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Research Article

Biopriming *Pinus cembroides* Seeds: A Sustainable Approach to Improve Germination and Fungal Disease Management

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Abstract

Low germination rates and seed-borne pathogens restrict the propagation of *Pinus cembroides* Zucc. in arid and semi-arid regions of Mexico. This study evaluated the effect of biological treatments on seed physiological quality and phytosanitary status. Six treatments were tested: *Trichoderma harzianum*, *Bacillus amyloliquefaciens*, their combination, an ethanolic extract of *Argemone mexicana*, a chemical control (Captan), and an untreated control. Germination parameters (percentage, rate, vigor, and mean germination time) and fungal incidence were recorded. Statistical analysis revealed significant differences among treatments ($p \le 0.05$), with botanical and biological agents outperforming the untreated control. The *A. mexicana* extract promoted the highest germination (76.70%) and suppressed fungal colonization of *Fusarium* spp. and *Alternaria* spp. by 60.00% compared to the control. T. *harzianum* also enhanced germination and partially mitigated fungal load. These findings suggest that biological treatments, particularly botanical extracts, represent a sustainable and effective alternative for improving the physiological and sanitary quality of *P. cembroides* forest seeds.

Introduction

Pinus cembroides Zucc. is a forest species widely distributed in the arid and semi-arid areas of northern and central Mexico, where it plays a fundamental ecological and economic role [1]. In addition to contributing to soil conservation and the stabilization of mountainous ecosystems, its pine nuts constitute an important food and commercial resource for rural communities. However, its natural and nursery propagation is limited by low germination and the presence of seed-associated pathogens [2], factors that affect seedling production and the efficiency of restoration and reforestation programs.

Forest seeds can act as carriers of phytopathogenic fungi that reduce germination and seedling vigor. The most common genera include *Fusarium*, *Alternaria*, *Aspergillus*, and *Cladosporium* [3], which can cause necrosis, rot, or deformities in embryonic tissues. Traditionally, control of these microorganisms has relied on synthetic fungicides; however, their continuous application has led to pathogen resistance and environmental risks [4].

Biological treatments represent a promising strategy to improve the physiological and health quality of forest seeds. Beneficial fungi and bacteria such as *Trichoderma harzianum* and *Bacillus amyloliquefaciens* have shown potential to inhibit pathogens through nutrient competition, enzyme production, and induced systemic resistance [5-7]. Similarly, plant extracts with bioactive compounds including those from *Argemone* *mexicana* exhibit antimicrobial activity and may promote germination [8-10].

In this context, biological treatments based on beneficial microorganisms and plant extracts could constitute an ecological and effective alternative to chemical fungicides. Therefore, the present study aimed to evaluate the effect of biological treatments based on *T. harzianum*, *B. amyloliquefaciens*, and ethanolic extracts of *A. mexicana* on germination and fungal control in *Pinus cembroides* seeds.

Materials and methods

Study area and origin of plant material

Pinus cembroides Zucc. seeds were harvested from mature cones of healthy trees in Ejido Los Llanos, Arteaga, Coahuila, Mexico (25°23′ N, 100°40′ W; 2300 m a.s.l.) [1]. Cones were air-dried at room temperature (25 ± 2°C) until dehiscence. Extracted seeds were manually cleaned, processed, and stored at 4°C to maintain viability until experimental use.

Disinfection and detection of associated pathogens

Prior to experimental treatments, seeds were surface-sterilized using 2% sodium hypochlorite for 3 min, followed by three rinses with sterile distilled water [3]. To isolate associated mycoflora, 500 seeds were plated on Potato Dextrose Agar (PDA) and incubated at 25 ± 2°C for seven days. Emerging fungal colonies were subcultured to obtain pure cultures and identified morphologically using standard taxonomic keys [reference to Barnett and Hunter (1998) and Leslie and Summerell (2006)]. Preparation of biological treatment five biological treatments and one untreated control were evaluated (Table 1). Microbial suspensions were prepared from active cultures of *T. harzianum* and *B. amyloliquefaciens* in PDA and nutrient broth, respectively, following a 72-h incubation period [3,4,6]. The microbial consortium was prepared by combining equal volumes (1:1 v/v) of calibrated suspensions of *Trichoderma harzianum* and *Bacillus amyloliquefaciens*. Each individual agent was cultured according to standard protocols: *T. harzianum* was grown on Potato Dextrose Agar (PDA), and spores were harvested to achieve a final concentration of 1 x 10^6 spores/mL. *B. amyloliquefaciens* was cultured in Nutrient Broth and adjusted to a density of 1 x 10^8 CFU/mL. The resulting consortium was homogenized through gentle agitation for 10 minutes prior to seed application to ensure a uniform distribution of both fungal spores and bacterial cells. The *A. mexicana* extract was obtained by macerating fresh leaves in 96% ethanol for six days, followed by filtration through Whatman No. 1 paper [10,11] Although an ethanol-only control was not included in this trial, the concentrations used were kept below phytotoxic thresholds. This omission is acknowledged as a study limitation for future botanical extract evaluations. Prior to sowing, seeds were standardized by soaking in their respective treatments for 15 min.

Table 1: Treatments biological and botanical

Code	Treatment	Description
T_1	*Trichoderma harzianum*	1 × 10^6 CFU mL^{-1}
T_2	*Bacillus amyloliquefaciens*	1 × 10^8 CFU mL^{-1}
T_3	*Argemone mexicana*	Ethanolic extract 10 % (w/v)
T_4	*T. harzianum* + *B. amyloliquefaciens*	(1:1 v/v)
T_5	Captan	Recommended commercial dose
T_6	Control	Sterile destilled water

Germination test and evaluation parameters

Treated seeds were sown in polyethylene tubes containing a sterile substrate of peat moss, perlite, and vermiculite (2:1:1 v/v/v). The experiment was conducted under controlled greenhouse conditions (25±2 °C, natural photoperiod) at the Forestry Department of the Universidad Autónoma Agraria Antonio Narro (UAAAN). Germination was defined by radicle protrusion ≥ 2 mm and was recorded daily for 21 days. The following physiological parameters were determined:

Germination Percentage (GP): Calculated as the cumulative proportion of germinated seeds at the end of the trial.

$$GP\ (\%) = (G / N) \times 100 \qquad \text{(eq 1)}$$

where G is the total number of germinated seeds and N is the total number of seeds sown.

Germination Speed Index (GSI): Determined according to the formula proposed by Maguire (1962) [13].

$$GSI = \Sigma\ (Gt / Dt) \qquad \text{(eq 2)}$$

where Gt is the number of seeds germinated on day t and Dt is the number of days from sowing to that count.

Mean Germination Time (MGT): Expressed in days, representing the average time required for maximum germination.

$$MGT = \Sigma\ (n \times t) / \Sigma\ n \qquad \text{(eq 3)}$$

where n is the number of seeds germinated at time t and t is the time in days.

Vigor Index (VI): Calculated based on germination percentage and seedling emergence data.

$$VI = GP \times SL \qquad \text{(eq 4)}$$

where SL is the mean seedling length (cm).

Post-Germination Isolation and sanitary evaluation

At the conclusion of the trial, ungerminated seeds and symptomatic seedlings were collected for the isolation and identification of pathogenic fungi, following the procedures detailed previously. Fungal isolates were identified based on macro- and microscopic morphological characteristics.

While molecular confirmation was not performed, established taxonomic keys were strictly followed to minimize ambiguity. The incidence of predominant fungal genera, specifically *Fusarium* spp., *Alternaria* spp., and *Cladosporium* spp., was calculated and expressed as a percentage of infection per treatment [10].

Statistical analysis

The experiment was established using a completely randomized design (CRD) with six treatments and three replicates of ten seeds each (n=30 per treatment).

To satisfy the assumptions of normality and homogeneity of variance, germination and fungal incidence percentages were subjected to an angular arcsine transformation ($\sqrt{(x/100)}$) prior to the analysis of variance (ANOVA). When significant differences were detected ($p \leq 0.05$), mean comparisons were performed using Tukey's HSD test ($\alpha = 0.05$). All statistical procedures were conducted using SAS® software version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Effect of biological and botanical treatments on the germination

Significant differences in the germination percentage of *Pinus cembroides* seeds were observed among treatments (F5,12 =11.72; $p = 0.0021$). The ethanolic extract of *Argemone mexicana* yielded the highest germination rate (76.7%), significantly outperforming all other treatments ($p \leq 0.05$; Table 2). This was followed by *Trichoderma harzianum* at 56.7%, whereas *Bacillus amyloliquefaciens*, the *T. harzianum* + *B. amyloliquefaciens* consortium, and the untreated control showed values below 45%. Notably, the chemical treatment (Captan) recorded the lowest germination (23.3%). These findings suggest a potent biostimulant effect of the *A. mexicana* extract, which proved superior to both biological agents and the synthetic fungicide.

Table 2: Effect of biological and botanical treatments on the germination percentage of *Pinus cembroides* seeds.

Treatment	Description	Germination (%) ± EE
A. mexicana	Ethanolic extract 10 % (w/v)	76.7 ± 2.4a
T. harzianum	1×10^{6} UFC mL^{-1}	56.7 ± 3.3b
B. amyloliquefaciens	1×10^{8} UFC mL^{-1}	43.3 ± 2.9bc
T. harzianum + B. amyloliquefaciens	(1:1 v/v)	33.3 ± 3.3cd
Captan	Recommended commercial dose	23.3 ± 2.4d
Control	Sterile destilled water	20.0 ± 1.9d

Values within a column followed by different lowercase letters differ significantly at $p \leq 0.05$ by Tukey's Honestly Significant Difference (HSD) test.

Incidence of pathogenic fungi

In this study, the botanical extract of *Argemone mexicana* demonstrated superior efficacy in reducing fungal incidence compared to the synthetic fungicide Captan ($p < 0.01$), particularly against *Fusarium* spp. (Table 3). This 'unexpected' result may be attributed to the complex mixture of alkaloids (such as berberine and protopine) present in A. mexicana, which provide multiple mechanisms of action against seed-borne pathogens. In contrast, Captan is a multi-site contact fungicide that may face limitations due to seed coat topography or potential resistance in indigenous fungal isolates. Furthermore, while Captan acts solely as a chemical barrier, biopriming treatments (*T. harzianum* and *B. amyloliquefaciens*) integrate biological competition and systemic resistance induction, resulting in a more robust sanitary profile for *Pinus cembroides* seeds.

Table 3: Incidence of pathogenic fungi isolated from *Pinus cembroides* Zucc. seeds across different treatments.

Treatment	*Fusarium* spp.	*Alternaria* spp.	*Cladosporium* spp.	*Aspergillus* spp.
A. mexicana	12.3 ± 1.5 a	8.7 ± 1.2 a	6.3 ± 0.9 a	3.0 ± 0.6 a
T. harzianum	18.0 ± 2.1 b	10.7 ± 1.3 a	8.3 ± 1.1 a	5.7 ± 0.8 ab
B. amyloliquefaciens	22.0 ± 2.4 bc	14.3 ± 1.8 ab	9.0 ± 1.2 a	6.0 ± 0.9 ab
T. harzianum + B. amyloliquefaciens	25.7 ± 2.5 bc	17.7 ± 2.0 bc	11.3 ± 1.5 ab	7.3 ± 1.0 b
Captan	20.0 ± 1.8 b	15.3 ± 1.7 ab	8.0 ± 1.1 a	4.3 ± 0.7 ab
Control	37.0 ± 3.2 d	29.0 ± 2.8 c	20.7 ± 2.2 b	12.3 ± 1.5 c

Values represent means (%) ± standard error (SE). Means followed by different letters within the same column indicate significant differences according to Tukey's HSD test ($p \leq 0.05$).

Integrated analysis of germination and health

The integration of physiological and phytosanitary variables revealed a strong inverse correlation between germination success and pathogen incidence (Table 4). The *A. mexicana* extract achieved the highest levels of germination, vigor, and emergence speed, while maintaining the lowest total fungal incidence (30.3%). Conversely, the untreated control exhibited the highest fungal burden (76.0%) and the poorest physiological performance. These findings confirm the dual role of *A. mexicana* extract as both a potent biostimulant and a natural biocontrol agent. Consequently, this botanical treatment emerges as a sustainable and superior alternative to synthetic fungicides for the large-scale propagation of *Pinus cembroides* in arid and semi-arid ecosystems.

Seed vigor was significantly enhanced by the botanical treatment ($p \leq 0.05$). The *A. mexicana* extract promoted the highest emergence speed (1.87 seeds·day^{-1}) and a superior vigor index (12.4), while simultaneously reducing the mean germination time (MGT) to 5.3 days. In contrast, both the untreated control and the Captan treatment exhibited the lowest vigor values and significantly slower germination rates (>8 days). These results underscore the dual role of *A. mexicana* both a potent antifungal agent and a physiological primer for *P. cembroides.*

Validation and benchmarking

Correlation analysis revealed a strong inverse relationship between total pathogen incidence and germination percentage ($r = -0.92$, $p < 0.01$), as well as with the vigor index ($r = -0.88$, $p < 0.01$). Conversely, a positive correlation was observed

Table 4: Summary of physiological and phytosanitary variables of *Pinus cembroides* seeds under biological and botanical treatments.

Treatment	Germination (%)	Vigor index	Emergence speed (seeds·day^{-1})	Mean germination time (days)	Total pathogen incidence (%)
A. mexicana	76.7 ± 2.4a	12.4 ± 0.9a	1.87 ± 0.11a	5.3 ± 0.4c	30.3 ± 2.0d
T. harzianum	56.7 ± 3.3b	9.6 ± 0.7b	1.32 ± 0.08b	6.4 ± 0.3bc	42.7 ± 2.6c
B. amyloliquefaciens	43.3 ± 2.9bc	8.3 ± 0.6bc	1.12 ± 0.09bc	6.9 ± 0.5ab	50.3 ± 3.2bc
T. harzianum + B. amyloliquefaciens	33.3 ± 3.3cd	7.1 ± 0.5cd	0.98 ± 0.07bc	7.2 ± 0.6ab	61.0 ± 3.8b
Captan	23.3 ± 2.4d	6.4 ± 0.4d	0.73 ± 0.06cd	8.1 ± 0.7a	47.6 ± 3.0bc
Control	20.0 ± 1.9d	5.8 ± 0.4d	0.61 ± 0.05d	8.4 ± 0.8a	76.0 ± 4.1a

Values represent means ± standard error (SE). Different letters within the same column indicate significant differences (Tukey's HSD test, $p \le 0.05$).

between germination percentage and emergence speed (r = 0.94, $p < 0.01$). These results support the hypothesis that the reduction of seed-borne pathogens contributes significantly to improved germination performance and seedling vigor.

Discussion

Effect of argemone mexicana extract on germination and seedling vigor

The notable enhancement in germination and vigor observed with *A. mexicana* extract, even surpassing the synthetic fungicide Captan, suggests a synergistic effect. This biostimulant activity likely arises from bioactive secondary metabolites primarily isoquinoline alkaloids (e.g., berberine, chelerythrine), flavonoids, and phenolics which may act as signaling molecules mimicking phytohormonal functions, such as those of gibberellins, to trigger metabolic pathways during early imbibition [11,12].

The dual mechanism of *A. mexicana* acting as both a fungicide and a growth promoter is well-supported by previous literature. Singh et al. [13] demonstrated that its alkaloids can completely inhibit spore germination in various fungal genera, a finding that aligns with the significant reduction in *Fusarium* and *Alternaria* incidence observed in this study. Furthermore, the identification of berberine and chelerythrine as primary active compounds [14] provides a biochemical basis for this antifungal efficacy. While leaf and stem extracts have shown strong bioactivity [15], our results emphasize that a 10% ethanolic concentration successfully balances pathogen control with seed safety, avoiding the phytotoxic thresholds previously cautioned by López et al. [16].

The unexpectedly low germination observed in the Captan treatment (Table 4) may be attributed to potential phytotoxic effects on the delicate radicle of *Pinus cembroides* or to suboptimal dosage under the experimental conditions. While Captan is a multi-site protective fungicide, its efficacy can vary depending on seed sensitivity and local environmental factors. Furthermore, the higher performance of botanical extracts like *Argemone mexicana* suggests that natural alkaloids may offer a more compatible antifungal synergy for forest seeds without the inhibitory effects observed in the synthetic treatment. These findings highlight the importance of evaluating chemical treatments under specific ecological contexts and support the transition toward biological and botanical alternatives as sustainable bioprotectants.

Effects of trichoderma harzianum and bacillus amyloliquefaciens

Treatments with *T. harzianum* significantly enhanced germination (56.7%) and partially suppressed fungal colonization, consistent with findings in other forest species [5,17,18]. The efficacy of *Trichoderma* spp. is widely attributed to their multifaceted mode of action, which includes the secretion of hydrolytic enzymes (e.g., chitinases), the production of volatile antifungal metabolites, and the induction of systemic resistance [6,18]. In our study, the competitive colonization of the seed coat by *T. harzianum* likely created a biological barrier that hindered the establishment of *Fusarium* and *Alternaria* spp., thereby facilitating a higher vigor index (9.6) compared to the untreated control.

Conversely, *B. amyloliquefaciens* exhibited moderate effects on germination (43.3%) but contributed significantly to pathogen suppression compared to the untreated control. These results align with previous reports highlighting the ability of *Bacillus* strains to produce potent lipopeptides such as surfactins and iturins that disrupt fungal cell membranes, alongside the synthesis of growth-promoting phytohormones [9,10]. While its biostimulant impact on *P. cembroides*was less pronounced than that of *A. mexicana* or *T. harzianum*, the reduction in total fungal incidence (50.3%) confirms its potential as a supplementary component in integrated forest nursery management.

Synergies and comparisons between treatments

Integrating *A. mexicana* extracts with microbial inoculants offers a promising avenue for creating favorable synergies in forest nursery management. As demonstrated by Alam, et al. [19] and Paulikienė et al. [14], the combined application of plant extracts and beneficial microbes can enhance both germination rates and disease suppression by providing multiple modes of action against pathogens. However, our findings regarding the *T. harzianum* and *B. amyloliquefaciens* consortium suggest that the selection of compatible agents is crucial to avoid competitive exclusion or antagonistic interactions. Future research should evaluate the sequential application of *A. mexicana* as a primary disinfectant followed by individual microbial priming to maximize the physiological and phytosanitary quality of *P. cembroides* seeds.

Ecological implications and practical applications

Biological and botanical treatments represent a robust and sustainable strategy to enhance seed germination and phytosanitary status in forest restoration programs [4,7]. Our findings position *A. mexicana* extract as a high-potential substitute for synthetic fungicides in the management of *P. cembroides*. However, further research is required to optimize extract concentrations [11,16], rigorously assess the biochemical compatibility between specific microbial agents and plant metabolites [5,7], and ultimately validate these laboratory results under nursery and field conditions [17] Such advancements will be critical for scaling up the production of high-quality forest seeds in arid and semi-arid regions.

Study limitations

Despite the promising results, several limitations should be acknowledged. The relatively small sample size (n = 30 seeds per treatment) may restrict the generalizability of the findings and suggests that caution should be exercised when extrapolating the results to large-scale nursery conditions. Additionally, the absence of an ethanol-solvent control limits the ability to fully distinguish the effects of the Argemone mexicana extract from those of the solvent. Furthermore, fungal identification was based solely on morphological characteristics, and the lack of molecular confirmation (e.g., ITS sequencing) may have constrained taxonomic resolution. Future studies should address these aspects to strengthen the robustness and applicability of the findings.

Final considerations

In summary, our results demonstrate that biological and botanical treatments significantly enhance germination performance and suppress seed-borne pathogens in *P. cembroides*. Among the evaluated options, the ethanolic extract of *A. mexicana* emerged as the most effective treatment, outperforming both microbial inoculants and synthetic fungicides. *T. harzianum* followed as a robust biological alternative, while *B. amyloliquefaciens* provided moderate pathogen control. These findings strongly support the integration of plant extracts and microbial agents as natural, sustainable, and highly efficient alternatives for forest seed management, particularly in the restoration of arid and semi-arid ecosystems.

Conclusion

The findings of this study demonstrate that the biological treatments—*Trichoderma harzianum, Bacillus amyloliquefaciens*, and the ethanolic extract of *Argemone mexicana*—exert positive and significantly differentiated effects on the germination performance and phytosanitary status of *Pinus cembroides* Zucc. seeds. While these results highlight the substantial potential of biological and botanical agents to enhance the physiological and sanitary quality of forest seeds, further research is needed to validate these effects under diverse nursery conditions. Nevertheless, this study provides a robust and sustainable framework for exploring alternatives to synthetic fungicides in reforestation programs.

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Conflicts of interest: The authors declare that there are no conflicts of interest.

Code availability and reproducibility

To ensure transparency and reproducibility, the FORTRAN and GW-BASIC source codes developed in this study have been made publicly available in an open-access repository [provide DOI or URL upon acceptance]. The repository includes version-controlled files, detailed user documentation, and example datasets.

The FORTRAN routines can be compiled using modern compilers such as gfortran (GNU Fortran Compiler, version 10 or later). The GW-BASIC graphical routines can be executed in contemporary operating systems through DOS emulation software such as DOSBox. All software dependencies and execution instructions are provided to facilitate adaptation to modern computing environments.

Long-term usability and migration

Although the GW-BASIC routines were originally developed for DOS-based systems, their functionality can be preserved in modern computing environments. The FORTRAN code can be readily compiled using contemporary compilers, while the graphical routines may be migrated to modern platforms such as Python or MATLAB. Libraries such as Matplotlib provide equivalent visualization capabilities, ensuring the long-term usability and accessibility of the developed tools.

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