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Evaluating *Trichoderma* strains as a pretreatment to break physical dormancy in *Piscidia piscipula* and *Enterolobium cyclocarpum* (*Fabaceae*)

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Conflicts of Interest
The authors declare no conflict of interest.

Abstract
Piscidia piscipula and *Enterolobium cyclocarpum* are two key tree species distributed across the agroforestry landscapes of the Yucatan Peninsula, Mexico, both displaying physical dormancy. Although it has been possible to break physical dormancy through chemical and mechanical scarification, the effect of biological treatments for overcoming this condition in these species remains unknown. *Trichoderma* fungi naturally inhabit agricultural soils and can degrade seed coats, potentially facilitating germination. We assessed some seed traits (fresh mass, dry mass, and moisture content) for both species. Additionally, seeds were inoculated in Petri dishes at 30 °C with three *Trichoderma* strains isolated from different crops: *Zea mays* (C2), *Lactuca sativa* (C4), and *Theobroma cacao* (C10). Germination percentages were evaluated and compared against a non-inoculated control group. All *Trichoderma* inoculation treatments resulted in low germination rates for both species, showing no significant differences from the control. *Enterolobium cyclocarpum* seed mass (fresh and dry) was significantly higher than in *P. piscipula*; however, *P. piscipula* seeds exhibited higher moisture content than *E. cyclocarpum*. Treatments with *Trichoderma* strains proved ineffective in breaking physical dormancy in both species.

Keywords: dry mass, fresh mass, germination, moisture content, Yucatan Peninsula

Resumen
Piscidia piscipula y *Enterolobium cyclocarpum* son dos especies arbóreas clave distribuidas en los paisajes agroforestales de la Península de Yucatán, México; ambas presentan dormancia física. Aunque se ha logrado romper la dormancia física mediante escarificación química y mecánica, se desconoce el efecto de los tratamientos biológicos para superar esta condición en dichas especies. Los hongos del género *Trichoderma* habitan de forma natural en suelos agrícolas y pueden degradar las cubiertas seminales, lo que potencialmente facilita la germinación. Evaluamos algunos rasgos de las semillas (masa fresca, masa seca y contenido de humedad) en ambas especies. Además, las semillas se inocularon en placas de Petri a 30 °C con tres cepas de *Trichoderma* aisladas de diferentes cultivos: *Zea mays* (C2), *Lactuca sativa* (C4) y *Theobroma cacao* (C10). Se evaluaron los porcentajes de germinación y se compararon con un grupo control no inoculado. Todos los tratamientos de inoculación con *Trichoderma* resultaron en tasas de germinación bajas para ambas especies, sin mostrar diferencias significativas respecto al control. La masa de las semillas (fresca y seca) de *E. cyclocarpum* fue significativamente mayor que la de *P. piscipula*; sin embargo, las semillas de *P. piscipula* presentaron un mayor contenido de humedad que las de *E. cyclocarpum*. Los tratamientos con cepas de *Trichoderma* resultaron ineficaces para romper la dormancia física en ambas especies.

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Palabras clave: masa seca, masa fresca, germinación, contenido de humedad, Península de Yucatán

Résumé

Piscidia piscipula et *Enterolobium cyclocarpum* sont deux espèces arborées clés présentes dans les paysages agroforestiers de la péninsule du Yucatán, au Mexique; toutes deux présentent une dormance physique. Bien que la levée de la dormance physique ait été obtenue par scarification chimique et mécanique, l'effet des traitements biologiques pour surmonter cette condition chez ces espèces reste inconnu. Les champignons du genre *Trichoderma* sont naturellement présents dans les sols agricoles et peuvent dégrader les téguments des graines, facilitant potentiellement ainsi la germination. Nous avons évalué certaines caractéristiques des graines (masse fraîche, masse sèche et teneur en eau) chez les deux espèces. Par ailleurs, les graines ont été inoculées dans des boîtes de Petri à 30 °C avec trois souches de *Trichoderma* isolées de différentes cultures: *Zea mays* (C2), *Lactuca sativa* (C4) et *Theobroma cacao* (C10). Les taux de germination ont été évalués et comparés à ceux d'un groupe témoin non inoculé. Tous les traitements d'inoculation par *Trichoderma* ont entraîné de faibles taux de germination pour les deux espèces, sans différence significative par rapport au témoin. La masse des graines (fraîche et sèche) de *E. cyclocarpum* était significativement supérieure à celle de *P. piscipula*; toutefois, les graines de *P. piscipula* présentaient une teneur en eau plus élevée que celles de *E. cyclocarpum*. Les traitements avec des souches de Trichoderma se sont révélés inefficaces pour lever la dormance physique chez les deux espèces.

Mots-clés: masse sèche, masse fraîche, germination, teneur en eau, péninsule du Yucatán

INTRODUCTION

The *Fabaceae* family (commonly known as legumes) is one of the most important plant families globally (Hasanuzzaman & al. 2020), comprising 770 genera and 19,581 species (LPWG 2017). *Fabaceae* species are used for diverse purposes, including food, medicine, living fences, shade, timber, fuel, and reforestation (Hasanuzzaman & al. 2020). In Mexico, the family is represented by approximately 155 genera and 1,903 species (Villaseñor 2016). Notably, *Fabaceae* species predominate in the Mesoamerican region, which includes the Yucatan Peninsula, accounting for approximately 78 genera and 228 species, 21 of which are endemic (Carnevali & al. 2010).

Seed germination is a crucial stage in the life cycle of most plants (Baskin & Baskin 2014), with water availability being a key factor for this process to occur (Gurvich & al. 2017). Physical dormancy (PY) is a prominent dormancy type occurring in 19 angiosperm families (Jaganathan 2022), comprising ~25 % of plant species worldwide. Several legume species display PY (Baskin & Baskin 2014) and are mainly distributed in tropical dry forests (Jaganathan & Harrison 2024). Characterized by a water-impermeable seed or fruit coat, PY restricts the access of water and, occasionally, oxygen to the embryo, thereby preventing seed germination (Radchuk & Borisjuk 2014, Smýkal & al. 2014). Consequently, PY plays a fundamental role in the regeneration and population dynamics of these plant species.

In nature, a combination of biotic and/or abiotic factors are involved in breaking PY. Abiotic factors, such as fire (Jaganathan 2015), and fluctuating soil surface temperatures (Geneve 2003) have been identified as integral mechanisms for PY release. Some morphophysiological seed traits could be linked to both PY intensity and alleviation. For instance, Rodrigues-Junior & al. (2018) provided evidence that large (in terms of seed mass) S*enna multijuga* (Rich.) H. S. Irwin & Barneby (*Fabaceae*) seeds achieved PY release relatively more quickly than small seeds. These authors attributed these results to variations in the internal pressure to which large and small *S. multijuga* seeds are subject. In addition, a relationship between moisture content and seed impermeability has been identified (Jaganathan 2016, 2022), wherein, although the critical moisture content at which the seeds become impermeable varies among species and families, seed impermeability has been suggested to occur at a range of 8 % to 15 % moisture content (Hay & al. 2010; Jaganathan & al. 2017, 2019). However, new studies are required to establish the PY-breaking mechanisms present in *Fabaceae* species, while considering some of seed traits described above.

Regarding biotic factors that promote the breaking of PY, Jaganathan (2016) described the passage of seeds, when consumed by herbivores, through the digestive tract and the acidic intestinal environment, both of which processes thin the seed coat. Specifically, *Trichoderma* (*Ascomycota*: *Hypocreaceae*) is a cosmopolitan genus of filamentous fungi known, among other benefits, for its ability to trigger plant defense and resistance mechanisms against both biotic and abiotic stresses (Yadav & al. 2019). While published research also has suggested that *Trichoderma* spp. act as a germination enhancer (Contreras-Cornejo & al. 2016), studies assessing the effects of *Trichoderma* spp. on the germination ecology of tropical forest species remain scarce (dos Santos & al. 2020), particularly in *Fabaceae* species that display PY.

Piscidia piscipula (L.) Sarg. and *Enterolobium cyclocarpum* (Jacq.) Griseb. are two representative *Fabaceae* species that are naturally distributed in the Yucatan Peninsula, where they play a significant ecological and socio-cultural role (Patiño & Villagómez 1976) as timber sources and living fences (Tellez & al. 2020) as well as for restoration and reforestation programs (Sudrajat & al. 2023). Although research evaluating the seed ecology of these species is limited, physical dormancy (PY) mediated by seed coat water-impermeability has been documented in both species (Viveros-Viveros & al. 2015, 2017, Sánchez-Rendón & al. 2019). Arceo-Gómez & al. (2022) assessed variations in seed permeability, imbibition rates, and the external and internal sections of *P. piscipula* and *E. cyclocarpum* seed coats, while also testing different pre-germinative treatments in both species. For *E. cyclocarpum*, mechanical scarification (a scalpel cut opposite the micropyle) yielded the highest germination percentage (92 %), whereas for *P. piscipula*, immersion in boiling water applied for 10 s resulted in the highest

germination (76 %). However, the implications of biological treatments, such as *Trichoderma* spp., in breaking PY in *P. piscipula* and *E. cyclocarpum* seeds are still unknown.

Some species of *Trichoderma* can erode or crack the hardened seed coat, reducing the mechanical resistance to germination. For example, Delgado-Sánchez & al. (2011) found that both old (nine-year-old) and young (freshly harvested) *Opuntia streptacantha* Lem. (*Cactaceae*) seeds inoculated with *Trichoderma koningii* Oudem. showed a high level of germination (91 % and 58 %, respectively). Similarly, Delgado-Sánchez & al. (2010) obtained respective germination levels of 40 % and 37 % in two-year-old *Opuntia leucotricha* DC. seeds inoculated with *Trichoderma harzianum* Rifai and *Trichoderma koningii*. It should be noted that these *Opuntia* species display physiological dormancy (namely that the embryo has low growth potential and requires a period of after-ripening to break dormancy; Orozco-Segovia & al. 2007). Thus, the *Trichoderma* strains described above are involved in the cracking of the seed coat and, consequently, in breaking seed dormancy in both cacti species of interest.

Given this context, the present study tested the following hypothesis: the inoculation of specific *Trichoderma* strains into *Piscidia piscipula* and *Enterolobium cyclocarpum* seeds triggers PY release, thereby increasing seed germination in both species. Consequently, this study aimed to assess the effect of *Trichoderma* inoculation on seed germination in *P. piscipula* and *E. cyclocarpum*. Additionally, we evaluated differences in basic seed traits, such as moisture content, as well as fresh and dry seed mass, to complement our understanding of their seed biology.

MATERIALS AND METHODS

Study species

Piscidia piscipula (L.) Sarg. is a tall tree (>20 m) producing slightly scented panicles with pink petals, during a flowering season occurring from January to April, followed by a fruiting season from February to June (CICY 2010). This species produces fruit with brown wings and yellowish-brown seeds that are 5 mm long and 3 mm wide (SDS 2019). *Piscidia piscipula* occurs in Mexico, from Tamaulipas to the Yucatan Peninsula as well as in South Florida in the United States, the Caribbean, and Central America (CICY 2010). In the Yucatan Peninsula, *P. piscipula* is especially abundant in the secondary vegetation of medium sub-evergreen forests and medium sub-deciduous forests (Pennington & Sarukhán 2005), a vegetation type commonly known as jabín (Patiño & Villagómez 1976).

Enterolobium cyclocarpum (Jacq.) Griseb. is an arboreal species which reaches heights of 20 to 30 m and produces green and bipinnate leaves and actinomorphic flowers with a light green corolla and a green tubular calyx (Lafont & al. 2019). Its flowering season occurs from February to June, followed by a fruiting season from April to July (Aguilera 2011), while its fruits are flattened coiled woody pods with a shiny dark brown color, sweet smell and taste, and numerous seeds (Pennington & Sarukhán 2005). The *E. cyclocarpum* seeds are large (1.5-2.3 cm long by 1.0-1.5 cm wide), brown, ovoid, and flattened and are found inside indehiscent kidney-shaped circular pods (Viveros-Viveros & al. 2015, Lafont & al. 2019). This species is found in deciduous forests ranging from southwestern Mexico to northern South America (Venezuela and Brazil). In the Gulf of Mexico, *E. cyclocarpum* is found from southern Tamaulipas to the Yucatan Peninsula and, in the Pacific coast, from Sinaloa to Chiapas (Viveros-Viveros & al. 2015); it is known as pich.

Seed harvest

The present study collected seeds from ten adult plants of both *Piscidia piscipula* and *Enterolobium cyclocarpum* obtained from the surroundings of the city of San Francisco de Campeche (1 m above sea level, 19º51'00" lat. N, 90º31'59" long. W), in the municipality of Campeche, Mexico, from April to June 2021. Approximately 1,000 seeds of each species were collected. The harvested seeds of each adult plant of both species were mixed and stored in airtight plastic bags (one bag for the seeds of each mother plant of each species) and kept at room temperature (25 °C ± 2 °C and 60-80 % relative humidity) under normal day/night conditions (Euan-Tun & al. 2021, Arceo-Gómez & al. 2022) until experimentation in September 2021.

Seed sterilization

A total of 460 seeds per species (*Piscidia piscipula* and *Enterolobium cyclocarpum*) were placed in a 20 % commercial chlorine solution and shaken on a plate with a stirrer for two minutes. They were then placed in 70 % ethyl alcohol and stirred on a plate with a stirrer for two minutes, after which they were washed three times with distilled water (Robles-Díaz & al. 2014, Arceo-Gómez & al. 2022).

Seed viability

To evaluate seed viability the present study used the tetrazolium (2,3,5-tryphenil tetrazolium chloride, or TTC) test, which is a rapid, accurate, and effective method commonly used to assess seed viability (ISTA 2017). Fifty seeds of *Piscidia piscipula* and *Enterolobium cyclocarpum* were sown in five replicates of ten seeds each. To facilitate the entry of the solution into the seed, an incision was made, with a scalpel, parallel to the micropyle axis (Euan-Tun & al. 2021, Arceo-Gómez & al. 2022).

Seeds from each group were placed in a beaker and soaked in 20 mL of distilled water for 24 h. Subsequently, the distilled water was removed from each beaker and 20 mL TTC was added at 1 %. Each beaker was covered and wrapped in aluminum foil, to keep the seeds in darkness, and stored at 25 °C ± 2 °C for 48 h. The seeds were cut transversally with a scalpel to observe the embryos and ascertain whether they were viable via observation under a stereomicroscope. The present study considered viable seeds as those with visible cotyledons and red-stained embryos without apparent damage (Baskin & Baskin 2014). Thus, viability percentage ± standard deviation (SD) was calculated for each study species (Euan-Tun & al. 2021, Arceo-Gómez & al. 2022).

Fresh/dry seed mass and moisture content

Ten *Piscidia piscipula* and *Enterolobium cyclocarpum* seeds were individually weighed with a Cole-Parmer analytical balance (precision ± 0.2 mg) to obtain the fresh seed mass values (mg). Subsequently, the seeds of each species were placed in a drying oven (brand: RIOSSA, series: HSML, cycles: 127V/IN-50/60) at 80 °C ± 2 °C for 72 h until reaching the constant weight required for obtaining the dry seed mass (mg). In addition, this data was used to obtain moisture content (% ± SD), as follows (ISTA 2017, Reyes-Reyes & al. 2024): $mc = ((fw - dw) / fw) \times 100$, where *mc* is the moisture content, *fw* is the fresh weight, and *dw* is the dry weight.

Inoculation of the *Trichoderma* strains and the effect on seed germination

We used three commercial fungal strains, obtained from the CEPEC/CEPLAC Biocontrol Laboratory, which had been isolated from crops such as *Zea mays* L. (C2), *Lactuca sativa* L. (C4), and *Theobroma cacao* L. (C10). Strains C2, C4, and C10 were grown on potato-dextrose agar (PDA) at 28 °C ± 2 °C for 3-4 days, with colonization determined by visual observation. The methodology proposed by Delgado-Sánchez & al. (2011) was applied for the inoculation of these fungal strains in *Enterolobium cyclocarpum* and *Piscidia piscipula* seeds. The mycelia and spores of strains C2, C4, and C10 were collected in sterile distilled water and stored for the subsequent inoculation of the study species.

Sterilized seeds were grown on water agar Petri dishes and inoculated with 2 µL of either a spore or mycelium solution (6×10^7 spores/mL) obtained from each fungal strain (Delgado-Sánchez & al. 2011). For each target species, ten independent replicates (each replicate represented by a Petri dish) were used per treatment (C2, C4 and C10), corresponding to ten seeds per replicate, and a control treatment based on PDA medium also used. The Petri dishes were kept at room temperature, at 30 °C ± 2 °C and 60-80 % relative humidity, with a photoperiod of 12 h. The number of seeds germinated (% ± SD) every day for 30 days were recorded (Arceo-Gómez & al. 2022).

Data analysis

Data processing and analysis were conducted in R within the RStudio interface (version 2025.05.1), employing a significance level of $\alpha = 0.05$ (R Core Team 2025). Viability was analyzed by one-way ANOVA for each species. For the biometric variables—fresh weight, dry weight, and moisture content—parametric assumptions of normality and homogeneity of variance were previously verified using Shapiro-Wilk and Levene tests, respectively. Mean comparisons were performed using an independent samples t-test in cases of homoscedasticity, or Welch's t-test in the presence of heteroscedasticity, adjusting the degrees of freedom to ensure the robustness of the contrast. Furthermore, the effect of *Trichoderma* strains on the germination was analyzed using Generalized Linear Models (GLMs) with a binomial error distribution and a logit link function, independently for each species. The response variable was defined as the proportion of germinated seeds per Petri dish (with a total of 10 seeds per replicate). To validate the use of binomial distribution, a goodness-of-fit test was performed based on the ratio of residual deviance to residual degrees of freedom, discarding overdispersion as this value was less than 1 in both cases (0.75 for *Enterolobium cyclocarpum* and 0.96 for *Piscidia piscipula*). The significance of the treatments was determined through an Analysis of Deviance, which measured the change in deviance between the null model and the treatment model using a Chi-square (χ^2) test. Post-hoc comparisons were performed using the emmeans package.

RESULTS

High viability values were found for both target species, with 90.0 % ± 30.30 % for *Enterolobium cyclocarpum* and 100 % for *Piscidia piscipula*. Thus, there were no differences between species for this variable ($p > 0.05$). On the other hand, *E. cyclocarpum* exhibited significantly higher values for most seed traits compared to *P. piscipula* (Table 1), except for moisture content, where *P. piscipula* presented the highest values (Table 1).

In both species, germination was low across all treatments, including the control. Without considering fungal inoculation treatments, the control groups for *Enterolobium cyclocarpum* and *Piscidia piscipula* showed final germination values of 1.0 % ± 3.16 % and 20.0 % ± 12.47 %, respectively (Fig. 1). No significant differences were found between treatments for *E. cyclocarpum* (D = 2.88, DF = 3, $p = 0.4103$) or *P. piscipula* (D = 1.77, DF = 3, $p = 0.6193$) (Fig. 1).

DISCUSSION

Our study demonstrated results contrary to the formulated hypothesis. While we expected that the inoculation of specific *Trichoderma* strains into *Piscidia piscipula* and *Enterolobium cyclocarpum* seeds would trigger PY release, favoring higher germination levels, no significant changes were found relative to the control group in either species. These results may be related to the hardness and impermeability of the seed coat in the studied species as well as the inability of the tested *Trichoderma* strains to degrade these

Table 1. Seed and moisture traits of *Enterolobium cyclocarpum* (n = 10) and *Piscidia piscipula* (n = 10). Different letters indicate significant differences between species ($p < 0.05$).

Variable	*E. cyclocarpum* (mean ± SD)	*P. piscipula* (mean ± SD)	t	Degrees of freedom	*p*
Fresh mass (mg)	1117.04 ± 96.4[a]	24.98 ± 2.67[b]	35.779	9.0138	$5.007e^{-11}$
Dry mass (mg)	1062.4 ± 94.8[a]	22.4 ± 2.22[b]	34.678	9.0099	$6.676e^{-11}$
Moisture content (%)	4.91 ± 1.56[b]	10.23 ± 2.14[a]	6.3429	18	$5.627e^{-6}$

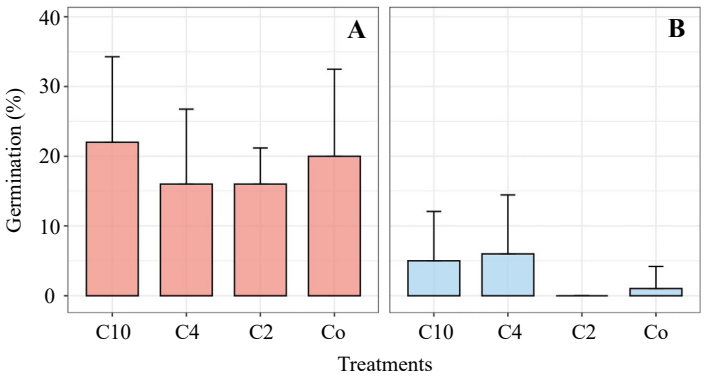


Figure 1. Final germination percentage of *Piscidia piscipula* (A) and *Enterolobium cyclocarpum* (B) inoculated with three *Trichoderma* strains (C10, C4, and C2) and a control treatment (Co). Bars represent mean germination after 30 days (± standard deviation; n = 10). Strain codes correspond to isolates from *Theobroma cacao* (C10), *Lactuca sativa* (C4) and *Zea mays* (C2).

protective layers and release physical dormancy. Published research assessing the effects of *Trichoderma* spp. on the seed ecology of tropical forest species remains scarce (dos Santos & al. 2020), particularly for *Fabaceae* species displaying PY. Conversely, under controlled laboratory, greenhouse, or field conditions, for agricultural purposes, seeds of *Lycopersicon esculentum* Mill. (Mastouri & al. 2010), *Capsicum annuum* L. (Asaduzzaman & al. 2010, Miguel-Ferrer & al. 2021), *Glycine max* (L.) Merr. (Tančić & al. 2013), *Cicer arietinum* L. (Kumar & al. 2014), *Triticum aestivum* L. (Hajieghrari & Mohammadi 2016), *Coffea arabica* L. (Ningsih & al. 2024), and *Zea mays* (Lahati & al. 2021), have consistently exhibited a faster, higher, or uniform germination.

Although *Trichoderma* spp. can erode the seed coat through enzymatic activity and mechanical pressure in species such as *Opuntia* spp. (Delgado-Sánchez & al. 2011), the efficacy of this mechanism in legumes with impermeable coats has not been sufficiently evaluated. Given the ability of this fungus to secrete a complex of lignocellulolytic enzymes—such as cellulases and xylanases—documented in studies on the degradation of structural polymers (Tengerdy & Szakacs 2003), this enzymatic activity could theoretically facilitate the breaking of physical dormancy by compromising seed coat integrity. To the best of our knowledge, the only studies describing a direct effect on seed coat morphology in wild species are those by (Delgado-Sánchez & al. 2010, 2011), who demonstrated physical erosion and cracking of the endocarp, which reduced mechanical resistance and improved germination in the genus *Opuntia*.

CONCLUSIONS

The biological treatments with *Trichoderma* strains used in this study were ineffective at breaking physical dormancy in either species. All *Trichoderma* inoculation treatments resulted in low germination rates for both species, showing no significant differences from the control. *Enterolobium cyclocarpum* seed mass (fresh and dry) was significantly higher than in *Piscidia piscipula*; however, *P. piscipula* seeds exhibited higher moisture content than *E. cyclocarpum*. Despite the negative results obtained in this work, it is pioneer and fills a significant gap in the ecophysiology of hard-seeded legumes such as studied species. Due to the scarcity of studies on species with physical dormancy, we propose that future research should evaluate the impact of different *Trichoderma* strains across a broader spectrum of legumes, integrating anatomical analyses to quantify specific changes in permeability and coat thickness following inoculation.

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