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Article

Effect of Hydropriming and Biopriming on Seed Germination and Growth of Two Mexican Fir Tree Species in Danger of Extinction

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Abstract: *Abies* spp. in general have been shown to need a period of cold stratification to break dormancy and germinate, but this can be very time consuming. In this study, hydropriming by itself and in combination with biopriming was carried out on *Abies hickelii* and *Abies religiosa* seeds. For biopriming, three species of plant growth promoting rhizobacteria (*Pseudomonas fluorescens*, *P. putida* and *Bacillus subtilis*) were tested. The purpose was to determine if germination and growth could be improved for these two endangered species. Our results demonstrated that treating *A. hickelii* and *A. religiosa* with both hydropriming and biopriming with certain strains of Plant Growth-Promoting Rhizobacteria (PGPR) could improve germination rates up to 91% for *A. hickelii* and up to 68% for *A. religiosa*. Importantly, these treatments showed no significant negative impact on the growth of *A. religiosa* and actually improved growth in *A. hickelii*. The application of

both hydropriming and biopriming offer possibly an alternative methodology to improve germination, survival and preservation of these fir tree species of Mexico that are at risk of extinction.

Keywords: germination; seedling growth; rhizobacteria; *Abies*

1. Introduction

Worldwide forest area has decreased by an estimated 40,000 km^2 from 2000 to 2005 resulting in net loss of 0.18% [1]. The majority of this loss has been due to land use changes [2], population increases [3], fires [4], overgrazing [5], air pollution [6], and the presence of pests and diseases [7]. Mexico is one of the 12 mega diverse countries in the world where its forest territory is occupied by broadleaf trees, as well as by coniferous forests, such as those of the genus *Abies* [8]. The forests with *Abies* species (Pinaceae, Coniferophyta) occupy less than 0.1% of Mexico [8]. This genus has about 40 species distributed in boreal and subalpine forest zones [9]. These forests are highly valued for their ecological importance, carbon capture and sequestration, groundwater recovery, oxygen generation, natural soil protection against erosion, and for the conservation of habitats of various species of flora and fauna [10,11]. The *Abies* species has been used for houses, doors, frames, pulp and paper, medicine, paint and varnish, flavoring in soaps, deodorants, perfumes, and for Christmas trees [12–14]. This has meant that the population is being reduced at an alarming rate due to excessive felling of trees [15–17]. There are presently only small areas of wooded places left of these species. They are located in inaccessible places like ravines, gullies and lower parts of the slopes [18,19]. Among *Abies* species, *Abies hickelii* Flous & Gaussen and *Abies religiosa* (Kunth.) Schltdl. & Cham. are in high demand for the quality of their wood. However, both are endangered species [20] due to overexploitation, low natural regeneration capacity and low levels of germination of seeds (of 10%–20%) [21]. In the Mexican list of endangered species [22] *A. hickelii* is recognized as an endemic tree that is at risk for extinction, but curiously *A. religiosa* does not appear in any category. However, the International Union for Conservation of Nature list both species as endangered in its Red list [23].

To make matters worse, these two species of trees are highly susceptible to plant and wood pathogens [24–27] and insects [14]. Forestry programs have not succeeded in re-afforestation of *A. hickelii* and *A. religiosa* in Mexico. Among the strategies that have been carried out to increase seed germination and seedlings of *Abies* spp. are: temperature treatments, scarification, elongation of adventitious shoots, organogenesis, somatic embryogenesis, callus formation and the use of growth hormones, among others [28–34].

Abies spp. in general have been shown to need a period of cold stratification to break dormancy and germinate. This can be very time consuming and in some cases takes up to several months. In recent years, priming has become a viable treatment of seeds with low germination; vigorous seedlings have been obtained with greater resistance to transplantation in the field. Studies have shown that seed priming can be used to improve germination, accelerate seedling emergence time, as well as increased seed longevity during storage and yield [35]. The favorable effects of priming have been shown for many

crops such as sugar beet [36], barley [37], chickpea, grass fox [38], chickpea [39], and lentil [40], amongst others.

Hydropriming is a technique for initiating germination without emergence of the radicle that involves soaking of seeds in a priming agent solution followed by drying [41]. Hydropriming allows the seeds to quickly reach a high level of moisture with a constant supply of oxygen, thus increasing the level of metabolites associated with the germination process (intermediate metabolites) and enzymes associated with the production of energy [42]. In general, seed hydration treatments have proven to be successful and are currently being investigated. More recent developments in seed priming with maize have shown that Moringa leaf extract significantly improves emergence time and final emergence [43,44].

Hydropriming has been used to increase the speed and uniformity of germination and improve final stand. Nonetheless, hydropriming should be undertaken with care in case seeds are infected with pathogens. If this is the case, fungal growth can be enhanced during hydropriming causing plant disease and stunting development. To respond to the possible negative effects of pathogens, biopriming was developed. Biopriming uses beneficial microorganisms to protect against pathogens and enhance plant growth. Many biopriming organisms are plant growth promoting rhizobacteria (PGPR) and include typical species of the genera *Pseudomonas* and *Bacillus*, among others. These placed on plant seeds help increase germination and seedling vigor as well as control disease from soil- and seed-borne pathogens. A study reported with cowpea that bioprimed seed treatment (with *Trichoderma harzianum*) reduced root rot incidence (caused by *Fusarium solani*, *Macrophomina phaseolina* and *Rhizoctonia solani*) and increased fresh pod yield from 44.0% to 36.1% compared with 19.5% to 11.2% in the case of chemical fungicide Rizolex-T [45]. Therefore, seed hydropriming in combination with biopriming or low dosages of fungicides can improve the rate and uniformity of seed emergence and reduce damping-off disease. Another study demonstrated that biopriming sun flower seeds with *Pseudomonas fluorescens* effectively controlled seed-borne infections of *Alternaria helianthi* [46]. A study discovered that bioprimed faba bean seeds (with *Trichoderma viride*, *T. harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens*) caused a complete reduction of root rot incidence at both pre- and post-emergence stages of plant growth compared with the control treatment [47]. In soybeans, it was found that biopriming with *Pseudomonas aeruginosa* was an effective treatment for controlling pre- and post-emergence soybean damping off [48]. Another study observed that biopriming with *Clonostachys rosea* controlled pre- and post-emergence death of carrot seed and seedlings caused by seedborne pathogens *Alternaria dauci* and *Alternaria radicina* as effectively as the fungicide iprodione [49].

Biopriming in combination with hydropriming has become a viable treatment for increasing seed germination rate and seedling vigor. This study investigated the effect of both hydropriming and biopriming on seed germination and seedling vigor of two Mexican fir tree species, *A. hickelii* and *A. religiosa*. This is the first report of the use of both treatments to evaluate its effect on germination and vigor of two Mexican fir tree species (*A. hickelii* and *A. religiosa*) that are endangered in Mexico.

2. Materials and Methods

2.1. Plant Material

Seeds of *Abies hickelii* and *Abies religiosa* were collected by the National Forestry Commission (Mexico). These seeds were made available to the Instituto de Ecología campus Xalapa, Veracruz, Mexico. These seeds had been collected eight months prior in Cofre de Perote National Park, Veracruz, Mexico. Collection was carried out manually by climbing trees to reach pine cones at mid-level tree height. *Abies* seeds for both species prior to the experiments were disinfected with a solution of sodium hypochlorite (NaClO) at 5% for 5 min. They were then were rinsed with sterile distilled water.

2.2. Rhizobacterial Strains

Three strains of rhizobacterias were selected. These were *Pseudomonas fluorescens* strain JUV8, *Pseudomonas putida* strain PpUV1and *Bacillus subtilis* strain BsUV. They were provided by the Laboratory of Parasitology and Biological Control, Faculty of Agricultural Sciences at the Universidad Veracruzana, Campus Xalapa. Rhizobacteria were grown in liquid culture medium B-King (Mast Group, Merseyside, United Kingdom) for 72 h at 28 °C, concentration was adjusted to 10^9 CFUs mL^{-1} using a digital spectrometer (Thermo Spectronic Genesys 20, Thermo Fisher Scientific, Inc., Waltham, MA, USA) calibrated to 660 nm wavelength and absorbance 1.0.

2.3. Substrate and Fertilization

Containers with a capacity of 200 mL of substrate were filled with a mixture of peat moss (57%), vermiculite (23%) and perlite (20%), previously sanitized with sodium hypochlorite (NaClO) at 5% for 5 min and wet sterilized at 100 °C for 3 h. Before planting seeds Osmocote® (12/07/17) was incorporated into mixture. The control seeds (without hydropriming or hydropriming plus biopriming treatments) for both species of *Abies* were fertilized with the recommended optimum dose used by many nurseries in Mexico of 4.75 $g \cdot L^{-1}$. Seed treatments that were hydroprimed or hydroprimed plus bioprimed were provided with only a half the dose of commercial fertilizer of 2.37 $g \cdot L^{-1}$ [50]. This was carried out to determine if hydroprimed and bioprimed seeds could grow as well or better with lower dosages of fertilizer than control group.

2.4. Experiment 1: Determining Germination Optimum Response Time with Hydropriming

To find an optimum hydropriming time that provides the largest percentage of germination, separate experiments were carried out using the two *Abies* spp. (*A. religiosa* and *A. hickelii*). The first independent factor was species, while the second factor was specified for seven hydropriming time periods (0, 12, 24, 36, 48, 60 and 72 h). Untreated seeds were used as the control. These were arranged in a completely randomized design with three replicates with each replicate consisting of 33 sterilized seeds per treatment ($7 \times 33 = 231$ seeds total used per experiment replica per species). For the experiment, a total of 693 seeds of *A. religiosa* and 693 seeds of *A. hickelii* were used.

For the actual treatments, the seeds were deposited in cheesecloth and hydroprimed by immersion in sterile distilled water for the required time period. Oxygenation was provided by using a Hagen air pump

that provided air with a constant flow of 2.5–4 $L \cdot min^{-1}$. Immediately thereafter, the seeds were deposited on to a sterile wet absorbent paper and rewetted with sterile distilled water every 3 days to keep moist at 20 °C. At 30 days, the percentage of seeds that germinated was evaluated. Germination was defined as having occurred when emergence of radicle through the seed coat was visually observable.

2.5. Experiment 2: Evaluating Germination Response with Hydropriming and Biopriming

The results of best hydropriming time from the first experiment above were used in combination with that of biopriming to evaluate germination in the two *Abies* species (*A. religiosa* and *A. hickelii*). The first independent factor was *Abies* species, while the second factor included the optimum hydropriming time period (*i.e.*, 12 h, the same for both species) and the three rhizobacteria strains (JUV8, PpUV1 strain BsUV). The group with no treatments was the control group for a total of five groups. These were arranged in a completely randomized design with three replicates. Each replicate consisted of 25 sterilized seeds per species, for a total of 375 seeds of *A. religiosa* and 375 seeds of *A. hickelii*.

All the seeds that were hydroprimed were for the same time period of 12 h (this condition had a higher germination percentage in both species in the previous experiment). Then, these seeds were bioprimed by depositing them in a suspension of 10 mL for 15 min with a rhizobacteria. Thereafter, the seeds were deposited in a petri dish with sterile moist paper towel and incubated for 30 days at 20 °C. At end of the experiment, the percentage of germination was evaluated.

2.6. Experiment 3: Measuring Morphological Growth Response with Hydropriming and Biopriming

From the previous experiment, 20 seedlings were selected of *A. hickelii* and 20 of *A. religiosa* from each treatment. For the five treatments of experiment 2, a total of 100 seedlings of each species was planted per replica. Since there were three replicas, a total of 300 seedlings of *A. religiosa* and 300 of *A. hickelii* were used. Radicle size of seedlings was about 1 mm and each was planted into a plastic-pot medium, kept for four months in a greenhouse at a temperature of 28 ± 5 °C and 65 ± 5% relative humidity. At the end of the experiment height, stem diameter, root length, biomass and root volume was quantified.

2.7. Data Analysis

For all experiments, one-way analysis of variance (one-way ANOVA) were carried out to compare treatments of seeds of two *Abies* spp. independently in terms of percentage of germination and morphological differences in growth variables, followed by a post hoc Tukey's Honestly Significant Difference (HSD) multiple range test at $p \leq 0.05$. All data from experiments were processed by using STATISTICA 10 (Statsoft Inc, Tulsa, OK, USA) software. Prior to one way ANOVA and Tukey test, normality and homoscedasticity was confirmed using Kolmogorov-Smirnov and Bartlett's tests, respectively. Hence, logit data transformation was not needed [51].

3. Results

3.1. Experiment 1: Germination Optimum Response Time with Hydropriming

The seed germination percentage of hydroprimed *Abies religiosa* and *A. hickelii* increased for both tree species relative to seeds without hydropriming (Figure 1). *A. religiosa* had the highest percentage of seed germination at 12, 24, 36 and 48 h of continuous hydropriming with values of 49%, 48%, 48% and 47%, respectively. For *A. hickelii* the highest percentage of seed germination was achieved at 12 h with 70%, with germination decreasing as hydropriming time increased. The germination of seeds without hydropriming was 33% for *A. religiosa* and 40% for *A. hickelii.*

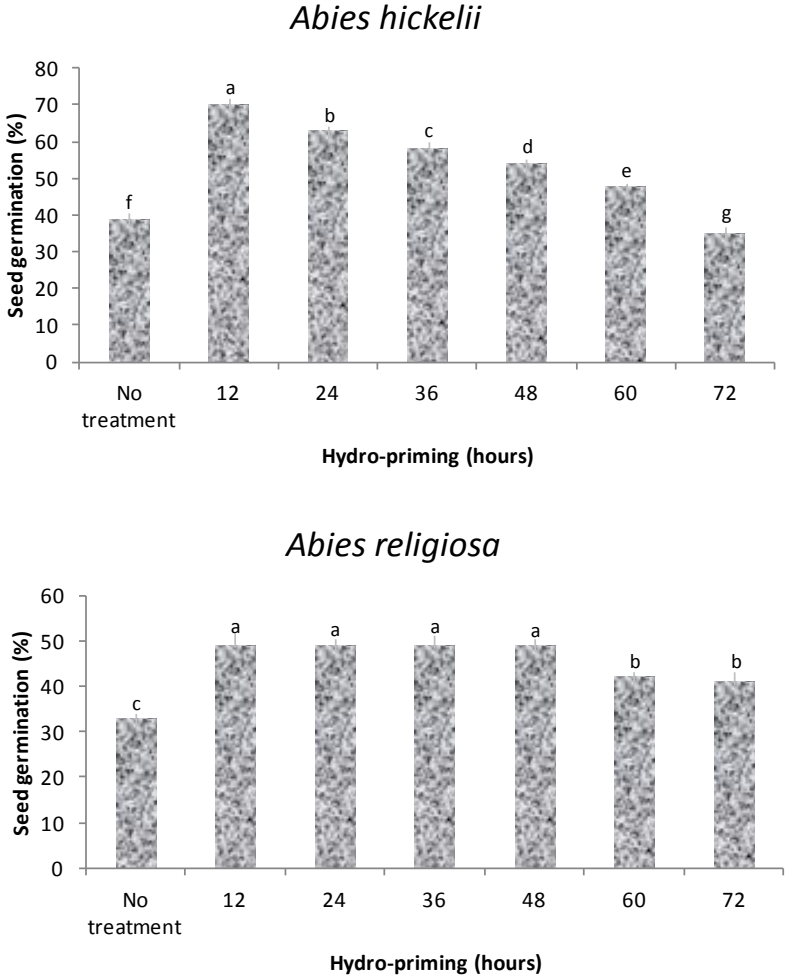

Figure 1. Germination percentage with only hydropriming for seeds of *Abies religiosa* and *A. hickelii.* No treatment = Control, only moistened with sterile distilled water. Remainder of seeds of *A. religiosa* and *A. hickelii* were treated with a constant flow of 2.5–4 $L \cdot min^{-1}$ distilled water and oxygen for seven time periods (0, 12, 24, 36, 48, 60 and 72 h); afterwards, seeds were deposited on to a sterile wet absorbent paper for 30 days at 20 °C to evaluate percent germination. The seeds with no hydropriming (0 h) are the control group. Different letters indicate statistical differences ($p \leq 0.05$). Error bars represent standard deviation values.

3.2. Experiment 2: Germination Response with Hydropriming and Biopriming

The seed germination percentages of hydroprimed and bioprimed *Abies hickelii* and *A. religiosa* were higher compared to the seeds without both treatments (Figure 2). *A. religiosa* inoculated seeds treated with rhizobacteria *Bacillus subtilis* strain BsUV had the highest germination percentage (68%). For *A. hickelii* the highest percentage of germination (91%) was achieved with hydroprimed seeds that were inoculated with rhizobacteria *Pseudomonas fluorescens* strain JUV8. Seed germination with hydropriming only reached 46% for *A. religiosa* and 62% for *A. hickelii*. The lowest germination rates for *A. religiosa* and *A. hickelii* were found for seeds without hydropriming and biopriming treatments (control) with values of 28% and 32%, respectively.

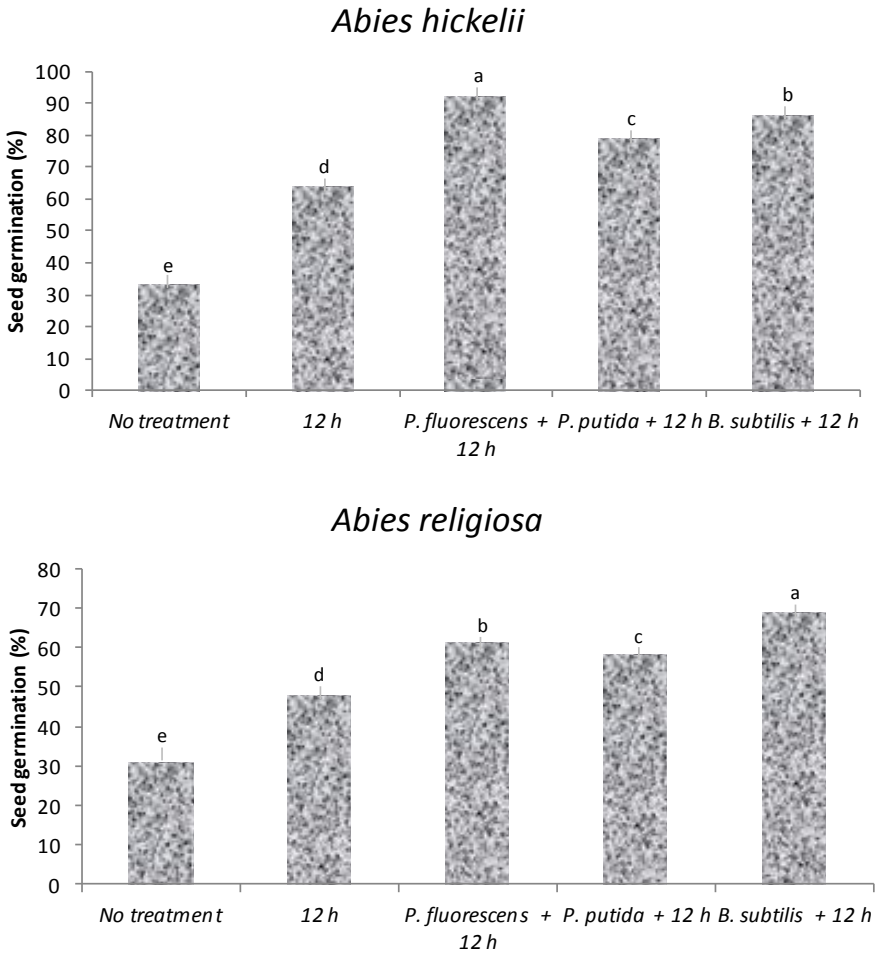

Figure 2. Germination percentage with both hydropriming and biopriming for seeds of *Abies religiosa* and *A. hickelii.* No treatment = Control, only moistened with sterile distilled water. 12 h = hydropriming with a constant flow of 2.5–4 $L \cdot min^{-1}$ distilled water and air, biopriming was carried out with *Pseudomonas fluorescens* strain JUV8, *P. putida* strain PpUV1 and *Bacillus subtilis* strain BsUV in suspension of 10 mL for 15 min. All biopriming seeds also included 12 h of prior hydropriming. Percentage of germination was evaluated after depositing seeds on sterile wet absorbent paper for 30 days at 20 °C. Different letters indicate statistical differences ($p \leq 0.05$). Error bars represent standard deviation values.

3.3. Experiment 3: Morphological Growth Response with Hydropriming and Biopriming

Seedlings of hydroprimed and bioprimed of *A. hickelii* had statistically higher growth rates compared to untreated seeds, while *A. religiosa* showed no differences in terms of growth variables (Figure 3). For the variable height (in cm), seeds were hydroprimed and inoculated with rhizobacteria *P. fluorescens* strain JUV8 and *P. putida* strain PpUV1, seedling height increased by 30% and 28%, respectively, compared to seeds without any treatment (Table 1).

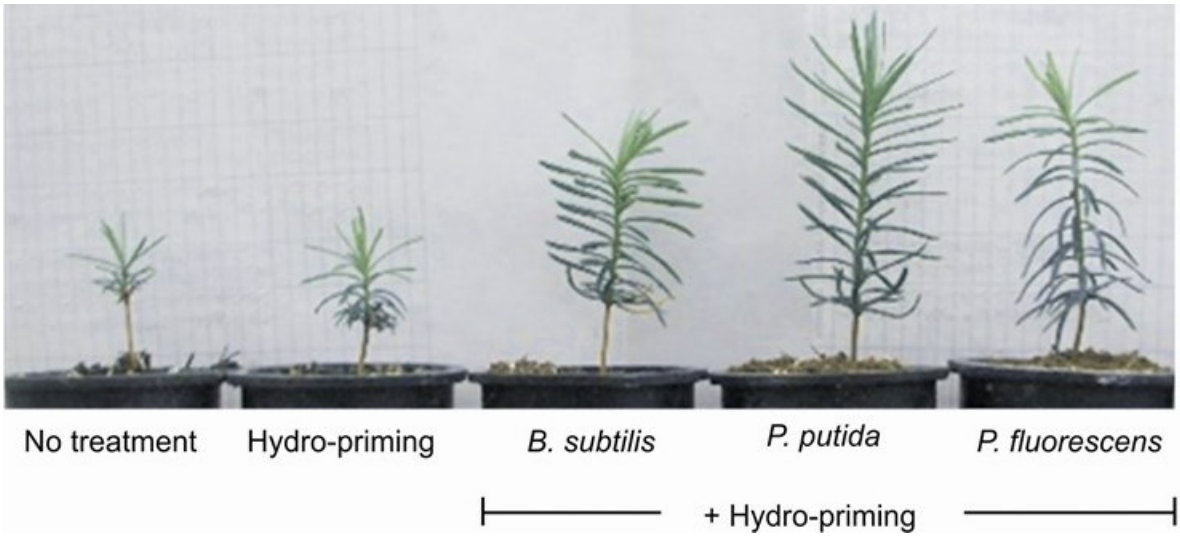

Figure 3. Photograph showing typical size of hydroprimed and bioprimed seedlings of *A. hickelii* (only) grown for four months in a greenhouse. No treatment = Control, only moistened with sterile distilled water. All hydropriming was for 12 h with distilled water with a constant air flow of 2.5–4 L·min^{-1}. Biopriming was carried out with *Pseudomonas fluorescens* strain JUV8, *P. putida* strain PpUV1and *Bacillus subtilis* strain BsUV in suspension of 10 mL for 15 min. All biopriming seeds also included 12 h of prior hydropriming as indicated in the picture. Note: *A. religiosa* showed no statistically significant differences in terms of growth variables with all treatments compared to the control, hence a photograph of typical size is not shown.

Table 1. Measurement of growth parameters of *Abies hickelii* (only) ǂ with hydropriming and biopriming treatments.

Seed Treatment	Height (cm)	Stem diameter (mm)	Root length (cm)	Total biomass (g)	Radicle volume (cm^3)
No treatment	7.1 bc	1.31 b	8.1 b	0.41 e	0.42 c
Hydropriming	7.8 bc	1.39 b	9.9 a	0.58 de	0.52 c
Hydropriming + *P. fluorescens*	9.2 a	1.65 a	10.2 a	0.80 bc	1.12 a
Hydropriming + *P. putida*	9.1 a	1.63 a	10.1 a	1.15 a	0.85 ab
Hydropriming + *Bacilus. subtilis*	8.2 ab	1.58 a	10.1 a	0.79 bc	0.76

Height, stem diameter, root length, biomass and root volume was kept was quantified at 4 months after treatments. ǂ Note: *A. religiosa* showed no statistically differences with untreated seeds in any of the growth variables tested (hence, data not shown). Different letters in columns indicate statistical differences ($p \leq 0.05$).

The response of seedlings that were hydroprimed and bioprimed were statistically greater in stem diameter. Seedlings with JUV8, PpUV1 and *B. subtilis* strain BsUV showed increases in stem diameter of 25%, 24% and 20%, respectively. This behavior was repeated with these treatments for root length. Roots increased in length 25% for seeds inoculated with JUV8 and 24% for seeds inoculated with PpUV1 and BsUV. In addition, biomass increased 80% with the hydroprimed and bioprimed seeds inoculated with the PpUV1 strain.

The root volume variable increased 66% with hydropriming and JUV8 strain inoculation. Although both species of *Abies* improved germination rates with the combination of hydro and biopriming, only *A. hickelii* had improved growth parameters. Hydroprimed and bioprimed seed treatment of *A. religiosa* showed no statistically significant differences to untreated seeds (control) in any of the growth variables tested.

4. Discussion

The results achieved when applying hydropriming in seeds of both *Abies hickelii* and *Abies religiosa* were favorable (Figure 1). It was found that germination of these *Abies* spp. was greater compared to untreated seeds. In addition, the germination rate further improved in both species of *Abies* with the application of both hydropriming and biopriming. PGPR increased germination compared to hydropriming alone for all bacteria (*Pseudomonas fluorescens*, *P. putida* and *Bacillus subtilis*) tested (Figure 2).

For *A. religiosa,* the best germination rate was 70% at 12 h of hydropriming and 91% with hydropriming (12 h) plus biopriming with *Pseudomonas fluorescens* strain, compared to 40% for the control. While for *A. hickelii* the best germination rate was 49% with 12 h of hydropriming and 70% with hydropriming (12 h) plus biopriming with *Bacillus subtilis*, compared to 33% for the control. Possible mechanisms for improved germination and release from dormancy by hydropriming and by using both hydropriming and biopriming are: activation of water induced metabolic processes, improved repair due to enzyme activity, production of hormones, increased availability of nutrients, biological control by production of antibiotics and/or siderophores.

In terms of growth, variables improved with the combined treatment of hydropriming and biopriming for *A. hickelii*; however, *A. religiosa* showed no improvement in growth variables measures compared to the control.

Hydropriming conditioning promotes the occurrence of pre-germinative metabolic events enabling embryo growth, and thus increasing the speed of germination and seedling vigor [43,52]. A study found that by adding enough oxygen seed, dormancy is broken and germination initiates [53]. Even with just the hydropriming conditioning, *Abies* seeds germinated sooner than untreated seeds. Moreover, germination percentage increased when the seeds were hydroprimed and bioprimed with different strains of rhizobacteria. This result is significant considering that *A. religiosa* is a recognized species with slow germination (listed as extremely slow) during their stay in the nursery [54]. In contrast, *A. hickelii* is reported to be low, between 10% and 20% of total seed germination [55]. In general, both species of *Abies* are considered to have low germination capacity [56]. Increased seed germination due to hydropriming and biopriming is caused in part by interruption of seed latency. The effects of different strains of rhizobacteria also improved germination capacity to different degrees depending on the strain.

Thus, the results reported here are similar to results of other studies, where they report the potential use of rhizobacteria in agricultural production systems to help increase crop yield while reducing the fertilization costs [57,58]. Previous studies have shown that soil bacteria are able to grow plants more successfully helping them respond to environmental stresses, compared with plants uninoculated [59,60]. This finding is also supported by other more recent studies as well. These studies assert that PGPR applied to seeds or roots provide large gains in growth and development. Possible mechanisms include production of plant and tree hormones, an increase in the availability of nutrients, as well as biological control due to the production of antibiotics and/or siderophores [61,62]. PGPR bacteria are able to promote the growth and biomass production in different plant species, including pines [63]. In this regard, one study in particular points out that some species of *Pseudomonas spp.* promote plant growth by increasing nutrient absorption (e.g., N, P, K) and providing hormones in the rhizosphere, while also protecting against phytopathogenic organisms [64,65].

5. Conclusions

In this study, it was found that the use of hydropriming and biopriming with PGPR bacteria improved germination rate for *A. religiosa* and *A. hickelii* under greenhouse conditions. *P. fluorescens*, *P. putida* and *B. subtilis* are potential tools for promoting biological germination in *A. religiosa* and *A. hickelii*, and growth at least in *A. hickelii*. Future work will be necessary to pinpoint the exact mechanisms hydropriming/biopriming play in improving the extent of germination. In particular, how this compares with the standard procedure of cold stratification used in interrupting dormancy and inducing germination in trees of cold latitudes would prove useful. Although preliminary, these results can be considered promising. They provide a possible methodology for reducing cold stratification periods, with some as long as 180 days or more in some species. The results may also play a role in allowing the shortening of the germination time period of these species in order to aid in reforestation programs.

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Author Contributions

Designed and conceived the experiments: Ramón Zulueta-Rodríguez, Luis G. Hernández-Montiel and Miguel V. Córdoba-Matson Performed the experiments: Ramón Zulueta-Rodríguez and Liliana Lara Capistrán Analyzed the data: Ramón Zulueta-Rodríguez, Miguel V. Córdoba-Matson, Luis G. Hernández-Montiel, Bernardo Murillo-Amador, Edgar O. Rueda-Puente, and Liliana Lara Capistrán Contributed reagents/materials/analysis tools/publication costs: Ramón Zulueta-Rodríguez and Bernardo Murillo-Amador Wrote, edited and contributed to revision of the manuscript: Ramón Zulueta-Rodríguez, Miguel V. Córdoba-Matson, Luis G. Hernández-Montiel, Bernardo Murillo-Amador, Edgar O. Rueda-Puente, Liliana Lara Capistrán and Enrique Troyo-Diéguez Approved the final version of the manuscript to be published: Ramón Zulueta-Rodríguez, Miguel V.

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Conflicts of Interest

The authors declare no conflict of interest.

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