ‘‘biotization’’ concept established by Nowak [1] who define it as: “a metabolic response of in vitro-grown plant material to a microbial inoculant(s), leading to the developmental and physiological changes enhancing biotic and abiotic stress resistance of the derived propagules”. There are some reports regarding to *in vitro* plants biotization that have employed microbial inoculants applied to plant micropropagation techniques (Hooker *et al.* [2]; Elmeskaoui *et al.* [3]; Nowak *et al.* [4]; Balla *et al.* [5]; Lazarovits and Nowak [6]; Murphy *et al.* [7]; Wilhelm et al. [8]. Particularly bacterization techniques have been applied and bacteria stimulate plantlet growth favoring developmental changes [4, 6, 9-12]; where live bacterial cells are required. Nowak *et al.* [4, 13]; Lazarovits and Nowak [6]; Pillay and Nowak [11] and Bensalim *et al.* [12] noted that both *in vitro* and *ex vitro* benefits of bacterization depended on plant species and growth conditions.

This study analyzed the effect on growth of a desert plant *Fouquieria splendens* bacterized callus with an endophyte bacterium against cadmium.

MATERIALS AND METHODS

Callus culture inoculants

The endophytes bacteria employed: *Staphylococcus pasteuri* strain Fs3, *Staphylococcus warneri* strain Fs4 and *Kocuria rhizophila* strain Fs7; were isolated by Salinas [14], from leaves of the desert plant *Fouquieria splendens* grown in the botanical garden of the Facultad de Estudios Superiores, Iztacala (FES)-UNAM. They were classified according to Khalid *et al.* [15] by their *in vitro* production of indole acetic acid (IAA) as medium producers (between 11 to 21µg/mL). Phytobacteria strains were maintained by culturing them on plates with nutritive agar (NA) medium for 48 h at 28ºC.

Fouquieria splendens callus culture

Callus were obtained from leaves of *F. splendens* plants, these were surface-sterilized with sodium hypochlorite solution (10%) for 45 seconds, followed by several rinses in sterile distilled water. Leaf explants were obtained aseptically cutting fractions of $1cm^2$. Five explants were placed separately in baby food flasks with Magenta SIGMA caps containing 25mL of Murashige and Skoog ¼ salts medium [16] supplemented with 30 g/L of sucrose, 1mg/L of naphthalene acetic acid (NAA), 1.5mg/L of kinetin (KIN) and 3 g/L phytagel named “MSE” medium, incubated at 28°C with photoperiod of 16 h light /8 h dark, for 50 days.

Biotization of *Fouquieria splendens* callus with endophyte strains exposed to cadmium

Biotization of *F. splendens* callus with the selected endophytes was done according to two sequential phases as follows: in the first phase, bacterial inoculum was obtained taken a sample of each endophyte strain cultured on plates with AN with calibrated loop (1/100 cells) and re-suspending in 5mL of sterile distilled water to adjust by optical density an inoculum with cell density of $7x10^7$ cells/mL. Callus biomass fractions ($5mm^2$) of *F. splendens* obtained after 50 days of culture were deposited in the 5mL of each phytobacterial suspensions at room temperature for one hour. The second phase of cultures initiated after the incubation of the callus biomass with endophyte strains. Twelve pieces of *F. splendens* callus where deposited in Petri dishes containing MSE medium without and with cadmium ($3CdSO_4 \cdot 8 \cdot H_2O$) 0.1, 0.5 and 1.0mM. Also, twelve fractions of no inoculated callus biomass deposited in plates with MSE medium without

cadmium and with cadmium concentrations were considered as controls. All the Petri dishes were sealed with Parafilm to prevent water loss and incubated at 28°C with photoperiod of 16 h light /8 h dark. Experiments were performed by triplicate and the effect of biotization and response to cadmium in culture was analyzed after 25 days, where all biomass fractions were weighted.

Statistical analysis

All data obtained were analyzed by one-way analysis of variance and the mean differences were compared applying a Tukey-Kramer Method using the statistics program Graph Pad Instat Ver. 2.03.

RESULTS

Growth of *F. splendens* callus biomass

Figure 1 shows the appearance of the progress of callus biomass development without phytobacteria and biotized with the selected endophytes; where in this study, only the callus cultured in MSE medium and MSE biotized with *Staphylococcus pasteuri* strain Fs3 showed no visible callus damage, no discoloration and an obvious not growth reduction both experimental conditions without cadmium (Figures 1a and 1b). There was a diminished growth and lightly browning color in callus biotized with *Kocuria rhizophila* strain Fs7 (Figure 1c) and the highest callus damage with obvious characteristics like tissue color change, cellular leakage, callus disintegration and severe growth reduction were present in callus biotized with *Staphylococcus warneri* strain Fs4 (Figure 1d).

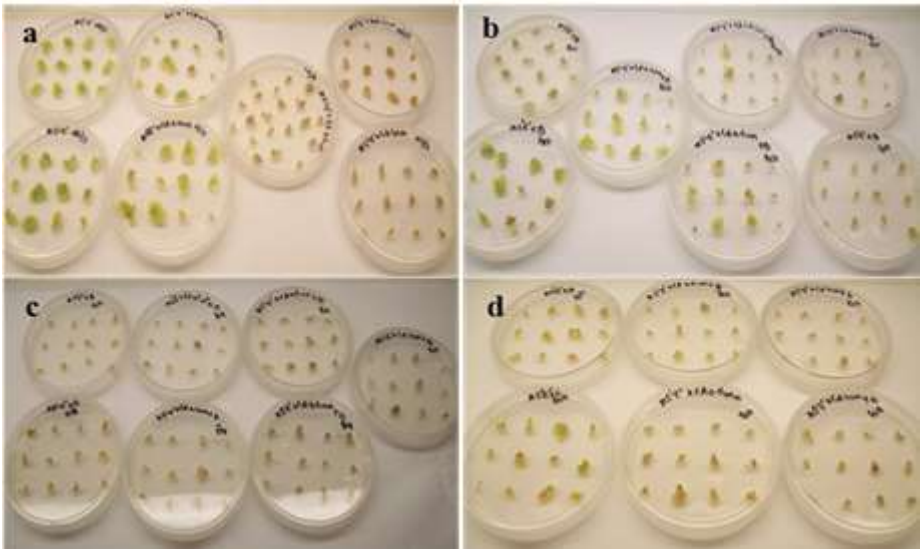

Figure 1. *In vitro Fouquieria splendens* callus development. **a)** MSE medium with and without Cd; **b)** MSE + Fs3 medium with and without Cd; **c)** MSE + Fs4 medium with and without Cd and **d)** MSE + Fs7 medium with and without Cd.

Figure 2 shows the variations of *F. splendens* callus growth for control fractions (Figure 2a), biotized with: strain Fs3 (Figure 2b), strain Fs4 (Figure 2c) and strain Fs7 (Figure 2d). There was a statistical significance between MSE, MSE + Cd 0.1mM and MSE+ Cd 0.5mM conditions ($p < 0.001$); between MSE, MSE + F3, MSE + F3 + Cd 0.5mM and MSE + Fs3 + 1.0mM ($p < 0.001$); between MSE, MSE + Fs4 + Cd 0.1mM and MSE + Fs4 + Cd 0.5mM ($p<0.001$) and finally, between MSE, MSE + Fs7 + Cd0.5mM and MSE + Fs7 + Cd 1.0 mM ($p<0.001$).

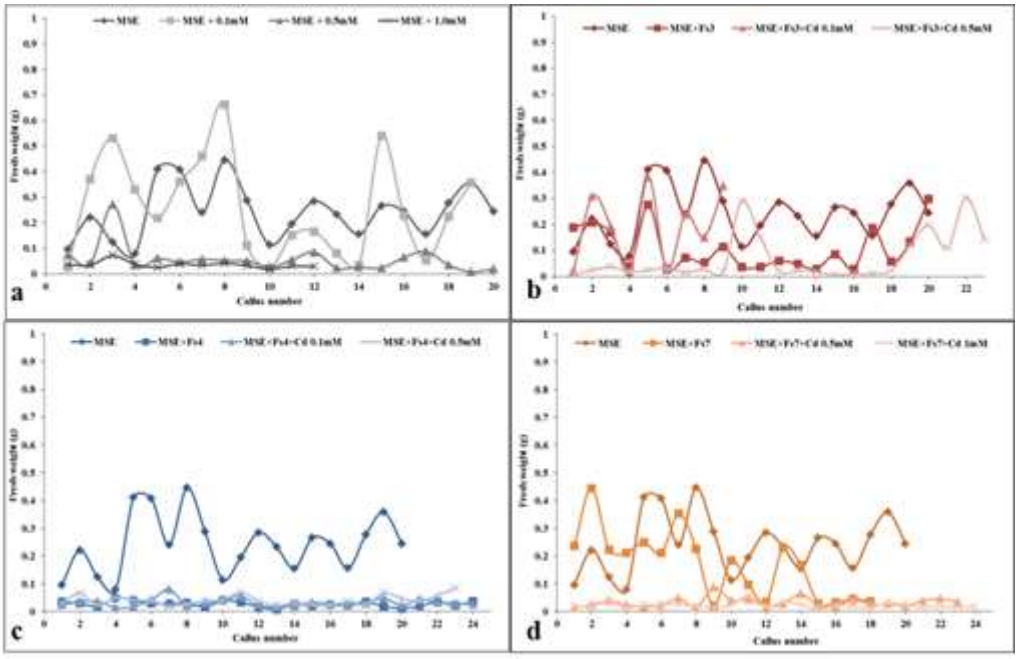

Figure 2. *In vitro Fouquieria splendens* callus variations of development: **a)** MSE medium with and without Cd; **b)** MSE + Fs3 medium with and without Cd; **c)** MSE + Fs4 medium with and without Cd and **d)** MSE + Fs7 medium with and without Cd.

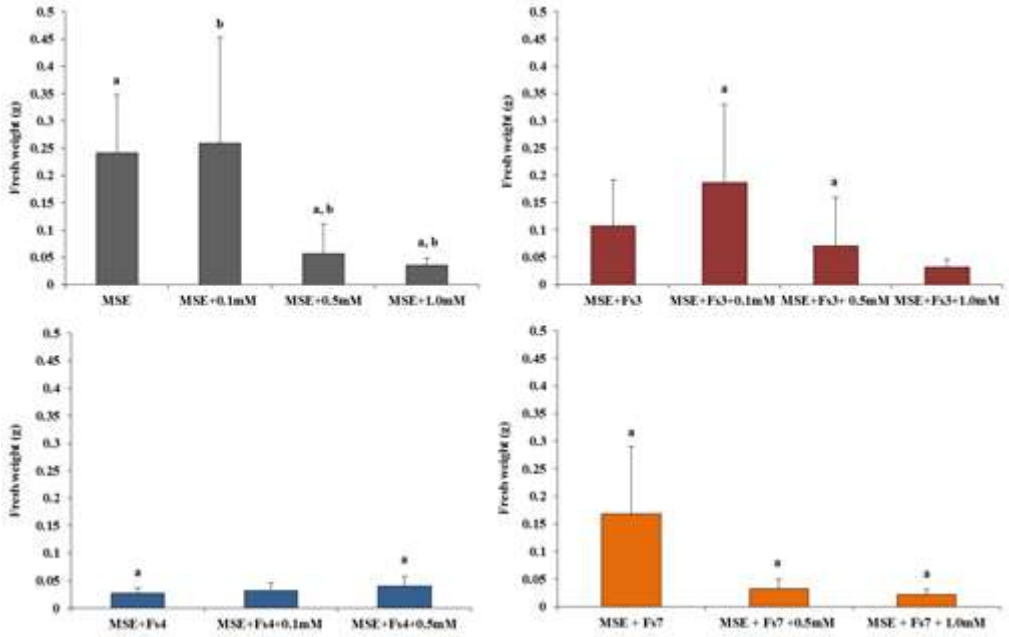

Figure 3. *Fouquieria splendens* average callus growth. **a)** MSE medium with and without Cd ($p < 0.001$); **b)** MSE + Fs3 medium with and without Cd ($p < 0.05$); **c)** MSE + Fs4 medium with and without Cd ($p < 0.01$) and **d)** MSE + Fs7 medium with and without Cd ($p < 0.001$). (n=36, the different lower-case letters shows the significant differences founded).

At the final time analyzed (50 days), the average callus growth response was significant ($p< 0.001$) in callus cultured in MSE medium compared to all cadmium conditions; there was a decrease in growth as cadmium concentration increased (Figure 3a); followed by the biotized condition with strain Fs3 (Figure 3b), with a slightly and significant

response of increase in growth of callus biomass in cadmium conditions ($p< 0.05$). The strain Fs7 (Figure 3d) only increased the callus biomass but this symbiotic association decrease when they were exposed to cadmium ($p<0.001$). There was an obvious deleterious response showed in callus biotized with the strain Fs4 (Figure 3c), even with and without the presence of cadmium ($p<0.01$).

DISCUSSION

Parray *et al.* [17] recommended the *in vitro* cells/tissues co-cultivation with bacterial strains for plant mass propagation; also this authors and Rennie and Larson [18] noted that the effects of plant growth promoting bacteria are considered to be highly specific with respect to plant and bacterial genotypic combination. Their hypothesis is further supported by Om *et al.* [19] who reported that inoculation of tissues with selected bacteria during *in vitro* micropropagation allowed a better adaptation of the host plants to environmental conditions and a higher survival rate of them. In this study, there was an evident effect of particular biotization of *F. splendens* cell biomass, where the endophyte strains tested showed a response as benefit and/or deleterious bacteria. *S. pasteuri* strain Fs3 and *K. rhizophila* strain Fs7 established a positive relationship with plant cells and *S. warneri* strain Fs4 a deleterious effect on the growth of callus biomass; this behavior was according to the fresh weights represented in the variation of each *F. splendens* callus biomass grown in each experimental conditions bacterized and/or exposed to cadmium concentrations. Melo [20] noted that the microbial symbiosis with plants can synthesize hormones similar to those produced by the plant as growth regulator such as auxins, gibberellins and cytokinins. In this study, even all the endophytes employed have been characterized as medium IAA producers; the obtained fresh biomass does not shown a notably effect on bacterized callus as a promotion response by them, but against the heavy metal there was a promotion response of cells growth with *S. pasteuri* strain Fs3. Frommel *et al.* [9] and Mahmood *et al.* [21] reported that under *in vitro* conditions the plant growth promoting bacteria can produce biochemical and histological modifications, growth promotion and reduction of hyperhydricity of *in vitro* cultured plants; also Pillay and Nowak [11] noted that *in vitro* growth responses depended on the degree of endophytic colonization and a certain threshold of the bacteria concentration is require to trigger the responses. According to this commentary, this study showed a particularly symbiotic relationship between *S. pasteuri* strain Fs3 and the *F. splendens* cells, favoring a cells protection against cadmium as Table 1 shows, regarding to the gain of callus biomass at Cd 0.01mM (0.23g), Cd 0.5mM (0.14g) and Cd 1.0mM (0.07g). *K. rhizophila* strain Fs7 also showed a positive response against the heavy metal with minor gain of callus biomass. These results were agree with the notes of Preininger *et al.* [22] and Lim *et al.* [23] whose comment that the interdependency of bacteria and in vitro plant tissues can be achieved in a symbiotic relationship that subsequently could promote a propagation of plant tissue.

Table 1. Gain of *F. splendens* callus biomass exposed to the experimental conditions*

	Callus biomass (g)			
	MSE	**Cd 0.1mM**	**Cd 0.5mM**	**Cd 1.0mM**
MSE	0.20+0.1(a)	0.26+0.17(a)	0.04+0.06(a)	0.01+0.017
MSE + Fs3	0.09+0.08(a,b)	0.23+0.91(a,b)	0.14+0.08(a)	0.007+0.004
MSE + Fs4	0.005+0.005(b)	0.013+0.01(b)	0.016+0.01(a)	----
MSE + Fs7	0.18+0.09	----	0.011+0.01	0.01+0.0

*Different lower-case letters shows the significant differences founded

Finally, the present study reports and suggest a positive biotization response of callus biomass of *Fouquieria splendens*; where even the endophytes do not increase at all the gain of biomass they were able to protect the cells against cadmium under *in vitro* conditions. Thus, these strains could be recommended as adequate endophytes for the establishment of *in vitro* plant-bacterium interactions.

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