UNIVERSIDAD AUTÓNOMA AGRARIA ANTONIO NARRO

SUBDIRECCIÓN DE POSGRADO

EFECTO DE ECTOMICORRIZAS EN EL CRECIMIENTO Y EXTRACCIÓN DE NUTRIENTES DE *Pinus greggii* var. *greggii* Engelm BAJO INVERNADERO

Tesis

Que presenta REBECA CASIQUE VALDÉS

como requisito parcial para obtener el Grado de

DOCTOR EN CIENCIAS EN AGRICULTURA PROTEGIDA

Saltillo, Coahuila Mayo, 2018

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Elaborada por REBECA CASIQUE VALDÉS como requisito parcial para obtener el grado de Doctor en Ciencias en Agricultura Protegida con la supervisión y aprobación del Comité de Asesoría

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DEDICATORIA

A mis hijos….nunca dejen de perseguir sus sueños

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INTRODUCCIÓN

En viveros forestales, las raíces de las plántulas se asocian con diversas comunidades de hongos ectomicorrícicos (ECM) que contribuyen al vigor de las plántulas (Menkis *et al*., 2005; Menkis y Vasaitis, 2011) y, posteriormente, mejoran su establecimiento y crecimiento después del trasplante en el campo (Ortega *et al*., 2004).

Se reconoce que la cointroducción de una especie de árbol exótica con su propia comunidad de ECM puede facilitar su supervivencia (Tedersoo *et al*., 2007). Una alternativa para recuperar zonas con elevada degradación de suelos, es establecer plantaciones donde el éxito de la sobrevivencia depende principalmente de la utilización de plantas con necesidades de suelos pobres de nutrientes (Lima-Rojas 2013; Van-Eerden, 2002). *Pinus greggii* var. *greggii* (ubicada al norte del país) se encuentra en los estados de Coahuila y Nuevo León; se considera importante por su plasticidad genética para adaptarse a suelos erosionados, pobres, con poca profundidad y materia orgánica; esta especie tiene gran potencial para plantiones forestales destinadas a recuperación de suelos erosionados (Dvorak et al., 1996). Es ampliamente utilizada en plantaciones de tipo comercial en México y en países como la India, Indonesia, Brasil, Sudáfrica y Colombia (Carrera-Nieva y López-Ríos, 2004).

En México se necesita información sobre las necesidades nutricionales de la mayoría de las especies forestales, especialmente las endémicas; esta falta de información dificulta el diagnóstico nutricional y como consecuencia el inicio de programas y estudios relacionados con nutrición la cual repercute en aplicaciones elevadas de fertilizantes, implicando pérdidas económicas y degradación ambiental (Iyer *et al*., 2002). La determinación de nutrientes en pino, se lleva a cabo por diferentes técnicas, entre las más usadas es la determinación de nitrógeno por digestión húmeda por el método de kjeldhal (Wells 1969; Gadgil 1976; Martínez-Reyes, *et al*., 2012) y las concentraciones de otros macronutrientes y micronutrientes se determinan por La espectroscopía de emisión óptica de plasma acoplado inductivamente (ICP-OES) (Fraga *et al*., 2012; Stein *et al*., 2014) o espectrometría de absorción atómica (Adams, 1973; Donagi *et al*., 1980), los cuales necesitan ser digeridos por ácidos a elevadas concentraciones. Estos procedimientos de

digestión en húmedo y en seco, aunque son excelentes para la descomposición de la muestra, implican etapas tediosas, laboriosas y demoradas, requieren el uso de reactivos peligrosos y con frecuencia conducen a errores y fracasos sistemáticos (Welna *et al*., 2013; Marguí *et al*., 2005). El objetivo del presente trabajo es estudiar la aplicabilidad del método de espectrometría por dispersión de energías de rayos X (EDS) en comparación con (ICP-OES) al análisis elemental en *Pinus greggii* var. *greggii* obtenido tanto en manejo tradicional o convencional en invernadero y evaluar el desarrollo de plántulas de *Pinus greggii* con inoculación de cuerpos fructíferos de hongos ectomicorrícicos de dos sitios en el campo, una población natural de *Pinus greggii* y otro bosque templado con *Abies religiosa* y *Pinus ayacahuite* principalmente.

OBJETIVO GENERAL

Determinar el efecto de ectomicorrizas (ECM) en el crecimiento de *Pinus greggii* var. *greggii* y evaluar por ICP-OES y EDS la extracción de nutrimentos en plántulas bajo invernadero

Objetivos específicos

Determinar qué especies de ectomicorrizas se asocian a *P. greggii* var. *greggii.*

Seleccionar ECM que generen mejor respuesta en calidad de la planta bajo invernadero a partir de cuerpos fructíferos colectados en campo.

Obtener curva de extracción de nutrientes de *P. greggii* bajo invernadero.

Estudiar la aplicabilidad del método de espectrometría por dispersión de energías de rayos X (EDS) en comparación con la espectroscopia de plasma acoplado inductivamente (ICP-OES) al análisis elemental en *Pinus greggii* var. *greggii* obtenido tanto en manejo tradicional y convencional en invernadero.

Hipótesis

La inoculación con cuerpos fructíferos de ECM aumentará la calidad de plántula en invernadero.

El cultivo en turba (peat moss) modificará la capacidad de extracción y acumulación de nutrientes en *Pinus greggii* obtenidos por ICP-OES y EDS siendo estas técnicas similares en la obtención de resultados.

REVISIÓN DE LITERATURA

Variedades de *Pinus greggii* (var. *greggii,* var. *australis*).

Pinus greggii Engelm. crece en dos regiones disjuntas en México (Fig. 1) separadas por aproximadamente 300 km, o cuatro grados de latitud. Las poblaciones del norte y del sur de *P. greggii* crecen en entornos distintos. Las poblaciones del norte se encuentran en los estados de Coahuila y Nuevo León, en el norte de México, en elevaciones que van desde 1900 a 2600 metros sobre el nivel del mar. La temperatura media anual en los sitios del norte es de 14 ° C, y reciben una precipitación media anual de 650 mm (Donahue y López-Upton, 1996). Los suelos superficiales en los sitios del norte de *P. greggii* son predominantemente neutros o ligeramente alcalinos (Donahue, 1993). Las poblaciones del sur de *P. greggii* ocurren en los estados del centro de México, Hidalgo, Puebla, Querétaro, San Luis Potosí y Veracruz en elevaciones que oscilan entre 1100 y 2400 metros sobre el nivel del mar. La temperatura media anual en los sitios del sur es de 17 ° C, y reciben una precipitación media anual de 800-1600 mm (Donahue y López-Upton, 1996). Los suelos superficiales en los sitios meridionales de *P. greggii* son predominantemente ácidos (Donahue, 1993).

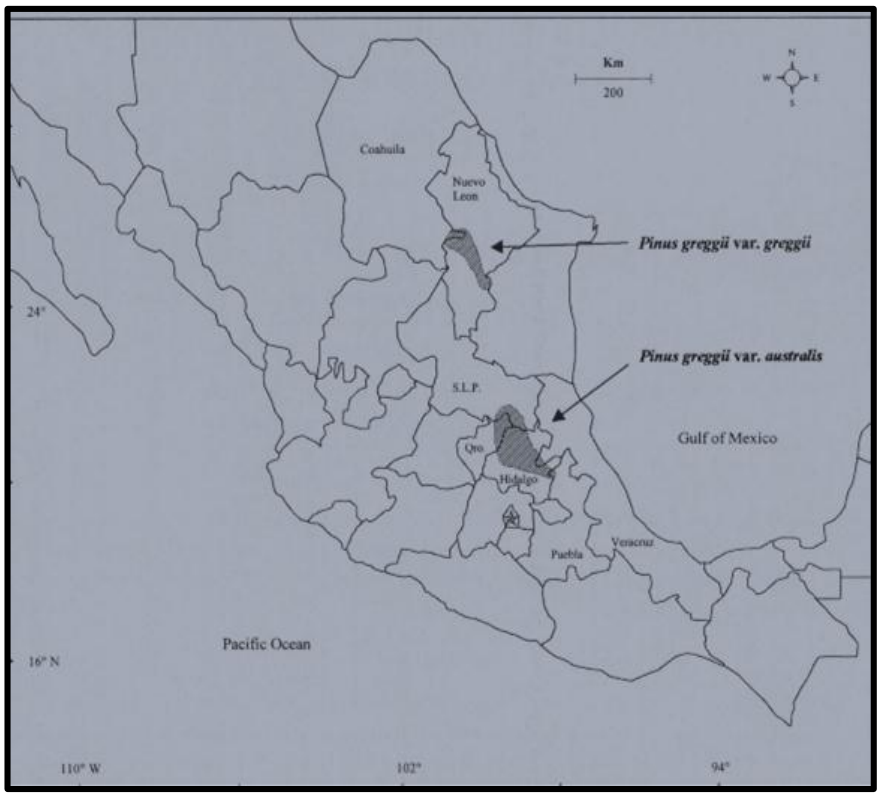

Figura 1. Distribución geográfica de *Pinus greggii* en México (Donahue y López-Upton, 1996).

Los resultados del estudio de morfología realizado por Donahue y Lopez-Upton (1996) mostraron que las poblaciones del sur tenían agujas significativamente más largas, más estomas por unidad de longitud de hoja, semillas más ligeras y cinco veces mayor frecuencia de canales internos de resina que las poblaciones del norte (Figura 2). Los árboles del norte tenían revestimientos de semillas más gruesos y semillas más pesadas. Es decir, la variedad *australis* tiene hojas en fascículos de 3, 10-15 cm de largo, 1.0-1.3 mm de ancho, con 36-41 estomas por 3 mm de longitud de hoja. La longitud del cono es de 8 - 13 cm y los anchos de cono son 3-5 cm. Las semillas miden 5-7 mm de largo y 3-4 mm de ancho. La longitud del ala de la semilla es 1 1-1 6 mm, y la anchura es 6-8 mm.

La variedad *greggii* tiene hojas en fascículos de 3, 7-12 cm de largo, 1.2-1.4 mm de ancho, con 34-37 estomas por 3 mm de longitud de hoja. La longitud del cono es de 8-12 cm y los anchos de cono son 3-5 cm. Las semillas miden 5-8 mm de largo y 3-4 mm de ancho. La longitud del ala de la semilla es de 13-16 mm y la anchura es de 5-7 mm.

La variedad del sur presentó mayor potencial de semillas y velocidad de emergencia que la var. del norte.

Figura 2. Comparación de las características foliares de (A) *Pinus greggii* var. *greggii* de La Tapona, Nuevo León, y (B) *Pinus greggii* var. *australis* de El Madroño, Querétaro (Donahue y López-Upton, 1996).

En un ensayo de procedencias (seis para *P. greggii* var. *greggii* y siete para var. *australis*) realizado por Valencia-Manzo *et al*., (2006) encontró que la var. *australis* presentó los mayores valores en crecimiento respecto a la var. *greggii*, lo cual es consistente con Dvorak *et al*., (1995) el cual reportó que la var. *australis* crece el doble que la var. *greggii* en un estudio de crecimiendo de *P. greggii* en diferentes tipo de suelo, la variedad del sur mostró mayores alturas que la variedad del norte. La var. *australis* crece con pH de suelo más ácido y la var. *greggii* con pH cercano al neutro (Valencia-Manzo *et al*., 2006); este mismo autor menciona que el pH donde crece la var. *australis* el cual puede ser causado por mayores precipitaciones, probablemente haya determinado que crezca más rápido que la var. *greggii*, además la var. *australis* se localiza a menores altitudes y latitudes, con mayor temperatura y precipitación media anual que la var. *greggii*. En un estudio realizado por Ramírez *et al*. 1997 en el cual estudiaron los loci polimórficos en ambas variedades encontró que la var. *australis* presentó mayor porcentaje de loci polimórficos (Parraguirre *et al*., 2002) y número de alelos por locus que la var. *greggii*. *P. greggii* var. *australis* presenta precocidad en la floración y altas tasas de crecimiento en altura y diámetro

(Azamar et al., 2000). En un ensayo realizado por Swaty *et al*., 1998, se examinaron los papeles que los cambios estacionales en la precipitación y la temperatura desempeñaron en la colonización ectomicorrízica (ECM) del pino piñonero (*Pinus edulis* Engelm). Los pinos que crecen en suelos cenizos experimentaron mucho más agua y estrés nutricional que los pinos que crecen cerca en suelos arenosos encontrando que la colonización de ECM fue significativamente mayor en el sitio de la ceniza. La temperatura del aire y la precipitación también se correlacionaron significativamente con la colonización de ECM. Los hongos son hiperdiversos pero poco conocidos, a pesar de sus impactos ecológicos y económicos. Tedersoo *et al*. (2014) colectó cerca de 15,000 muestras de tierra vegetal de 365 sitios en todo el mundo y secuenció sus genomas encontrando una notable disminución en la riqueza de especies de hongos con la distancia desde el ecuador. Por otra parte, Korkama *et al*., 2006, investigó los factores asociados con la estructura de la ECM en la picea Noruega quienes se formaron la hipótesis de que las comunidades de ECM asociadas con árboles individuales de la misma edad y especie varían significativamente, y que las diferencias en la estructura de la comunidad ECM covaría con las diferencias en las tasas de crecimiento del árbol huésped. En el cual se observó que la diversidad de ECM varió entre los grupos de clones, mostrando dos diferencias de crecimiento.

En un estudio de disertación de tesis sobre hongos ectomicorrízicos (Klavina, 2015) de pícea joven y madura (*Picea abies* (L.) Karst.) en relación con la tasa de crecimiento de los árboles, los parámetros del suelo y diferentes prácticas de manejo forestal aplicadas se observó que los sitios con alta incidencia de pudrición de raíces de Heterobasidion en suelos fértiles de turba con pH muy bajos se caracterizaron por especies de ECM *Amphinema* spp., *Inocybe* spp. y *Tylospora asterophora*. En plantaciones de árboles jóvenes, las especies de ECM *Thelephora terrestris, Amphinema byssoides* y *Wilcoxina* fueron los principales simbiontes. En contraste, los suelos maduros, principalmente en suelos de turba, fueron dominados por los géneros *Tylospora, Amphinema, Lactarius y Tomentella*. Los miembros de *Helotiales* hongos y géneros *Russula, Cortinarius, Inocybe* se observaron comúnmente en las raíces finas. Algunas diferencias observadas en la abundancia de especies pueden atribuirse a las condiciones del suelo (humedad del suelo, acidez y fertilidad). Por ejemplo, ECMf de los géneros *Amphinema, Tuber e Inocybe*

fueron específicos para parcelas fertilizadas con cenizas de madera con un aumento del pH del suelo, mientras que las especies de *Tylospora, Lactarius y Russula*, en bosques no fertilizados. Es decir, como se ha mostrado en estudios previos, es posible que por variedad, clon, madurez de los árboles y condiciones de suelo, la variedad en género de hongos ectomicorrícicos es distinta y puede ser muy diversa dependiendo de las condiciones en los que los árboles se encuentran.

Beneficios de las Ectomicorrizas.

El árbol suministra al hongo ECM hasta un 20% de su fotosíntesis derivada de carbohidratos, a cambio de hasta el 70% de sus necesidades de nitrógeno y fósforo, recibidos de las redes de hifas ECM que se extienden profundamente en el suelo. Durante el curso de la colonización de la raíz por un hongo ECM, la raíz de la planta experimenta una serie de cambios morfológicos, desde el cese del crecimiento hasta la alteración de las propiedades de las paredes celulares de las plantas y, por último, el control alterno de los transportadores. Durante el alojamiento de ECM dentro de la planta huésped, hay un cambio distinto en el metabolismo de las raíces de las plantas. A pesar de la importancia del transporte de nutrientes en simbiosis ECM, las variaciones de nutrientes no explican los mayores cambios observados en las reservas de metabolitos en la interacción entre *L. bicolor* y su anfitrión *P. Trichocarpa* (Tschaplinski y Plett, 2014). En esta interacción, un aumento en el giro de metabolitos asociados con la vía de degradación de benzoato en *L. bicolor* explica muchas de las mayores respuestas metabolómicas observadas (Tschaplinski y Plett, 2014). Estos cambios incluyen una disminución del alcohol bencílico que contiene glicósidos fenólicos, y la acumulación de ácido benzoico y muchos metabolitos hidroxilados del ácido benzoico. La desintoxicación del benzoato es un paso importante en una serie de otras relaciones simbióticas descritas (Liu *et al*., 2013). El papel de esta vía es actualmente desconocido, aunque se ha planteado la hipótesis de que esta vía puede ser para desintoxicar xenobióticos defensivos producidos por el organismo huésped.

Además, se encontró que *L. bicolor* indujo igualmente el perfil de metabolitos asociados con la defensa en las raíces de ambas plantas huésped sometidas a colonización. Estos

resultados sugieren que las plantas fácilmente colonizadas por hongos ECM son receptivas debido a la falta de metabolitos defensivos en comparación con los huéspedes recalcitrantes (Tschaplinski y Plett, 2014). Como con todos los organismos, los hongos micorrízicos presentan genes que codifican proteínas implicadas en la adquisición, almacenamiento y remobilización de elementos traza, para asegurar la homeostasis celular independientemente de las fluctuaciones naturales externas (por ejemplo, estacionales). Se requiere una concentración celular constante de oligoelementos esenciales para asegurar el crecimiento y la reproducción y para superar la toxicidad.

Algunos hongos ECM exudan compuestos polifenólicos y pigmentos asociados a paredes celulares como la melanina una vez expuesta a metales. La melanina y algunos otros pigmentos asociados a la pared celular tienen propiedades metalocelulares y antioxidantes. Sin embargo, con o sin pigmentos, se pueden unir oligoelementos a la pared celular. La composición química de la pared celular, con su alta abundancia de grupos carboxilo e hidroxilo, lo convierte en un excelente sitio de unión para los cationes. Esta unión no requiere energía celular, ya que no interviene ningún proceso enzimático catalizado, pero es reversible (Ruytinx *et al*., 2016).

En muchos hongos ECM, y especialmente en aquellos con extensas redes de hifas externas, la pared celular está cubierta con proteínas hidrófugas - las hidrofobinas. Esta capa hidrófoba es una capa de aislamiento, y probablemente restringe la pérdida de agua cuando el suelo se seca. Al mismo tiempo, las hifas hidrofóbicas ya no permiten la absorción de nutrientes (Courty *et al*., 2016). Los perfiles de expresión de un precursor de hidrofobina en diferentes aislados de *Suillus luteus* indican que la biosíntesis de hidrofobina podría estar regulada por la disponibilidad de Zn para reforzar o atenuar la barrera hidrofóbica (Muller *et al*., 2007).

Diferentes estructuras morfológicas se pueden distinguir dentro de una colonia fúngica (Figura 3). En los bosques, las colonias de ECM de hongos pueden extenderse por varios metros cuadrados. Típicamente, la mayor parte de la colonia está formada por hifas que crecen en el suelo y que exploran las capas minerales para obtener nutrientes. Sin embargo, existen especies con micelios menos desarrollados en el suelo. Algunos hongos ECM pueden transportar los nutrientes recolectados a largas distancias dentro de la colonia fúngica a través de rizomorfos. Estos rizomorfos también se utilizan para transferir

y distribuir los carbohidratos que entran en la interfaz planta-hongo ECM. En algunas épocas del año, las colonias de hongos pueden reproducirse sexualmente y dar lugar a esporocarpos. A lo largo del espacio y el tiempo, diferentes partes de la colonia de ECM permanecen funcionalmente interconectadas y las células de cada parte (hifas exploradoras del suelo, rizomorfos, ECM y esporocarpos) deben adaptar su propia homeostasis celular (Ruytinx *et al*., 2016).

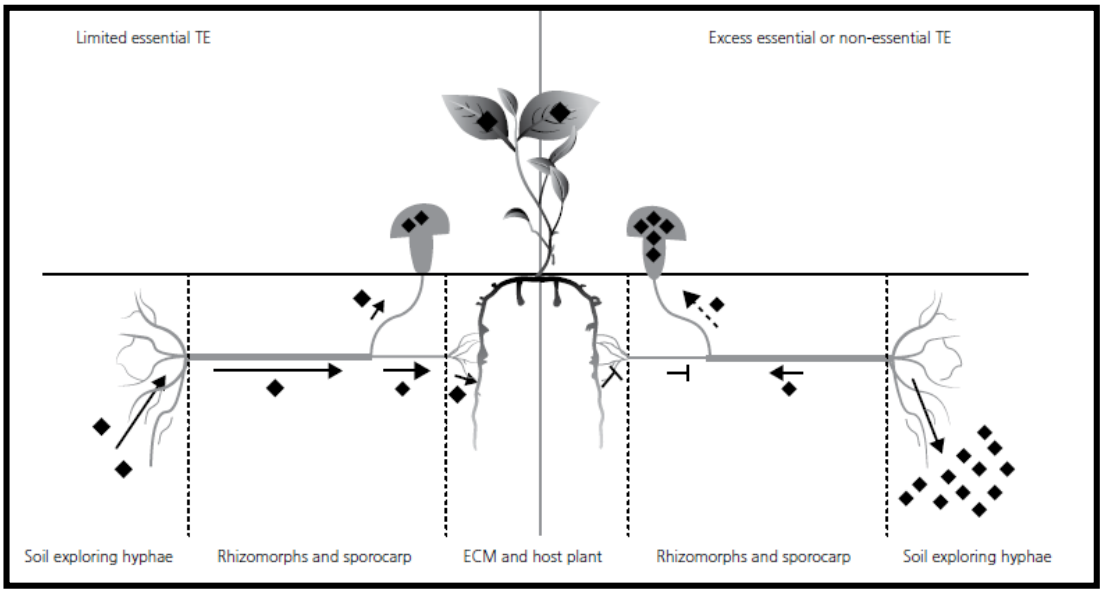

Figura 3. Modelo de las respuesta de la entidad hospedera fúngica-ECM a las fluctuaciones externas de elementos traza. Los elementos de rastreo entran en la colonia de hongos en las hifas que exploran el suelo. Desde allí se pueden transportar, también a través de largas distancias a través de rizomorfos, a esporocarpos y ECM. En la interfase planta-ECM, se intercambiarán por hidratos de carbono. La captación de elementos esenciales de trazas se estimula en las hifas y se facilita la transferencia a la planta huésped. Los elementos no esenciales o en exceso se excluyen de las hifas de exploración del suelo, y su transferencia a la planta huésped es limitada. Estos elementos se pueden acumular en los esporocarpos. Un TE en particular (por ejemplo, Zn) puede estar limitado para la entidad, mientras que otro puede estar presente en exceso (por ejemplo, Cu). ♦ TE, elemento traza; → dirección del transporte activo; ⊥ transporte inhibido (Ruytinx *et al*., 2016).

Inoculación de Pino con hongos ectomicorrícicos en vivero.

Estudios de ECM en vivero muestran diferente tipo de inóculo, desde materia orgánica, tal como suelo, hojarasca, formas variables de humus, madera podrida y ectomicorrizas recogidas de plantaciones forestales o bosques maduros sin embargo, el mayor

inconveniente es que las especies de hongos ECM que forman el inóculo no pueden ser controladas (Repáč, 2011).

Los esporocarpos y esporas de varios hongos se han usado como inóculo de ECM específicas, estos esporocarpos son en esencia inóculo en forma de espora (Marx y Kenney, 1982) el cual es fácil de colectar en grandes cantidades que aquellas producidas en forma artificial además de que esta pueden sobrevivir toda la temporada y la siguiente (Repáč, 2011).

Otro inóculo de tipo vegetativo son los propágulos de micelio el cual se realiza licuando el micelio con agua destilada, se pude inclusive producir en un fermentador.

He aquí algunos pasos a seguir antes de inocular con ECM:

- La fumigación del suelo del invernadero antes de la inoculación mejora el desarrollo de ECM, debido a que los microorganismos del suelo pueden colonizar el medio de inóculo, se combaten patógenos de raíz que pueden dañar la misma y puede coexistir otros microorganismos que compitan con hongos ECM. Martínez-Amores (1991) reporta el uso de Bromuro de Etidio.
- El inóculo debe realizarse después de autoclavar el sustrato de los semilleros.
- El inóculo se puede realizar antes de que las semillas emerjan o se siembren
- El inóculo se puede realizar cuando las semillas emergen o en etapa de plántulas.
- Se ha encontrado que las basidiosporas pueden inocularse en una mezcla rompiendo los esporocarpos o colocando las esporas directamente en el contenedor con un portador húmedo como vermiculita, kaolin o arena.
- Otra manera es asperjando las esporas secas alrededor de los semilleros y liberándolas en la zona radicular.
- Otra manera es la suspensión en agua inyectándola en el sustrato.

Para determinar la dosis depende del tipo de inóculo, hay autores que registraron la aplicación de esporas a la superficie del suelo, Marx *et al*. (1978) trabajó con 108, 324 o 648 mg de esporas por m^2. Rincón *et al*. (2001) mezcló esporas secas de *P. tinctorius* y *Scleroderma verrucosum* en concentraciones de 10^3- 10^8 esporas por cada 175 ml de sustrato. El método más utilizado es la aplicación de suspensión de esporas en agua directamente al sustrato. Marx and Bryan (1975) asperjaron basidisosporas *de P. tinctorius* (10 g suspendidos en 500 ml de agua destilada con una gota de Tween 20),

Theodorou (1984) inoculó semillas de *P. radiata* con 5.15 x 10^4 esporas por semilla de *Rhizopogon luteolus* 1-2 días antes de la emergencia de la semilla; este mismo autor reportó un inóculo de 4.46 x 107 esporas por ml después de la emergencia de las semillas. Rincón *et al*. (2001), y Hortal *et al*. (2008) después de un mes de emergencia inocularon en un rango de 10^2-10^8 esporas por semillero. Nuñez *et al*. (2006) aplicó una suspensión de *Tuber melanosporum* de forma manual en cada contenedor (400 ml de sustrato peat moss y vermiculita 2:1) aproximadamente 7.5 x 10^5 esporas. Machón *et al*. (2006), y Gange *et al*. (2005) añadieron 50 ml de vermiculita con 20 ml de mezcla licuada de micelio en cada contenedor de semilleros trasplantados.

Como se ha mencionado, las dosis de esporas o propágulos se muestra en un rango de 10^2-10^8 esporas por ml, semillero o contendor o licuado de propágulos en un rango de 2 hasta 10g de micelio seco por m^2; sin embargo para un procedimiento experimental es necesario identificar la micorriza de interés, aislarla si proviene de esporocarpos o raíz (Garza-Ocañas *et al*., 2002) y obtener cultivo puro de esta especie para tener suministro o si se carece de material e instalaciones para su aislamiento, colectar los esporocarpos para obtener suficiente inóculo para los experimentos a realizar, muchos autores reportan las tablas de micorrizas en el cual las esporas y fragmentos de micelio se mezclan en un acarreador (arena, perlas de alginato o arcilla (Turjaman *et al*. (2005, 2006).

Para ello, se puede trabajar en contenedor, área de trabajo o semilleros con las dosis experimentales mencionadas en el párrafo anterior para observar el desarrollo de la conífera y obtener la dosis correcta, he aquí un resumen del procedimiento:

1. Trasplantas las semillas de 4 semanas de edad en polybags de 10x15 cm. Las bolsas de polietileno también deben de fumigarse.

2. El sustrato debe ser esterilizado, el control debe tener el mismo inóculo pero esterilizado en autoclave. También en lugar de la fumigación, el sustrato esterilizado se cubre con una lámina de plástico durante dos semanas y después se airea durante cinco días (Restrepo-Llano *et al*., 2014). Después de este período de tiempo, el sustrato recibe fertilizante comercial ya sea de liberación lenta o convencional.

3. Para la obtención de dosis se debe seguir un rango de inóculo que abarque concentraciones mínimas y máximas como ya se mencionó anteriormente en un diseño al azar. Ciertos autores mencionan un segundo inóculo un mes posterior a la primera

aplicación. Las condiciones de humedad y temperatura deben ser controladas, Dalong *et al*. (2011) menciona 70% de humedad.

4. Se deben tomar al azar plántulas inoculadas después de 5-12 meses (Martínez-Amores, 1991).

5. Se debe reportar el porcentaje de colonización (Número de piezas radiculares con estructura ectomicorrízica / número total de piezas radiculares × 100%), tamaño, diámetro de tallo, peso seco. El volumen (cm^3) es importante como parámetro para obtener la respuesta de la inoculación en pinos (Ruehle *et al*., 1984) en donde se obtiene al multiplicar la altura por el diámetro del cuello de la raíz al cuadrado (Martínez-Amores, 1991).

6. Se debe considerar un número de repeticiones para su análisis de varianza y prueba de medias. Por ejemplo, Olivera *et al*., (2014) considera una unidad experimental de área de 6 × 10 m con seis plántulas plantadas en dos filas más la adición de tres plántulas plantadas al mismo tiempo entre ambas filas a extraer para el muestreo de raíz destructivo. Por otra parte Dalong *et al*., (2011) menciona 30 semilleros por tratamiento.

Ectomicorrizas y su beneficio en la reforestación.

La inoculación de árboles de importancia forestal con hongos micorrícicos representa una significativa herramienta en la producción de plantas en invernadero. Tras la transferencia y plantación de las plantas de semillero en el campo, se ha demostrado que las micorrizas promueven la supervivencia, el establecimiento y el crecimiento de los árboles jóvenes en las plantaciones forestales de reciente creación (Kropp y Langlois 1990; Perry *et al*. 1987; Stenström y Unestam, 1990). Incrementan la absorción de agua y nutrientes y protegen a la raíz contra el estrés ambiental como la sequía, los agentes patógenos y la contaminación por metales pesados (Menkis *et al*., 2005). Los hongos ectomicorrízicos (ECM) son indispensables para el establecimiento y funcionamiento de los bosques templados, algunos de ellos tienen esporas u otros propágulos resistentes y longevos; éstos se acumulan en el suelo forestal formando bancos de propágulos que constituyen la fuente de inóculo más importante después de disturbios severos (Garibay-Orijel *et al*., 2013). La colonización por hongos en los sistemas de raíces es un factor importante en la

determinación del vigor de las plántulas y por consiguiente, su calidad (Smith y Read 1997). Se ha demostrado que las ECM tienen un impacto positivo en la salud de las plántulas y su productividad en los viveros forestales (Jumpponen 2001; Stenström *et al*., 1997). Para el área fostestal resulta de gran interés la producción de inoculo que facilite el incremento en el crecimiento y sobrevivencia de las plántulas de coníferas que se cultivan bajo agricultura protegida para fines de plantaciones masivas en sitios con suelos pobres, erosionados o que han sido abandonados después de prácticas agrícolas improductivas.

El fracaso en la forestación ha sido previamente atribuido a la ausencia de hongos micorrícicos adecuado (Bjorkman 1970; Marx 1980). Una forma de la superación de este problema es la promoción de micorrización de plantas en condiciones de vivero (Menkis *et al*., 2005). Se ha encontrado que la co-introducción de una especie de árboles exóticos con su propia comunidad ECM puede facilitar su supervivencia (Tedersoo *et al* 2007; Vellinga *et al*., 2009; Dickie *et al*., 2010). La mayoría de los hongos ECM tienen huéspedes generales (Molina *et al*. 1992) y estos son menos propensos a ser afectados negativamente por la sustitución del hábitat de bosque nativo sin embargo, otros hongos ECM tales como miembros de los géneros *Suillus* y *Alnicola* muestran una alta especificidad con los géneros *Pinus* (Bruns *et al*., 2002) y *Alnus* (Moreau *et al*., 2006), respectivamente.

Especies de hongos ectomicorrícicos reportados para *Pinus* en México.

Pocos estudios han examinado las comunidades de hongos ectomicorrícicos (ECM) asociados con los pinos en México; aunado a ello el conocimiento de ECM asociado a *Pinus greggii* es escaso a pesar de su importancia económica y biológica (se adapta a condiciones edáficas nutricionalmente pobres y es de rápido crecimiento). En México sólo se han descrito las micorrizas *Suillus pseudobrevipes (*Carrera-Nieva y López-Ríos 2004) y *Pisolithus tinctorius* (García-Rodríguez *et al*., 2006, citado por Garibay-Orijel *et al*., 2013). (Garibay-Orijel *et al* (2013) Menciona algunas especies reportadas para especies de pino en México, entre ellos, *Pinus greggii* (tabla 1).

Tabla 1. Especies de hongos ectomicorrícicos reportados para *Pinus* en México.

Especie	Ectomicorriza	Referencia
Pinus montezumae	*Laccaria bicolor*	(Santiago-Martínez *et al*., 2003)
P. patula	*Boletus clavipes*	(Carrera-Nieva and López-Ríos, 2004)
	Laccaria laccata	
	Suillus pseudobrevipes	
P. greggii	S*uillus pseudobrevipes*	(Carrera-Nieva and López-Ríos, 2004)
P. greggii, *Eucalyptus urophylla*	*Pisolithus tinctorius*	(García-Rodríguez *et al*., 2006)
Quercus sp.	*Boletus rubropunctus*	(Smith and Pfister, 2009)
P. devoniana y *P. pseudostrobus*	*P. tinctorius*, *Scleroderma texense*	(Valdés Ramírez *et al*., 2010)
P. patula y *P. pseudostrobus*	*L. bicolor*, *L. laccata*, *L. proxima*, *Hebeloma alpinum*, *H. leucosarx* y *H. mesophaeum*	(Carrasco-Hernández *et al*., 2010)

Suillus pseudobrevipes es una especie de hongo comestible del género *Suillus.* Fue descrito por primera vez científicamente por los micólogos americanos Harry D. Thiers y Alexander H. Smith en 1964. Esta especie de hongos tiene un anillo fibrilar distintivo. De acuerdo al sistema BOLD (Boldsystems) en base de datos de hongos, el repositorio de datos de S. pseudobrevipes (10 ejemplares) indica que el 90% de estos se encuentran en México los cuales fueron obtenidos donados por el Instituto de Biología de la Universidad Nacional Autónoma de México: (http://www.boldsystems.org/index.php/Taxbrowser_Taxonpage?taxid=461112)

Por otra parte, *Pisolithus tinctorius* es un hongo micorrízico que no es del todo exigente con sus asociaciones de plantas y árboles. Por esta razón es utilizado con frecuencia por los silvicultores, crece bien en suelos pobres y arenosos (Kuo, 2006).

La presencia natural de *Pisolithus tinctorius* se ha confirmado en 33 países del mundo, se encuentra asociado con varias especies de árboles en viveros, zonas urbanas, huertos, bosques y despojos de minería a tiras. Los experimentos han demostrado que este hongo forma simbiosis con *Abies, Betula pendula, Carya illinoensis*, 11 especies

de *Eucalyptus*, 30 especies de *Pinus*, 2 especies de *Quercus*, es usado ampliamente debido a su producción masiva de esporas (Marx, 1977).

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ARTÍCULO

Determination of elements in *Pinus greggii* var. *greggii* obtained by SEM-EDS and ICP-OES cultivated in a conventional and traditional production in greenhouse

DETERMINATION OF ELEMENTS IN *Pinus greggii* var. *greggii* OBTAINED BY SEM-EDS AND ICP-OES CULTIVATED IN A CONVENTIONAL AND TRADITIONAL PRODUCTION IN GREENHOUSE

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Key words: Extraction curve, Nutrient uptake, EDX, *Pinus greggii*, Turba.

ABSTRACT

Pinus greggii var. *greggii*, an endemic species of northern Mexico, is adapted to nutritionally poor edaphic conditions, which is why it is used in forest plantations for reforestation; in spite of its importance, information on the nutritional needs of this specie is scarce, which leads to nutritional deficiencies and erroneous application of fertilizers. Currently, most of the elemental determination studies are carried out by ICP-plasma, however, this method requires acid digestions which involves the use of dangerous reactants, and tedious procedures in order to obtain an elemental analysis. The present study establishes the applicability of the X-ray diffraction spectrometry method (EDS) In comparison with the inductively coupled plasma spectrometry method(ICP-OES) in *P. greggii* samples obtained both in traditional soil management (TM), and conventional with peat moss (PM) in greenhouse.

It was proved that the nutritional absorption in both detection techniques, follows the same propensity, with a highly significant Pearson correlation for P, K, Ca, S, and Fe nutrients. Substrate based on peat moss, presented higher percentages of mineral

absorption in relation to mountain soil. Conventional management is recommended for the production of *P. greggii* in a nursery. It is possible to determine minerals by the EDS method, avoiding the use of corrosive and dangerous reactants resulting in tedious and arduous techniques.

Palabras clave: acumulación de nutrientes, EDX, peat moss, *Pinus greggii*.

RESUMEN

Pinus greggii var. *greggii* especie endémica del norte de México, se adapta a condiciones edáficas nutricionalmente pobres por lo que se usa en plantaciones forestales para recuperación de bosques; a pesar de su importancia, la información sobre las necesidades nutricionales de esta especie es escasa y conduce a fallas en el diagnóstico nutricional y aplicación errónea de fertilizantes. En este estudio se establece la aplicabilidad del método de espectrometría por dispersión de energías de rayos X (EDS) en comparación con la espectroscopia de plasma acoplado inductivamente (ICP-OES) al análisis elemental en *P. greggii* obtenido tanto en manejo tradicional con tierra de monte (TM) y convencional con turba (PM) en invernadero. Se demostró que la absorción nutrimental en ambos métodos de detección sigue la misma propensión con una correlación de Pearson altamente significativa para los nutrimentos P, K, Ca, S y Fe. Para la producción de *P. greggii* en vivero se recomienda el manejo convencional (sustrato a base de turba) ya que presentó porcentajes más elevados de absorción mineral respecto a tierra de monte. Es posible determinar minerales por EDS evitando el uso de reactivos corrosivos y peligrosos que conducen a técnicas tediosas y laboriosas.

INTRODUCTION

An alternative to mend areas with high degradation soil, is to establish plantations where survival success, depends mainly on the use of plants with poor nutrient soil needs (Lima-Rojas 2013, Van-Eerden 2002). *Pinus greggi* is an endemic Mexican pine, which adapts to nutritionally poor soil conditions; this specie has great potential for forest plantations, for the recovery of eroded soils (Dvorak et al 1996).

Most nurseries in Mexico, use forest soil for *P. greggii* production as the main component of the culture medium (Torres-Rojo and Velázquez-Sánchez 1996, Van-Eerden 2002). In the container, the soil is compacted and dense, which creates an inadequate drainage path for the root. (De la Garza-López 1995, Sandoval-Méndez, et al 2000) More than 60% of nursery-grown seedlings, are obtained under the traditional production system, using forest soil in polybags. Woodland plantations require enough of the essential elements in the soil, for a healthy and vigorous growth to be successful (Jenkinson 1995). However, the type of soils where this specie develops, are shallow slightly alkaline, with a pH of 7.0 to 8.0 (Hodge and Dvorak 2000). In Mexico, information on the nutritional needs for most forest species, especially endemics are required; the lack of this information, delays the nutritional diagnosis and, as a consequence, the initiation of nutrition-related programs. This has an impact on erroneous applications of fertilizer, involving economic losses, and environmental degradation (Iyer et al 2002). The fact finding of nutrients in pine, are carried out by different techniques, the most commonly used is, the nitrogen determination, via wet digestion using the method of kjeldal (Wells 1969. Gadgil 1976, Martínez-Reyes et al 2012) and the other macronutrients and micronutrients concentrations, are determined by ICP-OES (Fraga et al 2012, Stein et al 2014) or atomic absorption spectrometry (Adams 1973, Donagi et al 1980). These wet and dry digestion procedures, require to be dissolved using acids at high concentrations; whereas are excellent for sample break down, involve tedious, and time-consuming stages, which require the use of hazardous reactants, and often lead to errors and systematic failures. (Welna et al 2013, Marguí et al 2005). The aim of the present work, is to study the applicability of the X-ray diffraction spectrometry method (EDS) in comparison to the inductively coupled plasma spectrometry (ICP-OES) towards the elemental analysis of *Pinus greggii* var. *greggii* obtained both in conventional and traditional greenhouse management.

METHODS

Plant material and culture conditions

P. greggii seeds were obtained from cones of the Cañón de Caballos population (100 ° 54 '46 .61 "W and 25 ° 14' 47 .13" N) Saltillo, Coahuila, México. Which were treated with H_2O_2 at 2 % for four hours and planted in nurseries of 77 cavities on peat moss: perlite (1: 1) and were kept in a greenhouse under shade mesh.

Substrates management and sample processing

For conventional management, we worked with a mixture of peat moss and perlite in a ratio of 1: 1 (PM). In terms of traditional management, we used forest soil of horizon A (0-25 cm) (101° 1' 37.32" W, 25° 20' 44.68" N), silt and sand at a ratio of 2: 1: 1 (TM); both substrates were sterilized for one hour in an autoclave at 15 lb of pressure. The mixture of peat moss and perlite was fertilized with osmocote 14-14-14® at a rate of 1 $kg.m^{-3}$ (Dreesen et al 2002); the mixture of forest landwas used without any fertilization. The two substrates were sent to the soil laboratory of the Universidad Autónoma de Chapingo México to obtain the percentages of organic matter, clays, silt, sand, pH, macronutrients: N, P, K, Ca, and Mg.

The mixture of substrates was distributed in black polyethylene bags for nursery (polybags) of 10x20 cm in which 200 seedlings of *P. greggii* of two months of age were transplanted for each substrate with a total of 400 seedlings. 40 days after transplantation, the seedlings were treated with the systemic fungicide Mancolaxyl 72 WP ® to reduce the growth of pathogens associated with the root. Irrigation went directly to the rootball of each plant with a pH of 5.8 every 5 days.

Sample processing

The taking of destructive samplings was carried out 45 days after the application of the fungicide, and from the first intake; consecutive samplings were made every 25-35 days during 8 months, taking four repetitions per treatment (three plants as an experimental unit, UE).

Each plant was washed with running water to remove debris from the substrate, and then rinsed with distilled water. It was deposited on blotting paper and divided into roots and foliage (stem) with the help of pruning scissors. The parts of the plant were

placed in separate paper bags and dried at 52 ° C in an oven for 24-72 hours. The dry weight of both root and foliage of each plant was obtained per experimental unit and repetition of the treatments evaluated in each sampling.

Elemental Analysis by EDS

Two replicates (3 plants per UE), both root and foliage of each sample, were ground in a coffee mill Krups ® GX410011; approximately 1 g of each repetition was taken to muffle at 600 ° C for 3 hours acording to Buján-García (2009) to obtain ashes.

In each repetition, two spectra (a total of 4 readings) were performed, each sub-sample per repetition powder (ash), was pressed into a sample holder of 1 cm in diameter added to a double sided carbon tape (NEM TAPE Nisshin Co., Ltd.), it should be mentioned that the samples were not handling with coating.

The elemental composition of the pine samples was performed by X-ray diffraction energy (EDS) spectrometry, using an INCAx-sight model 7582 (Oxford Instruments, Oxfordshire, England) with resolution of 5.9 keV at 137 eV with a detection area of 10 mm^2 coupled to the scanning electron microscope column (SEM) JEOL JSM-639OLV Noran Six (Jeol Ltd. Japan); the electron beam emission was generated from a tungsten filament with a range of kV spectrum of , the working distance (WD) was 11mm with a voltage acceleration of 20 kV, and a beam diameter of electrons of 60-70 SS (SpotSize) and a magnification of 5000x, spectrum exposure time was 120 s. The elemental chemicalcomposition values determined in this test correspond to the average of four readings for each part of the plant, and each treatment and sampling.

Elemental analysis by ICP-OES

1 g of each dry sample (four replicates) was carried to ashes in porcelain crucible performing the previously described procedure, we followed the protocol technique (Belay 2014) modified, for which: 10 mL of nitric acid (65%) was deposited in the crucible with the ashes and boiled, once the sample was cold, 1 mL of hydrogen peroxide (30%) was added. Boiled once more cooled and filtered (Whatman® 42 paper). The sample was adjusted to 25 mL with deionized water (Milli-Q 18.2 MΩ

cm; Millipore, Bedford, MA, USA), and analyzed by ICP-OES Optima 8300 (Perkin Elmer, Inc., PA, USA). for the quantification of nutrimental analysis.

Determination of Nitrogen

As for the quantification of nitrogen by micro kjeldahl, 0.5 g of milled sample was placed in a digester tube with $CuSO_4$ and anhydrous Na_2SO_4 according to Page et al (1965) using 10 mL of concentrated H_2SO_4; sample was then processed in the micro kjeldahl distillation unit (Rapid Still II, 65200), Labconco), the aliquots were titrated with 0.1N HCl (Bremner 1965).

Analysis of data

The elementary percentage data were analyzed through an analysis of variance and comparison of Tukey means, using the statistical software R, version 3.3.0 (Venables and Smith 2014) ($p<0.05$); The analysis was complemented by a Pearson correlation in the SAS Enterprise 4.3 statistical program (SAS institute 1985). Comparisons between methods were analysed with data from all samples, to determine the relationship between both techniques on root and foliage (48 replicates per technique). Extraction curves were achieved in absorbed element per plant (mg) using SigmaPlot 11.0.

RESULTS

In the present study, no significant differences were observed according to the type of substrate (PM and TM)evaluated in the seedling obtained from *P. greggii* for the elemental percentage of N, K, Ca, Mg, S and Fe determined by the EDS technique;

except for the elements P and Mn, where the PM substrate presented higher percentages.

In relation to the elementary percentage estimated by ICP-OES, there are highly significant differences between both substrates, with the elementary percentage in PM being higher for all evaluated nutrients, except for calcium ($p< 0.0708$) (**Table I**). The absorption in nutriment in mg (mg per plant) throughout the monthly evaluation is presented in **Figures 2** to **6,** where TM substrate showed lower values than PM for all the elements; This shows the same propensity for the accumulation of biomass both aerial and root, in which it is observed that the best substrate for this variable (biomass) was PM, with values at 210 days after transplantation (300 days after germination of seed) of 3.49 ± 0.52 g in PM versus 0.60 ± 0.14g in TM in the shoot and 1.13 ± 0.27 g in PM compared to 0.31± 0.16 g in TM obtained in the root portion (**Fig. 1**).

The analysis of variance between elemental determination techniques (EDS and ICP-OES) in percentage of nutrients, indicates that there is no significant difference between methods for P, Mg and S values (**Table III**), and there is a highly significant correlation ($p<.0001$) between these methods in the determination of P, K, Ca, S and Fe (percentage) with Pearson values of 0.76, 0.54, 0.83, 0.59 and 0.77 respectively (**Table II**). Nitrogen absorption in foliage at 210 days after transplantation was 122.38 ± 11.40 mg per plant obtained in PM and 8.94 ± 0.12 mg per plant in TM by microkjeldal method; as the EDS technique revealed 17.47 ± 2.24 mg per plant in PM compared to 6.19 ± 1.99 mg per plant obtained in TM (**Fig. 2**).

For K absorption values of 21.11 ± 3.80 mg per plant were found determined by ICP-OES compared to 30.84 ± 4.56 mg per plant determined by EDS in PM substrate, 210 days after transplantation. (**Fig. 4**). Ca absorption was more eminent in the substrate TM (1.09 ± 0.52 % in ICP-OES and 0.71 ± 0.38% in EDS) In some studies, the standards of Ca are between 0.1 to 0.52%. for forest species; the highest accumulation of this element was presented at 145 days after transplantation (26.93 ± 14.62 mg per plant in ICP and 10.49 ± 5.32 mg per plant in EDS); despite its high standard error, the decrease in its accumulation can explain the higher amount of Ca in roots at 175, and 210 days after transplantation, from 12.49 to 29.14 mg per plant

in ICP and from 10.69 to 18.43 mg per plant in EDS respectively (**Fig. 5**). These results are similar to those reported by Martínez-Reyes et al (2012), where after 421 days of *P. greggii* plant emergence, the values of 15.01 ± 8.55 mg per plant were obtained.

The phosphorus percentage, varied from 0.22 ± 0.11% in ICP at 0.24 ± 0.17% in EDS; similar data were obtained by Lima-Rojas (2013) in P absorption in *P. greggii* seedlings, (0.24%) indicating that the EDS technique can be used to determine this element in forest plants. The highest level of absorption of P (mg per plant) is shown after 145 days of cultivation in both substrates; in root, the highest absorption of the element was given at 210 days in PM and for TM at 145 days, with values of (0.29 ± 0.11 in ICP versus 0.74 ± 0.33 in EDS) (**Fig. 3**). In calcareous soils, P is a limiting nutrient for the early growth of *P. greggii*, and the availability of Ca in the substrate, can hinder the absorption of P and K from the soil.(Calvo-Alvarado et al 2008).

The absorption of Mg in percentage by both described techniques, is very similar varying from 0.13 to 0.18%; similar data were found by Albaugh et al (2008), Sypert (2006) and Wallander and Nylund (1992) in some forest species; Figure 6 shows the accumulation curve of this nutrient. The percentage of sulfur obtained by EDS varied from 0.18% in EDS to 0.33% in ICP, data found between the reported by Iyer et al (2002) in P. strobus L. The greatest accumulation of this element, was in root on both substrates with a maximum of 14.79 ± 8.32 mg per plant.

DISCUSSION

Obtained results in PM substrates, were similar to those reported by Iyer et al (2002), in which obtained seedlings of P. strobus with slow-release fertilizers, ranged from 2.6 to 2.8 g at 210 days of growth. In a *P. greggii* production, in a forest nursery at 210 days of age, Sáenz-Reyes et al (2014), reported an accumulation of dry biomass, both shoot and root of 2.27 and 0.61 g respectively. Liu et al (2013), reported yields after 205 days of emergence in *P. pinaster* of 2.43 g in stem and 0.41 g in root (control), and using fertilizer, the average of 4.95 g per stem and root of 0.85 g was obtained; also in a *P. greggii* study, where the substrate was prepared from a mixture of sand, bark and forest soil, using a 2: 2: 1 ratio, Martínez-Reyes et

al. (2012), reported values of 0.92 ± 0.57 g in stem and 0.71 ± 0.37 g in root, after 421 days of evaluation. This data is lower than our results obtained in PM substrates. These differences in growth, may be manifested in the establishment phase at the field, where survival rates are affected by this response, and basically depends on genetic attributes and those acquired through management in the nursery (Alcalá et al 2002).

In an evolution of a field survival study, on different substrates, and levels of fertilization in *P. canariensis*, it was found that the plants cultivated in mountain soil, showed lower survivals and reached inferior heights. As far as the plants cultivated with peat, presented survivals of 90% (Díaz et al 2004). This is explained by the higher amount of organic matter that accompanies the root ball at the time of transplantation. The table 1 shows the characteristics of the mixtures where TM has a 3.92% of organic matter, compared to 50% in P.M. In forest plantations, these plants are bound to poor soils, which possess a low organic matter content, and high pH values ranging from 7.0 to 8.3 (Rodríguez-Laguna et al 2012). This decreases the cationic exchange capacity (Ubeda 2001). On the other hand, López-Locia (2005), indicates that the fertilization in the field, did not show significant differences regarding the control and the survival of the evaluated specie; Which presented the best growth response in height and diameter in three planting sites, even with the control treatment (not fertilized in nursery).

Bai et al (2007) evaluated the concentrations of minerals in EDS and ICP-AES, and did not find significant differences between methods with the exception of K, which reports to be underestimated by ICP-AES; the author states that the SEM-EDS technique can provide accurate concentration data for Al, Fe, Mg, Ca and K. The SEM-EDS method proposed by Marguí et al (2005), proved to be an efficient tool for the determination of metals in plants, since it allows to avoid the digestion stage, and the mistakes in the recovery of some elements; however, studies in the elementary nutritional contents in forest plants, for the comparison of both of these methods are scarce.

Data are comparable to the obtained by Liu et al. (2013), who reported 31.97 ± 3.05 mg per plant in *P. pinaster* and higher than referred by Martínez-Reyes et al (2012) in *P. greggii* (4.55 ± 2.73 mg per plant).

The micro nutrient data obtained by EDS are inferior to those found by ICP, or even undetected by this technique. Although in previous studies with the EDS method, the limits of detection and precision were satisfactory to evaluate this type of analysis (Fe and Mn) (Marguí et al 2005).

In this study, we can observe that trends in nutrient absorption are similar in both methods for mineral determination. It should be mentioned that the EDS technique does not differ significantly in most of the nutrients evaluated in relation to ICP-OES, and has the advantage of being less tedious; in fact this technique is quite fast, because the analysis to obtain ashes, can be carried out using the equipment, discarding laborious methods without using reactants that are dangerous, of high care and individual protection which does not require management time and experience.

For the *P. greggii* production seedlings in nursery, the conventional management is recommended, since low fertilization induces the nutritional absorption in a good proportion according to the research carried out in forest species.

CONCLUSIONS

For the creation of *P. greggii* seedlings inside the nursery, a conventional management (peat-based substrate) is recommended, since it presented higher percentages of mineral absorption compared to montain soil ($p < 0.005$) analyzed by ICP-OES. It was demonstrated that the nutrient absorption in both detection methods follows the same propensity with a highly significant Pearson correlation for the nutrients P, K, Ca, S and Fe. Consequently, it is possible to determine the elemental composition of *P. greggii* by the EDS technique, avoiding the use of corrosive materials that lead to laborious procedures and tedious processes in the analysis to obtain minerals by wet digestion according to ICP-OES.

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Tables and Figures

TABLE I. ANALYSIS OF VARIANCE OF THE ELEMENTAL CONTENT (%, ± SE) OBTAINED IN THE PRODUCTION OF *P. greggii* SEEDLINGS ON THE EVALUATED SUBSTRATES.

Method	P	K	Ca	Mg	S	Fe	Mn
EDS PM	0.24 ±0.17a	0.31±0.22a	0.57±0.52a	0.16±0.10a	0.23±0.35a	0.016±0.008a	0.009±0.009a
EDS TM	0.11 ± 0.08b	0.25±0.16a	0.71±0.38a	0.18±0.12a	0.19±0.16a	0.010±0.008a	0.004±0.001b
ANOVA P>F	1.45e-06 ***	0.142	0.169	0.437	0.528	0.938	8.44e-07 ***
ICP PM*	0.22±0.11a	0.44±0.10a	0.85±0.76a	0.13±0.03b	0.31±0.37a	0.015±0.01b	0.010±0.009a
ICP TM*	0.089±0.03b	0.36±0.12b	1.09±0.52a	0.18±0.03a	0.18±0.07b	0.025±0.02a	0.007±0.007a
ANOVA P>F	6.41e-12 ***	0.00118 **	0.0708	7.71e-13 ***	0.0231 *	0.00275 **	0.0519 *

EDS = X-ray diffraction spectrometry, ICP= inductively coupled plasma spectrometry, PM = Peat moss: perlite (1: 1), TM = Mountain soil, sand and silt (2: 1: 1); ICP PM*, ICP TM * = for nitrogen, this value was obtained by a Micro Kjeldahl method. Means with the same letter do not differ statistically from each other. n=48 replicates.

TABLE II. COMPOSITION OF THE EVALUATED SUBSTRATES.

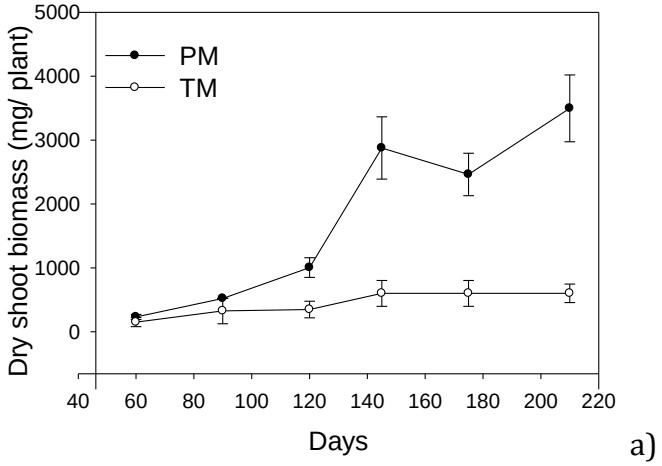
	pH	OM (%)	N (%)	P (ppm)	Ca ($mg.Kg^{-}$)	Mg ($mg.Kg^{1}$)	Clays (%)	Silt (%)	Sand (%)
PM	4.35	50.659	0.735	1055.17	2700	302.5	18.16	33.64	48.20
TM	7.83	3.922	0.210	0.936	8200	786.5	21.98	21.64	56.38

PM=peat moss, perlite (1:1), TM= Mountain soil, silt and sand (2:1:1), OM= organic matter.

Table III. Pearson correlation and analysis of variance for elemental composition (%, ± SE) derived from the method Micro Kjeldahl, ICP-OES and EDS in *P. greggii* seedlings obtained in two substrates.

	P	K	Ca	Mg	S	Fe	Mn
EDS PM	0.24±0.17a	0.31±0.22b	0.57±0.52b	0.16 ± 0.1a	0.22±0.35a	0.01±0.00 a	0.009±0.00a
ICP PM*	0.22±0.11a	0.44 ±0.10a	0.85±0.75a	0.13 ± 0.03a	0.3±0.37a	0.01±0.01 b	0.010±0.00a
PCC	**0.768**	**0.541**	**0.837**	0.036	**0.596**	**0.772**	0.193
P>F	**<0.0001**	**<0.0001**	**<0.0001**	0.806	**<0.0001**	**<0.0001**	0.189
ANOVA							
Pr>F	0.581	0.0004	0.045	0.084	0.242	0.034	0.759
EDS TM	0.11±0.08a	0.25±0.16b	0.70±0.38b	0.18±0.13a	0.19±0.16a	0.01±0.00b	0.004±0.00b
ICP TM*	0.09±0.03a	0.36±0.12a	1.09±0.52a	0.18±0.03a	0.18±0.07a	0.02±0.02a	0.007±0.00a
PCC	0.306	**0.569**	**0.699**	0.197	**0.396**	**0.449**	0.079
P>F	0.0345	**<0.0001**	**<0.0001**	0.179	**0.0054**	**0.0014**	0.595
ANOVA							
P>F	0.925	0.0003	<0.0001	0.736	0.871	<0.0001	<0.0001

EDS = X-ray diffraction spectrometry, ICP= inductively coupled plasma spectrometry , PM= Peat moss, perlite (1:1), TM= mountain soil, silt and clay (2:1:1), ICP PM*= for nitrogen, this value was obtained by micro kjeldahl, ICP TM * = for nitrogen, this value was obtained by micro kjeldahl, PCC = Pearson's correlation coefficient. The mean value represents 48 replicates. Highlighted fields represent elevated PCC highly significant ($p<0.01$)



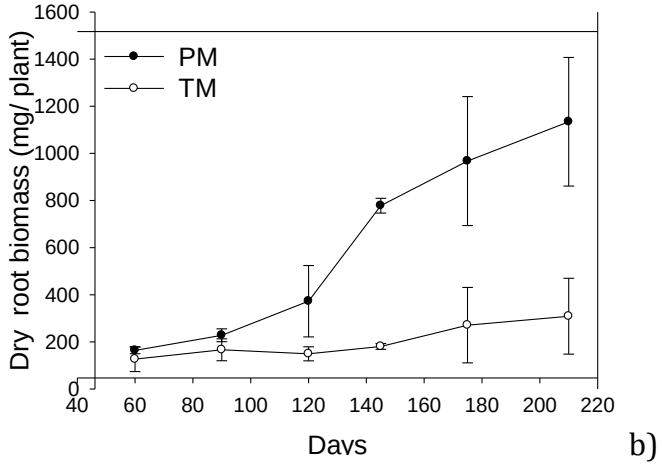


Fig 1. a) Dry biomass in aerial part and root (b) in seedlings of *P. greggii* var. *greggii* obtained on two substrates, PM= Peat moss: perlite (1:1), TM= Mountain soil, silt and sand (2:1:1)

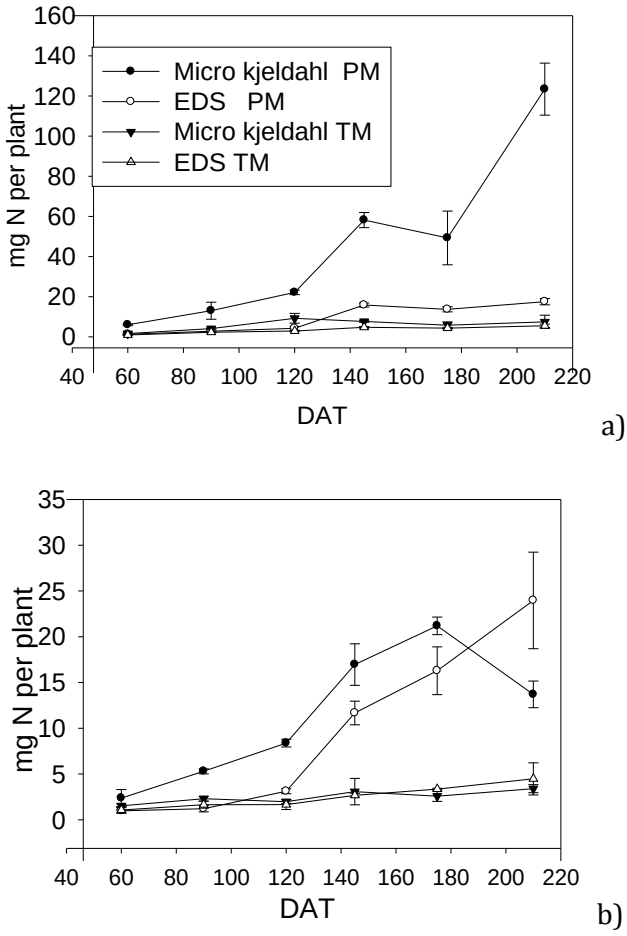
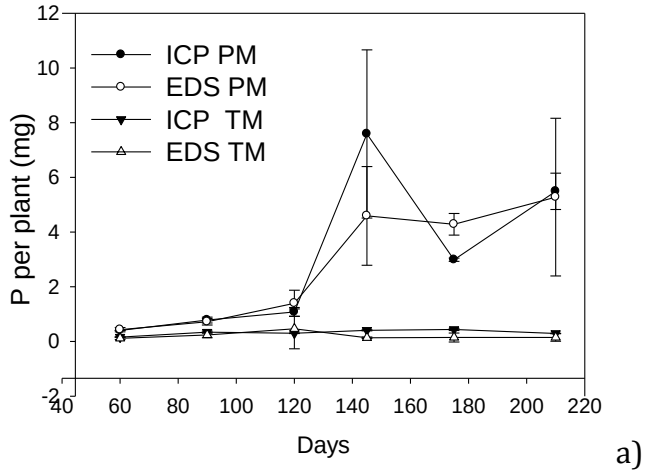
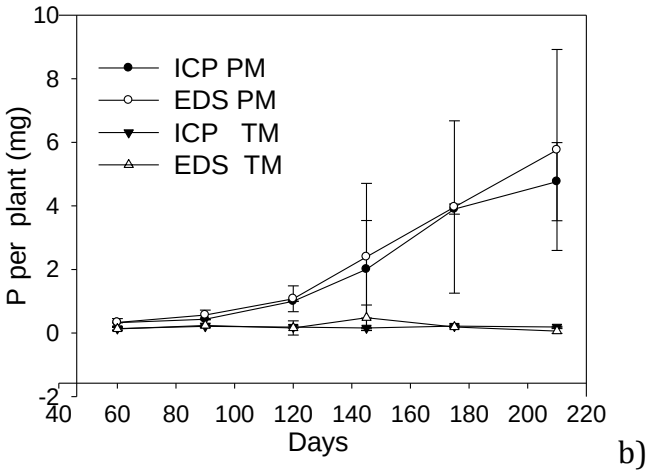


Fig 2. Nitrogen extraction curve (mg per plant) in stem (a) and root (b) in *Pinus greggii* var. *greggii* seedlings after 210 days of transplantation (DAT). EDS = X-ray energy diffraction spectrometry, ICP= inductively coupled plasma spectrometry, PM= Peat moss: perlite (1: 1), TM = Mountain soil, sand and silt (2: 1: 1)



a)



b)

Fig 3. Phosphorus extraction curve (mg per plant) in stem (a) and root (b) in *P. greggii* seedlings. EDS = X-ray diffraction spectrometry, ICP= inductively coupled plasma spectrometry, PM = Peat moss, perlite (1: 1), TM = Mountain soil, sand and silt (2: 1: 1)

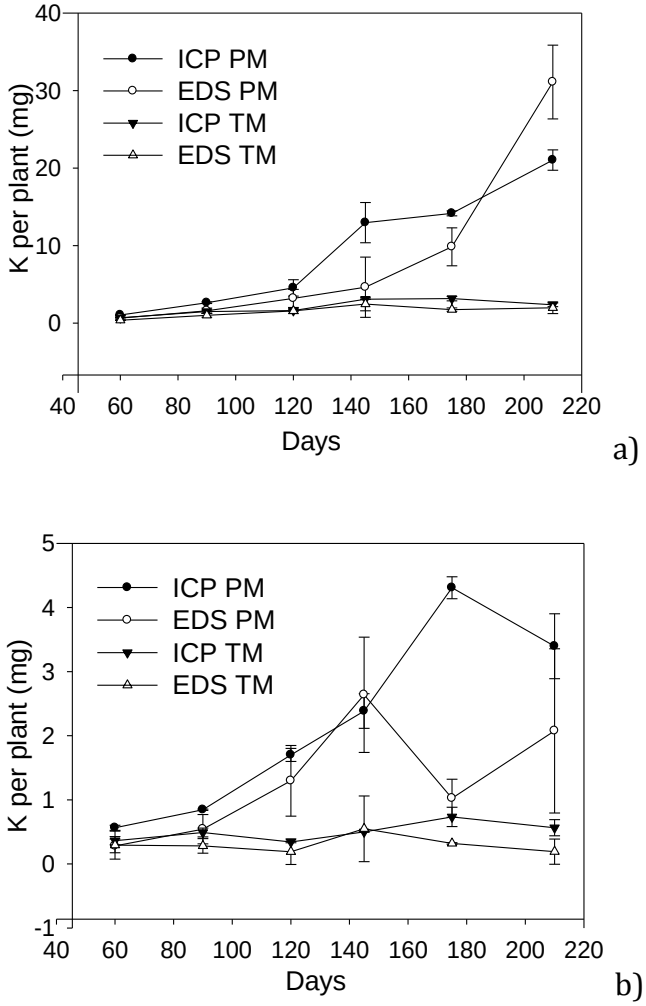
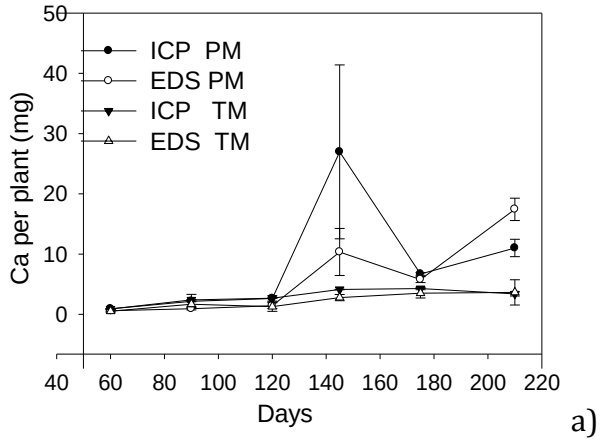


Fig 4. Potassium extraction curve (mg per plant) in aerial part (a) and root (b) in *P. greggii.* EDS = X-ray energy diffraction spectrometry, ICP= inductively coupled plasma spectrometry, PM = Peat moss, perlite (1: 1), TM = Mountain soil, sand and silt (2: 1: 1)



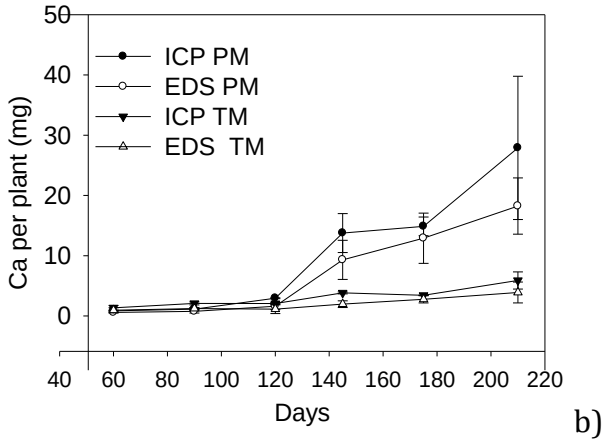
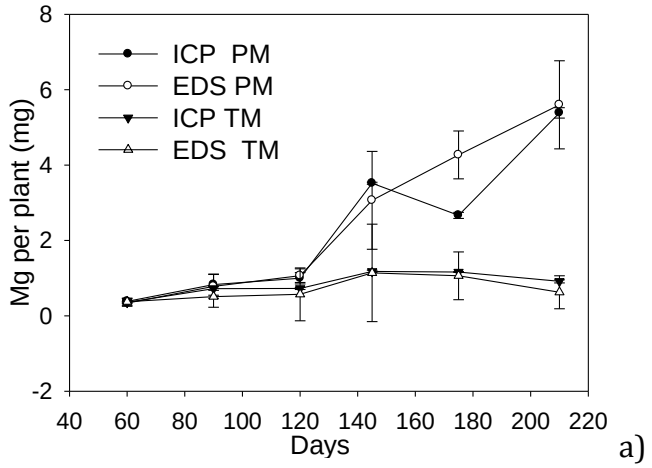


Fig 5. Calcium extraction curve (mg per plant) in stem (a) and root (b) in *P. greggii* seedlings. EDS = X-ray diffraction spectronomy, ICP= inductively coupled plasma spectronomy, PM = Peat moss, perlite (1: 1), TM = Mountain soil, sand and silt (2: 1: 1)



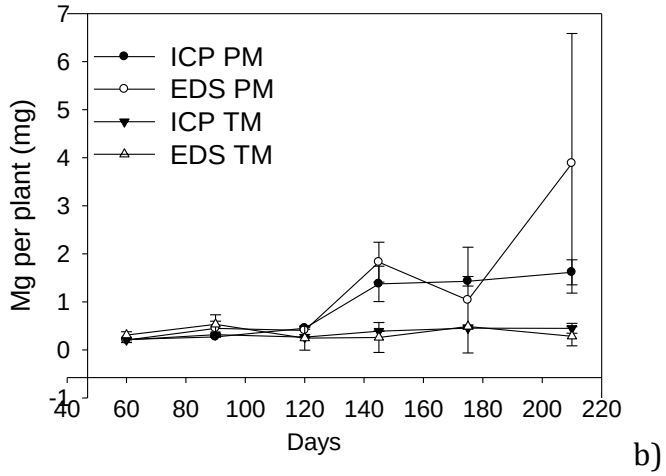


Fig 6. Magnesium (mg per plant) extraction curve in the stem (a) and root (b) in *Pinus greggii* var. greggii. EDS = X-ray diffraction spectrometry, ICP= inductively coupled plasma spectrometry, PM = Peat moss: perlite (1: 1), TM = Mountain soil, sand and silt (2: 1: 1)

ARTÍCULO 2

Improved parameters of *Pinus greggii* seedling growth and health after inoculation with ectomycorrhizal fungi

Improved parameters of *Pinus greggii* seedling growth and health after inoculation with ectomycorrhizal fungi.

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Abstract

Pinus greggii Engelm. ex Parl. is an endemic pine of Mexico with notorious adaptability to eroded, shallow and poor soils. It is widely used in reforestation programmes worldwide. The purpose of the present study is to develop an ectomycorrhizal fungi (ECM) treatment with different native ectomycorrhizal fungal species (collected in *P. gregii* and *Abies vejarii* stands) to improve seedling survival of *P. greggii.* Four month old *P. greggii* var *greggii* seedlings were inoculated with ground fruiting bodies of ECM species: *Astraeus* spp., *Boletus barrowsii*, *Geastrum minimum*, *Lactarius deliciosus* var. *deterrimus*, *Russula* cf. *atroglauca*, *Russula* spp., *Suillus caerulescens*, as well as a mixture of three species (*Russula* sp., *R. lutea* and *L. deliciosus*) and soil litter with mycelium from the base of a *L. deliciosus* fruiting body. Agronomic variables and glucosamine content were measured in *P. greggii* seedlings. Inoculated treatments with *B. barrowsii*, *Geastrum minimum,* and *S. caerulescens* showed increases in seedling height and basal diameter as well as dry shoot biomass compared to control plants. *G. minimum*, *B. barrowsii* and *R. xerampelina* significantly increased values of dry weight compared to control. Inoculation of soil litter from the base of *L. deliciosus* induced the maximum

glucosamine response (40.2 mg g^{-1} of dry root biomass); *G. minimum* a fungi collected in *P. greggii* stands, enhanced fresh root and shoot biomass and basal diameter of pine seedlings in the nursery. Fungal species *B. barrowsii, Suillus caerulescens, Russula* cf. *atroglauca* and *Russula* spp. from the *Abies* forest increased plant quality by measurig Dickson Index quality and root and shoot biomass compared to the control.

Key Words: ectomycorrhizae, *Geastrum minimum,* glucosamine, greenhouse, Index quality, *Pinus greggii, seedlings*.

1. Introduction

The role played by ectomycorrhizal fungal (ECM) associations in the nutritional status of forest trees, is currently recognized as crucial for ecosystem health. The network of fungal hyphae around the roots explores and draws water and nutrients from distant sites and transfers these to the host plant in exchange for photosynthetic carbohydrates (Van Der Heijden and Horton, 2009; Sousa et al., 2012). This tree-fungus symbiosis has been used in reforestation programs on a large scale, to improve nutrition, growth and adaptation of plants (Karkouri et al., 2005) and colonization of seedlings is important to their establishment and survival after transplant (Iwanski and Rudawska, 2006). For these reasons, studies of with ECM fungal inoculation in forests have been increasing (Cram et al., 1999). However ECM inoculation requires significant operational expenses; also, seedlings should be inoculated with fungal species best adapted to the environmental conditions of the final planting site (Menkis et al., 2007). The root association of tree seedlings with different communities of ECM fungi in forest nurseries can contribute to the quality of the plants (Menkis et al., 2005; Menkis and Vasaitis, 2011) and,

subsequently, improve their establishment and growth after transplantation in the field (Ortega et al., 2004).

Pinus greggii Engelm. ex Parl. is a Mexican endemic species, considered important due to its genetic plasticity and adaptation to eroded and otherwise poor soils (Dvorak and Donahue, 1993); it can be a dominant (or only) tree species in forests; therefore, in these ecosystems, diverse herbaceous and shrubby plants depend on the microenvironment generated by this species (Ramírez-Herrera et al., 2005). This pine is widely used in reforestation programmes for recovery of watersheds worldwide (Musálem and Martínez, 2003) and is tolerant to drought and certain pests and forest diseases (INIFAP, 2003). In addition, *P. greggii* has shown high growth rates in plantation trials (López et al., 1999; Salazar et al., 1999). These characteristics favor the use of *P. greggii* in commercial plantation programs in marginal areas where other *Pinus* species do not adapt. In Mexico, it is the fourth pine species in importance in plantations of the National Reforestation Program (Ramirez-Herrera et al., 2005). Cripps and Trusty (2007) confirmed the validity of using ground fruiting bodies (mushrooms) of ECM (a spore + mycelium slurry) as inoculum for establishment of ectomycorrhizae in pine seedlings. Martinez-Reyes (2012) assessed the use of an edible fungus, *Hebeloma mesophaeum,* on *P. greggii* seedlings in the nursery and Rentería-Chavez et al. (2017) evaluated the edible ECM fungi, *Laccaria laccata*, *L. bicolor* and *Hebeloma leucosarx,* on seedlings grown in two different substrates. A commercial product based on *Pisolithus tinctorius* is used in nurseries (unpublished data, SEDENA, Military Forest Nursery, 2017) however, there are no records of its effect on the growth and vigour of *P. greggii.* The purpose of this study was to evaluate the development of *P. greggii* seedlings after inoculation with ECM fungi

using fruiting bodies slurries; the mushrroms were collected from two field sites: one *P. greggii* natural stand and a natural temperate forest of *Abies vejarii* Martínez.

2. Materials and Methods

Fruiting bodies of ECM fungi present at 1.5 m from the trunk of *P. greggii* trees were collected in a mesic forest at Monterreal (25° 20' 38.68" N, 100° 34' 34.54" W) and xeric forest at Jamé (25° 21' 00" N, 100° 36' 00" W), municipality of Arteaga, state of Coahuila, Mexico, on 28-31 July 2015. Fruiting bodies were placed in Ziploc® bags and kept in a cooler for two hours, while taken to the laboratory of the Department of Horticulture, Universidad Autónoma Agraria Antonio Narro, Saltillo, Coahuila. Specimens were stored in the refrigerator at 4ºC for 24 hours before processing.

i. Molecular identification of the ECM fungi

Of the ECM fungi stored in refrigeration, 100 mg of each specimen were taken and macerated with liquid nitrogen to powder. About 20 mg were taken for DNA extraction using the Biobasic EZ-10 Spin Column kit (Biobasic Inc., Ontario, Canada) following the manufacturer's instructions. For PCR amplification 2µl of DNA (2 ng/ul) and primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) at 10 pmol/µl, plus 13 µL of double distilled sterilized H_20, 5x 4UL FirePol® Hot Mix Master Blend, 10 mM $MgCl_2$, and 2 mM dNTPs each in a final volume of 20 µl were used. For DNA samples that did not yield PCR products, amplification was repeated with ITS0F-T (5'-ACTTGGTCATTTAGAGGAAGT-3 ') in combination with a designed basidiomycete-specific LB-W primer (5'-CTTTTCATCTTTCCCTCACGG-3 ') using identical mixture

conditions (Tedersoo et al. 2008). The PCR reaction conditions were as follows: initial denaturation: 95° C, 15 min, 30 cycles of denaturation 95° C, 30 sec, annealing 55° C, 30 sec, elongation 72° C, 60 sec and final elongation at 72° C, 10 min. PCR products were deposited on 1% agarose gels in TBE 1x stained with ethidium bromide, the 100 bp DNA ladder (Solis BioDyne, Tartu Estonia) was used and examined under UV light and were sent to Estonian Genome Center (University of Tartu, Tartu, Estonia) for sequentiation. Sequences were edited and assembled with Sequencher 4.10.1 (Gene Codes, Ann Arbor, MI, USA). Blast searches (Camacho *et al.* 2009) were performed for the representative sequence using the NCBI Nucleotide Blast search.

ii. P. greggii seedling establishment

For *P. greggii* seedling production, seeds were obtained from pine cones collected in December 2014-January 2015 from two locations: Tapona, Galeana, Nuevo León, (100° 6' 44.33" W and 24° 43' 39.90" N), and Cañón de Caballos, Saltillo, Coahuila (100° 54' 46.61" W and 25° 14' 47.13" N). Cones from the two localities were placed in hot water (90° C) for 10 min, since *P. greggii* is a serotinous pine; then stored in plastic bags to keep them moist and placed in a glass chamber outdoors to favor the opening of cones under the heat of the sun for 16 hours per day for two days (Flores-López, 2015, unpublished data). Seeds were extracted from cones and disinfected with 2% hydrogen peroxide for 5 h. Once dried, seeds were sown in 77-cavity trays with 1:1 sterile peat moss and perlite medium (autoclaved twice at 120° C and 15 psi for 1 h); seedlings were kept in the nursery under shade mesh (90% black) and were watered three times/week with tap water (pH 6.2); temperature in the nursery was 28°-43° C. Two-month old seedlings of equal size were individually transplanted into black polyethylene bags (10 x 20 cm) containing 1:1

sterile peat moss and perlite. Osmocote 14-14-14® Classic (The Scotts Miracle-Gro Company, Marysville, OH, United States) was applied at 1 kg m^3 as a fertilizer. Seedlings were allowed to establish for a further two months prior to inoculation.

iii. Inoculation of seedlings with ECM fungi

Three different inoculum treatments of ECM fungi (Table 1) were evaluated in four-month old *P. greggii* seedlings, as follows: 50 g of ECM fruiting bodies from the two localities (Monterreal and Jamé); 50 g of a mixture of ECM fruiting bodies, or 50 g of soil litter from the base of the fruiting bodies. All three inocula were homogenized in 1 L of tap water (pH 6.2) in an Osterizer® 12-speed blender (Sunbeam-Oster, USA). Thirty mL of each treatment were added to the base of three seedlings (experimental unit) with four replicates per treatment. For non-inoculated (control seedlings) thirty mL of water were added.

iv. Plant growth response

Seedlings were harvested 14 weeks after inoculation. Care was taken to ensure that the root systems of individual plants were extracted as intact as possible. Plant growth variables examined were: fresh and dry biomass of stem and roots; plant height; basal diameter; the seedling Dickson quality index (QI) (which expresses the balance of the distribution of mass and robustness) (see Dickson et al., 1960) integrates the aspects of total plant mass, the sturdiness quotient and root length/shoot height ratio. The QI explains plant potential for survival and growth in the field. High index values are better (Ahmadloo, 2012); and avoids a bias for disproportionate plants whereas selecting sometimes for lower height but more vigor (García, 2007); and glucosamine and

phosphorus contentsin roots. Glucosamine, a saccharide found in the cell walls of fungi, is used as indicator of fungal colonization in roots (REF) (see next section). After measuring, shoots were dried at 60°C for 72 h to obtain dry mass. All three seedlings from each replicate (four replicates) were evaluated for the plant growth variables described, and four seedlings were randomly selected for determination of phosphorus and glucosamine content (Blix, 1948; AOAC, 1995)

v. Determination of glucosamine content in inoculated roots

For determination of fungal colonization, we used the modified technique of glucosamine content determination given by Blix (1948): 100 mg of dry sample of roots were placed in glass tubes (50 mL) and 5 mL of H_2SO_4 5N were added, stirred for 15 min and then centrifuged at 6000 rpm for 10 min. Each sample was filtered through Whatman No. 42 filter paper then washed twice with distilled water. The sample was transferred with spatula to a glass tube (13x100 mm) with screw cap and 500 µL of HCl 10M were added. Samples were incubated for 16 h at room temperature. They were then suspended in 4 mL of distilled water and autoclaved at 15 psi for 2 h. Samples were adjusted to pH 7 with NaOH 10N after cooling. An aliquot of 200 µL acetylacetone (1mL acetylacetone in 50mL of Na_2CO_3 0.5N) was added to 200 µL of each sample solution and incubated in a water bath at 96°C for 20 minutes. After cooling, 2 mL of 96% ethanol and 200 µL of Erlich reagent (1.6 g 4-(Dimethylamino) benzaldehyde (ACS, 99% Sigma-Aldrich,USA) in 60 mL of HCl:EtOH, 1:1) were added and vortexed. An hour later, absorbance was obtained at 530 nm. A standard curve of 0-200 µg.mL-1 was obtained with glucosamine (D-(+)-Glucosamine hydrochloride, Sigma-Aldrich, USA) as standard.

Determination of total phosphorus

For each analysis, plant material (0.5 g) was placed in a muffle furnace (650°C, 3 h). After this, samples were digested with 10 mL of nitric acid (66%) and boiled on a hot plate; 1 mL of 30% H_2O_2 was added after cooling and the sample was filtered (Whatman filter paper No. 42). Samples were diluted in 100 mL of deionized water. Thereafter a colorimetric technique was followed to measure total phosphorus, using the reducing agent aminonapthol sulphonic acid (ANSA) (AOAC, 1995); for the standard solution and calibration, 0.4394 g of potassium phosphate monobasic was dissolved in 300 ml of distilled water and 200 ml of 1N sulfuric acid was added and adjusted to 1L.

vi. Data analysis

The data (plant growth variables, glucosamine and phosphorus content) were analyzed using one-way analysis of variance (ANOVA) using a completely randomized design with ten treatments and four replicates. Means were compared using Tukey's multiple range test. All statistical analyses were performed using R 2.15.3 package. (R Core Team, 2013).

3. Results and Discussion

i. Identification of ECM fungi

The fungi used for inoculation, the Genbank accession and percentage identity with closest match, locality from which each fungus was collected and treatment code are listed in Table 1. A soil litter inoculum from the base of a *L. deliciosus* fruiting body (LDS), and a mixture of fungi: *Russula* sp. 1, *Russula* sp. 2, *R. lutea* and *L. deliciosus* (MixRL) was also used. With the possible exception of *G. minimum,* for which conflicting reports occur

(Trappe, 1962; Agerer and Beenken, 1998; Hosaka et al., 2006), all taxa above are well-known, confirmed ectomycorrhizal fungi.

Table 1. ECM treatment inoculation of *Pinus greggii* seedlings.

ECM Treatment	Species of ECM	Access No.	% Identity	% query coverage	e-value
A*	*Astraeus hygrometricus*	KC152070.1	96	97	2.9E-115
G*	*Geastrum minimum*	KC581957.1	99	97	1.4E-111
R+	*Russula cf. atroglauca*	KT934009.1	100	100	1.27E-92
B+	*Boletus barrowsii*	KC184479.1	100	100	7.94E-84
S+	*Suillus caerulescens*	EU486453.1	97	92	1.32E-80
RX+	*Russula xerampelina*	KJ146730.1	94	100	
LD+	*Lactarius deliciosus* var. *deterrimus*	EF685059.1	97	100	9.0E-101
MixRL+	Mixture of*: Russulla* sp*., Russulla* sp*., Russulua lutea, Lactarius deliciosus*	HQ604848.1ψ			
Control	Water				

ECM = Species of ectomycorrhizae, * = ECM species found and harvested at the Jamé, Coahuila locality; + = ECM species found and harvested at Monterreal, Coahuila locality; ψ = Accession number for *Russulla lutea* only*;* % Identity based on NCBI Nucleotide Blast search.

ii. Determination of agronomic variables

In this section, the treatments where significant differences were obtained compared to the control are shown. *Pinus greggii* seedlings inoculated with *Boletus barrowsii* from temperate stand and *Geastrum minimum* from Jame stand (natural *P. greggii* stand) showed an increase in basal diameter (3.67 ± 0.80 mm and 3.45 ± 0.19 mm, respectively) compared to non-inoculated plants (2.42 ± 0.25 mm; Fig.1a) the other treatments did not show differences respect to the control; however, *Astraeus hygrometricus* (obtained from natural *P. greggii* stand), *Russula* cf. *atroglauca*, *Russula xerampelina*, soil litter and the mixture of fruiting bodies (MixRL), did not

show differences between *G. minimum* and *Suillus caerulescens* (see Figs 1a and1b). Seedlings inoculated with *G. minimum, Lactarius deliciosus, R. xerampelina, S. caerulescens,* var. *deterrimus* and soil litter treatment showed statistical differences in height increase with respect to control (Fig 1b). The highest height was registered with the treatments *G. minimum* and *S. caerulescens* (32.1±3.84 cm) and (30.33±7.12 cm) respectively compared with the treatments that followed in increment: soil litter (28.1 ± 1.55 cm) and *L. deliciosus* var. *deterrimus* (28.08 ± 0.95 cm); in this response, the treatments in which no significant difference was observed with the control (22.21 ± 3.33 cm) were the mixture of fungi (MixRL) (24 ± 1.63 cm), *R. xerampelina* (25.37 ± 2.01 cm), *B. barrowsii* (25.75 ± 1.7), *R. atroglauca* (25.87 ± 2.56) and *A. hygrometricus* (25.75 ± 0.86) the last mentioned fungus together with *G. minimum*, belong to the natural locality of *P. greggii*; only *G. minimum* induced response for height and basal diameter; it has been demonstrated that height attained is a reliable predictor of pine seedling growth (Newton et al., 1993; Randall and Johnson, 1998). Only *G. minimum*, *B. barrowsii* and *S. caerulescens* significantly ($p<0.05$) increased dry shoot mass (2.45 ± 0.49, 2.34 ± 0.20, 2.32 ± 0.70 g of biomass, respectively) compared to non-inoculated plants (1.30 ±0.14 g) (Fig. 3); the figure 6 shows the comparison between *B. barrowsii* and control after 14 weeks of inoculation. In a similar experiment, Kipfer et al. (2012) found that *Suillus granulatus* considerably increased shoot biomass of *Pinus silvestris* seedlings compared to non-mycorrhizal plants.

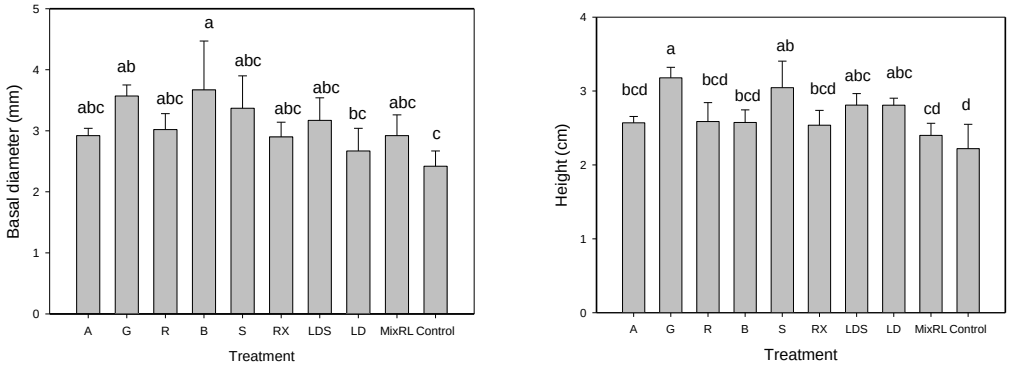

Fig. 1. Effect of different ECM fungi inoculated alone and in combinations on basal diameter ± sd (a) and height ± sd (b) of *P. greggii var greggii* seedlings. Means with the same letter are not significantly different (Tukey's test; $p<0.05$). *Astraeus hygrometricus* (A), *Geastrum minimum* (G), *Russula cf. atroglauca* (R), *Boletus barrowsii* (B), *Suillus caerulescens* (S), *Russula xerampelina* (RX), *Lactarius deliciosus* var. *deterrimus* (LD), soil litter from the base of *Lactarius deliciosus* fruiting bodies (LDS) and mixture of: *Russula sp., Russula sp., Russula lutea* and *Lactarius deliciosus* (MixRL), and Control.

Differences were recorded also between inoculated and non-inoculated seedlings for dry root biomass (Fig. 2) *B. barrowsii, G. minimum and Russula* cf. *atroglauca* increased biomass (0.69 ±0.17, 0.66 ±0.19, 0.69 ±0.27 g, respectively) versus the control (0.32 ±0.025 g) in the other treatments, no significant differences were found with the control and even the *L. deliciosus* var. *deterrimus* treatment had a lower root dry biomass (0.25 ± 0.049) than the control, plants treated with mixture of fungi (MixRL with four ECM species) were lower than those treated with a single fungal species (Figs 2 and 3). This might indicate antagonism or competition between ECM fungal species. These results are different to those reported by Sim and Eom (2006) who suggested that increasing the number of ECM species with which to inoculate will increase seedling growth.

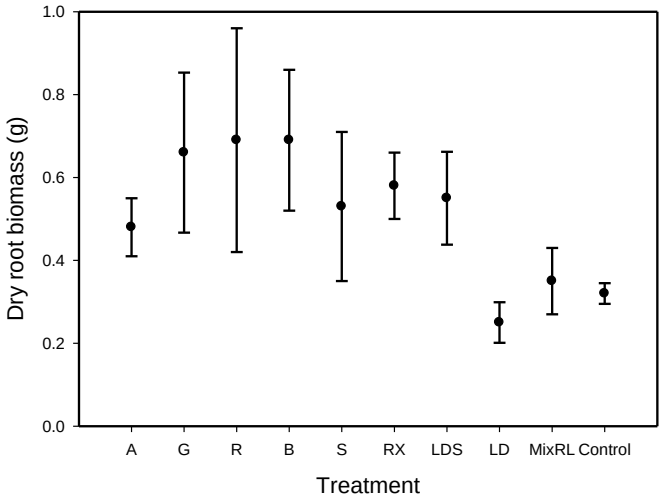

Figure 2. Effect of ECM inoculated treatments on dry root biomass ± sd of *P. greggii* seedlings. *Astraeus hygrometricus* (A), *Geastrum minimum* (G), *Russula cf. atroglauca* (R), *Boletus barrowsii* (B), *Suillus caerulescens* (S), *Russula xerampelina* (RX), *Lactarius deliciosus* var. *deterrimus* (LD), soil litter from the base of *Lactarius deliciosus* fruiting bodies (LDS) and mixture of: *Russula* sp., *Russula* sp., *Russula lutea* and *Lactarius deliciosus* (MixRL) and Control.

In generally, biomass increases were lower than those reported by Martínez-Reyes et al. (2012) who found that *P. greggii* plants inoculated with edible fungi were 2.9 times larger than non-inoculated plants. In terms of pine biomass production, beneficial effects have been reported before as a result of inoculation with other ectomycorrhizal mushrooms (Christopher et al., 2010; Dalong et al., 2011; Sim and Eom, 2006).

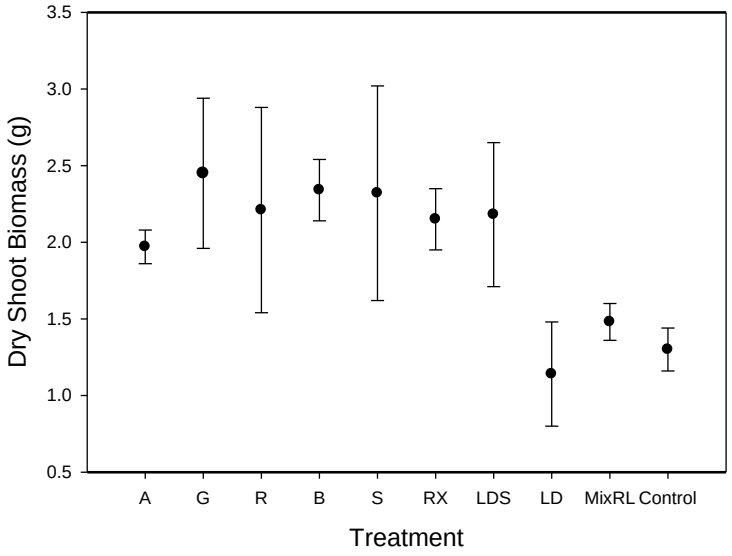

Figure 3. Effect of different ECM inoculated treatments on Dry Shoot Biomass ± sd of *P. greggii* seedlings*Astraeus hygrometricus* (A), *Geastrum minimum* (G), *Russula cf. atroglauca* (R), *Boletus barrowsii* (B), *Suillus caerulescens* (S), *Russula xerampelina* (RX), *Lactarius deliciosus* var. *deterrimus* (LD), soil litter from the base of *Lactarius deliciosus* fruiting bodies (LDS) and mixture of: *Russula* sp., *Russula* sp., *Russula lutea* and *Lactarius deliciosus* (MixRL) and Control.

Only the *B. barrowsii* ECM inoculation treatment produced superior plant quality (QI) (0.29 ± 0.073) compared to the control (0.124 ± 0.013, Fig. 5) nevertheless, *B. barrowsii* was not statistically different between the treatments *A. hygrometricus* (0.19 ± 0.01), *G. minimum* (0.24 ± 0.05), *R. cf. atroglauca* (0.24 ± 0.07), *S. caerulescens* (0.21 ± 0.07), *R. xerampelina* (0.22 ±0.03) and soil litter (0.22 ± 0.05).

Table 2. Effect of different ECM fungi inoculations and their combinations on Glucosamine and Phosphorus content of *P. greggii* seedlings.

Treatment	ECM Species	P (ppm) [a]		Glucosamine (mg/g) [a]	
A	*Astraeus hygrometricus*	25.92 ±5.0	a	7.29 ±1.2	de
G	*Geastrum minimum*	26.31 ±2.37	a	6.38 ±0.96	cde
R	*Russula cf. atroglauca*	18.32 ±0.72	bc	12.6 ±2.08	bcd
B	*Boletus barrowsii*	25.94 ±0.24	a	13.5 ±5.16	bcd
S	*Suillus caerulescens*	10.43 ±0.58	d	15.2 ±2.85	bc
RX	*Russula xerampelina*	27.89 ±2.47	a	16.2 ±8.02	b
LDS	*Lactarius deliciosus* (soil)	28.21 ±3.5	a	40.2 ±1.37	a
LD	*Lactarius deliciosus*	15.34 ±1.73	cd	3.9 ±1.34	e
MixRL	*Russulla* sp.,*Russulla* sp., *Russulua lutea, Lactarius deliciousus*	24.78 ±1.07	a	14.4 ±4.33	bcd
Control	Test	23.51 ±2.79	ab	0.26 ±0.076	e
CV		10.99		27.08	
MS Error		6.2		0.129	

[a] The values represent mean ± SE of four replicates (experimental unit of three plants). Different letters represent significant differences among inoculation treatments according to Tukey's test ($p<0.05$). *Astraeus hygrometricus* (A), *Geastrum minimum* (G), *Russula cf. atroglauca* (R), *Boletus barrowsii* (B), *Suillus caerulescens* (S), *Russula xerampelina* (RX), *Lactarius deliciosus* var. *deterrimus* (LD), soil litter from the base of *Lactarius deliciosus* fruiting bodies (LDS) and mixture of: *Russula* sp., *Russula* sp., *Russula lutea* and *Lactarius deliciosus* (MixRL). CV = coefficient of variation, MS Error = Mean square error.

Analysis of the Dickson quality index means allows for better evaluation of morphological differences between plants. Field-collected fruiting bodies from temperate areas in comparison to native fruiting bodies of this pine species (*G. minimum* and *A. hygrometricus*), could not survive in the absence of their host plants; some select or pure-culture inoculants may support high productivity in certain site conditions but may be less productive than native strains on other sites (Wilkinson, 2009).

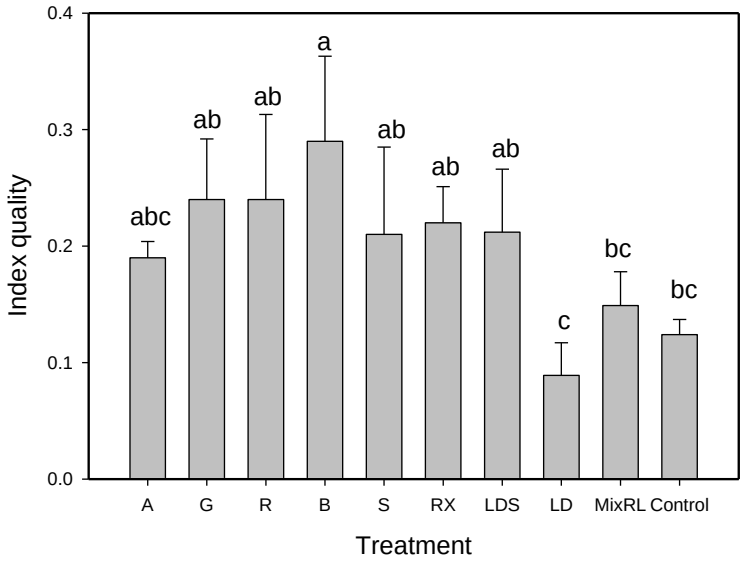

Figure 5. Dickson quality index ± sd evaluated in *P. greggii* seedlings. *Astraeus hygrometricus* (A), *Geastrum minimum* (G), *Russula cf. atroglauca* (R), *Boletus barrowsii* (B), *Suillus caerulescens* (S), *Russula xerampelina* (RX), *Lactarius deliciosus* var. *deterrimus* (LD), soil litter from the base of *Lactarius deliciosus* fruiting bodies (LDS) and mixture of: *Russulla* sp., *Russulla* sp., *Russulua lutea* and *Lactarius deliciosus* (MixRL). Means with the same letter between inoculation treatments are not statistical diferrent according to Tukey's test ($p<0.05$).

In spite of the evaluation made with fruiting bodies of ECM fungi from humid soils and temperate climates, it was observed that *G. minimum* affected the height, dry weight and quality of the *Pinus greggii* seedlings in positive way; this may be due to this fruiting body was collected in natural areas of this species of pine. At the Jamé site, *P. greggii* is a

dominant tree, while it is an uncommon tree at Monterreal, where *Pinus ayacahuite*, *Abies vejarii* and *Pseudotsuga menziesii* (spruce) dominate.

In a detailed review done by Renaldi et al. (2008), stated that *Geastrum* previously classified as saprophytic fungi, some authors have mentioned it as ectomycorrhizal; adding to the controversy the observation that the Harting net (typical of ECM) is missing in *G. minimum*; they pointed out that some saprotrophic fungi are exclusively associated with conifer species being sensitive to the enrichment of N. The right selection of ECM inoculum for a coniferous species like *P. greggii* is essential for maximizing the benefits that this practice may have on plant development at the nursery and subsequently in the field (Oliveira et al. 2010). The priority of inoculating containerized-grown seedlings is the extent of mycorrhizal success and the subsequent improvement in survival and growth in the field (Long-Chen et al., 2006). In this study, we showed that co-inoculation with various species of ECM fruiting bodies is less effective than individual inoculation, as proposed by the present results

iii. Determination of glucosamine content in inoculated roots

Inoculation with *L. deliciosus* var. *deterrimus*, *G. minimum* and *A. hygrometricus* , produced a reduced amount of glucosamine content (3.9 ±1.34, 6.38 ± 0.96 and 7.29 ± 1.29 mg.g^{-1}, respectively) with no differences between control (2.6 ± 0.76); seedlings inoculated with other ECM treatments showed no difference among them but were statistically different between control (Fig 4, Table 2). The highest level of glucosamine was present in those seedlings inoculated with the soil litter (40.2 ±1.37 mg g^{-1}) followed by *R. xerampelina* (16.2 ± 8.02 mg g^{-1}), S. *caerulescens* (15.2 ± 2.85 mg g^{-1}), MixRL(14.4 ± 4.33 mg g^{-1}), *B. barrowsii* (13.5 ± 5.16 mg g^{-1}) and *R. atroglauca* (12.6 ± 2.08 mg g^{-1})

(Fig 4). The higher glucosamine levels from soil litter treatment could indicate the presence of saprophytic and endophytic fungi which might not be mycorrhizal. Different methods (e.g. measurement of chitin and ergosterol) have been widely used to indirectly measure fungal biomass (Wallander et al., 2013) or level of fungal colonization (Castrillón et al., 2015; Rentería-Chavez et al., 2017). Free glucosamine can also be measured with chromatographic techniques (Ekblad et al., 1998) but the presence of glucosamine does not distinguish between saprotrophic and ECM fungi in samples, or separate living or dead mycelia (Wallander et al., 2013). The presence of glucosamine in the control indicated a low level of contamination in the seedlings (for example at the time of irrigation) and/or contamination from the in the environment by other fungi.

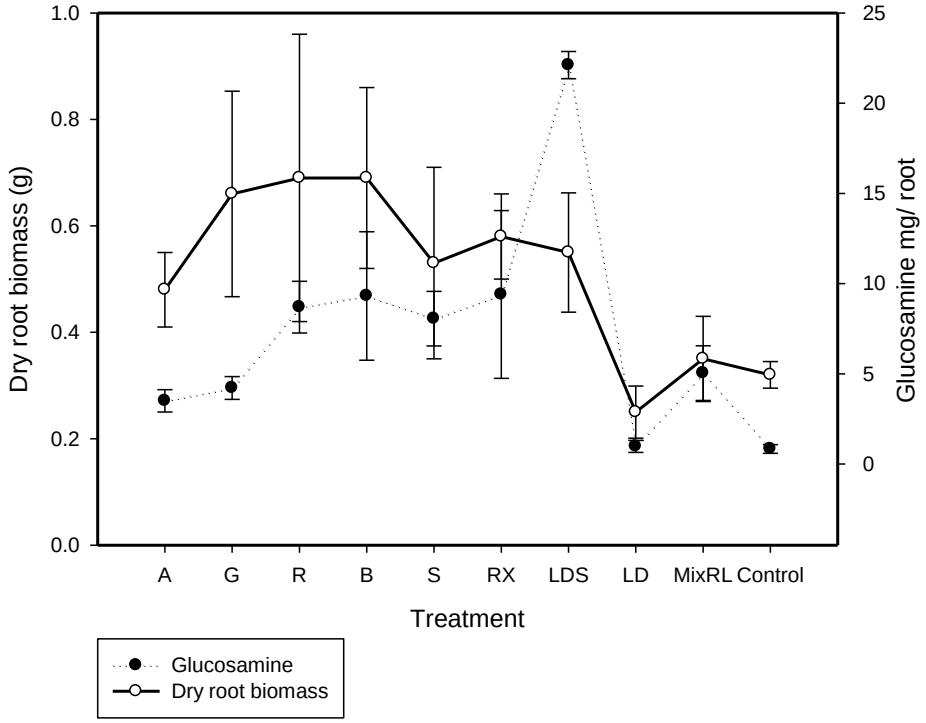

Figure 4. Glucosamine content (mg g^{-1}) of dry root biomass on ECM evaluated treatments. *Astraeus hygrometricus* (A), *Geastrum minimum* (G), *Russula cf. atroglauca* (R), *Boletus barrowsii* (B), *Suillus caerulescens* (S), *Russula xerampelina* (RX), *Lactarius deliciosus* var. *deterrimus* (LD), soil litter from the base of *Lactarius deliciosus* fruiting bodies (LDS) and mixture of: *Russulla* sp., *Russulla* sp., *Russulua lutea* and *Lactarius deliciosus* (MixRL) and Control.

iv. Determination of total phosphorus

The P level in the substrate was 1055.17 ppm. No treatment had higher phosphorus levels than the control (23.51 ±2.79 ppm, Table 2). Lower concentrations were recorded in some tested fungi: *L. delicious* (15.34 ±1.73 ppm), *R. cf. atroglauca* (18.32 ±0.72 ppm) and *S. caerulescens* (10.43 ±0.58 ppm) than control. While phosphorus content is an indicator of the effect of ECM fungi in nutrient uptake, there is a need to evaluate other macronutrients for comparison of the effect of the evaluated treatments. Some authors mention that the

growth of ectomycorrhizae can be delayed or inhibited by high levels of fertilization (Newton and Pigot, 1991), however doses reported for the management of coniferous trees was applied correctly.

Figure 6. Comparison between *B. barrowsii* single fungi inoculation (left) and control (right) after 14 weeks of inoculation.

4. Conclusions

The ECM fungi tested induced different responses in either biomass, plant quality, height or stem diameter in *P. greggii* seedlings. For instance, *G. minimum* (presented in *P. greggii* natural stands, ~~e.g. Jamé, Coahuila)~~ despite contradictory reports regarding its ectomycorrhizal status, improved the basal diameter, height and Dickson Index of *P. greggi* seedlings compared to *B. barrowsii*, a fungus collected in more humid temperate forests (e.g. Monterreal, Coahuila) where species of *Abies*, *Pseudotsuga* and *Pinus*

ayacahuite are dominant. Particularly *B. barrowsii*, *G. minimum* and *R.* cf. *atroglauca* induced a significant response in biomass and height; the levels of glucosamine in the roots were higher in the soil litter inoculum mix. This could indicate that level of fungal colonization (measured as glucosamine levels in roots in this study) might not be directly correlated with the effect of individual mycorrhizal species upon plant development. The present work on *P. greggii* confirms that pine seedlings can be inoculated in nurseries using fruiting bodies collected in the field as inoculum, specially those collected in stands of this endemic pine, to take advantage of possible preadaptation between symbionts. In particular, inoculation with *G. minimum* and *B. barrowsii* could improve the growth of the plants in the greenhouse, resulting in more vigorous plants for reforestation.

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CONCLUSIONES GENERALES

Los hongos ECM recolectados del campo indujeron una respuesta en las plántulas de *P. greggii* cultivadas en el invernadero después de 98 días de inoculación. Por ejemplo, *Geastrum mínimum* presentado en los rodales naturales de *P. greggii* (Jamé, Coahuila) mejoró el diámetro basal y la altura de las plántulas en comparación con el control. *Boletus barrowsii*, un hongo recolectado en bosques templados más húmedos (Monterreal, Coahuila) donde las especies de *Abies, Pseudotsuga* y *Pinus ayacahuite* son dominantes, indujeron valores altamente significativos en el Índice Dickson con respecto a otros tratamientos. La biomasa aumentó en 50% con los hongos evaluados, *Boletus barrowsii, Geastrum minimum y Russula cf. Atroglauca*, el nivel de glucosamina en las raíces de estos tratamientos fue menor que la materia orgánica incorporada a la base del cuerpo fructífero *L. deliciosus*. Esto podría indicar que el nivel de colonización fúngica (medido como niveles de glucosamina en las raíces) podría no estar directamente correlacionado con el efecto de las especies micorrízicas individuales en el desarrollo de la planta. El presente trabajo sobre *P. greggii* recomienda la inoculación de cuerpos de fructificación recolectados en el campo. Esto podría mejorar el crecimiento de las plantas en el invernadero, dando como resultado plantas más vigorosas para la forestación. Aunado al efecto de las ectomicorrizas en invernadero, para la producción de *P. greggii*, la mayor absorción de nutrientes se presentó en un manejo convencional (sustrato a base de turba) con respecto al suelo montano analizado por ICP-OES. La técnica de EDS (Espectroscopia de absorción de rayos X) es mejor determinar la composición elemental de *P. greggii* debido a que se evita el uso de materiales corrosivos y procedimientos laboriosos y tediosos en la digestión de muestras en húmedo (para el análisis ICP-OES).