UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE ECOLOGÍA

EFECTO DE LOS TRATAMIENTOS DE ACONDICIONAMIENTO PREGERMINATIVOS EN LA GERMINACIÓN Y MOVILIZACIÓN DE RESERVAS DE ESPORAS DE HELECHOS

TESIS

QUE PARA OPTAR POR EL GRADO

DOCTOR EN CIENCIAS

PRESENTA:

Luís Vidal Pedrero López

TUTORA PRINCIPAL DE TESIS: DRA. ALMA DELFINA LUCIA OROZCO SEGOVIA
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COORDINACIÓN GENERAL DE ESTUDIOS DE POSGRADO
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P r e s e n t e

Me permito informar a usted, que el Comité Académico, del Posgrado en Ciencias Biológicas, en su reunión ordinaria del día **24 de abril de 2023,** se aprobó el siguiente jurado para el examen de grado de **DOCTOR EN CIENCIAS** del estudiante **PEDRERO LÓPEZ LUÍS VIDAL** con número de cuenta: **302139918** con la tesis titulada: **"EFECTO DE LOS TRATAMIENTOS DE ACONDICIONAMIENTO PREGERMINATIVOS EN LA GERMINACIÓN Y MOVILIZACIÓN DE RESERVAS DE ESPORAS DE HELECHOS".** realizada bajo la dirección de la **DRA. ALMA DELFINA LUCIA OROZCO SEGOVIA, quedando** integrado de la siguiente manera:

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Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 01 de agosto de 2023

COORDINADOR DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

c. c. p. Expediente del alumno

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Resumen

Para la germinación de las semillas y de las esporas de helechos, el requerimiento ambiental más importante es la humedad, de ella depende que ocurra la movilización de las reservas y la activación celular. Las esporas de helechos, al igual que las semillas, inician la germinación con una rápida absorción de agua, seguida de una fase estacionaria sin captación de agua apreciable. Durante esta fase se realizan los procesos bioquímicos y metabólicos que preceden a la germinación. La subsecuente deshidratación, que evite la germinación, es parte esencial de los tratamientos de acondicionamiento en las semillas y esporas de helechos. Después de la deshidratación, las semillas mantienen sus avances metabólicos, lo que determina el desarrollo de una memoria hídrica. Como resultado, las semillas rehidratadas germinan rápidamente. En esta investigación, se aplicaron tratamientos de acondicionamiento, en esporas de helechos, con los objetivos de: 1) inferir cómo interactúan las esporas y sus reservas energéticas, durante su permanencia en el banco de esporas, y si desarrollan una memoria hídrica, de manera semejante a la de las semillas, en respuesta a los tratamientos de acondicionamiento hídrico y natural, y 2) conocer el efecto de los tratamientos de acondicionamiento hídrico y natural, sobre la germinación y movilización de las reservas energéticas presentes en las esporas. Para evaluar el efecto de la aclimatación, en seis especies de helechos, sus esporas fueron expuestas a: cuatro u ocho días de hidratación en agua (acondicionamiento hídrico) o en una matriz de suelo (acondicionamiento mátrico); o 1 mes de hidratación en el suelo del sitio de recolección (acondicionamiento natural). Los tratamientos de acondicionamiento acortaron el tiempo de inicio de la germinación y/o incrementaron la tasa y/o el porcentaje de germinación en relación con los controles. El acondicionamiento mátrico redujo el porcentaje de germinación de las esporas en tres de las especies. Los resultados obtenidos

aportan evidencia de que, después de los ciclos de hidratación-deshidratación en condiciones naturales, las esporas desarrollaron una memoria de hidratación que probablemente evolucionó en el banco de suelo, lo que podría proporcionarles ventajas para una germinación exitosa. Durante la germinación de las esporas, la hidrólisis de los lípidos es quien proporciona principalmente la energía para activar su metabolismo. En esta investigación, los ácidos grasos (linoleico, oleico, palmítico y esteárico) se cuantificaron en las esporas de cinco especies de helechos (dos de matorral xerófilo y tres de bosque nublado) expuestas o no al acondicionamiento. Proponemos que, durante la fase de hidratación del acondicionamiento, el perfil de ácidos grasos cambiaría en concentración, dependiendo del tipo de espora (no clorofílica y criptoclorofílica). Considerando las cinco especies y los tratamientos de acondicionamiento, el ácido oleico fue el más catabolizado. Después del acondicionamiento, identificamos dos patrones en el metabolismo de los ácidos grasos: 1) en especies no clorofílicas, los ácidos oleico, palmítico y linoleico fueron catabolizados durante la imbibición y 2) en las especies criptoclorofílicas, estos ácidos grasos aumentaron en concentración. Estos patrones sugieren que las esporas criptoclorofílicas con homoioclorofila (clorofila conservada después del secado) podría no requerir el ensamblaje de un nuevo aparato fotosintético durante la imbibición de las esporas en la oscuridad. Por lo tanto, estas esporas pueden requerir menos energía de los lípidos preexistentes y menos ácidos grasos como "bloques de construcción" para las membranas celulares, en comparación con las esporas no clorofílicas, que requieren la síntesis de novo y la reestructuración del aparato fotosintético.

Abstract

For the germination of fern seeds and spores, the most important environmental requirement is humidity, on which the mobilization of reserves and cell activation depend. Fern spores, like seeds, initiate germination with rapid water uptake, followed by a stationary phase with no appreciable water uptake. During this phase, the biochemical and metabolic processes that precede germination take place. Subsequent dehydration, which prevents germination, is an essential part of conditioning treatments on fern seeds and spores. After dehydration, seeds maintain their metabolic progress, which determines the development of a water memory. As a result, rehydrated seeds germinate rapidly. In this research, conditioning treatments were applied to fern spores with the objectives of: 1) inferring how spores and their energy reserves interact during their permanence in the spore bank, and if they develop a water memory, similar to that of seeds, in response to water and natural conditioning treatments, and 2) knowing the effect of water and natural conditioning treatments on germination and mobilization of the energy reserves present in the spores. To evaluate the effect of acclimatization in six fern species, their spores were exposed to: four or eight days of hydration in water (hydric conditioning) or in a soil matrix (matric conditioning); or one month of hydration in the soil of the collection site (natural conditioning). Conditioning treatments shortened germination initiation time and/or increased germination rate and/or percentage germination relative to controls. Matric conditioning reduced the percentage of spore germination in three of the species. The results obtained provide evidence that, after hydration-dehydration cycles under natural conditions, the spores developed a hydration memory that probably evolved in the soil bank, which could provide them with advantages for successful germination. During spore germination, lipid hydrolysis mainly provides the energy to activate their metabolism. In

this research, fatty acids (linoleic, oleic, palmitic and stearic) were quantified in the spores of five fern species (two from xerophytic scrub and three from cloud forest) exposed or not to conditioning. We propose that, during the hydration phase of conditioning, the fatty acid profile would change in concentration, depending on the type of spore (non-chlorophyll and cryptochlorophyll). Considering the five species and conditioning treatments, oleic acid was the most catabolized. After conditioning, we identified two patterns in fatty acid metabolism: 1) in non-chlorophyll species, oleic, palmitic and linoleic acids were catabolized during imbibition and 2) in cryptochlorophyll species, these fatty acids increased in concentration. These patterns suggest that cryptochlorophyll spores with homoiochlorophyll (chlorophyll preserved after drying) may not require assembly of a new photosynthetic apparatus during spore imbibition in the dark. Therefore, these spores may require less energy from pre-existing lipids and fewer fatty acids as "building blocks" for cell membranes compared to non-chlorophyll spores, which require de novo synthesis and restructuring of the photosynthetic apparatus.

Capítulo 1. Introducción

¿Qué son los helechos?

Los helechos representan un grupo ancestral de plantas vasculares verdaderas, que no producen semillas si no esporas. El clado Monilophyta (grupo monofilético) comprende a los helechos, es el grupo de plantas vasculares superiores más antiguo (Mehltreter *et al.* 2010; Nitta y Ebihara 2019), y es el segundo más diverso después de su grupo hermano las angiospermas (espermatofitas), se estima que hay de 10,000 a 12,000 especies en el mundo, en México se reporta la presencia de 1024 especies (Mickel y Smith 2004), están representados por cinco linajes principales, organizados en conjuntos de especies que comparten características morfológicas aparentes y relaciones genéticas, con aumento sucesivo en el grado de complejidad morfológica y con un incremento en el registro fósil: 1) Psilotales se caracterizan por poseer un esporofito muy simple: son plantas áfilas, con prófilos, sin raíces y con un tallo que se ramifica dicotómicamente. Muchas de ellas son epífitas, sus esporangios se desarrollan en conjuntos llamados sinangios. 2) Ophioglossales es un grupo basal de helechos, de tamaño pequeño, con hojas generadas estacionalmente. Además de las hojas fotosintéticas (trofóforos), tiene hojas hemidimórficas en las que una porción la hoja es exclusivamente fotosintética y la otra es fértil (esporóforo), desarrollan asociaciones micorrízicas tanto en el gametofito como en el esporofito (Pryer *et al.* 2004; Moran 2017). 3) Equisetales es un grupo monofilético, algunas especies tienen apéndices verticilados, un eustele, hojas micrófilas o licófilos, y esporangios que se agrupan en estróbilos (Kenrick y Crane 1997; Pryer *et al.* 2001, 2004), aunque su relación con el resto de las ramas de monilofitas aún no está claramente definida, algunos estudios basados en ADN de plástidos los ubican como grupo hermano de los Marattiidae (Pryer *et al*. 2004, Smith *et al*. 2006). 4) Marattiales poseen canales mucilaginosos en raíces, tallos y hojas.

Los tallos y las hojas carnosas presentan escamas. Tienen eusporangios en la cara abaxial de la hoja en estructuras alargadas que forman sinangios. El esporangio tiene una pared gruesa y carece de anillo, cada uno porta de 1000 a 7000 esporas. 5) Polypodiales o helechos leptosporangiados; son el grupo más diverso con alrededor de 11,000 especies en el mundo, sus esporangios generan un máximo de 64 esporas; y presentan un anillo definido (Judd *et al.* 1999; Pryer *et al.* 2004; Fig. 1).

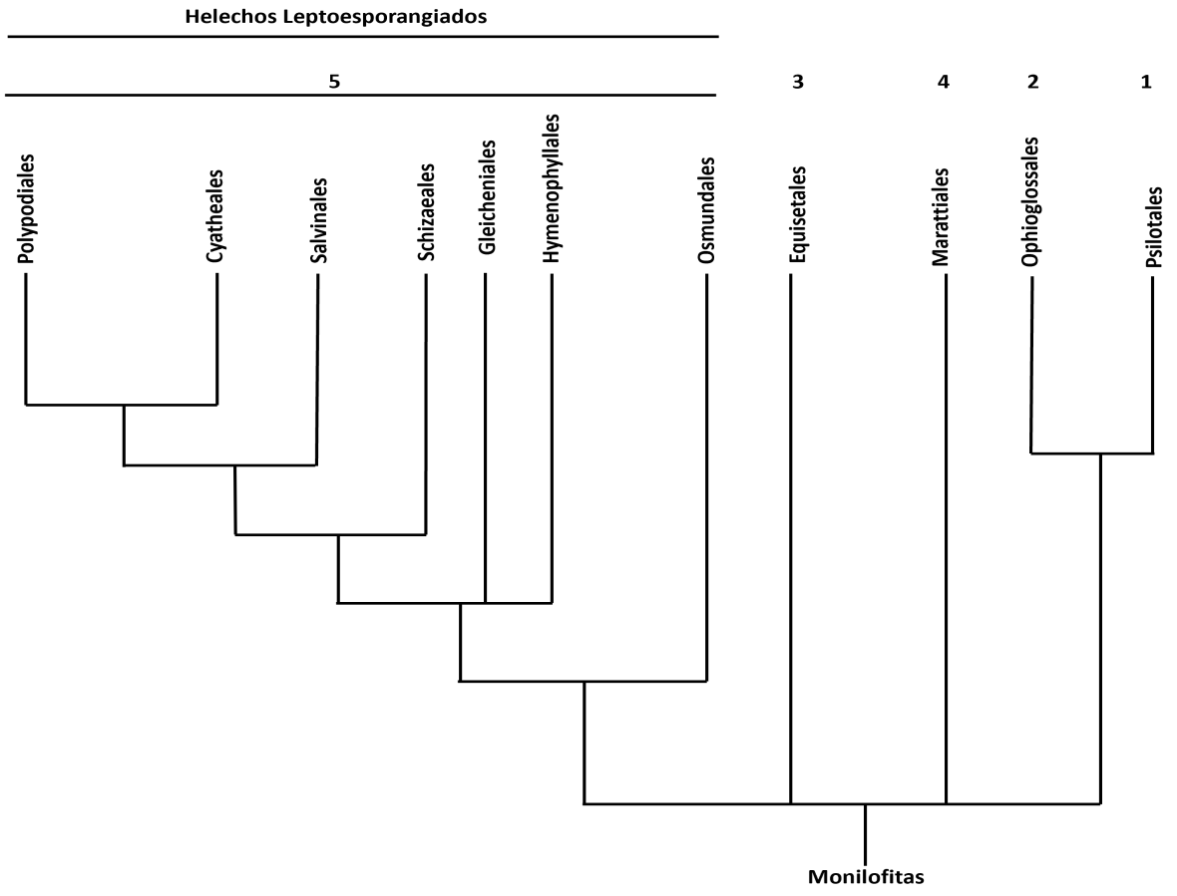

Fig. 1. Diagrama simplificado de las relaciones filogenéticas dentro del grupo de las Monilofitas. Se muestran los principales linajes de los helechos enumerados y las características sobresalientes de cada uno de ellos (Modificado por Luis Pedrero López, de: Judd *et al.* 1999; Schuettpelz y Pryer 2008).

El ciclo de vida de los helechos tiene dos fases heteromórficas, libres e independientes entre sí (Fig. 2). La fase gametofítica haploide (n) cuya función es formar gametangios da origen a los anteridios que forman anterozoides y a los arquegonios que forman a la oósfera u ovocélula. Al ocurrir la singamia (fusión de ambos gametos) se forma el cigoto, con un genoma derivado de ambos progenitores (recombinación genética), así comienza la fase esporofítica diploide (2n). Esta fase es la más aparente y dominante en el ciclo de vida de los helechos, la función del esporofito es formar las esporas. En la cara abaxial de la hoja del esporofito, se forman estructuras llamadas soros, que son conjuntos de esporangios, los cuáles contienen a las esporas (Evert y Eichhorn 2013) las que, al madurar y deshidratarse el esporangio, se dispersan de manera anemócora a cortas o largas distancias. Si las esporas encuentran las condiciones óptimas de luz, temperatura y humedad, germinan formando al gametofito, cerrando el ciclo de vida de los helechos. En caso contrario formaran parte del banco de esporas en el suelo (Dyer 1994; Esteves *et al.*, 2003).

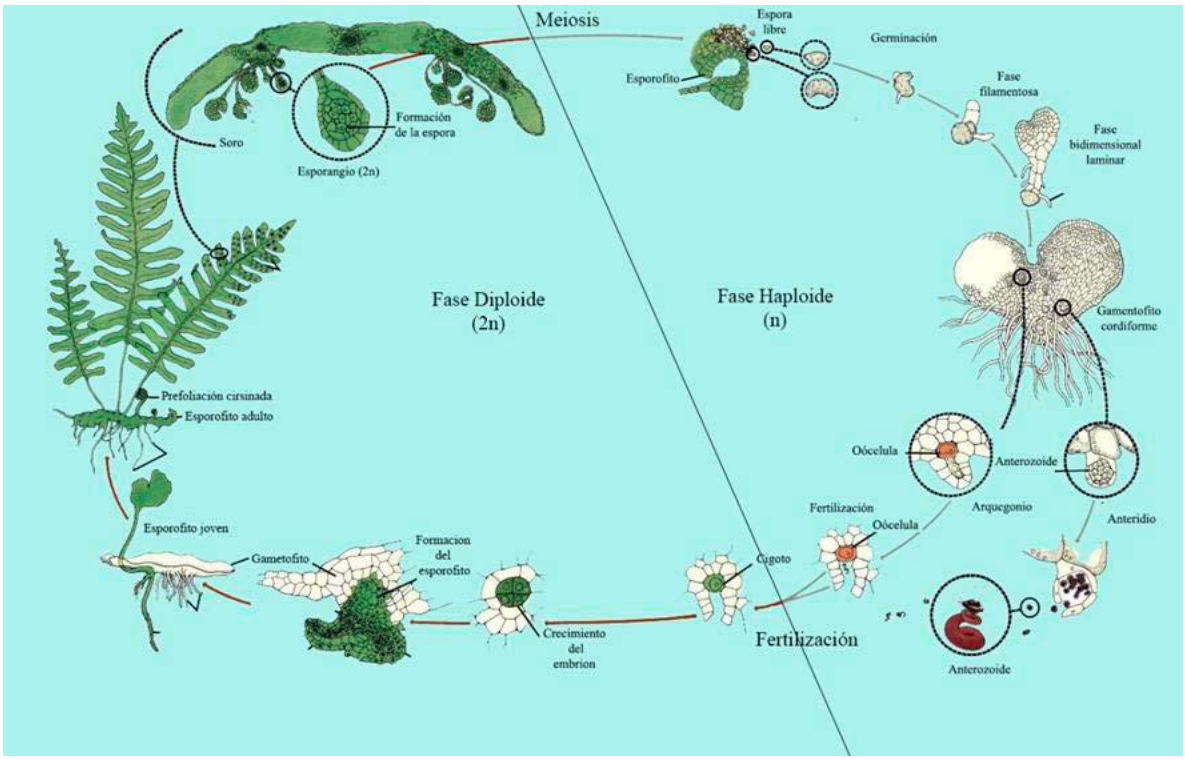

Fig. 2. Ciclo de vida de los helechos homospóricos (modificado de Evert y Eichhorn 2013).

La fisiología de la germinación de esporas de helechos

La espora de los helechos es una célula eucariótica, haploide, la cual es la unidad de dispersión y reproducción, se produce por meiosis, da origen al inicio de la fase gametofítica, sexual e inconspicua del ciclo de vida de los helechos (Raghavan 1992; Evert y Eichhorn 2013). La mayoría de las esporas pueden permanecer viables durante décadas, su viabilidad depende de la actividad metabólica de la espora, con base en esta característica las esporas se clasifican en dos tipos: las esporas no clorofílicas u ortodoxas y las clorofílicas o recalcitrantes (Pérez-García *et al.* 1994; Hock *et al.* 2006; Ballesteros 2010). Las esporas no clorofílicas son esporas de larga viabilidad con actividad metabólica reducida y germinación aletargada, su germinación comienza en promedio de 4 a 9 días con alta tolerancia a la deshidratación y altas probabilidades de conservación a temperaturas

bajas y pueden permanecer quiescentes o latentes durante años y quizá décadas (Raghavan 1989; Ballesteros 2010). Paul *et al.* (2014) reportan que las esporas de *Cheilanthes austrotenuifolia* H.M. Quirk & T.C. Chambers presentan una viabilidad durante 70 años bajo condiciones de herbario, mientras que Nagalingum *et al.* (2007) reportan que en capas de suelo de 100 años de antigüedad se encontraron esporas viables de Marsilea dentro de los esporocarpos 100-125 años, lo que sugiere una larga longevidad ecológica de las esporas de los helechos (Johnson, 1985). En contraste, el segundo tipo de esporas clorofílicas o recalcitrantes (Pérez-García *et al.* 1994; Ballesteros 2010) contienen clorofila y cloroplastos funcionales capaces de realizar la fotosíntesis a partir de su dispersión, su actividad metabólica es alta y consume sus reservas rápidamente, por lo que su viabilidad en promedio es reducida, de días a pocos meses. En consecuencia, su germinación es rápida, comienza en horas o en un par de días después de ser hidratadas. Alrededor del 7% de todos los helechos presentan este tipo de actividad metabólica. Las familias de helechos con esporas clorofílicas son Equisetaceae, Grammitidaceae, Hymenophyllaceae, Osmundaceae (Lloyd y Klekowski 1970; Moran 2017).

Se ha descrito un tercer grupo de esporas, las criptoclorofílicas (Sundue *et al.* 2011), las cuales también contienen clorofila, pero se presentan en familias de helechos en las que se refiere la presencia de esporas no-clorofilicas. En estas esporas la clorofila no puede observarse mediante microscopía óptica porque la cubierta de la espora presenta una pigmentación oscura; sin embargo, la microscopía de epifluorescencia permite observar la fluorescencia que emiten de los cloroplastos dentro de las esporas (Sundue *et al.* 2011). En este trabajo se demuestra la presencia de clorofila en esporas reportadas como no-clorofílicas, incluyendo 22 especies de *Elaphoglossum*, 10 especies de *Lomariopsis* y 12 especies de *Pleopeltis*; las cuales presentan esporas amarillentas o pardas con perisporio

aparentemente traslúcido. También Tseng *et al.* (2017) describen la presencia de esporas criptoclorofílicas en *Sphaeropteris lepifera* (Hook.) R.M. Tryon (Cyatheaceae), *Ceratopteris thalictroides* (L.) Brongn. (Pteridaceae), *Leptochilus wrightii* (Hook.) X. C. Zhang (Polypodiaceae), *Leptochilus pothifolius* (D.Don) Fraser-Jenk. (Polypodiaceae), *Lepidomicrosorum buergerianum* (Miq.) Ching & K. H. Shing (Polypodiaceae), *Osmunda banksiifolia* (Pr.) Kuhn (Osmundaceae) y *Platycerium grande* (A. Cunn.) J.Sm. en (Polypodiaceae).

La germinación de las esporas de helechos y su similitud con la germinación de las semillas

El origen y desarrollo de las esporas transcurre dentro del esporangio en el cual una célula somática se diferencia para dar origen a la espora. Una célula arqueospórica, después de cuatro divisiones mitóticas, da origen a la célula madre de la espora, que por división meiótica forma una tétrada de esporas, a este proceso se le llama esporogénesis (Raghavan 1989; Moran 2017). Las esporas obtienen sus nutrientes a partir del tapete que está dentro de cada esporangio (Lellinger 2002). Cuando las esporas están maduras, el esporangio cambia de coloración de verde a pardo oscuro. Al concluir la deshidratación del anillo del esporangio, éste se abre y libera a las esporas por el proceso de catapulta, después de lo cual el viento completa un proceso de dispersión secundaria. En condiciones ambientales adecuadas para germinar (humedad, luz, temperatura, principalmente) la activación de la espora comienza con la entrada de agua, por la lesura presente en la cubierta de la espora a la célula. El núcleo se activa y migra del centro de la célula al área proximal, desencadenándose la serie de procesos metabólicos que conducen a la germinación

(Raghavan 1989, 1992). La polaridad de la espora regula los movimientos celulares necesarios para la división celular durante la germinación.

Durante la imbibición también se inicia la movilización de reservas y del ARN (Fase I), se inicia la síntesis de proteínas y la reparación celular con la reparación de la membrana celular y de los organelos como las mitocondrias. Las reservas se utilizan en diferente orden, los primeros componentes son los carbohidratos que solo se encuentran en concentraciones muy pequeñas y con las cuales se activa la célula. Posteriormente, se inicia la Fase II con el uso de la reserva energética más abundante, los lípidos. Durante la Fase III se inicia la elongación de la célula rizoidal y, al protruir a través de las paredes de la espora ocurre la germinación. La replicación de ADN y demás eventos implicados en la división celular dan lugar a la formación de una pequeña célula en el área proximal de la espora, la cual da origen a la célula rizoidal que proporcionará agua y minerales para el desarrollo del gametofito; subsecuentemente la célula mayor comienza con otro proceso de división celular que da lugar a la célula protálica y a la formación de protoplastos y posteriormente se forma una fase filamentosa (Raghavan 1989, 1992; Bewley 1997). El criterio de germinación usado en este manuscrito es la protrusión de la célula rizoidal y/o la célula protálica, debido a que la espora se puede hinchar por absorción del agua, pero no germinar. Para la germinación de la espora el requerimiento ambiental más importante es la humedad, de ella depende que ocurra la movilización de las reservas y la activación celular. Los otros requerimientos son la luz y la temperatura, ésta última regula la actividad metabólica y delimita los intervalos de tolerancia para la supervivencia de las plantas (Miller 1968; Raghavan 1989). Por otro lado, las esporas tienen diversas características intrínsecas como

el genotipo, la edad, y/o diferencias funcionales debidas a distintas condiciones ambientales entre años de recolecta, por mencionar algunas (Ballesteros 2010).

Al ser liberadas, pueden presentar quiescencia que es una condición en la cual la espora puede germinar al encontrar las condiciones necesarias para hacerlo, o latencia, en la que la espora no germina, aunque existan las condiciones ambientales adecuadas, debido a un desequilibrio hormonal, lo que corresponde a una latencia fisiológica (Raghavan 1989), como la que presentan algunas semillas.

Viabilidad y reservas de las esporas

La viabilidad es la capacidad de las esporas de permanecer vivas conservando su capacidad para germinar. Está íntimamente relacionada con la longevidad. La viabilidad de las esporas está determinada por las condiciones ambientales como la humedad, la temperatura y la luz (Miller 1968; Banks 1999; Gabriel y Galán y Prada 2011). Los lípidos y proteínas en las esporas permanecen en el endosporio que es la pared interna de la espora, inmersos en el citoplasma de la espora, en donde se encuentran como glóbulos lipídicos y lipoproteínas (Templeman *et al.* 1987). La mayor proporción de lípidos son los triglicéridos, con contenidos variables de ácidos grasos; los principales ácidos grasos en las esporas (Gemmrich 1977), indican que la composición de lípidos difiere entre las esporas de las especies de helechos, las esporas pueden contener ácido oléico, linoléico, palmítico y otros ácidos poliinsaturados, con cadenas de 20 carbonos (Robinson *et al.* 1973; Gemmrich 1977; Seilheimer 1978). El contenido lipídico puede variar desde un 4% en *Ceratopteris thalictroides* (L.) Brongn., a un 79% en *Polypodium L*., (Gemmrich 1977; Koshiba *et al.* 1984; Sato y Fuyura 1984; DeMaggio y Stetler 1985; Gabriel y Galán y Prada 2011). Las proteínas se almacenan en glóbulos protéicos, compuestos principalmente por globulinas,

en el citoplasma (Templeman *et al.* 1988). Al comenzar la activación metabólica de las esporas, las proteínas se hidrolizan para satisfacer los procesos de desarrollo celular. Las proteínas en *Adiantum capillus-veneris L.*, son principalmente polipéptidos solubles con pesos moleculares de 14000 Da (Miller 1968; Koshiba *et al.* 1984; DeMaggio y Stetler 1985). Es difícil identificar a los carbohidratos, ya que su concentración en la espora es muy baja (Raghavan 1989). En las esporas los carbohidratos como la sacarosa y el almidón se han reportado en bajas concentraciones y son usados rápidamente durante el inicio de la germinación (Raghavan 1989; Towill 1985), cuando se movilizan las reservas lipídicas de la espora, las cuales pueden desdoblarse y formar moléculas de carbohidratos. La viabilidad de las esporas de helechos también es regulada por la temperatura a la que se encuentran en los bancos de esporas artificiales, en los cuales se controla la temperatura para prolongar la viabilidad, principalmente de esporas no-clorofílicas. La temperatura de los almacenes de esporas generalmente se mantiene a 4 °C o en almacenes criogénicos bajo decenas de grados centígrados (Ballesteros 2010; Mikula *et al.* 2010; Menéndez *et al.* 2011) también las esporas recalcitrantes pueden prolongar su viabilidad al almacenarse embebidas en la oscuridad a temperatura ambiente (Pérez-García *et al.* 1994).

Acondicionamientos pregerminativos (priming)

Los tratamientos de acondicionamiento pre-germinativos (priming en inglés) son usados en semillas con el fin de mejorar procesos agronómicos y de reproducción de especies para proyectos de restauración ecológica (González-Zertuche *et al.* 2001; Halmer 2004; Sánchez *et al.* 2001, 2005; Orozco-Segovia *et al.* 2014; Pedrero-López *et al.* 2016). Consisten en una hidratación regulada de la semilla, lo cual permite su activación metabólica. Subsecuentemente, la semilla se deshidrata para impedir su germinación, pero conserva los

avances metabólicos. Estas semillas pueden ser almacenadas y posteriormente hidratadas para su germinación. Este proceso da lugar a la vigorización de la respuesta germinativa y al incremento de la tolerancia al estrés de la semilla y la plántula (Sánchez *et al.* 2001; Pedrero-López *et al.* 2016). Los tratamientos de acondicionamiento (hidratación-deshidratación) abarcan las fases I y II de la imbibición de las semillas, por lo que permiten la movilización de las reservas almacenadas, reparar los daños de estructuras celulares como las membranas celulares, sintetizar y reparar el ADN y el ARN, y sintetizar proteínas como las LEA (Late Embryogenesis Abundant). La activación de estos avances pregerminativos que ocurren en las dos fases de imbibición reparan daños ocasionados durante la maduración y secado, que preceden a la dispersión de las semillas (Bray 1995, Orozco-Segovia y Sánchez-Coronado 2009; Varier *et al.* 2010). Los tratamientos de acondicionamiento terminan necesariamente con la deshidratación de las semillas, después de esto las semillas pueden ser almacenadas, ya que mantienen los avances metabólicos ocurridos durante el periodo de hidratación. La deshidratación al final del tratamiento constituye una señal de estrés, que sumada a los eventos ocurridos durante el período de hidratación dan como resultado el desarrollo de la "memoria hídrica" descrita por Dubrovsky (1996, 1998). En semillas de angiospermas el efecto del acondicionamiento repercute favorablemente el vigor y revigorización de las semillas y por lo tanto en la germinación, el crecimiento, el establecimiento y la sobrevivencia de las plántulas resultantes.

Se puede asumir que estos procesos metabólicos ocurren en cualquier célula de la semilla y por lo tanto se puede asumir que ocurren también en las esporas de los helechos, como lo sugiere la prolongación de la viabilidad en semillas (Villiers, 1973; Pedrero-López *et al.* 2019) y en esporas almacenadas embebidas (Pérez-García *et al.* 1994). Pedrero-López

et al. (2019) demostraron que los tratamientos de acondicionamiento también aumentan el vigor de las esporas durante la germinación. En la figura 3 se muestran las fases de la imbibición completa (fases I-III) o interrumpida durante los tratamientos de priming (fases I y II), y los avances metabólicos que suceden durante cada una de ellas.

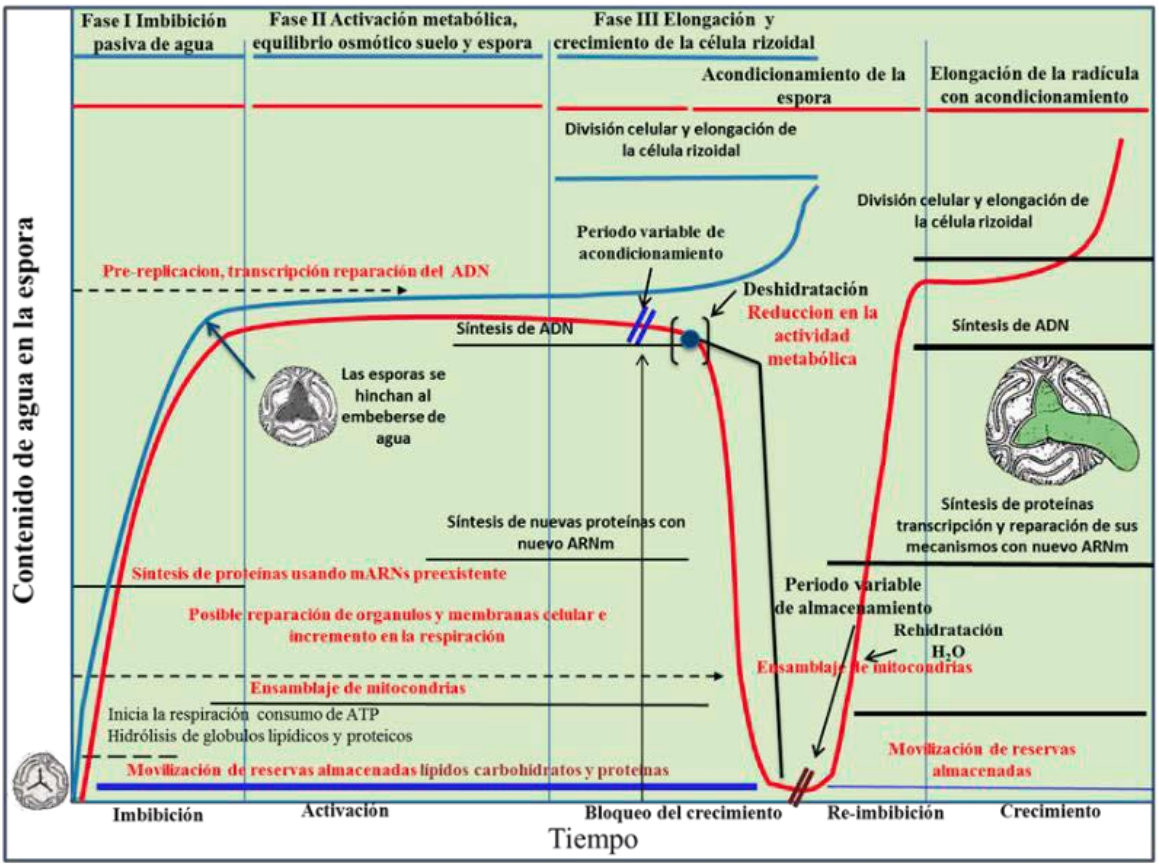

Fig. 3. Eventos metabólicos que ocurren durante el proceso de la germinación de las esporas de helechos, de acuerdo con el patrón trifásico de la absorción del agua (en azul) y los posibles eventos que acontecen durante la aplicación de los tratamientos de acondicionamiento pregerminativo (en rojo; Raghavan 1989; Bewley 1997; Varier *et al.* 2010 (modificado por Luis Vidal Pedrero-López).

El acondicionamiento en las semillas se logra mediante varios métodos de hidratación controlada; entre los más usados se encuentran los siguientes: 1) acondicionamiento

osmótico, que consiste en la hidratación de las semillas en soluciones osmóticas de solutos como polietilenglicol o NaCl entre otros, 2) acondicionamiento hídrico, consiste en uno o más ciclos de hidratación-deshidratación en agua 3) acondicionamiento mátrico, que se consigue con la hidratación de las semillas en una matriz húmeda, como la vermiculita, la agrolita o el suelo, en el laboratorio, entre otros. En este caso, la hidratación se lleva a cabo de manera muy similar a la forma en que las semillas toman el agua del suelo (Wuest 2007), y 4) acondicionamiento natural (AN), desarrollado por González-Zertuche *et al.* (2001), el cual consiste en enterrar en el campo, a las semillas en el suelo de la comunidad natural por un tiempo similar al que separa la dispersión de los propágulos de las primeras lluvias, para que perciban las condiciones ambientales. Durante este tiempo el ambiente prepara a la semilla para dar una respuesta germinativa rápida y sincrónica y genera una tolerancia o resistencia a condiciones de estrés ambiental, por lo que se ha propuesto que la respuesta de las semillas al acondicionamiento (realizado en el laboratorio) ha evolucionado de manera natural durante la permanencia de las semillas en el banco del suelo. La respuesta funcional de las esporas debe también estar asociada a procesos fisiológicos que les permiten percibir su ambiente y establecerse en condiciones adecuadas; un ejemplo de esto sería la percepción de que los requerimientos para la germinación están cubiertos en semillas quiescentes, o la percepción de las condiciones para romper o imponer latencia secundaria que son bien definidos en semillas y se han descritos someramente en esporas. La percepción de una señal ambiental está mediada por receptores que se unen a una cadena de transducción, la respuesta y el proceso en general está mediado por genes. La percepción del ambiente genera una mayor probabilidad de establecimiento en el ecosistema (Lüttge 2011). En esta investigación la aplicación de tratamientos de acondicionamiento en esporas de helechos tiene la intención de entender cómo interactúan las esporas con el suelo durante

su permanencia en el banco de esporas, a partir de los tratamientos de acondicionamiento hídrico y natural, y su efecto sobre su germinación.

Justificación del proyecto

La germinación de esporas de helechos ha sido poco estudiada desde un punto de vista ecofisiológico, es decir tomando en cuenta el ambiente en que germinan, por lo que es un tema de investigación original explorar su respuesta a tratamientos de acondicionamiento pregerminativos, la cual tiene como base la memoria hídrica definida en semillas por Dubrovsky (1996, 1998). Esta memoria, en semillas, proporciona una mayor oportunidad de establecimiento de las especies en los ecosistemas naturales, como lo describe Dubrovsky (1996, 1998) para semillas de cactáceas. Con respecto al acondicionamiento hídrico Pangua *et al.* (2009) mencionan que la memoria hídrica no se puede presentar en esporas ya que a diferencia de las semillas las esporas son unicelulares. Sin embargo, los procesos que ocurren en las semillas durante la hidratación son los mismos que regulan la hidratación de las células individuales. Por esto, la presente investigación que tiene una visión novedosa, pionera en la aplicación de tratamientos de acondicionamiento en esporas de helechos tiene como objetivo: 1) inferir cómo interactúan las esporas y sus reservas energéticas, previamente a su germinación en el suelo durante su permanencia en el banco de esporas, a partir de los tratamientos de acondicionamiento hídrico y natural, y evaluar si desarrollan una memoria hídrica semejante a la de las semillas, 2) conocer el efecto de los acondicionamientos pregerminativos sobre la germinación y movilización de sus reservas energéticas.

Hipótesis

• Si las esporas responden a los tratamientos de acondicionamiento pregerminativo hídrico, mátrico y/o natural, entonces las esporas germinarán con mayor vigor que las esporas control.

• Si la respuesta germinativa de las esporas al acondicionamiento mátrico y/o natural es análoga a la de las semillas, entonces la respuesta germinativa de las esporas, a este tratamiento, diferirá de acuerdo con las condiciones ambientales del hábitat que ocupan.

• Si, como ocurre en las semillas, la aplicación de tratamientos de acondicionamiento favorece la germinación de esporas de helechos, entonces se pueden esperar cambios en la movilización de sus reservas en respuesta a los tratamientos de acondicionamiento.

Objetivos

• Determinar el efecto de los tratamientos de acondicionamiento hídrico, mátrico y/o natural en el vigor de la germinación, con respecto a esporas control.

• Evaluar el efecto de los tratamientos de acondicionamiento, hídrico, mátrico y/o natural, en esporas de especies de helechos del bosque mesófilo y del matorral xerófilo.

• Comparar entre las especies estudiadas el efecto de los tratamientos de acondicionamiento hídrico y mátrico en la movilización de las reservas (lípidos y proteínas) de las esporas.

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Can fern spores develop hydration memory in response to priming?

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ABSTRACT

Fern spores and seeds initiate germination with fast water uptake, followed by a stationary phase with no appreciable water uptake and biochemical and metabolic processes that precede germination. After that, seed, germination is avoided by dehydration, as part of the priming treatments. After dehydration, seeds maintain their metabolic advances (hydration memory). As a result, rehydrated seeds germinate rapidly. We hypothesized that, as seeds, fern spores may be capable of developing hydration memory. To assess priming, spores of six fern species were exposed to: four or eight days of hydration in water (hydro-priming) or in a soil matrix (matrix-priming); or 1 month of hydration in the soil of the collection site (natural-priming). At the end of the treatments, the spores were dehydrated in the dark and germinated under laboratory conditions. Germination was evaluated using lag-time, germination rate and germination percentage. Priming treatments shortened lag time and/or increased germination rate or germination percentage in relation to the controls. Matrix-priming (8 days) reduced the spore germination percentage in three species. Our results provide evidence that fern spores possess a hydration memory that probably evolved in the soil bank and suggests that hydration-dehydration cycles within the natural soil might provide advantages for successful germination.

1. Introduction

Ferns present a heteromorphic life cycle with two independent phases. The gametophytic phase is initiated with the spore, which is the unit of dispersal. Spore germination begins with the imbibition of water, followed by activation of its metabolic process and ends with cellular elongation and protrusion of the rhizoidal cell. Thereafter, the gametophyte develops through diverse phases of growth until it reaches the sexual maturation phase when the antheridia (male) and/or the archegonia (female) are developed (Sharpe et al., 2010).

Germination is a triphasic physiologycal process that is similar in seeds and fern spores (Raghavan, 1989): Stage I, imbibition (passive intake of water); stage II with no appreciable intake of water but with metabolic activation, DNA and mRNA transcription, cell reserves mobilization (mainly lipids and proteins in spores) (Raghavan, 1989) and restoration of macromolecules, cellular membranes and organelles; and finally, stage III, which consists in the protrusion of the root (for seeds) or rhizoidal cell (in spores), cell elongation and in some cases, cell division (Bewley, 1997). These similarities are, however, analogous because fern spores are homologous to the pollen of the seed plants, which are both microspores (Sharpe et al., 2010).

Priming treatments are used to improve seed germination. The treatments allow the first two imbibition phases to occur but avoid germination through seed dehydration (Henckel, 1964). After priming, seeds can be stored and subsequently re-hydrated to germinate faster and more uniformly because they retain the metabolic advances of being in stage II. This effect of priming in seeds is called “hydration memory” (Dubrovsky, 1996, 1998). Consequently, priming is used to improve seed germination (early, fast and uniform), increase of tolerance to stress (e.g., drought salinity, heat, etc.) and crop performance (Bradford et al., 1989; Sánchez et al., 2001, 2003). Priming has also been used to improve efforts of ecological restoration (González-Zertuche et al., 2001; Pedrero-López et al., 2016). Despite the dehydration tolerance of spores (Hoekstra, 2002), it has been suggested that due to their unicellular nature, they may not develop hydration memory (Pangua et al., 2009).

Abbreviations: C, Control; HP, hydro-priming; MEXU, The National Herbarium of Mexico; MP, matrix-priming; NP, natural-priming; UAMIZ, The herbarium Universidad Autónoma Metropolitana Iztapalapa

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During laboratory priming, seeds can be imbibed in water (hydro-priming) in osmotic solutions of polyethylene glycol, salt or others (osmo-priming) or in solid matrices (vermiculite or soil, matrix-priming). In nature, seeds and spores may remain viable after dispersal and build up a seed or spore soil bank (Pérez-García et al., 1982; Dyer and Lindsay, 1992; Chambers and MacMahon, 1994; Dyer, 1994). In the soil bank, seeds and spores are exposed to cycles of partial or total hydration and dehydration. Because, these cycles have similar effects in seeds as those induced by priming, this process has been called "natural-priming" (González-Zertuche et al., 2000, 2001). Propagules in the soil sense the environment (moisture, temperature and light), and prepare the seeds and seedlings for the changing and heterogeneous environment that surrounds them (Castro-Colina et al., 2012; Benítez-Rodríguez et al., 2014; Orozco-Segovia et al., 2014). González-Zertuche et al. (2001) proposed that seed responses to priming have probably evolved in the soil as an adaptation to prepare seeds for rapid and uniform seed germination and successful establishment of the seedling.

After dispersal, both orthodox pluricellular seeds and unicellular pollen and spores are tolerant to dehydration. Although that in *Pleopeltis polypodioides* (Oliver et al., 2000) and other resurrection plants (Proctor and Pence, 2002), the tolerance to hydration-dehydration cycles has been studied from the biochemical, ecological and other diverse points of view, it has not been studied in fern spores during germination. This tolerance may have evolved from unicellular to pluricellular organisms during the colonization of land through similar morphological, biochemical and physical processes (Proctor and Pence, 2002; Graff and Oliver, 2013; López-Pozo et al., 2018). Although seeds and spores are not homologous dispersal units (Sharpe et al., 2010), both might develop hydration memory after priming (hydration-dehydration cycles), which may be an evolutionary advantage in the soil bank. However, it has not been studied in fern spores, and the development of this memory in the soil bank has been scarcely documented in seeds. Consequently, we hypothesized that unicellular fern spores may have hydration memory after priming, in natural and laboratory conditions, that might also be an advantage for fern spores remaining active in the soil bank (Chambers and MacMahon, 1994).

Therefore, we assessed the response of spores of six fern species from two environments (cloud forest and xerophyllous shrubland) to hydro-priming, matrix-priming and/or natural-priming by evaluating their germination percentage, germination rate (velocity) and lag time for germination in comparison to the control treatments of spores that were not subjected to priming.

2. Materials and methods

2.1. Spore collection

We collected spores from six fern species growing at three different locations: 1) *Llavea cordifolia* Lang., *Pellaea ovata* (Desv.) Weath. and *Thelypteris ovata* R. St. John in the year 2009 at Zacualtipán, Hidalgo, México; 2) *Sphaeropteris horrida* (Liebm.) R.M. Tryon and *Thelypteris rudis* (Kunze) Proctor. in 2012 and 2013 in Tlatlauquitepec, Puebla and 3) *Polypodium thyssanolepis* A. Braun ex Klotzsch., in 2012 and 2013 in the reserve "Parque Ecológico de la Ciudad de México", México City. Spores of all species were obtained from more than ten fertile individual plants. The corresponding voucher specimens for each species were stored in the herbarium of the Universidad Autónoma Metropolitana Iztapalapa (UAMIZ) as well as in the National Herbarium of Mexico (MEXU). To favour sporangia opening and subsequent spore liberation, pinnae were stored in paper envelopes and dried for one week in a dark room (21.6 ± 1.8 °C, relative humidity [RH] = 38.7%) and thereafter, spores were separated from leaf and sporangia fragments using a sieve of phytoplankton mesh with 074 mm openings.

2.2. Cultured of spores and observation of germination

Spores were sown with a brush on the cultivation medium. For each species and treatments, we sowed five replicates (five Petri dishes). The dishes were incubated at 20 °C in a germination chamber (Lab-Line Instruments, Inc., 844, Melrose Park, IL, U.S.A.) equipped with cool white fluorescent light (F20T12/CW, Sylvania, 20 W, Danvers, MA, U.S.A.). The photoperiod was 12/12 h (light/darkness). The percentage of spore germination was obtained by counting the number of germinated and ungerminated spores, in three randomly selected areas of 0.5 cm^2 for each dish and calculating the average of this number for each Petri dish. In all the dishes. The germination was evaluated every third day with a stereoscopic microscope (Nikon SMZ18, Japan), over the course of 60 days, regardless of whether the germination stopped earlier.

2.3. Natural priming (one month)

Before treatments, 100 mg of spores of each of the six species were placed inside phytoplankton mesh bags (20 µm opening). For the natural-priming treatment (NP), one bag of each of the six species was buried in the soil of their respective collection site for one month, unearthed and subsequently, dehydrated for two days in a dark room to avoid spore germination (21.6 ± 1.8 °C, relative humidity [RH] = 38.7%). The controls (C) were spores without treatment.

2.4. Hydro-priming and matrix-priming for four and eight days

Prior to treatments, 100 mg of spores of each of the six species were placed inside phytoplankton mesh bags. For hydro-priming (HP), the bags were soaked in sterile water for four (HP4) or eight (HP8) days and subsequently dehydrated in the laboratory for two days. For matrix-priming, individual bags of spores of each of the six species were buried in the soil of the collection site for four (MP4) or eight (MP8) days and then dug up and dehydrated for two days; dehydration was in the dark to avoid germination of spores. The controls were spores without priming treatment.

2.5. Cultivation medium and disinfection

After dehydration, spores that were collected in 2009 were sown on agar plates (at 1%, 10 g/L, agar/current water) in the incubation chamber at 25 °C, 12/12 h (day/night). In the case of spores collected in 2012–2013, due to the massive development of fungi on the culture medium, spores of the three species had to be disinfected prior to sowing and germinated on Thompson's medium (Klekowski, 1969) and germinated.

Due to a massive fungi development on the agar, the spores of *P. thyssanolepis*, *S. horrida* and *T. rudis* had to be disinfected with 1% calcium hypochlorite ($Ca[ClO]_2$, J. T. Baker, USA) with one droplet of Tween 20 (polyoxyethylene sorbitan monolaurate, Lipoquimia, Mexico) for each 100 mL of distilled and sterilized water. Spores were centrifuged once for five minutes in the disinfectant and twice for five-minute periods in distilled water. The spores were dried and sown, using a tiny paintbrush, in sterilized Petri dishes (5 cm in diameter) on Thompson's medium plates with 150 µL of Fluoconazole (Altia, Mexico, diluted in 150/98 *g*/mL of sterilized and distilled water to obtain a 10,000 ui/mL of antimycotic solution) and 100 µL antibiotic (from Cefotaxime, Sanofi, Mexico, diluted in 1/4, g/mL of water) for every 500 mL of agar (MCD LAB, Mexico, 1%) prior to gel solidification. After disinfection, the spores did not germinate on simple agar plates. The culture medium was autoclaved prior to sowing. The dishes were sealed with cellophane and kept inside hermetic sealed plastic bags.

2.6. Statistical analyses

Germination percentages were arcsine transformed (Zar, 2010) and the germination data were fitted to exponential sigmoid curves of the form $y = a/[a + b^{(-ex)}]$ with fittings done in TableCurve 2D, v3 (AISN Software, Chicago, IL, U.S.A.). The exponential sigmoid curve was used to estimate the lag time (time to initiate germination) and the germination rate (germination velocity) like the first derivative maximum (the slope at the inflection point). Both parameters and the final germination percentages were analysed. The assumptions of the parametric tests were assessed. The NP and control data were analysed using Student's t-tests or the Mann-Whitney U test for each species; with a significance level of $P \leq 0.05$. Data of the HP, MP and control treatments were analysed using the two-way Analysis of Variance (ANOVA, priming treatment and priming time). The post-hoc comparisons were done using the Tukey test.

3. Results

3.1. Natural-priming (one month)

In the three species collected in 2009, we observed that the application of NP invigorated germination, at least in a germination parameter (Table 1, Fig. 1). In the spores of *L cordifolia* and *P. ovata,* NP significantly shortened the lag time from 5.74 in the control to 5.21 in NP and from 3.98 in the control to 2.97 in NP, respectively. On the contrary, in *T. ovata*, lag time increased from 1.68 to 2.5 days. The germination rate was not significantly modified by NP in *L. cordifolia* and *P. ovata.* Only in *T. ovata*, NP decreased the germination rate, significantly, from 13.39% d^{-1} in the control to 9.42% d^{-1} in NP. The germination percentage was increased by NP in *L. cordifolia* and *P. ovata* from 97.69 to 100% and from 95.79 to 100%, respectively. The difference in *T. ovata* was that the NP did not affected the germination percentage.

In the spores of the three species that were collected in 2012–2013 and sterilised before being sown, NP had, in general, a significant favourable effect on the germination parameters (Table 1, Fig. 1). The lag time of *P. thyssanolepis* and *S. horrida* was reduced from 1.58 in the control to 1.35 days in NP and 1.40 in the control to 1.04 days in NP, respectively. Natural priming, however, had no effect on the spores of *T. rudis*. In terms of the germination rate, *P. thyssanolepis* did not exhibit the effects of the treatments but it increased in *S. horrida* from 8.62% d^{-1} in the control to 12.77% d^{-1} in NP and in *T. rudis*, from 6.98% d^{-1} in the control to 8.13% d^{-1} in NP. Finally, the germination percentage was not affected by the treatments on *P. thyssanolepis* but NP induced higher values in *S. horrida* (from 89.28% in the control to 100% in NP) and in *T. rudis* (from 98.29% in the control to 99.60% in NP).

3.2. Hydro-priming and matrix-priming for four and eight days

Hydro-priming and MP had favourable effects on the spore germination of the six studied species. However, the germination parameters were affected differentially between the species (Table 1; Fig. 2): priming treatments (MP and HP) shortened the lag time in *L. cordifolia* from 3.54 days in the control to 2.14 days in MP8 and in *P. ovata,* from 4.59 days in the control to 3.36 days in MP4. However, the exposure time to priming and its interaction with priming treatments, were not significant for these two species. While, in *T. ovata,* there were no significant effects of the treatments on lag time, the germination rate was significantly increased in *L. cordifolia* by all the priming treatments, in respect to the control. In *P ovata,* only the priming treatments significantly affected germination rate: HP4 and HP8 induced higher values (8.93 and 10.3% d^{-1} respectively) in relation to MP4 (4.55% d^{-1}) and the control (5.38% d^{-1}). Additionally, the interaction between the exposure time and priming treatment was significant; MP8 was not significantly different from the control. While, in *T. ovata,* the germination rate was increased significantly from 10.03% d^{-1} in the control to > 15% d^{-1} in all priming treatments without significant differences

Table 1
Effect of priming (Hydro-priming or Matrix) versus control treatments (results of two-way Analyses of Variance) on the germination parameters of the spores of six fern species from Zacualtipán (Hidalgo), Parque Ecológico de la Ciudad de México (Ciudad de México) and Tlatlauquitepec (Puebla). Sterilized spores were sown on Thompson medium. (n. s. = non significant).

Germination parameter	Spore	Collection		Priming		Days		Interaction	
Species	sterilization	year	site	$F_{(2,29)}$	P	$F_{(1,29)}$	P	$F_{(2,29)}$	P
Llavea cordifolia	No	2009	Hidalgo						
Lag time				26.55	< 0.001	0.01	n. s.	0.01	n. s.
Germination rate				37.68	< 0.001	4.15	0.05	8.05	0.002
Germination percentage				461.53	< 0.001	0.87	n. s.	0.87	n. s.
Pellaea ovata	No	2009							
Lag time				34.45	< 0.001	0.16	n. s	0.16	n. s.
Germination rate				12.28	< 0.001	1.39	n. s	0.35	n. s.
Germination percentage				6579.81	< 0.001	0.0	n. s.	0.0	n. s.
Thelypteris ovata	No	2009							
Lag time				3.28	n. s	0.11	n. s.	0.59	n. s.
Germination rate				78.14	< 0.001	1.21	n. s.	0.084	n. s.
Germination percentage				122.42	< 0.001	0.04	n. s.	0.04	n. s.
Polypodium thyssanolepis	Yes	2012–2013	Ciudad						
Lag time			de	16.94	< 0.001	0.03	n. s.	23.27	< 0.001
Germination rate			México	16.85	< 0.001	3.69	n. s	6.77	0.004
Germination percentage				27115.95	< 0.001	320.5	< 0.001	320.5	< 0.001
Sphaeropteris horrida	Yes	2012–2013	Puebla						
Lag time				38.87	< 0.001	10.51	0.003	7.24	0.003
Germination rate				136.52	< 0.001	92.97	< 0.001	23.40	< 0.001
Germination percentage				610.80	< 0.001	13.71	0.001	14.65	< 0.001
Thelypteris rudis	Yes	2012–2013							
Lag time				19.65	< 0.001	9.14	0.005	2.68	n. s
Germination rate				30.17	< 0.001	24.20	< 0.001	7.98	0.002
Germination percentage				493.63	< 0.001	80.53	< 0.001	81.83	< 0.001

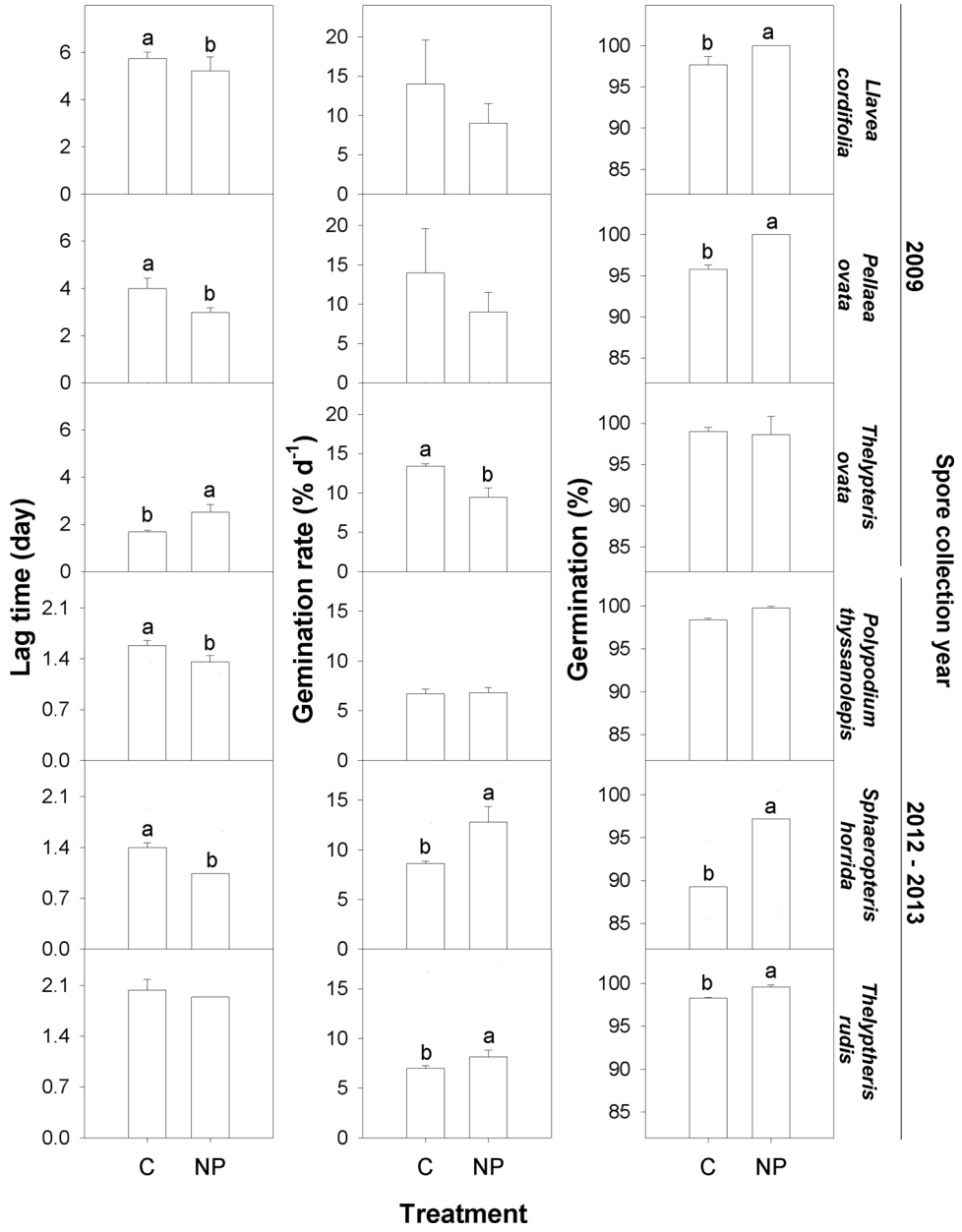

Fig. 1. Germination parameters of the spores of six fern species after different priming treatments before being sown in agar (1%) without sterilization. (C = control, NP = natural priming). The spores were collected at three different localities in México: 1) Zacualtipán, Hidalgo (*Llavea cordifolia*, *Pellaea ovata* and *Thelypteris ovata*), 2) Ciudad de Mexico (*Polypodium thyssanolepis*) and 3) Tlatlauquitepec, Puebla (*Sphaeropteris horrida* and *Thelypteris rudis*). Collection years are pointed out. Lowercase letters indicate significant differences between treatments. Mean ± SD (n = 5).

between treatments. The germination percentage was significantly improved only for priming treatments; primarily HP followed by MP. In *L. cordifolia* and *T. ovata*, HP induced the significantly highest value, (100%). In the control, the lowest values were found (90.05 and 84.41%, respectively). In *P. ovata*, both HP and MP induced 100% of germination, significantly higher than in the control (91.48%).

In the spores collected in 2012–2013, significant differences were found between priming treatments in the three species (Table 1, Fig. 3). The lag time of the spores of the three species was reduced for HP4 but in *P. thyssanolepis* and *T. rudis*, MP increased it. The lag time of spores of *S. horrida* was affected due to the priming treatments, the exposure time and their interaction. The significantly shortest value was found in HP4 (2.78 days) in respect to the control (six days). The lag time in *T. rudis* was affected by the priming and exposure time. HP4 significantly shortened the lag time (0.54 days) in comparison to the longest lag time due to MP8 (3.47 days) and the control (2.45 days).

The germination rate was affected, in spores collected in 2012–2013 by the priming treatment and its interaction with the exposure time (Table 1, Fig. 2). In *P. thyssanolepis*, HP8 (6.69% d^{-1}) and MP4 (5.96% d^{-1}) produced germination rates that were significantly higher than that of the control (5.09% d^{-1}). The germination rate in *S. horrida* and in *T. rudis* was affected by the priming, the exposure time and their interaction. In *S. horrida*, the significant highest rate was induced by HP8 (5.1% d^{-1}) and the lowest by MP4 (2.19% d^{-1}) and the control (2.62% d^{-1}). In *T. rudis*, the germination rate was significantly higher in MP8 (7.38% d^{-1}). In the other treatments, the germination rate was not different than that of the control (4.57% d^{-1}). The germination percentage of *P. thyssanolepis* was affected significantly by the priming treatments, the exposure time and their interaction. Spores in both the HP treatments and control, germinated at 100%. MP reduced the germination percentage: the lowest value was induced for MP8 (50.64%), which is significantly lower than in MP4 (64.56%). The germination

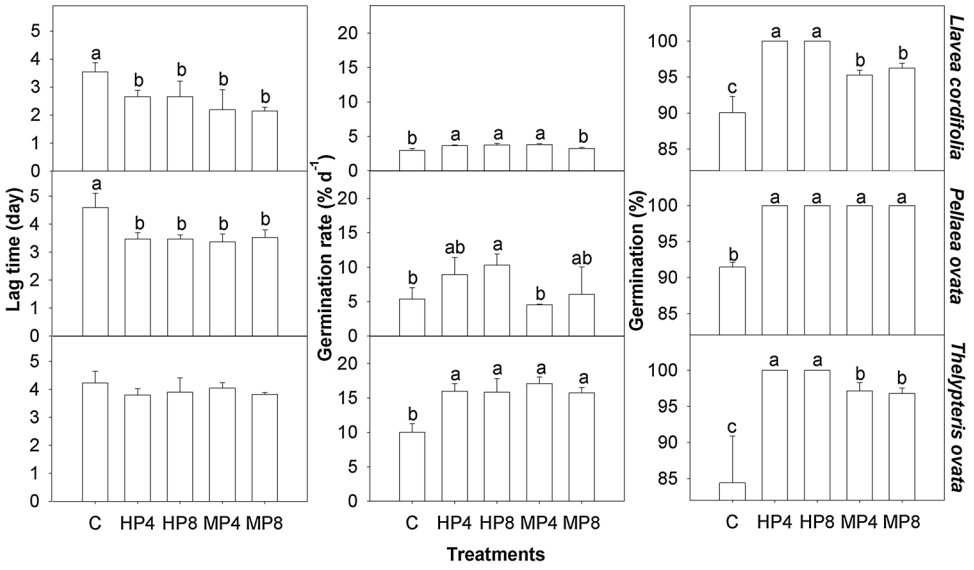

Fig. 2. Germination of the spores of three fern species collected in 2009 at Zacualtipán, Hidalgo. The number in the labels indicate the days that spores remain in the hydro-priming (HP) and matrix-priming (MP) treatments and control (C) before being sown in agar (1%) without sterilization. Lowercase letters indicate significant differences between treatments. Mean ± standard deviation (n = 5).

percentage in *S. horrida* and *T. rudis* was affected by the priming, the exposure time and their interaction. In *S. horrida*, HP induced the highest values, nevertheless, HP8 (92.7%) was significantly higher than HP4 (85.32%). The lowest values were found in MP (48.27 and 50.77%). In *T. rudis*, both HPs produced the highest values (95.23 and 95.26%) while MP8 produced the lowest (56.38%); this last value was significantly lower than the control (81.41%).

4. Discussion

Fern spores are tolerant to desiccation (López-Pozo et al., 2018), but the tolerance to hydration-dehydration cycles during priming treatment has not been studied in fern spores. In this work, we showed that after rehydration, spores of the six studied species responded favourably to all priming treatments on at least one germination parameter – usually in terms of reduction of the lag time and/or the increase in the germination rate. During priming treatments did not always, all fern spore species and the same germination parameters are favoured, as occur in seeds (Orozco-Segovia et al., 2014). Priming favours mainly the seed germination rate and/or lag time while the germination percentage can, sometimes, be reduced by priming (Bray, 1995). The germination advances are a result of what is called, "hydration memory". On the contrary, reduction in seed germination can be a result of advances in germination (closest to rhizoid or radicle emergence) reaching the "non-return point" (Schopfer and Plachy, 1984) and thereafter, dehydration results probably in spore death. In the fern spores collected in 2012–2013, only MP for four and eight days produced a pronunced germination percentage reduction. However, spores collected in 2009 survived NP after a month, additionally, has been reported that early germinated seeds can survive dehydration (Bruggink and Van der Toorn, 1995). Alternatively, secondary dormancy was probably acquired in MP for *P. thyssanolepis*, *S. horrida* and *T. rudis*, whose ungerminated spores remained apparently healthy on the agar layer for 60 days; this has been observed in other ferns' spores as a result of adverse environmental conditions in the imbibed state (Juárez-Orozco et al., 2013).

The fern spores are unicellular, but, as in seeds, imbibition is regulated by the gradient of water potential between cells and substrate. Thus, in the stationary phase (Bewley, 1997), in both spores and seeds, cell metabolic activation and macromolecules reparation occurs (such as in spores of *Adiantum capillus-veneris* L., Koshiba and Minamikawa, 1984; Raghavan, 1989; Bray, 1995). The germinative

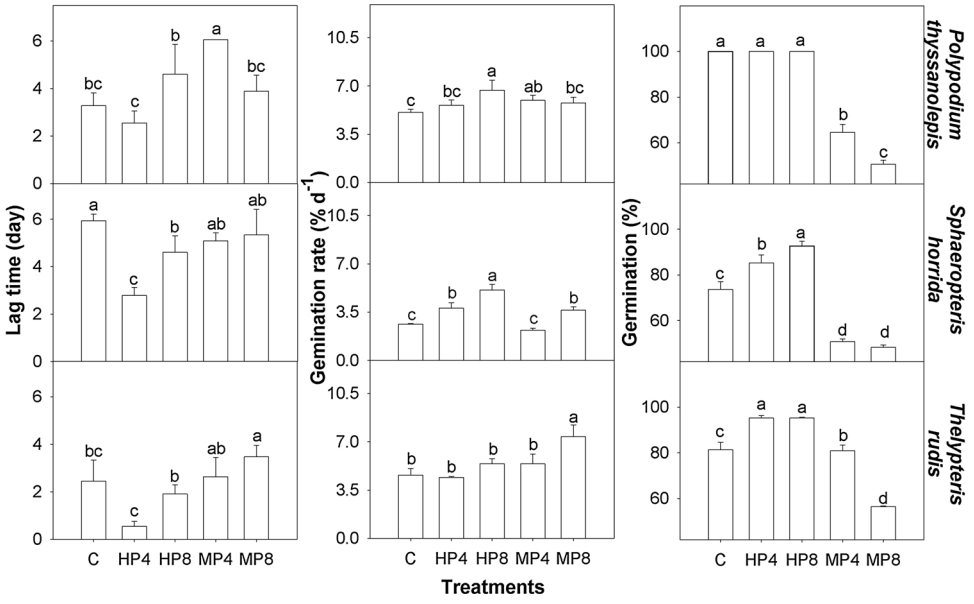

Fig. 3. Germination of the spores of three fern species collected in 2012–2013 at Ciudad de Mexico (*Polypodium thyssanolepis*) or Tlatlauquitepec, Puebla (*Sphaeropteris horrida* and *Thelypteris rudis*). The number in the labels indicate the days that spores remained in the hydro-priming (HP) and matrix-priming (MP) and control (C). The spores were sterilized before being sown in Thompson medium. Lowercase letters indicate significant differences. Mean ± standard deviation (n = 5).

advances occurring during the imbibed state, apparently, were also kept after fern spore dehydration at the end of the priming treatments, resulting in the developing of a hydration memory. This is a new discovery for plant science that refutes previous conjectures and suggestions about the lack of hydration memory in fern spores (Pangua et al., 2009) and open new questions in plant physiology and evolution: for e.g., are mechanisms that confer hydration memory to seeds the same as those that are present in fern spores? The fact that we found in both the spores that were sown on agar (non-sterilised) or on the Thompson medium (sterilized), a favourable effect of some of the priming treatments was found that emphasizes the relevance of hydration-dehydration treatments in the development of hydration memory and its consequences in spore physiology and, probably, in the ecology of fern spores.

Plants (pteridophytes *s.l.*, gymnosperm, and angiosperms) probably acquired tolerance to desiccation from the colonization of land and from cyanobacteria and unicellular algae (Proctor and Pence, 2002; Dekkers et al., 2015). Further, it has been suggested that the basic mechanisms that confer desiccation tolerance to seeds may have evolved from those present in the spores of precedent taxa, such as ferns (Graff and Oliver, 2013). And this could be the case in the response to priming. According to Sharpe et al. (2010) and Ballesteros et al. (2017), the morphological and the biochemical mechanisms involved in fern tolerance to desiccation might be homologous to those in lower and higher taxa of plants. Thus, it is not surprising that -in fern spores might be evolved the tolerance to hydration-dehydration cycles in the soil bank, as in seeds (González-Zertuche et al., 2001). This assumption results from the effect of NP on seed germination (burying the seeds in the soil, in natural conditions, dug up and drying). The germination response to NP, MP and HP exhibited by the studied species demonstrated that fern spores may have developed hydration memory.

After dispersal, the soil is the substrate where most propagules remain for variable intervals of time until adequate conditions for germination occur (Dyer and Lindsay, 1992; Chambers and MacMahon, 1994; Dyer, 1994; Ramírez-Trejo et al., 2004). The differences found between species also suggests that the requirement of water during priming also differs between species. In other words, the hydration memory (short lag time and highest germination rate and/or percentage), in some fern spore species, was developed under HP (*P. thyssanolepis*, *S. horrida* and *T. rudis*); while others had a better response to priming treatments that limited water availability (MP and/or NP). The dispersal of spores occurs in the rainy season, which favours germination and gametophyte establishment. Spores and seeds in the soil are exposed to the microenvironment on a very small scale (pico-scale, immediately neighbouring to fern spores) (Orozco-Segovia and Sánchez-Coronado, 2009). At this scale, soil water content is one of the factors that varies widely with small changes in the microtopography. Consequently, soil offers fern spores a wide intervals of water availability (windows of opportunities) to induce priming, germination and the establishment of gametophytes. In this context, the invigoration of spore germination in response to short (HP and MP, applied for four and eight days) and long (NP for a month) exposures to priming treatments is relevant because microsite protection by small rocks, leaves, and/or any other small soil particles may produce micro conditions of improved soil water retention, shade and other microenvironmental conditions that can favour imbibition, germination and/or survival (Greer, 1993; Maestre et al., 2003; Flinn, 2007). In this context, we can assume that fern spores are close to soil surface and exposed to hydration and dehydration cycles of different durations in the soil, which "might invoke stress-tolerance", as in seeds (sensu Chen and Arora, 2013) as a result of hydration memory.

The health of propagules is important to maintain and regenerate plant species populations. The fact that most of the studied fern spores maintained high germination percentages after one month of burial (NP) demonstrates that the six homosporous fern spores maintained viability in the soil for at least one month. The high germination, reduced lag time and increased germination rate indicates that spores are capable of a vigorous germination response, which might increase the probability of field establishment. It has been stated that early germination at the beginning of the rainy season, which might also occur in fern spores, provides a competitive advantage to seeds in seasonal environments (Thanos et al., 1995), and this may also apply to fern spores. However, for the spores with small-sizes (Raghavan, 1989) and tiny gametophytes, windows of opportunity for germination and gametophyte establishment might be shorter than that of the smallest seeds and seedlings. Consequently, even a small reduction in germination time might be an advantage in competing for resources (Greer, 1993). Some of the threats for spore germination and gametophytes establishment are being buried in the soil or covered by litter. However, fern spores are photoblastic positive, thus, germination can be inhibited by burial and under canopies, isolated leaves or litter (Pérez-García et al., 1994, 2007). Spores in the soil bank can eventually be brought to the surface by plants getting uprooted or the movements or eating habits of soil organisms such as worms, beetles and others (Traverse, 2007). In such conditions, germination and establishment opportunities ought also to be of curt time, after which the germinated fern spore could face competition for resources from seedlings and growing plants (Greer, 1993). Consequently, NP might provide spores that have advantages for fast and successful germination and establishment.

5. Conclusions

Priming treatments (HP, MP) invigorate germination responses in fern spores like those reported for seeds in other words, the lag time was shortened, and the germination rate was increased. These changes also occurred in NP and might increase the probability of successful germination and gametophyte establishment in the field. The similar response to priming in both seeds and fern spores suggests that the homologous processes occur during the stationary phase of imbibition and that the metabolic and biochemical advances in germination were also retained by spores after dehydration; i.e., priming induced hydration memory in fern spores. Additionally, the obtained results also suggest that, as in seeds (González-Zertuche et al., 2001), the response of fern spores to priming may have evolved in the soil bank.

Responses to NP, HP and MP of all the studied species had no pattern, whereas lag time and/or germination rate were the germination parameters that improved, more frequently. Similarly, NP was the treatment that enhanced the spore germination vigour, more frequently (increased lag time and germination rate) (Bewley and Black, 1994; González-Zertuche and Orozco-Segovia, 1996; Ranal and García de Santana, 2006).

More studies about the effect of priming, taking into account the ecological conditions about where seeds and fern spores remain in the soil, are necessary to understand how hydration–dehydration cycles determine the fate of seeds and fern spores in the soil bank: germination, secondary dormancy or death.

Declarations of interest

None.

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ORIGINAL RESEARCH

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Non-chlorophyllous and crypto-chlorophyllous fern spores differ in their mobilisation of fatty acids during priming

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Abstract

During fern spore germination, lipid hydrolysis primarily provides the energy to activate their metabolism. In this research, fatty acids (linoleic, oleic, palmitic and stearic) were quantified in the spores exposed or not to priming (hydration–dehydration treatments). Five fern species were investigated, two from xerophilous shrubland and three from a cloud forest. We hypothesised that during the priming hydration phase, the fatty acids profile would change in concentration, depending on the spore type (non-chlorophyllous and crypto-chlorophyllous). The fatty acid concentration was determined by gas chromatograph–mass spectrometer. Chlorophyll in spores was vizualised by epifluorescence microscopy and quantified by high-resolution liquid chromatography with a DAD-UV/Vis detector. Considering all five species and all the treatments, the oleic acid was the most catabolised. After priming, we identified two patterns in the fatty acid metabolism: (1) in non-chlorophyllous species, oleic, palmitic, and linoleic acids were catabolised during imbibition and (2) in crypto-chlorophyllous species, these fatty acids increased in concentration. These patterns suggest that crypto-chlorophyllous spores with homoiochlorophylly (chlorophyll retained after drying) might not require the assembly of new photosynthetic apparatus during dark imbibition. Thus, these spores might require less energy from pre-existing lipids and less fatty acids as 'building blocks' for cell membranes than non-chlorophyllous spores, which require de novo synthesis and structuring of the photosynthetic apparatus.

1 | INTRODUCTION

Ferns have two independent life cycle phases: the gametophyte (haploid) and the sporophyte (diploid). The germination of spores (gametophyte), is like seed (sporophyte) germination in angiosperms, represents the beginning of their life cycle (Dyer, 1994; Sharpe et al., 2010). Both fern spores (unicellular) and angiosperm seeds (pluricellular) are resistant structures that preserve genetic information, disperse and preserve it in space and time (Hoekstra, 2002). Germination of seeds and fern spores begins with water uptake, which initiates the activation of their metabolisms, such as the mobilisation of energetic stored reserves (lipids, proteins, and starch), cell division and growth, and finally, germination becomes evident by the protrusion of the radicle in seeds and of the primary rhizoid or prothallial initial cell in fern spores (Raghavan, 1989: Bewley, 1997).

Despite the differences in their life cycle and structure, fern spores and seeds have functional similarities (Ballesteros et al., 2017). In both propagules, lipid content provides high energy to activate the metabolism during germination. Lipids also are the main components for repairing old and synthesising new cell membrane systems, such as organelles and primary cell walls and maintaining the structural integrity of membranes and their functions (Bewley, 1997; Lüttge, 2011). Consequently, during germination, mainly the fatty acids decrease as they are easily assimilated (Gemmrich, 1977a; Naguib, 2019; Robinson et al., 1973). In contrast, the fatty acids are present in large quantities in dry spores, where they are the energy reserves for cellular metabolism. Fatty acids are divided into two main groups: unsaturated fatty acids (linoleic and oleic), used for their energetic metabolism, and the saturated fatty acids (palmitic and stearic) that generate new unsaturated acids or used in the structure of their cell membranes. The spore also contains phospholipids in very small amounts; their function is to maintain the cellular structure and the cell membrane permeability (Gemmrich, 1977a; Robinson et al., 1973; Seilheimer, 1978).

In general, fern spores germinate 4–9 days after hydration. The energy resources of spores are obtained from the mother plant during their development in the sporangium. The lipid reserves in the spores are found in the cytoplasm as lipid globules or mixed with other components as proteins and pigments (Raghavan, 1989; Templeman et al., 1987). In fern spores, the lipid content varies from 4% in the genus *Ceratopteris thalictroides* (L.) Brongn., to 79% *Drynaria meyeniana* (Schott) Christenh. (formerly *Polypodium meyenianum* (Schott) Hook.) (Gemmrich, 1977a).

Functionally, three main types of spores have been described: non-chlorophyllous, chlorophyllous and crypto-chlorophyllous. The presence of chlorophyll determines their metabolic activity: chlorophyllous spores show short viability and fast germination, while non-chlorophyllous spores have long viability, relative slow germination; these facts have consequences in gametophyte early establishment (Lloyd & Klekowski Jr, 1970). Although chlorophyll is present in the chlorophyllous and in the crypto-chlorophyllous spores, it is observable only in the chlorophyllous (green spores); chlorophyll being covered by the perispore pigments in the crypto-chlorophyllous spores, it is not visible to the naked eye or under optical or electronic microscopes (Sundue et al., 2011).

The hydration and activation of angiosperm seed metabolism is a triphasic process (Bewley, 1997); this occurs analogously in fern spores (Raghavan, 1989). During phase I, water diffuses into the spore to initiate its metabolism. The mobilisation of the reserves (proteins, lipids and carbohydrates) begins, and the mRNA is repaired and mobilised to repair cell components. During phase II, protein synthesis and reparation of cell membranes and mitochondria begin. In seeds and spores, carbohydrates are the first component to be metabolised followed by lipids. In spores, phase III is characterised by the protrusion of the first rhizoidal cell (Bewley, 1997; Raghavan, 1989). The events occurring in the three seed imbibition phases proposed by Bewley (1997) and the processes occurring during imbibition in spores (Raghavan, 1989) are homologous.

Priming treatments have been designed based on the stage of germination during seed and fern spore imbibition. These consist of one or more cycles of hydration–dehydration, where each cycle ends in a dehydration process to avoid radicle or rhizoid protrusion. All the biochemical and molecular changes occurring during imbibition phases I (fast water uptake) and II (imperceptible water uptake) remain even after storage in the dehydrated spore (Pedrero-López et al., 2021). During the spore hydration periods, metabolic advances occur. When propagules are re-hydrated, metabolism is re-started and germination occurs fast and synchronously (Bray, 1995; Gamboa-deBuen et al., 2006; Pedrero-López et al., 2019). Invigoration during germination of primed fern spores suggests that a similar metabolic process occurs in both seeds and fern spores during priming. There are several priming treatments that regulate water uptake rate, among others: imbibition in water (hydropriming, HP) and imbibition in a matrix, such as soil (matrix priming, MP) (González-Zertuche et al., 2001; Orozco-Segovia et al., 2014; Pedrero-López et al., 2019). These processes emulate the environmental conditions experienced in the soil by the small fern spores (40–60 μm) under natural conditions prior to spore germination (Pedrero-López et al., 2019, 2021). Because priming treatments in fern spores generate a functional response that favours the invigoration of fern spores during germination (Pedrero-López et al., 2019, 2021), we hypothesised that, as part of the metabolic and biochemical changes occurring during hydration, fern spore fatty acids will change in the amount and/or composition compared to non-primed spores. Lipids will be mobilised and/or catabolised because they are required during imbibition phases I and II to reinitiate spores metabolism. Non-chlorophyllous and crypto-chlorophyllous spores might exhibit a different lipid metabolism.

2 | MATERIAL AND METHODS

2.1 | Study area and plant material

Spores from five species of ferns were collected in 2012–2013 from two localities: *Pleopeltis thyssanolepis* (A. Braun ex Klotzsch) E.G. Andrews & Windham (formerly *Polypodium thyssanolepis*

Klotzsch, rupicolous and epiphyte), and *Pellaea ovata* (Desv.) Weath. (rupicolous) at the ecological reserve Parque Ecológico de la Ciudad de México (PECM) located in the municipality of Tlalpan, Mexico City, Mexico; and *Alsophila firma* (Baker) D.S. Conant (arborescent), *Sphaeropteris horrida* (Liebm.) R.M. Tryon (arborescent) and *Amauropelta rudis* (Kunze) Pic. Serm. (formerly *Thelypteris rudis* (Kunze) Proctor, terrestrial) at the municipality of Tlatlauquitepec, Puebla, Mexico. The studied species were selected based on their ability to produce a high number of spores. In the PECM, the vegetation is xerophilous shrubland growing on a lava field; the Köppen climate is Cb′(w2)(w) (temperate by elevation, with a long sub-humid fresh summer) with a mean annual temperature of 14°C and a mean annual precipitation of 880 mm (González-Hidalgo et al., 2002). In Tlatlauquitepec, the vegetation consists of a cloud forest under a Köppen climate (A)Ca(fm)(e) (subtropical with rains throughout the year) with a mean annual temperature of 20.2°C and a mean annual precipitation of 1243 mm (CONAGUA, 2022; Instituto de Geografia, 2004). All spores were obtained from fertile leaves of 5–10 individuals. Pinnae with closed sporangia were placed inside manila envelopes, labelled, and sealed. To dehydrate and liberate spores from the sporangia, the envelopes were placed on a flat surface in the laboratory in the dark (21.6 ± 1.8°C, relative humidity = 38.7%). Spores were sieved with a phytoplankton net with a mesh of 0.74 μm. Finally, they were placed in glass vials at 5°C. Vouchers of each species were deposited in the herbarium of the Universidad Autónoma Metropolitana-Iztapalapa (UAMIZ, LVPL 3–7).

2.2 | Priming treatments applied to fern spores

During the same year of spore collection, diverse treatments were applied to fern spores: hydropriming (HP), matrix priming (MP) and non-primed spores as control (C). Treatments were applied to each species in triplicate. Each replicate contained 100 mg of spores weighed in an analytical balance (OHAUS GA200, OHAUS Corp.) and placed inside phytoplankton net bags (25 μm in opening) (3 treatments × 5 species × 3 replications). For HP, net bags were immersed in tap water, while for MP they were buried 3 cm deep in pots (100 ml) filled with soil from the collection areas and watered to field capacity. Based on the lag time for germination of all studied species, hydration was applied for 8 days in the dark. After the hydration periods, bags were recovered from the hydration substrate and dehydrated for 2 days on a table surface inside the darkroom to avoid germination induction. Finally, spores were kept in closed glass vials until they were used for lipid extraction and quantification. Fatty acids extraction was carried out in a room at 4°C.

2.3 | Extraction of fatty acids

Two years after collection, spores were treated and then fatty acids were extracted from control and treated spores of *S. horrida* (C, HP and MP treatments), *Alsophila firma* (C, HP and MP), *Pleopeltis thyssanolepis* (C, HP and MP), *Pellaea ovata* (C and HP) and *Amauropelta rudis* (C and MP). The number of treatments differed among species because some samples were insufficient after spore trituration to perform all the fatty acids analyses. For each species, spores of the three replicates were mixed and an aliquot of 100 mg of spores was triturated at 4°C in a porcelain mortar containing glass powder. To the triturated sample, we added 1 ml of chloroform:methanol (2:1) solution. The supernatant was transferred to microcentrifuge tube vials that were shaken in a Vortex for 5 min to recuperate the organic layer. The supernatant was washed with 200 μl of NaCl at 9% and centrifuged at 18,000 × *g* at 10°C. To extract the fatty acids, we mixed 500 μl of the organic phase and 100 μl of the internal standard Heptadecanoic acid 1 mg ml^{-1} was used for fatty acid quantification and evaporated in a microcentrifuge tube vial (Brinkmann Instruments Inc., Centrifuge 5415C). Subsequently, 500 μl of BF_3 (boron trifluoride at 12% in methanol) was added. The tube was closed and sealed with sealing tape and placed in boiling water for 20 min. Finally, 500 μl of hexane and 1000 μl of distilled water were added to the tube, the tube was manually shaken, and the organic phase was recovered for the analysis of fatty acids, which was performed in triplicate.

For the analysis of the methyl esters of fatty acids, we followed the method of Cabrera-Santos et al. (2021). To identify the fatty acids, a gas chromatograph was used (GC; Agilent Technologies 6850), coupled with a mass spectrometer (MS; Agilent Technologies 5975C VL MSD). For the GC system, a DB-1 (dimethylpolysiloxane) capillary column was used (30 m length × 0.32 mm i.d., 5.00 μm film thickness, part number: 123-1035 E, Agilent Technologies 6850). The oven temperature was programmed as follows: from 100°C; ramp 1: to 250°C with 5°C min^{-1}. The injector temperature was 200°C in split mode. Helium was used as a gasifier fatty acids carrier at a linear flow velocity of 35 cm s^{-1} or 1.4 ml min^{-1}. The transfer line of the mass detector was kept at 250°C, the mass detector parameters ranged from 20 to 400 m/z, positive polarity, the ionisation energy of 70 eV, and a temperature of 200°C, with an injection volume of 2 μl. The results of the mass spectra were compared with the NIST/EPA/NIH Mass Spectral Library 2020 version.

2.4 | Determination of chlorophyll in spores

Crypto-chlorophyllous spores has been reported in tree ferns (i.e. Cyatheaceae; Sundue et al., 2011; Tseng et al., 2017); however, the presence of crypto-chlorophyllous spores was assessed in all the five species independent of the plant family. Ten years after spores collection, in viable spores of each species, images were acquired using a microscope with epifluorescence and laser microdissection functions (Arcturus XT—Nikon eclipse Ti, Applied Biosystems). The microdissection function was used at a wavelength of 349 nm to generate a cutting laser beam, of about 45 mW power, able to cut spores. To identify both chlorophylls *a* and *b* in the microdissected spores, the pieces were immediately observed under the microscope under a light source ranging from 330 to 750 nm wavelengths (halogen lamp) through a 20× objective lens, a set of filters was used to delimitate the specific excitation and emission spectra for both chlorophylls *a* and *b*. For chlorophyll *b*, we used an excitation filter with a

bandwidth of 475/15 nm, and for chlorophylls *a*, we used an excitation filter with a bandwidth of 375/15 nm, using a mercury lamp. Additionally, a dichromatic mirror cutoff of 395 nm long pass was used, the emission for both chlorophylls *a* and *b* of the spores was filtered by an emission filter with a bandwidth of 635/35 nm and collected by a CCD colour camera 1024 × 768 pixels (The Imaging Source). Extraction of photosynthetic pigments with methanol (including the accessory pigments) was performed 2 years after spore collection by high-resolution liquid chromatography (HPLC), using the reverse phase (Olives et al., 2006), with the following conditions: Allsphere ODS-1 column (250 × 4.6 mm) of 5 μm particle size, constant flow of 2 ml min^{-1}, the mobile phase consisted of a gradient containing acetonitrile:methanol:Tris–HCl buffer, 0.1 M pH 8 (75:12:4) as phase A, while phase B consisted of methanol:hexane (80:20). The gradient started with 100% A and held for 5 min, then changed gradually to 100% B during 2.5 min, and held for 7.5 min with 100% B, the total run time was 15 min. A diode array detector was used in a range of 380–800 nm and with detection at 663 nm. Finally, due to the lack of sufficient spores, an analytical quantification with an ultraviolet/visible light spectrophotometer of chlorophylls *a* and *b* was done only for *S. horrida* (UV/Vis spectrophotometer, Perkin Elmer Lambda S2, Perkin Elmer Corp.). The content of chlorophyll *a*, chlorophyll *b* and the total were determined using the models of Coombs et al. (1985).

2.5 | Data analyses

In control spores, the effect of the species, fatty acids type (sources of variation, categoric variables) and their interaction on the concentration (dependent variable, numeric) of the total of the fatty acids evaluated in this research were tested with a two-way analysis of variance (ANOVA).

For each species, the effect of priming treatments and fatty acid type (sources of variation, categoric variables) on fatty acid concentrations (dependent variable, numeric) were tested with a two-way ANOVA. In all cases, the normality of the data was tested with Shapiro–Wilk test ($p > 0.05$) and Tuckey's tests as post hoc test and homocedasticity was tested with Levene's tests ($p > 0.05$). Statistical analyses were performed with Statgraphics Centurion 15 v 15.2.05 software.

To estimate, in each species, the magnitude of the change in the concentrations of each fatty acids type, as an effect of the priming treatments, we also calculated the relative changes in the amount of each one of the fatty acids due to the treatments, in respect to the concentration in control spores (concentration of fatty acids in primed spores/control).

3 | RESULTS

3.1 | Fatty acid content

In control spores, the study found significant effects of the species, fatty acids type and their interaction ($F_{(4,59)} = 99.67$, $p < 0.001$; $F_{(3,59)} = 212.79$, $p < 0.001$ and $F_{(12,59)} = 70.60$, $p < 0.001$, respectively) on the total concentration of the four types of fatty acids evaluated in this research. Both *Pellaea ovata* and *Pleopeltis thyssanolepis* showed the highest concentration of fatty acids, mostly due to their significantly high concentration of oleic acid. *Amauropelta rudis* showed an intermediate trend with significant higher values for linoleic and oleic acids than *Alsophila firma* and *Sphaeropteris horrida*, which had the significantly lowest concentration of all fatty acid types (Table 1).

TABLE 1 Total concentration of the fatty acids included in this study (palmitic, linoleic, oleic and stearic acids), in control spores of five fern species collected in the ecological reserve Parque Ecológico de la Ciudad de México (*) or in the municipality of Tlatlauquitepec, Puebla, Mexico (ϕ)

Species	Total concentration of fatty acids (mg g^{-1})
Alsophila firma ϕ	10.66 ± 3.57c
Sphaeropteris horrida ϕ	12.01 ± 12.48c
Amauropelta rudis ϕ	69.91 ± 41.91b
Pellaea ovata *	131.07 ± 161.95a
Pleopeltis thyssanolepis *	121.49 ± 194.66a

Note: Mean values ± standard deviations. Different letters indicate significant differences ($p < 0.05$).

Significant differences were mainly found in oleic acid concentrations. In any case, stearic acid had the lowest concentrations (2.15–18.82 mg g^{-1}) and had no significant changes in any species or treatments.

In *Alsophila firma*, treatments, fatty acid type and their interactions induced significant differences in fatty acid concentrations ($F_{(2,35)} = 87.35$, $p < 0.001$; $F_{(3,35)} = 62.86$, $p < 0.001$ and $F_{(6,35)} = 38.8$, $p < 0.001$, respectively). Only oleic acid had an increase inconcentration after HP (14.65 times; 180.06 mg g^{-1}) compared to control (12.29 mg g^{-1}; Figure 1A).

In *S. horrida*, treatments, fatty acid type and their interaction induced significant differences in fatty acids concentrations ($F_{(2,35)} = 81.5$, $p < 0.001$; $F_{(3,35)} = 17.3$, $p < 0.001$ and $F_{(6,35)} = 10.34$, $p = 0.001$, respectively). After HP, the concentration in oleic (161.9 mg g^{-1}), linoleic (118.24 mg g^{-1}) and palmitic (104.09 mg g^{-1}) acids increased in relation to non-primed spores (7.18, 2.16, and 30.28 mg g^{-1}, respectively). When compared to controls, the increase in palmitic, linoleic and oleic acids in HP was 3.43, 54.74 and 22.54 times, respectively. In MP, only linoleic acid (39.66 mg g^{-1}) increased 18.36 times in relation to control (2.16 mg g^{-1}; Figure 1B).

For *Amauropelta rudis*, fatty acid determinations were done only for control and MP. In this species, priming, fatty acids type and their interaction were significant ($F_{(1,23)} = 231.2$, $p < 0.001$; $F_{(3,23)} = 83.11$, $p < 0.001$ and $F_{(3,23)} = 31.6$, $p < 0.001$, respectively). MP induced a decrease in the concentration of palmitic, linoleic, and oleic acids (42.92, 21.91 and 28.74 mg g^{-1}, respectively) compared to their concentrations in control (83.43, 94.60 and 94.04 mg g^{-1}, respectively) by 1.94 (palmitic acid), 4.31 (linoleic acid) and 3.27 (oleic acid) times (Figure 2A).

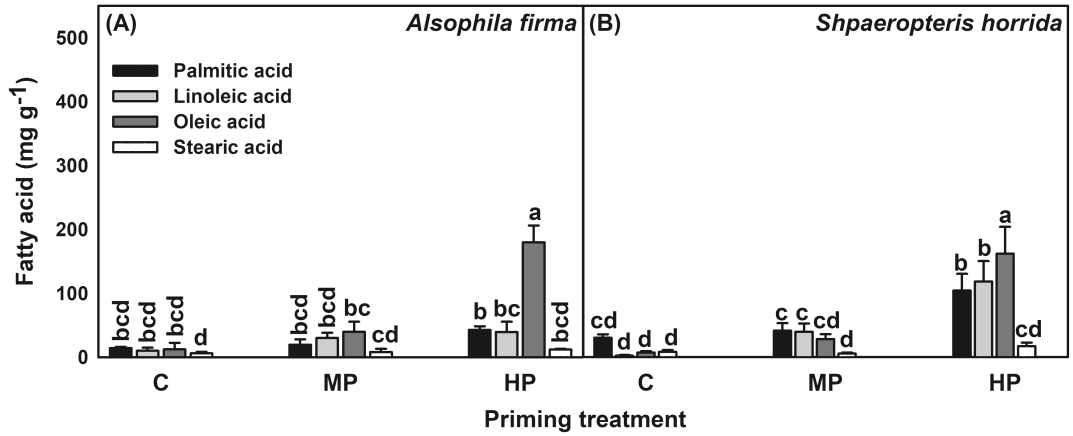

FIGURE 1 Effect of priming on fatty acid content on spores of (A) *Alsophila firma* and (B) *Sphaeropteris horrida*. Control (C), hydropriming (HP), matrix priming (MP). Data shown means ± SD, lowercase letters on bars indicate significant differences according Tukey test ($p < 0.05$, $n = 3$)

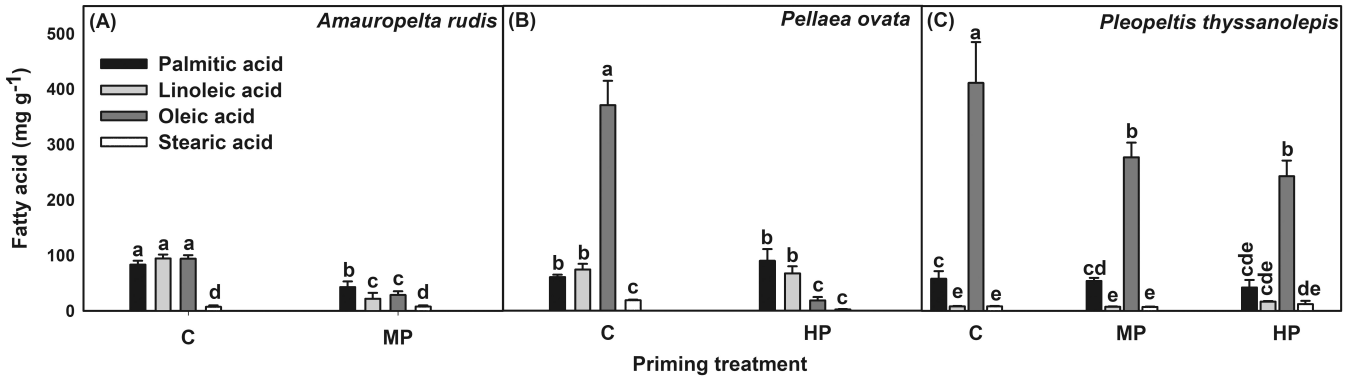

FIGURE 2 Effect of priming on fatty acid content on spores of (A) *Amauropelta rudis*, (B) *Pellaea ovata* and (C) *Pleopeltis thyssanolepis*. Control (C), hydropriming (HP), matrix priming (MP). Data shown means ± SD, lowercase letters on bars indicate significant differences according Tukey test ($p < 0.05$, $n = 3$)

For *P. ovata*, fatty acid determinations were done only for C and HP. Significant differences were induced for treatments, fatty acid type and their interaction ($F_{(1,23)} = 133.7$; $P < 0.001$, $F_{(3,23)} = 105.88$; $P < 0.001$ and $F_{(3,23)} = 141.5$; $P < 0.001$). Only oleic acid reduced its concentration after HP (18.43 mg g^{-1}) by 20.11 times compared to control (370.63 mg g^{-1}, Figure 2B).

For *Pleopeltis thyssanolepis*, treatments, fatty acid types and their interaction induced significant decreases in the fatty acid concentrations ($F_{(2,35)} = 10.23$, $p < 0.001$, $F_{(3,35)} = 307.23$, $p < 0.001$ and $F_{(6,35)} = 9.69$, $p < 0.001$, respectively). In control, oleic acid concentration was 411.35 mg g^{-1} and decreased 1.48 times in MP (277.07 mg g^{-1}) and 1.69 times in HP (243.05 mg g^{-1}) (Figure 2C).

3.2 | Determination of chlorophyll in spores

In the micro-dissected spores, we observed their emission for *S. horrida* and *Alsophila firma* (Figure 2). A second verification test of chlorophylls was carried out with a high-resolution liquid chromatography (HPLC). Both species presented emission spectra corresponding to chlorophylls *a* and *b* (Figures 3 and 4). *Sphaeropteris horrida* contained 7.975 μg g^{-1} of total chlorophyll, 4.67 μg g^{-1} of chlorophyll *a* and 2.72 μg g^{-1} of chlorophyll *b*. *Pellaea ovata*, *Pleopeltis thyssanolepis* and *Amauropelta rudis* did not present fluorescence associated to the chlorophyll presence and did not present emission spectra corresponding to chlorophylls *a* and *b*.

4 | DISCUSSION

The priming treatments induced different changes in the concentrations of the fatty acids, depending on the species, which suggests differential metabolism of fatty acids during priming, as it occurs in seeds (Espanany et al., 2016; Naguib, 2019). Based on fluorescence microscopy, HPLC and UV/Vis spectroscopy, *Pleopeltis thyssanolepis*, *Pellaea ovata* and *Amauropelta rudis* have non-chlorophyllous spores, while *S. horrida* and *Alsophila firma* have crypto-chlorophyllous spores. During priming, spores of both types stayed hydrated 8 days in darkness, thus absorbed water (imbibition phase

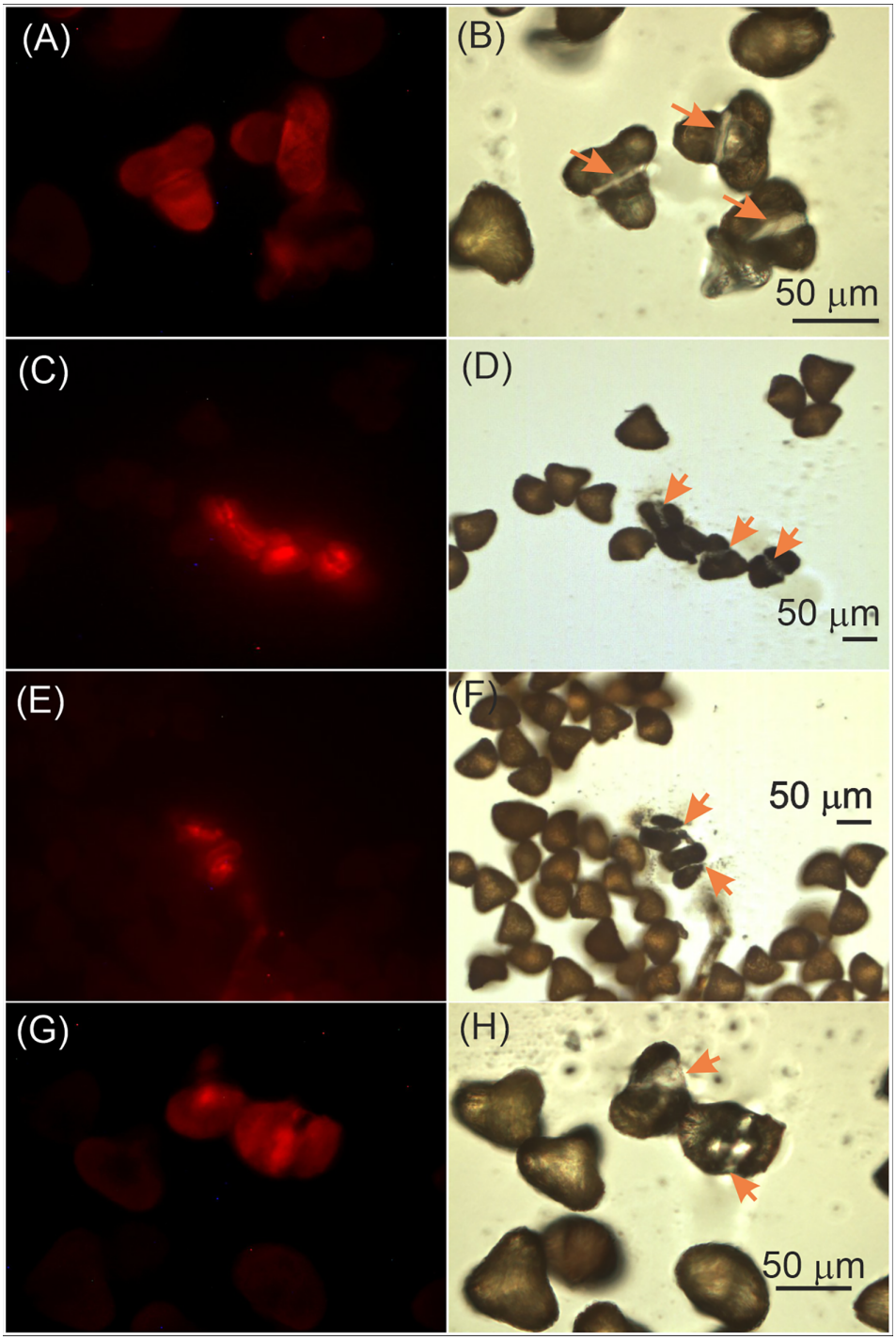

FIGURE 3 Photographic comparison of spores of: *Sphaeropteris horrida* (A–D) and *Alsophila firma* (E–H). Spores were photographed with: epifluorescence microscopy with UV light (A,E), epifluorescence microscopy with blue light (C,G) or white light microscopy (B,D,F,H)

l) and remained in imbibition phase II. As a result of priming, we observed two patterns in the fatty acids concentrations: one for non-chlorophyllous species and another one for the two crypto-chlorophyllous species.

Several of the processes occurring in spores, mainly during the activation of germination (sensu stricto; Bradford, 1995), might explain the contrasting patterns observed in fatty acids concentration after priming. In the non-chlorophyllous species, oleic acid was consumed. In *Amauropelta rudis*, palmitic acid and linoleic acid were also catabolised. The lipid reduction might be the result of the catabolic activities occurring in darkness during the priming treatments, as occurs in *Daucus carota* L. seeds (Zhao et al., 2021), or in fern spores during imbibition (Sato & Furuya, 1984). In general, monounsaturated fatty acids have a greater capacity to be metabolised during priming (Walters et al., 2005).

In contrast, in the primed crypto-chlorophyllous species, fatty acids' concentration increased. Oleic acid increased in *Alsophila firma*, and linoleic, oleic and palmitic acids increased their concentration in *S. horrida* after HP.

An explanation for these two patterns might be that spores of *S. horrida* and *Alsophila firma* have an initially low fatty acids content and might be using other type of reserves, such as globulin proteins

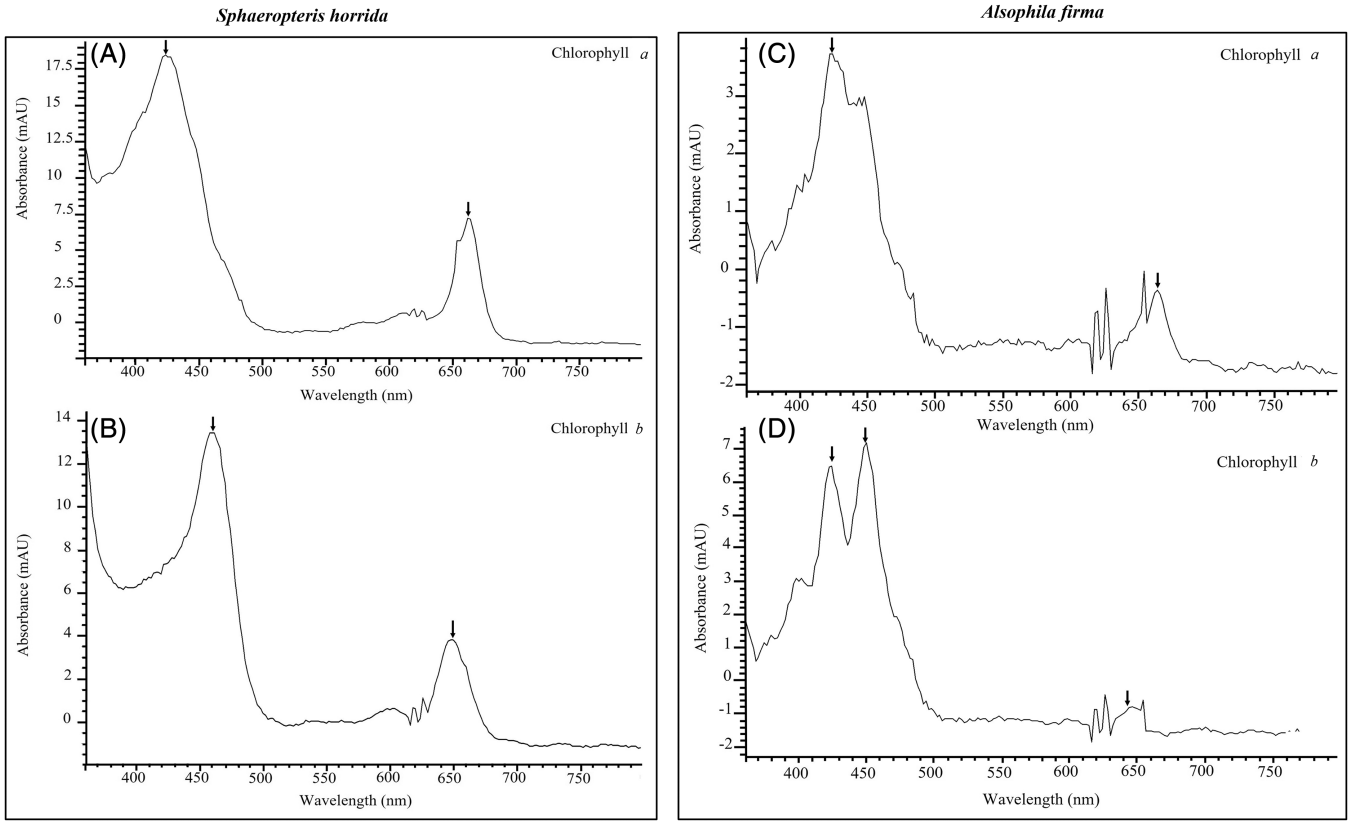

FIGURE 4 Absorption spectrum of chlorophyll *a* (A,C) and chlorophyll *b* (B,D), analysed by HPLC. Fern species are indicated on the top of the figure

(DeMaggio & Stetler, 1985). Thus, in these species, mobilisation and high synthesis de novo of fatty acids would occur during imbibition (phases I and II) to cover the subsequent gametophyte development and growth (Robinson et al., 1973). However, lipids, not proteins, are the main reserve in fern spores (Gemmrich, 1977b). Contrastingly, spores of non-chlorophyllous *Pleopeltis thyssanolepis*, *Pellaea ovata* and *Amauropelta rudis* have an initial high fatty acids content and their catabolism was mainly observed in primed spores compared to control. It is difficult to find research articles that could support this idea since fatty acids content has been determined in relatively few fern spores species. Only Gemmrich (1977a) includes the concentration of 8 fatty acids in spores of 17 species, 16 non-chlorophyllous species and one chlorophyllous.

The increase in fatty acids after priming also suggests that this response might be linked to biochemical metabolic differences occurring during imbibition phases I and II. This might be related to the initial chlorophyll content of the spores. Some species of tree ferns (Cyatheaceae), such as *Sphaeropteris lepifera* (Hook.) R.M.Tryon (Cyatheaceae), are crypto-chlorophyllous (Tseng et al., 2017), which makes an important functional difference compared to non-chlorophyllous fern spores. In our study, we showed that *S. horrida* and *Alsophila firma* were crypto-chlorophyllous.

In the germination activation phase (imbibition phase II), the requirements needed for the subsequent gametophyte or embryo development and growth ought to be initiated or covered (Bradford, 1995; Raghavan, 1989; Robinson et al., 1973). In the homoiochlorophylly found in desiccation-tolerant plant species, chlorophyll is retained after tissues or cells dehydration and reconstituted during rehydration (Shivaraj et al., 2021; Tuba et al., 1994; Tuba et al., 1998), as occurs in the homoiochlorophyllous sporophyte of *Pleopeltis polypodioides* (L.) E.G. Andrews & Windham (John & Hasenstein, 2018, 2020; López-Pozo et al., 2019). This might also occur in crypto-chlorophyllous and chlorophyllous spores; thus, chlorophyll reconstitution and repair of organelles might occur simultaneously during Phase II of imbibition of the priming treatments. Contrarily, in the imbibed state, the non-chlorophyllous fern spores require cell membrane repair, organelles assemblage and differentiation, and the synthesis of chlorophyll and other photosynthetic pigments, to complete photomorphogenesis during subsequent exposure to light and later produce functional chloroplasts (Gemmrich, 1980). In this research, the hydration phase of priming treatments occurred in darkness. For different taxa, including ferns, it has been reported that complete or partial development of photosynthetic apparatus occurs in darkness (Armstrong, 1998; Xue et al., 2017). The complete photosynthetic morphogenetic process might require lipids as energy source and/or as 'building blocks' of membrane lipids (sensu de Carvalho & Caramujo, 2018) from the pre-existing fatty acids, as it has been reported during chloroplast morphogenesis in darkness for the algae *Chromochloris zofingiensis* (Dönz) Fucíková & Lewis (Zhang et al., 2020).

Other processes linked to hydration–dehydration–rehydration treatments (as in priming treatments) might also explain the increase in fatty acids content observed after dehydration at the end of

the priming treatments in *Alsophila firma* and *S. horrida*. In the homoiochlorophyllous *P. polypodioides*, an increase in fatty acids content after dehydration has been considered as (1) an adaptation aimed to prevent lipid peroxidation caused by the increase of reactive oxygen species (ROS) in the components of the pre-existing photosynthetic apparatus, or (2) an adaptation to desiccation stress where fatty acids play a role of stress-responsive metabolites in cell membranes, depending on temperature (John & Hasenstein, 2018, 2020). Additionally, the unsaturated fatty acids increase also protect the photosynthetic apparatus from damage (Gombos et al., 1994; John & Hasenstein, 2020).

Nonetheless, increases in fatty acids concentration coming from the mobilisation of other lipids and synthesis of de novo fatty acids ought to be assessed to understand the increases in fatty acids concentrations found in these two tree ferns during priming. Because we identified *S. horrida* and *Alsophila firma* as species with crypto-chlorophyllous spores, it seems relevant to assess this spore trait in other fern species as well.

During priming treatments, it might occur catabolism, mobilisation or de novo synthesis of fatty acids that might increase the fern spore vigour (i.e. germination synchronisation, reduction in lag time and/or increase in germination rate) during germination as previously reported for primed fern spores of the species included in this research (Pedrero-López et al., 2019, 2021). Several authors have reported changes in the concentrations of fatty acids during fern spore germination (DeMaggio & Stetler, 1985; Gemmrich, 1977a; Raghavan, 1989; Seilheimer, 1978), but these changes are not clearly related to the biochemical changes occurring in the dark before germination, as in *Daucus carota* seeds during the imbibition (Zhao et al., 2021).

5 | CONCLUSION

In fern spores, the application of priming treatments caused changes in the concentrations of fatty acids, mainly oleic acid, which is an easily assimilable chemical component in fern spores, necessary for the metabolic activation of the spores and prepare them to germinate and to establish in their environment.

Changes in the fatty acids content in the studied species showed that these reserves are mobilised and/or catabolised during imbibition phases I and II to reinitiate the metabolism of spores. Fatty acids, in fern spores are the same as those stored in seeds, although, in fern spores, the lipids metabolism occurs on a smaller scale inside a single cell compartment. The fatty acids mobilisation differed between non-chlorophyllous and crypto-chlorophyllous spores as a result of priming. Non-chlorophyllous species catabolised oleic, palmitic, and linoleic acids, while crypto-chlorophyllous species increased their concentration, suggesting that crypto-chlorophyllous spores with homoiochlorophyll might not require fatty acids for the assembly of the photosynthetic apparatus during dark imbibition. Alternatively, fatty acids might prevent lipid peroxidation or be stress-responsive metabolites, as suggested by previous reports for sporophyte of *P. polypodioides*. However, it is necessary to assess de novo synthesis of fatty acids during dark imbibition at different temperatures in crypto-chlorophyllous spores.

Although matrix priming simulated the permanence of spores in the soil, hydropriming caused a more pronounced changes in fatty acids concentrations than matrix priming. This suggests that water availability in the soil (saturated soils vs. unsaturated soils) might be relevant for germination and the establishment of gametophytes in the field.

AUTHOR CONTRIBUTIONS

Luis V. Pedrero-López: conceptualisation, investigation, methodology, data collection and analysis, writing review and editing; César M. Flores-Ortiz: supervision, writing, review and editing, resources, validation; Blanca Pérez-García, Rocío Cruz-Ortega, Klaus Mehltreter: supervision, writing, review and editing; María E. Sánchez-Coronado: formal analysis, writing—review and editing; Luis Barbo Hernández-Portilla: data processing and methodology; Gastón Contreras-Jiménez: data collection and processing, final—draft; Alma Orozco-Segovia: conceptualisation, investigation, supervision, funding acquisition, methodology, resources, validation, writing—original draft, writing—review and editing.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

All data are available in the manuscript. Statistical analysis are available if they are required.

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Discusión General

Para la discusión de los temas abordados en esta tesis es importante reiterar los aspectos biológicos y ecológicos relacionados con las esporas, sobre los que se sustenta este trabajo. Existen estudios sobre el efecto que tienen algunos factores ambientales en campo sobre la germinación de las esporas, como el efecto de la calidad de la luz en la selva y en claros de ésta (Pérez-García *et al.* 1982) y del fuego (Ramírez-Trejo *et al.* 2010). Todos ellos se enfocan en la germinación del gametofito.

Las estructuras reproductivas, gametofitos y esporas, de los helechos son inconspicuas, por lo que su estudio en laboratorio es más común que su estudio en campo (Banks 1999). Además, la fase sexual es de corta duración y el gametofito es también una estructura de tamaño muy pequeño, en su fase de mayor expansión mide 1–2 mm (Banks 1999). En el aspecto reproductivo, los esporofitos producen las estructuras asexuales (esporas) que al germinar dan origen al gametofito (Evert y Eichhorn 2013). Para conocer la ecofisiología de las esporas es necesario considerar que éstas son dispersadas de forma autócora (catapulta) y posteriormente anemócora (secundaria), para finalmente caer al suelo, cerca de la planta madre o quedar sobre otras superficies, como troncos, ramas, etc., en donde pueden germinar (condición ventajosa para los helechos epífitos) o presentar una nueva etapa de dispersión hidrócora, al ser lixiviadas por la lluvia o por otros mecanismos, hasta llegar finalmente al suelo, estas etapas de dispersión consecutivas han sido descritas para semillas por Chambers y MacMahon (1994). En cualquiera de estos casos las esporas germinan si encuentran un sustrato de germinación húmedo, en forma relativamente continua, y condiciones adecuadas de luz y temperatura (Miller 1968; Menéndez *et al.* 2011). Si las esporas no germinan, en cualquiera de estos sustratos, al igual que en el caso de las semillas de las angiospermas, se forma un banco de esporas aéreo (en las ramas) o un

banco de esporas en el suelo (Lamont y Enright 2000; Steves *et al.* 2003). El banco de esporas que se forma en el suelo ha sido el más estudiado (Dyer 1994), principalmente en cuanto a su composición de especies y desarrollo del gametofito. Pero en ninguno de estos trabajos se consideran los periodos de hidratación-deshidratación a los que quedan expuestas las esporas en los distintos sustratos, ni la relación entre sustrato, hidratación, tipo de espora y longevidad

En los estudios sobre germinación de esporas es fundamental considerar el grupo de esporas al que pertenecen las distintas especies; las cuales se clasifican fundamentalmente por la presencia/ausencia de clorofila en esporas no clorofílicas y clorofílicas. La longevidad potencial de las esporas no clorofílicas es larga, tanto en condiciones de almacenamiento óptimas como subóptimas (sensu Vázquez-Yanes y Orozco-Segovia, 1993), por ejemplo, 70 años en herbario (Paul *et al.* 2014) y la longevidad se ha estimado hasta de 100-125 años resistiendo a la desecación (Johnson 1985; Esteves y Dyer 2003; Nagalingum *et al.* 2007), por lo que se les ha considerado similares a las semillas ortodoxas, de larga vida. Mientras que en las esporas clorofílicas la longevidad es corta, muy variable (dependiendo de las condiciones de hidratación y almacenamiento de las esporas), durar de días a pocos meses y se les considera equivalentes a las semillas recalcitrantes (Lloyd y Klekowski 1970; Pérez-García *et al.* 1994; Ballesteros 2010; López-Pozo 2019). Se ha descrito un tercer grupo de esporas, las criptoclorofílicas (Sundue *et al.* 2011), las cuales también contienen clorofila, pero ésta no puede observarse mediante microscopía óptica porque la cubierta de la espora presenta una mayor diversidad de tonalidades, entre ellas tonalidades oscuras (atropurpúreas) o pardas; por lo que se requiere microscopía de epifluorescencia para identificar su presencia (Sundue *et al.* 2011). Esto ha dado lugar a la reclasificación de algunas especies, de ser consideradas no clorofílicas a clasificarlas como

criptoclorofílicas; tales como: *Sphaeropteris lepifera* (Hook.) R.M. Tryon (Cyatheaceae), *Ceratopteris thalictroides* (L.) Brongn. (Pteridaceae), *Leptochilus wrightii* (Hook.) X. C. Zhang (Polypodiaceae), *Leptochilus pothifolius* (D.Don) Fraser-Jenk. (Polypodiaceae), *Lepidomicrosorum buergerianum* (Miq.) Ching & K. H. Shing (Polypodiaceae), *Osmunda banksiifolia* (Pr.) Kuhn (Osmundaceae), y *Platycerium grande* (A. Cunn.) J.Sm. (Polypodiaceae) (Tseng *et al.* 2016). Se requieren estudios cuidadosos sobre la viabilidad de estas especies.

También se ha podido establecer la similitud bioquímica, funcional y ecofisiológica en esporas de helechos y semillas de las angiospermas. Por ejemplo, similitud en su composición química (DeMaggio y Stetler 1985; Templeman *et al.* 1988; Raghavan 1989; Banks 1999), presencia de quiescencia, latencia y latencia secundaria (Juárez-Orozco *et al.* 2013) y la presencia de efectos maternos como en las semillas (Orozco-Segovia *et al.* 2000) y quizá en esporas, derivados de distintas condiciones ambientales entre años de producción de éstas, por mencionar algunas.

Desde el punto de vista de su composición química, las esporas de helechos fueron muy estudiadas en los años 60's y en los 80's, incluso se estableció una relación interesante entre la presencia de clorofila y la de ácido palmítico en esporas de *Polypodium vulgare* (Robinson *et al.* 1973). Estos estudios se han reiniciado en las últimas tres décadas y gracias al desarrollo de la tecnología, entre ellas el internet, se ha simplificado el acceso a la información generada en las primeras décadas, la cual contiene información original, que no había sido retomada. Para el presente trabajo es de gran relevancia la similitud de las esporas con las semillas con relación los procesos bioquímicos y moleculares que ocurren durante la imbibición, antes de la germinación (Raghavan 1989, 1992; Dyer 1994; Bray 1995) y el hecho de que, estando embebidas, tanto las esporas recalcitrantes como las

ortodoxas pueden permanecer viables en la oscuridad a temperatura ambiente (Pérez-García *et al.* 1994). De acuerdo con Bewley (1997) y Raghavan (1989) la imbibición de semillas y esporas de helechos es un proceso análogo que se presentan en tres fases: fase I, de imbibición; fase II, en la que se igualan el potencial osmótico de las semillas o las esporas con el del suelo, y hay una gran actividad metabólica, bioquímica y molecular. Es importante señalar que en esta fase se restauran las macromoléculas, membranas celulares y se reparan o ensamblan nuevos organelos (Bray 1995). La imbibición concluye con la fase III, en la que se continúan los procesos iniciados en la fase II la toma de agua se reinicia y protruye la radícula (en semillas) o la célula rizoidal que es la primera célula protálica (en esporas). Con base en los procesos que ocurren en estas tres etapas se diseñaron para semillas tratamientos de acondicionamiento (hidratación-deshidratación, priming) cuyo propósito metodológico inicial fue mejorar la germinación de semillas agrícolas (Bray 1995) incrementando el vigor de la semilla. Por lo tanto, podría incrementarse la oportunidad de germinar de la espora y el establecimiento del gametofito (Bray 1995; Pedrero-López *et al.* 2021). La germinación de esporas de helechos ha sido poco estudiada desde un punto de vista ecofisiológico, es decir tomando en cuenta el ambiente en el que permanecen en el suelo antes de su germinación. Dado que las esporas que se encuentran en el banco de esporas del suelo y las que se exponen a acondicionamiento en el laboratorio están sujetas a los mismos principios fisicoquímicos que regulan la hidratación y deshidratación en las semillas, en esta tesis pusimos a prueba si las esporas también desarrollan durante el acondicionamiento una memoria hídrica como la definida para semillas por Dubrovsky (1996; 1998). En la presente investigación se tuvo un enfoque pionero sobre la aplicación de tratamientos de acondicionamiento en esporas de helechos y en evaluar su efecto en la germinación y movilización de sus reservas lipídicas durante el acondicionamiento. Los

tratamientos de acondicionamiento utilizados fueron: natural (enterramiento en el suelo en el mismo sitio en donde las esporas fueron recolectadas) y/o acondicionamiento mátrico (enterradas en suelo de la zona de estudio, en el laboratorio), y/o acondicionamiento hídrico (hidratación en agua). Los tres tratamientos concluyeron con la deshidratación de las esporas. Con base en las respuestas de las semillas a estos tratamientos, en este trabajo se probaron tres hipótesis, cuyos resultados fueron novedosos:

• Si las esporas responden a los tratamientos de acondicionamiento pregerminativo hídrico, mátrico y/o natural, entonces las esporas germinarán con mayor vigor que las esporas control.

• Si la respuesta germinativa de las esporas al acondicionamiento mátrico y/o natural es análoga a la de las semillas, entonces la respuesta germinativa de las esporas, a este tratamiento, diferirá de acuerdo con las condiciones ambientales del hábitat que ocupan.

• Si, como ocurre en las semillas, la aplicación de tratamientos de acondicionamiento favorece la germinación de esporas de helechos, entonces se pueden esperar cambios en la movilización de sus reservas en respuesta a los tratamientos de acondicionamiento.

Para responder las dos primeras hipótesis se estudiaron cinco especies de helechos que crecen en bosque de niebla o en matorral xerófilo. Las especies se recolectaron en dos épocas de producción (2009 y 2012–2013). En 2009 se recolectaron *Llavea cordifolia*, *Pellaea ovata* y *Thelypteris ovata*; en 2012–2013 *Sphaeropteris horrida*, *Thelypteris rudis* y *Polypodium thyssanolepis*. Se expusieron a los tratamientos de acondicionamiento natural, mátrico e hídrico por distintos periodos de tiempo y se evaluó su efecto en el inicio, velocidad y porcentaje de germinación. Los fundamentos más importantes para la aplicación de tratamientos de acondicionamiento en esporas de helechos fueron: 1) Las esporas de los helechos son unicelulares, pero, como en las semillas, la imbibición debe

estar regulada por el gradiente de potencial hídrico entre las células y el sustrato (Bray 1995, Taiz y Zeiger 1991), 2) Se conocen los procesos bioquímicos y moleculares que ocurren durante la imbibición de las esporas los cuales son similares a los que ocurren en semillas (Raghavan 1989; Bewley 1997; Banks 1999), 3) Se espera que durante la hidratación en la oscuridad ocurran los mismos procesos que durante la hidratación a la luz, así como otros que modifiquen la posterior respuesta germinativa, la cual en la mayoría de las esporas de helechos requiere de luz. Por ejemplo, en esporas fotoblásticas positivas la sensibilidad a la luz se incrementa después de haberse hidratado en la oscuridad (Pérez-García *et al.* 2007). Los resultados mostraron que las esporas de las seis especies estudiadas respondieron favorablemente a todos los tratamientos de acondicionamiento, es decir la germinación se inició más pronto y/o hubo un incremento en la velocidad de germinación, al igual que ocurre en especies agrícolas y en especies de angiospermas silvestres (Orozco-Segovia *et al.* 2014). Los avances en el proceso de la germinación fueron el resultado de lo que se denomina "memoria de hidratación". En algunas especies y algunos tratamientos hubo un porcentaje menor en la germinación de esporas, que en las esporas control, probablemente porque los avances en la germinación, durante la fase II, alcanzaron el punto de no retorno (Schopfer y Plachy 1984), por lo tanto, al deshidratar a las esporas, en la etapa final del acondicionamiento se produjo su muerte. Esto ocurrió solo en las esporas de helechos recolectadas en 2012–2013, expuestas a acondicionamiento mátrico durante cuatro y ocho días. Sin embargo, las esporas recolectadas en 2009 expuestas al acondicionamiento natural, sobrevivieron después de un mes, lo que sugiere: 1) Las condiciones ambientales durante el desarrollo de las esporas en el esporofito pueden tener algún efecto en la velocidad a la cual se llevan a cabo los avances metabólicos de la fase II, de imbibición. Otros efectos ligados al ambiente en que se desarrollan las esporas han sido reportados por Ballesteros (2010); 2)

En forma alternativa se propuso que *P. thyssanolepis*, *S. horrida* y *T. rudis* adquirieron latencia secundaria en el acondicionamiento mátrico, ya que las esporas no germinadas permanecieron sanas durante 60 días; se ha reportado que cuando esporas embebidas están expuestas a un ambiente adverso entran en latencia secundaria (Juárez-Orozco *et al.* 2013).

Algunas especies de esporas de helechos respondieron mejor al acondicionamiento hídrico (*P. thyssanolepis*, *S. horrida* y *T. rudis*); mientras que otras respondieron mejor al acondicionamiento mátrico y/o al acondicionamiento natural. Esto refleja que las esporas de las distintas especies pueden tener distintas oportunidades de germinar y establecerse en la diversidad de micrositios, a escala pequeña, que se encuentran en el suelo. El potencial osmótico indudablemente debe cambiar dependiendo de la microtopografía del suelo y el grado de exposición de estos micrositios. En consecuencia, las ventanas de oportunidad para la germinación de las esporas y el desarrollo del gametofito deben variar ampliamente en el suelo, el cual es heterogéneo en el espacio vertical y horizontal y en el tiempo (Dyer y Lindsay 1992). Para resolver la hipótesis número tres se diseñó un experimento con cinco especies que crecen en bosque de niebla (*S. horrida*, *Alsophila firma* y *Amauropelta rudis*) o en matorral xerófilo (*Pellaea ovata* y *Polypodium thyssanolepis*). Se aplicaron tratamientos de acondicionamiento hídrico y mátrico durante 8 días. Se estudió la movilización de lípidos que ocurre durante la etapa de hidratación en la oscuridad, primera etapa del acondicionamiento. Se seleccionó la movilización de lípidos debido a que es la principal reserva en esporas de helechos 4–79% (Gemmrich 1977) y son los que principalmente aportan la energía utilizada por las esporas durante la germinación (Templeman *et al.* 1987; Raghavan 1989). Después de los tratamientos de acondicionamiento se determinó por cromatografía de gases la concentración de 4 ácidos grasos principales de las reservas lipídicas de las semillas y de las esporas de helechos:

ácidos esteárico, linoléico, oléico y palmítico. La presencia de clorofila en las esporas se identificó con microscopía de epifluorescencia y se confirmó con cromatografía líquida y en el caso de *S. horrida* también se cuantificó con espectrofotometría. Debido al tamaño de las esporas se requiere de una gran cantidad de ellas para poder estudiar la movilización de los lípidos. Además, las reservas de lípidos se encuentran en el citoplasma de la espora formando glóbulos lipídicos, por lo que para tener acceso a ellos es necesario triturar finamente a las esporas con vidrio molido. Los tratamientos de acondicionamiento produjeron cambios en las concentraciones de los ácidos grasos. Se identificaron dos patrones en el cambio en la concentración de los lípidos: los cuales se asociaron con la presencia o ausencia de clorofila en ellas. Las esporas no clorofílicas de *Pleopeltis thyssanolepis*, *Pellaea ovata* y *Amauropelta rudis* catabolizaron el ácido oléico. *Amauropelta rudis*, también catabolizó ácido palmítico y ácido linoleico, mientras que, las esporas criptoclorofílicas de *S. horrida* y *Alsophila firma* presentaron un incremento en la concentración de ácido oleico; además en *S. horrida*, después del acondicionamiento hídrico, también incrementaron la concentración de los ácidos linoleico y palmítico.

Estos dos patrones se asociaron a los procesos que ocurren durante la imbibición en la oscuridad, esto es, las esporas de *S. horrida* y *Alsophila firma* al tener un contenido inicial bajo de ácidos grasos requiere movilizar otro tipo de reservas y simultáneamente sintetizar de novo los ácidos grasos necesarios para el desarrollo y el crecimiento del gametofito (Robinson *et al.* 1973). Por el contrario, las esporas de *Pleopeltis thyssanolepis*, *Pellaea ovata* y *Amauropelta rudis* tuvieron un alto contenido inicial de ácidos grasos, los cuales fueron catabolizados durante la imbibición en la oscuridad, en comparación con el control. Este aumento de ácidos grasos después del acondicionamiento se relacionó con la presencia o ausencia de clorofila en las esporas (esporas criptoclorofílicas y no clorofílicas,

respectivamente); lo cual representa una diferencia funcional importante entre ambos tipos de esporas. Dado que en esporas clorofílicas y criptoclorofílicas la clorofila sintetizada durante el desarrollo y secado de las esporas en la planta madre, se asoció a la clorofila que se encuentra en los helechos de la resurrección, que son plantas tolerantes a la desecación, denominada homoioclorofila. A pesar de ser químicamente idéntica a la clorofila de cualquier otra planta se le denomina así porque se conserva y se reconstituye al rehidratarse, sin destruirse (Tuba *et al.* 1994; Tuba *et al.* 1998; Neeragunda-Shivaraj *et al.* 2021). La reconstitución de la clorofila y la reparación de organelos en esporas criptoclorofílicas pueden ocurrir simultáneamente durante la imbibición en la oscuridad en la primera etapa de los tratamientos de acondicionamiento. Por el contrario, las esporas no clorofílicas en estado embebido requieren reparación de la membrana celular, ensamblaje y diferenciación de organelos (como los cloroplastos) y la síntesis de clorofila y otros pigmentos fotosintéticos para completar la fotomorfogénesis durante la exposición posterior a la luz para que los cloroplastos sean funcionales (Gemmrich 1980). Para diferentes taxones, incluidos los helechos, el desarrollo completo o parcial del aparato fotosintético ocurre en la oscuridad (Armstrong 1998; Xue *et al.* 2017). El proceso morfogenético del aparato fotosintético, completo puede requerir de lípidos como fuente de energía y/o como parte de la estructura de los lípidos de membrana (“building blocks”, sensu deCarvalho y Caramujo 2018) a partir de los ácidos grasos preexistentes, como ocurre en algas durante la morfogénesis en la oscuridad del cloroplasto en *Chromochloris zofingiensis* (Dönz) Fucíková y Lewis (Zhang *et al.* 2020).

En *Alsophila firma* y en *S. horrida* el aumento en el contenido de ácidos grasos observado tras la deshidratación al final de los tratamientos de acondicionamiento se puede explicar de la misma manera que se ha hecho para el helecho homoioclorófilo, *Pleopeltis*

polypodioides; el aumento en el contenido de ácidos grasos es: 1) una adaptación dirigida a prevenir la peroxidación lipídica causada por el aumento de especies reactivas de oxígeno (ROS) en los componentes del aparato fotosintético preexistente, o 2) el aumento de ácidos grasos insaturados también protege al aparato fotosintético, como una adaptación al estrés por desecación donde los ácidos grasos juegan un papel de metabolitos que responden al estrés en las membranas celulares, dependiendo de la temperatura (Gombos *et al.* 1994; John y Hasenstein 2018; 2020).

Conclusiones Generales

Nuestros resultados demostraron que los avances pregerminativos ocurridos durante imbibición se mantuvieron después de la deshidratación de las esporas, al final de los tratamientos de acondicionamiento, lo que es conocido como memoria de hidratación, esto se expresó como inicio de germinación corto y mayor velocidad de germinación. Este nuevo descubrimiento refuta la propuesta de Pangua *et al.* (2009) de que las esporas de los helechos, por ser estructuras unicelulares, no responden a los tratamientos de acondicionamiento y por lo tanto no desarrollan memoria de hidratación.

La respuesta germinativa de las esporas estudiadas a los tratamientos de acondicionamiento confirma que los mecanismos desarrollados a lo largo de la evolución por los helechos en respuesta a la deshidratación son homólogos a los de los taxones inferiores y superiores de las plantas como sugieren Sharpe *et al.*, (2010) y Ballesteros *et al.* (2017). Además, el desarrollo de memoria hídrica en las esporas de helechos sometidos a acondicionamiento apoya la propuesta de González-Zertuche *et al.* (2001), de que la tolerancia a los ciclos de hidratación-deshidratación debieron evolucionar en el banco de diásporas del suelo.

Los cambios en el contenido de ácidos grasos mostraron que los lípidos, principalmente el ácido oléico, se movilizan y/o catabolizan en la oscuridad durante las fases de imbibición I y II. La movilización de ácidos grasos difirió entre las esporas no clorofílicas y criptoclorofílicas. Las especies no clorofílicas catabolizaron sus reservas lipídicas (los ácidos oléico, palmítico y linoléico), mientras que las especies criptoclorofílicas aumentaron su concentración, lo que sugiere que las esporas criptoclorófilas al tener homoioclorofila probablemente no requieren de ácidos grasos para el ensamblaje del aparato fotosintético durante la imbibición en la oscuridad. El incremento observado en ácidos grasos podría relacionarse con el hecho de que previenen la peroxidación de lípidos, protegen las membranas celulares y/o se comportan como metabolitos que responden al estrés, como en el esporofito de *P. polypodioides* (John y Hasenstein 2018; 2020).

El acondicionamiento mátrico simuló la permanencia de las esporas en el suelo, sin embargo, el acondicionamiento hídrico provocó cambios más pronunciados en las concentraciones de ácidos grasos. Esto sugiere que la cantidad de agua en los distintos micrositios del suelo podría ser una señal que indique a las esporas su disponibilidad para la germinación y el establecimiento de gametofitos en el campo. A pesar de este resultado no se encontró relación entre la respuesta al acondicionamiento de las esporas de las especies estudiadas y su hábitat, lo que refleja que la heterogeneidad del suelo, es decir la diversidad de micrositios en el suelo podría ser más relevante para la germinación de las esporas de las especies estudiadas

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