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ECOPHYSIOLOGICAL EFFECTS OF ARBUSCULAR MYCORRHIZAL INOCULATION ON *ARUNDO DONAX* UNDER MEDITERRANEAN CONDITIONS

Antònia Romero Munar

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CERTIFICAMOS:

Que el presente trabajo titulado *"ECOPHYSIOLOGICAL EFFECTS OF ARBUSCULAR MYCORRHIZAL INOCULATION ON ARUNDO DONAX UNDER MEDITERRANEAN CONDITIONS*" presentado por Antònia Romero Munar para optar al TÍTULO universitario oficial de DOCTORA por la *Universitat de les Illes Balears* dentro del programa de doctorado de Biología de las Plantas, se ha realizado bajo nuestra dirección.

Revisado el presente trabajo, autorizamos su presentación para que pueda ser juzgada por el tribunal correspondiente.

Palma de Mallorca, 14 de julio de 2017

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Felicidad… que bonito nombre tienes

Felicitat, molta, infinita, mai abans experimentada, d'haver arribat a la fita on comença una nova aventura. Aquí no s'acaba res, és un punt i seguit, amb la maleta plena d'un bagatge que només el trànsit pel viatge d'una tesi doctoral és capaç donar.

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Symbols and abbreviations list (Table I)

AM arbuscular mycorrhiza

A_N net CO_2 assimilation rate

AOP alternative oxidase pathway

AOX alternative oxidase

C_c chloroplastic CO_2 concentration

Ci substomatal CO_2 concentrations

COP cytrochrome oxidase pathway

COX cytochrome oxidase

DW dry weight

g_m mesophyll conductance

g_s stomatal conductance

J_{flu} the electron transport rate

J_{max} the potential light saturated electron transport rate

LA leaf area

LAR leaf area ratio

NM non-arbuscular mycorrhiza plants

P_N net CO_2 assimilation rate

R_d the leaf dark respiration in the light

R_n the respiratory rate in the absence of light

SLA specific leaf area

TCA tricarboxylic acid cycle

v_{alt} AOX activity

V_{cmax} the maximum ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate

v_{cyt} COX activity

V_t the total respiration

WUE water use efficiency

WUE_i intrinsec water use efficiency

WUE_p plant water use efficiency

Φ_{PSII} the photochemical efficiency of photosystem II

τ_a the electron partitioning to the alternative pathway

Justificación del compendio de artículos

La presente tesis doctoral se presenta en formato de compendio de cuatro artículos que son cada uno de los cuatro capítulos en los que está estructurada.

Capítulo / Chapter 1

Título: Leaf plasticity and stomatal regulation determines the ability of *Arundo donax* plantlets to cope with water stress
Estado: **Publicado** en **Photosynthetica**, **Q2**, **IF 1.558**. DOI 10.1007/s11099-017-0719-y
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Cita: Romero-Munar, A., Baraza, E., Cifre, J., Achir, C., & Gulías, J. (2017). Leaf plasticity and stomatal regulation determines the ability of *Arundo donax* plantlets to cope with water stress. Photosynthetica, 1-9.

Capítulo / Chapter 2

Título: Arbuscular mycorrhizal symbiosis with *Arundo donax* decreases root respiration and increases both photosynthesis and plant biomass accumulation
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Capítulo / Chapter 3

Título: Arbuscular mycorrhizal fungi confer salt tolerance in giant reed (*Arundo donax* L.) plants grown under low phosphorus by reducing leaf Na^+ concentration and improving phosphorus use efficiency
Estado: **En preparación**
Autoras/es: Antònia Romero-Munar, Elena Baraza, Javier Gulías y Catalina Cabot

Capítulo / Chapter 4

Título: Nursery preconditioning of *Arundo donax* L. plantlets determines biomass harvest in the first two years.

Estado: **En preparación**

Autoras/es: Antònia Romero-Munar, Maria Tauler, Javier Gulías y Elena Baraza

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Resumen

El interés por los biocombustibles como alternativa viable a las fuentes no renovables de energía ha ido en aumento en la última década. Sin embargo, los biocombustibles no han estado exentos de polémica generando controversia a nivel social, económico y ambiental, como la competencia por la tierra con los cultivos básicos para alimentación humana y animal. Los biocombustibles de segunda generación se basan en gramíneas perennes como *Arundo donax*, plantas que no forman parte de la base alimentaria, capaces de producir grandes cantidades de biomasa con pocos aportes externos y con capacidad para adaptarse a condiciones de tierras marginales, en las que no se producen cultivos para alimentación humana o animal. *A. donax* es capaz de adaptarse a diferentes condiciones del suelo y presenta una alta producción de biomasa en condiciones de baja fertilidad en las zonas mediterráneas. Sin embargo, una de las desventajas de esta especie es su sensibilidad a la falta de agua y nutrientes durante las primeras etapas de crecimiento. Se ha demostrado que la simbiosis entre plantas y hongos formadores de micorrizas arbusculares (HMA) aumenta la resistencia de las plantas al estrés biótico y abiótico. En este escenario, el objetivo general de esta tesis doctoral es analizar la ecofisiología de *A. donax* en simbiosis con HMA, y su comportamiento tanto en condiciones control como bajo estrés salino. Las respuestas de las plantas en simbiosis y sin ella se evaluaron mediante el estudio de la fotosíntesis, la respiración *in vivo* y el metabolismo primario, el estado nutricional y la acumulación de biomasa. Los efectos fisiológicos y nutricionales de la simbiosis de *A. donax* con HMA también fueron estudiados bajo estrés salino y escasez de nutrientes. Además, se analizó el uso de HMA, entre otras técnicas, durante la producción de planta, con la intención de mejorar la calidad del plantel de *A. donax* para asegurar la supervivencia y mejorar la producción de biomasa en el primer y segundo año de cultivo. Los resultados de esta tesis doctoral muestran que *A. donax* presentó tolerancia a la sequía (a través de cambios morfo-anatómicos y de ajuste osmótico, y alta eficiencia en el uso del agua), a pesar de que la producción de biomasa se redujo significativamente. Además, presentó tolerancia a la salinidad moderada y una alta eficiencia en el uso del fósforo. Los cambios observados a nivel fisiológico en el simbionte generado por la simbiosis con *Rizophagus irregularis* y *Funneliformis mosseae*, permitieron una mayor producción de biomasa en comparación con las plantas no simbiontes tanto en condiciones control como bajo condiciones de estrés salino. El uso de HMA durante el periodo de aclimatación mejoró la producción de plantel, con tasas de supervivencia del 100% en campo y un aumento de la producción durante los primeros años. Sin embargo, otras técnicas como el uso de alveolos de gran tamaño y sustratos de alta calidad también pueden asegurar la supervivencia y producción del cultivo. Considerando todos los resultados en conjunto, se puede concluir que si bien *A. donax* por si sólo presenta características de alta productividad: tasas fotosintéticas elevadas, alta eficiencia en el uso de recursos (tanto agua como nutrientes) y moderada capacidad de resistencia al estrés, manteniendo producciones considerables que le hacen ser una especie adecuada para la producción de biomasa en tierras marginales, la simbiosis con HMA representa una herramienta interesante para mejorar todas estas características y asegurar el éxito de su cultivo bajo las condiciones impuestas por este tipo de tierras.

Palabras clave: *Arundo donax*, micorrizas arbusculares, estrés hídrico, estrés salino, fotosíntesis, producción de planta, tierras marginales, biocombustibles de segunda generación

Resum

L'interès pels biocombustibles com a alternativa viable a les fonts no renovables d'energia ha anat en augment en l'última dècada. No obstant això, els biocombustibles no han estat exempts de polèmica generant controvèrsia a nivell social, econòmic i ambiental, com la competència per les terres de cultiu amb els cultius bàsics per a alimentació humana i animal. Els biocombustibles de segona generació es basen en gramínies perennes (com ara *Arundo donax*), plantes que no formen part de la base alimentària, amb capacitat de produir grans quantitats de biomassa, amb pocs aports externs i amb capacitat per adaptar-se a condicions de terres marginals. *Arundo donax* és capaç d'adaptar-se a diferents condicions del sòl i presenta una alta producció de biomassa en condicions de baixa fertilitat en les zones mediterrànies. No obstant això, una de les desavantatges d'aquesta espècie és la seva sensibilitat a la falta d'aigua i nutrients durant les primeres etapes de creixement. S'ha demostrat que la simbiosi entre plantes i fongs formadors de micorizes arbusculars (FMA) augmenta la resistència a l'estrès biòtic i abiòtic del sòl. En aquest escenari, l'objectiu general d'aquesta tesi doctoral va ser estudiar els efectes ecofisiològics de la simbiosi micorrícica en *A. donax*, així com el seu comportament tant en condicions control com d'estrès salí. Les respostes de les plantes en simbiosi i sense es van avaluar mitjançant l'estudi de la fotosíntesi, la respiració *in vivo* i els canvis en el metabolisme primari, així com l'estat nutricional i l'acumulació de biomassa. Els efectes fisiològics i nutricionals de la simbiosi d'*A. donax* amb FMA també van ser analitzats sota salinitat i carència de fósfor. A més, es van investigar diferents possibilitats per millorar la qualitat del planter d'*A. donax* per assegurar la supervivència i la producció de biomassa en el primer i segon any de cultiu. Els resultats d'aquesta tesi doctoral mostren que *A. donax* presenta tolerància a la sequera (a través de canvis morfo-anatòmics i d'ajust osmòtic, i alta eficiència en l'ús de l'aigua), malgrat la biomassa produïda es veu reduïda. A més, *A. donax* va presentar tolerància a la salinitat moderada i alta eficiència en l'ús del fòsfor. No obstant això, els canvis observats a nivell fisiològic en el simbiont generats per la simbiosi amb *R. irregularis* i *F. mosseae*, permeten una major producció de biomassa en comparació de les plàntules no simbionts. La simbiosi amb FMA representa una valuosa eina per augmentar l'èxit de la implementació a camp i augmentar la biomassa de cultiu d'*A. donax* en terres marginals. L'ús de FMA durant el període d'aclimatació va millorar la producció del plantel, amb taxes de supervivència del 100% en camp i un augment de la producció durant els dos primers anys. No obstant, altres tècniques com l'ús d'alvèols de gran mida i de substrats d'alta qualitat també poden assegurar la supervivència i la producció del cultiu. Considerant tots els resultats en el seu conjunt, es pot concloure que si bé *A. donax* presenta característiques pròpies d'una espècie d'elevada productivitat: elevades taxes de fotosíntesi, elevada eficiència en l'ús dels recursos (aigua i nutrients, principalment), així com una moderada capacitat de resistència a l'estrès, mantenint produccions considerables que la fan ser una espècie adequada per a la producció de biomassa en terres marginals, la simbiosi amb FMA representa una eina interesant per millorar aquestes característiques i assegurar l'èxit del cultiu en les condicions imposades per aquest tipus de terres.

Paraules clau: *Arundo donax*, micorizes arbusculars, estrès hídric, estrès salí, fotosíntesi, producció de planta, terres marginals, biocombustibles de segona generació

Summary

The interest of biofuels has increased in the last decade as an alternative to non-renewable energy sources. However, biofuels presented several social, economic and ecological costs, like competitiveness with food crops and high water and fertilizer needs. The second-generation biofuels based on perennial grasses such as *Arundo donax* (giant reed) are non-food plant species that should be able to produce large amounts of biomass with low inputs and presented high adaptability to marginal lands. Giant reed is able to adapt to different soil conditions and presents high biomass production under low fertility conditions in Mediterranean areas. However, one of the handicaps of giant reed may be its sensitivity to lack of water and nutrients during the early stages of growth. Arbuscular mycorrhizal fungi (AM) symbiosis has been demonstrated to increase plant resistance to biotic and abiotic soil stresses in a variety of host plants. In this scenario, the aim of this study was to study the ecophysiological responses of *A. donax* to AM inoculation under control and salt stress conditions. Plant responses were evaluated through the study of the photosynthesis and respiration in vivo and on the levels of primary metabolites, nutritional status and biomass accumulation. Physiological and nutritional AM effects were also tested under salinity stress and lower nutrient inputs. Furthermore, we investigated different possiblities to improve *A. donax* plantets quality to ensure survival and biomass production in the first and second year of crop. The results of this PhD thesis show that *A. donax* presents tolerance to drought (through morpho-anatomical and osmotic adjustment, and high water use efficiency). In addition, it presents tolerance to moderate salinity and high phosphorus use efficiency. However, changes observed at the physiological level in the symbiont generated by the symbiosis with *R. irregularis* and *F. mosseae*, allowed a higher production of biomass compared to non-symbiotic plantlets. AM inoculation represented a valuable tool to increase the successful implementation and the increase of crop biomass of giant reed on marginal lands. The inoculation of micropropagated *A. donax* plantlets with AM at the acclimation stage increased the quality of the plantlets, what produced an increment in biomass production the first and the second year in the field. However, the use of bigger cells and better substrates would also ensure the plant survival and the biomass production. I can conclude that despite *A. donax* is a high productivity species, showing high photosynthetic rates and resources (water and nutrients) use efficiency, as well as a moderate stress resistance even under marginal land conditions, the inoculation with AM may be helpful to enhance all these traits in order to ensure the success of *A. donax* crop in such stressful areas.

Key words: *Arundo donax*, arbuscular mycorrhiza, water stress, salinity stress, photosynthesis, plant production, marginal lands, second generation biofuels

Introducción

Marco Socioeconómico de la Energía: Del petróleo a los biocombustibles de segunda generación

El desarrollo de la sociedad industrializada se ha basado en el uso de fuentes de energía no renovables, principalmente petróleo y gas natural. El siglo XXI empezó con una de las mayores crisis mundiales provocada por la inminente llegada al pico de extracción del petróleo, que encareció hasta niveles nunca alcanzados su precio. Sumado al coste económico, el otro hándicap de esta fuente de energía no renovable es el coste ambiental que conllevan su extracción y procesado, particularmente los flujos de gases de efecto invernadero que se producen en todo el proceso y en su consumo. Desde 1970, con la revolución de los biocombustibles a base de aceite vegetal, la investigación en energías alternativas como intento de diversificar las fuentes de energía ha ido en aumento (IPCC, 2011).

Hasta hoy, la historia de los biocombustibles ha pasado por diversas fases no ausentes de polémica. Los biocombustibles de primera generación provienen de materias primas ricas en carbohidratos o de semillas oleaginosas, obtenidos de productos agrícolas. Su origen agrícola provocó una competencia por el uso de suelo fértil entre el mercado alimentario y el energético. Este conflicto de intereses provocó incrementos en los precios de algunas de estas materias primas usadas en la industria alimentaria, siendo éste el mayor hándicap de los biocombustibles de primera generación (Solomon, 2010; Zegada-Lizarazu *et al.*, 2010). Tras este conflicto social y natural (BBC, 2007; Friends of the Earth, 2007), las agencias políticas hicieron un llamamiento a la sostenibilidad de este tipo de cultivos (OECD/FAO, 2007; Renewable Fuels Agency, 2008). Como respuesta, la Unión Europea modificó su Directiva de 2003 sobre biocombustibles para incluir una cláusula de sostenibilidad en la Directiva sobre Energías Renovables de 2009, así como varios sistemas de certificación de su cumplimiento como la "Roundtable on Sustainable Biofuels" (Raman y Mohr, 2014). Éste fue el escenario en el que surgió la segunda generación de biocombustibles. En este caso, la materia prima vegetal proviene de restos agrícolas, forestales o se basa en cultivos que no forman parte de la cadena alimentaria ni humana ni animal, proponiéndose como alternativa energética de futuro a medio y largo plazo (Davis *et al.*, 2009; Suurs y Hekkert, 2009). Además, el uso de la biomasa como fuente de energía tiene un menor impacto ambiental ya que el carbono que emite a la atmósfera cuando se quema es compensado total o parcialmente por el carbono fijado por las plantas durante el cultivo (Royal Society,

2008). De esa propuesta de futuro a finales de la década de los 2000 se ha pasado a la realidad del pasado 2016, año en el que se produjeron 114 millones de barriles de biocombustibles de segunda generación solo en Estados Unidos, según los informes de la *United States Environmental Protection Agency* y la *Office of Air and Radiation* (US EPA, OAR). Bioetanol de celulosa, hidrobiodiésel, biometanol y diésel de madera representaron la mayor parte de dicha producción. En paralelo surgió una tercera generación de biocombustibles, basados en el uso de métodos de producción similares a los de segunda generación, pero empleando como materia prima biomasa específicamente adaptada o diseñada mediante biología molecular. Podríamos incluir a las microalgas en este grupo aunque hay autores que las engloban dentro de una cuarta generación de biocombustibles por su gran capacidad de crecimiento y su potencial energético. Sin embargo, la composición de estas materias primas para biocombustibles de segunda generación y sucesivos es rica en celulosa y lignina, implicando un mayor coste logístico y energético por la necesidad de una doble transformación. Actualmente la investigación en biocombustibles ha impulsado la implementación de nuevas tecnologías para economizar el proceso de transformación.

La inversión en la ingeniería industrial de la extracción de petróleo ha derivado en progresos tecnológicos que han permitido abaratar sus costes y la consecuente despresurización del mercado petrolero (Kuper, 2012; Pérez y Hierro, 2014). Aunque a nivel económico el problema está resuelto a medio plazo (hecho que ha supuesto una retirada de los fondos de inversión del gobierno europeo - que no del de Arabia Saudí, que sigue en la búsqueda de alternativas que les permitan seguir a la cabeza del lobby energético –) la realidad de un cambio climático global derivado, en parte, de las emisiones de gases de efecto invernadero (Basha *et al.*, 2009) sigue avanzando, generando graves problemas ambientales.

Marco Ambiental y consecuencias del Cambio Climático: El caso concreto de la Cuenca del Mediterráneo, el incremento de aridez de su clima y la desertificación de suelos

El cambio climático produce una alteración en el patrón global y regional de las precipitaciones, afectando a su intensidad, frecuencia o duración (Trenberth *et al.*, 2003). Estas modificaciones en el clima a nivel global suponen efectos importantes a nivel regional sobre las fuentes de disponibilidad hídrica (Seiler, 2007). El clima mediterráneo se caracteriza por la estacionalidad, con inviernos templados y húmedos, primaveras y otoños variables, tanto en temperatura como precipitaciones y veranos con elevadas temperaturas y secos (Giorgi y Lionello, 2008). En la cuenca mediterránea se prevé un aumento de aridez del clima debido a un incremento significativo de temperatura y un cambio en la distribución de las precipitaciones, tendiendo ésta hacia una mayor irregularidad con periodos de sequía prolongada y episodios de lluvias torrenciales (Giorgi y Lionello, 2008), lo que incrementará el riesgo de erosión y degradación del suelo y su desertificación (Geeson *et al.*, 2002). El proceso de desertificación conlleva, entre otras consecuencias, la salinización del suelo. La salinización es uno de los estreses abióticos más severos y provoca pérdidas de suelo fértil y su abandono. En efecto, más de la mitad de la superficie agrícola mundial afronta limitaciones de calidad de los suelos, dejando en un 12% la proporción de suelo catalogado cómo fértil. Esto representa 4.400 millones de hectáreas de tierra apta para la agricultura, de las que el 80% son de secano (FAO, 2011).

La pérdida de suelo útil para la producción agrícola, además de por problemas de salinización y escasez de agua derivados del cambio climático, se genera por los cambios de uso y agotamiento por sobreexplotación (Geeson *et al.*, 2002). Como consecuencia quedan grandes extensiones de suelo no cultivadas por falta de productividad tanto biológica como económica, las llamadas tierras marginales (Fagnano *et al.*, 2012). Las restricciones de agua y las escasas cosechas ocurridas al inicio de los años 90 (Parry *et al.*, 2000), ponen de manifiesto la vulnerabilidad de la región mediterránea, con un incremento de este tipo de suelos.

Proceso de selección de la materia prima para biocombustibles de segunda generación: gramíneas perennes adaptadas al estrés abiótico y con altas tasas de producción de biomasa

Ante el presente marco socioeconómico y ambiental de la producción de energía, la selección de las especies vegetales aptas para la producción de biocombustibles de segunda generación ha sido y es básica para su éxito. En este aspecto, la tolerancia y/o adaptación de estas especies a los estreses abióticos más frecuentes en las tierras marginales en las que se proyecta su cultivo, déficit hídrico y salinidad, es uno de los ejes centrales de la investigación para garantizar el éxito de esta fuente de energía alternativa. Por este motivo, en los últimos 10 años (tanto a nivel nacional como internacional) se ha centrado la atención en especies vegetales que presentan, entre otras características, una alta eficiencia en el uso del agua y de nutrientes, al tiempo que mantienen o reducen levemente la producción de biomasa en condiciones de estrés moderado.

Durante la segunda mitad del Siglo XX, la ecofisiología vegetal fue intensificando de forma gradual el estudio del efecto de la sequía y la salinidad sobre la fisiología y el crecimiento de las plantas en general y de los cultivos en particular. Se han identificado diversas adaptaciones tanto a nivel morfológico como fisiológico al estrés hídrico (tanto por falta de agua como a la sequía fisiológica en el caso de la salinidad), que caracterizan a la mayoría de especies vegetales de clima mediterráneo (Hsiao *et al.*, 1976; Reich *et al.*, 1997; Gulías *et al.*, 2003; Galmés *et al.*, 2011). Hoy en día, la investigación se centra en especies vegetales que, además de ser tolerantes a altas temperaturas y presentar alta eficiencia en el uso del agua, su cultivo conlleve pocos requerimientos de manejo, obteniendo grandes cantidades de biomasa con un elevado nivel de calidad que hagan que tanto su producción como su transformación sean rentables.

Las gramíneas perennes, en general, son cultivos resistentes a la sequía y con pocos requerimientos de cultivo, permitiendo reducir los inputs de los sistemas agrarios, como el suministro de riego y fertilizantes. Por este motivo algunas especies características de esta familia se han propuesto como fuente de biomasa para la transformación en biocombustibles de segunda generación. Tal es el potencial de estas especies que la Unión Europea impulsó cinco proyectos hace cinco años sobre manejo, gestión y producción de biocombustibles de segunda generación basados en dichas especies,

entre ellos el FP7 – KBBE – 2011– 5 OPTIMA "Optimization of Perennial Grasses for Biomass Production in the Mediterranean Environment" en el que se enmarca la presente tesis doctoral. Este proyecto se centró en el estudio de las potencialidades de las gramíneas perennes como cultivo energético en ambientes mediterráneos, además de hacer referencia al amplio y variado rango de beneficios del uso de gramíneas perenes como son su viabilidad a largo plazo bajo prolongados periodos de sequía, su papel como sumideros de carbono, así como su uso como fitoremediadoras, apuntando incluso a la potencialidad de su manejo con aguas residuales y lodos de depuradora.

Arundo donax y su pontencialidad real como cultivo energético

Dentro del citado proyecto se dividen dos grupos de especies de gramíneas perennes según su distribución: por una parte, las especies autóctonas de la cuenca mediterránea y, por la otra, gramíneas perenes de ámbito cosmopolita. En el primer grupo se incluyeron especies tales como *Ampelodesmos mauritanica* L., *Dactylis glomerata* L., *Piptatherum miliaceum* L., y *Phragmites australis* L., entre otras. En lo que refiere a las de ámbito cosmopolita, *Arundo donax* L. es la especie que más potencial presenta en zonas de clima Mediterráneo, por presentar una alta eficiencia en el uso del agua y tolerancia a altas temperaturas. Esta gramínea C_3 presenta una alta producción de biomasa con tasas fotosintéticas similares a las especies C_4 (Rossa *et al.*, 1998; Webster *et al.*, 2016). Además de su capacidad de adaptación a un amplio rango de temperaturas, puede crecer en tierras marginales con bajos aportes de agua y nutrientes. Estas características hacen que hoy en día se encuentre establecida prácticamente en todas las regiones templadas y subtropicales del mundo, lo que ha propiciado su catalogación como una de las diez especies invasoras más peligrosas (Mack, 2008). Sin embargo, además de sus características biológicas, su bajo coste de manejo y su alta producción de biomasa la convierten en un cultivo energético modelo (McKendy, 2002). De hecho, *A. donax* ha sido estudiada especialmente en Italia, donde es un cultivo ampliamente implantado y caracterizado a nivel agronómico para su uso en la industria bioenergética (Pilu *et al.*, 2012; Scordia *et al.*, 2012, 2014; Alexopoulou *et al.*, 2015; Monti *et al.*, 2015). Sin embargo, a pesar de presentarse como un prometedor cultivo energético, algunos aspectos de su biología deben tenerse en cuenta para optimizar su uso, evitando riesgos derivados de su potencial invasor, y asegurando su viabilidad económica. *A. donax* es una planta estéril cuya reproducción por propagación vegetativa (mediante la fragmentación de rizoma o esquejes de las yemas del tallo) y/o por cultivo *in vitro*

complica y encarece la implantación a gran escala del cultivo (Pilu *et al.*, 2013). Referente a este aspecto, Pilu et al. (2013) apuntan a que el momento más crítico del cultivo es precisamente el primer año de implantación, en el que la planta es más sensible al estrés ambiental y requiere grandes aportes de agua y nutrientes, empezando a ser rentable a partir del segundo o tercer año. Además, su esterilidad hace imposible su mejora genética mediante las técnicas habituales de hibridación, por lo que la selección de ecotipos así como las técnicas de mutagénesis química y física y la transgénesis son las alternativas para la mejora de este cultivo. Se ha mostrado que pueden existir diferencias en la respuesta fisiológica a estreses hídrico y salino entre ecotipos (Sánchez *et al.*, 2015), aunque aún quedan por descifrar los fundamentos ecofisiológicos de dicha tolerancia. Un mayor conocimiento de las respuestas fisiológicas al estrés abiótico, salino e hídrico principalmente, en las etapas tempranas de crecimiento permitiría una posibilidad de mejorar el manejo de esta especie en el momento crítico de su ciclo vital.

La sostenibilidad de los cultivos

La simbiosis entre plantas y hongos formadores de micorrizas arbusculares (HMA)

A lo largo de la historia evolutiva, los seres vivos han respondido al estrés de varias maneras para lograr su supervivencia. La simbiosis es una de las más antiguas a la par de compleja, presente como respuesta adaptativa desde los orígenes de la vida (Margulis 1981). La simbiosis entre plantas y hongos a nivel radicular se denomina micorriza (del griego “mycos”, hongo, y “rhiza”, raíz), y se distinguen dos grandes grupos: ecto y endomicorrizas. En las ectomicorrizas – predominantes en árboles – el componente fúngico queda fuera de las células vegetales, abrazando por fuera la estructura radicular, mientras que las endomicorrizas – Ericoides, Orquioides y Arbusculares– parte de las hifas del hongo se encuentra dentro de la célula vegetal. La micorriza arbuscular (MA) ha sido propuesta como una de las principales estrategias que aseguró el éxito de las plantas en el paso del medio acuático al terrestre (Strullu-Derrien y Strullu, 2007; Parniske, 2008; Bonfante *et al.*, 2010). Guether et al. (2009) clonaron siete genes vegetales involucrados en la reprogramación celular para la simbiosis con hongos formadores de micorrizas arbusculares (HMA). Esta predisposición marcada a nivel del material genético vegetal muestra la íntima y antigua relación entre las plantas vasculares y los HMA. La reprogramación implica la formación de un aparato pre-

simbiótico en la célula vegetal previo a que se produzca la interacción con el HMA (Parniske, 2008). Por parte del componente fúngico también se han descrito moléculas de señalización mediante las que el hongo percibe los exudados radiculares y la cercanía de la raíz, estimulando la germinación de las esporas o la quimiotaxis de las hifas extraradicales cercanas a la raíz (Bago *et al.*, 2000). En ambas situaciones, se forma el aprensorio, estructura fúngica que establece el contacto con el aparato pre-simbiótico de las células radiculares y se establece la primera conexión. Una vez establecida ésta, tiene lugar la fusión entre la célula vegetal y la fúngica, y se produce la formación del arbúsculo. Esta estructura debe su nombre a su semejanza con las ramas de un árbol, son exclusivas de los HMA (llamados arbusculares por este motivo) y constituyen la estructura simbiótica característica de la micorriza arbuscular, en la que se entremezclan estructuras del componente vegetal y fúngico a la vez. Desde el interior hacia el exterior, se compone de un citoplasma fúngico rodeado por una plasmamembrana fúngica, que se recubre por una membrana periarbuscular generada por la célula vegetal (PAM). Esta última es un continuo con la plasmamembrana de la célula vegetal, y mantiene separado el citoplasma vegetal del citoplasma fúngico. El espacio intermembrana se denomina espacio periarbuscular, y es el punto en el que ambos componentes, fúngico y vegetal, interactúan. El establecimiento de los arbúsculos pone de manifiesto la íntima relación que se establece entre los componentes de esta simbiosis, a pesar de pertenecer a dos reinos diferentes. Por esta íntima relación entre el HMA y la raíz vegetal, a partir de este momento en la presente introducción se hará referencia al "simbionte" para nombrar a la planta con MA (Smith y Read, 2008).

El arbúsculo es la estructura mediante la que se hace el intercambio de sustancias entre ambos componentes del simbionte (Bonfante *et al.*, 2010). Tradicionalmente esta simbiosis se ha descrito basándose en una obtención de carbohidratos procedentes de la fotosíntesis del vegetal a cambio de un aporte de nutrientes y agua por parte del componente fúngico del simbionte. Por una parte, se ha establecido que una media del 20% del carbono fijado mediante la fotosíntesis se destina íntegramente al metabolismo fúngico (Bago *et al.*, 2000). Este 20% ha sido ligado al incremento en la tasa fotosintética que se observa por regla general en las plantas en simbiosis. Por otra parte, el volumen de suelo explorado por la red de hifas extraradicales ha sido estimado en 10 cm de longitud por cada centímetro de raíz colonizada y por 100 metros de hifa por centímetro cúbico de suelo, muy superior a la capacidad de exploración que pueda

tener una raíz no micorrizada (Miller *et al.*, 1995). Así los HMA presentan una mayor capacidad de absorción de agua y nutrientes, pero no sólo debido a que aumentan la cantidad de suelo explorado, sino también por ser capaces de modificar las características biológicas y físicas del suelo; aumentar la solubilidad de nutrientes como el fósforo; y acceder a un *pool* de agua que una raíz no micorrizada no es capaz de aprovechar gracias a una menor tensión superficial de la hifa provocada por su reducido diámetro (Jeffries *et al.*, 2003; Singh *et al.*, 2017). Sin embargo, en la última década se ha demostrado que, basándose en la íntima interrelación que se establece a nivel celular entre la planta y el hongo, el simbionte presenta diferentes respuestas a nivel celular, genético, metabólico y fisiológico, en comparación a plantas con la raíz no micorrizada. Estas respuestas van más allá de la localización del HMA en la raíz, afectando tanto a la parte subterránea como a la aérea del simbionte (Hohnjec *et al.*, 2005; Aroca *et al.*, 2009; Rivero *et al.*, 2015; Schweiger y Müller, 2015; Romero-Munar *et al.*, 2017; Del-Saz *et al.*, 2017).

A pesar de que la mayoría (65-85%) de las especies de plantas terrestres conocidas en la actualidad presentan simbiosis con HMA (Gianinazzi-Pearson 1996; Van der Heijden et al. 1998; Yano-Melo et al. 2003; Smith et al. 2003; van der Heijden et al. 2008), se han descrito menos de 200 especies de HMA (Montesinos-Navarro *et al.*, 2012). Esta baja diversidad de hongos en comparación con la diversidad de especies de plantas hospedadoras llevó a la histórica presunción de la baja especificidad de esta simbiosis (Smith y Read, 2008). Varias especies de hongo pueden establecer simbiosis simultáneamente con la misma raíz, y un mismo hongo puede estar en simbiosis a la vez en más de una planta, conectando dos o más plantas hospedadoras (Montesinos-Navarro *et al.*, 2012).

Esta falta de especificidad ha favorecido la producción de múltiples inóculos comerciales basados en dos especies cosmopolitas *Rhizophagus irregularis* (Blaszk, Wubet, Renker y Buscot) y *Funnelifomis mosseae* (T.H. Nicolson y Gerd.) (Tabla 1). Estas dos especies, además de ser de las más estudiadas por la comunidad científica son las más habituales en los inóculos comerciales ya que se ha demostrado su potencial beneficio sobre el crecimiento de diversas especies de plantas. Además estas especies presentan una alta capacidad para formar simbiosis en condiciones de estrés abiótico del suelo (como es el caso de los suelos desertificados) (Requena *et al.*, 1996; Marulanda *et al.*, 2007).

Tabla 1. Composición especifica de algunos inóculos comerciales de endomicorrizas en España. En las páginas web de los productos siguen usando al nomenclatura antigua *Glomus mosseae* para *Funneliformis mosseae, Glomus intrarradices* para *Rhizophagus irregularis*. Muchos inóculos no especifican la composición, por tanto no se han incluído en esta tabla.

Empresa	Producto	Especies
MICOLOGIA FORESTAL & APLICADA	ENDOPLANT	*R. irregularis – F. mosseae*
BIOSIM EIRL-MYCOSYM INTERNATIONAL AG	MYCOSYM TRI-TON®	*R. irregularis*
AGROINECO	MICOTEC	*R. irregularis*
BATLLE	MICORRIZAS, HONGOS MEJORADORES ECO	*R. irregularis – F. mosseae*
MASSÓ	MYC 4000	*R. irregularis*
AgroGENIA	MYCO-STAR	*R. irregularis*

Sostenibilidad del proceso de producción de A. donax: potencialidad de la simbiosis con HMA bajo condiciones de estrés hídrico y salino

Tanto en el ecosistema natural como en el ecosistema agrícola, son varios los estreses a los que se ven sometidos sus componentes. Como se ha comentado anteriormente, en la Cuenca Mediterránea los mayores problemas vienen por estrés relacionado con la aridez del suelo. A pesar de que las plantas tienen sus propios mecanismos para combatir la sequía y la salinidad (desde adaptaciones morfológicas hasta procesos a nivel molecular), aumentan su tolerancia y adaptación gracias a simbiosis mutualistas con microorganismos, especialmente bacterias (como las promotoras del crecimiento o PGPRs) y HMA (Aroca y Ruiz-Lozano, 2009). De hecho, la respuesta del simbionte MA al estrés hídrico y salino ha sido y es ampliamente estudiada por su implicación y aplicación en la agricultura (Fitter, 1988; Marulanda *et al.*, 2003; Evelin *et al.*, 2009; Miransari, 2010; Ruiz-Lozano *et al.*, 2012; Estrada *et al.*, 2013). Se ha mostrado que diferentes grado de estrés (Aliasgharzad *et al.*, 2006) y la identidad tanto de la planta como de HMA implicados en la simbiosis pueden cambiar la respuesta del simbionte (Marulanda et al. 2003, 2007). No obstante respuestas fisiológicas como la reducción

del estrés oxidativo, el incremento del estatus hídrico o la regulación de la expresión de ciertos genes son atributos comunes observados en simbiontes MA (Augé, 2001; Aroca y Ruiz-Lozano, 2009; Evelin *et al.*, 2009; Ruiz-Lozano *et al.*, 2012; Miransari, 2014). El simbionte sometido a sequía presenta mayores tasas de traspiración, mayor conductividad hidráulica en la raíz y menor estrés oxidativo, presentando mayor crecimiento final, que la planta no micorrizada (Aroca y Ruiz-Lozano, 2009). No obstante, también se ha mostrado un incremento de estos parámetros fisiológicos bajo condiciones control (sin estrés) en simbiontes comparados con plantas no micorrizadas (Wu y Xia, 2006). Este hecho plantea el paradigma de que las plantas en simbiosis con HMA, o simbiontes MA, presentan un estatus fisiológico que les permite poder afrontar las futuras condiciones adversas. Los mecanismos por los cuales se produce este incremento tanto en condiciones control como bajo estrés son aún un enigma. Si bien es cierto que en los últimos años se han publicado diversos estudios sobre las diferencias de expresión de algunos genes y variaciones en el metabolismo primario entre plantas que no están en simbiosis y simbiontes MA, queda un amplio abanico de preguntas sin resolver. ¿Cuáles son las vías y/o mecanismos mediante los cuales se producen tales variaciones en el simbionte MA? Los estudios realizados hasta mediados de la década de los 90 atribuían la mejora fisiológica y de biomasa al incremento de exploración del suelo por el micelio extraradical, lo que permite una mayor captación y absorción de agua y nutrientes (en especial de fósforo). Fueron la base imprescindible para plantear preguntas que llevaron a la investigación molecular. Estudios basados en expresión génica y proteica mostraron cómo el incremento del estatus hídrico es consecuencia no solo de un mayor volumen explorado, sino también de un aumento en la conductividad hidráulica de la raíz mediante cambios en la expresión de acuaporinas (proteínas de membrana presentes en todos los organismos vivos, que facilitan el flujo de agua por difusión (Maurel *et al.*, 2008; Aroca *et al.*, 2009; Bárzana *et al.*, 2012; Navarro-Ródenas *et al.*, 2012, 2013; Ruiz-Lozano *et al.*, 2012). Referente a la captación de nutrientes, varios estudios han mostrado la existencia de una vía directa (a través de la raíz) y de una vía micorrícica (a través del micelio) (Fellbaum *et al.*, 2012a,b, 2014), y cómo la actividad de una y otra varían según las condiciones de crecimiento del simbionte MA (Watts-Williams *et al.*, 2015). El efecto de la simbiosis con MA incrementando la biomasa respecto a los no simbiontes se ha atribuido a la mejora del estado nutricional y del estado hídrico y su efecto sobre la fotosíntesis (A_N) y la conductancia estomática (g_s).

Bajo condiciones de estrés hídrico, se ha sugerido el incremento de la eficiencia en el uso del agua, así como los mecanismos mediante los cuales se produce ese incremento, como adaptación de diversas especies a climas semiáridos como el Mediterráneo (Flexas *et al.*, 2004; Galmés *et al.*, 2007; Galle *et al.*, 2009; Gulías *et al.*, 2009; Tomás *et al.*, 2012). Estos trabajos han mostrado cómo el estrés hídrico afecta tanto a la morfología foliar como al aparato fotosintético, viéndose afectada la conductancia del mesófilo (g_m), contribuyendo a las limitaciones fotosintéticas generadas por este estrés (Galmés *et al.*, 2007). Sin embargo, hasta la fecha, no se han desarrollado estudios que analicen en profundidad el efecto de la simbiosis con MA sobre estos parámetros y otros relacionados, abriéndose una nueva línea de investigación en la que se enmarca la presente tesis doctoral.

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Objetivos

Dentro del marco conceptual desarrollado entre los apartados 1 y 4 de la introducción, la presente tesis tiene como **objetivo general** analizar la ecofisiología de *A. donax* en simbiosis con HMA, y su comportamiento tanto en condiciones control como bajo estrés salino. Todos los experimentos realizados se han llevado a cabo durante los primeros estadios de crecimiento, excepto en el capítulo 4 en el que, además de las fases iniciales de crecimiento, se hizo el seguimiento del trasplante a campo hasta dos años después. Las fases iniciales de crecimiento son las más sensibles, y como se ha comentado al principio de la introducción, la capacidad de resistir las condiciones de estrés de las plantas provenientes de cultivo in vitro es aún menor. Por este motivo se parte de la **hipótesis** que el establecimiento de simbiosis con HMA puede tener efectos positivos y determinantes para el éxito productivo de *Arundo donax* en tierras marginales.

Rhizophagus irregularis y *Funneliformis mosseae* han sido seleccionadas para el desarrollo de todos los experimentos que conforman esta tesis doctoral por dos motivos: el primero, comentado en la introducción, por su ubicuidad y tolerancia a estrés moderado; y segundo, por ser las principales componentes de los inóculos comerciales disponibles.

El objetivo general se divide en 6 objetivos y preguntas específicos, que serán resueltos en uno o varios capítulos de la tesis, y que se enumeran a continuación:

1. Estudio de la respuesta a la sequía de *A. donax* ¿Cuál es el efecto del estrés hídrico sobre la biomasa de la planta y el intercambio de gases durante los primeros estadios de crecimiento? ¿Hay alguna posibilidad de ajustar el riego en base a la fisiología de la planta para optimizar el uso del agua en áreas mediterráneas? (Capítulo 1).

2. Estudio del efecto de la simbiosis con HMA sobre la biomasa, fotosíntesis, respiración y metabolismo primario de *A. donax*. ¿Existe un comportamiento diferente entre simbiontes MA y plantas sin simbiosis, en condiciones control? (Capítulo 2).

3. En los estadios iniciales de crecimiento, ¿Presenta *A. donax* tolerancia a la salinidad? Teniendo en cuenta que en tierras marginales es frecuente la combinación de diferentes estreses, ¿Es *A. donax* sensible a la carencia de

fósforo? ¿Cuál es su respuesta frente a la combinación de ambos estreses? (Capítulo 3).

4. ¿Cuál es papel de la simbiosis en la captación y translocación de nutrientes bajo condiciones control y de estrés salino? ¿Se modifica el crecimiento y la fisiología del simbionte bajo diferentes grados de estrés? (Capítulo 3).

5. ¿Puede la simbiosis con HMA suponer una mejora en la aclimatación previa al establecimiento del cultivo en campo de *A. donax*? ¿Existen diferencias entre las plantas procedentes de cultivo *in vitro* crecidas sin simbiosis MA y las que son inoculadas durante la aclimatación? (Capítulos 2, 3 y 4).

6. En el trasplante a campo, ¿Cuáles son los factores a tener en cuenta en la fase de aclimatación previa? ¿Se observan variaciones de crecimiento y acumulación de biomasa en el primero y segundo año después del establecimiento del cultivo? ¿Presenta el simbionte MA alguna ventaja frente a la planta sin simbiosis una vez trasplantadas? (Capítulo 4).

Chapter 1

Leaf Plasticity and Stomatal Regulation Determines the Ability of *Arundo donax* Plantlets to Cope with Water Stress

Leaf plasticity and stomatal regulation determines the ability of *Arundo donax* plantlets to cope with water stress

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Abstract

The objective of this study was to evaluate the response of the giant reed (*Arundo donax* L.) to drought stress at early stages, as well as to determine the effects of limited soil water availability on plant growth, gas exchange, and water-use efficiency. Plantlets of a commercial clone were grown in a greenhouse under two water treatments: at 100% of field capacity and progressive drought for 66 days (until 20% of field capacity). Soil water content, leaf elongation rate, plant water consumption and gas-exchange parameters were measured throughout the experiment. Total plant biomass, leaf water and osmotic potential were determined at the end of the experiment. Plant growth and leaf gas-exchange parameters were significantly affected by soil water availability, but only when it was below 40% of field capacity. At early stages, *Arundo donax* showed drought stress acclimation due to leaf plasticity, stomatal regulation and osmotic adjustment.

Additional key words: early stage; osmotic potential; stomatal conductance; water deficit.

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Abbreviations: C_c – chloroplastic CO_2 concentration; Chl – chlorophyll; C_i – substomatal CO_2 concentration; DAT – days after transplantation; FC – field capacity percentage; g_s – stomatal conductance; g_m – mesophyll conductance; H2Oc – total water consumption; J_{max} – the potential light- saturated electron transport rate; J_{flu} – the electron transport rate; P_N – net photosynthetic rate; R_D – respiration rate in the light; R_n – the respiratory rate in the absence of light; V_{cmax} – the maximum Rubisco carboxylation rate; WD – water-stressed; WUE – water-use efficiency; WW – well-watered.

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Introduction

Arundo donax (giant reed) is a perennial grass found in several regions throughout the world that has been proposed as a potentially high-yielding non-food crop under Mediterranean conditions (Arcidiacono and Porto 2012, Pilu *et al*. 2013, Sánchez *et al*. 2015). It is a rhizomatous C_3 grass species that shows a high growth capacity, and it is used to colonize several different soil conditions, especially flooded areas (Bell, 1997). The interest in this grass as a biomass source is based on its high biomass production under the low fertility conditions of Mediterranean areas and on its interesting second generation biofuel production (Pilu *et al*. 2012, Scordia *et al*. 2012). Moreover, its cropping life has been reported to be approximately 12–15 years with low maintenance costs due to low fertilizer and pesticide requirements (Pilu *et al*. 2013). Indeed, in addition to non-food crops, the use of marginal lands has been highlighted as a key point for biofuels sustainability. Although the concept of marginal land is under debate and may have different implications world-wide, it seems to be accepted that producing biomass under low water and fertilizer inputs in lands that are not currently used for agricultural production may be a sustainable source of bioenergy (Kang *et al*. 2013, Shortall 2013).

The Mediterranean climate is characterized by a strong seasonal variability in precipitation (Paredes *et al*. 2006) with severe summer droughts that last for two to six months and cool wet winters. In a climate change scenario, a decrease in the annual number of precipitation days in Mediterranean regions has been predicted (Stocker *et al*. 2013). In this region, more than 60% of the water resources are used for irrigation to meet crop water requirements. The giant reed shows potential traits to develop climatic tolerance to different abiotic stresses (Mann *et al*. 2013, Pilu *et al*. 2013). However, although it is well known that once established *A*. *donax* is highly drought tolerant (Perdue 1958, Lewandowski *et al*. 2003, Lambert and Dudley *et al*. 2010), water availability during the first year after plantation, when the root system is still underdeveloped, considerably limits plant growth and establishment, which endangers its future productivity (Arcidiacono and Porto 2012). Because of these issues, maximizing plant growth and resource-use efficiency at early stages appear to be key factors in ensuring future crop production and sustainability in *A. donax* under Mediterranean conditions. Although research of *A*. *donax* under water stress conditions has increased in recent years, little attention has been devoted to the study of early plant

acclimation mechanisms to cope with drought and the possibilities of increasing water-use efficiency (WUE) at this stage. Sánchez *et al*. (2015, 2016) recently reported that water deficit leads to tight stomatal closure and biomass decrease in different *A. donax* clones that showed lower leaf area ratio and specific leaf area in response to drought. However, little is known about the relationship among soil water availability, stomatal conductance, and biomass accumulation in *A. donax* plantlets. Indeed, no data is available about limitations of photosynthesis machinery under drought stress at early stages. Studies of the biochemical and diffusive parameters regulating the maximum rate of photosynthesis are thus required to explain the basis of the observed changes in photosynthetic rate (P_N) under water stress conditions.

Materials and methods

Plant material and experimental design

Twenty-four micropropagated plantlets of a commercial clone of giant reed (provided by *Piccoplant Inc*., Germany) were transplanted into 4-L pots with silicic sand and 25 g of slow-release fertilizer. All the transplanted plantlets had a similar size, with one tiller and 3–5 expanded leaves. The experimental trial was performed from October to January (during 12 weeks) under greenhouse conditions to avoid rainfall interference. The daily maximum temperatures showed little variation between the beginning and the end of the experiment (approximately 24ºC).

The plants were well-watered (at 100% of field capacity) for 20 d after transplanting (DAT). Afterwards, twelve plants were kept under well-watered conditions (WW) and twelve were subjected to water-deficit (WD) conditions by a reduction in irrigation levels during 62 DAT [from 100% to 20% field capacity (FC)]. The soil water regime was managed by weighing each pot three times per week and restoring either 100% or 80% of the consumed water in WW and WD plants, respectively (Fig. 1), which also allowed the determination of plant water consumption. The progressive WD treatment was imposed by restoring 80% of water losses until the soil water content achieved 20% FC. The pot mass at field capacity was previously determined after watering to saturation and allowing drainage for 24 h. The increase of whole plant mass during the experiment was not considered because it was negligible in comparison to the total pot mass.

Growth and biomass parameters

The leaf elongation rate (mm per day) was determined during the experiment (initial, 0–20 DAT; middle, 30–50 DAT; end, 70–80 DAT) by measuring the length of the youngest leaf of each plant until growth ceased. At the end of the experiment, the plant height, number of leaves per stem, and number of stems per plant were measured.

Each plantlet was separated into stems, roots, and leaves, and was separately dried in an oven. The total dry biomass per plant was determined by summing the aerial dry biomass and the root dry biomass. The shoot-root ratio, leaf mass ratio (LMR) and leaf area ratio (LAR) were also calculated. Three to five total expanded leaves were separately scanned at the moment of destructive analysis and sampling to dry to measure the specific mass and to determine the specific leaf area (SLA) and total leaf area (LA).

Leaf gas exchange and chlorophyll (Chl) a fluorescence

Simultaneous measurements of leaf gas exchange and Chl *a* fluorescence were conducted from 10:00 to 12:30 h on sunny days, twice per week along the experiment on young, fully expanded leaves in all plants. These measurements were performed with an open infrared gas-exchange analyzer system (*Li- 6400*; *Li-Cor Inc*., Lincoln, NE, USA) equipped with a leaf chamber fluorometer (*Li-6400-40*, *Li-Cor Inc.*). Measurements were performed under saturating PPFD of 1,500 µmol m^{-2} s^{-1}, with 10% of blue light, and a vapor pressure deficit of 2.0–3.0 kPa at a CO_2 concentration of 400 µmol mol^{-1}(air). The leaf temperature was set at 25ºC, and the relative humidity of the incoming air was approximately 50% throughout all measurements.

The gas-exchange measurements provided the net CO_2 assimilation (P_N) and the stomatal conductance (g_s) of the leaves. The Chl *a* fluorescence measurements allowed the determination of the photochemical efficiency of PSII (Φ_{PSII}) in the light-adapted stage (Peguero-Pina *et al*. 2012).

Mesophyll conductance and biochemical photosynthetic parameters

In order to conduct a thorough analysis of drought stress on photosynthesis, maximum Rubisco carboxylation rate (V_{cmax}), potential light-saturated electron transport rate (J_{max}), leaf dark respiration in the light (R_D), and mesophyll conductance (g_m) were estimated as explained below. After inducing steady-state photosynthesis for at least 30

min, the photosynthesis response to varying substomatal CO_2 concentration (C_i) was measured in all samples per treatment as explained in Galmés *et al.* (2007). Net photosynthesis-response curves to varying substomatal CO_2 concentration (P_N-C_i) consisted of 16 measurements per curve [stepwise in the range of 50–2,000 μmol(CO_2) mol^{-1}(air)] and were transformed to P_N *vs*. chloroplastic CO_2 concentration (C_c) curves (P_N-C_c) according to Peguero-Pina *et al.* (2012). The chloroplast CO_2-compensation point (Γ*) was estimated as described in Galmés *et al*. (2007).

Respiratory measurements in the absence of light (R_n) were measured by placing a leaf in the gas-exchange cuvette, without light source, after 30 min in the dark (time for transition from light-acclimated to dark-acclimated mitochondrial respiration). R_n was used as a proxy of dark respiration (R_D) in P_N-C_c curves (Bernacchi *et al.* 2002, Niinemets *et al.* 2006). Corrections for the leakage of CO_2 into and out of the leaf chamber of the *Li-6400* have been applied to all gas-exchange data, as described by Flexas *et al.* (2007).

Following method by Harley *et al.* (1992), based on combined gas exchange and Chl fluorescence, g_m and the chloroplastic CO_2 concentration were estimated.

Quantitative analysis of photosynthetic limitation

To determine the quantitative limitation of photosynthesis, P_N-C_i curves were performed as described in suporting information (SI) following the methodology used by different authors (Harley *et al.* 1992, Bernacchi *et al.* 2002, Niinemets *et al.* 2006, Flexas *et al.* 2007, Galmés *et al.* 2007, Peguero-Pina *et al.* 2012). P_N, g_s, g_{sc}, g_m, and V_{cmax} values (obtained with P_N-C_i analyse) were used to calculate the quantitative limitations of photosynthesis following the quantitative limitation analysis of Grassi and Magnani (2005). This method allows the determination of nonstomatal limitations, defined as the sum of the contributions due to mesophyll conductance and leaf biochemistry ($NS_L = MC_L + B_L$), and the diffusive limitations, defined as the sum of stomatal and mesophyll conductance components ($D_L = S_L + MC_L$). In the present work, the method modified by Grassi and Magnani (2005) was used to quantify the WD effect in each limitation component in comparison with WW conditions. In this sense, the values of each parameter of WW plants were used as a control in this analysis. The

values of each parameter used in the quantitative limitation analysis are the mean of 12 plantlets per treatment.

Leaf water and osmotic potential

To determine plant water status at the end of the experiment, the leaf water and osmotic potentials were measured at predawn and midday for each plant at the end of the experiment. The leaf water potential was determined on fully expanded leaves with a Scholander pressure chamber (*Soil moisture Equipment Corp.*, Santa Barbara, CA, USA); afterwards, a portion of each leaf was stored at –80ºC. The leaf osmotic potential was determined in those samples by using a vapor pressure deficit osmometer (*Wescor Inc*., Logan, UT, USA).

Water-use efficiency was assessed at the leaf and plant levels

The WUE at the leaf level was determined by intrinsic gas-exchange measurements as P_N/g_s (WUE_i, µmol mol^{-1}). At the end of the experiment, the WUE at the plant level (WUE_p, g L^{-1}) was calculated as a relation between plant dry biomass production and total water consumption (H_2Oc) of each pot during all the experiment.

Statistical analyses

A univariate analysis of variance (*ANOVA*) and *Duncan*'s test were performed to reveal the differences between groups in the studied parameters. The analyses were performed using *JMP®, Version 10* (*SAS Institute Inc.*, Cary, NC, 1989–2007).

Results

Evaluation of soil water content and plant water consumption: early response of *A. donax* to soil water content decrease

The soil water content of both WW and WD plants ranged between 80% FC from 0 to 20 DAT. From 20 DAT, after a progressive drought imposition, the soil water content of WD plants progressively dropped to 20% FC at 50 DAT, and it was kept at approximately this level until the end of the experiment (Fig. 1). The soil water content differences between treatments were significant from the first day of treatment application (20 DAT), which showed a progressive increase of differences along the experiment (Fig. 1). The water consumption increased along the experiment in WW

plantlets, while WD ones showed constant values of consumed water. From the day the treatments were completely imposed (50 DAT), the water consumption was five times higher in WW plantlets than that in the WD plantlets (Table 1). Over the whole experimental period, the WD plantlets consumed 62% less water than the WW plantlets (Table 1).

Table 1. Plant water consumption of well-watered (WW) and water-deficit (WD) plants throughout the experiment. Values of water consumption are the means of 12 replicates ± SE. * – significant differences between treatments at $p<0.05$.

Plants	First week	Last week	Whole period
WW [ml day^{-1}]	20 ± 2.0	166 ± 16.0	92 ± 8.0
WD [ml day^{-1}]	20 ± 2.0	29 ± 3.0*	35 ± 2.0*

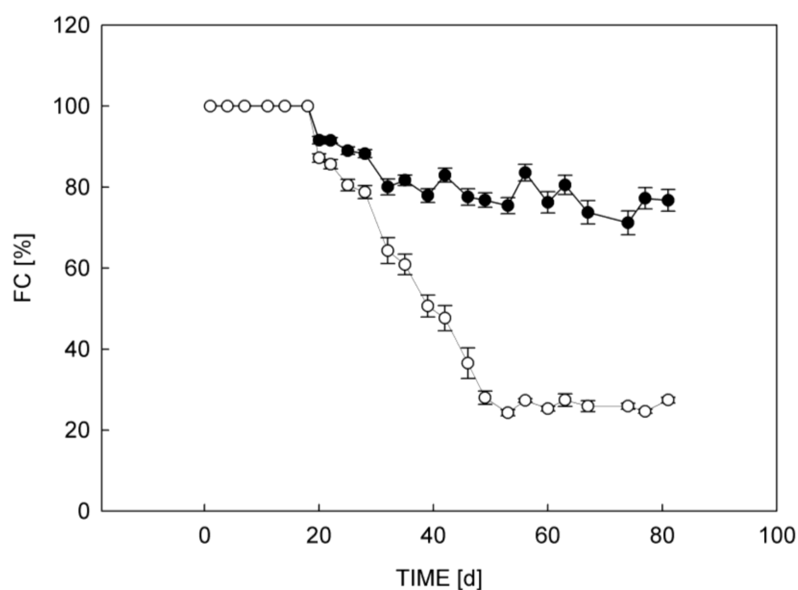

Fig. 1. Evolution over time of the soil water content (expressed as percentage of field capacity, FC) of well-watered plants (*black circles*) and water-deficit plants (*white circles*). Values are the means of 12 replicates ± SE.

Growth and biomass parameters

Both leaf length and leaf elongation rate (LER) were affected by the soil water content (Table 2). The WD plants possessed smaller leaves and lower LER than the WW plants at the middle and at the end of the experiment when the soil water content was significantly different between both treatments (Table 2).

The biomass production and allocation were affected by the water treatment (Table 3). Under WD conditions, the decrease of total biomass was mostly due to the reduction of shoot biomass because the root biomass was not significantly different between treatments. As a consequence, the shoot to root ratio (S/R) was significantly higher in the WW plants than that in the WD plants. The leaf area (LA), leaf mass ratio (LMR), leaf area ratio (LAR), and specific leaf area (SLA) were significantly lower in the WD plants than those in the WW plants (Table 3). The LA decrease in the WD plants was mainly due to both the lower number of shoots and the smaller leaves under the WD conditions than under the WW conditions, rather than to the number of leaves per shoot (Tables 2, 3). In spite of the lower biomass production, the WD plants showed a WUE_p increase (4.39 ± 0.460 g L^{-1} in WD and 2.89 ± 0.268 g L^{-1} in WW plantlets), as a consequence of the relatively high reduction in water consumption under the WD conditions.

Table 2. Air temperature, soil water content (% of field capacity, FC), leaf elongation rate (LER) and leaf length at three periods of the experiment: initial (days 0–20), middle (days 30–50), and end (days 70–80). Values are the means of 12 plants ± SE. *Different letters* within the same treatment indicate significant differences ($p<0.05$) between periods. Within the same period, * denotes significant differences ($p<0.05$) between treatments.

	Temperature [ºC]	FC [%]		LER		Leaf length	
		WW	WD	WW	WD	WW	WD
Initial	26.36	95.79 ± 0.47	93.59 ± 0.52	1.23 ± 0.07^a	1.21 ± 0.14^a	19.91 ± 1.35	17.40 ± 1.25
Middle	25.56	78.17 ± 1.80	34.11 ± 2.21 *	1.89 ± 0.09^b	1.07 ± 0.16^{a} *	23.98 ± 1.14	18.63 ± 1.72 *
End	23.56	77.90 ± 2.37	26.05 ± 0.73 *	1.27 ± 0.18^a	0.37 ± 0.07^{b} *	23.78 ± 1.79	11.70 ± 0.80 *

Water relations

Both pre-dawn and midday water potential were lower in the WD plants at the end of the experiment (Fig. 2A) when the soil water content was significantly different between treatments. Those differences were higher at midday than at pre-dawn. At midday, the WD plants showed leaf water potential of –2.3 MPa, whereas the WW plants only reached –1.2 MPa. Plants under both treatments rehydrated at night because both the relative water content (Fig. 2*B*) and the leaf water potential were significantly

higher at predawn than at midday. Similarly, the leaf osmotic potential was significantly lower in the WD plants than in the WW plants at both predawn and midday (Fig. 2*B*). At midday, the WD plants showed a leaf osmotic potential of –2.3 MPa, whereas the WW plants reached only –1.5 MPa.

Gas-exchange parameters

The P_N, g_s, and J_{flu} were significantly lower in the WD plants than those in the WW plants during most of the experiment (Fig. 3). However, the effect of the WD was observed in the g_s before the P_N. Indeed, the P_N was significantly lower in the WD plants only when the soil water content fell below 40% FC (20 DAT). In contrast, g_s was significantly different between treatments as soon as the soil water content fell below 60% FC (10 DAT). Electron transport rate (J_{flu}), was the latest parameter being affected by water stress, significant differences between WD and WW plants were only observed after 47 DAT (Fig. 3*C*). In all cases, the differences between treatments increased as the soil water content decreased in the WD pots. The estimation of biochemical parameters showed no effect of water deficit in the photosynthetic apparatus because no differences were observed in either $V_{c,max}$ or J_{max} (Table 4). The quantitative limitation analyses revealed that the photosynthesis of the WD plants was mostly limited by stomatal limitations, which accounted for more than 93.3% of the total limitations. Biochemical limitations represented 4.7% of the total limitations, and mesophyll conductance limitations accounted for 2% of the total limitations.

Table 3. Growth and biomass parameters of *Arundo donax* plantlets grown under well-watered (WW) and water-deficit (WD) conditions. Values of biomass parameters are reported as dry mass. Height of principal stem; number of stems per plant; stem biomass; number of leaves of the principal stem; total leaf biomass; shoot biomass; root biomass; shoot to root ratio (S/R); specific leaf area (SLA); plant leaf area (LA); leaf mass ratio (LMR); leaf area ratio (LAR). Values are the means of 12 replicates ± SE. * denotes significant differences between treatments at $p<0.05$.

	WW	WD
Height [cm]	51.6 ± 3.64	42.2 ± 2.47 *
Nº of stems	7.0 ± 0.60	5.0 ± 0.50 *
Stem biomass [g]	4.25 ± 0.28	2.04 ± 0.16 *
Nº of leaves	14.25 ± 0.74	12.67 ± 0.56
Leaf biomass [g]	6.38 ± 0.42	3.46 ± 0.21 *
Shoot biomass [g]	10.60 ± 0.69	5.50 ± 0.36 *
Root biomass [g]	5.63 ± 0.59	4.55 ± 0.53
Total biomass [g]	16.28 ± 1.17	10.06 ± 0.84 *
S/R	2.08 ± 0.16	1.27 ± 0.11 *
SLA [cm^2 g^{-1}]	238.1 ± 8.38	184.6 ± 3.48 *
LA [cm^2]	1525.0 ± 114.90	671.4 ± 37.97 *
LMR [g g^{-1}]	0.395 ± 0.01	0.344 ± 0.02 *
LAR [cm^2 g^{-1}]	94.09 ± 4.26	63.45 ± 2.86 *

Discussion

Petroleum and agricultural land scarcity triggered the research based in second generation biofuels. In this sense, *A. donax* had been demonstrated good traits regarding its crop productivity and acclimation to different soil conditions (Pilu *et al*. 2012). Webster *et al*. (2016) reported that the high productivity of *A. donax* is due to the higher photosynthetic capacity and intrinsic water-use efficiency. However, there is a lack of knowledge on *A. donax* ecophysiology under water-stress conditions that made important crop losses in the first stages of implantation. In this line, the aim of this study was to evaluate the response of *A. donax* to a moderate water deficit at early developmental stages as a critical determinant of the potential use of this species as biomass source for second generation biofuels in Mediterranean marginal lands. *A. donax* plantlets showed morphological, anatomical, and physiological changes as a consequence of the water deficit, which led to lower plant water consumption and higher water-use efficiency at both the leaf and plant levels. The present study supports the possibility of increasing the water-use efficiency with a low biomass decrease in *A. donax* plantlets during the first year of plantation, optimizing the water supplies during that critical moment in areas where water is the most limiting factor in plant production.

Regarding to the soil water conditions, the progressive water deficit imposed (thus a slow depletion of the soil water content, which allowed the acclimation of plants) and the low vapor pressure deficit (VPD) registered during the experiment led to a moderate water stress despite soil water content being as low as 20% FC. Those conditions, moderate water deficit and low VPD, are similar to those observed in several Mediterranean areas during autumn and early spring in dry years; the areas and seasons where and when *A. donax* is usually planted (Angelini *et al*. 2005).

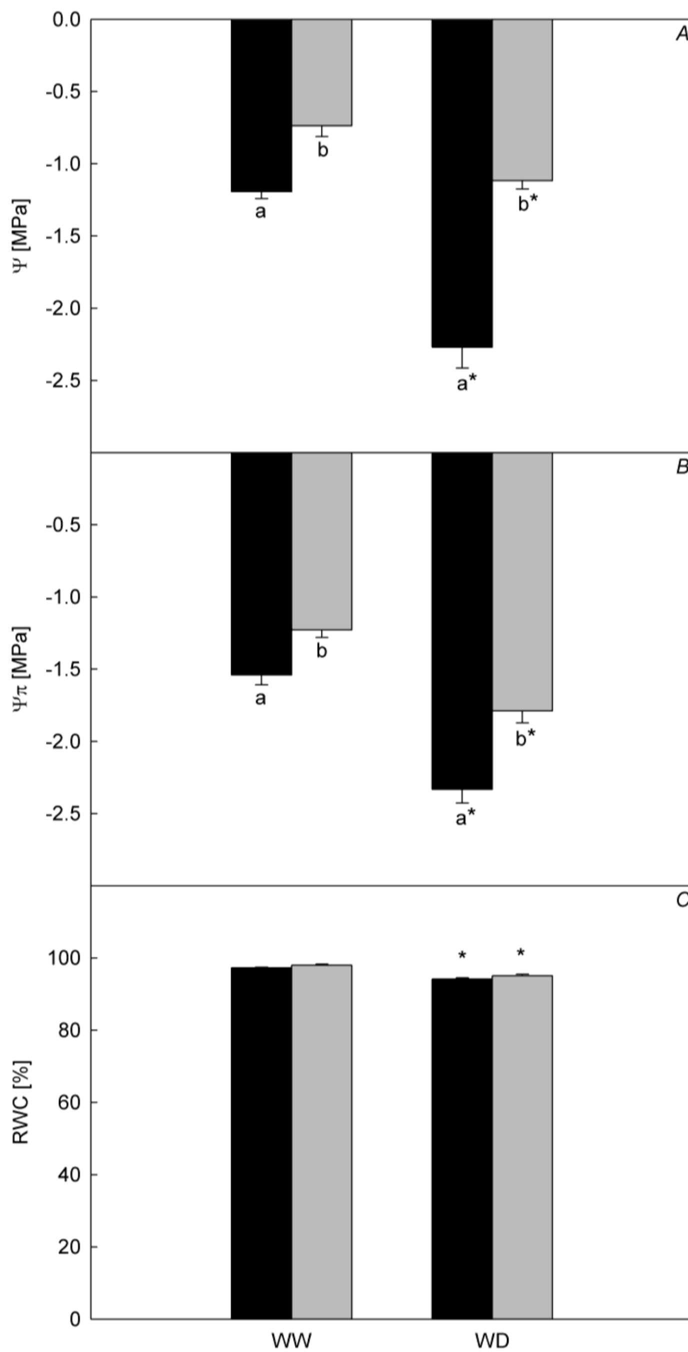

Fig. 2. Leaf water potential (Ψ, *A*), osmotic potential (Ψ_{π}, *B*) and relative water content expressed as a percentage (RWC, *C*) of *Arundo donax* under well-watered (WW) and water-deficit (WD) conditions at midday (*black bars*) and predawn (*grey bars*) at day 69. Values are the means of 12 replicates ± SE. *Different letters* within treatment denote significant differences ($p < 0.05$) between sampling times. * means significant differences ($p < 0.05$) between treatments within the same time of measurement.

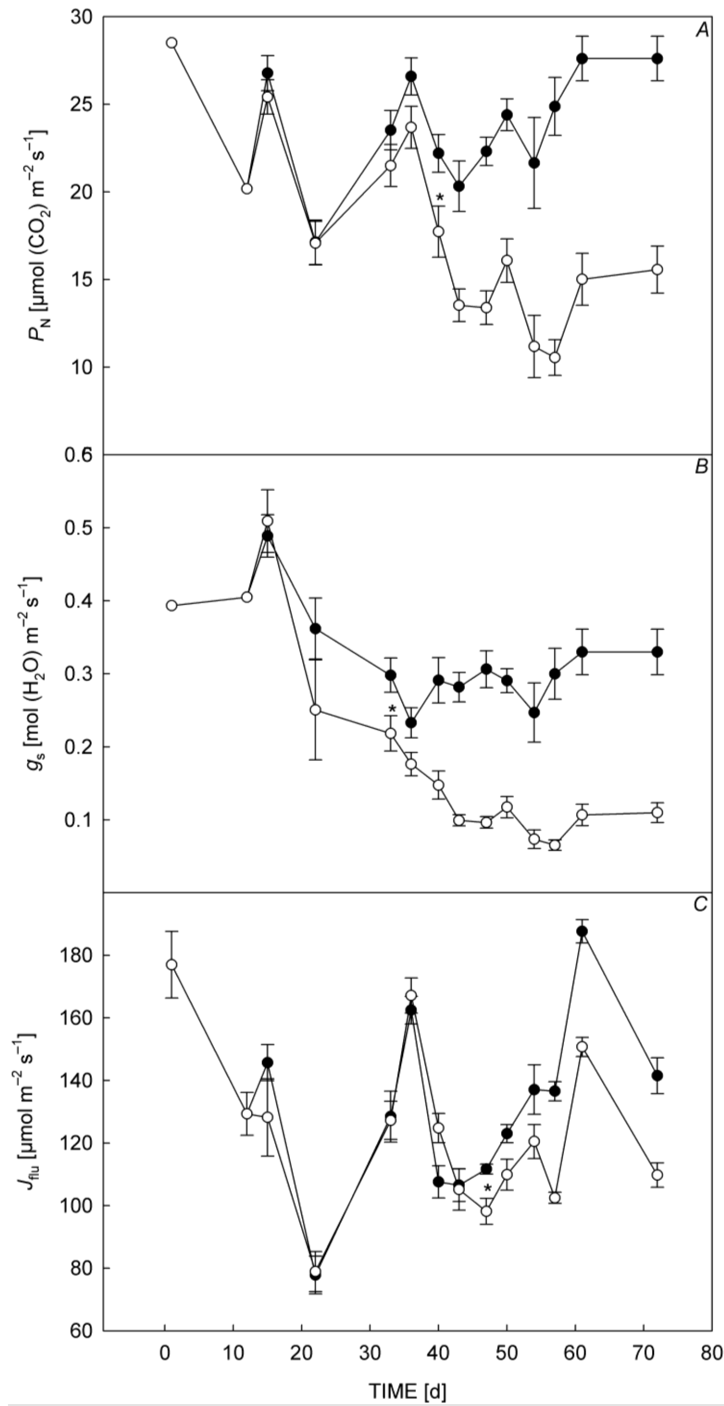

Fig. 3. Evolution over time of net photosynthetic rate (P_N, *A*), stomatal conductance (g_s, *B*), and electron transport rate (J_{flu}, *C*) along the experiment in well-watered plants (*black circles*) and water-deficit *Arundo donax* plants (*white circles*). Values are the means of 12 replicates. * means the first measure day in which values were significantly different between treatments ($p < 0.05$).

The biomass reduction under water deficit occurred mainly due to a decline in shoot biomass because root biomass did not significantly change under water deficit, which led to a lower shoot to root ratio in WD plants than in WW ones (Table 2). The shoot biomass reduction appeared to be a consequence of both a lower number of tillers per plant and smaller leaves in WD plantlets. Moreover, WD plantlets showed a significant lower SLA, as well as the reduction in plant leaf area and the leaf elongation rate, morphological and anatomical changes that led to a lower water consumption and an increased water-use efficiency in the WD plantlets (Hsiao *et al.* 1976, Meier and Leuschner 2008, Anjum *et al.* 2011). However, it is remarkable that such a reduction in *A. donax* plantlets was achieved by reducing the leaf size, with no effect on leaf number, because smaller leaves present lower transpiration requirements to reduce their temperature (Nobel 2009, Haworth *et al.* 2017).

Although the leaf elongation rates decreased under the WD conditions (Table 3), the leaves continued to grow until the end of the experiment under low soil water content and at a leaf water potential as low as –1.0 MPa at predawn and –2.5 MPa at midday. The osmotic potential differences between the WD and WW plants registered with relatively low changes in the relative water content (Fig. 2*B*,*C*) suggests that most of those differences were due to active solute accumulation, *i.e.* osmotic adjustment, in the WD plants (Wright *et al.* 1997, Babu *et al.* 1999). Osmotic adjustment has been reported to contribute to maintain turgor in cells, which is necessary to maintain an active growth in water stressed plants (Hsiao *et al.* 1976, Morgan 1984). Likewise, modification of turgor would contribute to the ability of *A. donax* plantlets to maintain leaf elongation and, thus, plant growth under low soil water content and leaf water potential. Indeed, the WD plantlets showed an estimated turgor pressure (water potential osmotic potential) of 0.7 MPa at predawn, although it was close to 0 MPa at midday.

The SLA reduction in the WD plants suggests an increase in leaf density and/or leaf thickness as acclimation to reduce the water losses (Liu and Stützel 2004, Villagra and Cavagnaro 2006, Wu et al. 2008). Such leaf anatomical acclimation used to be observed in drought-adapted species that are able to show phenotypic plasticity (Villagra and Cavagnaro 2006, Wu et al. 2008, Gulías et al. 2009). As a consequence of the effects of WD on the biomass partitioning and SLA, the relative biomass investment in the leaves (i.e. LMR) and the relative leaf area per plant biomass (i.e. LAR) significantly decreased in the WD plantlets (Table 2). However, LAR showed a higher reduction than LMR, 33% and 15%, respectively, due to the SLA decrease under the WD conditions.

Indeed, a low SLA has been linked to both low growth rates and high resource use efficiencies (Poorter et al. 1990, Reich et al. 1997, Wright et al. 2001). The plasticity of *A. donax* plantlets to produce smaller leaves with lower SLA under WD conditions would be a key component in its ability to cope with water stress. In this sense, the water-use efficiency at the plant level of the WD plants showed a significant increase in comparison to the WW plants.

Table 4. Mean values for the photosynthetic parameters analyzed. P_N – net photosynthesis [μmol(CO_2) m^{-2} s^{-1}]; g_s – stomatal conductance to H_2O [mol(H_2O) m^{-2} s^{-1}]; chloroplast CO_2-compensation point (Γ*); g_m – mesophyll conductance [mol(CO_2) m^{-2} s^{-1}]; C_i – substomatal CO_2 concentration [μmol mol^{-1}(air)]; C_c – chloroplastic CO_2 concentration [μmol mol^{-1} air]; $V_{c,max}$_C_c – maximum velocity of carboxylation [μmol m^{-2} s^{-1}] calculated from gas exchange on a C_c basis; J_{max}_C_c – maximum capacity for electron transport [μmol m^{-2} s^{-1}] calculated from gas exchange on a C_c basis; Four P_N-C_i curves per treatment were done. Data are means ± SE of 12 replicates per treatment. * means statistically significant differences ($p < 0.05$) between well watered (WW) and water-deficit (WD) plantlets.

	WW	WD
P_N [μmol (CO_2) m^{-2} s^{-1}]	25.88 ± 1.61	15.45 ± 3.22 *
g_s [mol (H_2O) m^{-2} s^{-1}]	0.170 ± 0.02	0.067 ± 0.01 *
Γ*	37.7	48.8
g_m fluo [mol (CO_2) m^{-2} s^{-1}]	0.626 ± 0.08	0.530 ± 0.15
g_m Sharkey [mol (CO_2) m^{-2} s^{-1}]	0.528 ± 0.04	0.418 ± 0.15
C_i [μmol mol^{-1} air]	218.15 ± 14.34	150.58 ± 4.871 *
C_c [μmol mol^{-1} air]	173.88 ± 14.93	117.23 ± 9.13 *
$V_{c,max}$_C_c Harley [μmol m^{-2} s^{-1}]	111.5 ± 4.57	106.1 ± 9.06
$V_{c,max}$_C_c Sharkey [μmol m^{-2} s^{-1}]	150.04 ± 9.28	126.08 ± 52.59
J_{max}_C_c Harley [μmol m^{-2} s^{-1}]	202.2 ± 6.90	152.1 ± 20
J_{max}_C_c Sharkey [μmol m^{-2} s^{-1}]	182.40 ± 1.93	137.85 ± 33.52

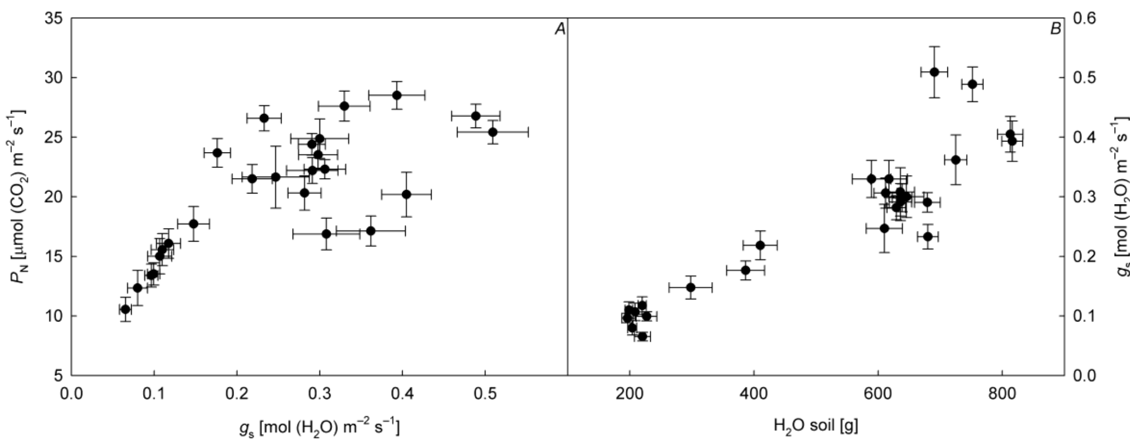

Fig. 4. Relationship between net photosynthetic rate (P_N) and stomatal conductance (g_s) (*A*) as well as between g_s and soil water content (*B*) in *Arundo donax* plantlets.

The WD plantlets showed an early response to drought through a reduction in g_s 10 d before the P_N reduction in the WD plantlets (Fig. 3*A*,*B*). Several studies have reported earlier stomatal closure than P_N depletion as a consequence of water deficit in various species (Medrano *et al*. 2002, Flexas *et al*. 2004), which lead to an increase in the intrinsic water-use efficiency (WUE_i), significantly increased WUE_i by a 43%, from

88.97 ± 5.29 to 154.78 ± 10.13 in the WW and WD plantlets, respectively (data not shown). The high WUE_i in WD plantlets is in accordance with low biochemical limitations to P_N. In this sense, Flexas *et al*. (2004) reported that WUE_i increased under moderate water deficit is dominated by diffusive limitations to P_N as a consequence of relatively higher g_s than P_N reduction. In contrast, WUE_i decreases greatly under severe water stress, when photosynthetic biochemical limitations are higher than diffusive ones (Flexas *et al*. 2004). The significant and negative correlation between SLA and both P_N/g_s and WUE_p (Table 1S, *supplement available online*) supports the important contribution of such an anatomical acclimation to the increase in WUE.

The high plantlet growth rate during the first year of plantation has been reported to be a key point to ensure high productivity during the following years in *A. donax* (Pilu *et al*. 2013). Such high growth rate used to be ensured by fertilization and irrigation under Mediterranean conditions. Nevertheless, if *A. donax* is to be a useful species in Mediterranean marginal lands, where water availability for irrigation is generally low, it is extremely important to maximize not only the plant biomass accumulation but also its efficient use of resources. In addition, the trade-off between growth rate and water-use efficiency within and among species is widely reported (French and Schultz 1984, Fernández and Reynolds 2000, Condon *et al*. 2002). However, our results showed that it is possible to reduce the stomatal conductance without a significant reduction of the net photosynthesis in *A. donax* plantlets (Fig. 4*A*). For instance, WUE_i would be highest when g_s values are approximately 0.2 mol m^{-2} s^{-1} (Fig. 4*A*). Similar opportunities of maximizing WUE_i have been reported in other crops (Pou *et al*. 2008, Tomás *et al*. 2012). In the present study, the g_s values were registered when the total soil water content was approximately 400 ml (Fig. 4*B*). The precise amount of soil water needed to maintain the g_s would depend on several factors, such as plant leaf area, VPD, and soil characteristics; however, the significant regression between g_s and soil water content reported in this study ($R^2 = 0.84$ and $p<0.0001$, Fig. 4*B*) suggests the possibility of a reliable estimation of g_s from the measurement of soil water content; a parameter that is more economically and practically favorable to leaf gas exchange analysis under field conditions.

Conclusions

Arundo donax has been reported to be a water-stress tolerant species at the adult stage, what is partially due to the ability of its rhizome to accumulate water and nutrients. However, little is known regarding the ability of *A. donax* to cope with water limited conditions at early stages, when the rhizome has not been developed yet. In this study, *A. donax* showed a great ability to tolerate moderate water stress. The plantlets reduced their water consumption to a larger extent than their biomass accumulation, which led to higher water-use efficiency in the water-deficit plantlets than in the well-watered ones. Such an increment was achieved by decreasing the leaf size and the specific leaf area in addition to the reduction of the stomatal conductance. As a result, both WUE_i and WUE_p significantly increased. Moreover, *A. donax* leaves showed an osmotic adjustment that clearly contributed to maintain leaf elongation under low soil water content. The reported reduction of g_s with a low variation in P_N and the highly significant regression between g_s and the soil water content suggest the possibilities of precise irrigation practices to optimize the water-use efficiency under field conditions in Mediterranean marginal lands, where water availability largely limits using *A. donax* as a biomass source.

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Suplementary information (SI) Chapter 1

SI Table 1. Pearson correlation between biomass accumulation (Shoot, Root and Total Biomass, g), anatomic (Leaf Area, LA [m^2]; Specific Leaf Area, SLA [$cm^2\ g^{-1}$]; Leaf Area Ratio, LAR [$cm^2\ g^{-1}$]; Leaf Mass Ratio, LMR [$g\ g^{-1}$]), and physiological parameters (Net Photosynthesis rate, P_N; stomatal conductance g_s; intrinsic Water Use Efficiency, P_N/g_s; Water Use Efficiency at plant level, WUE_p [$g\ l^{-1}$]) and water relations (soil water content, SWC [ml (H_2O) in the soil] and plant water consume [l]). Blue and red color mean positive and negative significant correlation respectively and grey color means no significant correlation. * Denotes significant Pearson correlation with $p < 0.0001$.

	P_N	g_s	P_N/g_s	WUEp	SWC	Water Consum	Shoot	Root	Total Biomass	LA	SLA	LAR	LMR
P_N	1												
g_s	0.93*	1											
P_N/g_s	– 0.87*	– 0.9*	1										
WUE_p	– 0.73*	– 0.78*	0.77*	1									
SWC	0.75	0.69	– 0.71	– 0.56	1								
Water Consum	0.77*	0.84*	– 0.81*	– 0.82*	0.67	1							
Shoot	0.69	0.71	– 0.67	- 0.53	0.71	0.86*	1						
Root	0.02	0.06	0.005	0.083	0.16	0.31	0.51	1					
Total Biomass	0.49	0.48	– 0.48	– 0.34	0.56	0.75*	0.93*	0.78*	1				
LA	0.73*	0.74*	– 0.69	– 0.63	0.72	0.90	0.96	0.46	0.88*	1			
SLA	0.72*	0.71	– 0.68	– 0.72	0.70	0.81*	0.68	0.23	0.58	0.85*	1		
LAR	0.81*	0.83*	– 0.76*	– 0.79*	0.69	0.78*	0.67	– 0.11	0.44	0.81*	0.87*	1	
LMR	0.63	0.65	– 0.58	– 0.59	0.43	0.45	0.42	– 0.49	0.09	0.45	0.39	0.78*	1

Chapter 2

Arbuscular Mycorrhizal Symbiosis with *Arundo donax* Decreases Root Respiration and Increases Both Photosynthesis and Plant Biomass Accumulation

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Original Article

Arbuscular Mycorrhizal Symbiosis with *Arundo donax* Decreases Root Respiration and Increases Both Photosynthesis and Plant Biomass Accumulation

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Abstract

The effect of arbuscular mycorrhiza (AM) symbiosis on plant growth is associated with the balance between costs and benefits. A feedback regulation loop has been described in which the higher carbohydrate cost to plants for AM symbiosis is compensated by increases in their photosynthetic rates. Nevertheless, plant carbon balance depends both on photosynthetic carbon uptake and respiratory carbon consumption. The hypothesis behind this research was that the role of respiration in plant growth under AM symbiosis may be as important as that of photosynthesis.

This hypothesis was tested in *Arundo donax* L. plantlets inoculated with *Rhizophagus irregularis* and *Funneliformis mosseae.* We tested the effects of AM inoculation on both photosynthetic capacity and *in vivo* leaf and root respiration. Additionally, analyses of the primary metabolism and ion content were performed in both leaves and roots. AM inoculation increased photosynthesis through increased CO_2 diffusion and electron transport in the chloroplast. Moreover, respiration decreased only in AM roots via the cytochrome oxidase pathway (COP) as measured by the oxygen isotope technique. This decline in the COP can be related to the reduced respiratory metabolism and substrates (sugars and tricarboxylic acid cycle intermediates) observed in roots.

Key-words: arbuscular mycorrhizal colonization; metabolomics; net assimilation rate; oxygen isotope fractionation; plant growth.

Abbreviations: AM, arbuscular mycorrhiza; A net, CO_2
assimilation rate; AOP, alternative oxidase pathway; AOX, alternative oxidase; C_c, chloroplastic CO_2 concentration; Ci, substomatal CO_2 concentrations; COP, cytrochrome oxidase pathway; COX, cytochrome oxidase; DW, dry weight; g_m, mesophyll conductance; g_s, stomatal conductance; J_{flu}, the electron transport rate; J_{max}, the potential light saturated electron transport rate; LA, leaf area; LAR, leaf area ratio; NM, non-arbuscular mycorrhiza plants; R_d, the leaf dark respiration in the light; R_n, the respiratory rate in the absence of light; SLA, specific leaf area; TCA, tricarboxylic acid cycle; v_{alt}, AOX activity; V_{cmax}, the maximum ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate; v_{cyt}, COX activity; V_t, the total respiration; Φ_{PSII}, the photochemical efficiency of photosystemII; τ_a, the electron partitioning to the alternative pathway.

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Introduction

The positive effect of arbuscular mycorrhiza (AM) symbiosis on plant growth has been widely reported; thus, the range of potential benefits are often viewed as prime candidates to aid in moving toward sustainable agricultural systems (Hodge & Storer, 2015). However, it is also known that the effect of AM on plant growth is variable and depends on the host plant and fungal species (Stribley *et al*., 1980). This variability is due to the complexity of the relationship between the plant and the fungi and the additional biotic and abiotic factors that affect such relationships.

The effect of AM symbiosis on plant physiology has been extensively studied beyond increases or decreases in biomass production based on the concept of a "true cost-benefit analysis" (Koide & Elliott, 1989). This approach takes into account not only plant growth but also other physiological parameters of plants that are affected by AM colonization. Previous studies mostly linked AM effects on plant growth to increases in photosynthetic rates (Xu & Xia, 2006; Fan *et al*., 2008). Moreover, it has been found that AM colonization affects both plant respiration (Baas *et al.,* 1989; Hodge, 1996; Johnson *et al.,* 2002; Atkin *et al.,* 2009) and metabolism (Hohnjec *et al*., 2005; Schliemann *et al*., 2008; Laparre *et al*., 2014).

Most of the effects of mycorrhiza on host plants have being linked to higher nutrient uptake. In fact, mycorrhiza can improve nutrient acquisition because AM fungi provide an additional means of nutrient uptake, the mycorrhizal nutrient uptake pathway (Smith *et al*., 2004; Bucher *et al*., 2014; Watts-Williams *et al*., 2015), which can bypass the pathway of direct nutrient uptake (Smith *et al*., 2011; Facelli *et al*., 2014). In this sense, both higher plant nutrient concentrations and water status have been linked to photosynthetic enhancement by AM (Wu & Xia, 2006; Ruiz-Lozano & Aroca, 2010). However, the mechanisms by which AM inoculation affects photosynthetic activity remain unclear. Studies of the biochemical and diffusive parameters regulating the maximum rate of photosynthesis are thus required to explain the basis of the observed changes in photosynthetic rate (A_N) under AM symbiosis.

As mentioned above, another essential physiological process affected by AM colonization is respiration. Several studies have shown increases in root respiration in AM plants (Baas *et al.,* 1989; Hodge, 1996; Johnson *et al.,* 2002; Atkin *et al.,* 2009), which was suggested to be associated with increased carbon demand to ensure the maintenance of the symbiosis (Hughes *et al*., 2008). Based on this reasoning, an

increase in respiration rates would be foreseen to occur at the level of the alternative oxidase pathway (AOP) because the AOP is related to the maintenance component of respiration (Florez-Sarasa *et al*., 2007). However, the effect of AM on the *in vivo* activities of the AOP and the cytochrome oxidase pathway (COP) has not yet been reported. Such a study should be performed by using the oxygen isotope fractionation approach because this is the only available technique that allows the measurement of the *in vivo* activities of both respiratory pathways (Day *et al*., 1996; McDonald *et al*., 2003). It is well known that plant metabolic status is intimately connected with physiological parameters such as photosynthesis and respiration (Fernie *et al*., 2004; Noguchi & Yoshida, 2008; Florez-Sarasa *et al*., 2012; Gago *et al*., 2016). Several studies have assessed the effects of AM symbiosis on plant metabolism through metabolomic analyses, showing the complexity of the relationship between the plant and the fungi (López-Ráez *et al*., 2010; Fester *et al*. 2011; Rivero *et al*. 2015). Despite the overall positive effect of AM on the host plant, the response of host plant metabolism has been shown to be complex. López-Ráez *et al*. (2010) reported changes in the content and biosynthesis of plant phytohormones involved in the maintenance of the symbiosis and showed that the effect on the host plant differed based on the specific AM fungi involved. Moreover, the effect of AM colonization on metabolite profiles has been studied in different plant tissues; leaf metabolites related to abiotic stress signaling were increased as well as root metabolites indicating the carbon sink effect of the AM symbiosis (Fester *et al.,* 2011; Rivero *et al.,* 2015). However, to the best of our knowledge, there are no studies that have combined both physiological and metabolomics analysis in AM plants; such a combined approach can provide new insights for a better understanding of the effect of AM symbiosis on plant carbon metabolism. In the present study, the effects of AM colonization by *Rhizophagus irregularis* and *Funneliformis mosseae* (formerly called *Glomus intraradices* and *Glomus mosseae*, respectively) on photosynthesis and respiration *in vivo* and on the levels of primary metabolites were evaluated to obtain a global picture of the impact of AM colonization on giant reed (*Arundo donax L.)* plantlets. *Arundo donax* was selected due to be considered one of the most promising species for energy, cellulose paste and second-generation biofuel production tanks to its high biomass productivity (Hidalgo & Fernandez, 2001; Shatalov & Pereira, 2001; Lewandowski *et al*., 2003). Moreover, *A. donax* is susceptibility of AM inoculation which could result in higher productivity

(Baraza *et al*., 2016). However, there is a lack of basic knowledge concerning its physiology that renders its cultivation difficult.

The following four specific questions were addressed: (i) What are the photosynthetic parameters and limitations that are affected by AM that could lead to improved photosynthesis? (ii) What is the effect of AM on the *in vivo* activities of AOP and COP in leaves and roots? (iii) Which primary metabolites change in response to AM colonization in leaves and roots? and (iv) What metabolic changes underlie the responses of photosynthesis and respiration to AM colonization?

Materials and methods

Plant and fungal material

Twenty plantlets of the *A. donax* K12 clone were provided by Biothek Ecologic Fuel S.L. (Spain). The micropropagated plants were received as bare roots and were immediately planted in trays of agricultural substrate previously tindalized for excluding other microorganisms present in the peat, which mostly consisted of nutrient-rich black peat (Kekkilä DSM 1 W, pH 5.9, 90% of organic material. Principal compounds: Sphagnum peat; additives: N-P_2O_5-K_2O (16-4-17, 0.60 g l^{-1}), wetting agent (0.10 g l^{-1}) and dolomite limestone (5.0 g l^{-1})). Two-week old plants were transplanted into 1 L pots in the same substrate (Kekkilä DSM 1 W). Roots of ten plants (AM plantlets) were sprinkled with 5 mL (5 g approx.) of a commercial AM fungi inoculum at the transplantation moment, in this way the inoculum is attached to the root or placed next to it at the time of transplantation to 1 L pots. Non-inoculated (NM) plantlets (the rest ten plantlets) were supplied with 5 mL of autoclaved inoculum plus 3 mL of an inoculum filtrate (< 20 μm) to provide a general microbial population that was free of AM propagules. In order to ensure the AM infection we used commercial inoculum (AEGIS® Sym Microgránulo, Atens Agrotecnologías Naturales S.L., Barcelona, Spain) that previous studies had determined as effective in the infection of *A. donax* (Tauler & Baraza 2015). Moreover that inoculum contained two generalist species, abundant in all

soil types and widespread uses as commercialized inoculum: *Rhizophagus irregularis* and *Funneliformis mosseae* (25 spores per g each).

The plants were grown for three months in a growth room under controlled conditions of 25/20 °C day/night temperature, greater than 40% relative humidity and a 12 h photoperiod (300 μmol m^{-2} s^{-1} of photosynthetic photon flux density, PPFD) and were regularly irrigated with deionized water.

Mycorrhizal development

The percentage of mycorrhizal root colonization was determined by the visual observation of fungal colonization after washing (10% KOH) and staining (0.05% trypan blue in lactic acid (v/v)) the roots according to Phillips and Hayman (1970). AM colonization was assessed using the magnified intersections method (McGonigle *et al*., 1990), in which the frequency of colonization (hyphae, arbuscules and/or vesicles) represents the ratio between the fragments of colonized root and the total number of root fragments examined. An average of 300 root pieces per plant and nine plants per treatment were examined.

Biomass measurements

Plant growth, which entailed the total number of leaves and stems and the length of the longest stem and root, was measured in 3-month-old plants. Four plantlets per treatment were randomly selected to perform destructive biomass measurements. The specific leaf area (SLA, m^2 g^{-1}) was calculated as the ratio of leaf area to dry mass. The fresh leaf area of four fully developed leaves was determined using the Image J (Wayne Rasband, NIH) program, and the leaf dry weight (DW) was obtained after drying for 48 h at 70 °C. Each plantlet was separated into stems, roots and leaves, which were dried separately to obtain the total dry biomass. The whole-plant leaf area (LA, cm^2) and leaf

area ratio (LAR, cm^2 g^{-1}) were calculated as the total leaf dry weight/specific leaf area and leaf area/total biomass respectively.

Ion concentration

The ion concentration (mg g^{-1}) was determined in the leaves, stems and roots of the four plantlets that were used previously in the monitoring of plant photosynthesis and respiration and the destructive biomass measurements. The plant tissues were dried at 70 °C for 48 h prior to analysis. One hundred milligrams of finely ground tissue was digested in 15 mL of 0.1 M nitric acid for 12 h and filtered for cation analysis. P_i, Ca^{2+}, Mg^{2+}, K^+ and Na^+ tissue contents were determined by inductively coupled plasma (ICP) spectrometry (Perkin-Elmer Plasma-2000, Perkin-Elmer Inc. Norwalk, CA, USA).

Photosynthetic measurements and analysis

Leaf gas exchange and chlorophyll a *fluorescence*

Simultaneous measurements of leaf gas exchange and chlorophyll *a* fluorescence were performed every day from 10:00 a.m. to 14:00 p.m. during the last two weeks of the experiment with an open infrared gas-exchange analyzer system (Li-6400; Li-Cor Inc., Lincoln, NE, USA) equipped with a leaf chamber fluorometer (Li-6400-40, Li-Cor Inc.). One young fully expanded leaf per plantlet (ten plantlets per treatment) and two replicate measurements were performed for each leaf over the last two weeks of the experiment.

The measurements were taken under a saturating PPFD of 1500 µmol m^{-2} s^{-1} with 10% blue light, a vapor pressure deficit of 2.0–3.0 kPa, and a CO_2 concentration (C_a) of 400 µmol mol^{-1} air. The leaf temperature was set at 25 °C and the relative humidity of the incoming air was approximately 50% for all measurements.

The gas exchange measurements provided the net CO_2 assimilation (A_N) and stomatal conductance (g_s) of the leaves. Chlorophyll *a* fluorescence measurements allowed the

determination of the photochemical efficiency of photosystem II (Φ_{PSII}) in a light-adapted state (Peguero-Pina *et al.*, 2012). From these values, the real quantum efficiency of photosystem II (ΦPSII) was calculated as

$\Phi PSII = (Fm' - Fs)/Fm$ (1)

and the electron transport rate (J_{flu}) through PSII was calculated as

$J_{flu} = \Phi PSII \cdot PPFD \cdot \alpha \cdot \beta$ (2)

where the PPFD value corresponded to the saturated photosynthetic photon flux density stated above, α was the absorbance of the leaf determined according to Schultz (1996) using a spectroradiometer (HR2000CG-UV-NIR; Ocean Optics Inc., Dunedin, FL, USA), and β was the theoretical partitioning of the absorbed PAR to PSII, which was assumed to be 0.5.

Mesophyll conductance and biochemical photosynthetic parameter estimation

To conduct a thorough analysis of the effect of AM inoculation on photosynthesis, the maximum ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate (V_{cmax}), the potential light saturated electron transport rate (J_{max}), the leaf dark respiration in the light (R_d) and the mesophyll conductance (g_m) were estimated as explained below. After inducing steady-state photosynthesis for at least 30 min, the photosynthetic response to varying substomatal CO_2 concentrations (Ci) was measured in nine samples per treatment as explained in Galmés *et al.* (2007). The net photosynthetic response curves to varying substomatal CO_2 concentrations (A_N–Ci) consisted of 17 measurements per curve (stepwise in the range of 0–2000 µmol CO_2 mol^{-1} air) and were transformed to A_N versus chloroplastic CO_2 concentration (C_c) curves (A_N-C_c) according to Peguero-Pina *et al.* (2012).

The chloroplast CO_2 compensation point (Γ^*) was estimated as is described in Galmés *et al.* (2007). All equations and estimates are described in the Supporting Information.

The respiratory measurements in the absence of light (R_n) were measured by placing a leaf in the gas-exchange cuvette without a light source after 30 min in the dark (time for transition from light-acclimated to dark-acclimated mitochondrial respiration). R_n was used as a proxy of dark respiration (R_d) in the A_N-C_c curves (Bernacchi *et al.*, 2002; Niinemets *et al.*, 2006). Corrections for the leakage of CO_2 into and out of the leaf chamber of the Li-6400 were applied to all gas-exchange data, as described by Flexas *et al.* (2007).

The following two different methods were used to estimate g_m and the chloroplastic CO_2 concentration: the method by Harley *et al.* (1992), which is based on the combined gas exchange and chlorophyll fluorescence, and the method by Ethier & Livingstone (2004), as modified by Buckley and Diaz-Espejo (2015), which is based on curve fitting. Both estimates were used to calculate V_{cmax} and J_{max} (Flexas *et al.* 2007). The values of g_m, V_{cmax} and J_{max} shown in the Results were obtained by the model described by Farquhar *et al.* (1980) after the values were validated through their use in both models (Supporting Information, Fig. S1).

Quantitative analysis of photosynthetic limitation

In stress experiments in which A_N is thoroughly analyzed, photosynthetic limitations are determined with A_N, g_s, stomatal conductance to CO_2 (g_{sc}), g_m and V_{cmax} values using the quantitative limitation analysis described by Wilson & Baldocchi as modified by Grassi & Magnani (2005). This method allows for the determination of non-stomatal limitations (defined as the sum of the contributions due to mesophyll conductance and leaf biochemistry ($NS_L = MC_L + B_L$) and diffusive limitations (the sum of stomatal and mesophyll conductance components ($D_L = S_L + MC_L$)). In the present work, the Grassi and Magnani-modified method was used to quantify the effect of AM inoculation on each limitation component in comparison with no inoculation. In this sense, the values

of each parameter from NM plantlets were used as a control in this analysis. Additionally, following the methodology used by Gallé et al. (2009), the calculations of B_L were confirmed using J directly instead of V_{cmax} as a surrogate for leaf biochemistry to account for possible errors in the determination of g_m and V_{cmax} (as V_{cmax} values are derived from the same A_N–C_c curves as g_m and depend on the validity of Rubisco kinetics as estimated by Bernacchi *et al.*, 2002). The values of each parameter used in the quantitative limitation analysis represent the mean of eight plantlets per treatment.

Respiration and oxygen isotope fractionation measurements

Oxygen uptake was measured after the A_N–Ci curve measurements in the same leaf. The root measurements were carried out immediately following the *in vivo* leaf respiration analysis. The tissues were harvested and placed in a 3-mL stainless steel closed cuvette, from which 250 μL of air was sequentially withdrawn and fed into the mass spectrometer sample bellows. The cuvette was maintained at a constant temperature of 25 °C using a copper plate and a serpentine coil around the cuvette in a temperature-controlled water bath (Gastón *et al.*, 2003).

Accurate measurements of the changes in the $^{18}O/^{16}O$ ratios and oxygen consumption are needed to obtain the oxygen isotope fractionation. Masses 34 ($^{18}O^{16}O$) and 32 ($^{16}O^{16}O$) were used to measure the oxygen isotope $^{18}O/^{16}O$ ratios. Masses 32 ($^{16}O_2$) and 28 ($^{14}N_2$) were used to calculate the total oxygen uptake (O_2/N_2) and therefore the total respiration (V_t). The values of m/z 34/32 ($^{18}O_2/^{16}O_2$) and m/z 32/28 ($^{16}O_2/^{28}N_2$) were obtained from a standard and the sample air using dual-inlet analysis with six replicate cycles for each respiration measurement using a dual-inlet mass spectrometer system (Delta XPlus, Thermo LCC, Bremen, Germany). The time between successive samples was 20 min, and the length of a full experiment varied between 90 and 120 min.

The calculations of the oxygen isotope fractionation were conducted as described by Guy *et al*. (1989) and Ribas-Carbó *et al*. (1995), and the electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy *et al*. (1989). The r^2 values of all linear regressions between ln f and $\ln(R/R_0)$, with a minimum of five data points, were at least 0.995, the minimal acceptable level (Ribas-Carbó *et al*., 1997). The electron partitioning to the alternative pathway (τ_a) was calculated as follows:

$$\tau_a = (\Delta_n - \Delta_c) / (\Delta_a - \Delta_c)$$

where Δ_n, Δ_c, Δ_a represent the oxygen isotope fractionation in the absence of inhibitors, in the presence of SHAM, and in the presence of KCN, respectively. For the KCN inhibitor treatments, the leaves and roots were incubated for 30 min by sandwiching them between medical wipes soaked with a water solution of 10 mM KCN. The values of Δ_a obtained in the leaves and roots of *A. donax* were 30.2 ± 0.3‰ (five replicates) and 26.0 ± 0.1‰ (four replicates), respectively. For the calculation of the oxygen isotope fractionation by cytochrome c oxidase (Δ_c), the leaves were incubated by being submerged in a water solution of 25 mM SHAM for 30 min. The values of Δc obtained in the leaves of *A. donax* were 19.5 ± 0.4‰ (four replicates). In the roots, an assumed value of 17‰ for the Δ_c was used for the electron partitioning calculations, as this has been shown to be fairly constant among the species examined up to date (Ribas-Carbó *et al*., 2005). All stock solutions were freshly prepared.

Metabolic profiling

Samples were harvested before the *in vivo* respiration measurements (and after 30 min in the dark for leaves). Metabolite extractions, derivatization and gas chromatography-time-of-flight mass spectrometry (GC-TOF-MS) analyses were carried out in the leaves

and roots of AM and NM *Arundo donax* plantlets as previously described (Lisec *et al*., 2006). The GC-TOF-MS system was composed of a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph and an LECO Pegasus III time-of-flight mass spectrometer running in EI+ mode. The metabolites were identified by comparison with database entries of standards (Kopka *et al*., 2005; Schauer *et al*., 2005). The data were normalized with respect to NM plantlets. The values presented are the mean of the relative values ± SE of four to six measurements.

Statistical analyses

Univariate analysis of variance (ANOVA) was performed to reveal the differences between groups in the studied parameters. Pearson correlations were applied to determine the relationship among the different variables studied. The analyses were performed using JMP®, version 10 (SAS Institute Inc., Cary, NC, 1989-2007).

Results

Arbuscular mycorrhizal colonization

The colonization of giant reed roots was examined at nine AM and nine NM plantlets. Non-inoculated plantlets were examined to ascertain the absence of AM, ensuring that they were completely free of structures typically of AM symbiosis. In AM plantlets, the colonization frequency was 48.6 ± 6.2%. Moreover, microscopic observations demonstrated the presence of typical AM structures, arbuscules, vesicles or inter- and intracellular hyphae in AM roots.

AM fungi increase biomass and nutrient content

AM inoculation significantly increased shoot development. This increase was due to the higher number of stems and leaves produced by AM plantlets, thus leading to higher leaf area (Table 1). However, no differences were found for either specific leaf area or

leaf area ratio between AM and NM plantlets. Root biomass did not differ between the AM and NM plantlets.

In addition, no difference was observed in nutrient concentration between the treatments (Table 2). However, AM plantlets showed a lower leaf Ca^{2+}/Mg^{2+} ratio in comparison to NM plantlets (Table 2).

Table 1. Effects of AM fungi on shoot and root biomass, as determined by the stem height, number of stems, total number of leaves, total leaf area (LA), specific leaf area (SLA), leaf area ratio (LAR), shoot dry weight and root dry weight and length. Values are means ± SE for four biological replicates and asterisks denote significant differences between non-AM plantlets (NM) and AM-inoculated plantlets (AM) ($p < 0.05$).

	NM	AM
	Shoot	
Height (cm)	12.3 ± 1.86	19.6 ± 1.25*
Stems (number)	3.3 ± 0.48	5.8 ± 0.75*
Leaves (number)	16.8 ± 3.82	31.0 ± 4.38*
LA (cm^2)	125.47 ± 33.78	260.59 ± 37.32*
SLA (cm^2 g^{-1})	372.56 ± 12.27	351.18 ± 14.75
LAR (cm^2 g^{-1})	189.41 ± 11.48	172.00 ± 5.66
Shoot DW (g)	0.58 ± 0.17	1.29 ± 0.18*
	Root	
Root DW (g)	0.11 ± 0.04	0.23 ± 0.04
Length (cm)	22.6 ± 2.54	25.6 ± 0.63

Table 2. Ion concentration (mg g^{-1}) in leaf and root tissues. Values are means and standard error of the measurements from four plants per treatment. An asterisk indicates a significant difference ($p < 0.05$) in ion concentration between non-AM (NM) and AM plantlets.

Tissue	**Leaf**		**Root**	
Treatment	NM	AM	NM	AM
P_i	3.27 ± 0.441	4.05 ± 0.302	1.84 ± 0.644	1.94 ± 0.088
Ca^{2+}	2.22 ± 0.189	1.97 ± 0.113	2.07 ± 0.534	1.61 ± 0.118
Mg^{2+}	1.14 ± 0.105	1.23 ± 0.051	1.69 ± 0.511	1.12 ± 0.033
Ca^{2+} / Mg^{2+}	1.97 ± 0.135	1.60 ± 0.026*	1.27 ± 0.077	1.45 ± 0.100
K^+	7.14 ± 0.599	9.75 ± 0.136	9.09 ± 2.566	5.58 ± 0.572
K^+ / Mg^{2+}	6.47 ± 0.914	7.83 ± 0.874	5.50 ± 0.277	4.97 ± 0.423
Na^+	0.43 ± 0.090	0.31 ± 0.049	1.17 ± 0.286	0.71 ± 0.093
Na^+/ K^+	0.06 ± 0.013	0.036 ± 0.012	0.14 ± 0.117	0.13 ± 0.025

Table 3. Mean values for the photosynthetic parameters analyzed. Two replicate measurements per sample for at least eight samples per treatment were taken. Eight A_N - C_i curves per treatment were generated. A mean value per plantlet was obtained, and the data represent the mean and standard error for the plantlets in each treatment. Asterisks indicate statistically significant differences ($p < 0.05$) between AM and non-AM (NM) plantlets. A_N, net photosynthesis; g_s, stomatal conductance to H_2O; g_{sc}, stomatal conductance to CO_2; C_i, sub-stomatal CO_2 concentration; C_c, chloroplastic CO_2 concentration; $V_{c,max}$_C_c, maximum velocity of carboxylation calculated from the gas exchange on a C_c basis; J_{max}_C_c, maximum capacity for electron transport calculated from the gas exchange on a C_c basis; J_{flu}, electron transport rate estimated by chlorophyll fluorescence. Amelioration of the limitations of A_N (expressed as a percentage) of AM with respect to NM plantlets: S_L, stomatal limitation; MC_L, mesophyll limitation; B_L, biochemical limitation using V_{cmax} estimation.

	NM	AM
A_N (µmol CO_2 m^{-2} s^{-1})	15.19 ± 0.80	20.42 ± 1.25 *
g_s (mol H_2O m^{-2} s^{-1})	0.252 ± 0.03	0.364 ± 0.029 *
g_{sc} (mol CO_2 m^{-2} s^{-1})	0.157 ± 0.019	0.228 ± 0.018 *
A_N / g_s CO_2 (µmol mol^{-1})	112.8 ± 9.3	95.6 ± 6.3
g_m Harley (mol CO_2 m^{-2} s^{-1})	0.114 ± 0.011	0.161 ± 0.023
C_i (µmol mol^{-1} air)	254 ± 9	264 ± 6
C_c (µmol mol^{-1} air)	109 ± 7	120 ± 8
V_{c,max_Cc} (µmol m^{-2} s^{-1})	356 ± 50	397 ± 28
J_{max_Cc} (µmol m^{-2} s^{-1})	152 ± 11	178 ± 6 *
J_{flu} (µmol m^{-2} s^{-1})	161 ± 11	195 ± 5 *
S_L	-	13
MC_L	-	18
B_L	-	4

AM fungi *R. irregularis* and *F. mosseae* have an effect on the yield of the photosynthetic apparatus

Higher stomatal conductance (g_s) and photosynthetic rates were observed in AM plantlets compared to NM plantlets (Table 3). However, mesophyll conductance (g_m) was not significantly different between the AM and NM plants, and both photosynthesis rate (A_N) *vs* CO_2 g_s and A_N *vs* g_m showed linear and significant correlations ($r^2 = 0.8$ $p = 0.0003$; and $r^2 = 0.84$ $p < 0.0001$, respectively).

The A_N-C_c curve analysis showed no differences in the maximum carboxylation capacity of Rubisco (V_{cmax}). However, the AM plantlets showed a higher chloroplastic electron transport rate, (J_{flu}), and a higher maximum activity (J_{max}) compared to NM plantlets (Table 3). Indeed, photosynthesis showed a positive and significant correlation with the electron transport rate (J_{flu} and its maximum activity J_{max} ($r^2 = 0.5207$ $p = 0.0321$ and $r^2 = 0.6248$ $p = 0.0073$, respectively).

The quantitative analysis of photosynthetic limitations showed that the absence of AM symbiosis led to photosynthetic limitation in NM plantlets. Non-AM plantlets displayed 25.68% lower rates of photosynthesis than AM plantlets. The increased rate of photosynthesis in AM plantlets (Table 3) is explained by diminishing stomatal and mesophyll conductance limitations (13% and 18%, respectively). Regarding biochemical limitations (B_L), which were determined independently for V_{cmax} and J_{max}, AM colonization reduced the limitations in these variables by 4% and 5%, respectively.

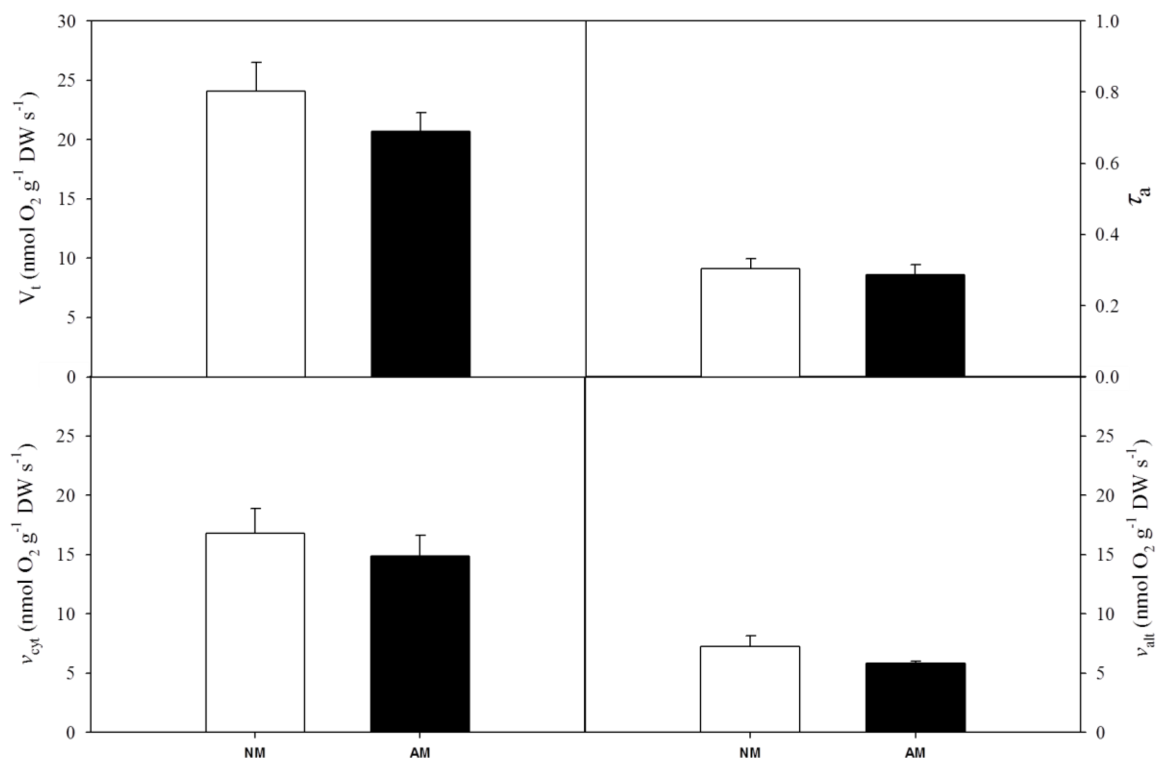

Figure 1. Total respiration (V_t), electron partitioning to the alternative pathway (τ_a), cytochrome pathway activity (v_{cyt}) and alternative pathway activity (v_{alt}) of fully expanded leaves from *A. donax* plantlets without (white bars) or with AM colonization (black bars). Bars represent means ± SE of five replicates.

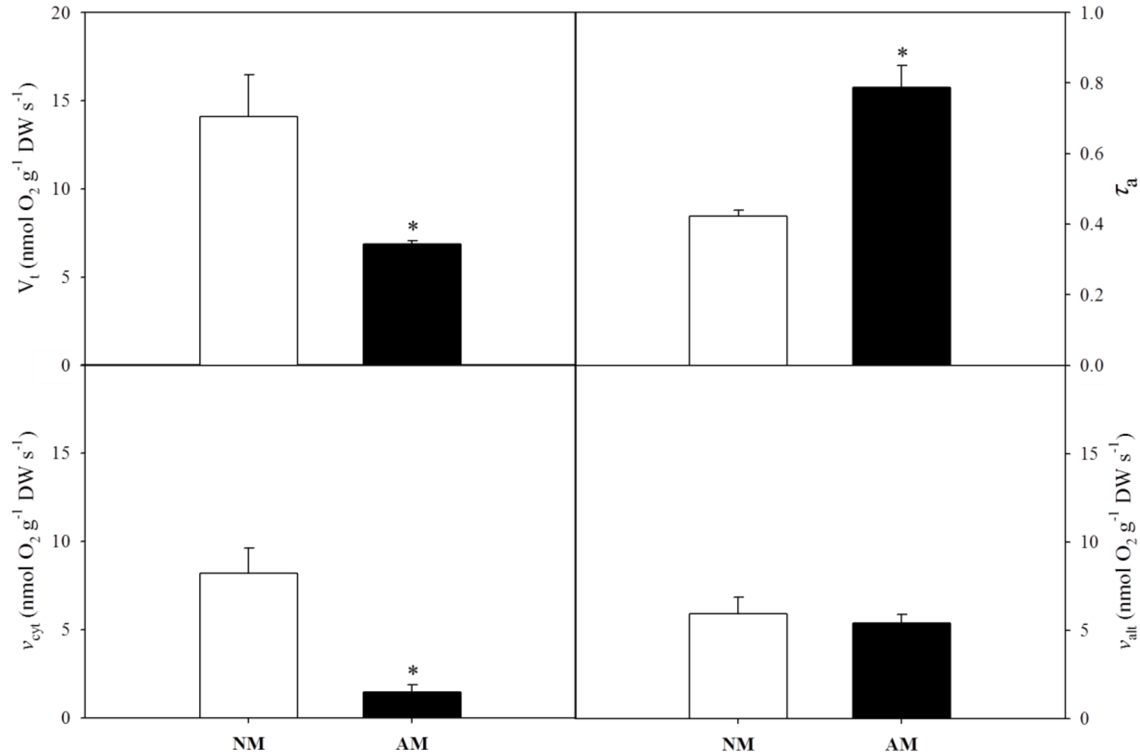

Figure 2. Total respiration (V_t), electron partitioning to the alternative pathway (τ_a), cytochrome pathway activity (v_{cyt}) and alternative pathway activity (v_{alt}) of *A. donax* plantlets roots without (white bars) or with AM colonization (black bars). Bars representmeans ± SE of four replicates, and asterisks denote significant differences between non-AM plantlets (NM) and AM plantlets (AM) (P<0.05).

AM colonization distinctly affects the respiratory activities of leaves and roots

In leaves, AM colonization induced no changes in the total respiration (V_t), cytochrome oxidase (COX) activity (v_{cyt}), or alternative oxidase (AOX) activity (v_{alt}). Furthermore, the electron partitioning through alternative oxidase (τ_a) remained constant, and hence the COX/AOX proportion was maintained in both AM and NM plantlet leaves (Fig. 1). In the roots, AM colonization decreased V_t and v_{cyt} by 51% and 82%, respectively, and v_{alt} remained invariable; consequently, τ_a increased by 88% in AM roots (Fig. 2).

AM colonization induces changes at the metabolite level

AM colonization caused significant changes in the levels of 11 of the 42 analyzed metabolites in leaves compared to NM plantlet levels (Table 4). The levels of malic acid, pyruvic acid, putrescine, aspartic acid and β-alanine decreased significantly by 76,

43, 42, 38 and 29%, respectively, in AM relative to NM plantlets. However, the levels of serine, ornithine, proline, threonine, phosphoric acid and myo-inositol increased significantly by 1.80, 1.71, 1.60, 1.60, 1.48 and 1.23 times, respectively, in AM relative to NM plantlets.

In the roots, AM colonization caused significant changes in 11 of the 42 analyzed metabolite levels when compared to NM roots (Table 4). The levels of sugars (and sugar derivatives) such as maltose, sucrose and glucuronic acid decreased significantly by 45, 39 and 43%, respectively, in AM relative to NM plantlets. Citrate and malate also decreased by 82 and 66%, respectively. The levels of amino acids generally decreased in AM plantlets, for example, asparagine (by 37%), cadaverine (by 56%), cysteine (by 71%), glutamine (by 58%) and glutamate (by 47%). In contrast, ornithine increased 2.25-fold in AM plantlets.

Table 4. Metabolite profiling in leaves and roots of *A. donax* treated with AM inoculation (AM). Plants were harvested for metabolite profiling after 30 min in dark (for details, see the Materials and Methods section). Relative values are expressed as fold changes with the levels in non-AM (NM) plantlets set to unity. Red and blue boxes denote significant increases and decreases, respectively, as assessed by Student's t-test at $p < 0.05$. The data are means ± SE of 4 to 6 replicates.

AM vs NM leaves	
Amino acids	
Alanine	0.96±0.13
Alanine, beta	0.71±0.07
Asparagine	1.09±0.35
Aspartic acid	0.62±0.13
Cysteine	1.35±0.29
Glutamate	0.96±0.03
Glutamine	0.87±0.22
Glycine	0.84±0.13
Lysine	0.83±0.10
Methionine	1.09±0.27
Ornithine	1.71±0.19
Phenylalanine	1.14±0.18
Proline	1.60±0.21
Serine	1.80±0.13
Threonine	1.60±0.15
Tyrosine	0.87±0.06
Tryptophan	0.79±0.16
Valine	0.93±0.09
Organic acids	
Aconitic acid	0.95±0.37
Benzoic acid	0.97±0.13
Butyric acid, 4-amino	0.79±0.20
Citric acid	0.83±0.04
Dehydroascorbic acid	1.00±0.22
Glutaric acid, 2-oxo	1.14±0.15
Lactic acid	0.74±0.09
Malic acid	0.24±0.10
Pyruvic acid	0.57±0.08
Succinic acid	0.77±0.09
Sugars and derivates	
Arabinose	0.86±0.27
Erythritol	0.98±0.13
Galactose	0.82±0.08
Galactinol	1.23±0.36
Glucuronic acid	0.93±0.11
Maltose	0.76±0.05
Palatinose	1.36±0.31
Sucrose	0.99±0.15
Others metabolites	
Glycerol-3-phosphate	0.92±0.20
Inositol, myo	1.23±0.07
Malonic acid, 2-amino	0.85±0.22
Phosphoric acid	1.48±0.13
Putrescine	0.58±0.10
Tyramine	1.28±0.08

AM vs NM roots	
Amino acids	
Alanine	0.93±0.18
Alanine, beta	0.96±0.08
Asparagine	0.67±0.08
Aspartic acid	1.09±0.19
Cadaverine	0.44±0.08
Cysteine	0.29±0.03
Glutamate	0.53±0.07
Glutamine	0.42±0.12
Glycine	1.08±0.10
Isoleucine	1.23±0.21
Lysine	1.03±0.10
Methionine	1.06±0.14
Ornithine	2.25±0.30
Phenylalanine	0.96±0.12
Proline	1.17±0.20
Pyroglutamic acid	0.94±0.12
Serine	0.80±0.09
Threonine	1.11±0.17
Tyrosine	1.05±0.09
Tryptophan	1.01±0.10
Valine	1.03±0.14
Organic acids	
Benzoic acid	0.91±0.32
Butyric acid, 4-amino	0.84±0.12
Citric acid	0.18±0.08
Dehydroascorbic acid	1.18±0.07
Glutaric acid, 2-oxo	0.64±0.32
Lactic acid	0.96±0.22
Malic acid	0.34±0.10
Pyruvic acid	0.95±0.15
Succinic acid	0.87±0.11
Sugars and derivates	
Fructose	0.97±0.27
Galactose	0.71±0.10
Glucuronic acid	0.67±0.05
Maltose	0.55±0.07
Sucrose	0.61±010
Others metabolites	
Benzoic acid, 3-hydroxy	0.91±0.32
Glycerol-3-phosphate	1.00±0.05
Inositol, myo	1.01±0.14
Malonic acid, 2-amino	0.97±0.04
Phosphoric acid	0.90±0.15
Putrescine	1.46±0.11
Tyramine	1.26±0.21

Discussion

The present study analyzes the effects of AM inoculation on growth, photosynthesis and respiration *in vivo* in the same leaves and roots that were used for the metabolite profiling analysis. We show that an increase in photosynthesis and slower root respiratory rate could leave more carbon for biomass accumulation in AM plants. Moreover, the observed differences in the physiological responses at the leaf and root levels coincide with tissue-specific changes in primary metabolites in AM plants.

Arbuscular mycorrhizal colonization increased the total dry biomass of *Arundo donax* plantlets, which is in accordance with previous studies on other species (Hayman & Mosse, 1971; Smith, 1982; Baas *et al*., 1989; Smith & Gianinazzi-Pearson, 1990; Liu *et al*., 2007). In contrast, several reports have shown no effect of AM on biomass accumulation (Smith & Smith, 2011b; Rivero *et al*., 2015) or have even reported a decrease in the biomass of AM plants (Koide *et al*., 1985; Smith & Smith, 2011a, b). Such differences in the effects of AM on plant growth have been related to the phenological stage of the plants (Johnson *et al*., 1997; Klironomos, 2003; Smith & Smith, 2015). Indeed, the cost of the establishment of the symbiosis should be high enough to decrease the accumulation of plant biomass at the early stages of development, such as in seedlings or plantlets (Smith & Smith, 2015). However, the increase in the total dry biomass of AM plantlets observed in this study is in line with previous observations of potted *A. donax* plantlets in symbiosis with *R. irregularis* and *F. mosseae,* in which a stronger positive effect of mycorrhization on biomass accumulation in the first weeks of plant development compared to later stages has been reported (Tauler & Baraza, 2015; Baraza *et al*., 2016).

The higher total biomass observed in AM plantlets was due to higher shoot biomass, with no significant differences in root biomass observed between the treatments.

Moreover, AM did not affect the phenological stage because both AM and NM plantlets were at the vegetative growth stage over the duration of the whole experiment. In accordance with the results of Miller *et al.* (1987), the higher biomass accumulation in the shoots of AM plantlets was due to increased tiller production and an increase in the number of leaves and total leaf area. In contrast and in accordance with previous results in studies of sunflower plantlets (Koide *et al.*, 1985), mycorrhizal colonization did not significantly affect some of the main traits related to biomass accumulation and relative growth rate, such as SLA and LAR, suggesting that mycorrhiza did not largely affect neither biomass partitioning nor leaf anatomy.

Previous reports have linked the increases in biomass caused by AM colonization to higher photosynthetic rates, which were explained by an improved nutrient and water status (Brown & Bethlenfalvay, 1988; Kaschuk *et al.*, 2009; Bárzana *et al.*, 2012; Nazeri *et al.*, 2013). Nevertheless, the present results show that an increased photosynthetic rate can be induced without changes in nutrient concentrations in AM plantlets (Table 2). In fact, the analysis of the quantitative limitations of photosynthesis reveals that AM colonization decreases photosynthetic limitations by improving diffusional rather than biochemical parameters. Diffusional limitation is a combination of stomatal and mesophyll conductance (Galmés *et al.*, 2007), and AM plantlets showed both higher stomatal conductance (g_s) and increases in mesophyll conductance (g_m). Several studies have reported g_s increases in AM plants (Wu & Xia 2006, Bárzana *et al.*, 2012) as a consequence of higher water availability in AM than in NM plants. However, no previous data on the effects of AM on g_m have been reported. Our results show a direct correlation between g_m and A_N, suggesting a key role of this parameter on the photosynthetic improvement in AM plantlets. Moreover, aquaporin expression has been shown to be induced in the roots of several species after AM colonization (Ruiz-

Lozano & Aroca, 2010; Bárzana *et al*., 2014). Therefore, the observed g_m increases in mycorrhizal plantlets may be triggered by aquaporin expression, which may contribute to regulate rapid variations in response to environmental changes. Despite some anatomical traits, such as cell wall thickness and leaf thickness and density may largely determine g_m (Flexas *et al*. 2012), specific leaf area was not affected by AM colonization in our plants, suggesting that those parameters are not the responsible of g_m increase in *A. donax* plantlets. However, further studies are needed to clarify the effect of mycorrhizas in aquaporin content and cell wall composition and their relationship with g_m increase.

The effects of AM on photosynthesis have been shown to be not only based on diffusion parameters but also on the electron transport rate (J_{flu}) and the potential light saturated electron transport rate (J_{max}). No previous data on the relationship between AM and the biochemical parameters of photosynthesis have been reported. Certainly, this is an area of research that deserves more attention.

The increase in photosynthesis in AM plantlets has also been related to an increased demand for the carbohydrates supplied to the fungus (Hughes *et al*., 2008; Kaschuk *et al*., 2009). Similarly, an increase in carbon flow through the symbiont to the belowground ecosystem has been directly related to fast respiratory rates of AM plants (Baas *et al.,* 1989; Hodge, 1996; Johnson *et al.,* 2002; Atkin *et al.,* 2009). In the present study, root respiration was quantified in NM and AM plantlets of *Arundo donax* using the oxygen isotope fractionation technique to determine not only the total respiration but also the *in vivo* activities of the cytochrome (COP) and alternative (AOP) respiratory pathways. Based on previous studies (Wright *et al.,* 1998; Douds *et al*., 2000; Graham, 2000), root respiration was expected to increase in the roots of AM plantlets. However, root respiration in the colonized *A. donax* plantlets decreased,

mainly due to a decrease in COX activity (v_{cyt}), while leaf respiration was unaffected. Moreover, root metabolism was affected by AM colonization in agreement with previous studies (Schliemann *et al*., 2008; Laparre *et al*., 2014; Rivero *et al*., 2015). Florez-Sarasa *et al*. (2012) related several metabolites with the *in vivo* activities of the two respiratory pathways in leaves, but this relationship had not been previously studied at the root level. In our study, AM colonization caused a decrease in the root levels of sugars, organic acids and amino acids, which could explain the low respiratory rate, especially of the COP. Such a decrease in sugars could affect glycolysis and consequently reduce the levels of organic acids in the TCA cycle (citrate and malate) and those derived from amino acids (asparagine, cadaverine, cysteine, glutamate and glutamine). Indeed, previous studies have shown that v_{cyt} in roots decreases together with the sugar content (Millenaar *et al*., 2000). However, the decrease in v_{cyt} can be related to a reduction in the ATP demand caused by the effects of AM on nutrient transport. Watts-Williams *et al.* (2015) recently reported a decrease in the direct pathway uptake activity of nutrient absorption in AM-colonized *Medicago truncatula* roots, whose in turn use the mycorrhizal nutrient uptake pathway (Smith *et al*., 2004). In addition, the decreased levels of citrate and malate observed in the present study (Table 4) may also indicate the reduced exudation of these carboxylates as previously observed (Ryan *et al*., 2012; Nazeri *et al*., 2013), which synthesis was related to AOP activity in cluster roots (Florez-Sarasa *et al*., 2014).

Although AM are morphologically restricted to the roots, AM colonization has been previously shown to affect the metabolic profiles of both roots and shoots (Fester *et al*., 2011). AM also affected the metabolite profile of leaves, reducing the levels of pyruvic, malic and aspartic acids. A similar decrease in these organic acids from the central catabolic pathways was also reported by Fester *et al.* (2011) in leaves of AM-colonized

Lotus japonicus plants. The observed decrease in organic acids could be due to an enhancement of leaf amino acid metabolism (Sweetlove *et al*., 2010). Indeed, the leaf metabolite profiles of AM plantlets showed a significant increase in serine, proline, ornithine and threonine. As previously proposed, the synthesis of several amino acids triggered by AM colonization could be related to the increased synthesis of secondary metabolites (Fester *et al*., 2011; Rivero *et al*., 2015). However, AM leaves showed lower malate levels and higher stomatal conductance. Malate and other organic acids have been shown to be related to stomatal control in TCA cycle-modified tomato plants (Araújo *et al*., 2011 and references therein) and in multiple species in general, based on a meta-analysis approach (Gago *et al*., 2016). In terms of leaf respiration, Florez-Sarasa *et al*. (2012) also showed that organic acids such as aconitic, dehydroascorbic, glutamic, 2-oxo-glutaric and lactic acids were significantly correlated with changes in leaf respiration in *Arabidopsis* plants. The reported changes in metabolic profile as a consequence of mycorrhizal colonization and their relationship with the observed physiological changes would suggest that the effects of mycorrhizas on plant performance are not only due to eventual increase in water and nutrient availability but also to deep biochemical changes in colonized plants.

Conclusions

This study provides relevant data to improve the knowledge about the effects of mycorrhiza on plants. Our results show that the higher biomass accumulation observed in AM plantlets of *A. donax* can not only be associated to increases in photosynthesis but also to a decrease in root respiratory rates. Photosynthesis increased mainly through both lowered diffusion limitations at the stomatal and mesophyll levels and increased light absorption, especially J_{max} and J_{flu}.

Root respiration mainly decreased in AM plantlets primarily due to a decrease in v_{cyt} in parallel with reductions in the metabolites related to respiration. The present research shows the importance of AM colonization in the *in vivo* mitochondrial electron partitioning in roots, and thus, the oxygen isotope fractionation technique in combination with metabolite profiling are presented as new approaches to elucidate the "black-box" (quoting Cameron *et al.*, 2008) that represents the respiratory behavior of the root-fungus matrix.

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Author contribution

A.R-M. and N.F-dS. conceived the research plan and designed the experiments. Performed all the respiration and the photosynthesis measurements and sampling for metabolic analyses. E.B. supervised the arbuscular mycorrhiza inoculation and the colonization assessment performed by A.R.-M.

J.F. and J.G. supervised the photosynthesis experiments and discussed the data. M.R.-C supervised the respiration experiments and discussed the data.

N.F-dS and I.F.S performed the GC-MS metabolite profiling analysis and data generated was analyzed by N.F-dS together with I.F.S and A.R.F.

A.R-M. and N.F-dS. wrote the manuscript.

E.B., J.F. M.R-C and J.G. assisted with the writing of the manuscript. All authors read and approved the final manuscript after critical revision.

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Suplementary information (SI) Chapter 2

Supporting Information

Arbuscular mycorrhizal symbiosis with *Arundo donax* decreases root respiration and increases both photosynthesis and plant biomass accumulation

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The following Supporting Information is available for this article:

Methods S1 Validation of method to estimate g_m, V_{cmax} and J_{max}

In order to deal actual controversial on mesophyll conductance modelling, and consequently biochemical photosynthetic parameters which are estimated from chloroplast CO_2 concentration, V_{cmax} and J_{max}, two independent models were used: by gas exchange and chlorophyll fluorescence (combined Harley method and The Farquhar, von Caemmerer & Berry (1980)) and estimation by curve-fitting model, using Ethier model modified by Buckley & Diaz-Espejo (2015). Estimations of g_m AMp and non-AMp were performed using the method of Harley *et al*. (1992), as follows: $g_m = A_N /(C_i - (\Gamma^* (J_{flu} + 8(A_N + R_l)) /(J_{flu} - 4(A_N + R_l))))$ (1) where A_N (net CO_2 assimilation) and C_i (substomatal CO_2 concentration) were taken from gas- exchange measurements at saturating light, whereas Γ^* (the chloroplastic CO_2 photocompensation point in the absence of mitochondrial respiration) was estimated for *Arundo donax* using Savoir (2009) equations, following Galmés personal comments. R_n (the respiration in night) was measured as described above, and was used to estimate R_l using the Laisk (1977) method, following the methodology described in Flexas *et al*. (2007c). Although this method allows the calculation of g_m across the full range of CO_2 concentrations, we decide to apply a constant g_m value, which was the g_m value obtained at 400 μmol CO_2 mol^{-1} air. This value of g_m (specifically calculated per each plantlet of each treatment), was used to convert A_N–C_i into A_N–C_c curves (where C_c is

the chloroplastic CO_2 concentration) using the equation $C_c = C_i - (A_N / g_m)$. The maximum carboxylation and J_{flu} capacities ($V_{c,max}$ and J_{max}, respectively) were calculated from the A_N–C_c curves, using Rubisco kinetic constants and the temperature dependence of Rubisco kinetic parameters described on a C_c basis by Bernacchi *et al*. (2002), following Peguero-Pina *et al*., (2012) methodology. The Farquhar *et al*. (1980) model was fitted to the data by applying iterative curve-fitting (minimum leastsquare difference) using Microsoft Excel Solver tool. The second one, is an adjust of A-C_i curves based on Ethier & Livingston method (2004), proposed by Buckley & Diaz-Espejo (2015), modified for not deciding a priori A_N limited by Rubisco-substrate dependence (A_c) and A_N limited by RuBP recycling velocity of light limitation (A_q) regions following Manter and Kerrigan (2004) and Dubois (2007). In addition of teorical and mathematical differences with the first model described before, (see Peguero-Pina *et al*., 2012), the principal new approach is that this model is obtained g_m, $V_{c,\,max}$ and J_{max} from reformuled equation of Farquhar, von Caemmerer & Berry (1980), integrated in the same equation, both three photosynthesis restricted estimations (Ethier *et al*., 2006) . In fact, this model is based on fitting $A_N - C_i$ curves with a nonrectangular hyperbola version of Farquhar's biochemical model of leaf photosynthesis (Farquhar *et al*. 1980). Thus, this method allows the calculation of the three parameters across the full range of CO_2 concentrations, which we have fitted an average g_m value over a C_i range (for mathematical and theoretical cross information, review Buckley & Diaz-Espejo, 2015). Positive correlation among values of g_m calculated by Harley and calculated by Ethier (Fig. 1) denoted robustness of both models. As a result of that correlation, Farquhar, von Caemmerer & Berry (1980) model were used to estimate $V_{c,max}$ and J_{max} (Table 3).

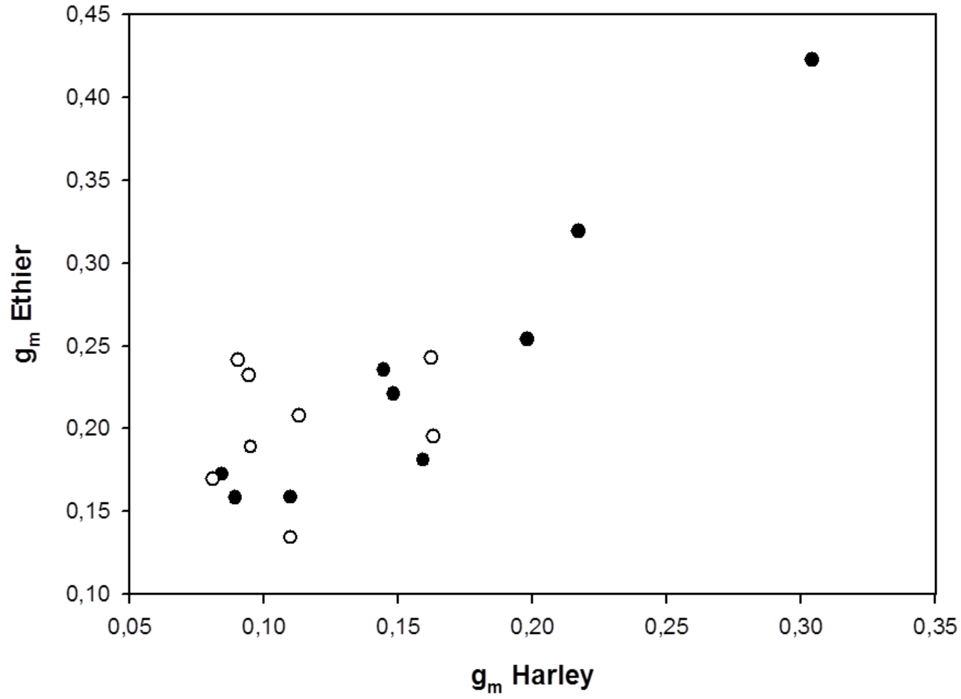

Fig. S1 Pairwise correlation among g_m values obtained by Harley and Ethier equations ($r^2 = 0.8476$ $p < 0.0001$).Values obtained per each plantlet and treatment are represented, non-AM plantlets (white circles) and AM plantlets (black circles).

Chapter 3

Arbuscular Mycorrhizal Fungi Confer Salt Tolerance in Giant Reed (*Arundo donax* L.) Plants Grown Under Low Phosphorus by Reducing Leaf Na^+ Concentration and Improving Phosphorus Use Efficiency

Arbuscular mycorrhizal fungi confer salt tolerance in giant reed (*Arundo donax* L.) plants grown under low phosphorus by reducing leaf Na^+ concentration and improving phosphorus use efficiency

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Abstract

To avert food crop displacement from fertile agricultural soils, second-generation energy crops are produced on marginal lands. In these barren soils, yields are lower and the potential for yield improvement in the near future limited. Salinization is worldwide one of the most important degradation processes decreasing soil quality. Arbuscular mycorrhizal fungi (AMF) as components of soil-plant systems ameliorate the plant's response to salinity and have beneficial effects on plant growth and yield. In this study, the relative importance of the inoculation with a commercial AMF in the response of *Arundo donax* plantlets to salt stress under limited phosphorus availability has been investigated. AMF-inoculated (AM) and non-inoculated plantlets of *A. donax* were grown at two P (0.25 and 0.025 mM) and three NaCl (1, 75 and 150 mM) concentrations in a room chamber under controlled conditions. After 5 weeks, AMF root colonization was 60, 26 and 15% in 1, 75 and 150 mM NaCl-treated plants, respectively. At 75 mM NaCl, AM plants showed increased growth, decreased Na^+ uptake and Na^+ root-to-shoot translocation and increased P use efficiency. Moreover, despite no differences in plant biomass were found under 150 mM NaCl, AM plantlets showed higher total leaf biomass and the higher phosphorus use efficiency was maintained. The possible mechanisms involved in the response of AM plants to combine low P - high salinity stress, as well as the role of AMF inoculation to ameliorate of *A. donax* as a second-generation energy crop are discussed.

key words: early stage; osmotic potential; phosphorus scarcity; salinity tolerance.

Introduction

Second-generation biofuels mainly developed in the second half of the 2000s in response to social concerns over environmental and food security issues raised by the first-generation biofuels. The second-generation energy crops include both woody and herbaceous plant species unsuitable for consumption by humans or animals (Mohr & Raman, 2013). To ensure a more sustainable used of agricultural soils and to prevent the displacement of food crops, second-generation energy crops can be grown on marginal lands, abandoned or unsuitable for food production. However, marginal lands have lower productivity and, regrettably, in the near future, the potential to breed energy crops for yield improvement when grown on barren soils is quite limited (Allen *et al.*, 2013).

Salinity is one of the most damaging degradation processes affecting soils, especially in arid and semi-arid regions, where salinization is considered a major cause of soil desertification. According to the FAO Soils Portal, globally, salinity affects 19.5 % of irrigated and 2.1 % of dry agricultural lands.

Cultivation of adequate energy crops on salinized soils, in addition to biomass production, have an important restoration potential improving amongst other carbon sequestration and soil structure. However, soil salinization negatively affects plant growth and yield. The high salt concentration in the soil solution decreases the soil osmotic potential that may result in loss of cell turgor in species unable to regulate their water potential. Additionally, the excess of ions, principally Na^+ and Cl^-, negatively affect plant metabolism by inducing ion toxicity or/and ion imbalance in plant tissues (Marschner, 2011). Nonetheless, plants have evolved multiple mechanisms to cope with salt stress that principally involve from control of water and ion homeostasis as well as Na^+ exclusion from the shoot and Na^+ tissue tolerance, until triggering the mechanisms to scavenge toxic compounds (Hasegawa *et al.*, 2000; Munns & Tester, 2008).

As components of soil-plant natural ecosystems, AMF can greatly ameliorate the plant's response to salinity and have beneficial effects on plant growth and yield, which made AMF suitable candidates to bio-ameliorate salinized soils (reviewed by Evelin et al. 2009). Although AMF are present in saline soils (Landwehr *et al.*, 2002), the osmotic and toxic effects of excess of salt also affect fungus growth, in a similar way as plants (Juniper & Abbott, 2006). Nonetheless, large variation in the response of AMF to

salinity dependent on species and isolates has been reported (Tian *et al.*, 2004; Juniper & Abbott, 2006; Zou & Wu, 2011).

Simple passive as well as complex active mechanisms participate in the AMF-mediated growth enhancement in salt-stressed plants. AMF improved plant water status by a decrease in the matrix potential (Rodríguez-Echeverría *et al.*, 2008), but AMF also regulated plant aquaporins increasing root hydraulic conductivity (Ruiz-Lozano & Aroca, 2010). Salt-stressed AMF-inoculated plants showed higher stomatal conductance and transpiration rate (Jahromi *et al.*, 2008). Different studies reported that the positive growth response in salt-stressed AMF-inoculated plants was due to an AMF-mediated amelioration of nutrient acquisition, especially phosphorous, as under saline conditions Pi absorption is greatly reduced (Shokri & Maadi, 2009). AMF-inoculation was also reported to reduce Na^+ uptake and translocation while favored the uptake of essential cations such as K^+, Ca^{2+} and Mg^{2+} and increased the K^+/Na^+ and Mg^{2+}/Na^+ ratios in shoots (Giri *et al.*, 2003; Giri & Mukerji, 2004; Colla *et al.*, 2008).

Arundo donax L. (giant reed) is one of the most promising species for energy, cellulose paste and second-generation biofuel production because its high biomass productivity (Hidalgo and Fernandez 2001; Shatalov and Pereira 2001; Lewandowski *et al.* 2003). This fast-growing perennial grass has also been highlighted as a promising crop for lignocellulosic biomass production in salinized soils (Sánchez *et al.*, 2016 and references herewith). Moreover, giant reed has also been reported to be an environmentally sustainable, low-cost, low-maintenance crop with very low fertilizer requirements (Lewandowski et al. 2003). However, to grow giant reed in salinized soils could affect its biomass quality for biofuel production. Reduced biomass quality would be principally related to the release of inorganic elements, amongst others K, Ca, Si and P, from the fuel to the gas phase during the biomass combustion process to generate heat and power (Novakovic *et al*. 2009). It is especially recommended that the ratios amongst K, Ca and Si should be taken into consideration when biomass quality is evaluated (Novakovic *et al*. 2009).

On the other hand, there are some bottlenecks regarding giant reed physiology and cultivation. Although due to the lack of viable seeds, in nature giant reed is principally propagated through rhizomes, plantlets obtained through micro-propagation of embryogenic callus are nowadays used for large-scale cultivation. As a consequence the

establishment and success of giant reed plantlets can be costly and challenging especially in marginal soils and more data on how plantlet establishment is affected by, amongst others, fertility factors are needed. In this line, the early inoculation of giant reed plantlets with AMF has been propose as an useful strategy to improve field establishment and first year crop production as well as improving plant tolerance to marginal lands (Baraza et al. 2016). Moreover, at long term, the ability of giant reed to establish symbiosis with AMF, could result in high productive crops (Romero-Munar *et al*. subbmited) and confer new physiological traits (Romero-Munar *et al*. 2017).

In this work the effect of AMF on the growth and biomass allocation, water relations, nutrient use efficiency and ion concentration of *A. donax* grown at different salinity regimes and phosphorous concentrations were tested. A commercial clone of *A. donax* and a commercial mycorrhiza fungi inoculum mix of *Funneliformis mosseae* and *Rhizophagus irregularis* were used as a model of usual and generalist organisms. On one hand, *A. donax* K12 is a usual commercial clone multiplied by micropropagation. On the other hand, both, *F. mosseae* and *R. irregularis* are generalist species with higher richness in all soil types. The role of AMF inoculation on this species potential for the restoration of salinized soils as well as on its quality for lignocellulosic biomass production was also assessed.

Material and Methods

Plant and fungi material

Fifty four plantlets of *Arundo donax* K12 clone (micropropagated plants provided by Biothek Ecologic Fuel S.L.) were received bare-root and immediately planted on trays of agricultural substrate (previously tindalized for excluding other microorganisms present in the peat) which mostly consisted of nutrient-rich black peat (Kekkilä DSM 1 W, pH 5.9, 90% of organic material. Principal compounds: Sphagnum peat; additives: $N\text{-}P_2O_5\text{-}K_2O$ (16-4-17, 0.60 g l^{-1}), wetting agent (0.10 g l^{-1}) and dolomite limestone (5.0 g l^{-1})).

One-week old plants were transplanted in sterilized silicic sand on 4L pots. Eighteen plantlets were inoculated in the transplanted moment with 5 mL (5 g aprox.) of commercial inoculum (AEGIS SYM®) which contained two different arbuscular mycorrhizal fungi (AMF) species, *Rhizophagus irregularis* (25 spores per g) and

Funneliformis mosseae (25 spores per g) (Schüssler & Walker, 2010). Inoculated plantlets were termed AM plantlets. The rest 36 non-inoculated plants were supplied with 5 mL (5 g aprox.) of autoclaved inoculum plus 3 mL of an inoculum filtrate (< 20 µm) to provide a general microbial population, free of AM propagules (Barzana *et al.* 2012).

Plants were grown for three months in growth room under controlled conditions at 25/20ºC day/night temperature, above 40% relative humidity and 12 h photoperiod (300 µmol m^{-2} s^{-1} of photosynthetic photon flux density, PPFD) and regularly irrigated with deionized water.

Treatments establishment

Pots were kept at field capacity by watering the plants with 25% modified Hoagland nutrient solution with 2.5 µM Pi and 1 mM Na for 7 weeks to allow AM fungi establishment. After AM colonization, two phosphorus and three salinity treatments where set up in a step-wise manner in sextuplicate, resulting in nine treatments: Control, C (non-inoculated plants growing with 2.5 µM P); Phosphorus plantlets, P (non-inoculated plants growing with 25 µM Pi); and arbuscular mycorrhiza plantlets, AM (colonized plants growing with 2.5 µM Pi); each one with the three levels of salt (1, 75 and 150 mM NaCl).

Mycorrhizal development

The percentage of mycorrhizal root colonization was determined by visual observation of fungal colonization. According to Philips and Hayman (1970) roots were washed with 10% KOH and stained with trypan blue (0.05% in lactic acid (v/v)). AM fungal colonization was assessed using the magnified intersections method (Abbott *et al.*, 1984) where the frequency of colonization represents the ratio between fragments of colonized root and the total number of root fragments examined. An average of 300 root pieces per plant and nine plants per treatment were examined. Percentage of mycelium, spores, vesicles, arbuscules and total infection were determined to evaluate salinity effect on the average of these structures.

Biomass measurements

Three-month old plantlets (after 2 month of treatments establishment), six plantlets per treatment of all treatments were processed to allow the total number of leaves, stems and length of the highest stem. Leaves (grouped in young leaves (YL – two leaves from

the top of the stem), mature leaves (ML – third or fourth leaf from the top of the stem) and old leaves (OL – leaves located at the bottom of the stem)), stems and roots (grouped in thick roots (TR – diameter > 5 mm) and fine roots (FR – diameter < 5 mm) were dried separately to obtain them specific dry biomass. Specific and total leaf area were determined (SLA, m^2 g^{-1} and LA, m^2 - respectively). SLA was measured in four fully developed leaves to determine the projected one-sided fresh area using Image J (Wayne Rasband / NIH) program. After drying for 72 h at 70 ºC, dry weight (DW) was measured, and SLA was calculated as the ratio of leaf area to dry mass. Whole-plant LA was calculated as the total leaf dry weight/specific leaf area.

The leaf greenness indices

The leaf greenness index was obtained from SPAD values using a SPAD-502 instrument (that measures the absorbance of a leaf in the red and near-infrared regions instrument). To determine SPAD values 3 measurements of the youngest fully developed leaves of each pot were took and calculated an average SPAD value for each pot at each sample time SPAD-502.

Chlorophyll and leaf protein

After 3 month of treatments establishment, ten borers (diameter=16 mm) per leaf were collected in all plantlets of each treatment. Half of them were dried to allow the dry weight and the rest were processed to determine the pigments and leaf protein concentration after being harvested in liquid nitrogen and kept in freezer at -80 °C until extraction. The leaf chlorophyll was extracted in 96% ethanol following Lichtenthaler and Wellburn (1983). The extract was then spun in a micro centrifuge to precipitate the cell debris. The absorbance (A) of the samples was measured at 665 nm (for chlorophyll a) and 649 nm (for chlorophyll b) by the Ultra Violet to Visible spectrophotometer. Chlorophyll a, chlorophyll b and total chlorophyll content were computed using the following equations (Lichtenthaler & Wellburn, 1983):

$C_a = 13.95A_{665} - 6.88A_{649}$

$C_b = 24.96A_{649} - 7.32A_{665}$

Leaf protein content was determined following Bradford method (1976) and absorbance (A) of the samples was measured at 595 nm.

Leaf osmotic potential

To determine plant osmotic status at the end of the experiment, the leaf osmotic potential was measured in two mature leaves per plant at the end of the experiment. The procedure was as follows: first, leaves removed from the plant were immediately frozen in liquid nitrogen, and then stored at −80 ◦C. Later, the leaves were thawed for 30s and a 10 μL volume of sap was obtained for the determination of solute concentration (Gucci et al., 1991). Leaf osmotic potential was then measured with a Wescor 5500 vapour pressure osmometer (Wescor Inc., Logan, Utah, USA).

Ion tissue concentration

Ion concentration was determined in young, mature and senescent leaves, apical and basal segment stem parts, thick (> 0.5 mm) and fine roots (< 0.5 mm). Dry tissue was finely powdered using an orbital shaker in seal tubes with glass balls. One hundred mg of dried tissue were dry ashes at 550ºC during three hours followed by digestion in 9.2:0.8 0.08M H_2SO_4: 40% HF. The suspension was shaken for 1 h and left overnight at room temperature. Prior analysis, the excess of HF was eliminated with 0.32% H_3BO_3. After filtering, the ion concentration was determined by Inductively Coupled Plasma (ICP) Spectrometry (Perkin-Elmer Plasma-2000, Perkin-Elmer Inc. Norwalk, CA, USA).

Statistical analyses

Two way analysis of variance (ANOVA) was performed to analyze the effect of the two treatment in the main studied parameters: biomass dry weight, leaf physiological parameters and ion tissue concentration. We performed a posteriori T-test to analyze differences among P and AM treatments with the control or among salt levels.

In the case of measures made in different periods General Mix Models were made considering the identity of the plant as a random factor and including time as a fix factor. The analyses were performed using the JMP®, Version 10 (SAS Institute Inc., Cary, NC, 1989-2007).

Results

Table 1. Summary of two-way analysis of variance (ANOVA) for the effects of nutrient treatment (N) and Salt treatment (S), with their interaction factor (N×S) on Bimass, physiological and nutrition- related traits in *Arundo donax* plants. *Values correspond to P values*. Abbreviations as shown in the text. Red numbers (no significance) had been highliht to easily view of the table.

Trait	*N*	*S*	*NxS*
Total Biomass	< 0.0001	< 0.0001	0.0025
Leaf Biomass	< 0.0001	< 0.0001	0.4504
Root Biomass	< 0.0001	< 0.0001	0.0005
LA (cm^2)	0.0053	< 0.0001	0.5157
Stems (number)/root (g)	0.3678	0.0023	0.0011
$\Psi\pi$	0.3700	< 0.0001	0.2468
SPAD	0.0135	0.0024	0.0155
Leaf protein	0.4119	0.7362	0.0011
Chla	0.4049	0.0975	0.0074

Trait	*N*	*S*	*NxS*
Na	0.0001	< 0.0001	0.0043
K	0.4642	0.0032	0.0842
Na/K (leaf)	0.1221	< 0.0001	0.0297
P_i	< 0.0001	0.3232	0.0165
Mg^{2+}	0.4631	0.0723	0.5191
Ca^{2+}	0.2670	0.0489	0.8620
Si	0.0715	0.3612	0.9733
KUE	0.0004	0.0003	0.0146
MgUE	0.2418	0.0091	0.1976
CaUE	0.2332	0.8814	0.7825
SiUE	0.4385	0.1014	0.4896
PUE	0.0022	0.0659	0.5616
PUE_e Leaf	< 0.0001	< 0.0001	0.0264
PUE_e Shoot	< 0.0001	< 0.0001	0.0143
PUE_e Root	< 0.0001	0.0004	0.1323
PUE_i Leaf	< 0.0001	< 0.0001	0.0080
PUE_i Shoot	< 0.0001	< 0.0001	0.0050
PUE_i Root	< 0.0001	< 0.0001	0.0002

Salinity severely decreased AMF growth

The colonization of *Arundo donax* roots by *Rhizophagus irregularis* and *Funneliformis mosseae* reached values of 54% ± 9.98 in non-salinized plants. Root colonization was greatly reduced by salinity, with values of 23.50% ± 6.22 and 12.29% ± 4.33 in 75 and 150 mM NaCl-treated plants, respectively (Fig. 1). Salinity also induced differences in the presence of AMF structures, especially arbuscules and vesicles. The amount of arbuscules was highly reduced (47%) at moderate salt stress (75 mM NaCl), while it greatly increased (59%) at severe salt stress (150 mM NaCl) with respect non-salinized roots.

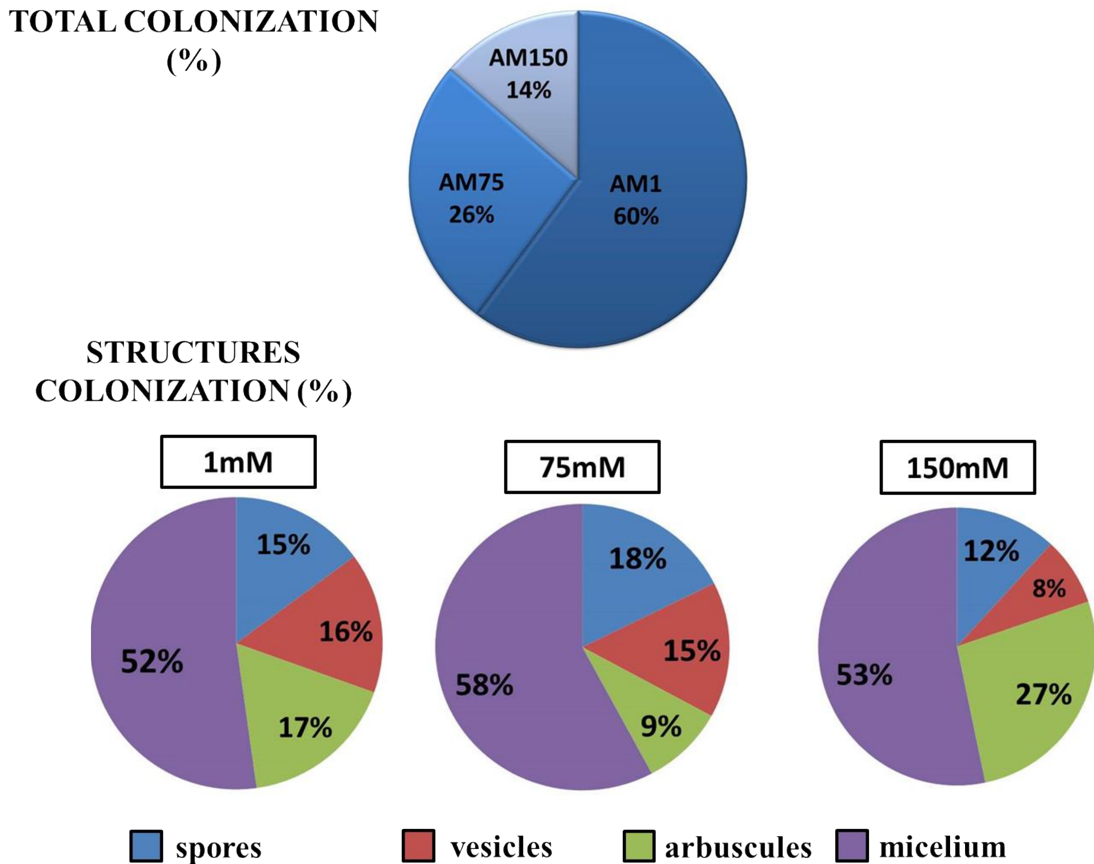

Figure 1. Root total colonization and AMF structures percentages found in AMF-inoculated plantlets grown at different salt concentrations.

AMF increased plant growth in non-salinized and moderately salinized plantlets

Plant biomass and its distribution were significantly affect by both factors (nutrition, N factor - C, P and AM - and salinity treatments, S factor – 1, 75 and 150 mM NaCl), and also by their interaction (Table 1).

In non-salinized plantlets, phosphorus addition increase total plant dry weight (DW) by 11% compared with C plants due to higher root rather than shoot biomass (Table S1 and Fig. 2A). AM plantlets produced 41% more biomass than the C ones (Fig. 2A) due to increased stem (Table S1), leaves and root DW (Fig. 2B and C respectively). Under mild salinity (75 mM NaCl), the positive AM effect on plant growth was maintained, and AM plantlets showed a 14% higher total dry biomass, while the positive effect of phosphorus was revoked by salt. No differences on total biomass production between C, P and AM plantlets grown at 150 mM NaCl were found (SI Table 1, Fig. 2A).

Phosphorus deprivation did not trigger root production in C plants, whilst AM plantlets showed increased root biomass at 1 mM NaCl (Fig 2C) and higher root-to-shoot ratio (0.49 ± 0.05 and 0.74 ± 0.06, respectively, $p = 0.0013$ – as significance value of N

factor for root-to-shoot variable). In non-salinized plantlets, P addition also increased root-to-shoot ratio compared with C (P plants root-to-shoot ratio 0.64 ± 0.03, $p = 0.0013$). C, P and AM plantlets showed the same leaf number, however C plantlets had a higher number of stems compared with the other two treatments (SI Table 1, Fig. 2E).
Regarding to stems production (number) and root biomass investment ratio, C plants presented a different behavior in comparison with AM and P plantlets; while C plants decreased the ratio number of stems number / root biomass along salinity increment, AM and P plantlets increased it (Fig. 1E).
No differences in biomass distribution parameters such as specific leaf area (SLA), leaf area ratio (LAR) and leaf mass ratio (LMR) between treatments were found (Table SI 2). Nonetheless, AM plantlets showed statistically higher total leaf area (LA, cm^2) than P and C plantlets at 1 mM NaCl ($p < 0.05$). Those significant differences disappeared at 75 and 150 mM NaCl (Fig. 2D). This resulted from the significant effect on LA of both, nutrient treatment and salinity; however, their interaction was not significant allowing any differences under salinity stress conditions between C, P and AM plantlets (Table1).

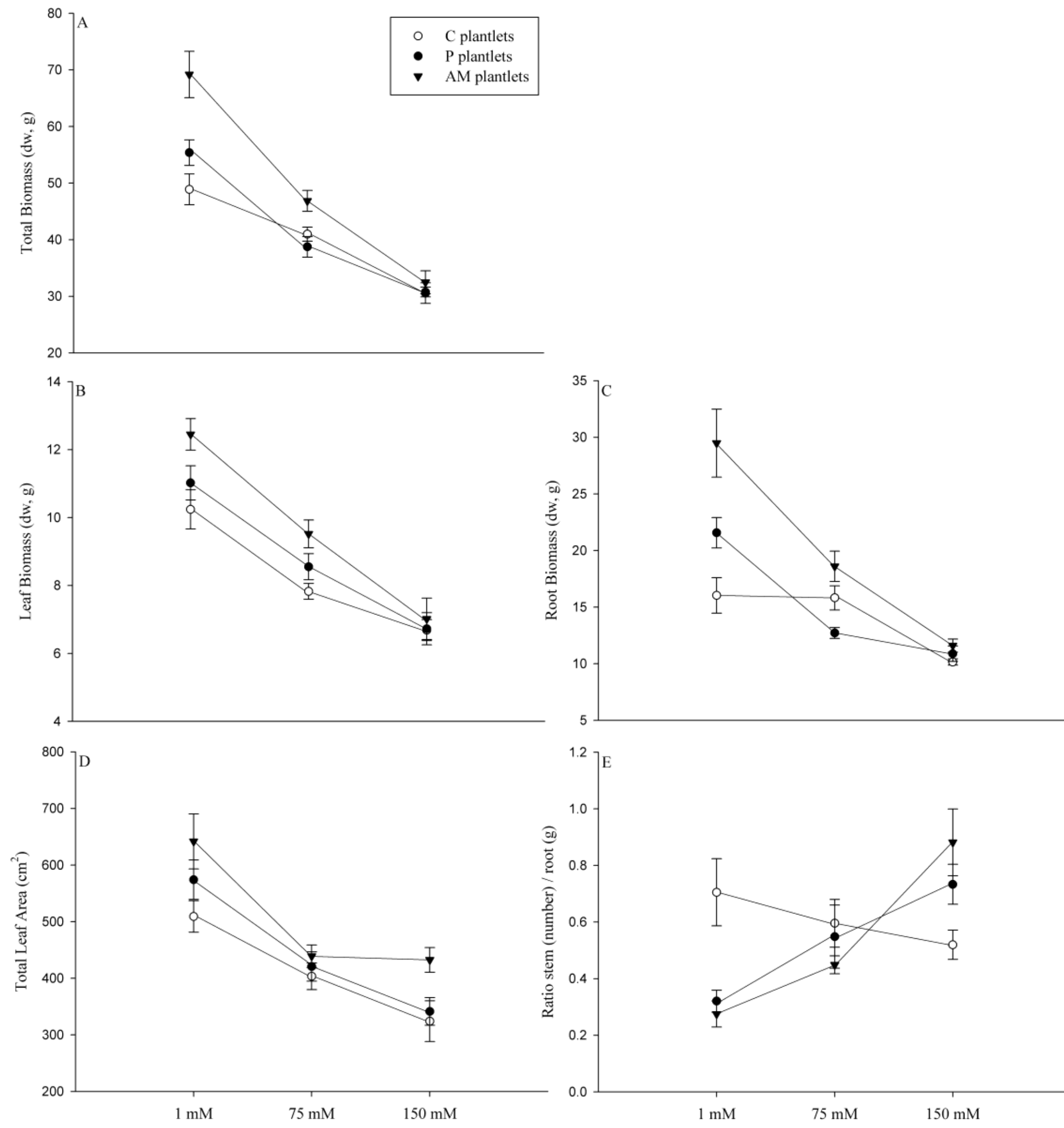

Figure 2. Total biomass dry weight (dw) (A), leaf and root biomass dw (B and C, respectively), total leaf area (D) and the ratio of number of stems per root dw (E) of control plantlets (C, white circle), phosphorus plantlets (P plantlets, blank circle) and arbuscular mycorrhiza plantlets (AM, black triangle) under 1, 75 and 150 mM NaCl. Values are mean ± SE of six replicates. Statistical analysis are given in Table 1.

Physiological leaf responses of C, P and AM plantlets to different NaCl concentrations

Leaf physiological responses of C, P and AM plantlets under 1, 75 and 150 mM NaCl are showed in Figures 3 and 4. Osmotic potential was significantly affected by salinity treatment but not by nutritional factor and by factor interaction neither (Table 1). Analyzing the values obtained in the post-hoc T-test we discuss the behavior of C, P and AM plants at each salt level separately. Under non-salinized conditions, C plantlets showed statistically significant more negative osmotic potential values than P and AM plantlets, while under salt stress conditions, osmotic potential decreased with no differences among treatments (Fig. 3A). Regarding the leaf protein concentration (Fig. 3B), only the interaction between both factors (NxS) was significant (Table 1). AM and P plantlets showed lower leaf protein with respect C plantlets (post-hoc T-test) under non-salinized conditions. Despite the decrease observed on leaf protein in C and the increase in P and AM plantlets under moderate and severe stress, the differences were not significant.

SPAD was significantly affected by both factors and their interaction (Table 1). Post-hoc T-test showed no differences among C, P and AM plantlets at 1 mM NaCl (Fig. 3C), but at 75 mM NaCl, SPAD values were higher in AM plantlets in comparison to C and P plantlets. At 150 mM no differences were observed among C, P and AM plantlets.

Chlorophyll a was significantly affected by the interaction of both factors, but not by each factor separately (Table 1). Analyzing C, P and AM plantlets under each salinity treatment with the T-test values, AM and P plantlets showed higher chlorophyll a (Chl a) concentration under non-salinized conditions (Fig. 3D), although the differences were only significant between C and AM plantlets. Moderated stress did not affect Chl a in C and P plantlets, whilst AM plantlets showed the lowest concentration (post-hoc T-test). No differences in Chl a were found among C, P and AM plantlets at 150 mM NaCl.

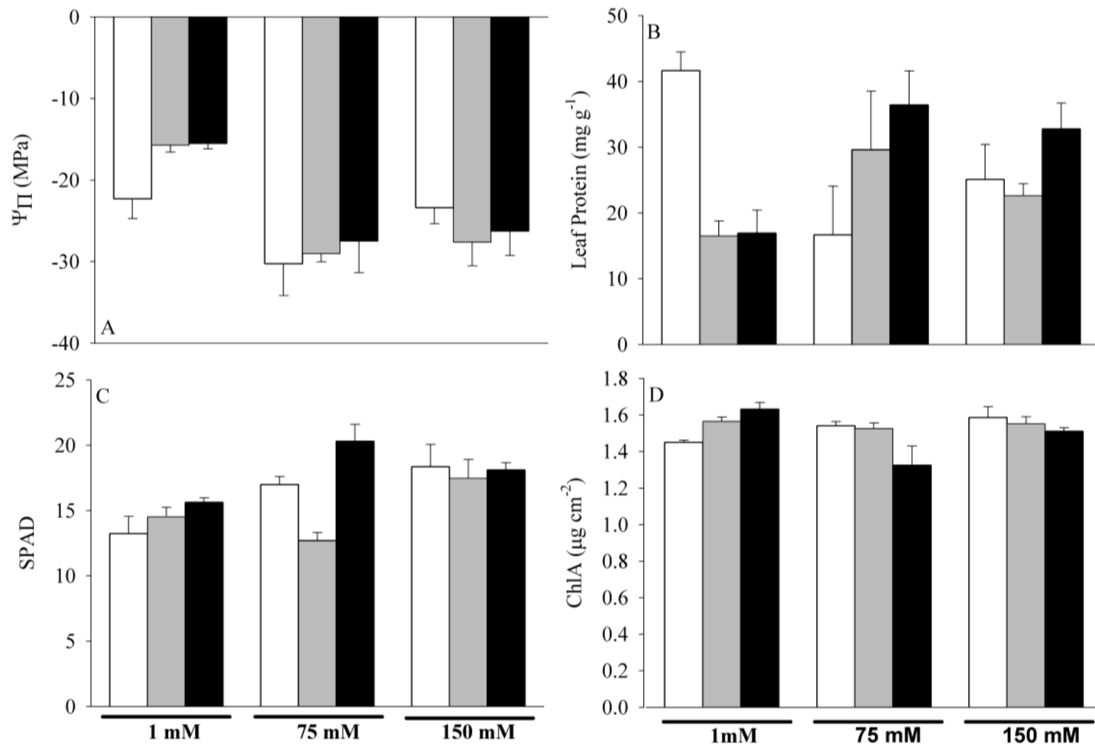

Figure 3. Leaf physiological traits (osmotic potential (Ψ_π), A; Leaf protein, B; SPAD, C; and Chlorophyll a concentration (Chl a), D) of C, P and AM plantlets (white, grey and black bars, respectively) at 1, 75 and 150 mM NaCl. Values are mean and SE of six replicates. See statistical analysis in table 1.

AMF modulated salinity and phosphorus scarcity effects on ion uptake and allocation

Na^+ concentration and distribution

The Na^+ and K^+ concentration in different organs of C, P and AM plantlets is shown in Figure 4. Sodium concentration decreased in the order root > stem > leaf in all treatments (Fig. 4A, B, C). AMF inoculation or supplemental P did not affect root Na^+ concentrations that reached similar values of about 2% on dry weight basis in plantlets exposed to either 75 or 150 mM NaCl. However, leaf Na^+ concentration increased with Na^+ supply reaching the highest values in plantlets treated with 150 mM NaCl ($p < 0.0001$). In both salinity treatments, AM plantlets showed a higher Na^+ exclusion capacity and lower leaf Na^+ concentrations than the corresponding non-inoculated ones (ANOVA one way $p = 0.0049$ and $p = 0.0280$ at 75 and 150mM NaCl respectively, Fig. 4). Moreover, at plant level, AM plantlets showed lower Na^+ concentration compared with C and P plantlets under sever salt stress conditions (SI Fig. 1A).

*K^+ concentration and distribution*Potassium concentration was not affected for NaCl addition and all plantlets showed the same tissue distribution (Fig. 5D, E, F). The highest concentration was observed at shoot level, particularly in the stem tissue.

Regarding mature leaves Na^+/K^+ ratio (Fig. 5), C showed the highest value compared with P and AM plantlets at 75 mM NaCl (C > P > AM, $p < 0.05$). At 150 mM NaCl Na^+/K^+ P plantlets showed the highest value and significantly different from C and AM plantlets ($p < 0.05$).

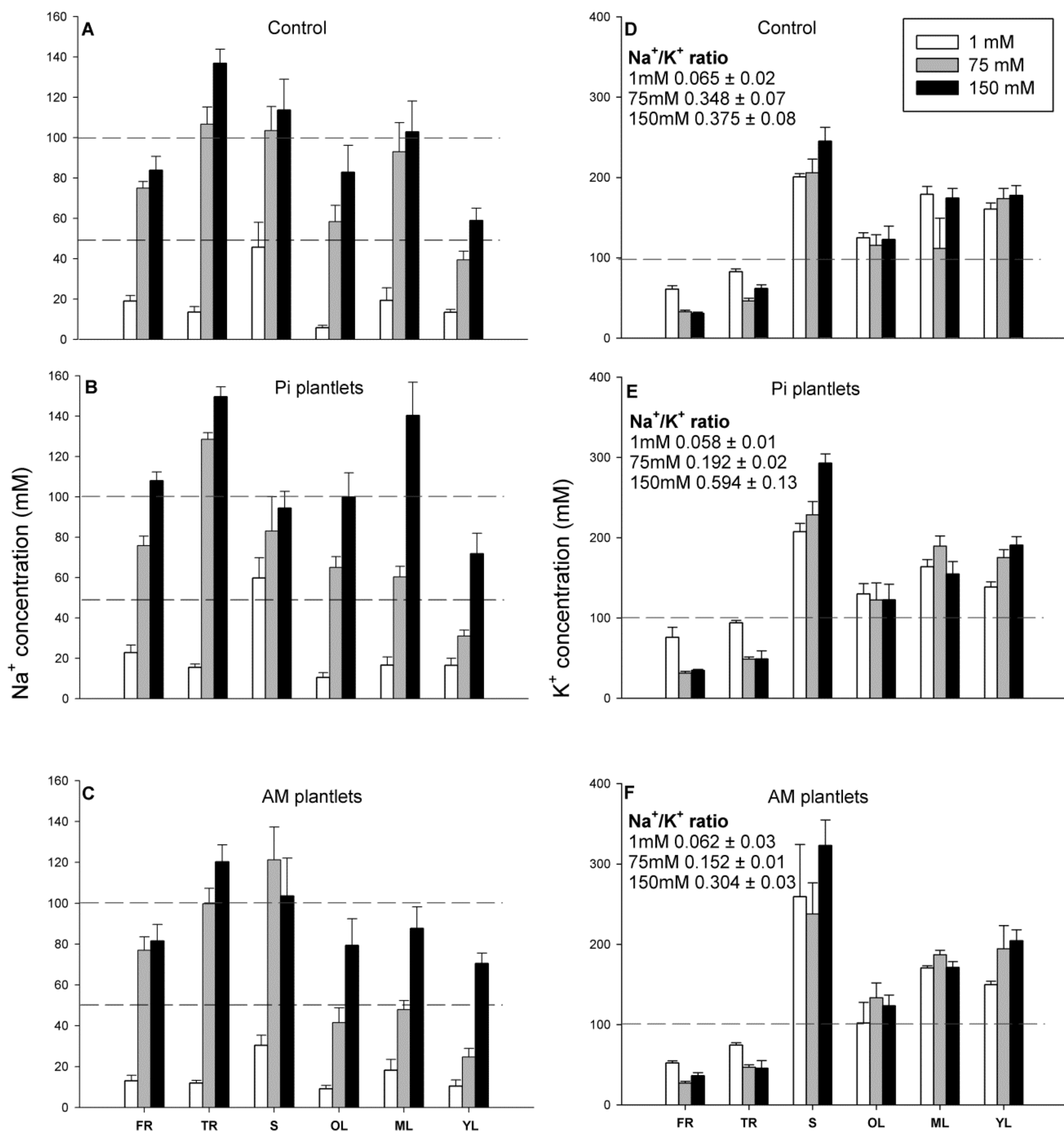

Figure 4. Sodium and potassium concentration (A, B and C show Na^+ concentration, and D, E and F show K^+ concentration) of different tissues (fine root – FR; thick root – TR; stem – S; old leaf – OL; mature leaf – ML and young leaf – YL) of C, P and AM plantlets, at 1, 75 and 150 mM NaCl (white, grey and black bars, respectively). Values of ML Na^+/K^+ ratio are shown. Values are mean and SE of 6 replicates.

P concentration

Total plant phosphorus concentration was significant higher in P than in C and AM plantlets at 1, 75 and 150 mM NaCl ($p = 0.0019$; $p = 0.0002$; $p = 0.0001$ respectively; SI Fig 1C). No differences in P concentration were observed between C and AM plantlets. Salinity stress did not affect P concentrations in any nutritional treatment.

Mg^{2+}, $Ca^{2+}$and Si concentration

No differences were found on Ca^{2+}, $Mg^{2+}$and Si total plant concentration among different nutritional or salt treatments (SI Table 2, SI Fig. 1D-F). Regarding to the Ca^{2+} distribution, it detection in the stem was hardly undetectable, highly concentrated in leaves and less in roots, and it was not affected by nutritional and either salinity treatments (Table 1, Table SI 3B and Fig SI 1E).

Statistical analysis showed no significantly effects of N and S factor and their interaction (Table 1) on total plant Mg^{2+} concentration, and all plantlets showed similar values under 1, 75 and 150 mM NaCl (SI Fig. 1D). As it can be expected, Mg^{2+} distribution was hardly in leaves than stem and roots (SI Fig.1D). By contrast, Si distribution was strongly storage in roots, with lower concentration in shoot (SI Fig. 1F). Moreover, no Si plant concentration did not increased under salinity stress conditions.

AM symbiosis increased the nutrient use efficiency

The nutrient use efficiency was calculated dividing the total plant biomass by the plant nutrient concentration (Fig. 5) (Siddiqi & Glass, 1981). The results showed that K^+ and P use efficiency (KUE and PUE respectively) were affected by AM treatment (Fig 5A and B respectively).

KUE (Fig 5A) was significantly affected by both factors and their interaction (Table 1). Analyzing each level of salt separately (ANOVA one way, analyzing N factor effect split by each salt level) both AM and P plantlets showed higher values compared with C ones at 1 and 150 mM NaCl ($p = 0.037$ and $p = 0.038$ respectively), while no differences were observed under moderate stress conditions.

Statistical analysis showed Mg^{2+} se efficiency (MgUE) only had been affected by S factor, however, analyzing split by each salt level, at 1 mM NaCl, N factor had

significant effect (p = 0.0082), where AM plantlets showed the highest MgUE (post-hoc T-test).

Regarding Ca^{2+} use efficiency (CaUE) and silicon use efficiency (SiUE), despite figure 5D and E respectively, apparently showed variations, no statistical differences were found among treatments, and any statistical effect of N and S factor and their interaction (Table 1).

Phosphorus use efficiency (PUE) was significantly affected by N factor (Table 1). The post-hoc T-test showed that AM plantlets presented higher PUE compared with C and P plantlets in all saline treatments (1, 75 and 150 mM NaCl), (Fig 6B) (ANOVA one way, analyzing N factor effect split by each salt level, p = 0.0053; p= 0.0098; p = 0.033 respectively).

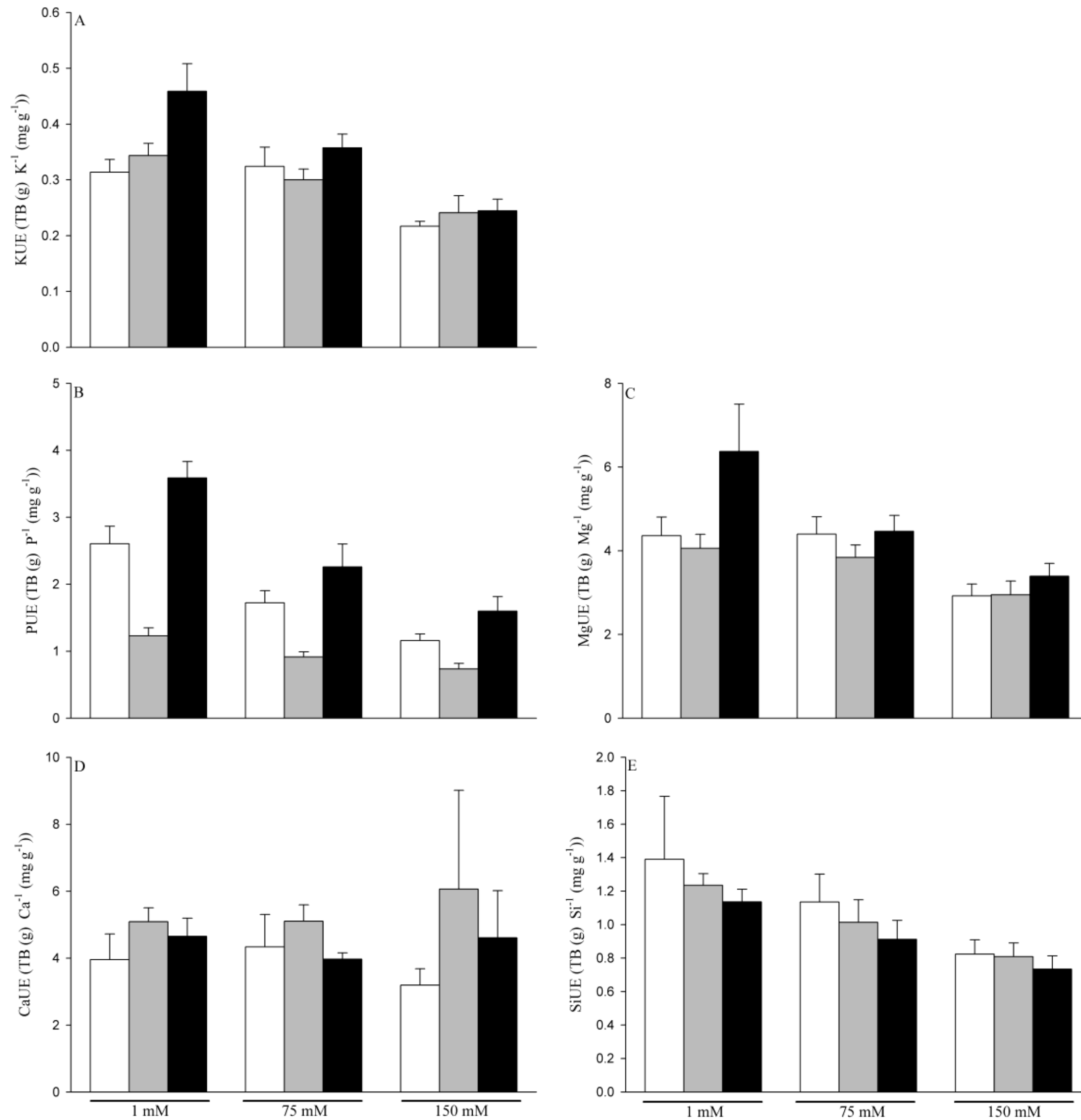

Figure 5. Nutrient Use Efficiency (Total Biomass, TB (g) / nutrient concentration (mg g^{-1})) K^+ (KUE, A), P (PUE, B), Mg^{2+} (MgUE, C), Ca^{2+} (CaUE, D) and Si (SiUE, E). Values are mean and SE of six replicates of C (white bars), P (grey bars) and AM plantlets (black bars) at the three salinity levels (1, 75 and 150 mM NaCl).

External and internal PUE (PUE_e and PUE_i respectively) were calculated as follows, for leaf, shoot and root tissues:

PUE_e= Tissue DW/plant P concentration, following Zhang et al. (2009)

PUE_i = Tissue DW/tissue P concentration, following Hammond et al. (2009)

The analysis of PUE_e and PUE_i showed that AM plantlets had higher values of both parameters under each salt level and in each tissue, followed by C plantlets, while P ones always showed the lowest values.

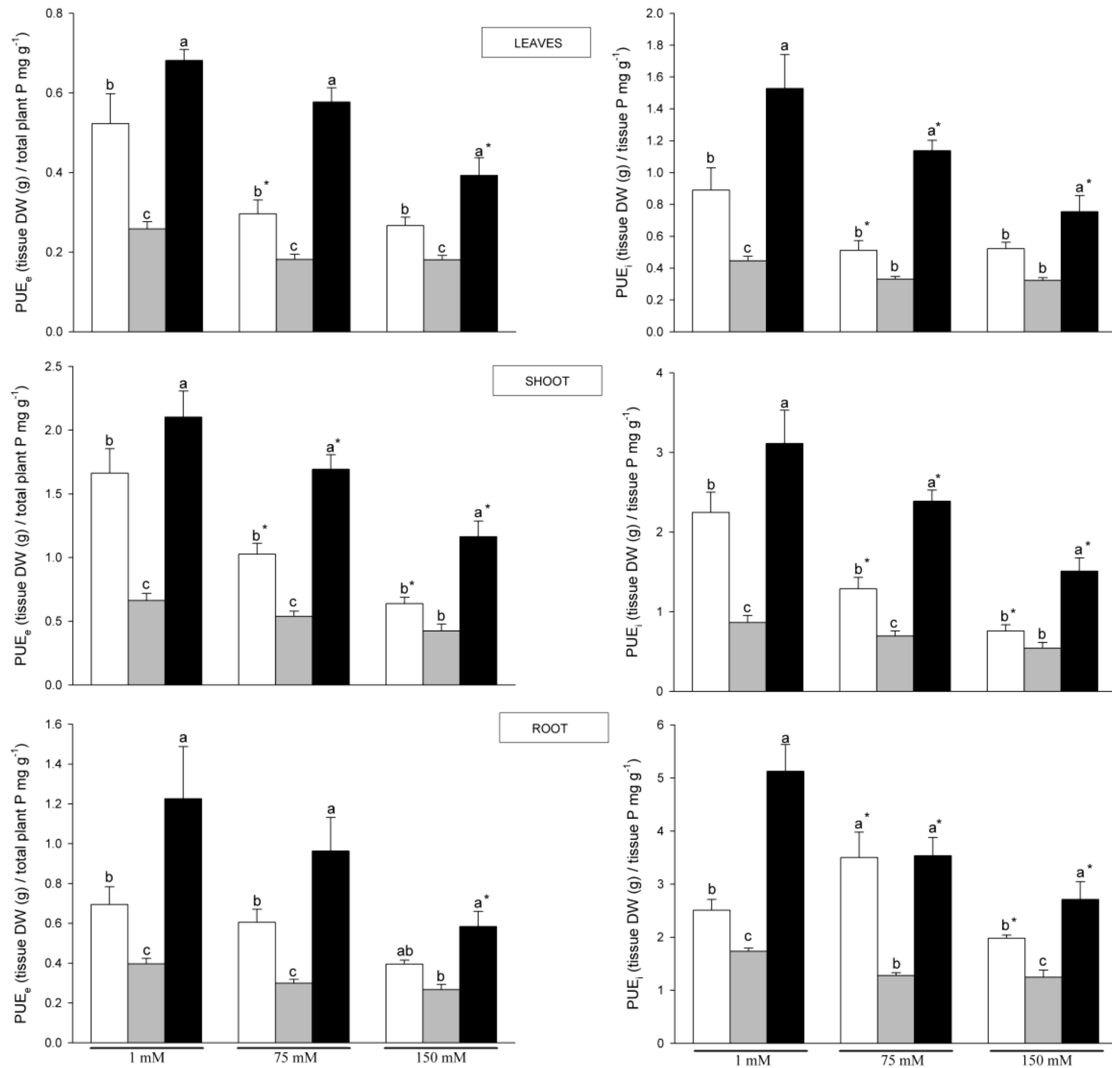

Figure 6. Phosphorus use efficiency external and internal (PUE_e and PUE_i respectively), of leaves, shoots and roots, for C, P and AM plants (white, grey and black bars respectively) under 1, 75 and 150 mM NaCl. Values are mean and standard error of at least 4 replicates per treatment. Different letter mean significant differences ($p < 0.05$) between nutritional treatment (C, P and AM) within the same salt level. * means significant differences ($p < 0.05$) between salt levels within the same nutrient treatment.

Discussion

Arundo donax is one of the most promising bioenergy crops producing high yields even when grown on marginal lands (Lewandowski et al., 2003a; Pilu et al., 2012). In arid and semiarid regions, combined phosphorus deficiency and high salinity are often soil-borne stress conditions that limit plant production. Nowadays, few controversial studies have investigated the effect of salinity on the physiology and biomass production of *A. donax*, and even fewer of these researches focused on the early stages of this crop (Sánchez et al., 2015; Pompeiano et al., 2016), when it shows a higher susceptibility to stress conditions (Pilu et al. 2013).

On the other hand, AM fungi are well known to enhance nutrient acquisition and ameliorate plant responses to saline conditions (reviewed by Evelin et al. 2009). The effectivity of the AM association depends, amongst others, on the inherent plant mechanisms for acquiring nutrients. A previous study using *A. donax* plantlets has shown a beneficial AM effect on plant growth (Baraza et al. 2016).

Here, marginal land stress conditions were mimicked by growing AM and non-AM plantlets at low and sufficient phosphorous concentrations under increasing salinity. To the best of our knowledge, there is no previous literature report on the interactive effects of phosphorus starvation and salinity on the physiology and biomass production on *A. donax*. The results showed a remarkable positive AM effect on the plantlets growth when they were submitted to low or moderate saline conditions even though AM plantlets were treated with a commercially available AM-based inoculum (AEGIS SYM®) that showed a susceptibility to salt stress.

Arundo donax has a high nutrient use efficiency that is further increased by AM inoculation

In our growing conditions, *A. donax* showed a remarkable response to Pi scarcity, and despite the marked differences in the total P tissue concentration, within the high range in P plantlets (>5 mg P/g DW) and close or just below critical values (3 mg P/g DW) in C plantlets (Veneklaas et al. 2012 and references herewith), the growth of C and P plantlets was similar and only significant higher P than C biomass values were found for roots of plants grown at 1 mM NaCl. Therefore, *A. donax* response to low Pi cannot be related to an increased root biomass. However, external and internal PUE were significantly higher in C than in P plants, which, amongst others, could be related to the

capability of vacuolar P to buffer cytoplasmic P concentration to maintain cellular homeostasis at low Pi supply (Yang et al. 2017). The lack of response to low P, that includes amongst others increased root/shoot biomass and anthocyanin production (Hammond et al. 2011), indicates that the Pi concentration supplied to *A. donax* plantlets in this study was not low enough to trigger Pi signaling pathways to low P availability which would explain why C kept growth rates like P plants.
AM symbiosis has frequently been reported as a biological method to promote plant growth by increasing nutrient uptake, especially P (Wright et al. 1998; Richardson et al. 2009; Smith and Smith 2011). From our results, it cannot be established whether AM-inoculation increased Pi uptake by the roots (µg root P/cm^2 root surface), as AM plantlets also showed higher root growth, which could have been caused by the additional Pi taken up. Nonetheless, AM-inoculation further increased external and internal PUE in low Pi supplied *A. donax* plantlets. Like in C plantlets, no low P signaling-triggered response were found in AM plantlets. Moreover, AM plantlets, despite their lower tissue P concentration, exhibited higher growth values than P plantlets. The growth increase in AM plantlets could be related to AM-produced plant hormonal factors, such as cytokinins (CKs) that could have counteracted any low P signaling triggered by the low Pi in the growing media.

Internal PUE is commonly applied to the leaves as it is a key factor to prevent a reduction in photosynthesis due to increased sucrose concentrations in response to low P. Nonetheless, as *A. donax* propagation occurs through the regrowth of rhizome fragments (reproduce solely via asexual fragments (vegetative propagules: stem and rhizome fragments), rhizomatous growth habit), the higher values in root PUE found in C but more markedly in AM plants with respect to P plants sustains the high physiological capability of this species to grow on low Pi media and the additive effect on this response exerted by AM inoculation.

Moreover, in the highly AM-colonized plantlets grown at 1 mM NaCl, the higher external K and Mg use efficiency could have benefited, amongst others, the leaf's water relations and photosynthesis and consequently plant growth.

AM symbiosis enhances A. donax salt tolerance

Although in nature *A. donax* principally colonizes freshwater riparian areas, its capacity to grow on marginal soils with limited water availability has been reported (Cosentino

et al. 2014, Haworth et al. 2017). Recently, it has been classified as a moderately salt tolerant species, as in a glasshouse experiment, *A. donax* plantlets showed 50% relative growth values at 120 mM NaCl and survive at salt concentrations up to 420 mM NaCl (Nackley and Kim 2015). Similarly, our results showed 63% relative growth values in *A. donax* plantlets grown at 150 mM NaCl irrespectively of Pi supply.

Salinity tolerance for grasses has been attributed to Na^+ exclusion from the shoot and to Na^+ tissue tolerance (Flowers et al. 1977). Due to their lower storage capacity, grasses have higher K^+ and compatible solute needs for cytosol and cell organelles than dicotyledonous species (Flowers and Yeo, 1988). *A. donax* maintained high tissue K^+ concentration under salt stress, which could be a positive trait for salt tolerance (Maathuis and Amtmann, 1999). Moreover, *A. donax* showed a high selectivity for K^+ and in salt-treated plants, tissue K^+ concentrations were maintained independently of Na^+. Therefore, it is unlikely that the salt-induced changes in plant growth could be related to changes in K^+ uptake by the roots or to tissue K^+ concentration. Despite *A. donax* Na^+ exclusion capacity indicated by the independence of tissue Na^+ concentration from the NaCl supplied in the media, leaf Na^+ concentrations were sometimes above 100 mM. Nonetheless, up to know there is little information available on the plant/cell Na^+ concentration toxicity threshold. In leaves of salt-stressed durum wheat, non-stomatal limitation of CO_2 assimilation was associated with leaf Na^+ concentrations above 200 mM (James et al. 2002). In barley, considered a salt-tolerant species, plant dry weight was found to decrease at shoot Na^+ concentrations above 9.2 mg g^{-1} DW (Tavakkoli et al. 2011). Regarding, another key parameter for salt tolerance, the Na^+/K^+ ratio (Tester et al. 2003), its expression differed depending on the P concentration supplied. A significant increase in Na^+/K^+ ratio with increase NaCl supply was found in P plantlets, while no differences between mild and high salinity treatments occurred in C plantlets.

AM fungi modulate the plant's response to salt stress (Evelin et al. 2009). The effect of salt excess on plant colonization by AM fungi depends on the fungus tolerance to salinity (Yamato et al. 2008). Root colonization by *Rhizophagus irregularis* and *Funneliformis mosseae* was severely decreased by salt. Reduced root colonization by AM fungi in saline environments has been related to a salt effect on primary infection as more inhibition has been reported at the early stages of AM symbiosis (Wilson 1984,

MacMillen et al. 1998). However, as salinity treatments were started after AM colonization was achieved, the reduction in AM colonization of *A. donax* roots was more likely due to a salt effect on secondary infection. It has been reported that 100 mM NaCl reduces the number of spores produced by AM fungi as well as the hyphal length and the number of branched absorbing structures (Jahromi et al. 2008). Nonetheless the mycelium and spore percentage was similar in all salt treatments, while for vesicles and arbuscules was independent of salt supply. What does that mean?

The ameliorated plant growth response to saline conditions was directly related to plant colonization by AM fungi, and therefore phenotypic effects were only manifested in inoculated plants treated with 75 mM NaCl. Salinity tolerance marker values for leaf Na^+ and Na^+/K^+ were lower in AM than in P plants, which outstand the potential of AM inoculation as a biological tool to increase *A. donax* yield under saline conditions. Increased K^+ and decreased Na^+ tissue concentrations has been reported in AM-treated plants grown under saline conditions (Evelyn et al. 2012). The AM-induced ameliorated growth response of *A. donax* to mild salinity was related to a decrease in Na^+ activity in the leaves rather to an enhancement on K^+ uptake. Nonetheless, the capacity to maintain tissue K^+ concentrations under saline conditions has been reported as a key trait for salt tolerance (Shabala and Cuin, 2008). In AM-inoculated plants the intraradical hyphae could provide the plant with an additional space for Na^+ allocation and help to prevent its translocation to the shoots (Cantrell and Linderman 2001). Recently, an AM symbiosis-dependent differential expression of three transport systems, AKT2, a phloem located K^+ channel, SKOR, involved in xylem K^+ uploading and SOS1 and PM Na^+/H^+ antiporter has been reported in *Zea mays* (Estrada et al. 2013).

Taken together our results highlight the potential of AM fungi inoculation to increase *A. donax* biomass under combined soil stress conditions, nutrient scarcity and mild salinity, during the plant's early developmental stages. The amelioration in growth found in AM-inoculated plantlets was mainly due to increased root and shoot PUE and to the maintenance of a low shoot Na^+/K^+ ratio.

Conclusions

Arundo donax plantlets showed higher PUE which was even increased by AM symbiosis. Furthermore, AM symbiosis increased biomass production more than Pi under non salinity conditions. Those symbiotic effects could be related with plant

hormone regulation by AM fungi, but more studies will be required. Regarding to salt stress, our results demostrated that *A. donax* is a moderated salinity tolerant species due to Na^+ exclusion, however, biomass was reduced. AM symbiosis ameliorated biomass losses and AM plantlets showed higher biomass production under moderate salt stress conditions compared with the other treatments. The present study showed that AM symbiosis could be a good tool to enhance *A. donax* phyisiological traits and biomass productions in early stages under salinity and Pi soil starvation.

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Suplementary information (SI) Chapter 3

Biomass distribution and leaf anatomical distribution

Table S1. Phosphorus addition (P) and arbuscular mycorrhiza (AM) effect on biomass distribution of plantlets growth under phosphorus starvation and under three salt levels, expressed as relative values (fold changes). Plantlets grown under phosphorus starvation (C) set to unity. Green and red boxes denote significant increase and decrease ($p < 0.05$ Students t-test). Data are means ± SE of 5 to 6 replicates.

Treatment NaCl (mM)	1		75		150	
	P	AM	P	AM	P	AM
# leaves	1.12±0.07	1.15±0.23	1.12±0.10	1.19±0.10	1.03±0.06	1.09±0.07
# stems	0.65±0.08	0.72±0.14	0.73±0.14	0.88±0.03	1.47±0.12	1.88±0.20
Leaf (g)	1.08±0.05	1.24±0.23	1.09±0.05	1.22±0.05	1.01±0.07	1.05±0.09
Stem (g)	1.01±0.03	1.20±0.22	1.01±0.07	1.08±0.05	0.93±0.06	1.00±0.07
Fine Root (g)	1.51±0.25	1.13±0.09	0.66±0.04	0.59±0.09	0.94±0.08	1.05±0.09
Thinck Root (g)	1.31±0.12	1.98±0.23	0.84±0.04	1.31±0.09	1.10±0.07	1.16±0.06
Total Plant (g)	1.13±0.05	1.42±0.27	0.95±0.04	1.14±0.05	0.99±0.06	1.06±0.07

Table S2. Biomass parameters of C, P and AM plantlets growing at 1, 75 and 150 mM NaCl. Specific leaf area (SLA, [cm^2 g^{-1}]); leaf area ratio (LAR, [cm^2 g^{-1}]) and leaf mass ratio (LMR, [g g^{-1}]). Data are means ± SE of 5 to 6 replicates.

Treatment NaCl (mM)	1			75			150		
	C	P	AM	C	P	AM	C	P	AM
SLA (cm^2 g^{-1})	53.062 ± 2.87	51.742 ± 2.85	51.762 ± 4.23	51.459 ± 2.29	49.311 ± 2.65	46.169 ± 1.47	51.784 ± 4.13	50.890 ± 2.14	57.100 ± 2.68
LAR (cm^2 g^{-1})	11.084 ± 0.47	10.416 ± 0.86	9.483 ± 1.22	9.918 ± 0.73	10.999 ± 0.92	9.399 ± 0.42	11.359 ± 1.22	11.178 ± 0.57	12.284 ± 0.87
LMR (g g^{-1})	0.211± 0.01	0.199 ± 0.01	0.183 ± 0.01	0.191 ± 0.01	0.222 ± 0.01	0.203 ± 0.01	0.217 ± 0.01	0.219 ± 0.004	0.215 ± 0.01

No significant effects of nutrient treatment (C, P and AM), salinity and their interaction ($p > 0.05$) in SLA and LAR, while salinity treatment had an effect on LMR despite of it desapeared in the interaction between treatments ($p = 0.0234$ and $p = 0.0868$ respctively).

Ion concentration and distribution

S3 Table. Ion concentration (mg g^{-1}) obtained in each tissue analyzed by ICP, were SU = stem up (distal part of the stem); YL = young leaf; ML = mature leaf (fully expanded leaf); OL = old leaf (leaf in senescence); SD = stem down (proximal part of the stem); TR = thick root and FR = fine root. Values are mean ± SE of at least four replicates per treatment.

A. Phosphorus plant distribution

P mg g^{-1}	1			75			150		
Tissue	C	P	AM	C	P	AM	C	P	AM
SU	2.29 ± 0.18	6.31 ± 0.35	2.12 ± 0.65	4.85 ± 0.81	7.51 ± 0.79	4.81 ± 1.21	5.92 ± 0.67	7.33 ± 0.41	4.40 ± 0.47
YL	4.23 ± 0.51	7.69 ± 0.35	4.03 ± 0.39	5.67 ± 0.64	7.90 ± 0.65	5.34 ± 0.68	7.30 ± 1.57	7.30 ± 0.37	4.93 ± 0.49
ML	2.73 ± 0.40	7.95 ± 0.44	2.53 ± 0.18	4.15 ± 0.06	7.20 ± 0.44	3.06 ± 0.30	4.37 ± 0.33	10.5 ± 0.74	3.60 ± 0.17
OL	2.94 ± 0.50	9.01 ± 1.00	2.67 ± 0.23	3.01 ± 0.60	7.53 ± 0.94	3.55 ± 0.49	3.41 ± 0.40	6.62 ± 1.25	3.30 ± 0.53
SD	0.55 ± 0.05	1.37 ± 0.13	1.02 ± 0.38	0.72 ± 0.05	2.91 ± 0.51	0.95 ± 0.14	1.63 ± 0.34	2.09 ± 0.30	1.26 ± 0.17
TR	1.88 ± 0.20	4.48 ± 0.23	2.42 ± 0.08	1.61 ± 0.08	3.69 ± 0.12	2.33 ± 0.30	2.21 ± 0.32	3.53 ± 0.14	1.97 ± 0.06
FR	2.88 ± 0.28	6.18 ± 0.50	3.63 ± 0.23	2.97 ± 0.15	6.27 ± 0.18	2.96 ± 0.14	2.89 ± 0.16	5.85 ± 0.24	2.61 ± 0.22

B. Calcium distribution

Ca^{2+} mg g-1	1			75			150		
Tissue	C	P	AM	C	P	AM	C	P	AM
SU	0.43 ± 0.13	0.64 ± 0.19	0.79 ± 0.05	0.41 ± 0.14	0.47 ± 0.23	0.24 ± 0.07	0.40 ± 0.14	0.59 ± 0.16	0.40 ± 0.12
YL	1.07 ± 0.29	1.06 ± 0.33	0.68 ± 0.16	0.92 ± 0.42	0.28 ± 0.07	0.12 ± 0.08	0.64 ± 0.38	0.56 ± 0.30	0.70 ± 0.17
ML	2.07 ± 0.57	2.37 ± 0.49	1.78 ± 0.61	2.12 ± 1.30	1.26 ± 0.21	1.24 ± 0.07	2.44 ± 0.57	1.89 ± 0.38	2.31 ± 0.43
OL	7.35 ± 0.79	6.11 ± 0.92	5.85 ± 0.48	6.16 ± 1.26	4.58 ± 0.98	6.38 ± 1.28	5.58 ± 0.92	5.59 ± 0.75	4.36 ± 0.96
SD	0.10 ± 0.08	0.44 ± 0.13	1.35 ±	0.09 ± 0.03	0.20 ± 0.07	0.15 ±	0.20 ± 0.05	0.19 ± 0.05	0.94 ±
TR	0.90 ± 0.17	0.63 ± 0.20	0.70 ± 0.17	0.47 ± 0.16	0.65 ± 0.11	0.29 ± 0.14	0.60 ± 0.06	0.90 ±	0.83 ± 0.11
FR	1.13 ± 0.13	1.03 ± 0.11	2.19 ± 0.48	0.80 ± 0.15	0.38 ± 0.14	1.18 ± 0.20	1.06 ± 0.29	0.43 ± 0.12	0.84 ± 0.23

C. Magnesium distribution

Mg^{2+} mg g-1	1			75			150		
Tissue	C	P	AM	C	P	AM	C	P	AM
SU	1.12 ± 0.10	1.39 ± 0.04	1.13 ± 0.06	1.10 ± 0.09	1.17 ± 0.12	1.32 ± 0.09	1.03 ± 0.18	1.21 ± 0.17	1.19 ± 0.11
YL	0.89 ± 0.04	1.18 ± 0.07	0.73 ± 0.05	0.95 ± 0.11	0.75 ± 0.05	0.74 ± 0.03	0.85 ± 0.09	0.61 ± 0.04	0.87 ± 0.09
ML	1.86 ± 0.34	1.97 ± 0.24	1.85 ± 0.31	1.89 ± 0.85	1.61 ± 0.33	1.48 ± 0.14	2.47 ± 0.48	2.23 ± 0.36	2.05 ± 0.54
OL	5.18 ± 0.42	5.48 ± 0.28	4.42 ± 0.54	4.76 ± 0.69	4.07 ± 0.53	5.29 ± 0.70	4.47 ± 0.47	4.03 ± 0.63	4.27 ± 0.87
SD	0.45 ± 0.07	0.48 ± 0.03	0.70 ± 0.34	0.37 ± 0.05	0.53 ± 0.07	0.46 ± 0.03	0.42 ± 0.04	0.45 ± 0.05	0.48 ± 0.04
TR	0.57 ± 0.06	0.95 ± 0.03	0.90 ± 0.03	0.50 ± 0.07	0.86 ± 0.05	0.69 ± 0.10	0.61 ± 0.05	0.69 ± 0.05	0.60 ± 0.09
FR	1.34 ± 0.08	1.36 ± 0.07	1.80 ± 0.23	1.05 ± 0.06	1.28 ± 0.07	1.23 ± 0.09	1.11 ± 0.10	1.39 ± 0.09	1.08 ± 0.15

D. Silicon distribution

Si mg g^{-1}	1			75			150		
Tissue	C	P	AM	C	P	AM	C	P	AM
SU	2.5 ± 0.19	2.36 ± 0.19	2.26 ± 0.28	2.50 ± 0.29	3.20 ± 0.52	3.16 ± 0.48	3.18 ± 0.43	4.81 ± 0.41	4.19 ± 0.41
YL	1.42 ± 0.46	1.33 ± 0.21	1.31 ± 0.19	1.83 ± 0.24	1.62 ± 0.15	1.85 ± 0.19	2.33 ± 0.33	2.01 ± 0.20	1.76 ± 0.15
ML	2.15 ± 0.23	1.78 ± 0.20	2.30 ± 0.27	2.40 ± 0.61	2.54 ± 0.46	2.51 ± 0.32	3.00 ± 0.42	4.00 ± 0.37	2.45 ± 0.41
OL	5.77 ± 0.83	4.72 ± 0.71	4.23 ± 0.48	5.97 ± 1.47	5.07 ± 1.12	6.36 ± 1.14	5.39 ± 0.78	5.75 ± 0.90	5.18 ± 1.28
SD	1.69 ± 0.11	1.88 ± 0.14	1.65 ± 0.29	1.61 ± 0.15	2.14 ± 0.31	1.99 ± 0.19	1.69 ± 0.05	2.17 ± 0.18	1.76 ± 0.04
TR	9.89 ± 3.18	10.7 ± 1.69	14.2 ± 3.20	9.07 ± 2.24	6.93 ± 1.15	14.1 ± 2.65	6.37 ± 1.20	7.40 ± 0.97	8.53 ± 1.39
FR	24.6 ± 9.07	25.1 ± 2.31	31.1 ± 3.22	12.8 ± 5.14	20.1 ± 5.30	26.7 ± 5.93	17.7 ± 3.51	13.3 ± 1.53	28.4 ± 6.80

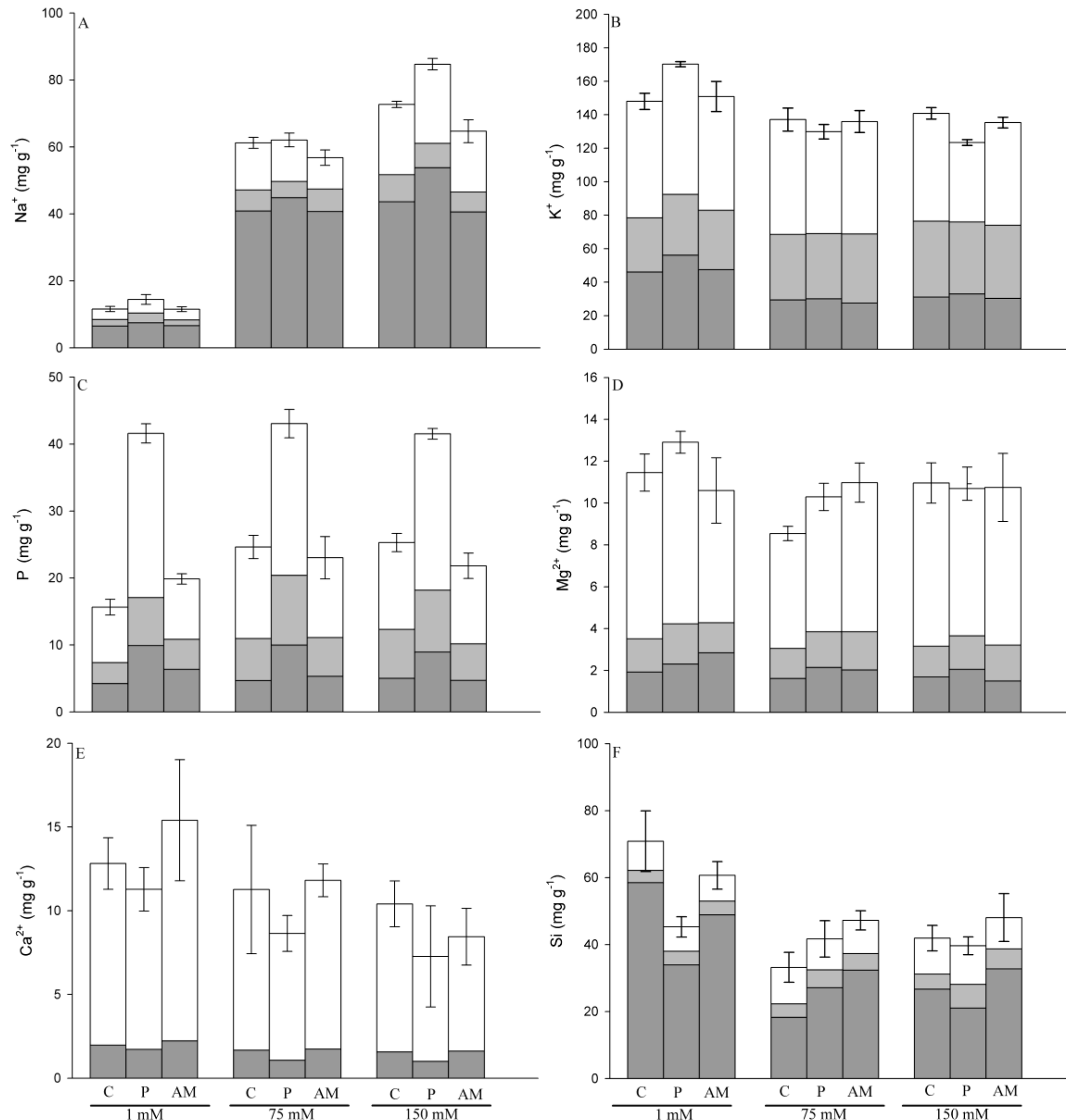

Figure S1. Stacked bars of mean and standard error of total plant concentration, mg g^{-1} (height of bar) of Na^+, K^+, P, Mg^{2+}, Ca^{2+} and Si (a, b, c, d, e and f respectively). Total plant concentration was represented at the sum of root concentration (dark gray), stem (grey) and leaves concentration (white).

Nutrient concentration (mg g^{-1}) was not affected by NaCl addition, thus C, P and AM plantlets showed the same values under 1, 75 and 150 mM NaCl (Fig. 1). Pi concentration was always higher in P plantlets than in C and AM ones, due to it higher concentrations in nutritional solution apply to P plantlets. Nonetheless, salinity stress was not affect P concentrations in any case.

As it was expected, Na^+ concentration increased in the salt treatments respect 1 mM NaCl in all plantlets, however, under 150 mM NaCl, AM plantlets showed low Na^+ concentration compared with C and P plantlets ($p = 0.0002$).

Chapter 4

Nursery Preconditioning of *Arundo donax* L. Plantlets Determines Biomass Harvest in the First Two Years

Nursery preconditioning of *Arundo donax* L. plantlets determines biomass harvest in the first two years

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Abstract

Arundo donax L. has become one of the most promising species for energy production in several subtropical and Mediterranean areas. This species can thrive across a wide range of soil types and is tolerant of many stressful conditions. However, establishment of the crop is critical for the cultivation of *A. donax.* In this study, we evaluated the effect of nursery preconditioning on survival during transfer to the field and harvests in the first and second year for *A. donax*. We used early arbuscular mycorrhizae (AM) inoculation, two different substrates, and two pot sizes to generate different nursery conditions that resulted in micropropagated plantlets of *A. donax* of different sizes and quality. In total, we had 3 treatments. Treatment inoculation: mycorrhizal (AM) plantlets were inoculated with commercial inoculum (AEGIS SYM ®), which contained *Rhizophagus irregularis* and *Funneliformis mosseae*, at the time of transplantation, whereas Control plantlets were not inoculated. Substrate treatment: half of the plantlets were acclimatized with commercial nutrient-rich agricultural substrate (AS) and half with a mix of agricultural substrate:sand (1:1, v/v) (MIX). Cell size treatment: after 100 days of acclimatization, 20 plants per treatment were grown in 50 cm^3 cells (SC), whereas 20 plants per treatment were transplanted into trays with 300 cm^3 cells (BC) in order to generate bigger plants. Our results showed that after acclimatization, AM plantlets presented a greater height, number of leaves, biomass and chlorophyll content than non-mycorrhized (Control) plantlets. In the same way, the results showed a positive effect of the use of AS versus MIX. At the time of transplanting into the field, the plants that remained in small cells were significantly smaller than those that were transplanted into large cells. Additionally, mycorrhizal plants that had been grown in AS had a taller initial height. Plant height at the time of transplantation significantly affected the first- and second-year harvests. Inoculated plants showed higher biomass accumulation than non-inoculated plants after 11 months of growth in an open field. In the second year, only the tray cell size had significant effects on dry biomass harvest. These results demonstrate the potential benefits of producing more vigorous and larger plants in order to increase plant survival and biomass harvest in the first and second year.

Key words: Giant reed, Arbuscular mycorrhizal fungi, Micropropagated plants, Stressful conditions, Crop harvest.

Introduction

Among the strategies to reduce fossil fuel dependence and greenhouse gas emissions (GHG), bioenergy crops have been one of the most promising alternatives due to their low carbon footprint (Fagnano et al., 2015; Zucaro et al., 2015), especially second-generation biofuels, which not only do not compete for arable soils with food crops but also allow a reduction in farm emissions and an increase in C storage in the soil (Ballesteros 2015). Such energy crops are based on perennial crops with high yield that are cultivated using low-input cropping systems with low cost to produce biomass as raw material for energy (Fagnano *et al*., 2015). One of the most promising species for energy, cellulose paste, fiber and second-generation biofuel production is *Arundo donax* L. (giant reed) (Shatalov and Pereira, 2002; Lewandowski *et al.*, 2003; Pilu *et al.*, 2013, Corno *et al*., 2014; 2015; Fagnano et al., 2015).

Arundo donax is a rhizomatous perennial grass that has been introduced worldwide due to its ability to adapt to a wide variety of environments. Its low input requirements after establishment (Cosentino *et al.*, 2008; Pilu *et al.*, 2013; Fagnano et al., 2015) and its high production of biomass, approximately 40 t $year^{-1}$ ha^{-1} of dry biomass (Angelini *et al.,* 2005; Mantineo *et al.*, 2009; Angelini *et al.*, 2009), makes it an important bioenergy crop. The rhizome and its leaf plasticity allow *A. donax* to be drought tolerant once established in the field (Perdue, 1958; Lewandowski *et al.*, 2003; Lambert *et al.*, 2010; Romero-Munar *et al*., 2017). Its tolerance to environmental stresses allows the use of marginal or submarginal lands, thus reducing competition with food crops, which generally require better-quality lands (Sims *et al.*, 2010; Di Nasso *et al.*, 2013). Nevertheless, the establishment period for this crop is a critical moment in which irrigation and fertilization are advised (Pilu *et al*., 2013).

A crucial factor that limits *A. donax* cultivation is the difficulties in its propagation due to its sterility, which increases the labor required for propagation techniques (Pilu *et al.*, 2013). In vitro culturing methods have been proposed as a solution for large-scale propagation to facilitate obtaining large amounts of high-quality genetically homogeneous plants in a moderate amount of time that are guaranteed to be free of pathogens (Cavallaro *et al.*, 2011; 2014). However, plants resulting from micropropagation may not be prepared to resist transplantation to Mediterranean or semi-arid marginal farmlands. Obtaining a resistant plant capable of surviving transplantation to the field and maintaining good growth under the new conditions can imply a long-term benefit, ensuring greater production (Sarangi et al., 2015). In fact, for other perennial species, it has been shown that conditioning techniques used in the nursery during seedling production are crucial for the establishment, survival and subsequent growth of plants after transplanting (Franco *et al.*, 2006; Sarangi et al., 2015). Environmental conditions and cultivation techniques in the nursery could be manipulated to produce robust seedlings. Water stress preconditioning is the most commonly used technique to produce high-quality seedlings in Mediterranean and semi-arid areas (e.g.Ruiz-Sanchez et al., 2000; Vilagrosa et al., 2003). However, nursery conditions can also be manipulated by using different-sized containers and substrates with different water retention characteristics (Narciso et al., 1997), fertilization (Trubat et al., 2011) or inoculating arbuscular mycorrhizal fungi (Abbaspour et al., 2011; García et al., 2011). Accordingly, symbiosis manipulation and variation of microclimatic conditions can be used to control growth to produce high-quality seedlings which could resist transplanting shock and be able of rapid establishment and resumption of growth under stress conditions (Franco *et al.*, 2006).

Poorter *et al.* (2012) reported, on average, that doubling the pot size increased seedling

biomass production by 43% in their meta-analyses of 65 studies that analyzed the effect of pot size on growth. Pot size affects root growth but also causes the down-regulation of photosynthesis and a decrease in total plant growth (Hurley and Rowarth 1999; Ronchi *et al.*, 2006). In tree species, a significant effect of plant size has been detected on plant survival and subsequent growth over several years (Puértolas et al., 2003; Villa-Salvador et al 2012). Nevertheless, no information has been found about plant size effects on biomass production over multiple years in perennial crop species. The use of bigger cell traits could generate bigger plants but increase the cost of plant production, so it is important to evaluate the real benefit of initial size on crop establishment and yields.

The use of arbuscular mycorrhizal fungi (AMF) has been proposed to improve the micropropagation process in many plant species (Alarcón and Ferrera-Cerrato, 2000; Estrada-Luna *et al.*, 2000; Kapoor *et al.*, 2008 and references therein). Inoculation with AMF during hardening enhances the development of the root system, increases nutrient uptake, water conducting capacity and photosynthetic rates and promotes defense against harmful pathogens and the synthesis of growth hormones (Kapoor *et al.*, 2008; Parkash *et al.*, 2011; Singh *et al.*, 2012). The positive effects of AMF became more important when seedlings were exposed to stressful conditions (Abbaspour et al., 2011; García et al., 2011). For energy crops, da Silva Folli-Pereira *et al.* (2012) reported that arbuscular mycorrhization enhances the production system of micropropagated plantlets of *Jatropha curcas*, increasing phosphorus uptake and growth during the acclimatization period. Mirshad and Puthur (2016) found that AMF associations alleviate drought stress in *Saccharum arundinaceum*. Regarding *A. donax*, Tauler and Baraza (2015) reported that early inoculation of micropropagated plants with an AMF biofertilizer is an effective method to improve the acclimatization process and plantlet

quality. However, the effect of AMF inoculation on biomass production over multiple years has been poorly studied.

The aim of the present study was to investigate how plantlet quality and size could affect *A. donax* field transfer and harvest in the first two years. First, we analyzed how AMF inoculation and substrate type affect the survival and development of micropropagated plantlets of *Arundo donax* during the acclimatization period. Second, we studied the effect of those nursery treatments and cell size on plant survival and growth during field transfer and on the first- and second-year harvest.

Materials and Methods

The plantlets used to perform the present work were *Arundo*-K12 clones provided by BIOTHEK ECOLOGIC FUEL S.L. The study had three phases: acclimatization, crop field establishment and crop harvest, and a schematic description of the methodology can be found in Table 1.

Table 1. Schematic diagram of the methodology: D = days; DAT = days after transplantation in the field MAT month after trasnplantation

Time	No. Plants	Action
D0	260 bare-root micropropagated	Plantlet preselection
D7-D107	55 per treatment in 50 cm^3	Treatment establishment: Two substrates: agricultural substrate (AS) peat base or a mix with sand (MIX). Inoculated with arbuscular mycorrhizae (AM) or not (C; control)
D107	15 per treatment	Destructive measures
D107–D150	20 per treatment in 50 cm^3 20 per treatment in 300 cm^3	Transplantation of 20 plants per treatment into 300 cm^3 tray cells with AS
D151	20 per treatment	Field transfer
0DAT-52DAT	20 per treatment	Height and stem number measurements
9 MAT	8 per treatment	Aerial biomass production
21 MAT	6 per treatment, did not cut previous year	Aerial biomass production

1. First phase: acclimatization

All 220 micropropagated plantlets were received bare-root and were immediately planted in a tray filled with agricultural substrate (AS) (KEKKILÄ DSM 1 W ©, pH 5.9, 90% organic material; principal compound: sphagnum peat; additives: N-P_2O_5-K_2O (16-4-17, 0.60 g l^{-1}), wetting agent (0.10 g l^{-1}) and dolomite limestone (5.0 g l^{-1})). After one week of acclimatization, plantlets were transferred to a cell tray (50 cm^3 of volume per cell) with two different substrates: 110 plantlets were planted in a cell tray containing the same AS, while the other 110 plantlets were planted in a cell tray containing a mix of agricultural substrate:sand (1:1, v/v) (MIX). MIX was considered to be a stressful treatment since it provides only 5.7% of the total N contained in the agricultural substrate (Kjeldahl measures), and the water-holding capacity was lower (35.71% and 59.40% for MIX and AS, respectively). To ensure plant survival, 24 ml per plant of a modified Hoagland solution that contained half of the phosphorus intake were added for the plants in MIX.

At the moment of transplantation, 5 cm^3 of AMF inoculum was added to 55 plantlets (AM plants) in each tray. The commercial inoculum used was AEGIS® Sym Microgránulo (Atens Agrotecnologías Naturales S.L., Barcelona, Spain), which previous studies had determined was effective in the infection of *A. donax* (Tauler and Baraza 2015). The inoculum contained two generalist species that are abundant in all soil types and commonly used as commercialized inoculum: *Rhizophagus irregularis* and *Funneliformis mosseae* (25 spores per g each). To imitate the soil texture of the inoculated cells, we added the same quantity of sterile zeolite to the other plantlets, which were used as a Control. Plantlets were randomly distributed in the planting tray. In addition, we moved the tray periodically to avoid a position effect. The plantlets were watered every three days. The daily maximum temperature and relative humidity

showed little variation between the beginning and end of the experiment, approximately 18°C and 86%, respectively.

During the acclimatization period, the height (up to the highest leaf) and number of green leaves were measured regularly every two weeks.

After 100 days of planting, plant biomass was determined for 15 plantlets per treatment. Each plantlet was separated into leaves, stems and roots to obtain the total, aerial and radicular fresh biomass. After that, the tissues were dried separately (78 h at 60°C), and the aerial, radicular and total dry biomass were determined. The fresh leaf area of four fully developed leaves was determined using the ImageJ program (Wayne Rasband, NIH). The shoot:root ratio and leaf mass per area (LMA g cm^{-2}, calculated as leaf dry weight/leaf area) were also calculated.

The same 15 plantlets were used to determinate chlorophyll content. A circle of 50.27 mm^2 of fresh leaf tissue was sampled, and pigments were extracted with 2 mL of 90% acetone. Absorbance values at 664, 647, 630 and 750 nm were read using a UV-visible spectrophotometer (CARY®50). Chlorophyll concentrations were calculated using the equations provided by Jeffrey and Humphrey (1975):

$$Chlorophylla\left(\frac{\mu g}{l}\right) = 11.85E_{664} - 1.54E_{647} - 0.08E_{630}$$

$$Chlorophyllb\left(\frac{\mu g}{l}\right) = -5.43E_{664} + 21.03E_{647} - 2.66E_{630}$$

where E_{664}, E_{647} and E_{630} represent the absorbance values read at 664, 647 and 630 wavelengths, respectively.

1.1 Determination of mycorrhizal colonization

The percentage of mycorrhizal root colonization was determined by the visual observation of fungal colonization after washing (10% KOH) and staining (0.05%

trypan blue in lactic acid (v/v)) the roots according to Phillips and Hayman (1970). AM colonization was assessed using the magnified intersections method (McGonigle *et al*., 1990), in which the frequency of colonization (hyphae, arbuscules and/or vesicles) represents the ratio between the fragments of colonized root and the total number of root fragments examined. Approximately 0.2 g of fresh root was used to determinate the degree of colonization.

2. Second phase: greenhouse maintenance and crop establishment

After 100 days of acclimatization, to generate different plantlet sizes, 80 plantlets, of which 40 plantlets were from each substrate tray - 20 AM and 20 Control - were transplanted to cells of 300 cm^3. In this way, we generated 8 treatments (2 substrate types × 2 inoculation treatments × 2 cell sizes) and 20 plantlets per treatment.

In April, when climatic conditions were favorable, the 20 plantlets of each treatment were transferred to an agricultural field located southeast of Palma de Mallorca (Balearic Islands, west Mediterranean Basin). In the field, plants from the eight treatments were systematically distributed and planted using a 1 m × 1 m planting distance (thus, a plant density of 10,100 plants per hectare). The experimental plants were grown surrounded by non-experimental *A. donax* plants in order to avoid edge effects. The length of the main stem and the number of stems of all plants were measured at day 0 and 52 days after transplantation (DAT).

3. Third phase: crop harvest

Plants were watered with treated waste water (mean value ± standard error of water characteristics between April 2014 and January 2016: DBO < 10 mg l^{-1} O_2; 1.47 ± 0.656 P mg l^{-1} and 15.46 ± 6.152 N mg l^{-1}), but they were not fertilized. During the first

two months, mechanical weed control was implemented, but the great and rapid proliferation of weeds made the use of herbicides necessary. Sixty-four DAT in the field, a systemic and selective post-emergent herbicide (CALLISTO® 480 SC) was applied in order to control weeds, primarily hardwoods. No more control was necessary.

Plant fresh biomass was determined on two different dates: 9 months after transplantation, using eight plants per treatment, and 21 months after transplantation, using 6 plants per treatment. Dry plant biomass was estimated after oven drying a plant subsample with the same proportion of leaves, stems and panicles for 78 h at 60°C.

Statistical analysis

Each plantlet/plant was considered as an experimental unit. All datasets were tested for a normal distribution and variance homogeneity ($P < 0.05$), and variables were log transformed when necessary. The statistical design adopted to analyze the change in plant height over time was two-way-repeated measures ANCOVA (rmANCOVA), considering the AM and substrate treatments as factors and the initial height as a covariate. Because of the distribution of the data, variation in the number of leaves was analyzed using a generalized linear mixed model using the *lmer* function in the *lme4* package in R. We considered AM treatment, substrate type and time as fixed factors and the identity of the plant as a random factor with a Poisson distribution of residuals. When analyzing the variation in the number of stems between two sampling events, we used a generalized linear model with Poisson or quasi-Poisson distribution depending on the overdispersion of residuals (Zuur *et al*., 2009), with AM treatment and substrate type as factors. The significance of fixed factors was calculated using likelihood ratio tests for nested models (Zuur *et al*., 2009). Two-way ANOVA with substrate type and AM treatment as factors was performed for biomass, shoot-root ratio, LMA and chlorophyll content. Means were compared using *a posteriori* Tukey tests. Because of

the lack of homogeneous variance (AMF colonization percentage), we used Kruskal-Wallis tests to compare AMF colonization percentages. Three-way ANOVA with cell size, substrate type and AM treatment was performed for biomass harvest from the field. The effect of plant size at the time of transplantation in the field on harvests was evaluated using linear regression. Experimental data were analyzed with the R and JMP® statistical programs.

Results

AMF colonization

Typical structures of arbuscular mycorrhizal fungi, such as vesicles, arbuscules, hyphae and spores, were observed in all inoculated plants. Although MIX plants presented a higher percentage of infection than AS plants (32.5% ± 4.8 and 23.6 ± 4.8, respectively), no significant effect of substrate type was detected (P = 0.11 Kruskal-Wallis test).

AM structures were observed in 46% of Control plants, most likely from contamination by the presence of a few AMF in the substrate used or by the proximity of plantlets from the other treatment. However, the mean percentage of AMF infection in the Control plants was very low (2%), so we can consider that no effects of mycorrhization occurred in this group of plants.

In addition to AMF, an obligate biotrophic root-associated fungus, *Olpidium* sp., colonized the AM roots. *Olpidium* infection appeared in some of the AM roots because it was likely present in the environment. The effects of *Olpidium* on plants have been reported to be relatively neutral (Powell 1993; Weber and Webster 2000).

Acclimatization period

Plantlet survival was 100% for all treatments during the acclimatization period.

The change in plant height was significantly affected by substrate type ($p < 0.0001$), AM treatment ($p < 0.0001$) and initial height ($p < 0.0001$). The effects of substrate type and AM treatment varied over time (time × substrate × AM, $p < 0.0001$). The final measurements showed that, although their growth was slower at the beginning, AM plants in MIX reached heights similar to those of Control plants in AS (Fig. 1A). Control plants in MIX had a significantly lower height than the other plants, while AM plants in AS showed the greatest increase in height (Fig. 1A).

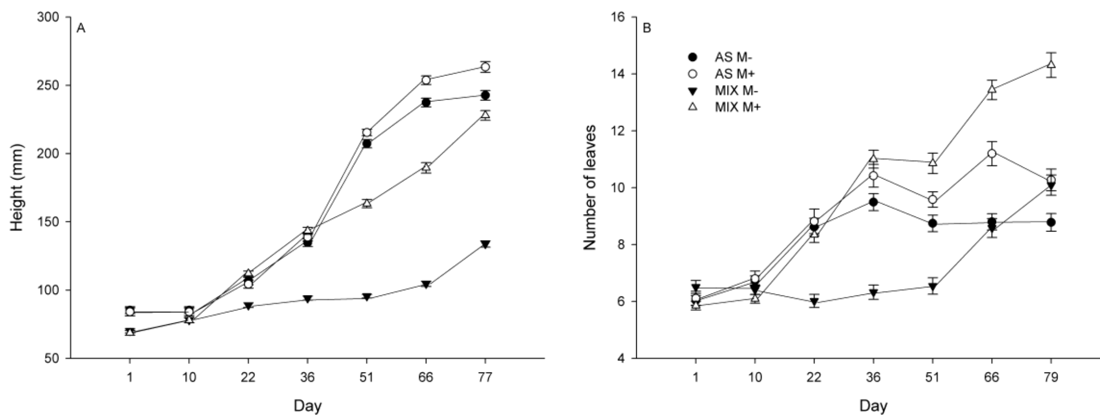

Figure 1. Effect of inoculation with mycorrhizae and substrate type on plant height (A) and the number of leaves (B) during acclimatization. Mean and standard error values are shown for AM plants (M+) and Control plants (M-) in agricultural substrate (AS) and agricultural substrate:sand (MIX).

There was a significant effect of AM inoculation on the change in the number of leaves ($p < 0.0001$) during acclimatization (Fig. 1B). Substrate type did not have a significant effect, but there was a significant interaction between substrate type and AM ($p < 0.0001$) since differences between Control and AM plantlets were greater for MIX plants. Moreover, the change differed depending on the two treatments (AM × substrate × time, $p < 0.0001$). AM plants in MIX showed a greater number of leaves at the end of the measurements (Fig. 1B). Control plants in MIX had the lowest number of leaves

until day 51 DAP, after which they experienced a greater increase in leaves than the others until reaching values similar to those of AS plants.

Table 1. Effects of AMF inoculation and substrate type (AS = agricultural substrate and MIX = agricultural substrate:sand) on total and aerial dry biomass, shoot:root ratio, leaf mass area (LMA) and chlorophyll *a* and *b* content (Chl *a* and *b*, respectively). Different letters mean significant differences ($p < 0.05$, post hoc Tukey test).

	AS		**MIX**	
	Control	**AM**	**Control**	**AM**
Total dry biomass (g)	0.55 ± 0.047a	0.58 ± 0.056a	0.30 ± 0.014b	0.56 ± 0.020a
Aerial dry biomass (g)	0.41 ± 0.037a	0.44 ± 0.048a	0.20 ± 0.009b	0.46 ± 0.019a
Shoot:root ratio	3.10 ± 0.161b	3.30 ± 0.243b	2.21 ± 0.173c	4.42 ± 0.313a
LMA (g/m^2)	44.97 ± 0.937a	39.58 ± 0.963b	46.65 ± 1.053a	38.41 ± 0.790b
Chl *a* (mg Chl/g plant)	1.28 ± 0.056b	1.57± 0.042a	1.32 ± 0.057b	1.60 ± 0.073a
Chl *b* (mg Chl/g plant)	0.30 ± 0.014bc	0.37± 0.012ab	0.32 ± 0.016c	0.39 ± 0.023a

Mycorrhizal inoculation positively affected plant total and aerial dry biomass (p = 0.0003 and $p < 0.0001$, respectively) in MIX, which increased by 86% and 130%, respectively. However, no significant differences between AM and Control plantlets were reported in the agricultural substrate (Table 1) since the interaction between substrate type and AM treatment was significant (total biomass $p = 0.0032$; aerial biomass $p = 0.0008$). Substrate type had a significant effect on both total biomass (p = 0.001) and aerial biomass ($p = 0.002$), although the values for AM plantlets were similar between the two substrates. Meanwhile, plants in AS showed greater biomass than plants in MIX in the case of Control plantlets (Table 1). The shoot:root ratio was significantly affected by the AM treatment ($p < 0.0001$) and the interaction between AM treatment and substrate type ($p < 0.0001$). The shoot:root ratio was higher in AM plantlets grown in MIX than in the other treatments (Table 1). The AM treatment had a significant effect on LMA ($p < 0.0001$), but LMAwas not affected by substrate type (p = 0.78). LMA was lower in AM plants in both substrate types (Table 1).

AM plants had significantly higher chlorophyll *a* and *b* contents than Control plants in both substrates ($p < 0.0001$ and $p < 0.0001$, respectively), while substrate type did not have a significant effect on the chlorophyll content (Table 1).

Establishment and biomass production in the field

During crop field establishment, only four Control plants from small cells died. AM treatment, substrate type and cell size had significant effects on the height of plants the day of transplantation and 52 DAT ($p < 0.0001$ in all cases). Plants from big (i.e., 300 cm^3) cells, AS and the AM treatment were significantly taller than those from small (i.e., 50 cm^3) cells, MIX or the Control treatment, showing similar differences between treatments the day of transplantation and 52 DAT (Fig. 2). Plants from small cells barely grew or even decreased in height because of the death of the principal stem (Fig. 2).

The increase in the number of stems between one and 52 DAT was significantly ($p < 0.0001$) higher in plants from big cells (9.16 ± 0.42) than in plants from small cells (3.58 ± 0.18). However, no significant effects of substrate, AM treatment or interactions among treatments were found ($p > 0.05$ in all cases). The increases in the number of stems were very variable, with a maximum of 20 and a minimum of 0.

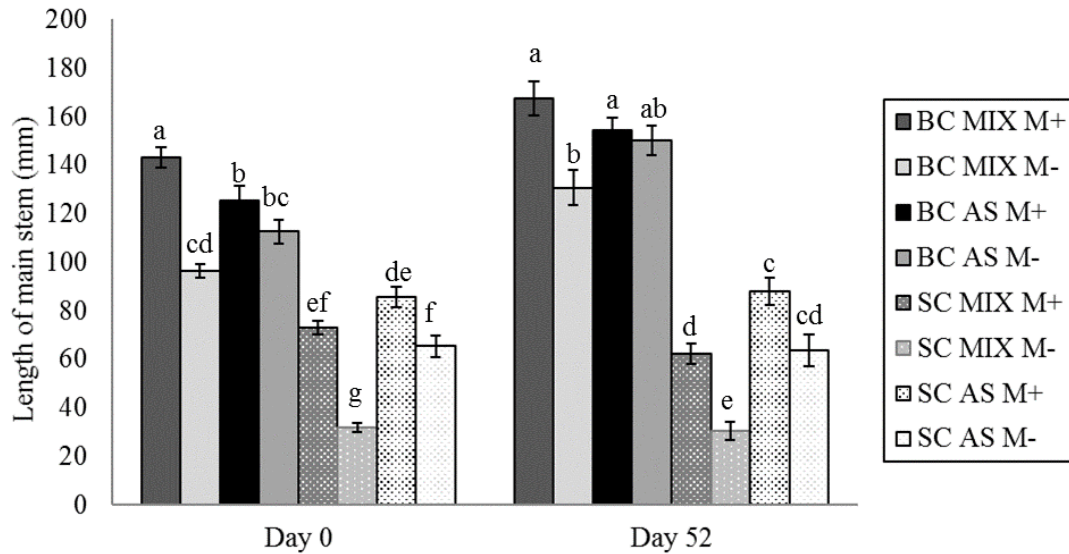

Figure 2. Effect of the inoculation with mycorrhizae, substrate type and cell size on the height the day of transplantation and 52 days after transplantation in the field. Mean and standard error values for AM plants (M+) and Control plants (M-) in agricultural substrate:sand (MIX) and agricultural substrate (AS) after growing in small cells (SC) or big cells (BC) are shown. Different letters mean significant differences on the same day (*$P < 0.05$,* post hoc Tukey test).

The first-year harvest was significantly affected by the initial height ($R^2 = 0.41$, $p < 0.0001$), and significant effects of the three factors were detected ($p < 0.003$ in all cases). In general, plants form bigger cells, AS and the AM treatment accumulated more aerial biomass than plants form small cells, MIX or the Control treatment (Fig. 3).

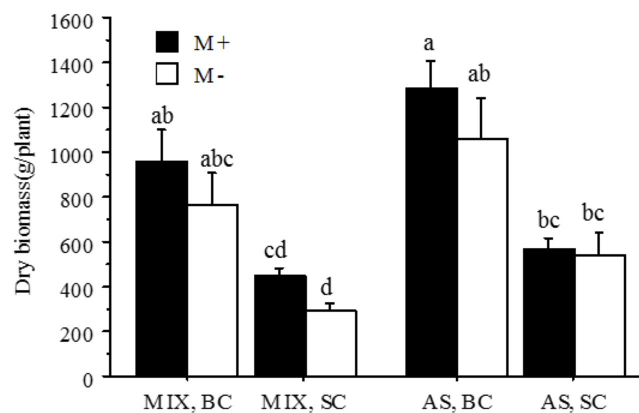

Figure 3. Effect of inoculation with mycorrhizae (M+) or no inoculation (M-), substrate type (agricultural substrate:sand (MIX) or agricultural substrate (AS)) and cell size (small cells (SC) or bigger cells (BC)) on the total dry biomass per plant after 9 months of growth in the field. Means and standard errors are shown. Different letters mean significant differences (*$p < 0.05$*, post hoc Tukey test).

Bigger cells resulted in a mean increase of 547.8 g/plant of dry biomass with respect to plants from small cells. Plants acclimatized in MIX produced 215.64 g/plant less than plants from AS. In addition, inoculation with AM during acclimatization increased the first-year harvest by 188 g/plant.

Second-year production was lower but still significantly influenced by the initial height ($R^2 = 0.15$, $p = 0.004$). A significant increase in dry biomass was detected in plants from trays with big cells (4.42 ± 0.34 kg/plant from big cells and 2.49 ± 0.35 kg/plant from small cells, $P = 0.0002$). Although no significant differences in the mean dry biomass per plant were detected for the AM ($p = 0.16$) and substrate treatments ($p = 0.08$), AM-inoculated plants from AS tended to be larger than Control plants from MIX.

Discussion

Our results demonstrate the better performance of *A. donax* cultivation when plants of a greater size and better quality were used at the time of planting. In fact, in the second year of harvest, the maximum yield would be obtained from large-cell plants acclimated with high-quality substrate and inoculated with AM, with an average estimated value of 53.36 t/ha. In contrast, the minimum harvest value, 18.78 t/ha on average, would be obtained from small-cell plants acclimated with sparsely nutritious substrate with low water-holding capacity.

AMF inoculation increases plant size and quality.

In accordance with the effects of AMF when tested in other energy crops (Clark *et al.*, 1999; Kumar *et al.*, 2010 and da Silva Folli-Pereira *et al.*, 2012, Mirshad and Puthur 2016), our results showed a positive effect of inoculation with AMF since better development was observed in plants inoculated with AM not only during acclimatization but also during the first and second year of crop production. This is in

agreement with Tauler and Baraza (2015), who reported that during acclimatization in agricultural substrate (AS), *A. donax* mycorrhized plantlets (AM) presented a higher increase in biomass compared to non-mycorrhized plantlets. In the present study, the use of a sparsely nutritious substrate and with low water-holding capacity (MIX, agricultural substrate:sand, 1:1) generated somewhat stressful conditions that significantly increased the differences between plants inoculated with AM and non-inoculated ones. During the colonization process, AM symbionts results in different responses enviromental conditions than those in non-symbiotic plantlets (Schweiger *et al*., 2014; Watts-Williams *et al*., 2015), which confers the capacity to avoid stressful soil conditions (Monticelli *et al*. 2000; Munier-Lamy *et al*. 2007; Huang *et al*. 2009). This effect of mycorrhizal colonization is conditioned by the growth environment (Porcel and Ruiz-Lozano, 2004). In fact, our results show that inoculation with AMF attenuates the effects of a sparsely nutritious substrate, allowing plants to exhibit the same growth as plants that had grown in agricultural substrate, while Control plantlets were 50% smaller. Therefore, the use of AMF facilitates the acclimatization conditions because it diminishes the need for a specific and expensive substrate.

However, AMF not only generated bigger plants, but the leaf morphology and chlorophyll content, which are directly related with nutrient and water acquisition, were different in AM and Control plantlets. It has been reported that AM symbiosis increases root hydraulic conductivity due to the promotion of aquaporin gene expression and by allowing the enhancement of apoplastic water flow (Ruiz-Lozano and Aroca, 2010; Bárzana et al., 2012). This enhancement of root development allows the capacity to improve uptake nutrient flow and, among other factors, increases chlorophyll synthesis and content, which was observed in the present work. Nonetheless, recent studies reported that AM symbiosis in micropropagated *A. donax* plantlets not only enhances

photosynthesis but also decreases root respiration and causes changes to primary metabolism (Romero-Munar et al., 2017). Taken all together, the results indicate that the plantlets that grew in symbiosis with AMF had a greater size and an increased capability to avoid stressful conditions. In fact, the effect of AM was still significant after 9 months of growth in the field and, although not statistically significant, differences in dry plant biomass were detected in the second-year harvest; AM can cause an increase of 19.5% in the dry biomass yield, which results in a mean increase of 6.25 t/ha.

Bigger cells generated bigger plants and greater plant biomass production.

In regard to the pot size, Poorter *et al*. (2012) reported in a review that small pots caused a reduction in photosynthesis per unit leaf area rather than changes in leaf morphology or biomass allocation. Thus, pot size selection in the nursery is critical for obtaining optimal plants for field establishment. During the period of establishment in the field, larger plant sizes allow greater initial growth, which facilitates competition with weeds and ensures establishment. In fact, only 4 plants died after transplantation, and all of them were from small cells. Interestingly, the effect of cell size was not only evident at the time of plant transfer to the field, but after 21 months of growth in the field, there were still significant differences in the total biomass of plants from cells of different sizes. The biomass of plants from 300 cm^3 cells compared to those from 50 cm^3 cells increased by 77.45% (44.63 t/ha of mean production for bigger cells for all other treatments combined and 25.15 t/ha mean production for small cells for all other treatments combined). In other crops, such as strawberry, an important effect of explant size on fruit harvest has already been observed (Torres-Quezada et al., 2015). Likewise, Puértolas et al. (2003) found a positive effect of seedling size on the development of *Pinus halepensis* three years after planting. This positive effect has been associated with

a larger root size, which allows the plant to further explore soil resources by generating positive feedback (Villar-Salvador et al., 2011).

Stressful conditions during plant acclimatization have permanent negative effects on plant growth.

Similar to AM inoculation, the substrate used during acclimatization not only affected plant development during this period but also had permanent effects that resulted in significant differences after 9 months of growth in the field. Plants acclimatized in suboptimal substrate (MIX) produced 25.23% less aerial dry biomass than plants acclimatized in AS. The suboptimal substrate likely generated two types of stress, water deficit and nutrient limitation. Water deficit has been traditionally used to harden perennial plants to generate seedlings that are more prepared to sustain transplant stress (Ruiz-Sanchez et al., 2000; Bañon et al., 2015); however, contradictory results have been reported (Vilagrosa et al., 2003; Franco et al., 2006). Similarly, it could be possible to find positive (Puértolas et al., 2003) or negative (Trubat et al., 2011) effects of fertilization in the nursery on seedling performance in the field among different perennial species. In both cases, the field environmental conditions are key in determining the effects of water and nutrient deprivation on plants. Under stressful conditions (e.g., degraded areas, as in Trubat et al., 2011), stressed plants may have advantages, whereas under more mesic conditions (such as former arable land, as in Puértolas et al., 2003) a larger size may be advantageous. In our case, the stress generated during acclimatization had negative subsequent effects likely because the cultivation conditions were favorable since plants were watered during the summer. Perhaps in more stressful situations, such as in non-irrigated areas or those with more degraded soil, the implementation of stressful conditions during acclimatization could

become an advantage. However, under these conditions, *A. donax* productivity is lower (Fagnano et al., 2015) with less economic viability (Testa et al., 2016).

Conclusions

Arundo donax plantlet size and quality, regulated by nursery conditions, not only determine survival and growth after transplantation to the open field but also influence first- and second-year biomass yield. Better nursery conditions resulted in larger plants that produce more biomass during the first and second year. Early inoculation of *A. donax* plantlets with AMF enhanced plant growth, especially under stressful conditions, allowed plant performance to mitigate ex vivo stress conditions and furthermore significantly improved areal biomass production in the first year. The peat-based agricultural substrate generated bigger plants not only during plant acclimatization but also increased the first- and second-year dry biomass production in comparison with the more stressful substrate. Pot size appeared to be the principal factor determining plantlet size, which was significantly related to first- and second-year biomass yield. Bigger cells resulted in a 77% increase in the second-year biomass harvest. The increased cost of generating bigger plants in the nursery could be offset by increases in biomass yield that can compensate for the initial financial investment.

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Discusión

La presente tesis doctoral aporta conocimiento sobre las características ecofisiológicas de *Arundo donax* que le confieren su gran potencialidad como especie para la producción de biomasa, por su alta capacidad de producción, pero también por la demostrada capacidad de tolerar estrés hídrico y salino y la baja necesidad de P, características todas ellas comunes a muchas de las tierras marginales en las que se proyecta su cultivo. Sin embargo, estas mismas características podrían estar relacionadas con su carácter invasor (Mack, 2008), cosiderándola una de las especies invasoras más amenazante, ya que puede superar diversos tipos de estrés aumentando su capacidad competitiva. Estos resultados remarcarian la necesidad del establecimiento de protocolos para minimizar el riesgo de invasión por esta especie. Asi Virtue et al (2010) recomiendan la ubicación de las plantaciones fuera de las zonas sujetas a inundaciones, creación de zonas de amortiguación alrededor de las plantaciones, encuestas anuales para detectar y eliminar las fugas y establecimiento de protocolos de higiene para la cosecha, el transporte y el procesamiento. Considerando el elevado coste económico de diversos programes de erradicación de la especie (Seawright eta al 2009; Torro et al 2012) estos protocolos deberian establecerse como obligatorios para dar los permisoso pertinentes para el cultivo de la especie.

Además, los resultados presentados en esta tesis demuestran la capacidad de *A. donax* para formar simbiosis con hongos formadores de micorrizas arbusculares (HMA), y las ventajas que dicha simbiosis supone para el desarrollo de las plantas generadas por micropropagación. La inoculación con HMA en la producción de plantel a partir de plantas micropropagadas se considera una técnica adecuada para mejorar la calidad de la planta y su futura tolerancia al estrés. La presente tesis demuestra la potencialidad de su uso en estadios muy primarios del desarrollo vegetal, ya que el simbionte generado muestra una aceleración del desarrollo de la planta con una mayor tasa de crecimiento inicial basada en una gestión de los recursos diferente a las plantas sin micorrizar. Es importante recalcar que la simbiosis debe desarrollarse en los primeros estadios de producción de planta durante la aclimatación.

El aumento de producción obtenido gracias al uso de plantas de mayor tamaño durante el primer y segundo año del cultivo en campo demuestra que una de las claves del éxito del cultivo de *A. donax* se basa en la generación del plantel de alta calidad. El estudio en profundidad de la biología de los estadios primarios del desarrollo vegetal de *A.*

donax que se ha llevado a cabo en la presente tesis ha permitido ampliar el conocimiento básico sobre esta especie, conocer mejor el funcionamiento de la simbiosis micorrícica y generar conocimiento para la mejora de la producción de plantel para su cultivo.

A. donax en los primeros estadios de desarrollo bajo condiciones de estrés hídrico y salino: tolerancia y adaptación a través de cambios fisiológicos, morfológicos y anatómicos

La sequía afecta negativamente a gran variedad de procesos fisiológicos y bioquímicos provocando una reducción del crecimiento y una menor producción final del cultivo (Ashraf *et al.*, 2011). Las plantas han desarrollado mecanismos para hacer frente al estrés hídrico, como la prevención de la sequía, evitar la deshidratación o incluso la tolerancia a la deshidratación. Estos mecanismos provienen de cambios a nivel bioquímico, fisiológico y morfo-anatómico. En la presente tesis doctoral, el establecimiento de la sequía progresiva permitió reconocer la capacidad de adaptación al déficit hídrico de *A. donax*, observando la plasticidad foliar que presenta, reduciendo el tamaño de las hojas y de su área foliar específica (*specific leaf area*, SLA), pero manteniendo el número de hojas producidas. Hojas más pequeñas presentan menores requerimientos de transpiración para refrigerarse (Nobel, 2009; Haworth *et al.*, 2017). Esta plasticidad en la morfología foliar bajo condiciones de estrés hídrico, resaltando la reducción del SLA, se ha relacionado con una alta eficiencia en el uso de recursos (Poorter y Remkes 1990; Wright y Westoby 2001). En concreto, es la plasticidad que presenta en las hojas formadas bajo estrés hídrico moderado la que le permiten la alta eficiencia en el uso del agua que ha mostrado en estadios tempranos de desarrollo bajo condiciones de restricción moderada de agua. Aparte de adaptaciones de tipo morfológico y anatómico, *A. donax* presenta cambios a nivel fisiológico, como su capacidad de ajuste osmótico, el cual le permitiría mantener el turgor celular y, por tanto, la elongación foliar en condiciones de sequía moderada.

Por lo que se refiere al aparato fotosintético, trabajos recientes han mostrado las altas tasas fotosintéticas y de conductancia estomática que presenta esta especie (Webster *et al.*, 2016; Sánchez *et al.*, 2016), características que ya presenta entre los dos y tres meses de desarrollo. Además, frente al déficit hídrico, *A. donax* muestra un rápido control estomático y un incremento de la eficiencia en el uso del agua. Esto se traduce en un mantenimiento de la tasa fotosintética (A_N) en un rango amplio de conductancia

estomática (g_s), entre 0.500 mol H_20 m^{-2} s^{-1} - en condiciones óptimas de riego - y 0.200 mol H_20 m^{-2} s^{-1}, punto a partir del cual la A_N se reduce. A nivel bioquímico, *A. donax* protege el aparato fotosintético, manteniendo intactos los valores de velocidad de carboxilación de la rubisco ($V_{c,max}$) así como la capacidad máxima de la cadena de transporte de electrones (J_{max}), lo que implica que toda la limitación fotosintética observada en sequía se debe únicamente al cierre estomático. Así pues, frente a bajas cantidades de agua disponible en el sustrato (20-30% de la capacidad de campo de la maceta), *A. donax* presenta un nivel de sequía que puede ser caracterizado como moderado o leve, reajustando rápidamente a nivel fisiológico pero incluso morfo-anatómico para garantizar el mantenimiento del crecimiento, a pesar de una reducción de biomasa final en comparación con las plantas crecidas sin restricciones hídricas.

En lo que se refiere al estrés salino, a pesar de que diferentes especies, incluso variedades dentro de la misma especie, puedan manifestar diferentes grados de tolerancia a la salinidad, en general ésta afecta a la germinación de semillas, al crecimiento vegetativo, a la adquisición de nutrientes y al metabolismo debido a la sequía fisiológica generada a nivel osmótico, así como los efectos tóxicos de los iones de la sal y el consecuente desequilibrio nutricional que causan (Munns y Termaat 1986; Pugnaire et al. 1999; Munns y Tester 2008). En el estudio sobre la respuesta de *A. donax* a la salinidad realizado en esta tesis doctoral (capítulo 3), *A. donax* muestra una respuesta general al estrés que se resume a nivel de biomasa, reduciéndola significativamente al someter a la planta a estrés moderado y severo (75 mM y 150 mM NaCl respectivamente). A diferencia de la respuesta a la sequía, en la que se observaron cambios morfo-anatómicos a nivel foliar, bajo los niveles de salinidad a los que se sometieron las plantas, no se observó este reajuste. Sin embargo, se observó un ajuste osmótico a través de la utilización del Na^+ disponible, sin que éste desplazara al K^+ en ningún caso. A pesar del aumento de la concentración de Na^+ en hoja, el hecho de que los valores de K^+ se mantengan, demuestra una gestión activa del exceso de Na^+, moderando el nivel de toxicidad. A pesar de que al tratarse de estadios juveniles en los que todavía no se había formado el rizoma, la raíz de *A. donax* actuó como un órgano importante para la retención Na^+, reduciendo la translocación de éste a las hojas.

Con el fin de simular condiciones típicas de tierras marginales en ambiente mediterráneos, además del estrés salino se añadió un tratamiento de limitación de fósforo, aplicándolo en una concentración 10 veces menor respecto a las plantas

crecidas con la solución nutricional control. Como ya se ha comentado al principio de esta discusión, es ampliamente conocido el bajo requerimiento nutricional de *A. donax* una vez que el cultivo se ha implantado (Pilu *et al.*, 2013; Scordia *et al.*, 2014). Durante los primeros estadios de desarrollo en maceta estudiados en la presente tesis, *A. donax* ha mostrado una alta eficiencia en el uso del fósforo inorgánico (P_i), ya que, a pesar de la diferencia de concentración de P_i en tejido, la diferencia de biomasa producida solo fue significativamente menor a las plantas crecidas con concentraciones de P_i control en el tratamiento de baja salinidad (1 mM NaCl) en raíces. La falta de respuesta de *A. donax* a la carencia de fósforo, que incluye entre otras un aumento de la razón raíz/tallo y de la producción de antocianos (Hammond *et al.*, 2011), indica que la cantidad suministrada de P_i no fue lo suficientemente baja como para que se iniciara la señalización en esta especie. Al someter a estas plantas a niveles moderados y severos de salinidad, las respuestas mostradas fueron muy similares a las explicadas anteriormente. Los valores de eficiencia en el uso del P_i de estas plantas fue significativamente superior a las plantas sin carencia, pudiéndose apuntar a una gestión del P_i a nivel vacuolar (Yang *et al.*, 2017), lo que pondría de manifiesto que *A. donax* no sólo presenta un bajo requerimiento de fósforo, sino también una elevada eficiencia en su uso (PUE). Esta elevada PUE se observó tanto a nivel de hoja como de raíz, lo que tiene una importancia relevante para el éxito de la propagación y supervivencia de la planta, al tratarse de una especie de reproducción asexual que en el medio natural se propaga tanto por fragmentos de tallo como fragmentos de rizoma.

Los resultados extraídos de la presente tesis doctoral de la descripción de la fisiología y respuestas al estrés de *A. donax*, muestran que la clave del éxito de esta especie como cultivo para biomasa en zonas marginales de ambientes mediterráneos radica en la capacidad de adaptación a los estreses principales del suelo, déficit hídrico, salinidad y carencia de nutrientes, gracias a la plasticidad foliar que presenta, la capacidad de ajuste osmótico y una alta eficiencia en el uso del fósforo.

La simbiosis con HMA: "el frágil equilibrio de la vida del simbionte, basada en el intercambio y la solidaridad máxima en la fusión de organismos" *Dr. López-Otín*

Dentro de la controvertida historia de las micorrizas arbusculares (MA), está la interpretación darwiniana de la competencia por la supervivencia, o bien el paradigma

de la simbiosis tal como la describió en 1981 la Dra. Margulis (Margulis, 1981) y que resume muy bien la cita que encabeza esta sección, del catedrático genetista Dr. López-Otín.

Como se comentó en la introducción, en los ecosistemas naturales el 80% de especies terrestres establecen simbiosis con hongos formadores de micorrizas (Phillips & Hayman, 1970). Los motivos del éxito de esta simbiosis y por qué se ha dado y se sigue dando son varios y de diversa índole, desde procesos evolutivos que permitieron el paso de las plantas al medio terrestre (Bonfante & Genre, 2008; Parniske, 2008), hasta la capacidad de tolerar estreses bióticos y abióticos que presentan las plantas micorrizadas o simbiontes, como se les denomina en esta tesis (Aroca y Ruiz-Lozano 2009; Mohammadi et al. 2011; Lenoir et al. 2016, y todas las referencias que contienen). Existe mucha literatura sobre esta simbiosis y aún hoy sigue creando debate.

En una revisión muy personal sobre la historia de la investigación de micorrizas arbusculares, Koide y Mosse (2004) fechan la primera descripción de los HMA en 1842, por Nägeli. En 1943 fue la primera vez que se demostró que las plantas micorrizadas crecían más rápido (Koide y Mosse 2004), aunque aún quedaba un largo camino hasta entender los procesos por los cuáles esto sucede, y que aún hoy siguen generando inquietudes en la comunidad investigadora. Ya en 1968, Scannerini y Bellando describieron un espacio entre la membrana de la célula vegetal y la pared del hongo que contenía material vegetal, definiendo así lo que se conoce hoy como membrana periarbuscular (Pumplin y Harrison 2009). En ese espacio es donde se genera el intercambio planta-hongo y es un espacio que sólo existe durante la simbiosis. En ese espacio radica la íntima relación simbiótica, y seguramente donde residen gran parte de las primeras respuestas sobre el efecto de la simbiosis en la respuesta del vegetal. Una de las características básicas de cualquier relación simbiótica es la generación de propiedades emergentes en el simbionte. Por ende, la simbiosis planta-HMA (simbiosis MA a partir de ahora) genera un simbionte con tales propiedades (como la generación de estructuras propias que no poseen los componentes por separado, como, por ejemplo, el espacio periarbuscular) y que se reflejan en respuestas del vegetal (porque generalmente es el centro de estudio) diferentes de los no simbiontes. Para que esas propiedades se den, se producen cambios a nivel anatómico, fisiológico y bioquímico. Con el fin de analizar esos cambios y definir el funcionamiento de la simbiosis entre *A. donax* y HMA, se realizó el experimento del capítulo 2 de esta tesis. Con él se ha

contribuido no sólo a aportar nuevos datos sobre el efecto de la simbiosis MA en el incremento de la biomasa de las plantas de *A. donax*, el aumento de su tasa fotosintética, así como de los cambios a nivel del metabolismo primario de la hoja y la raíz observados en el simbionte, sino que, hasta donde llega mi conocimiento, es la primera vez que se muestran datos de una disminución de la respiración de la raíz del simbionte derivada de una disminución de la actividad de la citocromo oxidasa (v_{cyt}). La figura 1 muestra la relación de la disminución de v_{cyt} con los citados cambios del metabolismo de la raíz, que unido a los resultados de Watts-Williams et al. (2015) permite establecer una relación de la disminución de v_{cyt} con una disminución de la vía directa de absorción de nutrientes y una mayor actividad de la vía de absorción a través de la micorriza. Hasta el momento, los estudios sobre el efecto de MA sobre la respiración habían mostrado incrementos de la tasa respiratoria de la raíz del simbionte asociada al mantenimiento del hongo (Baas *et al.*, 1989; Wright *et al.*, 1998; Grimoldi *et al.*, 2006; Atkin *et al.*, 2009). Florez-Sarasa et al. (2007) relacionaron la actividad de la vía alternativa de la respiración con el componente de mantenimiento de ésta, por lo que, si el incremento de respiración de la raíz observado en los citados trabajos es debido al gasto energético que implica el mantenimiento del hongo, debería esperarse un aumento de la actividad de la oxidasa alternativa (v_{alt}). Sin embargo la técnica de fraccionamiento de isótopos (Ribas-Carbo *et al.*, 2005) usada en esta tesis, muestra como v_{alt} no varía respecto a las plantas no simbiontes, y v_{cyt} se ve reducida en la raíz del simbionte. Podría interpretarse como una interrelación de fuente y sumidero (Engels *et al.*, 2012), sobre todo en los primeros estadios del desarrollo, dado que como se demuestra en el capítulo 2 de esta tesis, la parte fúngica del simbionte desarrolla un papel clave en el ahorro de energía y su reinversión en biomasa para el componente vegetal. Si esta relación cambia en los diferentes estadios de desarrollo (desde el establecimiento de la simbiosis hasta la finalización del ciclo vital del simbionte) es una línea de investigación que queda por explorar y que aportaría conocimiento sobre la dinámica de esta simbiosis. El aumento de biomasa del simbionte respecto a las plantas no simbióticas y la relación de esto con la disminución de la v_{cyt}, puede estar relacionado con la eficiencia en la captación de nutrientes de la vía micorrícica (Smith *et al.*, 2011; Watts-Williams *et al.*, 2015) En la figura 1, elaborada a partir de la interpretación de los resultados del capítulo 2, esta hipótesis (indicada con flechas grises) está añadida como siguiente paso en el estudio de esta línea de investigación. Como se ha comentado

anteriormente, estos cambios morfológicos, fisiológicos y bioquímicos son la base de las propiedades emergentes o respuestas diferentes observadas en los simbiontes.

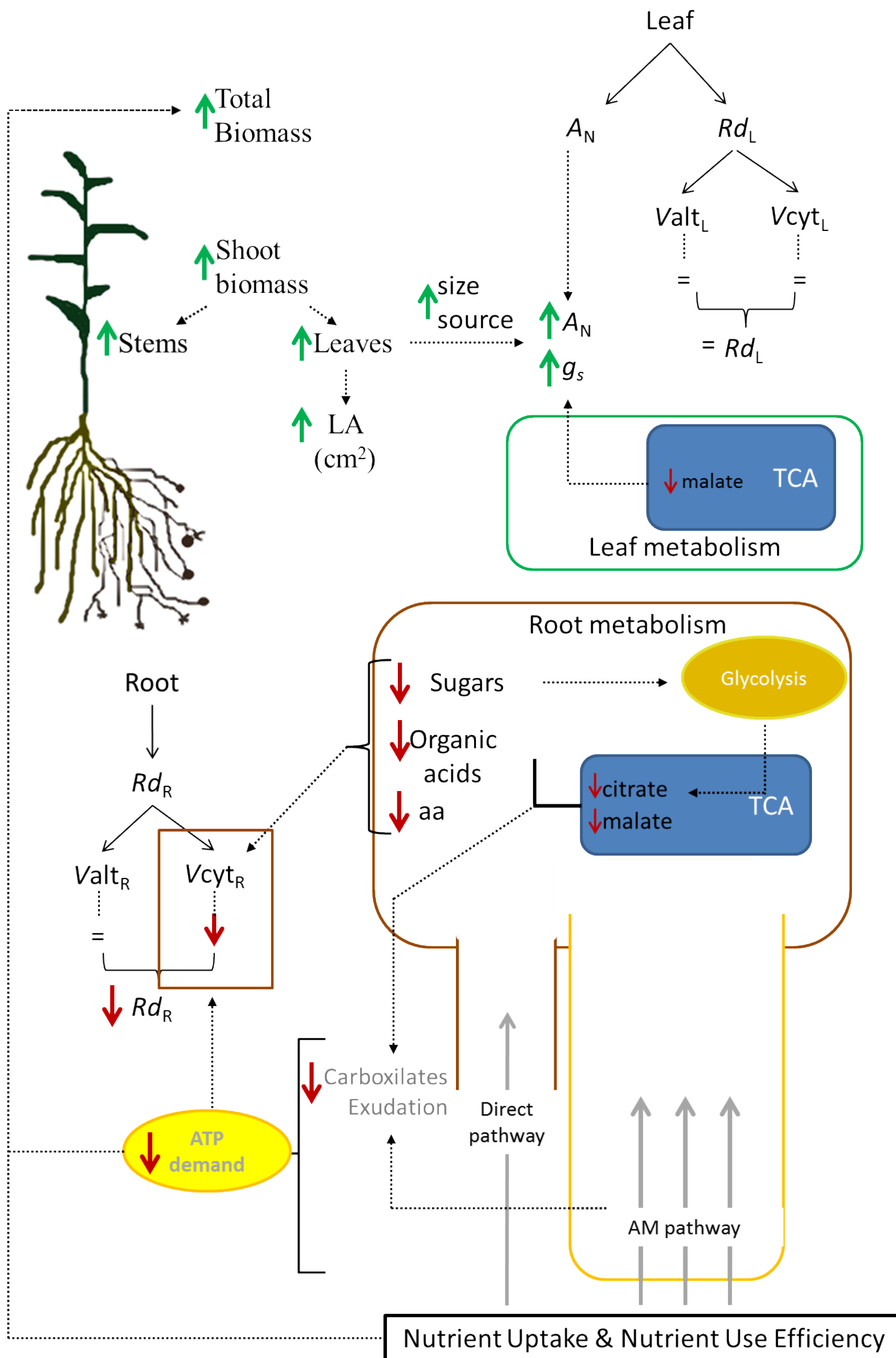

Figura 1. Esquema de la relación entre la disminución de la actividad de la vía citocrómica de la respiración de la raíz, el cambio en el metabolismo primario y el incremento de biomasa del simbionte. Las flechas negras indican parámetros medidos en esta tesis, mientras que las flechas grises conforman el marco teórico en el que se desarrolla la explicación de la conexión entre los cambios producidos en respiración y biomasa. Las flechas de color verde representan incrementos estadísticamente significativos, mientras que las rojas indican disminuciones

significativamente diferentes respecto a no simbiontes. (Abreviaturas según aparecen en la sección de símbolos y abreviaturas de esta tesis).

Bajo condiciones de combinación de dos estreses comunes en suelos marginales como la salinidad y la falta de fósforo (capítulo 3), el simbionte presenta una mayor biomasa, incluso por encima de las plantas crecidas sin carencia de fósforo en niveles de 1 mM y 75 mM NaCl, a pesar de la reducción de colonización observado bajo condiciones salinas. La figura 2 muestra como a pesar de presentar la misma concentración total de Na^+, a 1 y 75 mM NaCl, el simbionte ha desarrollado una mayor área y biomasa foliar (también observado en los resultados del capítulo 2 y en la figura 2 de esta sección), y una mayor biomasa de raíz. El simbionte muestra una mayor retención de Na^+ a nivel radicular, lo que conlleva una menor translocación hacia hojas fotosintéticamente activas. Esta menor actividad de Na^+ en la hoja, sumada a la mayor translocación de K^+, permite la mayor producción de biomasa del simbionte bajo 75 mM NaCl respecto a las plantas de los otros dos tratamientos.

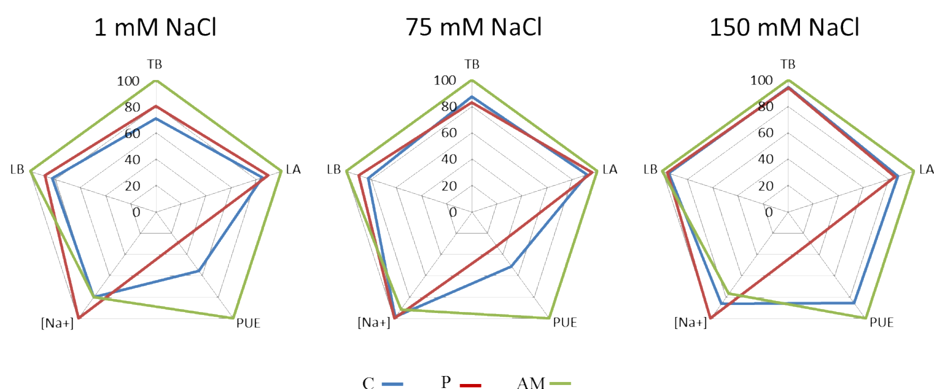

Figura 2. Resumen de los resultados más representativos analizados en el capítulo 3 de los tres tratamientos con NaCl (biomasa total – TB; área foliar – LA; eficiencia en el uso del fósforo – PUE; concentración de sodio total – [Na^+] y biomasa foliar – LB), siendo 100 el valor de la media más alta al que se referencian los demás valores. Las líneas azul, roja y verde corresponden respectivamente a las plantas control, C (deficitarias en Pi); plantas crecidas con fósforo suficiente, P; y plantas en simbiosis con micorrizas arbusculares y con déficit de fósforo, AM.

Las plantas han desarrollado estrategias para aumentar la adquisición de fósforo (entre las que se incluye la simbiosis MA) y otras adaptaciones como el aumento de la eficiencia del uso metabólico del fósforo dentro de la planta. Entre éstas últimas, destacan la disminución del ratio de crecimiento, el incremento del crecimiento por unidad de fósforo absorbido, la removilización del fósforo interno, modificaciones del metabolismo del C y de las vías respiratorias alternativas (Lynch *et al.*, 2005). Como se ha comentado en el primer apartado de esta sección, *A. donax* presenta una alta eficiencia en el uso del fósforo. Los resultados de esta tesis muestran que la simbiosis MA confiere una mayor eficiencia en el uso de fósforo del simbionte, así como de otros nutrientes, como el Mg^{2+} y el K^+. Smith et al. (2011) mostraron que las plantas asociadas con HMA (en esta tesis llamadas simbiontes), presentan una vía específica

para la adquisición de fósforo que empieza por las hifas extraradicales en el suelo, por donde el P_i es translocado hacia las raíces y liberado a través del arbúsculo al espacio periarbuscular. Ese P_i es transportado a través de la membrana periarbuscular por transportadores específicos de Pi, que aunque forman parte de la familia de los Pht1, sólo se expresan en el simbionte (Maeda *et al.*, 2006; Javot *et al.*, 2007). En un reciente estudio sobre la expresión de transportadores específicos de *Rhizophagus irregularis* y *Funneliformis mosseae*, tras analizar los resultados sobre la expresión de transportadores específicos de Pi y su papel en la liberación de éste al espacio periarbuscular, Walder et al. (2016) se atrevieron a lanzar la hipótesis de que la adquisición de P_i a través de la vía micorrícica podría estar más regulada por el hongo o por la planta, dependiendo de las especies implicadas. Ese "baile" entre los componentes del simbionte se podría trasladar no solo a la identidad de los componentes, sino también al lugar donde ocurre ese "baile". Es decir, según las condiciones de crecimiento y las especies implicadas en la simbiosis, variará la eficiencia en la adquisición de P_i. En el caso del simbionte *A. donax* - *R. irregularis* - *F. mosseae*, ha resultado en una alta eficiencia no solo en la adquisición del poco Pi disponible en la solución nutritiva, sino que ha habido cambios en la eficiencia de su uso. Una hipótesis podría estar relacionada con la rizoeconomía del coste de carbono que supone la adquisición de Pi (Lynch *et al.*, 2005), y que se ligaría con la disminución de la actividad de la vía citocrómica en la raíz observada en el capítulo 2 de esta tesis, junto con el aumento de biomasa final, tanto en el capítulo 2 como en el 3. Se puede especular con que el gasto de ATP que conlleva la absorción a través de la vía micorrícica del simbionte es mucho menor que la del a vía directa de la raíz. A través del análisis de transportadores, junto con la técnica de fraccionamiento de isótopos, en un estudio a lo largo del ciclo fenológico del simbionte, se podría arrojar luz de los cambios de actividad de los dos componentes, y observar el cómputo final a través de la fisiología del simbionte.

El frágil equilibrio al que hace referencia el encabezado de esta segunda parte de la discusión radica precisamente en que estos cambios en la fisiología del simbionte no siempre conllevan un mayor éxito del simbionte respecto de las plantas no simbióticas (Carling & Brown, 1980; Hayman, 1980; Azcón & Ocampo, 1981; Allen & Boosalis, 1983; Augé, 2001). Esa mayor o menor eficiencia del simbionte (en términos de producción de biomasa, por ejemplo) vendrá determinada por la identidad de los

componentes de la simbiosis, así como por las condiciones ambientales en las que se haya desarrollado la simbiosis. Por esta razón, el estudio integral de la simbiosis MA desde sus procesos moleculares hasta la implantación del simbionte en campo, arrojaría grandes ventajas para su manejo en agricultura.

De la investigación básica a la aplicada, ¿Es posible mejorar el cultivo de *Arundo donax*?

A lo largo de los estudios realizados en esta tesis, *Arundo donax* ha demostrado ser una especie de crecimiento rápido y resistente. De hecho, las plantas micropropagadas de *A. donax* presentan altas tasas de supervivencia durante el período de aclimatación y en el proceso de trasplante a campo (capitulo 4). Sin embrago, si bien la supervivencia es alta, hay importantes descensos en la producción de biomasa de las plantas de *A. donax* que crecen en condiciones de estrés (capitulo 1 y 3). *Arundo donax* puede tolerar una gran diversidad de condiciones de estrés como la sequía, la salinidad, las inundaciones y los metales pesados (Lewandowski *et al.*, 2003b; Papazoglou *et al.*, 2005; Nassi o Di Nasso *et al.*, 2013). Sin embargo, los resultados de esta tesis doctoral muestran su sensibilidad a la falta de agua (bien debido a déficit hídrico bien a estrés salino) durante las primeras etapas de crecimiento. Esto es consistente con Perdue (1958), quien reportó que el crecimiento de *A. donax* puede ser retardado seriamente por la falta de humedad durante su primer año. En los ensayos en condiciones controladas, la inoculación con HMA ha demostrado ser efectiva para incrementar la velocidad de crecimiento y aumentar la tolerancia al estrés de los juveniles de *A. donax* (capítulos 2, 3 y 4). Nuestros estudios han demostrado que un inóculo comercial de dos especies comunes (Tabla 1 de la introducción) no sólo es capaz de establecer simbiosis con *A. donax*, sino que produce mejoras en su crecimiento y capacidad de tolerancia al estrés durante la fase de aclimatación de plantas micropropagadas y el crecimiento de los juveniles (capítulos 3 y 4).

A pesar de la multitud de estudios que demuestran los potenciales beneficios de HMA sobre las plantas hospedadoras en condiciones controladas, existen menos datos sobre su influencia en condiciones de cultivo (Tawaraya y Turjaman 2014). Hijri (2016) demuestra aumentos en las cosechas de patata inoculada con *Rhizophagus irregularis* por encima del umbral económicamente rentable en casi el 79% de un conjunto de 231 ensayos de campo durante un período de 4 años en América del Norte y Europa bajo

condiciones de campo. Douds y colaboradores (2016) demuestran un efecto positivo del uso de un inóculo multiespecifico no comercial sobre la producción de 7 variedades de tomate. En general para los cultivos hortícolas Baum y colaboradores (2015) en una revisión bibliográfica muestran que en suelos con bajo inóculo MA nativo y condiciones de crecimiento bastante desfavorables, la inoculación puede dar lugar a un incremento del crecimiento, una mayor tolerancia al estrés abiótico, una resistencia mejorada a los patógenos y una mejor calidad del producto del simbionte. En algunos casos, el uso de inóculo se ha combinado con la fertilización mostrando que el simbionte es capaz de hacer un mejor uso de los fertilizantes (caso de la soja) o requerir menores dosis de los mismos (caso del algodón) (Cely *et al.*, 2016).

En el caso de cultivos de gramíneas perenes de ciclo largo para biomasa existen algunos ensayos sobre los beneficios del uso de micorrizas en las primeras fases de cultivo (Tauler y Baraza 2015), pero poco se sabe de su efecto a largo plazo en cultivos que pueden durar hasta 20 años.

En nuestro caso, los resultados de campo (capitulo 4) mostraron la importancia de la calidad de la planta para el establecimiento en el campo y la producción del primero y segundo año en el cultivo de *A. donax*. Si bien las mayores producciones de biomasa se dieron en plantas de gran tamaño en el momento del trasplante, obtenidas gracias al uso de alveolos de mayor volumen, se detectó un efecto positivo y significativo del uso de inóculo comercial hasta el primer año de cosecha y una tendencia a una mayor producción de biomasa de plantas inoculadas durante el segundo año.

Los resultados de esta tesis indican que el uso estandarizado del inóculo de HMA puede ser muy útil para la producción a gran escala de *A. donax*, no sólo para generar plantel de alta calidad que asegure el establecimiento en el campo, sino también para mejorar el rendimiento de los primeros años. Sin embargo, se necesitan más estudios para comprobar el mantenimiento del efecto a medio y largo plazo, ya que se trata de un cultivo perenne que suele durar entre 15 y 20 años.

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Conclusiones

1. *Arundo donax* es una especie con una alta eficiencia en el uso del agua: bajo condiciones de estrés hídrico moderado, presenta una mayor reducción del consumo de agua en comparación a la reducción de biomasa, lo que incrementa significativamente su eficiencia en el uso del agua.

2. *Arundo donax* presenta plasticidad foliar y capacidad de ajuste osmótico, que le confieren tolerancia a la sequía moderada.

3. *Arundo donax* presenta un alto control estomático, con una rápida respuesta de la g_s frente al déficit hídrico, con leves reducciones de la tasa fotosintética.

4. La regresión significativa observada entre g_s y la cantidad de agua en el suelo, permiten la aplicación de un riego controlado y de precisión, una herramienta clave para el manejo eficiente del agua en este cultivo en condiciones de clima mediterráneo.

5. La simbiosis MA entre *A. donax* - *R. irregularis* y *F. mosseae* da lugar a un simbionte que presenta mayores tasas fotosintéticas y una reducción de la respiración radicular en comparación con plantas no simbióticas.

6. El simbionte presenta mayor A_N debido a menores limitaciones fotosintéticas de tipo difusivo (a nivel estomático y del mesófilo) y a un aumento en la capacidad de la cadena de transporte de electrones a nivel de J_{max} y J_{flu}.

7. La reducción de la tasa respiratoria observada en la raíz del simbionte, en comparación con las plantas no simbióticas, es debida a una disminución de v_{cyt} y, paralelamente, a una reducción de los metabolitos relacionados con la respiración.

8. *Arundo donax* es tolerante al estrés salino moderado, presentando una gestión activa del Na^+ y mecanismos de exclusión, a pesar de lo cual la producción de biomasa se ve reducida.

9. *Arundo donax* posee una elevada eficiencia en el uso del fósforo (PUE), sin reducciones significativas de su producción de biomasa en comparación con plantas crecidas sin restricciones nutricionales.

10. El simbionte crecido en condiciones de bajo fósforo presenta valores de biomasa y PUE mayores que las plantas no simbiontes crecidas en las mismas condiciones, y también en comparación con plantas crecidas en niveles suficientes de fósforo.

11. El simbionte presenta mayor producción de biomasa también bajo condiciones de estrés combinado de salinidad moderada y escasez de fósforo, consecuencia de una menor razón de Na^+/K^+ y una mayor PUE tanto a nivel de tallo como de raíz.

12. El tamaño y la calidad de las plantas de *A. donax* provenientes de microprogación aclimatadas en condiciones controladas en semillero, no sólo determinan la

supervivencia y el crecimiento después del trasplante al campo abierto sino que también influyen en el rendimiento de la biomasa del primero y segundo año.

13. El sustrato agrícola basado en turba generó plantas más grandes no sólo durante la aclimatación de la planta, sino que también aumentó la producción de biomasa seca de primero y segundo año en comparación con el sustrato más estresante.

14. El tamaño de las plantas antes de ser transplantadas es fuertemente determinado por el tamaño de alveolo donde se produce la aclimatación, y está altamente corelacionado con el rendimiento de la biomasa del primero y segundo año.

15. La inoculación con HMA en estadios tempranos de las plantas generadas por micropropagación incrementa el crecimiento, especialmente bajo condiciones de estrés generadas por el sustrato, dando lugar a un simbionte capaz de sobrevivir en condiciones ex vivo y aumentar la biomasa producida el primer año de cultivo, en comparación con plantas no simbióticas.