EXCELLENT PUBLISHERS

International Journal of Current Research in
Biosciences and Plant Biology

Volume 7 • Number 6 (June-2020) • ISSN: 2349-8080 (Online)

Journal homepage: www.ijcrbp.com

Original Research Article **doi:** https://doi.org/10.20546/ijcrbp.2020.706.001

Biotization of *Fouquieria splendens* callus as protective strategy against cadmium damage

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Article Info

Date of Acceptance:
21 May 2020

Date of Publication:
06 June 2020

Keywords

Artificial symbiosis
Biotization
Cadmium
Callus culture
Endophytes

ABSTRACT

"Biotization" defined as a metabolic response of plant cells to microbial inoculants. There are some reports that this process has employed plant growth promoting bacteria to establishing an artificial symbiosis between them and tissue culture of plants. The aim of this work was analyze the protecting effect against cadmium damage by biotization of *Fouquieria splendens* callus with two endophyte bacterium. The effect of plant growth promoting bacteria were considered to be highly specific with respect to plant and bacterial genotypic combination; thus, *Kocuria rhizophila* strain Fs7 biotization does not showed a good response compared to *Staphylococcus pasteuri* strain Fs3, because there was an inconsistent growth of *F. splendens* callus. The results showed that there were also several factors influencing the growth and differentiation of callus, like the quality and especially the age of the callus employed. This study reports the positive biotization response of *F. splendens* callus with *S. pasteuri* strain Fs3 and it was able to protect callus against cadmium under *in vitro* conditions. This available strategy of biotization with the endophyte could be recommended as an adequate biostimulant and protective bacteria.

Introduction

Unorganized cell masses ("callus")grown under culture conditions by the induction of cell proliferation with great plasticity are considered as a good *in vitro* experimental models (Ikeuchi et al., 2013, 2015a, 2015b, 2016, 2017; Efferth, 2019; Féher, 2014, 2019; Niazan et al., 2019; Popielarska-Konieczna et al., 2020). It has been also reported that *in vitro* co-culture of tissue explants with beneficial microbes may enhance protection and tolerance against heavy metals contamination. Biotization has been defined by Nowak (1998) and Herman (1996a,b) as "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 tolerance and resistance". Plant growth promoting bacteria (PGPB) are important microorganisms involved in plant growth promotion by direct and indirect effects. Direct effects occur when they produces substances such as

phytohormones (Dubeikovsky et al., 1993; Janzen et al., 1992; Srinivasan et al., 1996; Siddiquiz, 2006; Ertuk et al., 2010). There are some reports regarding to *in vitro* plants biotization that have employed microbial inoculants establishing an artificial symbiosis between tissue culture of plants and PGPB (Liu et al., 1995; Nowak et al., 1995; Balla et al., 1997; Lazarovits and Nowak,1997; Pillay and Nowak, 1997; Preininger et al., 1997). This study analyzed the protecting effect against cadmium damage by biotization of *Fouquieria splendens* callus with two endophyte bacterium.

Materials and methods

Callus culture inoculants

The endophyte bacteria employed: *Staphylococcus pasteuri* strain Fs3 (access numberMG920268) and *Kocuria rhizophila* strain Fs7 (access number MG920272); were isolated by Salinas-Patiño(2018), from leaves of the desert plant *Fouquieria splendens* grown in the botanical garden of the Facultad de Estudios Superiores, Iztacala (FES)-UNAM. 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 1 cm^2. Five explants were placed separately in baby food flasks with Magenta SIGMA caps containing 25mL of Murashige and Skoog (1962) ¼ salts medium 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 7×10^7 cells/mL. Callus biomass fractions (5 mm^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 as protecting agent against cadmium in culture was analyzed after 25 days.

Fouquieria splendens callus damage analysis

After incubation, each callus was visualized for their damage analysis occasioned by the experimental conditions and the presence of cadmium according to Souissi and Kremer (1994, 1998) criteria based on rating description: 0: no discoloration, no visible damage and no growth reduction; 1: slight discoloration and no visible growth reduction; 2: tissue color change and slight growth reduction; 3: tissue color change, obvious growth reduction and frequently manifested by tissue shrinkage and 4: tissue color change, cellular leakage, callus disintegration and severe growth reduction. Also, as Visarada et al. (2002) mention callus variation based on their morphology could analyzed to show the changes related with their development and plants regeneration. Selected fractions were prepared for scanning electron microscopy (SEM) for the analysis of morphological changes induced in *F. splendens* callus according to Corona-Álvarez et al. (2018) as follows: samples were cut into 4 or 5mm pieces, fixed in 2.5% gluataraldehyde for 1h at room temperature (27°C), then, washed three times in a phosphate buffer, post fixed in 1% OsO_4 for 1h at room temperature, washed and dipped into distilled water for three times. The samples were

undergone a series of dehydration processes: 30% ethanol for 10 min; 40% ethanol for 10 min; 50 % ethanol for 10 min; 60% ethanol for 10 min; 70% ethanol for 10 min; 80 % ethanol for 10 min; 90% ethanol for 10 min and finally 100% ethanol for 10 min (three times). The material was dried in the critical point dryer apparatus, mounted and sputter-coated with gold in an ion coater for 60 seconds. Finally, the samples were ready for viewing under field scanning electron microscope JSM 5800 LV for their examination and photography.

Statistical analysis

A numerical comparative analysis of each experimental conditions biotized or no biotized and exposed to cadmium was done; a distance matrix built using the conventional standard distance coefficient and a phenogram was resolved using the unweighted pair group method of arithmetic averages (UPGMA) method, and finally a correlation coefficient of Pearson was obtained using the version 2.11T Numerical Taxonomy and Multivariate Analysis System (NTSyS-PC) software.

Results and discussion

Damage variation of *Fouquieria splendens* callus

Fig. 1 shows the variations of *F. splendens* callus damage for no biotized conditions and exposed to cadmium (Fig. 1a), biotized with *S. pasteuri* strain Fs3 (Fig. 2b) and *K. rhizophila* strain Fs7 (Fig.1c); according to the rating system proposed by Souissi and Kremer (1994, 1998) there was a diverse behavior of damage effect; not only by the presence of endophytes, it also was demonstrated an additive effect with cadmium exposition. For the evaluation of the phytobacteria presence producing a protective effect against cadmium based on visual observations; the progress of callus development under no biotized and biotized with phytobacteria is showing in Figs. 2, 4 and 6. Callus grown in MSE medium showed cell damage categorized between rating 0 to 2 with no visible callus damage, no discoloration and an obvious not growth reduction. Callus grown in MSE+Cd 0.1mM, MSE+Fs3 and MSE+Fs7 showed cell damage rating between 0 to 4 and as cadmium concentration increased, cell damage was determined between 3 and 4 rating, where the number of shrinking callus increased at highest concentrations of cadmium. These results showed that there were also several factors influencing the growth and differentiation of callus, like the quality and especially the age of the callus employed; because some young callus lines are considered more resistant and possess a great regeneration potential.

Morphological changes induced in *Fouquieria splendens* callus

A particular response of callus without biotized process was obtained; only in callus grown in MSE medium there was the presence of a clear extracellular matrix covering all the mass of cells (Figs. 3a to 3f). As Pilarska et al. (2014) mention, this kind of cell cultures (callus)induces different cellular responses according to each experimental conditions, including the formation of extracellular strands, fibrils and/or a continuous layer over callus that implies the induction of morphogenesis; this structure has been named extracellular matrix (ECM) or extracellular matrix surface network (ECMSN). Popielarska et al. (2006) and Popielarska-Konieczna et al. (2008 a,b) describe it as a discontinuous amorphous layer of complex non-cellular material on the callus surface as a layer covering the outer cell wall. Also has been reported that this structure possess regulatory and coordinating functions during the early stages of morphogenesis (Verdeil et al., 2001; Bobák et al., 2003; Konieczny et al., 2005; Popielarska-Konieczna et al., 2008a; Blehová et al., 2010). Pilarska et al. (2014) also noted that this extracellular matrix formed against stress response provide protection against external conditions or factors. In this study, SEM of *F. splendens* callus showed heterogeneous material covering the callus surface; because some callus sections were coated by a smooth membranous layer and others by fibrillary and granular structures similar to the *in vitro* organogenesis reported in different species (Verdeil et al., 2001; Popielarska-Konieczna et al., 2008a; Pilarska et al., 2013). Selected *F. splendens* callus shows morphological changes induced in biotized callus; there was presence of the *S. pasteuri* strain Fs3 associated to the callus mass with a slightly appearance of early shoots in culture without cadmium (Figs. 5a to 5l).

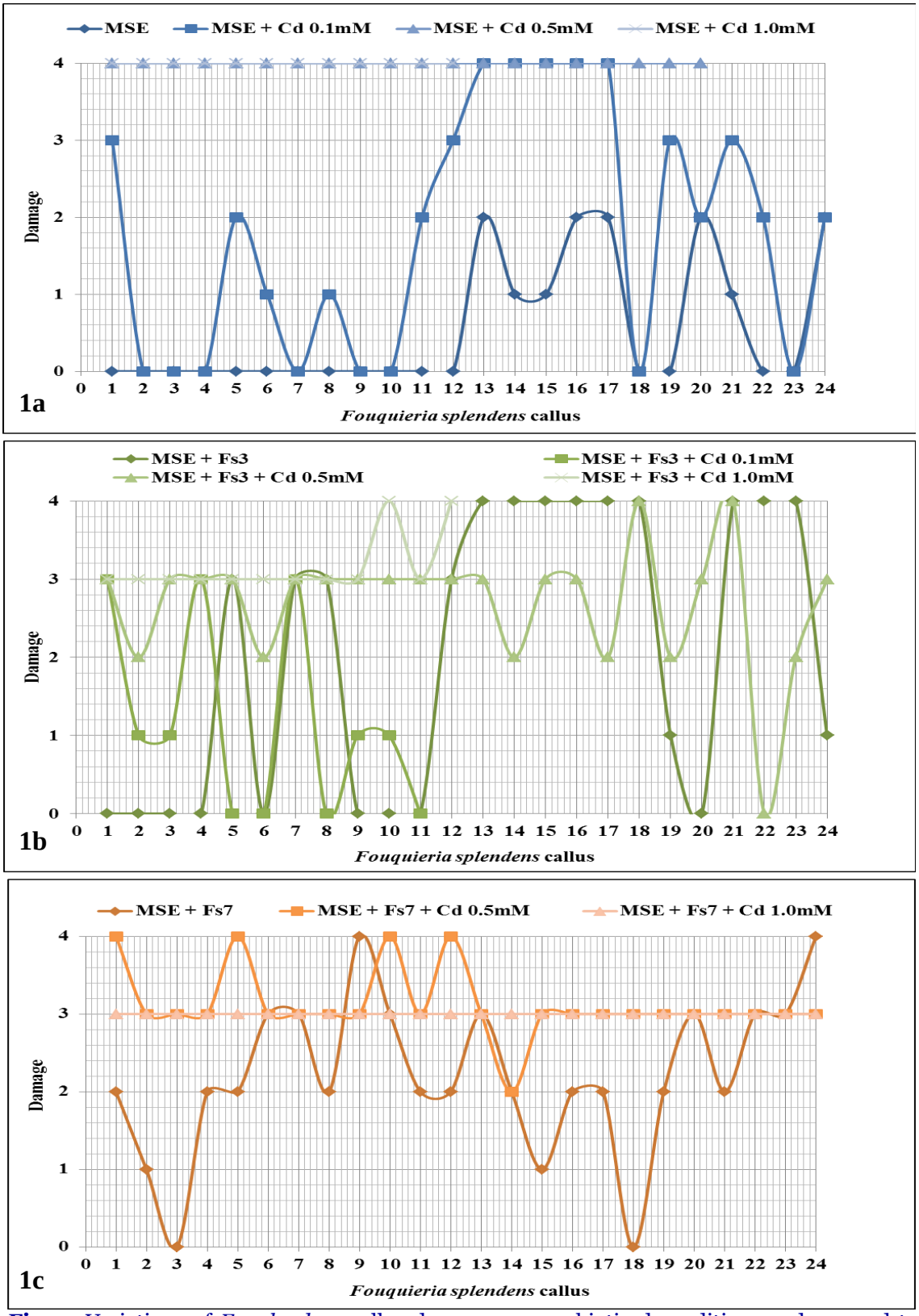

Fig. 1: Variations of *F. splendens* callus damage: **1a:** no biotized conditions and exposed to cadmium, **1b:** biotized with *Staphylococcus pasteuri* strain Fs3 and **1c:** biotized with *Kocuria rhizophila* strain Fs7.

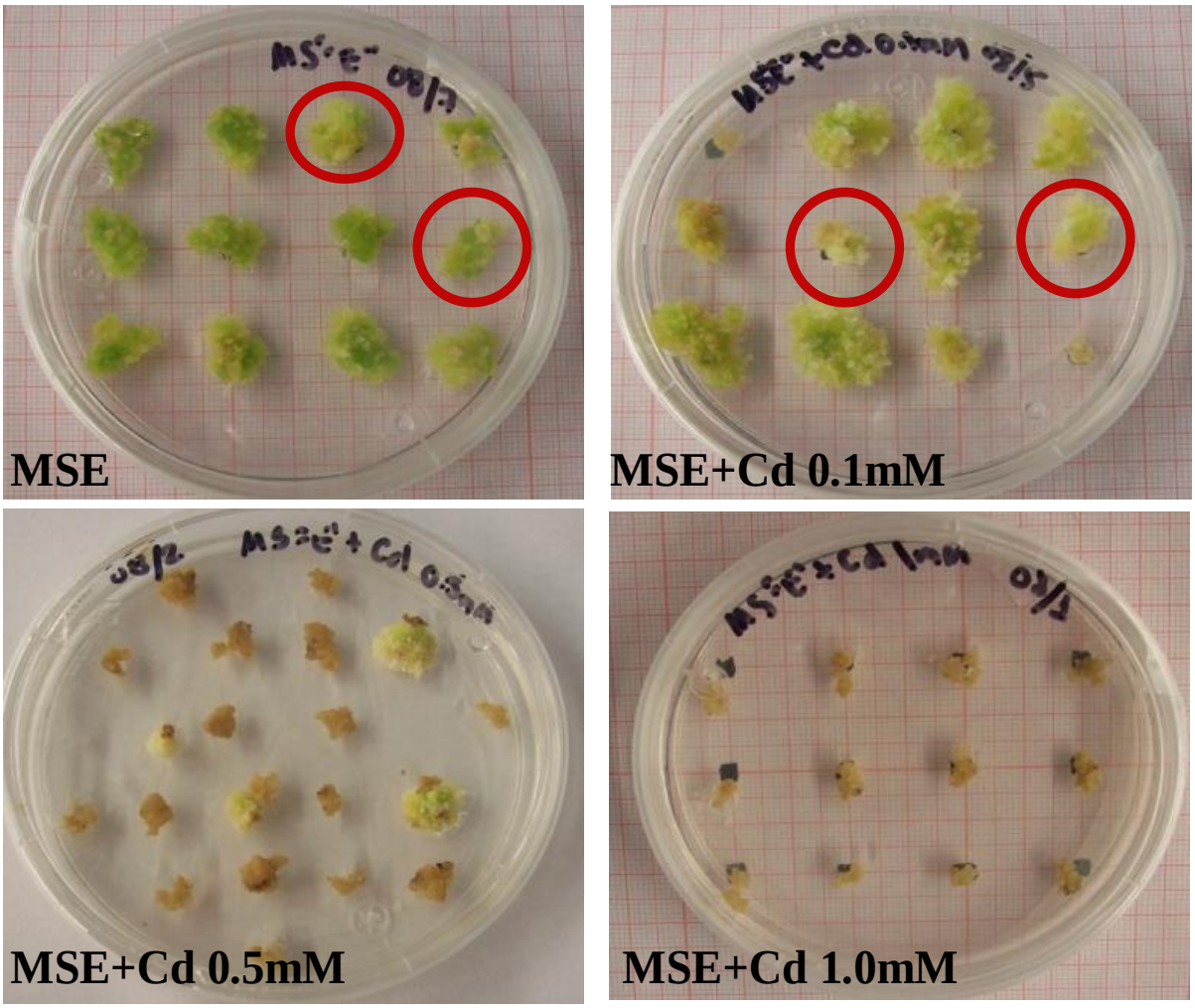

Fig. 2: *In vitro Fouquieria splendens* callus development grown in MSE medium without bacterization and exposed to Cd (selected callus in red circle were analyzed by SEM).

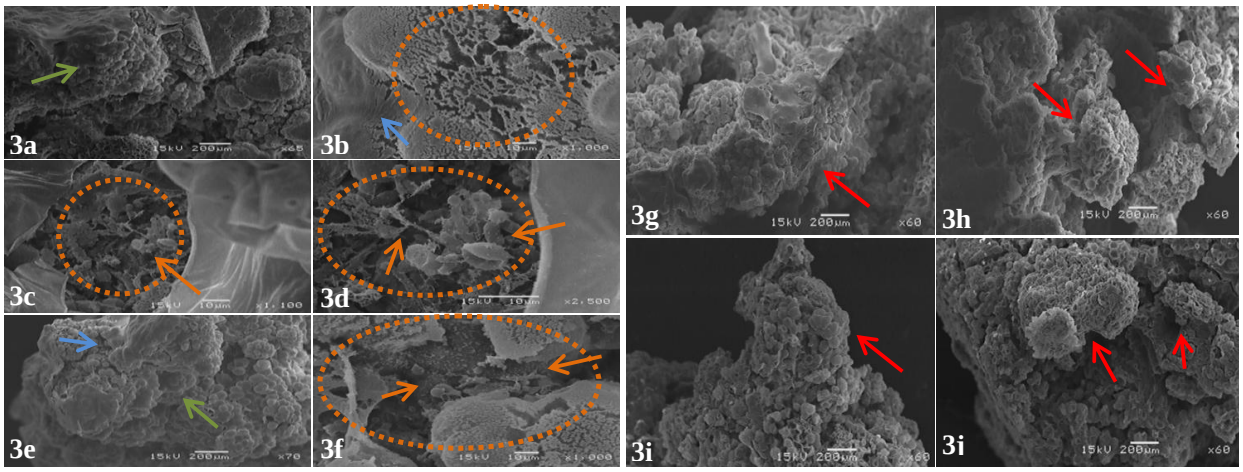

Fig. 3: Scanning electronic micrographs of selected *Fouquieria splendens* callus grown in MSE medium, where: **sequence 3a to 3f** shows the appearance of early shoots (green arrows) and the presence of a smooth membranous layer coated some parts of the callus surface (blue arrows); other zones (dotted orange circles and orange rows)covered by fibrillar structures and granules. **Sequence 3g to 3i** of callus grown in MSE+Cd 0.1 mM shows callus damage (red arrows).

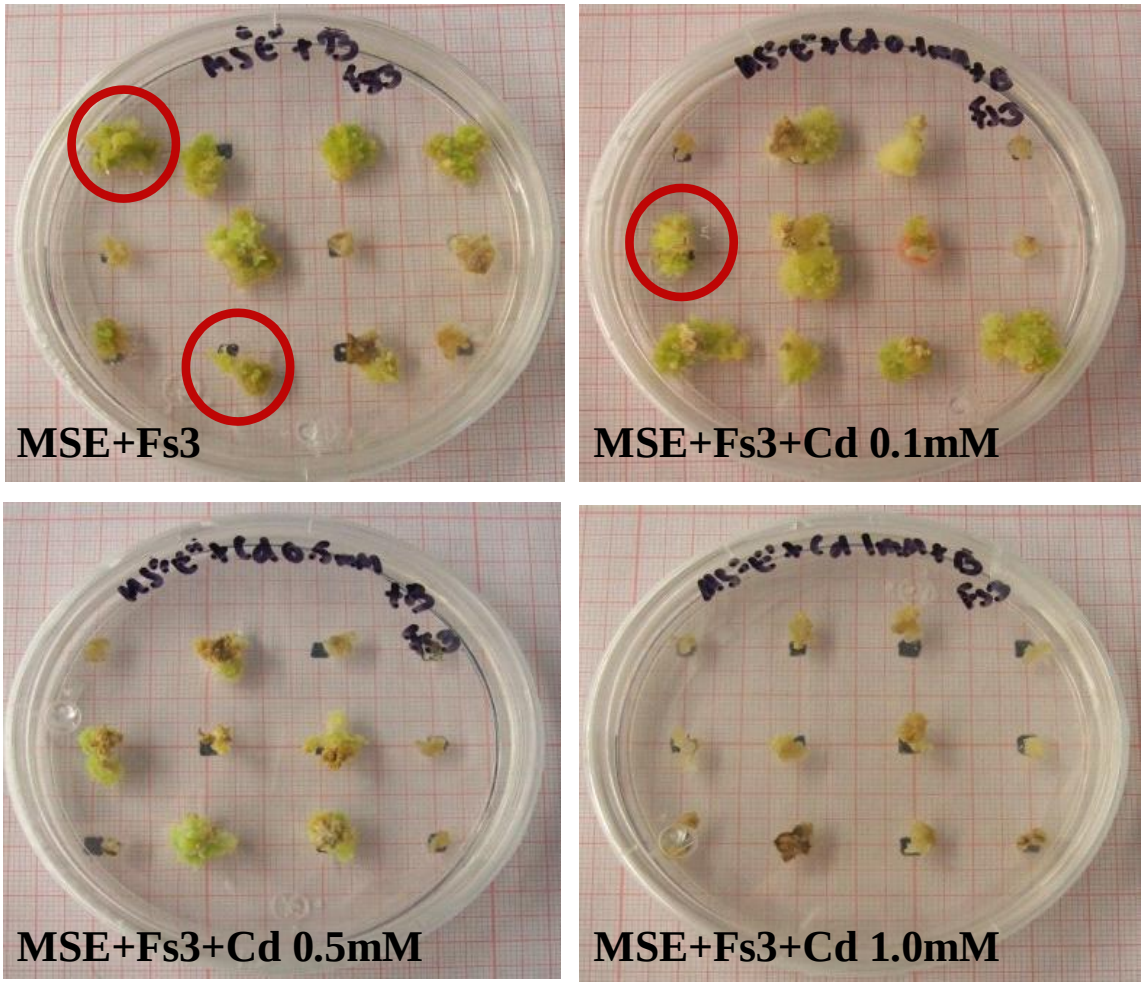

Fig. 4: *In vitro Fouquieria splendens* callus development grown in MSE medium biotized with *Staphylococcus pasteuri* strain Fs3 and exposed to Cd (selected callus in red circle were analyzed by SEM).

Bacterial cells were observed on the surface as well as in the intercellular spaces of the treated callus, agree with the reported by Lim et al. (2016) where the surface of *Elaeis guineensis* samples were colonized by *Herbaspirillum seropedicae* strain Z78. A slightly cellulose microfibril movement "rosette" was also observed in the micrograph founded in the surface of *F. splendens* callus as Mariani and Erlangga (2014) noted, this structure is related to protoderm formation. These results also were agree with Bennett and Lynch (1981) and Schmidt et al. (2011), whose mention that presence of bacteria in the intercellular spaces of the plant cells could be due to the efficiency of colonization; plant cells may supply the bacteria with carbon and energy sources by transforming the sugars available in the media during incubation period in order to favored a symbiotic relationship.

Colonization of bacteria and the attachment toward plant tissues will give also benefits to the callus (Azlin et al., 2007, 2009). It could be that the attachment of *S. pasteuri* strain Fs3 and *K. rhizophila* strain Fs7 (Figs. 6a to 6l) to *F. splendens* callus could associated to certain "quorum sensing" signal in the internal part of the plant tissues and exchange the signal molecules between them enhancing cell development as Lim et al. (2016) noted. According to Parray et al. (2015), the effect of plant growth promoting bacteria are considered to be highly specific with respect to plant and bacterial genotypic combination. *K. rhizophila* strain Fs7 biotization does not showed a good response compared to *S. pasteuri* strain Fs3; because there was an inconsistent growth of *F. splendens* callus.

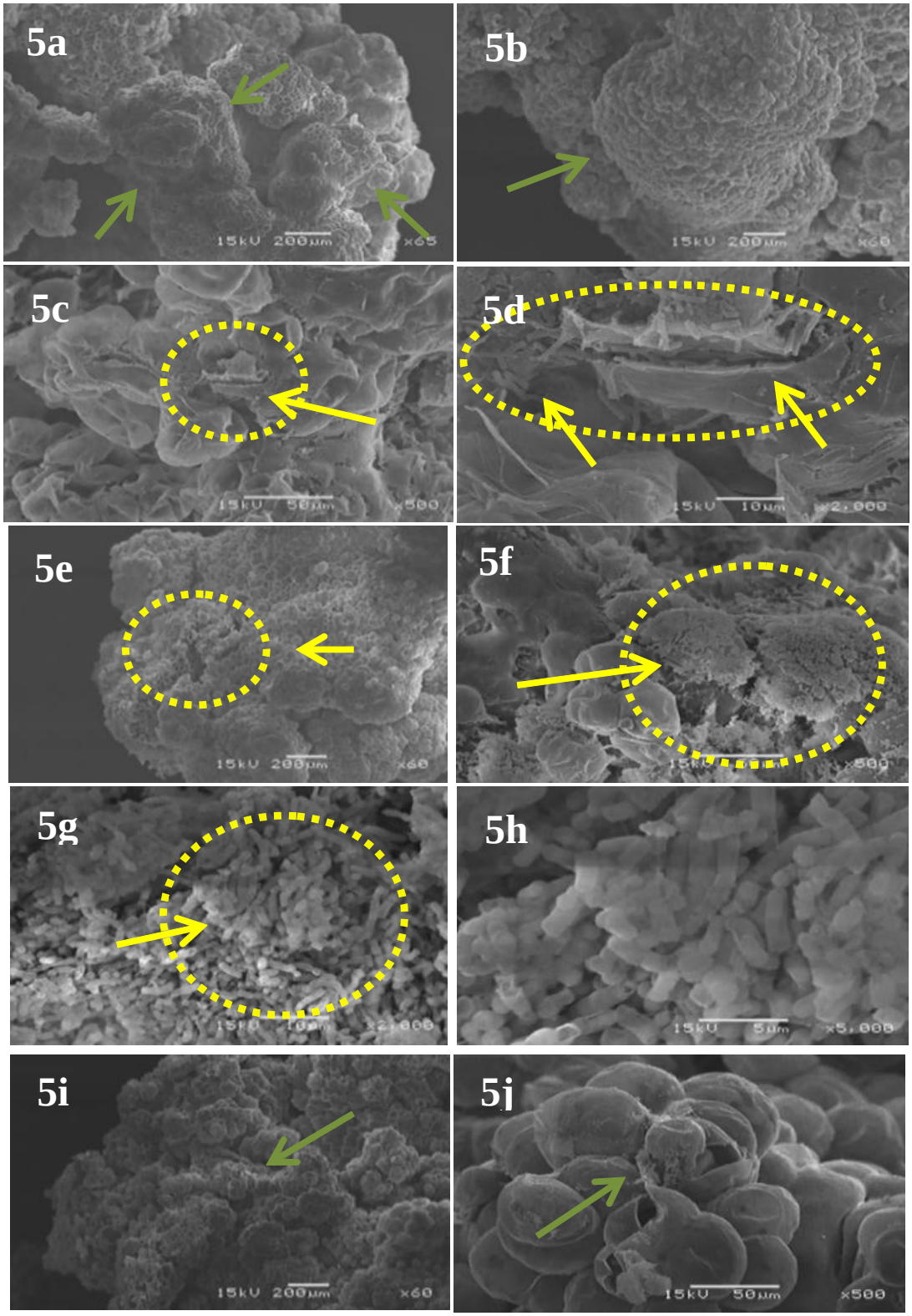

Fig. 5: Scanning electronic micrographs of selected *Fouquieria splendens* callus biotized with *Staphylococcus pasteuri* strain Fs3, where: **sequence 5a to 5h** shows early shoots formed (green arrows). Dotted yellow circles and yellow rows show the sequence of *S. pasteuri* strain Fs3 located near the surface of callus. **Sequence 5i to 5l** of callus grown in MSE+Fs3+Cd 0.1 mM shows detailed early shoots (green arrows).

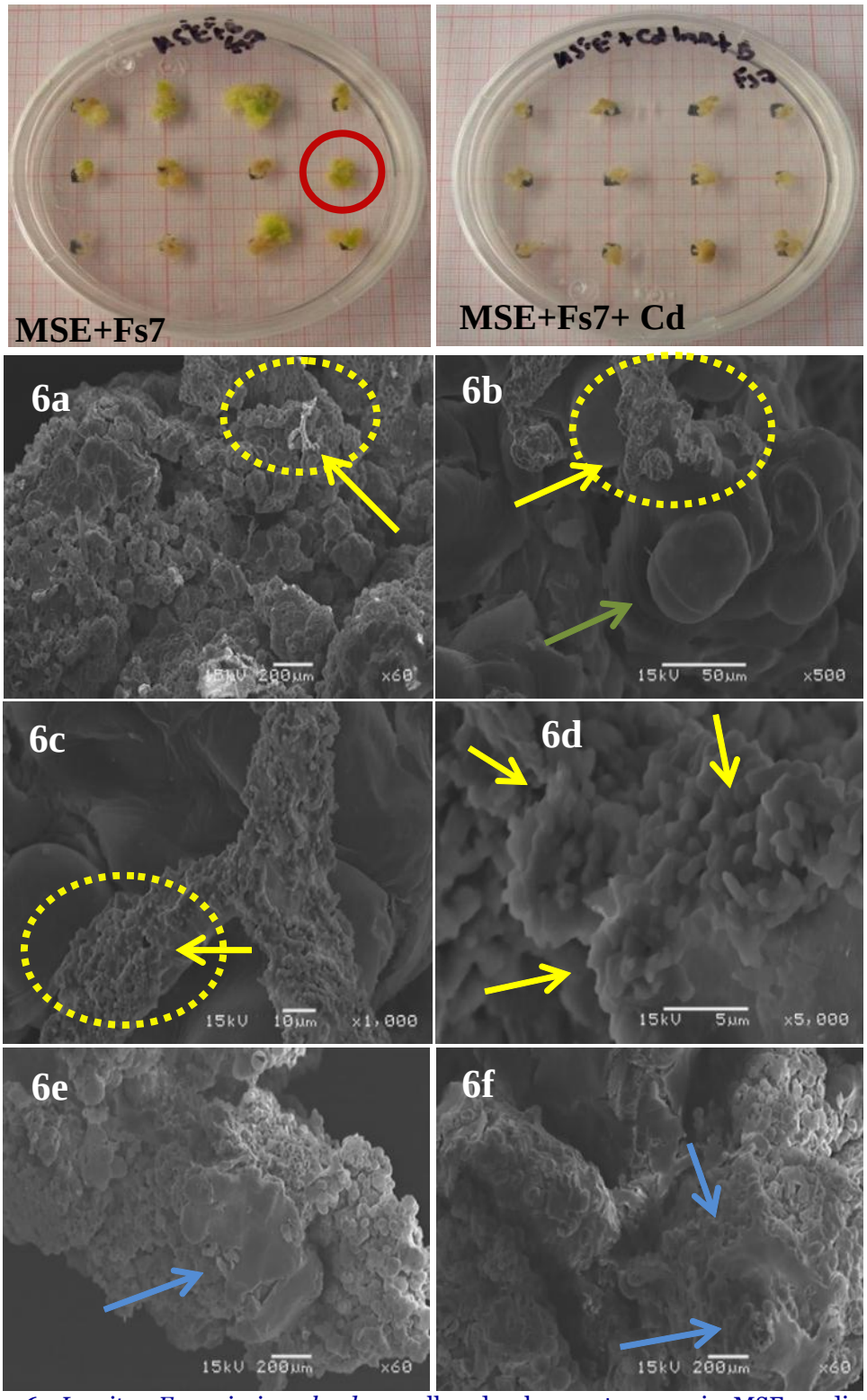

Fig. 6: *In vitro Fouquieria splendens* callus development grown in MSE medium biotized with *Kocuria rhizophila* strain Fs7 and exposed to Cd. Selected callus in red circle was analyzed by SEM as follows: **sequence 6a to 6f** shows early shoots (green arrows); and the presence of a smooth membranous layer coated some parts of the callus surface (blue arrows), finally dotted yellow circles and yellow arrows show the sequences of endophyte bacteria surrounding the callus surface with particular details.

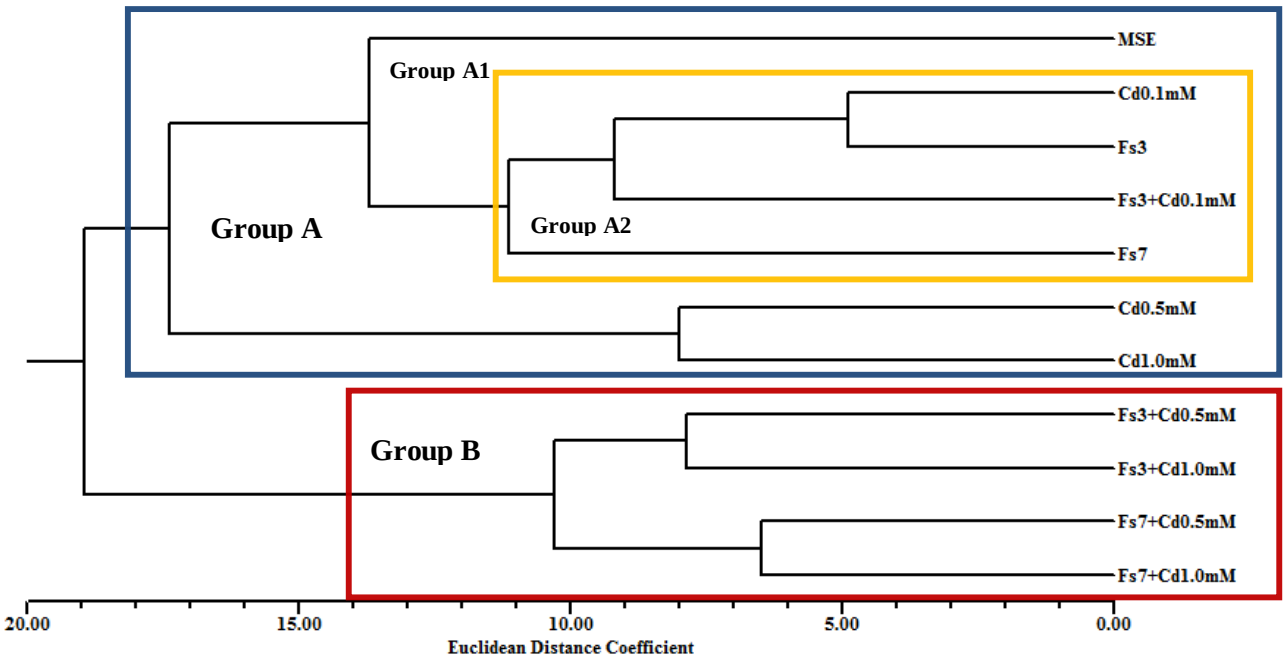

Fig. 7: Phenogram comparing each experimental conditions biotized or no biotized and exposed to cadmium (r= 0.65).

Comparison of callus damage and biotization

The phenetic analysis (Fig. 7) considering the callus number for each experimental conditions related to damage rate according to Souissi and Kremer (1994, 1998) showed at first two groups "A" and "B" clearly separated by the slightly and highest damage, respectively. Group "A" divided in two groups: "A1" conformed by MSE alone as a particular group related to Cd 0.1mM, Fs3, Fs3+Cd 0.1 mM and Fs7, showing a good number of callus tested rating between 0 to 2 damage classifications (MSE: 24 callus, Cd 0.1 mM: 19, Fs3: 11, Fs3+Cd 0.1 mM: 8 and Fs7: 15) and separately the response of *F. splendens* callus exposed to Cd 0.5 mM and Cd 1.0 mM named group "A2" with the highest callus damage ("4"). Group "B" associated the callus biotized and exposed to highest cadmium concentrations response with cell damage between 3 and 4. In this study, there was an evident effect of particular biotization of *F. splendens* callus, where the endophyte *S. pasteuri* strain Fs3 showed a significant response establishing a positive relationship with callus protecting them to the cadmium damage between 0.5 and 1.0mM concentrations. This response was agree to Pillay and Nowak (1997) whose noted that *in vitro* growth responses are related to the degree of endophytic colonization and bacteria concentration to induce the callus responses and according to Lim et al. (2016) whose established that the interdependency of bacteria and *in vitro* plant tissues consequently promotes a propagation of plant tissue; it was viewed by the appearance of the early shoot development in *F. splendens* callus.

Conclusion

This study reports the positive biotization response of *Fouquieria splendens* callus with *Staphylococcus pasteuri* strain Fs3 showing no visible callus damage, no discoloration and an obvious not growth reduction, thus it was able to protect callus against cadmium under *in vitro* conditions. This available strategy of biotization with the endophyte could recommend it as an adequate biostimulant and protective bacteria.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

Authors are grateful to the Research Projects SIP-IPN: 20181504 and SIP-20202094, of the

Secretaría de Investigación y Posgrado del Instituto Politécnico Nacional, for providing the facilities to carry out this work and also wish to thank for the fellowships from Comisión de Operación y Fomento de Actividades Académicas (COFAA, I.P.N.), EDI (Estímulo al Desempeño de los Investigadores, I.P.N.) and SNI-CONACYT.

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How to cite this article:
Espinoza-Mellado, M. R., García-Pineda, M., Rodríguez-Dorantes, A., 2020. Biotization of *Fouquieria splendens* callus as protective strategy against cadmium damage. Int. J. Curr. Res. Biosci. Plant Biol. 7(6), 1-12. **doi:** https://doi.org/10.20546/ijcrbp.2020.706.001