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ARTICULOS

¿El sombreamiento es capaz de potenciar el crecimiento de plantas de *Myrocarpus frondosus* en el campo?

Is shading able to potentiate the growth of *Myrocarpus frondosus* seedlings in the field?

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SUMMARY

Light is an important environmental factor in the establishment of vegetation. The knowledge of the behavior of the species in response to light demand for survival and growth in the field becomes essential. Thus, the present study aimed at evaluating the survival and initial growth of *Myrocarpus frondosus* seedlings under different levels of shade in the field. The treatments used were: 0 % (full sun); 18 %; 50 % and 70 % shading in a randomized block design. The survival of seedlings was evaluated 30 and 540 days after planting (d.a.p.) and the morphological attributes height (H), stem diameter (DC) and H / DC ratio every 90 days. Leaf area, leaf dry matter, dry matter of stem and branches and dry matter of shoot were obtained 540 d.a.p. The analyses of the physiological attributes fluorescence of chlorophyll *a* and photosynthetic pigments were performed 180, 360 and 540 d.a.p. The use of shading in the planting of *Myrocarpus frondosus* influences the survival and the morphological and physiological attributes of the species. The morphological and physiological attributes of *Myrocarpus frondosus* plants show that the species requires shading of 50 to 70 % in its initial growth phase in the field (540 d.a.p.). It is recommended to use the species in sub-forest enrichment plantations and in consortium with other species more demanding in light.

Key words: tree species, morphological and physiological attributes, forest enrichment, light demand.

RESUMEN

La luminosidad es un factor ambiental importante en el establecimiento de la vegetación, siendo imprescindible el conocimiento del comportamiento de las especies en respuesta a la demanda de luz para supervivencia y crecimiento en el campo. El presente estudio tuvo como objetivo evaluar la supervivencia y el crecimiento inicial de plantas de *Myrocarpus frondosus* bajo diferentes niveles de sombreamiento en el campo. Los tratamientos utilizados fueron 0 % (pleno sol), 18 %, 50 % y 70 % de sombreamiento en un diseño de bloques al azar. La supervivencia de las plantas fue evaluada a los 30 y 540 días después de la plantación (ddp) y los atributos morfológicos altura (H), diámetro del cuello (DAC) y relación H/DC cada 90 días. A los 540 ddp se obtuvo el área foliar, materia seca foliar, materia seca del tallo y ramas y la materia seca de la parte aérea. Los análisis de los atributos fisiológicos fluorescencia de la clorofila α y los pigmentos fotosintéticos fueron realizados a los 180, 360 y 540 ddp. La utilización de sombreamiento en la plantación de *Myrocarpus frondosus* evidencian que la especie necesita de 50 y 70 % de sombra en la fase inicial de crecimiento en el campo (540 ddp). Se recomienda el uso de la especie en plantaciones de enriquecimiento de sub bosque y en consorcio con otras especies más exigentes en luminosidad.

Palabras clave: especie forestal, atributos morfológicos y fisiológicos, enriquecimiento forestal, demanda de luz.

INTRODUCCIÓN

Un factor importante para el éxito en programas de restauración forestal es la utilización de plantas de calidad de especies adaptadas a las condiciones edafoclimáticas. Las especies poseen adaptaciones fisiológicas complejas que permiten soportar niveles de luminosidad desfavorables, falta de agua y otros factores ambientales (Yu *et al.* 2018). En ecosistemas naturales la disponibilidad de luz presenta variaciones en el tiempo y en el espacio, siendo muy importante para el establecimiento y crecimiento (Begon *et al.* 2007, Valladares *et al.* 2016). Las especies arbóreas presentan respuestas diferentes en relación con la luminosidad, muchas veces asociada al grupo ecológico.

Dependiendo de la condición de luminosidad a que las plantas son expuestas, pueden ocurrir cambios en la morfología foliar para mejorar la eficiencia de captación de luz (Larcher 2000, Martins *et al.* 2015) siendo eso suficiente para dar continuidad al crecimiento.

Conforme Souza *et al.* (2009) la capacidad de utilizar y adaptarse a diferentes luminosidades es fundamental para la distribución de las especies en la regeneración de claros. No obstante, poco se conoce sobre las respuestas morfológicas y fisiológicas de las especies arbóreas en diferentes condiciones de luz, observándose que algunas expresan su mejor potencial de crecimiento en pleno sol o en la sombra.

En ese sentido, varias especies con potencial de uso en programas de forestación y reforestación necesitan de investigaciones sobre su desarrollo en diferentes niveles de sombreamiento en el campo, siendo que la mayoría de los estudios fueron desarrollados solamente en la fase de vivero, es decir, en la fase que las plantas son menos susceptibles a daños causados por factores ambientales. Como es el caso de la especie *Myrocarpus frondosus* Allemão, que no posee informaciones sobre el desarrollo inicial de plantas en relación a la demanda de luz en el campo.

La especie pertenece a la familia Fabaceae, estando presente en las principales formaciones forestales, en Bolivia, Paraguay y Brasil, excepto en el cerrado (Lorenzi 2002). Se destaca entre las principales especies arboles nativas de mayor producción comercial de madera, por ser muy resistente, por la alta densidad y durabilidad, aparte de ser utilizada en la recomposición de ecosistemas alterados, reposición de bosques de galería, arborización urbana, en la industria de perfumerías y medicamentos (Carvalho 2003, Santi *et al.* 2017). La especie presenta diferentes clasificaciones en cuanto a su grupo sucesional como secundaria inicial (Vaccaro *et al.* 1999), secundaria tardía (Durigan y Noguera 1990) y semi-heliofita (Carvalho 2003).

Esa divergencia en la clasificación en cuanto al grupo sucesional puede ocasionar problemas en la supervivencia y crecimiento inicial de la especie, evidenciando la necesidad de investigaciones que evalúen su crecimiento y desarrollo en diferentes niveles de sombreamiento, para viabilizar su uso en plantaciones. El trabajo formuló la siguiente hipótesis: en el campo, dentro del área de distribución natural de la especie, el sombreamiento facilita el establecimiento y crecimiento de plantas de *Myrocarpus frondosus*. De esa manera, este estudio tiene como objetivo evaluar la supervivencia y el crecimiento inicial de plantas en campo, bajo diferentes niveles de sombreamiento.

MÉTODOS

El experimento fue instalado en octubre de 2015, en un área próxima al Vivero Forestal (29º 43' 13'' S e 53º 43' 17'' O) de la Universidad Federal de Santa Maria (UFSM), municipio de Santa Maria, RS, Brasil, que se encuentra dentro del área de distribución natural de la especie. De acuerdo a la clasificación de Köppen, el clima de la región es subtropical, del tipo "Cfa", con lluvias bien distribuidas durante todos los meses del año, presentando precipitación media anual de 1.620 mm y temperatura media del mes más frio -3 °C y del mes más caliente superior a 22 °C (Alvares *et al.* 2013).

Inicialmente fue realizada la colecta de muestras de suelo en las profundidades de 0 a 20 cm y de 20 a 40 cm, para la caracterización del área, siendo enviadas para el Laboratorio de Análisis de Suelos de la UFSM. De forma general, el análisis de suelo indicó pH muy bajo a medio (5,3), correspondiendo a un suelo ácido, contenido de materia orgánica (MO) bajo (1,1 %), nivel de fósforo considerado muy bajo a medio (4,5 mg dm^{-3}), potasio alto (69,3 mg dm^{-3}), magnesio alto (1,8 cmolc dm^{-3}), calcio de medio a alto (5,2 cmolc dm^{-3}), índice SMP de 6,3 y CTC pH7 de 10,5 cmolc dm^{-3} (CQFS/SBCS-RS/SC 2004). A pesar del valor bajo de pH el encalado no fue realizado, teniendo en cuenta que los tenores estaban dentro de los previstos para especies forestales.

El experimento fue conducido en un diseño de bloques al azar, con cuatro repeticiones en cada tratamiento, las evaluaciones se hicieron en diferentes momentos. Cada unidad de muestreo fue representada por seis plantas en espaciamiento 1,0 m x 1,0 m, totalizando 96 plantas. Los niveles del factor de sombreamiento fueron (0 % - pleno sol, 18, 50 y 70 %) y los niveles del factor tiempo fueron diferentes evaluaciones para atributos morfológicos a los 0, 90, 180, 270, 360, 450 y 540 días después de la plantación (ddp), para la supervivencia a los 30 y 540 ddp y atributos fisiológicos a los 180, 360 y 540 ddp. En los tratamientos con sombreamiento fueron utilizadas mallas de nylon, dispuestas sobre arcos de metal recubiertos con caños plásticos con estructura sustentada por estacas de madera. La dimensión de cada armazón fue de 2,5 m x 2,5 m en la base y 2,20 m de altura en el área cubierta para no comprometer los tratamientos.

Las plantas de *Myrocarpus frondosus* utilizadas en la plantación fueron producidas en recipientes de 180 cm^3 con sustrato Carolina Soil® a base de turba de *Sphagnum* y fertilizante de liberación controlada, Osmocote®, 18-05-09 ($N\text{-}P_2O_5\text{-}K_2O$) en la dosis de 6 g L^{-1}, en el Vivero Forestal de la UFSM. Las mismas fueron seleccionadas de un

lote que presentó altura promedio de 20,9 cm y diámetro del cuello de 4,4 mm, 300 días después de la emergencia.

Previamente fue realizada la eliminación semimecanizada (desmalezadora costal) de la vegetación herbácea. La abertura de los hoyos para la plantación fue realizada con la ayuda de un perforador de suelo, acoplado a un tractor, en las dimensiones de 0,3 m de diámetro x 0,3 m de profundidad (0,02 m^3). La fertilización de base fue utilizada con 8 L de estiércol bovino seco por planta, incorporado al suelo en el hoyo.

Posterior a la plantación, las plantas fueron regadas, para la acomodación del suelo en su entorno. La irrigación de las plantas durante los tres primeros meses fue realizada cada tres días con regaderas, con aproximadamente 2 L de agua por planta, en la ausencia de precipitación, posterior a ese periodo no hubo más necesidad de irrigaciones, debido a que la precipitación fue suficientemente distribuida para mantener el suelo húmedo.

El control de hormigas cortadoras se realizó cuando fue necesario aplicándose hormiguicida granulado. El control de malezas, alrededor de las plantas, fue realizado con carpida manual (coronado) y entre los bloques con limpieza semimecanizada (desmalezadora costal). La fertilización de cobertura fue realizada a los 180 ddp con 8 L de estiércol bovino seco por planta, siendo la misma fertilización de base. No obstante, a los 360 ddp, no fue posible obtener el estiércol en el local, así que fue realizada fertilización química con fertilizante de liberación controlada Polyblen® (N-P-K 18-08-18 + azufre y boro), con aproximadamente 130 g por planta.

La supervivencia de las plantas fue evaluada a los 30 y 540 ddp y a los 0, 90, 180, 270, 360, 450 y 540 ddp fueron realizadas las mediciones de los atributos morfológicos altura (H) con regla milimetrada (cm) y diámetro del cuello (DC) con un pie de metro digital de precisión (mm). A partir de esas variables fue posible obtener la relación H/DC.

Además, a los 540 ddp fueron obtenidos los atributos del área foliar (AF), materia seca foliar (MSF), materia seca del tallo y ramas (MSTR) y materia seca de la parte aérea (MSPA). Las colectas fueron realizadas considerando la H y el DC promedios de cada repetición, muestreando una planta de cada repetición. Los tallos de las plantas fueron cortados en la altura del cuello, con la ayuda de un serrucho y las hojas fueron separadas del tallo principal y ramas, y utilizadas para la determinación del área foliar. Para AF fueron colectadas aleatoriamente 30 hojas de las plantas, las mismas fueron distribuidas sobre papel A4 con escala milimétrica, prensadas en vidrio y fotografiadas con cámara digital con zoom de 1,4x. Las imágenes fueron procesadas con la ayuda de software Image J. Posteriormente el material fue acondicionado en bolsas de papel tipo Kraft y llevado a una estufa con circulación de aire a 65°C hasta alcanzar peso constante y, posteriormente pesadas en balanza analítica, para la determinación de la MSF, MSTR y MSPA. El AF fue obtenida de acuerdo a adaptaciones de la metodología de Coelho-Filho *et al.* (2012), utilizando la siguiente fórmula:

$$AF = \frac{(MSTH * AF30H)}{MS30H} \quad [1]$$

En que: AF= área foliar; MSTH= materia seca de todas las hojas; AF30F= área foliar de 30 hojas; y MS30H= materia seca de 30 hojas.

Los análisis fisiológicos de fluorescencia de clorofila α y cuantificación de los pigmentos fotosintéticos fueron realizados a los 180, 360 y 540 ddp en el campo. La cuantificación de los pigmentos fotosintéticos clorofila α, clorofila *b*, relación α/*b* y carotenoides fue realizada en el Laboratorio de Fisiología Vegetal, del Departamento de Biología de la UFSM. Se colectó la cuarta hoja expandida de tres plantas por tratamiento que fueron congeladas en nitrógeno líquido y, posteriormente, almacenadas en ultra freezer a -80 °C, hasta la cuantificación. La cuantificación de los pigmentos fotosintéticos fue realizada de acuerdo a la metodología de Hiscox e Israelstam (1979), donde las muestras frescas de hojas (0,05 g) fueron incubadas a 65 °C con dimetilsulfóxido (DMSO) por dos horas, posteriormente fue realizada la lectura de las absorbancias en espectrofotómetro (Celm E-205D), en las longitudes de onda 663, 645 y 470 nm, para Chl α, Chl *b* y carotenoides, respectivamente. Posteriormente las medias fueron estimadas utilizando la fórmula de Lichtenthaler (1987).

La fluorescencia de la clorofila α fue evaluada en cinco plantas por tratamiento, utilizando fluorómetro portátil de luz modulada Junior-Pam (Walz, Germany). La medición fue realizada en días soleados (entre 7h30min y 10h30min), en el tercio medio de la planta en las hojas expandidas representativas de cada tratamiento. Las hojas fueron adaptadas al oscuro por 30 minutos con papel aluminio, para la medición de la fluorescencia inicial (Fo) y, posteriormente sometidas al pulso de luz saturante (10.000 µmol m^{-2} s^{-1}) por 0,6 s, determinándose de esta manera la fluorescencia máxima (F_m). El rendimiento cuántico máximo PSII (F_v/F_m) fue obtenido por la razón de la fluorescencia variable ($F_v=F_m-F_o$) y la fluorescencia máxima. Además de eso, fue obtenida la tasa de transporte de electrones (ETR).

Los datos fueron evaluados en cuanto a los presupuestos de normalidad por el test de Shapiro-Wilk y homogeneidad por el test de Bartlett. Cuando fue observada diferencia significativa entre los tratamientos por el test de F, las medias fueron comparadas por la prueba de Tukey y/o análisis de regresión polinomial, al 5 % de probabilidad de error. Los análisis estadísticos de los datos fueron realizados utilizando el paquete estadístico Sisvar v. 5.3 (Ferreira 2014).

RESULTADOS

Los datos meteorológicos de precipitación, temperatura media, máxima y mínima registradas en el municipio de Santa María fueron obtenidos en la Estación Meteorológica de Santa María, localizada en el Campus de la UFSM (figura 1).

Los datos fueron normales y homogéneos. La tasa de supervivencia de las plantas de *Myrocarpus frondosus* en campo, a los 30 días después de la plantación (ddp) fue de 100 % en los tratamientos 18, 50 y 70 % de sombreamiento, difiriendo del tratamiento a pleno sol (97 % de supervivencia). A los 540 ddp la supervivencia fue menor en los tratamientos a pleno sol (75 %), difiriendo del sombreamiento con 18 y 50 % (92 %) y del sombreamiento con 70 %, que presentó mayor supervivencia (96 %) (cuadro 1).

Se verificó interacción ($P < 0,05$) para los atributos altura (H), diámetro de cuello (DC) y relación H/DC. Para H, el crecimiento fue cuadrático creciente en todos los niveles de sombreamiento (figura 2A), con las mayores medias observadas bajo 70 % y las menores a pleno sol. A los 540 ddp la media de altura fue de 209,7 cm con 70 % de sombreamiento, mientras que en pleno sol la media fue de 100,1 cm. Para DC también fue observado crecimiento cuadrático creciente, en que las mayores medias fueron

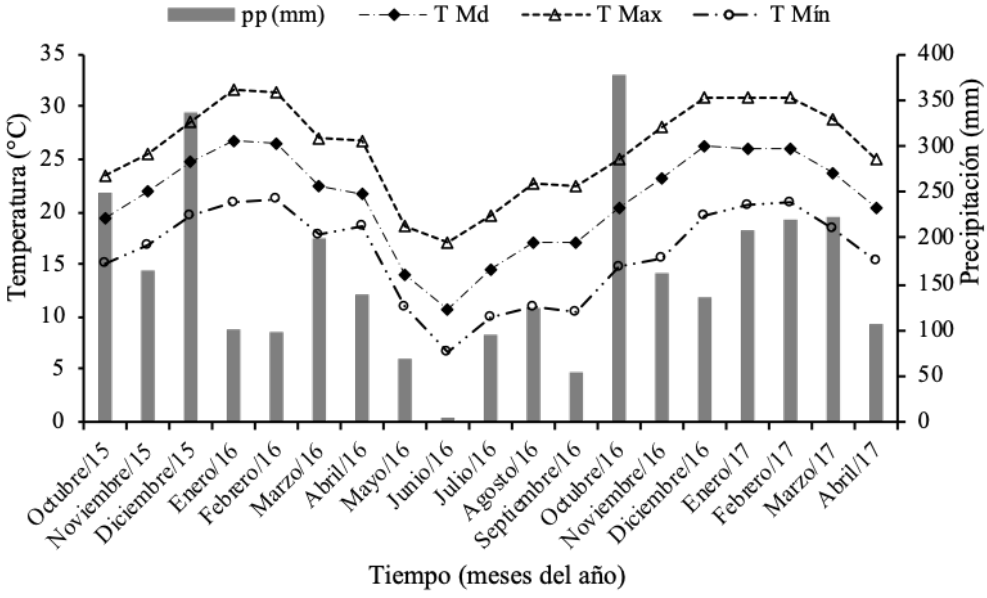

Figura 1. Precipitación (pp), temperatura media (T Md), temperatura máxima (T Max) y temperatura mínima (T Min) registradas en el municipio de Santa Maria, RS, durante la conducción del experimento de plantas de *Myrocarpus frondosus* en campo. Fuente: BDMET/INMET (2018).

Precipitation (pp), average temperature (T Md), maximum temperature (T Max) and minimum temperature (T Min) registered in the municipality of Santa Maria, RS, during the conduct of the experiment of seedlings from *Myrocarpus frondosus* in the field.

Cuadro 1. Tasa de supervivencia, área foliar (AF), materia seca foliar (MSF), materia seca de tallo y ramas (MSTR) y materia seca de la parte aérea (MSPA) de plantas de *Myrocarpus frondosus*, a los 540 días después de la plantación en campo, Santa María, RS.

Survival rate, leaf area (AF), leaf dry matter (MSF), dry matter of stems and branches (MSTR) and aboveground dry matter (MSPA) plant *Myrocarpus frondosus*, 540 days after planting in the field, Santa Maria, RS.

Nivel de Sombreamiento (%)	Supervivencia días después de la plantación (%)		AF (m²)	MSF (g)	MSTR (g)	MSPA (g)
	30	540				
0	97 b*	75 c	1,310 b*	115,0 b	244,5 b	359,5 b
18	100 a	92 b	3,178 a	192,4 b	340,7 b	533,0 b
50	100 a	92 b	2,915 a	257,2 a	466,8 a	724,0 a
70	100 a	96 a	4,368 a	354,7 a	648,5 a	1.003,2 a
Media general	99,25	88,7	2,94	229,8	425,1	654,9
CV (%)	0,88	33,46	26,04	5,96	5,20	4,77

*Medias seguidas de la misma letra no difieren entre sí, según la prueba de Tukey al 5 % de probabilidad de error. CV: Coeficiente de variación.

observadas con 70 % de sombreamiento y las menores en pleno sol (figura 2B). A los 540 ddp las medias fueron de 24,4 mm en el sombreamiento con 70 % y 19,6 mm en pleno sol. Para la relación H/DC el crecimiento fue cuadrático decreciente en todos los sombreamientos (figura 2C). Las mayores medias fueron observadas con 70 % de sombreamiento y las menores a pleno sol.

Para los atributos área foliar (AF), materia seca foliar (MSF), materia seca del tallo y ramas (MSTR) y materia seca de la parte aérea (MSPA) hubo diferencia ($P < 0,05$)

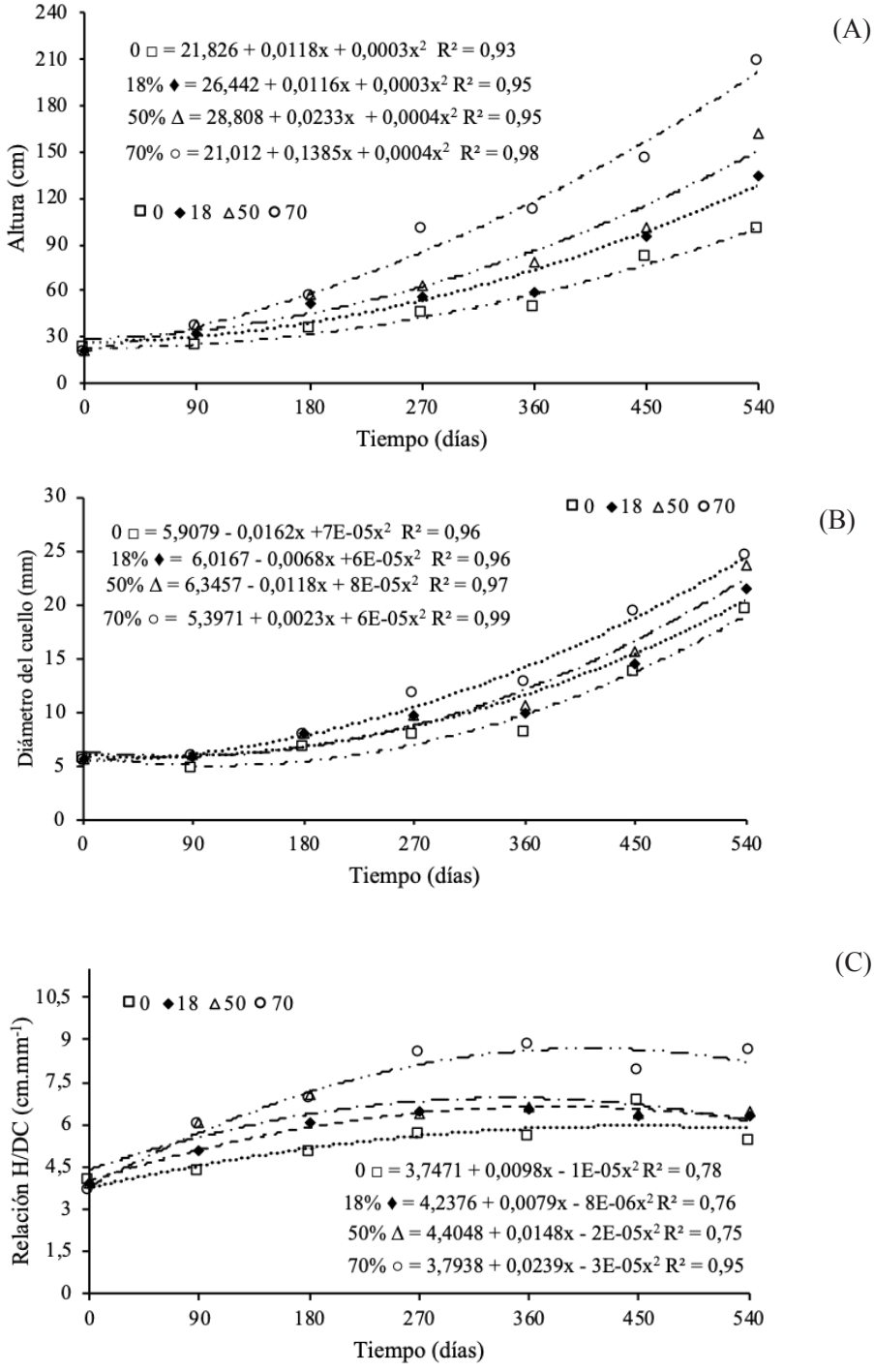

Figura 2. Crecimiento en altura (A), diámetro de cuello (B) y relación H/DC (C) de plantas de *Myrocarpus frondosus*, en diferentes intensidades de sombreamiento y tiempos de evaluación, en campo, Santa Maria, RS.

Height growth (A), stem diameter (B) and ratio H/DC (C) seedlings of *Myrocarpus frondosus*, on different intensity of shading and evaluation times, in the field, Santa Maria, RS.

entre los sombreamientos. La mayor AF fue observada con 70 % de sombreamiento, no difiriendo de los sombreamientos con 18 y 50 %, la menor media fue a pleno sol, difiriendo de los demás sombreamientos (cuadro 1). La MSF, MSTR y la MSPA presentan comportamientos semejantes en los diferentes sombreamientos, las mayores medias fueron encontradas en el sombreamiento con 70 %, no difiriendo del tratamiento con 50 % y las menores medias fueron observadas en el tratamiento a pleno sol.

Para los atributos clorofila α, clorofila *b* y razón α/*b* no hubo interacción, solamente efecto significativo aislado para los factores. La tasa de transporte de electrones no presentó diferencia significativa para el sombreamiento (P = 0,9944) apenas para el tiempo a los 360 ddp. La clorofila α fue mayor a 50 y 70 % de sombreamiento, difiriendo los demás tratamientos y la menor media fue a pleno sol (cuadro 2). En el tiempo, las mayores medias fueron observadas a los 180 y 360 ddp. Para la clorofila *b* la mayor media fue observada con 70 % de sombreamiento y en las evaluaciones a los 180 y 360 ddp. La razón clorofila α/*b* también presentó la misma tendencia en el tiempo, no obstante, las mayores medias fueron observadas con 50 y 70 % de sombreamiento. Para los carotenoides no hubo diferencia para el sombreamiento (P = 0,0988), solo para los tiempos de evaluación, siendo que la mayor media fue observada a los 540 ddp.

Hubo interacción (P < 0,05) entre los factores sombreamiento x tiempo para la fluorescencia inicial (F_o), fluorescencia máxima (F_m) y rendimiento cuántico máximo PSII (F_v/F_m). Para Fo hubo solo diferencia en el sombreamiento con 50 % y 70 % a los 540 ddp (cuadro 3).

Con relación a los tiempos de evaluación las mayores medias fueron observadas a los 540 ddp para pleno sol, 50 % y 70 % de sombreamiento, no difiriendo de 18 % a los 180 ddp. Para F_m las mayores medias fueron observadas a los 180 y 540 ddp a pleno sol y con 18 % de sombreamiento no difiriendo de los sombreamientos a los 540 ddp. Las mayores medias de F_v/F_m fueron observadas a los 360 ddp (cuadro 3).

DISCUSIÓN

En el establecimiento inicial a campo de las plantas de *Myrocarpus frondosus* es necesario sombreamiento para que ocurra mayor supervivencia (cuadro 1). La tasa de supervivencia inicial observada puede ser considerada alta para especies nativas, demostrando adecuado establecimiento en campo. Resultados semejantes fueron observados en estudios con sombreamiento con *Apuleia leiocarpa* (Vogel) J.F. Macbr (Aimi *et al.* 2017). En ese sentido, se destaca las variaciones de las especies arbóreas, en cuanto a condiciones de sombreamiento, y la necesidad de estudios considerando la luminosidad como un factor de selección en el bosque, durante los estadios sucesionales (Freitas *et al.* 2012).

Para los atributos morfológicos H, DC, y relación H/DC fueron observadas las mayores medias para plantas sometidas a 70 % de sombreamiento (figura 2). A los 540 ddp las plantas presentaron media de H de 210 cm, lo que está de acuerdo con Lorenzi (2002) que describe a la especie con crecimiento medio de 2,5 m a los dos años. Además de eso, cuanto mayor la H y el DC, mejor será la supervivencia y el establecimiento de las plantas en campo.

Cuadro 2. Tasa de transporte de electrones (ETR), clorofila α, clorofila *b*, razón clorofila α/clorofila *b* y carotenoides de plantas de *Myrocarpus frondosus* en diferentes sombreamientos y tiempos de evaluación en campo, Santa Maria, RS.

Electron transport rate (ETR), chlorophyll *a*, chlorophyll *b*, chlorophyll *a*/chlorophyll *b* and carotenoids *Myrocarpus frondosus* seedlings in different shades and evaluation times in the field, Santa Maria, RS.

Nivel de sombreamiento (%)	Tasa de transporte de electrones – ETR (μmol m^{-2} s^{-1})	Clorofila *a* (mg g^{-1})	Clorofila *b* (mg g^{-1})	Razón clorofila *a*/*b*	Carotenoides (mg g^{-1})
0	139,353 ^{ns}	1,357 b	0,411 b	3,181 b	0,436^{ns}
18	139,799	1,402 b	0,417 b	3,272 b	0,444
50	133,466	1,530 a	0,506 b	3,672 a	0,480
70	135,864	1,550 a	0,615 a	3,921 a	0,474
Tiempo (días)					
180	77,242 b*	1,569 a	0,583 a	3,892 a	0,446 b
360	265,598 a	1,556 a	0,530 a	3,765 a	0,444 b
540	68,521 b	1,253 b	0,348 b	2,878 b	0,486 a
Media general	137,120	1,459	0,487	3,511	0,459
CV (%)	2,77	12,17	29,15	15,57	10,83

* Medias seguidas de la misma letra no difieren entre sí, según la prueba de Tukey al 5 % de probabilidad de error. ^{ns} = no significativo y CV: Coeficiente de variación.

La mayor capacidad de crecimiento de las plantas en ambientes sombreados es un mecanismo importante de adaptación de la especie para sobrevivir en esa condición de menor intensidad luminosa (Siebeneichlen *et al.* 2008). Cuando eso ocurre, no necesariamente significa que la planta demanda sombreamiento durante la plantación, pero que hasta determinado punto responde favorablemente a tal condición, como fue observada para las plantas de *M. frondosus* a los 540 ddp. A partir de eso, otros atributos morfológicos evaluados pueden ser utilizados para verificar la calidad de las plantas, lo que puede ser comprobado con el AF y MSPA.

Las medias de los atributos morfológicos en los mayores niveles de sombreamiento (50 % y 70 %) demostraron que las plantas de *M. frondosus* no presentan etiolamiento, que es una respuesta morfogénica que ocurre por la menor condición de luminosidad durante el crecimiento vegetal e incluye el alargamiento caulinar envés de aumentar en espesor (Taiz *et al.* 2017). Además de eso, esa alteración ocurre principalmente en las especies arbóreas con poca tolerancia al sombreamiento en el bosque, que de esa forma alcanzan, con mayor rapidez, superar en altura las plantas que las sombrean (Poorter 1999).

El AF y la MSA también fueron mayores en el sombreamiento con 50 % y 70 % lo que puede ser explicado por el hecho de la necesidad de aumentar la captación de luz ocasionando hojas con área foliar mayor (Larcher y Boeger 2009). Generalmente, el incremento del AF con el sombreamiento es una de las maneras de la planta aumentar a superficie fotosintética, asegurando un aprovechamiento más eficiente de las menores intensidades luminosas y, compensando las bajas tazas de fotosíntesis en las hojas de sombra (Jones y McLeod 1990). En pleno sol fueron encontradas las menores medias para AF y MSA, así la mayor disponibilidad de luz en las hojas se refleja en el espesamiento y menor área foliar, pues la luz no es un factor limitante (Larcher y Boeger 2009). El aumento en la espesura de la hoja en especies en pleno sol ocurre debido a la mayor cantidad de células que forman la camada empalizada, protegiendo la hoja en contra

Cuadro 3. Fluorescencia inicial (F_o), fluorescencia máxima (F_m) y rendimiento cuántico máximo (F_v/F_m) de plantas de *Myrocarpus frondosus* en diferentes sombreamientos y tiempos de evaluación en campo, Santa Maria, RS.

Initial fluorescence (F_o), maximum fluorescence (F_m) and maximum PSII in quantum yield (F_v/F_m) of seedlings from *Myrocarpus frondosus* in the field in different shades and times, Santa Maria, RS.

Atributos	Nivel de Sombreamento (%)	Tiempo (días)		
		180	360	540
F_o	0	192,0 Ba*	153,5 Ba	244,2 Ab
	18	226,7 Aa	142,25 Ba	309,5 Ab
	50	240,7 Ba	154,5 Ca	460,0 Aa
	70	177,7 Ba	157,25 Ba	537,3 Aa
Media general		216,31		
CV (%)		6,76		
F_m	0	533,5 Aa	186,5 Ba	353,2 Ac
	18	544,0 Aa	184,0 Ba	705,0 Ab
	50	591,5 Ba	238,7 Ca	919,0 Aa
	70	561,5 Ba	248,0 Ca	1165,0 Aa
Media general		519,1		
CV (%)		5,48		
F_v/F_m	0	0,73 Aa	0,71 Aa	0,31 Bb
	18	0,59 Ba	0,78 Aa	0,53 Ba
	50	0,60 Ba	0,76 Aa	0,45 Ca
	70	0,69 Ba	0,78 Aa	0,54 Ba
Media general		0,62		
CV (%)		15,26		

*Medias seguidas de la misma letra mayúscula en la línea y minúsculas en la columna no difieren entre sí, según la prueba de Tukey al 5 % de probabilidad de error. CV: Coeficiente de variación.

de posibles daños causados por la elevada luminosidad (Taiz *et al.* 2017).

Para la clorofila α y clorofila *b* las mayores medias fueron observadas con 50 % y 70 % de sombreamiento. De forma general, las clorofilas y los carotenoides tienden a aumentar con la reducción de la intensidad luminosa. La clorofila α es el principal pigmento utilizado en la etapa fotoquímica de la fotosíntesis, pues constituye el centro de reacción del fotosistema, en cuanto a la clorofila *b* actúa en el complejo antena colector de luz, auxiliando en la observación y transferencia de energía para el PSII (Streit *et al.* 2005). El aumento de las clorofilas en los niveles de mayor sombreamiento puede ser debido a la compensación de la especie a la menor cantidad de radiación disponible. En hojas sombreadas, como es el caso de las especies no pioneras en su estadio inicial de crecimiento, puede ocurrir alargamiento del parénquima lagunoso. También, puede ocurrir aumento en la síntesis de clorofila *b* y carotenoides, intensificando la capacidad de absorción de luz en el ambiente sombreado (Taiz *et al.* 2017).

Para los carotenoides no hubo diferencia entre los sombreamientos, apenas para el tiempo (cuadro 2). Normalmente, los carotenoides tienden a aumentar con la reducción de la intensidad luminosa y durante la fotosíntesis pueden desempeñar dos funciones distintas: absorción de luz (pigmentos accesorios) y foto protectores del aparato fotoquímico (Kerbauy 2004). Conforme Streit *et al.* (2005) por la estructura química ser inestable, las clorofilas son fácilmente degradadas y sintetizadas, en la misma proporción. Así, hojas de sombra presentan mayor cantidad de pigmentos que hojas a pleno sol.

La menor media de la fluorescencia inicial (F_o), obtenida con 70 % de sombreamiento a los 180 ddp con 18 % de sombreamiento a los 360 ddp y a pleno sol a los 540 ddp demuestran plantas en mejores condiciones en relación a los otros sombreamientos, pues cuanto menor el valor de F_o, menor es la pérdida de energía. El aumento en los valores de F_o a los 180 y 540 ddp indica disminución en la capacidad de transferencia de energía del complejo antena para los centros de reacciones (Baker y Rosenqvist 2004). Además, el aumento en la F_o indica que hubo daño en la proteína D1 del centro de reacción del PSII, ocasionando daños irreversibles al complejo de evolución de oxígeno (Bertamini *et al.* 2004).

Con relación a la fluorescencia máxima (F_m), a los 180 ddp esos valores permanecieron próximos en todos los sombreamientos, pero con el correr del tiempo hubo alteraciones y las menores medias fueron observadas con 18 % de sombreamiento y a pleno sol, principalmente a los 540 ddp. La reducción de F_m ha sido atribuida a la disipación no fotoquímica, principalmente por medio del ciclo de la xantofila. De ese modo, el exceso de energía absorbida por la hoja es drenado para los carotenoides del ciclo de la xantofila, que la disipa en la forma de calor, protegiendo el fotosistema II contra posibles daños oxidativos causados por la exposición a pleno sol (Demmig-Adams y Adams 2006).

La reducción de F_v/F_m puede estar asociada al tiempo de evaluación, pues a los 540 ddp correspondió al mes de abril, es decir, posterior al término del verano, periodo con menor incidencia de luz y temperaturas (Figura 1). Esa reducción indicó menor cantidad de energía absorbida por los pigmentos fotosintéticos en el complejo ante de la planta, que es utilizada para reducir el carbono y producir materia seca. En plantas saludables esa relación debe estar entre 0,75 y 0,85, valores inferiores a 0,75 indican reducción del potencial fotosintético de la planta en función de alguna situación de estrés (Ritchie *et al.* 2010).

En plantas de la especie *Cordia superba* Cham. con 12 meses de edad, en dos ambientes con diferentes luminosidades (pleno sol y 85 % de sombreamiento), Souza *et al.* (2009) verificaron que las hojas en el ambiente sombreado mostraron mayores valores para F_v/F_m que hojas a pleno sol, pudiendo ser considerada una característica deseable en ambiente con baja disponibilidad de luz. Disminución del F_v/F_m posterior a la exposición a alta irradiación de las plantas aclimatadas a condición de sombreamiento también fueron observados en otras especies arbóreas (Tobita *et al.* 2010, Aimi *et al.* 2017). La reducción del valor de ETR a los 540 ddp puede indicar disminución en la actividad del fotosistema II, con el aumento de la fotoinhibición.

Myrocarpus frondosus es una planta decidua (Lorenzi 2002), lo que puede explicar esa variación en las medias de fluorescencia de la clorofila α. A los 360 ddp las evaluaciones fueron realizadas en octubre (invierno-primavera). Mientras que las evaluaciones a los 180 y 540 ddp fueron realizadas en abril (verano-otoño). Las plantas deciduas son aquellas que evolutivamente fueron sensibles a la reducción del fotoperiodo y desencadenan mecanismos de abscisión de las hojas en el invierno (Larcher 2000). Con el aumento del periodo de luz en la primavera, las plantas liberan las hojas nuevas producidas en el final del otoño y mantenidas protegidas por brácteas durante todo el invierno, iniciando un nuevo periodo de primavera y verano de intensa actividad fotosintética (Taiz *et al.* 2017). En este estudio no fue observado pérdida de hojas de las plantas de *M. frondosus*, no obstante, a los 360 ddp hubo mayor taza de transporte de electrones y relación F_v/F_m.

En ambientes forestales, el aparato fotosintético debe ser capaz de utilizar la energía luminosa disponible de forma eficiente, una vez que esa disponibilidad se altera de acuerdo al periodo de exposición y la intensidad luminosa (Way y Pearcy 2012). Conforme Taiz *et al.* (2017) esa utilización eficiente de la luz está relacionada al mantenimiento de la capacidad de activación del aparato fotosintético, y envuelve factores bioquímicos y estomáticos, una vez que la luz actúa en la activación de enzimas relacionadas a la fijación de carbono y en el control de abertura y cierre de estomas.

Myrocarpus frondosus es clasificada como secundaria inicial (Vaccaro *et al.* 1999) a secundaria tardía (Durigan y Nogueira 1990) y semi-heliofita que tolera sombreamiento de media intensidad (Carvalho 2003), esa plasticidad po-

siblemente esté asociada a su amplia distribución geográfica. Mientras tanto, en regiones con las cuatro estaciones bien definidas, la especie presentó mejor crecimiento con 50 y 70 % de sombreamiento. Así, se recomienda el uso de la especie en plantaciones de enriquecimiento del subbosque y en asociación con otras especies.

CONCLUSIONES

La hipótesis planteada: en el campo, el sombreamiento facilita el establecimiento y crecimiento de plantas de *Myrocarpus frondosus*, es aceptada.

La utilización de sombreamiento en la plantación de *Myrocarpus frondosus* favoreció la supervivencia de las plantas y a los atributos morfológicos y fisiológicos.

Los atributos morfológicos y fisiológicos de las plantas evidenciaron que la especie necesita de sombreamiento de 50 a 70 % en la fase inicial de su crecimiento en campo (540 ddp).

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Germinación y efectos del almacenamiento de frutos de *Prosopis ruscifolia* (Fabaceae)

Germination and effects of storage of fruits of *Prosopis ruscifolia* (Fabaceae)

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SUMMARY

Prosopis ruscifolia is a promising forest species for restoration of saline areas that produces high quality wood and nutraceutical interest fruits. Its germination requirements and optimal storage conditions are unknown. The objectives of the work were 1) to evaluate different methods to stimulate germination, 2) to establish if there is an interaction between pregerminative treatments and the substrate used in laboratory tests (paper or sand), and 3) to analyze the effect of three different conditions of fruit storage on germination. Normal seedlings (PG), abnormal seedlings, hard and dead seeds were recorded. Three fruit samples were stored for eight months at 7 ± 2 °C, -17 ± 2 °C and the control at 25 °C. Afterwards, germination tests were carried out and the speed indexes (IVG) and mean germination time (TMG) were determined. Results indicated that chemical scarification with sulfuric acid was the most efficient pregerminative treatment to stimulate germination in both substrates. Immersion in water at room temperature showed the lowest percentage of germination in both substrates. The seeds of fruits stored at 7 ± 2 ° C had the highest germination percentage. Low temperatures improved velocity indices (IVG) and mean germination time (TMG). Results indicated the need for methods to overcome seed dormancy in *P. ruscifolia* and the excellent tolerance to low temperatures during fruit storage.

Key words: dormancy, seed test, storage, vinal.

RESUMEN

Prosopis ruscifolia es una especie forestal promisoria para restauración de áreas salinas, que produce madera de calidad y frutos de interés nutraceútico. Sus requerimientos germinativos y condiciones de almacenamiento óptimas no se han establecido aún. Los objetivos del trabajo fueron 1) evaluar diferentes métodos para estimular la germinación, 2) establecer si hay interacción entre los tratamientos pregerminativos y el sustrato empleado en ensayos de laboratorio (papel o arena), y 3) analizar el efecto de tres diferentes condiciones de almacenamiento de los frutos sobre la germinación. Se registraron plántulas normales (PG), plántulas anormales, semillas duras y muertas. Tres muestras de frutos se almacenaron durante ocho meses a 7 ± 2 °C, -17 ± 2 ºC y el control a 25 °C. Luego se realizaron ensayos de germinación y determinaron los índices de velocidad (IVG) y tiempo medio de germinación (TMG). Los resultados indicaron que la escarificación química con ácido sulfúrico fue el tratamiento pregerminativo más eficiente para estimular la germinación en ambos sustratos. La inmersión en agua a temperatura ambiente mostró el menor porcentaje de germinación en ambos sustratos. Las semillas de los frutos almacenados a 7 ± 2 °C presentaron el máximo porcentaje de germinación. Las bajas temperaturas mejoraron los índices de velocidad (IVG) y tiempo medio de germinación (TMG). Los resultados indicaron la necesidad de métodos para superar la dormición de las semillas en *P. ruscifolia* y la excelente tolerancia a bajas temperaturas durante el almacenamiento de los frutos.

Palabras clave: dormición, análisis de semillas, almacenamiento, vinal.

INTRODUCCIÓN

Las especies de *Prosopi*s son comunes en ambientes áridos y semiáridos de América, donde representan recursos genéticos valiosos por los servicios ambientales que brindan (Villagra y Álvarez 2019). Es sabido que dichas regiones experimentan fuertes procesos de degradación, con pérdidas crecientes de recursos forestales, que afectan la producción de bienes y servicios ecosistémicos (Villagra *et al*. 2010). La recuperación productiva de áreas degradadas incluye planes de forestación y la implementación de sistemas de uso múltiple como los silvopastoriles. *Prosopis ruscifolia* Griseb. conocida como "vinal", es una especie leñosa nativa del Chaco semiárido y subhúmedo que en los últimos años se ve revalorizada tanto por la calidad de su madera, como por el valor forrajero y alimenticio de sus frutos y semillas (González Galán *et al*. 2008). Su tolerancia a la salinidad (Meloni *et al*. 2008, Taleisnik y López Launestein 2011), la convierte en una especie promisoria desde el punto de vista productivo ya que permite el desarrollo

de la actividad ganadera, con buena producción de pasturas bajo su cubierta (Astrada y Adámoli 2005). Se la propone además como pie de injerto de otras especies de *Prosopis* que son sensibles a la salinidad (Felker *et al*. 2000).

La germinación y el establecimiento de las plántulas es un proceso crítico en estos ambientes áridos y semiáridos, donde hay un marcado déficit hídrico y las plantas están expuestas a condiciones climáticas adversas a lo largo de varios meses. Las especies de *Prosopis* se propagan habitualmente de manera sexual, por lo cual es importante conocer cuáles son los métodos más adecuados para promover la germinación. En general, se considera que las fabáceas poseen semillas con dormición física debido a la presencia de sustancias hidrófobas en la cutícula, de macroescleridas en empalizada o ambas características a nivel de la cubierta seminal (Abraham de Noir *et al.* 2004, Ferreira y Borghetti 2004, Bravo *et al*. 2011, Villarreal Garza *et al*. 2013, Baskin y Baskin 2014). Lopes de Oliveira *et al*. (2017) sostienen que la dormición impuesta por la cubierta seminal es parte de una estrategia de supervivencia y perpetuación de las especies.

Entre los tratamientos pregerminativos más utilizados para superar la dormición física, se encuentran la escarificación mecánica, térmica y la química (Sanabria *et al*. 2004, Dutra *et al*. 2007, Muñoz *et al*. 2009, Pereira *et al*. 2016, Trombin Souza *et al*. 2018). Algunos autores describen los requerimientos germinativos de diferentes especies de *Prosopis* (Bravo *et al*. 2011, Sobrevilla Solís *et al*. 2013, Rodríguez Araujo *et al.* 2017) y sugieren la importancia del estudio particular para cada especie y ambiente. Los métodos para estimular la germinación de *P. ruscifolia* es información necesaria para planes de manejo en plantación. Así también, la evaluación del comportamiento germinativo en diferentes sustratos es un tópico escasamente abordado en trabajos de esta temática con especies forestales. Son escasos los trabajos donde se mencionan diferencias en el número de plántulas normales germinadas en distintos sustratos (Bravo *et al*. 2011, Lopes de Oliveira *et al*. 2017). Estos datos son de gran importancia al momento de evaluar en laboratorio la calidad de lotes de semillas, sobre todo en especies no domesticadas. Antecedentes en otras especies de *Prosopis* indican que presentan dormición impuesta por la cubierta seminal (Bravo *et al*. 2011, Sobrevilla-Solís *et al*. 2013, Rodríguez Araujo *et al*. 2017). La distribución de la humedad en el sustrato de siembra y la presencia de compuestos tóxicos pueden alterar la germinación e inclusive el número de plántulas normales en un lote de semillas (Bravo *et al*. 2011, ISTA 2015, Lopes de Oliveira *et al.* 2017).

Las semillas de *Prosopis* spp. se comportan como ortodoxas en el almacenamiento (Bonner 2008). La maduración de los frutos de esta especie se concentra durante diciembre y enero dentro de su área de distribución (Abraham de Noir y Bravo 2014). La producción de plantines en almácigos o contenedores habitualmente se realiza a partir de octubre, por lo cual los frutos suelen almacenarse durante varios meses previos a la siembra. La liberación de las semillas del fruto y la selección de aquellas que reúnen las características deseables de calidad, es una labor que demanda mucho tiempo y costos. El almacenamiento de frutos y semillas de esta especie, así como de otras especies de *Prosopis*, presenta dificultades debido al ataque de lepidópteros, coleópteros y hemípteros (Mazzuferi y Conles 2005). Una alternativa que disminuye las pérdidas por estos ataques es el almacenamiento de los frutos a largo plazo a temperaturas entre 0 y 20 °C (Piotto y Fallieri 2001, Rojas Espinoza 2004, Were *et al*. 2004). La germinación, el porcentaje de plántulas normales y los índices de germinación se reducen con un incremento de la humedad y de la temperatura de almacenamiento (Azadi y Younesi 2013). Se necesitan investigaciones tendientes a conocer las condiciones óptimas de almacenamiento de frutos de esta especie y su tolerancia a temperaturas bajo cero.

Aun cuando presenta mayores dificultades logísticas (espacio, tamizado, esterilización, entre otros), la arena como sustrato es recomendado para algunas especies que presentan inconvenientes en la germinación en papel (Dousseau *et al.* 2011, ISTA 2015). El presente trabajo plantea las siguientes hipótesis: a) las semillas de *P. ruscifolia* presentan dormición impuesta por la cubierta seminal, que puede superarse con la aplicación de procedimientos pregerminativos que disminuyen su dureza y/o interrumpen la barrera de macroesclereidas, b) el sustrato en el que se realizan las pruebas de germinación en laboratorio influye en el número de semillas germinadas con plántulas normales, y c) el almacenamiento de frutos de esta especie a temperaturas inferiores a 7 °C y -17 °C no afecta el porcentaje de germinación.

Los objetivos de este trabajo son: 1) evaluar diferentes tratamientos pregerminativos para superar la dormición de las semillas en *P. ruscifolia*; 2) establecer si hay interacción entre los tratamientos pregerminativos (inmersión en ácido sulfúrico, corte a nivel de cubierta seminal seminal, escarificación física, inmersión en agua a temperatura ambiente y en agua caliente) y el sustrato empleado en ensayos de laboratorio (papel o arena), y 3) analizar el efecto de tres diferentes condiciones de almacenamiento de frutos sobre la germinación. Estos datos son esenciales para mejorar la producción de plantines de esta especie destinados a tareas de restauración ecológica y productiva, en áreas del Chaco semiárido afectadas por salinidad.

MÉTODOS

Recolección de frutos. Se recolectaron frutos maduros de bosques secundarios de *P. ruscifolia*, en la localidad de Maco, Departamento Capital, Provincia de Santiago del Estero, Argentina a 27º51’50,14” S, 64º13’11,29” O (figura 1). Esta área se encuentra incluida en la región Chaqueña Occidental de Argentina. La cosecha se realizó durante los meses de diciembre de 2011 y enero de 2012. Se seleccionaron 10 ejemplares maduros y vigorosos. Del material

cosechado se separaron los frutos destinados a la obtención de semillas para los ensayos de germinación y para el ensayo de almacenamiento a diferentes temperaturas.

Evaluación de tratamientos pregerminativos e interacción con el sustrato. Las semillas se extrajeron manualmente de los frutos maduros usando pinzas de corte, y se mantuvieron a 7 ± 2 °C hasta su análisis para prevenir el ataque de insectos. Se realizó una selección previa descartando semillas que pudieran estar dañadas o vanas. Los ensayos se llevaron a cabo dos meses luego de la cosecha, en el Laboratorio de Semillas del Instituto de Silvicultura y Manejo de Bosques (INSIMA) perteneciente a la Facultad de Ciencias Forestales de la Universidad Nacional de Santiago del Estero. Se siguieron protocolos establecidos por ISTA (2015), para los ensayos de germinación se aplicaron los siguientes tratamientos pregerminativos: corte a nivel de cubierta seminal en el ápice distal de las semillas (opuesto al hilo; utilizando pinza de corte), abrasión colectiva de las semillas de cada repetición con discos de lija de granulometría Nº 80 adheridos a la superficie de una caja de Petri con igual tiempo de procesamiento, inmersión en agua a temperatura ambiente (25 °C, durante 24 horas), inmersión en agua caliente a temperatura inicial de 100 °C (dejando enfriar progresivamente durante 24 horas previas a la siembra), inmersión en ácido sulfúrico (H_2SO_4) concentrado (98 g mol^{-1}, durante 3 minutos y posterior lavado durante 15 minutos en agua corriente para su neutralización), más un lote de semillas sin tratar, al que se consideró como control.

Antes de la siembra las semillas fueron desinfectadas con una solución de hipoclorito de sodio (60 g L^{-1}) al 1 % durante 5 minutos, enjuagándose posteriormente con agua destilada, durante 30 minutos. Los sustratos papel y arena se esterilizaron en autoclave antes de la siembra. La misma se realizó entre toallas de papel, a las cuales se agregó un volumen de agua destilada 2,5 veces su peso. La arena estéril se humedeció uniformemente con 125 mL de agua destilada, disponiendo 2 kg en cada contenedor. Para las muestras sembradas en papel así como para la siembra en arena, se utilizaron contenedores de poliestireno con tapas herméticas, de un volumen de 3.250 mL.

Para las pruebas de germinación, se realizaron cuatro repeticiones de 50 semillas cada una por tratamiento. Se llevaron a cámara de germinación a 25 °C, con fotoperíodo de 8 horas de luz y 16 horas de oscuridad. Las evaluaciones se realizaron cada tres días, registrando el número de semillas germinadas que produjeron plántulas normales y anormales, número de semillas muertas y número de semillas duras. Las semillas se consideraron germinadas cuando los cotiledones emergieron de la cubierta seminal (germinación epígea) con desarrollo de plántula normal. La duración del ensayo fue de 21 días, considerando los antecedentes de Bravo *et al*. (2011) en *Prosopis kuntzei* Harms de la misma área de estudio.

Los ensayos de germinación se llevaron a cabo bajo un diseño factorial completamente aleatorizado. El análisis estadístico de datos se realizó con el programa Infostat (2017). El efecto de los tratamientos pregerminativos

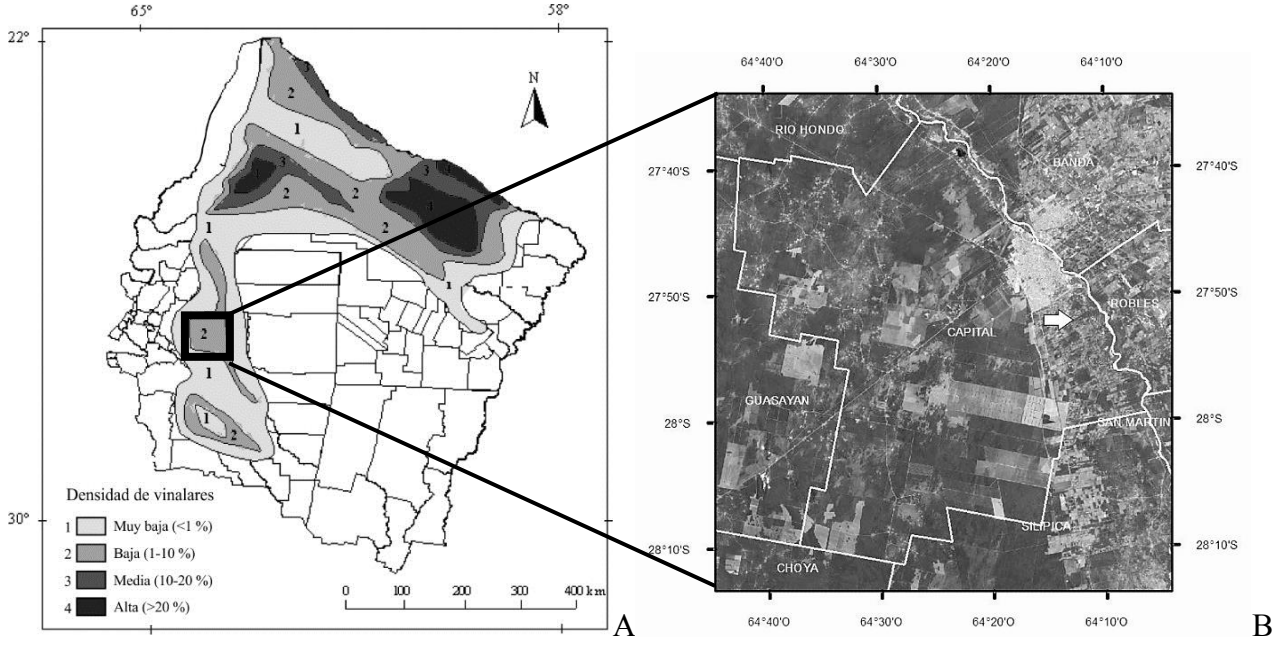

Figura 1. Distribución de *Prosopis ruscifolia* (vinal) en la Región Chaqueña de Argentina y área de recolección. A: distribución según grados de densidad tomado de Astrada y Adámoli (2005). B: Área de recolección de frutos en Santiago del Estero, Dpto. Capital.

Distribution area of *Prosopis ruscifolia* (vinal) in Chaco Region of Argentina and collection area. A: distribution according to degrees of density taken from Astrada and Adámoli (2005). B: Fruit collection area in Santiago del Estero, Capital Department.

se evaluó por medio de Modelos Lineales Generalizados Mixtos (GLMM) con distribución binomial. Los tratamientos (control, corte, escarificado, agua fría, agua caliente y ácido) y los sustratos (papel y arena) se consideraron efectos fijos, y las repeticiones como aleatorios, tanto para el análisis de semillas germinadas con plántulas normales como para semillas no germinadas (duras y muertas). La significancia de los tratamientos, representados en este trabajo como la combinación de pretratamientos y sustratos, fueron determinadas a través de pruebas secuenciales y marginales (Wald), y los contrastes de los promedios fueron analizados con la prueba de Di Rienzo, Guzmán y Casanoves al 5 %.

Efectos de diferentes temperaturas durante el almacenamiento de frutos. Para el estudio de las condiciones de almacenamiento se tomaron aleatoriamente ocho muestras de 100 g cada una y se secaron en estufa a 35 °C hasta peso constante. Luego las muestras se almacenaron en envases herméticos de vidrio, en tres condiciones diferentes: a) control: temperatura ambiente 25 ±2 °C, b) 7 ±2 °C y c) -17± 2 °C.

El material de estudio se almacenó durante ocho meses hasta el inicio de los ensayos de germinación siguiendo protocolos generales de Reglas ISTA (2015), con cuatro repeticiones de 25 semillas cada una, por tratamiento. Como método pregerminativo se utilizó la escarificación química con ácido sulfúrico (98 g mol^{-1}) siguiendo idéntico procedimiento al empleado en el ensayo de germinación y entre toallas de papel como sustrato. El ensayo de germinación posterior al almacenamiento de frutos a diferentes temperaturas tuvo una duración de 12 días y al final del mismo se registraron solo plántulas normales y semillas muertas. Bajo un diseño completamente aleatorizado, se evaluó el efecto de los diferentes tratamientos con modelos lineales generalizados mixtos (GLMM) con distribución binomial. Los tratamientos a bajas temperaturas y el control fueron considerados efectos fijos, y su significancia se determinó con las pruebas secuenciales y marginales (Wald).

Se determinó el porcentaje de germinación y para evaluar el vigor de las semillas en los distintos tipos de almacenamientos se consideraron dos índices: 1-índice de velocidad de germinación (IVG) (Maguire 1962), que expresa la velocidad en número de semillas germinadas por día, evaluándose con modelos lineales generales y mixtos; 2- el tiempo medio de germinación (TMG) (Nakagawa 1999), que mide la velocidad y dispersión de las semillas germinadas, estimado bajo modelos generalizados mixtos, con distribución binomial y enlace *logit.*

RESULTADOS

Evaluación de tratamientos pregerminativos e interacción con el sustrato. La escarificación con ácido sulfúrico fue el método más efectivo para estimular la germinación en semillas *P. ruscifolia*, ya que se obtuvo el mayor porcentaje de semillas germinadas con plántulas normales (99 %) sin diferencias significativas entre sustratos, pero con diferencias significativas de ambos respecto a los controles (cuadro 1). Los ensayos de germinación en ambos sustratos permitieron identificar plántulas normales y anormales y semillas duras y muertas de vinal a 21 días de

Cuadro 1. Porcentajes promedio de semillas germinadas con plántulas normales, plántulas anormales, semillas muertas y semillas duras, con diferentes tratamientos pregerminativos y diferentes sustratos.

Average percentages of seeds germinated with normal seedlings, abnormal seedlings, dead seeds and hard seeds, with different pregerminative treatments and different substrates.

Tratamiento pregerminativo	Sustrato	Plántulas normales	Plántulas anormales	Semillas muertas	Semillas duras
Control	Arena	16e	0	0	84_A
	Papel	43d	0	1	56_B
Ácido	Arena	98a	0	2	0_B
	Papel	99a	0	1	0_B
Corte	Arena	92b	0	8	0_B
	Papel	86b	4	10	0_B
Escarificado	Arena	33d	0	4	63_B
	Papel	37d	0	0	63_B
Agua caliente	Arena	69d	0	5	25_B
	Papel	50c	2	3	45_B
Agua a temperatura ambiente	Arena	20e	0	1	79_A
	Papel	9 e	0	0	91_A

Medias con una letra común no son significativamente diferentes ($P > 0,05$).

la siembra. La aparición de semillas germinadas en papel fue al cabo de tres días mientras que la aparición de los cotiledones en la siembra en arena se produjo a los seis días de la siembra. Se obtuvieron diferencias significativas en el porcentaje de semillas germinadas con plántulas normales y la interacción método*sustrato fue significativa ($P < 0,0001$, cuadro 1).

El número de semillas germinadas con plántulas normales con el método de corte de la cubierta seminal en el extremo distal de la semilla, no mostró diferencias significativas entre sustratos, pero si de ambos respecto a los controles. Sin embargo, presentó el mayor porcentaje de semillas muertas.

La inmersión en agua caliente utilizando arena como sustrato, mostró mayor porcentaje de germinación que en sustrato papel y hubo diferencias significativas entre ellos y respecto al control en arena.

La germinación de las semillas luego de la escarificación mecánica fue similar en los dos sustratos, sin diferencias significativas entre ellos, tampoco respecto al control en sustrato papel. Sin embargo, se observaron diferencias significativas respecto al control en arena.

La inmersión de las semillas en agua a temperatura ambiente produjo la menor proporción de plántulas normales, en los dos sustratos, sin diferencias estadísticamente significativas entre ambos, pero sí respecto al control en papel.

Todos los tratamientos para estimular la germinación produjeron un bajo porcentaje plántulas anormales y semillas muertas. El corte de la cubierta seminal en el extremo distal del embrión mostró el mayor porcentaje de plántulas anormales y de semillas muertas en papel. Sin embargo, no existe efecto significativo del método, del sustrato y de la interacción de ambos, bajo el análisis de las pruebas secuenciales y marginales ($P > 0,99$).

Las mayores proporciones de semillas duras se observaron con el método de inmersión en agua a temperatura ambiente (cuadro 1). La inmersión en agua caliente disminuyó significativamente el número de semillas duras, sin que existan tampoco diferencias entre sustratos. El método de escarificación mecánica es el segundo en orden de importancia en relación al número de semillas duras al final del ensayo, con igual tendencia respecto a los sustratos y al control en papel, que la mencionada para inmersión en agua fría. No se registraron semillas duras con los métodos de corte y ácido.

Efectos de diferentes temperaturas durante el almacenamiento de frutos. El inicio de la germinación se observó al tercer día desde la siembra (DDS), en las semillas cuyos frutos se almacenaron a -17 °C y al quinto DDS, para aquellas semillas cuyos frutos se almacenaron a 25 °C y a 7 °C (figura 2). En el primer caso el PG al tercer día fue del 3 %, pero por razones de escala y tamaño del gráfico este valor no se representa en dicha figura.

Los porcentajes de germinación, según de los diferentes tratamientos se muestran en el cuadro 2 y representan la germinación de *P. ruscifolia* a 12 DDS. No hubo diferencias significativas en los porcentajes de germinación de semillas entre control y -17 °C, por lo cual se compararon como un bloque con respecto a los resultados obtenidos del material almacenado a 7 °C se confirmó dichas diferencias ($P = 0,0016$).

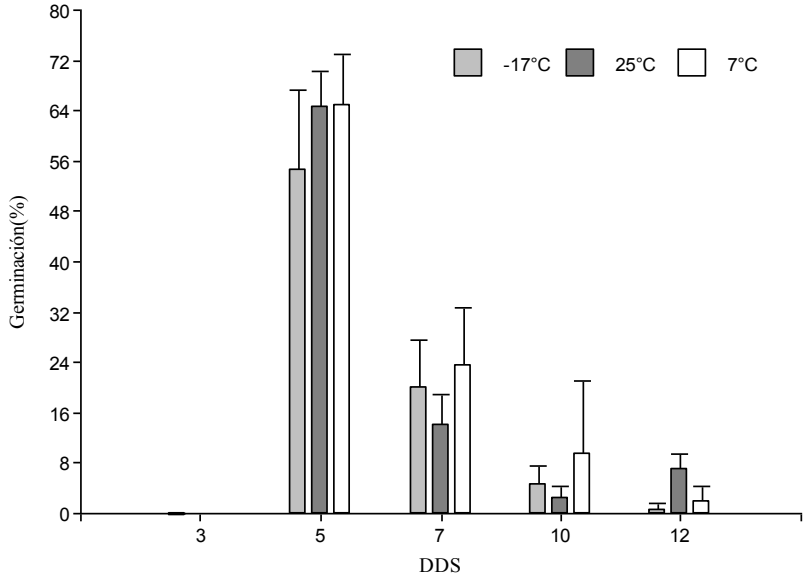

Figura 2. Porcentaje de germinación (no acumulado) de *Prosopis ruscifolia* en diferentes días desde la siembra (DDS), con distintas temperaturas de almacenamiento de frutos.

Germination percentage (not accumulated) of *Prosopis ruscifolia* on different days after sowing, at distinct fruit storage temperatures.

Al finalizar el ensayo (12 DDS) no se observaron semillas duras en ninguno de los tratamientos, mientras que se detectó un 11 % y 12 % de semillas muertas en el control y -17 °C, respectivamente. Las bajas temperaturas de almacenamiento mejoraron el IVG y el TMG, sin diferencias significativas ($P < 0,05$) entre el almacenamiento a una temperatura de -17 °C y a una temperatura de 7 °C, pero significativamente diferentes al control (cuadro 2).

DISCUSIÓN

Germinación. Los resultados obtenidos en laboratorio para superar la dormición de las semillas de *P. ruscifolia* coinciden con los comunicados para otras especies de fabáceas, arbóreas y arbustivas, en las que se utilizaron diferentes métodos para estimular la germinación a través de desgaste, abrasión o interrupción de la continuidad y/o dureza de la cubierta seminal (Abraham de Noir *et al.* 2004, Baskin y Baskin 2014). Entre los tratamientos pregerminativos analizados, la inmersión en ácido sulfúrico muestra los mejores resultados, probablemente debido a la desintegración de la laminilla media de las macroesclereidas presentes en la cubierta seminal. Esta barrera de macroesclereidas es considerada responsable de las dificultades en la imbibición, en ausencia de algún método de escarificación (Villarreal Garza *et al.* 2013, Baskin y Baskin 2014). Pereira *et al.* (2016) observaron en *Schinus areira* L. que el ácido sulfúrico escarifica las paredes de las células tornándolas irregulares, acelerando la tasa de imbibición de las semillas. Las diferencias significativas en los porcentajes de germinación con este tratamiento pregerminativo, en relación a los controles en ambos sustratos, reafirman la presencia de una dormición física impuesta por la cubierta seminal en vinal.

Los resultados concuerdan con las diversas investigaciones en *Prosopis* realizadas por Villarreal Garza *et al.* (2013), Sobrevilla-Solís *et al.* (2013) y Rodríguez Araujo *et al.* (2017) quienes aplicaron el método de escarificación química con ácido sulfúrico para elevar el porcentaje de germinación en estas especies. El elevado porcentaje de germinación de semillas con plántulas normales y la baja incidencia de semillas muertas y duras obtenidos en este trabajo, sugieren que el tiempo de inmersión de 3 minutos en ácido sulfúrico es el apropiado para vinal. En este sentido, Sanabria *et al*. (2004) informaron un aumento en el porcentaje de germinación de *Cassia moschata* Kunth con la inmersión en ácido sulfúrico, disminuyendo además de 88 al 2 % las semillas duras.

Los resultados indican que los controles producen un número significativamente menor de semillas germinadas, con diferencias entre sustratos. El mejor PG en el control se obtiene en papel, en concordancia con observado por Lopes de Oliveira *et al.* (2017) en *Acacia auriculiformis* A. Cunn. ex Benth., y a diferencia de lo señalado por Bravo *et al.* (2011) en *Prosopis kuntzei*, en la cual los porcentajes de semillas germinadas fueron mayores en arena.

En relación con los sustratos, el papel es uno de los más adecuados para realizar ensayos de germinación en laboratorio ya que está presente en la rutina de análisis de varias especies, permitiendo visualizar fácilmente el desarrollo de la raíz para una mejor la evaluación de las plántulas.

El sustrato arena puede presentar problemas en su uso relacionados a la distribución del tamaño de partícula y la capacidad de retención de agua, lo que puede causar un drenaje excesivo y sequedad de la superficie (Bravo *et al.* 2011). Otras dificultades que presenta la utilización de la arena como sustrato son la estandarización del peso volumétrico, el pH y la conductividad eléctrica (Trombin Souza *et al*. 2018).

La ausencia de diferencias significativas en la germinación, de las semillas tratadas con ácido sulfúrico entre arena y papel sugiere que ambos sustratos son apropiados para ensayos en laboratorio.

El corte en el extremo de la cubierta seminal como método para estimular la germinación en vinal, parece también apropiado con valores superiores al 85 %, aunque se observa una mayor incidencia de semillas muertas en ambos sustratos. Este último aspecto desfavorable podría atribuirse a pudriciones durante el curso del ensayo, producto de la interrupción abrupta de la continuidad de la cubierta seminal. Bravo *et al.* (2011) observaron en *P. kuntzei* un menor porcentaje de germinación con el método de corte en papel, atribuyéndolo a una excesiva acumulación de

Cuadro 2. Porcentaje promedio de germinación de semillas de *Prosopis ruscifolia* bajo diferentes temperaturas de almacenamiento de los frutos.

Average germination percentages of *Prosopis ruscifolia* seeds after different fruit storage temperatures.

Temperatura de almacenamiento	Inicio de la germinación (DDS)	Germinación (%)	Índice de velocidad de germinación	Tiempo medio de germinación
25 ºC	5	89±2b	7,93b	6,23b
7 ºC	5	99±1a	8,75a	6,08a
-17 ºC	3	88±4,9b	8,21a	5,53a

Medias con una letra común no son significativamente diferentes ($P > 0,05$).

agua alrededor de las semillas y a una baja aireación entre las toallas de papel. La ausencia de semillas duras con el método de corte, en ambos sustratos, reafirman la dormición impuesta por la cubierta seminal en *P. ruscifolia*.

Los porcentajes de germinación obtenidos con escarificación mecánica con lija en ambos sustratos son significativamente menores a los obtenidos con los métodos de inmersión en ácido sulfúrico, corte de la cubierta seminal e inclusive sin diferencias significativas respecto al control en papel. El elevado porcentaje de semillas duras al finalizar el ensayo indica que este método es insuficiente para superar la barrera física de la cubierta seminal, ya que las semillas escarificadas sembradas en ambos sustratos no mostraron diferencias significativas respecto al control en papel. Sin embargo, Sobrevilla-Solís *et al.* (2013) propusieron la escarificación mecánica como un método adecuado para estimular la germinación en *Prosopis*, extremando cuidados para no afectar la calidad fisiológica de las semillas (Dutra *et al*. 2007) Además, Rodríguez Araujo *et al.* (2017) indicaron que la técnica de escarificación puede variar según la persona que la realiza, obteniendo con estos métodos elevados porcentajes de germinación en distintas especies de *Prosopis*. Por lo tanto, esto podría explicar los altos porcentajes de semillas duras obtenidas en la presente investigación. Sin embargo, según de Menezes Silva *et al.* (2011) la escarificación con lija resultó el método más eficiente en *Sesbania virgata* (Cav) Pers. comparado con la inmersión en ácido sulfúrico. La distribución de la humedad propia del riego inicial es probablemente diferente entre las partículas de arena que en papel.

Los porcentajes de semillas germinadas con plántulas normales fueron mayores con el método de inmersión en agua caliente que en agua a temperatura ambiente y existen diferencias significativas entre sustratos solo en el primero. Esto podría sugerir la existencia de inhibidores químicos termolábiles a nivel de cubierta seminal o algún tipo de daño al embrión. Es común la presencia de inhibidores de naturaleza hidrofóbica como cutinas, suberinas, pectinas, lípidos y ligninas (Ferreira y Borghetti, 2004). Los resultados obtenidos con inmersión en agua caliente sugieren la eliminación de inhibidores de naturaleza lipídica, que no produce la inmersión en agua a temperatura ambiente. Lopes de Oliveira *et al.* (2017) determinaron que la inmersión en agua a temperatura ambiente no permite superar la barrera impuesta por la cubierta seminal a la imbibición.

Almacenamiento. Los elevados porcentajes de germinación observados en el ensayo de almacenamiento indican el excelente vigor del lote de semillas. Los resultados sugieren que almacenamiento de frutos de *P. ruscifolia* a 7 ºC permiten un óptimo porcentaje de germinación en esta especie. Bonner (2008) describió el comportamiento de las semillas de especies de *Prosopis* como ortodoxas, en congruencia con resultados del presente trabajo. Valores de porcentaje de germinación semejantes a los observados en este trabajo, se comunicaron para las semillas ortodoxas de *Anadenanthera colubrina* (Vell.) Bren almacenadas bajo condiciones similares (Rojas Espinoza 2004). Los resultados obtenidos con el almacenamiento a -17 °C podrían representar una alternativa más segura, sobre todo cuando se almacenan frutos completos ya que podría reducir el ataque de insectos (Mazzuferi y Conles 2004).

El incremento en IVG y TMG en semillas provenientes de frutos almacenados en condiciones de bajas temperaturas, podría atribuirse a una mejor conservación del sistema enzimático que participa en la movilización de azúcares (Azadi y Younesi, 2013). Si bien el contenido de humedad no ha sido considerado en este trabajo, el almacenamiento a temperatura ambiente de los frutos de vinal podría haber generado un envejecimiento en el lote de semillas, justificando el menor IVG y el mayor TMG obtenido durante el ensayo.

Debido a que las legumbres de especies del género *Prosopis* contienen un elevado porcentaje de azúcares (González Galán *et al*. 2008), los estudios en relación a las condiciones óptimas para el almacenamiento son útiles y necesarios para viveristas.

CONCLUSIONES

Los resultados de este trabajo identifican que la inmersión en ácido sulfúrico es el mejor método para estimular la germinación de semillas en *P. ruscifolia* y el papel, el sustrato más adecuado para la obtención de plántulas normales en ensayos de laboratorio. Este resultado permitirá optimizar las evaluaciones de calidad de semillas para esta especie. Se confirma la necesidad de escarificación para romper la dormición.

El almacenamiento de frutos de vinal a 7 °C es el método más apropiado para alcanzar los mayores porcentajes de germinación. Esta información es de importancia para viveristas, ya que confirma la posibilidad de almacenamiento de frutos de *P. ruscifolia*, en medios de baja tecnología y disminuye la urgencia de las tareas de limpieza y liberación de semillas. El alto porcentaje de germinación de semillas provenientes de frutos almacenados a -17 °C indica tolerancia de las mismas a temperaturas bajo cero. Se plantea la necesidad de continuar las pruebas considerando periodos de almacenamiento más prolongados.

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Microplants production of *Eucalyptus cloeziana* from indirect organogenesis

Producción de microplantas de *Eucalyptus cloeziana* por organogénesis indirecta

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SUMMARY

Few studies have focused on the *in vitro* multiplication methods for *Eucalyptus cloeziana*. In this work we developed an indirect organogenesis protocol to obtain micropropagated plants. The interactions of plant growth regulators in juvenile tissues were evaluated. Hypocotyl, cotyledon and root tissues obtained from *in vitro* germinated seedlings were subjected to combinations of α-naphthaleneacetic acid (NAA), thidiazuron (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D). *In vitro* callogenesis, adventitious bud induction, shoot elongation, and *ex vitro* survival and rooting were evaluated. Callogenic structures were observed in all evaluated tissues. Morphogenic characteristics related to the meristematic center was observed. The tissues that presented the callus mass were subcultured in a regeneration culture medium supplemented with 1.0 mg L^{-1} of 6-benzylaminopurine (BAP). Only hypocotyl and cotyledon induced adventitious bud and shoot elongation. Hypocotyl subjected to 2.0 mg L^{-1} TDZ had the highest number of elongated shoots. The two tissues (*i.e.*, hypocotyl and cotyledon) combined with thidiazuron were characterized by the *ex vitro* survival of microcuttings and by adventitious rooting. Results confirmed tissue culture efficiency for microplants production by indirect organogenesis from hypocotyl and cotyledon cultivated with TDZ, and its implementation can be an alternative for forest tree breeding programs of *E. cloeziana*.

Key words: adventitious rooting, callogenesis, indirect regeneration, adventitious bud.

RESUMEN

Pocos estudios se han centrado en los métodos de multiplicación *in vitro* para *Eucalyptus cloeziana*. En este trabajo se desarrolló un protocolo de organogénesis indirecta para obtener plantas micropropagadas. Se evaluaron las interacciones de los reguladores del crecimiento en tejidos juveniles. Los tejidos del hipocótilo, cotiledón y raíces obtenidos de plántulas germinadas *in vitro* se sometieron a combinaciones de ácido α-naftaleneacético (ANA), tidiazurón (TDZ) y ácido 2,4-diclorofenoxiacético (2,4-D). Se evaluaron la callogénesis *in vitro*, la inducción adventicia de yemas, el alargamiento de brotes, y la sobrevivencia y enraizamiento *ex vitro*. Se observaron estructuras calogénicas en todos los tejidos evaluados. Se observaron características morfogénicas relacionadas con la formación de centros meristemáticos. Los tejidos que presentaron la masa del callo fueron subcultivados en medio de cultivo de regeneración suplementado con 1,0 mg L^{-1} de 6-bencilaminopurina (BAP), y solo los tejidos del hipocótilo y cotiledón formaron yemas adventicias y brotes alargados. Los tejidos del hipocótilo sometidos a 2,0 mg L^{-1} de tidiazuron obtuvieron mayor número de brotes alargados. Los dos tejidos (hipocótilo y cotiledón) combinados con TDZ se caracterizaron por sobrevivencia y el enraizamiento adventicio *ex vitro* de las microestacas. Los resultados confirmaron la eficiencia del cultivo de tejidos para la producción de microplantas por organogénesis indirecta a partir de tejidos del hipocótilo y cotiledón cultivados con TDZ y su implementación puede ser una alternativa para los programas de mejoramiento forestal de *E. cloeziana*.

Palabras clave: enraizamiento adventicio, callogénesis, regeneración indirecta, brote adventicio.

INTRODUCTION

The genera *Eucalyptus* and *Corymbia* are the most cultivated exotic species of the forest sector. They are well recognized for their silvicultural characteristics, fast growth, great ecological plasticity and wood properties, making them attractive for numerous industrial applications (Brondani *et al*. 2018, Arriel *et al*. 2019). Among these genera, several species still have been evaluated for commercial plantation establishment, because of the growing demand for forest products. Such species include *Eucalyptus cloeziana* F. Muell., which stands out for its wide applications, including the production of raw materials for furniture, construction, energy and pulp and paper industries, and has established itself in the industrial sector in Brazil (Alves *et al*. 2017).

In vitro plant regeneration techniques have been used as complementary tools to forest tree breeding, where the development of a tissue culture protocol is essential to obtain whole plants on a large scale. The phases of tissue regeneration, bud multiplication, shoot elongation and subsequent rooting and acclimatization can only be performed after the establishment of aseptic cultures with good vegetative vigor (Silva *et al*. 2019). One of the current methodologies adopted as an alternative to obtain complete plants is indirect organogenesis by the induction of callus, and it has several applications, such as the production of transgenic plants (Silva *et al*. 2019).

The use of indirect organogenesis allows the formation of adventitious bud and shoot elongation for producing large quantities of microplants in a short period and enables the study of different morphogenetic and physiological phenomena (Hesami and Daneshvar 2018), production of secondary metabolites (Mutawil *et al*. 2016), transgenic plants, polyploidy, and somaclonal variations (Hesami and Daneshvar 2018) and callus culture for growing cells in suspension (Silva *et al*. 2019).

Callogenesis and bud induction occur from the redifferentiation of the cells forming new tissue, as these processes are directly controlled by morphophysiological events that regulate endogenous mechanisms, proteins and DNA methylation (Pan *et al*. 2010). Thus, the influence of the incubation conditions has been studied in several *Eucalyptus* species (Fernando *et al*. 2016) during organogenesis. These changes may occur due to the composition of the culture medium, nutrition, balance between the plant growth regulators, type and ontogenetic age of the explant and determination of the tissues (Brondani *et al*. 2012, 2018, Oliveira *et al*. 2015, Mittal and Sharma 2017, Souza *et al*. 2019).

Eucalyptus cloeziana is important in the forest sector, therefore obtaining an efficient and reproducible protocol for indirect tissue regeneration is essential to propagate improved genotypes. Furthermore, the knowledge generated may contribute to plants production by specialized forest companies, producers of improved plants and research institutions in different world regions. In this context, the present study aimed at establishing an indirect organogenesis protocol for *E. cloeziana* from hypocotyl, cotyledon and root tissues, regarding: (i) tissue competence and plant growth regulator concentrations for the induction of callus structures; (ii) presence of meristematic center; (iii) *in vitro* adventitious bud induction and shoot elongation; (iv) *ex vitro* survival of microcuttings and adventitious rooting.

METHODS

Source of tissues. Hypocotyl, cotyledon and root tissues were collected from *E. cloeziana* F. Muell seedlings germinated *in vitro*. The seeds originated from a Seed Producing Area (SPA) in Anhembi, state of São Paulo, Brazil, from cultivar LCFA026 of the Forest Science and Research Institute (IPEF).

Seed disinfection and in vitro germination. Seeds were pre-treated by imbibition in distilled water for 24 hours. Subsequently, seeds were washed under tap water for 5 minutes and sterilized first in a 70 % ethanol solution for 90 seconds and then in sodium hypochlorite (NaOCl, 2.0-2.5 % active chlorine) containing one drop (0.05 mL) of tween-20 for 25 minutes. After the disinfection process, seeds were washed three times with autoclaved distilled water. The seeds selected for cultivation were those that showed a state of turgidity and white color (Oliveira *et al*. 2015). Seeds were *in vitro* inoculated in glass test tubes (20 × 100 mm) containing 6 mL of the culture medium composed only of distilled water and agar. Seeds were kept under ambient growth room conditions until tissue was collected.

Preparation of the culture medium. The culture medium was prepared with deionized water, 6 g L^{-1} of agar and 30 g L^{-1} of sucrose. The pH of the medium was adjusted to 5.8 with 0.1 N - HCl and 0.1 N - NaOH before the agar was added, and afterwards, the mixture was autoclaved at 121 °C (~1.0 kgf cm^{-2}) for 20 minutes. Before the culture medium was autoclaved, a plant growth regulator was added.

Culture conditions under growth room. The growth room had a temperature of 25 °C (± 2 °C), photoperiod of 16 hours of light and irradiance from cool white fluorescent lamps of 32 µmol m^{-2} s^{-1}.

Callus culture. Seedlings obtained *in vitro* after 20 days of germination were used to collect the tissues (*i.e.*, explants). Fragments of root (middle portion measuring 0.5 cm in length), hypocotyl (middle portion measuring 0.5 cm in length) and cotyledons (sectioned at 50 % of the area) were individually inoculated in glass test tubes (20 × 100 mm) containing 6 mL of Woody Plant Medium

(WPM) (Lloyd and McCown 1980). The culture medium was supplemented with different combinations and concentrations of plant growth regulators, namely, 0.0, 2.0 and 4.0 mg L^{-1} of α-naphthaleneacetic acid (NAA); 0.0, 1.0 and 2.0 mg L^{-1} of thidiazuron (TDZ); and 0.0, 1.0 and 2.0 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D). Each of the treatments consisted of 10 replicates (the experimental unit was test tubes containing one explant). The experiment was conducted in a completely randomized design in a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon and root) and 27 combinations of plant growth regulators (*i.e.*, NAA × TDZ × 2,4-D). After inoculation, explants were kept in a growth room in the dark for 30 days. After treatments had been applied for 30 days, the percentage of callogenesis was evaluated.

Adventitious bud induction. Callus were transferred to the adventitious bud induction medium, constituted by WPM culture medium and supplemented with 1.0 mg L^{-1} of 6-benzylaminopurine (BAP). Explants were inoculated in glass containers (6 × 7 cm) containing 40 mL of a culture medium. Subcultures were performed every 30 days under growth room conditions. The experiment was conducted in a completely randomized design in a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon, and root) and 27 combinations of plant growth regulators (NAA × TDZ × 2,4-D). The percentage of adventitious buds induced in 90 days was evaluated.

Shoot elongation. Only the explants that regenerated adventitious buds were used for the shoot elongation phase. Explants with initiation of 3 to 5 shoots for culture were inoculated in WPM culture medium supplemented with 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} of indole-3-butyric acid (IBA). Explants were inoculated in glass containers (6 × 7 cm) containing 40 mL of culture medium. Subcultures were performed every 30 days under growth room conditions. The experiment was conducted in a completely randomized design with a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon, and root) and 27 combinations of plant growth regulators (NAA × TDZ × 2,4-D). At the end of the experiment, the number of elongated shoots (≥ 1 cm) in 90 days was evaluated.

Ex vitro survival, rooting and acclimatization. In vitro elongated shoots (*i.e.*, microcuttings with a length equal to or larger than 1 cm) were collected and transplanted *ex vitro* to a mini-incubator system (Brondani *et al*. 2018). The substrate used to promote the rooting of the microcuttings was a mixture of decomposed *Pinus* bark and medium-sized vermiculite, at a 1:1 (v v^{-1}) ratio. The mini-incubator system was kept under growth room conditions. The survival percentage and the adventitious roots of the microcuttings were evaluated at 20 days of *ex vitro* incubation. For acclimatization, the rooted plants were transplanted to individual containers (250 mL) containing the same proportion of the substrate used for rooting. In this phase, a nutrient solution composed of MS half-strength (Murashige and Skoog 1962) culture medium was applied every 7 days. The acclimatization period lasted 20 days. After acclimatization, the plants were transplanted to larger plastic cups (500 mL) and transferred to a greenhouse (50 % natural light). The growth phase in the greenhouse lasted 80 days, and plant survival was evaluated. The experiment was conducted in a completely randomized design with a factorial arrangement (3 × 27), where the factors were three types of explants (*i.e.*, hypocotyl, cotyledon and root) and 27 combinations of plant growth regulators (*i.e.*, NAA × TDZ × 2,4-D). The steps outlined in the methods section are briefly illustrated in figure 1.

Histological analyses. Samples of the callus were fixed in a modified formaldehyde and glutaraldehyde solution (glutaraldehyde 1 %, paraformaldehyde 4 % in sodium phosphate buffer, $NaH_2PO_4.H_2O$; 0.1 M; pH 7.2) (Karnovsky 1965) and were submitted to six vacuum series (-600 mmHg) for 30 minutes each. Samples were subsequently stored for 30 days at 4 °C and dehydrated by ethanol-alcohol series in increasing concentrations (*i.e.*, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 %, v v^{-1}), remaining in each solution for 15 minutes. Samples were placed in an infiltration medium (Historesin®, Leica) for 24 hours and prepared according to the manufacturer's instructions, remaining 28 days at 24 °C. The blocks containing the samples were sectioned longitudinally or transversally to 5 µm thickness using a Microm HM 355S automatic rotary microtome (Thermo Scientific®). Each section was stained with toluidine blue (0.05 %, v v^{-1}) in sodium phosphate buffer and citric acid (Sakai 1973) for 30 minutes and mounted on histological slides with synthetic resin (Entellan®). The histological sections were analyzed and photographed under an optic microscope (Opton®), and the images were captured at a micrometric scale. A descriptive analysis was performed on each sample, aimed at identifying the disposition of the tissues connecting the axillary buds and nodes, the region of the adventitious root emergence and evident meristematic zones.

Statistical analyses. The data measured in all experiments were analyzed using Hartley's test ($P > 0.05$) and the Shapiro-Wilk's test ($P > 0.05$) to assess the homogeneity of the variances and the normal distribution of data, respectively. Data were transformed as needed by the Box-Cox test. Next, an analysis of variance (ANOVA, $P < 0.05$) was performed. According to the significance of ANOVA, the mean values of the treatments were compared by the Duncan's test ($P < 0.05$). The steps outlined in the methods section are briefly illustrated in figure 1.

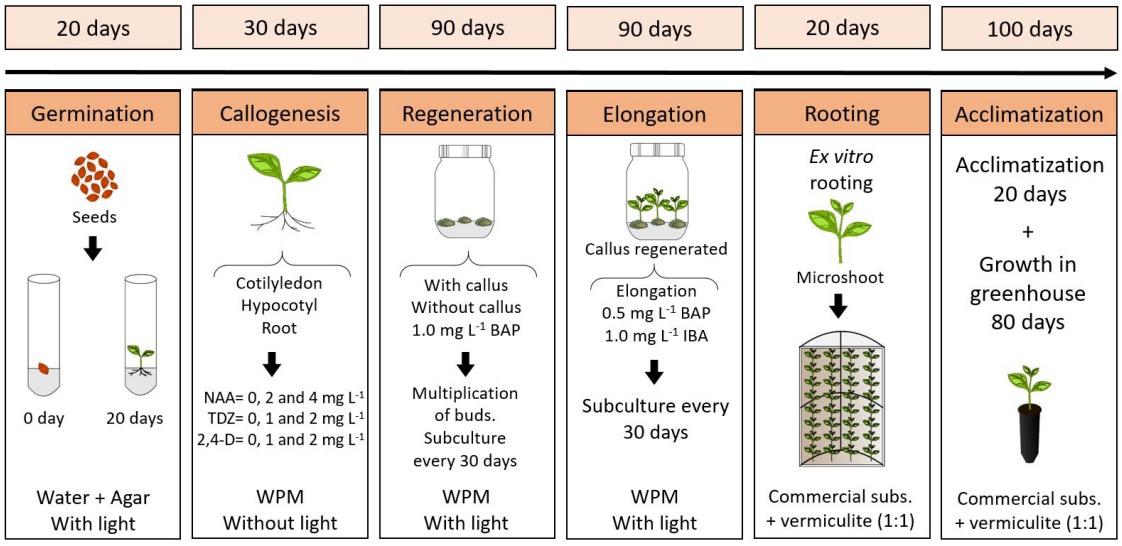

Figure 1. Flowchart of the experiment with detail of the *in vitro* seed inoculation phase until the acclimatization of microplants of *E. cloeziana* obtained by indirect regeneration in 350 days.

Diagrama de flujo del experimento con detalles de la fase de inoculación de semillas *in vitro* hasta la aclimatización de microplantas de *E. cloeziana* obtenidas por regeneración indirecta en 350 días.

RESULTS

Callus culture. Seeds of *E. cloeziana* were inoculated *in vitro* to obtain seedlings as a source of explants, and cultures with no visible microbial infection (bacteria and/or fungi) were selected. After 30 days of the callogenesis process with different tissues and combinations of plant growth regulators, induction to callogenesis was observed in all treatments, with an overall mean callogenesis of 78.4 %. Percentages of callogenesis of 30-100 % were observed for the hypocotyl, 40-100 % for the cotyledon and 20-100 % for the root (table 1).

Intense meristematic activities related to the organogenic competence of the tissues induced by combinations of plant growth regulators were observed. Cellular areas of meristematic competence were identified in the cells of the superficial tissues of callus structures, characterized by thin cell walls, reduced cell size, isodiametric cells and a high nuclear/cytoplasmatic ratio (figure 2A). In addition, the formation of a vascular bundle and the presence of a region with intense meristematic activity denotes cell competence for the formation of new tissues (figure 2A). High accumulation of ergastic substances in cells, intense meristematic activity (figure 2B) and the presence of meristemoids (figure 2C) were observed in several anatomical sections, indicating morphogenic potential.

Applications of 1.0 mg L^{-1} 2.4-D and 4.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2,4-D provided an effective hormonal balance for the initiation of the adventitious root formation from the callus in the hypocotyl and cotyledon, respectively (figure 2D).

Adventitious root formation was observed in the hypocotyl and cotyledon (figure 3A), showing the formation of a meristematic center with a connection to the callus mass (figure 3B). High nuclear/cytoplasmic ratio was observed in isolated cells for the combination of NAA and 2,4-D in all tissues tested (figure 3C). In addition, meristematic centers were also observed in the upper ends of the callus structures (figure 3D), indicating intense morphogenic activity.

Adventitious bud induction. Tissues that formed a callus were transferred to the culture medium supplemented with 1.0 mg L^{-1} BAP. Only 1.5 % of the callus demonstrated organogenic competence for adventitious bud induction, where the best responses occurred for the combinations of 1.0 mg L^{-1} TDZ, 2.0 mg L^{-1} TDZ and 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ. Hypocotyl and cotyledon tissues were able to form adventitious buds (table 2).

Callus formed from hypocotyl did not present a significant difference between applications of 2.0 mg L^{-1} TDZ and 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ, which resulted in mean shoot values of 22.2 % and 20.0 %, respectively (table 2). On the other hand, among the callus obtained from the cotyledon, a significant difference was observed between the treatments that were composed by the combinations of 1.0 mg L^{-1} TDZ and 2.0 mg L^{-1} TDZ, with the latter being more responsive to the regeneration medium, resulting in 55.5 % of the callus regenerating buds (table 2). The other tissues that did not show adventitious bud regeneration in the 90-day period were discarded.

The histological sections confirmed the regeneration of adventitious buds, which originated from the callus that presented organogenic competence, thereby indicating meristematic regions (figure 4A) and the vascular connection of shoots with the cell mass of origin (figure 4B).

Table 1. Percentage of callogenesis in *E. cloeziana* explants according to the combinations of plant growth regulators and explants at 30 days of *in vitro* cultivation.

Porcentaje de calogénesis en explantes de *E. cloeziana* de acuerdo con las combinaciones de reguladores del crecimiento de las plantas y explantes a los 30 días de cultivo *in vitro*.

NAA (mg L^{-1})	TDZ (mg L^{-1})	2,4-D (mg L^{-1})	Explant		
			Hypocotyl	Cotyledon	Root
0.0	0.0	0.0	MP	66.7^{Ba} (±16.7)	25.0^{DEb} (±16.4)
0.0	0.0	1.0	40.0^{CDb} (±16.3)	88.9^{Aa} (±11.1)	37.5^{CDb} (±18.3)
0.0	0.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	1.0	0.0	50.0^{Cc} (±16.7)	85.7^{Aa} (±14.3)	70.0^{Bb} (±15.3)
0.0	1.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	1.0	2.0	50.0^{Cc} (±16.7)	55.6^{Bb} (±17.6)	66.7^{Ba} (±16.7)
0.0	2.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	2.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
0.0	2.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	0.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	0.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	0.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	1.0	0.0	80.0^{Bb} (±13.3)	100.0^{Aa} (±0.0)	MP
2.0	1.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	1.0	2.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	20.0^{Ec} (±13.3)
2.0	2.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	2.0	1.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
2.0	2.0	2.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
4.0	0.0	0.0	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
4.0	0.0	1.0	50.0^{Cb} (±16.7)	100.0^{Aa} (±0.0)	MP
4.0	0.0	2.0	90.0^{ABa} (±10.0)	90.0^{Aa} (±10.0)	20.0^{Eb} (±13.3)
4.0	1.0	0.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	40.0^{Cc} (±16.3)
4.0	1.0	1.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	50.0^{Cc} (±16.7)
4.0	1.0	2.0	90.0^{ABb} (±10.0)	100.0^{Aa} (±0.0)	100.0^{Aa} (±0.0)
4.0	2.0	0.0	40.0^{CDb} (±16.3)	100.0^{Aa} (±0.0)	MP
4.0	2.0	1.0	MP	90.0^{Aa} (±10.0)	MP
4.0	2.0	2.0	30.0^{Db} (±15.3)	40.0^{Ca} (±16.3)	MP

Means followed by the same uppercase letter in the columns and means followed by the same lowercase letter in the rows do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error). MP = missing parcel due to bacterial manifestation.

Despite the high callus formation intensity of the tissues by the application of plant growth regulators, the root did not present competence for the induction of adventitious buds in the regeneration medium.

Shoot elongation. Only the callus showing adventitious bud regeneration (table 2) were subdivided into standard explants with initiation of 3 to 5 shoots for culture (figure 4A). The elongation medium was supplemented with 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} IBA to establish microstumps *in vitro*. Hypocotyl originating from the application of 2.0 mg L^{-1} TDZ showed the highest number of elongated shoots at 90 days of culture (table 3), resulting in a mean of 41.5 shoots per explant. All callus showed the formation of shoots (*i.e.*, microcuttings), which were collected for the rooting phase.

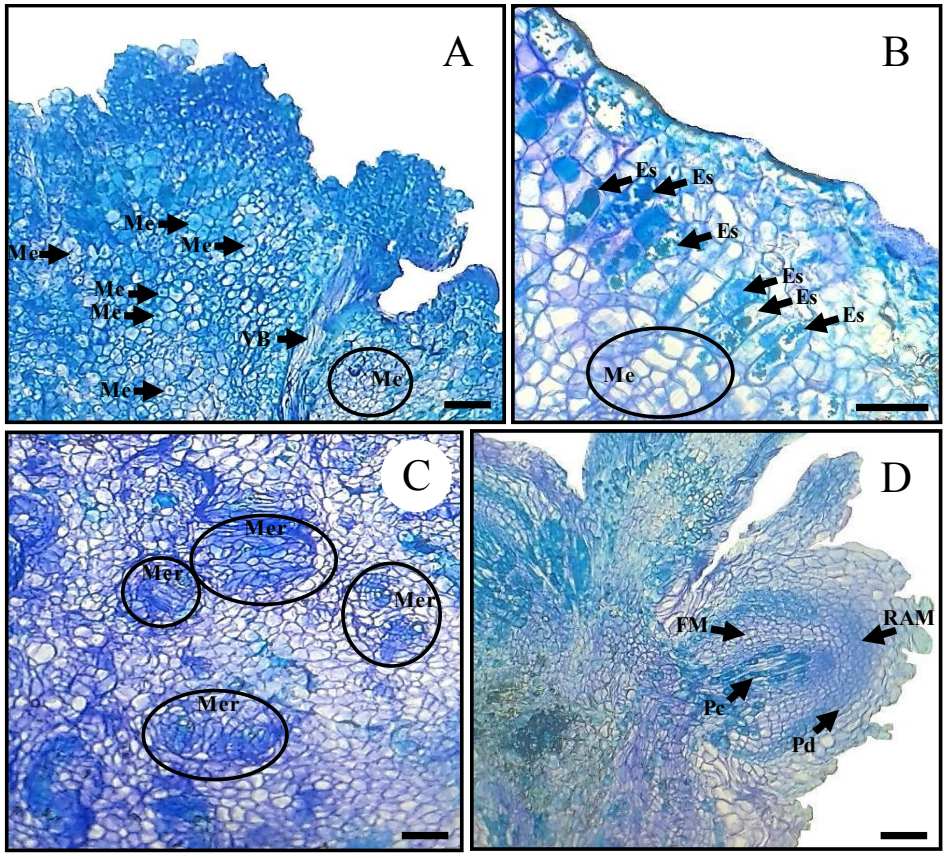

Figure 2. Histology of callus of *E. cloeziana*. A) Callus showing formation of a vascular bundle (arrow) and area with meristematic activity (arrow and region) supplemented with 2.0 mg L^{-1} TDZ and cotyledon. B) Detail of cells showing high accumulation of ergastic substances (arrows) and a region with meristematic activity in the presence of 2.0 mg L^{-1} TDZ and cotyledon. C) Region of callus showing formation of meristemoids with 1.0 mg L^{-1} TDZ and cotyledon. D) Detail of the formation of adventitious roots from the callus, with the root apical meristem with 1.0 mg L^{-1} 2,4-D and hypocotyl. Vascular bundle (VB), meristematic activity (Me), ergastic substances (Es), meristemoids (Mer), root apical meristem (RAM), fundamental meristem (FM), procambium (Pc), and protoderm (Pd). Bar = 100 µm.

Histología de callos de *E. cloeziana*. A) Callo que muestra la formación de un haz vascular (flecha) y área con actividad meristemática (flecha) con suplementación de 2,0 mg L^{-1} de TDZ y cotiledón. B) Detalle de las células que muestran una alta acumulación de sustancias ergásticas (flechas) y una región con actividad meristemática en presencia de 2,0 mg L^{-1} de TDZ y cotiledón. C) Región del callo que muestra la formación de meristemoides con 1,0 mg L^{-1} de TDZ y cotiledón. D) Detalle de la formación de raíces adventicias del callo, con el meristemo de la raíz apical con 1,0 mg L^{-1} de 2,4-D e hipocotilo. Haz vascular (VB), actividad meristemática (Me), sustancias ergásticas (Es), meristemoides (Mer), meristemo de la raíz apical (RAM), meristemo fundamental (FM), procambio (Pc) y protodermo (Pd). Barra = 100 µm.

Ex vitro survival, rooting and acclimatization. Microcuttings were collected (figure 5B) from the microstumps rooted in the shoot elongation phase (figure 5A), and these microcuttings were placed in a mini-incubator system for rooting. The mini-incubator system was maintained in a growing room under ambient conditions.

Survival and *ex vitro* rooting of the microcuttings (figure 5C) were evaluated at 20 days. Cotyledon subjected to 1.0 mg L^{-1} TDZ and hypocotyl subjected to 2.0 mg L^{-1} TDZ showed satisfactory survival and rooting, and complete plants were obtained (table 4). The remaining combinations of plant growth regulators tested in this phase did not result in rooting, and tissue mortality was observed (table 4).

Rooted microcuttings were acclimatized for 20 days and afterwards grown in a greenhouse, where they showed normal development and growth (figure 5D), enabling completion of the adventitious regeneration protocol of plants in 350 days.

DISCUSSION

Several studies have reported on the efficiency of callus induction from hypocotyl and cotyledon with *Eucalyptus* species, such as those developed for *E. camaldulensis* Dehnh. (Dibax *et al*. 2010), *E. saligna* Smith. (Silva *et al*. 2015) and *E. globulus* Labill. (Salla *et al*. 2018). In the present study with *E. cloeziana*, callus formation occurred

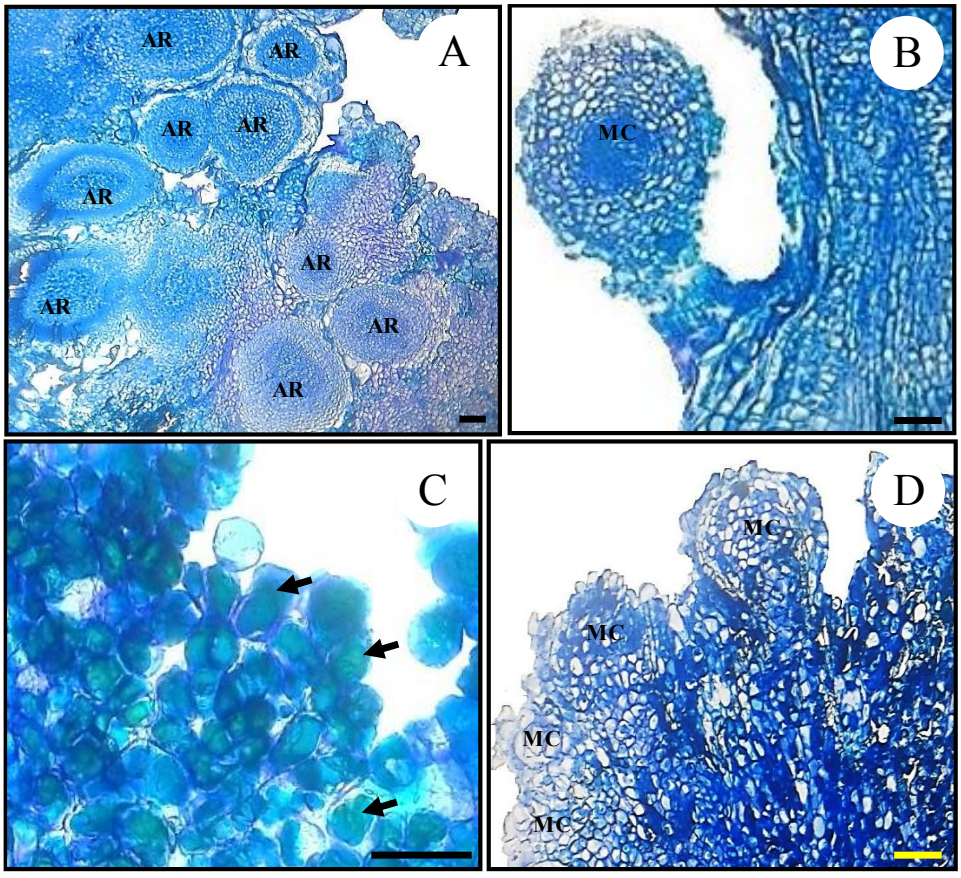

Figure 3. Histology of callus of *E. cloeziana*. A) Callus structure showing meristematic activities and initiation of adventitious root formation with 4.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2,4-D and cotyledon. B) Evidence of meristematic center showing connection with the callus mass with 1.0 mg L^{-1} TDZ and cotyledon. C) Presence of isolated cells with high nuclear/cytoplasmic ratio (arrows) with 4.0 mg L^{-1} NAA + 2.0 mg L^{-1} 2,4-D and cotyledon. D) Evidence of meristematic center at the upper ends of the callus mass with 2.0 mg L^{-1} TDZ and cotyledon. Adventitious root (AR), meristematic center (MC). Bar = 100 µm.

Histología de callos de *E. cloeziana*. A) Estructura del callo que muestra actividades meristemáticas e iniciación de la formación de raíces adventícias con 4,0 mg L^{-1} de ANA + 2,0 mg L^{-1} de 2,4-D y cotiledón. B) Evidencia de centro meristemático que muestra conexión con la masa de callos con 1,0 mg L^{-1} de TDZ y cotiledón. C) Presencia de células aisladas con alta relación nuclear/citoplasmática (flechas) con 4,0 mg L^{-1} de ANA + 2,0 mg L^{-1} de 2,4-D y cotiledón. D) Evidencia de centro meristemático en los extremos superiores de la masa del callo con 2,0 mg L^{-1} de TDZ y cotiledón. Raíces adventicias (AR), centro meristemático (MC). Barra = 100 µm.

in all tissues tested, independent from the plant growth regulator combinations; however, the highest percentages of callogenesis were observed when cotyledon tissue was used (table 1).

Hypocotyl and cotyledon extracted from *E. cloeziana* seedlings were the sources of explants most suitable for callus formation, including evidence of adventitious root induction and buds at this organogenic phase. Vascular bundle formation and an area with meristematic activity in the callus were observed with the combination of 2.0 mg L^{-1} TDZ and the cotyledon (figure 2A). Aggarwal *et al*. (2010) found intense meristematic activity in cells of the superficial layer in tissues of *E. tereticornis* Smith., and these cells were later organized into buds. This observation was also reported in *E. cloeziana*, both for root and bud formation (figures 2A-D). In addition, a high accumulation of ergastic substances in callus from cotyledon combined with 2.0 mg L^{-1} TDZ (figure 2B) and callus showing the formation of meristems in the combination of 1.0 mg L^{-1} TDZ and cotyledon (figure 2C) were observed. In a study on the induction of callus with BAP and NAA and/or IAA (indole-3-acetic acid) in *E. dunnii* Maiden., Oberschelp *et al*. (2015) observed the presence of subepidermal meristemoids and meristematic areas developing at the end of the hypocotyl and evidence of a high number of druses in buds or ergastic substance, observations similar to those made in the present study with *E. cloeziana*. Initiation of adventitious root occurred from the callus in the hypocotyl and cotyledon (figures 2D and 3A).

The presence of a meristematic center with a vascular connection to the callus mass was observed in cotyledon treated with 1.0 mg L^{-1} TDZ (figure 3B) and 2.0 mg L^{-1}

Table 2. Percentage of induction of adventitious buds in *E. cloeziana* explants according to the combinations of plant growth regulators and explants at 90 days of *in vitro* cultivation.

Porcentaje de inducción de yemas adventicias en explantes de *E. cloeziana* de acuerdo con las combinaciones de reguladores del crecimiento de las plantas y explantes a los 90 días de cultivo *in vitro*.

NAA (mg L^{-1})	TDZ (mg L^{-1})	2,4-D (mg L^{-1})	Explant		
			Hypocotyl	Cotyledon	Root
0.0	0.0	0.0	MP	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	0.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	0.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	1.0	0.0	0.0^{Bb} (±0.0)	42.8^{Ba} (±20.2)	0.0^{Ab} (±0.0)
0.0	1.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	1.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	2.0	0.0	22.2^{Ab} (±14.7)	55.5^{Aa} (±17.5)	0.0^{Ac} (±0.0)
0.0	2.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
0.0	2.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	0.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	0.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	0.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	1.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP
2.0	1.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	1.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	2.0	0.0	20.0^{Aa} (±13.3)	0.0^{Cb} (±0.0)	0.0^{Ab} (±0.0)
2.0	2.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
2.0	2.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	0.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	0.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP
4.0	0.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	1.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	1.0	1.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	1.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	0.0^{Aa} (±0.0)
4.0	2.0	0.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP
4.0	2.0	1.0	MP	0.0^{Ca} (±0.0)	MP
4.0	2.0	2.0	0.0^{Ba} (±0.0)	0.0^{Ca} (±0.0)	MP

Means followed by the same uppercase letter in the columns and means followed by the same lowercase letter in the rows do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error). MP = missing parcel due to bacterial manifestation.

Table 3. Number of elongated shoots per *E. cloeziana* explant according to combination of plant growth regulators and explants at 90 days of *in vitro* culture.

Número de brotes alargados por explante de *E. cloeziana* de acuerdo con la combinación de reguladores del crecimiento de las plantas y explantes a los 90 días de cultivo *in vitro*.

NAA (mg L^{-1})	TDZ (mg L^{-1})	Explant	Shoot number (shoot $explant^{-1}$)
0.0	1.0	Cotyledon	20.0^{B} (±3.5)
0.0	2.0	Hypocotyl	41.5^{A} (±16.5)
0.0	2.0	Cotyledon	12.0^{B} (±2.9)
2.0	2.0	Hypocotyl	9.5^{C} (±3.5)

Means followed by the same uppercase letter do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error).

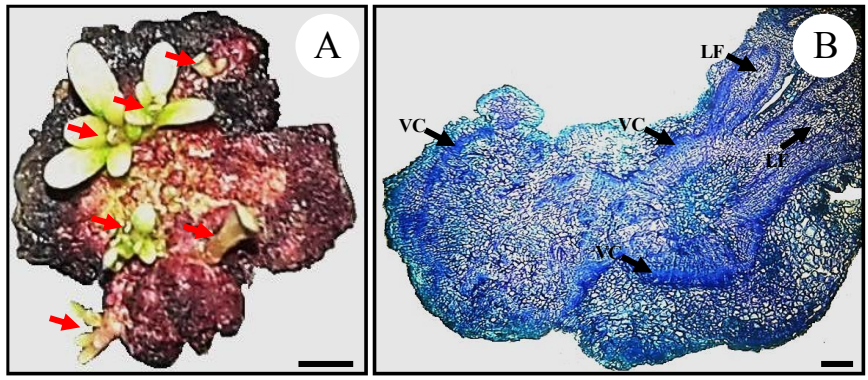

Figure 4. Histology of organogenic callus of *E. cloeziana* obtained from the combination of 2.0 mg L^{-1} TDZ and hypocotyl. A) Details on the induction of adventitious buds with shoot initiation, leaves and apical meristem (arrows). Bar = 1 cm. B) Adventitious leaf primordia showing vascular connection with the callus mass. Adventitious leaf primordia (LF), vascular connection (VC). Bar = 100 µm.

Histología de callos organogénicos de *E. cloeziana* obtenidos de la combinación de 2,0 mg L-1 de TDZ y hipocotílico. A) Detalles sobre la inducción de yemas adventicias con iniciación de brotes, hojas y el meristemo apical (flechas). Barra = 1 cm. B) Primordios de hojas adventicias que muestran conexión vascular con la masa del callos. Primordios de hojas adventicias (LF), conexión vascular (VC). Barra = 100 µm.

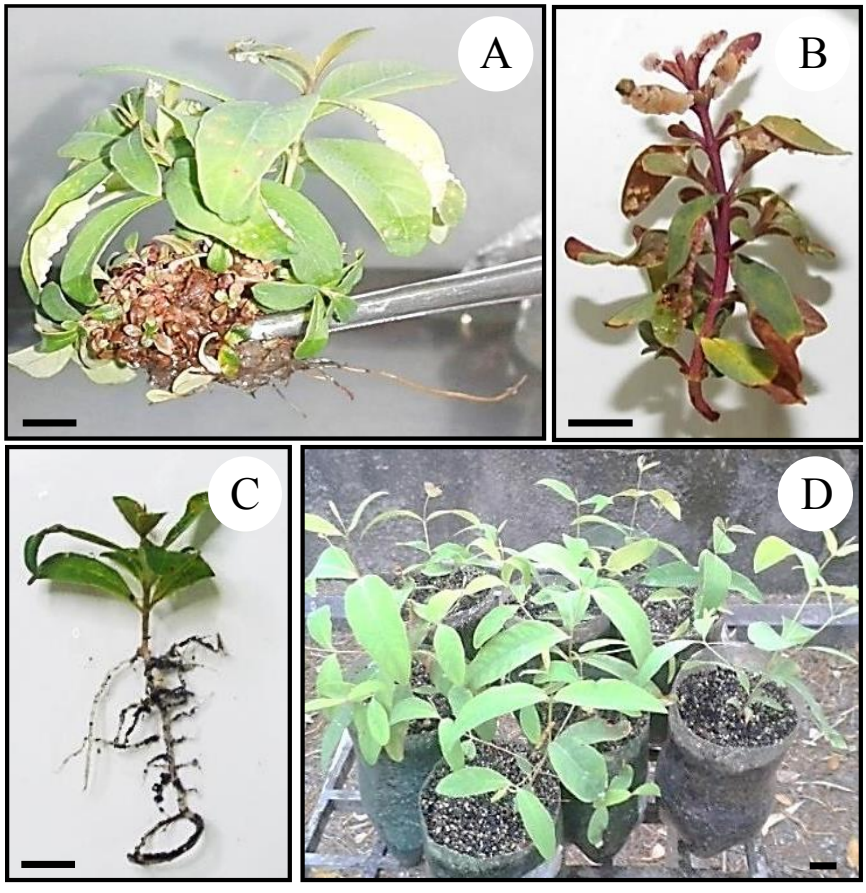

Figure 5. Stages of shoot elongation and *ex vitro* rooting of *E. cloeziana*. A) Details of *in vitro* rooted microstump, with visible formations of shoots. Bar = 1 cm. B) Details of a shoot collected (*i.e.*, microcutting) from a microstump where callus structures were present on the leaves. Bar = 1 cm. C) Details of microcutting rooted under *ex vitro* conditions at 20 days. Bar = 1 cm. D) Acclimatized plants grown in a greenhouse at 100 days. Bar = 2 cm.

Etapas de alargamiento de brotes y enraizamiento *ex vitro* de *E. cloeziana*. A) Detalles del microcepa enraizado *in vitro*, con formaciones visibles de brotes alargados. Barra = 1 cm. B) Detalles de un brote recogido (microestaca) de una microcepa donde las estructuras de callos estaban presentes en las hojas. Barra = 1 cm. C) Detalles de microestaca enraizado en condiciones *ex vitro* a los 20 días. Barra = 1 cm. D) Plantas climatizadas cultivadas en un invernadero por 100 días. Barra = 2 cm.

Table 4. Percentage of survival and *ex vitro* rooting of *E. cloeziana* microcuttings according to the concentration of plant growth regulators and explants at 20 days of cultivation.

Porcentaje de supervivencia y enraizamiento *ex vitro* de microestaca de *E. cloeziana* de acuerdo con la concentración de reguladores del crecimiento de las plantas y explantes a los 20 días de cultivo.

NAA (mg L^{-1})	TDZ (mg L^{-1})	Explant	Survival (%)	Rooting (%)
0.0	1.0	Cotyledon	66.7^{B} (±12.6)	53.3^{A} (±13.3)
0.0	2.0	Hypocotyl	100.0^{A} (±0.0)	55.6^{A} (±17.6)
0.0	2.0	Cotyledon	0.0^{C} (±0.0)	0.0^{B} (±0.0)
2.0	2.0	Hypocotyl	0.0^{C} (±0.0)	0.0^{B} (±0.0)

Means followed by the same uppercase letter in the columns do not differ significantly by the Duncan's test at the 5 % probability level. Data are expressed as the mean (±standard error).

TDZ (figure 3D), which can regenerate buds. This characteristic is important for the plant regeneration, such as reported by Mycock and Watt (2012), when evaluating the callus anatomy in *E. grandis* W. Hill ex Maiden × *E. urophylla* S. T. Blake observed that the roots developed from the stem region immediately above the callus or from the callus itself and that of the primordial meristems of the root appear to have been derived from the pericycle of the stem, *i.e.*, from the parenchyma layer between the endodermis and the phloem.

The efficiency of *in vitro* organogenesis depends primarily on the source of the explant, on the components of the culture medium and on the environmental conditions (Hesami and Daneshvar 2018, Silva *et al*. 2019). Another factor influencing the organogenic responses in *Eucalyptus* is the endogenous and exogenous hormonal balance between the cytokinin and auxin present in the plant tissue, which serve as inducing agents for *in vitro* morphogenesis (Silva *et al*. 2019, Souza *et al*. 2019). Many studies report a favorable effect of TDZ at different stages of callus formation (Jafari *et al*. 2017), with TDZ concentrations below 2.5 mg L^{-1}, being recommended for effective applications to the tissue of woody species, since high concentrations of this plant growth regulator may limit the indirect organogenesis by reducing the induction of buds and increasing hyperhydricity (Hesami and Daneshvar 2018). At 90 days after the start of the experiment, the combination of plant growth regulators and type of explants had an effect on the indirect organogenesis in *E. cloeziana*. In this context, the use of 1.0 and 2.0 mg L^{-1} TDZ, considering cotyledon and hypocotyl, respectively, produced the best responses due to the higher percentage of adventitious buds compared to the other evaluated treatments (table 2, figures 4A-B).

As for the number of elongated shoots arising from bud regeneration from callus, the best results were observed with the use of 2.0 mg L^{-1} TDZ, using hypocotyl (table 3). Data reported in literature corroborate those found in this study, since TDZ promotes cell division (Fernando *et al*. 2016, Jafari *et al*. 2017). Some studies have identified TDZ as one of the most effective cytokinins for bud regeneration in *Eucalyptus*. The efficiency of TDZ was demonstrated in other species of the genus *Eucalyptus*, such as in *E. camaldulensis* (Dibax *et al*. 2010). This ability of the callogenic tissues to redifferentiate into buds is essential for the shoot elongation phase (Salla *et al*. 2018).

Hypocotyl and cotyledon combined with TDZ (*i.e.*, 1.0 and 2.0 mg L^{-1} TDZ) favored the *ex vitro* microcuttings survival (66.7-100.0 %) and adventitious rooting (53.3-55.6 %) (table 4). These results confirm the need for an adequate choice of plant growth regulator and tissue for the development of a protocol aiming at whole plant regeneration. The successful regeneration of plants through indirect organogenesis has been reported for a limited number of commercially important eucalypt species.

Hypocotyl and cotyledon of *in vitro* cultured seedlings were the most responsive tissues as explants for the regeneration of new *E. cloeziana* plants via indirect organogenesis, showing a response similar to that of other studies (Mittal and Sharma 2017). A possible cause for this tissue differentiation, considering that these events may vary according to the plant genotype (Salla *et al*. 2018), is related to the regulation of physiological processes that favor the formation of new tissues because they contain cells with high juvenility and cellular competence (Wendling *et al*. 2015), leading to increased organ production and development (Gupta and Karmakar 2017).

Ex vitro survival and rooting of *E. cloeziana* microcuttings obtained by indirect organogenesis is difficult to achieve; however, the efficiency of this protocol was observed in 350 days (figures 5A-D). Rooting is one of the most difficult phases of micropropagation of woody species and is usually conducted under *ex vitro* conditions (Brondani *et al*. 2012, 2018). However, modifications in the rooting procedures have allowed results to be obtained at adequate levels (Brondani *et al*. 2012), whereby various combinations of plant growth regulators can be effective in promoting survival and plant rooting through

indirect organogenesis (Aggarwal *et al*. 2010, Oliveira *et al*. 2015).

The use of the mini-incubator system was adequate for the survival, rooting and initial acclimatization of *E. cloeziana* plants (table 4). These results corroborate other studies using the same type of system, where higher survival and rooting in microcuttings of *Corymbia citriodora*, *E. urophylla*, *E. benthamii* (Brondani *et al*. 2012, 2018) and *E. cloeziana* (Oliveira *et al*. 2015) were obtained.

Regarding the *in vitro* culture time, considering the phases of germination (20 days), callogenesis (30 days), bud regeneration (90 days), shoot elongation (90 days), *ex vitro* survival and rooting (20 days), and acclimatization (20 days) and hardening (80 days), the protocol proposed (350 days) can be considered an alternative for the propagation of the species for numerous applications within forest tree breeding.

The developed methodology can be tested in other *Eucalyptus* and *Corymbia* species that present rooting difficulties, considering that, in the current processes of *in vitro* rejuvenation, between 12 and 19 successive subcultures (360 to 523 days) are commonly used to obtain juvenility/reinvigoration of the mature tissues and, consequently, to increase the rhizogenic competence of the propagules. Notably, the protocol was developed for explants collected from seedlings obtained by *in vitro* germination, and thus, the feasibility of applying the technique to selected adult plants needs to be tested.

In conclusion for *E. cloeziana*, (*i*) tissue competence and plant growth regulator concentrations were effective for the induction of callus structures; (*ii*) meristematic center and high nuclear/cytoplasmic ratio were observed in the combination of NAA and 2,4-D; (*iii*) *in vitro* adventitious bud induction and shoot elongation occurred with 1.0 mg L^{-1} TDZ combined with cotyledon, 2.0 mg L^{-1} TDZ combined with hypocotyl and cotyledon, and 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} TDZ combined with hypocotyl; (*iv*) cotyledon combined with 1.0 mg L^{-1} TDZ and hypocotyl combined with 2.0 mg L^{-1} TDZ were characterized by the *ex vitro* survival of microcuttings (66.7-100.0 %) and by adventitious rooting (53.3-55.6 %).

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Native and exotic plant species diversity in forest fragments and forestry plantations of a coastal landscape of central Chile

Diversidad de plantas nativas y exóticas en fragmentos de bosque y plantaciones forestales en un paisaje costero de Chile central

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SUMMARY

Native forest replacement by exotic forestry plantations and fragmentation may have different consequences for biodiversity. In the transition zone between the Mediterranean and Temperate Regions of Chile, native forests have been replaced and fragmented, and currently are surrounded by *Pinus radiata* plantations. However, the effects of these changes on biodiversity are still poorly understood. In this study, we evaluated how the replacement and fragmentation of these native forests have affected plant communities of a coastal area of the Maule Region in central Chile. We compared plant communities between three types of landscape units: pine plantations, small forest fragments and a continuous native forest. On each landscape unit, we evaluated richness and composition of native and exotic species on 100 m^2 plots located in two positions: edge and interior. Native species richness decreased and exotic invasion increased in plantations compared to fragments and continuous forests. Fragmentation increased invasion of exotic species, nonetheless did not affect native species richness. Small fragments were significantly similar to the continuous forest in native species composition (~52 % similarity). Exotic species composition of the plantation was significantly similar to edges of fragments (> 53 % similarity). Our results suggest that, although several native species may inhabit pine plantations, these are not able to support as many native plants as do native forests. Furthermore, plantations facilitate exotic invasion. Despite that fragments have been invaded, the fact that many native species are growing there suggests that small fragments of native forest may be considered as high-value areas for conservation.

Key words: alien species, biodiversity, invasion, replacement, forestry plantation, fragmentation.

RESUMEN

Los bosques nativos de la zona transicional entre las regiones de clima mediterráneo y templado del sur de Chile han sido reemplazados y fragmentados, y actualmente están rodeados de plantaciones de *Pinus radiata*. Sin embargo, aún se conocen poco los efectos de estos cambios en la biodiversidad. En este estudio evaluamos el efecto del reemplazo y fragmentación del bosque nativo sobre las comunidades vegetales de un área costera de la región del Maule, Chile. Para esto, comparamos las comunidades vegetales entre tres tipos de unidades de paisaje: plantaciones de pino, pequeños fragmentos de bosque nativo, y un bosque nativo continuo. En cada unidad de paisaje evaluamos la riqueza y composición de especies en parcelas de 100 m^2 ubicadas en dos posiciones: borde e interior. La riqueza de especies nativas decreció y la invasión de exóticas aumentó en las plantaciones comparado a fragmentos y bosque continuo. La fragmentación aumentó la invasión de plantas exóticas, pero no afectó la riqueza de especies nativas. Los fragmentos fueron significativamente similares al bosque continuo en composición de nativas (~52 %). La composición de exóticas de la plantación fue significativamente similar a bordes de fragmentos (> 53 %). Los resultados sugieren que, aunque diversas especies nativas pueden habitar en plantaciones de *P. radiata*, estas no pueden albergar tan alta diversidad como los bosques nativos, y en cambio facilitan la invasión. Además, aunque los fragmentos han sido invadidos, poseen una importante riqueza de especies nativas y pueden ser considerados como áreas de alto valor de conservación.

Palabras clave: biodiversidad, especies exóticas, fragmentación, invasión, plantaciones forestales, reemplazo de bosque nativo.

INTRODUCTION

Changes in land uses of forest ecoregions have produced landscapes that may include contrasting ecosystem types, such as native forest fragments of different sizes, forestry exotic plantations, agricultural crops, urban areas and roads (Groom and Schumaker 1993, Bustamante and Castor 1998). The resulting habitat loss and fragmentation

have been considered as the main factors producing loss of biodiversity (Fahrig 2017).

Deforestation of native forests may result in strong area reductions and increases in isolation and edges of remnant fragments (Groom and Schumaker 1993). These spatial changes in habitats may produce important effects on biodiversity by mean of different ecological mechanisms (Fahrig 2017). For example, effects on genetic and reproductive processes, alterations in species interactions and changes in abiotic conditions, such as increase of air temperature and light, and decrease in soil moisture, have frequently been related to modifications in species richness and composition (Murcia 1995, Chen *et al.* 1995, Didham and Lawton 1999, Hobbs 2001, Fahrig 2017). For plant species, reductions in fragment size and increases of edge habitats in forest patches usually decrease native species richness (*e.g.* Laurance *et al.* 1998, Honnay *et al.* 1999, Benítez-Malvido and Martínez-Ramos 2003, Guirado *et al.* 2006) and enhance plant invasion (Brothers and Spingarn 1992, Halpern and Spies 1995, Hobbs 2001, Rojas *et al.* 2011).

Another important human-caused change in landscapes of forest ecoregions is the installation of exotic forestry plantations (Groom and Schumaker 1993). These plantation ecosystems share some environmental conditions with the original native forests (*e.g.* shade produced by trees, leaf litter, some cover of understory, etc) and can also support an important number of native species (Brockerhoff *et al*. 2003, Gómez *et al.* 2009, Heinrichs *et al.* 2018). However, depending on the management of plantations, disturbance regime is very different from that of native forests. Especially when plantations are managed using the clear-cutting method, disturbances and strong environmental changes caused by this harvest method may produce detrimental effects on native species richness and enhance invasion of exotic species (Halpern and Spies 1995, Decocq *et al.* 2004). After plantations have been established, recolonization of native species may also depend on distance to native forests that function as sources of propagules (González-Moreno *et al.* 2013).

In addition to changes in species richness of native and exotic species, fragmentation and installation of forestry plantations may also produce variations in species composition. A possible pattern to expect in these landscape types is community composition nestedness that results from extinction and colonization of species among plant communities (Honnay *et al.* 1999, Patterson 1990). A nested pattern occurs when the species composition of areas with lower richness corresponds to a subset of species present in richer areas (Patterson 1990). This ecological pattern may emerge among continuous forests, fragments and plantation areas. For native species, this may occur because shade-tolerant and hygrophilous species might go locally extinct in plantations; therefore, only some of them remain in small fragments and finally all of them remain in continuous forests. Thus, native species composition in plantations would be nested in small fragments, and both of them nested in continuous forests (Honnay *et al.* 1999). A different pattern may occur through species colonization processes. For example, a continuous forest may be nested in fragments if some native species colonize fragments and no extinction occurs in them. A nested pattern would also be possible to expect for the community of exotic species. In this case, if invasion occurs mainly in plantation areas, then in small fragments, and less in continuous forests, the composition of exotic species in the continuous forests may be a subset of the species found in small fragments, and the composition of small fragments would correspond to a subset of the species found in plantations. Although nestedness patterns have been examined in different native assemblages, this pattern has been less evaluated in exotic plant species (see Higgins *et al.* 2006 for a review). Current evidence about patterns of species richness and nestedness in fragmented landscapes suggests that native and exotic plant species may respond oppositely to fragmentation and forest replacement. However, few studies have compared patterns of native and exotic species richness and nestedness across a fragmented landscape (*e.g.* Guirado *et al.* 2006, Rojas *et al.* 2011).

Coastal forests of the transition area between Mediterranean and Temperate climates in Chile (35-38°S) hold a rich and endemic biodiversity (Armesto *et al.* 1995). However, in this region many native forests have been replaced by other land uses, especially farmlands and commercial forestry plantations of exotic trees such as *Pinus radiata* D. Don. As a result, native forests remain as small remnant fragments. Few large and continuous forests are still present in the region, most of them corresponding to protected areas (Bustamante and Castor 1998, Echeverría *et al.* 2007). One of these protected areas is Los Queules National Reserve, a 145 ha protected area, immersed within a large tract of approximately 600 ha of native forest located in the coastal range of Maule Region of Chile. This continuous forest is surrounded by pine plantations and scattered fragments of native forest. Pine plantations are managed using even-age silviculture. Pine plantations are typically harvested using clear-cutting, and stands are established using replanting. After replanting native vegetation is allowed to grow, either by resprouting or dispersal from adjacent native forests (García *et al.* 2016).

We evaluated the variation of the plant species richness and composition across a landscape dominated by pine plantations, small fragments of native forest and a continuous forest corresponding to Los Queules National Reserve. We compared richness and composition of native and exotic species between these types of landscape units as well as edges and interiors of them. We expect native species richness to be negatively --while exotic species positively-- affected by fragmentation and presence of pine plantations. We also expect these changes in species richness to occur in a nested way across different landscape units and positions. As consequence, we expect changes

in species composition of both native and exotic species between these landscape units and positions in this forest landscape of this region of Chile.

METHODS

Study area. The study was conducted in a coastal forest landscape of the transition area between the Mediterranean and Temperate climate regions in Chile (35°59'S, 72°41'W) (figure 1). The area has between three and five arid months, the mean annual temperature is 12.7 °C and the mean annual precipitation is 918 mm (di Castri and Hajek 1976). The landscape is currently composed by native forest fragments of different sizes, and many large stands of pine (*Pinus radiata*) plantations, the dominant land cover in the landscape. Native forests corresponded to a mixed forest of deciduous and evergreen species, dominated by the deciduous species *Nothofagus glauca* (Phil.) Krasser and *N. obliqua* (Mirb.) Oerst. and by evergreen tree species such as *Cryptocarya alba* (Molina) Looser, *Aextoxicon punctatum* Ruiz *et* Pav., and *Gevuina avellana* Molina. The studied plantation stands substituted the original native forest and were installed shortly after cutting the native forest. Studied plantations corresponded to the first generation after substitution, and were approximately 15 years old at the sampling moment, with 400 individuals per hectare, systematically spaced, and a height of approximately 15 m. Every twenty years, all these plantations are completely harvested by clear-cutting. For this reason, the post-harvest environment is an open habitat, although it is immediately planted with pine seedlings. Based on growth rate of pines, during approximately five years there is very few tree cover (of pines) in the plantations. The height of the plantation reaches the height of the native forest approximately at 10-12 years.

Sampling design. During spring and summer of 2006-2007, 60 100 m^2 (10 x 10 m) plots were installed in three types of landscape unit (continuous forest, fragments and plantation). A continuous forest corresponded to an area of native forest with a continuous cover of native trees and a surface larger than 100 ha, which was the minimal area used by Laurance *et al*. (1998) to define a non-fragmented and continuous forest. A fragment corresponded to an area of forest with a continuous cover of native trees and a surface smaller than 100 ha. However, in this study we used only small fragments (hereafter fragments), all of them smaller than 5 ha to extreme differences in size with continuous forests. We found one continuous forest (600 ha); part of this forest fall within "Los Queules" National Re-

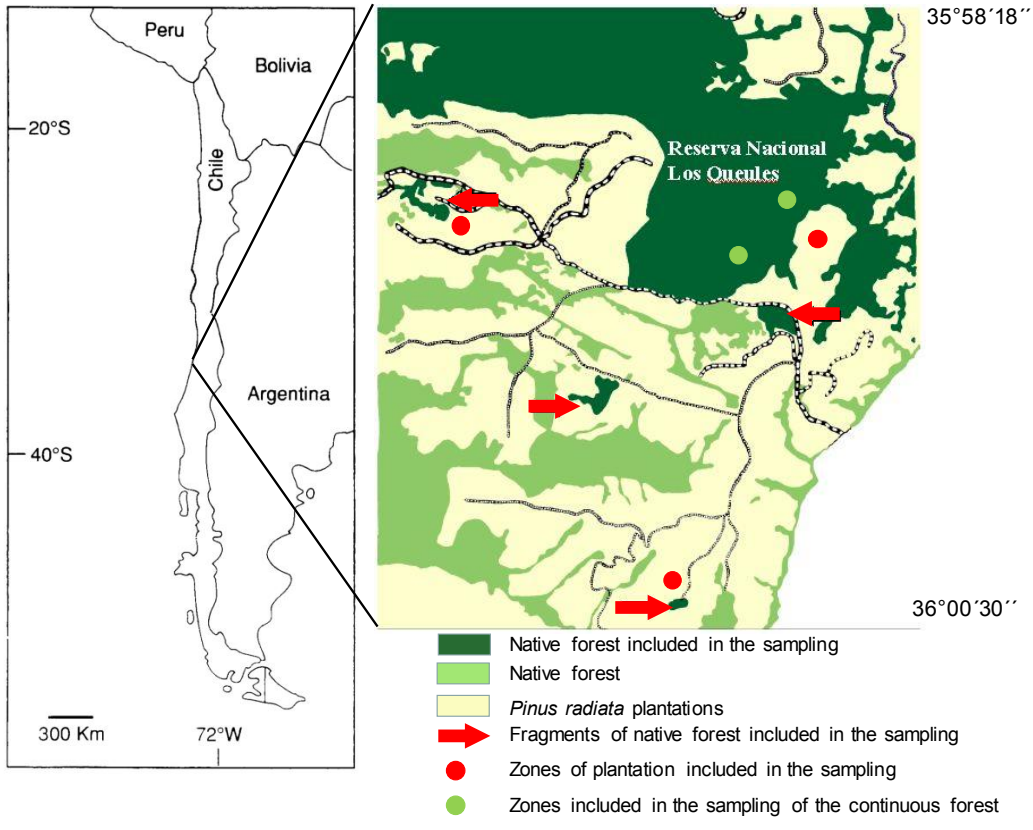

Figure 1. Location of the study area. Sampled zones of the continuous forest, fragments and pine plantations are shown.
Ubicación del área de estudio. Se muestran el bosque continuo, fragmentos y plantaciones de *Pinus radiata*.

serve (145 ha). We used four fragments in the study and corresponded to nearby patches of the same native forest. Areas of fragments varied between 0.8 and 3.4 ha, approximately. Distance between fragments and among these and the continuous forest varied between 0.2 and 2.7 km (figure 1). All the fragments had similar habitat conditions since all of them were on flat areas or south and southwest-facing gentle slopes. The studied zones of the continuous forest and plantation were also flat areas or south-facing slopes.

To quantify plant communities on each landscape unit, we located ten plots in the edges and ten plots in the interior (hereafter positions). Plots at edges were located just at the beginning of the tree cover of each unit. The edges of fragments and continuous forest were located adjacent to plantations. The edges of plantation were located adjacent to fragments and continuous forest (figure 1). The plots in the interior were located approximately 40 m into fragments, a distance approximately twice the height of trees in fragments. In the continuous forest and plantation, interior plots were located 100 m from the closest edge. Because of the size of the small fragments selected for this study, the maximum distance between interior sites and edges in them could not be larger than 40 m. Instead, in the continuous forest and plantation we preferred to use larger distance (100 m) between interior sites and edges to avoid any environmental influence of edges in interior sites (Laurance *et al.* 1998). This different distance between edges and interior positions in fragments regarding the other landscape units implies that possible observed edge effects (contrasting results between edges and interior positions) should be carefully interpreted, considering separately edge effects between landscape units.

We sampled the continuous forest in two zones per position 200 m apart (figure 1), each with five plots (five plots at the edge and five at the interior). In fragments, we sampled two plots in each position in two fragments, and three plots per position in the other two fragments (figure 1). Because the plantation was compared to the continuous forest as well as fragments, we sampled the plantation (edges and interior) in three zones: one zone adjacent to the continuous forest and the other two zones adjacent to each of two different fragments (figure 1). In the zone adjacent to the continuous forest, we installed five plots at the edge and five plots in the interior. In one zone adjacent to one fragment we installed two plots at the edge and two plots at the interior of the plantation. The third zone sampled in the plantation was located adjacent to another fragment, with three plots at the edge and three plots at the interior. All plots in each position in all landscape units were separated by 10 m along a transect parallel to the nearest edge. The fact of separating the sampling of the plantation in three zones, the continuous forest in two zones and fragments in four small parts (figure 1) allowed us to reduce the level of pseudoreplication. However, plots sampled within each zone and within a specific fragment were effectively pseudoreplicates, which is a limitation of this study.

Data analyses. All analyses were carried out separately for the total of vascular plants, native species and exotic species. Nomenclature and biogeographic origin of species were established according to Rodríguez *et al.* (2018). Differences in species richness between landscape units and positions were evaluated by a two-way analysis of variance (ANOVA) as data of richness satisfied normality of distribution. Richness of exotic species was square root transformed for normality of data. We considered the landscape unit (continuous forest, fragments and plantation) and the position (edge and interior) as factors. Replicates were ten plots per combination of landscape unit and position, and 60 in total. We performed Tukey tests to examine specific comparisons.

We also assessed the nestedness between landscape locations (landscape units and positions). To do this, we evaluated the order and disorder of the distribution pattern of species through the landscape by Temperature Analyses (*sensu* Atmar and Patterson 1995) for each group of species (total, native, exotic). The temperature analysis allows to establish whether there is some general pattern among locations in the landscape, either nestedness or checkboard pattern (Atmar and Patterson 1995). Temperature analyses were carried out according to Nestedness Temperature Calculator program (Atmar and Patterson 1995), in which an observed Temperature Value (*T*) is obtained and compared to a randomly determined *T* value by a Monte Carlo simulation process. Values of *T* significantly lower than expected by chance indicate nestedness, and *T* values significantly higher than expected by chance indicate a checkboard pattern (Atmar and Patterson 1995).

Finally, differences in species composition between landscape units and positions were assessed by cluster analyses pooling all species from the 10 plots installed per each combination of landscape unit and position. We used the Jaccard's coefficient for comparison between each combination of landscape unit and position. This index does not account for abundance of species, and thus, probably these values overestimate the similarity between landscape units and positions. A value of 1 indicates equal composition and a value of zero indicates completely different composition. Cluster analyses were performed by an Unweighted Pair Group Method using Arithmetic averages (UPGMA) procedure. We determined critical values of similarity lower and higher than expected by chance. These values were obtained by a Monte Carlo simulation process from a randomly calculated matrix of similarity. We allowed a completely random number of species per location and number of location per species for the random process. We produced 1,000 random values of Jaccard's coefficient for each comparison and 15,000 in total from all pairs of comparisons. From these 15,000 values, we obtained the 5^{th} and the 95^{th} percentiles for determining the significant value below and above which clusters had significantly lower and higher values than expected by chance, respectively.

RESULTS

Flora of native and exotic species. We found 95 species of vascular plants corresponding to 88 genera and 50 families. Eighty-nine species were Magnoliophyta, one species was Pinophyta and five species were Pteridophyta. The flora is composed mostly of herbs and less by lianas, and 79 (83.2 %) species were natives while 16 (16.8 %) were exotics (table 1). Exotic species were mainly herbs, while natives were mainly and equally represented by shrubs and trees (table 1).

Patterns of species richness. Pooling the total number of species from the ten plots per combination of landscape unit and position, total species richness of vascular plants was higher at the edges of fragments and lower at the interior of the plantation (table 2). In native species, total species richness was higher in the edge of the continuous forest and the lowest value was observed in the interior of the plantation (table 2). Total exotic species richness was higher at the edge of the plantation and the lowest value occurred in the interior of the continuous forest where no exotic species was observed (table 2).

Regarding richness at a plot level, species richness of all vascular plants significantly varied between landscape units nonetheless not between positions, and the statistical interaction between these factors was not significant (table 3, figure 2A). Specifically, richness of vascular plants was significantly higher in the continuous forest and fragments than in the plantation, with no significant difference between the first two landscape units (figure 2A). Richness of native species per plot significantly differed between landscape units though not between positions, and the statistical interaction between these factors was not statistically significant (table 3). In this case, species richness was significantly higher in the continuous forest and fragments than in the plantation, with no significant difference between the first two landscape units (figure 2B). Richness of exotic species per plot significantly differed between landscape units and positions, and the statistical interaction between these factors was significant (table 3). We observed significantly higher exotic species richness in the plantation than in the continuous forest, both at edges

Table 1. Percentage (%) of species belonging to each biogeographic origin per growth form.

Porcentaje (%) de especies pertenecientes a cada origen biogeográfico por forma de vida.

Origin	Trees	Shrubs	Herbs	Lianas
Total	22.1	24.2	43.2	10.5
Exotics	6.3	18.7	75.0	0.0
Natives	25.3	25.3	36.7	12.7

Table 2. Total species richness (pooling all plots) for each species type per landscape unit and position. (C: continuous forest, F: fragments, P: plantation, E: edge, I: interior).

Riqueza total de especies (combinando todas las parcelas) por cada tipo de especies y por posición y unidad de paisaje (C: bosque continuo, F: fragmentos, P: plantación, E: borde, I: interior).

Variable	CE		CI		FE		FI		PE		PI	
	N°	%	N°	%	N°	%	N°	%	N°	%	N°	%
All vascular plants	56	-	41	-	57	-	44	-	48	-	36	-
Natives	52	92.9	41	100	47	82.5	42	95.5	35	72.9	27	75.0
Exotics	4	7.1	0	0.0	10	17.5	2	4.5	13	27.1	9	25.0

Table 3. Results from two-way ANOVAs for the effect of landscape unit, position and its interaction on total richness, exotic species richness and native species richness. Richness of exotic species was square root transformed for normality of data.

Resultados de ANOVA de dos vías para los efectos de la unidad de paisaje, posición y su interacción sobre la riqueza total de especies, riqueza de especies exóticas y de especies nativas. Riqueza de especies exóticas fue transformada para normalidad de datos con la raíz cuadrada.

Richness	Landscape unit		Position		Unit x Position	
	$F_{(2,54)}$	*P*	$F_{(1,54)}$	*P*	$F_{(2,54)}$	*P*
All vascular plants	14.76	<0.001	2.36	0.131	0.11	0.891
Native	33.04	<0.001	0.25	0.622	0.12	0.880
Exotic	19.54	<0.001	28.96	<0.001	2.73	0.050

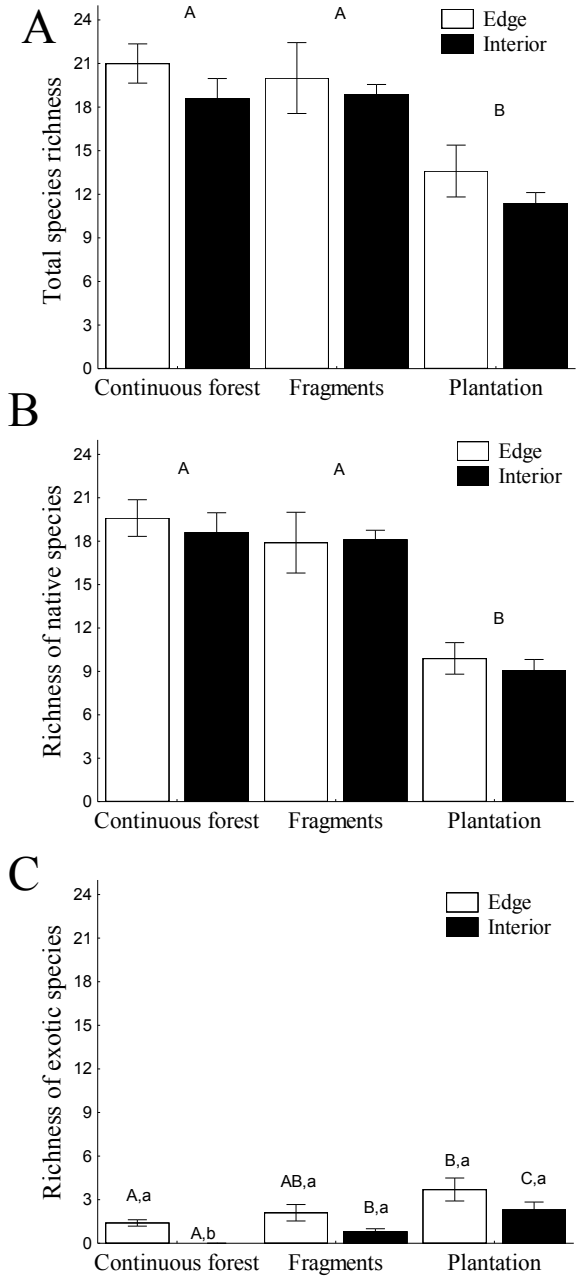

Figure 2. Species richness of all vascular plants (A), native species (B) and exotic species (C) per position (interior and edge) and landscape unit (continuous forest, fragments, and plantation). Different uppercase letters indicate significant differences (Tukey, $P < 0.05$) between landscape units for a single position, and different lowercase letters indicate significant differences (Tukey, $P < 0.05$) between positions for a single landscape unit.

Riqueza de especies total de plantas vasculares (A), nativas (B) and exóticas (C) por posición (interior y borde) y unidad de paisaje (bosque continuo, fragmentos, and plantación). Diferentes letras mayúsculas indican diferencias significativas (Tukey, $P < 0,05$) entre unidades de paisaje, y diferentes letras minúsculas indican diferencias significativas (Tukey, $P < 0,05$) entre posiciones.

and interiors. Likewise, species exotic richness was significantly more important in the plantation than in fragments in interior positions nevertheless not in edges (figure 2C). Fragments had significantly larger exotic species richness than that present in the continuous forest in interior positions, though not at edges (figure 2C). In addition, only in the continuous forest there was a significant difference between positions, being exotic species richness at edges significantly higher than in the interior (figure 2C). In the other landscape units, exotic species richness was also superior at edges than in interiors, although without significant statistical differences (figure 2C).

Nestedness pattern. Regarding all vascular plants, there was no a significant nestedness pattern (table 4). By contrast, native species were significantly nested across landscape units and positions considered in this study (table 4). This indicates that positions and landscape units with lower native species richness were composed of a subset of species present in the richer ones (see table 2). In this case, this pattern was mainly produced by several species which were present in both fragments and continuous forest, although not in the plantation (and not the inverse), such as *Aextoxicon punctatum* Ruiz *et* Pav., *Chusquea quila* Kunth, *Citronella mucronata* (Ruiz *et* Pav.) D. Don, *Viola portalesia* Gay, *Raphitamnus spinosus* (Juss.) Moldenke, *Greigia sphacelata* (Ruiz *et* Pav.) Regel, *Lomatia dentata* (Ruiz *et* Pav.) R. Br., *Senna stipulacea* (Aiton) H.S. Irwin *et* Barneby, *Lardizabala biternata* Ruiz *et* Pav. (table 5). Similarly, the nestedness in native species was generated by some native species which were present in edges, though not in interior sites (and not the inverse). For example, in the continuous forest, this occurred in *Adiantum chilense* Kaulf. *Lepechinia chilensis* (Molina) R. Morales, *Lithrea caustica* (Molina) Hook. *et* Arn., *Muehlenbeckia hastulata* (Sm.) I.M. Johnst., *Nassella gigantea* (Steud.) Muñoz-Schick, *Ribes punctatum* Ruiz *et* Pav. In fragments, this occurred in *Chiropetalum tricuspidatum* (Lam.) A. Juss., *Greigia sphacelata* (Ruiz *et* Pav.) Regel, *Senna stipulacea* (Aiton) H.S. Irwin *et* Barneby, *Uncinia macloviana* Gaudich.

Table 4. Nestedness (Temperature value, *T*) among locations in the landscape for each species type. Values of observed *T* and *T* under a null model (mean ± 1 S.D.) from Monte Carlo simulation are shown.

Anidamiento (Valor de Temperatura, *T*) entre posiciones en el paisaje por cada tipo de especie. Se muestran valores observados de *T* y de *T* usando un modelo nulo (media ± 1 D.S.) a través de una simulación tipo Monte Carlo.

Species group	Observed *T*	Null *T*	*P*
All vascular plants	47.41	50.50 ± 4.72	0.257
Natives	40.70	50.43 ± 5.10	0.028
Exotics	11.22	38.30 ± 9.35	0.002

On the other hand, exotic species also showed a significantly nested distribution (table 4). This pattern was mainly produced by several exotic species which were present in the plantation, nonetheless not in fragments or continuous forest (and not the inverse) (*e.g. Rumex acetosella* L., *Holcus lanatus* L., *Prunella vulgaris* L.), and by other exotic species present in the plantation and fragments, though not in the continuous forest (and not the inverse) (*e.g. Cynosurus echinatus* L., *Rosa rubiginosa* L.) (table 5). Correspondingly, this pattern was generated by exotic species which were present in edges, nevertheless not in interior sites (and not the inverse), either in fragments and continuous forest (*e.g. Rubus ulmifolius* Schott, *Agrostis capillaris* L.), or in the plantation (*e.g. C. echinatus, R. acetosella, H. lanatus*) (table 5).

Table 5. Origin (N: native, E: exotic), climatic region of distribution of native species in Chile (CR) (T: temperate, M: Mediterranean), Life form (A: tree, N: shrub, H: herb, L: liana), and frequency (% of plots with presence) of species growing in each landscape unit and position (C: continuous forest, F: fragment, P: plantation, E: edge, I: interior). Only exotic and native species with mean frequency > 10 % are shown. Values for *Pinus radiata* correspond to naturally growing individuals (naturalized).

Origen (N: nativo, E: exótico), región climática de distribución de las especies nativas en Chile (CR) (T: templada, M: mediterránea), forma de vida (A: árbol, N: arbusto, H: hierba, L: liana), y frecuencia (% de parcelas) de las especies en cada unidad y posición de paisaje (C: bosque continuo, F: fragmento, P: plantación, E: borde, I: interior). Se muestran solo especies nativas o exóticas con frecuencia > 10 %. Valores para *Pinus radiata* corresponden a individuos naturalizados.

Species	Origin	CR	LF	CE	CI	FE	FI	PE	PI
Agrostis capillaris L.	E		H	10	0	30	0	30	10
Cynosurus echinatus L.	E		H	0	0	20	0	20	0
Digitalis purpurea L.	E		H	10	0	0	0	0	0
Gastridium ventricosum (Gouan) Schinz *et* Thell.	E		H	0	0	10	0	10	0
Holcus lanatus L.	E		H	0	0	0	0	10	0
Hypericum perforatum L.	E		H	0	0	10	0	20	0
Hypochaeris radicata L.	E		H	0	0	0	0	0	10
Lactuca virosa L.	E		H	0	0	10	0	30	20
Pinus radiata D. Don	E		A	0	0	40	20	30	10
Plantago lanceolata L.	E		H	0	0	20	0	20	20
Prunella vulgaris L.	E		H	0	0	0	0	10	0
Rosa rubiginosa L.	E		N	0	0	10	0	40	40
Rubus ulmifolius Schott	E		N	40	0	10	0	50	60
Rumex acetosella L.	E		H	0	0	0	0	10	0
Sanguisorba minor Scop.	E		H	0	0	0	0	0	10
Teline monspessulana (L.) K. Koch	E		N	80	0	50	60	90	50
Adiantum chilense Kaulf.	N	MT	H	40	0	10	40	10	0
Aextoxicon punctatum Ruiz *et* Pav.	N	MT	A	100	100	40	40	0	0
Aristotelia chilensis (Molina) Stuntz	N	T	A	90	50	100	90	100	100
Azara integrifolia Ruiz *et* Pav.	N	T	A	50	40	70	80	20	50
Blechnum hastatum Kaulf.	N	T	H	80	100	80	90	50	10
Bomarea salsilla (L.) Herb.	N	MT	H	10	70	20	80	0	10
Chiropetalum tricuspidatum (Lam.) A. Juss.	N	T	H	50	40	30	0	0	0
Chusquea quila Kunth	N	T	N	40	50	10	30	0	0
Cissus striata Ruiz *et* Pav.	N	M	L	90	60	60	50	50	70
Citronella mucronata (Ruiz *et* Pav.) D. Don	N	M	A	10	30	30	20	0	0
Cryptocarya alba (Molina) Looser	N	M	A	90	100	80	70	80	70

Continue

Table 5 Continued

Gevuina avellana Molina	N	T	A	50	100	70	100	20	30
Greigia sphacelata (Ruiz *et* Pav.) Regel	N	T	N	50	90	20	0	0	0
Herreria stellata Ruiz *et* Pav.	N	T	L	30	50	50	40	40	0
Lapageria rosea Ruiz *et* Pav.	N	T	L	70	100	40	80	20	0
Lardizabala biternata Ruiz *et* Pav.	N	MT	L	70	60	10	20	10	0
Laurelia sempervirens (Ruiz *et* Pav.) Tul.	N	T	A	50	10	20	20	20	20
Lepechinia chilensis (Molina) R. Morales	N	T	N	20	0	20	10	20	50
Lithrea caustica (Molina) Hook. *et* Arn.	N	M	A	10	0	80	80	0	0
Lomatia dentata (Ruiz *et* Pav.) R. Br.	N	T	A	80	100	50	40	0	10
Luma apiculata (DC.) Burret	N	T	A	100	70	100	70	80	80
Luzuriaga radicans Ruiz *et* Pav.	N	T	L	70	80	0	0	0	0
Muehlenbeckia hastulata (Sm.) I.M. Johnst.	N	M	N	30	0	0	0	20	100
Nassella gigantea (Steud.) Muñoz-Schick	N	T	H	30	0	60	50	40	40
Nothofagus glauca (Phil.) Krasser	N	M	A	0	0	40	60	0	0
Nothofagus obliqua (Mirb.) Oerst.	N	MT	A	70	40	100	90	60	30
Persea lingue (Ruiz *et* Pav.) Nees	N	MT	A	40	90	80	70	20	40
Peumus boldus Molina	N	M	A	0	0	40	30	40	40
Proustia pyrifolia DC.	N	M	L	10	10	30	10	20	10
Quillaja saponaria Molina	N	M	A	0	0	40	20	20	10
Raphitamnus spinosus (Juss.) Moldenke	N	MT	N	70	20	20	30	0	0
Ribes punctatum Ruiz *et* Pav.	N	MT	N	10	0	50	40	50	30
Senna stipulacea (Aiton) H.S. Irwin *et* Barneby	N	T	N	40	80	20	0	0	0
Ugni molinae Turcz.	N	T	N	20	10	40	60	20	10
Uncinia macloviana Gaudich.	N	T	H	90	20	20	0	0	10
Viola portalesia Gay	N	T	H	10	30	50	80	0	0

Patterns of species similarities. Regarding all vascular plants as well as native species, no similarity value was lower than expected by chance (figure 3). Interior sites and edges presented higher similarity values than expected by chance in each landscape unit (figure 3), and fragments were significantly more similar to the continuous forest than expected by chance (figure 3). In turn, similarity between the plantation and any other landscape unit was not lower or higher than expected by chance (figure 3). In exotic species, we found that similarity between the interior of the continuous forest and the other combinations of landscape unit and position were lower than expected by chance (figure 3). In addition, edges and interiors of the plantation and edges of fragments were significantly more similar than expected by chance (figure 3).

DISCUSSION

Expansion of exotic forestry plantations and fragmentation of native forests have produced important effects on plant communities of our study area. These effects have been different for native and exotic species. Regarding native species, our results suggest that, in contrast to our predictions, reductions of area of the native forest (from continuous forest to small fragments) and presence of edges had no important impact on the native species richness at a plot scale. These results contrast to many other studies documenting that fragmentation negatively affects native species diversity (*e.g.* Laurance *et al.* 1998, Honnay *et al.* 1999, Hobbs 2001, Benítez-Malvido and Martínez-Ramos 2003, Guirado *et al.* 2006, Echeverría *et al.* 2007) at a patch scale (sensu Fahrig 2017). Habitat fragmentation frequently produces higher radiation and lower soil moisture in edges and small fragments compared to interior sites and continuous forests (Chen *et al.* 1995, Murcia 1995, Didham and Lawton 1999). However, in our study area, when plantations reach tree cover and height similar to that of the native forest (approximately 10 years after cutting and planting), environmental changes in edges and small fragments may be ameliorated, which could allow

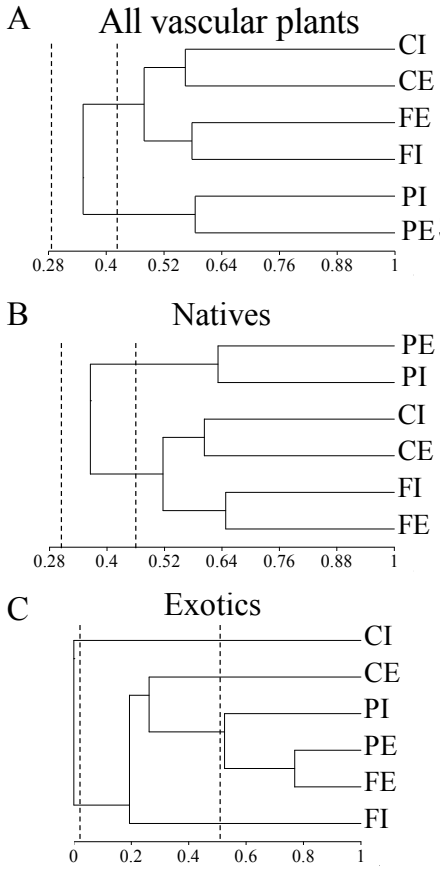

Figure 3. Species similarities for all vascular plants (A), native species (B) and exotic species (C) among locations in the landscape (CI: continuous forest interior, CE: continuous forest edge, FI: fragments interior, FE: fragments edge, PI: interior of pine plantation, PE: edge of pine plantation) using Jaccard's coefficient and a UPGMA procedure. Dashed lines indicate level of similarity below (left line) and above (right line) which cluster are significantly more different and more similar than expected by chance respectively.

Similitud de especies para todas las plantas vasculares (A), especies nativas (B) y especies exóticas (C) entre ubicaciones en el paisaje (CI: interior de bosque continuo, CE: borde de bosque continuo, FI: interior de fragmentos, FE: borde de fragmento, PI: interior of plantación de pino, PE: borde de plantación de pino) usando el coeficiente de Jaccard y procedimiento UPGMA. Líneas segmentadas indican el nivel de similitud bajo y sobre el cual las agrupaciones son significativamente más diferentes y más similares que lo esperado por azar respectivamente.

the survival of native species present in adjacent edges of fragments and continuous forest (*e.g.* Vandermeer and Carvajal 2001). Furthermore, certain habitat continuity produced by adult plantations could reduce any isolation effect on native species communities of fragments (Fahrig 2017). Thus, the presence of forestry plantations surrounding fragments may explain the absence of significant fragmentation effects on native species richness in this area. Instead, in other cases, for example Echeverría *et al.* (2007) and Rojas *et al.* (2011) in the south of Chile, fragments were surrounded by agricultural crops, a radically different environment from native forest fragments, which could have produced strong edge and isolation effects and thus explained the negative effects of fragmentation on native species richness observed in these studies.

Nevertheless, edges and interior sites in fragments as well as in the continuous forest were not identical in native species composition. Although native species similarity between edges and interior sites was higher than expected by chance both in fragments and continuous forest, we observed values only around ~60 %. This is consistent with the nestedness produced by the presence of several species in edges, however not in interior sites in both landscape units, which could be related to typical increases in radiation and temperature in edges compared to interior sites (Chen *et al.* 1995, Murcia 1995).

Regarding native species, patterns observed in the continuous forest were quite similar to those observed in fragments, despite different distance edge-interior between these two landscape units. Moreover, native species richness at a plot scale and species composition of interior sites did not significantly differ between fragments and continuous forest. This suggests that, at least in terms of native species, areas 40 m into the interior of native forests are still behaving as interior habitats.

On the other hand, native species richness in edges and interior sites of fragments and continuous forest were significantly larger than edges and interiors of the pine plantation. This is consistent with the nested pattern observed in native species, mainly produced by several species present in fragments and continuous forest, however not in the pine plantation. As consequence, native species composition in the plantation was not more similar than expected by chance to edges or interiors of fragments and continuous forest. This was probably triggered by many native species which were not able to resprout or regenerate from seeds after pine plantations were installed. These results suggest that plantations cannot hold native plant assemblages similar to those held by the native forests in this area, which has also been observed in other studies (Halpern and Spies 1995, Heinrichs *et al*. 2018). Decreased native species richness in the plantation may be related to direct effects of disturbance when native forests were clear-cut (Brockerhoff *et al.* 2003), or environmental changes after cutting, that inhibit their recolonization (Decoq *et al.* 2004). However, 15 years after cutting and planting (age of studied plantations), native species richness in the plantation reached near half of the richness observed in native forests, indicating that the environment of a pine plantation is not completely detrimental for growing native species. In fact, some studies report that *Pinus radiata* plantations may be recolonized by many native species after planting even reaching, in older plantations, levels of species richness similar to those reached in adjacent native forests (Brockerhoff *et al.* 2003). A possible cause of this important native diversity within plantations could be

the fact that these plantations directly substituted the original native forest and were installed shortly after cutting it. Hence, probably some native species could resprout or regenerate from seed banks and thus recolonize these plantations. Additionally, native species richness and composition did not differ between interior sites and edges in the plantation, which could be produced by efficient seed dispersal (García *et al.* 2016), at least up to 100 m into the interior. However, García *et al.* (2016) suggested that beyond 160 m very few seeds may access into plantation stands in this same locality, and probably beyond 160 m native diversity might decrease. On the other hand, despite native diversity can grow within plantations, clear-cutting system applied in them may limit long-term conservation of the native flora in them (Heinrichs *et al*. 2018). Survival levels of individuals of native species after applying clear-cutting in pine plantations are unknown. It is also unknown how long and how many rotations of pine plantations, using this management system, native species may survive, resprout or regenerate from seed banks.

Richness of exotic species was enhanced by fragmentation. We found higher exotic richness in small fragments than in the continuous forest in interior sites, but not in edges. Furthermore, exotic richness was higher in edges than interiors in the continuous forest, though not in fragments. These patterns suggest that, in terms of plant invasion, interior sites are behaving more similar to edges in fragments than in the continuous forest. This could be related to the shorter distance edge-interior in fragments than in the continuous forest. Furthermore, interior sites and edges of both fragments and continuous forest presented less exotic richness than that presented by plantations. This pattern is consistent with the nested pattern observed in exotic species, in which, many exotic species were present in the plantation, though not in fragments and continuous forest. As a result of this species distribution, exotic species composition in edges of fragments was more similar than expected by chance to exotic composition in edges and interior of the plantation. These results indicate that reduction of area and presence of edges in native forests, as well as replacement of the native forest by pine plantations, have facilitated the invasion of exotic plants. Furthermore, our results suggest that exotic species invade from the plantation toward the native forest, first along edges, and then the interior of fragments, although only two species have been able to invade interior sites of fragments, and no exotic species the interior of the continuous forest. All these results about exotic species are consistent with previous studies. Invasion of exotic plants has generally been observed to be higher within small fragments and edges compared to larger fragments and interior sites (*e.g.* Brothers and Spingarn 1992, Hobbs and Huenneke 1992, Richardson *et al.* 1994, Hobbs 2001, Rojas *et al.* 2011), probably due to resource release, especially light (Chen *et al.* 1995). On the other hand, positive effects on exotic invasion produced by logging and clear-cutting in plantations have been a widely documented pattern (Halpern and Spies 1995, Silveri *et al.* 2001, Becerra and Simonetti 2013). Especially when plantations are open habitats (for 7-10 years after cutting and planting), increased radiation (Chen *et al.* 1995) and nutrient release (Hobbs and Huenneke 1992) may enhance plant invasion (Silveri *et al.* 2003, Decocq *et al.* 2004). Although we did not use edges next to roads, roads probably were the main way for exotic species to immigrate into our study area, firstly into the plantation (Heinrichs *et al*. 2018).

Because this landscape is mainly composed of native species, patterns of richness and similarity of the total plant community followed the patterns observed in native species. Nevertheless, total community did not show nestedness among landscape units and positions, mainly because native and exotic species showed relatively different nestedness patterns. Our results also suggest that native and exotic species respond oppositely to the presence of pine plantations. Instead, native and exotic species respond differently but not oppositely to fragmentation as natives were not affected while exotics were positively influenced by it. Therefore, these results contrast with studies carried out in other temperate forest of south of Chile (Rojas *et al.* 2011) and Europe (Guirado *et al.* 2006), where native and exotic species richness responds oppositely to fragmentation. This suggests that exotic species were more sensitive than native species to the same environmental modification of the native forest.

Our study had different limitations and hence our inferences should be considered with caution. The use of only one large continuous forest impedes us to generalize our results for other continuous forests of the region. Thus, further research is necessary to examine if our findings also occur in other large fragments of native forest. On the other hand, although pine plantation and continuous forest were sampled in different zones within the study area, reduced number of zones and fragments, also constrains our potential to generalize our results. However, very few studies have examined the variability of plant communities across landscapes dominated by pine plantations, native forest fragments and more continuous native forests in this region of Chile (Bustamante and Castor 1998, Bustamante *et al.* 2005, Becerra and Simonetti 2013, Heinrichs *et al*. 2018). Therefore, our study shows possible patterns that may be expected in other similar landscapes where very little native forests remain. Other limitations in our study were the different isolation levels between fragments. This was an uncontrolled variable and probably contributed to the variability between fragments.

In conclusion, our results suggest that the replacement of this native forest by pine plantations has had a more important effect on native plant communities than fragmentation in this region. The fact that native species have not strongly been affected by fragmentation reinforces the idea that small fragments are of high conservation value at a landscape scale (sensu Fahrig 2017), as they hold high

levels of native biodiversity and ought to be included in conservation plans of the Chilean temperate forests (Grez *et al.* 2006). Finally, our results also suggest that *Pinus radiata* plantations may be inhabited by a high proportion (at least 50 %) of native species of this area, and hence, because pine plantations will continue being present in this region, an appropriate management system in them may also contribute to conservation of native flora.

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Efecto de la concentración de fósforo y calcio sobre atributos morfo-fisiológicos y potencial de crecimiento radical en plantas de *Aextoxicon punctatum* producidas a raíz cubierta en la etapa de endurecimiento

Effect of phosphorous and calcium concentration on morpho-physiological attributes and root growth potential of *Aextoxicon punctatum* plants produced by covered root during hardening stage

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SUMMARY

The success of restoration programs through the establishment of a vegetable cover on altered areas is directly related to morpho-physiological attributes of plants established on field conditions. These attributes have been mainly studied on many species at national and international level, while research on native species is still incipient. The objective of this research is to determine the effect of different concentrations of phosphorous and calcium during nursery hardening stage, on morphological attributes (stem length, stem diameter, leaf area), physiological (nutritional status) and root growth potential (number and length of new roots) in olivillo (*Aextoxicon punctatum*) plants produced by covered root. Nine fertilization schemes were designed with three different concentrations of phosphorous and calcium each (0, 150 and 300 mg L^{-1}). After 13 weeks, corresponding to the hardening period, no significant differences were observed among treatments for morphological variables. However, treatments with applications of 300 mg L^{-1} of phosphorous induced higher nitrogen and lower calcium foliar concentrations. Higher calcium concentrations (300 mg L^{-1}) induced higher foliar concentrations of this element and higher number and length of new roots.

Key words: olivillo, autumn fertilization, nutritional status, roots development.

RESUMEN

El éxito de programas de restauración por medio del establecimiento de una cubierta vegetal en áreas que han sido alteradas, se relaciona directamente con atributos morfo-fisiológicos de las plantas que se establecen en condiciones de campo. Estos atributos han sido estudiados principalmente para diversas especies forestales a nivel nacional e internacional, no obstante, para especies nativas la investigación aun es incipiente. El objetivo de este estudio es determinar el efecto de diferentes concentraciones de fósforo y calcio en la etapa de endurecimiento en vivero, sobre atributos morfológicos (longitud de tallo, diámetro de cuello, área foliar), fisiológicos (estatus nutricional) y potencial de crecimiento radical (largo y número de raíces nuevas) de plantas de olivillo (*Aextoxicon punctatum*) producidas a raíz cubierta. Se definieron nueve esquemas nutricionales que variaron fósforo y calcio en tres concentraciones cada uno (0, 150 y 300 mg L^{-1}). Luego de 13 semanas, periodo de endurecimiento, no se observaron diferencias significativas entre los tratamientos para las variables morfológicas y de potencial de crecimiento raíces. Sin embargo, tratamientos con aplicaciones de 300 mg L^{-1} de fósforo generaron las mayores concentraciones a nivel foliar de nitrógeno y las menores de calcio. Dosis de 300 mg L^{-1} de calcio aplicadas en el medio determinaron una mayor concentración foliar del mismo elemento, y un mayor número de raíces y más largas.

Palabras clave: olivillo, fertilización otoñal, estatus nutricional, desarrollo radical.

INTRODUCCIÓN

El manejo de plantas a nivel de vivero considera habitualmente un acondicionamiento de ellas que incluye niveles de humedad más restrictivos y fertilización diferenciada en su fase de endurecimiento, de manera que la planta sea capaz de soportar el estrés de plantación, así como el déficit hídrico que existe en los primeros meses post plantación.

Al respecto, la incidencia del contenido de nutrientes en la morfología de la planta, es uno de los aspectos que ha

sido mayormente estudiado. Un ejemplo de ello es el nitrógeno y fósforo, que contribuyen a mejorar los parámetros morfológicos de la planta, aumentar las concentraciones nutricionales internas de la planta y estimular el desarrollo del sistema radical (Razaq *et al.* 2017). Diversos autores confirman también, una relación positiva entre la concentración de fósforo y la producción de nuevas raíces (Valdecantos *et al.* 2006, Oliet *et al.* 2008), lo que sin duda tiene una importancia fundamental en la supervivencia de las plantas. Además, Dussan *et al*. (2016) señalan que uno de los principales efectos de la deficiencia de fósforo en las plantas se traduce en una reducción en el crecimiento de la hoja, así como en el número de hojas. Por otra parte, Sanz *et al.* (2001) concluyen que el calcio es esencial en la planta a nivel radical, pues incide en el número y longitud de los pelos radicales, fundamentales en la absorción de nutrientes.

En general, en la etapa de endurecimiento se reducen las prácticas culturales de fertilización y riego; sin embargo, Boivin *et al.* (2004) destacan que, en esta etapa se produce un crecimiento considerable de la raíz, y translocación de macro y microelementos, lo que provoca una dilución y un descenso de la concentración de nutrientes en los tejidos, por tanto, las labores debieran estar orientadas a aportar cantidades adicionales de algunos elementos, de manera de conservar o aumentar el nivel de reservas de la planta sin alterar significativamente su morfología (Monsalve *et al*. 2009, Villar-Salvador *et al*. 2012).

De esta forma, los atributos morfológicos y fisiológicos de las plantas producidas en vivero, se relacionan con el éxito de la restauración de cubiertas vegetales en áreas que han sido alteradas (Villar-Salvador 2003), por lo tanto, la manipulación de sus características morfo-funcionales durante el proceso de viverización, resultaría en gran medida en un incremento de la capacidad de las plantas para superar la fase de establecimiento, especialmente el primer año (Andivia-Muñoz *et al.* 2011).

En virtud de lo anterior, la hipótesis planteada dice relación con que, plantas de *Aextoxicon punctatum* Ruiz *et* Pavon (olivillo), fertilizadas con altas dosis de fósforo y calcio en la etapa de endurecimiento generarán respuestas morfo-fisiológicas y de potencial de crecimiento radical superiores que aquellas que no consideran fertilización con fósforo y calcio en la etapa de endurecimiento. En este sentido, el objetivo es determinar el efecto de concentraciones variables de fósforo y calcio en la etapa de endurecimiento en vivero, sobre atributos morfológicos, fisiológicos y potencial de crecimiento radical de plantas de *A. punctatum*, producidas a raíz cubierta.

MÉTODOS

El ensayo se estableció en septiembre del año 2012, en el Centro Tecnológico de la Planta Forestal, del Instituto Forestal, ubicado en la comuna de San Pedro de la Paz, provincia de Concepción, región del Biobío (18 H 666583 E; 5920348 S). Las semillas fueron colectadas en abril del mismo año, desde ejemplares de *A. punctatum* que sobrevivieron al incendio ocurrido en el cerro Cayumanque, comuna de Quillón (18 H 720735,04 E; 5934792,84 S). Se emplearon almacigueras de poliestireno expandido de 84 cavidades con un volumen por cavidad de 130 mL, el sustrato utilizado fue corteza de pino compostada con una porosidad total de 49,3 %; porosidad de aireación 25,2 % y porosidad de retención 24,1 %.

El riego se aplicó siguiendo un criterio de riego evaluado mediante el monitoreo de la pérdida de peso de la bandeja, según etapa de desarrollo de la planta: en establecimiento, este se realizó a capacidad de campo; y pleno crecimiento, entre un 60 – 70 % de contenido hídrico del sustrato.

La fertilización se realizó durante las etapas de establecimiento (15 de octubre a 15 diciembre de 2012) y pleno crecimiento (15 diciembre a 15 de abril de 2013) de las plantas mediante fertirrigación, controlando la concentración de macroelementos en función de la etapa de desarrollo de las plantas (cuadro 1). Las soluciones nutritivas fueron preparadas según la metodología propuesta por Landis (2000) a partir de fertilizantes de la línea Ultrasol de Soquimich ® (cuadro 1). Las labores de fertilización se realizaron en forma alternada a las de riego, y la aplicación se realizó mediante regadera.

En la etapa de endurecimiento (15 abril a 15 de julio de 2013), la frecuencia de fertilización se realizó de manera alternada a los riegos, todos realizados a saturación del sustrato. Considerando como criterio de riego, la pérdida del 50 % del peso de agua retenida por el sustrato. Se utilizaron soluciones nutritivas que fueron preparadas según la metodología propuesta por Landis (2000). Para evaluar el efecto de los nutrientes en esta etapa, se definieron nueve esquemas nutricionales que variaron en las concentraciones de fósforo y calcio, en tres concentraciones de 0, 150 y 300 mg L^{-1} cada uno (cuadro 2). La generación de los tratamientos, se realizó mediante la utilización de las siguientes sales: $MgSO_4$; K_2CO_3; $NaNO_3$, KH_2PO_4, $NH_4H_2PO_4$, $Ca(NO_3)^2$; $CaCO_3$. Las concentraciones del resto de los macroelementos fueron manejadas en forma constate: nitrógeno, 100 mg L^{-1}; potasio, 100 mg L^{-1}; magnesio, 40 mg L^{-1}; azufre, 90 mg L^{-1}.

Evaluaciones morfológicas. Con una frecuencia quincenal, desde el inicio del endurecimiento, se realizaron evaluaciones de longitud de tallo (LT, cm) medidas desde el cuello hasta el ápice de la planta, con cinta métrica, hasta el término del proceso de producción de plantas. La variable diámetro a la altura del cuello (DAC, mm) se evaluó al inicio y término de la etapa de endurecimiento, con un pie de metro digital, a las mismas plantas evaluadas en longitud de tallo. Con estas variables, fue posible calcular los incrementos en longitud de tallo (INC_LT, cm) e incremento en diámetro a la altura del cuello (INC_DAC, mm). Las mediciones se realizaron sobre las 18 plantas centrales

Cuadro 1. Concentración de macroelementos (mg L^{-1}) aplicados en las etapas de establecimiento y pleno crecimiento a plantas de *Aextoxicon punctatum* producidas a raíz cubierta.

Macroelements concentration (mg L^{-1}) applied during establishment and rapid growth phases of *Aextoxicon punctatum* plants produced by covered root.

Etapa de crecimiento	Fertilizante*	Concentración (mg L^{-1})					
		Nitrógeno (N)	Fósforo (P)	Potasio (K)	Calcio (Ca)	Magnesio (Mg)	Azufre (S)
Establecimiento	Ultrasol Inicial	150	131,0	124,5	0,0	3,0	7,0
	Ultrasol Inicial	200	174,6	166,0	0,0	4,0	9,3
Pleno crecimiento	Ultrasol Crecimiento	250	43,7	83,0	0,0	3,0	8,0
	Ultrasol Crecimiento	300	52,4	99,6	0,0	3,6	9,6
	Ultrasol Desarrollo	200	29,1	166,0	0,0	6,7	66,7

* Producto comercial de la empresa Sociedad Química y Minera S.A. (Soquimich)

Cuadro 2. Concentración de macroelementos (mg L^{-1}) aplicados en la etapa de endurecimiento por tratamiento a plantas de *Aextoxicon punctatum* a raíz cubierta.

Macroelements concentration (mg L^{-1}) of each treatment applied to *Aextoxicon punctatum* plants during hardening phase, produced by covered root.

Tratamientos	Concentración (mg L^{-1})	
	Fósforo (P)	Calcio (Ca)
1	0	0
2	150	0
3	300	0
4	0	150
5	150	150
6	300	150
7	0	300
8	150	300
9	300	300

de cada almaciguera, monitoreando un total de 486 plantas (18 plantas x 3 repeticiones x 3 concentraciones de fósforo x 3 concentraciones de calcio).

La evaluación del área foliar (cm^2), se realizó al final del período de endurecimiento, con un planímetro electrónico modelo LI-3000 (Licor Instruments Co USA), con precisión de 0,05 cm^2. Para esta evaluación se utilizaron 54 plantas en total a las cuales se les midió y pesó el follaje fresco y seco completo por planta, así como el número de hojas de cada planta (2 plantas x 3 repeticiones x 3 concentraciones de fósforo x 3 concentraciones de calcio). El follaje colectado se secó en un horno de ventilación forzada por 48 horas a 60 °C. La determinación del peso seco del follaje se realizó con balanza digital, con precisión de ± 0,0001 g. De esta forma fue posible calcular la esclerofilia en g dm^{-2}, cociente entre el peso seco del follaje y el área foliar. Cabe señalar, que este es un índice que mide el carácter escleromorfo de las hojas, es decir, el índice aumenta al acrecentarse los caracteres xeromórficos de la hoja frente a los mesomórficos.

Evaluaciones de potencial de crecimiento radical (PCR). La evaluación del PCR, se realizó al final del período de endurecimiento. Las raíces de las plantas fueron lavadas, extrayendo las raíces nuevas no suberizadas. Estas plantas fueron instaladas en una cámara aeropónica, a temperatura de llovizna de 22 °C y 18 °C, manteniendo en ambas la misma frecuencia de riego cada 10 minutos, inyectando agua sin fertilizantes hacia el sistema radical durante seis segundos y fotoperíodo de 16 horas, proporcionado por tubos fluorescentes instalados sobre la cámara, los cuales produjeron un flujo fotónico promedio a nivel de follaje de 100 μmol m^{-2} s^{-1}. Para ello se utilizaron 108 plantas en total (2 plantas x 3 repeticiones x 3 concentraciones de fósforo x 3 concentraciones de calcio x 2 temperaturas de llovizna). El ensayo se realizó en 60 días y las variables evaluadas fueron: número de raíces nuevas totales, y largo de raíces nuevas, en este caso se promediaron las tres raíces nuevas más largas, de longitud ≥ 0,5 cm.

Evaluaciones nutricionales. La evaluación nutricional de los tejidos se realizó al final del período de endurecimiento. Esta consideró la extracción aleatoria de tres muestras compuestas de follaje de tres plantas por tratamiento. Para ello se utilizaron 81 plantas en total (3 plantas x 3 repeticiones x 3 concentraciones de fósforo x 3 concentraciones de calcio), a las cuales se les determinaron los niveles de nitrógeno (N), fósforo (P), potasio (K), calcio (Ca) y magnesio (Mg). Para la determinación de nitrógeno total se siguieron los métodos de digestión de Kjeldahl y determinación mediante método colorimétrico; para el fósfo-

ro mediante calcinación por vía seca, disolución en ácido clorhídrico 1N y determinación por método colorimétrico; en el caso del potasio mediante calcinación por vía seca, disolución con ácido clorhídrico 1N y determinación por espectrofotometría de emisión atómica; finalmente, para el calcio y el magnesio, mediante calcinación por vía seca, disolución en ácido clorhídrico 1N y determinación por espectrofotometría de absorción atómica.

Diseño experimental y análisis estadístico. El diseño experimental correspondió a un diseño factorial en bloques completamente aleatorizado, de dos factores (Little y Hills 1978), concentración de fósforo (tres niveles) y concentración de calcio (tres niveles), con tres réplicas (bloques), generando un total de 27 unidades experimentales. La unidad experimental estuvo conformada por una almaciguera de 42 plantas, con tres repeticiones. Los resultados fueron analizados estadísticamente mediante análisis de varianza (ANDEVA), comprobando su independencia, normalidad y homogeneidad de varianzas. Las diferencias entre medias fueron contrastadas con el método de comparación múltiple de Tukey (Montgomery 1991). Se utilizó el programa InfoStat versión 2012 (Di Rienzo *et al*. 2012) extensión R-proyect (V.2.15.0).

RESULTADOS

Respuestas morfológicas. Al finalizar el período de endurecimiento, los factores evaluados no generaron un efecto sobre los incrementos en longitud de tallo y diámetro de cuello ($P > 0,05$) (cuadro 3). Respecto a las variables foliares, no hubo efecto significativo de los factores evaluados para el número de hojas y área foliar. Mientras que, para la esclerofilia, el factor calcio generó un efecto significativo ($P = 0,0026$). Aquellas plantas que se fertilizaron con alguna concentración de calcio (150 y 300 mg L^{-1}), generaron los valores significativamente más bajos de esclerofilia, con 1,14 y 1,11 g dm^{-2}, respectivamente (figura 1).

Respuestas de potencial de crecimiento radical. Se observó un efecto de interacción significativa ($P = 0,0151$) para los factores concentración de calcio y temperatura de llovizna sobre la variable número de raíces nuevas producidas (cuadro 4). Para la longitud de las raíces nuevas producidas se obtuvo una respuesta de los efectos principa-

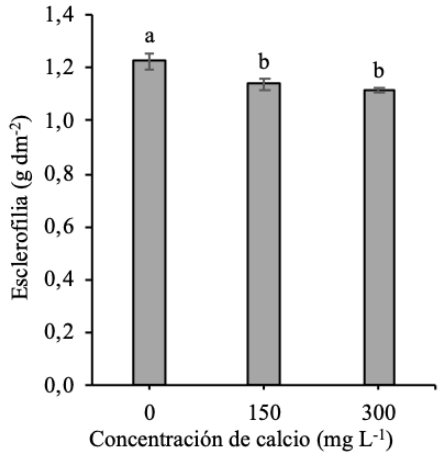

Figura 1. Esclerofilia (g dm^{-2}) promedio (barras indican + desviación estándar) en plantas de *Aextoxicon punctatum* producidas a raíz cubierta, en respuesta a la concentración de calcio aplicado durante la etapa de endurecimiento. Diferentes letras, según Tukey, difieren significativamente, $P < 0,05$.

Mean sclerophyllia (g dm^{-2}) (Bars + standard deviation) on *Aextoxicon punctatum* plants produced by covered root according to different calcium concentrations applied during the hardening stage. Different letters, according to Tukey, indicate significant differences, $P \leq 0.05$.

Cuadro 3. Valores medios ± desviación estándar del incremento en longitud de tallo (INC_LT) e incremento en diámetro de cuello (INC_DAC) en plantas de *Aextoxicon punctatum* cultivadas a raíz cubierta y sometidas a diversas dosis de fósforo y calcio en la etapa de endurecimiento (n = 3).

Mean values (± standard deviation) of stem length increment (INC_LT) and stem diameter increment (INC_DAC) of *Aextoxicon punctatum* plants produced by covered root with different concentrations of phosphorous and calcium during the hardening stage (n = 3).

P (mg L^{-1})	Ca (mg L^{-1})	INC_LT (cm)	INC_DAC (mm)
0	0	7,75 ± 0,42	1,84 ± 0,15
0	150	9,76 ± 0,41	2,43 ± 0,11
0	300	8,24 ± 1,09	1,61 ± 0,23
150	0	9,37 ± 0,95	2,07 ± 0,16
150	150	9,01 ± 1,47	2,02 ± 0,28
150	300	10,23 ± 0,82	2,08 ± 0,36
300	0	9,02 ± 1,36	1,64 ± 0,25
300	150	9,36 ± 1,03	1,93 ± 0,29
300	300	11,24 ± 1,54	1,74 ± 0,30

les, altamente significativa para la temperatura de llovizna (P = 0,0001), y significativa para la concentración de calcio (P = 0,0370).

Con respecto a la interacción sobre el número de raíces nuevas, las plantas fertilizadas con 300 mg L^{-1} de calcio y dispuestas a una temperatura de llovizna de 22 °C, generaron en promedio 41 nuevas raíces (figura 2); las demás interacciones de calcio o temperatura, generaron en promedio 12 nuevas raíces. Para la variable longitud de raíces nuevas, se observó que plantas fertilizadas con 300 mg L^{-1} de calcio generaron en promedio raíces nuevas de 2,6 cm (figura 3A), el resto de las concentraciones evaluadas indujo a que dichas plantas generaran en promedio raíces nuevas de 1,36 cm. Por otra parte, cuando el sistema radical de plantas de *A. punctatum* fue expuesto a temperatura de 22 °C, se generaron en promedio raíces de 2,6 cm, mientras que, al exponerlas a temperatura de 18° C, el largo promedio de dichas raíces, disminuyó a 1,4 cm (figura 3B).

Cuadro 4. Significancia estadística del análisis de varianza (ANDEVA) para los valores medios de las variables de potencial de crecimiento radical de las plantas de *Aextoxicon punctatum*. *Efectos significativo ($P \leq 0,05$).

Statistical significance of the variance analysis (ANDEVA) for mean values of the root growth potential of *Aextoxicon punctatum* plants. Significant effects ($P \leq 0.05$) highlighted with bold font and underlined.

Factor	Número raíces	Largo raíces
Bloque	0,8265	0,5637
Temperatura (T)	0,0006*	0,0001*
Fósforo (P)	0,2627	0,4487
Calcio (Ca)	0,0018*	0,0370*
T x P	0,1218	0,0831
T x Ca	0,0151*	0,4328
P x Ca	0,1228	0,1315
T x P x Ca	0,7973	0,4259

Respuestas nutricionales. Al evaluar la significancia estadística de los factores testeados, las aplicaciones de fósforo en el medio, generaron un efecto estadísticamente significativo en las concentraciones foliares de nitrógeno, fósforo y calcio en las plantas de *A. punctatum*. A su vez, las aplicaciones de calcio en el medio, generaron un efecto significativo en las concentraciones foliares de calcio y potasio. No se registraron interacciones significativas entre los factores fósforo y calcio (cuadro 5). Se obtuvo una mayor concentración foliar de nitrógeno y una menor concentración de calcio en aquellas plantas fertilizadas con 300 mg L^{-1} de fósforo (cuadro 6). Por su parte, el

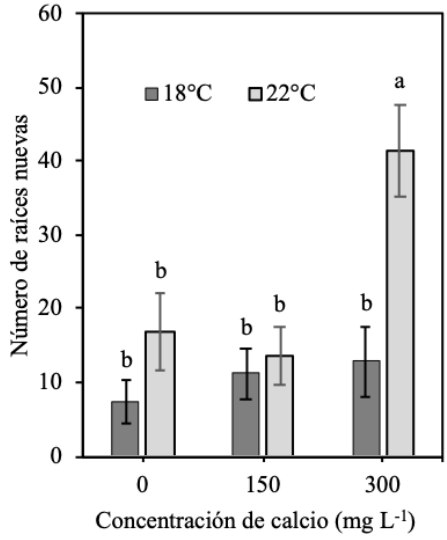

Figura 2. Efecto de la concentración de calcio y temperatura de llovizna de la cámara aeropónica sobre el número de raíces nuevas promedio (barras indican + desviación estándar) producidas por plantas de *Aextoxicon punctatum* cultivadas a raíz cubierta. Diferentes letras, según Tukey, difieren significativamente, $P \leq 0,05$.

Effect of calcium concentration and aeroponic chamber drizzle temperature on average number of new roots (bars + standard deviation) on *Aextoxicon punctatum* plants produced by covered roots. Different letters, according to Tukey, indicate significant differences, $P \leq 0.05$.

Cuadro 5. Resumen de significancia estadística (valor P > F) para los efectos fijos bloque, fósforo y calcio sobre las concentraciones foliares de macronutrientes de plantas *Aextoxicon punctatum* cultivadas a raíz cubierta y sometidas a diversas concentraciones de fósforo y calcio. *Efectos significativo ($P \leq 0,05$).

Summary of statistical significance (P value > F) for fixed effects block, phosphorous and calcium on leaf macronutrient concentration of *Aextoxicon punctatum* plants, produced by covered root with different phosphorous and calcium concentrations. Significant effects ($P \leq 0.05$) highlighted with bold font and underlined.

Factor	Nitrógeno (N)	Fósforo (P)	Potasio (K)	Calcio (Ca)	Magnesio (Mg)
Bloque	0,0247*	0,1613	0,1858	0,1274	0,0946
Fósforo (P)	0,0055*	<0,0001*	0,2242	0,0057*	0,2357
Calcio (Ca)	0,0506	0,1305	0,0429*	<0,0001*	0,6773
P x Ca	0,5472	0,9016	0,1698	0,7504	0,1012

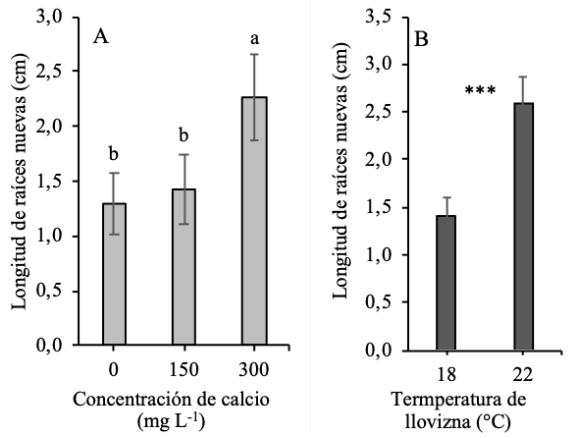

Figura 3. A) Longitud de raíces nuevas promedio (+ desviación estándar) en plantas de *Aextoxicon punctatum* en repuesta a distintas concentraciones de calcio suministradas durante la etapa de endurecimiento en vivero. B) Longitud de raíces nuevas promedio (+ desviación estándar) en plantas de *Aextoxicon punctatum* en repuesta a distintas temperaturas de llovizna de la cámara de potencial de crecimiento radical. Diferentes letras, según Tukey, difieren significativamente, $P \leq 0,05$. *** Significativo a $P \leq 0,001$.

(A) Average length of new roots (+ standard deviation) on *Aextoxicon punctatum* plant according to different calcium concentrations applied during nursery hardening stage. (B) Average length of new root (+ standard deviation) on *Aextoxicon punctatum* plants according to the drizzle temperature of the root growth potential chamber. Different letters, according to Tukey, indicate significant differences, $P \leq 0.05$. *** significant at $P \leq 0.001$.

incremento de la concentración de calcio en el medio, generó un aumento significativo y en relación directa con los niveles de calcio foliares (cuadro 7). Todas las concentraciones de calcio suministradas, generaron una disminución significativa de los niveles foliares de fósforo (cuadro 7).

DISCUSIÓN

Diversos autores como Monsalve *et al.* 2009, Oliet *et al*. 2016 y Razaq *et al.* 2017 entre otros, reportan incrementos en la longitud de tallo, en plantas de leñosas exóticas, al realizar fertilizaciones nitrogenadas junto con otros elementos, como el fósforo y potasio. En cuanto a estudios con especies nativas, Bustos *et al.* (2008) reportaron que altas concentraciones de fertilizantes que incluyen nitrógeno, fósforo y potasio generan un aumento en todos los parámetros morfológicos evaluados tanto para coigüe (*Nothofagus dombeyi* (Mirb.) Oerst.), raulí (*Nothofagus nervosa* (Mirb.) Oerst.) y ulmo (*Eucryphia cordifolia* Cav.). Cabe señalar, que el manejo de la fertilización en este estudio con *A. punctatum* fue diferenciado solo en la etapa de endurecimiento, no en las etapas anteriores (pleno crecimiento y establecimiento), por lo que las diferencias en crecimiento señalada por estos autores se originan desde el inicio de la producción. Además, se debe considerar que la etapa de endurecimiento se realiza bajo condiciones

Cuadro 6. Valores promedios (%) ± desviación estándar de la concentración foliar de macroelementos en función de las concentraciones de fósforo aplicadas en el medio, en plantas de *Aextoxicon punctatum* cultivadas a raíz cubierta (n = 3). En una columna, diferentes letras, indican diferencias significativas, según Tukey, $P \leq 0,05$.

Mean values (%) ± standard deviation of macroelements leaf concentration according to different phosphorous concentrations, on *Aextoxicon punctatum* plants produced by covered root (n = 3). On the same column, different letters indicate significant differences, according to Tuckey, $P \leq 0.05$.

Concentración de fósforo (mg L^{-1})	Niveles foliares obtenidos para los tratamientos (%)				
	Nitrógeno (N)	Fósforo (P)	Potasio (K)	Calcio (Ca)	Magnesio (Mg)
0	1,32 ± 0,04 b	0,24 ± 0,01 b	1,37 ± 0,04 a	0,70 ± 0,06 a	0,32 ± 0,01 a
150	1,31 ± 0,04 b	0,35 ± 0,02 a	1,40 ± 0,05 a	0,68 ± 0,07 a	0,32 ± 0,01 a
300	1,48 ± 0,05 a	0,37 ± 0,02 a	1,32 ± 0,02 a	0,59 ± 0,06 b	0,30 ± 0,02 a

Cuadro 7. Valores promedios (%) ± desviación estándar de la concentración foliar de macroelementos en función de las concentraciones de calcio aplicadas en el medio, en plantas de *Aextoxicon punctatum* cultivadas a raíz cubierta (n = 3). En una columna, diferentes letras, indican diferencias significativas, según Tukey, $P \leq 0,05$.

Mean values (%) ± standard deviation of macroelements leaf concentration according to different calcium concentrations, on *Aextoxicon punctatum* plants produced by covered root (n = 3). On the same column, different letters indicate significant differences, according to Tuckey, $P \leq 0.05$.

Concentración de calcio (mg L^{-1})	Niveles foliares obtenidos para los tratamientos (%)				
	Nitrógeno (N)	Fósforo (P)	Potasio (K)	Calcio (Ca)	Magnesio (Mg)
0	1,37 ± 0,04 a	0,32 ± 0,02 a	1,44 ± 0,05 a	0,46 ± 0,02 c	0,31 ± 0,01 a
150	1,31 ± 0,05 a	0,30 ± 0,02 a	1,33 ± 0,02 b	0,65 ± 0,03 b	0,32 ± 0,01 a
300	1,44 ± 0,05 a	0,35 ± 0,02 a	1,32 ± 0,04 b	0,86 ± 0,03 a	0,31 ± 0,01 a

ambientales menos favorable para el crecimiento, como disminución del fotoperiodo y de la temperatura, por lo que no es probable que se produzcan cambios morfológicos durante esta etapa. Al respecto Chirino-Miranda *et al.* (2004), señalan que variaciones morfológicas son esperables cuando los periodos en los que se aplican los tratamientos abarcan un tiempo superior al de la etapa de endurecimiento. Resultados similares a los de este estudio, lo señalan Boivin *et al.* (2004), Rikala *et al.* (2004) y Oliet *et al.* (2008) en donde indican que la fertilización en el periodo otoñal no genera mayores incrementos en variables dasométricas de las plantas, pero sí en las concentraciones nutricionales en las plantas.

En este estudio con plantas de *A. punctatum*, si bien no se detectan diferencias significativas a nivel de área foliar, es posible apreciar que, a medida que la concentración de fósforo en el medio aumenta, la superficie foliar es mayor, con valores entre 166,8 y 196,4 cm^2 para dichas dosis, respecto a los 156 a 165 cm^2 por planta, para aquellas con omisión de fósforo. Al respecto, Dussan *et al.* (2016) señalan que, uno de los principales efectos de la deficiencia del fósforo y nitrógeno en plantas de *Psidium guajava* L. fue la reducción en el área foliar y número de hojas, similar a lo observado con *A. punctatum* para los tratamientos con omisión de fósforo, no obstante, estos mismos autores reportan aumentos en el número de hojas cuando la fertilización es realizada con concentraciones deficientes de calcio. En cuanto al número de hojas, si bien en este estudio con plantas de *A. punctatum*, estas no fueron influenciadas por las diversas dosis de fósforo y calcio aplicadas en el medio, Monsalve *et al.* (2009), en plantas de *Eucalyptus globulus* Labill. sometidas a concentraciones crecientes de nitrógeno reportaron un aumento del número de hojas de dichas plantas.

Respecto a la evaluación de la esclerofilia, la fertilización con fósforo, no indujo diferencias significativas para este parámetro en las plantas de *A. punctatum*, no obstante, la aplicación de calcio, produjo una menor esclerofilia que aquellas plantas que no fueron fertilizadas con calcio. Squeo *et al.* (2004), evaluaron la esclerofilia en árboles de *A. punctatum* ubicados dentro de bosquetes y en situación de borde, en el Parque Nacional Fray Jorge en la Región de Coquimbo. Estos autores encontraron que aquellos árboles ubicados en bosquetes con menor captura de neblina y en situación de borde (condición más xérica), presentaron una mayor esclerofilia, en comparación a los bosquetes más húmedos y en las hojas bajo dosel. Estos autores concluyen, que el bosque remanente de *A. punctatum* que se desarrolla en sectores alterados presenta un mayor ángulo foliar, mayor esclerofilia y alta eficiencia en el uso del agua respecto a bosques no perturbados dentro del mismo Parque Nacional. Respecto a la relación con la disponibilidad de nutrientes en el suelo, Sereda *et al.* (2016) evaluó que plantas *Calophyllum brasiliense* Cambess aumentaron el índice de esclerofolia en suelos con menor disponibilidad de nitrógeno. Considerando los resultados obtenidos, se observa que aplicaciones reducidas de calcio en plantas de *A. punctatum* inducirían una mayor esclerofilia, atributo importante para plantas que deben ser establecidas en campo en condiciones de mayor restricción hídrica.

En este estudio, al finalizar el periodo de endurecimiento, las plantas de *A. punctatum* que fueron fertilizadas con mayores concentraciones de calcio y fósforo, generaron una concentración foliar significativamente superior en nitrógeno, fosforo y calcio, por lo que esta carga nutricional, la cual se refleja en el resultado de PCR, debería generar un mejor desempeño de las plantas de *A. punctatum* en campo. Por otra parte, todos los tratamientos en que fueron aplicados fósforo y calcio, resultaron con una concentración foliar de nutrientes baja en potasio y mayor en nitrógeno respecto al tratamiento con omisión de dichos elementos. Cabe señalar que, para este estudio, los niveles nutricionales foliares de nitrógeno se encontraron bajo el rango recomendado como óptimo (1,7 a 3,0 %) de acuerdo a lo indicado por Escobar *et al.* (2002). Por tanto, estas plantas se encontrarían en un intervalo de deficiencia llamado "necesidad oculta" según lo propuesto por Landis (2000), debido a que no se observaron síntomas de deficiencia. Sin embargo, los tratamientos que consideraron la aplicación con las dosis más altas de nutrientes (300 mg L^{-1} de fósforo y 300 mg L^{-1} de calcio) presentaron las mayores concentraciones de nitrógeno foliar (cuadros 6 y 7). Esto indicaría una mejor capacidad fotosintética de las plantas sometidas a altas concentraciones de fósforo y calcio, respecto a las que fueron fertilizadas con bajas dosis de los mismos elementos, dado que existe una correlación positiva entre la capacidad fotosintética de las hojas y el contenido de nitrógeno foliar, esto porque las proteínas del ciclo de Calvin y de los tilacoides representan la mayor parte del nitrógeno foliar (Gibson 2005).

Para las concentraciones foliares de fósforo, en todos los tratamientos, excepto con omisión de fósforo, se obtuvo concentraciones foliares sobre el rango óptimo recomendado. Escobar *et al.* (2002), recomiendan un rango para el fósforo de 0,16 – 0,26 %. Al respecto, Sardans y Peñuelas (2005) reportaron que la fertilización con fósforo en condiciones controladas realizadas a plantas de *Quercus ilex* spp *rotundifolia* (Lam) O. Schwarz ex Tab. Mor. y *Pinus halepensis* Mill generó aumentos en las concentraciones foliares de fósforo, magnesio, calcio, potasio y azufre. Para el caso del potasio, todos los tratamientos presentaron valores dentro de los recomendados por Escobar *et al.* (2002), lo que refleja que las dosis no produjeron efectos sinérgicos ni antagónicos en su absorción, no obstante, al aumentar las concentraciones de calcio en el sustrato, las concentraciones foliares de potasio disminuyeron. Al respecto, se asocia el potasio a la capacidad de ajuste osmótico y apertura de estomas en las plantas, por lo que una mayor concentración de potasio en el follaje, favorecería su establecimiento en condiciones restrictivas de agua y altas temperaturas (Navarro-Sandoval 2013). En cuanto al calcio, el tratamiento con omisión de este elemento, pre-

sentó valores bajo lo recomendado como óptimo, mientras que las mayores concentraciones foliares se presentaron en el tratamiento fertilizado con 300 mg L^{-1} de calcio, encontrándose dentro del rango óptimo. Las concentraciones foliares de magnesio en todos los tratamientos fueron similares estadísticamente y dentro de los niveles óptimos recomendados por Escobar *et al.* (2002).

Respecto al ensayo de potencial de crecimiento radical, se destaca la mayor respuesta tanto en el número de raíces como en el largo de ellas para temperaturas de llovizna de la cámara aeropónica de 22 °C. Esto indicaría que las raíces, en el caso de *A. punctatum*, promueven su formación cuando la temperatura del suelo se acerca a ese valor; es decir, durante los meses de primavera – verano, lo que concuerda con lo señalado por Santelices (2005) respecto del éxito de plantaciones de *E. globulus* establecidas en primavera.

En general, las plantas de *A. punctatum* de los tratamientos con omisión de los elementos testeados (fósforo y calcio) presentaron los menores valores en cuanto al número de raíces nuevas, así como el largo de ellas. Por su parte, los tratamientos con mayores concentraciones nutricionales, presentaron un potencial de regeneración de raíces mayor que las menos fertilizadas. Estos resultados coinciden con lo obtenido por Andivia-Muñoz *et al.* (2011) en plantas de *Quercus ilex spp. Ballota* (Desf.) Samp., quienes determinan que plantas fertilizadas con una mayor dosis de nitrógeno presentan un potencial de regeneración de raíces mayor que el de las plantas con fertilización baja. Por su parte, autores como Oliet *et al.* (2008) y Villar-Salvador *et al.* (2012) menciona que para diversas especies mediterráneas existe una correlación positiva entre el nivel de fertilización, la capacidad para generar nuevas raíces y su éxito en campo. No obstante, lo anterior, Navarro-Sandoval (2013) no encontró diferencias significativas en el número total de raíces nuevas al realizar la evaluación del potencial de crecimiento radical probando distintas dosis de nitrógeno, fósforo y potasio aplicadas en concentraciones desde un 50 % menor a un 100 % más de nutrientes, en plantas de *Abies religiosa* (HBK) Schlecht. *et* Cham. En este estudio, las plantas de *A. punctatum* fertilizadas con distintas dosis de fósforo establecidas en la cámara hidropónica a distintas temperaturas, no presentaron diferencias entre las diversas dosis de fósforo, no así en temperaturas. En este sentido, Bakker *et al.* (2009) determinaron que la fertilización realizada con fósforo, así como con una mezcla de nutrientes, redujo la densidad y el grosor de raíces finas en parcelas de *Pinus pinaster* Ait. Considerando lo obtenido en este estudio, en donde plantas de *A. punctatum* fertilizadas con mayores concentraciones de macroelementos presentaron un potencial de crecimiento radical superior que las menos fertilizadas, es esperable que, en campo la respuesta en términos de crecimiento y supervivencia de las plantas mejor nutridas sea mayor que las que fueron menos fertilizadas. Si bien es cierto, diversos autores señalan una relación positiva entre la concentración de fósforo y la producción de nuevas raíces (Valdecantos *et al.* 2006, Oliet *et al*. 2008), en este estudio, no fue posible comprobar que aquellas plantas de *A. punctatum* fertilizadas con fósforo producían mayor cantidad de raíces, no obstante, las aplicaciones de calcio, generaron un mayor número de raíces y estas fueron más largas, lo que concuerda con una de las funciones del calcio en la planta, que dice relación con que este tiende a reducir la permeabilidad de las raíces jóvenes, provocando la elongación de ellas y la de pelos radicales, aumentando la exploración del suelo (Sanz *et al.* 2001).

Al igual que lo observado por Monsalve *et al.* (2009) y Acevedo *et al*. (2020), en plantas de *E. globulus* fertilizadas con dosis creciente de nitrógeno, obtuvieron un número de raíces por sobre las 20 para el ensayo de potencial de crecimiento radical. En este estudio, se obtuvieron más de 40 raíces totales solo para las fertilizaciones realizadas con 300 mg L^{-1} de calcio. Esto denota un potencial de formación de raíces interesante para *A. punctatum*, a pesar de que la respuesta se manifiesta luego de un tiempo más prolongado comparado con *E. globulus*, ya que en esta última especie el potencial de crecimiento radical se ha evaluado al mes de establecerse el ensayo.

En general, en varias especies leñosas se pone de manifiesto que fertilizaciones con dosis crecientes de macronutrientes, generan valores superiores en términos de parámetros morfológicos, nutricionales y de potencial de crecimiento radical (Bustos *et al.* 2008, Monsalve *et al.* 2009, Oliet *et al.* 2016, Razaq *et al.* 2017), comportamiento observado de manera similar en el presente estudio. Además, fertilizaciones en la etapa de endurecimiento (otoñales) generan diversos atributos de calidad de planta relacionados principalmente con el aumento de reservas en las plantas que implican un desempeño en campo exitoso de las mismas (Boivin *et al*. 2004, Grossnickle y Macdonald 2017).

CONCLUSIONES

La fertilización con 300 mg L^{-1} de fósforo y calcio en la etapa de endurecimiento de plantas de *A. punctatum* no genera incrementos diferenciales significativos de parámetros morfológicos, salvo para la esclerofilia, en donde bajos niveles de calcio inducirían una mayor esclerofilia, atributo importante para plantas que deben ser establecidas en condiciones de restricción hídrica.

Dosis alta de fósforo (300 mg L^{-1}) durante la etapa de endurecimiento de plantas de *A. punctatum* producen las mayores concentraciones nutricionales foliares de nitrógeno y las menores de calcio. En cambio, dosis alta de calcio (300 mg L^{-1}) en la etapa de endurecimiento provocan mayores concentraciones nutricionales foliares del mismo elemento y las menores de potasio; y generan un mayor número de raíces y estas son más largas.

De acuerdo a lo experimentado, 22 °C es la temperatura de la zona radical que genera el mayor número de raíces nuevas y de mayor longitud en plantas de *A. punctatum*.

Concentraciones entre 150 y 300 mg L^{-1} de fósforo y calcio, complementadas con aportes de macronutrientes

como nitrógeno, potasio, magnesio y azufre, aplicadas a plantas de *A. punctatum* en la etapa de endurecimiento, generan plantas de un estado nutricional y vigor, superior que aquellas que omiten la aplicación de estos elementos en la etapa de endurecimiento.

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Performance rating on silvicultural activities of state forest enterprises using fuzzy TOPSIS and SAW methods

Calificación de desempeño en actividades silvícolas de empresas forestales estatales que utilizan métodos difusos TOPSIS y SAW

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SUMMARY

Silvicultural activities are the guiding practices carried out in the forest to achieve the objectives of forest enterprises. The suitability of these activities directly affects the level of achievement of the forest enterprise and thus the performance level. This article discusses how to evaluate the performance of silvicultural activities in forest enterprises. The study was carried out at the state forest enterprises belonging to Denizli forestry regional directorate in Turkey. Eight criteria were generated and also used in the study, which was related to cost, size, and amount of silvicultural activities, natural regeneration, regeneration care, thicket tending care, forest rehabilitation, artificial regeneration, cultivation care and number of staff. These criteria have been weighted by experienced forest engineers through the application of a well-structured surveying method. The criteria are weighted for they were not equally effective on the silvicultural performance of the enterprise. Forest enterprises are open to uncertainties because they work in natural environments, therefore criteria and data are defined as triangular fuzzy numbers. Study results show that the fuzzy technique for order preference by similarity to ideal solution and fuzzy simple additive weighting methods could be used to evaluate the performance of silvicultural activities of state forest enterprises. These methods have been successful in ranking enterprises from the best to the worst. Due to their advantages, these methods have the potential to be used to evaluate the performance of other forestry works.

Key words: silvicultural performance, forest management, Fuzzy TOPSIS, Fuzzy SAW, Turkey.

RESUMEN

Las actividades silvícolas son prácticas orientadoras que se efectúan en el bosque para alcanzar los objetivos de las empresas forestales. La idoneidad de estas actividades afecta directamente el nivel de logro de la empresa forestal y, por lo tanto, el nivel de rendimiento. Este artículo discute cómo evaluar el desempeño de actividades silvícolas en las empresas forestales. El estudio se realizó en las empresas forestales estatales de la Dirección Regional Forestal de Denizli en Turquía. Se generaron y usaron ocho criterios en este estudio relacionados con el costo, tamaño y cantidad de actividades silvícolas, regeneración natural, cuidado de regeneración, cuidado de matorrales, rehabilitación forestal, regeneración artificial, cuidado de cultivo y cantidad de personal. Estos criterios fueron ponderados por ingenieros forestales experimentados mediante la aplicación de un método de reconocimiento bien estructurado. Los criterios fueron ponderados, porque no fueron igualmente efectivos en el desempeño silvícola de la empresa. Las empresas forestales están abiertas a las incertidumbres, porque trabajan en entornos naturales, por lo que los criterios y los datos se definen como números difusos triangulares. Los resultados del estudio mostraron que la técnica difusa para la preferencia de orden por similitud con la solución ideal y los métodos simples de ponderación aditiva difusa podrían usarse para evaluar el desempeño de las actividades silvícolas de las empresas forestales estatales. Estos métodos han tenido éxito en clasificar a las empresas de mejor a peor. Debido a sus ventajas, estos métodos tienen el potencial de ser utilizados para evaluar el desempeño de otros trabajos forestales.

Palabras clave: desempeño silvícola, manejo forestal, Fuzzy TOPSIS, Fuzzy SAW, Turquía.

INTRODUCTION

Forestry is a multidisciplinary profession that consists of biological, technical, economic, social and administrative works to meet continuous society's demand for forest products and services (Helms 1998). As the local conditions are very variable and there are diversified sectorial objectives, forest enterprises have to meet the goals of the national forestry targets and those of the society's multiple demands. In this context, in forest resource management, silvicultural activities are gaining importance. As forestry enterprises have many objectives and very complex structure, the determination of the silvicultural performance level of these enterprises is very challenging.

Silvicultural practices can be designed for any management objective, such as timber, wildlife or biological

diversity (Meadows and Stunturf 1997). The primary objective of silvicultural activities is to achieve better wood quality. The silvicultural decisions are affected by the forest species, field conditions, species composition, species associations, diameter distribution and ecological characteristics of forests such as the age of slaughter, management objectives (FAO 2017). In Turkey, state forest enterprises are executing silvicultural activities and various supporting operations to conserve and tend the existing forested lands, utilize and regenerate them and also create new forest areas.

Turkey's forest growing stock is 1.66 billion cubic meters, 98.9 % of this is the high forest, and 1.1 % is the coppice forest. Therefore, 19.08 million cubic meters of industrial wood is produced annually from these forests managed and operated by the state. In Turkey in 2018, natural regeneration of 30,735 ha, artificial regeneration of 10,102 ha, tending (regeneration tending, release cutting and tending) of 463,159 ha, and the rehabilitation of 170,425 ha were made. A total investment of $ 43.78 million was made for these activities (GDF 2019). The activity of state forest enterprises is independent in terms of budget, staff, etc. The performance measurement of these enterprises is mostly made by comparing the activities of other forest enterprises operating under similar conditions (Geray 1982). Methods such as the data envelopment analysis in Kao (2000a), the financial statements analysis in Altunel Açıkgöz (2003), and the ratio analysis in Hajdúchová *et al.* (2017) were used for evaluating the performance of forest enterprises.

On the other hand, multi-criteria decision making (MCDM) techniques are widely used both in performance evaluations and in overcoming problems related to planning in forestry (Diaz-Balteiro and Romero 2008). MCDM techniques such as Analytical Hierarchy Process (AHP) (Yılmaz *et al.* 2004, Kaya and Kahraman 2011), Analytic Network Process (ANP) (Wolfslehner *et al.* 2005), ELimination Et Choix Traduisant la REalité (ELECTRE) (Pauwels *et al.* 2007), The Technique for Order Preference by Similarity to Ideal Solution (TOPSIS) (Stoms *et al.* 2009, Korkmaz and Gürer 2018), Simple Additive Weighting (SAW) (Yılmaz *et al.* 2011) and Data Envelopment Analysis (DEA) (Kao 2000b) have been commonly utilized for planning of forest resources in recent years.

At the evaluation stage of the MCDM method, it is assumed that values and weights of criteria were known exactly (Chu and Lin 2009). However, some preferences including information/knowledge such as human judgments were inadequate for modeling the real-life situations. Besides, preferences for types of data such as bounded data, interval data, ordinal data and fuzzy data cannot be assessed with exact numeric data (Jahanshaloo *et al.* 2006). In such environments, decisions are being given using the fuzzy logic system that is developed by Zadeh (Zadeh 1965). The uncertainty problems were investigated with interval-valued fuzzy data/sets (Zadeh 1965, Liu 2011), triangular membership function (Chen 2000, Sun 2010, Sagar *et al.* 2013) and trapezoidal membership functions (Liu 2011).

Because they work in open conditions, forest enterprises have to work with a large number of input and output variables that are uncertain. This study is aimed at evaluating the performance of state forest enterprises in Turkey, and since there are few studies on silvicultural performance, it has been decided to examine silvicultural performance. In this context, in literature, DEA (Zhang 2002), fuzzy DEA (Şafak *et al.* 2014), ELECTRE III (Pauwels *et al.* 2007), Stochastic Frontier Analysis (SFA) (Lien *et al.* 2007) and fuzzy TOPSIS (Prato and Paveglio 2014) techniques have been used to evaluate the performance of silvicultural activities. Shape, size, violence, financial burden, advantages of the silvicultural activities differ depending on to the geographical and economic structure of each enterprise. Therefore, it is important to perform a silvicultural performance assessment using MCDM methods that take into account fuzzy logic.

In this study, it was tested whether fuzzy TOPSIS and fuzzy SAW methodology are suitable to evaluate the silvicultural performance of forest enterprises. In this context, both internal and external evaluations of the state forest enterprises affiliated to the same top institution in terms of silvicultural aspects were made. For this purpose, it was aimed to i) show which criteria can be used based on silvicultural activities, ii) determine the weights of these criteria, iii) on a real-world scale by applying these criteria determine the performance of state forest enterprises in a forest region, and iv) evaluate the applicability of these methods within the scope of the silvicultural performance analysis.

METHODS

Study area. Turkey's forest assets are 22.62 million hectares, which covers 29 % of the surface area of the country. High forest corresponds to 94.70 % of these forests and 5.30 % are operated as the coppice forest. State forests in Turkey are divided into 28 forest regions. In addition, each region is divided into an average of nine forest enterprises and each enterprise into an average of five forest districts (GDF 2019). In this study, a performance evaluation was made on the scale of forest enterprises. Denizli Forestry Regional Directorate (DFRD) has been chosen as the study area due to its physical characteristics, being in the transition zone and performing many silviculture activities. This area in the western part of Turkey has divided Acıpayam, Çal, Çameli, Denizli, Eskere, Tavas and Uşak State Forest Enterprises.

Method. To evaluate the silvicultural performance of these forest enterprises between 2016-2018, the criteria set was firstly developed to determine which activities were important and symbolize silvicultural operations. The ac-

tivities of Denizli Forest Enterprise Directorate in the field of silviculture were taken into consideration in the creation of the criteria set. Expert forest engineers were used to determine the weights of these criteria. Afterwards, the fuzzy TOPSIS and fuzzy SAW methods were used to determine the silvicultural score of the highest enterprise. Which of these methods is effective was determined by producing a solution set according to both methods.

Developing criteria set. The following eight criteria were used to evaluate the silvicultural performance of these forest enterprises:

- Cost of silvicultural activities (x_1): It covers all of the cost of activities such as natural regeneration, regeneration tending, release cutting, forest rehabilitation, artificial regeneration and tending (as United States dollar ($)).
- The amount of natural regeneration area (x_2): It expresses the area (hectare) of natural regeneration that is achieved by a forest enterprise. Natural regeneration is a process by which woodlands are restocked by trees that develop from seeds that fall and germinate *in situ*.
- The amount of regeneration tending area (x_3): It expresses the area (hectare) of regeneration tending (natural growth) that is achieved by a forest enterprise. Regeneration tending is the maintenance process in young forest areas established by natural regeneration.
- The amount of release cutting area (x_4): It expresses the area (hectare) of release cutting that is achieved by a forest enterprise. Release cutting is the process of reducing the number of individuals (trees) in forest areas according to the objectives of the forest enterprise.
- The amount of forest rehabilitation area (x_5): It expresses the area (hectare) of forest rehabilitation that is achieved by a forest enterprise. Reforestation etc. silvicultural studies conducted in forests whose natural structure has been damaged for various reasons in the past.
- The amount of artificial regeneration area (x_6): It expresses the area (hectare) of artificial regeneration (reforestations) that is achieved by a forest enterprise. Artificial regeneration is generally the process of planting saplings grown in nurseries in forest areas.
- The amount of tending area (x_7): It expresses the area (hectare) of tending that is achieved by a forest enterprise. Tending is the maintenance process in young forest areas established by artificial regeneration.
- Number of staff (x_8): It expresses the total number of personnel (engineer, forest ranger, worker, etc.) in the forest enterprise. Since it is desired to have a low value in the performance evaluation of the variable of the number of staff, this variable has been normalized within the scope of cost minimization.

While these criteria show the performance of an enterprise, the expenses are expected to be the lowest and the other activities are expected to be the highest. In this context, in this study, cost minimization was taken into account in normalization of the variables of cost of silvicultural activities (x_1), and number of staff (x_8). Benefit maximization was performed in the normalization of other variables. In table 1, the data for the silvicultural performance of forest enterprises in 2016-2018 are seen.

Weighting the criteria set. An order of importance/priority was needed to find out which of the developed criteria set content was more dominant, effective or important in measuring silvicultural activities. For this; expert forest engineers -who have professional experience and can understand the technical, economic and ecological effects of forestry activities- were used. Contact was made with forest engineers (21 persons) who were considered to be in accordance with these characteristics and 14 of them voluntarily participated in this study. These expert forest engineers (14 forest engineers) consist of one forest enterprise manager, two deputy enterprise managers, three forest districts chiefs, three engineers of branch managers, four branch managers and one deputy regional director. The average working time of the expert forest engineers participating in the assessment was 17.93 years.

The expert forest engineers were asked to sort out which of the above eight criteria was more important and what the importance of the others should be. These importance data were used as weight data in Fuzzy TOPSIS and Fuzzy SAW methodology (table 2).

Fuzzy TOPSIS method. The TOPSIS method is one of MCDM methods which allows choosing among alternatives according to certain criteria. In the application of the TOPSIS method, there are some processes that numerically determine the relative importance of criteria and the performance of each alternative in terms of these criteria (Park *et al.* 2011). The method has been commonly used in recent years for solving MCDM problems. In this paper, the fuzzy TOPSIS method that was developed by Chen (2000) was used to evaluate the silvicultural performance of forest enterprises. This algorithm of fuzzy TOPSIS is explained below with general headings.

Step 1. Criteria and their weights are determined. In the determination of the importance level of each criterion were used linguistic values such as very good (0.9; 1; 1), good (0.7; 0.9; 1), medium good (0.5; 0.7; 0.9), fair (0.3; 0.5; 0.7), medium poor (0.1; 0.3; 0.5), poor (0; 0.1; 0.3) and very poor (0; 0; 0.1).

$$\widetilde{w} = [\widetilde{w}_1, \widetilde{w}_2, \ldots, \widetilde{w}_n]$$

Table 1. The silvicultural data of forest enterprises between 2016-2018 years.

Datos silvícolas de las empresas forestales entre los años 2016-2018.

Forest enterprises	x_1*			x_2			x_3			x_4		
	2016	2017	2018	2016	2017	2018	2016	2017	2018	2016	2017	2018
Acıpayam	258,502	284,834	345,163	238	375	186	1,150	1,429	1,485	3,415	2,513	2,475
Çal	332,538	263,592	360,451	239	90	207	550	704	677	1,549	2,690	2,090
Çameli	216,031	218,498	185,436	292	265	211	1,250	1,238	1,054	1,172	824	646
Denizli	429,681	383,707	649,896	147	195	190	751	756	930	3,095	4,051	4,676
Eskere	127,676	126,903	144,286	144	140	160	550	638	814	552	635	631
Tavas	310,419	269,357	287,774	217	276	284	1,250	1,154	1,225	2,972	3,595	3,310
Uşak	405,970	331,107	373,671	439	312	148	1,200	1,355	1,513	2,660	2,036	2,041
Forest enterprises	x_5			x_6			x_7			x_8		
	2016	2017	2018	2016	2017	2018	2016	2017	2018	2016	2017	2018
Acıpayam	145	121	306	80	103	183	1,000	1,181	691	80	79	77
Çal	1,811	290	271	109	64	91	1,500	1,313	1,360	75	75	73
Çameli	266	154	231	1	1	1	60	49	41	44	44	43
Denizli	1,112	557	1,329	176	127	501	2,200	1,820	2,086	172	171	167
Eskere	125	105	67	22	9	1	240	188	130	40	45	42
Tavas	275	258	323	143	76	92	700	589	701	69	65	61
Uşak	684	455	518	74	161	124	1,100	990	770	147	138	146

*The average exchange rates are 3.0213 $/TL in 2016, 3.6449 $/TL in 2017, and 4.8134 $/TL in 2018.

Table 2. Criteria weights.

Criterios de ponderación.

Criteria	Lower (L)	Median (M)	Upper (U)
x_1	0.9	1.0	1.0
x_2	0.7	0.9	1.0
x_3	0.5	0.7	0.9
x_4	0.5	0.7	0.9
x_5	0.3	0.5	0.7
x_6	0.3	0.5	0.7
x_7	0.5	0.7	0.9
x_8	0.3	0.5	0.7

Step 2. Fuzzy decision matrix ($\tilde{D}$) is formulated. In Formula 1, fuzzy ($\tilde{x}_{ij}$) variables refer to the silvicultural data of forest enterprises between 2016-2018.

$$\tilde{D} = \begin{bmatrix} \tilde{x}_{11} & \tilde{x}_{12} & \dots & \tilde{x}_{1n} \\ \tilde{x}_{11} & \tilde{x}_{22} & \dots & \tilde{x}_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ \tilde{x}_{m1} & \tilde{x}_{m2} & \dots & \tilde{x}_{mn} \end{bmatrix} \quad [1]$$

$$\tilde{x}_{ij} = (a_{ij}, b_{ij}, c_{ij}) \qquad i = 1,2,\dots,m; \;\; j = 1,2,\dots,n$$

Step 3. Normalized fuzzy decision matrix ($\tilde{R}$) is formulated.

$$\tilde{R} = [\tilde{r}_{ij}]_{m\times n} \quad [2]$$

$$\tilde{r}_{ij} = \left(\frac{a_{ij}}{c_j^*}, \frac{b_{ij,}}{c_j^*}, \frac{c_{ij}}{c_j^*}\right), \qquad j \in B;$$

$$\tilde{r}_{ij} = \left(\frac{a_j^-}{c_{ij}}, \frac{a_j^-}{b_{ij}}, \frac{a_j^-}{a_{ij}}\right), \qquad j \in C;$$

$$c_j^* = \max_i c_{ij} \qquad if\ j \in B;$$

$$a_j^- = \min_i a_{ij} \qquad if\ j \in C;$$

Step 4. Weighted normalized fuzzy decision matrix ($\tilde{V}$) is formulated.

$$\tilde{v}_{ij} = \tilde{r}_{ij} \times \tilde{w}_j \quad [3]$$

Step 5. The fuzzy positive-ideal solution (PIS) (A^*) and the fuzzy negative-ideal solution (NIS) (A^-) are formulated.

$$A^* = [(1;1;1);(1;1;1);\dots;(1;1;1)] \quad [4]$$

$$A^- = [(0;0;0);(0;0;0);\dots;(0;0;0)] \quad [5]$$

Step 6. Distances of each alternative from A^* and A^- are formulated.

$$d_i^* = \sum_{j=1}^{n} d(\tilde{v}_{ij}, \tilde{v}_j^*), \qquad i = 1,2,\dots,m \qquad [6]$$

$$d_i^- = \sum_{j=1}^{n} d(\tilde{v}_{ij}, \tilde{v}_j^-), \qquad i = 1,2,\dots,m \qquad [7]$$

Step 7. The closeness coefficients (CC_i) of each alternative are formulated.

$$CC_i = \frac{d_i^-}{d_i^* + d_i^-} \qquad i = 1,2,\dots,m \qquad [8]$$

Fuzzy SAW method. Fuzzy SAW method was used in addition to fuzzy TOPSIS to test whether the determined criteria set yields the same result in terms of performance. The SAW method is probably among the known and widely used method in MDMC. The method is based on the weighted average using the arithmetic mean (Abdullah and Rabiatul Adawiyah 2014). This method aims at selecting the alternative that provides the highest value (Ezquerro *et al.* 2016). Fuzzy SAW method is the combination of both fuzzy MDMC method and SAW method (Sagar *et al.* 2013).

The various steps of Fuzzy SAW method are presented as follows (Kao 2000b, Demircioğlu 2010).

Step 1. Choosing the criteria ($\mathbf{C_j}$) that will be used as a reference in decision-making.

Step 2. The suitable rating of the criteria weights was assigned in terms of linguistic variables by the experts.

Step 3. Equivalents of weights in terms of triangular fuzzy numbers are determined and a fuzzy weight matrix is defined. The elements of the fuzzy weight matrix are defuzzified. In formula (9), a_j, b_j, and c_j denote the elements of the triangular fuzzy numbers in the fuzzy linguistic weight matrix.

$$d(\tilde{w}_j) = \frac{1}{3}(a_j + b_j + c_j) \;;\; j = 1,2,\dots,n \qquad [9]$$

In formula 3, a, b, and c denote the elements of a triangular fuzzy number.

Step 4. After the defuzzified operation, the normalized values of the weights ($\mathrm{w_j}$) are obtained. Here the sum of the weights is equal to 1.

$$W_j = \frac{d(\tilde{w}_j)}{\sum_{j=1}^{n} d(\tilde{w}_j)} \;;\; j = 1,2,\dots,n \qquad [10]$$

$$\sum_{j=1}^{n} W_j = 1 \qquad [11]$$

Step 5. Minimization and maximization targets are taken into account, and the triangular fuzzy number equivalents of the factors are determined. Later, fuzzy decision matrix $(\tilde{D})$ is created. In formula (12), fuzzy ($\tilde{x}_{ij}$) variables refer to the silvicultural data of forest enterprises between 2016-2018.

$$\tilde{D} = \begin{bmatrix} \tilde{x}_{11} & \tilde{x}_{12} & \dots & \tilde{x}_{1n} \\ \tilde{x}_{11} & \tilde{x}_{22} & \dots & \tilde{x}_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ \tilde{x}_{m1} & \tilde{x}_{m2} & \dots & \tilde{x}_{mn} \end{bmatrix} \qquad [12]$$

$$\tilde{x}_{ij} = (a_{ij}, b_{ij}, c_{ij}) \qquad i = 1,2,\dots,m;\; j = 1,2,\dots,n$$

Step 6. Fuzzy decision matrix elements $(\tilde{D})$ are multiplied by factor weights $(\mathrm{w_j})$. The calculated matrix $(\mathrm{d}(\tilde{\mathrm{f}}_\mathrm{j}))$ is then defuzzified. As a result, the total results scores for each decision alternative were determined by the Fuzzy SAW method. In formula (14), r_j, s_j, and t_j denote the elements of the triangular fuzzy numbers in the calculated matrix.

$$\tilde{F} = \begin{bmatrix} \tilde{x}_{11} & \tilde{x}_{12} & \dots & \tilde{x}_{1n} \\ \tilde{x}_{11} & \tilde{x}_{22} & \dots & \tilde{x}_{2n} \\ \vdots & \vdots & \vdots & \vdots \\ \tilde{x}_{m1} & \tilde{x}_{m2} & \dots & \tilde{x}_{mn} \end{bmatrix} \otimes \begin{bmatrix} w_1 \\ w_2 \\ \vdots \\ w_n \end{bmatrix} = \begin{bmatrix} \tilde{f}_1 \\ \tilde{f}_{12} \\ \vdots \\ \tilde{f}_n \end{bmatrix} \qquad [13]$$

$$(d(\tilde{f}_j) = \frac{1}{3}(r_j + s_j + t_j) \;,\; j = 1,2,\dots,n)$$

Step 7. The total result values for each decision alternative are sorted from large to small. The alternative with the highest value is considered the highest priority alternative.

In this study, matrices in the Fuzzy TOPSIS and Fuzzy SAW methods are solved using Microsoft Office Excel 2016 according to the order of operation.

RESULTS

The data of the normalized fuzzy decision matrix (table 3), which was calculated by taking into consideration the criteria for the silvicultural performance of forest enterprises in 2016-2018, were used in both the fuzzy TOPSIS and the fuzzy SAW methods as the same.

In the analysis conducted within the scope of Fuzzy SAW, minimization and maximization targets are taken into account, and the triangular fuzzy number equivalents of the factors are determined. Later, a fuzzy decision matrix $(\tilde{D})$ is created. Fuzzy decision matrix elements $(\tilde{D})$ are multiplied by factor weights $(\mathrm{w_j})$. In this context, the normalized criteria weights presented in table 4 were used as factor weights $(\mathrm{w_j})$. The calculated matrix $(\mathrm{d}(\tilde{\mathrm{f}}_\mathrm{j}))$ is then defuzzified. As a result, in table 5, the total silvicultural performance scores for each forest enterprises were determined by fuzzy SAW method.

In the analysis conducted within the scope of Fuzzy TOPSIS, $\tilde{D}$ and $\tilde{R}$ matrices were created to evaluate the data of the silvicultural performance of the forest enter-

prises between 2016-2018. Later, $\tilde{V}$ matrix was formed. In this context, the weight values presented in table 2 were used in the weighted normalized fuzzy decision matrix ($\tilde{V}$). The distances of each alternative to the A^* and A^- were calculated separately with the vertex method. Calculated distance values d^* and d^- are expressed. In the final step, in table 5, the ranking order of silvicultural performance of forest enterprises was obtained. Closeness coefficients of each alternative were calculated with the equation (no 8). Thus, the ranking order of silvicultural performance of forest enterprises was obtained (table 5).

As a result (table 5), the total silvicultural performance scores for each forest enterprise were determined by fuzzy TOPSIS and fuzzy SAW method. As expected, both fuzzy TOPSIS and fuzzy SAW methods achieved the same results. In terms of silvicultural activities, Denizli forest enterprise was the best forest enterprise between 2016-2018. After Denizli forest enterprise the ranking order was Tavas, Acıpayam, Uşak, Çal, Çameli and Eskere respectively. In this period, Uşak forest enterprise has shown the worst performance in terms of silvicultural activities.

Table 3. Data of normalized fuzzy decision matrix used in fuzzy TOPSIS and fuzzy SAW methods.
Datos de la matriz de decisión difusa normalizada utilizada en los métodos difusos TOPSIS y SAW.

Forest enterprises	x_1			x_2			x_3			x_4		
	L	M	U	L	M	U	L	M	U	L	M	U
Acıpayam	0.418	0.446	0.494	0.542	0.655	1.000	0.920	0.981	1.000	0.529	0.620	1.000
Çal	0.384	0.400	0.481	0.240	0.544	0.729	0.440	0.447	0.493	0.447	0.454	0.664
Çameli	0.581	0.591	0.778	0.665	0.707	0.743	0.697	0.866	1.000	0.138	0.203	0.343
Denizli	0.222	0.297	0.331	0.335	0.520	0.669	0.529	0.601	0.615	0.906	1.000	1.000
Eskere	1.000	1.000	1.000	0.328	0.373	0.563	0.440	0.446	0.538	0.135	0.157	0.162
Tavas	0.411	0.471	0.501	0.494	0.736	1.000	0.808	0.810	1.000	0.708	0.870	0.887
Uşak	0.314	0.383	0.386	0.521	0.832	1.000	0.948	0.960	1.000	0.436	0.503	0.779
Forest enterprises	x_5			x_6			x_7			x_8		
	L	M	U	L	M	U	L	M	U	L	M	U
Acıpayam	0.080	0.217	0.230	0.365	0.455	0.640	0.331	0.455	0.649	0.500	0.545	0.557
Çal	0.204	0.521	1.000	0.182	0.398	0.619	0.652	0.682	0.721	0.533	0.575	0.587
Çameli	0.147	0.174	0.276	0.002	0.005	0.006	0.020	0.026	0.027	0.909	0.977	1.000
Denizli	0.614	1.000	1.000	0.789	1.000	1.000	1.000	1.000	1.000	0.233	0.251	0.257
Eskere	0.050	0.069	0.189	0.002	0.056	0.125	0.062	0.103	0.109	0.978	1.000	1.000
Tavas	0.152	0.243	0.463	0.184	0.472	0.813	0.318	0.324	0.336	0.580	0.677	0.689
Uşak	0.378	0.390	0.817	0.248	0.420	1.000	0.369	0.500	0.544	0.272	0.288	0.319

Table 4. Normalized criteria weights used in fuzzy SAW method.
Pesos de criterios normalizados utilizados en el método difuso SAW.

Criteria	Weight value
x_1	0.178
x_2	0.159
x_3	0.129
x_4	0.129
x_5	0.092
x_6	0.092
x_7	0.129
x_8	0.092

Table 5. Results of Fuzzy TOPSIS and Fuzzy SAW.
Resultados de Fuzzy TOPSIS y Fuzzy SAW.

Forest enterprises	Fuzzy TOPSIS		Fuzzy SAW	
	CC_n	Ranks	Final scores	Ranks
Acıpayam	0.398	3	0.586	3
Çal	0.345	5	0.512	5
Çameli	0.323	6	0.478	6
Denizli	0.448	1	0.648	1
Eskere	0.306	7	0.444	7
Tavas	0.402	2	0.592	2
Uşak	0.388	4	0.575	4

DISCUSSION

Forest enterprises are active to meet the forest products and services demands of human societies. Hörnfeldt and Ingemarson (2006) stated that the primary objectives of forest enterprises are to produce valuable products, to preserve biodiversity and to take into account public interests. During the fulfillment of duties and responsibilities, many decisions that have biological, technical, social, cultural, economic etc. aspects are taken by managers. During the planning and preparing of these decisions, multi-criteria techniques are usually used. The fuzzy TOPSIS and fuzzy SAW methods are the commonly used techniques in forestry.

In this study, the effectiveness of the decisions of seven forest enterprise directorates under similar conditions on silviculture is evaluated by fuzzy TOPSIS and fuzzy SAW methods. Thus, in terms of silvicultural activities, forest enterprises are ranked from the best to the worst. Forest enterprise directorates, which are in the last place (for example, Eskere ve Çameli), should take the example of the best forest enterprises (for example, Denizli and Tavas) to be more effective. This forest enterprise should plan its activities taking the example of the best forest enterprises.

The determination of the highest performance forest enterprise directorate is important in terms of giving an answer to some questions of interest groups about whether these enterprises are operating effectively. The interest groups demand that the taxes they pay be spent in appropriate activities by the state forest enterprise directorates, which is a public institution. Therefore, it is desirable for managers to control all the criteria used in performance measurements. However, forest resource managers may not have a direct impact on some fixed criteria such as "amount of forest area" (also some criteria in natural conditions) that affect performance. Such criteria affect the managerial ability of forest resource managers positively or negatively depending on the situation. For this reason, forest resource managers have to choose the most appropriate technology in the criteria (such as silviculture costs) that is correctly related to the work done to work effectively. Such appropriate decisions will increase the effectiveness of the managers and also forest enterprise directorates.

McKenney (2000) and FAO (2017) stated that the severity of silvicultural decisions affects topics such as the growth rate of forests, the quality of forests, the level of technology use, environmentally friendly practices, accessibility, marketing opportunities, product quality, management objectives and ownership. For this reason, forest resource managers have to choose among the combinations that provide the lowest costs, use the highest technology, take into account the most environmentally friendly practices and support rural development. This choice requires both to produce higher quality data and to use different multi-criteria decision-making techniques.

Silvicultural practices, such as natural generation or artificial regeneration, care of regeneration and cultivation, pre-commercial thinning care, rehabilitation practices and control of stand density affect both product quality and the sustainability of forest resources. Hörnfeldt and Ingemarson (2006) reported that suitable silvicultural practices help protect nature, while severe silvicultural practices increase soil erosion. As emphasized in this study, the variety and intensity of silvicultural activities directly affect the success of forest enterprises.

Nilsson *et al*. (2016) states that the decisions regarding forest resources must take into account not only economic aspects but also factors such as ecological and social values. In the analysis with multi-criteria decision-making techniques, the best silviculture plan decision is affected by targets, criteria and the weight or priority level of stakeholders. In this study, the criteria weighted by forest engineers were used to determine the forest enterprise directorates that gave the best silvicultural decision.

As with most of the multi-criteria decision-making techniques, the fuzzy TOPSIS and fuzzy SAW techniques are not desired to have too many variables. The number of variables should not be more than 9, which makes the human brain difficult to compare (Geray *et al*. 2007). For this reason, the number of variables was determined as 8 in this study. Similarly, the number of forest enterprise directorates whose performance will be measured is not desired to increase. As a matter of fact, the sum of the variable values of enterprises is converted to 1 by the normalization process. This process causes the variable values to be too close or equal due to decimal rounding in data close to each other. This situation reduces the separation ability of the fuzzy TOPSIS and fuzzy SAW techniques.

The method presented in this study has brought a useful approach to the comparison of forest enterprises that perform almost the same activity in line with the same criteria, and to determine the degree of success and ranking of success. This study for silviculture activities can be applied to other activities of forestry, and can be a guide for calculating the success of enterprises and determining the success rankings.

CONCLUSIONS

In this study, how silvicultural decisions taken by forest enterprises can be compared with MCDM techniques is discussed. In this context, the silvicultural decisions of seven state forest enterprises operating under similar conditions were compared with fuzzy MCDM techniques. For this purpose, Fuzzy TOPSIS and Fuzzy SAW methodology were used in this study. The same performance ranking was achieved in both methods.

In this study, eight criteria related to the cost of silvicultural activities, the amount of the natural regeneration area, the regeneration tending area, the release cutting area, the forest rehabilitation area, the artificial regenera-

tion area, the tending area and the number of staff were determined and these criteria were used. The results of the study show that fuzzy TOPSIS and fuzzy SAW methods can be used to evaluate the performance of silvicultural decisions of state forest enterprises with these criteria. These methods have been successful in ranking businesses from the best to the worst. Because of their advantages, these methods have the potential to be used to evaluate the performance of forest enterprises' decisions on other issues such as wood production, forest protection, wildfire fighting, biodiversity, recreation and clean water supply.

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Environmental factors variation in physiological aspects of *Erythroxylum pauferrense*

Variación de factores ambientales en los aspectos fisiológicos de *Erythroxylum pauferrense*

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SUMMARY

Ecophysiological studies are highly important to seek understanding of the plant-environment relationship. The objective of this work was to evaluate the influence of environmental factors on physiological aspects in young plants of *Erythroxylum pauferrense* throughout the day. A completely randomized design with 10 treatments and 4 replicates was used. Treatments were at different evaluation times throughout the day (8:00 h-17:00 h) with a 60-minute interval between them. The physiological variables evaluated were: photosynthesis rate, internal CO_2 concentration, transpiration, stomatal conductance, vapor pressure deficit, instantaneous water use efficiency, intrinsic water use efficiency, instantaneous carboxylation efficiency and leaf temperature. Internal and external temperature, relative humidity of the internal and external air of the greenhouse and photosynthetically active radiation were measured as climatic variables. The climatic variables presented correlations with the physiological parameters, with a strong association between photosynthetically active radiation and internal and external temperature of the greenhouse with the rate of photosynthesis, vapor pressure deficit, leaf temperature, transpiration, intrinsic efficiency of the use of water, stomatal conductance and instant water use efficiency. The influence of climatic factors on the physiological aspects in plants of *E. pauferrense* can be observed. The most suitable period of the day for conducting the physiological evaluations in species is between 11:00 h and 13:00 h.

Key words: gas exchange, guarda-orvalho, irradiance, photosynthesis.

RESUMEN

Los estudios ecofisiológicos son de gran importancia para buscar la comprensión de la relación planta-ambiente. El objetivo de este trabajo fue evaluar la influencia de los factores ambientales en los aspectos fisiológicos en plantas jóvenes de *Erythroxylum pauferrense* a lo largo del día. Se utilizó un diseño completamente al azar con 10 tratamientos y cuatro repeticiones. Los tratamientos fueron diferentes tiempos de evaluación a lo largo del día (8:00 h-17: 00 h) con un intervalo de 60 minutos entre ellos. Las variables fisiológicas evaluadas fueron: tasa de fotosíntesis, concentración interna de CO_2, transpiración, conductancia estomática, déficit de presión de vapor, eficiencia de uso de agua instantánea, eficiencia de uso de agua intrínseca, eficiencia de carboxilación instantánea y temperatura foliar. Para las variables climáticas se midió: temperatura interna y externa, humedad relativa del aire interno y externo del invernadero y la radiación fotosintéticamente activa. Las variables climáticas presentaron correlaciones con los parámetros fisiológicos, con una mayor asociación entre la radiación fotosintéticamente activa y la temperatura interna y externa del invernadero con la tasa de fotosíntesis, déficit de presión de vapor, temperatura de la hoja, transpiración, eficiencia intrínseca del uso del agua, conductancia estomática y eficiencia de uso instantáneo del agua. Se puede observar la influencia de los factores climáticos en los aspectos fisiológicos en plantas de *E. pauferrense*. El período del día más adecuado para realizar las evaluaciones fisiológicas en especies es entre las 11:00 h y las 13:00 h.

Palabras clave: intercambios de gas, protector de rocío, irradiancia, fotosíntesis.

INTRODUCTION

Popularly known as guarda-orvalho, *Erythroxylum pauferrense* Plowman is a species of understory, belonging to the family Erythroxylaceae, presenting a size of 1.5 to 4 m in height. It is found in the northeastern region of Brazil with a geographic distribution exclusively in the state of Paraíba, from where the specimen-type is derived, in the city of Areia (city), at an altitude varying between 500 and 660 m. Its occurrence is more frequent in forested environments in the

interior of these regions, known as Wetland Altitude (Loiola and Costa-Lima 2015). This species is of fundamental ecological importance for the regions, where the fruits serve to feed the local fauna, acting directly on the dispersion and propagation of seeds, in disturbed and threatened environments such as Wetland Altitude (Ribeiro *et al*. 2019).

Given the importance of the species, studies are needed to evaluate the effect of abiotic factors that influence the behavior and physiology of forest species. Among the main abiotic factors that affect the photosynthetic activity of plants are temperature, relative humidity and solar radiation (Taiz *et al*. 2017). These factors can be altered according to the different conditions of the environment, and for this reason can significantly modify the ecophysiological responses of the plants (Silva *et al*. 2010).

From the physiological point of view, plants respond rapidly to variations in solar radiation throughout the day. The luminous intensity mainly influences the process of opening and closing the stomata, controlling the absorption of CO_2 in the leaves, acting directly on the development and productivity of the plant (Kaiser and Paoletti 2014).

The effects of air temperature on the physiological aspects of plants can be classified as direct and indirect (Way and Oren 2010). The direct effect occurs in the processes of photosynthesis, providing changes in the activity of Rubisco and regeneration of the enzyme 1,5-ribulose-bisphosphate (RuBP) in the Calvin cycle (Kerbauy 2012, Duca 2015). The indirect effect is associated with the functioning of the stomata, which may lead to a reduction of water loss through transpiration.

In this context, gas exchange evaluations are highly important to assess the adaptation and stability of plants under certain environmental conditions, since this information will provide subsidies for other investigations that seek to understand the physiological behavior of *E. pauferrense* regarding climatic variation. The tested hypothesis is that the ecophysiological aspects of *E. pauferrense* change according to climatic variations at different times of the day, with a reduction in photosynthetic efficiency in the period of low temperature and less irradiance (photosynthetically active radiation).

Therefore, the objective of the present study was to evaluate the influence of environmental factors on physiological aspects of *E. pauferrense* plants throughout the day, and to indicate the best time for physiological evaluations.

METHODS

The experiment was conducted in a greenhouse, belonging to the Department of Plant Science and Environmental Sciences, Federal University of Paraíba (Campus II), located in Areia city, in the microregion of wetland and mesoregion of wild regions in Paraiba, state of Paraíba, Brazil (6°57'59" S and 35°42'57" W). The region presents variable altitude, between 400 and 600 m, with average temperature of 22 °C and precipitation around 1,400 mm (Ribeiro *et al.* 2018). The climate is tropical, classified according to Peel, Finlayson and McMahon (2007) as Aw', and it is warm and humid with autumn-winter rains.

The seeds of *E. pauferrense* were collected from mother plants in the Mata do Pau-Ferro State Park, Areia (city), state of Paraíba, Northeast Brazil (6°58'12" S and 35°42'15" W). For the production of the seedlings, the pulp was removed from the fruits and the seeds were exposed to running water for a period of 5 minutes. Subsequently, the seeds were disinfested with 2% sodium hypochlorite solution for five minutes before planting.

Planting was carried out in plastic containers with a capacity of 5 dm^3, using a substrate composed of vegetal soil and vermiculite in the proportion of 3:1. Samples of the substrate were collected for the analysis of soil chemical attributes found in table 1.

Three seeds per pot were used and the plants thinned after reaching 10 cm in height, selecting the uniform individuals. During the conduction of the experiment, the plants were irrigated daily and the water regime of the pots was maintained with pot capacity in 80 %, according to Souza *et al.* (2000).

A completely randomized design was used, with 10 treatments and five replicates. Each replicate was composed of three plants, totaling 15 seedlings. The treatments were constituted by different schedules of evaluation throughout the day (between 8:00 h and 17:00 h) with intervals of 60 minutes between them.

The physiological variables evaluated were the rate of photosynthesis (A) ($\mu mol\, m^{-2} s^{-1}$), internal CO_2 concentration (Ci) ($\mu mol\ mol^{-1}$), transpiration (E) (mmol of $H_2O\ m^{-2}\ s^{-1}$), stomatal conductance (gs) ($mol\ m^{-2}\ s^{-1}$), vapor pressure deficit (VPD) (kPa), instantaneous water use efficiency (EUA: A/E) [($\mu mol\ m^{-2}\ s^{-1}$) / (mmol of $H_2O\ m^{-2}\ s^{-1}$)], intrinsic water use efficiency (EiUA: A/gs) [($\mu mol\ m^{-2}\ s^{-1}$) /

Table 1. Chemical characteristics of the substrate used in the experiment.
Características químicas del sustrato utilizado en el experimento.

pH in H_2O	P	K	Na	H+Al	Al	Ca	Mg	BS	CEC	O.M.
	mg dm^{-3}		$cmol_c\ dm^{-3}$							g kg^{-1}
5.5	4.7	110.4	0.19	4.27	0.65	2.73	0.59	4.47	5.48	29.86

BS: base sum; CEC: cation exchange capacity; O.M: organic matter.

(mol m^{-2} s^{-1})], instantaneous carboxylation efficiency (EiC: A/Ci) [(µmol m^{-2} s^{-1}) / (µmol mol^{-1})] and leaf temperature (Tleaf) (°C). The evaluations were done on healthy, undifferentiated and fully expanded leaves located in the middle third of the plants, using a portable infrared gas analyzer (IRGA) (Licor, model Li-6400XT). The protocol for IRGA measurements was: 6 cm² leaf chamber with coupled natural light sensor, air humidity between 50-60 %, air flow of 300 µmol s^{-1} and 400 µmol mol^{-1} of atmospheric CO_2.

Regarding climatic variables, internal and external temperature (Tin and Tex) (°C), and the relative humidity of the internal and external air (RHin and RHex) (%) of the greenhouse were evaluated using an integrated thermo-hygrometer digital sensor (Hygrotherm). For the determination of the photosynthetically active radiation (PAR) (µmol m^{-2} s^{-1}), a quantum sensor coupled to IRGA (Li-6400XT) was used.

Measurements were performed 255 days after emergence, under full daylight conditions (zero cloudiness), thus allowing the real effect of the climatic parameters of the environment on the physiological aspects of the plants.

Data were submitted to a canonical correlation analysis (CCA) and principal components analysis (PCA), to observe the associations between climatic (PAR, Tin, Tex, RHin and RHex) and physiological (A, gs, E, Ci, VPD, WUE, iWUE, ICE and Tleaf) variables. The significance of the canonical roots together was analyzed from Wilks' Lambda multivariate test of significance (approximation of the F distribution). These statistical analyses were carried out using SAS® 9.3.5 software (Cody 2015).

RESULTS

Regarding the Wilks's Lambda significance test, it was found that the physiological variables evaluated showed correlations with the environmental variables through the CCA, in which the first and second canonical pair were highly significant, presenting R^2 of 0.98 and 0.96, respectively (table 2).

In relation to the first canonical pair (R^2 = 0.98), it is observed that the most important climatic variables were photosynthetically active radiation and internal and external temperature of the greenhouse (cc of 0.82, 0.72 and 0.70, respectively), presenting positive correlations with the rate of photosynthesis (cc = 0.89), vapor pressure deficit (cc = 0.85), leaf temperature (cc = 0.78), transpiration (cc = 0.76), intrinsic water use efficiency (cc = 0.71), stomatal conductance (cc = 0.69) and instantaneous water use efficiency (cc = 0.55) (table 3).

According to the analysis of principal components (PCA), it is observed that the dimensions of the two compo-

Table 3. Canonic correlations and canonical pair between climate and physiological variables.

Correlaciones canónicas y pares canónicos entre el clima y las variables fisiológicas.

Variables	Canonical pair
Climate	
Photosynthetically active radiation (PAR)	0.82
Internal temperature (Tin)	0.72
External temperature (Tex)	0.70
Relative humidity - internal (RHin)	- 0.46
Relative humidity - external (RHex)	- 0.48
Physiological	
Rate of photosynthesis (A)	0.89
Stomatal conductance (gs)	0.69
Transpiration (E)	0.76
Internal CO_2 concentration (Ci)	-0.53
Vapor pressure deficit (VPD)	0.85
Instantaneous water use efficiency (WUE)	0.55
Intrinsic water use efficiency (iWUE)	0.71
Instantaneous carboxylation efficiency (ICE)	0.45
Leaf temperature (Tleaf)	0.78
R^2	0.98

Table 2. Wilks's Lambda multivariate test (F distribution approximation).

Prueba multivariada Lambda de Wilks (aproximación de distribución F).

Canonical function	R^2	Fa	GL1	GL2	*P*-value
1	0.98	15.602	45	208.8	< 0.0001
2	0.96	7.624	32	174.9	< 0.0001
3	0.55	1.627	21	138.3	0.051
4	0.45	1.175	12	98.0	0.312
5	0.18	0.347	5	50.0	0.882

Fa: approximate F value; GL_1: degrees of freedom regarding treatments; GL_2: degrees of freedom regarding error.

nents (axes), with 60.23 in the first and 23.25 in the second, concentrated 83.48 % of the total variability of data (figure 1). Thus, it was possible to register strong correlations among rate of photosynthesis, stomatal conductance, transpiration, intrinsic water use efficiency, instantaneous water use efficiency and instantaneous carboxylation efficiency with photosynthetically active radiation and between vapor pressure deficit and leaf temperature with the internal and external temperature of the environment (figure 1). Along the first axis (PC1), the values of the eigenvectors of the physiological variables, except for Ci, are distributed in the most extreme portion to the right (with positive values), while RHin and RHex are arranged in the left portion (with negative values), thus evidencing the separation of these variables and the others evaluated (figure 1).

Regarding the recorded climatic variations, it was observed that the photosynthetically active radiation (PAR) presented an expressive increase throughout the day, reaching values ranging from 32.75 to 1570.72, in the period between 11 h and 17 h. It was verified that the maximum internal and external temperature (Tin and Tex) had the highest values between 13 h and 15 h, reaching values of 39.7, 39.9 and 38.7 °C inside the greenhouse and 37.7, 38.9 and 36.7 °C in the external environment (figure 2).

The relative humidity of the air inside and outside the greenhouse showed decrease during the day. The highest values were recorded at 8 h (66 % in both locations) and the lowest values at 13 h (28 and 31 %, respectively) (figure 2).

Rate of photosynthesis (A) showed a rapid increase along with photosynthetically active radiation (PAR) in the first hours of the day, reaching the maximum values at 11 h and 12 h (5.23 and 5.19 μmol m^{-2} s^{-1}, respectively), and declining at 13 h (4.46 μmol m^{-2} s^{-1}), with the lowest values that were recorded at 17 h (0.73 μmol m^{-2} s^{-1}), when PAR and temperature were also with very low values (figure 3A). The values of stomatal conductance (gs) showed a behavior similar to that of the photosynthesis rate, increasing gradually during the day, with a considerable decline at 13 h, with values varying between 0.0662 and 0.0223 mol m^{-2} s^{-1}, recorded at 11h and 17 h, respectively (figure 3B).

The values of transpiration (E) presented the same tendency as rate of photosynthesis and stomatal conductance, with values varying between 3.2854 mmol m^{-2} s^{-1} (12 h) and 0.7958 mmol m^{-2} s^{-1} (17 h) (figure 3C).

The internal CO_2 concentration (Ci) showed an inverse trend in relation to the other variables, with the highest recorded values of late afternoon at 17 h (figure 3D). Ci values ranged from 221.36 μmol mol^{-1} (12 h) to 320.71 μmol mol^{-1} (17 h) (figure 3D).

The instantaneous efficiency of water use (WUE) showed considerable reduction during the day. The reduction between the highest and lowest value found was of 59 %, with higher values between 8 h and 10 h with 2.37 and 2.13 [(μmol m^{-2} s^{-1}) (mmol m^{-2} s^{-1})$^{-1}$], and the lowest values observed at 17 h with 0.97 [(μmol m^{-2} s^{-1}) (mmol m^{-2} s^{-1})$^{-1}$] (figure 4A).

The intrinsic efficiency of water use (iWUE) recorded maximum values at 12 h and 13 h, with 85.39 and 82.82 [(μmol m^{-2} s^{-1}) (mol m^{-2} s^{-1})$^{-1}$]. The lowest values were observed at 17 h, with 34.97 [(μmol m^{-2} s^{-1}) (mol m^{-2} s^{-1})$^{-1}$] (figure 4B).

The instantaneous efficiency of carboxylation (ICE) showed increasing behavior throughout the day, with the highest values recorded at 11h and 12h, with 0.0192 and 0.0238 [(μmol m^{-2} s^{-1}) (μmol mol^{-1})$^{-1}$]. The lowest values were observed in the period of lowest irradiance at 17 h, with 0.0022 [(μmol m^{-2} s^{-1}) (μmol mol^{-1})$^{-1}$] (figure 4C).

The vapor pressure deficit (VPD) showed the same trend as Tleaf, with maximum values recorded at 12 h and 13 h, reaching 2.00 and 2.19 kPa (figure 4D). Leaf temperature (ºC) increased gradually during the day, presenting values that varied from 27.70 to 40.07 °C, registered in the period between 7 h and 13 h (figure 4E). The lowest values of Tleaf and VPD were found in periods with low light and temperature availability (7 h and 17 h) (figure 4D and 4E).

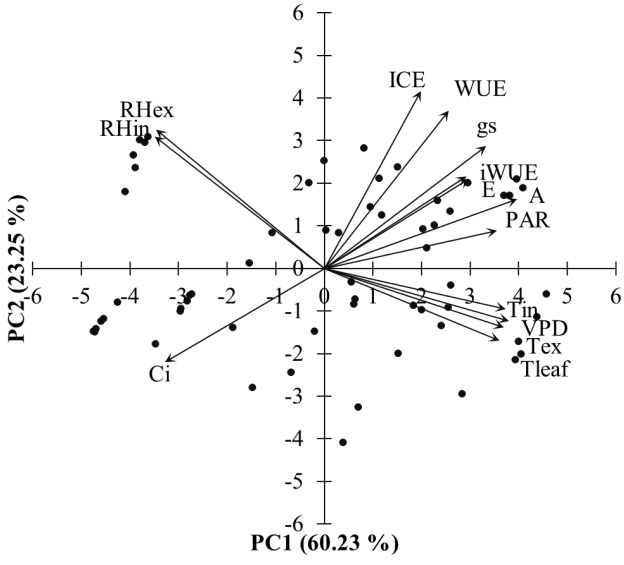

Figure 1. Principal Component Analysis (PC1 and PC2) among the climatic and physiological variables in plants of *E. pauferrense*.

Análisis de componentes principales (PC1 y PC2) entre las variables climáticas y fisiológicas en plantas de *E. pauferrense*.

DISCUSSION

The results obtained through the canonical correlation analysis (CCA) is indicative of the influence of climatic factors on the physiological characteristics of *E. pauferrense* along the day. According to Hair *et al*. (2009), the higher the canonical coefficient (cc), the more important is the variable in the group (climatic and physiological). By means of the positive correlations between the different groups of variables, the influence of PAR and temperature

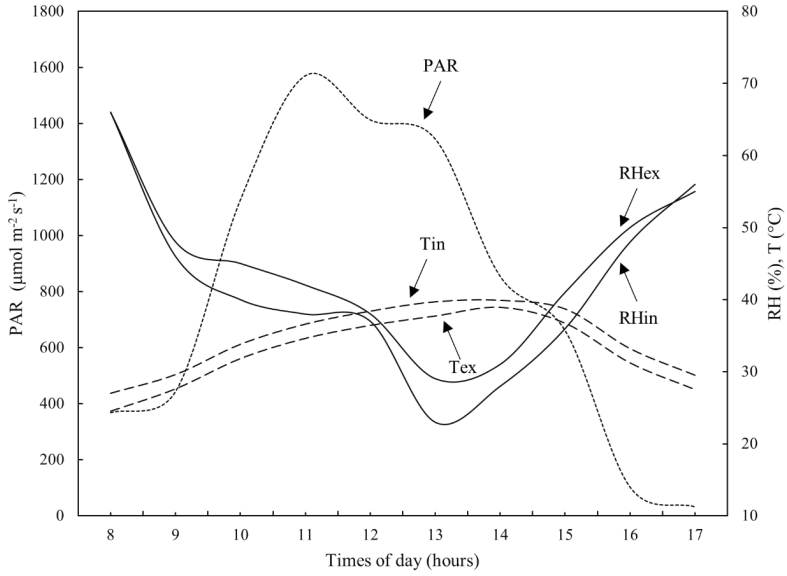

Figure 2. Photosynthetically active radiation (PAR), internal (Tin) and external (Tex) temperature, and internal (RHin) and external (RHex) relative humidity in greenhouse during the conduction of the experiment.

Radiación fotosintéticamente activa (PAR), temperatura interna (Tin) y externa (Tex), y humedad relativa interna (RHin) y externa (RHex) en invernadero durante la conducción del experimento.

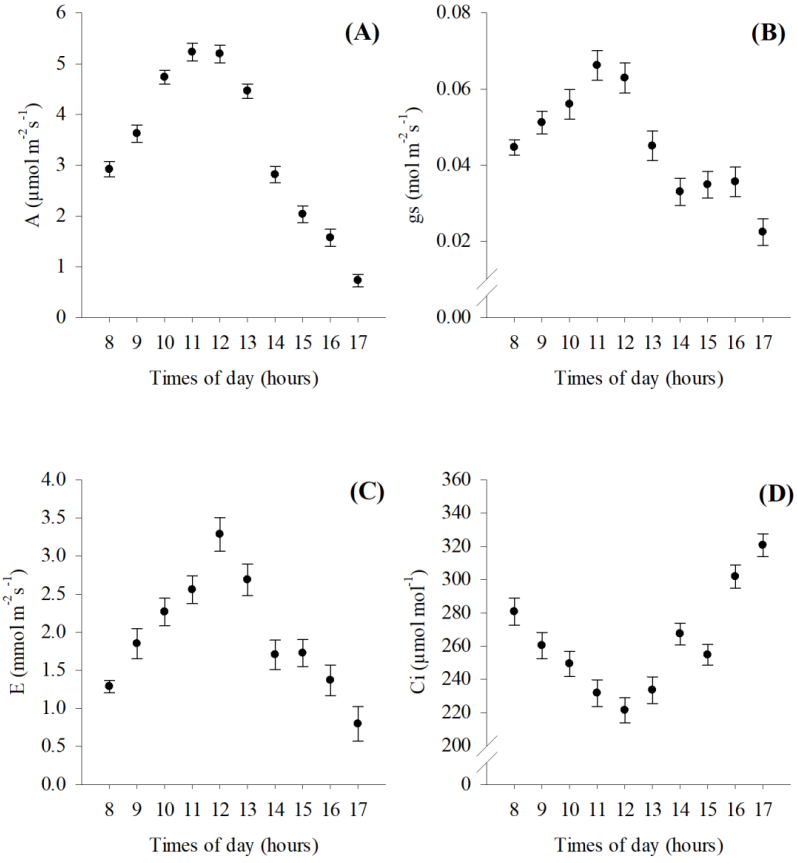

Figure 3. Mean values of rate of photosynthesis (A) (A), stomatal conductance (gs) (B), transpiration (E) (C) and internal CO_2 concentration (D) in plants of *E. pauferrense* as a function of time of the day.

Valores medios de la tasa de fotosíntesis (A) (A), conductancia estomática (gs) (B), transpiración (E) (C) y concentración interna de CO_2 (D) en plantas de *E. pauferrense* en función de hora del día.

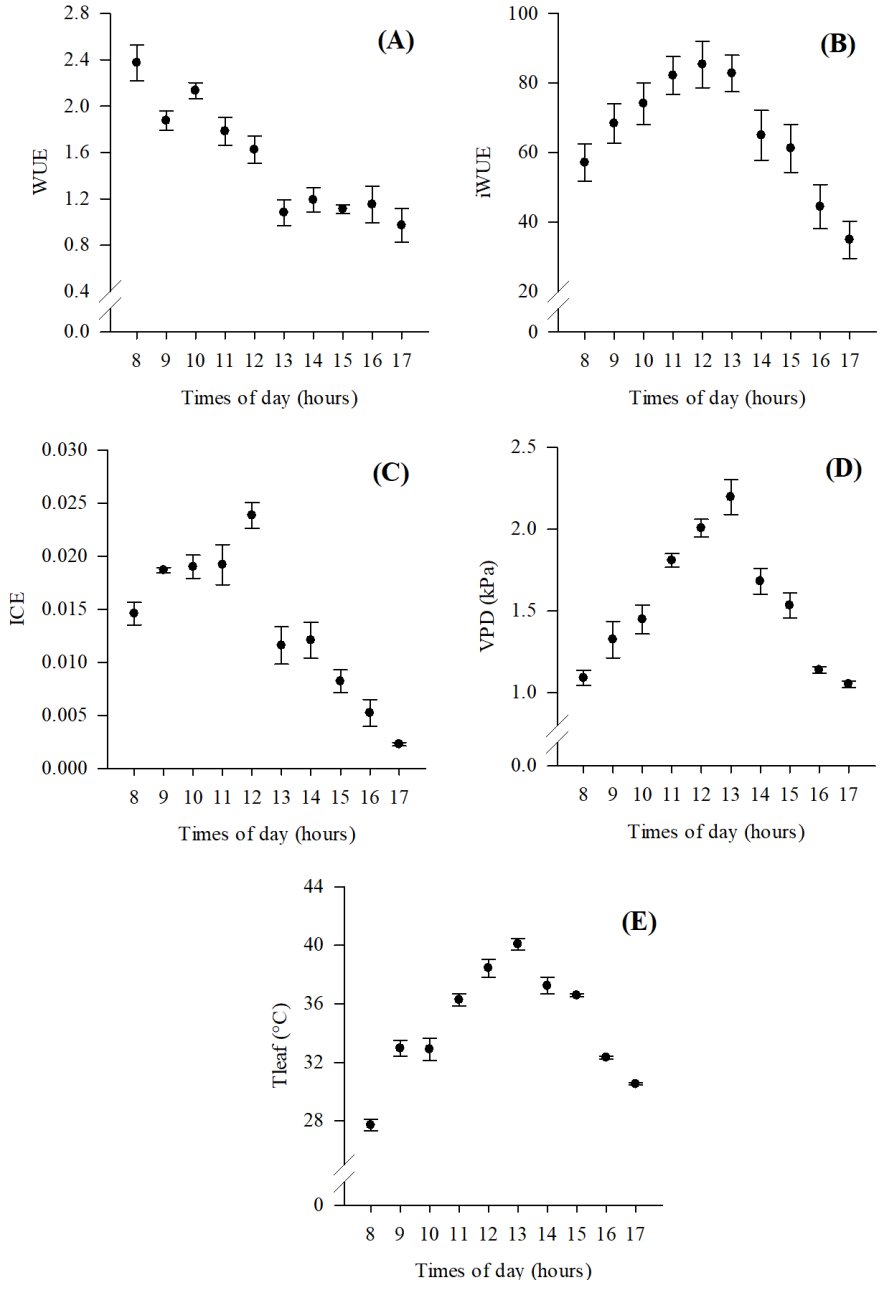

Figure 4. Mean values of instantaneous water use efficiency (WUE) (A), intrinsic water use efficiency (iWUE) (B), instantaneous carboxylation efficiency (ICE) (C), vapor pressure deficit (VDP) (D) and leaf temperature (Tleaf) (E) in plants of *E. pauferrense* as a function of time of the day.

Valores medios de eficiencia de uso de agua instantánea (WUE) (A), eficiencia de uso de agua intrínseca (iWUE) (B), eficiencia de carboxilación instantánea (ICE) (C), déficit de presión de vapor (VDP) (d) y temperatura de la hoja (Tleaf) (e) en plantas de *E. pauferrense* en función de la hora del día.

(Tin and Tex) on physiological aspects in young plants of *E. pauferr*ense is evidenced throughout the day (Kerbauy 2012, Taiz *et al*. 2017).

Jadoski *et al*. (2005) in their study found similar values in young *Capsicum annuum* L. plants during the day, with maximum PAR (photosynthetically active radiation) values recorded between 11:30h and 13:30h and maximum temperatures between 13:30 h and 15:30 h. This decrease observed in the relative humidity of the air is due to the high values recorded in the photosynthetically active radiation and the air temperature, throughout the day (Dalmago *et al*. 2006).

The highest values of A and g were recorded at similar times in the hottest period of the day (> PAR; > Tin

and Tex), confirming the direct relationship between these variables (Dias and Marenco 2007). Such climatic conditions, possibly, provided superior CO_2 entry in the leaves, directly influencing the physiological processes in the photosynthetic apparatus of the plants (Baroli *et al*. 2008). The low values of these variables at 08 h and 17 h are related to the stomatal closure, due to the fact that plants are not under ideal conditions of irradiation and temperature, thus reducing the efficiency in the CO_2 fixation process (Kerbauy 2012).

The closure of stomata at the end of the afternoon may happen due to the strategy of the plants to maintain the turgescence of the leaves during the respiratory period (Marenco and Lopes 2007, Taiz *et al*. 2017). In *Aleurites fordii* plants, Caron *et al*. (2017) observed similar behavior in the transpiration curves, in which the transpiratory rate decreased with the stomatal closure.

Regarding Ci, Dalastra *et al*. (2014) found different results from those of the present study, in which plants submitted to favorable conditions present high concentrations of CO_2 and higher photosynthetic rate, and as concentration decreased, photosynthesis became limited.

WUE is associated with water saving by the plants, that was measured from the relationship between the rate of photosynthesis and transpiration, in which the measured values relate the amount of carbon gained by the plant, per unit of water lost (Jaimez *et al*. 2005). According to Shimazaki *et al*. (2007) the assimilation of CO_2 from the environment causes the loss of water, and the reduction of this loss reduces the entry of CO_2 into the plants. Therefore, the gradual reduction of WUE throughout the day may be associated with observed increases in the rate of photosynthesis and transpiration in young plants of *E. pauferrense*.

The increase in iWUE throughout the day is a reflection of the increases in the rate of photosynthesis and stomatal conductance, indicating that high A values associated with the increase of gs provide an increase in the intrinsic efficiency of water use (Wieser *et al*. 2018).

ICE is a variable that allows analyzing nonstomatic factors that interfere with the photosynthesis rate of plants (Ferraz *et al*. 2012). For Machado, Schmidt, Medina and Ribeiro (2005), ICE has little relation with the internal concentration of CO_2 and with the rate of photosynthesis. Considering the behavior of ICE throughout the day in the present work (figure 4C), it is possible to observe that in addition to stomatal factors, such as WUE and iWUE, nonstomatic factors were affected by the climatic parameters of the environment, due to irradiance and temperature, causing the absence of ATP (anaerobic glycolysis) and NADPH (Nicotinamide adenine dinucleotide) derived from the electron transport chain of photosystem II (PSII) (Silva *et al*. 2015). According to this author, to have the photosynthetic process, ICE depends on the amount of light, temperature, CO_2 availability in the leaf mesophyll and the enzymatic activity.

The increase of Tleaf and VPD in the hottest period of the day (12 h and 13 h) (> PAR; > Tin and Tex) may be associated with a larger stomatal opening, promoting a higher flow of gas exchange in the leaves (Souza *et al*. 2016). The association between these variables can be observed in figures 4D and 4E; a fact that happens because the PVD depends on both the temperature and humidity of the air as well as the leaf temperature (Marenco and Lopes 2005, Taiz *et al*. 2017).

Regarding the influence of climatic factors on physiological parameters of *E. pauferrense* from the CCA and PCA, it was verified that photosynthetically active radiation (PAR) and temperature (Tin and Tex) had stronger influence on the rate of photosynthesis, vapor pressure deficit, transpiration, leaf temperature, intrinsic water use efficiency, stomatal conductance and efficiency instant carboxylation.

Erythroxylum pauferrense presented oscillations in the physiological aspects evaluated as a function of time of day and climatic conditions of the environment.

Low temperatures and less irradiance provided lower physiological development of *E. pauferrense* plants during the day.

The most suitable period of the day to carry out the physiological evaluations in young plants of *E. pauferrense* is between 11 h and 13 h.

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Valoración económica del secuestro y almacenamiento de carbono en la puna seca del suroeste del Perú

Economic valuation of carbon capture and storage in the puna dry of southwestern Peru

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SUMMARY

In the face of a global warming scenario, carbon capture and storage has become an international strategy to mitigate the effect of greenhouse gases. Oceans and Tropical forests are considered the largest carbon sinks in the world, however a few studies have evaluated the relative importance of other plant formations as carbon sinks in the Neotropical Region. This study documents the economic value of carbon capture and storage by five plant formations representative in the Puna Seca of southwestern Peru. Sampling plots were established to estimate the biomass contained in seedlings of Pajonal, Tolar, Bofedal, Yaretal and Queñual, in Salinas y Aguada Blanca National Reserve (Arequipa, Peru). Later, carbon stored and carbon captured as CO_2 was estimated. The Peruvian average carbon exchange price per ton of CO_2 was used for the economic evaluation of environmental services. It was found that the Reserve contains at least 13,507,104.16 equivalent metric tons of CO_2 worth U$D 86,310,395.58. Bofedal was the main vegetal formation that contributed in these values (52.48 %), in spite of being little represented in the total area of the Reserve (2.58 %). It is expected that the economic value obtained will serve as a frame of reference in decision-making for various purposes (environmental awareness, environmental management instruments, national accounting, among others).

Key words: Arequipa, biomass, CO_2 sink, ecosystem service, Salinas y Aguada Blanca National Reserve.

RESUMEN

Ante un panorama de calentamiento global, la captura y secuestro de carbono se ha convertido en una estrategia internacional para mitigar el efecto de los gases invernadero. Los océanos y los bosques tropicales son considerados como los mayores sumideros de carbono en el mundo, sin embargo, pocos estudios han evaluado la importancia relativa de otras formaciones vegetales como sumideros de carbono en la región Neotropical. El presente estudio documenta el valor económico de la captura y secuestro de carbono por cinco formaciones vegetales representativas de la Puna Seca del suroeste del Perú. Se establecieron parcelas de muestreo para estimar la biomasa contenida en plántulas del Pajonal, Tolar, Bofedal, Yaretal y Queñual, en la Reserva Nacional Salinas y Aguada Blanca (Arequipa, Perú), posteriormente, se estimó el carbono almacenado y el carbono capturado como CO_2. Se utilizó el precio promedio de la tonelada de CO_2 equivalente, establecido en el mercado peruano, para obtener el valor económico del servicio ambiental. Como resultados, se observó que la Reserva contiene al menos 13.507.104,16 toneladas métricas de CO_2 equivalente, valoradas en U$D 86.310.395,58. El Bofedal fue la principal formación vegetal que contribuyó en dichos valores (52,48 %), a pesar de estar poco representado en la superficie total de la Reserva (2,58 %). Se espera que el valor económico obtenido sirva de marco de referencia en la toma de decisiones para fines diversos (concientización ambiental, instrumentos de gestión ambiental, contabilidad nacional, entre otros).

Palabras clave: Arequipa, biomasa, Reserva Nacional de Salinas y Aguada Blanca, servicio ecosistémico, sumidero de CO_2.

INTRODUCCIÓN

Uno de los problemas ambientales más severos al que nos enfrentamos en el presente siglo es el Cambio Climático, el cual se debe al incremento en las emisiones antropogénicas de gases de efecto invernadero, tales como dióxido de carbono (CO_2), monóxido de carbono, clorofluorocarbonados, óxidos de nitrógeno y metano, siendo el CO_2 uno de los gases más importantes por las grandes cantidades en las que se emiten a la atmósfera (Phillips *et al.* 2017). La preocupación mundial por mitigar el efecto de dichos gases ha dado lugar a una política internacional dirigida a entender los procesos de generación y absorción de ellos, buscando mantener el aumento

de la temperatura media mundial muy por debajo de 2 °C con respecto a los niveles preindustriales, y proseguir los esfuerzos para limitar ese aumento de la temperatura a 1,5 °C con respecto a los niveles preindustriales (Trinidad y Ortiz 2019). Esto ha permitido reconocer la importancia de los ecosistemas terrestres y, en particular, el papel que tiene la vegetación para captar el CO_2 atmosférico por medio de la fotosíntesis, incorporándolo a las estructuras vegetales, y mitigando a largo plazo el cambio climático (Sullivan *et al.* 2017).

La medición y el monitoreo de carbono en ecosistemas de alta montaña se ha tornado un importante tópico de investigación en los años recientes, siendo importante su cuantificación para disponer de datos empíricos en las negociaciones para reducir las emisiones de gases efecto invernadero asociadas a deforestación y degradación forestal (REDD+). La fijación de un precio al carbono viene siendo una política climática cada vez más prioritaria en los países de América Latina (Trinidad y Ortiz 2019). El Perú, como país miembro de la Convención Marco de las Naciones Unidas para el Cambio Climático y comprometido con la reducción gases de efecto invernadero a través del Protocolo de Kioto, ha adoptado una serie de instrumentos económicos y de mercado (Bonos de carbono) para mitigar las emisiones de gases de efecto invernadero (Phillips *et al.* 2017).

A nivel regional, el Perú se encuentra en una categoría de alto riesgo a los impactos asociados al cambio climático (Trinidad y Ortiz 2019). Dicha ubicación es de singular importancia debido a los procesos de deforestación, tala ilegal, cambio en el uso de suelo e incendios forestales vienen originado cinco puntos focales (Hotspot) de deforestación en la Amazonía del Perú, ocasionando la pérdida de 59 millones de toneladas métricas de carbono entre 2013 a 2017 (Martel y Cairampoma 2012).

Estudios sobre la valoración del servicio ecosistémico de captura y almacenamiento de CO_2 en Perú han sido desarrollados principalmente en el Amazonía (Glave y Pizarro 2001, Martel y Cairampoma 2012). En cambio, estudios en regiones altoandinas son escasos, los cuales han sido realizados de forma particular en formaciones vegetales como Pajonal, Bofedal o Queñual, haciendo difícil la incorporación de su valor económico en la toma de decisiones para el manejo de recursos naturales altoandinos (Glave y Pizarro 2001, Gibbon *et al.* 2010, Medrano *et al.* 2012, Vásquez *et al.* 2014, Crispin 2015, Morales 2015, Limache 2016, Flores 2017, Sarcca 2017).

Por consiguiente, la pregunta de investigación que fundamenta este estudio es cuál es la importancia relativa del Pajonal, Tolar, Bofedal, Yaretal y Queñual en la puna seca del suroeste del Perú, a base del valor económico que representan por el servicio de secuestro y almacenamiento de CO_2. Se planteó como objetivo estimar el valor económico asociado al secuestro y almacenamiento de CO_2 en los principales tipos de vegetación de la Reserva Nacional Salinas y Aguada Blanca (Arequipa, Perú), considerando como hipótesis que el valor económico del servicio ecosistémico debería variar según la formación vegetal y la superficie que esta ocupa en la RNSAB. Para cumplir con lo propuesto, se estimó la biomasa y carbono capturado en parcelas de muestreo y se utilizó el método de precio de mercado, aplicando el valor asignado por tonelada de CO_2 equivalente implementado en el Estado Peruano.

MÉTODOS

Área de estudio. La Reserva Nacional de Salinas y Aguada Blanca (RNSAB) es una de las principales Áreas Naturales Protegidas del Perú que resguarda una muestra representativa de la puna seca en el suroeste del país. Se ubica en los departamentos de Arequipa y Moquegua, ocupando un área de 366.936 ha, en un rango altitudinal que va de los 2.800 a más de 6.000 m de altitud (figura 1).

El clima de toda la RNSAB está dominado por dos condiciones, la altura y la sequedad, que le confieren un clima de montaña notablemente árido. La precipitación en el área varía entre 80 a 1.000 mm, presentándose marcada estacionalidad lluviosa, mayormente restringida a los meses de verano, con sequías frecuentes. Hacia el oeste de la Reserva llueve menos de 250 mm, por lo que se le podría considerar como desértico, mientras que la mayor parte del área es semidesértica. La fuerte insolación es característica de ambientes montañosos. La humedad relativa es el principal factor que limita la distribución de las plantas y los animales, llegando a alcanzar un 60% en promedio. La temperatura promedio en la zona varía entre los 2 y 8°C, con mínimas de hasta -10°C (Zeballos *et al.* 2010).

Desde el punto de vista geomorfológico, es una planicie elevada (meseta), por sobre la cual se encuentran estructuras volcánicas que son parte de la cordillera volcánica del sur peruano. La geomorfología actual del área es el resultado del efecto combinado de fuerzas endógenas y exógenas que se desarrollaron durante el terciario y el cuaternario gracias a procesos morfodinámicos que en sus fases agradacionales corresponden al dimorfismo vertical y al vulcanismo Plio-Pleistoceno, y en sus fases gradacionales a la acción erosiva de períodos pluvio glaciales (Zeballos *et al.* 2010).

La flora de la RNASB consta de más de 463 especies de plantas vasculares, siendo las Asteraceae y Poaceae las familias con mayor número de especies; mientras que, los vertebrados suman 207 especies, conformados por 37 mamíferos (34 nativos y tres introducidos en estado silvestre), 158 aves, cinco reptiles, cuatro anfibios y tres peces (dos nativos y uno introducido) (Zeballos *et al.* 2010).

Las principales formaciones vegetales que ocurren en la RNSAB, según su representatividad y estado de conservación, son: Pajonal, dominado por *Stipa* spp. Linnaeus y *Festuca orthophylla* Pilg. (con una superficie de 317.134,84 ha); Tolar, dominado por *Parastrephia* spp. Nutt. y *Baccharis* spp. Linnaeus (48.623,80 ha); Bofedal,

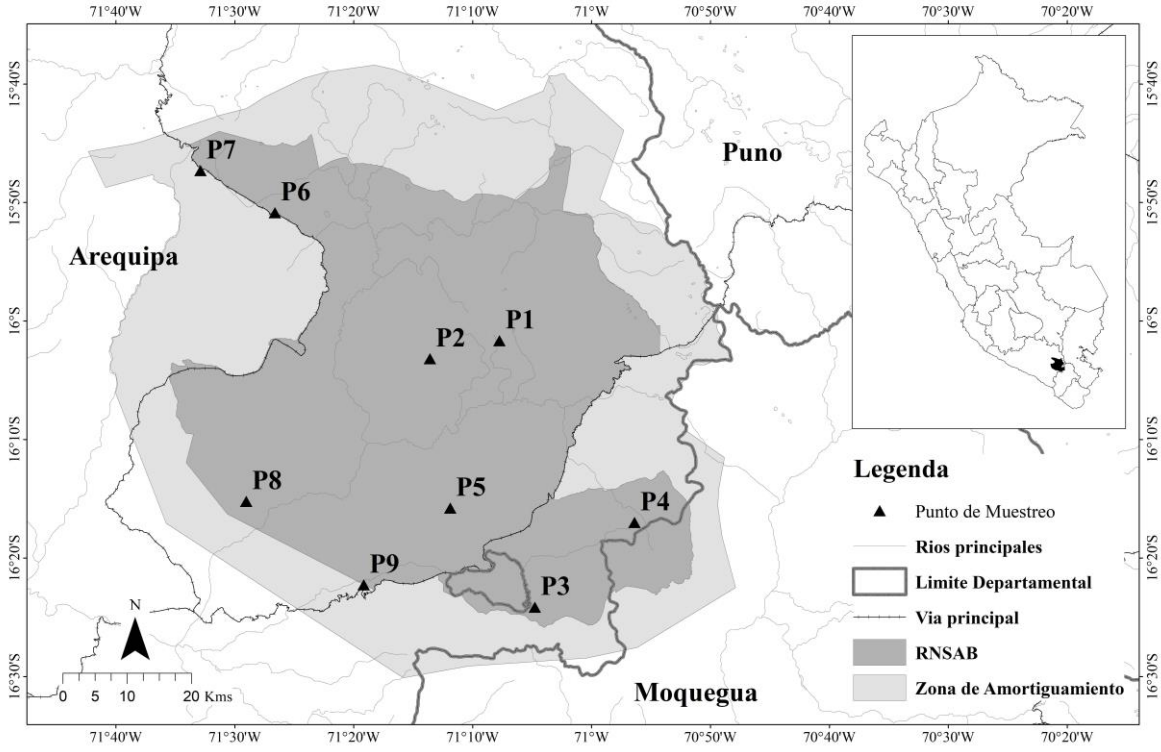

Figura 1. Mapa de ubicación de los puntos de muestreo evaluados en la Reserva Nacional Salinas y Aguada Blanca.
Location of sampling points assessed in Salinas y Aguada Blanca National Reserve.

dominado por *Alchemilla diplophylla* Diels y *Distichia muscoides* Nees *et* Meyen (11.085,31 ha); Yaretal, dominado por *Azorella* spp. Lamarck y *Pycnophyllum* spp. J. Rémy (8.146,16 ha); y queñual, dominado por *Polylepis rugulosa* Bitter (4.295,48 ha) (Zeballos *et al.* 2010).

Muestreo. Los trabajos de campo se realizaron durante los meses de enero y febrero de 2017. Se ubicaron nueve puntos de muestreo en la RNSAB (cuadro 1), siguiendo las recomendaciones de MINAM (2015) y diseños de muestreo de estudios previos (Crispin 2015, Morales 2015, Limache 2016, Flores 2017, Mango 2017, Sarcca 2017). A continuación, se detalla las formaciones vegetales evaluadas:

- Pajonal. Se instalaron seis parcelas de 3*3 m, en las cuales se colectó la totalidad de plántulas de los géneros *Stipa* y/o *Festuca*. La parte aérea y las raíces fueron pesadas en fresco y luego empaquetadas en bolsas de papel para su traslado al laboratorio. Se pesó un total de 75,757 kg de plántulas frescas, las cuales presentaron en promedio 66,8 % de materia seca.
- Tolar. Se instalaron seis parcelas de 50*20 m, en las cuales se realizó el conteo total de plántulas de los géneros *Parastrephia* y/o *Baccharis*. Luego, se colectó al azar una muestra de 10 plántulas (parte aérea y raíces) por parcela, las cuales fueron pesadas en fresco y luego empaquetadas para su traslado al laboratorio. Se pesó 35,126 kg de plántulas frescas, provenientes de 60 individuos, los cuales presentaron en promedio 59,71 % de materia seca; además se registró una densidad promedio de 8.085 individuos/ha.
- Bofedal. Se instalaron seis parcelas de 1*1 m, cada una de las cuales fue dividida en 4 sub-parcelas de 50*50 cm y ubicadas en forma cruz, a una distancia de 7 m desde el punto central de la parcela. La parte aérea y las raíces, hasta una profundidad de 30 cm, fueron colectadas, pesadas en fresco, y luego empaquetadas para su traslado al laboratorio. Se pesó un total de 1.189,446 kg de muestra fresca, presentando en promedio 17,21 % de materia seca.
- Yaretal. Se instalaron seis parcelas de 50*20 m, en las cuales se realizó un conteo total de plántulas del género *Azorella*. Luego, se escogió al azar seis plántulas por parcela para estimar el volumen de su parte aérea, en función a su altura, diámetro mayor y diámetro menor. Finalmente, en las mismas plántulas medidas se colectó una sub-muestra de volumen conocido (prisma rectangular de 7 cm de lado y "z" de profundidad), la cual fue pesada en fresco y luego empaquetadas para su traslado al laboratorio. Se pesó 33,609 kg de muestra

fresca, provenientes de 36 individuos, los cuales presentaron en promedio 43,62 % de materia seca y densidad promedio de 981,7 individuos/ha.

- Queñual. Se instalaron cuatro parcelas de 50*20 m, en las cuales se midió la totalidad de plántulas de *Polylepis rugulosa*. Se obtuvo la altura total y el diámetro de la copa para cada individuo, información necesaria para aplicar la fórmula dasométrica propuesta Sarcca (2017) para *P. rugulosa*. Se midió 206 individuos y se registró una densidad promedio de 515 individuos/ha.

Carbono almacenado. Se utilizó el método calorimétrico para obtener el carbono almacenado en las muestras (Eduarte y Segura 1998). Para ello, las muestras colectadas en las parcelas de Pajonal, Tolar, Bofedal y Yaretal fueron desecadas sobre un plástico negro expuesto a la radiación solar, por períodos de tres horas a 62 °C, durante cinco a seis días consecutivos. Luego, dichas muestras fueron llevadas al laboratorio para terminar el proceso a 80 °C durante 24 h, hasta alcanzar un peso seco constante, valor conocido también como Biomasa. En el caso del Yaretal, la biomasa contenida en cada submuestra fue extrapolada al volumen total de cada plántula. En el caso del Queñoal, la biomasa fue estimada con la fórmula dasométrica propuesta por Sarcca (2017) [1]:

$$Bio = 0{,}16496\ [A + D]\ 2{,}667785 \qquad [1]$$

Donde:
Bio = Biomasa (kg)
A = altura total de la plántula (m)
D = diámetro de la copa (m)

La cantidad de carbono almacenado en las parcelas evaluadas fue estimada con la fórmula [2]. En el caso del Tolar, el valor de carbono almacenado contenido en las 10 plántulas colectadas en cada parcela fue extrapolado con la densidad de plántulas contabilizadas en la misma parcela. De forma similar, en el Yaretal, el valor de carbono almacenado contenido en las seis plántulas medidas en cada parcela fue extrapolado a la densidad de plántulas contabilizadas en la misma parcela.

$$CA = Bio * FC \qquad [2]$$

Donde:
CA = Carbono almacenado
Bio = Biomasa (Mg)
FC = Factor de carbono en biomasa (0,5), siguiendo a Eggleston *et al.* (2006).

Carbono capturado (CC). La cantidad de carbono capturado en cada parcela fue estimada con la fórmula [3]:

$$CC = CA * Kr \qquad [3]$$

Donde:
CC = Carbono capturado
CA = Carbono almacenado
Kr = Factor de conversión de carbono a CO_2 (3,66), siguiendo a Eggleston *et al.* (2006).

Valoración económica. A pesar de que el Perú no aplica un impuesto al carbono nacional, se aplicó el precio de mercado voluntario de USD 6,39 por tonelada de CO_2 equivalente, estimado a partir de la evaluación de la rentabilidad social de proyectos de inversión pública llevados a cabo en el territorio peruano en el año 2018 y 2019 (Trinidad y Ortiz 2019). Dicho monto fue multiplicado a la cantidad promedio de CO_2 capturado en las parcelas evaluadas y posteriormente, el valor obtenido fue extrapolado a toda el área de la RNSAB con apoyo de cartografía disponible para las formaciones vegetales presentes en la Reserva (Zeballos *et al.* 2010).

Cuadro 1. Puntos de muestreo ubicados en la Reserva Nacional Salinas y Aguada Blanca.
Sampling points located in Salinas y Aguada Blanca National Reserve.

Puntos de Muestreo	Localidad de Referencia	Unidades de vegetación	Coordenadas geográficas		
			S	W	m s.n.m
P1	Condori	Pajonal, Tolar	16° 01,652’	71° 07,746’	4.353
P2	Huayllacucho	Tolar	16° 03,179’	71° 13,597’	4.460
P3	Santa Lucia de Salinas	Pajonal, Bofedal	16° 24,145’	71° 04,781’	4.322
P4	Vía Cancosani	Bofedal, Yaretal	16° 16,998’	70° 56,390’	4.425
P5	Salinas Huito - Cerro Pucasaya	Tolar, Yaretal	16° 15,783’	71° 11,892’	4.987
P6	Pampas de Tocra	Pajonal, Bofedal	15° 50,851’	71° 26,635’	4.344
P7	Cerro Chucura	Yaretal	15° 47,299’	71° 32,891’	4.642
P8	Pampa Cañahuas - Cabrerías	Pajonal, Queñual	16° 15,219’	71° 29,053’	4.727
P9	El Simbral	Queñual	16° 22,264’	71° 19,160’	3.810

Finalmente, el valor económico total asociado al secuestro y almacenamiento de CO_2 en la RNSAB fue calculada por la suma de los valores económicos obtenidos para el Pajonal, Tolar, Bofedal, Yaretal y Queñual.

RESULTADOS

Los resultados muestran que el servicio de secuestro y almacenamiento de CO_2 fluctúa según el tipo de formación vegetal y el área que esta ocupa en la Reserva Nacional de Salinas y Aguada Blanca (cuadro 2).

Respecto a la cantidad de carbono almacenado, se observó una fluctuación de 4,66 a 639,39 Mg/ha de CO_2 en el área de estudio, siendo el Bofedal la formación vegetal donde se concentró la mayor cantidad de carbono almacenado (639,39 Mg/ha de CO_2), seguido del Yaretal (98,63), Pajonal (16,84), Queñual (11,6) y Tolar (4,66). Dichos valores al ser extrapolados a la superficie total de cada formación vegetal en el área de estudio, mostró que la RNSAB resguarda al menos 13.507.104,16 toneladas métricas de CO_2 equivalente.

Respecto al valor económico del servicio de secuestro y almacenamiento de CO_2 por formación vegetal, se observó una fluctuación de U$D 318.350,12 a U$D 45.291.274,34, siendo el Bofedal la formación vegetal donde se obtuvo el mayor valor económico (U$D 45.291.274,34), seguido del Pajonal (34.119.364,04), Yaretal (5.133.964,15), Tolar (1.447.442,93) y Queñual (318.350,12). El valor económico del servicio de secuestro y almacenamiento de CO_2 en la RNSAB fue estimado en U$D 86.310.395,58.

DISCUSIÓN

Los resultados confirman la gran importancia que tiene la vegetación alto-andina en cuanto al servicio ecosistémico de secuestro y almacenamiento de CO_2, en función al tipo de formación vegetal y su extensión.

Cuadro 2. Diseño de muestreo y estimación del valor económico del servicio ecosistémico de secuestro y almacenamiento de CO_2 en la Reserva Nacional Salinas y Aguada Blanca.

Sampling design and estimation of economic value of the ecosystem service of carbon capture and storage in Salinas y Aguada Blanca National Reserve.

Característica	Pajonal	Tolar	Bofedal	Yaretal	Queñual
Dimensiones de parcelas evaluadas (m)	3*3	50*20	1*1	50*20	50*20
Densidad de plántulas, promedio ± SD (individuos/parcela)	-	808,50 ± 338,90	-	98,17 ± 23,06	51,5 ± 23,13
Área de parcelas evaluadas (ha)	0,0009	0,1	0,0001	0,1	0,1
Réplicas (n)	6	6	6	6	4
CO_2 capturado, promedio ± SD (Mg/parcela)	0,015153 ± 0,004889	0,465856 ± 0,237645	0,063939 ± 0,027761	9,862773 ± 10,13338	1,159825 ± 0,610465
CO_2 capturado, promedio ± SD (Mg/ha)	16,837 ± 5,432	4,659 ± 2,376	639,39 ± 277,610	98,62773 ± 101,334	11,59825 ± 6,105
CO_2 capturado, promedio ± SD (Mg/km^2)	1.683,667 ± 543,222	465,856 ± 237,645	63.939,340 ± 27.759,650	9.862,773 ± 10.133,38	1.159,825 ± 610,465
Área total de la Unidad de Vegetación (ha) en la RNSAB	317.134,84	48.623,80	11.085,31	8.146,16	4.295,48
CO_2 capturado por Unidad de vegetación (Mg) en la RNSAB	5.339.493,59	226.516,89	7.087.836,36	803.437,27	49.820,05
Precio de tonelada de CO_2 (U$D)	6,39	6,39	6,39	6,39	6,39
Valor económico por Unidad de vegetación (U$D) en la RNSAB	34.119.364,04	1.447.442,93	45.291.274,34	5.133.964,15	318.350,12
Valor económico del servicio ecosistémico (U$D) en la RNSAB			86.310.395,58		

Por un lado, la cantidad de carbono almacenado obtenida para el Bofedal (639,39 Mg/ha de CO_2) fue mayor a lo reportado por Crispin (2015) con 518,8 Mg/ha de CO_2, en Huancavelica, Perú (13° 19,779' S, 74° 58,902' W); sin embargo fue menor a la cantidad registrada por Mango (2017) con 1.173,95, en Arequipa, Perú (15° 43,154' S, 71° 18,823' W). Dichas variaciones pueden ser consecuencia del manejo que realizan los pobladores locales en los bofedales para aumentar su productividad y sirvan de forraje para su ganado. Mientras que, la cantidad de carbono almacenado obtenida para el Pajonal (16,84 Mg/ha de CO_2) fue relativamente similar a lo reportado por la FAO (2010) y Medrano *et al.* (2012), con valores de 16,5 y 15,43, respectivamente; en cambio Gibbon *et al.* (2010) y Flores (2017) reportaron valores inferiores (7,5 a 10,78). Dichas variaciones pueden ser explicadas debido a que los pajonales evaluados en cada estudio estuvieron dominados por distintas especies de Poaceae (*Stipa*, *Festuca* y/o *Deyeuxia*) y por variaciones en el diseño de muestreo (Gibbon *et al.* 2010 solo evalúan biomasa aérea).

Aquí, se documenta por primera vez la cantidad de carbono almacenado para las formaciones vegetales de Yaretal (98,63 Mg/ha de CO_2) y Tolar (4,66), dejando una fuente de referencia para valorar su importancia como sumidero de carbono en futuras investigaciones.

La cantidad de carbono almacenado obtenida para el queñual (11,6 Mg/ha de CO_2) fue menor a lo reportado por FAO (2010), con 56,3 Mg/ha de CO_2, y Vásquez *et al.* (2014), con 439; sin embargo, fue mayor a lo reportado por Glave y Pizarro (2001), Morales (2015) y Sarcca (2017), con valores de 3,55, 0,69 y 7,57, respectivamente. Dichas variaciones podrían ser explicadas a la especie y densidad de árboles en cada zona de estudio.

Cabe resaltar, que los bosques son particularmente importantes debido a que los árboles almacenan más carbono por unidad de área (en forma de madera) que otros tipos de vegetación (Houghton 2007), llegando a almacenar en algunos casos hasta 335,1 Mg/ha de CO_2 en bosques húmedos tropicales de tierras bajas (Martel y Cairampoma 2012). Sin embargo, los resultados demuestran que el Bofedal puede almacenar una mayor cantidad de carbono (639,39 Mg/ha de CO_2), ya que este utiliza otras estructuras como sumideros de carbono (raíces y/o masa muerta en el suelo). Un panorama similar fue observado por Castañeda-Martín y Montes-Pulido (2017) quienes reportaron un almacenamiento de 119 a 397 Mg/ha de CO_2 en los primeros 40 cm de profundidad del suelo del Páramo.

Por otro lado, respecto al valor económico, a pesar de que el Bofedal está representado en una pequeña área de la superficie total de la RNSAB (2,58 %), este aportó un poco más de la mitad del valor económico total de secuestro y almacenamiento de CO_2 en la RNSAB (52,48 %) (figura 2), corroborando su relevancia en la lucha contra la mitigación del cambio climático (Hribljan et al. 2015). El Pajonal se ubicó en segundo lugar de importancia de contribución al valor económico total del servicio de secuestro y almacenamiento de CO_2, el cual se encuentra ampliamente representada en la superficie total de la RNSAB (81,47 %). Mientras que, el Yaretal, Tolar y Queñual fueron las formaciones vegetales que menos aportaron al valor económico total (5,95 %, 1,68 % y 0,37 %, respectivamente), las cuales se encuentra poco representadas en la superficie total de la RNSAB.

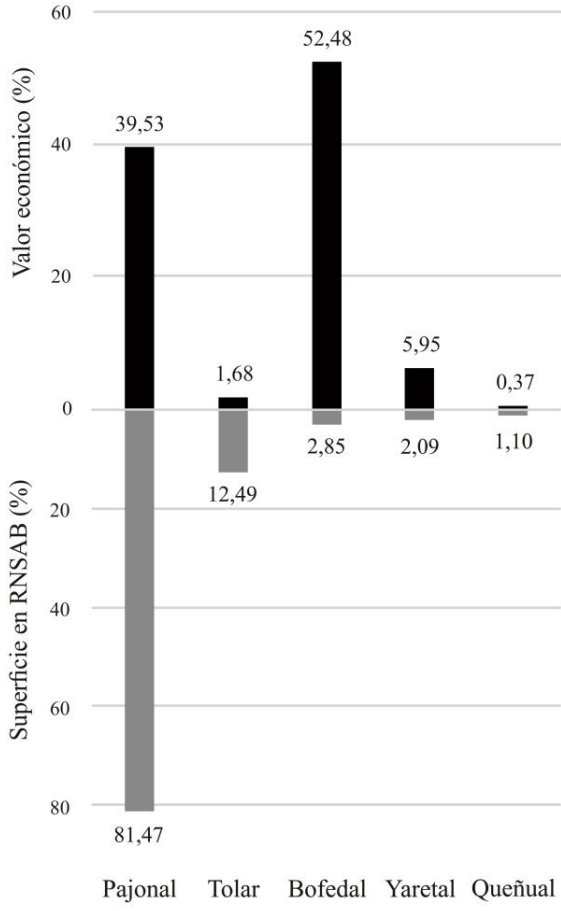

Figura 2. Relación entre el valor económico estimado y el área de cada formación vegetal en la Reserva Nacional Salinas y Aguada Blanca.

The relationship between economic value estimated and area of each vegetal formation in Salinas y Aguada Blanca National Reserve.

En las últimas cuatro décadas, los departamentos de Apurímac, Arequipa, Cusco y Puno han agrupado el 80 % de superficie afectada por fuego en la Región Andina del Perú (Manta *et al.* 2018). Un ejemplo de ello, son los recientes incendios forestales significativos provocados en la región de Arequipa (Andagua, Miraflores, Mariano Melgar, Pocsi, Yura, entre otros), los cuales ocasionaron la pérdida de pajonales, matorrales y queñuales, afectando la situación socioeconómica, desarrollo y bienestar de la población. Ante dicho panorama, se espera que el presente estudio sirva de marco de referencia en temas de concientización ambiental (conservación de la cobertura vegetal),

elaboración de instrumentos de gestión ambiental (diseño e implementación de sanciones por destrucción de la cobertura vegetal), contabilidad nacional, entre otros., con miras hacia una adecuada gestión ambiental de los ecosistemas desérticos de la puna seca del suroeste del Perú.

A nivel de la región de Arequipa, solo se conoce dos estudios relacionados a la valoración económica de servicios ambientales proporcionados por la RNSAB. El primero, estimó un valor económico de U$D 2.132.502,57 por el servicio de provisión de agua para Arequipa Metropolitana (Loyola 2007); mientras que el segundo, estimó un valor económico de U$D 384.209,60 por concepto de aprovechamiento de fibra de vicuña (Medina *et al*. 2018). Siguiendo dichas iniciativas, se insta el desarrollo de investigaciones que involucren la valoración de otros bienes y servicios ecosistémicos que brinda la RNSAB, tales como: ecoturismo, fijación de nitrógeno atmosférico, medicina tradicional, entre otros.

CONCLUSIONES

El valor económico de secuestro y almacenamiento de carbono por la vegetación de la RNSAB asciende a U$D 86.310.395,58, siendo el Bofedal quien aportó el 52,48 % de dicho valor, seguido del Pajonal (39,53 %), Yaretal (5,95 %), Tolar (1,68 %) y Queñual (0,37 %).

Considerando la importancia relativa del Bofedal, Pajonal, Yaretal, Tolar y Queñual como sumideros de carbono en Los Andes, se recomienda la implementación de un programa de monitoreo a largo plazo para evaluar las fluctuaciones del secuestro y almacenamiento de carbono en la puna seca del suroeste del Perú.

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Characterization and evaluation of *Flammulina mexicana* growth in lignocellulosic residues

Caracterización y evaluación del crecimiento de *Flammulina mexicana* en residuos lignocelulósicos

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SUMMARY

Flammulina mexicana is a fungus species endemic to high mountains and has potential for commercial cultivation. Four strains were isolated from wild fruit bodies and its growth was evaluated in two culture media. There were no morphological differences among these strains; however, the choice of culture media did produce differences. Growth rate was superior in strains IE 974 and IE 986 at 0.65 cm $day^{-1.}$ These strains also presented the highest biomass production at 6.9 and 7.7 g L^{-1}, respectively; with a period of 35 days in production with solid spawn and 20 days with liquid spawn. All the four strains have the ability to fructify in agricultural and forestry wastes. Strains IE 986 and IE 974 presented better biological efficiency at 34.5 % and 31.9 %, respectively, with maize stubble as a substrate. A nutritional analysis of the fructifications obtained showed a high percentage of fiber (52.51 %) and low carbohydrate content (9.03 %). For this reason, culture of these species represents an alternative for their *ex situ* conservation and use.

Key words: native strains, liquid spawn, maize stubble, fruit bodies, hemicellulose.

RESUMEN

Flammulina mexicana es una especie fúngica endémica de alta montaña con potencial para ser cultivada. A partir de esporomas silvestres, se aislaron cuatro cepas y se evaluó su crecimiento en dos medios de cultivo. No se presentaron diferencias morfológicas entre cepas, pero sí entre medios de cultivo. La velocidad de crecimiento fue mayor en las cepas IE 974 y IE 986 con 0,65 cm/día y la mayor producción de biomasa con 6,9 y 7,7 g/L, respectivamente; con un tiempo de 35 días para la producción de inóculo sólido y 20 para inóculo líquido. Las cuatro cepas tienen capacidad de fructificar en desechos agrícolas y forestales. Las cepas IE 986 e IE 974 presentaron mayor eficiencia biológica con 34,5 % y 31,9 %, respectivamente, en rastrojo de maíz como sustrato. El análisis nutricional de las fructificaciones obtenidas muestra un alto porcentaje de fibra (52,51 %) y bajo contenido de carbohidratos (9,03 %). Por lo que, el cultivo de esta especie representa una alternativa para su conservación *ex situ* y su aprovechamiento.

Palabras clave: cepas nativas, inóculo líquido, rastrojo de maíz, cuerpo fructífero, hemicelulosa.

INTRODUCTION

In Mexico, 371 species of wild mushrooms are consumed (Garibay-Orijel and Ruan-Soto 2014); however, not more than five of these species are cultivated at commercial scale and experimentation with new cultures is relatively recent and rare (Garibay-Orijel *et al*. 2010). Wild mushrooms represent a very important biological and genetic resource that offers great potential for the cultivated mushroom productive sector. They are organisms that develop in given substrates and environmental conditions; which provide the genetic variation necessary to produce the characteristics desired by the commercial sector. Given their importance to the ecosystem and high economic potential, exploitation of wild mushrooms requires sustainable management to guarantee conservation of the wild germplasm (Salmones and Mata 2012).

Cultivation of new species of wild mushrooms represents a strong potential for the preservation of traditional knowledge and acquisition of new genes and functional

properties (Morales *et al*. 2010). Cultivation of species valued by regional populations helps to promote their consumption and commercialization, ensuring the commercial viability of new businesses. These mushroom cultures can be an alternative for the reduction of contamination caused by organic agricultural, forestry or agro-industrial wastes, converting them into nutritious foods (Royse 1996, Philippoussis *et al*. 2001) and contributing to solving problems such as food insufficiency. One example of this is mushroom *Flammulina mexicana* Redhead, Estrada *et* R.H. Petersen, commonly known as "hongo de jara", whose consumption has been documented in five locations from Nevado de Toluca, Mexico and mentioned within the 20 species of mushrooms of higher cultural importance according to the traditional knowledge generated from research carried out in the area (Franco-Maass *et al*. 2012). In addition, it has already been tested in an *in vitro* culture from a wild strain (Arana-Gabriel *et al.* 2014). This species is endemic to the high mountains (Redhead *et al*. 2000), grows at elevations above 2,700 m, at temperatures below 18 °C (Redhead *et al*. 2000, Franco *et al*. 2012, Arana-Gabriel *et al*. 2014). *Flammulina mexicana* presents sexual reproduction of the bifactorial heterothallic type (Redhead *et al*. 2000) and saprotrophic habit, fructifying on *Senecio cinerarioides* A. Rich., a shrub that grows in high mountain forests and commonly known as "jara". In general, the genus *Flammulina* is considered cosmopolitan in deciduous forests and tolerant to low temperatures, for these reasons it is recognized as a winter mushroom. *Flammulina velutipes* (Curtis) Singer is the best-known species of the genus, it is one of the most cultivated mushrooms of the world due to its nutritional value and its therapeutic use (Sharma *et al*. 2010, Royse *et al*. 2017).

For *Flammulina mexicana*, the environmental conditions and the substrates required for the formation of fruiting bodies had not been previously described. Therefore, in this research it was necessary to use strains of different fruit bodies, to contribute to genetic selection, and thereby identify germplasm with desirable characteristics for the commercial sector (Savoie and Mata 2016). This genetic variety of the strains is expressed in variables such as growth rate, biomass production, fruit body production and biological efficiency. The characterization and evaluation of these variables help to have better strains, this is because not all of them have the capacity to form fruiting bodies, in addition to the fact that the growth rate and the biomass of the strains are not necessarily reflected in the biological efficiency (De León *et al.* 2012, León-Avendaño *et al.* 2013, Saboya and Mata 2016).

Due to this problem, the objective of the present investigation is to compare the biological efficiency of wild strains of *F. mexicana* on various lignocellulosic residues under controlled conditions at the laboratory level.

Because the nutritional and environmental conditions of the *F. mexicana* strains modify their growth rate and the production of fruiting bodies, as an expression of the genetic variety, it is expected that the strains with a higher growth rate and production of biomass on *in vitro* culture will present superior biological efficiency and short culture cycles.

METHODS

Collection and characterization of fruit bodies. Fruit bodies were collected from a sample directed to different secondary vegetation sites of *Senecio cinerarioides* from Nevado de Toluca Flora and Fauna Protection Area in Mexico. The specimens collected were characterized based on Delgado *et al*. (2005) and were identified according to Redhead *et al*. (2000) and Franco *et al*. (2012). Voucher specimens were deposited in the fungal collection of the National Herbarium of Mexico, at the Biology Institute of the UNAM (MEXU), under the following registry numbers: 27411, 28146, 28150, 28151.

Acquisition and morphological characterization of strains. Strains of *F. mexicana* were obtained from mushrooms collected on both dead material and live branches in different shrubs of *S. cinerarioides* by vegetative isolation in a dog food agar (DFA) medium and subsequent incubation at 18 °C in darkness. Tissue fragments were submerged in oxygenated water for 30 minutes and washed with sterile distilled water to avoid contamination. The strains obtained were deposited in the strain collection of the Ecology Institute (INECOL) in Xalapa, Veracruz, Mexico, with the keys: IE 984, IE 985, IE 986 and evaluated together with the strain IE 974 of *F. mexicana* obtained in a previous study (Arana-Gabriel *et al.* 2014). The sequences of each strain were also deposited in GenBank under the accession numbers: KY794659, KY794660, KY794661 and KY794662, respectively.

Once the mycelium had completely invaded the Petri dish, macroscopic characterization was conducted, based on Cruz-Ulloa (1995) and using the color key HTML Color Codes (2017). For the microscopic characterization, temporary preparations were made with 10 % Congo Red to verify the presence of clamps connections. The diameter of the hyphae was measured at 100x (20 hyphae per treatment) using the program Motic digital Microscope DMB3-223 (Motic China Group Co., Ltd., 2001-2004).

Growth rate of strains in a solid medium. With a borer, 5 mm diameter samples were removed from the agar containing mycelium and placed in the center of Petri dishes (9 cm in diameter) in two culture media. One non-conventional medium: DFA (15 g of agar, 15 g of dog croquettes of the brand GANADOR®, 2 g of yeast extract, 1 g of gelatin peptone in one liter of distilled water) (Stamets 2000, Arana-Gabriel *et al*. 2014) and one conventional medium: malt extract agar with added peptone and yeast (MEA-PY) (31.9 g of malt extract agar, 2 g of yeast extract, 1 g of ge-

latin peptone in one liter of distilled water). Five replicates were conducted per treatment and strain. The strains were incubated in darkness at 18 °C. Strain diameter was measured every third day with a Vernier (metal vernier caliper 5" Pretul®, ver-6p, China). The growth rate (GR) was obtained using the equation of Huerta *et al*. (2009) GR= (Fd-Id)/(Ft-It) (where "Fd" is the final diameter of growth, "Id" is the initial diameter of growth and "Ft-It" is the duration of the period of mycelial growth in days).

Biomass production of strains in a solid medium. Once the incubation period of the strains was over, the agar of the samples was eliminated by immersion in boiling water. The mycelium was recovered, filtered and rinsed with hot water. It was then oven-dried at 80 °C for 24 hours and weighed to obtain the dry biomass (Arana-Gabriel *et al*. 2014). Five replicates were conducted per treatment and strain.

Biomass production of strains in a liquid medium. One hundred ml of the dog food medium with peptone and yeast (DF-PY) and malt extract with peptone and yeast (ME-PY) were placed in 250 ml Erlenmeyer flasks (five replicates per treatment). All the culture media of the different treatments were sterilized in an autoclave at 121 °C, 15 lb psi for 15 minutes and 0.05 g of Chloramphenicol (SIGMA®) added as an antibiotic. The media inoculated with 0.5 cm in diameter of mycelium were incubated for 20 days at 18 °C and at 120 rpm in a water bath with agitation (PolyScience®). Five replicates were conducted per treatment and strain. At the end of this incubation period, the pH of the medium was measured, and the mycelium filtered and left to dry at 80 °C for 24 hours prior to weighing (Arana *et al*. 2014).

Statistical analyses. The statistical analysis was conducted with the program Statgraphics® Centurion XVI; for the evaluation of the strains, the data are applied to a multivariate analysis of variance (MANOVA) to determine whether the interaction between the strains and the culture media affects growth and/or biomass production. In addition, a correlation analysis was performed between the biomass production and the growth rate in solid medium.

For the biological efficiency and fruit bodies size in the fruiting bioassay, an analysis of variance (ANOVA) was performed; and the significant differences for both analyzes were determined with a Tukey multiple rank test ($P \leq 0.05$).

Solid spawn production. Wheat seeds were subjected to a process of cleaning, washing and hydration for 24 hours, subsequently boiled for ten minutes. Once drained, 200 g were weighed and slaked lime [$Ca(OH)_2$] (3.5 g of slaked lime per kg of seeds) added. The moisture content of the seeds was 77 % and the pH was 8.5. The seeds were then placed in flasks (five replicates per treatment) for sterilization in an autoclave at 121 °C, 15 lb psi for 1 h (Guzmán *et al*. 2013). Once the flasks had cooled, the seeds were inoculated with three 1 cm^2 fragments of mycelium from the four strains developed in the two media (DFA and MEA-PY); with a factorial design of 4 x 2 x 5 (4 strains, 2 culture media, 5 replicates). For the secondary spawn, wheat seeds were subjected to the procedure described above and inoculated with five percent of the primary spawn.

Liquid spawn production. The same procedure described for biomass production in a liquid medium was followed; following 20 days in agitation, the pellets were washed and homogenized in a liquidizer for 10 s with 100 ml of sterile distilled water (Stamets 2000). Five replicates were conducted per treatment and strain.

Fructification bioassays production. Four different formulations were used as substrates: 1) *Quercus* sp. sawdust 100 % (QS), 2) *Senecio cinerarioides* sawdust 100 % (SSC), 3) *S. cinerarioides* sawdust with maize stubble 50:50 (SSC+MS) and 4) Maize stubble 100 % (MS). The different combinations of substrates were hydrated to 70 % and slaked lime [$Ca(OH)_2$] (0.1%) and gypsum ($CaSO_4$) (0.1 %) added. Two kg of each of the four combinations of substrates were placed in low density plastic bags REYMA$^{M.R.}$ with a factorial design 4 x 4 x 2 x 5 (4 strains x 4 substrates x 2 spawn types x 5 replicates). The substrates were sterilized for two hours at 121 °C and 15 lb psi for 15 min. Once the bags had cooled, they were inoculated with 5 % (based on the substrate moist weight) of solid or liquid spawn. For inoculation with the seeds, an orifice was made in the center of the substrate in which the spawn was placed and for inoculation with liquid, sterile 60 ml syringes were used. The bags were incubated at 18 °C in darkness, until the mycelium had invaded the substrate. They were then placed in a fructification chamber, under the following conditions: incubation temperature of 18 °C (Thermo-hygrometer Vaisala®), relative humidity of 90-100 % (humidifier Vitallys Plus®), 70-100 lux of light (luxometer BK Precision®) and exchange of fresh air for one hour twice a day.

Characterization of harvested fruit bodies. Once the mature fruit bodies were obtained, the biological efficiency (BE) was determined using the equation BE = fresh weight of harvested fungi / dry weight of the substrate used x 100 (Guzmán *et al*. 2013). The fruit bodies were characterized macro- and micro-morphologically and compared with those collected in the field. A bromatological analysis of the cultivated mushrooms was conducted; moisture content was calculated based on NOM-116-SSA1-1994, ash content based on NMX-F-607-NORMEX-2013, fat content based on NMX-F-615-NORMEX-2004, protein content (factor 6.25) based on NMX-F-608-NORMEX-2011, raw fiber content based on NMX-F-613-NORMEX-2003 and carbohydrate content by calculation. Three replicates of each treatment were randomly selected.

Quantification of substrate fiber components. Quantification of fiber components (cellulose-hemicellulose and lignin) for the different treatments was conducted at three stages: prior to inoculation, at the end of incubation and at the end of final mushrooms production, along with a control without spawn for each substrate. Using the detergent method (Goering and Van 1970), the fiber was extracted both in neutral detergent (FND) and in acid detergent (FAD). Lignin was determined with sulfuric acid (72 %), cellulose content was estimated directly from the FDA-lignin and hemicellulose content was calculated as FND-FAD (Goering and Van 1970). All analyses were conducted in triplicate and the results presented were based on dry material (%). Three replicates of each treatment were randomly selected.

RESULTS

Characterization of wild fruit bodies. Pilei were flat-convex in form, of 2.3 (1-2-2.6) cm in size, pale yellow (#F2F5A9) in color, which as they matured changed into dark yellow (#8A4B08) and to brown (#61380B) coloration in the center of the pilei, with a translucent margin, moist-viscous surface with a small umbo in the center. Gills were free of the stipe and pale yellow (#F2F5A9) in color. Stipes were cylindrical and slightly flattened in form, 2.9 (2.4-3.7) x 0.2 (0.1-0.3) cm in size, cartilaginous and with a fibrous surface, the basal part was brown (#61380B) and as it reached the pilei the color vanished until becoming pale yellow (#F2F5A9).

Morphological characterization of strains. Morphologically, no differences were found among strains though there were differences among the growth patterns observed in culture media. The mycelium of the four strains in the DFA medium was white (#FFFFFF) with uniform growth, fimbriate margin, aerial mycelium, cotton-like texture and a flat surface. In contrast, the mycelium of the strains developed in the medium MEA-PY was from light greyish yellow (#F7F8E0) to dark yellow (#B18904) and brown (#61380B), with an irregular shape and prostrate or aerial type growth. Microscopically, the four strains developed hyphae that were branched, septate, smooth and with the presence of clamps. The diameter of the hyphae was similar among strains however differed between culture media; in the DFA medium, the hyphae were slightly thicker [1.9 (1.7-2.7 µm)] than in the MEA-PY medium [1.7 (1.2-2.5 µm)].

Growth rate of strains in solid medium. According to the statistical analysis, the culture medium ($P < 0.0001$) affected growth rate, with the mycelium reaching 9 cm in diameter in a maximum of 15 days in the DFA medium, for average growth rate of 0.65 cm day^{-1}. This contrasts with the medium MEA-PY, where this value was 0.38 cm day^{-1}. In addition to the culture medium, strain ($P \leq 0.0001$) was also a factor that affected growth rate, as observed in table 1. The Tukey multiple ranges test shows that strains IE 974 and IE 986 presented the highest growth rate, followed by IE 984 and IE 985. However, there was no statistically significant interaction ($P \geq 0.0568$) between strains and culture media.

Biomass production of strains in solid medium. Biomass production in the solid medium presented significant differences in relation to the strain ($P \leq 0.0001$) and culture medium ($P \leq 0.0001$). There was an interaction ($P \leq 0.0040$) between these two factors that also affected biomass production. Regarding the Tukey multiple ranges test, biomass production was significantly higher in the medium MEA-PY with strains IE 984, IE 985 and IE 986 nonetheless lower in the DFA medium (table 1). In general, the strains that developed the highest biomass presented lower growth rate (r = -0.6134).

Biomass production of strains in a liquid medium. The statistical analysis did not reveal significant differences regarding strains ($P \geq 0.2077$); however, there were differences between culture media ($P \leq 0.0001$) and no interaction ($P \geq 0.4718$) between the two factors. The Tukey multiple ranges test revealed that there was superior biomass production in strains IE 984, IE 985 and IE 986 with the medium DF-PY, and lower production in strains IE 974 and IE 984 with the medium ME-PY (table 1). The pellets formed in the liquid medium presented a globose form and fibrous surface. The final pH of the liquid medium in the four strains changed from 6.1 to 6.4 in the medium DF-PY and from 5.3 to 5.8 in the medium ME-PY.

Inoculation with solid spawn. In all the treatments, the mycelium was dense, white (#FFFFFF) and presented 100 % invasion of the seeds within 20 days. For the production of secondary spawn, the time of colonization was 15 days with dense mycelium and 100 % invasion of the seeds.

Fructification bioassay. By day 30 of incubation, the strain IE 986 inoculated with liquid spawn had invaded 100 % of the MS substrate, followed by the strain IE 974 within 40 days and the rest of the treatments in 42 days. In the case of strains IE 986 and IE 974 in MS with liquid spawn, the primordia developed in darkness four days after the mycelium invaded the substrate. For the rest of the treatments, fruit bodies formation was induced by removing the bags and leaving the substrate blocks exposed to a relative humidity of 90 to 100 %, with continuous light (70-100 lux) for 24 h per day and exchange of air for one hour twice per day. When the stipes reached 2 cm in length, a PVC tube of 4.5 cm in length by 11 cm in diameter was placed (this is part of a commercial method that allows fruiting bodies to remain firm, since the stipes are usually thin and fragile). They reached their state of maturation in 15 days. The first harvest was made before the pilei margin began to curve

Table 1. Growth rate and biomass production in four strains of *F. mexicana* (means ± SD).

Velocidad de crecimiento y producción de biomasa de cuatro cepas de *F. mexicana* (promedio± DS).

	Solid medium			Liquid medium	
Strain	Culture medium	Growth day (cm day^{-1})	Biomass (g Petri dish)	Culture medium	Biomass (g L^{-1})
IE974	MEA-PY	0.36 ± 0.00c*	0.08 ± 0.01c*	ME-PY	2.3 ± 0.04c*
	DFA	0.65 ± 0.00a	0.07 ± 0.00c	DF-PY	6.9 ± 0.21ab
IE 984	MEA-PY	0.26 ± 0.02d	0.18 ± 0.03a	ME-PY	2 ± 0.22c
	DFA	0.57 ± 0.00b	0.10 ± 0.00c	DF-PY	8.7 ± 0.25a
IE 985	MEA-PY	0.29 ± 0d	0.16 ± 0.02ab	ME-PY	4.7 ± 0.28abc
	DFA	0.58 ± 0.03b	0.10 ± 0.00c	DF-PY	8.5 ± 0.27a
IE 986	MEA-PY	0.38 ± 0.01c	0.18 ± 0.01a	ME-PY	3.1 ± 0.18bc
	DFA	0.65 ± 0a	0.11 ± 0.04bc	DF-PY	7.7 ± 0.05a

DFA: Dog Food Agar, MEA-PY: Malt Extract Agar-Peptone and Yeast, DF-PY: Dog Food-Peptone and Yeast, ME-PY: Malt Extract-Peptone and Yeast.
*Different letters in the same column indicate significant differences (Tukey, $P \leq 0.05$).

upwards, with average production of a 100 g per bag. The culture cycles stopped at the end of the third harvest, with a period of 6 days between harvests for induction of the fruit bodies and 15 days for their maturation.

Strains IE 974, IE 984 and IE 985 with liquid spawn in SSC+MS, and IE 986 in SSC+MS with solid spawn began to form small mushrooms at 13 days after the process of induction; however, these did not mature and therefore their BE was not reported. The rest of the treatments, mainly those with *S. cinerarioides* and *Quercus* sp. sawdust with solid and liquid spawn, presented slow mycelial growth and did not invade completely, which allowed the substrates to become contaminated with *Trichoderma* sp., and those that were invaded did not form mushrooms (IE 984 and IE 986).

Strains presented differences in BE ($P \leq 0.0001$); strains IE 974 and IE 986 with MS inoculated with liquid medium presented the highest BE compared to the rest of the strains and treatments, with up to 31.9 and 34.5 %, respectively. Strains IE 986 and IE 985 fructified in MS with solid and liquid spawn. As shown in table 2, in both cases the liquid spawn is more efficient than the solid spawn, both in terms of fruit bodies production and times of colonization of the substrate. IE 986 was the only strain to fructify in two different substrates (MS and SSC+MS), of which the latter substrate presented the lowest BE (5.8 %). In total, the culture cycle for the strain IE 986 in MS with liquid spawn was 91 days, while for the strain IE 974 this was 101 days, beginning from incubation of the substrate until acquisition of a third harvest with mature mushrooms. With solid spawn, the culture cycle was 117 days. The rest of the treatments had a cycle culture of 103 days.

Characterization of cultivated fruit bodies. Morphologically, the cultivated mushrooms were very similar to their wild counterparts (figure 1). Pilei reached an average size of 2 (1.3-3.7) cm, were flat-convex in form and pale yellow in color (#F2F5A9, #F7D358). As they matured, the center of the pilei changed from dark yellow (#8A4B08) to brown **(**#61380B) coloration in its center, with a translucent margin, moist-viscous surface with a small umbo in the center. Gills were free of the stipe, joined and pale yellow in color (#F2F5A9, #F5DA81). Stipes were cylindrical and slightly flattened in form, 7.2 (3-10) x 0.2 (0.1-0.5) cm in size, cartilaginous and with a fibrous surface, the basal part was brown (#61380B) and as it reached the pilei, the color vanished until reaching a pale yellow (#F2F5A9). Regarding size, the cultivated mushrooms had larger pilei and stipes than those presented by their wild counterparts. The pilei diameter presented statistical differences among treatments and strains (twenty fruit bodies were measured per treatment bag) (table 2).

In terms of the bromatological analysis of the cultivated mushrooms, which was conducted only with the mushrooms of the strain IE 986 harvested in MS, the following results were obtained based on 6.42 % of dry material: moisture content 93.58 %, ashes 12.72 %, fats 1.64 %, protein (factor: 6.25) 17.68 %, raw fiber 52.51 % and carbohydrates 9.03 %.

Quantification of the substrate fiber components. To identify the component(s) of the fiber that are capable of degradation by *F. mexicana*, a fiber analysis of the substrates that were completely colonized by mycelium, and those from which fruit bodies were obtained, was conducted. In the different treatments, in both periods of quantification (substrates colonized by mycelium and spent-ground) the percentages of hemicellulose decreased contrasted with the control; however, in the final stage after the third harvest, this component decreased by more than half, while

Table 2. Size of wild and cultivated mushrooms and biological efficiency of *F. mexicana* (means ± SD).

Tamaño de los hongos silvestres y cultivados y eficiencia biológica de *F. mexicana* (promedio ± DS).

Treatment	Type of spawn	Pilei	Stipe		Biological Efficiency
		Diameter (cm)	Length (cm)	Diameter (cm)	
Wild fruit bodies		2.3 (1.6-2.6)	2.9 (2.4-3.7)	0.2 (0.1-0.3)	-
IE 974/MS	L	2.15 (1.5-3)± 0.60ab*	7.52 (5.5-9) ± 1.38ab*	0.24 (0.2-0.4) ± 0.08ab*	31.9 ± 2.91a*
IE 986/MS	L	1.86 (1.5-2.7) ± 0.40b	7.82 (6-10) ± 1.23a	0.24 (0.2-0.4) ± 0.08ab	34.5 ± 4.09a
IE 986/MS	S	1.98 (1.3-2.5) ± 0.41ab	6.86 (3-9) ± 2.31abc	0.23 (0.15-0.25) ± 0.05ab	14.8 ± 1.96b
IE 986/SSC+MS	L	2.15 (1.5-3) ± 0.45ab	6.02 (4.3-7) ± 0.79abc	0.17 (0.1-0.25) ± 0.05b	5.84 ± 1.00d
IE 985/MS	L	1.91 (1.5-2.2) ± 0.20b	5.99 (4.2-7.2) ± 0.84bc	0.22 (0.2-0.3) ± 0.03b	8.7 ± 0.50cd
IE 984/MS	L	2.55 (1.7-3.7) ± 0.65a	5.68 (4-7) ± 0.96c	0.34 (0.15-0.5) ± 0.16a	10.8 ± 0.76bc
IE 985/MS	S	1.76 (1.5-2.1) ± 0.22b	5.65 (4.2-7) ± 1.07c	0.18 (0.1-0.25) ± 0.04b	7.18 ± 0.72cd

MS: Maize stubble, SSC+MS: *S. cinerarioides* sawdust and maize stubble, L: liquid spawn, S: solid spawn. *Different letters in the same column indicate significant differences (Tukey, $P \leq 0.05$).

Figure 1. *Flammulina mexicana.* A) Wild mushrooms growing on *S. cinerarioides*, B) cultivated mushrooms on maize stubble (MS).

Flammulina mexicana. A) hongos silvestres creciendo sobre *S. cinerarioides*, B) Cultivo sobre rastrojo de maíz (RM).

the quantity of cellulose increased slightly and that of lignin doubled (table 3).

This trend was clearly observed in the MS substrate, which presented the highest quantity of hemicellulose and lowest quantity of cellulose and lignin among the four different substrates. In the strain IE 986 with MS, which presented the highest BE, hemicellulose decreased from 28.23 to 12.02 %, cellulose increased slightly from 30.83 to 35.86 % and lignin increased from 5.08 to 12.86 %. Therefore, it is inferred that *F. mexicana* could be a species of brown-rot fungus.

For the rest of the treatments, data pertaining to the consumption of hemicellulose were obtained after the incubation period, when substrates were completely invaded by the mycelium (table 2). For example, for SSC+MS (IE 984), hemicellulose decreased from 27.21 to 22.34 %, cellulose remained the same as with the strain IE 984 or decreased from 32.39 to 28.73 % in IE 986 and lignin stayed the same or decreased from 13.68 to 7.44 in IE 985. In SSC, hemicellulose decreased from 23.79 to 18.12 % in the strain IE 986, as did the cellulose from 32.48 to 30.89 % in IE 984. Concerning lignin, statistically there were no changes. In the substrate QS, with the strain IE 984, hemicellulose and cellulose decreased from 21.42 to 17.49 % and from 42.24 to 38.37 %, respectively, and there were no differences in the lignin contrasted with the control.

Table 3. Fiber analysis in colonized substrates with fruit bodies production (means ± SD).

Análisis de fibra en sustratos colonizados y con producción de cuerpos fructíferos (promedio ± DS).

Strain	Substrate	Type of spawn	Invaded substrate			Harvested substrate		
			Hemicellulose (%)	Cellulose (%)	Lignin (%)	Hemicellulose (%)	Cellulose (%)	Lignin (%)
Control	MS	-	28.23 ± 0.01a*	30.83 ± 0.31fgh	5.08 ± 0.89j	28.23 ± 0.01a	30.83 ± 0.31ef	5.08 ± 0.89 f
Control	MS+SSS	-	27.21 ± 0.13ab	32.39 ± 0.10ef	13.68 ± 0.19de	27.21 ± 0.13b	32.39 ± 0.10de	13.68 ± 0.19d
Control	SSC	-	23.79 ± 0.29cd	32.48 ± 0.87ef	18.1 ± 0.30c	23.79 ± 0.29c	32.48 ± 0.87de	18.1 ± 0.31b
Control	QS	-	21.42 ± 0.37efg	42.24 ± 0.37a	23.13 ± 0.67a	21.42 ± 0.37d	42.24 ± 0.37a	23.13 ± 0.67a
IE 974	MS	L	13.66 ± 0.54j	35.52 ± 0.92 cd	9.13 ± 0.39h	10.95 ± 0.26g	37.32 ± 0.26bc	12.79 ± 0.26d
IE 984	MS	L	24.33 ± 0.63cd	25.50 ± 0.81i	7.42 ± 0.17i	13.12 ± 0.00e	33.6 ± 1.35d	10.06 ± 0.56e
IE 985	MS	S	21.65 ± 0.45efg	19.97 ± 0.50j	9.40 ± 0.23gh	10.26 ± 0.04gh	24.83 ± 0.68g	10.84 ± 0.36e
IE 985	MS	L	25.16 ± 0.84bc	30.01 ± 0.76gh	6.57 ± 0.13i	12.42 ± 0.12ef	30.42 ± 0.35f	10.57 ± 0.18e
IE 986	MS	S	13.92 ± 0.41i	34.67 ± 0.41de	10.45 ± 0.24g	12.10 ± 0.47f	30.07 ± 0.15f	10.58 ± 0.69e
IE 986	MS	L	25.17 ± 0.90bc	25.65 ± 0.83i	7.63 ± 0.17i	12.02 ± 0.27f	35.86 ± 0.75 c	12.86 ± 0.42d
IE 986	SSC+MS	L	23.51 ± 0.72cde	28.73 ± 0.79h	13.06 ± 0.28ef	10.19 ± 0.03h	37.94 ± 0.25b	15.45 ± 0.11c

QS: *Quercus* sp. sawdust, SSC: *S. cinerarioides* sawdust; MS: maize stubble, SSC+MS: *S. cinerarioides* sawdust and maize stubble, L: liquid spawn, S: solid spawn. *Different letters in the same column indicate significant differences (Tukey, $P \leq 0.05$).

DISCUSSION

For the experimental cultivation of *F. mexicana* from native strains, the DFA culture medium, considered a non-conventional source of carbon is the most suitable medium for *in vitro* culture. Chegwin and Nieto (2013) report that some non-conventional carbon sources generate good biomass production, with more important growth rate and higher production of metabolites, and can also be used in the culture of other species of fungi, thus decreasing process cost.

Using a solid medium, *F. mexicana* presented maximum biomass production in the medium MEA-PY at 0.18 g Petri dish. Using a liquid medium, this was presented at 8.7 g L^{-1} in the DF-PY medium. These results are similar to those of *F. velutipes*, where biomass production of 4.16-4.82 g L^{-1} was reported after 15 days at 25 °C (Kim *et al*. 2002) and 5.5 g L^{-1} by day 10 (Kozhemyakina *et al*. 2010). Biomass production in a liquid medium depends on species, intensity of agitation and the nutrients in the culture medium. It has been reported that biomass production increases 1.5 to 2 times in liquid media relative to production in solid media (Kozhemyakina *et al*. 2010), as was demonstrated in this study with *F. mexicana*. Because superior quantity of biomass of higher quality is produced in a shorter time in a liquid medium (Frieal and McLoughlin 2000) it can be used as spawn.

Spawn type (solid or liquid) affected the time of substrate colonization; the liquid spawn developed dense mycelium and was the first to invade, compared to the substrates inoculated with seed. Mushrooms production was more rapid in substrates with liquid spawn. These trends agree with that reported for other species, such as *Agaricus blazei* Murill (Lin and Yang 2006) and *Pleurotus ostreatus* (Jacq. ex. fr) Kummer. (Silveira *et al*. 2008). Due to the fact that the liquid medium can be inoculated directly into the substrate, incubation time is reduced and there is better density of mycelium, more uniform distribution in the substrates and fewer problems of contamination (Frieal and McLoughlin 2000).

The moisture content of the substrates and ambient temperature are important factors for mycelium growth. All substrates were adjusted to 70 % of moisture content; however, this percentage could have affected the sawdust-based substrates, slowing the growth rate and preventing the mycelium from completely invading the substrate, leading to problems of contamination. Rapid invasion of substrates would avoid contamination by other microorganisms. For *F. velutipes,* the optimum moisture content is 60-65 % (Sharma *et al*. 2010), therefore, it is suggested that the moisture content for sawdust-based substrates should be less than 70 %.

For fruit bodies production, the best strain was IE 986 in MS with liquid spawn. This strain invaded in a maximum of 30 days, a period similar to that of *F. velutipes*, which invades in approximately 25 days (Royse 1996). Fruit bodies formation began at day 34 of incubation in darkness at a temperature of 16 to 18 ºC. To stimulate formation, it is important to consider factors such as light and moisture content of both the substrate and the environment. The temperature conditions under which *F. mexicana* fructified were very different to those for *F. velutipes*, which had to

be subjected to different temperature changes for a period of 10 to 14 days (Royse 1996). However, for both species, the primordia are induced in conditions of darkness, which explains why there were blocks that did not fructify in the presence of light or in some cases even failed to mature.

Once the fruit bodies had developed, exchange of fresh air was carried out twice a day with alternating 12 h periods of light and darkness. Light, exchange of fresh air and moisture content are all essential elements for fruit bodies maturation (Sakamoto *et al*. 2004).

The aerial growth of the mycelium that *F. mexicana* presented in culture at strain level suggests that it is a species that must be subjected to conditions of sufficient oxygen as with *F. velutipes*. This condition is reflected in the size of the fruit bodies, which presented pilei with smaller diameters and longer stipes than those found in wild specimens of this species. The pilei of the wild mushrooms measure 2.2 x 2.3 cm and the stipes 2.9 x 0.2 cm, while those obtained in culture present pilei of 1.9 (1-5-2.7) x 1.7 (1-2.9) cm and stipes of 7.2 (3-10) x 0.2 (0.1-0.4) cm.

These changes are affected by both the exchange of fresh air and quantity of light. In a commercial cultivation of *F. velutipes*, the concentrations of CO_2 and intensity of light are altered in order to modify the size of the pilei and stipe. When CO_2 concentrations increase, pilei diameters decrease and the stipes elongate to 14-18 cm (Sharma *et al*. 2010). In conditions of darkness, the stipes are elongated and thin and present small pilei (pinhead fruit body) that are whiter in color than those that formed in the presence of light (Sakamoto *et al*. 2004).

In terms of the BE, strains IE 986 and IE 974 had a percentage in three harvests that exceeded that of the other two strains (table 2); however, when compared with the BE of *F. velutipes*, it was very low. For *F. velutipes*, Harith *et al*. 2014 report BE values between 48-138 % in supplemented substrates and up to 150 % in fiber of palm and rice.

Despite the low BE found compared to that of *F. velutipes*, it is important to note that this study represents the first experimental cultivation conducted with *F. mexicana* and that the substrates utilized by this study were not supplemented. The supplementation substrates will have a better biological efficiency (Harith *et al*. 2014). The mycelial growth and the fruit bodies production in the experimental cultivation depend of the genetic characteristics of each species, strains, substrates and spawn types used (Philippoussis *et al*. 2001). The liquid spawn is more efficient in the substrate colonization; strains IE 986 and IE 974 showed higher production of biomass and growth rate *in vitro* culture, hence those with a shorter culture cycle are more capable of producing fruit bodies.

Regarding the nature of the substrates, the decrease in hemicellulose and cellulose in the four different substrates prior to formation of fruit bodies and after the third harvest indicated that *F. mexicana* has the capacity to selectively degrade cellulosic and hemicellulosic components. In this way, the substrates MS and MS+SSC that presented a higher percentage of hemicellulose and lignin also had the highest rate of mycelial growth and mushrooms production. The capacity for degradation is therefore influenced by the nature of the substrate, environmental and genetic factors among species or even among strains of the same species, enzymes and the quantity and resistance of the cellulose, hemicellulose and lignin (Economou *et al*. 2017).

The substrates used for the cultivation of mushrooms are generally derived from agricultural, agro-industrial or forestry wastes, which are found with varying availability in different regions (Salmones and Mata 2012). The success of the *F. mexicana* culture in maize stubble is highly important, considering that this substrate is one of the most commonly available agricultural wastes in the central region of Mexico. This also contributes to the reduction of costs and avoids the use of its natural substrate (*S. cinerarioides*) in the context of a commercial cultivation process.

In terms of the nutrient content of the cultivated *F. mexicana* mushrooms (ashes 12.72 %, fats 1.64 %, protein 17.68 %, raw fiber 52.51 % and carbohydrates 9.03 %), values are within the ranges reported for other species (Sharma *et al*. 2010) with the exception of fiber content. The cultivated mushrooms of the *F. mexicana* strain IE 986 in MS presented high percentage of fiber and low percentage of carbohydrates; this trend supports that reported for *Ganoderma tsugae* Murrill, which presents high percentage of fiber (73.4) and, as the mushrooms mature, the quantity of fiber increases and the contents of other components are reduced (Tseng *et al*. 2005). These results allow *F. mexicana* to be considered a good source of fiber. This fiber is mostly composed of chitin (25 %), hemicelluloses, mannans and β-glucans (4-13 %), which are all components of the cell wall (Manzi and Pizzoferrato 2000). These components confer anti-allergen activities and participate in modulation of the immune system, prevention of the promotion and progression of certain types of cancer and reduction of levels of cholesterol in the blood, among other functional properties (Manzi and Pizzoferrato 2000).

It is possible to obtain fructifications at an experimental culture level of *F. mexicana* from wild strains. Mycelial growth and production of fruiting bodies in the different substrates depend on the strain and the type of spawn, being more efficient by the time of colonization the liquid spawn. Strains IE 986 and IE 974 present higher production of biomass and growth rate in *in vitro* culture, hence, for the production of fruiting bodies are more appropriate those that have a shorter culture cycle (91 days) and better biological efficiency with maize stubble as a substrate. The production of fruiting bodies occurs at a temperature of 16-18 ºC, light intensity of 70-100 lux, exchange of air for one hour twice per day and a relative humidity of 90-100 %. Cultivated fruit bodies are high in fiber (52.51 %) and low in carbohydrates (9.03 %).

In this way, cultivation is an excellent alternative for the conservation of *F. mexicana*, which by developing only on *S. cinerarioides*, at elevations above 2700 meters and being

intolerant to heat, as well as being considered a species endemic to the high mountains, is currently restricted to small isolated populations or "islands" (Redhead *et al*. 2000).

These types of studies, focused on the experimental cultivation of wild edible mushrooms, form the basis for continued research that helps to identify the strains, culture media, environmental conditions and substrates that can be used and/or modified to optimize the culture process and obtain superior yields. Safeguarding the germplasm and the culture of new native species represents a viable alternative for their *ex situ* conservation (Salmones and Mata 2012, Alvarado-Castillo *et al*. 2015).

CONCLUSIONS

It is possible to obtain fructification at an experimental culture level of *F. mexicana* from wild strains. There are differences in the biological efficiency related to the genetic variability of the four strains of *F. mexicana*. Mycelial growth and the production of fruiting bodies in the different substrates depending on the strain and the type of spawn are more efficient by the time of colonization in the liquid spawn. Strains IE 986 and IE 974 present higher production of biomass and growth rate in *in vitro* culture, hence, for the production of fruiting bodies are those that have a shorter culture cycle (91 days) and better biological efficiency with maize stubble as a substrate. The production of fruiting bodies occurs at a temperature of 16-18 ºC, light intensity of 70-100 lux, exchange of air for one hour twice per day and a relative humidity of 90-100 %. Cultivated fruit bodies are high in fiber and low in carbohydrates.

Strains could be used for research focused on breeding programs for strains, to obtain higher yields and desirable characteristics in the commercial sector; allowing the use and conservation of germplasm of native species.

The cultivation of *F. mexicana* could represent a viable alternative in the diversification of the cultivated species and the use of the natural resources of each region, including agricultural, forestry or agroindustrial wastes and the specific environmental conditions required by each species. Such is the particular case of corn stubble, one of the most available and low-cost waste in central Mexico.

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The dynamics of primary growth in woody species from rain and transitional forests of Argentinean north Patagonia

Dinámica del crecimiento primario de especies leñosas en bosques lluviosos y de transición de la Patagonia Norte, Argentina

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SUMMARY

Rainforests (RF) and transitional forests (TF) of Argentinean north Patagonia develop under such different climatic conditions that key aspects of seasonal growth may be assumed to differ among the woody plants that characterize these forests. This study was aimed at evaluating primary growth dynamics in tree species typical of RF and TF. Two common-garden essays were performed in Bariloche, Argentina, one with RF species and the other with TF species. The times of extension initiation and end, the duration of the extension period and the relative and absolute extension rates (RER and AER, respectively) were registered for each species. The relation between AER and air temperature was compared between species. In general, RF species had less variable times of extension initiation, extension end and time of maximum RER, and longer-lasting extension than TF species. Among RF species, extension duration was the longest for *Caldcluvia paniculata* (26.9 weeks, on average) and the shortest for *Luma apiculata* (18.9 weeks). Among TF species, the longest and shortest extension durations corresponded, respectively, to *Diostea juncea* (18.2 weeks) and *Maytenus boaria* (13.0 weeks). The extension rates of RF species tended to be more related to temperature than those of TF species. This study provides some evidence that endogenous control of growth dynamics would be tighter in TF than in RF species; the former would be better adapted to more severe climatic conditions during the primary-growth period.

Key words: Patagonian forest, plant architecture, primary growth, annual shoots, seasonal growth.

RESUMEN

Los bosques lluviosos (RF) y los bosques de transición (TF) de Patagonia Norte argentina se desarrollan bajo condiciones climáticas contrastantes, por lo que puede esperarse que las especies leñosas que caracterizan estos dos tipos de bosque difieran en aspectos clave del crecimiento estacional. Este estudio tuvo como objetivo evaluar la dinámica de crecimiento primario de especies arbóreas típicas de RF y de TF, mediante dos ensayos en jardín común en Bariloche, Argentina. Para cada especie se registraron las fechas del inicio y fin, y duración del alargamiento. Se calcularon las tasas de alargamiento relativas (RER) y absolutas (AER), relacionando las últimas con la temperatura. En general, las especies de RF tuvieron momentos de inicio del alargamiento, fin de alargamiento y RER menos variables y períodos de alargamiento más largos que las de TF. Entre las especies de RF, *Caldcluvia paniculata* tuvo el período de alargamiento más largo y *Luma apiculata* el más corto (26,9 y 18,9 semanas, respectivamente). Entre las especies de TF, *Diostea juncea* tuvo el período de alargamiento más largo y *Maytenus boaria* el más corto (18,2 y 13,0 semanas, respectivamente). Las AER de las especies de RF estuvieron más relacionadas con la temperatura que las de TF. Este estudio aporta evidencia que sugiere que la dinámica de crecimiento en especies del TF está sujeta a mayor control endógeno que en especies de RF; las especies del TF estarían mejor adaptadas a condiciones climáticas más estrictas durante el período de crecimiento primario.

Palabras clave: bosques patagónicos, arquitectura vegetal, crecimiento primario, brotes anuales, crecimiento estacional.

INTRODUCTION

Axis length growth in woody plants, also known as primary growth (organogenesis followed by extension), is controlled by the interaction of endogenous and environmental forces, and has a key influence on the ability of a species to adapt to external conditions (Barthélémy and Caraglio 2007). Primary-growth patterns of woody plants have been extensively studied for many purposes, *e.g.* to understand the way plant architecture takes form, and to unravel the adaptive physiological mechanisms underlying plant architecture (Isik *et al.* 2002). The interaction between endogenous and environmental effects on extension and architecture has been investigated using different scientific approaches (Gordon *et al.* 2006). A species basic extension pattern may be analyzed through field studies that include populations at dissimilar areas within the species range, although severe restrictions derive from multiple variability sources. For instance, the primary growth of annual shoots is highly variable within and between species as a result of both the expression of ontogenetic gradients, and the influence of environmental factors (Barthélémy and Caraglio 2007, Magnin *et al.* 2016). Common-garden experiments are useful in this regard, as ontogenetic, environmental and size-related variability sources would be better controlled (Premoli *et al.* 2007). Since common-garden experiments allow all plants to be subject to even conditions, growth attributes driven mainly by endogenous or genetic factors would be evidenced.

Two basic patterns of primary growth dynamics have been described: continuous and rhythmic. Continuous growth is defined by the lack of a clearly identifiable growth period due to the absence of endogenous limitations, so that plants grow whenever external conditions are favorable (Barthélémy and Caraglio 2007). Rhythmic growth is characterized by an endogenous determination of a period of shoot growth followed by a resting period, which occurs even when external conditions are favorable for vegetative growth. In general terms, the primary growth of trees from temperate and cold regions takes place in the period of the year when climatic conditions are most favorable to the species concerned (Stecconi *et al.* 2000). In some temperate regions, the limits of that period may not be easy to establish as climatic conditions exhibit relatively smooth seasonal variations. It may be hypothesized that primary-growth patterns could change, in evolutionary time, from continuous to rhythmic or vice-versa. This hypothesis may be tested by means of the evaluation of endogenous and environmental influences on the primary growth of woody plants (Buissart *et al.* 2018). Such information is highly interesting, considering that current global tendencies point to increments in climatic variability for temperate regions (Matthews *et al.* 2016), which would impact on plant fitness in future scenarios.

In Argentinean northern Patagonia, a sharp decrease in precipitations from west to east, ranging from 3500 to 600 mm/year, is evident within 50 km east of the Andes Mountains due to a rainshadow effect (Conti 1998). Plant communities vary, respectively, from temperate rainforests to steppe. Forests at the western end of this gradient are included in the so-called "Valdivian rainforest", and are considered a hotspot of biodiversity (Myers *et al.* 2000), with diverse evergreen tree species, some of which belong to Neotropical lineages and others to Gondwanan lineages (Aizen and Ezcurra 1998). Two of the main climatic factors that condition primary-growth dynamics, rainfall and temperature, have increased their ranges of within-year variation from these rainforests eastwards (Donoso 2013, Stecconi *et al.* 2017), due to the buffering effects of the humid winds from the Pacific Ocean since the elevation of the Andes, about 25 m.y. ago (CONAMA 2008). Towards the dry end of this precipitation gradient, those forests that are transitional between the rainforests and the steppe (hereafter transitional forests) are often subject to below-zero temperatures in spring and summer, and to long summer periods of water deficit (Kitzberger 2012). These factors would have a major influence on the lower richness of woody species of transitional forests compared to that in the nearby rainforests.

Climate models for Patagonia predict a trend towards decreasing water inputs (Bates *et al.* 2008), with more frequent and/or intense drought periods. It has been shown for several tree species that fitting primary growth to the time window with suitable conditions has key importance in the likelihood of inhabiting a particular region (Sow *et al.* 2018). A better understanding of which factors are more involved in the regulation of tree growth may contribute in the prediction of species responses to eventual climatic changes in this or other regions, and would mean a step forward in explaining evolutionary variations in the extension patterns of plants. In the present study, we evaluated the extension dynamic of annual shoots (times of extension start and end, rates and duration) and the relationships between extension rate and mean temperature fluctuations for woody species typical of transitional forests (four species) and rainforests (four species) of Argentinean North Patagonia. We hypothesized that intra-annual variations in precipitations and temperatures are strong selective forces that contribute to modifying endogenous patterns in the primary growth dynamics of tree species. Since rainforests in Argentinean Patagonia are subject to less sharp seasonal changes in temperature and humidity than transitional forests, we expect that primary-growth patterns of rainforests species would express tighter responses to environmental conditions during the growth season than those of transitional forest species; the latter would exhibit stronger endogenous controls of shoot extension.

METHODS

Study species. The study included individuals from eight native woody species, which were assigned to one of two

groups in accordance to their natural habitat. All of them were produced from seeds in greenhouse and maintained in nursery with periodic watering up to the beginning of this study:

- Transitional forest species: comprised five-year-old individuals of the following species: *Aristotelia chilensis* (Molina) Stuntz (Elaeocarpaceae; n = 25), *Maytenus boaria* Molina (Celastraceae; n=25), *Schinus patagonicus* (Phil.) I.M. Johnst. (Anacardiaceae; n = 25) and *Diostea juncea* (Gill. *et* Hook.) Miers (Verbenaceae; n = 25), and
- Rainforest species: comprised three- to five-year-old individuals in all cases of: *Luma apiculata* (DC.) Burret (Myrtaceae; n = 20), *Gevuina avellana* Molina (Proteaceae; n = 22), *Caldcluvia paniculata* (Cav.) D. Don. (Cunoniaceae; n = 15) and *Weinmannia trichosperma* Cav. (Cunoniaceae; n = 20). The minimum number of individuals per species was 15, taking into account the number of plants commonly used in primary growth dynamics research (*e.g.* Poorter *et al.* 2006; Stecconi *et al.* 2017; Buissart *et al.* 2018).

Transitional forest species are native to transitional communities between the steppe-forest ecotone and the rainforests. *Aristotelia chilensis* is wintergreen, *M. boaria* and *S. patagonicus* are evergreen, and *D. juncea* is deciduous (though it has green stems). All four selected rainforest species are evergreen trees typical of the temperate rainforests of Chile and Argentina. Every specimen derived from seeds obtained from Argentinean natural populations. We assumed that all plants were at the same architectural stage based on architectural characteristics, the absence of flowers and the clearly defined vertical trunk (Gerea 2008; Salgado 2018). These two species groups were established in different nurseries in San Carlos de Bariloche (transitional forests: 41º 08' S 71º 18' W, 880 m a.s.l.; rainforests: 41º 11' S 71º 44' W, 818 m a.s.l.). The reason for growing transitional forest and rainforest species in two different nurseries was based on the fact that these two species groups differ in their optimal levels of water and light.

Measurements. Data regarding each group was collected independently, in different growth seasons: 2006-2007 for rainforests and 2016-2017 for transitional forests. At the beginning of the respective growth season (August), the base of the apical bud of each plant main axis (the most vigorous and vertical) was marked with a paint pen. During the growth season, the length of the shoot developed from each marked bud, from the basal mark to the distal end of the apical bud, was measured weekly with a measuring tape (to the nearest mm; figure 1).

Data analyses. The dates of extension initiation and cessation were registered for each shoot. Weekly absolute extension rate (AER) and relative extension rate (RER) were calculated for each plant according to equations [1] and [2]:

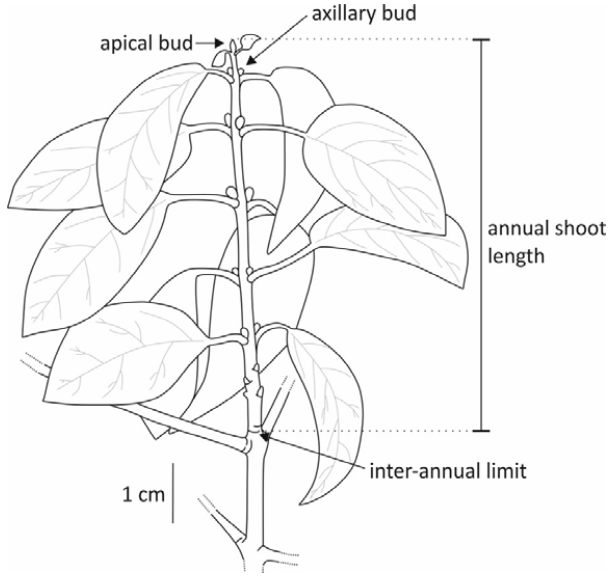

Figure 1. Diagrammatic representation of an annual shoot of *Aristotelia chilensis*. The apical bud, an axillary bud and the inter-annual limit between two successive shoots are indicated.

Diagrama de un brote anual de *Aristotelia chilensis*. Se indican la yema apical (apical bud), una yema axilar (axillary bud) y el límite interanual entre dos brotes sucesivos (inter-annual limit).

$$AER = \frac{L2 - L1}{t2 - t1} \times 7 \quad [1]$$

$$RER = \frac{\ln(L2) - \ln(L1)}{t2 - t1} \times 7 \quad [2]$$

where L1 and L2 are shoot lengths at dates t1 and t2, respectively. So that t2−t1 is the number of days between these dates.

Extension duration was calculated as the number of weeks from extension initiation to extension end. The intra-specific standard errors (SE) of the moments of extension initiation, maximum RER (RER_{max}) and extension end were calculated for each species. Within each species group, inter-specific variations in RER and extension duration were analyzed by means of one-way ANOVA. Spearman correlations between centered and standardized data of average weekly AER and temperature were computed for each species (mean temperatures obtained from *Dirección de Aguas Rionegrinas*). To qualitatively evaluate the relationship between AER and temperature variations, an analysis of the proportion of coincidences of weekly increases or decreases of both variables during the extension period of each plant was performed. These data were com-

pared between species (main factor) by means of Generalized Linear Models, assuming a binomial error distribution and a log-link function. This model was contrasted against the null model by means of a chi-square test of sequential deviance followed by Tukey's pairwise comparisons on the *multcomp* package. All statistical analyses were performed with R software (R Development Core Team 2014), assuming a 5 % significance level in all comparisons.

RESULTS

For most species, shoot extension started by early September, except for *W. trichosperma,* which had a slightly later budbreak (figure 2). *Gevuina avellana*, *M. boaria* and *S. patagonicus* showed the highest standard errors for the date of extension initiation, whereas lower variations were observed for the other species. Some individuals

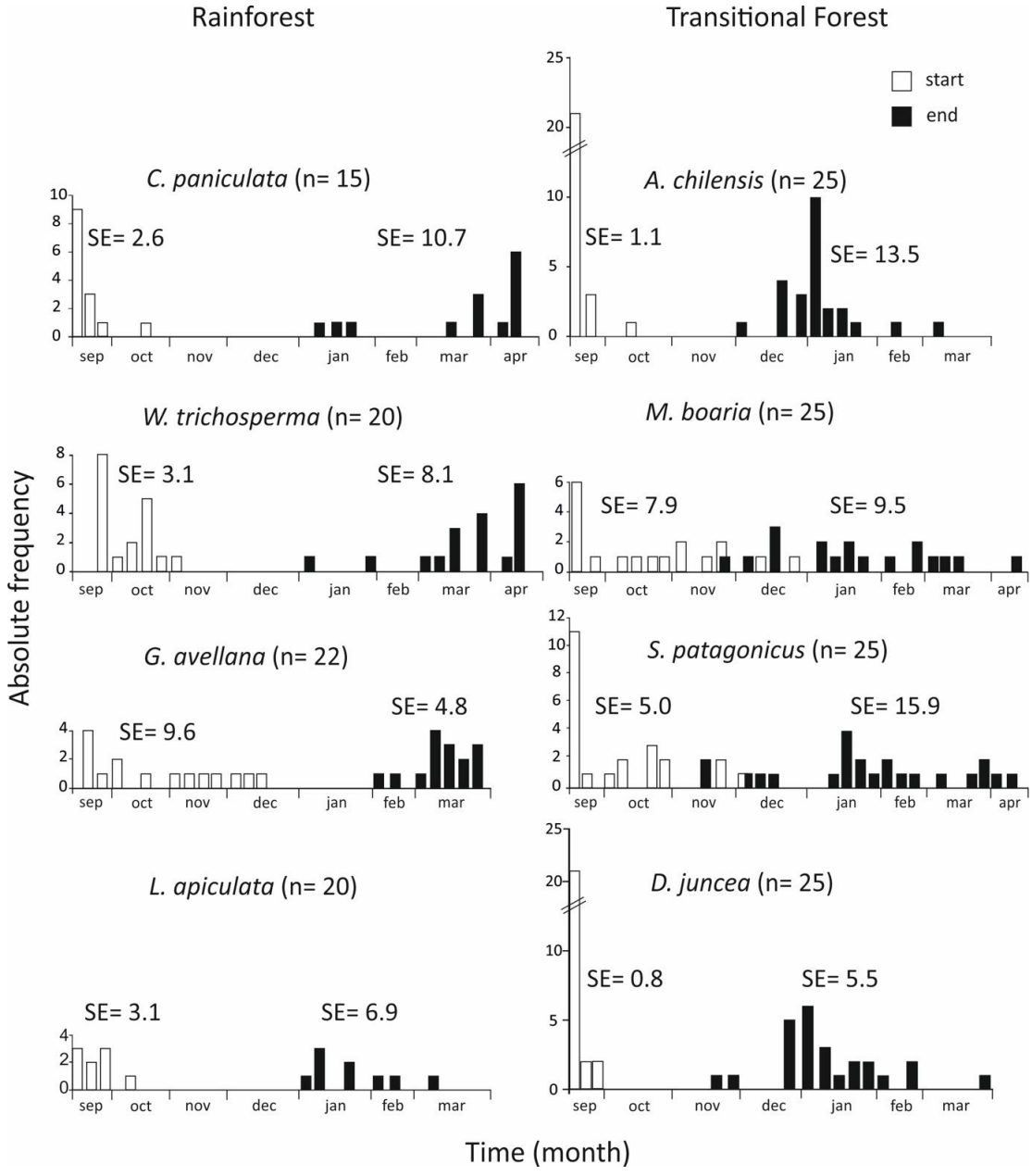

Figure 2. Frequency distributions of the times of shoot extension initiation (white bars) and end (black bars) for each of the eight species under survey. Above the white and black bars are the standard errors (SE) of extension initiation and end, respectively. Next to each species name are the number of individuals (n) studied. Left graphs: rainforest species, right graphs: transitional forest species.

Distribuciones de frecuencias de los momentos de inicio (barras blancas) y fin (barras negras) del crecimiento del brote para cada especie estudiada. Sobre las barras blancas y negras se encuentran los errores estándar (SE) del inicio y del fin del crecimiento, respectivamente. Al lado del nombre cada especie está el número de individuos (n) estudiados. Izquierda: especies de bosque lluvioso; derecha: especies de bosque de transición.

of *M. boaria* and *S. patagonicus* initiated their extension at a time when other co-specific individuals had already ended their extension periods. For the remaining species, extension initiation and end were several weeks apart. The time of RER_{max} was little variable in most rainforest species (considering the SE of this variable) and increased in this order: *D. juncea* (SE = 2.0), *A. chilensis* (SE = 2.9), *W. trichosperma* (SE = 3.0), *C. paniculata* (SE = 3.3), *L. apiculata* (SE = 3.6), *S. patagonicus* (SE = 6.0), *M. boaria* (SE = 6.2), *G. avellana* (SE = 6.7). Among rainforest species, *C. paniculata* and *W. trichosperma* presented the latest extension ends (up to April). Most *L. apiculata* shoots ended growing between January and February, whereas in the case of *G. avellana,* extension end was mostly concentrated in March. The highest variations in extension end were observed for two transitional forest species (*A. chilensis* and *S. patagonicus*) and the lowest one, for a rainforest species (*G. avellana*).

Among rainforest species (figure 3A) extension duration was longer for *C. paniculata* and *W. trichosperma* (26.9 and 24.3 weeks on average, respectively) than for *L. apiculata* and *G. avellana* (18.9 and 20.7 weeks). Significant variations between these species were confirmed by statistical analyses (F= 8.38; $P < 0.001$). For transitional forest species, extension duration tended to be shorter than 20 weeks; *Maytenus boaria* and *S. patagonicus* presented, on average, shorter extension durations (13.0 and 15.7 weeks, respectively) than those presented by *A. chilensis* and *D. juncea* (18.0 and 18.2 weeks; F= 5.82; $P < 0.001$; figure 3C). The lowest RER_{max} among RF species was that for *W. trichosperma*; all other rainforest species did not differ in this variable (figure 3B; F= 8.94; $P < 0.001$). Regarding transitional forest species, RER_{max} was the lowest for *A. chilensis* and the highest for *S. patagonicus* (figure 3D; F= 17.28; $P < 0.001$).

Weekly variations in absolute extension rate (AER) were associated with mean temperature variations during a limited period for most species (figure 4). AER was significantly correlated with weekly temperature only for *W. trichosperma* and *G. avellana* (Spearman's coefficient = 0.45 and 0.54, respectively). *Gevuina avellana* had only one peak in AER, which occurred during the period of highest temperatures. *Caldcluvia paniculata, A. chilensis, L. apiculata* and *D. juncea* had their highest AER during

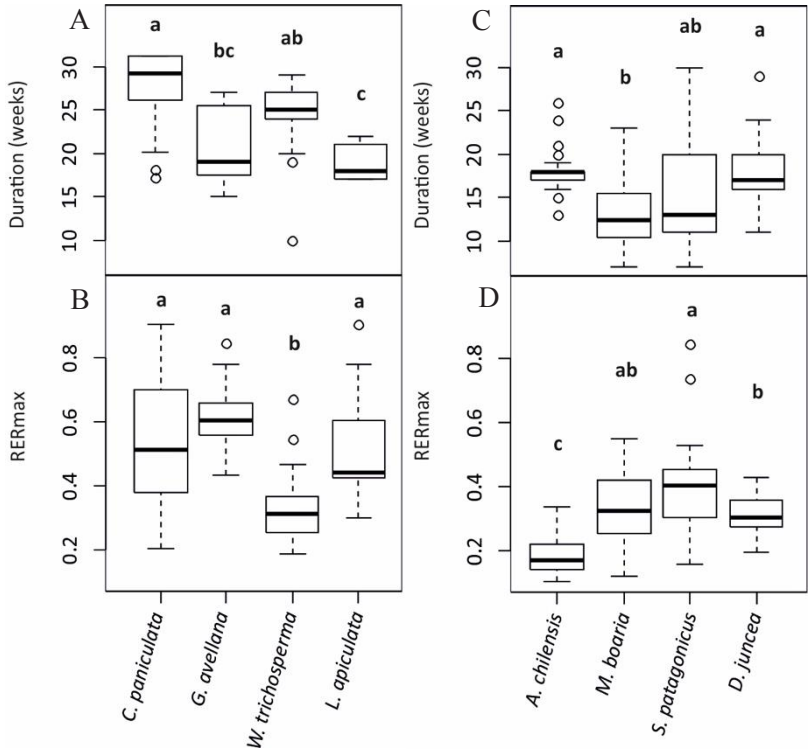

Figure 3. Extension duration and maximum relative extension rate for rainforest (A and B) and transitional forest (C and D) species. Boxes represent the range between 25 % and 75 % percentiles. Circles represent outlying data. The line in the box represents the median and whiskers mark the 5 % and 95 % percentiles. Different letters indicate significant differences obtained by statistical analyses.

Duración del alargamiento y tasa de alargamiento relativo máxima (RERmax) para las especies del bosque lluvioso (A y B) y para las de bosque de transición (C y D). Las cajas representan el rango entre los percentiles de 25 % y 75 %. Los círculos representan datos atípicos. La línea horizontal dentro de la caja representa la mediana y los bigotes marcan los percentiles del 5 % y 95 %. Las especies con letras iguales encima de sus cajas no difieren estadísticamente.

the first half of their respective extension periods, coinciding with increments in mean temperature. Increases in AER during the second half of the extension periods of these species did not go along with increases in temperature. It is worth noticing the increment in AER registered for *C. paniculata* close to the end of its extension period, which corresponded to a second growth flush in most individuals (figure 4). A second peak in the AER of *W. trichosperma* paralleled a December-January temperature increment. *Maytenus boaria* and *S. patagonicus* had several peaks in AER during the growth season. The degree of coincidence of weekly increases and decreases in temperature and AER, as evaluated through a binomial model, was variable among species (residual deviance null model = 186.36, residual deviance model with species= 145.9; $P < 0.001$). Overall, higher coincidences were observed for rainforest species than for transitional forest species, although the most contrasting results were those observed for *W. trichosperma* and *D. juncea,* for which the highest and the lowest levels of coincidence, respectively, were observed (table 1). Statistical differences were confirmed between *D. juncea* and all rainforest species, between *D. juncea* and *A. chilensis*, and between *A. chilensis* and *W. trichosperma*.

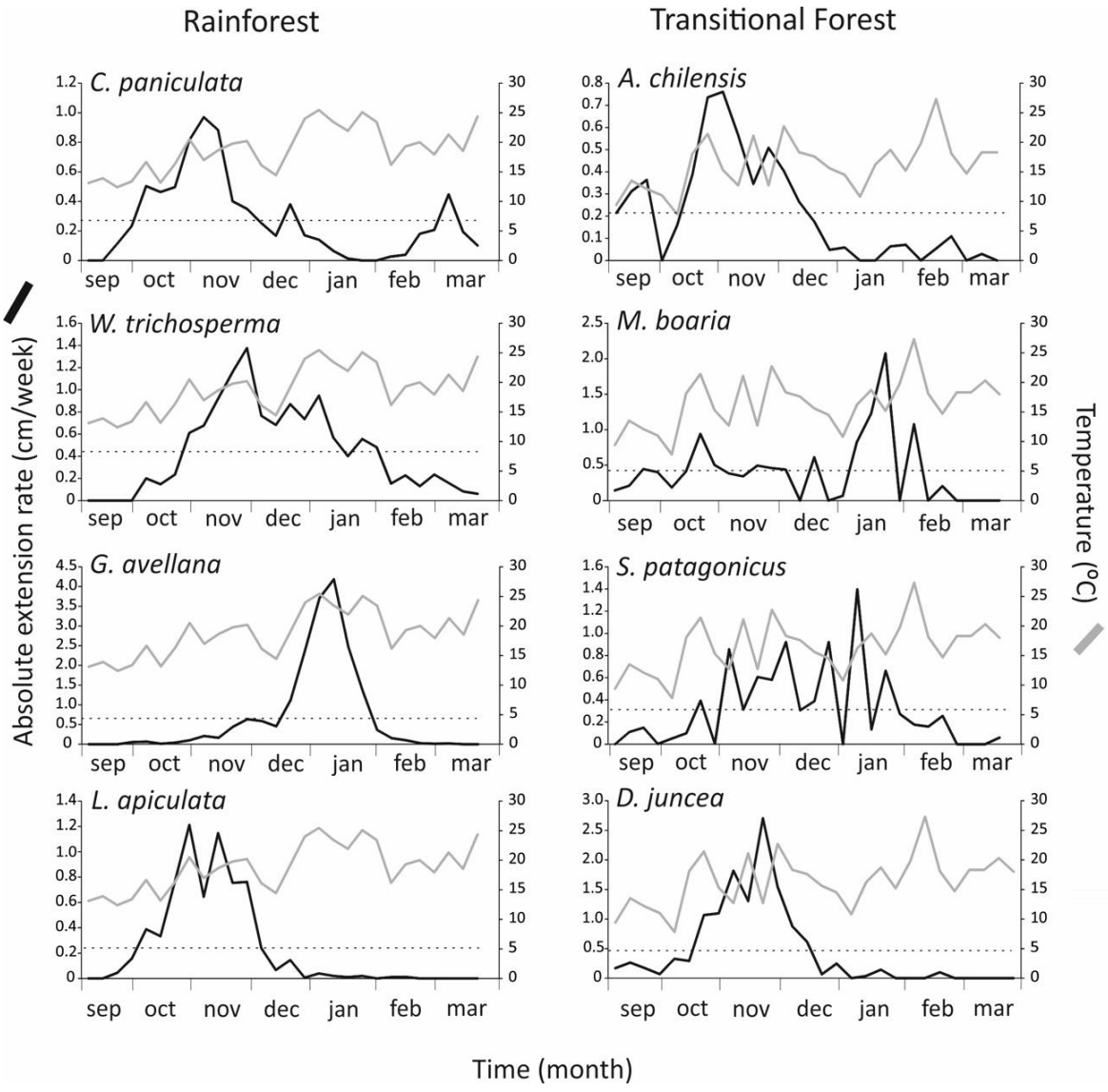

Figure 4. Mean absolute extension rate (AER) for each species (in black) and mean weekly temperature (in gray) for the observation periods. In each graph the average AER of each species is shown with a straight dashed line. Left graphs: rainforest species; right graphs: transitional forest species.

Tasa de alargamiento absoluto (AER) media de cada especie (en negro) y la temperatura semanal media (en gris) para los períodos de observación. En cada gráfico se muestra la AER promedio de cada especie como línea recta punteada. Gráficos a la izquierda: especies del bosque lluvioso; y gráficos de la derecha: especies del bosque de transición.

Table 1. Mean proportion of coincidence between weekly variations in absolute extension rate (AER) and temperature for the studied species (RF: rainforest species; TF: transitional forest species). Results of pairwise Tukey's comparisons are provided.

Proporción media de coincidencia entre variaciones semanales en la tasa de alargamiento absoluta (AER) y temperatura para cada especie estudiada (RF: especies de bosque lluvioso; TF especies de bosque de transición). Se proveen los resultados de comparaciones de a pares de Tukey.

Species	Group	Proportion of coincidence	Tukey's groups
Gevuina avellana	RF	0.60	AB
Caldcluvia paniculata	RF	0.56	AB
Weinmannia trichosperma	RF	0.65	A
Luma apiculata	RF	0.65	AB
Aristotelia chilensis	TF	0.54	B
Schinus patagonicus	TF	0.54	ABC
Maytenus boaria	TF	0.53	ABC
Diostea juncea	TF	0.42	C

DISCUSSION

Growth dynamics parameters. This study was aimed at evaluating extension patterns of groups of woody species typical of rainforests and transitional forests in North Patagonia; these two forest types are subject to contrasting climatic conditions, nonetheless are geographically close to one another (less than 50 km apart). Our study includes the assessment of primary growth of plants of these two forests in different common gardens and growth seasons, which prevents us from making direct comparisons between both species groups regarding certain growth parameters such as extension rate and duration. Since transitional forest species are adapted to higher seasonal climatic variations, we had expected that their extension patterns would be more endogenously fixed and less environmentally affected than those of rainforest species, which inhabit areas with longer periods of suitable conditions for growth, and are less exposed to extreme conditions. Although high levels of variation among species were observed concerning most aspects of growth dynamics analyzed, our predictions were only partially fulfilled. The beginning of shoot extension was highly variable for two of the transitional forest species, *M. boaria* and *S. patagonicus*, and for one of the rainforest species, *G. avellana*. These intra-specific variations in budbreak may also have an ontogenetic component, as indicated in studies on *Nothofagus* species (Stecconi *et al.* 2000); such a component of variability was dealt with in this study by restricting plant age and architectural stage, axis category and position of those shoots that were measured. Except for *G. avellana,* the time of extension initiation was less variable than the time of extension end. In temperate climates, the start of shoot extension is highly relevant as it determines the risk of damage by late spring frosts (Fady *et al.* 2003). This trait would have a complex genetic-environmental basis (Parker *et al.* 2017), therefore it has been proposed as a main signal of a species adaptation to local conditions (Fady *et al.* 2003). Low intra-specific variability of budbreak in a common-garden experiment could mean that plants are synchronized by an external signal and might exhibit high plasticity to adapt their extension periods to different conditions (Csilléry *et al.* 2019). Thus, co-specific individuals of those species with the lowest variations in budbreak*, i.e. C. paniculata, W. trichosperma, L. apiculata, D. juncea* and *A. chilensis*, would be able to respond coordinately to external signals such as increasing temperatures or daylength. In contrast, *M. boaria* and *S. patagonicus* had high variabilities in the time of budbreak, which could be related to genetic variability, and also had high variations in the time of RER_{max}. The latter results could imply that the time of RER_{max} may be conditioned by the time of budbreak in these species, and be little affected by environmental conditions. The contrary was observed for most rainforest species, in which RER_{max} was highly synchronized at the intra-specific scale.

In general terms, extension end was more variable than extension initiation and exhibited, contrary to our prediction, higher intra-specific variation for transitional forest than for rainforest species. The latest extension end was observed for *W. trichosperma* and *C. paniculata*: several plants of these species grew until early autumn, whereas most shoots of all studied species ended their extension during the summer. In a study on xylem development in conifers, it has been shown that the effect of photoperiod on cambial activity prevents shoot damage due to early frosts. Growth stops near the summer solstice (the warmest period of the year), which consequently allows lignification and cell wall production to finish early enough (Rossi *et al.* 2008). In the present study, although the growth season could extend beyond the warmest month of the year (January in this region; Conti 1998), the proportion of plants that did so was notably higher in the case of rainforest species with the exception of *L. apiculata* (but

see Puntieri *et al*. 2018). Thus, our results provide moderate support to the idea that rainforest species are adapted to conditions in which early frosts are unlikely.

Our initial hypothesis suggested that the growth dynamics of Patagonian rainforest species is adapted to regions in which environmental limitations (frosts and droughts) are less severe in the summer. Thus, longer extension durations would be expected in rainforest than in transitional forest species. Such hypothesis could be fully confirmed by means of experimental assessment including controlled environmental conditions. This is not the case of the present study, although we observed plants in similar ontogenetic stages and under non-limiting water conditions; that means extension durations recorded for rainforest species were higher (up to 15 weeks) than those for transitional forest species. For rainforest species, our results are similar to those reported for other Patagonian rainforest species in a recent common-garden study (Sosa and Puntieri 2016). Consequently, preliminarily, these results are in accordance with our initial hypothesis. The shorter extension duration of transitional forest species could be related to shoot protection against early frosts (Fady *et al.* 2003), which are more common towards the eastern limit of the Argentinean Patagonian forests, and to the avoidance of the driest summer periods. Nonetheless, the transitional forest species *S. patagonicus* and *M. boaria* showed high intra-specific variability in extension initiation, end and duration, which would explain the wide distributions of these species within the E-W precipitation gradient that characterizes north-western Patagonia (Donoso 2013). These intra-specific variations could be due to genetic factors. In addition to having longer extension durations, rainforest species had higher relative extension rates than those shown by transitional forest species. It is possible that rainforest species are more adapted to interspecific competition for light, so that they may be favored by having high shoot-extension rates (Poorter *et al.* 2006).

Extension rates and temperatures. In the present study we had predicted that those species characteristic of regions with narrower ranges of within-year temperature/rainfall variations (rainforest species) would be more responsive to temperatures in terms of shoot extension rates. This prediction was confirmed for two of the studied rainforest species (*W. trichosperma* and *G. avellana*), in which extension rates were correlated to air temperatures. On the other hand, weekly variations in extension rates tended to be more responsive to temperature variations in the case of rainforest species than in transitional forest species (although *W. trichosperma* was the only RF species that was significantly different from all transitional forest species in this regard). This result complies with the idea on which this study was based and it is worth remarking that all species under survey are distributed within a similar latitudinal range (Donoso 2013). More notably so, the extension rates of *W. trichosperma* and *G. avellana* not only increased with temperature, but also mirrored temperature changes in magnitude. This could imply that these species would be more affected by low temperatures during the growth season than any of the other investigated species.

The only species that showed a clear peak in growth rate towards the end of the growth season was the rainforest species *C. paniculata*. The second peak constituted evidence that this species is capable of growing late in the growth season, as long as external conditions are suitable. The possibility that the extension of *C. paniculata* could be regulated by external rather than endogenous factors is supported by the fact that its apical buds are devoid of specialized leaf-derived organs (Sosa 2019). Frosts might be a weak selective pressure in rainforests; therefore, it is plausible that some species exhibit growth rate peaks near the end of the growth season. This may explain the high rates of shoot apex deaths recorded for this and other Patagonian rainforest species when growing in areas with more seasonal climate (Sosa 2019).

Gevuina avellana was the only species that had the highest AER concentrated in a few weeks during the period of the growth season when temperatures were the highest, despite having highly variable RER. We observed that the time of RER_{max} was variable among individuals, nonetheless that those individuals whose RER_{max} coincided with the highest temperatures were those with the highest rates. *Gevuina avellana* stood up from the rest of the rainforest species in its high intra-specific variations in extension initiation and time of RER_{max}. This could be explained by its biogeographic origin instead of its current habitat, since *G. avellana* may, like other species of Gondwanan origin, belong to a linage with colder requirements (Villagrán 2018). The Gondwanan flora faced climatic changes after the breakage of the supercontinent, which caused the extinction of more than half of the species (Villagrán and Hinojosa 1997). Therefore, although *G. avellana* currently lives in the rainforests of Patagonia, it may have withheld functional traits that are frequent in regions under more seasonal climate, such as a highly variable RER_{max} or a short extension period. However, the other two rainforest species of Gondwanan origin that were considered here, *i.e*. *W. trichosperma* and *C. paniculata* (Aizen and Ezcurra 1998, Vasconcelos *et al*. 2017), differed notably from *G. avellana* in their growth patterns. When considering the Gondwanan vs Neotropical biogeographical origin of the species included in this study (based on Villagrán and Hinojosa 1997, Marx *et al*. 2010, Phoon 2015) apparently contradictory results arise concerning growth dynamics. Biogeographical origin, linage-history past and current selective pressures are to be considered to reach a better understanding of variability in the primary growth of trees.

Conclusions: extension patterns and climate in woody species. The distinction between rhythmic and continuous growth in trees appears, in theory, straightforward (Barthélémy and Caraglio 2007). However, seasonal cycles and

other periodical environmental factors may blur this distinction in many world regions. The primary growth dynamics of all species under survey here could, *a priori*, be categorized as rhythmic; nevertheless, there are reasons to believe that environmental factors may have a significant influence on the setting of resting periods, at least for some of them. The extension features investigated in this study, such as extension initiation and end, time of RER_{max}, extension duration and temperature effects on extension rate, exhibited high intra- as well as inter-specific variations under common garden conditions. Among the four rainforest species studied and despite inter- and intra-specific variability, we could establish that *W. trichosperma* and, to a lesser extent, *C. paniculata*, exhibit higher levels of variation in growth dynamics relative to some of the general trends that are typical to trees from temperate regions (see Kozlowski 1971).

We had proposed that, as climatic seasonality becomes sharper, the extension pattern of trees would become more 'conservative', since selection pressures would tend to reduce the possibility that sensitive newly-formed organs would be exposed to extremely cold, dry and/or dark periods of the year. Drought events and early frosts are highly likely during the summer in the region currently occupied by transitional forests, so that a strict endogenous control of growth dynamics was expected for the tree species that inhabit these forests. The less restrictive conditions under which rainforest species inhabit led us to expect a tighter growth adjustment to external conditions than to endogenous control. The differences were not as sharp as predicted, although the results found for *W. trichosperma*, *S. patagonicus* and *M. boaria* provided some support to our hypothesis. Future surveys on the growth dynamics of tree species from different forest types should not only consider current climatic conditions, but also biogeographic origin.

The results of this work show a variety of responses of the growth dynamics of tree species due to both endogenous and environmental causes. This knowledge helps to understand the adaptation of the species to the environment they occupy and allows us to predict the possible responses of the species to future climate changes. This information could be applied so as to determine sites with high conservation value and is relevant for restoration plans of the Patagonian forests. In this context, species with high endogenous variations regarding growth initiation (*e.g. M. boaria* and *S. patagonicus*) could be suitable for restoration programs in a variety of environments. The description of endogenous patterns of primary growth could be also a valuable tool for establishing protocols of nursery care for each species; for example, the length of the extension period allows determining the time-lag when irrigation or risk of frost damage are more important.

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Visualization of water transport pathways in various organs on the fruit-bearing shoot of walnut trees

Visualización de vías de transporte de agua en varios órganos del brote frutal de nogales

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SUMMARY

To reveal the regular patterns of water transport in fruit-bearing shoots of walnut (*Juglans regia*), water transport pathways for various organs were observed using dye-tracing technique; water potential, water status and water transport rate were determined for each of these organs. Water potential in the pedicel, petiole, lateral shoot and main shoot was -2.59, -2.85, -1.68 and -0.37 MPa, respectively. Significant differences were noted among various organs in terms of water status. The ratio of bound water to free water in the petiole was the highest (1.93 RBC), followed by the main shoot, lateral shoot and pedicel. Water transport speed in the petiole was the fastest (3.13 cm/min), followed by the lateral shoot, pedicel and main shoot. However, the water transport rate of the main shoot was the highest, and higher than the sum of the other three organs. The xylem in the petiole was separated from the main shoot; at the top of the main shoot, the xylem was divided into two parts, one of which was connected to the xylem of the pedicel and the other to the lateral shoot. During the shell-hardening period of fruits, the dye was found at the edge of the fruit vascular bundle, although, it was not at the central vascular bundle. No dye was found in the main and accessory buds, instead, only a small part of the xylem was dyed between the accessory bud and the main shoot. Water transport pathways were different for various organs. Thus, the transport and distribution of water in various organs was affected by their water potential, tissue structure and water requirement.

Key words: Juglans regia, water, dye-tracing, water potential, xylem.

RESUMEN

Para revelar pautas regulares de transporte de agua en brotes frutales de nogal (*Juglans regia*), se observaron vías de transporte de agua de diversos órganos mediante rastreo de tintes; se determinó: potencial hídrico, estado del agua y tasa de transporte de agua de cada órgano. El potencial hídrico en el pedicelo, peciolo, brote lateral y brote principal fue de -2,59, -2,85, -1,68 y -0,37 MPa, respectivamente. El estado del agua mostró diferencias significativas entre los órganos. La relación entre el agua ligada y el agua libre en el pecíolo fue la mayor (1,93 RBC), seguida por: brote principal, brote lateral y pedicelo. La velocidad de transporte de agua en el pecíolo fue la mayor (3,13 cm/min), seguida del brote lateral, pedicelo y brote principal. Pero la tasa de transporte de agua del brote principal fue la mayor y más alta que la suma de los otros tres órganos. El xilema del peciolo estuvo separado del brote principal; en su parte superior estuvo conectado al xilema del pedicelo y al brote lateral. Durante el período de endurecimiento de la cáscara del fruto, el colorante estuvo en el borde del haz vascular del fruto, pero no en el haz vascular central. No se encontró colorante en yemas principales ni accesorias, en cambio, solo se tiñó una pequeña parte del xilema entre la yema accesoria y el brote principal. Las vías de transporte de agua fueron diferentes para los distintos órganos. El transporte y distribución del agua en varios órganos fueron afectados por su potencial hídrico, la estructura de los tejidos y las necesidades de agua.

Palabras clave: Juglans regia, agua, rastreo de colorante, potencial hídrico, xilema.

INTRODUCTION

Water is the carrier of plant nutrients during plant functioning, and a variety of physiological and metabolic reactions take place in water, directly or indirectly. Therefore, water is essential for the material circulation and energy metabolism in plant operation. Water plays a decisive role in the activities of plants, and the study of plant water physiology remains a key issue in plant physiology (Johnson *et al.* 1965, Blackman *et al.* 1985, Cochard *et al.*

2002, Javaux *et al.* 2016). The process of water transport in plants is driven not only by the negative pressure generated by leaf transpiration, and the growth and metabolic activities of tissues and organs, but also by the external environment, the tissue structure and water requirement of organs (Markhart *et al.* 1979, Ford *et al.* 2004, Martorell *et al.* 2013). Much of the current research is primarily focused on studying the influence of external environmental factors on plant water transport. In contrast, there are very few reports on the structure of water transport pathways for various plant organs (Xie *et al.* 2016).

With the developments in the study of transport physiology, it has been established that the long-distance transport of water from the roots to the leaves is carried out mainly through the xylem, a process in which xylem vessels play an important role (Fricke 2017). The driving force for conducting both water and dissolved mineral ions from the roots to the leaves is primarily composed of two forces: the transpiration pull from leaves and root pressure (Tyree *et al.* 2002). Vessels show a great variety of structural features that could affect water transport in xylem of plants (Taneda *et al.* 2007). Drought as well as freeze-thaw events can induce embolism in the plant xylem. Embolisms can impede water transport in plants, in severe cases leading to the death of roots, branches and even whole plants (Schenk 2014). Hydraulic conductivity, number of vessels, average diameter of vessels and vessel length, all show a gradual exponential decrease from the base of the plant to higher up the stem (Nijsse 2001). Sperry (1991) suggested that in rapidly growing *Populus tremuloides* Michx. branches, the vessels of the outer growth ring were functional, whereas vessels in older xylem were mostly embolized. Water transport in plants is not just a simple flow through xylem vessels and sieve tubes, but also includes xylem-phloem exchange during transport (Patrick *et al.* 2001). A study by Ohya (2008) showed that the net volume of water escaping from xylem vessels was not dependent on the transpiration rate of the plant. The self-diffusion effect of water was strong for lateral water movement, although another driving force besides thermal motion was included in the process, and the process was also affected by the water permeability of the plasma membrane. Transport across the cell-to-cell pathway can involve water crossing plasma membranes, and thus, the rate of water uptake can be influenced by the abundance and activity of aquaporins (Gambetta 2013).

Walnut (*Juglans regia* L.) is a deciduous tree of the Juglandaceae, and is one of the famous "four nuts" in the world. Walnut forests provide several ecological and economic benefits (Gauthier *et al.* 2011). Walnut cultivation has become an important pillar industry for mountain farmers to improve their economic situation, and it has been listed as a national strategic economic forest tree by the State Forestry Administration in China. It has been shown that the yield and quality of the walnut depend directly on the status of water supply (Cristofori *et al.* 2009). Shoots are the main parts of walnut trees during the growing season, and fruit-bearing shoots support many other organs such as leaves and buds. Shoots are not only a part of tree structure, but also the main organ of water consumption through the leaves growing on shoots. These organs represent key avenues of water use in tree bodies, even though there are some differences in their water use patterns and water needs, due to the fact that the differences would be structural in terms of wood anatomy. Understanding the xylem structure and water utilization patterns of various organs on the fruit-bearing shoot can provide a theoretical basis for rational irrigation and efficient water use in walnut trees.

In this study, the water transport pathways for each organ were observed using the dye-tracer method for elucidating the fruit-bearing shoot water utilization patterns in walnut. In addition, we investigated water potential in different organs across time and developmental stages by obtaining data on water potential, water status and water transport rate. We combined different approaches to comprehensively map out the potential routes for water transport and the physiology behind them. Results would be especially relevant in an ecological context, primarily in cases where there is limited water availability.

METHODS

Site description. The study was conducted in the trial orchard of Hebei Agricultural University, which is located in the Lianchi District of Baoding City, Hebei Province (38°49′–38°56′N, 115°21′–115°34′E). This area belongs to the warm temperate continental monsoon climate, with four distinct seasons. Annual average temperature is 12.7 °C, the highest temperature is 43.3 °C, and the lowest temperature is -26.8 °C. Annual sunshine time is 2,447–2,871 hours, the frost-free period lasts 165–210 days, average annual rainfall is 575.4 mm, and average annual evaporation is 1,758.3 mm. The soil type is sandy loam.

Experimental materials and methods. The walnut trees were seedlings of the precocious walnut cultivar Juglans regia "lvling" which were sowed in the spring of 2015. The Walnut trees were about 1.0 cm in diameter and 1.5 m in height. The row spacing was 20 cm × 40 cm, and the direction of planting was east-west.

At the shell-hardening period, 18 fruit-bearing shoots with 1–2 fruits and one lateral shoot were selected to carry out the experiments from 120 total samples. Six of them were used to determine physiological indexes (plant water potential, plant bound water and free water), and the other 12 were used for dye-tracer observation. Dye-tracer observation was carried out at about 9:00 am on June 20, 2017 (temperature is 29 ºC), and related physiological indexes were measured at the same time. Specific test contents are as follows:

1. Water potential: Water potential was determined using a Psypro dew point water potential measurement system (Wescor, Logan, America).

2. Plant water contents: Total, free, and bound water were determined by Abbe refractometer according to the method described by Slavik (1974).

3. Water transport rate: Cut the organs off the walnut tree and place the cut end immediately inserted into a plastic bottle with the dye solution. After 5 min, the transport distance of the dye in the organs could be observed. Through the distribution of dyes in different organs, the cross-sectional area of water passing through can be calculated. Transport rate = transport distance / time×the cross-sectional area of water passing.

4. Observation of water transport pathway: The water transport pathway of organs was observed by the dye-tracing technique, following Bhaska *et al.* (2005). The dye used in the experiment was 0.2 % (m/V) basic fuchsin solution. The cutting end of the fruit branch of walnut was put into the dye and removed after 2 hours. The distribution of dye in various organs or tissues were observed under an Olympus SZ61 stereo microscope (Olympus, Tokyo, Japan) and a Motic BA210 basic biological microscope (Motic, Amoy, China).

Statistical analyses. The differences among organs were detected using one-way analysis of variance (ANOVA). The statistical evaluation of data was performed by the DPS 7.05 program ($P \leq 0.05$).

RESULTS

Water transport between the main shoot and the leaf. At the node between the petiole and the shoot, the water transport pathway in the petiole was associated with the main shoot by the xylem that separated from the main shoot, and the xylem of petiole came from the inner layer of the main shoot xylem (figure 1A–E). Radial distribution of the dye indicated that the xylem of the petiole came from the

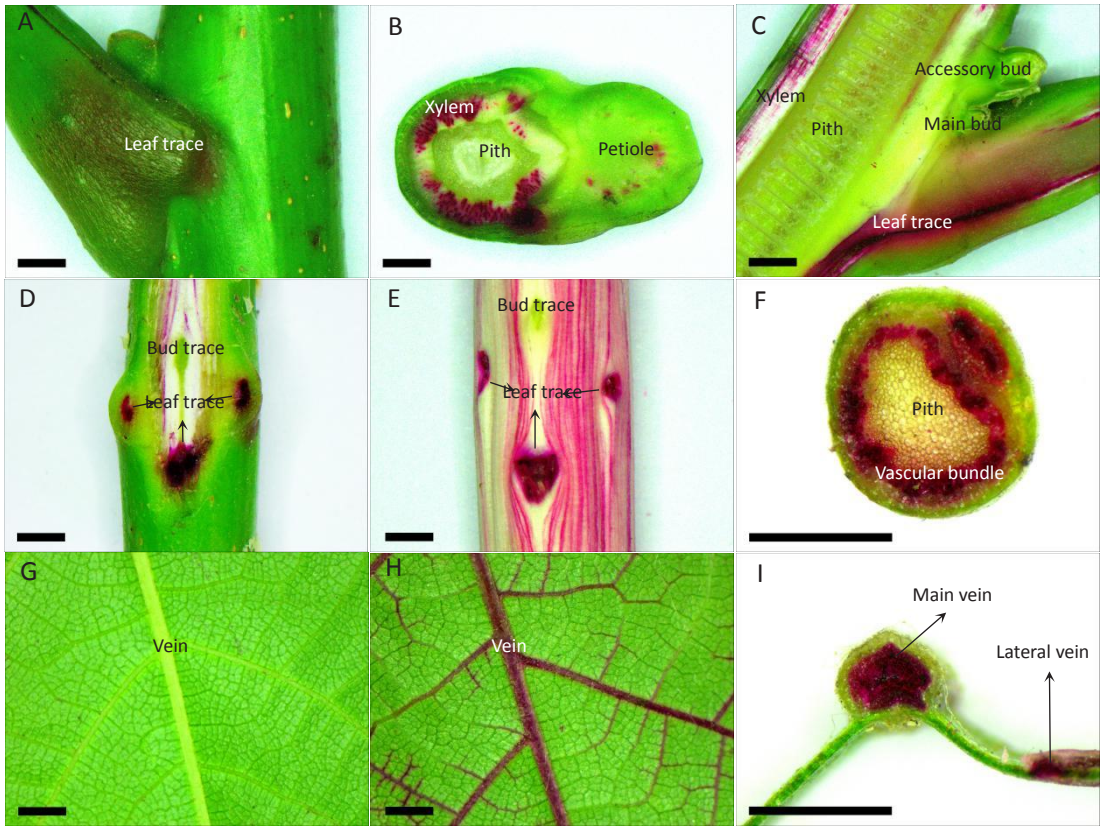

Figure 1. Distribution of the dye in the main shoot and the leaf. A: The junction of the main shoot and the leaf; B: The cross-section of the junction of the main shoot and the leaf; C: The Longitudinal section of the junction of the main shoot and the leaf; D: The Lateral section of the junction of the main shoot and the leaf; E: The xylem of the junction of the main shoot and the leaf; F: The cross-section of the petiole; G: The leaf before and after the entering of dye; H: The cross-section of the vein. The length of the scale in the graph is 2.5 mm.

Distribución del colorante en el brote principal y en la hoja. A: Unión del brote principal y la hoja. B: Sección transversal de la unión del brote principal y la hoja. C: Sección longitudinal de la unión del brote principal y la hoja. D: Sección lateral de la unión del brote principal y la hoja. E: Xilema de la unión del brote principal y la hoja. F: Sección transversal del peciolo. G: Hoja antes y después de la entrada del tinte. H: Sección transversal de la vena. La longitud de la escala del gráfico es de 2,5 mm.

primary xylem at the base of the internode of the main shoot (figure 1C). From the distribution of the dye in the leaves, it was clear that the regular pattern of water entering the leaves is: the petiole → main leaf veins → lateral leaf veins → mesophyll cells (figure 1G and H). In the vascular bundles of the petiole, xylem and a part of the phloem were dyed red (figure 1F), the vascular bundles and pith of the veins were also dyed red (figure 1I), while the dye could not be seen clearly in the phloem of the main shoot. This phenomenon indicated that the rate of lateral diffusion of water in various organs was different.

Water transport between the main shoot and the lateral shoot, buds and fruit. The lateral shoot was developed from the bud on the main shoot, and the connection between the main shoot and the lateral shoot was similar to that between the main shoot and the pedicel (figure 2A and B). The dye color of the longitudinal section at the junction of the main shoot and the pedicel suggested that the water transport rate in the lateral shoot was similar to that in the petiole, whereas both of them were clearly faster compared with the pedicel.

In the dye-tracer experiment, the dye was not seen in the main bud (figure 2D–F), which indicated that the main bud did not establish a direct contact with the xylem of the main shoot during the shell-hardening period, which retained a relatively independent status. Although no dye was found in the accessory bud either, a small portion of the xylem between the accessory bud and the main shoot was dyed (figure 2E and F). The accessory bud had just sprouted, and the main bud was in dormancy at this time. It can be concluded that the xylem in the bud was formed after the sprouting.

From the longitudinal section of the junction of the main shoot and other organs, it was found that the pith of the main shoot was curved towards the pedicel (figure 2B). The xylem of the pedicel was connected directly with that of the main shoot, and water flowed in sideways into the fruit at the junction of the main shoot and pedicel (figure 2G–I). From the distribution of the dye in the pedicel, only the

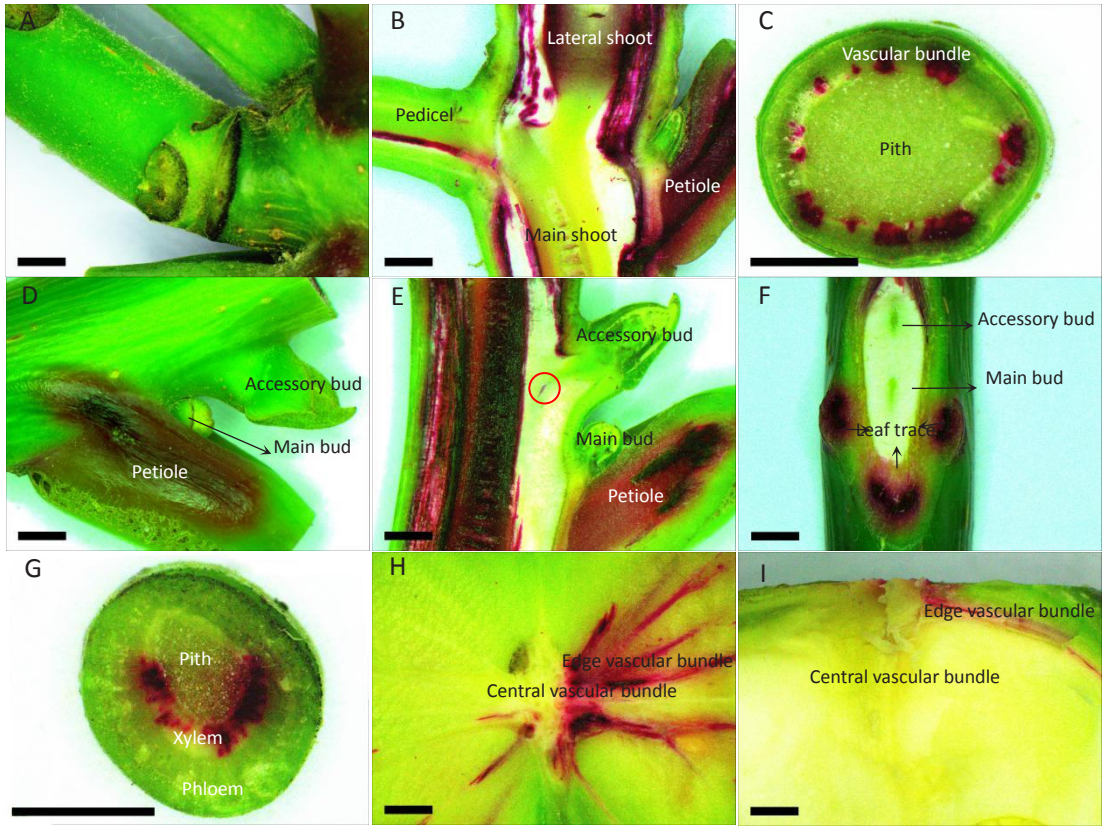

Figure 2. Distribution of the dye in the main shoot, lateral shoot, fruit and buds. A-B: Distribution of the dye at the junction of the main shoot and the pedicel, lateral shoot and leaf; C: Longitudinal section of the lateral shoot; D-F: Distribution of the dye at the junction of the main shoot and the bud; G: Longitudinal section of the pedicel; H-I: Distribution of the dye in the fruit. The length of the scale in the graph is 2.5 mm.

Distribución del colorante en el brote principal, el brote lateral, el fruto y las yemas. A-B: Distribución del colorante en la unión del brote principal y el pedicelo, el brote lateral y la hoja. C: Sección longitudinal del brote lateral. D-F: Distribución del colorante en la unión del brote principal y la yema. G: Sección longitudinal del pedicelo. H-I: Distribución del colorante en el fruto. La longitud de la escala del gráfico es de 2,5 mm.

xylem in the underside of the pedicel was dyed (figure 2B and G), which indicated that the xylem at the underside of the pedicel was the main water transport pathway to the fruit. The dye was found at the dorsal vascular bundles of the fruit (figure 2H and I) nevertheless, it was not found in the central vascular bundle of the fruit (figure 2I). The result clearly indicated that the water requirement for kernel development was low, and the kernel maintained its internal water balance through water diffusion with the husk.

Structural characteristics of water transport channel. Structural characteristics of the water transport channel of each part of the fruit-bearing shoot are presented in figure 3. The diameter of vessels in the pedicel was the least, about 0.02 mm, and was significantly lower compared with the other parts. The density of vessels in the petiole was the highest, which was about the same as that in the pedicel, whereas lateral shoot had lowest density of vessels. From the distribution of the dye in the cross-section of various organs, it could be seen that the dye color of various xylem vessels in the same organ was different and no dye was found in some of the xylem vessels. The result indicated that the water transport rate among various xylem vessels was also different, and it was not necessarily associated with the diameter and density of the catheter.

Water potential in organs. The water potential of the various organs on the fruit-bearing shoot exhibited significant differences (figure 4). The water potential of the main shoot was the highest, followed by the lateral shoot and the pedicel, whereas the petiole showed the lowest value. There was a large difference in the water potential between the main shoot and the pedicel and petiole, although the difference between the main shoot and the lateral shoot was relatively much lower.

Water status in organs. Water status in various organs on the fruit-bearing shoot is presented in figures 5 and 6. The free water content in the lateral shoot was the highest, which was significantly higher than that of the main shoot and the petiole. The free water content in the pedicel was not significantly different from that in the lateral

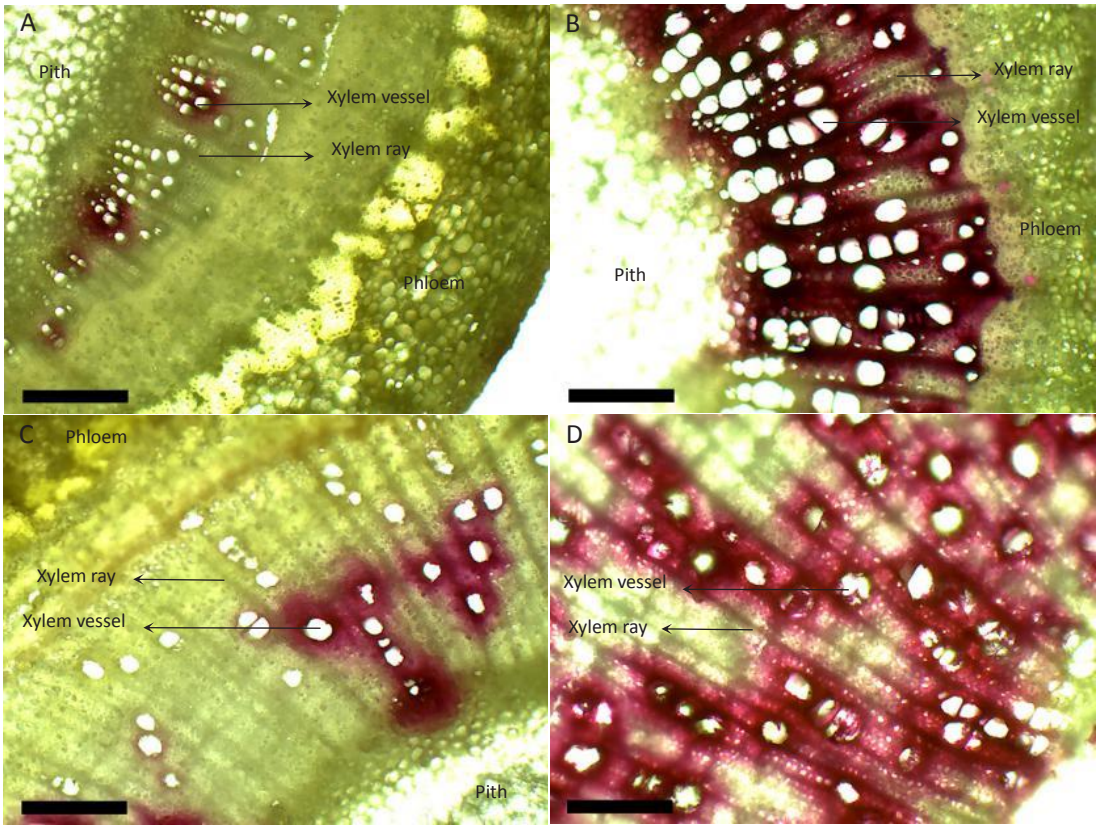

Figure 3. Distribution of the dye in xylem of the pedicel, petiole, lateral shoot and main shoot. A: Distribution of dye in the xylem of pedicel; B: Distribution of dye in the xylem of petiole; C: Distribution of dye in the xylem of lateral shoot; D: Distribution of dye at the xylem of shoot. The length of the scale in the graph is 0.25 mm.

Distribución del colorante en el xilema del pedicelo, el peciolo, el brote lateral y el brote principal. A: Distribución del colorante en el xilema del pedicelo. B: Distribución del colorante en el xilema del peciolo. C: Distribución del colorante en el xilema del brote lateral. D: Distribución del colorante en el xilema del brote. La longitud de la escala del gráfico es de 0,25 mm.

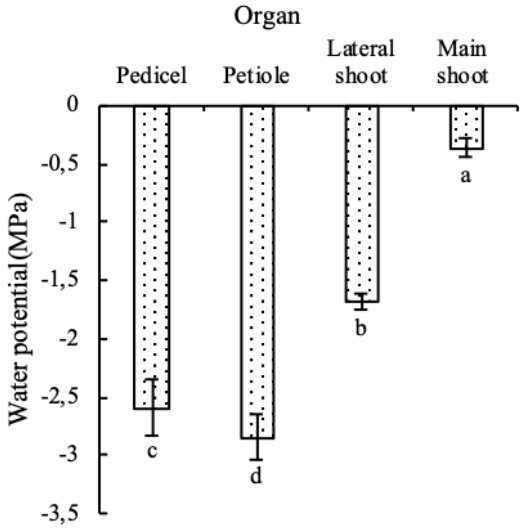

Figure 4. The water potential of various organs on the fruit-bearing shoot. Different lowercase letters indicate that there are significant differences among the treatments ($P < 0.05$).

Potencial hídrico de varios órganos en el brote del fruto. Diferentes letras minúsculas indican que hay diferencias significativas entre los tratamientos ($P < 0,05$).

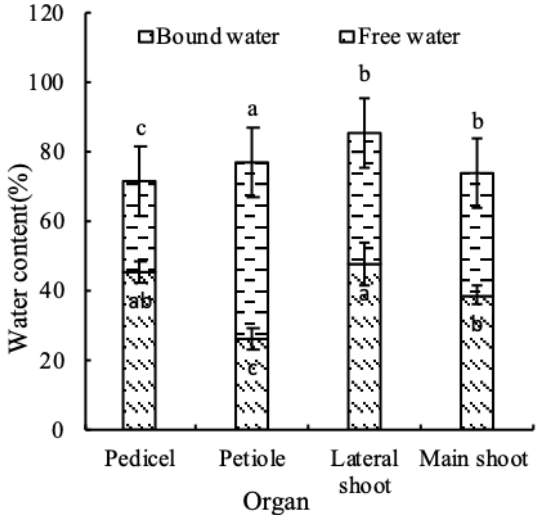

Figure 5. The water status in various organs on the fruit-bearing shoot.

Estado hídrico de varios órganos en el brote frutal.

shoot. However, the highest bound water content was observed in the petiole, which was significantly higher than the others, whereas the bound water content in the pedicel was the lowest. The total water content in the lateral shoot was the highest, which was significantly higher than the others. There was no significant difference among the pedicel, the lateral shoot and the main shoot regarding the ratio of bound water to free water, and these were significantly higher than the ratio of bound water to free water of the petiole.

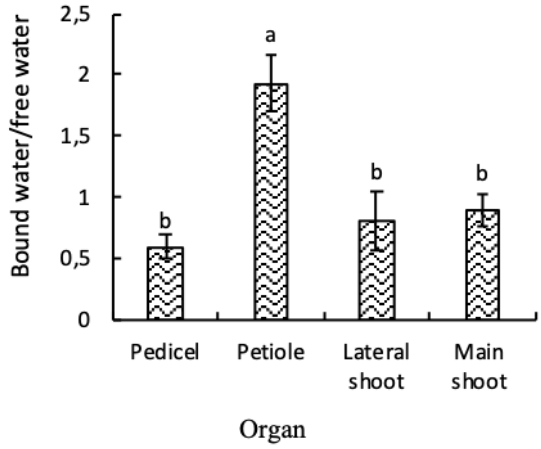

Figure 6. The ratio of bound water to free water in various organs on the fruit-bearing shoot.

Relación entre el agua ligada y el agua libre en varios órganos del brote frutal.

Water transport rate in organs. The leaf, bud, fruit and lateral shoot were growing on the main shoot, which is the last passageway of water into these organs through long distances. The water in the leaf, bud, fruit and lateral shoot was directly derived from the main shoot, and therefore, these organs form a competitive relationship with each other, such that the water transport rate in one part would directly affect the distribution of water in others. The water transport speed in various organs exhibited significant differences ($P < 0.05$) (figure 7). The water transport speed in the petiole was the highest, and it was significantly higher compared with that in the main shoot and the pedicel. The water transport speed in the lateral shoot showed no significant difference with that in the petiole. It was found that the higher the degree of lignification, the slower was the water transport rate. However, the water transport rate of the main shoot was the highest, and higher than the sum of the other three organs.

DISCUSSION

There was a significant difference in the water transport rate for the various organs on the fruit-bearing shoot due to the difference in their anatomic structure and water requirement. In this experiment, the water transport pathway from the main shoot to the other organs of the fruit-bearing shoot could be clearly seen using the dye-tracing technique. During the longitudinal transport of water, the water transport pathways from the main shoot to the other parts were independent from each other, and the corresponding xylem was not connected with each other. The position of the lateral shoot, leaf and fruit was close to the

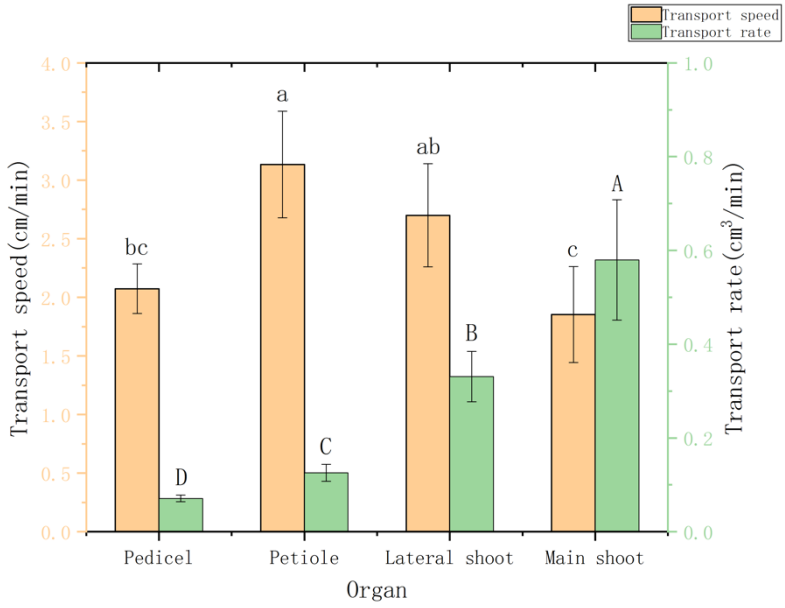

Figure 7. The water transfer speed and transfer rate in various organs on the fruit-bearing shoot.

Velocidad de transferencia de agua y la tasa de transferencia en varios órganos del brote frutal.

main shoot, and the water competition relationship among them was mainly related to their water potential (Tyree and Zimmermann 1983) and structure (Jožica *et al.* 2017). The traditional cohesion-tension theory held that the low water potential produced by transpiration was the main driving force for water transport in the xylem (Tyree 1997). Zhang *et al.* (2006) found that there are stomata on different green organs, though the number of stomas exhibited significant differences. The difference in the number of stomata caused differential transpiration pull, which may be the main reason for the difference in water transport rate between different organs on the fruit-bearing shoot, although it is also limited by the diameter and structure of the conduit. If the transport rate was determined only by the potential difference, the water transport rate should have been higher in the pedicel than in the lateral shoot, as the water potential in the former was lower than that in the latter, though the result was the opposite. The differences in the structure of the two organs (the water transport pathway at the junction between the main shoot and the lateral shoot was straight, while the water transport pathway between the main shoot and the pedicel was curved) were suspected to make the difference in terms of resistance to water. Zimmermann *et al.* (2004) also mentioned that in addition to transpiration pull, other factors play an important role in the long-distance transport of water. It was found that the pith of the petiole was all dyed, a part of the pith of the lateral shoot was dyed, and the pith of the pedicel and the main shoot were not dyed (figure 6B). This indicated that there was a horizontal transport of water in plants, nonetheless there were differences in the horizontal transport rate of water in different organs. Using hydrogen isotope tracer technique, Liu *et al.* (1998) confirmed that water can be transported laterally in plants. The difference in the rate of lateral diffusion of water may be related to water loss in the stomata of the epidermis and metabolic processes in the cell.

Water is an important factor in the growth and development of organs or tissues. At the beginning of sprouting, the accessory buds had no xylem, and there was a small part of the xylem found between the accessory bud and the main shoot (figure 2E). However, there was no xylem between the accessory bud and the main shoot before sprouting (figure 1C). Result indicated that the connecting xylem between the shoot and buds was formed after sprouting, and the water requirement for the organs was different in different developmental stages. When Drazeta *et al.* (2004) and Dichio *et al.* (2003) studied the water transport pathway in apple fruit, it was found that the water requirement of fruits was different at different developmental stages. The water transport efficiency in the xylem of the fruit decreased with the growth and development of the fruit. This could explain why the dye was found only in the edge vascular bundle of the fruit though no dye was found in the central vascular bundle of the fruit (figure 2H and I). Choat *et al.* (2009) and Wang *et al.* (2015) extensively discussed the reasons for the decline in xylem water transport rate in the fruit, and found that, during the development of fruits, the expansion of parenchyma cells in the xylem caused the main vascular bundle to be extruded and deformed, which resulted in the dispersion of some vascular bundles and the dislocation between the vessels that lose the collaboration

function, such that the water transport rate in the xylem decreased. Understanding the water requirement of the fruit at different developmental stages could help make the irrigation strategies more scientific.

Not only the width increments but also the anatomy of the xylem and phloem differed in the stem and branches. The xylem vessel is the main channel for longitudinal water transport, and vessel features strongly influence the amount of water that can be transported in a living tree. There is a strong vessel size–conductivity relationship because the hydraulic efficiency of the vessel increases proportionally to the fourth power of its radius (Hagen–Poiseuille law); therefore, even small differences in vessel size would drastically change water transport efficiency and security (Lynch *et al.* 2014). Jožica *et al.* (2017) found an inverse correlation between vessel density and diameter in all sampling parts, which can be explained by hormonal regulation (Aloni 2015). In addition to the auxin radial distribution pattern, steep concentration gradients of soluble carbohydrates (particularly sucrose) across developing vascular tissues in plants suggest a role for sugar signaling in vascular development (Uggla *et al.* 2001).

CONCLUSIONS

During the longitudinal transport of water, the water transport pathways from the main shoot to the other parts were independent of each other, and no connection was observed among their xylem tissues. Water was transported vertically and horizontally in plants, and there was a significant difference in the transport rates for different organs. The longitudinal water transport rate was determined mainly by water potential, and the organizational structure of organs also showed a significant effect. Moreover, the water requirement of the organs at different developmental stages was different. However, the water transport rate in organs was determined in vitro using the dye-tracer method, and the dye also had some effect on the water transport rate. We need to look for better technical means to determine the real water transport rate in organs under normal growth. In addition, we also need to further study the structure of various organs to better explain the mechanism of water transport, for example, the proportion of different types of vessels and the water transport rate in various types of vessels.

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Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aceptado: 25/05/20

Instrucciones para los autores de la revista Bosque, proceso de publicación y políticas para los árbitros

Actualización de fecha: agosto 2011

Instrucciones para los autores

Bosque es una revista científica que publica trabajos originales relacionados con el manejo y producción de recursos forestales, ciencias y tecnología de la madera, silvicultura, ecología forestal, conservación de recursos naturales y desarrollo rural asociados con los ecosistemas forestales. Las fechas de publicación son en abril, agosto y diciembre de cada año. Las contribuciones podrán ser en las modalidades de artículos, revisiones, notas u opiniones, en castellano o inglés.

- *Artículos.* Informan acerca de investigaciones inéditas de carácter científico que proyectan el conocimiento actualizado en un campo particular contemplado en los ámbitos de la revista y están sustentados en datos procedimentales propios o generados a partir de otros estudios publicados. La extensión máxima de los manuscritos será de 8.000 palabras, considerando todo su contenido (incluye todos los archivos del manuscrito con sus contenidos completos).
- *Revisiones*. Síntesis y discusión de la información científica más actual con respecto a un tema relevante en el ámbito de la revista. La extensión máxima de los manuscritos será de 8.000 palabras, considerando todo su contenido.
- *Opiniones.* Analizan, desde un punto de vista personal o con apoyo bibliográfico, un tema de actualidad relacionado con el carácter de la revista. La extensión máxima de los manuscritos será de 3.000 palabras, considerando todo su contenido.
- *Notas.* Describen metodologías o técnicas nuevas en el ámbito de la revista, o bien informan acerca de investigaciones en desarrollo, con resultados preliminares. La extensión máxima de los manuscritos será de 3.000 palabras, considerando todo su contenido.

Estructura de los manuscritos

La organización de artículos y notas debe seguir la siguiente estructura:

- *Título*. El título debe ser preciso y conciso. Elegir con mucho cuidado todas las palabras del título; su asociación con otras palabras debería ser cuidadosamente revisada. Debido al acceso internacional de la revista, se recomienda incluir en el título información relevante sobre la localización geográfica del estudio cuando corresponda.
- *Autores*. Indicar el nombre y apellido de todos los autores con letras minúsculas, con las letras iniciales en mayúscula. Separar los autores con coma. Ordene cada dirección mencionando los datos necesarios, primero la institución matriz (por ejemplo, la universidad) y luego las dependencias dentro de aquella en orden decreciente (por ejemplo, facultad, departamento, laboratorio); a continuación indique la ciudad y el país de residencia del autor. Aplique el formato del siguiente ejemplo:

Nombre1 Apellido1 [a], Nombre2 Apellido2 [b]*, Nombre3 Apellido3 [a,b]

[a] Universidad Uuu, Facultad Ffff, Departamento de Dddd, Ciudad, País.

*Autor de correspondencia: [b] Instituto de Iiiii, Departamento de Dddddd, Nombre de calle y número, Ciudad, País, tel.: 56-63-2221056, correo@electronico.cl

- *Resumen*. Debe contener el planteamiento del problema, el objetivo, fundamentos metodológicos, resultados y conclusiones más relevantes, con un máximo de 250 palabras. Evite descripciones largas de métodos y no incluya citas bibliográficas ni los niveles de significancia estadística.
- *Palabras clave*. Como máximo cinco palabras (puede incluir una o dos frases breves de un máximo de tres palabras) que identifiquen claramente el tema del trabajo. Se sugiere usar nuevas palabras no incluidas en el título del manuscrito.
- *Introducción.* Comprende planteamiento del problema, importancia del tema, hipótesis si compete, objetivos, alcances del trabajo y limitaciones para su desarrollo, si es que las hubo. En este capítulo se realizará una síntesis e interpretación de la literatura relacionada directamente con el título y objetivos del trabajo.
- *Métodos.* Proveerá información suficiente y concisa de manera que el problema o experimento pueda ser reproducido o fácilmente entendido por especialistas en la materia. Deberán señalarse claramente las especificaciones técnicas y procedencia de los materiales usados, sin describir materiales triviales. Los organismos bióticos deberán ser convenientemente identificados de acuerdo con las normas internacionales que correspondan. En los métodos empleados se deberá señalar claramente el procedimiento experimental o de captación de datos y los métodos estadísticos, así

como los programas computacionales. Si el método no fuese original, se indicará bibliográficamente; si fuera original o modificado se describirá convenientemente. En cualquier caso, la presentación de varios métodos será cronológica.

- *Resultados.* Incluye la presentación sintética, ordenada y elaborada de la información obtenida. Entrega resultados en forma de texto escrito con apoyo de cuadros y figuras, si corresponde, conjuntamente con análisis e interpretación de los datos. Se deberá evitar tanto la repetición de detalles dados en otros capítulos como la descripción de aquello que sea evidente al examinar los cuadros o figuras que se presenten.
- *Discusión.* Incluye la interpretación integrada de los resultados y, cuando corresponda, la comparación de ellos con los de publicaciones previas. Es un análisis crítico de los resultados de acuerdo con los objetivos y la hipótesis, si fuera el caso. Debe comentarse el significado y la validez de los resultados, de acuerdo con los alcances definidos para el trabajo y los métodos aplicados. En este capítulo no deberán repetirse los resultados obtenidos.
- *Conclusiones.* Podrán ser incluidas en un capítulo único de conclusiones o bien integradas en la discusión. En caso de presentarlas como un capítulo, se incluirán allí en forma precisa y concisa aquellas ideas más relevantes que se deriven directamente de lo aportado por el trabajo. Deben dar respuesta a las hipótesis o a los objetivos planteados en la introducción. Deben redactarse en forma clara y objetiva sin incluir citas bibliográficas. Pueden incluir recomendaciones para trabajos futuros.
- *Agradecimientos.* En este acápite se deberán mencionar brevemente a personas e instituciones que contribuyeron con financiamiento u otro tipo de colaboración para la realización del trabajo.
- *Referencias.* Se indicarán las referencias de todas las citas bibliográficas señaladas en el texto, ordenadas alfabéticamente. La precisión y la veracidad de los datos entregados en las referencias bibliográficas son responsabilidad del o los autores de las contribuciones y deben corresponder a publicaciones originales. El número máximo de referencias será de 25 para artículos, notas y opiniones, y de 40 para revisiones. Utilice literatura moderna, relevante y directamente relacionada con su trabajo. Por lo menos 2/3 de las referencias deberán corresponder a revistas científicas de corriente principal.

Para las modalidades de revisión y opinión no se exige seguir la estructura indicada anteriormente. En todo caso, deben contener las secciones de título, autores, resumen, palabras clave, introducción, el desarrollo del trabajo adecuadamente dividido en capítulos, agradecimientos y referencias.

Estilo y formato

En general, el resumen, métodos y resultados del manuscrito deberán estar redactados en tiempo pasado, y la introducción, discusión y conclusiones en tiempo presente. Use tiempo presente cuando se refiera a resultados publicados previamente, esto ayuda a diferenciar entre los hallazgos de su estudio (tiempo pasado) y los hallazgos de otros estudios. En el texto no utilice acrónimos ni abreviaturas, escriba el nombre completo de las cosas; las excepciones que se pueden utilizar son aquellas de dominio global como, por ejemplo, ADN, pH, CO_2 y muy pocas otras. Tampoco utilice en el texto los símbolos de los elementos químicos. Acate las reglas gramaticales en todo el manuscrito, incluidos cuadros y figuras.

El trabajo debe estar escrito en hojas tamaño carta (279 x 216 mm), con márgenes de 2 cm por lado, interlineado a espacio y medio, letra Times New Roman, tamaño 12 puntos, con numeración de página en el extremo inferior derecho y número de línea correlativo para todo el trabajo, a la izquierda. Separar los párrafos a renglón seguido y con sangría de ocho caracteres a la izquierda de la primera línea. Debe presentarse en archivos electrónicos con procesador de texto Word o formato RTF.

El título principal se escribirá con letras minúsculas y negritas, centrado. En él deberá omitirse la mención de los autores de nombres científicos, los que, sin embargo, se presentarán la primera vez que se mencionen en el texto a partir de la introducción. En el encabezado superior derecho de cada página debe incluirse un título abreviado con un máximo de 60 caracteres y espacios.

Las ecuaciones se numerarán en el margen derecho con paréntesis cuadrados "[ ]"; en el texto se mencionarán de acuerdo con esta numeración.

Las unidades de medidas deberán circunscribirse al Sistema Internacional de unidades (SI). En la notación numérica, los decimales deberán ser separados por coma (,) y las unidades de miles por punto (.). En los textos en inglés, los decimales separados por punto y las unidades de miles por coma. Usar cero al comienzo de números menores a una unidad, incluyendo valores de probabilidad (por ejemplo, $P < 0{,}001$).

La descripción de los resultados de cada prueba estadística en el texto debe incluir el valor exacto de probabilidad asociado *P*. Para valores de *P* menores que 0,001, indique como $P < 0{,}001$. En cuadros y figuras usar asteriscos para señalar el nivel de significancia de las pruebas estadísticas: * = $P < 0{,}05$; ** = $P < 0{,}01$; *** = $P < 0{,}001$; ns = no significativo.

Debe indicarse el nombre científico de todos los organismos biológicos que aparezcan en el texto, de acuerdo con la nomenclatura internacional respectiva. Si un nombre común es usado para una especie, la primera vez que cite en el texto, a partir de la introducción, se debe dar a continuación su nombre científico en cursiva entre paréntesis, por ejemplo, coihue (*Nothofagus dombeyi* (Mirb.)

Oerst.). Citas posteriores pueden aparecer con el nombre del género abreviado seguido del adjetivo del nombre científico (por ejemplo, *N. dombeyi*), siempre y cuando no produzca confusiones con otras especies citadas en el manuscrito. Al iniciar una oración con el nombre de una especie, escriba su género completo y no lo abrevie con su inicial. En el resumen y en el título no mencione los autores de nombres científicos.

En los cuadros se deben incluir los datos alfanuméricos ordenados en filas y columnas, escritos con fuente Times New Roman de 12 puntos (mínimo 9 puntos de tamaño), sin negritas. Sólo los encabezamientos de las columnas y los títulos generales se separan con líneas horizontales; las columnas de datos deben separarse por espacios y no por líneas verticales. En las figuras se incluyen otras formas de presentación de datos o información, como gráficos, dibujos, fotografías y mapas. En cuadros y figuras se deben incluir los títulos auto explicativos en castellano e inglés numerados en forma consecutiva (cuadro 1., cuadro 2., …; figura 1., figura 2., …). Las figuras llevan el título en el margen inferior y los cuadros en el margen superior. Los cuadros y figuras deben tener una resolución tal que permitan ser reducidos sin perder legibilidad. Sólo se trabaja en blanco, negro y tonos de grises. Sin embargo, podrán usarse colores en las figuras si ello es imprescindible para su comprensión. La inclusión de figuras con colores deberá acordarse previamente con el editor. El espacio que ocupen cuadros y figuras en el trabajo deberá ser menor al 50 % del total del impreso. Incluya en el archivo de texto principal los cuadros con sus respectivos títulos, ubicándolos lo más próximo posible después de haberlos citado por primera vez en el texto. Los cuadros deben estar en el formato de tablas (editables, no como imágenes). Las figuras deben ser entregadas en un archivo aparte, con un formato editable; su ubicación en el texto principal debe ser informada, incluyendo su título, al igual que los cuadros.

En las figuras todos los rótulos y leyendas deben estar escritos con letra Times New Roman de tamaño 9 a 12 puntos, sin negrita y respetando la gramática y normas de escritura de la revista. Las figuras pequeñas deberán estar diseñadas con un ancho máximo de 8 cm (una columna en la revista) y las grandes con un máximo de 16 cm de ancho (dos columnas en la revista). Excepcionalmente, una figura podrá tener 23 cm de ancho (y máximo 14 cm de alto) para presentarla en formato apaisado. Organice las figuras reuniendo en una sola aquellos objetos afines (por ejemplo, gráficos de un mismo tipo de información) e identifíquelos con una letra mayúscula (A, B, C…), la que se explicará en el título de la figura.

Los manuscritos en castellano deben incluir en un archivo separado las respectivas traducciones al inglés de:

- Título del manuscrito.
- Summary: debe ser equivalente en contenido al resumen en castellano.
- Key words: equivalentes a las palabras clave en castellano.
- Títulos de cuadros y de figuras.

En el caso de manuscritos en inglés, se debe incluir el respectivo texto en castellano.

Citas y referencias

Las citas bibliográficas se indicarán en el texto por el apellido del o los autores, seguido del año de publicación. Algunos ejemplos de citas bibliográficas más frecuentes son:

- Citas bibliográficas de uno y dos autores:

Santamaría (2010) constata que el crecimiento...
... están influidos por el sitio en cuestión (Santamaría 2010, López y Castro 2011).

- Citas bibliográficas de más de dos autores:

Barría *et al*. (2009) señalan como factor más importante...
... entre otros, el diámetro y la altura (Barría *et al.* 2009, Morán *et al.* 2010).

- Citas bibliográficas de un mismo autor, publicadas en un mismo año:

Rodríguez (2009abd) observa que en cada unidad de muestreo...
... lo que es coincidente con estudios anteriores (Rodríguez 2009ab, Morán *et al.* 2010acd).

- Citas de más de una publicación a la vez, se ordenan cronológicamente:

Cerón (2007), García y Villanueva (2009) y Suárez *et al.* (2010) analizan los componentes edafoclimáticos...

En el capítulo de referencias, las referencias bibliográficas deben incluir apellido paterno e inicial del o los nombres de todos los autores, el año de publicación, el título y la información complementaria que permita localizar la fuente del documento en cuestión; si cuentan con DOI, debe agregarlo al final de la respectiva referencia. Algunos ejemplos de los formatos de las referencias bibliográficas más frecuentes son:

- Referencias de artículos en revistas periódicas (escriba con cursiva los nombres completos de las revistas, sin abreviar):

Guddants S. 2008. Replicating sawmill sawing with top-saw using CT images of a full-length hardwood log. *Forest Products Journal* 48(1): 72-75.

Kogan M, C Alister. 2010. Glyphosate use in forest plantations. *Chilean Journal of Agricultural Research* 70(4):652-666. DOI: 10.4067/S0718-58392010000400017.

Karzulovic JT, MI Dinator, J Morales, V Gaete, A Barrios. 2009. Determinación del diámetro del cilindro central defectuoso en trozas podadas de pino radiata (*Pinus radiata*) mediante atenuación de radiación gamma. *Bosque* 26(1):109-122.

- Referencias de libros como un todo:

Morales EH. 2005. Diseño experimental a través del análisis de varianza y modelo de regresión lineal. Santiago, Chile. Andros. 248 p.

CONAF (Corporación Nacional Forestal, CL). 2007. Estadísticas de visitantes e ingresos propios de áreas silvestres protegidas de la Décima Región de Los Lagos. 52 p. (InformeEstadístico Nº 47).

- Referencias a partes o capítulos de libros:

Gutiérrez B, R Ipinza. 2010. Evaluación de parámetros genéticos en *Nothofagus*. *In* Ipinza R, B Gutiérrez, V Emhart eds. Domesticación y mejora genética de raulí y roble. Valdivia, Chile. Exion. p. 371-390.

- Referencias a memorias, tesis, seminarios de titulación o trabajos de titulación:

Emhart V. 2006. Diseño y establecimiento de un huerto semillero clonal de *Eucalyptus nitens* (Deane *et* Maiden) con fines de producción, investigación y docencia. Tesis Ingeniero Forestal. Valdivia, Chile. Facultad de Ciencias Forestales, Universidad Austral de Chile. 79 p.

Aparicio J. 2008. Rendimiento y biomasa de *Eucalyptus nitens* con alternativas nutricionales para una silvicultura sustentable en suelo rojo arcilloso. Tesis Magíster en Ciencias. Valdivia, Chile. Facultad de Ciencias Forestales, Universidad Austral de Chile. 234 p.

- Referencias a documentos en internet:

De Angelis JD. 2009. European pine shoot moth. Oregon State University Extension (Urban Entomology Notes). Consultado 10 jul. 2009. Disponible en http://www.ent.orst.edu/urban/home.html.

Para mayor información respecto de otros casos específicos relacionados con las citas bibliográficas y referencias bibliográficas, se pueden consultar los documentos que a continuación se señalan. No obstante, el orden y la tipografía de los elementos constituyentes de las citas y referencias bibliográficas deberán ajustarse a la reglamentación de la revista Bosque.

Biblioteca Conmemorativa Orton (IICA/CATIE). 2011. Normas para citar referencias bibliográficas en artículos científicos 4 ed. Consultado13 abr.2011. Disponible en http://biblioteca.catie.ac.cr/index.php?option=com_content&task=view&id=18&Itemid=50

The Council of Biology Editors (CBE). 1994. Scientific style and format: The CBE manual for authors, editors, and publishers. 6 ed. Cambridge, New York. Cambridge University Press. 704 p.

Carta de envío

Los autores deberán acompañar su manuscrito con una carta de envío que indique que el trabajo es original, no ha sido publicado previamente y no está siendo considerado para publicación en otro medio de difusión. También deberán declarar cualquier posible conflicto de intereses que pudiesen tener. Se deberá señalar el tipo de contribución del manuscrito (artículo, revisión, opinión, nota). La carta deberá ser firmada al menos por el autor líder del manuscrito.

Envío de documentos

Los archivos deberán ser nombrados según el tipo de información contenida en el archivo. Por ejemplo, los archivos digitales del manuscrito se etiquetarán de la siguiente forma:

Texto.doc: texto principal del trabajo (incluye cuadros).
Figuras.doc: figuras con sus títulos en castellano.
Ingles.doc: textos en inglés con el siguiente orden: título del trabajo, summary, key words, títulos de cuadros y de figuras.
Carta: carta de presentación y envío del manuscrito.

Los archivos digitales del manuscrito deben ser remitidos por correo electrónico a revistabosque@uach.cl. El autor de correspondencia recibirá una carta de acuse de recibo del Editor.

Proceso de publicación

El cabal cumplimiento de las instrucciones para los autores se refleja en menores tiempos del proceso editorial. El comité editor revisa el manuscrito para verificar la pertenencia al ámbito de la revista y el cumplimiento de las instrucciones para los autores. Cuando no se cumplen tales condiciones, el manuscrito es devuelto al autor de correspondencia, informándole su situación. Cuando se ha verificado el cumplimiento de dichas condiciones, se registra esa fecha como recepción del manuscrito y el comité editor envía el manuscrito a un mínimo de dos árbitros o revisores externos, en un sistema de doble ciego. A los árbitros se les solicita declinar la revisión de un manuscrito cuando sientan que presentan conflictos de interés o que no podrán realizar una revisión justa y objetiva. Los

árbitros evalúan el manuscrito de acuerdo con la pauta que proporciona la revista. Si los árbitros o el comité editor lo estiman pertinente, podrán solicitar a los autores, a través del editor, información adicional sobre el manuscrito (datos, procedimientos, etc.) para su mejor evaluación. La respuesta de los árbitros puede ser: publicar con modificaciones menores, publicar con modificaciones mayores o no publicar. Las observaciones de los árbitros son evaluadas por el comité editor, el cual informa por escrito al autor de correspondencia la decisión de continuar o no en el proceso de publicación y si su manuscrito deberá ser nuevamente evaluado por árbitros. Cuando el manuscrito es aceptado, el comité editor envía al autor de correspondencia una carta de aceptación de su manuscrito, indicando el tipo de modificación necesaria. En no más de ocho semanas el autor de correspondencia debe devolver una versión modificada a la revista, para que el comité editor analice el manuscrito corregido. El comité editor decide el orden en que aparecerán los trabajos publicados en cada número. Una contribución puede ser rechazada por el comité editor en cualquiera de las instancias del proceso de publicación, ya sea por cuestiones de fondo o de forma que no cumplan con las instrucciones para los autores. Ante sospecha de conducta poco ética o deshonesta por parte de los autores que han sometido su manuscrito al proceso de edición, el editor se reserva el derecho de informar a las instituciones patrocinadoras u otras autoridades pertinentes para que realicen la investigación que corresponda.

Los trabajos publicados en Bosque están bajo licencia Creative Commons Chile 2.0.

Ante cualquier duda se sugiere contactarse con el editor (revistabosque@uach.cl) o revisar la información adicional de nuestra página web www.revistabosque.cl

La versión electrónica de libre acceso de los trabajos completos publicados por Bosque se encuentran en: http://mingaonline.uach.cl/scielo.php, http://www.scielo.cl, y http://redalyc.uaemex.mx/.

Políticas para los árbitros

Los árbitros o revisores de los manuscritos son integrantes clave del proceso editorial de la revista. Tienen la misión de contribuir a que la ciencia avance a través de su aporte en garantizar la alta calidad de los trabajos antes que estos se publiquen. Su trabajo es altruista y anónimo con respecto a los autores de los manuscritos.

El editor envía cada manuscrito a por lo menos dos árbitros que considera idóneos para el tema y así el comité editor puede considerar diversas opiniones de especialistas para decidir sobre el proceso editorial.

La responsabilidad de los árbitros es la de evaluar rigurosamente los manuscritos dentro del plazo propuesto por la revista.

Los árbitros deberán declinar la revisión del manuscrito cuando sientan que presentan conflictos de interés o que no podrán realizar una revisión justa y objetiva, los árbitros deberán declinar la revisión del manuscrito. Un arbitraje apropiado incluye virtudes y debilidades del manuscrito, sugerencias para su mejoramiento, preguntas precisas para que los autores puedan responderlas y orientaciones para que el trabajo sea de mejor calidad y mayor aceptación por los futuros lectores. Los árbitros deben mantener la confidencialidad de los manuscritos que reciben para revisión y nunca utilizar o difundir datos o información de ellos; el hacerlo es una conducta reñida con la ética. Los árbitros deberán abstenerse de solicitar la inclusión de aspectos que el manuscrito no busca responder, como también de insinuar que sean citados sus propios trabajos.

Frente a la revista, los árbitros deberán velar por la calidad y rapidez de sus revisiones y evitar los conflictos de intereses. Los árbitros deben cumplir los plazos y formatos solicitados por la revista. Cuando ello no sea posible, deberán declinar oportunamente el arbitraje. Cuando requieran de un tiempo adicional para la revisión de un manuscrito, deberán informar al editor. Si un árbitro presenta conflicto de intereses con respecto a un manuscrito, deberá abstenerse de realizar la revisión, informando al editor. Cuando un árbitro propone no publicar un manuscrito o hacerlo sólo después de cambios mayores, podrá recibir una nueva versión corregida por los autores que haya acogido las sugerencias de mejoramiento. El arbitraje es una herramienta eficaz para mejorar la calidad de los trabajos.

El editor podrá difundir informes de arbitrajes entre los revisores (conservando el anonimato) para promover el buen desempeño, resolver controversias y mejorar el proceso de edición.

Los árbitros serán informados del destino del manuscrito que revisaron. Como una forma de retribuir sus valiosos aportes, el editor les enviará una carta de agradecimiento por cada arbitraje y publicará sus nombres a inicios del año siguiente a su colaboración.

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