Open Access Research Journal of
Biology and Pharmacy
Journals home page: https://oarjbp.com/
ISSN: 2782-9979 (Online)

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(RESEARCH ARTICLE)

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EFFECT OF ENDOPHYTIC BIOTIZATION ON GROWTH AND MORPHOMETRIC DESCRIPTORS OF FOUQUIERIA SPLENDENS CALLI UNDER CADMIUM STRESS

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Open Access Research Journal of Biology and Pharmacy, 2026, 18(01), 036–043

Publication history: Received on 01 September 2026; revised on 24 October 2026; accepted on 28 October 2026

Article DOI: https://doi.org/10.53022/oarjbp.2026.18.1.0053

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ABSTRACT

In vitro plant tissue culture serves as a pivotal platform to evaluate heavy metal phytotoxicity and the mitigation mechanisms mediated by 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 establish an artificial symbiosis between them and tissue culture of plants. In this study, the impact of cadmium (Cd: 0.1, 0.5, and 1.0 mM) and biotization with *Staphylococcus pasteuri* strain Fs3 and *Kocuria rhizophila* strain Fs7 on the growth and cellular morphometry of *Fouquieria splendens* calli was non-destructively evaluated. Utilizing computer-assisted digital morphometric analysis and image segmentation, baseline morphometric descriptors and relative growth rate parameters were quantified at 52 and 76 days of culture. Principal Component Analysis (PCA) revealed a strain and dose-dependent geometric reprogramming under heavy metal stress. Under moderate stress, the Fs3 + Cd 0.1 mM treatment exhibited a prominent morphometric hormesis response, achieving the maximum area growth percentage, driving also the highest area and perimeter relative growth rates. Conversely, severe exposure to Cd 1.0 mM induced net cellular collapse and negative growth rates related to this extreme toxicity, biotization with *K. rhizophila* strain Fs7 acted as a structural biological rescue system, reversing tissue damage by maintaining a positive growth percentage and promoting a strong three-dimensional spherical compaction, as evidenced by the highest distance dispersion index value. These findings demonstrate that endophytes plastically modulate callus geometry as an adaptive strategy for cell tolerance mechanisms under severe chemical stress.

Keywords: Callus culture, Endophytes, Biotization, Heavy Metals, Image Processing, Plant hormesis

1 INTRODUCTION

Callus is a mass of unorganized thin-walled parenchyma cells [1-3] grown under culture conditions by the induction of cell proliferation with great plasticity are considered as a good *in vitro* experimental model in cell or tissue culture studies [4-8]. 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 and has emerged as a promising biological strategy to re-establish functional plant-microbe associations [9,10] for investigating morphogenetic responses contributing to plant regeneration [11]. Known as biotization [12,13], beneficial microorganism included in micropropagation, providing a mechanistic basis for the morphogenesis analysis and resilience observed in biotized cultures. There are some reports regarding *in vitro* plant biotization that have employed microbial inoculants establishing an artificial symbiosis between tissue culture of plants and them [14-18]. Image processing is a powerful method in callus studies because it can distinguish intermediate levels of callus growth and provide detailed information of them than conventional counting [19-21]. Because biotization induces some structural and developmental changes; quantitative morphological dispersion metrics (variation in cell size, shape, spatial distribution) are appropriate for detecting and characterizing these responses. Thus, plant morphogenesis studies include the segmentation of callus tissues to measure their geometry and heterogeneity for understanding callus differentiation. Morphometric analysis via image processing offers a powerful, non-invasive alternative to traditional destructive harvesting methods (like fresh/dry weight measurements) for monitoring plant callus growth and by using the two-dimension surface area and perimeter measurements, it can precisely quantify growth and calculate the relative growth rate over time. The aim of the present work was to evaluate the effect of cadmium stress and biotization with endophytic bacteria *Staphylococcus pasteuri* strain Fs3 and *Kocuria rhizophila* strain Fs7 on the growth and cellular architecture of *Fouquieria splendens* calli, using a non-destructive digital morphometric analysis.

2 MATERIAL AND METHODS

2.1 Callus culture inoculants

The endophyte bacteria employed: *Staphylococcus pasteuri* strain Fs3 (access number MG920268) and *Kocuria rhizophila* strain Fs7 (access number MG920272); were isolated by Salinas-Patiño [22], from leaves of the desert plant *Fouquieria splendens*. Phytobacteria strains were maintained by culturing them on plates with nutritive agar (NA) medium for 48 h at 28ºC.

2.2 Culture and biotization of *Fouquieria splendens* callus with endophyte strains exposed to cadmium

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 [23] 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 *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 Nutritive Agar 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 suspension at room temperature for one hour. The second phase of cultures initiated after the incubation of the callus biomass with endophyte strains, where twelve pieces of *F. splendens* callus were 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 76 days.

2.3 Morphometric analysis and growth kinetics of Fouquieria splendens callus

To non-destructively evaluate the impact of cadmium (Cd) stress and biotization with endophytic bacteria on the cellular growth of *F. splendens*, digital top-down photographs of the calli were captured employing the Zeiss-Stemi SV11 microscope at 6X with an Axiocam HRC camera, for each experimental condition at 52 days (t_1) and 76 days (t_2) of culture, establishing an evaluation period (Δ_t) of 24 days. Image processing and digital segmentation were performed using morphometric analysis software ImageJ to extract two primary pixel-based metrics: Projected Area (A) and Perimeter (P); from these primary data, the following morphological and growth descriptors were calculated: Maximum and Minimum Area Variation ($AV=A_{max}-A_{min}$): the absolute difference between the final area (t_2) and initial area (t_1); Area Growth Percentage (AGP): the proportional increase in area relative to the initial dimension, expressed as a

percentage (%); Area Relative Growth Rate (ARGR): evaluated using a natural logarithmic scale per unit of time ($ARGR=\ln_{A2}-\ln_{A1}/ t_2-t_1$); Maximum and Minimum Perimeter Variation ($PV=P_{max}-P_{min}$): the absolute difference between the final and initial perimeter; Perimeter Growth Percentage (PGP): the proportional increase of the peripheral boundary expressed as a percentage (%); Perimeter Relative Growth Rate (PRGR): calculated as the logarithmic change of the boundary per unit of time ($PRGR=\ln_{P2}-\backslash\ln_{P1}/t_2-t_1$) and finally the Distance Dispersion Index measured according to Uozumi et al. [24] with the formula: $DD = (Dn-Dav)^2/A$. Where: Dn is the distance between the "x" and "y" values obtained from the centroid analysis; Dav: is the average of Dn values and A: is the final area of each explant.

2.4 Statistical analysis

A numerical comparative analysis of each experimental conditions biotized or no biotized and exposed to cadmium was done, 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. Finally, to evaluate the global interactions between the calculated kinetic and geometric descriptors, a Principal Component Analysis (PCA) was done using a standardized correlation matrix with the PAST software (Paleontological Statistics Software Package) Ver. 5.3.

3 RESULTS AND DISCUSSION

3.1 Variation in the response of *F. splendens* callus

Figure 1 shows the representative appearance of *F. splendens* callus for no biotized and biotized conditions and exposed to cadmium (**Figures 1a-4a**), biotized with *S.pasteuri* strain Fs3 (**Figures 1b-4b**); with a diverse response not only by the presence of endophytes, it also was demonstrated an additive effect with cadmium exposition, showing that several factors influencing the growth and differentiation of callus, particularly the fast and progressive response of *F. splendens* callus growth. The *F. splendens* callus growth was monitored using image analysis techniques that make it possible to investigate the evolution of calli without destructive interaction. Considering that the growth of the calli is not isotropic and may contain protuberances that correspond to the parts of the calli that grow faster than others; the lack of uniformity can be explained by the irregular pattern of cell differentiation. Callus culture dispersion was analyzed by combining morphometric measurements, growth metrics, automated image-based segmentation, variation in callus area, perimeter, or colony spacing; heterogeneity in color, texture, or necrosis patterns; distribution of morphogenetic centers which all together quantify spatial distribution and analyze the developmental variation in callus tissues as follows.

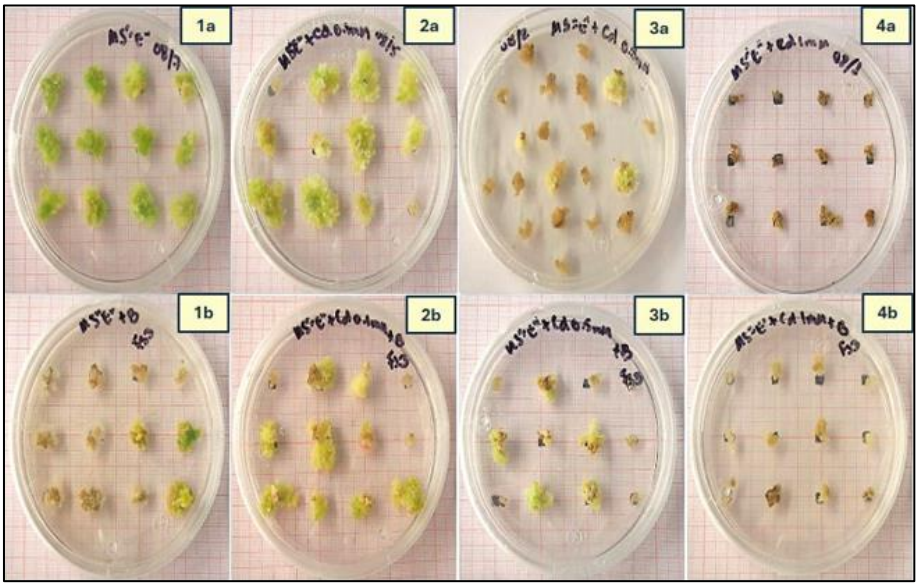


Figure 1: *F.* splendens callus representative final response: 1a: no biotized callus grown in MS medium; 2a: callus grown in MS+Cd 0.1mM; 3a: callus grown in MS+Cd 0.5mM; 4a: callus grown in MS+Cd 1.0mM; 1b: biotized with *S. pasteuri* strain Fs3; 2b: biotized with *S. pasteuri* strain Fs3+Cd0.1mM; 3b: biotized with *S. pasteuri* strain Fs3+Cd0.5mM; and 4b: biotized with *S. pasteuri* strain Fs3+Cd1.0mM.

3.2 *F. splendens* non-destructive image analysis and callus distance dispersion analysis

In this work the morphometric analysis derived from the image processing by the employ of ImageJ software, considered two principal shape descriptors: the area and centroid dimensions of the explants associated by the measurement of distance dispersion data. In plant tissue culture, distance dispersion index quantified from the centroid (x,y) and total area calculates how spread out the tissue mass is relative to its geometric center [25,26]. **Figure 2** describes how these measurements were taken particularly, the centroid analysis (**Figures 2a-2c**) and the graphic presentation of the basic morphometric descriptors analyzed in *F. splendens* callus (**Figures 2d-2f**).

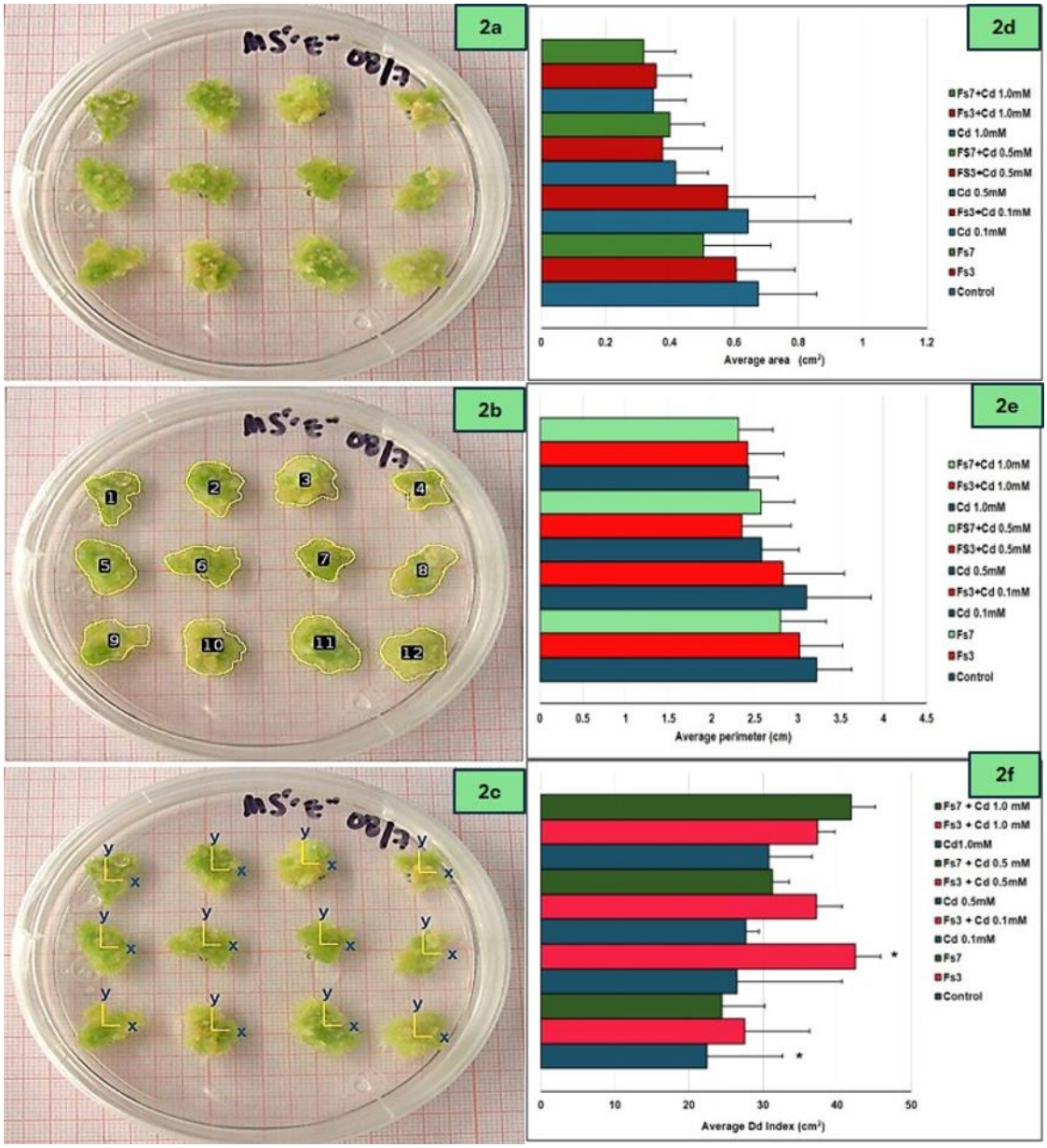


Figure 2: Digital morphometric analysis and macroscopic growth descriptors of *F. splendens* calli. 2a: illustrates the initial callus mass; 2b: the digital boundary segmentation workflow and 2c: the axis projection for the Distance Dispersion Index calculation (centroid x,y). 2d: the mean values for average area; 2e: the mean values for average perimeter, and 2f: the mean values for Dd Index.

Combining morphometrics + growth rate is methodologically strong integrating both approaches allows us to describe how callus grows and how its form changes-two distinct but complementary dimensions of development. Morphometrics captures the shape variation independent of size, the structural heterogeneity and developmental patterning like compactness, lobing and surface irregularity; meanwhile the growth rate analysis captures the biomass accumulation, expansion dynamics and physiological responses to treatments. **Table 1** shows both results in a single presentation, where quantitative digital morphometric analysis revealed that cadmium stress and endophytic biotization exert a significant allosteric and kinetic regulation on *F. splendens* calli as follows.

Table 1: Morphometric parameters associated to *F. splendens* callus growth response

Experimental condition	Amax-Amin variation (cm^2)	Area growth percentage (%)	RGR Area (cm^2/day)	DDI (A.U.)
Control	0.842±0.36c	260.1±128.08a,c	1.226±0.33c	22.376±10.28a
Fs3	0.532±0.42c	133.083±112.9a	0.708±0.53b,c	27.548±8.77
Fs7	0.174±0.46c	71.958±97.55c	0.282±0.66b,c	24.453±5.74
Cd 0.1mM	0.857±0.75c	258.375±209.32c	1.061±0.70c	26.486±14.19
Fs3+Cd 0.1mM	0.827±0.68b,c	293.75±288.48a,c	1.102±0.79b,c	42.421±3.47a
Cd 0.5mM	0.127±0.27c	39.659±71.09a,c	0.210±0.40c	27.649±1.83
Fs3+Cd 0.5mM	0.208±0.40b,c	74.291±108.04c	0.261±0.66b,c	37.159±3.47
Fs7+Cd 0.5mM	-0.140±0.10c	0a,c	-0.359±0.26b,c	31.259±2.25
Cd 1.0mM	-0.140±0.13c	0.791±2.79a,c	-0.421±0.35b,c	30.746±5.82
Fs3+Cd 1.0mM	-0.039±0.03a,c	1.333±3.14c	-0.099±0.09b,c	37.360±2.35
Fs7+Cd 1.0mM	-0.066±0.10c	6.727±17.80c	-0.204±0.33c	41.877±3.23
Experimental condition	**Pmax-Pmin variation (cm)**	**Perimeter growth percentage (%)**	**RGR Perimeter (cm/day)**	
Control	1.944±0.65c	83.620±31.70a,c	0.594±0.16c	
Fs3	1.186±0.89b,c	47.875±38.77a,b,c	0.350±0.27b,c	
Fs7	0.447±1.09a,c	27.791±32.60c	0.142±0.32b,c	
Cd 0.1mM	1.914±1.33c	80.625±53.76a,c	0.537±0.33c	
Fs3+Cd 0.1mM	1.819±1.52b,c	85.416±75.92c	0.528±0.43b,c	
Cd 0.5mM	0.355±0.62c	16.1+23.34c	0.116±0.19a,b,c	
Fs3+Cd 0.5mM	0.458±1.11c	29.25±41.50c	0.120±0.37a,b,c	
Fs7+Cd 0.5mM	-0.403±0.38a,c	0c	-0.162±0.15a,b,c	
Cd 1.0mM	-0.469±0.44a,c	1±4.69c	-0.199±0.17b,c	
Fs3+Cd 1.0mM	-0.087±0.11c	0.916±2.31c	-0.034±0.05b,c	
Fs7+Cd 1.0mM	-0.217±0.45c	4.541±8.13b,c	-0.096±0.19c	

Where: a: $p \leq 0.05$; b: $p \leq 0.01$; c: $p \leq 0.001$

Optimal tissue growth in the Control group (MS medium), characterized by the maximum increase in area (260.1%) and the highest relative growth rates (ARGR: 1.226 cm^2/day and PRGR: 0.594cm/day), establishing the baseline of cell proliferation free from chemical or biological perturbations. Interestingly, exclusive bacterial inoculation in the absence of heavy metal triggered a deceleration in the growth parameters of the callus. The reduction in area growth percentage to 133.08% by *S. pasteuri* strain Fs3 and to 71.95% by *K. rhizophila* strain Fs7 suggests the existence of an initial energetic or metabolic cost associated with establishing the plant-microorganism interaction in closed *in vitro* systems. This behavior is characteristic of symbiotic systems where the endophyte modulates the uncontrolled cell proliferation of the callus mass, redirecting resources toward secondary metabolic pathways or promoting tissue pre-differentiation [10]. Under moderate heavy metal stress, a phenomenon of morphometric hormesis became evident in the *S. pasteuri* strain Fs3 + Cd 0.1 mM treatment. This condition outperformed even the absolute control, achieving the highest area growth percentage in the entire study (293.75%). When transitioning to severe phytotoxicity scenarios (Cd 0.5 and 1.0 mM), non-biotized treatments collapsed, showing negative area variations (Amax-Amin: -0.140 cm^2) and negative growth rates (ARGR: -0.421cm^2/day), which denotes a strict arrest of the mitotic cell cycle combined with heavy metal-induced tissue necrosis and lysis processes. Faced with this lethal damage, biotization played a strain-specific structural rescue role. *K. rhizophila* strain Fs7 proved to be the most efficient entity to mitigate extreme cadmium toxicity at 1.0 mM, successfully maintaining a positive area growth percentage (6.727%) and mitigating the drop in the relative growth rate (ARGR: -0.204 cm^2/day) compared to the metal alone. Furthermore, this treatment triggered the DDI to one of its most prominent values (41.877). This increase in callus geometry reflects a coordinated architectural adaptation; the

endophyte induces a three-dimensional cell compaction that minimizes the surface of the callus exposed to the toxic medium. The multidimensional morphometric analysis demonstrated that cadmium (Cd) stress and biotization with endophytic bacteria exert a severe geometric and growth reprogramming on the cell populations in *F. splendens* calli. **Figure 3** shows the high explanatory resolution by the Principal Component Analysis (PCA), which accounts for 98.92% of the total variance (PC1: 86.60%, PC2: 12.32%), reveals that the fate of cellular development undergoes a homeostatic trade-off between biomass expansion and structural compaction for survival.

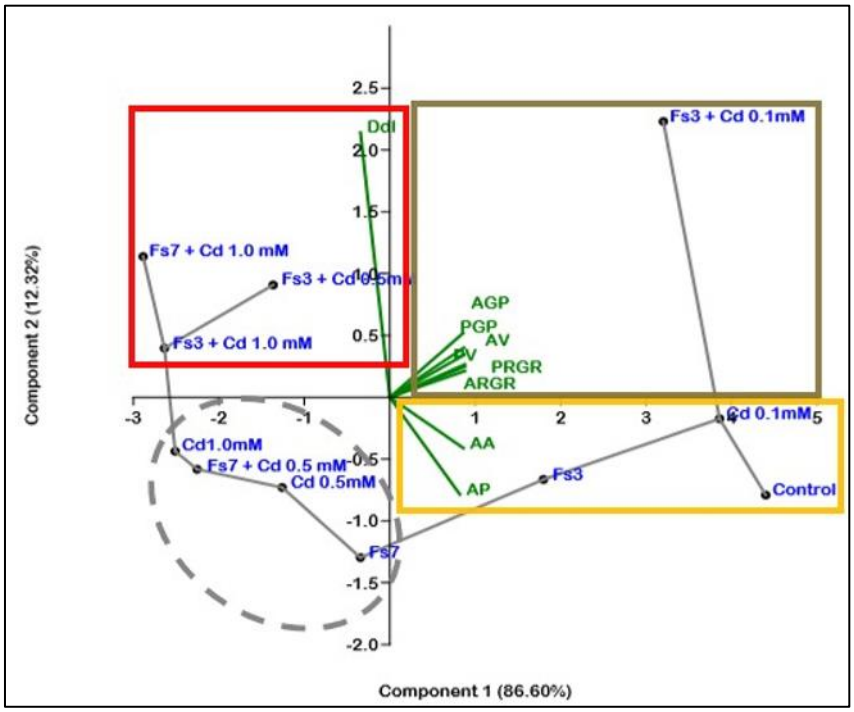


Figure 3: Principal Component Analysis (PCA) of the morphometric response of *F. splendens* calli under interaction with endophytes strains and chemical stress by Cd. Accounts for 98.92% of the cumulative variance (PC1: 86.60%, PC2: 12.32%). The green vectors correspond to absolute size variables (AA, AP); morphometric variations (AV, PV); growth increment percentages (AGP, PGP); relative growth rates (ARGR, PRGR) and Distance Dispersion Index (DdI).

3.3 Morphometric synergy and hormesis at low cadmium doses

A remarkable finding of this research is located in the upper-right quadrant of the PCA, where the Fs3 + Cd 0.1 mM treatment segregated uniquely and parallel to the vectors of relative rates and growth percentages (ARGR, PRGR, AGP, and PGP), as well as absolute variations in area and perimeter (AV and PV). This phenomenon describes a plant hormesis response, a process where sub-toxic concentrations of a xenobiotic act as a eustress (positive stress). Cadmium at 0.1 mM, in synergy with the IAA produced by *S. pasteuri* strain Fs3 induces the most accelerated proliferation and elongation rates along the callus periphery observed in the entire experiment, reflected as drastic and simultaneous increases in area and perimeter within the 52 to 76-day interval.

3.4 The biostimulant role of IAA in baseline stability

Under optimal culture conditions, the absolute Control group and the treatment inoculated exclusively with *S. pasteuri* strain Fs3 positioned themselves within the quadrant of maximum correlation with Average Area (AA) and Average Perimeter (AP). Biologically, this is attributed to the capacity of the endophytic bacteria to biosynthesize Indole-3-Acetic Acid (IAA). The constant flux of this microbial auxin triggering the baseline processes of synchronous cell division and elongation within the callus, thereby maintaining a stable vegetative ontogeny, confirming that optimal status favors absolute horizontal mass expansion without altering instantaneous growth rates.

3.5 Physiological investment under severe stress: structural shape protection

As heavy metal concentrations scale to severe phytotoxic levels (Cd 0.5 and 1.0 mM), the trajectory of the biotized calli exhibits a critical adaptive response. While cadmium treatments without bacteria (Cd 0.5 and 1.0 mM alone) projected diametrically opposite to all biological growth variables evidencing net cellular damage, mitotic cycle arrest, and probable tissue necrosis the addition of endophytes caused a radical shift of the treatments (Fs3 + Cd 1.0 mM, Fs7 + Cd 1.0 mM, and Fs3 + Cd 0.5 mM) toward the upper-left quadrant, aligning directly with the Distance Dispersion Index.

This behavior demonstrates a bacterium-mediated buffering and morphometric investment strategy. Under heavy metal toxicity that threatens callus viability, microbially driven interaction forces a shift in cell growth patterns from a friable and irregular nature to an extreme sphericity or compaction.

Finally, most notably as cadmium concentrations escalated to severe or lethal levels (0.5 and 1.0 mM), the addition of the bioinoculants forced a radical shift of the treatments (Fs7 + Cd 1.0 mM, Fs3 + Cd 1.0 mM, and Fs3 + Cd 0.5 mM) this topological distribution evidences a bacteria-mediated morphological investment strategy; under critical chemical stress, the endophytes redirect the callus developmental pattern away from dimensional extension to establish a dense, protective cellular arrangement for the cell aggregates. Thus, uninoculated toxic treatments (Cd 1.0 mM) and the low-buffering combination (Fs7 + Cd 0.5 mM) clustered in the opposite lower-left quadrant, away from all growth dynamics, statistically validating the severe mitotic arrest and net phytotoxic tissue damage caused by the free metal.

4 CONCLUSIONS

Cadmium stress and endophytic biotization exert a coordinated reprogramming on the growth and cell geometry of *Fouquieria splendens* calli, where *Staphylococcus pasteuri* strain Fs3 demonstrated a superior phytoprotective capacity related to a morphometric hormesis response, inducing the highest area growth percentage and strongly relative growth rates, which demonstrates an adaptive synergy at sub-lethal heavy metal concentrations; meanwhile inoculation with *Kocuria rhizophila* strain Fs7 under conditions of extreme phytotoxicity, acted as a biological rescue system, mitigating biomass loss and redirecting the cellular architecture toward high symmetrical circularity, thereby minimizing the cellular exposure surface against environmental stress. Finally, the endophytes tested not only adapted to the nutritional conditions of the culture media; they also established a strategic combination offering a synergistic framework for enhancing the morphogenesis and growth of *Fouquieria splendens* callus under several chemical stress.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Acknowledgments

Authors wish to thank for the fellowships from BEIFI- IPN; COFAA-IPN; EDI-IPN and SNI-CONAHCYT.

Funding Source

Authors are grateful to the Research Projects SIP-IPN: 20211499 and SIP-20221730, of the SPI-IPN, for providing the facilities to carry out this work.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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