Salinidad y trigo duro: Firmas isotópicas, actividad enzimática y expresión génica

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SALINIDAD Y TRIGO DURO:
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Memoria presentada por Salima Yousfi para optar al título de Doctor por la *Universitat de Barcelona*. Este trabajo se enmarca dentro del programa de doctorado "Biología de las Plantas en Condiciones Mediterráneas", correspondiente al bienio 2005/2007 de la *Universitat de Barcelona*. Este trabajo se ha realizado en el Departamento de Biología Vegetal de la Facultad de Biología de la *Universitat de Barcelona* bajo la dirección del Dr. Josep Lluís Araus Ortega y la Dra M. Dolors Serret Molins.

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Abreviaciones y símbolos

A_{sat}, Light saturated net CO_2 assimilation rate

ABA, Abscisic acid

ANOVA, Analysis of variance

*c*a, Ambient CO_2 concentration (µmol mol^{-1})

*c*i, Intercellular CO_2 concentration (µmol mol^{-1})

C_T, The threshold cycle

dm, Dry matter

dSm^{-1}, DeciSiemens por metro

DI, Deficit irrigation (35% pot capacity) with normal Hoagland solution

DI-12 dSm^{-1}, Deficit irrigation with Hoagland solution at 12 dS m^{-1}

EA, Elemental analyser

FI, Full irrigation (i.e. control) with normal Hoagland solution

FI 12 dSm^{-1}, Full irrigation with Hoagland solution at 12 dS m^{-1}

FI 17 dSm^{-1}, Full irrigation with Hoagland solution at 17 dS m^{-1}

*F*v'/*F*m', Efficiency of excitation energy capture by open PSII reaction centres

GS, Glutamine synthetase (GS; EC 6.3.1.2)

g_s, Stomatal conductance

GS1, Cytosolic isoforms glutamine synthetase

GS2, Plastic isoforms Glutamine synthetase

IAEA, International atomic energy agency

IR, Induction ratio

IRGA, Infrared gas analyzer

IRMS, Isotope Ratio Mass Spectrometer

N, Nitrogen content

NHX1, Na^+/H^+ antiporters

NR, Nitrate reductase (NR; EC 1.6.6.1)

P_i, Presión parcial intercelular de CO_2

P_a, Presión parcial de CO_2 en la atmósfera

ROS, Reactive oxygen species

SCT, Serveis Científico-Tècnics

SPAD, Special Products Analysis Division

T, Transpiration rate

TaDREB, Triticum aestivum dehydration responsive element binding

RIL, Recombinant inbred line

VPDB, Vienna Pee Dee Belemnite calcium carbonate

VSMOW, Vienna Standard Mean Oceanic Water

wsf, Water soluble fraction

WUE, water use efficiency

δ_p, Product isotope composition

δ_r Reactive isotope composition

$\delta^{13}C$, Carbon isotope composition

$\delta^{15}N$, Nitrogen isotope composition

$\delta^{18}O$, Oxygen isotope composition

$\Delta^{13}C$, Carbon isotope discrimination

INTRODUCCIÓN

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Los cereales ocupan aproximadamente una superficie de 127 millones de hectáreas en el continente europeo (FAOSTAT, 2009). Entre todos los cereales, el trigo y la cebada son los dos cultivos de mayor importancia al nivel mundial, así como los dos principales en la región del mediterráneo, representando un 55% y un 27% respectivamente de la superficie cultivada de cereales durante los últimos 40 años (FAOSTAT, 2009). El trigo duro es el cultivo principal en el área mediterránea, ya que el 60 % de la producción mundial se localiza en esta región, siendo la Unión Europea la principal productora (Morancho, 2000). Le sigue Argelia, Marruecos, Siria, Túnez y Turquía que en conjunto cultivan cerca de un tercio de la superficie mundial, pero que también tienen una demanda interna bastante alta y figuran entre los principales países importadores (Morancho, 2000). En España el cultivo de trigo duro se concentra en Andalucía, Extremadura y Aragón, cultivándose mayoritariamente en condiciones de secano. Como ocurre en toda el área Mediterránea, los cultivos de secano se caracterizan por tener importantes fluctuaciones del rendimiento, debido normalmente a estreses abióticos como la sequía y la salinidad.

La salinidad afecta aproximadamente un 6 % del área total cultivada del mundo, que asciende a 930 millones de hectáreas (Munns y Tester, 2008). A dicha área habría que añadir un incremento del 10% cada año (Pannamieruma, 1984). En un campo agrícola, la salinidad del suelo no es natural, sino que tiene, sobre todo, un origen antropogénico. En el pasado, este tipo de estrés era relativamente poco importante y se localizaba sólo en áreas cercanas a la costa o en determinados ambientes con abundancia de sales en el suelo procedentes de la evaporación de aguas cargadas de sales. Sin embargo el desarrollo experimentado por las actividades agrícolas en los últimos años, unido a una mala gestión de los recursos medioambientales, ha provocado que el estrés salino sea hoy en día uno de los principales problemas con los que se enfrenta la agricultura. La irrigación de cultivos con aguas de mala calidad, o con aguas de alto contenido salino, o un sistema de drenaje deficiente, provoca, tras la evapotranspiración, la acumulación de sales en el suelo. Además el empleo indiscriminado de grandes cantidades de fertilizantes

químicos y la sobreexplotación de los acuíferos han ocasionado un drástico aumento de la superficie afectada por la salinidad. Este fenómeno convierte en improductivos muchos suelos agrícolas. En la actualidad, más del 20% de los suelos cultivados y aproximadamente el 50% de las tierras irrigadas, están catalogados como potencialmente salinos (Rhoades y Loveday, 1990) y gran parte de ellos se localizan en la cuenca mediterránea. En España, se estima que unas 840.000 ha están afectadas por la salinidad; la mayoría de ellas se encuentran en el valle del Ebro (Alberto et al., 1986).

El aumento de la frecuencia de los períodos de sequía en muchas regiones del mediterráneo y los problemas asociados con la salinidad del agua de riego, a menudo, son consecuencia de la aparición consecutiva de la sequía y la salinidad en las tierras cultivadas, y plantean serios problemas que limitan la productividad de los cultivos (Isla et al., 1998). El riego inadecuado, a largo plazo y en condiciones de elevada demanda evapotranspirativa, junto con el empleo de aguas de mala calidad (por ejemplo recicladas), un abonado excesivo y la falta de un drenaje adecuado inducen frecuentemente la salinización de las tierras cultivables.

Resumiendo, la salinidad junto con la sequía son dos factores ambientales muy importantes que limitan la producción de los cultivos en muchos lugares del mundo, pero sobre todo en regiones áridas y semiáridas como las del mediterráneo (Araus et al., 2002, Isla et al., 1998). En estas condiciones, donde se cultiva frecuentemente el trigo duro, la mejora para la tolerancia a la salinidad en condiciones de riego deficitario puede ser una de las estrategias para aliviar este problema (Araus, 2004).

1 - Salinidad y estrés hídrico: efectos y respuestas

Las respuestas de las plantas al estrés salino e hídrico tienen mucho en común. La salinidad reduce la capacidad de las plantas para absorber agua, y esto de manera rápida da lugar a una reducción en la tasa de crecimiento además de causar cambios metabólicos idénticos a los causados por el estrés hídrico (Munns, 2002). Esta reducción inicial del crecimiento de las plantas en medios salinos se debe probablemente en una primera fase a un estrés osmótico causado por la sal, disminuyendo el potencial hídrico

de las raíces. Es posible que más tarde los efectos específicos de la sal tengan un impacto sobre el crecimiento. Como se observa en la Figura 1, la respuesta de una planta al estrés salino se produce en dos fases: inicialmente hay una respuesta rápida a la disminución del potencial hídrico del substrato (asociada a una disminución del potencial osmótico causada por un efecto iónico), y posteriormente se da una respuesta más lenta debido a la toxicidad asociada a la acumulación de Na^+ en las células.

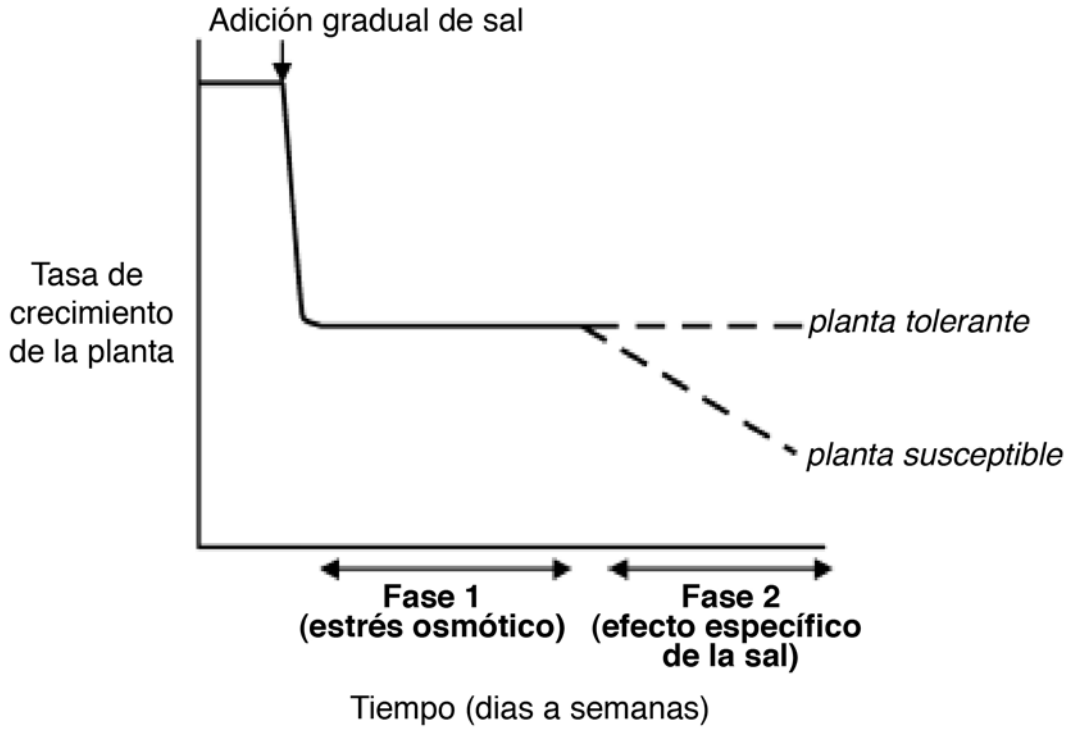


***Figura 1**. Esquema de las dos fases de respuesta de las plantas a la salinidad. Durante la Fase 1, la reducción del crecimiento de ambos genotipos (tolerantes y sensibles) se debe a los efectos osmóticos de las sales solubles en el medio de la raíz. Durante la Fase 2: las hojas de los genotipos sensibles envejecen, reduciendo la capacidad fotosintética. Es en esta fase cuando se ejerce el efecto especifico de las sales sobre el crecimiento de las plantas (Imagen adaptada de Munns, 2005).*

1 - 1 Crecimiento y productividad

Según Munns (1993) el efecto más claro de la salinidad sobre el crecimiento de las plantas es una disminución del potencial hídrico de la hoja. Generalmente, esta disminución va acompañada por una disminución del potencial de turgencia de las hojas. El crecimiento de las células se correlaciona con la presión de turgencia de las hojas y por lo tanto la disminución del turgor es la causa principal de la inhibición del crecimiento de las células bajo condiciones salinas (Greenway y Munns, 1980). Hu y Schmidhalter (1998) demostraron que el trigo que crecía en solución salina reaccionó disminuyendo un 25% su crecimiento, pero aumentando la cantidad de Na^+ en las células. Al mismo tiempo, Ball (1988) encontró que la disminución de la extensión de la hoja se relacionaba con una pérdida en la presión del turgor, posiblemente como resultado de un cambio hormonal provocado por una señal de las raíces a las hojas. La salinidad influye sobre el número de tallos y su aspecto en plantas de trigo (Mass y Poss, 1989). Como consecuencia, también se ve afectada la producción de materia seca total, aunque esta reducción sea un parámetro que depende del genotipo y de la concentración de sal (Pessarakli y Huber, 1991). Aloy (1992) encontró que el peso de 1000 granos en cebada fue el parámetro más fuertemente afectado por la salinidad; más que el número de granos por espiga y el número de espigas por planta, mientras que en arroz ocurría al revés (Zeng et al., 2002).

El efecto de la toxicidad de la sal se observa en genotipos sensibles a la salinidad, que acumulan cantidades tóxicas de Na^+ en las hojas, dando por resultado la muerte prematura de hojas viejas y daños permanentes en hojas jóvenes, así como la aparición de hojas nuevas con características suculentas (Munns y James, 2003). Por lo tanto, en una planta de trigo crecida en condiciones de elevada salinidad, es esperable una disminución en el número de hojas verdes y sanas y en el tamaño de las semillas (Mass y Poss, 1989).

La sensibilidad de la planta a la salinidad y al estrés hídrico cambia según su etapa de desarrollo fenológico (Neumann, 1995, Araus et al., 2008). Por ejemplo, muchas plantas muestran poca tolerancia a la salinidad durante la germinación de la

semilla, y sin embargo presentan una mayor tolerancia durante las últimas etapas de crecimiento, mientras que ocurre al revés en otras plantas. En el caso del trigo, los resultados han demostrado que es más sensible durante la fase vegetativa, y menos durante el llenado del grano (Mass y Poss, 1989). Por lo tanto, conocer las respuestas de las plantas a la salinidad y al estrés hídrico según su fase del crecimiento es muy importante para poder adoptar las estrategias de mejora genética más convenientes para el estudio de la resistencia de las plantas a dichos factores. Así por ejemplo, en el caso de la salinidad, si un genotipo es más sensible durante un determinado estadio de crecimiento, podríamos irrigar con agua salina durante las etapas más tolerantes del crecimiento y utilizar agua con concentración baja de sales solamente durante etapas más sensibles del crecimiento.

1 - 2 Nutrición mineral

La adquisición y la utilización de algunos elementos necesarios para las plantas, particularmente K^+ y Ca^{2+}, también puede verse alterada bajo condiciones salinas o estrés hídrico. Una cantidad razonable de ambos iones es necesaria para mantener la integridad de la membrana celular y sus funciones (Wei et al., 2003).

La disponibilidad de K^+ y Ca^{2+} para la planta disminuye cuando disminuye la cantidad de agua en el suelo, debido a la disminución de la movilidad de ambos elementos. Kuchenbuch et al. (1986) mostraron que la falta de humedad en el suelo reduce el crecimiento radicular y la tasa de absorción del potasio por las plantas. En condiciones de estrés hídrico, el envejecimiento de las plantas es consecuencia de una posible deficiencia de K^+ (Beringer y Trolldenier, 1978). De igual forma, la absorción del calcio se reduce en condiciones de sequía (Hu y Schmidhalter, 2005), provocando efectos sobre el crecimiento de las plantas. La disminución de la asimilación de K^+ y de Ca^{2+} bajo salinidad podría deberse a un antagonismo de Na^+ y K^+ o Ca^{2+} en los sitios de asimilación en las raíces, causando cambios en los cocientes K^+/Na^+ y Ca^{2+}/Na^+ y afectando al crecimiento y la productividad de las plantas (Greenway y Munns, 1980).

La toxicidad específica de la sal se produce con el tiempo (ver Figura 1), después que el Na^+ alcanza altas concentraciones en las hojas más adultas (Munns y Tester, 2008), causando la senescencia de las mismas, debido a la alta concentración de Na^+ y a que no se tolera su acumulación dentro de la planta. No se conoce totalmente como actúa la toxicidad de Na^+ dentro de la planta, pero se cree que la sal podría acumularse en el apoplasto y deshidratar las células. También podría ser que el Na^+ se acumulara en el citoplasma y así inhibir las enzimas que participan en el metabolismo de los carbohidratos o también podría ser que se acumulara en los cloroplastos y de esta forma ejercer un efecto tóxico directo sobre la fotosíntesis.

1 - 3 Limitaciones estomáticas y daño fotosintético

La fotosíntesis disminuye como respuesta tanto a la salinidad como al déficit hídrico (Munns, 2002). La conductancia estomática responde rápidamente a los cambios del potencial hídrico del suelo, sean éstos causados por estrés hídrico o salino, y su variación es la principal causa de la disminución de la fotosíntesis y el crecimiento de la planta (Munns y Tester, 2008; Munns et al., 2010). Williams et al. (1994) encontraron una elevada correlación entre el contenido de agua del suelo y la conductancia estomática. Cuando las plantas se encuentran bajo déficit hídrico, el principal factor limitante es el cierre estomático y por lo tanto la disminución de la conductancia. Se sabe que el cierre estomático se puede inducir antes de cualquier cambio detectable en el potencial hídrico y en el contenido relativo de agua de las hojas, y actualmente se acepta la existencia de una señal proveniente de las raíces (Flexas y Medrano, 2002). El fenómeno está vinculado con el aumento de los niveles xilemáticos del ácido abscísico (ABA), aunque la intensidad de la respuesta puede ser modulada por otros factores tales como el gradiente de la presión parcial del vapor de agua (Tardieu y Simonneau, 1998) (Figura 2). En cebada se detecta un aumento rápido de ABA en los tejidos fotosintéticos durante los 10 primeros minutos después de añadir 100 mM NaCl (Fricke, 2004).

Como en el caso del déficit hídrico, la salinidad puede afectar la fotosíntesis por causas de reducción de conductancia estomática y por otras causas metabólicas, pero

los efectos son más agresivos en algunos casos de salinidad en comparación con los observados bajo estrés hídrico debido al efecto tóxico de la sal dentro de la planta (Heuer y Plaut, 1989). Se encontró una relación negativa entre la actividad de la fotosíntesis y el contenido de Na^+ y Cl^- en hojas de muchas especies (Yeo, 1998), incluido en trigo (James et al., 2002).

En algunos casos, las tasas de fotosíntesis por unidad de área foliar en plantas tratadas con sal no cambian mucho en comparación con plantas control, aunque se reduzca la conductancia estomática (James et al., 2002). Esta paradoja se explica por los cambios de la anatomía celular ya que en condiciones salinas se forman hojas más pequeñas y más gruesas y por tanto hay una mayor densidad de cloroplastos por unidad de área foliar.

Se han observado también cambios en la ultraestructura de los cloroplastos bajo condiciones salinas (Suleyman et al., 2002; Salama et al., 1994), un descenso en las reacciones fotoquímicas y de carboxilación (Sharma y May 1992; Miteva et al., 1992) y un aumento del nivel de azúcares solubles en los tejidos (Dubey t Singh 1999; Flower et al., 1977). La salinidad también afecta a la respiración. Así una alta concentración de sal afectaría las funciones de determinados enzimas (Walker et al., 1981; Seemann y Critchly, 1985), provocando un aumento en la respiración. Este aumento es mucho mayor en las especies sensibles a la sal que en las tolerantes (Semikhatova et al., 1993).

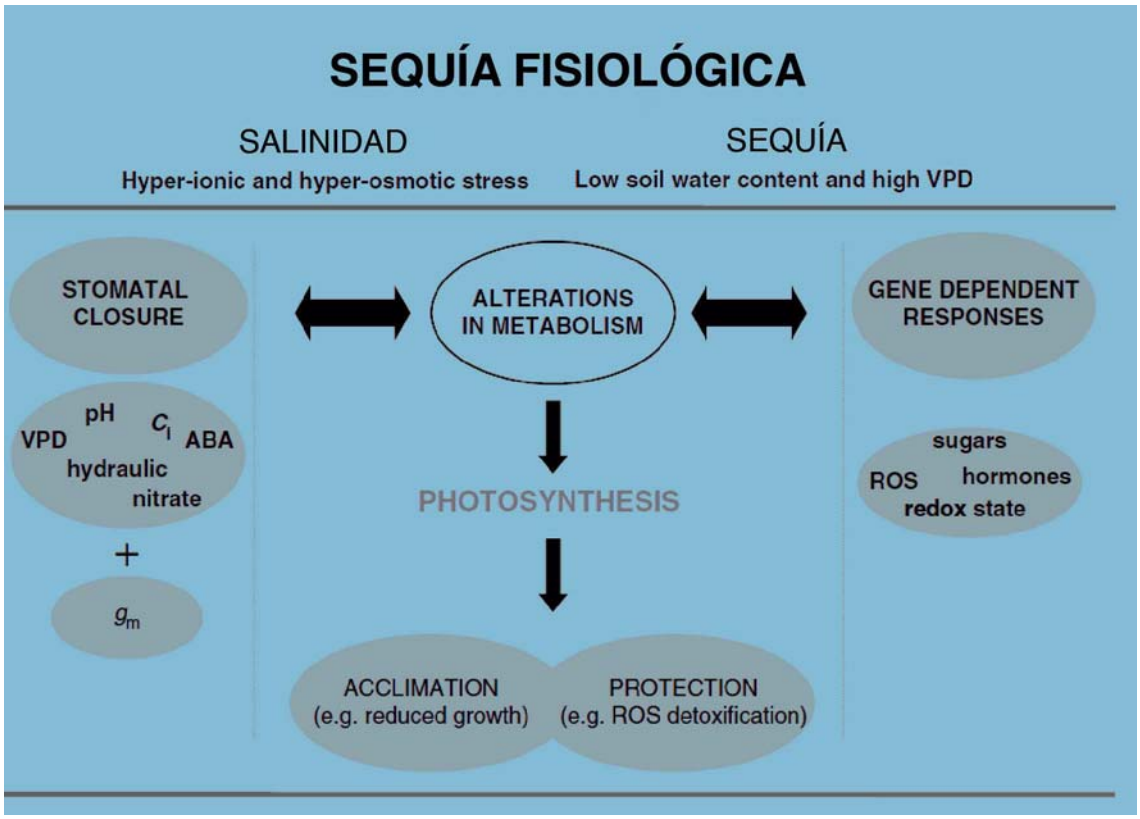


Figura 2. *Efectos directos de la salinidad y la sequía sobre la conductancia estomática y el metabolismo fotosintético (Imagen adaptada de Chaves et al., 2009)*

1 - 4 Daño oxidativo y bioquímico

La disminución de la tasa fotosintética favorece la formación de especies reactivas del oxígeno (ROS), y aumenta la actividad de las enzimas que desintoxican estas especies (Foyer y Noctor, 2005; Logan, 2005). Cualquier condición ambiental que afecta la fotosíntesis tales como la salinidad o la sequía podrían favorecer la producción excesiva de los radicales libres del oxígeno (moléculas de oxígeno o grupos hidroxilo con un electrón extra) que son altamente tóxicos y pueden oxidar compuestos esenciales, en particular los lípidos de la membrana (Foyer et al., 1994).

En el caso de la salinidad, diversos estudios han demostrado que la salinidad puede generar una acumulación de compuestos tóxicos en las plantas tales como especies reactivas de oxigeno, entre las que se incluyen los peróxidos, los superóxidos y los radicales del oxidrilo (Burdon et al., 1996; Shen et al., 1997; Tsugane et al., 1999). Estas moléculas tóxicas podrían dañar las membranas celulares, interferir en las actividades de las enzimas y el DNA especialmente en las mitocondrias y los cloroplastos, y por tanto podrían afectar muy negativamente el crecimiento vegetal y la supervivencia de las plantas (Allen, 1995). Según Naya et al. (2007), la sequía directamente o asociada a otros estreses (como la salinidad) produce un incremento en la expresión de genes involucrados en la detoxificación de radicales de O_2 como la CuZn-superóxido dismutasa (SOD) citosólica y la glutatión reductasa.

Además del daño fisiológico, la salinidad y el déficit hídrico podrían causar un daño bioquímico en las plantas. Ambos estreses provocan una disminución de los niveles de auxinas, de giberelinas y de citoquininas en la parte aérea, y un aumento en ABA (Moorby y Besford, 1983). Tales cambios en los niveles hormonales conducen a una reducción del crecimiento. Parece ser que las alteraciones en los niveles de hormonas y las actividades enzimáticas y el metabolismo de las proteínas causados por la salinidad son una consecuencia del déficit del agua (Blum, 1988).

1 - 5 Efectos en la reducción y asimilación del nitrógeno

El nitrógeno es un elemento muy importante para las plantas y cualquier alteración en su suministro puede afectar al crecimiento y la productividad de muchos cereales como la cebada (Ali et al., 2001, Britto y Kronzucker, 2002.) y el trigo (Cox y Reisenauer, 1973; Cramer y Lewis, 1993). El nitrógeno es el elemento mineral que las plantas necesitan en mayor cantidad y forma parte de muchos componentes de las células vegetales, incluyendo los aminoácidos y los ácidos nucleicos (Bernard y Habash, 2009). Por lo tanto su deficiencia inhibe rápidamente el crecimiento de las plantas y altera muchos procesos metabólicos esenciales en la productividad de los cultivos.

Las plantas captan y utilizan diferentes formas de nitrógeno (N) de los suelos, aunque la fuente más importante es el amonio inorgánico (NH_4^+) y el nitrato (NO_3^-) (Marschner, 1995). La nitrato reductasa (NR; CE 1.6.6.1) y la glutamina sintetasa (GS; CE 6.3.1.2) son las principales enzimas responsables de la asimilación del N y del metabolismo de los hidratos de carbono (Bernard y Habash, 2009, Solomonson y Barber, 1990; Lam et al., 1996; Hirel et al., 2005). Después de la absorción del nitrato por la raíz, el primer paso de su asimilación es su reducción a nitrito (NO_2^-) por la nitrato reductasa (Tcherkez y Hodges, 2008). El nitrato se reduce en el citoplasma y el nitrito resultante entra en los cloroplastos, donde se reduce aún más en amoníaco y luego se incorpora en los aminoácidos (Carillo et al., 2008.). La glutamina sintetasa es la enzima responsable de la primera etapa de la asimilación del amonio en las plantas (Miflin y Lea, 1977). La asimilación del amoníaco en aminoácidos se lleva a cabo por la glutamina sintetasa y la glutamato sintasa (GOGAT). Juntas, estas dos enzimas catalizan la conversión de 2-oxoglutarato (2-GO) y amoníaco a glutamato, que luego se utiliza en una amplia variedad de reacciones biosintéticas (Lancien et al., 2000).

La salinidad puede interferir con la nutrición nitrogenada de una manera directa e indirecta, aunque por lo general afecta de forma simultánea varios puntos de la vía de la asimilación del nitrógeno. Sin embargo, como pauta general las plantas en condiciones salinas prefieren más la vía de asimilación del amonio que la reducción del nitrato (Ullrich, 2002). Así, Botella et al., (1997) han observado en plantas de trigo una inhibición de las tasas de absorción del nitrato superior al 50% en condiciones de 60 mM de NaCl, mientras que la absorción del amonio parece ser mucho menos sensible (sólo el 8% de inhibición). La actividad de la NR en hojas depende en gran parte del flujo del nitrato de las raíces (Ferrario et al., 1998) y se ve muy afectada por el estrés osmótico inducido por NaCl (Rao y Gnanam, 1990; Viégas y Silveira, 1999; Abd-El Baki et al., 2000). A tal efecto, bajo condiciones de restricción en el flujo del nitrato inducida por el estrés salino o el déficit hídrico, la actividad de la NR podría reducirse inicialmente en cuenta de la degradación/inactivación de las enzimas y la reducción en la expresión génica y la síntesis de proteínas por la NR (Plaut, 1974; Ferrario et al., 1998). El estado de activación de la NR y la actividad máxima de la NR se reduce rápidamente en respuesta al estrés osmótico (Hall et al., 1998) y a la salinidad (Rao y Gnanam, 1990; Carrillo et al., 2005).

Sin embargo, varios trabajos indican que los niveles de salinidad moderada (100-150 mM de NaCl) aumentan la actividad de la glutamina sintetasa del trigo mientras que disminuyen la actividad de la nitrato reductasa (Wang et al., 2007; Carrillo et al., 2008). Ahora bien, la asimilación del amonio por la GS se ve muy limitada en condiciones de concentraciones muy altas de sal, tales como 300 mM de NaCl (Wang et al., 2007).

2 - Mecanismos de resistencia y tolerancia al estrés salino e hídrico

La tolerancia a la salinidad y al estrés hídrico se refiere a la capacidad de las plantas de mantener su crecimiento bajo estas condiciones. El estudio de las respuestas de plantas al estrés es un aspecto fundamental de la fisiología, el cual se propone conocer cómo las plantas funcionan en sus ambientes naturales y cuáles son los patrones que determinan su tolerancia, supervivencia y crecimiento (Kramer y Boyer, 1995; Lambers et al., 1998; Ackerly et al., 2002). Cuando una planta está sometida a unas condiciones bastante diferentes de las óptimas para su crecimiento se dice que está sometida a un *estrés.* Las diferentes especies o variedades difieren en sus requerimientos óptimos y por tanto en su susceptibilidad a un determinado estrés (Hsiao, 1973; Levitt, 1980). El conocimiento de los mecanismos de resistencia al estrés permite comprender los procesos evolutivos implicados en la adaptación de las plantas a un ambiente adverso como el mediterráneo. Este conocimiento puede ayudar a predecir la respuesta de la planta al incremento de los niveles de estrés, lo que tiene potenciales aplicaciones tanto en mejora como en manejo de cultivos.

Como hemos apuntado más arriba la disponibilidad hídrica se considera como el principal factor que afecta al crecimiento o actividad de las plantas en los ecosistemas mediterráneos (Di Castri et al., 1981; Blondel y Aronson, 1999). Cuando hay déficit hídrico se produce una tensión o estrés que actúa sobre las plantas, y toda tensión produce dos tipos de respuesta en los organismos: respuestas que tienden a evitar o prevenir la tensión (mecanismos de evitación) y mecanismos o adaptaciones que permiten soportar o resistir el estrés (mecanismos de tolerancia). Según Hickman (1970), las especies evitadoras del estrés hídrico serían especies homeohídricas (comportamiento hídrico estable) o bien “reguladoras”, ya que regulando la

transpiración evitarían tensiones excesivas en el xilema, mientras que las especies tolerantes serían "conformistas", según este mismo autor. Según Levitt (1980), las especies con estrategia tolerante serían las plantas que tienen mecanismos que minimizan o eliminan los efectos que pueden sufrir como consecuencia del estrés, alcanzando un equilibrio termodinámico con el mismo sin sufrir daños. Un aspecto importante de esta estrategia son los mecanismos reparadores de ese estrés, que la planta tiene que poner en funcionamiento cuando éste ha dejado de actuar. En la estrategia evitadora del estrés, las plantas previenen o minimizan el efecto del estrés en sus tejidos, ya que éstos son muy sensibles a la deshidratación. Las especies que siguen esta estrategia o bien maximizan la absorción de agua (e.g. sistemas radicales profundos) o bien minimizan las pérdidas de agua con el cierre de estomas rápido y sensible a ligeros descensos del contenido hídrico de los tejidos, y en consecuencia presentan valores en general bajos de conductancia estomática, paredes celulares poco elásticas que inducen cambios rápidos del potencial hídrico en respuesta a pequeñas pérdidas de agua, hojas pequeñas y bajas tasas de transpiración. Otro tipo de estrategia, no considerado por muchos autores como de auténtica resistencia al déficit hídrico, es la estrategia de escape de la sequía, que es aquella en que las plantas completan su ciclo vital antes de la llegada del estrés hídrico y, por lo tanto, el período desfavorable lo pasan en forma latente (por ejemplo de semilla).

Igual que ocurre con el estrés hídrico, las plantas sometidas a un estrés salino tienen sus propios mecanismos de resistencia. Los mecanismos desarrollados por las plantas tolerantes a la sal se basan en la exclusión del Na^+ del citoplasma, mediante su almacenamiento en las vacuolas o mediante la inhibición de su entrada o la estimulación de su salida de la célula (Kramer, 1984). La exclusión de la sal significa que las plantas poseen una capacidad para restringir la asimilación de iones tóxicos (Munns, 2002). En contraste a la exclusión de la sal, el acúmulo de la misma en grandes cantidades puede causar muchos problemas fisiológicos y bioquímicos en las células (resumidos más arriba). La capacidad que las plantas tienen de mantener la concentración de iones tóxicos en el citoplasma a niveles bajos es una de las características de la tolerancia a la sal (Yeo, 1998; Ashraf, 2002). Por lo tanto, dicha tolerancia a la sal está relacionada con la exclusión de la misma y con la capacidad de

mantener una concentración homeostática de iones en el citoplasma (Ungar, 1991). Cuando consideramos tolerancia a la salinidad existe una relación muy cercana entre la exclusión de la sal y la regulación de la selectividad en la asimilación de los iones, particularmente la discriminación de Na^+/K^+ (Gorham, 1994). La captación de Na^+ puede limitar el proceso de asimilación de K^+. Altos niveles de K^+ en tejidos jóvenes foliares se asocian a la tolerancia a la sal en muchas especies (Bhandal y Malik, 1988). Por lo tanto, es posible que la discriminación de Na^+/K^+ esté asociada a la tolerancia a la sal. Se ha identificado el locus genético que determina la discriminación de Na^+/K^+ en el trigo, observándose que cuando se inserta en el genoma de genotipos sensibles a la sal, se aumenta la tolerancia (Dvorak et al., 1994).

Como se ha indicado anteriormente, junto con los mecanismos de exclusión y el acúmulo inerte de la sal, el ajuste osmótico se considera como una característica de adaptación muy importante de las plantas a la salinidad, haciendo que se mantenga la turgencia de la célula. Durante el ajuste osmótico, la célula tiende a acumular la mayor parte de los iones absorbidos en vacuolas al mismo tiempo que se sintetizan y se acumulan los elementos orgánicos compatibles en el citoplasma para mantener el equilibrio osmótico entre estos dos compartimentos (Serrano y Gaxiola, 1994; Hasegawa et al., 2000).

3 - Mejora del trigo al estrés salino e hídrico

3 - 1 Estrategias y herramientas de mejora: criterio(s) de selección

Hasta ahora, la mejora del trigo se ha realizado de una forma bastante empírica, atendiendo exclusivamente al rendimiento de los genotipos que se pretenden mejorar (Loss y Siddique, 1994; Royo et al., 2005; Garcia del Moral et al., 2005b; Araus et al., 2008). Aunque esta aproximación empírica ha sido bastante eficaz para el aumento del rendimiento del trigo (Rajaram, 2001), la ganancia genética en la producción potencial del rendimiento en grano no ha sido homogénea en los distintos países a lo largo del pasado siglo (Royo et al., 2008). Igualmente, aunque el rendimiento de los cereales en ambientes no limitantes ha aumentado considerablemente en los últimos 50 años

(Austin et al., 1989), el incremento bajo condiciones de estrés ha sido mucho más moderado (Perry y D'Antunuo, 1989) e incluso ha disminuido en los últimos años (Braun et al., 1998). La mejora del rendimiento del trigo ha de mantenerse al mismo ritmo que hasta ahora o incluso aumentar, para poder satisfacer la demanda de alimentos de una población que se estima alcanzar los 800 millones en el año 2025 (ESA, 2006).

Las alternativas potenciales para poder satisfacer la demanda mundial de cereales pasarían por poder incrementar la superficie agrícola por una parte y por otra, el rendimiento por unidad de superficie (Edgerton, 2009). Sin embargo, la falta de expansión, y en algunos casos la reducción de las tierras cultivables disponibles, maximiza la importancia de aumentar el rendimiento potencial y la adaptación de los cereales (Slafer et al., 1999; Araus et al., 2002, 2008; Reynolds et al., 2009). La mejora de la adaptación de los cereales a diferentes estreses (como la salinidad o el estrés hídrico) es un proceso muy importante que puede ser una de las estrategias para aliviar este problema (Araus, 2004). Durante muchos años, determinados caracteres agronómicos como la producción de biomasa, la supervivencia al estrés y el rendimiento han sido los criterios más comúnmente usados para la identificación de la tolerancia a la sal y al estrés hídrico en programas de mejora. Esto se debe principalmente a la facilidad de medición de estos parámetros y porque al final lo importante (tanto en términos absolutos como relativos) en condiciones de estrés es el rendimiento. Sin embargo debido a la baja heredabilidad y la alta interacción entre genotipo y ambiente (GXE) del rendimiento, se ha sugerido el uso de caracteres secundarios basándose en una respuesta fisiológica más representativa de las interacciones GXE, y su aplicación ha demostrado ser útil para mejorar el rendimiento y la adaptación de los cereales al estrés (Araus et al., 2002, 2008; Reynolds et al., 2001). Además, estos caracteres fisiológicos secundarios pueden ser empleados como objetivos en la mejora asistida por marcadores (Araus et al., 2008).

La mejora para aumentar la tolerancia a los estreses puede ser más eficiente, si la selección fenotípica se basa directamente en los criterios fisiológicos pertinentes (Yeo et al., 1990; Araus et al., 2002; Zeng et al., 2002). La identificación de nuevos

criterios para la evaluación del rendimiento y la adaptación al estrés en los cereales y otras plantas es un tema de investigación activo y de gran importancia (Araus et al., 2008). En este sentido, el uso de los isótopos estables ha surgido recientemente como una de las herramientas más poderosas para evaluar las relaciones entre las plantas y su ambiente. El uso de los mismos como criterio en estudios de investigación en plantas ha crecido en forma sostenida durante las últimas dos décadas. Esta tendencia se mantendrá en un futuro ya que los investigadores se han dado cuenta de que los isótopos estables pueden servir como valiosos trazadores de cómo las plantas han interactuado y responden a sus ambientes abióticos y bióticos en el transcurso de su ciclo (Dawson et al., 2002). Los isótopos estables son criterios integradores (en tiempo y nivel de organización de la planta) que proporcionan información a largo plazo del rendimiento de la planta. Quizás el más importante sea la composición isotópica del carbono ($\delta^{13}C$), que frecuentemente se expresa como la discriminación ($\Delta^{13}C$) en materia seca (Araus et al., 2002, 2008), aunque tampoco se pueden despreciar la composición isotópica del oxígeno ($\delta^{18}O$) (Cabrera et al., 2009a) y nitrógeno ($\delta^{15}N$) (Lopes et al., 2004).

3 - 2 Uso de los isótopos estables en la mejora del trigo

3 - 2 - 1 Isótopos estables: concepto y nomenclatura

Los isótopos son átomos del mismo elemento que difieren únicamente en el número de neutrones, manteniendo idénticas sus propiedades químicas. Dentro de cada elemento encontramos isótopos radioactivos (inestables) y otros no radioactivos (estables). La mayoría de los elementos de interés biológico (ver Tabla 1) son el carbono (C), nitrógeno (N), hidrógeno (H) y el oxígeno (O) presentándose el ^{13}C, $^{2}H(D)$, ^{18}O y ^{15}N como los isótopos más pesados (mayor número de neutrones).

Tabla 1: *Isótopos estables y abundancia relativa de los elementos más abundantes en la biosfera y usados en estudios ambientales y de ecología (adaptado de Mateo et al., 2004).*

Elemento	Isótopo estable	Abundancia relativa
Hidrógeno	^{1}H	99,985 %
	$^{2}H(D)$	**0,015 %**
Carbono	^{12}C	98,892 %
	^{13}C	**1,108 %**
Nitrógeno	^{14}N	99,635 %
	^{15}N	**0,365 %**
Oxígeno	^{16}O	99,759 %
	^{17}O	0,037 %
	^{18}O	**0,204 %**

La composición isotópica de una muestra dada se determina por espectrometría de masas, y se expresa generalmente en diferentes notaciones. La ecuación (I) muestra una de las formas de calcular la composición isotópica usando la notación "delta" (Coplen, 2008):

$$\delta = (R_{muestra}/R_{estándar}) - 1 \qquad \text{(Ec. I)}$$

donde $R_{muestra}$ y $R_{estándar}$ son las relaciones molares de los isótopos pesados y ligeros (por ejemplo, $^{l3}C/^{12}C$) en la muestra y en la norma internacional (Tabla 2) respectivamente. Debido a que las diferencias en las relaciones entre la muestra y el estándar son muy pequeñas, los valores de δ se expresan por mil (‰).

Tabla 2. *Valores Estándar, abundancia, rangos y error analítico de los isótopos estables más utilizados en fisiología vegetal. Adaptado de Mateo et al. (2004). Los datos originales son de Barbour et al. (2001), Epstein et al. (1977), Farquhar et al. (1989) y Handley y Raven (1992).*

	Isótopos			
	$^{13}C/^{12}C$	**$^{15}N/^{14}N$**	**$^{18}O/^{16}O$**	**$^{2}H(D)/1H$**
Estándar	PDBb	Air N2	SMOWc	SMOWc
Notaciones	$\delta^{13}C$	$\delta^{15}N$	$\delta^{18}O$	δD
Media de abundancia (‰)	1,1	0,37	0,20	0,015
Rangos observados (‰)	-35 a -5	-10 a +10	+15 a +35	300 a +20
Error analítico (‰)	0.1	0.2	0.05-0.2	4-7

Durante los procesos físicos y químicos, la diferencia isotópica entre la fuente y el producto se refleja por el fraccionamiento isotópico que se expresa generalmente por el factor de fraccionamiento α (Ecuación II, la relación entre el producto dividido por la fuente) y la discriminación de isótopos (Δ) (Ecuación III: en algunos estudios, el fraccionamiento isotópico se refiere a la discriminación isotópica, sin el signo negativo):

$$\alpha = R_p \,/\, R_r \qquad \text{(Ec. II)}$$

$$\Delta\ (‰) .= \alpha - 1 = (\delta_r - \delta_p) / (1 + \delta_P/1000) \qquad \text{(Ec. III)}$$

donde δ_r y δ_p son la composición isotópica de los reactivos y productos, respectivamente.

3 - 2 - 2 Isótopos estables del carbono

En la naturaleza existen dos isótopos estables del carbono: ^{12}C y ^{13}C, con proporciones respectivas de 98,9% y 1,1%. La proporción relativa de ^{13}C en tejidos vegetales es inferior a la presente en la atmósfera, indicando que existe discriminación isotópica de carbono en la incorporación de CO_2 en la planta. Durante el proceso de la fotosíntesis debido a la discriminación isotópica que ocurre en los procesos físicos y químicos de la incorporación del CO_2 a la biomasa vegetal, los isótopos estables del carbono se distribuyen en cantidades desiguales. Las plantas discriminan contra el ^{13}C durante el proceso fotosintético (O'Leary, 1981), lo que se manifiesta en la composición isotópica de sus tejidos. Se habla entonces de discriminación isotópica del carbono ($\Delta^{13}C$) que se formula con la Ecuación III, donde δ_r y δ_p son la composición isotópica del CO_2 en el aire que rodea a la planta (frecuentemente escrito como δ_r) y la de los tejidos vegetales, respectivamente. La discriminación isotópica del carbono es uno de los parámetros fisiológicos recientemente desarrollado y que en condiciones de estrés mantiene una relación inversa con la eficiencia del uso del agua. El fundamento de este parámetro fue establecido por Farquhar y Richards (1984) y está basado en las proporciones relativas de los isótopos del carbono. La $\Delta^{13}C$ puede proporcionar una medida indirecta de la eficiencia de la transpiración y podría ser utilizada como criterio de selección en programas de mejora (Farquhar et al., 1982; Farquhar y Richards, 1984). La teoría predice que el valor de $\Delta^{13}C$ está determinado por el cociente (P_i/P_a) (entre la presión parcial intercelular de CO_2 (P_i) y la presión parcial de CO_2 en la atmósfera (P_a) que a su vez viene controlada por la tasa de asimilación neta (A_{sat}) y la conductancia estomática (g_s). El cociente P_i/P_a permite diferenciar entre las plantas debido al grado de abertura estomática y a la variación en la demanda del cloroplasto para el CO_2.

Hace algunos años, se propuso la discriminación isotópica del carbono como indicador del metabolismo fotosintético (Farquhar et al., 1989) y de la refijación del CO_2 procedente de la respiración (Araus et al., 1992). Desde los trabajos pioneros de Farquhar y sus colaboradores (Farquhar et al., 1982; Farquhar y Richards, 1984), se ha demostrado que en varias especies C_3, la variación en $\Delta^{13}C$ refleja la variación en la eficiencia del uso del agua (Condon et al., 2004). Por lo tanto, $\Delta^{13}C$ se ha propuesto

como un criterio de selección en muchos programas de mejora. En ambientes mediterráneos, $\Delta^{13}C$ (especialmente cuando se mide en granos maduros) frecuentemente se correlaciona positivamente con el rendimiento en grano (Araus et al., 1998, 2003; Villegas et al., 2000; Condon et al., 2004). Una de las razones de esta relación positiva es que los genotipos con alta $\Delta^{13}C$ muestran una mayor capacidad para mantener un estado mejor de agua y por lo tanto los estomas más abiertos (Araus et al., 2002; Condon et al., 2004). Como cada vez hay menos disponibilidad de agua para las plantas, los estomas se cierran para reducir la pérdida de agua por transpiración (Lawlor y Cornic 2002; Flexas y Medrano 2002). Esta tensión inducida por el cierre estomático hace que exista un flujo reducido de CO_2 en el espacio interior de las plantas (Zengh et al., 2008). Por ello, la fotosíntesis y la actividad de la fijación del carbono son limitados (Flexas et al., 2004). La combinación de estos dos efectos da lugar a un fraccionamiento isotópico bajo y por lo tanto una disminución en la discriminación del carbono $\Delta^{13}C$ en la materia orgánica de las plantas (Farquhar et al., 1989, 2007). Una disminución de la $\Delta^{13}C$ debido a salinidad o sequía puede ser evidencia de una limitación a largo plazo de la fotosíntesis mediada básicamente por un cierre estomático (Medrano et al., 2002).

Debido a la respuesta integradora de $\delta^{13}C$ a varias limitaciones ecofisiológicas a través del tiempo, la composición isotópica del carbono se puede utilizar para evaluar muchas características relacionadas con el intercambio de gases y las relaciones hídricas, incluida la eficiencia del uso del agua (Farquhar y Richards, 1984; Henderson et al., 1998), la capacidad fotosintética (Virgona y Farquhar, 1996), la conductancia estomática (Condon et al., 1987; Ehleringer, 1990; Ehleringer et al., 1990; Virgona et al., 1990; Meinzer et al., 1992), el contenido en nitrógeno de la hoja (Sparks y Ehleringer, 1997; Schulze et al., 1998), la masa de hoja por área (Vitousek et al., 1990; Williams y Ehleringer, 2000) y la tasa relativa de crecimiento (Ehleringer,1993; Poorter y Farquhar, 1994).

Muchos estudios en trigo duro y otros cereales han demostrado que la $\Delta^{13}C$ disminuye (o la composición isotópica del carbono, $\delta^{13}C$, aumenta) en condiciones de estrés hídrico (Araus et al., 1997, 2003; Condon et al., 2002). Experimentalmente se ha encontrado una relación lineal y negativa entre $\Delta^{13}C$ y la eficiencia de transpiración

(Farquhar y Richards, 1984; Masle y Farquhar, 1988; Condon y Richards, 1992). Además, se han observado relaciones lineales y positivas entre $\Delta^{13}C$ y el rendimiento para diferentes cultivos como el trigo (Condon et al., 1987; Masle y Farquhar, 1988; Araus et al., 1993) y la cebada (Austin et al., 1990; Voltas et al., 1999). Muchos parámetros climáticos influyen en la $\delta^{13}C$ de las plantas, y esto hace que se pueda aplicar como criterio de selección en muchos programas de mejora bajo diferentes condiciones de crecimiento (Figura 3). En este sentido, se han estudiado ampliamente los efectos de la limitación de agua en la $\delta^{13}C$ (o en la $\Delta^{13}C$) de los cereales C_3 y por regla general todos reafirman que un déficit hídrico tiene un efecto negativo en $\Delta^{13}C$ (o positivo en $\delta^{13}C$) (Farquhar y Richards, 1984, Araus et al., 1997, 1998, 2003; Voltas et al., 1999; Monneveux et al., 2005).

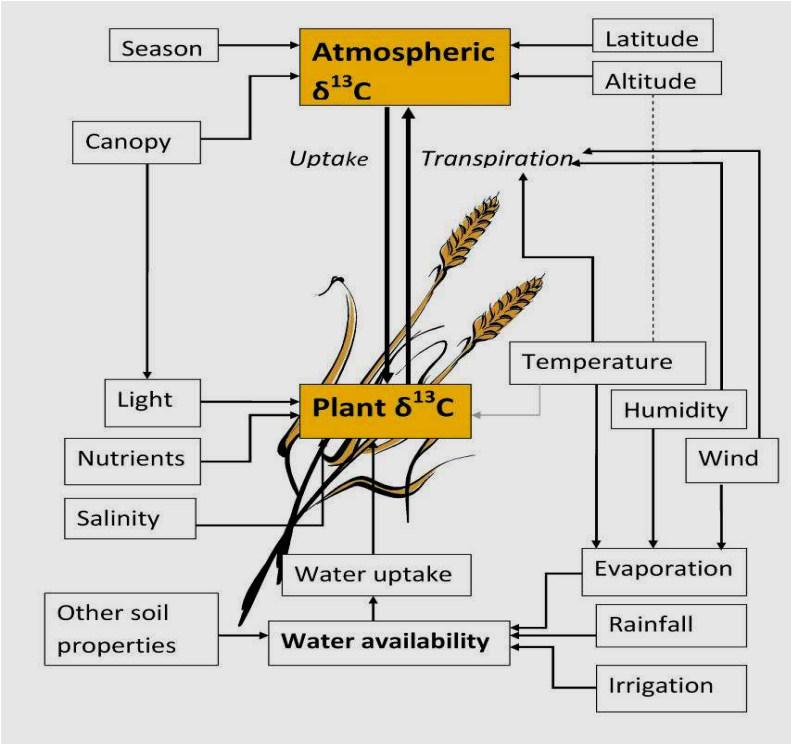


Figura 3. *Influencia de los parámetros ambientales sobre la composición isotópica del carbono en plantas (Fuente Flohr et al., 2009)*

La $\delta^{13}C$ (o la $\Delta^{13}C$) se ha utilizado también como parámetro para ver la respuesta de la planta frente a diferentes condiciones de salinidad (Condon et al., 1990; Isla et al., 1998; Rasmuson y Anderson, 2002; Shaheen y Hood, 2005). Un aumento en la salinidad da lugar a un aumento linear y significativo de $\delta^{13}C$ (o una disminución en $\Delta^{13}C$), posiblemente debido al efecto del estrés hídrico asociado a la salinidad. Una disminución de $\Delta^{13}C$ debida a la salinidad es una respuesta común en trigo (Ansari et al., 1988) y cebada (Isla et al., 1998). En otros trabajos se ha estudiado el efecto de la salinidad analizando la relación entre la discriminación isotópica y los intercambios de gases fotosintéticos; paralelamente, otros han propuesto como objetivo ver el efecto de la salinidad mediante la discriminación isotópica en relación con algunos parámetros agronómicos como el índice de cosecha, el peso de 1000 granos y el rendimiento (Shaheen y Hood, 2005).

3 - 2 - 3 Isótopos estables del oxígeno

Los isótopos estables del oxígeno en la naturaleza están en su mayoría en forma de ^{16}O y ^{18}O y se encuentran principalmente en el H_2O y CO_2 en las plantas. En general, se sabe que la composición isotópica del oxigeno ($\delta^{18}O$) de la materia orgánica refleja la variación en (i) la composición isotópica de las fuentes de agua, (ii) el enriquecimiento por evaporación en las hojas debido a la transpiración, y (iii) el fraccionamiento bioquímico durante la síntesis de materia orgánica (Yakir, 1992; Farquhar y Lloyd, 1993). Además de la variación en función de la fuente de agua, la composición isotópica del oxígeno en la materia vegetal varía en función de la humedad y la temperatura (Barbour y Farquhar, 2000; Barbour et al., 2000b; Scheidegger et al., 2000; Helliker y Ehleringer, 2002), de la humedad del suelo (Yakir et al., 1990; Saurer et al., 1997; Ferrio et al., 2007) e incluso en función de la disponibilidad del nitrógeno (Cernusak et al., 2007; Bassin et al., 2009).

Aunque la composición isotópica del oxigeno no se ha estudiado tanto como la del carbono, la $\delta^{18}O$ es potencialmente muy relevante como criterio para la mejora de plantas. Se ha demostrado que $\delta^{18}O$ (a veces expresado como enriquecimiento respecto al agua empleada por la planta, $\Delta^{18}O$) es un buen indicador de las condiciones de

transpiración de la hoja durante todo el ciclo del cultivo (Barbour et al., 2000a, Barbour, 2007). Por otra parte, al comparar diferentes plantas que crecen en condiciones óptimas idénticas, $\delta^{18}O$ refleja diferencias en la conductancia estomática. Por lo tanto, se ha propuesto como un criterio que integra en el tiempo y a escala de la planta entera las diferencias genéticas en la conductancia estomática y la transpiración en el trigo y arroz (Barbour y Farquhar, 2000; Barbour et al., 2000a). Además, en especies en las que se correlaciona la conductancia estomática y el rendimiento del grano, $\delta^{18}O$ puede reflejar diferencias en dicho rendimiento (Barbour et al., 2000a; Ferrio et al., 2007; Cabrera-Bosquet et al., 2009b).

Un aumento en $\delta^{18}O$ puede ser el resultado de una disminución en la conductancia estomática (g_s) y en la transpiración (*T*) (Barbour 2007; Farquhar et al., 2007; Sharma y Williams, 2009). Durante la última década se ha estudiado en el trigo y otros cereales cultivados bajo diferentes regímenes de agua el aumento de la $\delta^{18}O$ en las plantas como respuesta al déficit hídrico (Ferrio et al., 2007; Cabrera-Bosquet et al., 2009a, 2011; Araus et al., 2010). Sin embargo, debemos hacer notar que con anterioridad a este trabajo de tesis no había estudios sobre la $\delta^{18}O$ en plantas de trigo bajo condiciones de salinidad.

3 - 2 - 4 Isótopos estables del nitrógeno

El nitrógeno es el elemento que más limita el crecimiento de las plantas en muchos ecosistemas. La variación en los isótopos estables N ($^{15}N/^{14}N$) se considera a menudo como una herramienta para estudiar la dinámica del N de la planta. La forma isotópica del nitrógeno absorbido por las plantas puede tener distinta composición isotópica (Yoneyama, 1996) por lo que el tipo de nitrógeno asimilado por la planta junto con otros procesos metabólicos posteriores serán los determinantes de la composición isotópica del nitrógeno ($\delta^{15}N$) en la planta. Muchos estudios indican que la $\delta^{15}N$ en niveles de abundancia natural actúa como un marcador, es decir, la proporción de isótopos de nitrógeno de origen se conserva en cierta medida durante la absorción, la asimilación y la posterior translocación del nitrógeno, y que la $\delta^{15}N$ de los distintos tejidos de la planta refleja la fuente del nitrógeno del suelo (Dawson et al., 2002; Serret

et al., 2008). Sin embargo, procesos como la absorción del nitrógeno y sus vías de asimilación, así como el reciclaje del nitrógeno en la planta, pueden discriminar positivamente o negativamente contra el ^{15}N (Evans, 2001). Así pues la $\delta^{15}N$ de las plantas depende no sólo de la firma isotópica (^{15}N) de la fuente(s) del nitrógeno, sino también del equilibrio entre la actividad enzimática y la concentración externa de N (Mariotti et al., 1982; Handley y Raven, 1992). Se sabe que el fraccionamiento isotópico se produce durante la asimilación del nitrato por la NR y la absorción y asimilación del amonio por la GS (Evans, 2001; Tcherkez, 2011). Estas dos enzimas tienen aparentemente *in vitro* factores similares de discriminación (Ledgard et al., 1985; Yoneyama et al., 1993). La sensibilidad de los valores de $\delta^{15}N$ a las tasas fotorespiratorias (asociadas con la actividad de la GS) y a la captación de N (reducción de NO_3^- por NR) parece bastante alta (Tcherkez 2011), y hace que el uso de $\delta^{15}N$ sea primordial en estudios relacionados con el metabolismo del nitrógeno en plantas.

La abundancia natural de la $\delta^{15}N$ de las plantas refleja el efecto de una serie de procesos muy complicados y difíciles de explicar (revisados por Handley y Scrimgeour, 1997; Robinson, 2001; Evans, 2001; Unkovich et al., 2001; Tcherkez, 2011). La presencia de múltiples fuentes de N con distintos valores isotópicos, la variación temporal y espacial en la disponibilidad de N, y los cambios en la demanda de las plantas pueden influir en $\delta^{15}N$ de las plantas. Además, de estos parámetros, otras condiciones y variables ambientales pueden también influir en la $\delta^{15}N$ de la planta (Figura 4). Sin embargo, estudios con plantas en condiciones hidropónicas y la presencia de una sola fuente de N inorgánico han permitido avanzar en esta problemática. Cuando el NH^+_4 es la única fuente del nitrógeno hay poca, o ninguna, discriminación cuando la concentración de N es limitante (Evans et al., 1996). Sin embargo, puede haber un gran empobrecimiento en ^{15}N de toda la planta en concentraciones altas de N (Yoneyama et al., 2001). En cambio, concentraciones externas de NO_3^- parece que tienen un efecto sobre la $\delta^{15}N$ de la planta entera (Mariotti et al., 1982; Kohl y Shearer, 1980; Bergersen et al., 1988; Yoneyama y Kaneko, 1989; Evans et al., 1996; Yoneyama et al., 2001). Las plantas pueden estar ligeramente enriquecidas o ligeramente empobrecidas en ^{15}N en comparación con la fuente de NO_3^- (Yoneyama et al., 2001). Una concentración externa

alta de NO_3^-, un estrés osmótico o la sequía pueden inducir un fraccionamiento isotópico de N, a pesar de que los mecanismos de este fraccionamiento no se conocen muy bien (Handley et al., 1994, 1997).

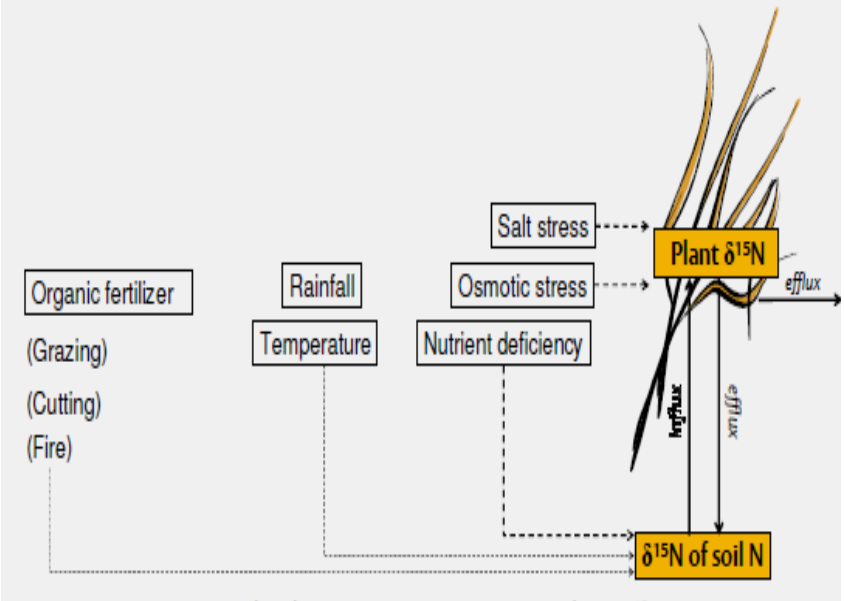


Figura 4: *Influencia de los factores ambientales sobre la composición isotópica del nitrógeno en las plantas (Fuente de Flohr et al., 2009)*

A pesar de que no hay un conocimiento preciso de los mecanismos bioquímicos implicados en la firma isotópica del nitrógeno, la variación natural en la $\delta^{15}N$ es potencialmente útil para la detección de diferencias genotípicas en condiciones de sequía o salinidad, ya que está vinculada con el metabolismo del nitrógeno de la planta (Handley et al., 1997.; Robinson et al., 2000; Ellis et al., 2002; Pritchard y Guy, 2005; Coque et al., 2006). Sin embargo, el sentido de los cambios (aumento o disminución) en $\delta^{15}N$ es frecuentemente contradictorio. De hecho tanto, la salinidad como la sequía pueden disminuir (Ellis et al., 1997; Handley et al., 1997; Robinson et al., 2000) como aumentar la $\delta^{15}N$ (Ellis et al., 2002; Lopes et al., 2004; Lopes y Araus, 2006) con respecto a las plantas control.

OBJETIVOS

OBJETIVOS

La tolerancia a la salinidad es un objetivo importante en muchos programas de mejora de trigo duro. El empleo de criterios específicos y eficientes de fenotipeado que permitan detectar variabilidad genotípica y seleccionar los genotipos más tolerantes a la sal podrá acelerar el avance genético. Para alcanzar este objetivo es necesario poner a punto protocolos multidisciplinarios de selección.

De acuerdo con esto, el objetivo general de esta tesis fue estudiar el uso potencial de la composición isotópica del carbono, el oxígeno y el nitrógeno como herramientas ecofisiológicas para la evaluación de genotipos de trigo duro en diferentes condiciones de salinidad y compararlos con otros criterios de selección más convencionales. Para ello, este objetivo general se divide en los siguientes objetivos específicos:

1- Hacer un análisis del efecto de diferentes combinaciones de salinidad y aporte hídrico sobre el crecimiento y características morfo-fisiológicas clásicas en estudios de salinidad en un conjunto de genotipos de trigo duro (y especies relacionadas) con respuestas contrastadas a la salinidad. Las características evaluadas fueron: contenido en clorofila y concentración de iones, junto con parámetros de intercambio de gases fotosintéticos y transpiratorios.

2- Estudiar la respuesta de las firmas isotópicas del ^{13}C, ^{18}O, y ^{15}N en las mismas condiciones y genotipos que en objetivo anterior y relacionarlos con los criterios clásicos.

3- Comparar la eficiencia de las diferentes firmas isotópicas frente a los criterios convencionales para determinar tolerancia genotípica frente a diferentes niveles de salinidad.

4- Determinar la eficiencia relativa de los tres isótopos estables en diferentes niveles de salinidad, fase de crecimiento y duración del tratamiento.

5- Desarrollar un modelo que combine los tres isótopos estables con el metabolismo nitrogenado para explicar las diferencias genotípicas en tolerancia a diferentes condiciones de salinidad.

6- Estudiar las relaciones entre expresión de diferentes categorías de genes asociados con la respuesta a estreses salinos e hídricos y del metabolismo del nitrógeno con las diferencias genotípicas en el crecimiento, firmas isotópicas y actividades enzimáticas.

Informe de los Directores de Tesis sobre el factor de impacto de los artículos publicados y participación de la doctoranda

El Dr. José Luis Araus Ortega y la Dra. Maria Dolors Serret como Directores de la Tesis que lleva por título: "**Salinidad y trigo duro: *Firmas isotópicas, actividad enzimática y expresión génica***" que ha desarrollado la doctoranda Salima Yousfi,

INFORMAN sobre el índice de impacto y la participación de la doctoranda en cada uno de los artículos incluidos en la memoria de esta Tesis Doctoral.

Capítulo 1. Artículo: "**Shoot $\delta^{15}N$ gives a better indication than ion concentration or $\Delta^{13}C$ of genotypic differences in the response of durum wheat to salinity"** publicado en la revista *Functional Plant Biology* con un índice de impacto en 2009 de 1.678. En este estudio se analizaron las variaciones genotípicas para la tolerancia a la salinidad en una población de trigo duro. Se evaluó la composición isotópica de carbono ($\delta^{13}C$) y nitrógeno ($\delta^{15}N$) y el acúmulo de iones en un subconjunto de genotipos de dicha población seleccionados por tolerancia contrastada a la salinidad. Este fue el primer experimento realizado por el doctorando y supone un primer paso para asimilar los fundamentos científicos y metodológicos que la doctoranda desarrollará a lo largo de su tesis. La cantidad de trabajo invertido en este estudio, incluyendo el cultivo durante un periodo de varios meses de centenares de plantas en condiciones de hidroponía, fue muy elevado.

Capítulo 2. Artículo: **"Effect of salinity and water stress during the reproductive stage on growth, ion concentrations, $\Delta^{13}C$, and $\delta^{15}N$ of durum wheat and related amphiploids"** publicado en la revista *Journal of Experimental Botany* con un índice de impacto en 2010 de 4.818. Como continuación del Capítulo 1, en este artículo se estudió el efecto de la salinidad en la composición isotópica del carbono ($\delta^{13}C$) y el nitrógeno ($\delta^{15}N$) de genotipos de trigo duro y de dos amfiploides (un tritórdeo y un triticale). En este segundo experimento y al contrario del primero, la salinidad se aplicó más tarde en el ciclo de crecimiento de las plantas, es decir, durante la floración y el llenado del grano por unas pocas semanas. Este trabajo representó la puesta a punto del estudio del comportamiento fisiológico del trigo duro durante la fase reproductiva y bajo diferentes combinaciones de salinidad y riego. Junto con las evaluaciones de

biomasa y los análisis de isótopos estables y concentraciones de iones se evaluaron las actividades fotosintéticas y transpiratoria de todas las plantas estudiadas.

Capítulo 3. Artículo: **"Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit",** publicado en la revista *New Phytologist* con un índice de impacto en 2011 de 6.516. Como continuación de los capítulos 1 y 2 y en vista de los resultados obtenidos en el uso de las firmas isotópicas como criterio de evaluación bajo condiciones salinas, se planteó evaluar el uso combinado de la composición isotópica del carbono ($\delta^{13}C$), oxigeno ($\delta^{18}O$) y el nitrógeno ($\delta^{15}N$) en materia seca para observar las respuestas genotípicas de plantas de trigo duro sometidas a diferentes combinaciones de salinidad. Como contribución original, se elaboró un modelo conceptual de las tres firmas isotópicas juntas ($\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$) junto con características del metabolismo nitrogenado para explicar las diferencias genotípicas en tolerancia a distintas condiciones de salinidad y estrés hídrico. También se han evaluado las características fotosintéticas en relación con las firmas isotópicas y las actividades de enzimas claves del metabolismo nitrogenado.

Capítulo 4. Artículo: **"A comparative effect of salinity and drought on growth, ion concentration and $\delta^{13}C$ and $\delta^{15}N$ in barley"** publicado en la revista *Aspects of Applied Biology***,** de la AAP (Association of Applied Biologists). En este estudio se evaluó el comportamiento de un genotipo de cebada bajo diferentes combinaciones de salinidad y regímenes de riego para confirmar los resultados obtenidos en las anteriores publicaciones referente al efecto de los estreses sobre la $\delta^{13}C$ y $\delta^{15}N$.

Capítulo 5. Artículo: **"Comparative response of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in dry matter and the water-soluble fraction of durum wheat exposed to salinity at the vegetative and reproductive stages"** enviado a la revista *Plant Cell and Environment*, con un índice de impacto en 2011 de 5.145. Este estudio compara la eficiencia de las firmas isotópicas de carbono, oxígeno y nitrógeno en la materia seca y fracción soluble evaluando diferencias genotípicas en tolerancia a diferentes condiciones de salinidad y régimen hídrico aplicadas en diferentes estadios fisiológicos de las plantas.

Capítulo 6. Artículo: **Gene expression and physiological responses of resistant and sensitive durum wheat genotypes under salinity and water stress.** Artículo preparado para enviar a la revista *New Phytologist* con un índice de impacto en 2011 de 6.516. En este último capitulo de la tesis se analizó la respuesta genética de plantas de trigo duro a la salinidad a través las tasas de transcripción de genes específicos asociados a tolerancia a salinidad y estrés hídrico, junto a otros que codifican por enzimas claves del metabolismo nitrogenados. Así mismo se han estudiados las relaciones entre estas tasas de transcripción y las diferencias genotípicas en crecimiento, firmas isotópicas y actividades de enzimas del metabolismo nitrogenado.

Cabe destacar que la Ingra. Salima Yousfi, desde su llegada a Barcelona se ha integrado perfectamente en nuestro equipo. Ha colaborado de forma autónoma en la realización de todos los experimentos de su tesis doctoral, participando en el diseño de los mismos, toma de datos, análisis químicos y moleculares, tratamiento estadístico, elaboración de tablas y figuras, discusión de los resultados y redacción de las publicaciones. La doctoranda ha demostrado una gran capacidad de trabajo, tanto en el trabajo de invernadero y cámaras de cultivo de condiciones controladas como en todos los trabajos de laboratorio. Fruto de estos años de trabajo ha alcanzado un gran conocimiento sobre el tema, además de haber demostrado una iniciativa y capacidad de trabajo probados.

Y, para que así conste a los efectos oportunos,

Dr. José Luis Araus Ortega Dra. M. Dolors Serret Molins

Tabla de los experimentos realizados

Tabla de los experimentos realizados

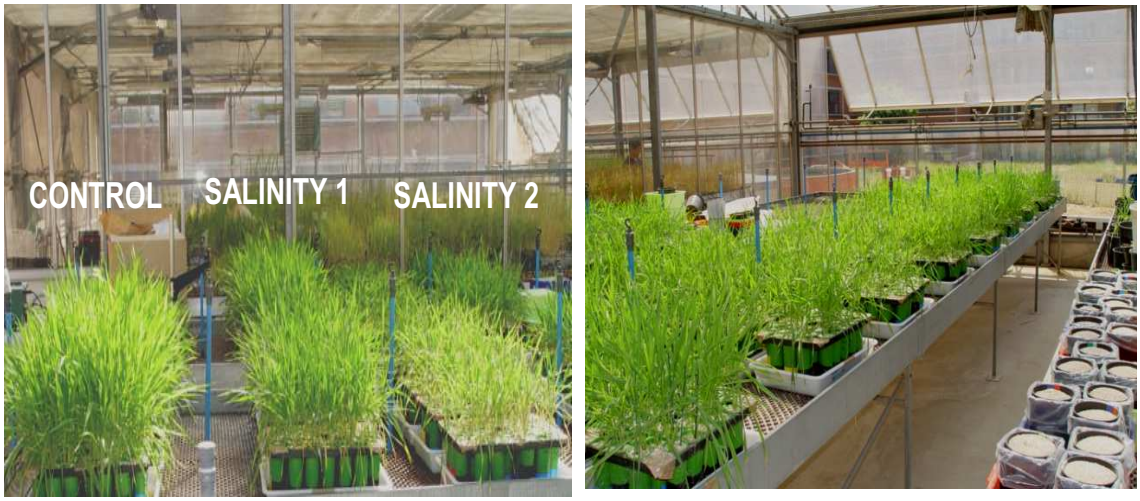
Ensayos	Capítulo	Localización	Condiciones experimentales	Material vegetal	Tratamientos	Análisis
I	1	UB	- Invernadero - Condiciones hidropónicas - Estrés aplicado en estado vegetativo de la planta - Estrés a largo plazo	114 genotipos: Población de 112 RILs de trigo duro y sus parentales	- Salinidad	- Parámetros morfológicos - Análisis de iones - Intercambio de gases - Isótopos estables ^{13}C, ^{15}N
II	2	UB	- Cámara de cultivo - Estrés aplicado en estado reproductivo de la planta - Estrés a corto plazo	6 genotipos: 4 trigo duro, 1 tritordeum, 1 triticale	- Salinidad - Déficit hídrico - Combinación de ambos	- Parámetros morfológicos - Análisis de iones - Intercambio de gases - Isótopos estables ^{13}C, ^{15}N
III	3	UB US	- Cámara de cultivo - Estrés aplicado en estado vegetativo de la planta - Estrés a corto plazo	4 genotipos de trigo duro	- Salinidad - Déficit hídrico - Combinación de ambos	- Parámetros morfológicos - Análisis de iones - Intercambio de gases - Isótopos estables ^{13}C, ^{15}N, ^{18}O - Actividad enzimática: Glutamina sintetasa y nitrato reductasa.
	4	UB	- Cámara de cultivo - Estrés aplicado en estado vegetativo de la planta - Estrés a corto plazo	1 genotipo de cebada	- Salinidad - Déficit hídrico - Combinación de ambos	- Parámetros morfológicos - Análisis de iones - Intercambio de gases - Isótopos estables ^{13}C, ^{15}N
	6	UB CSIC	- Cámara de cultivo - Estrés aplicado en estado vegetativo de la planta - Estrés a corto plazo	4 genotipos de trigo duro	- Salinidad - Déficit hídrico - Combinación de ambos	- Parámetros morfológicos - Isótopos estables ^{13}C, ^{15}N , ^{18}O - Transcripción: PCR en tiempo real en genes de salinidad y de déficit hídrico
I + II	5	UB	Comparación de las condiciones de los experimentos I y II	20 genotipos de trigo duro del experimento I (10 sensibles y 10 tolerantes a la salinidad) 4 genotipos de trigo duro del experimento II	- Salinidad - Déficit hídrico - Combinación de ambos	- Comparación entre los isótopos ^{13}C, ^{15}N , ^{18}O en materia seca y fracción soluble

RESULTADOS

Capítulo 1

Shoot $\delta^{15}N$ gives a better indication than ion concentration or $\Delta^{13}C$ of genotypic differences in the response of durum wheat to salinity

Yousfi, S., Serret, M.D., Araus, J.L. 2009. *Functional Plant Biology* 36: 144-155.



Genotipos de trigo duro en el invernadero de los Servicios Experimentales de la Facultad de Biología, Universidad de Barcelona. Año 2006. Foto: S. Yousfi

Resumen

En este estudio se comparó el comportamiento de diferentes características fisiológicas que revelan las variaciones genotípicas a la tolerancia a la salinidad en trigo duro. Se cultivaron un total de 114 genotipos en condiciones hidropónicas durante 3 meses, sometidos a tres tratamientos: control, salinidad moderada (12 dS m^{-1}) y severa (17 dS m^{-1}), se mantuvieron durante casi 8 semanas y se muestrearon al final del alargamiento del tallo. La biomasa de los genotipos en condiciones control se correlacionaba positivamente con la biomasa en los dos niveles de salinidad. Posteriormente, se seleccionaron dos subconjuntos de 10 genotipos cada uno, en base a diferencias extremas de biomasa en los dos niveles de salinidad, pero que mostrasen una biomasa relativamente similar bajo condiciones control. La discriminación isotópica del carbono ($\Delta^{13}C$), la composición isotópica del nitrógeno ($\delta^{15}N$) y la concentración del nitrógeno, fósforo y varios iones (K^+, Na^+, Ca^{2+}, Mg^{2+}) se analizaron en los dos subgrupos y en los tres tratamientos. En la salinidad moderada (12 dS m^{-1}) la concentración de K^+, el cociente K^+/Na^+, $\Delta^{13}C$ y $\delta^{15}N$ se correlacionaban positivamente y Na^+ negativamente con la biomasa. Bajo condiciones del control y en 17 dS m^{-1} no se observaron correlaciones (excepto por $\delta^{15}N$ a17 dS m^{-1}). Sin embargo, se vio que $\delta^{15}N$ es el criterio que correlaciona mejor con las diferencias genotípicas en biomasa bajo las condiciones de salinidad moderada (12 dS m^{-1}). Este criterio ($\delta^{15}N$) fue la primera variable elegida en cada uno de los dos niveles de salinidad en análisis del Stepwise. Se discuten los mecanismos que relacionan $\delta^{15}N$ con la biomasa y el uso de la firma isotópica del nitrógeno como un criterio de selección.

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Shoot $\delta^{15}N$ gives a better indication than ion concentration or $\Delta^{13}C$ of genotypic differences in the response of durum wheat to salinity

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Abstract. We compared the performance of different physiological traits that reveal genotypic variations in tolerance to salinity in durum wheat. A set of 114 genotypes was grown in hydroponics for over 3 months. Three conditions: control, moderate (12 dS m^{-1}) and severe (17 dS m^{-1}) salinity, were maintained for nearly 8 weeks before harvest. The genotype biomass in control conditions correlated with the biomass at the two salinity levels. Subsequently, two subsets of 10 genotypes each were selected on the basis of extreme differences in biomass at the two salinity levels while showing relatively similar biomass in control conditions. Carbon isotope discrimination ($\Delta^{13}C$), nitrogen isotope composition ($\delta^{15}N$), and the concentration of nitrogen, phosphorus and several ions (K^+, Na^+, Ca^{2+}, Mg^{2+}) were analysed in the two subsets for the three treatments. At 12 dS m^{-1}, K^+ concentration, K^+/Na^+ ratio, $\Delta^{13}C$ and $\delta^{15}N$ correlated positively and Na^+ correlated negatively with shoot biomass. Under control conditions and at 17 dS m^{-1} no correlation was observed. However, the trait that correlated best with genotypic differences in biomass was $\delta^{15}N$ at 12 dS m^{-1}. This trait was the first variable chosen at each of the two salinity levels in a stepwise analysis. We consider the possible mechanisms relating $\delta^{15}N$ to biomass and the use of this isotopic signature as a selection trait.

Additional keywords: NaCl, photosynthesis, potassium, salt, sodium, stable isotopes, *Triticum turgidum* ssp. *durum*.

Introduction

Drought is the main abiotic factor limiting productivity in Mediterranean environments. Durum wheat is the most cultivated herbaceous crop in the south and east Mediterranean basin (www.fao.org/statistics/yearbook, accessed 4 December 2008). One of the ways of increasing productivity in these semiarid environments is irrigation; however, this may expose soils to progressive salinisation (World Bank 2007). Selecting genotypes that have a higher salt tolerance is one way of palliating this problem (Munns *et al*. 2002; Munns 2008).

Salt tolerance is dependent on several traits, and presumably controlled by many genes which have yet to be discovered (cf. Flowers 2004). The complexity of salt tolerance may explain the slow progress that has been made in developing salt-tolerant crops. In this context, more efficient screening techniques need to be established to identify salt-tolerant progeny in breeding programs in which genes for salt tolerance have been introduced by either conventional breeding or genetic engineering (Munns *et al*. 2002). This process is particularly important in durum wheat, since it is much more salt-sensitive than other cereals like barley or even bread wheat (Munns *et al*. 2002). However, the lack of reliable, large-scale field screening facilities is a major problem in the genetic improvement of salt-tolerance in durum wheat (Srivastava and Jana 1984) and other cereals (Isla *et al*. 1998). Fast and effective glasshouse screening techniques that identify genetic variation in salinity tolerance may represent a feasible alternative (Munns and James 2003).

Among the classic traits of salt tolerance in cereal crops suitable for large scale evaluation in greenhouses (Colmer *et al*. 2005) is ion 'exclusion' (Munns and James 2003; Tester and Davenport 2003). This is defined as the ability to restrict the rate of entry of potentially toxic Na^+ (and Cl^-) into the shoots, combined with the maintenance of K^+ uptake, even when Na^+/K^+ is very high in the soil solution (Gorham 1993). Ion 'exclusion' may be considered the 'first defence' against salinity. To prevent ions accumulating to toxic concentrations in the shoots, the roots must exclude almost all the Na^+ and Cl^- dissolved in the nutrient solution (Munns 2008). In wheat, Cl^- 'exclusion' shows little correlation with genotypic differences in salt tolerance. Therefore, only Na^+ accumulation in the shoots is usually measured (Colmer *et al*. 2005).

Even when most of the Na^+ and Cl^- in a soil is excluded, these ions still accumulate to some extent in transpiring plant organs. Thus, as well as enhancing transpiration efficiency (i.e. the ratio of photosynthesis to transpiration), a low stomatal conductance may reduce the rate at which ions concentrate in the soil solution (Munns 2008), the movement of salts into the root zone

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(Rengasamy 2002), and, ultimately, their accumulation in the root tissues. However, the reverse may also be true: a genotype with higher stomatal conductance under salinity may be the consequence of higher tolerance of this stress (James *et al*. 2002, 2008). Screening for high stomatal conductance (Munns and James 2003), although valuable for selecting parentals, is not feasible for the routine screening of many genotypes (but see Rebetzke *et al*. 2000). As an alternative, when transpiration efficiency is measured as carbon isotope composition ($\delta^{13}C$) – frequently expressed as discrimination ($\Delta^{13}C$) in dry matter – it can be used for large scale assessments. Discrimination of $^{13}C/^{12}C$ in C_3 species can be interpreted using a well established physiological model (Farquhar *et al*. 1982). Conditions which induce stomatal closure (e.g. water stress, either directly or through salinity) restrict the CO_2 supply to carboxylation sites and can be detected by reduced $\Delta^{13}C$. Foliar $\Delta^{13}C$ (or $\delta^{13}C$) values have been used in wheat as an integrated measure of the response of photosynthetic gas exchange to salinity (Arslan *et al*. 1999; Rivelli *et al*. 2002). In addition, genotypic variability of this trait under salinity has been reported (Ellis *et al*. 1997, 2002; Isla *et al*. 1998; Shaheen and Hood-Nowotny 2005). Ellis *et al*. (2002) report a weak ($r = 0.38$) but significant positive relationship between $\delta^{13}C$ and shoot biomass in barley, which suggests that the genotypes with higher transpiration efficiency (and probably lower stomatal conductance) were the most tolerant to salinity. A comparable trend was also reported by Pakniyat *et al*. (1997), working with different barley mutants. However, the same study (Pakniyat *et al*. 1997) reports that shoot $\delta^{13}C$ was positively correlated with shoot Na^+.

The 'last defence' against salinity is tolerance of high internal Na^+. Thus, leaf injury and extended survival at high salinities may also be a convenient test, and has been used in several studies (Kingsbury and Epstein 1984; Sayed 1985). In these studies, it was assessed indirectly by measuring chlorophyll content (Munns and James 2003).

Natural variation in plant N isotope composition ($\delta^{15}N$) has been proposed as a useful trait for screening, as it is linked to plant N metabolism, even though there is no precise knowledge of the underlying mechanisms or function (Handley *et al*. 1997; Robinson *et al*. 2000; Ellis *et al*. 2002; Pritchard and Guy 2005; Coque *et al*. 2006). Different nitrogen uptake mechanisms and assimilation pathways, as well as the recycling of nitrogen in the plant can discriminate against ^{15}N (Evans 2001). Moreover, plants depend not only on the $\delta^{15}N$ of N source(s), but also on the balance between enzyme activity and external concentration (Mariotti *et al*. 1982). Different reports indicate that abiotic stresses such as salinity and drought can either decrease (Ellis *et al*. 1997; Handley *et al*. 1997; Robinson *et al*. 2000) or increase (Ellis *et al*. 2002; Lopes *et al*. 2004; Lopes and Araus 2006) $\delta^{15}N$ relative to controls. Such patterns contrast with the well established decrease in $\Delta^{13}C$ (or increase in $\delta^{13}C$) associated with these stresses in the same studies, and illustrate the relative complexity of the mechanisms determining $\delta^{15}N$ signatures in plants. Furthermore, in comparison to $\Delta^{13}C$, relatively few studies have addressed genotypic variation in plant $\delta^{15}N$ in response to stress conditions, and mostly to drought (Robinson *et al*. 2000; Evans 2001; but c.f. Peuke *et al*. 2006). Measuring the natural abundance of plant $\delta^{15}N$ has been proposed as a way of studying the physiology of salt tolerance in barley grown in hydroponics (Ellis *et al*. 1997, 2002; Handley *et al*. 1997). Coque *et al*. (2006), working with a set of 99 recombinant inbred lines of maize grown at low and high N-input, concluded that two mechanisms could explain genetic variation in ^{15}N discrimination ability: morphophysiological differences, in particular in the root system, and differences in the activity of the main enzymes in nitrogen metabolism. In the same way, Pritchard and Guy (2005) propose that genotypic differences in $\delta^{15}N$ of white spruce are caused by differences in demands on N assimilation and in uptake capacity. Hypothetically, genotypes with a high N demand, high N assimilation capacity or a low uptake capacity will all have a low substrate-to-enzyme ratio and therefore discriminate less against ^{15}N. These same variables influence plant nitrogen use efficiency (Pritchard and Guy 2005). In contrast, processes such as loss of ammonia or nitrous oxide through stomata may also affect the final signature (Farquhar *et al*. 1980; Smart and Bloom 2001).

Robinson *et al*. (2000) proposed that measuring the natural abundance of both ^{13}C and ^{15}N may give an indication of responses to stresses such as drought and nitrogen starvation. Moreover, both $\delta^{13}C$ and $\delta^{15}N$ have been used to phenotype the response of (barley) mapping populations to salinity, as the natural abundances of these isotopes are strongly affected by salinity and there is genotypic variability in both stable isotopes (Ellis *et al*. 1997, 2002; Handley *et al*. 1997). However, to date, only a weak positive genotypic relationship between ^{13}C signature and biomass has been reported (Ellis *et al*. 2002), whereas other studies failed to find any significant relationship (Pakniyat *et al*. 1997; Isla *et al*. 1998; Shaheen and Hood-Nowotny 2005). The situation is even more complicated for $\delta^{15}N$. No clear relationship between $\delta^{15}N$ and biomass and/or grain yield has been reported (Ellis *et al*. 1997, 2002; Handley *et al*. 1997), despite of the fact that $\delta^{15}N$ has been used to map the phenotype of populations exposed to salinity (Ellis *et al*. 2002). This lack of relationship may be related to the experimental conditions, as the genotypes were only grown for short periods of time (from one to a few weeks) in saline conditions (Munns *et al*. 2002; Rivelli *et al*. 2002). For example, in some of the studies, plants were exposed to the final salt concentration for 8 days (Ellis *et al*. 1997; Handley *et al*.1997), 2 weeks (Ellis *et al*. 2002) and 4 weeks (Pakniyat *et al*. 1997). In other studies, the saline conditions did not even result in a decrease in shoot biomass compared with the control (Handley *et al*. 1997; Shaheen and Hood-Nowotny 2005).

The objective of this study was to perform a comparative evaluation of a wide set of screening traits for selecting salt-tolerant progeny in a recombinant inbred line population of durum wheat. These traits included ion concentration and ratios, chlorophyll levels and the natural abundance of the stable isotopes ^{13}C and ^{15}N. Salinity tolerance was defined as genotypic differences in 'absolute' shoot biomass after growing plants for 2 months in saline conditions. In controlled environments, differences in salt tolerance among species are frequently assessed as the percentage of biomass production in saline *v.* non-saline conditions (e.g. Munns and James 2003; Colmer *et al*. 2005). However, this approach does not take into account genotypic variability in yield potential. Instead, it may result in selecting the genotypes that have the lowest yield

potential. We postulate that natural abundance signatures of ^{13}C and ^{15}N measured in dry matter indicate genotypic tolerance to salinity better than other more conventional parameters, such as ion concentration, do. This is because the former directly reflect the effect of salinity on carbon and nitrogen metabolisms and thus on plant growth. We also discuss the potential mechanisms that relate $\delta^{15}N$ to biomass. Although $\delta^{15}N$ in plants has been proposed as an indicator of genetic variation that reflects a balance between N uptake and assimilation (Handley *et al*. 1997; Robinson *et al*. 2000; Pritchard and Guy 2005; Coque *et al*. 2006), the pattern of relationships between $\delta^{15}N$ and both $\Delta^{13}C$ and biomass among genotypes and across growing conditions may provide some clues as to how salinity affects $\delta^{15}N$ (Pritchard and Guy 2005).

Materials and methods

Plant materials and growth conditions

The durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] population used in this study consisted of 112 recombinant inbred lines (RILs) derived by single seed descent from the cross ICD-MN91–0012 between Jennah Khetifa and Cham 1. This cross was made in 1991 at the Tel Hadya research station (Aleppo province, Syria) by the CIMMYT/ICARDA durum breeding program for Mediterranean dryland (Nachit *et al*. 2001). Jennah Khetifa is a landrace that shows specific adaptation to the North African continental drylands, being tall and moderately resistant to drought and cold. Cham 1 is a variety that has been released for commercial production in several countries of the Mediterranean basin. It is highly adaptable, has high yield potential and yield stability and shows high osmotic adjustment.

Plants were grown in a hydroponic system within a greenhouse at the Experimental Fields of the University of Barcelona, Spain. The hydroponic system consisted of 48-L tanks covered with perforated plates. Each tank contained 28 pots of 300 mL each. The experimental design involved a randomised complete block with three salinity levels (control, 1.8 dS m^{-1}; moderate salinity, 12 dS m^{-1}; severe salinity, 17 dS m^{-1}), 114 genotypes (112 RILs plus the 2 parental lines) and four replicates per genotype and growing condition. The total number of pots was 1368. The pots were filled with fine particle (size B6) perlite and separated from the nutrient solution by a mesh. Two seeds were planted in each pot and watered to field capacity to facilitate germination. After a week, only one plant per pot was left. From germination to the 4–5 leaf stage, all plants were grown with a half-strength Hoagland solution (Hoagland and Arnon 1950). Subsequently, the different growing conditions were established. Control plants continued to be grown in the same Hoagland solution and no salt was added. For the two salinity levels, NaCl was added progressively to the nutrient solution, starting with a salt concentration of 4 dS m^{-1}. This concentration was increased 1 week later to the final salt levels of 12 dS m^{-1} (corresponding to ~120 mM NaCl) and 17 dS m^{-1} (~170 mM NaCl). Then, plants were grown for a further 2 months until the end of jointing, when they were harvested. The total duration of the growing experiment was 3 months. The electrical conductivity, pH and O_2 concentration of all solutions were monitored weekly and solutions were changed every 2–3 days, except for the last 20 days of the experiments when they were changed daily. The average temperature during the experiment was 26/16°C day/night. Relative humidity ranged from 40 to 65% and the maximum PPFD was ~1200 µmol m^{-2} s^{-1}.

Gas-exchange measurements

Leaf gas exchange was measured in the two parental lines three times during the experiment: before starting the salinity treatments, after nearly a month of treatment with salt, and a few days before harvest. Measurements were made with an open IRGA LI-COR 6400 system (Li-Cor Inc., Lincoln, NE, USA) They were conducted in leaves that had recently fully expanded, at 10–14 h (solar time) under saturated PPFD conditions (beyond 1000 µmol m^{-2} s^{-1}), a temperature of around 25°C and a CO_2 concentration within the chamber of 400 µmol mol^{-1}. For each genotype, growing condition and date of measurement four leaves were assayed. The gas exchange parameters were light-saturated net CO_2 assimilation rate (A_{sat}); transpiration rate (T); and stomatal conductance (g_s). Subsequently, the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) was calculated, according to Sharkey and Raschke (1981).

Plant growth and leaf chlorophyll content

Chlorophyll content was measured in the last fully expanded leaf of all the plants assayed. Measurements were performed just before harvesting using a portable meter (Minolta SPAD 502 Meter, Plainfield, IL, USA). Four measurements were performed per leaf from the middle of the blade.

The height of the highest shoot of each plant was measured with a ruler before harvest. After harvesting, the total number of shoots per plant was counted. Shoots were then oven-dried at 85°C for 24 h and the weight of the dry aerial biomass determined. The pots with the roots were stored in a cold chamber (4°C) for further analyses (see below).

Two subsets of extreme genotypes were chosen according to their aerial biomass under the two saline conditions. One subset included 10 genotypes with a biomass that was among the top 20 genotypes of the entire population in each of the two salinity levels. The other subset was made up of another 10 genotypes ranked among the 20 genotypes with the lowest biomass values under the two saline conditions. Whenever possible, both sets of genotypes were also selected to have similar biomass under control conditions. A total of 240 plants (20 genotypes per three treatments and four replications) were chosen.

The aerial parts of these plants were ground. Subsequently, the amount of different ions and the composition of stable carbon and nitrogen isotopes were analysed. Roots of the same plants were washed with deionised water, dried in the oven at 85° for 24 h and ground for further ion analyses.

Ion analysis

For each leaf or root sample analysed, 100 mg of dry material was digested with 3 mL concentrated HNO_3 and 2 mL H_2O_2. The samples were placed overnight in a microwave at 90°C. After digestion, each sample was then brought up to 30 mL final volume with pure water. The amount of Na^+, Ca^{2+}, K^+, P, and Mg^{2+} in the sample was then determined with an Inductively Coupled Plasma

Emission Spectrometer (L3200RL, Perkin Elmer, Uberlingen, Germany) at the Scientific Facilities of the University of Barcelona. Ion concentrations were expressed as mol per g of dry weight.

Total N concentration and stable carbon and nitrogen isotope composition

Total nitrogen concentration and the stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios in the aerial part of the plant were measured using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Breman, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan), operating in continuous flow mode. Samples of ~1 mg and reference materials were weighed into tin capsules, sealed, and then loaded into an automatic sampler (ThermoFinnigan) before EA-IRMS analysis. Measurements were conducted at the Scientific Facilities of the University of Barcelona.

Nitrogen was expressed as a concentration (mol per g of dry weight) and as total nitrogen content (g) of the aerial part. The $^{13}C/^{12}C$ ratios were expressed in δ notation (Coplen 2008) determined by: $\delta^{13}C = (^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1$ (Farquhar *et al*. 1989), where sample refers to plant material and standard to Pee Dee Belemnite (PDB) calcium carbonate. The same δ notation was used for the $^{15}N/^{14}N$ ratio expression ($\delta^{15}N$), but in this case the standard referred to N_2 in air.

Atropine was used as a system check in the elemental analyses of nitrogen. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH_7 polyethylene foil, IAEA CH_6 sucrose and USGS 40 L-glutamic acid) were used for calibration to a precision of 0.1‰. For nitrogen, isotope secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N_1 and IAEA N_2 ammonium sulfate and IAEA NO_3 potassium nitrate) were used. Mean $\delta^{15}N$ of the fertilizers provided by the Hoagland solution was 0.6‰.

The carbon isotope discrimination ($\Delta^{13}C$) of shoots was calculated as: $\Delta^{13}C(‰) = (\delta^{13}C_a - \delta^{13}C_p)/[1 + (\delta^{13}C_p)]$, where *a* and *p* refer to air and the plant, respectively (Farquhar *et al.* 1989). Air samples inside the greenhouse were taken and analysed by the GC-C-IRMS technique, as previously described in Nogués *et al*. (2004). Air analyses were conducted at the Scientific Facilities of the University of Barcelona. The $\delta^{13}C_a$ was –11.3‰. The N isotope discrimination ($\Delta^{15}N$) of a plant compared with fertiliser ^{15}N may be formulated in the same manner as $\Delta^{13}C$ (Evans 2001). Nevertheless, in our study we used $\delta^{15}N$ rather than $\Delta^{15}N$, because the latter does not provide any relevant advantage when considering the effect of salinity on the ^{15}N signature of the plant. In fact most publications dealing with ^{15}N use $\delta^{15}N$ not $\Delta^{15}N$. By contrast, $\Delta^{13}C$ gives more comprehensive information than $\delta^{13}C$ on the level of stress suffered by plants.

Statistical analysis

Analysis of variance (ANOVA) was performed using the GLM procedure to calculate the effects of salinity level and genotype. Means were compared by the Duncan's test ($P < 0.05$). A bivariate correlation procedure was used to calculate the Pearson correlation coefficients. Multiple linear regression analysis (stepwise) was used to analyse the relationship between the studied variables. Data were analysed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using a Sigma-Plot 6.0 program (SPSS Inc.).

Results

The effect of salinity and genotype on growth parameters

The increase in salinity significantly affected all three growth traits studied (Table 1). However, the trait that was most affected was total aerial biomass, which decreased to about half the control values at 12 dS m^{-1}, and to a quarter of control values at 17 dS m^{-1}. Plant height and the number of tillers per plant were also affected by salinity, but to a lesser extent. In particular, there was much less difference between these traits under 12 dS m^{-1} and 17 dS m^{-1} conditions. Chlorophyll content, calculated on the basis of leaf area, was also affected significantly by salinity. Compared with the control it was higher at 12 dS m^{-1} but lower at 17 dS m^{-1}.

The effect of genotype was highly significant for all the four traits studied (Table 1), even though it was far lower than that attributed to salinity. Biomass was the only growth trait for which no interaction between salinity regime and genotype (S × G) was observed. The error in ANOVA was the smallest for this trait. There was no significant S × G interaction for chlorophyll content, but the error in the ANOVA was very high in this case. Shoot biomass was used as a criterion for selecting the subset of extreme genotypes, in terms of their response to salinity.

Growth in control conditions and genotype performance under salinity

The average biomass attained by the 114 genotypes in the absence of stress (control) correlated positively and significantly ($P < 0.001$) with the biomass of these genotypes at each of the two salinity levels (Fig. 1). Although the slope of the relationship between the control biomass and the biomass at 17 dS m^{-1} was

Table 1. Effect of different levels of salinity on the aerial biomass, plant height, number of tillers per plant and leaf chlorophyll content of durum wheat
Values shown are the means ± s.e. for all the 114 genotypes of the RIL population. Means followed by different letters were shown to be significantly different ($P < 0.05$) by the Duncan's test. Analysis of variance for the same variables is shown for the salinity regime (S), genotype (G) and interaction (S × G) effects. The associated percentage of the sum of squares and probabilities (ns, not significant; ***, $P < 0.001$) are shown

	Control	12 dS m^{-1}	17 dS m^{-1}	Salinity (S)	Genotype (G)	S × G	Error
Biomass (g)	11.12 ± 0.16a	5.13 ± 0.06b	2.70 ± 0.02c	72.9***	4.0***	4.7ns	18.4***
Plant height (cm)	40.06 ± 0.29a	31.29 ± 0.15b	28.20 ± 0.12c	57.2***	13.3***	9.2***	20.3***
Tillers/plant	5.04 ± 0.10a	2.88 ± 0.03b	2.68 ± 0.03c	37.6***	16.8***	13.6***	31.7***
Leaf chlorophyll (SPAD units)	36.35 ± 0.15b	38.51 ± 0.09a	34.00 ± 0.17c	11.3***	11.5***	13.7ns	63.5***

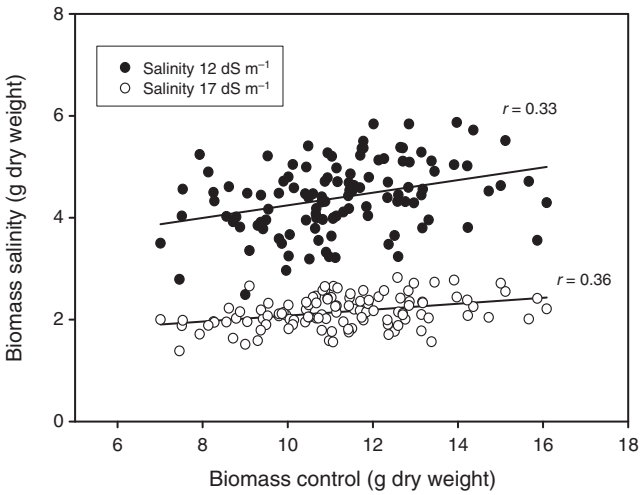

Fig. 1. Relationship across the set of 114 genotypes of durum wheat between shoot biomass in control conditions and shoot biomass in the two levels of salinity. Each point is the average value of one genotype. Correlations were highly significant ($P < 0.001$).

lower than that of the control biomass against the 12 dS m^{-1} biomass, the correlation coefficients were very similar. Biomass at 12 dS m^{-1} was also positively and significantly ($P < 0.001$) correlated with that at 17 dS m^{-1}, even when the correlation coefficient ($r = 0.26$) was lower than in the other two cases.

In order to assess the roles of growth potential and adaptive responses to salinity on genotypic differences in biomass for each of the two levels of salinity, we performed a regression analysis with successive steps (stepwise analysis). The dependent variables were biomass at either 12 dS m^{-1} or 17 dS m^{-1} for all genotypes. The independent variables were biomass, plant height, number of tillers and leaf chlorophyll, measured in the absence of stress and at salinity levels other than that of the dependent variable (Table 2). For biomass at both moderate salinity and severe salinity, the independent variable that was chosen first by the model was biomass under optimum conditions (an indicator of potential growth), followed by other variables related to salinity tolerance (plant height and number of tillers in the other level of salinity). Chlorophyll content was not chosen by the model in any case.

Table 2. Stepwise analysis with biomass for the whole set of 114 genotypes of durum wheat in either of the saline conditions as a dependent variable, and biomass, plant height, number of tillers per plant and leaf chlorophyll content measured in the other two growth conditions (control and the other salinity level) as independent variables

Independent variables chosen in either of the analyses contributed significantly to the models. BC, biomass at control; HC, plant height in control; HS1, plant height under 12 dS m^{-1}; HS2, plant height under 17 dS m^{-1}; TS2, number of tillers under 12 dS m^{-1}. Significance: ***, $P < 0.001$

Biomass	Variable chosen	r	R^2	Significance
At 12 dS m^{-1}	BC	0.337	0.114	***
	BC, TS2	0.459	0.211	***
	BC, TS2, HS2	0.495	0.245	***
	BC,TS2, HS2, HC	0.539	0.291	***
At 17 dS m^{-1}	BC	0.399	0.160	***
	BC, HS1	0.486	0.237	***

Effect of salinity on photosynthetic and transpiratory gas exchange

The magnitude of stress caused by the two different levels of salinity was further evaluated by measuring the gas exchange traits (A_{sat}, g_s, C_i/C_a, T) of the two parents of the population just before the start, at the middle and at the end of the saline treatment. Since both genotypes showed a similar pattern of response, only the average values (including all replications of the two genotypes) of the different traits were pooled (Table 3). In the controls, the values of the four gas exchange traits did not change significantly throughout the experiment. In contrast, A_{sat} rates declined gradually during growth at both 12 dS m^{-1} and 17 dS m^{-1}. Just before harvest, values were five times lower (12 dS m^{-1}) and 10 times lower (17 dS m^{-1}) than in control plants. Conductances decreased to around 5 times lower values by the middle of the 12 dS m^{-1} treatment. The values then remained unchanged until before harvesting. Under 17 dS m^{-1} conditions, rates decreased progressively to attain 20-fold lower values by the end of the treatments. The ratio C_i/C_a decreased progressively during growth to attain values that were 60% and 75% lower than control plants at 12 dS m^{-1} and 17 dS m^{-1}, respectively. Rates of T also decreased. By harvest, values were nearly four times lower in 12 dS m^{-1} conditions than in the control and almost eight times lower in 17 dS m^{-1} conditions than in the control.

Table 3. Effect of different levels of salinity on leaf net CO_2 assimilation (A_{sat}), stomatal conductance (g_s), the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) and the transpiration rate (T) in durum wheat

Measurements were performed in mature, recently expanded leaf blades on three occasions: before starting the treatment (date 1), after one month (date 2) and after 2 months, just before harvesting (date 3). The values shown are the means ± s.e. for the two parents of the RIL population. The means followed by different letters were shown to be significantly different ($P < 0.05$) by the Duncan's test

	First date			Second date			Third date		
	Control	12 dS m^{-1}	17 dS m^{-1}	Control	12 dS m^{-1}	17 dS m^{-1}	Control	12 dS m^{-1}	17 dS m^{-1}
A_{sat} (μmol CO_2 m^{-2} s^{-1})	26.9 ± 0.9a	26.4 ± 0.5a	25.7 ± 0.6a	23.3 ± 0.7a	11.4 ± 0.4b	6.8 ± 0.6c	23.9 ± 0.8a	6.8 ± 0.6b	1.2 ± 0.2c
g_s (mmol CO_2 m^{-2} s^{-1})	402 ± 67a	416 ± 35a	424 ± 75a	322 ± 25a	91 ± 16b	56 ± 1b	412 ± 49a	92 ± 1b	15 ± 32b
C_i/C_a	0.71 ± 0.05a	0.73 ± 0.04a	0.69 ± 0.04a	0.70 ± 0.02a	0.46 ± 0.04b	0.29 ± 0.03c	0.68 ± 0.03a	0.32 ± 0.12b	0.16 ± 0.01c
T (mmol H_2O m^{-2} s^{-1})	6.36 ± 0.56a	6.43 ± 0.27a	6.06 ± 0.34a	6.07 ± 0.39a	2.69 ± 0.11b	1.39 ± 0.12c	5.22 ± 0.32a	1.56 ± 0.10b	0.66 ± 0.18b

Effect of salinity on the ion and N concentrations, $\Delta^{13}C$ and $\delta^{15}N$ of plants

As explained in 'Materials and methods', out of the population of 114 genotypes, we selected a subset of the 10 most tolerant and the 10 most susceptible genotypes. The selection was made according to biomass at the two levels of salinity (whenever possible, the differences in biomass under control were small). We analysed the ion concentration in the leaves and roots of plants in these subsets. We also measured the nitrogen concentration, stable carbon discrimination ($\Delta^{13}C$) and nitrogen isotope composition ($\delta^{15}N$) of leaves.

Regardless of the treatment, K^+, Mg^{2+} and P concentration was higher in leaves than roots. In contrast, Ca^{2+} concentration was lower in leaves than roots, but Na^+ had quite similar values in both organs. Consequently, the ratio K^+/Na^+ was much higher in the leaves than in the roots, whilst the opposite occurred for the ratio Ca^{2+}/Na^+ (Table 4).

The growing conditions significantly affected the ion concentration in both the leaves and roots (Table 4). In leaves, salinity decreased the K^+, Ca^{2+} and Mg^{2+} concentration to values that were about half those of control plants, while P decreased far less and Na^+ increased more than 10-fold. Consequently, K^+/Na^+ and Ca^{2+}/Na^+ ratios decreased in response to salinity. In general, the most notable difference in ion concentration was found when plants under 12 dS m^{-1} conditions were compared with controls. A further increase to 17 dS m^{-1} levels of salinity had much less effect on ion concentration. The pattern of response to salinity was similar in roots and leaves. However, one exception was K^+. When analysed in roots, K^+ was found to be almost three times higher in saline conditions than in the control. Moreover, Na^+ accumulated in a more progressive manner in roots than in leaves as the salinity increased.

There was a progressive reduction of almost 6 ‰ in $\Delta^{13}C$ and close to 3.5 ‰ in $\delta^{15}N$ (Table 5) from control to 17 dS m^{-1}. Therefore, when considering all three growing conditions together, we found a positive relationship between $\Delta^{13}C$ and $\delta^{15}N$, even though this relationship was negative for each of the individual growing conditions considered separately (Fig. 2). Nitrogen concentration was ~2% less at 12 dS m^{-1} than control, but no further decrease occurred at 17 dS m^{-1} (Table 5). The total nitrogen content of the aerial part was three times less at 17 dS m^{-1} than control. The largest difference was found between the control and 12 dS m^{-1}.

Differences between saline tolerant and susceptible genotypes

Table 6 shows the mean biomass values for each of the genotype groups (tolerant and susceptible), plus the parameters from Tables 4 and 5 that revealed differences between the two groups of genotypes in at least one of the three growing conditions. As might be expected, there were highly significant differences ($P < 0.001$) in biomass between the two groups at each of the two levels of salinity. The tolerant group had values nearly 40% higher than the susceptible group at 12 dS m^{-1} and close to 50% higher at 17 dS m^{-1}. The tolerant group had a 15% higher biomass than the susceptible group ($P < 0.05$) under control conditions. In addition, the two groups of extreme genotypes showed significant differences ($P < 0.001$) in the number of tillers and the plant height (data not shown). The total N accumulated in the aerial part was ~40% higher ($P < 0.001$) in the tolerant group *v.* the susceptible group at both salinity levels, and there were no differences between the groups under control conditions. The K^+ concentration in the aerial part was ~10% higher ($P < 0.05$) in the tolerant group than in the susceptible genotype under the 12 dS m^{-1} condition. There were no differences in the other two growing conditions. The ratio K^+/Na^+ was ~15% higher in the tolerant group *v.* the susceptible group under control and at 12 dS m^{-1}, and no differences where observed at 17 dS m^{-1}. There was no difference between the two groups in the ion concentration of roots. The $\Delta^{13}C$ was smaller (0.36 ‰) in the tolerant group than in the susceptible group ($P < 0.05$) at 12 dS m^{-1}. No differences occurred in the other two growing conditions. The $\delta^{15}N$ was higher in the tolerant group for both 12 dS m^{-1} (0.68 ‰, $P < 0.001$) and 17 dS m^{-1} (0.34 ‰, $P < 0.01$), and no differences existed in the control conditions.

Genotypic correlations between biomass and physiological parameters

For each of the three growing conditions and across the set of 20 genotypes, we studied the linear correlations of biomass against the different physiological parameters included in Table 6 ($\Delta^{13}C$, $\delta^{15}N$, K^+ and K^+/Na^+) (Table 7). The total N content of the shoot was not considered, as this parameter is calculated using the shoot biomass. The K^+ concentration and the ratio K^+/Na^+ in the aerial part correlated positively with shoot

Table 4. Effect of different levels of salinity during growth on the ion concentration of shoots and roots of durum wheat
Data, expressed as a mmol per g of dry weight, are means ± s.e. of the 20 genotypes and four replications per genotype. Values followed by different letters are different by the Duncan's test ($P < 0.05$).

	K^+	Ca^{2+}	Na^+	P	Mg^{2+}	K^+/Na^+	Ca^{2+}/Na^+
				Aerial part			
Control	1.47 ± 0.02a	0.16 ± 0.00a	0.12 ± 0.00c	0.27 ± 0.00a	0.09 ± 0.01a	12.48 ± 0.40a	1.37 ± 0.04a
12 dS m^{-1}	0.70 ± 0.01b	0.07 ± 0.00b	1.44 ± 0.02b	0.25 ± 0.00b	0.06 ± 0.00b	0.50 ± 0.01b	0.04 ± 0.00b
17 dS m^{-1}	0.60 ± 0.01c	0.06 ± 0.02c	1.54 ± 0.05a	0.25 ± 0.00b	0.05 ± 0.00c	0.40 ± 0.00b	0.04 ± 0.00b
				Roots			
Control	0.04 ± 0.00c	0.62 ± 0.03a	0.17 ± 0.01c	0.09 ± 0.00a	0.06 ± 0.00a	0.28 ± 0.02a	5.37 ± 0.54a
12 dS m^{-1}	0.08 ± 0.01b	0.17 ± 0.00b	0.83 ± 0.03b	0.08 ± 0.02b	0.03 ± 0.00b	0.09 ± 0.01b	0.23 ± 0.01b
17 dS m^{-1}	0.13 ± 0.01a	0.16 ± 0.01b	1.46 ± 0.06a	0.08 ± 0.00b	0.02 ± 0.00b	0.09 ± 0.00b	0.12 ± 0.00b

Table 5. Effect of different levels of salinity on stable carbon isotope discrimination ($\Delta^{13}C$), stable nitrogen isotope composition ($\delta^{15}N$), the nitrogen concentration as mmol per g of dry weight (N) and the total N content of the shoot as g of nitrogen (Total N)

Data, are means ± s.e. of the 20 genotypes in durum wheat and four replications per treatment. Values followed by different letters were shown to be different ($P < 0.05$) by Duncan's test

	$\Delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	N (mmol g^{-1} DW)	Total N (g)
Control	20.81 ± 0.05a	3.14 ± 0.07a	3.78 ± 0.04a	0.55 ± 0.02a
12 dS m^{-1}	17.23 ± 0.07b	1.43 ± 0.07b	2.71 ± 0.04b	0.25 ± 0.01b
17 dS m^{-1}	15.02 ± 0.08c	–0.17 ± 0.06c	2.66 ± 0.03b	0.18 ± 0.15c

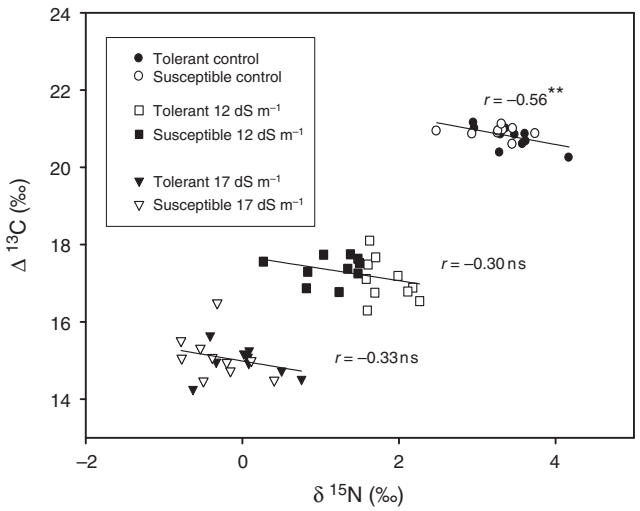

Fig. 2. Relationship between $\delta^{15}N$ and $\Delta^{13}C$. For each of the three growing conditions, relationships across the 20 genotypes of durum wheat is shown. Each point is the average of four plants of a given genotype and growing condition.

biomass under 12 dS m^{-1} conditions, but no correlation was observed for the other two growing conditions. $\Delta^{13}C$ was negatively correlated ($P < 0.05$) with biomass at 12 dS m^{-1}, while there was no correlation under control conditions or at 17 dS m^{-1}. $\delta^{15}N$ was the parameter that correlated best with shoot biomass. It was strongly and positively correlated at 12 dS m^{-1} and still positively (but not as strongly) correlated under control conditions (Table 7; Fig. 3). $\delta^{15}N$ also correlated best with total nitrogen accumulated in the shoot (data not shown). In this case, positive correlations ($P < 0.05$) were observed for the set of 20 genotypes in control and 12 dS m^{-1} conditions ($R^2 = 0.46$ and 0.60 for control and 12 dS m^{-1}, respectively).

In addition, a stepwise analysis was performed. The biomass of the set of 20 genotypes at either 12 dS m^{-1} or 17 dS m^{-1} was used as the dependent variables. The different ions analysed in both plant parts, their ionic ratios, $\Delta^{13}C$, $\delta^{15}N$ and the nitrogen concentration of shoots under any of the three growth conditions were used as independent variables (Table 8). The $\delta^{15}N$ measured at 12 dS m^{-1} was the first variable selected by the model. It alone accounted for around 70% of the genotypic variation in biomass at 12 dS m^{-1} conditions and 60% of the variation at 17 dS m^{-1}. The $\delta^{15}N$ at 17 dS m^{-1} was the second variable chosen by the model to explain the biomass in 12 dS m^{-1}. Ion concentration and ion ratios had a minor role in explaining the differences in biomass. Adding biomass under either of the other two growth conditions as a further independent variable to the stepwise analysis did not modify the primacy of $\delta^{15}N$ as the first variable chosen by the model.

Discussion

The effects of our experimental conditions on plant growth are comparable to those reported before in bread wheat. Thus, shoot biomass decreased by half when the level of salinity reached 12 dS m^{-1} (Ayers and Westcott 1989). In addition, plant height and the final tiller number decreased in saline conditions (Nicolas *et al*. 1993; Maas *et al*. 1994). We found genotypic variability in biomass, plant height and final tiller number. Moreover, plant height and the number of tillers under saline conditions were chosen as independent variables, after biomass under control conditions, in the stepwise analysis to predict genotypic differences in biomass under salinity. Plant height and tiller number can be considered screening criteria in durum wheat (Islam and Sedgley 1981; Munns and James 2003).

Chlorophyll content, calculated on the basis of leaf area and measured *in situ* (with a portable device), has been proposed as a screening criterion for wheat survival under high salinity (Munns and James 2003). In our study, chlorophyll content did not

Table 6. Effect of growing conditions on the different traits analysed in the two groups of genotypes from the RIL population of durum wheat, selected for the difference in their biomass within each of the two levels of salinity

Data shown are means ± s.e. of the 10 genotypes of each subset (tolerant and susceptible). Beside biomass and Na^+ concentration in roots, the table includes those physiological parameters that showed differences between groups in at least one of the three growth conditions

	Control			12 dS m^{-1}			17 dS m^{-1}		
	Tolerant	Susceptible	*P*	Tolerant	Susceptible	*P*	Tolerant	Susceptible	*P*
Biomass (g)	11.53 ± 0.53	9.64 ± 0.50	0.015	5.80 ± 0.19	4.17 ± 0.18	0.000	3.21 ± 0.13	2.19 ± 0.10	0.000
K^+ aerial part (mmol g^{-1} DW)	1.45 ± 0.02	1.49 ± 0.03	0.358	0.72 ± 0.01	0.68 ± 0.01	0.021	0.62 ± 0.01	0.59 ± 0.01	0.171
Na^+ aerial part (mmol g^{-1} DW)	0.11 ± 0.00	0.13 ± 0.00	0.006	1.40 ± 0.04	1.47 ± 0.04	0.225	1.61 ± 0.06	1.47 ± 0.08	0.119
K^+/Na^+ aerial part	13.73 ± 0.57	11.75 ± 0.67	0.013	0.52 ± 0.01	0.47 ± 0.02	0.043	0.39 ± 0.01	0.42 ± 0.01	0.205
Na^+ roots (mmol g^{-1} DW)	0.16 ± 0.01	0.19 ± 0.03	0.311	0.85 ± 0.05	0.82 ± 0.06	0.737	1.56 ± 0.10	1.34 ± 0.08	0.109
$\Delta^{13}C$ (‰)	20.76 ± 0.07	20.87 ± 0.08	0.306	17.06 ± 0.11	17.40 ± 0.08	0.013	14.99 ± 0.12	15.05 ± 0.11	0.663
$\delta^{15}N$ (‰)	3.43 ± 0.08	3.26 ± 0.10	0.227	1.84 ± 0.08	1.16 ± 0.08	0.000	0.04 ± 0.07	–0.30 ± 0.08	0.003
Total nitrogen per shoot (g)	0.55 ± 0.02	0.48 ± 0.02	0.104	0.20 ± 0.01	0.14 ± 0.006	0.000	0.11 ± 0.00	0.08 ± 0.00	0.000

Table 7. Linear correlation coefficients of the relationships within each of the three growing conditions and across the set of 20 genotypes of biomass of durum wheat against K^+ concentration and the K^+/Na^+ ratio, carbon isotope discrimination ($\Delta^{13}C$) and nitrogen isotope composition ($\delta^{15}N$) of the shoot

Levels of significance: ns, not significant; *, $P < 0.05$; **, $P < 0.01$

	K^+	K^+/Na^+	$\Delta^{13}C$	$\delta^{15}N$
Biomass control	0.091ns	0.068ns	–0.359ns	0.593**
Biomass 12 dS m^{-1}	0.753**	0.669**	–0.474*	0.832**
Biomass 17 dS m^{-1}	0.202ns	–0.394ns	–0.075ns	0.377ns

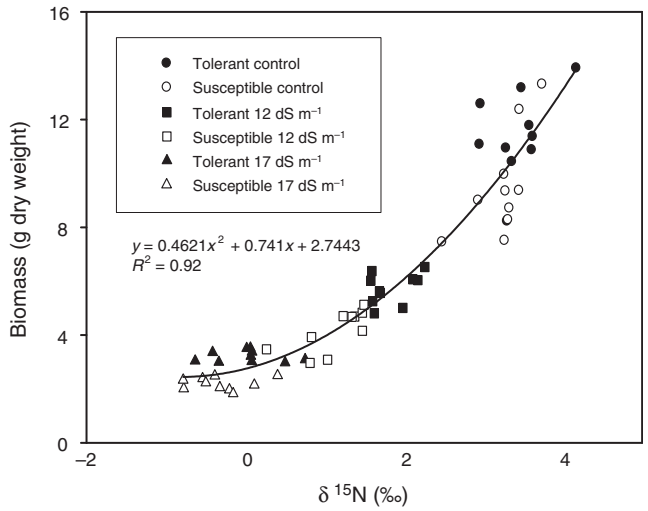

Fig. 3. Relationship between $\delta^{15}N$ and shoot biomass. For each of the three growing conditions, relationships across the 20 genotypes of durum wheat is shown, along with the general relationship pooled data for all 20 genotypes and the three different growing conditions together. Each point is the average of four plants of a given genotype and growing condition.

correlate with biomass at either of the two salinity levels. This may be because we measured chlorophyll in leaves that had expanded after the saline conditions had been well established. Moderate salinity may lead to thicker or denser leaves (i.e. with a more compact mesophyll), as reported in wheat (Passioura and Munns 2000), therefore, increasing the chlorophyll content per unit leaf area. Thus, we found that mean chlorophyll content was higher in 12 dS m^{-1} than in the control. However, the chlorophyll content at 17 dS m^{-1} was lower than the control values. This may be due to the combination of changes in leaf anatomy together with an accelerated senescence caused by high salinity.

The positive genotypic relationship between the biomass in the control and the two salinity levels suggests that greater intrinsic constitutive (i.e. in absence of stress) biomass may confer better adaptation to a wide range of salinity levels. Stepwise analysis further reinforced the evidence that genotypes with a higher growth potential perform better over a wide range of salinity levels. Isla *et al*. (1998) found that the highest yielding barley cultivars under non-saline conditions were the highest yielding under moderate salinity, but not under high salinity (with a soil EC of 22 dS m^{-1}). Nevertheless, they concluded that grain yield under salt stress remains the only reliable means of identifying higher salt tolerance in barley. However, Richards (1983) states that higher yields of durum and bread wheat in saline fields can be obtained by breeding for yield under non-saline conditions. However, he supports this approach with an agronomical consideration: as salinity is usually variable within a field, and most of the yield comes from the least saline areas, selecting for performance in a high-salinity environment alone may not be productive (Richards 1983).

The response of ion concentration to salinity, the balances within the plant and the existence of genotypic variability are in accordance with the results of previous studies in durum and bread wheat and other crops species. Thus, as reported before, Na^+ concentration in shoots of durum wheat did not increase linearly with increasing salinity, but reached a plateau around 50 mM NaCl treatment, which is less than our moderate salinity (Husain *et al*. 2004). Further, Na^+ exclusion and K^+/Na^+ discrimination, usually from leaves and shoots rather than roots, have been proposed as screening traits for tolerance to moderate salinity (e.g. Dvorak *et al*. 1994; Chhipa and Lal 1995; Colmer *et al*. 2005). However, in contrast to our study, Munns and James (2003) reported in a set of 21 tetraploid wheat genotypes that Na^+ correlated with shoot biomass far better than K^+/Na^+ and K^+. Nevertheless, maintenance of K^+ uptake – and, thus, a high K^+/Na ratio, even in the face of high external Na^+ concentration – is vital, since K^+ is a macronutrient that is essential for enzyme

Table 8. Stepwise analysis between the biomass of the 20 genotypes of durum wheat in each of the two levels of salinity as a dependent variable, and all the physiological parameters (ion concentration and ratios, N concentration and stable isotope signatures) measured in the three growing conditions as independent variables

Independent variables chosen in any of the two analyses contributed significantly to the models. S1, 12 dS m^{-1}; S2, 17 dS m^{-1}. Significance: ***, $P < 0.001$

Model	Variable chosen	r	R^2	Significance
Biomass 12 dS m^{-1}	$\delta^{15}N$ S1	0.832	0.692	***
	$\delta^{15}N$ S1, $\delta^{15}N$ S2	0.875	0.766	***
	$\delta^{15}N$ S1, $\delta^{15}N$ S2, Mg^{2+} aerial part S2	0.905	0.819	***
Biomass 17 dS m^{-1}	$\delta^{15}N$ S1	0.765	0.585	***
	$\delta^{15}N$ S1, Ca^{2+}/Na^+ aerial part S2	0.875	0.766	***

function. It is also a major osmoticum (Cuin *et al*. 2003; Colmer *et al*. 2005).

Effect of salinity on gas exchange, $\Delta^{13}C$, $\delta^{15}N$ and N concentration

Salinity may reduce the rate of photosynthesis through stomatal and non-stomatal effects (Heuer and Plaut 1989). In our results, both salinity treatments induced a strong decrease in photosynthetic rates, mainly due to stomatal limitation, as concluded from the parallel drop in the ratio C_i/C_a. This is in agreement with previous reports on wheat (Ouerghi *et al*. 2000) and other species (Rasmuson and Anderson 2002). Furthermore, both levels of salinity decreased the $\Delta^{13}C$ of shoot biomass. This may be evidence of long-term stomatal limitation of photosynthesis, and is in agreement with previous studies on wheat and other cereals (Isla *et al*. 1998; Ouerghi *et al*. 2000; Shaheen and Hood-Nowotny 2005). Thus, Isla *et al*. (1998) concluded for barley under field conditions that the $\Delta^{13}C$ of mature grains decreased by 0.2‰ per unit (dS m^{-1}) increase in soil solution electrical conductivity (EC). Decreases of ~0.1‰ have been reported for wheat leaves in pots (Shaheen and Hood-Nowotny 2005) and barley shoots under hydroponics (Handley *et al*. 1997). However, no differences in total plant dry matter between control and salinity were reported in either of these two studies under controlled conditions. In contrast, we found a decrease of 0.34‰ in the $\Delta^{13}C$ of shoot biomass per unit increase in ECs. The faster decrease in our study than that found by Isla *et al*. (1998) may be due to a set of different factors, such as the plant part analysed (whole shoot *v.* kernels), growing conditions (hydroponics and soil), the range of salinity levels (maximum ECs of 17 and 22 dS m^{-1}) and plant species (durum wheat *v.* the more tolerant barley). In the study by Handley *et al*. (1997), the salinity (a final concentration of 175 mol m^{-3} of NaCl for just 1 week) was probably too moderate.

We found, on average, a decrease of nearly 0.2‰ in shoot $\delta^{15}N$ per unit increase in ECs. Several studies on barley and hydroponics have reported that shoot $\delta^{15}N$ (Ellis *et al*. 1997; Handley *et al*. 1997) decreased at a rate near half per unit increase in ECs. Again, this is probably because barley is more tolerant to salinity than durum wheat (Munns *et al*. 2002). In addition, the salinity stress in these studies was mild. Other stresses, such as drought, may also reduce $\delta^{15}N$, but to a lesser extent than salinity (Robinson *et al*. 2000). However, owing to the purely hydroponic nature of this study (Robinson *et al*. 2000), drought stress was imposed by exposing roots to air for 3 h daily. In studies under natural conditions (Handley *et al*. 1999) or of plants in pots (Lopes *et al*. 2004; Lopes and Araus 2006) water stress caused an increase in $\delta^{15}N$, which suggests drought affects plant $\delta^{15}N$ in a different way to salinity.

Fractionation of ^{14}N and ^{15}N could occur at uptake from the medium into root cells, or during subsequent enzymatic assimilation into other N forms. Further fractionation may occur if biochemical components of varying isotopic composition are lost through translocation or exudation (Evans 2001; Pritchard and Guy 2005). Considering the three growing conditions together, $\Delta^{13}C$ and $\delta^{15}N$ were positively related (Fig. 2). Reduced stomatal conductance due to salinity should lead to a reduction in the loss of ammonia and nitrous oxide, and this should reduce ^{15}N enrichment, as light-isotope nitrogen compounds are lost more readily than compounds containing the heavy isotope (Farquhar *et al*. 1980; Smart and Bloom 2001). However, Lopes and Araus (2006) reported the opposite relationship between the isotopic signatures for durum wheat growing with different N sources and water regimes. In fact, other mechanisms (discussed in the next section) may also lead to salinity increasing discrimination against ^{15}N (and thus decreasing plant $\delta^{15}N$).

The decrease in N concentration and $\delta^{15}N$ found in saline conditions suggests that this stress also affects nitrogen uptake and/or assimilation (Ellis *et al*. 2002). Thus, plant $\delta^{15}N$ seems to be dependent upon the external concentration and isotopic signature of nitrogen, as well as the enzyme activity on source $\delta^{15}N$ (Mariotti *et al*. 1982). A high external N concentration relative to a modest demand can induce discrimination against ^{15}N. Consequently, the suboptimal growing conditions associated with any stress may produce a decrease in demand relative to a constant external N concentration. This may have the same effect as increasing the external concentration (Mariotti *et al*. 1982), leading to greater isotopic discrimination (Vitousek *et al*. 1989; Handley *et al*. 1997). In this regard, Handley *et al*. (1997) suggest that 'stress' would make $\delta^{15}N$ more negative than in controls, due to downregulation of the assimilating enzyme – in this case nitrate reductase. Further, $^{15}N/^{14}N$ fractionation may occur during nitrate assimilation by nitrate reductase or ammonium assimilation by glutamine synthetase (Evans 2001). These two enzymes have apparently similar *in vitro* discrimination factors (Ledgard *et al*. 1985; Yoneyama *et al*. 1993) and in wheat the activity of both is decreased by salinity (Carillo *et al*. 2005; Wang *et al*. 2007). Regardless of the mechanism affecting plant $\delta^{15}N$, this trait seems to reflect the capacity of the plant to use available nitrogen. Thus, when we combined all of the genotypes across the three growing conditions, $\delta^{15}N$ strongly positively correlated with total shoot biomass (Fig. 3) and with total shoot nitrogen content ($R^2 = 0.93$, data not shown). Handley *et al*. (1997) found no such relationships, probably because their salinity treatment was too short to modify nitrogen content and shoot biomass.

Nevertheless, since our $\delta^{15}N$ data only cover the shoot it could be argued that it is not possible to extrapolate to an assumption regarding discrimination during uptake and assimilation throughout the whole plant; the change in $\delta^{15}N$ in the shoot might be accompanied by the opposite change in the roots and overall plant $\delta^{15}N$ might not change at all. Changes in shoot $\delta^{15}N$ might be related to changes in partitioning of N assimilation between shoots and roots. Further, Lopes and Araus (2006) reported that water stress affected (increased) leaf rather than root $\delta^{15}N$ in durum wheat grown with a mixture of ammonium and nitrate (as in the present study).

Genotypic variability in $\Delta^{13}C$ and $\delta^{15}N$ and their relationships with biomass under salinity

Munns and James (2003) suggest that screening for high g_s may be the most effective way of selecting genotypes that will grow fast in saline soil. Isla *et al*. (1998) reported that $\Delta^{13}C$ was positively correlated with grain yield in the absence of stress, suggesting that the genotypes with higher g_s were the most

productive. However, this correlation was absent under highly saline conditions. For durum wheat, when moderate water stress is the factor limiting yield, a positive correlation between $\Delta^{13}C$ and yield usually emerges (Araus *et al*. 2003). However, we found a negative relationship between biomass and $\Delta^{13}C$ at 12 dS m^{-1}, meaning than the genotypes with lower g_s (and thus less transpiration) were the most tolerant. Nevertheless, we observed no differences in Na^+ concentration between tolerant and susceptible genotypes. Thus, it appears that toxic ions do not accumulate more in the susceptible genotypes due to higher transpiration. Alternatively, leaves that maintain an intrinsically higher photosynthetic capacity, due, for example, to less senescence, would have a smaller C_i/C_a (and, thus, lower $\Delta^{13}C$). Likewise, we found that at moderate salinity tolerant genotypes had higher chlorophyll content per unit leaf area than susceptible genotypes (39.0 *v.* 36.1, $P < 0.05$).

The range of genotypic variability in shoot $\delta^{15}N$ found in this study is similar or somewhat higher than that of previous studies with barley under control, salinity and N starvation conditions (Ellis *et al.* 1997; Handley *et al*. 1997; Robinson *et al*. 2000), but clearly less than that reported for root $\delta^{15}N$ in a mapping population of 156 barley double haploids (Ellis *et al*. 2002). Besides the existence of genotypic variability in plant $\delta^{15}N$, and the fact that this trait has been used to phenotype barley mapping populations (Ellis *et al*. 2002), there are no reports on genotypic relationships between $\delta^{15}N$ and biomass or yield under salinity. We found a positive relationship between shoot $\delta^{15}N$ and dry matter under control conditions and under moderate salinity in particular (Fig. 3) and comparable relationships were found between $\delta^{15}N$ and total shoot nitrogen content. Contrary to our results, in barley under hydroponics and short-term drought or N starvation, Robinson *et al*. (2000) reported a negative correlation between root $\delta^{15}N$ and plant dry weight, while shoot and whole-plant $\delta^{15}N$ were not correlated with dry weight. These authors conclude that the most productive and stress tolerant genotypes (based on a small stress index), were those that probably retained most N, therefore, they had the lowest plant $\delta^{15}N$. Conversely, genotypes expressing the least discrimination against ^{15}N were smaller and contained less N. However, the authors only used 18 genotypes for drought and eight genotypes for N-starvation out of the total set of 30 analysed in their study. More importantly, they related $\delta^{15}N$ with a stress index [calculated as (weight unstressed – weight stressed)/weight unstressed] rather than the absolute value of biomass under stress. Even if they excluded the least productive and least responsive genotypes, the relationship was still biased by the fact that the least productive genotypes under control conditions were those showing a smaller stress index and vice versa (see fig. 1 in Robinson *et al*. 2000). Recently, a positive genotypic relationship between the $\delta^{15}N$ of aerial plant parts against (absolute values) of grain yield and N grain yield have been reported in a set of 99 recombinant inbred lines of maize at low N input (Coque *et al*. 2006). These and other authors (Evans 2001; Coque *et al*. 2006) suggest that two mechanisms could explain genotypic variation in ^{15}N discrimination ability: morphophysiological differences, in particular in the root system; and the activity of the main enzymes in nitrogen metabolism. There should be a positive relationship between enzyme activity and discrimination abilities.

Some insights into the mechanism behind the genotypic relationship between $\delta^{15}N$ and biomass

If isotopic fractionation depends on the substrate-to-enzyme ratio (Mariotti *et al*. 1982) then higher enzyme activity or lower N supply reduces discrimination. Therefore, genotypes with a high N demand, high N assimilation capacity or low uptake capacity will all have a low substrate-to-enzyme ratio and therefore should discriminate less against ^{15}N (Robinson *et al*. 2000; Pritchard and Guy 2005). In accordance with this, Coque *et al*. (2006) reported a positive (albeit weak) relationship between glutamine synthetase activity and $\delta^{15}N$ in adult maize plants. If growth tends to be limited by N uptake, then genotypic variation in $\delta^{15}N$ should reflect uptake capacity, and correlations between biomass and $\delta^{15}N$ will therefore be negative (Pritchard and Guy 2005). In contrast, if growth is limited by assimilation capacity and/or growth-driven N demand, then genotypic correlations between biomass and $\delta^{15}N$ will be positive. The positive relationship we found between $\delta^{15}N$ and biomass (Fig. 3) suggests that genotypic differences in biomass under saline conditions are associated with assimilation capacity and N demand.

In agreement with this hypothesis, we found that the relationship between $\delta^{15}N$ and $\Delta^{13}C$ among genotypes within each growing condition tended to be negative at the two salinity levels and achieved statistical significance under control conditions (Fig. 2). Such negative relationships might result from genotypes with improved N nutrition exhibiting higher intrinsic photosynthetic capacity and enhanced water use efficiency (which result in a lower $\Delta^{13}C$). Alternatively, they may arise from differences in N and C demand for active growth, which would decrease discrimination against ^{15}N, thus, increasing $\delta^{15}N$ in plants (Pritchard and Guy 2005). Moreover, $\delta^{15}N$ correlated positively with shoot N content under control and 12 dS m^{-1} conditions. However, the genotypic relationship between $\delta^{15}N$ and $\Delta^{13}C$ was not as strong under salinity as under control conditions, despite the fact that $\delta^{15}N$ correlated with biomass better at 12 dS m^{-1} than under control conditions. This suggests other processes affecting $\Delta^{13}C$ may weaken the relationship between $\delta^{15}N$ and $\Delta^{13}C$ under salinity. Increasing salinity affects water use efficiency and $\Delta^{13}C$ through a decrease in stomatal conductance. Hence, an effect on $\delta^{15}N$ mediated by differences in stomatal conductance and subsequent loss of light N compounds should be ruled out since genotypic relationships between $\delta^{15}N$ and $\Delta^{13}C$ tended to be negative.

Further studies are required to clarify the mechanisms by which salinity affects $\delta^{15}N$. Regardless of the mechanism involved, genotypic differences in plant $\delta^{15}N$ seem to reflect the extent to which plants retain N in their tissues (Robinson *et al*. 2000). Likewise, Coque *et al*. (2006), working with maize under well fertilised conditions, found 10 QTLs for $\delta^{15}N$ that coincided with the QTLs involved in nitrogen use efficiency (grain yield, N-uptake and N remobilisation) and the root system.

We conclude that increased potential growth (i.e. in the absence of stress) may be a trait to consider when selecting genotypes that are better adapted to salinity. Moderate, rather than severe, salinity was the most adequate growing condition in which to evaluate genotypic performance under salinity. Nitrogen isotope composition was the trait that best correlated with genotypic differences in shoot biomass under moderate

salinity, and the only trait that correlated at high salinity. Changes in $\delta^{15}N$ in response to salinity seem to reflect the effect of such stress on N assimilation or N demand for plant growth, rather than on N uptake and loss of N compounds by the plant. In contrast, other variables that are classically associated with adaptation to salinity, such as ion concentration and ion ratios, explained the genotypic variability in biomass to a lesser extent. Our experimental conditions are comparable to those of previous studies in terms of the effect of salinity on plant growth, ion concentration, gas exchange and $\Delta^{13}C$. This reinforces the potential validity of $\delta^{15}N$ as a criterion to screen genotypes for tolerance to salinity. The possibility of measuring $\delta^{15}N$ in early stages of the plant cycle and then having a good prediction of differences in biomass between genotypes would further strengthen the potential validity of $\delta^{15}N$ as a criterion. In our study, we analysed $\delta^{15}N$ from plants sampled before the heading stage, and, therefore, long before anthesis. Moreover, the whole aerial part of the plant developed, rather than just the last leaf, was analysed. Therefore, examining $\delta^{15}N$ in even earlier plant stages (e.g. in seedlings) may still result in a reasonable prediction, providing the $\delta^{15}N$ signature of the kernel which initiates the seedling is diluted enough and the seedling has been exposed to moderate salinity for weeks. The possible inference of genotype performance under saline conditions from the $\delta^{15}N$ of young plants growing under non-saline conditions should also be considered. Here, we observed that $\delta^{15}N$ of control plants correlated significantly not just with biomass of control plants (Table 7) but also with biomass at 12 dS m^{-1} ($r = 0.545$, $P < 0.05$) and in a nearly significant manner with biomass at 17 dS m^{-1} ($r = 0.395$, $P < 0.1$). Even if $\delta^{15}N$ of plants at 12 dS m^{-1} correlated far better than $\delta^{15}N$ of control plants with biomass under salinity, it may still be reasonable to consider the constitutive value of $\delta^{15}N$ (i.e. in the absence of stress) as a selection trait.

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Capítulo 2

Effect of salinity and water stress during the reproductive stage on growth, ion concentrations, $\Delta^{13}C$, and $\delta^{15}N$ of durum wheat and related amphiploids

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Genotipos de trigo duro, triticale y tritordeum en el invernadero de los Servicios Experimentales de la Facultad de Biología, Universidad de Barcelona. Año 2007. Foto: S. Yousfi

Resumen

En este trabajo se estudia el comportamiento fisiológico del trigo duro y dos amfiploides relacionados durante la fase reproductiva y bajo diferentes combinaciones de salinidad y riego. Para ello se cultivaron un genotipo de triticale, un tritordeum y cuatro genotipos de trigo duro en macetas en ausencia de estrés hasta la fase de espigado, momento en que se aplicaron de manera progresiva seis tratamientos distintos. Dichos tratamientos resultaron de la combinación de dos regímenes de riego (100% y el 35 % de capacidad de contenedor) y tres niveles de salinidad (1.8, 12 y 17 dS m^{-1} respectivamente) y se mantuvieron durante tres semanas. Se hicieron medidas de intercambio de gases y fluorescencia de la clorofila antes de la cosecha, y posteriormente, se calculó la biomasa, la altura de la planta, la $\Delta^{13}C$ y $\delta^{15}N$ y la concentración de nitrógeno (N), fósforo (P); además se analizaron otros iones (K^+, Na^+, Ca^{2+}, Mg^{2+}) en las hojas. En comparación con las condiciones de control (riego completo con solución de Hoagland normal), todos los tratamientos presentaban una inhibición de la fotosíntesis debida al cierre de estomas, aceleración de la senescencia y disminución de biomasa. El riego completo con 12 dS m^{-1} superó a los otros tratamientos de estrés en cuanto a biomasa y comportamiento fisiológico. La biomasa se correlacionaba positivamente con N y $\delta^{15}N$ y negativamente con Na^+ en los genotipos y los tratamientos de riego completo. En cambio, las correlaciones en condiciones de riego deficitario fueron más débiles o ausentes. $\Delta^{13}C$ no se correlacionó con la biomasa en todos los tratamientos, pero fue el mejor criterio correlacionado con las diferencias fenotípicas en biomasa entre tratamientos. Tritordeum produjo más biomasa que el trigo duro en todos los tratamientos. Una baja $\Delta^{13}C$ y alta K^+/Na^+, junto con un elevado potencial de crecimiento pueden estar en la base de esta respuesta del tritordeum. Finalmente, el trabajo discute los mecanismos que relacionan $\delta^{15}N$ y $\Delta^{13}C$ con la biomasa.

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RESEARCH PAPER

Effect of salinity and water stress during the reproductive stage on growth, ion concentrations, $\Delta^{13}C$, and $\delta^{15}N$ of durum wheat and related amphiploids

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Abstract

The physiological performance of durum wheat and two related amphiploids was studied during the reproductive stage under different combinations of salinity and irrigation. One triticale, one tritordeum, and four durum wheat genotypes were grown in pots in the absence of stress until heading, when six different treatments were imposed progressively. Treatments resulted from the combination of two irrigation regimes (100% and 35% of container water capacity) with three levels of water salinity (1.8, 12, and 17 dS m^{-1}), and were maintained for nearly 3 weeks. Gas exchange and chlorophyll fluorescence and content were measured prior to harvest; afterwards shoot biomass and height were recorded, and $\Delta^{13}C$, $\delta^{15}N$, and the concentration of nitrogen (N), phosphorus, and several ions (K^+, Na^+, Ca^{2+}, Mg^{2+}) were analysed in shoot material. Compared with control conditions (full irrigation with Hoagland normal) all other treatments inhibited photosynthesis through stomatal closure, accelerated senescence, and decreased biomass. Full irrigation with 12 dS m^{-1} outperformed other stress treatments in terms of biomass production and physiological performance. Biomass correlated positively with N and $\delta^{15}N$, and negatively with Na^+ across genotypes and fully irrigated treatments, while relationships across deficit irrigation conditions were weaker or absent. $\Delta^{13}C$ did not correlate with biomass across treatments, but it was the best trait correlating with phenotypic differences in biomass within treatments. Tritordeum produced more biomass than durum wheat in all treatments. Its low $\Delta^{13}C$ and high K^+/Na^+ ratio, together with a high potential growth, may underlie this finding. Mechanisms relating $\delta^{15}N$ and $\Delta^{13}C$ to biomass are discussed.

Key words: $\Delta^{13}C$, $\delta^{15}N$, durum wheat, leaf photosynthesis, potassium, salinity, sodium, triticale, tritordeum, water limitation.

Introduction

Water scarcity is the main factor limiting agricultural productivity in the Mediterranean region (Araus, 2004). This limitation is likely to increase in the future as climatic change is expected to decrease precipitation and increase evapotranspiration (World Bank, 2007; Lobell *et al.*, 2008), and at the same time competition for water resources due to population growth and the development of economical sectors other than agriculture (e.g. industry or tertiary activities such as tourism) will also grow (Araus, 2004). Under such circumstances agriculture will be limited by reduced water supply and water of lower quality, particularly for crops with a water productivity (i.e. cash per unit water consumed) lower than that of horticultural or other intensive crops (Hsiao *et al.*, 2007). Deficit irrigation,

Abbreviations: A_{sat}, light-saturated net CO_2 assimilation rate; C_i/C_a, ratio of intercellular to ambient CO_2 concentration; DI, DI-12 dS m^{-1} and DI-17 dS m^{-1}, deficit irrigation with normal nutrient solution, and with 12 dS m^{-1} and 17 dS m^{-1} conductivity nutrient solutions, respectively; FI, FI-12 dS m^{-1} and FI-17 dS m^{-1}, full irrigation with normal nutrient solution, and with 12 dS m^{-1} and 17 dS m^{-1} conductivity nutrient solutions, respectively; g_s, stomatal conductance; F_v'/F_m', efficiency of excitation energy captured by open PSII reaction centres; T, transpiration rate; $\Delta^{13}C$, carbon isotope discrimination; $\delta^{15}N$, nitrogen isotope composition.

defined as the application of water below full crop water requirements, is one of the alternatives to sustain productivity (Fereres and Soriano, 2007). For durum wheat under Mediterranean conditions, deficit irrigation may improve water use efficiency (biomass produced per unit water applied) as well as water productivity compared with purely rainfed or fully irrigated crops (Oweis *et al.*, 1998; Hsiao *et al.*, 2007).

Durum wheat is one of the most cultivated herbaceous crops in the south and east Mediterranean basin (www.fao.org/statistics/yearbook). These environments, where durum and other cereals are cultivated, are characterized by 'terminal stress' in the sense that drought develops during the last part of the crop cycle. As this stress occurs during the reproductive period, it may affect yield, either impeding grain set or further affecting grain filling (García del Moral *et al.*, 2003). Under such circumstances, one possible way of increasing (or at least, stabilizing) productivity in semi-arid environments is to apply supplemental irrigation during the reproductive part of the crop cycle. As a drawback, the available water could be of low quality, which may compromise yield and critically expose soils to progressive salinization (World Bank, 2007). Therefore it would be advisable to use genotypes with improved salt tolerance (Munns *et al.*, 2002; Munns, 2008). This is particularly important in durum wheat, since it is much more salt sensitive than other cereals such as barley or even bread wheat (Munns *et al.*, 2002).

Morphophysiological traits for breeding of salt tolerance are extensively reviewed elsewhere (Munns and Tester, 2008, and references herein). They are based on the understanding that the mechanisms of salinity tolerance fall into any of the following categories: tolerance to osmotic stress; Na^+ exclusion from leaves; or tolerance of tissue to accumulated Na^+ (Munns and Tester, 2008). Moreover, salinity also affects N metabolism, reducing, for example, the levels and activity of nitrate reductase (Rao and Gnanam, 1990; Foyer *et al.*, 1998; Carillo *et al.*, 2005). In that regard, the stable nitrogen isotope signature ($\delta^{15}N$) of dry matter might be useful as a screening tool (Yousfi *et al.*, 2009), even though the mechanisms underlying the genotypic and environmental relationships between $\delta^{15}N$ and biomass are not fully elucidated.

Nevertheless, salt tolerance is a complex phenomenon where plant response depends on the phenological stage at which stress is experienced (Munns *et al.*, 2006). In this context, the suitability of screening techniques may depend not only on the severity of the saline conditions, but also on the plant stage at which salinity is imposed (Leland *et al.*, 1989). Moreover, the interaction between deficit irrigation and salinity may exacerbate the effect of salinity. Thus, while a toxicity-mediated effect may take time to develop (Munns, 2002), a premature senescence may also be produced if the drought effect is severe enough.

In a previous study (Yousfi *et al.*, 2009), the genotypic performance was evaluated during the first part of the plant cycle under full irrigation with different saline conditions. It was shown that nitrogen isotope composition ($\delta^{15}N$) was better for tracking genotypic differences in salinity tolerance than carbon isotope discrimination ($\Delta^{13}C$) and other widely accepted traits such as the accumulation and ratios of ions such as Na^+, K^+, and Ca^{2+}. However, for growth conditions such as those resembling supplemental (either fully or deficit) irrigation with brackish water during the later stages (i.e. anthesis and grain filling) of the crop cycle, the plant's response to salinity and, therefore, the traits that best reflect its performance, may be different. In fact, there are differences in the specific stage at which the plant first encounters salinity, and the duration of the stress may also be variable.

In this work the response of durum wheat and related amphiploids to either deficit irrigation or salinity, or both in combination, that was imposed at anthesis was evaluated. The main objective was to determine the most informative physiological traits on genotypic performance within, as well as across, growing conditions. To this end, two outstanding recombinant inbred lines (RILs), along with their common parents, of a population tested in an earlier study under continuous salinity during the vegetative stage were evaluated (Yousfi *et al.*, 2009). These two RILs were among the most salt tolerant in the entire population, while they exhibited a high growth in the absence of stress. One genotype each of the amphiploids triticale and tritordeum was also included. These two cereals were obtained after interspecific hybridization, having durum wheat as one of the parents, and have been reported to be comparatively better adapted to drought and salinity conditions than durum wheat (Gallardo and Fereres, 1989; Giunta *et al.*, 1993; Martín *et al.*, 1999, 2000; Villegas *et al.*, 2010), and therefore may represent a genetic bridge for the introgression of useful stress adaptation traits into wheat. Salinity tolerance was defined as genotypic differences in 'absolute' shoot biomass after growing plants under several saline conditions. Traits evaluated were the same as in Yousfi *et al.* (2009). It is postulated that natural abundance signatures of ^{13}C and ^{15}N measured in plant dry matter may be better at tracking genotypic performance within and across treatments than more conventional parameters such as ion concentration (Yousfi *et al.*, 2009).

Materials and methods

Plant material and growth conditions

The different species used in this study were durum wheat, triticale, and tritordeum. Four durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] genotypes were tested: two RILs (here termed as RIL47 and RIL85), obtained by single seed descent from the cross between Jennah Khetifa (also termed 'Lahn') and Cham 1 (hereafter Cham), and the two parents. This cross was performed in 1991 at the Tel Hadya research station (Aleppo province, Syria) by the CIMMYT/ICARDA durum breeding programme for Mediterranean dryland (Nachit *et al.*, 2001). These two RILs are among the best lines selected from a set of 112 belonging to the Jennah Khetifa×Cham population evaluated in a previous study (Yousfi *et al.*, 2009) for tolerance to different levels of continuous salinity during the vegetative stage (comprising from shortly after planting to booting). Jennah

Khetifa is a landrace that shows specific adaptation to the North African continental drylands, being tall and moderately resistant to drought and cold. Cham is a variety that has been released for commercial production in several countries of the Mediterranean basin. It exhibits broad adaptation and has both high yield potential and yield stability. A hexaploid tritordeum (×*Tritordeum* Asch & Graeb) was also included, which is a fertile amphiploid derived from crosses between *Hordeum chilense* Roem. et Schult. and durum wheat. The genome of *H. chilense* confers tritordeum with a certain degree of drought and salt tolerance (Martín *et al.*, 1999, 2000). The line tested was HT621 (reg. no. GP-7, PI 636334) developed and released in 2001 by the Institute for Sustainable Agriculture (CSIC), Córdoba, Spain. Although HT621 is not suitable for commercial cultivation due to its brittle rachis, hard glumes, excessive height, and therefore a low harvest index, it is very adapted to Mediterranean environments (Ballesteros *et al.*, 2005). Triticale (×*Triticosecale* Wittm.) is an allopolyploid obtained from combining the chromosomes of wheat (*Triticum* spp.) and rye (*Secale cereale* L.). The variety tested was Imperioso, a hexaploid triticale having durum wheat as a parental line, registered in 2006 by Agrovegetal, S.A., Seville, Spain, and characterized by high and stable productivity and good grain quality.

Plants were grown in a greenhouse at the Experimental Fields of the University of Barcelona, Spain. Plants were planted in a mixture of peat, perlite, and vermiculite (2:1:1). The average temperature during the experiment was 26/18 °C day/night. Relative humidity ranged from 50% to 68% and the maximum photosynthetic photon flux density (PPFD) was ~1000 μmol m^{-2} s^{-1}. Two seeds were planted in 3 dm^3 pots and watered to field capacity to facilitate germination. After a week, only one plant was left per pot.

The combinations of two water regimes and three levels of salinity were tested, accounting for a total of six different treatments. Water regimes corresponded to full irrigation (FI) (100% of container capacity) and deficit irrigation (DI) (35% of container capacity), respectively. The three salinity levels were 1.8 dS m^{-1} (which corresponds to half-strength normal Hoagland solution; Hoagland and Arnon, 1950), 12 dS m^{-1}, and 17 dS m^{-1}. A completely randomized design was used to accommodate the three-way factorial experiment, with genotype, water level, and salinity stress as factors. Three single-pot replicates per factorial combination were used, totalling 108 pots. All plants were grown in the absence of water stress and supplied with a half-concentrated Hoagland solution until heading. The DI regime was imposed progressively over 1 week by decreasing irrigation, and then the two salinity treatments were imposed by adding NaCl progressively to the nutrient solution, starting with a salt concentration of 4 dS m^{-1}. This concentration was increased progressively during 1 week to reach the final salt levels of 12 dS m^{-1} (~120 mM NaCl) or 17 dS m^{-1} (~170 mM NaCl). A total of six treatments were studied: (i) FI, full irrigation (i.e. control) with normal Hoagland solution; (ii) FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dS m^{-1}; (iii) FI-17 dS m^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; (iv) DI, deficit irrigation with normal Hoagland solution; (v) DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}; and (vi) DI-17 dS m^{-1}, deficit irrigation with Hoagland solution at 17 dS m^{-1}. The different treatments were fully established at anthesis and then the plants were grown for ~3 weeks when they were harvested. Plants were grown for a total of 4 months. Except for triticale, which was 2–3 d earlier, all other genotypes reached anthesis simultaneously.

Gas exchange measurements

Leaf gas exchange was measured at the end of treatments. Measurements were made with an open IRGA LI-COR 6400 system (LICOR Inc., Lincoln, NE, USA). For each treatment and genotype, measurements were carried out in three randomly chosen, fully expanded flag leaf blades, each one from a different pot, at 10–15 h (solar time) under saturated PPFD conditions (>1200 μmol m^{-2} s^{-1}), at a temperature of 25 °C, and chamber CO_2 concentration of 400 μmol mol^{-1}. The gas exchange parameters were light-saturated net CO_2 assimilation (A_{sat}), transpiration rate (T), and stomatal conductance (g_s). Subsequently, the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) was calculated according to Sharkey and Raschke (1981). The efficiency of excitation energy captured by open PSII reaction centres (F_v'/F_m') was also estimated in the same leaves.

Plant growth and leaf chlorophyll content

Chlorophyll content was measured in the same flag leaves monitored for gas exchange. Four measurements were performed from the middle of the leaf blade just before harvesting using a portable meter (Minolta SPAD 502 Meter). The height of the main shoot of each plant was measured with a ruler prior to harvest, with a precision of ~1 mm. After harvesting, shoots were oven dried at 70 °C for 48 h, weighed, and finely ground for subsequent analyses.

Ion analysis

For each shoot sample analysed, 100 mg of dry material was digested with 3 ml of concentrated HNO_3 and 2 ml of H_2O_2. The samples were placed overnight in a microwave at 90 °C. After digestion, each sample was then brought up to 30 ml final volume with pure water. The amount of Na^+, Ca^{2+}, K^+, P, and Mg^{2+} in the sample was then determined with an Inductively Coupled Plasma Emission Spectrometer (L3200RL, Perkin Elmer, Germany) at the Scientific Facilities of the University of Barcelona. Ion concentrations were expressed as mmol per g of dry weight.

Total nitrogen concentration and stable carbon and nitrogen isotope signatures

Total nitrogen concentration and the stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios in the shoots were measured using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan, Germany), operating in continuous flow mode. Samples of ~1 mg and reference materials were weighed into tin capsules, sealed, and then loaded into an automatic sampler (ThermoFinnigan, Germany) prior to EA-IRMS analysis. Measurements were carried out at the Scientific Facilities of the University of Barcelona.

Nitrogen was expressed as either concentration (mmol per g of dry weight) or total content (g) of the aerial part (shoot nitrogen). The $^{13}C/^{12}C$ ratios were expressed in δ notation (Coplen, 2008):

$$\delta^{13}C = (^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1 \text{ (Farquhar et al., 1989)}$$

where 'sample' refers to plant material and 'standard' to Pee Dee Belemnite (PDB) calcium carbonate. The same δ notation was used for the $^{15}N/^{14}N$ ratio ($\delta^{15}N$), but in this case the standard referred to N_2 in air. Atropine was used as a system check in the elemental analyses of nitrogen. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose, and USGS 40 L-glutamic acid) were used for calibration to a precision of 0.1‰. For nitrogen, isotope secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N_1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate) were used. The mean $\delta^{15}N$ of the fertilizer provided by the Hoagland solution was 0.6 ‰.

The carbon isotope discrimination ($\Delta^{13}C$) of shoots was calculated as:

$$\Delta^{13}C(‰) = (\delta^{13}C_a - \delta^{13}C_p)/[1+(\delta^{13}C_p)/1000],$$

where the subscripts a and p refer to air and the plant, respectively (Farquhar *et al.*, 1989). Air samples were taken inside the greenhouse and analysed by the GC-C-IRMS technique, as previously described in Nogués *et al.* (2004). Air analyses were carried out at the Scientific Facilities of the University of Barcelona. The $\delta^{13}C_a$ was –11.3‰.

Statistical analysis

Data for the set of morphophysiological traits were subjected to factorial analyses of variance (ANOVAs) to test for the effects of treatment (irrigation, salinity), genotype, and their first- and second-order interactions. Means were compared by Duncan's test (P <0.05). The morphophysiological data set was then subjected to stepwise discriminant analyses to ascertain which traits best discriminated between genotypic or treatment groups. The two procedures made use of neither the 'biomass' variable (since it was used as a dependent variable in subsequent stepwise regression analyses) nor the 'total shoot N' variable (whose values were tightly related to those of 'biomass'). The 'control' (i.e. full irrigation with normal nutrient solution) records were not included in the discriminate analysis of treatment groups so as to highlight differences in the data set that had only arisen from deficit irrigation or salinity effects. The significance level corresponding to the F-value for developing or retaining a specific trait was set at P=0.15. All traits that remained in the models once the stepwise regression processes stopped were considered to discriminate significantly between groups (either genotype or treatment). For the selected traits, Hotelling's T^2 statistics were calculated to test for significance of between-group differences. Canonical discriminant analysis was then used to perform graphical representations of the classifications. Either treatment means (for distinguishing between genotype groups) or genotype means (for distinguishing between treatment groups) across replicates were used as input for the canonical analyses. In order to test the association between biomass and the set of morphophysiological traits measured, further linear stepwise models across genotypes were constructed that were independent for each growing condition, with P=0.05 as the criterion for variables to be either included or removed from the model. Data were analysed using SPSS (SPSS Inc., Chicago, IL, USA) and SAS (SAS Institute Inc., Cary, NC, USA) statistical packages.

Results

The effect of treatments and genotypes on growth parameters

Growing conditions other than control (FI) significantly decreased aerial biomass, plant height, leaf chlorophyll, nitrogen concentration, and total shoot nitrogen content (Table 1). The combination of deficit irrigation and salinity most affected all growth traits under study. Thus FI-12 dS m^{-1} limited growth less than DI, while no significant differences existed between the latter treatment and FI-17 dS m^{-1}. Genotypes significantly differed for all traits, whereas significant interaction between genotype and growing conditions (G×T) only existed for chlorophyll and N concentration (Table 1). Overall, Cham was the genotype with the lowest biomass and shoot N concentration across the four most stressful treatments (FI-17 dS m^{-1}, DI, DI-12 dS m^{-1}, and DI-17 dS m^{-1}), while tritordeum and triticale showed the highest values at DI and DI-12 dS m^{-1}(Supplementary Table S1 available at *JXB* online). Tritordeum and triticale also showed the highest biomass in the absence of stress (FI). The relative decrease (i.e. compared with FI) in biomass for each of the six genotypes at any of the five stress treatments was also studied. Overall, RIL47 was the genotype least affected (i.e. showed the least reduction in biomass) by the various treatments, followed by RIL85. In contrast, Cham and triticale were usually the most affected genotypes (except for DI-12 dS m^{-1}), while tritordeum and Lahn showed intermediate responses (Fig. 1).

The effect of irrigation, salinity, and genotype on photosynthesis and stable isotope signatures

Compared with control, all the other treatments strongly decreased in A_{sat}, g_s, C_i/C_a, T, F_v'/F_m', $\Delta^{13}C$, and $\delta^{15}N$. FI-17 dS m^{-1} and DI- 17 dS m^{-1} were the treatments that most affected these traits, and DI was the treatment with the least effect (Table 2). There were no differences for A_{sat} among treatments other than control, even though g_s and T were slightly, but significantly, higher at FI- 12 dS m^{-1} as well as for DI. Treatment effect was higher than genotypic effect for $\delta^{15}N$, while the opposite occurred for $\Delta^{13}C$. Cham showed the highest $\Delta^{13}C$ value and the lowest records for other traits, apart from g_s. In turn, titordeum, followed by triticale, showed the lowest $\Delta^{13}C$ values. Transpiration, g_s, $\delta^{15}N$, and $\Delta^{13}C$ were the only traits not showing a significant G×T intercation.

The effect of irrigation, salinity, and genotype on ion concentration

Treatments significantly affected the concentration of ions in the shoots. Thus, all treatments with saline water strongly increased Na^+, and slightly decreased K^+, Ca^{2+}, Mg^{2+}, and P, as compared with control conditions. Therefore, the ratios K^+/Na^+ and Ca^{2+}/Na^+ decreased markedly. DI was the treatment that affected ion concentration the least. There were also genotypic differences for all traits, with triticale exhibiting the highest K^+/Na^+ and Ca^{2+}/Na^+ ratios (Table 3). Except for Ca^{2+}, all ions and their selected ratios showed significant genotype by treatment interactions. Thus, for example, tritordeum and triticale showed the highest K^+/Na^+ ratios under FI, FI-12 dS m^{-1}, and DI, but this was not the case for the other treatments (Supplementary Table S1 at *JXB* online).

Overall differences across treatments

The stepwise discriminant analysis indicated that 10 traits contributed the most to the differentiation among treatments. These were (ranked by order of inclusion in the model): Na^+, K^+/Na^+, leaf chlorophyll (SPAD), K^+, $\delta^{15}N$, F_v'/F_m', Ca^{2+}, T, N concentration, and plant height. Hotelling's T^2-statistic testing for between-treatment differences was significant for all pairwise comparisons. This was also suggested by the outcome of a canonical discriminant

Table 1. Effect of different levels of salinity, water stress, and the combination of the two stresses on the shoot biomass and nitrogen (N) concentration, the total shoot nitrogen content, plant height, and chlorophyll content of the flag leaf of durum wheat (Cham, Lahn, RIL47, and RIL85), triticale (Imperioso), and tritordeum (HT621)

For each genotype values shown are the means of three repetitions. The means followed by different letters were significantly different (P <0.05) by Duncan's test. The associated sum of squares and probabilities (ns, not significant; **P <0.01; ***P <0.001) are shown.

	Biomass (g)	Plant height (cm)	Leaf chlorophyll (SPAD units)	Shoot N concentration (mmol g^{-1} DW)	Total shoot N (g)
Genotype					
Cham	51.08 a	66.36 ab	20.74 b	2.32 a	1.42 a
Lahn	64.88 b	66.65 ab	23.37 bc	2.57 b	1.88 abc
RIL47	63.39 b	74.94 c	28.07 d	2.72 b	1.84 abc
RIL85	62.27 ab	70.05 b	25.44 cd	2.20 a	1.55 ab
Triticale	68.56 b	81.50 d	21.49 b	2.65 b	2.07 c
Tritordeum	67.50 b	64.52 a	15.86 a	2.82 b	2.00 bc
Treatment					
FI	121.81 d	78.65 c	48.90 d	3.94 e	4.75 d
FI-12 dS m^{-1}	73.00 c	73.89 bc	31.12 c	2.76 d	2.00 c
FI-17 dS m^{-1}	58.33 b	68.33 a	17.81 b	2.30 bc	1.35 b
DI	60.06 b	70.07 ab	15.67 b	2.11 b	1.29 b
DI-12 dS m^{-1}	39.17 a	68.76 a	14.45 b	2.41 c	0.95 a b
DI-17 dS m^{-1}	29.18 a	65.06 a	10.12 a	1.79 a	0.55 a
ANOVA					
G	3693.27**	3506.95***	924.81***	6.40***	6.93***
T	73367.21***	1916.70***	15364.23***	42.60***	161.98***
G×T	10805.36 ns	1335.46 ns	3036.73***	6.54***	12.33 ns

FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}; DI-17 dS m^{-1}, deficit irrigation with Hoagland solution at 17 dS m^{-1}; G, genotype; T, treatment; G×T: genotype by treatment interaction.

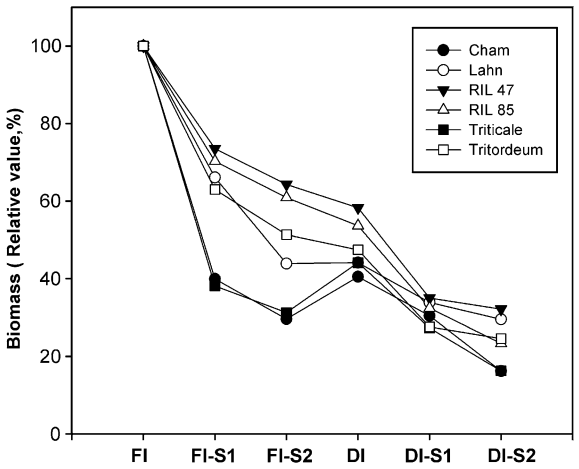

Fig. 1. Relative decrease in shoot biomass of the six different genotypes under the different growing conditions compared with control conditions. For each genotype and growing condition values are expressed as a percentage of the average of the same genotype under full irrigation with Hoagland solution (FI). FI-S1, full irrigation with Hoagland solution at 12 dS m^{-1}; FI-S2, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation with normal Hoagland solution; DI-S1, deficit irrigation with Hoagland solution at 12 dS m^{-1}; DI-S2, deficit irrigation with Hoagland solution at 17 dS m^{-1}.

analysis (Fig. 2), with most of the between-treatments to within-treatments variability explained by the first two canonical axes (CAN1 and CAN2). The discriminant loadings for each variable (or simple correlations of the variable and the discriminant scores for each axis), imposed on the plot representation as attribute points, provided a more complete interpretation of the analysis. Following the direction denoted by these points, CAN1 was mainly concerned with Na^+, hence separating FI-17 dS m^{-1} (with high Na^+) from the other, lower Na^+, treatments, which tended to exhibit higher Ca^{2+}, K^+/Na^+, T, F_v'/F_m', and $\delta^{15}N$ values and correspond to the three deficit irrigation treatments. In turn, CAN2 corresponded closely to Ca^{2+}, K^+, N concentration, and leaf chlorophyll, with FI-12 dS m^{-1} as the only group having higher values of these attributes.

Relationships between biomass and physiological traits across treatments

Relationships between biomass and physiological variables were assessed across full irrigation (i.e. combining FI, FI-12 dS m^{-1}, and FI-17 dS m^{-1}) and deficit irrigation (i.e. DI, DI-12 dS m^{-1}, and DI-17 dS m^{-1}) treatments, independently. Overall, biomass was negatively related to Na^+ concentration and positively related to the ratio K^+/Na^+. However, relationships were not linear and they followed

Table 2. Effect of different levels of salinity, water stress, and the combination of the two stresses on leaf net CO_2 assimilation (A_{sat}), stomatal conductance (g_s), the ratio of intercellular to ambient CO_2 concentration (C_i/C_a), the transpiration rate (T), efficiency of excitation energy capture by open PSII reaction centres (F_v'/F_m'), stable carbon isotope discrimination ($\Delta^{13}C$), and stable nitrogen isotope composition ($\delta^{15}N$) of durum wheat (Cham, Lahn, RIL47, and RIL85), triticale (Imperioso). and tritordeum (HT621)

Gas exchange measurements were performed in flag leaf blades and stable isotopes analysed in shoots sampled ~3 weeks after anthesis. Abbreviations for treatments and ANOVA analysis are as defined in the footnotes of Table 1. Means followed by different letters were significantly different (P <0.05) by Duncan's test. The associated sum of squares and probabilities (ns, not significant; $^{**}P$ <0.01; $^{***}P$ <0.001) are shown.

	A_{sat} (μmol CO_2 m^{-2} s^{-1})	g_s (mol CO_2 m^{-2} s^{-1})	C_i/C_a	F_v'/F_m'	T (mmol H_2O m^{-2} s^{-1})	$\Delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Genotype							
Cham	4.62 a	0.07 ab	0.29 a	0.18 a	0.98 a	21.12 c	3.41 a
Lahn	5.70 bc	0.09 ab	0.32 a	0.30 bc	1.66 bc	20.93 bc	5.03 cd
RIL47	5.74 bc	0.06 ab	0.37 ab	0.25 ab	1.47 ab	21.47 d	5.96 e
RIL85	5.11 ab	0.05 a	0.34 a	0.22 ab	1.10 ab	20.90 bc	5.37 de
Triticale	6.89 c	0.10 b	0.51 c	0.39 d	2.19 c	20.84 b	4.44 bc
Tritordeum	5.55 b	0.06 ab	0.46 bc	0.36 cd	1.20 ab	20.34 a	4.13 ab
Treatment							
FI	22.14 b	0.35 b	0.80 d	0.66 d	6.03 d	21.30 c	7.27 c
FI-12 dS m^{-1}	2.76 a	0.02 a	0.33 b	0.22 b	0.75 bc	20.89 ab	4.31a
FI-17 dS m^{-1}	1.99 a	3×10–3 a	0.16 a	0.02 a	0.08 a	20.72 a	3.60 a
DI	2.68 a	0.04 a	0.61 c	0.38 c	1.38 c	21.12 bc	5.61 b
DI-12 dS m^{-1}	2.72 a	0.02 a	0.20 a	0.36c	0.51ab	21.06 bc	4.46 a
DI-17 dS m^{-1}	1.80 a	0.02 a	0.20 a	0.09 a	0.38 ab	20.75 a	3.59 a
ANOVA							
G	53.43***	0.02 ns	0.68***	0.66***	18.95***	9.61***	70.70***
T	4441.68***	1.46***	5.42***	4.45***	368.02***	3.4 ***	125.51***
G×T	145.82**	0.07 ns	1.18***	1.33***	21.45 ns	5.38 ns	42.94 ns

a different pattern under full irrigation compared with deficit irrigation (Fig. 3A, B). Nitrogen concentration was related positively to biomass, but the linear relationship was different across the two irrigation regimes (Fig. 4). $\delta^{15}N$ also correlated positively with total biomass, but only when full irrigation treatments were combined, while no correlation existed across deficit irrigation treatments (Fig. 5A). $\Delta^{13}C$ did not relate to biomass across full irrigation or deficit irrigation treatments (Supplementary Fig. S1A at *JXB* online). However the ratio C_i/C_a was positively related to biomass across treatments, although again full and deficit irrigation followed different patterns (Supplementary Fig. S1B at *JXB* online).

Overall genotypic differences

The stepwise discriminant analysis indicated that nine attributes contributed the most to the differentiation among genotypes. These were (ranked by order of inclusion in the model): $\Delta^{13}C$, plant height, P, K^+/Na^+, $\delta^{15}N$, C_i/C_a, Ca^{2+}, Mg^{2+}, and N concentration. However, Hotelling's T^2-statistic testing for between-genotypes differences was in some cases not significant. In particular, Lahn was not statistically distinguishable from RIL85 (P=0.25), nor was RIL47 from RIL85 (P=0.12). This was also suggested by the outcome of a canonical discriminant analysis (Fig. 6), with most of the between-genotypes to within-genotypes variability explained by the first two canonical axes (CAN1 and CAN2). The discriminant loadings for each variable, imposed on the plot as attribute points, provided a better interpretation of the analysis. The first dimension correlated closely (and negatively) with $\Delta^{13}C$ and P, and separated the two amphiploids (tritordeum and triticale) from the durum wheat genotypes satisfactorily. Differences between durum wheat genotypes were smaller. Even so, Cham was placed away from the other three durum wheat genotypes. On the one hand, RIL47 and RIL85 (as well as tritordeum) had centroids with positive values of CAN2, which was basically related to higher plant height, $\delta^{15}N$, and Mg^{2+} values. On the other hand, Cham (and also triticale) displayed a negative value for this dimension, while Lahn occupied an intermediate position in the figure.

Both tritordeum and triticale exhibited the highest biomass and the lowest $\Delta^{13}C$, $\delta^{15}N$, and Na^+ content, and the highest K^+/Na^+ ratio under FI and DI (Supplementary Table S1 at *JXB* online). Tritordeum also exhibited the highest biomass at FI-12 dS m^{-1} and FI-17 dS m^{-1}, followed by Lahn and the two RILs, respectively. Under FI-12 dS m^{-1} a high biomass was again accompanied by low $\Delta^{13}C$ and Na^+, together with high K^+/Na^+ and C_i/C_a ratios. Triticale and tritordeum also showed the highest biomass at DI-12 dS m^{-1} and DI-17 dS m^{-1}, respectively, while Lahn ranked second in both cases. Triticale also showed the highest C_i/C_a and A_{sat} at DI-12 dS m^{-1}, together with the lowest Na^+ and the highest K^+/Na^+ ratio. In turn, tritordeum showed the highest N concentration and A_{sat} at DI-17 dS m^{-1}, together with the lowest $\Delta^{13}C$. Cham was the genotype with the least growth in most treatments (Supplementary Table S1 at *JXB* online). On the other hand, Cham and triticale were the genotypes exhibiting the

Table 3. Effect of different levels of salinity, water stress, and the combination of the two stresses during growth on the ion concentration of shoots of durum wheat (Cham, Lahn, RIL47, RIL85), tritordeum (Imperioso), and triticale (HT621)

Measurements were performed ~3 weeks after anthesis. Abbreviations for treatments and ANOVA analysis are as defined in the footnotes of Table 1. The values shown are the means of three replicates of each genotype. Concentrations are expressed as mmol per g of dry weight. Means followed by different letters are different by Duncan's test (P <0.05). The associated sum of squares and probabilities (ns, not significant; ** P <0.01; *** P <0.001) are shown.

	Na^+	K^+	Ca^{2+}	Mg^{2+}	P	K^+/Na^+	Ca^{2+}/Na^+
Genotype							
Cham	0.87 a	1.14 ab	0.20 d	0.14 a	0.24 d	5.11 a	0.94 a
Lahn	1.07 b	1.22 bc	0.16 b	0.15 a	0.22 c	6.90 a	1.04 a
RIL47	0.73 a	1.19 bc	0.18 c	0.14 a	0.19 b	6.14 a	1.03 a
RIL85	0.85 a	1.18 ab	0.19 c	0.15 a	0.20 c	5.24 a	0.91 a
Triticale	0.82 a	1.26 c	0.14 a	0.15 a	0.18 b	23.20 b	3.17 b
Tritordeum	1.05 b	1.13 a	0.19 c	0.18 b	0.16 a	6.90 a	1.20 a
Treatment							
FI	0.07 a	1.32 d	0.27 d	0.18 d	0.22 c	31.50 d	5.59 c
FI-12 dS m^{-1}	0.92 c	1.23 c	0.20 c	0.17 d	0.18 ab	1.94 ab	0.30 a
FI-17 dS m^{-1}	3.31 d	1.10 b	0.11 a	0.01 a	0.17 a	0.34 a	0.03 a
DI	0.09 a	1.27 cd	0.17 b	0.15 c	0.21 c	17.40 c	2.25 b
DI-12 dS m^{-1}	0.34 b	1.00 a	0.15 b	0.14 bc	0.20 b	3.30 b	0.52 a
DI-17 dS m^{-1}	0.45 b	1.20 c	0.14 b	0.13 b	0.21 c	3.88 b	0.49 a
ANOVA							
G	1.16***	0.18**	0.04***	0.02***	0.05***	7548.82***	142.24***
T	133.61***	1.07***	0.20***	0.07***	0.03***	16304.85***	473.80***
G×T	7.78***	1.47***	0.01 ns	0.01**	0.03***	20877.91***	472.97***

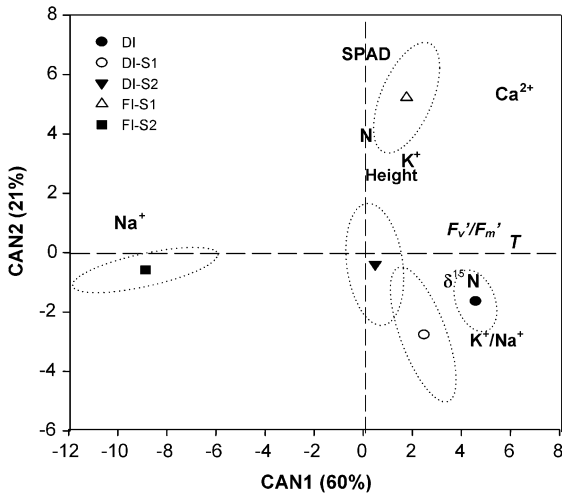

Fig. 2. Plot of the centroids (mean values) and their 95% confidence ellipses for the first two canonical variables of full and deficit irrigation treatments. Rescaled discriminant loadings of the explanatory variables are included in the plots. Abbreviations for treatments are as defined in the legend of Fig. 1. Full irrigation with normal Hoagland solution was not included in the analysis. Abbreviations of variables are as follows: $\delta^{15}N$, stable nitrogen isotope composition; F_v'/F_m', efficiency of excitation energy capture by open PSII reaction centres; height, plant height; K^+, Na^+, Ca^{2+}, dry matter concentrations of K^+, Na^+, and Ca^{2+}, respectively; K^+/Na^+, ratio of K^+ to Na^+ concentrations in dry matter; N, nitrogen concentration; SPAD, leaf chlorophyll content; T, transpiration rate. Except for ion concentrations and ratios, which were analysed in the whole shoot, and plant height, all other parameters were measured in the flag leaf blade.

highest relative decrease in biomass at both fully and deficit irrigation conditions as a response to Na^+ accumulated in the plant (Supplementary Fig. S2 at *JXB* online).

Relationships between biomass and physiological traits across genotypes

Stepwise regressions were performed for each growing condition using biomass as the dependent variable and combining all individual measurements for the six genotypes (Table 4). Except for the most severe treatment (DI-17 dS m^{-1}), the first trait selected was related to plant photosynthetic performance; either g_s (FI), $\Delta^{13}C$ (FI-12 dS m^{-1} and FI-17 dS m^{-1}), or C_i/C_a (DI and DI-12 dS m^{-1}). Moreover, $\Delta^{13}C$ ranked second in the selection process under DI and DI-12 dS m^{-1}, and C_i/C_a also ranked second at DI-17 dS m^{-1} (Table 4). $\Delta^{13}C$ and biomass were negatively correlated within each treatment except for FI and DI-17 dS m^{-1} (Fig. 7A, B). C_i/C_a had a positive influence on biomass under DI and DI-12 dS m^{-1}, while it had a negative influence under DI-17 dS m^{-1}, the most severe treatment. A positive influence of K^+/Na^+ on biomass was only detected under FI-17 dS m^{-1}. A high N content was chosen as the first trait at DI-17 dS m^{-1}.

Discussion

Growing conditions and genotypes both differed significantly for biomass and plant height; traits that can be considered useful for screening durum wheat germplasm

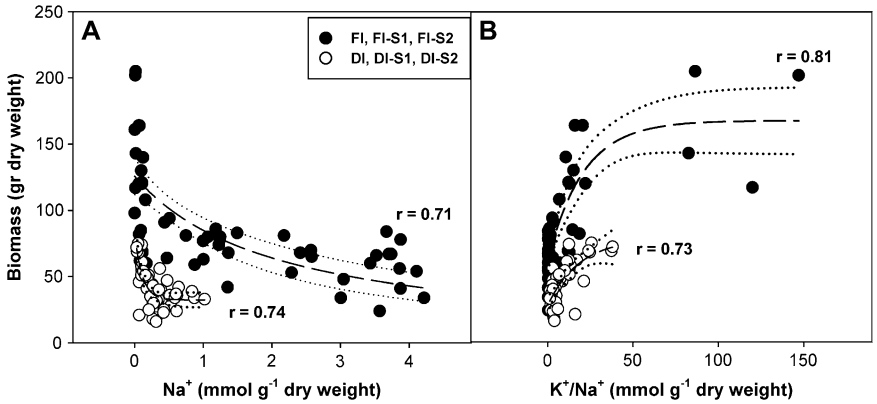

Fig. 3. Exponential relationship between biomass and (A) the Na^+ concentration and (B) the K^+/Na^+ ratio in shoots across the six genotypes (four of durum wheat, one of triticale, and one of tritordeum) assayed under full irrigation (filled circles: FI, FI-S1, and FI-S2) and deficit irrigation (open circles: DI, DI-S1, and DI-S2) conditions. Each point represents the individual value for a given replication and genotype within a growing condition. Plants were sampled ~3 weeks after anthesis after 3 weeks of treatments. Abbreviations for treatments are as defined in the legend of Fig. 1.

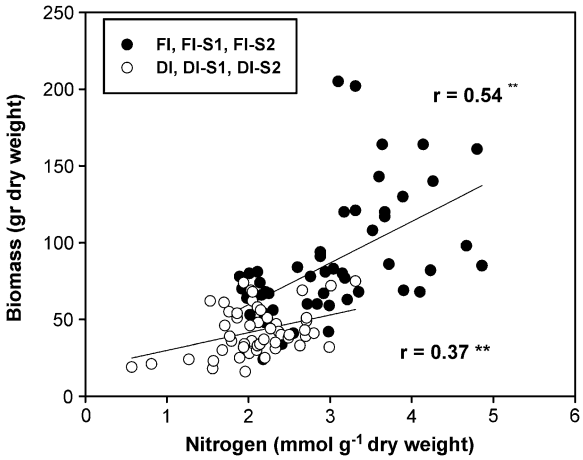

Fig. 4. Relationship between biomass and the nitrogen concentration in the shoot across the six genotypes (four of durum wheat, one of triticale, and one of tritordeum) assayed under full irrigation (filled circles: FI, FI-S1, and FI-S2) and deficit irrigation (open circles: DI, DI-S1, and DI-S2) conditions. Each point represents the individual value for a given replication and genotype within a growing condition. The coefficient of regression for the linear relationship combining all data of the six treatments together was 0.66. Abbreviations of treatments are as defined in the legend of Fig. 1. $^{**}P$ <0.01

under salinity and water stress (Islam and Sedgley, 1981; Nicolas *et al.*, 1993; Munns and James, 2003). Compared with control conditions (FI), all treatments decreased plant height and shoot biomass, but plant height was far less affected than biomass because salinity and water stress were applied during the reproductive stage (i.e. late in the crop cycle). Thus, while biomass at DI-17 dS m^{-1} was reduced by ~75% relative to the control, plant height was reduced by just 17%. Even shoot biomass decreased by 40% under FI-12 dS m^{-1}, which is in line with the ~50% decrease reported in previous studies using similar levels of salinity (Ayers and Westcot, 1989; Yousfi *et al.*, 2009), but represented a reduction in height of only 6%.

FI-12 dS m^{-1}, after the control (FI), was the second best treatment in terms of biomass, while the most stressful treatments resulted from a combination of deficit irrigation with saline water (DI-12 dS m^{-1} and DI-17 dS m^{-1}). A major difference between the full irrigation treatments with saline solution versus deficit irrigation (even if with no saline solution) is the total amount of water available. Under fully irrigated treatments, a large (essentially unlimited) amount of water at a constant, low water potential is available. Durum wheat and other cereals may adjust osmotically under saline conditions through the incorporation of available ions such as Na^+, which allows plants to access water in the substrate for growth (Munns, 2002; Cuin *et al.*, 2009). Thus in the present study, Na^+ concentration in shoots increased as the amount of salt provided in the growing medium increased, reaching the highest value at FI-17 dS m^{-1}. These Na^+ concentrations at least doubled those reported in previous studies with durum and bread wheat exposed to saline conditions during the vegetative stage (Husain *et al.*, 2004; Yousfi *et al.*, 2009). In the current work, salinity treatments were imposed on plants with fully developed leaves, so no dilution effect was produced as new leaves appeared.

On the other hand, the two most stressful treatments in terms of biomass (DI-12 dS m^{-1} and DI-17 dS m^{-1}) induced very low Na^+ concentration (0.34–0.45 mmol g^{-1} DW) and K^+/Na^+ ratios far higher (between 3 and 4) than those reported by Yousfi *et al.* (2009) under FI-12 dS m^{-1} and FI-17 dS m^{-1}. Only FI-17 dS m^{-1} produced a K^+/Na^+ ratio <0.5, comparable with those reported previously for vegetative durum wheat at FI-12 dS m^{-1} (Yousfi *et al.*, 2009).

Plant growth responds to salinity in two phases: a rapid, osmotic phase that parallels that of drought stress; and a slower, ionic phase that accelerates the senescence of mature leaves (Munns and Tester, 2008). In spite of higher Na^+ accumulation and lower K^+/Na^+ ratios, full irrigation with saline solutions delayed senescence compared with

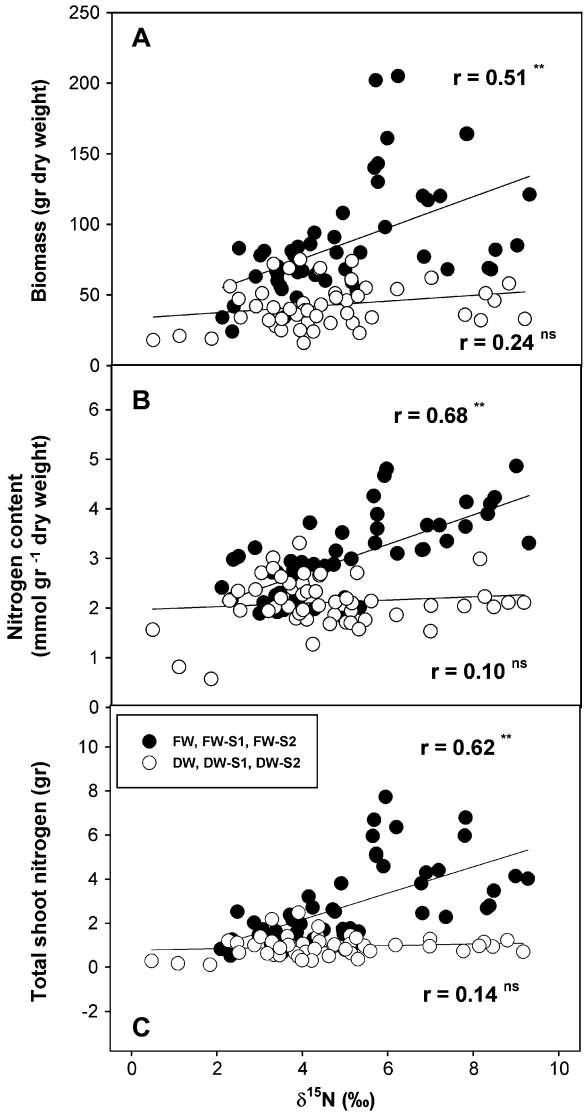

Fig. 5. Relationship between the nitrogen isotope composition ($\delta^{15}N$) of shoots and (A) the shoot biomass, (B) the nitrogen concentration, and (C) the total nitrogen content of shoots across the six genotypes assayed under full irrigation (filled circles: FI, FI-S1, and FI-S2) and deficit irrigation (open circles: DI, DI-S1, and DI-S2) conditions. Each point represents the individual value for a given replicate and genotype within a growing condition. Abbreviations of treatments are as defined in the legend of Fig. 1. ns, not significant; $^{**}P$ <0.01

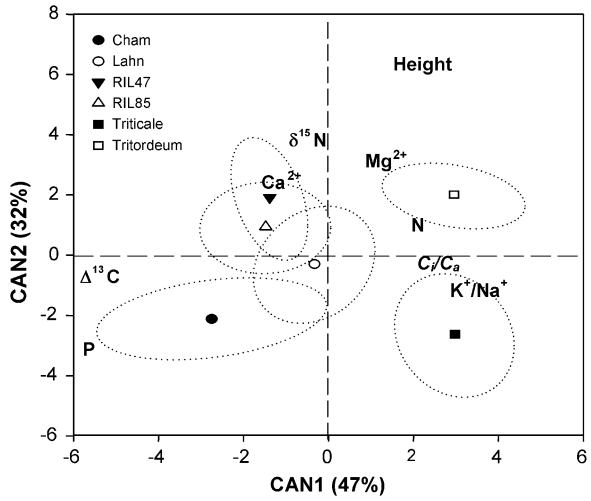

Fig. 6. Plot of the centroids (mean values) and their 95% confidence ellipses for the first two canonical variables of durum wheat, triticale, and tritordeum genotypes. Rescaled discriminant loadings of the explanatory variables are included in the plots. Abbreviations of variables are as in Fig. 2 and Tables 2 and 3.

deficit irrigation with similar saline solutions; this is supported by a higher N concentration and SPAD values in the former treatments. Moreover, salinity is reported to have a strong effect of accumulating amino acids (mostly proline and glycine betaine) in durum wheat leaves, which may act as protective compounds (Carillo *et al.*, 2008).

Effect of treatments on gas exchange and $\Delta^{13}C$

Compared with control, all the other treatments induced a decrease in photosynthetic rates. Besides an accelerated senescence, the drop in photosynthesis also seems to be due to stomatal limitation, as concluded from the drop in the C_i/C_a ratio. This is in agreement with previous reports on durum and bread wheat (Ouerghi *et al.*, 2000; Zheng *et al.*, 2008; Yousfi *et al.*, 2009), triticale (Morant-Manceau *et al.*, 2004), and other species (Isla *et al.*, 1998; Rasmuson and Anderson, 2002; Shaheen and Hood-Nowotny, 2005). Flexas *et al.* (2004) conclude that salt and drought stress predominantly affect diffusion of CO_2 in the leaves through a decrease of stomatal and mesophyll conductances, but not the biochemical capacity to assimilate CO_2.

The C_i/C_a measured at the end of the treatment showed a positive relationship with biomass (Supplementary Fig. S1B at *JXB* online). However, a time-integrative estimate of C_i/C_a such as $\Delta^{13}C$ did not correlate with biomass across treatments (Supplementary Fig. S1A at *JXB* online). In the present study, salinity decreased $\Delta^{13}C$ by just 0.03‰ and 0.02‰ per unit increase in electrical conductivity (EC) under full irrigation and deficit irrigation conditions, respectively. These values are about one order of magnitude lower than those reported for durum wheat leaves (Yousfi *et al.*, 2009) and barley kernels (Isla *et al.*, 1998) under long-term salinity conditions, and even 3–5 times smaller than those reported for wheat leaves grown in pots (Shaheen and Hood-Nowotny, 2005) and barley shoots under hydroponics (Handley *et al.*, 1997) exposed to salinity for short periods. It is likely that in the present study, shoot $\Delta^{13}C$ reflects the plant carbon assimilation history as weighed by the impact of the pre- and reproductive phases (i.e. before and after imposing the treatment) on plant functioning.

Effect of treatments on $\delta^{15}N$

In the present study $\delta^{15}N$ decreased about 0.2‰ and 0.12‰ per unit increase in ECs under full irrigation and deficit

Table 4. Multiple linear regressions (stepwise) explaining biomass variation across genotypes in each growing condition as a dependent variable, and all the physiological parameters (plant height, ion concentrations and ratios, gas exchange traits, nitrogen concentration, and chlorophyll content and stable isotope signatures) measured (excluding total shoot N) in the same particular growing condition as independent variables

Abbreviations for treatments and ANOVA analysis are as defined in the footnotes of Table 1. For the calculations all the individual measurements of the six different genotypes were used (n= 8). **P <0.01; ***P <0.001.

Model treatments	Variable chosen	R^2	Final stepwise model
Biomass FI	g_s	0.44***	199.7 g_s+53.7
Biomass FI-12 dS m^{-1}	$\Delta^{13}C$	0.52***	–16.6 $\Delta^{13}C$–2.6 A_{sat}+434.8
	$\Delta^{13}C$, A_{sat}	0.64 ***	
Biomass FI-17 dS m^{-1}	$\Delta^{13}C$	0.61***	–38.8 $\Delta^{13}C$+75.7 K^+/Na^++858.4
	$\Delta^{13}C$, K^+/Na^+	0.74***	
Biomass DI	C_i/C_a	0.23**	27.6 C_i/C_a–10.1 $\Delta^{13}C$+261.8
	C_i/C_a, $\Delta^{13}C$	0.50**	
Biomass DI-12 dS m^{-1}	C_i/C_a	0.51***	43.5 C_i/C_a–7.9 $\Delta^{13}C$+202.5
	C_i/C_a, $\Delta^{13}C$	0.73***	
Biomass DI-17 dS m^{-1}	N	0.42***	11.45 N–16.4 C_i/C_a+11.96
	N, C_i/C_a	0.57***	

The table show only the parameters significantly entering in the models. g_s, stomatal conductance; $\Delta^{13}C$, shoot carbon isotope discrimination; K^+/Na^+, ratio of potassium to sodium, C_i/C_a, the ratio of intercellular to ambient CO_2 concentration; N, leaf nitrogen concentration.

irrigation conditions, respectively. Yousfi *et al.* (2009) reported a nearly 0.2‰ decrease in shoot $\delta^{15}N$ per unit increase in ECs. Several studies on barley under hydroponics have reported rates of shoot $\delta^{15}N$ that decreased by nearly half (Ellis *et al.*, 1997; Handley *et al.*, 1997), probably due to the fact that barley is more tolerant to salinity than durum wheat, and salinity conditions were mild (Munns *et al.*, 2002). Deficit irrigation also reduced $\delta^{15}N$ as compared with control conditions (Robinson *et al.*, 2000; Raimanová and Haberle, 2010). However, in other studies with different species including cereals (Handley *et al.*, 1999; Lopes *et al.*, 2004; Lopes and Araus, 2006) water stress caused an increase in $\delta^{15}N$, which suggests that drought affects plant $\delta^{15}N$ in a different way from salinity.

Isotope fractionation of nitrogen may occur during uptake from the medium into root cells, or during subsequent enzymatic assimilation into other N forms. Further fractionation may also occur if biochemical components of varying isotopic composition are lost through translocation, exudation, or volatilization (Evans, 2001; Pritchard and Guy, 2005). Reduced stomatal conductance, due to either salinity or water stress, or a combination of both factors, should lead to a reduction in the loss of ammonia and nitrous oxide, decreasing $\delta^{15}N$ (Farquhar *et al.*, 1980; Smart and Bloom, 2001). In fact, $\Delta^{13}C$ and $\delta^{15}N$ were positively related across treatments, and the same pattern was observed considering either full or deficit irrigation (Supplementary Fig. S3A at *JXB* online). A positive relationship between $\Delta^{13}C$ and $\delta^{15}N$ has also been reported for durum wheat seedlings across full irrigation treatments (Yousfi *et al.*, 2009). Moreover, $\delta^{15}N$ correlated positively with C_i/C_a (Supplementary Fig. S3B at *JXB* online), as well as with g_s and transpiration (r=0.61 and 0.69, P <0.01, respectively) across full irrigation treatments. C_i/C_a may integrate leaf permeability together with the functional status of a major N pool (carboxylation enzyme), which would explain why $\delta^{15}N$ correlates with C_i/C_a better than with g_s or with transpiration.

Mechanisms other than N loss may also lead to salinity increasing the discrimination against ^{15}N, such as a high external N concentration relative to a modest demand (Mariotti *et al.*, 1982). Consequently, the suboptimal growing conditions associated with any stress may produce a decrease in demand relative to a constant external N concentration. This may have the same effect as increasing the external concentration (Mariotti *et al.*, 1982), leading to greater isotopic discrimination (Vitousek *et al.*, 1989; Handley *et al.*, 1997). Thus the positive relationship found between $\delta^{15}N$ and biomass might be due to differences across treatments in $\delta^{15}N$ being associated with assimilation capacity and N demand (Robinson *et al.*, 2000; Pritchard and Guy, 2005; Coque *et al.*, 2006). In this regard, a positive relationship across growing conditions between $\delta^{15}N$ and biomass has already been reported in durum wheat (Yousfi *et al.*, 2009). Alternatively, Handley *et al.* (1997) suggest that salt stress would make $\delta^{15}N$ less positive than in controls due to down-regulation of assimilating enzymes. Thus the N concentration decreases with increased salinity under full irrigation, and this would agree with a decrease in plant enzyme activity. In fact $^{15}N/^{14}N$ fractionation may occur during either nitrate assimilation by nitrate reductase or ammonium assimilation by glutamine synthetase (Evans, 2001). These two enzymes have apparently similar *in vitro* discrimination factors (Ledgard *et al.*, 1985; Yoneyama *et al.*, 1993), and their activity is reduced by salinity in wheat (Carillo *et al.*, 2005). However, other works indicate that moderate salinity levels (100–150 mM NaCl) in wheat increase activities of glutamine synthetase while decreasing

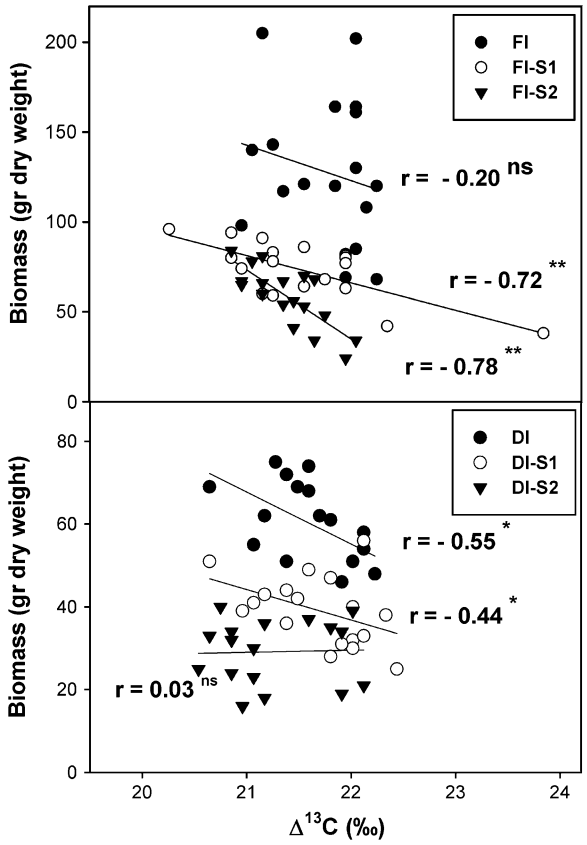

Fig. 7. Relationship between stable carbon isotope discrimination ($\Delta^{13}C$) and shoot biomass across the six genotypes within each of the six growing conditions assayed. Each point represents the individual value for a given replication and genotype within a growing condition. ns, not significant; *P <0.05; **P <0.01. Abbreviations of treatments are as defined in the legend of Fig. 1.

levels of nitrate reductase (Wang *et al.*, 2007; Carrillo *et al.*, 2008), whereas at 300 mM the activity of glutamine synthetase decreases (Wang *et al.*, 2007). In agreement with $\delta^{15}N$ changes mediated by a decrease in enzyme activity due to salinity, when genotypes were combined across growing conditions under full irrigation, $\delta^{15}N$ positively correlated not just with biomass but also with N concentration (Fig. 5B) and total shoot nitrogen content (Fig. 5C). Similar results were reported by Yousfi *et al.* (2009), but Handley *et al.* (1997) did not find such relationships; in this latter case, the salinity treatment imposed was probably too short to modify nitrogen content and shoot biomass.

It may be concluded that regardless of the mechanism(s) affecting plant $\delta^{15}N$, this trait seems to relate to the plant's ability to use available nitrogen. Moreover, under the present experimental conditions, N concentration and $\delta^{15}N$ are better at reflecting differences in biomass caused by short-term treatments applied at the end of the crop cycle than $\Delta^{13}C$. This reflects the far less mobile nature of carbon, mostly accumulated in support structures (i.e. cell walls) that were already developed before imposition of treatment, compared with the N invested in metabolism, which is more affected by differences in the current growing conditions, including accelerated senescence and remobilization to growing grains.

Relationships of biomass to physiological traits across treatments and genotypes

Relationships of biomass to the physiological traits included in this study (Na^+, Na^+/K^+, C_i/C_a, N, and $\delta^{15}N$) were different across full irrigation from deficit irrigation treatments, and usually stronger in the first case. This may reflect the different nature of drought and salinity (Chaves *et al.*, 2009), and consequently the potential interactions between both stresses.

In a previous study $\delta^{15}N$, rather than $\Delta^{13}C$, was the best informative trait on genotypic differences in tolerance to salinity (Yousfi *et al.*, 2009). In the present study, the genotypic effect for $\Delta^{13}C$ was much more relevant than the treatment factor, while the reverse was true for $\delta^{15}N$. The phenotypic negative association between $\Delta^{13}C$ and biomass for each stress treatment suggests that genotypes with lower g_s (and, thus, less transpiration) were most tolerant. A negative relationship between biomass and $\Delta^{13}C$ has also been reported by Yousfi *et al.* (2009) at 12 dS m^{-1}. For barley, Isla *et al.* (1998) reported a positive correlation between $\Delta^{13}C$ and grain yield in the absence of stress, whereas no association was observed under highly saline conditions. Munns and James (2003) suggest that screening for high g_s may be the most effective way of selecting genotypes that will grow fast in saline soil. In the present study, however, g_s was not chosen as a variable related to biomass in the stepwise analysis, which may be due to the fact that all treatments other than control induced very low g_s values. It was only under control conditions that g_s entered the model, with genotypes having higher g_s yielding more. Instead, C_i/C_a, probably associated with g_s, was included as the first trait in the stepwise models for DI and DI-12 dS m^{-1}.

Physiological mechanisms of genotypic performance

The results suggest that a superior growth potential (i.e. in the absence of stress) may confer a better performance in terms of total biomass under the different salinity and drought combinations. Thus, tritordeum and Cham were genotypes with the most and least biomass, respectively, under control conditions, but also under the different stress treatments. Previous studies also support a positive role for constitutive high growth, rather than high physiological tolerance (i.e. small phenotypic plasticity) (Rawson *et al.*, 1988; Isla *et al.*, 1998; Yousfi *et al.*, 2009), conferring adaptation to salinity.

Besides its high potential growth under control conditions, tritordeum also exhibited the lowest $\Delta^{13}C$ across treatments, while durum wheat genotypes, particularly Cham, showed the highest values (Supplementary Table S1 at *JXB* online). Tritordeum has higher water use efficiency than wheat (Martin *et al.*, 1999) probably associated with a constitutively (i.e. in the absence of stress) low g_s (Aranjuelo *et al.*, 2009). On the other hand, the genome of *H. chilense* seems to confer a certain degree of tolerance to drought and salt on this cereal (Martín *et al.*, 2000). Thus,

tritordeum has been reported to maintain greater g_s than wheat and triticale under water deficit conditions (Gallardo and Fereres, 1989), which agrees with the present results under DI. Triticale was, together with tritordeum, the best genotype in terms of biomass at DI and DI-12 dS m^{-1} (Supplementary Table S1 at *JXB* online). Giunta *et al.* (1993) conclude that triticale is more drought resistant than durum wheat due (at least in part) to its greater ability to extract water from the soil. Aranjuelo *et al.* (2009) also reported higher g_s values in triticale compared with wheat and tritordeum. In the present study, however, it did not show a consistently higher g_s than durum wheat genotypes at the end of treatments DI and DI-12 dS m^{-1}. Rather the opposite was seen, as triticale (as tritordeum) showed lower $\Delta^{13}C$ than durum wheat, which suggests lower g_s during the period of treatment.

Differences in Na^+ accumulation may also be involved in genotypic performance under saline conditions (Zheng *et al.*, 2008), which suggests that toxic ions may accumulate more in the susceptible genotypes due to higher transpiration. Thus, and except for the two treatments with the highest salinity level (17 dS m^{-1}), tritordeum and triticale exhibited a lower accumulation of Na^+ and higher K^+/Na^+ ratio than the four durum wheat genotypes. Na^+ exclusion and a subsequent high K^+/Na^+, usually from leaves and shoots, have been proposed as screening traits for tolerance to moderate salinity (e.g. Dvorak *et al.*, 1994; Chhipa and Lal, 1995; Colmer *et al.*, 2005; Yousfi *et al.*, 2009). Moreover, both triticale and tritordeum showed the highest N concentration in the most stressful treatments (17 dS m^{-1}) while, compared with control, $\delta^{15}N$ decreased the least across saline treatments. In fact, triticale is reported as tolerant to salinity (Morant-Manceau *et al.*, 2004), with thylakoid functions being preserved and senescence delayed in salt stress conditions.

Among durum wheat genotypes the two RILS performed slightly better in terms of biomass and senescence than Lahn, and consistently better than Cham, the other parent. In fact, Cham showed higher $\Delta^{13}C$ together with lower K^+/Na^+ ratios and concentrations of ions other than Na^+. This genotypic ranking agrees with Yousfi *et al.* (2009), and suggests that genetic variability for salinity tolerance in durum wheat is maintained through the entire crop cycle. Therefore, genotypic evaluation during the first part of the crop cycle (perhaps even at the seedling stage) may be a valid option to select for salt tolerance in durum wheat. This study also points to tritordeum and, to a lesser extent, triticale as potential sources to introgress genes for tolerance to salinity, drought, or, what is most common under field conditions, both stresses in combination.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Relationship between biomass and (A) the carbon isotope discrimination ($\Delta^{13}C$) of shoots and (B) the intercellular to ambient CO_2 concentration (C_i/C_a) of flag leaves across the six genotypes assayed under full irrigation (filled circles: FI, FI-S1, and FI-S2) and deficit irrigation (open circles: DI, DI-S1, and DI-S2) conditions. Each point represents the individual value for a given replicate and genotype within a growing condition. Gas exchange measurements and shoot sampling were performed ~3 weeks after anthesis. Abbreviations of treatments are as defined in the legend of Fig. 1.

Figure S2. Relationship between biomass and Na^+ values across treatments. Biomass is expressed as a percentage of the value reached by each genotype grown in the absence of salinity, and Na^+ is expressed as the absolute difference between Na^+ concentration in shoot dry matter in the presence or absence of salinity for a given genotype. Left: full irrigation treatments (normal Hoagland, 12 dS m^{-1}, and 17 dS m^{-1}). Right: deficit irrigation treatments (normal Hoagland, 12 dS m^{-1}, and 17 dS m^{-1}). Circles represent the average genotypic value for each treatment.

Figure S3. Relationship between the nitrogen isotope composition ($\delta^{15}N$) of shoots and (A) the carbon isotope discrimination ($\Delta^{13}C$) of shoots and (B) the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) of flag leaves at the end of the treatment across the six genotypes assayed under full irrigation (filled circles: FI, FI-S1, and FI-S2) and deficit irrigation (open circles: DI, DI-S1, and DI-S2) conditions. Each point represents the individual value for a given replication and genotype within a growing condition. Abbreviations of treatments are as defined in the legend o Fig. 1. ns, not significant; $^{**}P < 0.01$

Table S1. Effect of different levels of salinity, water stress, and the combination of the two stresses on the shoot biomass, concentration of Na^+, the ratio K^+/Na^+, stable carbon isotope discrimination ($\Delta^{13}C$), stable nitrogen isotope composition ($\delta^{15}N$), and nitrogen concentration in shoots, leaf net CO_2 assimilation (A_{sat}) and the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) of durum wheat (Cham, Lahn, RIL47, and RIL85), triticale (Imperioso), and tritordeum (HT621). The data shown are the mean of the three replicates for each genotype in each treatment. Abbreviations for treatments and ANOVA are as defined in Table 1. Means followed by different letters were significantly different ($P < 0.05$) by Duncan's test.

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Supplementary data (published at *JXB* online)

Table S1. Effect of different levels of salinity, water stress and the combination of the two stresses on the shoot biomass, concentration of Na^+, the ratio K^+/Na^+, stable carbon isotope discrimination ($\Delta^{13}C$), stable nitrogen isotope composition ($\delta^{15}N$) and nitrogen concentration in shoots, leaf net CO_2 assimilation (A_{sat}) and the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) of durum wheat (Cham, Lahn, RIL47, RIL85), triticale (Imperioso) and tritordeum (HT621). The data show is the mean of the three replicates for each genotype in each treatment. Abbreviations for treatments and Anova analysis as defined in Table 1. Means followed by different letters were significantly different ($P < 0.05$) by the Duncan's test

		Genotype					
Treatments	**Variable**	**Cham**	**Lahn**	**RIL47**	**RIL85**	**Triticale**	**Tritordeum**
FI	Biomass (g)	119.33 ^{ab}	123 ^{ab}	104.66 ^{a}	110 ^{a}	159.50 ^{bc}	174 ^{c}
	Na^+ (mmol g^{-1} dry weight)	0.12 ^{c}	0.07 ^{b}	0.08 ^{bc}	0.11 ^{c}	0.01 ^{a}	0.02 ^{a}
	K^+/Na^+(mmol g^{-1} dry weight)	11.58 ^{a}	19.05 ^{a}	15.79 ^{a}	11.55 ^{a}	133.54 ^{c}	84.49 ^{b}
	$\Delta^{13}C$ (‰)	21.60 ^{b}	21.06 ^{ab}	21.60 ^{b}	21.28 ^{b}	21.05 ^{ab}	20.58 ^{a}
	$\delta^{15}N$ (‰)	5.94 ^{a}	7.86 ^{a}	8.22 ^{a}	7.75 ^{a}	6.29 ^{a}	5.96 ^{a}
	N concentration (mmol g^{-1} dry weight)	3.69 ^{a}	4.05 ^{a}	3.99 ^{a}	3.82 ^{a}	3.49 ^{a}	3.35 ^{a}
	A_{sat} (µmol CO_2 m^{-2} s^{-1})	20.66 ^{a}	22.73 ^{a}	24.06 ^{a}	21.03 ^{a}	24.45 ^{a}	21.50 ^{a}
	g_s (mol CO_2 m^{-2} s^{-1})	0.36 ^{a}	0.42 ^{a}	0.30 ^{a}	0.28 ^{a}	0.52 ^{a}	0.41 ^{a}
	C_i/C_a	0.82 ^{b}	0.83 ^{b}	0.75 ^{a}	0.77 ^{ab}	0.80 ^{ab}	0.73 ^{a}
FI-12 dS m^{-1}	Biomass (g)	47.66 ^{a}	81.33 ^{c}	77 ^{c}	77.33 ^{c}	61 ^{b}	93.66 ^{d}
	Na^+ (mmol g^{-1} dry weight)	1.06 ^{b}	1.10 ^{b}	1.18 ^{b}	1.23 ^{b}	0.50 ^{a}	0.43 ^{a}
	K^+/Na^+(mmol g^{-1} dry weight)	1.05 ^{a}	1.25 ^{a}	1.04 ^{a}	0.79 ^{a}	3.74 ^{b}	3.75 ^{b}
	$\Delta^{13}C$ (‰)	21.11 ^{b}	21.04 ^{ab}	22.09 ^{c}	20.32 ^{ab}	20.68 ^{ab}	20.09 ^{a}
	$\delta^{15}N$ (‰)	2.83 ^{a}	3.65 ^{a}	6.10 ^{b}	4.05 ^{a}	4.62 ^{ab}	4.63 ^{ab}
	N concentration (mmol g^{-1} dry weight)	2.97 ^{bc}	3.04 ^{bc}	3.41 ^{c}	2.01 ^{a}	2.60 ^{ab}	2.52 ^{ab}
	A_{sat} (µmol CO_2 m^{-2} s^{-1})	0.79 ^{a}	2.01 ^{a}	2.13 ^{a}	2.04 ^{a}	7.21 ^{b}	2.40 ^{a}
	g_s (mol CO_2 m^{-2} s^{-1})	1x10^{-3} ^{a}	9x10^{-3} ^{a}	2x10^{-3} ^{a}	8x10^{-4} ^{a}	0.06 ^{b}	0.05 ^{b}
	C_i/C_a	0.06 ^{a}	0.11 ^{a}	0.32 ^{b}	0.18 ^{ab}	0.52 ^{c}	0.77 ^{d}
FI-17 dS m^{-1}	Biomass (g)	35.33 ^{a}	54 ^{ab}	67.33 ^{bc}	67 ^{bc}	50 ^{ab}	76.33 ^{c}
	Na^+ (mmol g^{-1} dry weight)	3.20 ^{bc}	3.89 ^{d}	2.29 ^{a}	2.89 ^{b}	3.83 ^{d}	3.76 ^{cd}
	K^+/Na^+(mmol g^{-1} dry weight)	0.35 ^{b}	0.26 ^{a}	0.49 ^{c}	0.38 ^{b}	0.29 ^{a}	0.30 ^{a}
	$\Delta^{13}C$ (‰)	20.79 ^{b}	20.69 ^{b}	21.29 ^{c}	20.56 ^{ab}	20.74 ^{b}	20.29 ^{a}
	$\delta^{15}N$ (‰)	2.74 ^{a}	3.57 ^{ab}	4.42 ^{b}	3.54 ^{ab}	3.45 ^{ab}	3.86 ^{ab}
	N concentration (mmol g^{-1} dry weight)	2.26 ^{a}	2.33 ^{a}	2.11 ^{a}	2.05 ^{a}	2.32 ^{a}	2.76 ^{b}
	A_{sat} (µmol CO_2 m^{-2} s^{-1})	1.01 ^{a}	2.46 ^{a}	1.61 ^{a}	2.00 ^{a}	2.53 ^{a}	2.34 ^{a}
	g_s (mol CO_2 m^{-2} s^{-1})	3 x 10^{-3} ^{a}	8x 10^{-3} ^{a}	1x 10^{-3} ^{a}	1x10^{-3} ^{a}	4 x 10^{-3} ^{a}	3 x 10^{-3} ^{a}
	C_i/C_a	0.11 ^{a}	0.08 ^{a}	0.11 ^{a}	0.24 ^{a}	0.17 ^{a}	0.28 ^{a}
DI	Biomass (g)	48.33 ^{a}	54.33 ^{a}	61.00 ^{ab}	59.00 ^{ab}	70.66 ^{b}	70.50 ^{b}
	Na^+ (mmol g^{-1} dry weight)	0.17 ^{c}	0.08 ^{b}	0.10 ^{b}	0.11 ^{b}	0.04 ^{a}	0.03 ^{a}
	K^+/Na^+(mmol g^{-1} dry weight)	7.59 ^{a}	16.97 ^{b}	12.84 ^{ab}	11.25 ^{ab}	29.27 ^{c}	31.02 ^{c}
	$\Delta^{13}C$ (‰)	21.34 ^{b}	20.89 ^{ab}	21.41 ^{b}	21.37 ^{b}	20.97 ^{ab}	20.55 ^{a}
	$\delta^{15}N$ (‰)	4.82 ^{ab}	6.96 ^{bc}	7.39 ^{c}	6.09 ^{abc}	3.97 ^{a}	3.83 ^{a}
	N concentration (mmol g^{-1} dry weight)	1.89 ^{ab}	1.94 ^{ab}	2.09 ^{ab}	1.69 ^{a}	2.46 ^{bc}	2.83 ^{c}
	A_{sat} (µmol CO_2 m^{-2} s^{-1})	2.48 ^{ab}	3.40 ^{ab}	2.76 ^{ab}	1.74 ^{a}	1.45 ^{a}	5.04 ^{b}
	g_s (mol CO_2 m^{-2} s^{-1})	0.02 ^{ab}	0.05 ^{bc}	0.05 ^{bc}	0.02 ^{a}	0.05 ^{bc}	0.08 ^{c}
	C_i/C_a	0.48 ^{a}	0.56 ^{a}	0.66 ^{a}	0.59 ^{a}	0.70 ^{a}	0.69 ^{a}
DI-12 dS m^{-1}	Biomass (g)	36.33 ^{a}	41.66 ^{a}	36.66 ^{a}	35.66 ^{a}	43.66 ^{a}	41.00 ^{a}
	Na^+ (mmol g^{-1} dry weight)	0.43 ^{c}	0.57 ^{d}	0.34 ^{bc}	0.29 ^{ab}	0.20 ^{a}	0.23 ^{ab}
	K^+/Na^+(mmol g^{-1} dry weight)	2.29 ^{a}	2.17 ^{a}	3.01 ^{ab}	4.25 ^{ab}	5.04 ^{b}	3.03 ^{ab}
	$\Delta^{13}C$ (‰)	20.88 ^{ab}	21.24 ^{b}	21.41 ^{b}	21.49 ^{b}	20.91 ^{ab}	20.44 ^{a}
	$\delta^{15}N$ (‰)	3.02 ^{a}	4.37 ^{a}	5.28 ^{a}	6.09 ^{a}	4.12 ^{a}	3.90 ^{a}
	N concentration (mmol g^{-1} dry weight)	2.12 ^{a}	2.25 ^{a}	2.62 ^{b}	2.16 ^{a}	2.58 ^{b}	2.73 ^{b}
	A_{sat} (µmol CO_2 m^{-2} s^{-1})	2.45 ^{ab}	1.29 ^{a}	1.88 ^{ab}	1.22 ^{a}	5.41 ^{b}	4.10 ^{ab}
	g_s (mol CO_2 m^{-2} s^{-1})	0.02 ^{a}	0.01 ^{a}	0.03 ^{a}	9 x 10^{-3} ^{a}	1 x 10^{-3} ^{a}	0.03 ^{a}
	C_i/C_a	0.24 ^{a}	0.20 ^{a}	0.19 ^{a}	0.16 ^{a}	0.30 ^{a}	0.10 ^{a}
DI-17 dS m^{-1}	Biomass (g)	19.33 ^{a}	36.33 ^{c}	33.66 ^{bc}	25.66 ^{ab}	26.00 ^{ab}	36.50 ^{c}
	Na^+ (mmol g^{-1} dry weight)	0.21 ^{a}	0.73 ^{b}	0.37 ^{a}	0.43 ^{a}	0.37 ^{a}	0.68 ^{b}
	K^+/Na^+(mmol g^{-1} dry weight)	7.76 ^{a}	1.70 ^{a}	3.68 ^{a}	3.24 ^{a}	4.11 ^{a}	2.25 ^{a}
	$\Delta^{13}C$ (‰)	21.21 ^{b}	20.84 ^{ab}	20.99 ^{ab}	20.50 ^{ab}	20.56 ^{ab}	20.24 ^{a}
	$\delta^{15}N$ (‰)	1.13 ^{a}	3.47 ^{b}	4.35 ^{b}	4.71 ^{b}	4.30 ^{b}	3.57 ^{b}
	N concentration (mmol g^{-1} dry weight)	0.98 ^{a}	1.83 ^{bc}	2.13 ^{cd}	1.50 ^{b}	2.01 ^{bc}	2.56 ^{d}
	A_{sat} (µmol CO_2 m^{-2} s^{-1})	0.35 ^{a}	2.31 ^{a}	1.98 ^{a}	2.65 ^{a}	0.75 ^{a}	3.23 ^{a}
	g_s (mol CO_2 m^{-2} s^{-1})	9 x 10^{-3} ^{a}	0.01 ^{a}	0.02 ^{a}	0.01 ^{a}	0.05 ^{b}	0.03 ^{ab}
	C_i/C_a	0.10 ^{ab}	0.05 ^{a}	0.15 ^{ab}	0.09 ^{a}	0.57 ^{c}	0.26 ^{b}

Figure S1. Relationship between biomass and (A) the carbon isotope discrimination ($\Delta^{13}C$) of shoots and (B) the intercellular to ambient CO_2 concentration (C_i/C_a) of flag leaves across the six genotypes assayed under full irrigation (filled circles: FI, FI-S1, FI-S2) and deficit irrigation (open circles: DI, DI-S1, DI-S2) conditions. Each point represents the individual value for a given replicate and genotype within a growing condition. Gas exchange measurements and shoot sampling were performed about three weeks after anthesis. Abbreviations of treatments as defined in Figure 2

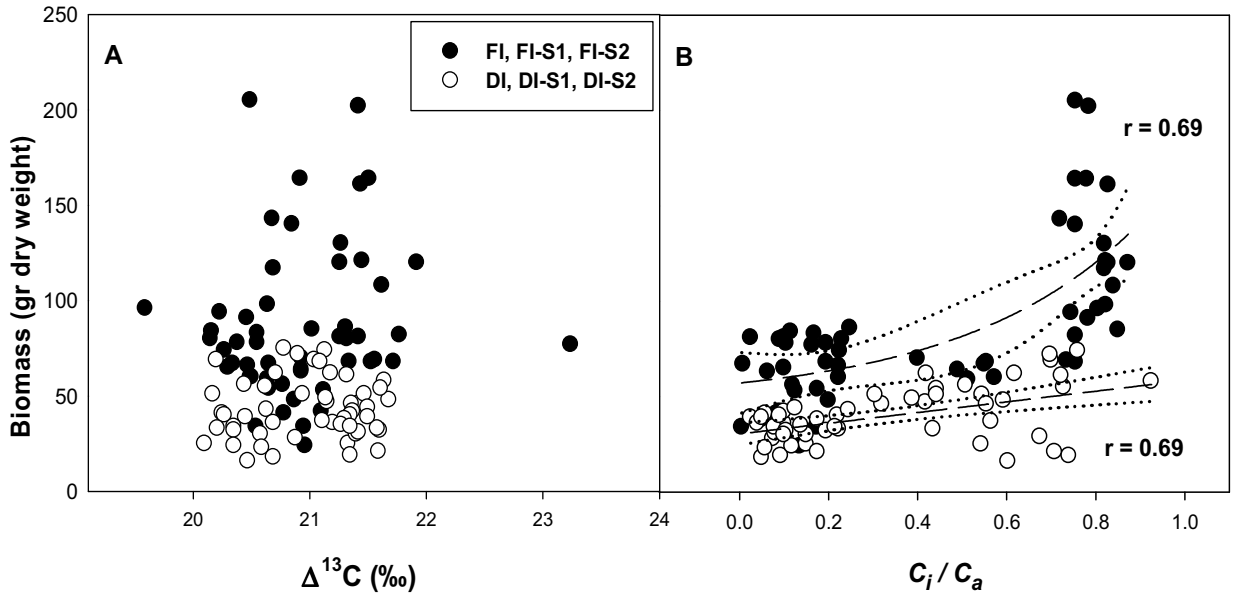


Figure S2. Relationship between biomass and Na^+ values across treatments. Biomass is expressed as a percentage of the value reached by each genotype grown in the absence of salinity, and Na^+ is expressed as the absolute difference between Na^+ concentration in shoot dry matter under presence or absence of salinity for a given genotype. Left: fully irrigation treatments (normal Hoagland, 12 dS m^{-1} and 17 dS m^{-1}). Right: deficit irrigation treatments (normal Hoagland, 12 dS m^{-1} and 17 dS m^{-1}). Circles represent the average genotypic value for each treatment

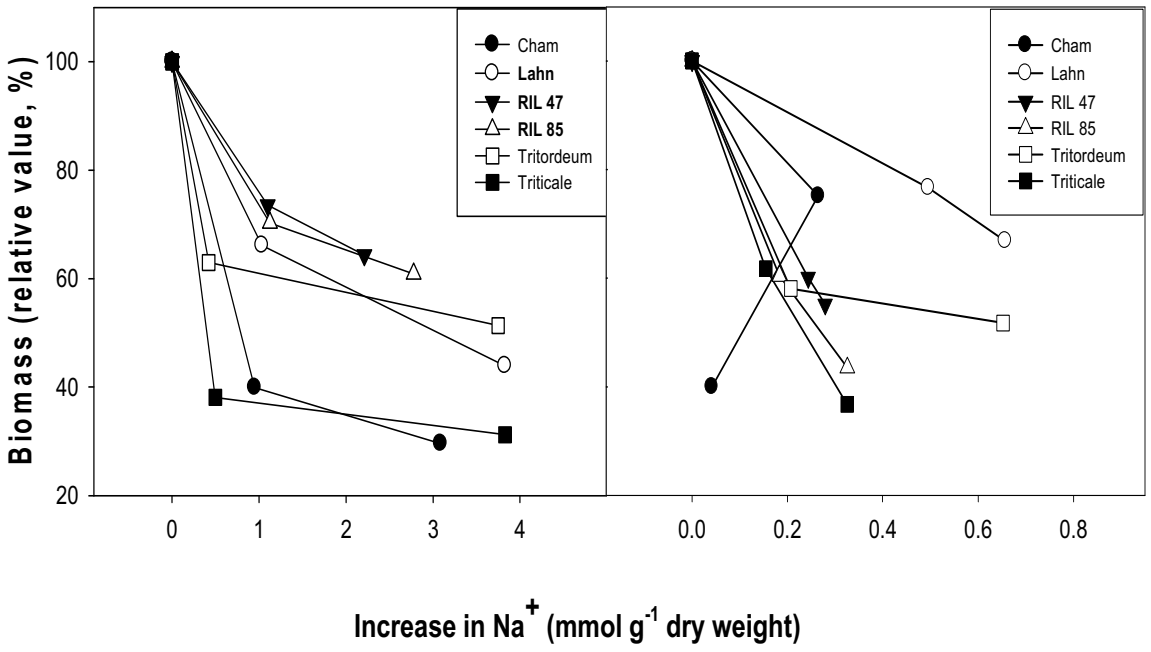
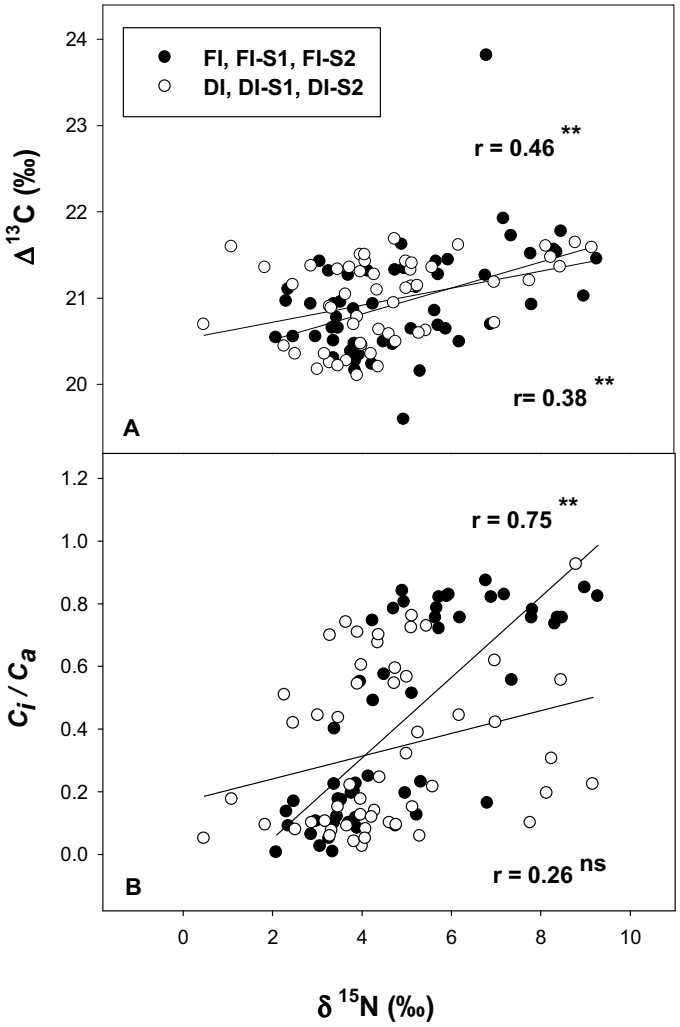


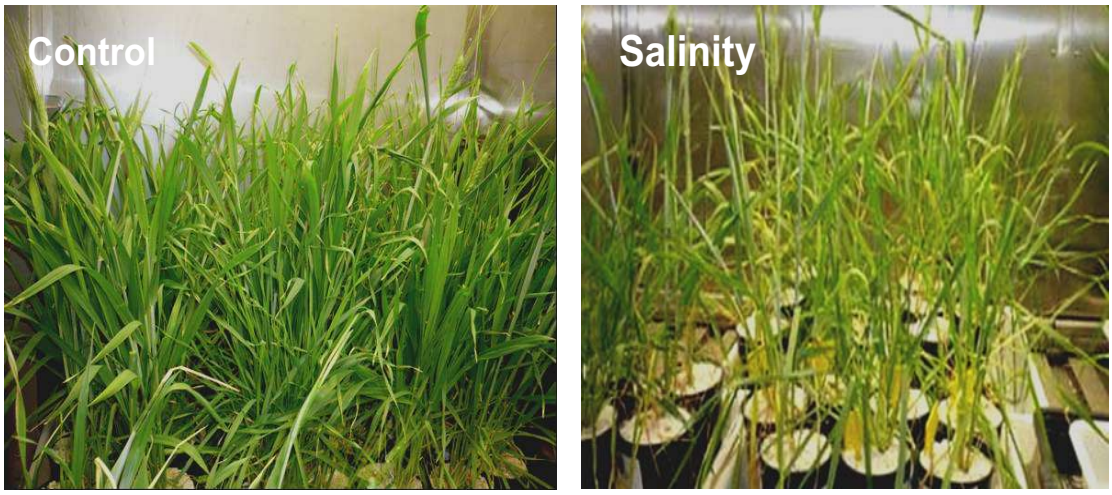
Figure S3. Relationship between the nitrogen isotope composition ($\delta^{15}N$) of shoots and (A) the carbon isotope discrimination ($\Delta^{13}C$) of shoots and (B) the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) of flag leaves at the end of the treatment across the six genotypes assayed under full irrigation (filled circles: FI, FI-S1, FI-S2) and deficit irrigation (open circles: DI, DI-S1, DI-S2) conditions. Each point represents the individual value for a given replication and genotype within a growing condition. Abbreviations of treatments as defined in Figure 2. ns, not significant; $^{**}P < 0.01$.



Capítulo 3

Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit.

Yousfi, S., Serret, M.D., Márquez, A.J., Voltas, J., Araus, J.L. *2012. New Phytologist* 194: 230-244.



Genotipos de trigo duro en una cámara de cultivo de los Servicios Experimentales de la Facultad de Biología, Universidad de Barcelona. Año 2008. Foto: S. Yousfi

Resumen

Implementar un fenotipeado de precisión sigue siendo uno de los principales factores que limitan la mejora de la resistencia a la salinidad y la sequía. En este estudio, el uso combinado de la composición isotópica del carbono ($\delta^{13}C$), oxigeno ($\delta^{18}O$) y el nitrógeno ($\delta^{15}N$) en materia seca es el objetivo de la evaluación de las respuestas genotípicas de plantas de trigo duro sometidas a diferentes combinaciones de los estreses mencionados más arriba. Dos genotipos tolerantes y dos susceptibles a la salinidad fueron cultivados bajo cinco combinaciones de diferentes niveles de salinidad y régimen de riego. Se hicieron medidas de biomasa, $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$, diferentes parámetros de intercambio de gases, concentración de iones y nitrógeno y actividad enzimática de la nitrato reductasa (NR) y la glutamina sintetasa (GS). Comparado con el control todos los estreses establecidos afectaron significativamente a todos los parámetros estudiados. Sin embargo, solo $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$, las actividades de la GS y la NR y la concentración de nitrógeno separaron adecuadamente entre genotipos tolerantes y susceptibles. En base a estos resultados el presente estudio desarrolla un modelo conceptual para explicar sobre la base de estas 5 características, las diferencias en biomasa para cada condición de cultivo. Las diferencias en la respuesta a la aclimatación de los genotipos de trigo duro sometidos a diferentes tratamientos de estrés se asociaron con $\delta^{13}C$. Sin embargo, a excepción del estrés más severo, $\delta^{13}C$ no tiene una relación directa (negativa) con la biomasa, sino que depende de factores que afectan ya sea a la $\delta^{18}O$ o al metabolismo del nitrógeno. Estos resultados subrayan el papel clave del metabolismo del nitrógeno en la adaptación del trigo duro a la salinidad y al estrés hídrico.

New Phytologist

Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit

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Key words: $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, durum wheat (*Triticum turgidum* ssp. *durum*), glutamine synthetase, nitrate reductase, salinity, water deficit.

Summary

- Accurate phenotyping remains a bottleneck in breeding for salinity and drought resistance. Here the combined use of stable isotope compositions of carbon ($\delta^{13}C$), oxygen ($\delta^{18}O$) and nitrogen ($\delta^{15}N$) in dry matter is aimed at assessing genotypic responses of durum wheat under different combinations of these stresses.
- Two tolerant and two susceptible genotypes to salinity were grown under five combinations of salinity and irrigation regimes. Plant biomass, $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$, gas-exchange parameters, ion and N concentrations, and nitrate reductase (NR) and glutamine synthetase (GS) activities were measured.
- Stresses significantly affected all traits studied. However, only $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$, GS and NR activities, and N concentration allowed for clear differentiation between tolerant and susceptible genotypes. Further, a conceptual model explaining differences in biomass based on such traits was developed for each growing condition.
- Differences in acclimation responses among durum wheat genotypes under different stress treatments were associated with $\delta^{13}C$. However, except for the most severe stress, $\delta^{13}C$ did not have a direct (negative) relationship to biomass, being mediated through factors affecting $\delta^{18}O$ or N metabolism. Based upon these results, the key role of N metabolism in durum wheat adaptation to salinity and water stress is highlighted.

Introduction

Durum wheat is the most cultivated crop in the south and east Mediterranean basin (http://www.fao.org/docrep/006/y4011e/y4011e04.htm), where drought and irrigation-induced salinity are the main constraints limiting productivity (Araus, 2004). Selecting genotypes that are either more drought-resistant (Araus *et al.*, 2002) or more salt-tolerant (Munns & Tester, 2008) are complementary ways of improving durum wheat adaptation. However, effective phenotyping remains a bottleneck in breeding for adaptation to such abiotic stresses (Araus *et al.*, 2008). Frequently, the traits that are most effective for phenotyping may differ with type and amount of stress. Moreover, while most studies aimed at elucidating traits and tools for phenotyping have addressed each stress individually, drought and salinity usually occur together under field conditions. This is the case, for example, for crops grown under deficit irrigation with brackish water.

Both drought and salinity induce water stress (Munns, 2002). Water stress affects plant growth via reduced carbon assimilation, tissue expansion and cell number (Hsiao, 1973; Tardieu *et al.*, 2000), and strongly affects nitrogen metabolism (Hirel *et al.*, 2007). Apparently, these are essentially uncoupled mechanisms (Tardieu *et al.*, 2011), but feedback pathways between them may exist.

Natural ^{13}C abundance in plant matter provides time-integrated information on the stress effects on photosynthetic carbon assimilation of C_3 species (Farquhar *et al.*, 1982; Tcherkez *et al.*, 2011). Conditions inducing stomatal closure (e.g. water deficit, either directly or through salinity) restrict the CO_2 supply to carboxylation sites, which then increases the carbon isotope composition ($\delta^{13}C$) of plant matter (Farquhar & Richards, 1984; Rivelli *et al.*, 2002; Yousfi *et al.*, 2010). The assessment of whether variation in $\delta^{13}C$ is the result of changes in intrinsic photosynthetic capacity (A) or stomatal conductance (g_s) remains challenging (Scheidegger *et al.*, 2000; Farquhar *et al.*, 2007). Oxygen isotope composition ($\delta^{18}O$) is a useful trait for this purpose, as it is largely unaffected by photosynthesis (Barbour & Farquhar, 2000; Farquhar *et al.*, 2007). Rather, the $\delta^{18}O$ of plant matter integrates the evaporative conditions throughout the crop cycle (Barbour *et al.*, 2000) and, consequently, it has been

proposed as a proxy for estimating g_s, transpiration (Sheshshayee *et al.*, 2005; Cabrera-Bosquet *et al.*, 2009a, 2011; Cernusak *et al.*, 2009a) and thus water status. To our knowledge, however, a thorough examination of $\delta^{18}O$ changes under salinity or as a result of the combined effect of both drought and salinity is currently absent.

Nitrate reductase (NR; EC 1.6.6.1) and glutamine synthetase (GS; EC 6.3.1.2) are key enzymes responsible for N assimilation and are also connected with carbon metabolism (Masclaux-Daubresse *et al.*, 2010). GS activity is one of the best physiological markers describing plant N status, whatever its developmental stage and N nutrition (Kichey *et al.*, 2006; Bernard & Habash, 2009). Higher NR activity has been correlated with enhanced plant growth (Lam *et al.*, 1996; Chen *et al.*, 2003). In principle, the activity of both GS and NR decreases under salinity or drought (Foyer *et al.*, 1998; Carillo *et al.*, 2005). However, some studies in wheat indicate that moderate salinity decreases NR activity while increasing GS activity (Wang *et al.*, 2007; Carillo *et al.*, 2008). GS is involved not only in using ammonium produced by nitrate reduction, but also in the reassimilation of ammonium generated from photorespiration, proteolysis and processes that are increased by stress (Tsai & Kao, 2002; Hirel *et al.*, 2007). Thus, rice tolerance to salinity has been related to increased GS activity (Sahu *et al.*, 2001), and genotypic tolerance to salinity in foxtail millet has been associated with proline accumulation coupled with an increase in salt-induced GS activity (Veeranagamallaiah *et al.*, 2007).

The natural variation in plant N isotope composition ($\delta^{15}N$) is potentially useful for genotypic screening under drought or salinity because it is linked to N metabolism, even though a complete knowledge of the underlying biochemical mechanisms is lacking (Cernusak *et al.*, 2009b; Tcherkez, 2011). Isotope fractionation may occur during enzymatic assimilation of nitrate or ammonium into other N forms. Further fractionation may take place as a result of N recycling in the plant or through translocation, exudation or volatilization (Evans, 2001; Tcherkez & Hodges, 2008). Isotopic fractionation occurs during nitrate assimilation by NR, and ammonium absorption and assimilation by GS, so that there is, on average, a 2–3‰ depletion in plant ^{15}N compared with source nitrate (Evans, 2001). $\delta^{15}N$ sensitivity to photorespiratory rates (associated with GS activity) and N-input levels (NO_3^- reduction by NR) seems quite high (Tcherkez, 2011).

Here we characterized the response of four durum wheat genotypes with contrasting performances under salinity to different combinations of salinity and irrigation. Growth, the isotopic signatures of C, O and N, gas exchange, ion accumulation, total N concentration and the activities of NR and GS were analysed. The main objective was to examine the effectiveness of $\delta^{15}N$, $\delta^{13}C$ and $\delta^{18}O$, alone or in combination, to track genotypic differences in biomass and N concentration for different arrangements of salinity and water treatments. In addition, this study is unique in that it proposes a conceptual model relating genotypic variability in the three isotopic signatures with N metabolism and above-ground biomass. Understanding the relationships between $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$ and plant growth under a wide range of abiotic stresses may help design more efficient breeding strategies, avoiding phenotyping redundancies by choosing in each case the most suitable trait(s). Moreover, studies examining the physiological basis of $\delta^{18}O$ and $\delta^{15}N$ and their potential breeding implications are indeed scarce and even contradictory, and the same applies to the relationships of $\delta^{13}C$ and $\delta^{18}O$ with nitrogen metabolism.

Materials and Methods

Plant material and growth conditions

Four recombinant inbred lines (RILs) of durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.), here designated as RIL24, RIL30 (salt-susceptible) and RIL47, RIL85 (salt-tolerant), were chosen on the basis of their contrasting shoot biomass among a set of 112 RILs evaluated in a previous study for tolerance to continuous salinity during the vegetative stage (Yousfi *et al.*, 2009). Plants were grown in controlled chambers (Conviron E15, Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at the Experimental Fields of the University of Barcelona for > 1 month. Experimental growing conditions were as follows: 400 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 70% relative humidity, 25°C day : 15°C night temperature and a 14 h photoperiod. Two seeds per pot were planted in 3 dm^3 pots containing perlite and watered to field capacity with complete Hoagland solution (Hoagland & Arnon, 1950). After 1 wk, seedlings were thinned to one plant per pot. Subsequently, five different growth conditions were imposed:

- FI or control (fully irrigated, 100% of container capacity, with complete Hoagland solution, 1.8 dS m^{-1})
- FI-12 (fully irrigated with saline Hoagland solution, 12 dS m^{-1})
- FI-17 (fully irrigated with saline Hoagland solution, 17 dS m^{-1})
- DI (deficit irrigated to 35% of container capacity, with normal Hoagland solution)
- DI-12 (deficit irrigated to 35% of container capacity with saline Hoagland solution, 12 dS m^{-1})

DI-17 has been shown to be too severe a treatment (Yousfi *et al.*, 2010). Solutions were changed every 2–3 d. A completely randomized design was used to accommodate the two-way factorial experiment, with genotype and salinity–drought arrangement as main factors. Four single-pot replicates per factorial combination were used, totalling 80 pots. Water deficit was imposed progressively over 1 wk by decreasing irrigation. Salinity treatment was also imposed by adding NaCl progressively to the nutrient solution, starting with a salt concentration of 4 dS m^{-1} to reach the final concentrations of 12 dS m^{-1} (*c.* 120 mM NaCl) or 17 dS m^{-1} (*c.* 170 mM NaCl).

Gas exchange, plant biomass and leaf Chl

An infrared gas analyser (LI-6400 system, Li-Cor, Inc., Lincoln, NE, USA) was used to measure gas exchange just before harvesting (end of tillering stage) in the fully expanded upper leaf of the

main plant tiller. Parameters measured were as follows: net CO_2 assimilation (A), transpiration (T) and stomatal conductance (g_s). Subsequently, the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) was calculated. The efficiency of excitation energy captured by open photosystem II (PSII) reaction centres (F_v'/F_m') was also estimated in the same leaves. Chl content was estimated in the same leaf blades monitored for gas exchange using a portable meter (Minolta SPAD 502 Meter, Plainfield, IL, USA). Additional leaf blades were harvested and stored at −80°C for analysis of enzyme activity. The rest of the plant was then harvested; the shoots were oven-dried at 70°C for 48 h, weighed and ground finely. Roots of the same plants were washed with deionized water and then treated in the same way as shoots.

N concentration and stable isotope signatures

The total N concentration and the stable carbon (^{13}C : ^{12}C) and nitrogen (^{15}N : ^{14}N) isotope ratios in shoots and roots were measured using an elemental analyser (Flash 1112 EA; Thermo-Finnigan, Bremen, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan) (EA-IRMS), operating in continuous flow mode. Samples of *c.* 1 mg and reference materials were weighed into tin capsules, sealed and loaded into an automatic sampler before EA-IRMS analysis (Table 1). Values were expressed in δ notation (Coplen, 2008): $\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard}] - 1$, where 'sample' refers to plant material and 'standard' to Pee Dee Belemnite (PDB) calcium carbonate (Table 1). The same δ notation was used for the ^{15}N : ^{14}N ratio ($\delta^{15}N$), but in this case the standard referred to N_2 in air (Table 2). In addition, the ^{18}O : ^{16}O ratios (expressed as $\delta^{18}O$) were determined in shoot samples of all plants tested (Table 1). Samples of *c.* 1 mg and reference materials were weighed into silver capsules, sealed, oven-dried at 60°C for not less than 72 h to remove moisture and loaded into an automatic sampler. The IRMS used was a Europa Scientific Geo 20-20 (Crewe, UK).

Ion analysis

Ion analysis was performed in shoots and roots (Table 3) by inductively coupled plasma emission spectrometry (L3200RL, Perkin Elmer, Rodgau, Germany).

Enzyme activity determinations

For the assay of GS and maximum NR enzyme activities, leaf samples were ground to a fine powder in liquid N_2 and resuspended (5 ml g^{-1}) in the following extraction buffer: 100 mM

Table 1 Genotype and treatment effects on biomass, gas-exchange parameters and carbon and oxygen isotope composition of four durum wheat genotypes grown under different combinations of salinity and water supply and their combinations

	B_{shoot}	LC	A	g_s	C_i/C_a	T	F_v'/F_m'	$\delta^{18}O_{shoot}$	$\delta^{13}C_{shoot}$	$\delta^{13}C_{root}$
Genotype										
Tolerant RIL47	31.64b	43.68a	5.13b	0.17ab	0.41a	1.83a	0.58ab	27.47ab	−29.52a	−29.43a
Tolerant RIL 85	34.42b	44.26a	7.82c	0.23b	0.42a	2.36a	0.63b	27.24a	−29.62a	−29.73b
Susceptible RIL 24	24.12a	41.72a	4.76a	0.16a	0.40a	2.06a	0.55a	28.12c	−28.92b	−28.99c
Susceptible RIL 30	24.95a	44.24a	5.11ab	0.13a	0.35a	1.43a	0.56a	27.80bc	−27.88c	−27.54d
Treatment										
FI	54.33c	42.12ab	16.04c	0.47c	0.78c	5.85b	0.70b	27.00a	−30.76a	−29.99a
FI- 12 dS m^{-1}	27.00b	43.22ab	2.54a	0.18b	0.33b	1.57a	0.55a	27.96c	−28.68c	−28.88b
FI-17 dS m^{-1}	15.15a	40.84a	2.53a	0.04a	0.20a	0.52a	0.52a	27.79bc	−27.79d	−28–29c
DI	30.20b	48.63b	5.07b	0.12ab	0.40b	0.83a	0.58a	27.98c	−29.27b	−28.81b
DI- 12 dS m^{-1}	14.08a	41.53ab	2.45a	0.02a	0.28ab	1.03a	0.52a	27.34ab	−28.46c	−28.71b
Level of significance										
Genotype (G)	0.000	ns	0.002	0.049	ns	ns	0.001	0.000	0.000	0.000
Treatment (T)	0.000	0.011	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.002
G × T interaction	0.000	ns	ns	ns	ns	0.010	ns	0.003	ns	ns

B_{shoot}, shoot biomass (g DW); LC, leaf Chl content (Special Products Analysis Division, SPAD, units); A, leaf net CO_2 assimilation (μmol CO_2 m^{-2} s^{-1}); g_s, stomatal conductance (mol CO_2 m^{-2} s^{-1}); C_i/C_a, ratio of intercellular to ambient CO_2 concentration; T, transpiration rate (mmol H_2O m^{-2} s^{-1}); F_v'/F_m', efficiency of excitation energy capture by open photosystem II (PSII) reaction centres; $\delta^{13}C$, stable carbon isotope composition (‰) of shoots ($\delta^{13}C_{shoot}$) and roots ($\delta^{13}C_{root}$); $\delta^{18}O_{shoot}$, stable oxygen isotope composition of shoots (‰).

Genotype values are the means of 20 measurements (five treatments and four replications per treatment), while treatment values are the means of the 16 measurements (four genotypes and four replications per genotype). Means followed by different letters are significantly different ($P < 0.05$) according to Tukey's honestly significant difference (HSD) test. Gas-exchange measurements were performed at 1200 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 25°C, and a CO_2 concentration of 400 μmol mol^{-1}. Measurements of $\delta^{13}C$ and $\delta^{18}O$ were carried out at the Scientific-Technical Services of the University of Barcelona and at Iso-Analytical Limited (Crewe, Cheshire CW2 8UY, UK), respectively. International isotope secondary standards of known ^{13}C : ^{12}C ratios (IAEA-CH7, IAEA-CH6 and USGS 40) and ^{18}O : ^{16}O ratios (IAEA-CH-6, IAEA-C-3 and IAEA-601) were used for calibration to a precision of 0.1 and 0.2‰, respectively.

Treatments: FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. ns, not significant.

Table 2 Genotype and treatment effects on nitrogen concentration, nitrogen isotope composition, glutamine synthetase and nitrate reductase activities of four durum wheat genotypes grown under different combinations of salinity and water supply and their combinations

	N_{shoot}	N_{root}	$\delta^{15}N_{shoot}$	$\delta^{15}N_{root}$	GS_{shoot}	NR_{shoot}
Genotype						
Tolerant RIL47	4.39c	1.49a	2.47b	3.45a	544.54b	2.08c
Tolerant RIL 85	4.34c	1.54a	2.92c	3.57a	442.71b	1.83bc
Susceptible RIL 24	3.45b	1.63a	2.12b	3.57a	280.99a	1.57b
Susceptible RIL 30	2.91a	1.48a	1.68a	3.22a	249.37a	0.99a
Treatment						
FI	4.95d	2.14b	3.13b	2.20a	488.02c	3.82c
FI- 12 dS m^{-1}	4.20c	1.21a	1.83a	3.41bc	420.16bc	1.25b
FI-17 dS m^{-1}	2.98b	1.19a	1.84a	4.68c	312.28ab	0.78a
DI	3.95c	2.00b	2.81b	3.17b	386.27bc	1.43b
DI- 12 dS m^{-1}	2.36a	1.07a	1.89a	3.92bc	241.61a	0.68a
Level of significance						
G	0.000	ns	0.000	ns	0.000	0.000
T	0.000	0.000	0.000	0.000	0.000	0.000
G × T	0.000	ns	ns	ns	ns	ns

N, nitrogen concentration (mmol g^{-1} DW) of shoots (N_{shoot}) and roots (N_{root}); $\delta^{15}N$, nitrogen isotope composition (‰) of shoots ($\delta^{15}N_{shoot}$) and roots ($\delta^{15}N_{root}$); GS_{shoot}, shoot glutamine synthetase activity (μmol g^{-1} FW h^{-1}); NR_{shoot}, shoot nitrate reductase activity (μmol g^{-1} FW h^{-1}).
Genotype values for nitrogen content and N isotope composition are the means of 20 measurements (five treatments and four replications per treatment), while treatment values are the means of the 16 measurements (four genotypes and four replications per genotype). Genotype and treatment values for enzyme activities are the means of 15 and 13 measurements, respectively. Means followed by different letters are significantly different ($P < 0.05$) according to Tukey's honestly significant difference (HSD) test. N concentration and stable isotope analyses were carried out at the Scientific-Technical Services of the University of Barcelona. Secondary isotope standards of known $^{15}N : {}^{14}N$ ratios (IAEA N_1 and IAEA N_2 and IAEA NO_3) were used for calibration to a precision of 0.2‰. The mean $\delta^{15}N$ of the fertilizer provided by the Hoagland solution was 0.6‰. GS and NR activities were determined at the Department of Plant Biochemistry and Molecular Biology, Faculty of Chemistry, University of Sevilla.
Treatments: FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. ns, not significant.

Tris-HCl supplemented with 1 mM dithiothreitol (DTT), 0.1 Triton X-100, 10 mM phenylmethylsulphonyl fluoride (PMSF) 10 mM EDTA. To determine actual NR activity, ethylendiaminetetraacetic acid (EDTA) was replaced in the buffer by 10 mM $MgCl_2$. The leaf powder was mixed with the buffer for 10 s at 4°C with a pellet homogenizer. The homogenates were centrifuged for 15 min at 27 000 ***g*** and the supernatants were immediately assayed.

Total GS activity was assayed using the 'biosynthetic' reaction (Márquez *et al.*, 2005). The standard reaction mixture (final volume 100 μl) contained: 10 μmol of Tris-HCl (pH 7.50 at 25°C), 200 μmol of L-glutamate, 5 μmol of NH_4Cl, 5 μmol of $MgCl_2$, 0.3 μmol of ATP (from a 100 mM stock solution, pH 7.5) and 5–10 μl of a 1 : 100 dilution of the crude extract. Duplicates of each enzyme assay were carried out and incubated at 37°C for 15 and 30 min to confirm that the amount of product obtained was linear over time. Inorganic phosphate released by ATP hydrolysis was determined using the malachite green method as described previously (Márquez *et al.*, 2005).

Nitrate reductase activity was determined as described previously (Pajuelo *et al.*, 2002). The standard reaction mixture (final volume 500 μl) contained 25 μmol of Tris-HCl (pH 7.50 at 25°C), 10 nmol of flavin adenine dinucleotide (FAD), 50 μmol of KNO_3, 0.15 μmol of nicotinamide adenine dinucleotide (NADH; from a freshly made 3 mM stock solution in 10 mM Tris-HCl, pH 7.50) and 15–30 μl of crude extract plus 220–235 μl extraction buffer. The reaction was done in duplicate and incubated at 30°C for 30 and 60 min to confirm that the amount of product obtained was linear over time. The amount of nitrite produced was determined as described previously (Márquez *et al.*, 2005). Since both maximum and actual NR activities were strongly correlated ($r = 0.93$) with a slope near one, only maximum NR activity was subsequently used in the study.

Statistical analysis

Data were subjected to factorial ANOVA to test for the effects of treatment (irrigation-salinity regime, genotype, and their interaction). Mean comparisons for genotype effects were performed using contrasts of tolerant vs susceptible material and Tukey's honestly significant difference (HSD) test. In order to test the association between shoot biomass and the set of physiological traits, linear stepwise models across genotypes were constructed that were independent for each treatment, with $P = 0.05$ as the criterion for variables to be either included or removed from the model. The set of physiological parameters was also divided into three categories as follows: photosynthetic traits (including $\delta^{13}C$ and $\delta^{18}O$), N metabolism traits (including $\delta^{15}N$), and ion concentrations. For each trait category, the genotype–treatment combinations (i.e. four genotypes crossed with five treatments) were subjected to unweighted pair group method with arithmetic mean (UPGMA) cluster analysis to summarize the relative merit

Table 3 Genotype and treatment effects on ion concentration of shoots and roots of four durum wheat genotypes grown under different combinations of salinity and water supply and their combinations

	Shoot							Root						
	Na^+	K^+	Ca^{2+}	P	Mg^{2+}	K^+/Na^+	Ca^{2+}/Na^+	Na^+	K^+	Ca^{2+}	P	Mg^{2+}	K^+/Na^+	Ca^{2+}/Na^+
Genotype														
Tolerant RIL47	0.98b	0.78a	0.07a	0.17a	0.06a	2.72b	0.18a	0.75a	0.46a	0.20ab	0.13a	0.04a	1.52a	0.65a
Tolerant RIL 85	1.04b	0.79a	0.09b	0.20b	0.06a	2.09a	0.21ab	0.69a	0.46a	0.19ab	0.13a	0.04a	1.41a	0.55a
Susceptible RIL 24	0.78a	1.01b	0.09b	0.21b	0.07b	2.94b	0.26c	0.59a	0.47a	0.17a	0.11a	0.04a	1.78a	0.57a
Susceptible RIL 30	0.96b	0.84a	0.07a	0.18a	0.06a	2.82b	0.21ab	0.72a	0.51b	0.22b	0.14a	0.04a	1.47a	0.53a
Treatment														
FI	0.19a	1.55c	0.11b	0.25d	0.07c	8.25c	0.59c	0.15a	0.72b	0.21c	0.21d	0.04b	4.73c	1.38c
FI- 12 dS m^{-1}	1.48c	0.54a	0.07a	0.20c	0.06b	0.36a	0.04a	0.90b	0.27a	0.15b	0.12b	0.02a	0.30a	0.18a
FI-17 dS m^{-1}	1.58c	0.46a	0.06a	0.18b	0.05a	0.29a	0.03a	1.16c	0.33a	0.09a	0.08ab	0.03a	0.29a	0.08a
DI	0.33b	1.22b	0.11b	0.19bc	0.08d	3.69b	0.35b	0.34a	0.71b	0.35d	0.16c	0.06c	2.06b	1.05b
DI- 12 dS m^{-1}	0.98b	0.55a	0.05a	0.10a	0.06ab	0.58a	0.05a	0.86b	0.31a	0.13ab	0.05a	0.04b	0.36a	0.15a
Level of significance														
G	0.025	0.001	0.000	0.000	0.004	0.000	ns	ns	ns	0.003	ns	ns	ns	ns
T	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G × T	ns	0.021	0.001	0.001	0.000	0.000	ns	ns	ns	ns	ns	ns	ns	ns

Genotype values are the means of 20 measurements (five treatments and four replications per treatment), while treatment values are the means of the 16 measurements (four genotypes and four replications per genotype). Concentrations are expressed as mmol g^{-1} DW. Means followed by different letters are significantly different ($P < 0.05$) according to Tukey's honestly significant difference (HSD) test. For each sample, 100 mg of dry material was digested with 3 ml of concentrated HNO_3 and 2 ml H_2O_2. Samples were placed overnight in an oven at 90°C. After digestion, each sample was then brought up to 30 ml final volume with deionized water. The amounts of Na^+, Ca^{2+}, K^+, P, and Mg^{2+} per sample were determined with an inductively coupled plasma emission spectrometer (L3200RL) at the Scientific-Technical Services of the University of Barcelona.
Treatments: FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. ns, not significant.

of genotypic effects and growing conditions as causes of changes in the observed plant responses. Finally, we performed path analyses (Li, 1975) to quantify the relative contributions of direct and indirect effects of stable isotopes on above-ground biomass. This methodology offers the possibility of building associations between variables on prior knowledge. A path analysis determines simple correlations between independent factors (in this case, $\delta^{13}C$ and $\delta^{18}O$), and regresses them on each intermediary (NR and GS activities, $\delta^{15}N$, N concentration) or dependent factor (biomass) to obtain direct effects in the form of partial regression coefficients (i.e. path coefficients). In this way, direct (i.e. from $\delta^{13}C$ and $\delta^{18}O$) and indirect (i.e. through the pathway of enzyme-based N metabolism) mechanisms that play potential roles in biomass variation and involving traits that exhibited genotypic differences were proposed, as detailed in the conceptual model displayed in Fig. 6(a). This model was aimed at understanding biomass responses to genotypic differences in water status and photosynthetic carbon and nitrogen performance, and was tested under each growth condition. For each condition, a reduced model was identified as reproducing the original correlation matrix nearly as well as the full, saturated model (i.e. in which there is a direct path from each variable to every other variable), as shown by goodness-of-fit tests. The reduced models were also compared with the independence model (one where all possible paths are deleted) by a comparative fit index (CFI) (Arbuckle, 1997). Data were analysed using the SAS package (SAS Institute Inc., Cary, NC, USA).

Results

Growth, photosynthetic traits and carbon and oxygen isotope signatures

Treatments significantly affected biomass, leaf Chl, gas exchange, and the stable carbon and oxygen isotope signatures (Table 1). The most severe treatments in terms of decreasing biomass were full irrigation with highly saline water (FI-17) and the combination of deficit irrigation with moderately saline water (DI-12). Both showed shoot biomass that was < 30% of the values of the control (FI). Intermediate treatments were full irrigation with moderately saline water (FI-12) and deficit irrigation with non-saline water (DI), with 50 and 48% reductions in biomass compared with FI, respectively. Leaf Chl was far less affected, and it even increased in DI compared with FI. The gas-exchange traits A, g_s, C_i/C_a, T and the ratio F_v'/F_m strongly decreased, compared with FI, in response to all stress treatments. As for biomass, FI-17 and DI-12 showed the lowest g_s and C_i/C_a, while FI-12 and DI had intermediate values. Stress treatments increased shoot $\delta^{18}O$ compared with FI, but the most severe treatments (FI-17 and DI-12) showed the least change. Root and shoot $\delta^{13}C$ also increased in the stress treatments compared with FI, but in this case the highest values were observed under the most stressful conditions.

Tolerant genotypes exhibited significantly higher biomass (*c.* 35%) than susceptible genotypes. Tolerant genotypes also

showed higher A, g_s, C_i/C_a and F_v'/F_m, and lower shoot $\delta^{18}O$ and root and shoot $\delta^{13}C$ (Table 1). The genotype × treatment interaction (G × T) was only significant for biomass, T and $\delta^{18}O$. Genotypic differences between pairs of tolerant and susceptible genotypes were also examined for each growing condition, and especially for traits displaying significant G × T interaction (Supporting Information, Table S1). Except for $\delta^{13}C$ of shoots and roots (lower in the tolerant genotypes), in the absence of stress (FI) there were no significant differences for any of the traits. By contrast, genotype differences in biomass (higher in tolerant lines) were observed for the stress treatments, and also in $\delta^{18}O$ and F_v'/F_m, but only under the least stressful conditions (FI-12 and DI). A crossover interaction was observed for T: susceptible genotypes exhibited higher transpiration than tolerant genotypes under DI-12, but lower values for the salinity-only treatments.

Nitrogen concentration, $\delta^{15}N$ and enzyme activities

Treatments significantly affected N concentration, $\delta^{15}N$, and GS and NR activities (Table 2). Stress treatments decreased N concentration in shoots and roots compared with FI. As for biomass, the most severe treatments were FI-17 and DI-12. Root $\delta^{15}N$ increased in all stress treatments, showing the highest values in the two most stressful treatments. By contrast, stress treatments significantly decreased shoot $\delta^{15}N$. The activities of GS and NR also decreased in response to stress compared with FI, especially in FI-17 and DI-12. NR activity was much more affected in relative terms than GS by stress conditions, with reductions of 60% and 14% in the least severe treatments (FI-12 and DI), as compared with FI.

The genotype effect was significant for N concentration and $\delta^{15}N$, but only in shoots, with values for both traits being higher in tolerant than in susceptible genotypes. Activities of GS and NR were also higher in tolerant genotypes. Except for shoot N, no significant G × T interaction was detected. Genotypic differences between pairs of tolerant and susceptible genotypes were also examined for each growing condition (Table S2). Under full irrigation, no differences existed for the set of traits, except for GS activity (higher in tolerant lines). By contrast, the N concentration of susceptible genotypes was reduced to a greater extent than in tolerant lines for all stress treatments, thus causing a significant G × T interaction. Also, $\delta^{15}N$ in shoots was significantly higher in tolerant genotypes for each stress treatment, but there were no differences in either N concentration or $\delta^{15}N$ in roots. GS and NR activities were also higher in tolerant genotypes for each stress treatment.

Ion concentrations

Treatments significantly affected ion concentrations in both shoots and roots (Table 3). Stress treatments involving salt application strongly increased Na^+ and decreased K^+, Ca^{2+}, Mg^{2+}, and P, and the ratios K^+ : Na^+ and Ca^{2+} : Na^+ in both plant parts. DI also increased Na^+ in shoots and roots compared with FI, but this was to a much smaller extent than was observed with saline treatments. In shoots, K^+ was also lower under DI than under FI, while no differences were observed in roots. The genotype effect was significant for all ions in shoots, but only for Ca^{2+}in roots. However, no clear pattern of differences between tolerant and susceptible genotypes was found for most ions in shoots, except for K^+ (with susceptible genotypes showing a higher concentration) and Na^+ (with tolerant genotypes showing a higher concentration). G × T interactino was significant for all ions in shoots except for Na^+, while there was no G × T interaction in roots. In most cases, there were no significant differences between pairs of tolerant and susceptible genotypes for ions measured in each growing condition and plant part (Table S3).

Effect of genotype and growing condition on physiological traits

Cluster analyses (Fig. 1) were performed as a way of summarizing the relevance of genotypic effects and growing conditions on changes in traits related to gas exchange (Table 1), N metabolism (Table 2) and ion accumulation (Table 3). Gas-exchange traits (including $\delta^{13}C$ and $\delta^{18}O$) provided a good separation of treatments but did not allow for clear genotype differentiation (Fig. 1a). By contrast, traits related to N metabolism (including $\delta^{15}N$) discriminated tolerant from susceptible genotypes (Fig. 1b). Finally, the cluster based on ion concentrations allowed for an almost perfect separation among treatments (Fig. 1c).

Relationships of biomass and nitrogen concentration with physiological traits across treatments

Nitrogen concentration was positively related to biomass across growing conditions and genotypes up to values near 5 mmol N g^{-1}, with a saturation response observed afterwards (Fig. 2). Biomass was negatively associated with shoot $\delta^{13}C$ and positively with shoot $\delta^{15}N$, whereas no clear trend was observed between biomass and shoot $\delta^{18}O$ (Fig. 3), except when the most severe treatments (FI-17 and DI-12) were discarded (inset Fig. 3c). N concentration was linearly correlated with $\delta^{13}C$ (negatively) and with $\delta^{15}N$ (positively), and also with $\delta^{18}O$, albeit in a weaker manner. The association between biomass and either GS or NR activity was positive (Fig. 4), but tighter in the latter case. N concentration was also positively correlated with the activities of both enzymes, either linearly (GS) or exponentially (NR). Both GS and NR activities correlated positively with shoot $\delta^{15}N$ and negatively with both $\delta^{13}C$ and $\delta^{18}O$, but, in this last case, weakly (Fig. 5). Overall, NR activity was related to both isotopic signatures more strongly than GS activity.

Relationships between biomass and physiological traits across genotypes

A multiple linear regression (stepwise) explaining biomass variation across genotypes as a function of traits related to gas exchange, N metabolism and ion accumulation was fitted independently for each growing condition (Table 4). $\delta^{13}C$ was chosen as the first explanatory variable in the control (FI, positive effect) and the

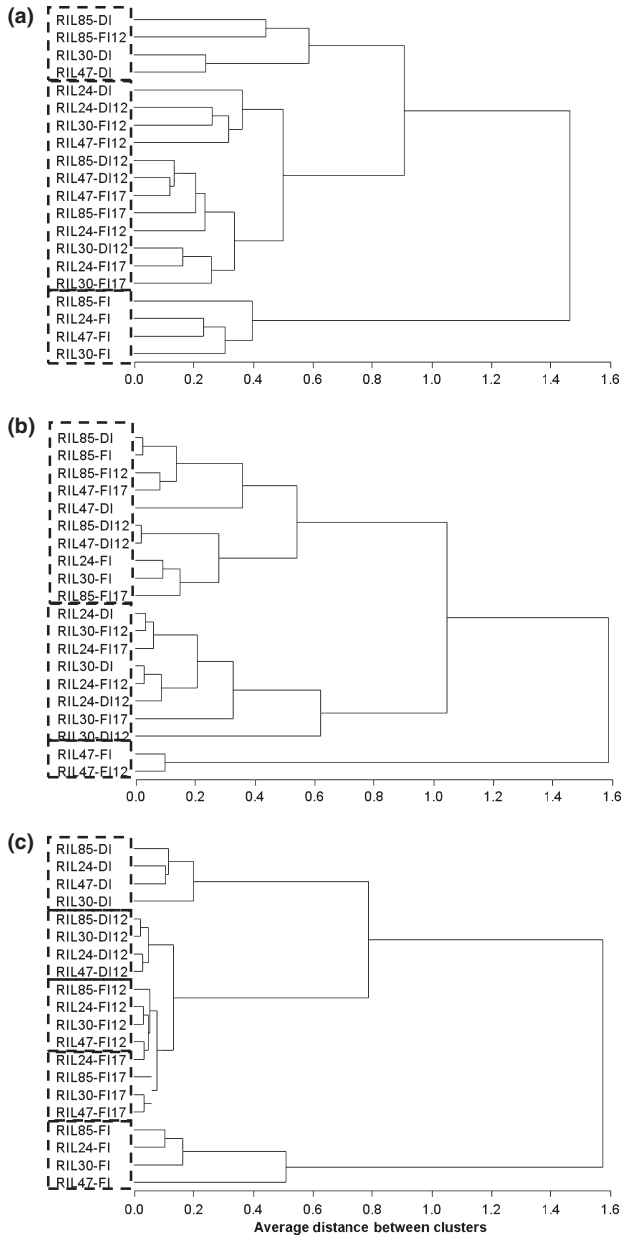

Fig. 1 Cluster analysis of the five growing conditions assayed, using the complete set of physiological parameters studied in this work as variables. These parameters were divided into three categories as follows: (a) photosynthetic traits and C and O isotopic signatures; (b) N metabolism traits (including N isotopic signatures, N content and glutamine synthetase (GS) and nitrate reductase (NR) activities); and (c) mineral nutrition traits. FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dSm^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}.

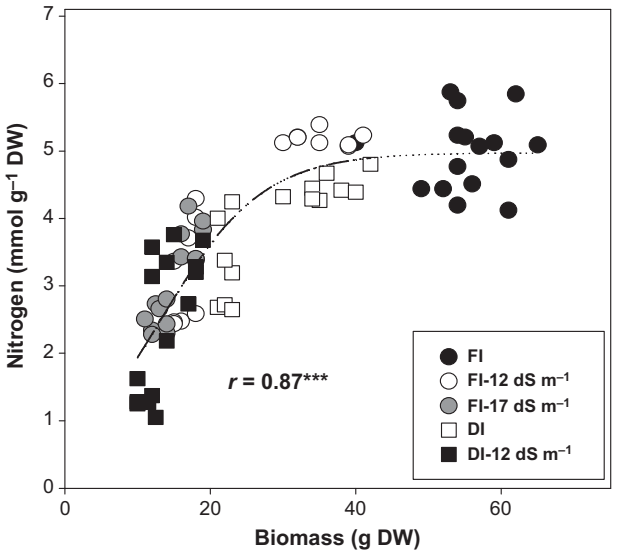

Fig. 2 Relationship between nitrogen content and shoot biomass across the four genotypes within each of the five growing conditions assayed. Each point represents the individual value for a given replication and genotype within a growing condition. Variables mentioned in this figure are from the aerial parts of plants. FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. ***$P < 0.001$.

most stressful treatment (DI-12, negative effect). Additional variables chosen in the FI model were also photosynthetic traits (A and g_s), while no other traits were included in the DI-12 model. By contrast, the first variable entering the model in other stress treatments was related to N metabolism: N concentration in the FI-12 model and GS activity in the FI-17 and DI models. $\delta^{18}O$ was selected as the second variable in two out of three models. Overall, > 80% of biomass variability was explained by the combination of two to three independent variables in four of the models, whereas 66% of the total variability was explained by just one variable in the remaining model (DI-12).

A conceptual model was proposed (Fig. 6a) separating direct acclimation responses in biomass related to water status (through $\delta^{18}O$) and photosynthesis (through $\delta^{13}C$) from those likely to correspond to indirect effects linked to N metabolism (through GS and NR activities, N and $\delta^{15}N$). The five path models provided an acceptable fit to the data (Fig. 6; CFI > 0.9 and $P > 0.05$ in all cases; as the objective here is to develop models that fit the data well, a nonsignificant χ^2 is preferred). In all cases, $\delta^{13}C$ had a strong negative effect on NR activity. Conversely, $\delta^{13}C$ had a direct positive effect on biomass only in FI, while it exhibited a direct negative effect in DI-12. Significant paths corresponding to a direct (negative) effect of $\delta^{18}O$ on biomass were observed in the mildest salinity treatment (FI-12) and also in DI. The indirect explanation of biomass via N metabolism traits showed significant directional effects of NR on GS for those cases (FI-17, DI, DI-12) where biomass was significantly associated with changes in N concentration (i.e. the third possible path to biomass determination after $\delta^{13}C$ and $\delta^{18}O$). On the other hand, GS was only significantly (and negatively) affected by $\delta^{13}C$ in DI-17, but in this case (and also in FI) N metabolism effects on biomass seemed negligible. Under FI, DI and FI-12, $\delta^{15}N$ was directionally affected by GS (positively) and, to a lesser extent, by NR (either positively or negatively), but only in FI-12 was there a significant directional effect of $\delta^{15}N$ on N concentration, which

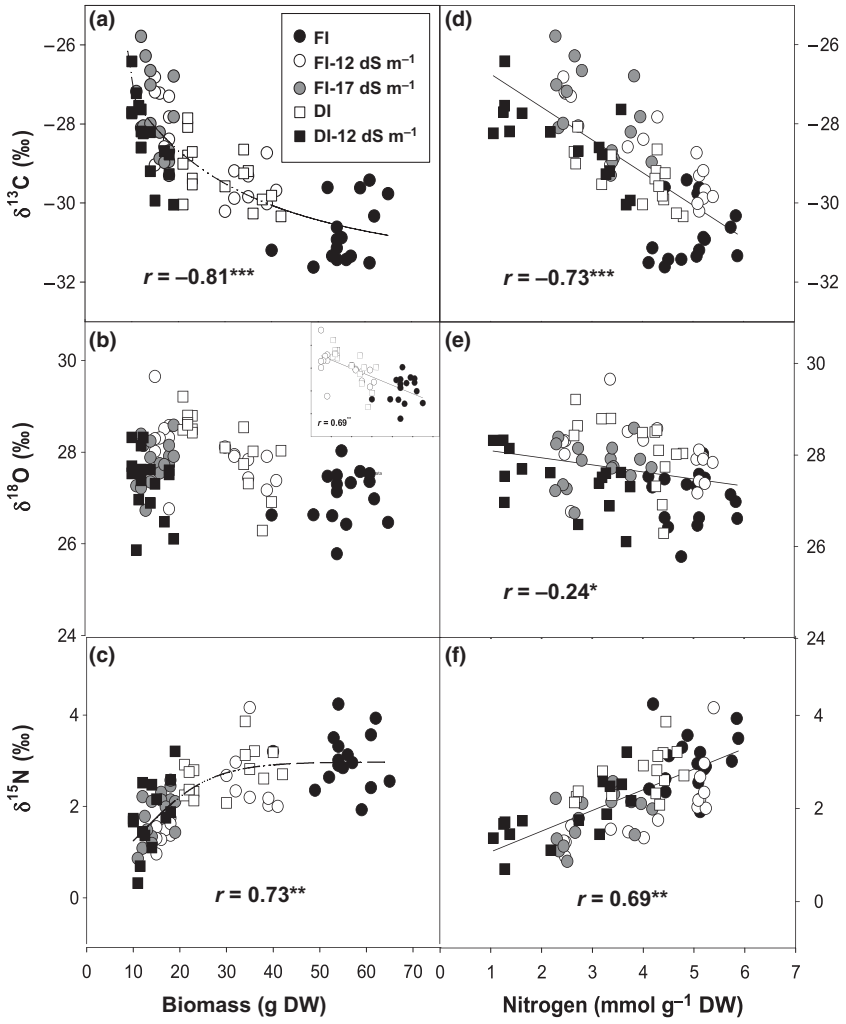

Fig. 3 The relationship of shoot biomass with carbon isotope composition ($\delta^{13}C$) (a), oxygen isotope composition ($\delta^{18}O$) (b) and nitrogen isotope composition ($\delta^{15}N$) (c). The relationship of nitrogen content with carbon isotope composition ($\delta^{13}C$) (d), oxygen isotope composition ($\delta^{18}O$) (e) and nitrogen isotope composition ($\delta^{15}N$) (f). Variables mentioned in this figure are from the aerial parts of plants. The five treatments and the four genotypes are plotted together. Each point represents the individual value for a given replication and genotype within a growing condition. FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. *P < 0.05, **P < 0.01, ***P < 0.001.

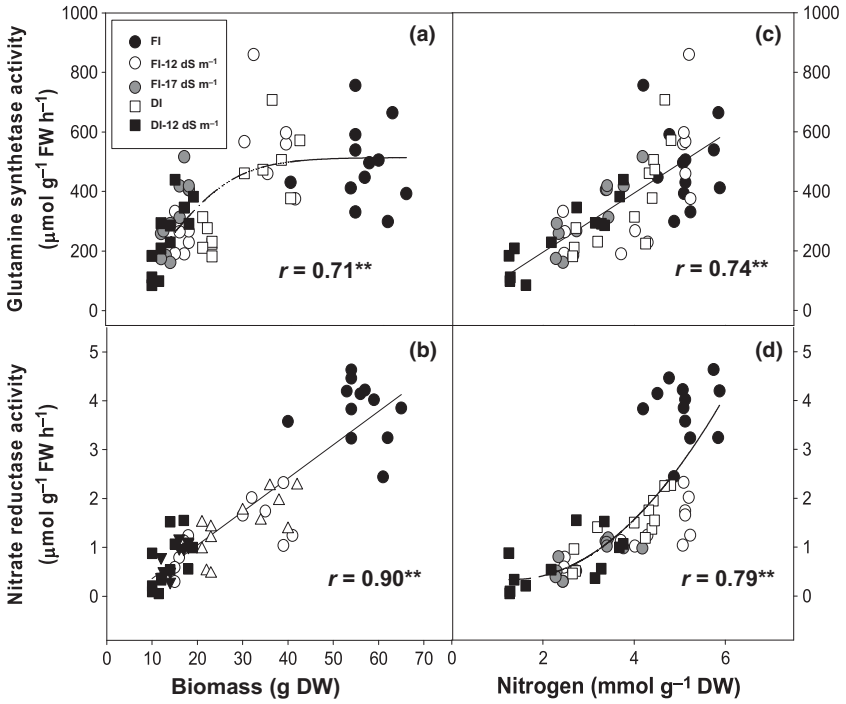

Fig. 4 The relationship of shoot biomass with glutamine synthetase activity (a) and nitrate reductase activity (b). The relationship of nitrogen content with glutamine synthetase activity (c) and nitrate reductase activity (d). Variables mentioned in this figure are from the aerial parts of plants. The five treatments and the four genotypes are plotted together. Each point represents the individual value for a given replication and genotype within a growing condition. FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. **P < 0.01.

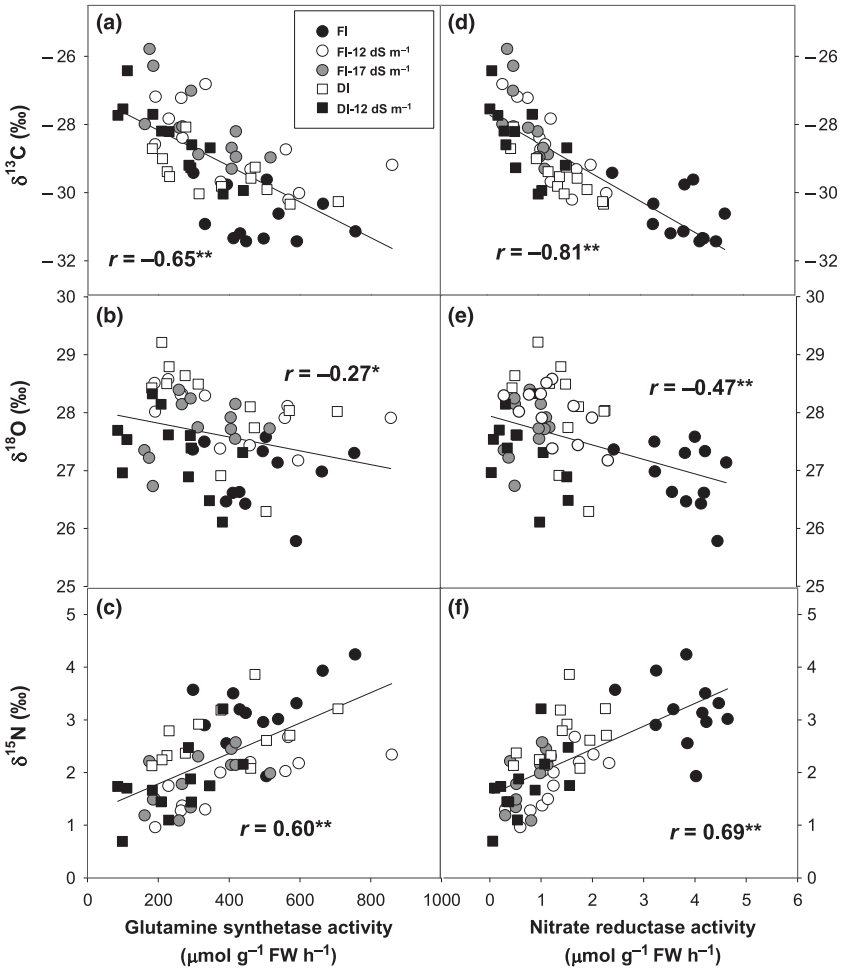

Fig. 5 The relationship of glutamine synthetase activity with carbon isotope composition ($\delta^{13}C$) (a); oxygen isotope composition ($\delta^{18}O$) (b) and nitrogen isotope composition ($\delta^{15}N$) (c). The relationship of nitrate reductase activity with carbon isotope composition ($\delta^{13}C$) (d); oxygen isotope composition ($\delta^{18}O$) (e) and nitrogen isotope composition ($\delta^{15}N$) (f). Variables mentioned in this figure are from the aerial parts of plants. The five treatments and the four genotypes are plotted together. Each point represents the individual value for a given replication and genotype within a growing condition. FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. $^{**}P < 0.01$.

in turn affected biomass. In summary, the mildest stress treatments (DI and FI-12) pointed to the dependence of biomass on both $\delta^{18}O$ and N metabolism traits, whereas under more severe growing conditions the influence of either N metabolism (in FI-17) or $\delta^{13}C$ (in DI-12) on biomass appeared relevant. Under control conditions, only $\delta^{13}C$ was directly associated with biomass, albeit with an opposite sign to that found in DI-12.

Discussion

Treatments that most affected growth were full irrigation with high amounts of salinity or a combination of deficit irrigation and salinity, in agreement with previous studies (Ayers & Westcott, 1989; Yousfi *et al.*, 2009, 2010). Moreover, consistent differences in biomass were observed between tolerant and susceptible genotypes regardless of the stress conditions (Yousfi *et al.*, 2009, 2010), although such differences vanished in the absence of stress.

Are ion concentrations, Chl content and short-term photosynthetic parameters good indicators of genotypic tolerance to salinity?

Changes in shoot ion concentrations under distinct salinity–irrigation arrangements are in agreement with previous studies (Munns & Tester, 2008). However, it has been reported that lower Na^+ concentrations and higher K^+ : Na^+ and Ca^{2+} : Na^+ ratios improve resistance to salinity (Hu & Schmidhalter, 2005) and, therefore, selecting for low Na^+ uptake and enhanced K^+ : Na^+ ratios has been proposed as a screening strategy (Munns *et al.*, 2000). In clear contrast, shoots from tolerant genotypes in this study did not exhibit lower Na^+ and higher K^+, Ca^{2+} and Mg^{2+} than susceptible ones, or higher K^+ : Na^+ and Ca^{2+} : Na^+ ratios, but rather had the opposite tendency (Table S3). This observation points to increased osmotic adjustment of tolerant lines to saline conditions through the incorporation of available ions such as Na^+ (Munns, 2002; Cuin *et al.*, 2009).

Chlorophyll content per unit leaf area has been proposed as a screening criterion for wheat tolerance to salinity (Munns & James, 2003). However, the expected decrease in leaf Chl as a result of salt toxicity was probably offset in our study by an increase in leaf thickness or packing of mesophyll cells as a response to water stress, which eventually translated into constant Chl readings (James *et al.*, 2006; Yousfi *et al.*, 2009; Munns *et al.*, 2010).

Salinity, water stress and their combined effect induced a strong decrease in photosynthesis and transpiration through a decrease in g_s (Ouerghi *et al.*, 2000; Yousfi *et al.*, 2009, 2010). Several studies (James *et al.*, 2008; Rahnama *et al.*, 2010) suggest that screening

Table 4 Multiple linear regressions (stepwise) explaining shoot biomass (B_{shoot}) variation across genotypes for each of five growing conditions, with traits related to photosynthetic performance (Table 1), nitrogen metabolism (Table 2) and ion accumulation (Table 3) used as independent variables

Treatments	Variable chosen	R^2	Final stepwise model
FI	$\delta^{13}C$	0.38	B_{shoot} = 212.91 + 6.27 $\delta^{13}C$
	$\delta^{13}C$, A	0.65	+ 1.59 A + 20.99 g_s
	$\delta^{13}C$, A, g_s	0.86***	
FI -12 dS m^{-1}	N	0.75	B_{shoot} = 334.96 + 5.32 N
	N, $\delta^{18}O$	0.93***	− 11.82 $\delta^{18}O$
FI -17 dS m^{-1}	GS	0.80	B_{shoot} = 7.55 + 0.20 GS
	GS, A	0.83***	+ 0.55 A
DI	GS	0.66	B_{shoot} = 156.76 + 0.03 GS
	GS, $\delta^{18}O$	0.83***	− 4.89 $\delta^{18}O$
DI -12 dS m^{-1}	$\delta^{13}C$	0.66***	B_{shoot} = −56.31− 2.45 $\delta^{13}C$

Treatments: FI, full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}.
Only parameters entering the models are shown: $\delta^{13}C$, carbon isotope composition (‰); A, leaf net CO_2 assimilation (μmol CO_2 m^{-2} s^{-1}); g_s, stomatal conductance (mol CO_2 m^{-2} s^{-1}); N, leaf nitrogen concentration (mmol g^{-1} DW); $\delta^{18}O$, oxygen isotope composition (‰); GS, glutamine synthetase activity (μmol g^{-1} FW h^{-1}); $\delta^{15}N$, nitrogen isotope composition (‰). ***, $P < 0.001$. All variables chosen by the different models refer to shoots.

for high g_s may be the most effective way of identifying fast-growing genotypes in saline soils. In our study, however, either g_s or other gas-exchange parameters could not differentiate between tolerant and susceptible genotypes (Table S1). Yousfi *et al.* (2010) found comparable results in wheat during the reproductive stage.

Growing conditions and genotypic effects on $\delta^{18}O$ and $\delta^{13}C$ under salinity and drought conditions

Plant $\delta^{13}C$ increased under water-limiting and salinity conditions, as reported elsewhere (Isla *et al.*, 1998; Condon *et al.*, 2002; Araus *et al.*, 2003; Yousfi *et al.*, 2009, 2010). The increase in $\delta^{13}C$ was not completely a consequence of a stomatal limitation to photosynthesis, since the association between $\delta^{13}C$ and $\delta^{18}O$ across environments, although positive, was weak (0.281 $P < 0.05$). Increases in plant $\delta^{18}O$ as a response to drought have been observed in cereals (Ferrio *et al.*, 2007; Cabrera-Bosquet *et al.*, 2009a; Araus *et al.*, 2010). A higher $\delta^{18}O$ may be linked to a decrease in g_s, lower transpiration and reduced leaf cooling, therefore resulting in lower ^{18}O enrichment at evaporation sites (Barbour, 2007; Farquhar *et al.*, 2007). To the best of our knowledge this is the first study in wheat reporting on genetic differences in plant $\delta^{18}O$ under salinity. In agreement with a higher long-term g_s, tolerant genotypes exhibited lower $\delta^{13}C$ and $\delta^{18}O$ than susceptible ones. Our results, therefore, highlight the advantage of time-integrative traits over instantaneous (but time-consuming) gas-exchange measurements for genotype evaluation (Araus *et al.*, 2002, 2008). Moreover, tolerant genotypes exhibited constitutively (i.e. in absence of stress) lower $\delta^{13}C$ (and also a tendency to lower $\delta^{18}O$) than susceptible ones (Table S1), in agreement with previous studies using $\delta^{13}C$ (Araus *et al.*, 2003; Yousfi *et al.*, 2009, 2010) and $\delta^{18}O$ (Cabrera-Bosquet *et al.*, 2009b), as well as reviews on yield potential and crop performance under water stress (Blum, 2005, 2009; Araus *et al.*, 2008), which conclude that drought resistance and yield potential are compatible.

Growing conditions and genotypic effects on N concentration and NR and GS activities

The effects of salinity and deficit irrigation that decreased shoot N concentration paralleled those obtained for shoot biomass, with tolerant genotypes exhibiting higher N concentration than susceptible ones. Besides a direct osmotic effect on plant water availability, the parallels between the changes in biomass and N concentration suggest that salinity and water deficit also affected growth through an effect on N metabolism (Hirel *et al.*, 2007). In relation to this, NR and GS activities also decreased under water deficit, salinity and in combination, but overall, tolerant genotypes exhibited higher GS and NR activities. A number of studies have reported decreased NR activity in response to water stress (Foyer *et al.*, 1998; Correia *et al.*, 2005) and salinity (Wang *et al.*, 2007; Carillo *et al.*, 2008). Salt was observed to inhibit nitrate transport to the leaf mainly because of nitrate/chloride competition, consequently affecting NR activity (Rao & Gnanam, 1990; Abd-El Baki *et al.*, 2000). There is also evidence that photosynthesis regulates nitrate reduction by modulating NR activity (Kaiser & Förster, 1989; Kaiser & Brendle-Behnisch, 1991), which is in accordance with current results showing that stress treatments decrease both photosynthesis and NR activity. Wang *et al.* (2007) reported that GS activity of wheat genotypes also decreased under salinity. GS plays a key role under osmotic stress, reassimilating nitrogen from increased amino acid catabolism, and producing protective nitrogen compounds (Brugière *et al.*, 1999; Diaz *et al.*, 2010). Thus, the higher GS activity in tolerant genotypes could be related to the fact that proline and glycine betaine accumulate in durum wheat under salinity (Carillo *et al.*, 2008). Several quantitative trait loci for important agronomic traits in wheat colocalize with the GS marker (Li *et al.*, 2011). Furthermore, overexpression of GS results in enhanced tolerance to salt stress and high light intensity through an improved capacity for photorespiration, therefore avoiding photoinhibition (Kozaki & Takeba, 1996; Hoshida *et al.*, 2000).

Effect of growing conditions and genotype on $\delta^{15}N$ of shoots and roots

An array of salinity–drought combinations modifies $\delta^{15}N$ in shoots and roots relative to controls, with tolerant genotypes exhibiting higher shoot $\delta^{15}N$ than susceptible genotypes (Table S1), suggesting that stress conditions influence N uptake and/or assimilation (Handley *et al.*, 1997; Ellis *et al.*, 2002; Yousfi *et al.*, 2009, 2010). Decreases in shoot $\delta^{15}N$ have been

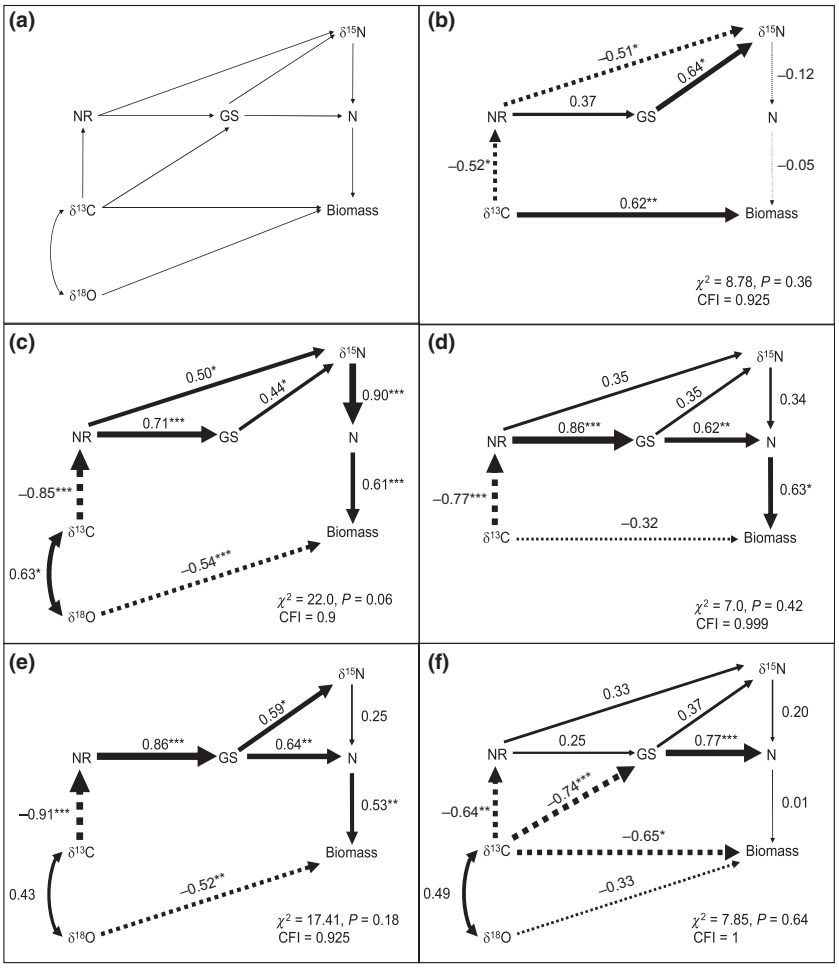

Fig. 6 Path analyses of four durum wheat (*Triticum turgidum* ssp. *durum*) genotypes grown under five combinations of drought and salinity. The conceptual model quantifying the relative contributions of direct and indirect effects of stable isotopes on above-ground biomass is shown in (a). The different combinations of drought and salinity are as follows: (b) FI, full irrigation with normal Hoagland solution; (c) FI-12 dSm^{-1}, full irrigation with Hoagland solution at 12 dSm^{-1}; (d) FI-17 dSm^{-1}, full irrigation with Hoagland solution at 17 dS m^{-1}; (e) DI, deficit irrigation (35% pot capacity) with normal Hoagland solution; (f) DI-12 dSm^{-1}, deficit irrigation with Hoagland solution at 12 dS m^{-1}. Variables mentioned in this figure are from aerial plant parts. A single-headed arrow between two variables denotes a hypothesis of direct causation, whereas a double-headed arrow reflects correlation without necessarily a direct causal relationship. Dashed lines indicate negative relationships. The width of arrows is proportional to the path coefficient values. Overall fit statistics for each path model (chi-squared and comparative fit index, CFI), the latter useful for small sample sizes (with values > 0.9 taken as indicative of a good fit), are shown at the bottom right of each panel. NR, nitrate reductase; GS, glutamine synthetase. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

reported in cereals as a response to salinity (Yousfi *et al.*, 2009, 2010) and deficit irrigation (Robinson *et al.*, 2000; Raimanová & Haberle, 2010). Reduced g_s as a result of either salinity or water stress, or a combination of both, would lead to a reduction in the loss of ammonia and nitrous oxide, hence decreasing $\delta^{15}N$ (Farquhar *et al.*, 1980; Smart & Bloom, 2001). A high external N concentration relative to a modest demand would also lead to salinity-induced depletion in plant ^{15}N (Mariotti *et al.*, 1982). Consequently, the suboptimal growing conditions associated with abiotic stresses would produce a decrease in demand relative to a constant N supply. This may have the same effect as increasing the external N concentration (Mariotti *et al.*, 1982), leading to greater isotopic discrimination (Vitousek *et al.*, 1989; Handley *et al.*, 1997). The higher $\delta^{15}N$ of shoots compared with roots under control conditions could be related to fractionation processes during assimilation by NR, with $\delta^{15}N$ of unassimilated nitrate becoming enriched relative to organic N (Evans, 2001; Tcherkez, 2011). Thus, NO_3^- that was not reduced in roots and exported to the shoots would be enriched in ^{15}N, allowing for an increased $\delta^{15}N$ of shoots relative to roots (Yoneyama & Kaneko, 1989; Evans *et al.*, 1996). However, stress conditions would limit NO_3^- export from the roots to the shoot (Kronzucker *et al.*, 1998), therefore decreasing $\delta^{15}N$ of shoots while increasing it in the roots compared with control. This would explain the opposite $\delta^{15}N$ response patterns to stress in shoots (^{15}N depletion relative to full irrigation) compared with roots (^{15}N enrichment). Water stress would limit the uptake and further transfer of N to the upper plant parts, subsequently increasing $\delta^{15}N$. Hence, when N availability is limited, a rapid decrease in N translocation from the root to the shoot has been reported (Kronzucker *et al.*, 1998). Moreover, tolerant genotypes tended to exhibit constitutively higher $\delta^{15}N$ (Table S1), which agrees with previous studies on durum wheat (Yousfi *et al.*, 2009).

Gas-exchange and N metabolism traits involved in genotypic performance

While changes in ion concentrations and ratios perfectly separated treatments (Fig. 1), they were unsuited for assessing genotypic tolerance to salinity and water stress (Tables 3, S3). By contrast, stable isotope signatures related to plant photosynthesis ($\delta^{13}C$) and transpiration ($\delta^{18}O$), and traits related to N metabolism (N, NR, GS and $\delta^{15}N$), were more appropriate for genotype differentiation. As these traits were almost the only ones selected in the stepwise models (Table 4) that explained genotypic differences in biomass, they were used to further interpret the physiological mechanisms underlying distinct genotypic performances under each growing condition.

Nitrogen uptake and assimilation is a complex event that depends on many factors, such as the coordination of nitrogen with carbon, energy, and other types of metabolism during plant development (Masclaux-Daubresse *et al.*, 2010; Rana *et al.*, 2010). N assimilation requires NADH for NR-driven and ATP for GS-driven reactions, as well as carbon skeletons derived from photosynthesis for the synthesis of amino acids. In this respect, we observed a decrease in NR and GS activities associated with an increase in $\delta^{13}C$ (Fig. 5), which in turn can be interpreted as the result of restricted photosynthetic activity mediated by lower g_s. Moreover, $\delta^{15}N$ is also affected by changes in photosynthetic activity driven by stress conditions (Lopes *et al.*, 2004; Lopes & Araus, 2006). Thus, NR and GS activities were positively associated with $\delta^{15}N$. It is well known that $^{14}N/^{15}N$ isotope fractionation occurs during nitrate and ammonium assimilation by plants (Tcherkez & Farquhar, 2006).

Recently, Tcherkez (2011) proposed a model explaining $\delta^{15}N$ variability in leaves in which a high sensitivity of $\delta^{15}N$ values to both photorespiratory and N input (e.g. reduction by NR) is demonstrated. Overall, this model shows a clear link between photosynthesis and N metabolism (affecting energy balance of key enzymes or through photorespiration fluxes) and might explain why $\delta^{15}N$ and $\delta^{13}C$ are covariates across growing conditions in this study ($r = -0.69^{**}$). Alternatively Cernusak *et al.* (2009b) hypothesized that whole-plant $\delta^{15}N$ should vary as a function of the transpiration efficiency of nitrogen acquisition, both factors being positively correlated.

Stress conditions, particularly saline treatments, were shown to cause a much stronger reduction in NR than in GS activity. An inhibition of nitrate uptake rates exceeding 50% has been observed under 60 mM NaCl in wheat (Botella *et al.*, 1997), whereas ammonium uptake seems much less affected (Ullrich, 2002). Furthermore, wheat plants have shown a preferential ammonium uptake in saline media (Botella *et al.*, 1993, 1997). This would justify the lower reduction in GS than in NR activity in response to stress. Moreover, NR catalyses a high energy-consuming metabolic reaction and NR activity is reported as strongly affected by stress conditions influencing photosynthesis (Tcherkez, 2011). The path analysis showed that NR activity was negatively associated with $\delta^{13}C$, regardless of the growing conditions. Simple correlations also showed that biomass and N concentration were better associated with NR than with GS activity. However, the path analysis revealed a direct effect of $\delta^{13}C$ on GS (Fig. 6) in the most severe treatment (DI-12), whereas the directional effect of NR on GS activity diminished as compared with other stress treatments. This realization suggests that if N uptake and assimilation are very low, N metabolism is basically driven by a high photorespiration. In fact, the photorespiratory flux of ammonium in C_3 plants can be 10 times higher than that originating from nitrate reduction (Hirel *et al.*, 2007).

The negative relationships of $\delta^{18}O$ with NR and GS activities observed across treatments are consistent with a dependence of $\delta^{18}O$ on g_s values mediated by particular stress conditions. Nevertheless, the relationships of enzymatic activities to $\delta^{18}O$ were far weaker than those observed for $\delta^{13}C$, which agrees with the fact that $\delta^{18}O$ is not as directly associated with photosynthesis as $\delta^{13}C$. Even so, $\delta^{18}O$ (as $\delta^{13}C$ and $\delta^{15}N$) correlated better with NR activity than with GS activity, in accordance with a more direct link of NR to photosynthesis.

Combined isotope signatures track genotypic performance under stress

Genotypes with lower $\delta^{13}C$ exhibited higher biomass under stress. However, the path analysis revealed that, except for the two most severe growing conditions (FI-17 and DI-12), $\delta^{13}C$ did not have a direct effect on biomass, but was mediated through $\delta^{18}O$ or N metabolism. Genotypes with lower $\delta^{18}O$ were directly associated with higher biomass in the two moderate stress treatments (FI-12 and DI; Table S1), with $\delta^{18}O$ being significantly related to g_s ($r = -0.66$ $P < 0.01$) at least in DI, which agrees with previous results on durum wheat where $\delta^{18}O$ reflected transpiration patterns (Cabrera-Bosquet *et al.*, 2009a, 2011). Results also showed that stress affected N metabolism in all growing conditions and, except in the most severe treatment (DI-17), it translated into genotypic differences in biomass, with a higher shoot $\delta^{15}N$ being a favourable trait. Besides that, because progressive water stress decreases tissue expansion before closure of stomata (Hsiao, 1973), any water stress increasing $\delta^{18}O$ would have already affected tissue expansion, and thus biomass. Tardieu *et al.* (2011) concluded that water deficit affects plant growth via reduced tissue expansion, carbon photosynthesis and cell number, the first process being the most crucial. However, our study highlights that N metabolism also plays a key role in genotypic performance under salinity and water stress.

Implications for breeding

This study examines the potential usefulness of C, O and N stable isotopes in shoots, alone or in combination, to assess genotypic performance of durum wheat to salinity and/or water deficit. Multiple isotope measurements may provide additional discrimination among phenotypes. Indeed, while selection of low-$\delta^{13}C$ genotypes arises as a positive choice in any of the stress conditions assayed, a low genotypic $\delta^{18}O$, along with a high $\delta^{15}N$, may represent a sensible combination to aid identifying genotypes better adapted to moderate stresses. The interest of selecting tolerant genotypes at early stages of the crop cycle stems from the possibility of scheduling pollinations at anthesis and saving resources at harvest. This is particularly evident for salinity, which, in contrast to water stress under Mediterranean conditions, may already be present at planting, thus affecting crop establishment and early vigour, and therefore limiting grain yield early in the crop cycle. Moreover, it may be worth selecting for a constitutively low $\delta^{13}C$ (together with lower $\delta^{18}O$ and higher $\delta^{15}N$) when screening for stress tolerance under near-optimal conditions, since it may obviate the need to manage salinity stress, which is a very difficult and costly task in field phenotyping. This study also highlights the importance of N metabolism, and particularly of NR and GS activities, as key components of genotypic performance under water and/or salinity stress. However, selection based on enzymatic activities

may not be feasible for large-scale field phenotyping, whereas it is perhaps realistic using high-throughput phenomic platforms. Further studies would be valuable to assess the performance of these stable isotopes for discerning genotypic variability in grain yield, preferably under field conditions.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Growth parameters, gas exchange, and carbon and oxygen stable isotope composition of two subsets (tolerant and susceptible) of durum wheat genotypes exposed to different levels of salinity, water stress and the combination of the two stresses

Table S2 Nitrogen content, nitrogen stable isotope composition, and glutamine synthetase and nitrate reductase activities of two subsets (tolerant and susceptible) of durum wheat genotypes exposed to different levels of salinity, water stress and the combination of the two stresses

Table S3 Ion concentration in shoots and roots of two subsets (tolerant and susceptible) of durum wheat genotypes exposed to different levels of salinity, water stress and the combination of the two stresses

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Supporting information

Table S1. Growth parameters, gas exchange, and carbon and oxygen stable isotope composition of two subsets (Tolerant and Susceptible) of durum wheat genotypes exposed to different levels of salinity, water stress and the combination of the two stresses. Data shown are the means of two genotypes per subset. Abbreviations and units of variables measured as well as treatments are as defined in Table 1. Probabilities (ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) are shown.

	FI			FI- 12 dSm^{-1}			FI- 17 dSm^{-1}			DI			DI- 12 dSm^{-1}		
	Tolerant	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*p*
Bshoot	54.50	54.20	ns	36.00	16.20	***	17.20	13.10	***	38.00	22.40	***	17.00	11.16	**
LC	43.57	40.96	ns	44.20	42.06	ns	42.10	39.58	ns	49.64	47.62	ns	38.50	44.56	ns
A	17.17	15.14	ns	3.28	1.66	ns	3.33	1.74	ns	6.81	3.33	ns	3.70	1.21	ns
$\mathbf{g_s}$	0.54	0.41	ns	0.24	0.10	ns	0.04	0.04	ns	0.13	0.10	ns	0.04	0.007	ns
C_i/C_a	0.78	0.79	ns	0.41	0.22	**	0.23	0.17	ns	0.39	0.41	ns	0.31	0.26	ns
T	5.76	5.93	ns	2.60	0.43	ns	0.90	0.15	ns	0.77	0.88	ns	0.49	1.57	ns
Fv'/Fm'	0.71	0.70	ns	0.60	0.50	*	0.55	0.50	ns	0.63	0.53	**	0.57	0.53	ns
$\delta^{18}O_{shoot}$	26.65	27.28	ns	27.65	28.34	**	27.81	27.77	ns	27.40	28.56	**	26.86	27.81	ns
$\delta^{13}C_{shoot}$	-31.17	-30.34	*	-29.60	-27.61	***	-28.44	-27.13	***	-29.62	-28.92	*	-29.01	-27.83	**
$\delta^{13}C_{root}$	-30.64	-29.34	**	-29.65	-28.00	***	-29.09	-27.49	***	-29.47	-28.14	**	-29.06	-28.32	**

Table S2. Nitrogen content, nitrogen stable isotope composition, and glutamine synthetase and nitrate reductase activities of two subsets (Tolerant and Susceptible) of durum wheat genotypes exposed to different levels of salinity, water stress and the combination of the two stresses. Data shown are the means of two genotypes per subset. Abbreviations and units of variables measured as well as treatments are as defined in Table 2. Probabilities (ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) are shown.

	FI			FI- 12 dSm^{-1}			FI- 17 dSm^{-1}			DI			DI- 12 dSm^{-1}		
	Tolerant	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*
$\mathbf{N_{Shoot}}$	4.80	5.08	ns	5.13	3.07	***	3.47	2.49	***	4.54	3.36	**	3.43	1.33	***
$\mathbf{N_{Root}}$	2.16	2.13	ns	1.20	1.23	ns	1.08	1.30	ns	2.05	1.94	ns	1.13	1.00	ns
$\mathbf{\delta^{15}N_{Shoot}}$	3.42	2.91	ns	2.24	1.35	***	2.32	1.37	***	3.11	2.50	*	2.52	1.26	*
$\mathbf{\delta^{15}N_{Root}}$	1.95	2.41	ns	3.53	3.26	ns	4.97	4.39	ns	2.84	3.50	ns	4.24	3.61	ns
$\mathbf{GS_{Shoot}}$	582.90	412.12	*	569.41	241.06	***	392.06	232.50	***	527.03	245.50	***	319.66	163.56	**
$\mathbf{NR_{Shoot}}$	4.26	3.50	ns	1.67	0.81	**	1.08	0.52	***	1.87	1.01	**	1.02	0.42	ns

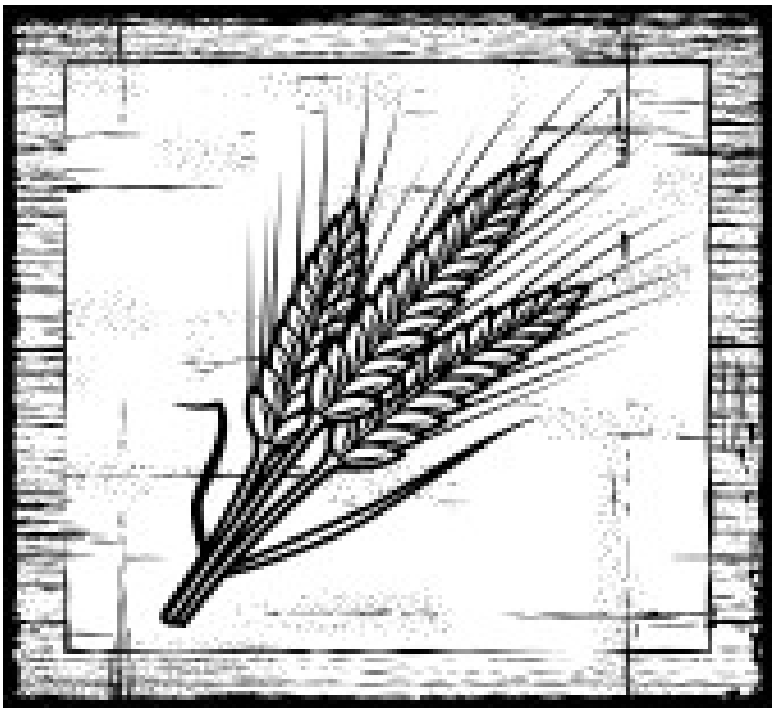
Table S3. Ion concentration in shoots and roots of two subsets (Tolerant and Susceptible) of durum wheat genotypes exposed to different levels of salinity, water stress and the combination of the two stresses. Concentrations are expressed as mmol per g of dry weight. Data shown are the means of two genotypes per subset. Probabilities (ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$) are shown.

	FI			FI- 12 dSm^{-1}			FI- 17 dSm^{-1}			DI			DI- 12 dSm^{-1}		
	Tolerant	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*	**Tolerant**	**Susceptible**	*P*
							Shoot								
Na^+	0.18	0.20	ns	1.58	1.37	ns	1.66	1.50	ns	0.34	0.33	ns	1.03	0.93	ns
K^+	1.51	1.58	ns	0.51	0.53	ns	0.42	0.50	ns	1.08	1.36	**	0.51	0.57	ns
Ca^{2+}	0.11	0.12	ns	0.06	0.06	ns	0.06	0.06	ns	0.10	0.13	ns	0.06	0.05	ns
P	0.25	0.26	ns	0.19	0.20	ns	0.18	0.18	ns	0.18	0.20	ns	0.10	0.10	ns
Mg^{2+}	0.06	0.07	ns	0.06	0.05	ns	0.05	0.05	ns	0.08	0.09	ns	0.06	0.05	ns
K^+/Na^+	8.57	8.00	ns	0.32	0.39	ns	0.26	0.33	*	3.22	4.16	**	0.51	0.64	ns
Ca^{2+}/Na^+	0.58	0.59	ns	0.04	0.05	*	0.04	0.04	ns	0.31	0.38	*	0.06	0.06	ns
							Root								
Na^+	0.14	0.16	ns	0.92	0.87	ns	1.22	1.11	ns	0.34	0.36	ns	0.90	0.82	ns
K^+	0.69	0.75	ns	0.28	0.26	ns	0.36	0.31	ns	0.67	0.76	ns	0.32	0.30	ns
Ca^{2+}	0.22	0.20	ns	0.16	0.16	ns	0.08	0.10	ns	0.37	0.33	ns	0.13	0.13	ns
P	0.24	0.19	ns	0.11	0.12	ns	0.08	0.08	ns	0.17	0.16	ns	0.05	0.04	ns
Mg^{2+}	0.04	0.04	ns	0.03	0.03	ns	0.03	0.03	ns	0.07	0.07	ns	0.04	0.03	ns
K^+/Na^+	4.88	4.61	ns	0.30	0.30	ns	0.30	0.28	ns	1.95	2.16	ns	0.35	0.38	ns
Ca^{2+}/Na^+	1.54	1.25	*	0.18	0.19	ns	0.07	0.10	*	1.16	0.94	ns	0.14	0.16	ns

Capítulo 4

A comparative effect of salinity and drought on growth, ion concentration and $\delta^{13}C$ and $\delta^{15}N$ in barley

Yousfi, S., Serret, M.D., Voltas, J., Araus, J.L. *2010. Aspects of Applied Biology* 105:73-81.



Resumen

La relación entre distintos parámetros fisiológicos y las firmas de los isótopos estables del carbono y el nitrógeno se evaluó en plantas de cebada bajo diferentes combinaciones de salinidad y regimenes de riego. Las plantas fueron cultivadas aproximadamente un mes en macetas dentro de una cámara de cultivo. Se evaluó la combinación de dos regimenes de agua (FI, riego total y DI riego deficitario al 35 % de la capacidad del contenedor) y de tres niveles de salinidad (solución Hoagland con agua desionizada, 12 dS m^{-1} y 17 dS m^{-1} respectivamente), con un total de cinco tratamientos diferentes (DI-17 dS m^{-1} no fue estudiado). Los tratamientos de estrés se establecieron una semana después de la germinación aumentando progresivamente la salinidad y disminuyendo el contenido de agua en las macetas hasta llegar a los niveles propuestos. Se midió el intercambio de gases y una vez cosechadas se midió la biomasa y se analizaron, las concentraciones de iones y de nitrógeno (N) y la composición isotópica del carbono ($\delta^{13}C$) y del nitrógeno ($\delta^{15}N$) de las plantas. Los tratamientos afectaron todos los parámetros estudiados. En comparación con las condiciones del control, DI-12 dS m^{-1} y FI-17 dS m^{-1} fueron los tratamientos en los que la biomasa y la concentración del nitrógeno en hojas y raíces disminuyeron de una manera más acusada, mientras el tratamiento DI (riego deficitario con la solución Hoagland normal) fue el menos afectado. Todos los tratamientos de estrés inhiben la fotosíntesis y la transpiración principalmente a través del cierre estomático. En consecuencia, $\delta^{13}C$ de las hojas y raíces aumentó como respuesta al estrés. Por el contrario $\delta^{15}N$ siguió un patrón diferente, dependiendo de la parte de la planta estudiada: $\delta^{15}N$ en hojas disminuyó en los tratamientos de salinidad mientras que no se modificó como respuesta a DI. En la raíces, $\delta^{15}N$ aumentó en todos los tratamientos de salinidad pero también, aunque en menor medida, en DI. La concentración de iones fue afectada no sólo por los tres tratamientos de salinidad, sino también por DI. Los dos tratamientos de salinidad bajo FI afectaron de una manera muy diferente las concentraciones de Na^+ y K^+, tanto en las hojas como en las raíces, siendo inferiores y superiores respectivamente en FI-17 dS m^{-1} que en FI-12 dS m^{-1}. En general $\delta^{13}C$ y $\delta^{15}N$ de las hojas fueron los parámetros más relacionados con las diferencias de biomasa y

de la concentración del N en los diferentes tratamientos. En consecuencia, $\delta^{13}C$ correlacionaba negativamente y $\delta^{15}N$ positivamente con el crecimiento de las plantas y la concentración del nitrógeno. Este estudio en plantas de cebada apoya la utilidad potencial de la $\delta^{13}C$ y del $\delta^{15}N$ en los tejidos como indicadores del rendimiento de las plantas bajo un amplio rango de condiciones de salinidad y de estrés hídrico.

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Water and nitrogen use efficiency in plants and crops

A comparative effect of salinity and drought on growth, ion concentration and $\delta^{13}C$ and $\delta^{15}N$ in barley

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Abstract

A relationship between various physiological parameters and stable isotope signatures of carbon and nitrogen was evaluated in barley under different combinations of salinity and water regimes. Plants were grown in pots within a growth chamber for about one month. The combination of two water regimes (FI, fully irrigation and DI, deficit irrigation to 35% of pot available water capacity) and three salinity levels (Hoagland solution formulated with deionized water, 12 dS m^{-1} and 17 dS m^{-1} water, respectively) were assayed, accounting for a total of five different treatments (DI-17 dS m^{-1} was not tested). Stress treatments were imposed one week after germination by progressively increasing salinity and decreasing water content for one week. Shoot biomass, ions and nitrogen concentration (N), gas exchange, and stable isotope compositions of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were analysed. Treatments significantly affected all the parameters studied. Compared with control conditions DI-12 dS m^{-1} and FI-17 dS m^{-1} were the treatments in which shoot biomass and shoot and root N concentration decreased the most while DI with normal solution was the least severe treatment. All stress treatments inhibited photosynthesis and transpiration mostly through stomatal closure. Consequently $\delta^{13}C$ of shoots and roots increased as a response to stress. $\delta^{15}N$ followed a different pattern depending on the plant part studied. In shoots $\delta^{15}N$ decreased in salinity treatments and did not change as response to DI. In roots $\delta^{15}N$ increased in all salinity treatments but also to a lesser extent under DI. Concentrations of ions were affected not just by the three different salinity treatments but also by DI. The two salinity treatments under FI were affected in a very different manner, with Na^+ and K^+ concentrations in both shoots and roots being lower and higher, respectively, at FI-17 dS m^{-1} than at FI-12 dS m^{-1}. Overall $\delta^{13}C$ and $\delta^{15}N$ of shoots were the traits best matching the differences in shoot biomass and N concentration across growing treatments. Thus $\delta^{13}C$ correlated negatively and $\delta^{15}N$ positively with plant growth and N concentration. This study supports the potential usefulness in barley of the ^{13}C and ^{15}N signatures in tissues as indicators of plant performance under a wide range water stress and salinity conditions.

Key words: Nitrogen content, $\delta^{13}C$, $\delta^{15}N$, barley, sodium, photosynthesis, salinity, water deficit

Introduction

Barley is one of the most extensively cultivated crops in the Mediterranean regions. This crop is usually placed in marginal areas, where water stress (Araus *et al*., 2002) and N availability (Oweis *et al*., 1999; Passioura, 2002) are the main constraints on productivity. Predicted climate change

will exacerbate the negative effects of such constraints. Deficit irrigation may alleviate drought but at the expense of a progressive salinization, particularly if low quality water is used (Ayers & Westcot, 1989; The World Bank, 2007). Selecting barley genotypes more resistant to drought (Araus *et al*., 2002) or with a higher salt tolerance (Munns, 2008) together with proper agronomical practices are complementary ways of improving present and future adaptation of this crop to the Mediterranean. Numerous studies have focused on the effect of drought or salinity on plant growth and physiological parameters of barley aiming to elucidate traits potentially useful for breeding and/or crop management. However studies comparing the effects of both stresses alone as well as in combination are far less abundant. Moreover some selecting traits such as concentration of ions are specific for salinity but do not apply to drought and may give misleading results when assessing the combined effect of both stresses. In that context integrative (time and organization level) traits (Araus *et al*., 2002, 2008) such as the carbon isotope composition ($\delta^{13}C$; frequently expressed as discrimination, $\Delta^{13}C$) in dry matter have been proposed for large scale assessments. Beside $\delta^{13}C$, nitrogen isotope composition ($\delta^{15}N$) is also affected. Foliar $\Delta^{13}C$ (or $\delta^{13}C$) values have been used in wheat and barley as an integrated measure of the response of photosynthetic gas exchange to salinity and drought (Arslan *et al*., 1999; Rivelli *et al*., 2002, Yousfi *et al*., 2010). In addition, genotypic variability of this trait under salinity has been reported (Ellis *et al*., 1997, 2002; Isla *et al*., 1998; Shaheen & Hood-Nowotny, 2005). Moreover, features related to the nitrogen (N) metabolism, such as the stable nitrogen isotope signature, have been recently proposed as screening tools (Yousfi *et al*., 2009). Natural variation in plant N isotope composition ($\delta^{15}N$) has been proposed as a useful trait for screening, as it is linked to plant N metabolism, even though there is no precise knowledge of the underlying mechanisms (Handley *et al*., 1997; Robinson *et al*., 2000; Ellis *et al*., 2002; Pritchard & Guy, 2005; Coque *et al*., 2006; Yousfi *et al*., 2009). Robinson *et al*. (2000) proposed that the combined measurements of $\delta^{13}C$ and $\delta^{15}N$ may give an indication of responses to stresses such as drought and nitrogen starvation. Moreover, both $\delta^{13}C$ and $\delta^{15}N$ have been used to phenotype the response of (barley) mapping populations to salinity, as the natural abundances of these isotopes are strongly affected by salinity (Ellis *et al*., 1997, 2002; Handley *et al*., 1997). The two stable isotope $\delta^{13}C$ and $\delta^{15}N$ were also evaluated under salinity, drought and the combination of the two stresses in durum wheat genotypes (Yousfi *et al*., 2009, 2010). In this study we compared the response of barley genotype to either deficit irrigation or salinity, or both in combination, imposed at vegetative stage of growth. The main objective was to determine the most informative physiological traits to detect differences between growing conditions. These traits included gas exchange parameters, chlorophyll levels, ion concentration, nitrogen content and the natural abundance of the stable isotopes ^{13}C and ^{15}N.

Material and Methods

The genotype used in this study was a two-row barley (*Hordeum vulagre* L. cv. Hispanic). This cultivar, characterized by a good tillering and precocity habit, has been the most planted in Spain during recent years. Seeds were planted in 3 dm^3 pots containing perlite. Plants were grown in a growth chamber at the Experimental Field Station of the University of Barcelona, for over 1 month. Plants were watered to field capacity to facilitate germination. Then, five different growth conditions were imposed one week after germination: (i) FI or control (fully irrigated, 100% of container capacity, with normal Hoagland solution), (ii) FI-12 dS m^{-1} (fully irrigated with saline Hoagland solution, 12 dS m^{-1}), (iii) FI-17 dS m^{-1} (fully irrigated with saline Hoagland solution, 17 dS m^{-1}), (iv) DI (deficit irrigated to 35% of container capacity, with normal Hoagland solution) and (v) DI-12 dS m^{-1}, the combination of the two stresses: (deficit irrigated to 35% of container capacity with saline Hoagland solution, 12 dS m^{-1}). Water deficit was imposed progressively during about 1 week by decreasing irrigation. Salinity treatment was also imposed by adding NaCl progressively to the nutrient solution, starting with a salt concentration of 4 dS m^{-1} to reach the final salt levels of 12 dS m^{-1} (~ 120 mM NaCl) or 17 dS m^{-1} (~ 170 mM NaCl). Leaf gas exchange was measured in all plants just before harvest. Measurements were made with an open IRGA LI-COR 6400. The

gas exchange parameters were light-saturated net CO_2 assimilation rate (A_{sat}); stomatal conductance (g_s) and transpiration rate (T). Subsequently, the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) was calculated according to Sharkey & Raschke (1981). The efficiency of excitation energy captured by open PSII reaction centres (Fv'/Fm') was also estimated in the same leaves. Leaf chlorophyll content (LC) was measured with a portable device (SPAD). After harvest roots were washed with deionized water and then both roots and shoots dried in an oven and further grounded. Then the amount of ions (Na^+, K^+, $Ca2^+$, Mg^{+2}, P) in shoots and roots was determined with an Inductively Coupled Plasma Emission Spectrometer at the Scientific Facilities of the University of Barcelona as detailed before (Yousfi *et al*., 2009, 2010). Total nitrogen concentration, stable carbon isotope composition ($\delta^{13}C$) and stable nitrogen isotope composition ($\delta^{15}N$) were measured in shoots and roots using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan, Germany), operating in continuous flow mode (Yousfi *et al*., 2009, 2010).

Results

Growing conditions significantly affected all the parameters evaluated in this study (Tables 1, 2, 3). Treatments other than control (FI) significantly decreased aerial biomass as well as the photosynthetic and transpiratory gas exchange traits A_{sat}, g_s, T and C_i/C_a, and the chlorophyll fluorescence ratio Fv'/Fm' (Table 1). The treatment which most affected biomass was the combination of water deficit and salinity (DI-12 dS m^{-1}) while the least affected was the water deficit with normal solution (DI). Deficit irrigation was also the stress treatment which least affected the gas exchange and the chlorophyll fluorescence. Leaf chlorophyll only decreased in the two water stress treatments (DI and DI-12 dS m^{-1}). Therefore gas exchange and chlorophyll-related traits did not fully match the variation in biomass caused by the different treatments (Table 1). Not just the three saline treatments but also DI significantly affected the concentration of different ions in both shoots and roots (Table 2). The amount of Na^+ in FI-12 dS m^{-1} (Table 2) strongly increased as compared with control conditions, whereas concentration of Na^+ in FI-17 dS m^{-1} was in between these two treatments (Table2). All the other ions analyzed in shoots and roots (K^+, Ca^{2+}, Mg^{2+}) decreased in stress conditions, DI included, while P content in the shoot remained almost unchanged. Paradoxically the treatment which less affected the concentrations of all the ions other than Na^+ was FI-17 dS m^{-1} (Table 2) in spite the fact it was one of the most severe treatments preventing biomass accumulation (Table 1).

Table 1. *Biomass, chlorophyll content, gas-exchange and chlorophyll fluorescence parameters of barley exposed to different levels of salinity and water stress. Treatments as defined in Material and Methods.Values shown are the means of four replicates of each treatment. Means followed by different letters were significantly different (*P *< 0.05) by the Tukey test. The* P *values of Anova test for treatments are shown: ns, no significant; *,* P *< 0.05; **,* P *< 0.01; ***,* P *< 0.001*

Treatments	Biomass	LC	A_{sat}	g_s	T	C_i/C_a	Fv'/Fm'
FI	134.33^a	45.20^a	16.33^a	0.39^a	3.07^c	0.85^a	0.64^a
FI-12 dS m^{-1}	27.00^c	44.26^a	1.91^c	0.01^b	0.30^a	0.46^b	0.52^b
FI-17 dS m^{-1}	24.00^c	48.02^a	0.82^c	0.005^c	0.08^a	0.37^b	0.54^b
DI	36.25^b	35.75^b	2.29^b	0.09^b	1.06^b	0.62^a	0.59^a
DI-12 dS m^{-1}	17.50^d	31.95^b	2.89^b	0.02^b	0.44^{ab}	0.39^b	0.50^b
Anova	***	*	***	***	***	*	*

Biomass: shoot biomass (g dry weight); LC, leaf chlorophyll content (SPAD units); A_{sat}, leaf net CO_2 assimilation (μmol CO_2 m^{-2} s^{-1}) ; g_s, stomatal conductance (mol CO_2 m^{-2} s^{-1}); T , transpiration rate (mmol H_2O m^{-2} s^{-1}); C_i/C_a, the ratio of intercellular to ambient CO_2 concentration; Fv'/Fm', efficiency of excitation energy capture by open PSII reaction centres.

Table 2. *Ion concentrations in shoots and roots of barley exposed to different levels of salinity and water stress. Treatments as defined in Material and Methods.The values shown are the means of four replicates of each treatment. Concentrations are expressed as mmol per g of dry weight. Means followed by different letters are different by the Tukey test (*$P < 0.05$*). The* P *values of Anova test for treatments are shown: ns, no significant; *,* $P < 0.05$*; **,* $P < 0.01$*; ***,* $P < 0.001$

	Na^+	K^+	Ca^{2+}	P	Mg^{2+}	K^+/Na^+	Ca^{2+}/Na^+
				Shoot			
Treatments							
FI	0.31^{a}	1.75^{a}	0.12^{a}	0.16^{a}	0.14^{a}	5.65^{a}	0.37^{a}
FI-12 dS m^{-1}	2.02^{d}	0.72^{b}	0.06^{b}	0.15^{a}	0.07^{c}	0.60^{c}	0.05^{c}
FI-17 dS m^{-1}	0.79^{bc}	1.59^{a}	0.11^{a}	0.12^{b}	0.10^{b}	4.12^{b}	0.29^{b}
DI	0.39^{ab}	0.56^{b}	0.05^{b}	0.15^{a}	0.05^{c}	0.31^{c}	0.03^{c}
DI-12 dS m^{-1}	1.21^{c}	0.78^{b}	0.06^{b}	0.09^{c}	0.06^{c}	0.98^{c}	0.08^{c}
Anova	***	***	***	***	***	***	***
				Root			
Treatments							
FI	0.40^{a}	0.34^{a}	0.72^{a}	0.18^{a}	0.10^{a}	0.84^{a}	1.78^{a}
FI-12 dS m^{-1}	1.79^{b}	0.08^{c}	0.10^{c}	0.07^{b}	0.04^{c}	0.04^{c}	0.06^{c}
FI-17 dS m^{-1}	0.69^{a}	0.25^{b}	0.50^{b}	0.08^{b}	0.07^{b}	0.36^{b}	0.76^{b}
DI	0.58^{a}	0.04^{c}	0.08^{c}	0.09^{b}	0.03^{c}	0.03^{c}	0.04^{c}
DI-12 dS m^{-1}	1.78^{b}	0.09^{c}	0.10^{c}	0.04^{c}	0.05^{c}	0.05^{c}	0.06^{c}
Anova	***	***	***	***	***	***	***

All the stress treatments increased $\delta^{13}C$ of shoots and roots compared with FI (Table 3). Increases in shoots followed the same pattern as for the decreases in biomass (Table 1), with DI and DI-12 dS m-1 being the treatments the least and the most affecting $\delta^{13}C$, respectively. By contrast $\delta^{13}C$ from roots was affected in different way, with $\delta^{13}C$ from DI-12 dS m^{-1} (the most stressful treatment) and FI-12 dS m^{-1} being not significantly different from control, while the treatment which most affected $\delta^{13}C$ was FI-17 dS m^{-1}. (Table 3). Roots showed smaller (i.e. closer to control) values of $\delta^{13}C$ compared with shoots for the three saline treatments, while the opposite occurred under DI. All the stress treatments caused a significant decrease in the nitrogen concentration of shoots and roots, with plants under DI being the less affected. The pattern of response to the other three treatments differed considering the plant part, with root N tracking quite well the differences across treatments in shoot biomass. Shoots showed higher N concentration than roots regardless the treatment.

Shoot δ15N also decreased as response to the three different salinity treatments, while DI did not significantly affect this trait (Table 3). By contrast, and compared with control, $\delta^{15}N$ in roots increased as response to all the stress treatments, with DI the least affected and the two fully watering treatments with saline solution (FI-12 dS m^{-1} and FI-17 dS m^{-1}) the most affected. Differences across treatments in shoot $\delta^{15}N$ rather than in root $\delta^{15}N$ matched differences in biomass.

A principal component analysis (PCA) combining all data for the five treatments together was performed (Fig. 1). The two first components explained together almost 80% of the total variance. PCA showed that a higher biomass was associated with a higher photosynthetic and transpirative activities (A_{sat}, g_s, T, Fv'/Fm'), smaller (i.e. more negative) $\delta^{13}C$ in shoots, more N concentration in shoots and roots and higher $\delta^{15}N$ in shoots. However traits such as C_i/C_a, root $\delta^{13}C$ and even less root $\delta^{15}N$ were not related with the differences in shoot biomass. Pooling together all the individual plant measurements of the five treatments (n = 20) shoot $\delta^{13}C$ was strongly and negatively correlated with shoot biomass and N concentration (Fig. 2). Additionally shoot $\delta^{15}N$ was strongly but positively correlated with biomass and N concentration. Beside that, the relationships of both stable

Table 3. *Nitrogen content, stable carbon and nitrogen isotope composition in shoot and root and total shoot nitrogen of barley exposed to salinity and drought. Treatments and abbreviations for traits as defined in Material and Methods.The values shown are the means of four replicates of each treatment. The means followed by different letters were significantly different (*$P < 0.05$*) by the Tukey test. The* P *values of Anova test for treatment are shown: ns, no significant; *,* $P < 0.05$*; **,* $P < 0.01$*; ***,* $P < 0.001$

	Shoot			Root		
	$\delta^{13}C$	N	$\delta^{15}N$	$\delta^{13}C$	N	$\delta^{15}N$
Treatment						
FI	-29.90^{d}	5.13^{a}	3.96^{a}	-28.58^{c}	2.31^{a}	3.08^{c}
FI-12 dS m^{-1}	-27.40^{b}	3.51^{c}	2.22^{b}	-28.10^{bc}	1.63^{b}	7.91^{a}
FI-17 dS m^{-1}	-26.46^{a}	1.57^{e}	1.68^{b}	-26.95^{a}	1.63^{b}	6.03^{a}
DI	-28.46^{c}	4.43^{b}	3.61^{a}	-27.43^{ab}	1.99^{a}	3.85^{b}
DI-12 dS m^{-1}	-26.09^{a}	2.70^{d}	2.23^{b}	-28.27^{c}	1.10^{c}	4.74^{b}
Anova	***	***	***	**	***	**

$\delta^{13}C$, Stable carbon isotope composition (‰); N , nitrogen concentration (mmol g^{-1} dry weight); and $\delta^{15}N$ stable nitrogen isotope composition (‰).

isotopes with N concentration were linear while those with biomass were not. Root $\delta^{13}C$ and $\delta^{15}N$ correlated far weaker (or were not correlated at all) than shoot $\delta^{13}C$ and $\delta^{15}N$ with shoot biomass and N concentration (data not shown).

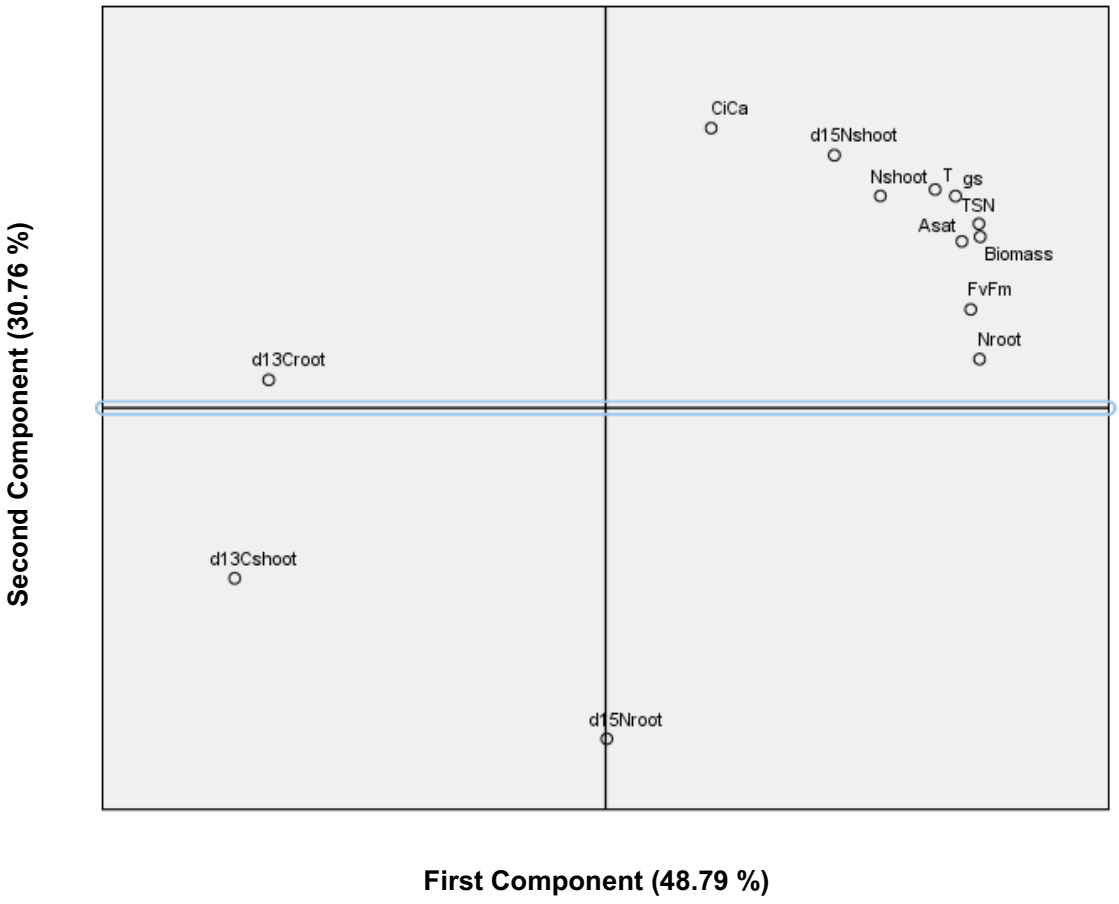

Fig. 1. Principal component analysis (PCA) of different water stress and salinity conditions (as defined in Material and Methods) using as a variables growth, photosynthetic and transpirative parameters and the stable isotope compositions of C and N in shoot and roots. Abbreviations of variables are as defined in the legends of Table 1 and Table 3.

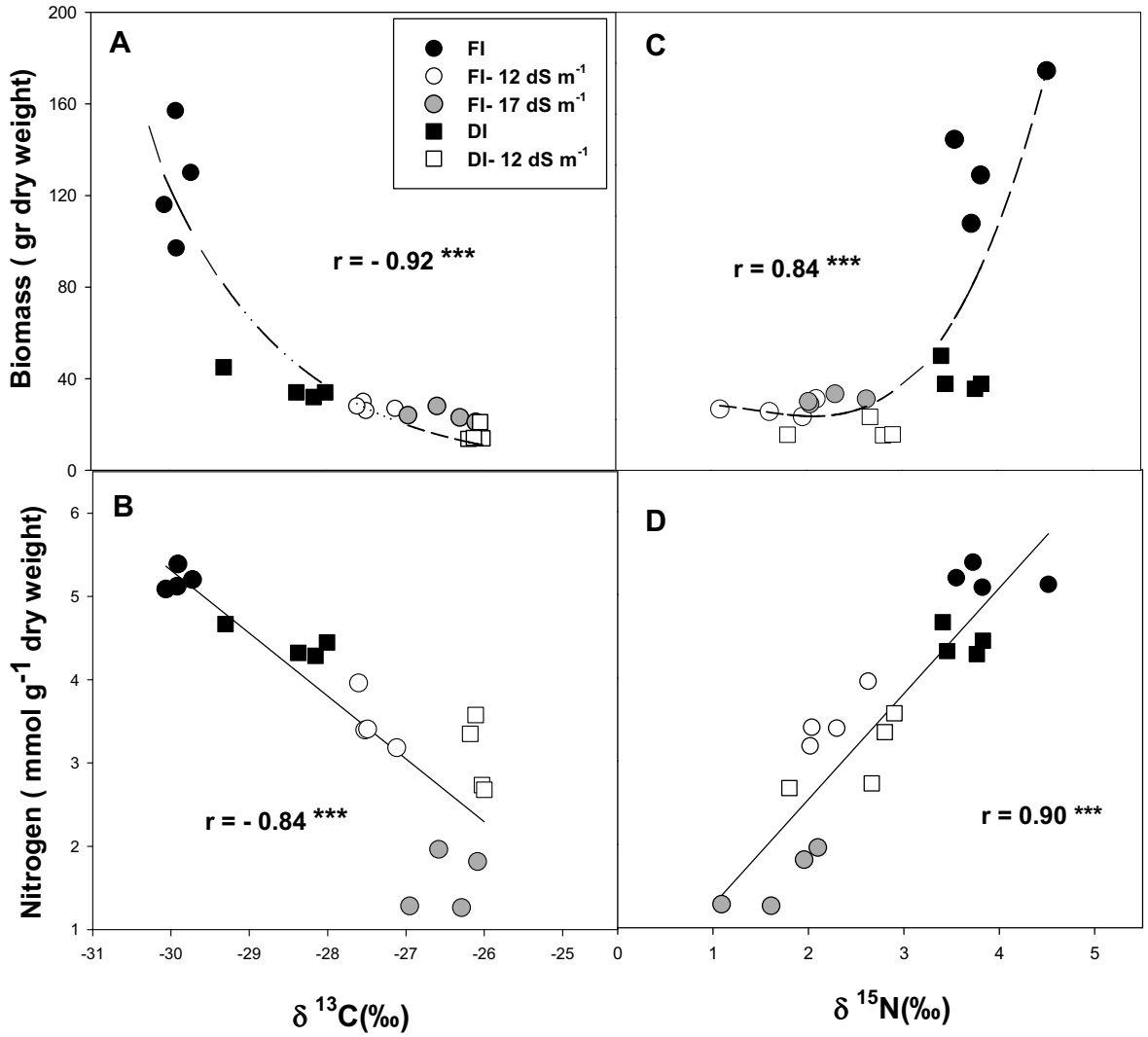

Fig. 2. Relationship of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope composition in shoots with shoot biomass and nitrogen concentration. Each point represents the individual value for a given replicate within a growing condition. Abbreviations of treatments are as defined in the legend of Table 1.

Discussion

The treatment which most affected growth was the combination of deficit irrigation and salinity, which is in line with the results reported in a previous study with durum wheat using the same range of irrigation treatments (Yousfi *et al*., 2010). Barley is a more salt-tolerant crop than other species like bread and durum wheat (Maas & Hoffman, 1977; Munns, 2008). Even so fully irrigation with a moderately saline solution (12 dS m^{-1}) reduced growth by 80% compared with non-stress conditions. Beside that full irrigation with the more saline solution (17 dS m^{-1}) did not affect growth significantly and even the most severe stress (DI-12 dS m^{-1}) only decreased biomass a further 30%. This contrast with our previous studies in durum wheat, where biomass at high salinity was lower than at moderate salinity (Yousfi *et al*., 2009, 2010). It is well known that plants growing in saline environments exclude salts from or tolerate them inside their cells. In our experiment, lower concentration of Na^+ of shoots and roots and higher values of the K^+/Na^+ and Ca^{2+}/Na^+ ratios were found at DI-17 dS m^{-1} compared with DI-12 dS m^{-1}. Some characteristics of barley cell membrane might be involved preventing Na^+ accumulation under high levels of salinity in the medium. Muller & Santarius (1978) suggested that a decrease in the content of galactolipids in membranes is one of the factors causing increased salt tolerance of barley plants exposed to extreme salinity.

Salinity may lead to thicker or denser leaves (i.e. with a more compact mesophyll), as reported in wheat (Passioura & Munns, 2000; Munns & Tester, 2008; Yousfi *et al*., 2009), therefore increasing the chlorophyll content per unit leaf area. This effect may offset eventually the negative effect of salinity decreasing chlorophyll concentration in tissues. By contrast drought stress may have a lesser effect of leaf structure while affecting chlorophyll concentration and therefore a clear decrease in chlorophyll content on area basis was observed in spite the fact N concentration was higher than under salinity. Overall these results indicate chlorophyll content is not a good indicator of plant performance under salinity and again support studies in durum wheat (Yousfi *et al*., 2009).

Salinity and drought stress predominantly affect diffusion of CO in the leaves through a decrease in stomatal conductance (Heuer & Plaut, 1989). Our results showed that both salinity and drought induced a strong decrease in photosynthetic and transpiration rates, mainly due to stomatal limitation. The drop of C_i/C_a together with the increase in $\delta^{13}C$ as response to stress conditions supports a stomatal limitation on photosynthesis and agrees with previous reports on wheat (Ouerghi *et al*., 2000; Shaheen & Hood-Nowotny, 2005; Yousfi *et al*., 2009, 2010). Moreover as for durum wheat salinity alone or combined with deficit irrigation caused a more severe stomatal limitation than water stress (Yousfi *et al*., 2010).

Shoot $\delta^{13}C$ was strongly negatively correlated with biomass and N concentration, while the correlation of root $\delta^{13}C$ with these growth traits was far lower. Differences in assimilate allocation between shoots and roots due to the specific features of water stress and salinity (the last one causes a toxic effect in top of an osmotic-driven water stress) may be behind such differences patter of correlations. Even when this is not supported by literature (Munns *et al*., 2006; Munns & Tester, 2008) we suggest that salinity conditions prevent adequate root growth, while the effect on shoots is comparatively less severe. Consequently assimilates produced after imposing salinity will be mostly allocated in the shoots causing a clear decrease in the $\delta^{13}C$ of shoots compared with roots for all three salinity treatments. For water stress alone the effect may tend toward the opposite. It is well known that under deficit irrigation plants develop roots comparatively more than shoots (Motzo *et al*., 1993), which implies new assimilates (produced already under stress conditions) are preferentially allocated to the growing root system. In agreement with that DI plants showed higher (i.e. less negative) $\delta^{13}C$ in roots than in shoots.

The decrease in N concentration as well as the significant changes in $\delta^{15}N$ in response to different stress treatments suggests that salinity and to a lesser extent water deficit affect nitrogen uptake and/or assimilation (Handley *et al*., 1997; Ellis *et al*., 2002; Yousfi *et al*., 2009). In our study, deficit irrigation reduced N and shoot $\delta^{15}N$ less than salinity which provides evidence that salinity has a stronger effect on N metabolism than water stress. Uptake and accumulation of Na^+ and Cl^- associated with salinity may cause toxicity effects in plants and therefore affect N metabolism more than water stress alone. In a previous study with durum wheat (but tested at different phenological stage) we also found that $\delta^{15}N$ was less affected by water stress alone than due to different saline treatments (Yousfi *et al*., 2010). However other studies on barley and durum wheat report a higher decrease in N as response to deficit irrigation than in the present study (Robinson *et al*., 2000; Raimanová & Haberle, 2010).

Handley *et al*. (1997) suggest that stress would decrease $\delta^{15}N$ compared with controls, due to down-regulation of the assimilating enzyme in this case nitrate reductase. Further, $^{15}N/^{14}N$ fractionation may occur during nitrate assimilation by nitrate reductase or ammonium assimilation by glutamine synthetase (Evans, 2001). Activities of these two enzymes were decreased by salinity (Carillo *et al*., 2005; Wang *et al*., 2007). However in our results only shoot $\delta^{15}N$ decreased as response to stress treatments while the $\delta^{15}N$ in the roots increased. The opposite pattern in the effect of $\delta^{15}N$ between shoots and roots suggests that both salinity and drought affect differentially N metabolism of these two plant parts or that N translocation from roots to shoots may further discriminate against the heavier isotope.

Regardless of the mechanism affecting plant $\delta^{15}N$, this trait seems to reflect the capacity of the plant to use available nitrogen. In accordance with this, our results showed a strong correlation of shoot $\delta^{15}N$ with N concentration and biomass.

We may conclude that the strong correlation of both shoot $\delta^{13}C$ and $\delta^{15}N$ with N concentration and biomass sustain the potential value these isotopic signatures revealing differences in the effect of salinity and water stresses on plant growth.

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Capítulo 5

Comparative response of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in dry matter and the water-soluble fraction of durum wheat exposed to salinity at the vegetative and reproductive stages

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Enviado para su publicación

Genotipos de trigo duro en dos diferentes estados de crecimiento (vegetativo y reproductivo) en el invernadero de los Servicios Experimentales de la Facultad de Biología, Universidad de Barcelona. Años 2006-2007. Foto: S. Yousfi

Resumen

Este estudio pretende comparar las composiciones isotópicas estables del carbono ($\delta^{13}C$), oxígeno ($\delta^{18}O$) y nitrógeno ($\delta^{15}N$) mediante el seguimiento de la respuesta y la variabilidad genotípica de plantas de trigo duro a diferentes condiciones de salinidad. Para ello, se analizaron $\delta^{13}C$, $\delta^{18}O$ y $\delta^{15}N$ en la materia seca (MS) y en la fracción soluble (FS) de hojas de plantas sometidas a distintas condiciones de salinidad, en dos estadios distintos del desarrollo de la planta: en fase vegetativa, poco después de la emergencia de la plántula o durante la floración y el llenado del grano. La $\delta^{13}C$ y la $\delta^{18}O$ de la FS registran las condiciones del cultivo, incluyendo los últimos cambios en las condiciones evapotranspirativas, mientras que $\delta^{15}N$ de la FS parece depender de los diferentes compuestos nitrogenados que contiene cada una de las dos fracciones (MS o FS). Independientemente de la fracción analizada, $\delta^{13}C$ y $\delta^{18}O$ aumentaron y $\delta^{15}N$ disminuyó en respuesta al estrés. Cuando las condiciones de estrés se establecieron inmediatamente después de la germinación, $\delta^{15}N$ seguido de $\delta^{13}C$ se correlacionaron positivamente con las diferencias genotípicas en la biomasa, y la FS fue el mejor elemento para el análisis isotópico que la MS. Por contra, $\delta^{18}O$ correlacionó peor con las diferencias genotípicas en biomasa. Cuando las condiciones de estrés se impusieron durante la etapa reproductiva, las relaciones entre las tres firmas isotópicas y la biomasa fueron significativas y positivas sólo dentro de los tratamientos más severos, con MS siendo el componente más adecuado para el análisis de isótopos.

Comparative response of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in dry matter and the water-soluble fraction of durum wheat exposed to salinity at the vegetative and reproductive stages

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Running title: 'Salinity and $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ in durum wheat'

ABSTRACT

This study aims to compare the performance of the stable isotope compositions of carbon ($\delta^{13}C$), oxygen ($\delta^{18}O$) and nitrogen ($\delta^{15}N$) by tracking plant response and genotypic variability of durum wheat to different salinity conditions. To that end, $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ were analysed in dry matter (*dm*) and the water-soluble fraction (*wsf*) of leaves from plants submitted to salinity, either soon after plant emergence or during flowering and grain filling. The $\delta^{13}C$ and $\delta^{18}O$ of the *wsf* recorded the recent growing conditions, including changes in evaporative conditions, while $\delta^{15}N$ of the *wsf* also seems to depend on the nitrogenous-containing compounds of this fraction. Regardless of the plant part (*dm* or *wsf*), $\delta^{13}C$ and $\delta^{18}O$ increased and $\delta^{15}N$ decreased as response to stress. When the stress conditions were established just after emergence, $\delta^{15}N$ followed by $\delta^{13}C$ correlated positively with genotypic differences in biomass, and *wsf* was the best component for analysis rather than *dm*. By contrast $\delta^{18}O$ performed poorly. When the stress conditions were imposed during the reproductive stage, relationships between the three isotope signatures and biomass were only significant and positive within the most severe treatments, with *dm* being the most adequate component for isotope analysis.

Keywords: $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$, dry matter, durum wheat (*Triticum turgidum* ssp. *Durum*), reproductive stage, salinity, vegetative stage, water-soluble fraction.

INTRODUCTION

Durum wheat is among the most cultivated crops in the south and east Mediterranean basin, with irrigation-induced salinity being a main constraint limiting productivity. This limitation is likely to increase in the future as climatic change is expected to decrease precipitation and increase evapotranspiration in the Mediterranean region (Lobell *et al.* 2008), therefore making cultivation more dependent on irrigation. At the same time competition for water resources among different social and economic sectors is growing, with agriculture being progressively forced to use lower quality water (Araus 2004). In that context, selecting more salt-tolerant genotypes is a way of improving durum wheat performance in the Mediterranean and other dry areas (Munns & Tester 2008).

The identification of new phenotypic traits to assess yield performance and stress adaptation in wheat and other crop plants is a topic of active research in crop breeding (Araus *et al.* 2002, 2008). The use of stable isotope techniques as traits in plant research has grown steadily during the past two decades. This trend will continue as researchers realise that stable isotopes can serve as time-integrated indicators of how plants interact with and respond to their abiotic and biotic environments (Dawson *et al.* 2002). Natural abundances of the stable isotopes of carbon (^{12}C, ^{13}C), oxygen (^{16}O, ^{18}O) and nitrogen (^{14}N, ^{15}N) are achieving greater interest in crop physiology studies. However, to date, only carbon isotopes have been largely investigated as phenotyping tools and proposed for practical applications in crop breeding.

Plants discriminate against the heavier carbon isotope (^{13}C) during photosynthesis and the extent of this discrimination depends on the ratio of intercellular versus external CO_2 concentration (C_i/C_a) in photosynthetic organs (Farquhar, O'Leary & Berry 1982; Farquhar, Ehleringer & Hubick 1989). Therefore carbon isotope composition ($\delta^{13}C$; frequently expressed as discrimination from the source air, $\Delta^{13}C$), when measured in dry matter, provides information on the long-term water-use efficiency of C_3 plants (Farquhar & Richards 1984; Farquhar, Ehleringer & Hubick 1989). Consequently, the carbon isotope signature has been used as a selection trait for high

water-use efficiency in commercial (bread) wheat breeding for water-limited environments (Rebetzke *et al.* 2002; Condon *et al.* 2004). Conditions that induce stomatal closure, such as water deficit or salinity, restrict the CO_2 supply to carboxylation sites, which then increases the $\delta^{13}C$ (or decreases $\Delta^{13}C$) of plant matter (Farquhar, Ehleringer & Hubick 1989). Thus increases in $\delta^{13}C$ have been observed in wheat as a response to water stress (Farquhar & Richards 1984; Araus *et al.* 1997, 2003; Yousfi *et al.* 2010) and salinity (Arslan, Zapata & Kumarasinghe. 1999; Poss *et al.* 2000; Yousfi, Serret & Araus. 2009; Yousfi *et al* 2010, 2012). Moreover genotypic variability for $\delta^{13}C$ in wheat under drought and salinity has also been reported (Shaheen & Hood-Nowotny 2005; Yousfi, Serret & Araus 2009; Yousfi *et al.* 2010).

The oxygen isotope signature (usually expressed as a composition, $\delta^{18}O$) of plant matter integrates the evaporative conditions throughout the crop cycle (Barbour *et al.* 2000) and it is largely unaffected by photosynthesis (Barbour & Farquhar 2000; Farquhar, Cernusak & Barnes 2007). Consequently, $\delta^{18}O$ has been proposed as a proxy for estimating stomatal conductance (g_s), transpiration (Sheshshayee *et al.* 2005; Cernusak, Winter & Turner 2009a; Cabrera-Bosquet *et al.* 2009, 2011) and thus plant water use in different crop species including wheat (Barbour *et al.* 2000; Cabrera-Bosquet *et al.* 2009, 2011). In fact $\delta^{18}O$ depends on environmental factors that affect transpiration, either through water vapour gradients such as air humidity and temperature (Barbour & Farquhar 2000; Barbour *et al.* 2000; Scheidegger *et al.* 2000; Helliker & Ehleringer 2002) or by decreasing g_s via water availability (Yakir, Deniro & Gat 1990; Saurer, Aellen & Siegwolf 1997; Ferrio *et al.* 2007) and salinity (Yousfi *et al.* 2012). Thus $\delta^{18}O$ has been used for assessing the effect of treatments and genotypic variability in yield in wheat (Barbour *et al.* 2000; Ferrio *et al.* 2007; Cabrera-Bosquet *et al.* 2009) under water stress. Moreover by considering concurrent variations in $\delta^{13}C$ and $\delta^{18}O$, it is possible to distinguish between biochemical and stomatal limitations to photosynthesis in response to a change in environmental conditions (Scheidegger *et al.* 2000; Dawson *et al.* 2002).

The natural variation in the plant N isotope signature (again commonly expressed as a composition $\delta^{15}N$), is potentially useful for genotypic screening under drought

(Robinson *et al.* 2000; Ellis, Forster & Gordon 2002) or salinity (Handley *et al.* 1997; Yousfi, Serret & Araus 2009; Yousfi *et al.* 2010) because it is linked to N metabolism (Yousfi *et al.* 2012), even though a complete knowledge of the underlying biochemical mechanisms is still lacking (Cernusak, Winter & Turner 2009b; Tcherkez 2011). Thus discrimination may occur during N uptake, assimilation and redistribution within the plant (Högberg 1997; Robinson, Handley & Scrimgeour 1998). Recycling of nitrogen in the plant associated, for example, with photorespiration can also discriminate ^{15}N (Evans 2001; Werner & Schmidt 2002; Tcherkez 2011). In fact, a change of environmental conditions that impact on metabolic commitments can cause a substantial change in the isotopic content of metabolites. This is the case of photorespiration, the rate of which responds to the intracellular CO_2/O_2 ratio which in turn depends upon stomatal closure: photorespiration tends to cause a relative ^{15}N-enrichment in glutamate and glutamine (Tcherkez 2011), precursors in many of the N metabolic pathways (Werner & Schmidt 2002). However there is no clear trend in the pattern of the response of plant $\delta^{15}N$ to salinity and drought. Thus there are reports indicating that $\delta^{15}N$ may either decrease (Ellis *et al.* 1997; Handley *et al.* 1997; Robinson *et al.* 2000, Yousfi, Serret & Araus 2009; Yousfi *et al.* 2010) or increase (Ellis, Forster & Gordon 2002; Lopes, Nogués & Araus 2004; Lopes & Araus 2006) relative to controls. Moreover, the response may vary depending on the plant part studied (Yousfi *et al.* 2012).

Besides the above considerations, when measured in dry matter either $\delta^{13}C$ or $\delta^{15}N$ (Yousfi, Serret & Araus 2009; Yousfi *et al.* 2010), as well as $\delta^{18}O$ (Yousfi *et al.* 2012), track genotypic performance within given salt stress conditions better than more conventional parameters such as ion concentration or gas exchange measurements. Nevertheless, the combined use of the three isotopes may provide more precise explanation of the physiological mechanisms responsible for the genotypic variability observed with a given stress. Thus in a previous study with durum wheat seedlings, genotypic differences in acclimation responses under different combinations of water regime and salinity treatment were associated with $\delta^{13}C$ (Yousfi *et al.* 2012). However, except for the most severe stress, $\delta^{13}C$ did not have a direct (negative) relationship to biomass, but was mediated through factors affecting

transpiration (and thus $\delta^{18}O$) or N metabolism (therefore $\delta^{15}N$). In fact it was already recognised over a decade ago that development of dual-isotope $\delta^{13}C$:$\delta^{15}N$ analysis approaches has the potential to enhance our understanding of plant ecological and physiological phenomena on all spatial and temporal scales (Robinson *et al.* 2000; Dawson *et al.* 2002) including the genotypic performance to salinity and water stress. However, except in the case of our recent study (Yousfi *et al.* 2012), integration of a third stable isotope like $\delta^{18}O$ has not been attempted previously.

Durum wheat may be exposed to salinity at very different phenological stages, which may affect either the response of the plants and their final yield as well as the performance of phenotyping indicators. Thus a crop may be exposed from the very beginning to salinity if it is planted in saline soils and/or irrigated throughout its life cycle with brackish water. Later exposure to salinity may occur for example if deficit irrigation with brackish water is applied well ahead of the crop cycle with the aim of preventing crop failure during the reproductive stage.

As stated above, stable isotope signatures when analysed in dry matter may integrate in-time the response of a plant to the growing conditions. This is a priori a potentially powerful aspect of isotope application when monitoring long-term plant performance challenged by any stress condition. However, such analyses may perform poorly in tracking variability in genotypic responses to later stages in the crop cycle or subtle changes in growing conditions. In that sense, the analysis of $\delta^{13}C$ and $\delta^{18}O$ in the recently produced assimilates might track the current photosynthetic and transpiratory performance of the plant, respectively (Brandes *et al.* 2007; Gessler *et al.* 2007a; Cabrera *et al.* 2011). In the same sense, the $\delta^{15}N$ of the water soluble fraction may inform on current N metabolism in response to stress conditions. The water-soluble organic matter fraction from leaves reflects the assimilates produced and exported by the photosynthetic organs and consists mainly of sugars but with some organic acids and amino acids (Brandes *et al.* 2006).

However, comparatively few studies have used the 'water-soluble fraction' (*wsf*) for isotope analyses, in spite of the relatively easy protocol for extraction. Except for some

recent reports analysing the $\delta^{13}C$ and $\delta^{18}O$ of the *wsf* (e.g. Gessler *et al.* 2007b; Cabrera *et al.* 2011), most of the studies focus only on the $\delta^{13}C$ of either the *wsf* (Brandes *et al.* 2006; Gessler, Rennenberg & Keitel 2004; Gessler *et al.* 2007a) or a particular component of it, such as sugars and/or starch (Brugnoli *et al.* 1988; Scartazza *et al.* 2004). In the case of $\delta^{18}O$ studies, the focus has been on the use of the whole phloem exudate from tree species (Cernusak *et al.* 2003; Keitel *et al.* 2003; Gessler, Rennenberg & Keitel 2004; Brandes *et al.* 2006, 2007) or the analysis of a purified fraction like cellulose (Barbour *et al.* 2000; Barbour & Farquhar 2000), which requires a time-consuming protocol of extraction and purification. Moreover, such approaches do not represent the recent assimilates. In the case of $\delta^{15}N$, the few reports existing on the analysis of the *wsf* or a specific chemical component of it deal with studies related to phloem transport and $\delta^{13}C$ gradients (Gessler, Rennenberg & Keitel 2004) or the effect of drought (Peuke, Gessler & Rennenberg 2006) in tree species. However the protocol of extracting the *wsf* (or even purifying a specific compound such as soluble sugar or starch) has potential methodological drawbacks that may jeopardise the adequacy of this approach. Thus, for example, the potential exchange of oxygen atoms between assimilates and the water used for extraction (Marino & DeNiro 1987), or the fact that *wsf* represents a pool of diverse components (e.g. not just sugars but also N compounds) that may vary depending on the growing conditions and phenology, may bias this approach.

This study aims to compare the $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ of the shoot dry matter (*dm*) and the *wsf* of durum wheat plants exposed to different saline stresses (i) soon after plant emergence (experiment 1) and (ii) during flowering and grain filling (experiment 2). Our aim was to test in each of the two cases which isotope ($\delta^{13}C$, $\delta^{18}O$ or $\delta^{15}N$) and which method (i.e. from total *dm* or *wsf*) performed better in explaining plant growth and genotypic variability under different salinity treatments. To that end we analysed $\delta^{13}C$ and $\delta^{15}N$ of the *wsf* and the $\delta^{18}O$ of both shoot *dm* and *wsf* from samples of two previous studies (Yousfi, Serret & Araus 2009; Yousfi *et al.* 2010) where the $\delta^{13}C$ and $\delta^{15}N$ of the shoot dry matter were already analysed.

MATERIALS AND METHODS

Plant material and growth conditions: experiment 1

The durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.] genotypes used in this study consisted of two sets of recombinant inbred lines (RILs), here designated as susceptible and tolerant, chosen on the basis of their contrasting shoot biomass among a set of 112 RILs evaluated in a previous study for tolerance to continuous salinity (12 dS m^{-1} and 17 dS m^{-1}) during the first part of the crop cycle (Yousfi, Serret & Araus 2009). The tolerant subset included 10 genotypes with biomasses that were among the top 20 genotypes of the entire population in each of the two salinity levels. The subset of susceptible RILs was made up of another 10 genotypes from the 20 genotypes with the lowest biomass values under the two saline conditions. Whenever possible, both sets of genotypes were also selected to have similar biomass under control conditions. Plants were grown in a hydroponic system within a greenhouse at the experimental fields of the University of Barcelona, Spain. Daily temperature and relative humidity are shown in Supporting Information Fig. S1 and the maximum photosynthetic photon flux density (PPFD) was ~1200 µmol m^{-2} s^{-1}. The experimental design was a randomised complete block with three salinity levels and four replicates per genotype and growing condition. Pots were filled with fine particle (size B6) perlite and separated from the nutrient solution by a mesh. Two seeds were planted in each pot and watered to field capacity to facilitate germination. After a week, only one plant per pot was left. From germination to the 4–5 leaf stage, all plants were grown with a half-strength Hoagland solution (Hoagland & Arnon 1950). Control plants continued to be grown in the same Hoagland solution and no salt was added. Stress treatments were imposed one week after germination. For the two salinity levels, NaCl was added progressively to the nutrient solution, starting with a salt concentration of 4 dSm^{-1}. This concentration was increased 1 week later to the final salt levels of 12 dSm^{-1} (corresponding to ~120mM NaCl) and 17 dSm^{-1} (~170mM NaCl). In total, three different growth conditions were imposed: (i) FI or control (fully irrigated, 100 % of container capacity, with complete Hoagland solution, 1.8 dS m^{-1}); (ii) FI-12 (moderate salinity: fully irrigated with saline Hoagland solution, 12 dS m^{-1}), (iii) FI-17 (severe

salinity: fully irrigated with saline Hoagland solution, 17 dS m^{-1}). Plants were grown for a further 2 months until the end of jointing, when they were harvested.

Plant material and growth conditions: experiment 2

Four durum wheat genotypes were tested, two RILs here termed as RIL47, RIL85 (salt-tolerant) and the two parents (Lahn and Cham). These two RILs are among the most tolerant lines selected from the set of 112 RILs lines of durum wheat evaluated in experiment 1 (Yousfi, Serret & Araus 2009). Plants were grown in a greenhouse at the Experimental Fields of the University of Barcelona, Spain (Yousfi *et al.* 2010). Daily temperature and relative humidity are shown in Supporting Information Fig. S1 and the maximum PPFD was ~1000 µmol m^{-2} s^{-1}. Plants were planted in a mixture of peat, perlite, and vermiculite (2:1:1). Two seeds were planted in 3 dm^3 pots and watered to field capacity to facilitate germination. After a week, only one plant was left per pot. All plants were irrigated with a Hoagland solution and grown in the absence of saline water stress until heading. Then water deficit was imposed progressively over approximately one week by decreasing irrigation. Salinity treatment was also imposed by adding NaCl progressively to the nutrient solution, starting with a salt concentration of 4 dS m^{-1} to reach the final salt levels of 12 dS m^{-1} (~ 120 mM NaCl) or 17 dS m^{-1} (~ 170 mM NaCl). In total, six different growth conditions were imposed: (i) FI or control (fully irrigated, 100 % of container capacity, with complete Hoagland solution, 1.8 dS m^{-1}); (ii) FI-12 (fully irrigated with saline Hoagland solution, 12 dS m^{-1}), (iii) FI-17 (fully irrigated with saline Hoagland solution, 17 dS m^{-1}); (iv) DI (deficit irrigated to 35 % of container capacity, with normal Hoagland solution); (v) DI-12 (deficit irrigated to 35 % of container capacity with saline Hoagland solution, 12 dS m^{-1}) and (vi) DI-17 (deficit irrigated to 35 % of container capacity with saline Hoagland solution, 17 dS m^{-1}). A completely randomised design was used to accommodate the two-way factorial experiment, with genotype and salinity-drought arrangement as main factors. Three single-pot replicates per factorial combination were used, totalling 72 pots. The different treatments were fully established at anthesis and then the plants were grown for 2 weeks when they were harvested. Plants were grown for a total of 4 months.

After harvesting, shoots of the two experiments were oven dried, weighed, and finely ground to extract the water soluble fraction and perform the stable isotopes analyses.

Gas exchange measurements

Leaf gas exchange was measured at the end of treatments in experiment 2. Measurements were made with an open IRGA LI-COR 6400 system (LICOR Inc., Lincoln, NE, USA). For each treatment and genotype, measurements were carried out in three randomly chosen, fully expanded flag leaf blades. The gas exchange parameters measured were light-saturated net CO_2 assimilation rate (A_{sat}), the transpiration rate (T) and the stomatal conductance. Subsequently, the ratio of intercellular to ambient CO_2 concentration (C_i/C_a) was calculated, according to Sharkey & Raschke (1981).

Water-soluble fraction

The protein-free water-soluble fraction (*wsf*) was extracted from shoots as described previously (Nogués *et al.* 2004; Cabrera *et al.* 2011). Briefly, 50 mg of fine shoot powder was suspended with 1 mL of Milli-Q water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany) for 20 minutes at about 5°C. After centrifugation (12.000 g for 5 min at 5°C), the pellet was discarded and the supernatant containing the *wsf* was then collected and heated at 100°C for 3 minutes to precipitate proteins. Samples were then centrifuged again (12.000 g for 5 min at 5°C) in order to separate the denatured proteins from the soluble fraction.

Stable carbon, nitrogen and oxygen isotope signatures

Stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios were measured using an elemental analyser (Flash 1112 EA; ThermoFinnigan, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan, Germany), operating in continuous flow mode. Dry matter samples of about 1 mg and reference materials were weighed into tin capsules, sealed, and then loaded into an automatic sampler (ThermoFinnigan, Germany) prior to EA-IRMS analyses as describe elsewhere (Yousfi, Serret & Araus 2009; Yousfi *et al.* 2010). Concerning the *wsf* and after the last centrifugation, an aliquot of 40 µl of the supernatant containing the protein-free *wsf*

was transferred to tin capsules and dried at 60 °C for carbon and nitrogen isotope analysis. Measurements were carried out at the Scientific Facilities of the University of Barcelona. The $^{13}C/^{12}C$ ratios were expressed in δ notation (Coplen 2008): $\delta^{13}C = (^{13}C/^{12}C)_{sample}/ (^{13}C/^{12}C)_{standard} -1$ (Farquhar, Ehleringer & Hubick 1989), where 'sample' refers to plant material and 'standard' to Pee Dee Belemnite (PDB) calcium carbonate. The same δ notation was used also for the $^{15}N/^{14}N$ ratio ($\delta^{15}N$), but in this case the standard referred to N_2 in air. Atropine was used as a system check in the elemental analyses of nitrogen. International isotope secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose and USGS 40 L-glutamic acid) were used for calibration to a precision of 0.1‰. For nitrogen, isotope secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N_1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate) were used for calibration to a precision of 0.2‰.

The $^{18}O/^{16}O$ ratios were determined in the same shoot dry samples and *wsf* of all plants tested for stable carbon and nitrogen isotopes. Analyses were carried at Iso-Analytical Limited (Crewe, Cheshire CW2 8UY, United Kingdom). Dry shoot samples of about 1 mg and reference materials were weighed into silver capsules, sealed, oven dried at 60 °C for no less than 72 hours to remove moisture and then loaded into an automatic sampler. Concerning the *wsf* a volume of 100 µl from the same supernatant as above was transferred to silver capsules and dried at 60 °C for oxygen isotope analysis. The reference material used for analysis was IAEA-CH-6 (sucrose, $\delta^{18}O_{V\text{-}SMOW}$ = 36.4 ‰). Test samples of IAEA-CH-6 and IAEA-C-3 (cellulose, $\delta^{18}O_{V\text{-}SMOW}$ = 32.2 ‰) and IAEA-601 (benzoic acid, $\delta^{18}O_{V\text{-}SMOW}$ = 23.3 ‰) were measured as quality control checks for calibration to a precision of 0.2 ‰. The IRMS used was a Europa Scientific Geo 20-20 with triple Faraday cup collector array to monitor the masses 28, 29 and 30. IAEA-CH-6, IAEA-C-3 and IAEA-601 are inter-laboratory comparison standards distributed by the International Atomic Energy Agency, for which there are generally agreed $\delta^{18}O$ values.

The $\delta^{18}O$ of the Milli-Q water used for the extraction of the *wsf* was analysed by a Picarro L2120-I Analyzer (Picarro, Inc, Sunnyvale, CA, USA) at the Scientific and Technical Facilities of the University of Lleida (Spain).

Statistical analysis

Data from the set of different stable isotope compositions ($\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$) measured in dry matter and the water-soluble fraction were subjected to factorial analyses of variance (ANOVA) to test the effects of treatment, genotype and their interaction. Means were compared by Tukey's HSD test and were performed using contrasts of tolerant vs. susceptible genotypes in experiment 1 and across the four different genotypes in experiment 2. A bivariate correlation procedure was constructed to analyse the relationship between biomass and the different stable isotopes analysed. To assess the performance of each isotope composition in distinguishing between treatment groups in each of the two experiments, the $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ analysed by the two methods were subjected to UPGMA (Unweighted Pair Group Method with Arithmetic) cluster analysis. Statistical analyses were performed using the SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA). Figures were created using a Sigma-Plot 11.0 program (SPSS Inc.).

RESULTS

Effects of treatment and genotype on stable isotopes

Stress applied from emergence (experiment 1)

Genotype and growing conditions significantly affected the stable isotope composition of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and oxygen ($\delta^{18}O$) from both the total shoot dry matter (*dm*) and the water-soluble fraction (*wsf*) (Table 1). The main effect was associated with the treatment regardless of the isotope and plant fraction analysed, but the relative importance of the genotype effect as well as the error component increased from $\delta^{13}C$ to $\delta^{18}O$ and was in between for $\delta^{15}N$.

The leaf constituent analysed (either *dm* or *wsf*) did not affected the relative importance of genotypic and treatment effects for $\delta^{13}C$ and $\delta^{15}N$, while for $\delta^{18}O$, genotypic and error effects were almost four times higher and the treatment effect decreased by more than half.

Table 1. Genotype and treatment effects on carbon ($\delta^{13}C$), oxygen ($\delta^{18}O$) and nitrogen isotope ($\delta^{15}N$) composition of dry matter (dm) and the water soluble fraction (wsf) of a set of 20 recombinant inbred lines of durum wheat grown under different levels of salinity during the vegetative stage (experiment 1). FI: Full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}: full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dS m^{-1}: full irrigation with Hoagland solution at 17 dS m^{-1}. Treatment values are the means of 80 measurements (20 genotypes and 4 replications per genotype). For simplicity genotypes assayed are displayed in the table clustered in two subsets (tolerant and susceptible) based on their contrasting biomass under salinity stress (Yousfi et al., 2009). Therefore, genotype values are the means of 120 measurements (10 genotypes in each subset, tolerant and susceptible, 3 treatments, and 4 replications per genotype). The associated percentage of the sum of squares and probabilities calculated through the analysis of variance (Anova) is shown for treatments (T), genotype (G) and interaction (GxT) effects. In addition, differences between the tolerant and susceptible subsets of genotypes were tested by another ANOVA and only probabilities are shown. Levels of significance are: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Treatment means followed by different letters are significantly different ($P < 0.05$) according to Tukey's HSD test.

	$\delta^{13}C_{dm}$ (‰)	$\delta^{13}C_{wsf}$ (‰)	$\delta^{18}O_{dm}$ (‰)	$\delta^{18}O_{wsf}$ (‰)	$\delta^{15}N_{dm}$ (‰)	$\delta^{15}N_{wsf}$ (‰)
Genotype						
Tolerant	-27.63	-28.15	29.72	26.58	1.86	4.88
Susceptible	-27.86	-28.45	29.83	26.26	1.39	3.84
	**	**	ns	**	***	***
Treatment						
FI	-30.90 [a]	-31.51 [a]	28.27 [a]	25.96 [a]	3.78 [a]	8.00 [a]
FI- 12 dS m^{-1}	-27.34 [b]	-28.21 [b]	30.17 [b]	26.11 [a]	1.43 [b]	3.52 [b]
FI- 17 dS m^{-1}	-25.08 [c]	-25.17 [c]	30.94 [c]	27.38 [b]	-0.17 [c]	1.35 [c]
Anova						
G	1.53***	1.31***	4.29***	16.71***	3.40***	3.66***
T	87.45***	87.66***	74.98***	29.48***	78.61***	78.81***
GxT	1.35*	1.24 [ns]	5.46**	21.03***	3.30***	1.74 [ns]
Error	2.55	3.27	9.81	32.08	6.87	7.00

The genotype by treatment (G x T) interaction was significant for $\delta^{13}C$ and $\delta^{15}N$ of *dm* ($\delta^{13}C_{dm}$ and $\delta^{15}N_{dm}$) as well as for $\delta^{18}O$ of the two fractions ($\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$), whereas no interaction existed for $\delta^{13}C$ and $\delta^{15}N$ of the *wsf* ($\delta^{13}C_{wsf}$ and $\delta^{15}N_{wsf}$). Rising salinity increased $\delta^{13}C$ and $\delta^{18}O$, whereas it decreased $\delta^{15}N$ regardless of the fraction

analysed. The range of the change between extreme treatments was far higher for $\delta^{13}C$ (about 6 ‰ for both $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$) and $\delta^{15}N$ (4 and 6.5 ‰ for both $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$, respectively) than for $\delta^{18}O$ (2.5 and 1.5 ‰ for $\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$, respectively). Within each treatment, the $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ were higher than $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$, while the opposite occurred for $\delta^{15}N$ (Tables 1, 2). Under control conditions, no differences existed between the two subsets of tolerant and susceptible genotypes for each of the three different isotopes and the two leaf constituents analysed. However, within each of the two stress conditions, susceptible genotypes showed smaller $\delta^{13}C_{dm}$, $\delta^{13}C_{wsf}$, $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ values than the tolerant ones (Table 2), whereas for $\delta^{18}O$, differences were significant only in one case ($\delta^{18}O_{wsf}$ at FI-17).

Stress applied during the reproductive stage (experiment 2)

Genotype and growing conditions significantly affected $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ of both leaf constituents (Table 3). Except for $\delta^{13}C_{dm}$, the main effect was associated with treatment, even when the relative effect of genotype, G x T interaction and the error were, in general, higher than in the first experiment and, at least for the error, relatively similar across isotopes and fractions. The $\delta^{13}C_{wsf}$ exhibited smaller genotypic and larger environmental effects than $\delta^{13}C_{dm}$, while the opposite trend was observed between $\delta^{18}O_{wsf}$ and $\delta^{18}O_{dm}$, and no clear differences were observed between $\delta^{15}N_{wsf}$ and $\delta^{15}N_{dm}$. The G x T interaction was significant for $\delta^{13}C_{dm}$, $\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$. Stress conditions slightly increased $\delta^{13}C_{dm}$ (less than 0.5 ‰ from extreme treatments) but far more than $\delta^{13}C_{wsf}$ (more than 1 ‰). The $\delta^{18}O$ showed a similar pattern as $\delta^{13}C$, increasing with the severity of stress and showing a larger range between extreme treatments for $\delta^{18}O_{wsf}$ (above 3 ‰) compared with $\delta^{18}O_{dm}$ (slightly larger than 1 ‰). Moreover, within each growing condition, $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ values were higher than those of $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$, with differences increasing in the more severe stress conditions. While $\delta^{15}N_{dm}$

Table 2. Effect of growing conditions during the vegetative stage on the different stable isotope compositions analysed in the two durum wheat genotype subsets (experiment 1). Data shown are means of the 10 genotypes of each subset (tolerant and susceptible). Means are significantly different ($P < 0.05$) according to the factorial analysis of variance (ANOVA). Levels of significance are: ns, not significant; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$. Abbreviations for variables and treatments as defined in Table 1.

	FI			FI- 12 dS m^{-1}			FI-17 dS m^{-1}		
	Tolerant	**Susceptible**	***P***	**Tolerant**	**Susceptible**	***P***	**Tolerant**	**Susceptible**	***P***
$\delta^{13}C_{dm}$ (‰)	-30.86	-30.94	ns	-27.18	-27.47	**	-24.23	-25.24	ns
$\delta^{13}C_{wsf}$ (‰)	-31.51	-31.50	ns	-28.07	-28.68	**	-24.87	-25.47	***
$\delta^{18}O_{dm}$ (‰)	28.33	28.20	ns	30.19	30.16	ns	30.85	31.03	ns
$\delta^{18}O_{wsf}$ (‰)	25.92	26.00	ns	26.30	25.94	ns	27.77	26.99	**
$\delta^{15}N_{dm}$ (‰)	3.43	3.26	ns	1.84	1.16	***	0.04	-0.30	***
$\delta^{15}N_{wsf}$ (‰)	8.24	7.75	ns	4.17	2.98	***	1.66	1.04	***

and $\delta^{15}N_{wsf}$ decreased as the severity of stress increased, with a range of about 4 ‰ from extreme treatments, values increased within each growing condition between 3.5 to more than 6 ‰ from $\delta^{15}N_{dm}$ to $\delta^{15}N_{wsf}$ (Table 3; Supporting Information Table S1). Of the two resistant genotypes, only RIL47 showed clear differences compared with the two parents, with $\delta^{13}C_{dm}$ at FI-12 and FI-17 showing lower values and $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ at FI-12 showing higher values. In addition, the two RILs exhibited higher $\delta^{18}O_{wsf}$ than the parents at FI-17 (Supporting Information Table S1).

Table 3. Genotype and treatment effects on carbon, oxygen and nitrogen isotope composition of dry matter and the water soluble fraction of four genotypes of durum wheat grown under different combinations of salinity and water supply during the reproductive stage (experiment 2). Genotypes assayed were the parents (Cham and Lahn) and two of the best performing recombinant inbred lines (RIL47 and RIL85) from the population tested in experiment 1. DI: Deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}: deficit irrigation with Hoagland solution at 12 dS m^{-1}; DI-17 dS m^{-1}: deficit irrigation with Hoagland solution at 17 dS m^{-1}. Additional abbreviations for treatments and variables are as defined in the legend of Table 1. Genotype values are the means of 18 measurements (6 treatments and 3 replications per treatment), while treatment values are the means of 12 measurements (4 genotypes and 3 replications per genotype). Analysis of variance for the same variables is shown for treatments (T), genotype (G) and interaction (GxT) effects. The associated percentages of the sum of squares and probabilities (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) are shown. Treatment means followed by different letters are significantly different ($P < 0.05$) according to Tukey's HSD test.

	$\delta^{13}C_{dm}$ (‰)	$\delta^{13}C_{wsf}$ (‰)	$\delta^{18}O_{dm}$ (‰)	$\delta^{18}O_{wsf}$ (‰)	$\delta^{15}N_{dm}$ (‰)	$\delta^{15}N_{wsf}$ (‰)
Genotype						
Cham	-32.12 ^{b}	-31.00 ^{bc}	30.17 ^{a}	31.07 ^{a}	3.41 ^{a}	8.14 ^{a}
Lahn	-31.93 ^{b}	-30.83 ^{c}	30.82 ^{b}	33.36 ^{c}	5.03 ^{b}	11.06 ^{c}
RIL47	-32.49 ^{a}	-31.48 ^{a}	30.49 ^{ab}	32.54 ^{b}	5.96 ^{c}	10.51 ^{bc}
RIL 85	-31.90 ^{b}	-31.34 ^{ab}	30.72 ^{b}	32.46 ^{b}	5.37 ^{bc}	9.08 ^{ab}
Treatment						
FI	-32.39 ^{a}	-31.72 ^{a}	29.98 ^{a}	30.40 ^{a}	7.44 ^{d}	11.53 ^{b}
FI- 12 dS m^{-1}	-32.25 ^{a}	-30.73 ^{c}	30.64 ^{b}	33.07 ^{b}	4.32 ^{ab}	8.83 ^{a}
FI- 17 dS m^{-1}	-31.83 ^{b}	-30.96 ^{bc}	30.77 ^{bc}	33.77 ^{b}	3.57 ^{ab}	7.12 ^{a}
DI	-32.21 ^{a}	-31.63 ^{a}	30.35 ^{ab}	30.98 ^{a}	6.19 ^{c}	12.39 ^{b}
DI- 12 dS m^{-1}	-32.23 ^{a}	-31.41 ^{ab}	30.36 ^{ab}	32.74 ^{b}	4.83 ^{b}	11.00 ^{b}
DI- 17 dS m^{-1}	-32.00 ^{b}	-30.56 ^{c}	31.16 ^{c}	33.00 ^{b}	3.11 ^{a}	7.49 ^{a}
Anova						
G	23,66***	12,03**	8,61**	18,33***	17,34***	14,73***
T	13,72**	34,70***	20,06***	40,57***	47,24***	43,02***
GxT	25.32**	8.83 ^{ns}	28.96**	20.27***	6.88 ^{ns}	12.92 ^{ns}
Error	33.39	38.73	32.74	20.37	25.45	27.08

Relationships between isotope signatures in *dm* and *wsf*

Stress applied from emergence (experiment 1)

Relationships for each of the three stable isotopes between the isotopic composition in the *wsf* against that of the *dm* are shown in Figure 1. Combining the three growing conditions together, $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$ as well as $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ correlated strongly and positively, while the relationship between $\delta^{18}O_{wsf}$ against $\delta^{18}O_{dm}$ was also positive but far weaker. In the three isotopes the slope of the relationship was lower than one, but the slope for $\delta^{13}C$ was far higher than for $\delta^{15}N$ and $\delta^{18}O$ (0.91, 0.48 and 0.49, respectively). Moreover, the $\delta^{13}C$ relationship showed quite similar $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$ for each plant analysed. For both of the $\delta^{13}C$ and $\delta^{18}O$ relationships, the isotopic compositions in the *dm* and *wsf* became similar as values increased, while the $\delta^{15}N$ relationship showed the opposite trend (Fig. 1).

Within each growing condition the relationships across individual plants for foreach of the three isotope signatures between *dm* and *wsf* were significant at the two stress conditions, with $\delta^{13}C$ and $\delta^{15}N$ showing stronger relationships than $\delta^{18}O$ (Table 4). Under control conditions, the only isotope exhibiting a significant (albeit very weak) relationship between the two shoot components was $\delta^{18}O$.

Combining all three growing conditions, the relationships between $\delta^{13}C_{dm}$ and $\delta^{15}N_{dm}$ and $\delta^{13}C_{wsf}$ and $\delta^{15}N_{wsf}$ were negative and strong (Table 5). Relationships between $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ were positive and relatively high, while the relationship between $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ was also positive but far weaker (Table 5).

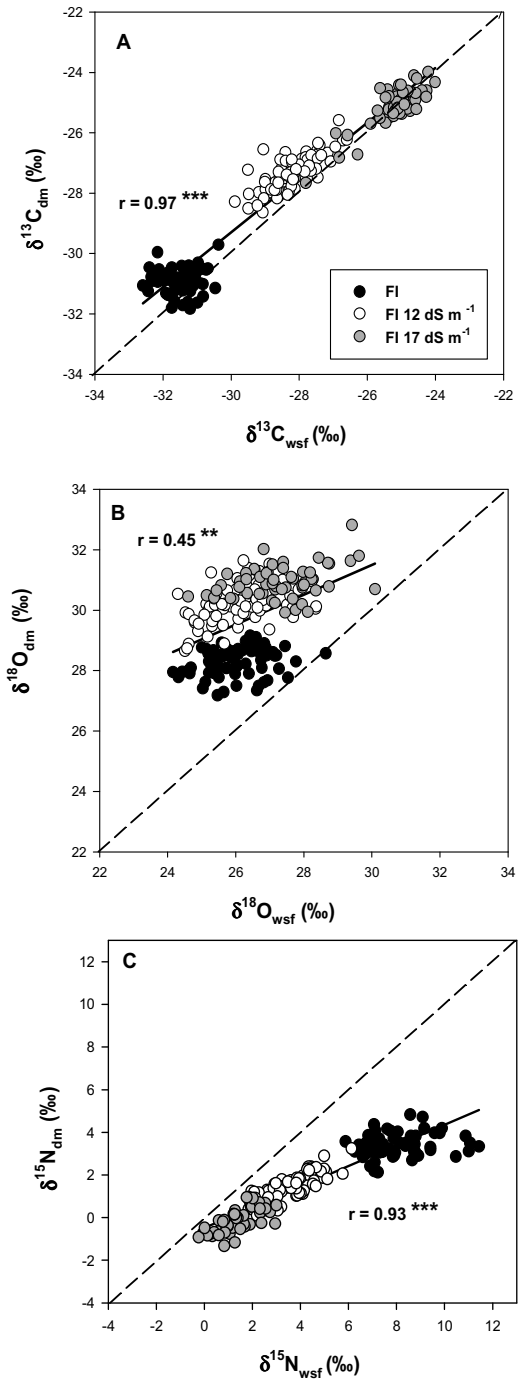

Figure 1. Relationship between (A) carbon isotope composition of the dry matter ($\delta^{13}C_{dm}$) and carbon isotope composition of the water soluble fraction ($\delta^{13}C_{wsf}$), (B) oxygen isotope composition of dry matter ($\delta^{18}O_{dm}$) and oxygen isotope composition of the water soluble fraction ($\delta^{18}O_{wsf}$) and (C) nitrogen isotope composition of dry matter ($\delta^{15}N_{dm}$) and nitrogen isotope composition of the water soluble fraction ($\delta^{15}N_{wsf}$) of plants growing during the vegetative stage under different salinity levels (experiment 1). The three treatments and the twenty genotypes are plotted together. Each point represents the individual value for a given replication and genotype within a growing condition. FI: Full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}: Full irrigation with Hoagland solution at 12 dS m^{-1} and FI-17 dSm^{-1}: full irrigation with Hoagland solution at 17 dS m^{-1}. For each figure the slope 1:1 is represented by a broken diagonal line.

Table 4. Correlation coefficients of the linear regressions between the isotope compositions analysed in dry matter and the water soluble fraction for each of the three stable isotopes and within each of the different growing conditions of experiments 1 (vegetative stage) and 2 (reproductive stage). For experiment 1, twenty genotypes by 4 replications per genotype were used, while for experiment 2, four genotypes and 3 replications per genotype were pooled. Abbreviations of parameters and treatments as in Tables 1 and 3. Probabilities (ns, not significant; *P <0.05; **P <0.01; ***P <0.001) are shown.

	$\delta^{13}C_{dm}$ *vs* $\delta^{13}C_{wsf}$	$\delta^{18}O_{dm}$ *vs* $\delta^{18}O_{wsf}$	$\delta^{15}N_{dm}$ *vs* $\delta^{15}N_{wsf}$
Vegetative stage			
FI	0.083 ^{ns}	0.244 *	0.156 ^{ns}
FI- 12 dS m^{-1}	0.765 ***	0.403 **	0.799 ***
FI-17 dS m^{-1}	0.840 ***	0.354 **	0.719 ***
Reproductive stage			
FI	0.135 ^{ns}	0.057 ^{ns}	0.760**
FI- 12 dS m^{-1}	-0.734 ***	0.673 **	-0.173 ^{ns}
FI-17 dS m^{-1}	-0.269 ^{ns}	0.361 ^{ns}	0.569 *
DI	0.315 ^{ns}	0.814 ***	0.293 ^{ns}
DI- 12 dS m^{-1}	0.041 ^{ns}	0.272 ^{ns}	0.389 ^{ns}
DI-17 dS m^{-1}	-0.300 ^{ns}	0.101 ^{ns}	0.046 ^{ns}

Stress applied during the reproductive stage (experiment 2)

Combining the six growing conditions together, relationships between $\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$ and $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ were significant and positive, while $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$ were not related (Fig. 2). The slopes of the relationships were far smaller than one (0.38 for $\delta^{15}N$ and 0.23 for $\delta^{18}O$, respectively), with differences between isotopic signatures of *dm* and *wsf* growing as values increased (Fig. 2).

Within each growing condition, relationships between $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$ were only significant (albeit negative) at FI-12, while relationships between $\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$ were only significant at FI-12 and DI, as were those between $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ at FI and FI-17 (Table 4). Combining all the three growing conditions the relationships between $\delta^{13}C$ and $\delta^{15}N$ were negative and weak between $\delta^{13}C_{dm}$ and $\delta^{15}N_{dm}$ but higher between $\delta^{13}C_{wsf}$ and $\delta^{15}N_{wsf}$ (Table 5). The relationships between $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ and $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ were both positive but weak.

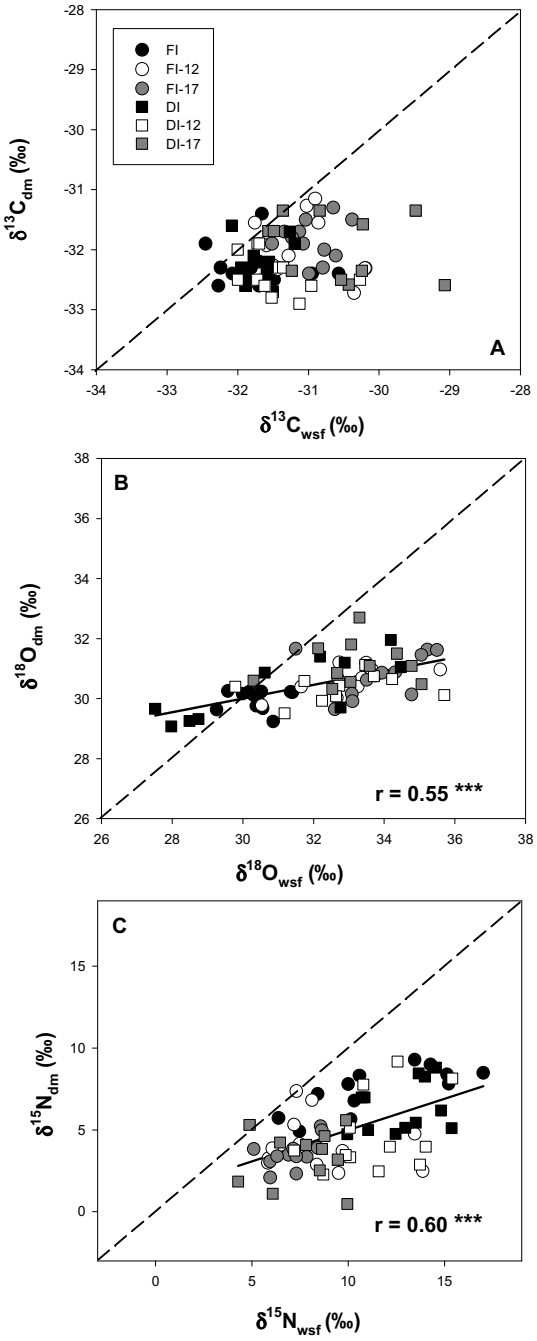

Figure 2. Relationship between (A) carbon isotope composition of the dry matter ($\delta^{13}C_{dm}$) and carbon isotope composition of the water soluble fraction ($\delta^{13}C_{wsf}$), (B) oxygen isotope composition of the dry matter ($\delta^{18}O_{dm}$) and oxygen isotope composition of the water soluble fraction ($\delta^{18}O_{wsf}$) and (C) nitrogen isotope composition of the dry matter ($\delta^{15}N_{dm}$) and nitrogen isotope composition of the water soluble fraction ($\delta^{15}N_{wsf}$) of plants growing under different combinations of salinity and irrigation levels during the reproductive stage (experiment 2). The six treatments and the four genotypes are plotted together. Each point represents the individual value for a given replication and genotype within a growing condition. DI: deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m^{-1}: deficit irrigation with Hoagland solution at 12 dS m^{-1} and DI-17 dS m^{-1}: deficit irrigation with Hoagland solution at 17 dS m^{-1}. The remaining abbreviations for treatments are defined in the legend of Fig. 1. For each figure the slope 1:1 is represented by a broken diagonal line.

Table 5. Correlation coefficients of the linear regressions across treatments and genotypes between carbon isotope composition of the dry matter versus oxygen and nitrogen isotope composition of the dry matter and carbon isotope composition of the water soluble fraction versus oxygen and nitrogen isotope composition of the water soluble fraction. For experiment 1, 240 points (20 genotypes x 4 replications per genotype x 3 treatments) and for experiment 2, 72 points (4 genotypes x 3 replications per genotype x 6 treatments) were pooled. Abbreviations for variables as defined in Table 1. Levels of significance: **, $P < 0.01$; ***, $P < 0.001$.

	Experiment 1 (vegetative stage)	**Experiment 2 (reproductive stage)**
$\delta^{13}C_{dm}$ (‰) *vs* $\delta^{18}O_{dm}$ (‰)	0.85***	0.30**
$\delta^{13}C_{wsf}$ (‰) *vs* $\delta^{18}O_{wsf}$ (‰)	0.46**	0.27**
$\delta^{13}C_{dm}$ (‰) *vs* $\delta^{15}N_{dm}$ (‰)	-0.88***	-0.22**
$\delta^{13}C_{wsf}$ (‰) *vs* $\delta^{15}N_{wsf}$ (‰)	-0.91***	-0.68**

Comparative evaluation of the isotopes dissecting genotypic performance and growing conditions

Stress applied from emergence (experiment 1)

The relationships between the different isotope signatures and biomass across genotypes within each growing condition were studied (Fig. 3). The isotope signature best correlated with biomass was $\delta^{15}N$, with $\delta^{15}N_{wsf}$ performing better than $\delta^{15}N_{dm}$. Thus $\delta^{15}N_{dm}$ correlated positively with biomass at FI and FI-12, while $\delta^{15}N_{wsf}$ also correlated positively with biomass, but within each of the three growing conditions. Carbon isotope composition performed less well with $\delta^{13}C_{dm}$ correlating positively with biomass only at FI-12, while $\delta^{13}C_{wsf}$ was positively correlated at FI-12 and FI-17. Oxygen isotope composition performed the worst with only $\delta^{18}O_{dm}$ correlating negatively with biomass at FI-17. The cluster analysis clearly showed that $\delta^{13}C$ was the best isotope in terms of separating growing conditions, regardless of the genotype considered (Fig. 4).

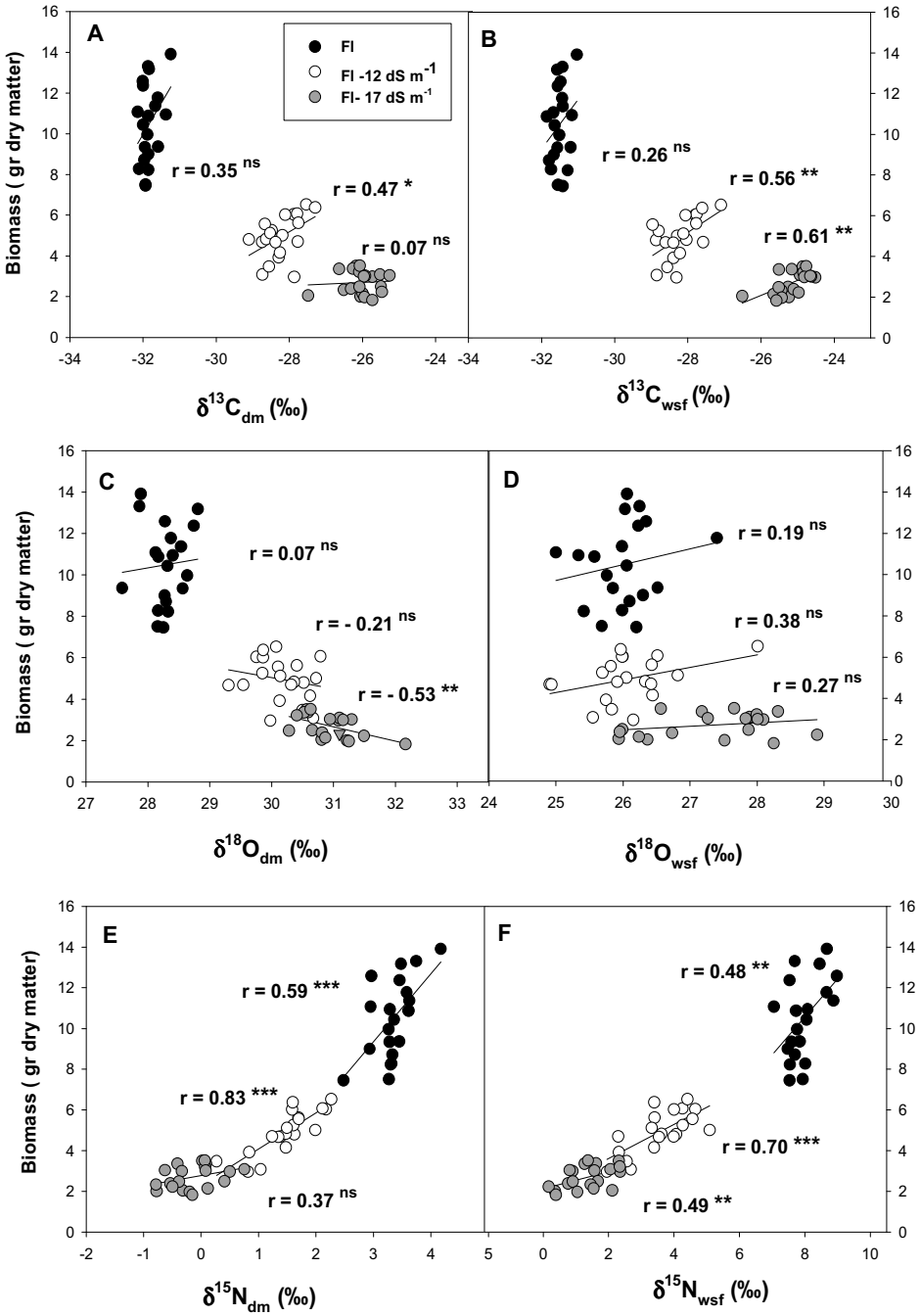

Figure 3. Relationship of shoot biomass with (A) carbon isotope composition of the dry matter ($\delta^{13}C_{dm}$), (B) carbon isotope composition of the water soluble fraction ($\delta^{13}C_{wsf}$), (C) oxygen isotope composition of the dry matter ($\delta^{18}O_{dm}$), (D) oxygen isotope composition of the water soluble fraction ($\delta^{18}O_{wsf}$), (E) nitrogen isotope composition of the dry matter ($\delta^{15}N_{dm}$) and (F) nitrogen isotope composition of the water soluble fraction ($\delta^{15}N_{wsf}$) of plants growing during the vegetative stage under different salinity levels (experiment 1). For each growing condition the twenty genotypes are plotted together. Each point represents the mean value for the four replications per genotype within a growing condition. Abbreviations for treatments are as in the legend of Fig. 1.

Despite both $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$ performing similarly in terms of the incorrect placement of genotypes, with only one among the 20 genotypes being incorrectly assigned, the correct placement of the three treatments (from FI – FI-12 – F-17) was only achieved with $\delta^{13}C_{dm}$. Nitrogen isotope composition performed less efficiently but was still separated in a quite clear manner between treatments, particularly $\delta^{15}N_{wsf}$ (Supporting Information Fig. S2). Oxygen isotope composition was again the worst performing isotope, particularly $\delta^{18}O_{wsf}$, while $\delta^{18}O_{dm}$ still clearly separated control from stress treatments (Supporting Information Fig. S3).

Stress applied during the reproductive stage (experiment 2)

The different stable signatures only correlated with biomass at the most severe stress conditions (FI-17 and DI-17) with isotopic signatures from *dm* performing somewhat better than those from *wsf*. Thus $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ correlated positively with both FI-17 and DI-17 while $\delta^{15}N_{dm}$ correlated positively with DI-17 (Supporting Information Table S2). Except for a positive relationship between $\delta^{18}O_{wsf}$ and biomass at DI-17, no relationships were found between any isotope composition in the *wsf* and biomass. The cluster analysis did not separate adequately across treatments, regardless of the isotope and the fraction assayed, even if only the treatments with the same irrigation regime (i.e. full irrigation or deficit irrigation) were considered (data not shown).

DISCUSSION

Effect of growing conditions on $\delta^{13}C$ and $\delta^{18}O$

Implementation of stress conditions increased both $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$. This agrees with the widely reported effect of water stress (Farquhar & Richards 1984) and salinity (Isla, Aragues & Royo 1998; Yousfi *et al.* 2010) on $\delta^{13}C$ mediated through a stomatal closure and a subsequent decrease in the C_i/C_a ratio (Farquhar, O'Leary & Berry 1982). However, increases in $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$ from control to the most severe stress were far higher in the first experiment, where stress conditions were already established very early during the plant cycle. In the second experiment, where stresses were imposed after heading, the range in $\delta^{13}C_{dm}$ across treatments was very low and only attained significance between control and the two most severe treatments. The range

in $\delta^{13}C_{wsf}$ across treatments in the second experiment was somewhat higher than in $\delta^{13}C_{dm}$, but far smaller than for the first experiment, which suggests that some of the carbon of the *wsf* was assimilated before the stress was imposed. Both $\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$ also increased in the two experiments in response to stress, which agrees with the effect of stress limiting g_s and transpiration (Barbour & Farquhar 2000; Farquhar, Cernusak & Barnes 2007; Cabrera-Bosquet *et al.* 2009, 2011; Yousfi *et al.* 2012). As for the $\delta^{13}C$, the range of $\delta^{18}O_{dm}$ between extreme treatments was far smaller in the second experiment compared with the first, but the opposite was observed for $\delta^{18}O_{wsf}$. In the second experiment, the far lower range in $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ compared with the first experiment and its much higher range of $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ of values agreed with the different duration and time (during the plant cycle) when the treatments were imposed.

The assessment of whether variation in $\delta^{13}C$ is the result of changes in intrinsic photosynthetic capacity (*A*) or g_s remains challenging (Scheidegger *et al*. 2000; Farquhar, Cernusak & Barnes 2007). To separate the independent effects of *A* and g_s on C_i/C_a, Scheidegger *et al.* (2000) have proposed measuring both $\delta^{13}C$ and $\delta^{18}O$ in leaf organic matter. Whereas $\delta^{13}C$ reflects C_i/C_a, $\delta^{18}O$ generally varies with ambient humidity, which in turn reflects changes in g_s and water use and (Ball, Woodrow & Berry 1987; Grantz 1990; Mott & Parkhurst 1991, Monteith 1995). In support of the stomatal effect of stress conditions increasing $\delta^{13}C$, the relationship across treatments between $\delta^{13}C$ and $\delta^{18}O$ in the first experiment was positive and strong (Scheidegger *et al.* 2000; Dawson *et al.* 2002). However, this relationship for the second experiment, even if positive and significant, was far weaker. This suggests that besides a decrease in the g_s, $\delta^{13}C$ may also be affected by a decrease in the intrinsic photosynthetic capacity mediated through an accelerated senescence of photosynthetic tissues, since the stress was imposed during the last past of the crop cycle.

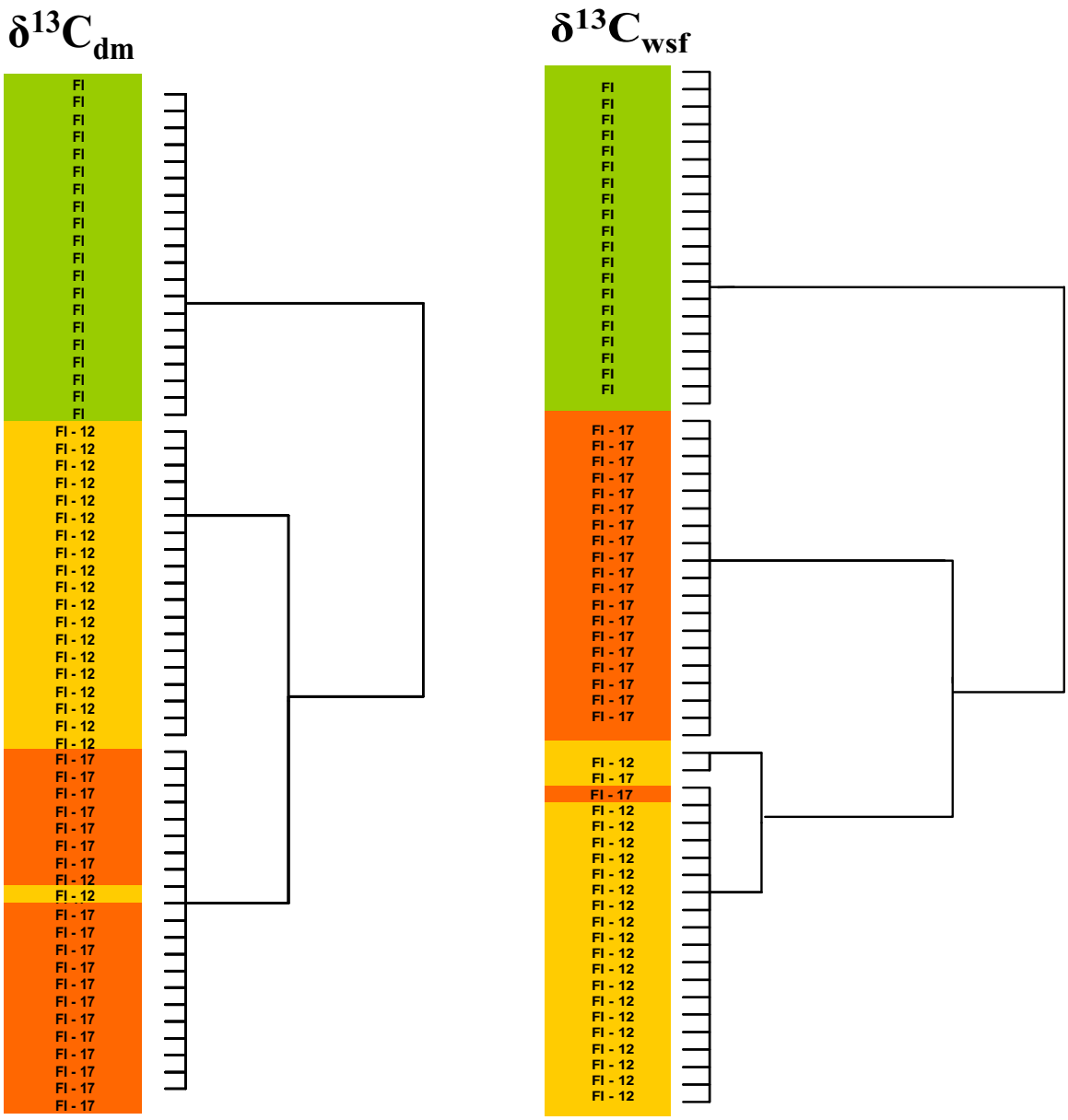

Figure 4. Cluster analysis of the three growing conditions assayed in experiment 1, using as variables (A) carbon isotope composition of dry matter ($\delta^{13}C_{dm}$) and (B) carbon isotope composition of the water soluble fraction ($\delta^{13}C_{wsf}$). Abbreviations for treatments are as in the legend of Fig. 1.

Effect of growing conditions on $\delta^{15}N$

The $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ decreased in response to stress in both experiments. A decrease in $\delta^{15}N_{dm}$ has been reported in durum wheat shoots as a response to salinity and water stress (Yousfi, Serret & Araus 2009; Yousfi *et al.* 2010). However, Peuke, Gessler & Rennenberg (2006) reported for beech that both the $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ of leaves were not affected by drought stress in spite of the fact that $\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$ clearly increased. Salinity and water stress, besides inducing stomatal closure and therefore increasing $\delta^{13}C$, also affected processes related to N uptake, assimilation, release and internal recycling, consequently affecting plant $\delta^{15}N$ (Cernusak, Winter & Turner 2009b; see Yousfi *et al.* 2012 and references herein). This would explain the strong negative relationships between $\delta^{13}C_{wsf}$ and $\delta^{15}N_{wsf}$ across treatments. It has been proposed that variability in the $\delta^{15}N$ of plants might originate from subtle changes in metabolic fluxes or environment-driven effects, such as stomatal closure, which in turn change the Rubisco-catalysed oxygenation rate (Tcherkez & Hodges 2008; Tcherkez 2011). In that sense, photorespiration tends to cause a relative ^{15}N-enrichment in N-metabolism precursors (such as glutamic acid and glutamine) and therefore positive relationships between $\delta^{15}N$ and transpiration efficiency have been reported (Cernusak, Winter & Turner 2009b). However in our study, as well as in previous studies (Yousfi *et al.* 2012) relationships between $\delta^{13}C$ and $\delta^{15}N$ were negative, which means that transpiration efficiency is negatively related to $\delta^{15}N$. This suggests that alternative mechanisms responsible for the bulk $\delta^{15}N$ of the leaf might be involved. Thus, the $\delta^{15}N$ in leaf metabolites is also predicted to depend on the reduced N-input (Tcherkez 2011). Typically, glutamic acid and glutamine are expected to be more ^{15}N-enriched when the N input is proportionally lower. Such a situation is believed to occur, for example, in the absence of abiotic stresses (such as salinity or water stress). This would be associated with a low balance of N availability versus uptake and assimilation by the plant (Mariotti *et al.* 1982; Vitousek *et al*. 1989; Handley *et al.* 1997), propitiated by the high activities of key enzymes N metabolism like nitrate reductase and glutamine synthase (Evans 2001; Yousfi *et al.* 2012). In addition, other fractionation processes may occur during the export of nitrogen-containing compounds from the roots to the shoot (Kronzucker *et al.* 1998), nitrogen recycling, including photorespiration, or an increase in the loss of ammonia and nitrous

oxide due to a higher g_s (Farquhar *et al.* 1980; Smart & Bloom 2001). In any case, the cluster analysis using $\delta^{15}N_{dm}$ or $\delta^{15}N_{wsf}$ clearly separated the growing conditions of the first experiment, reflecting that this isotope signature is consistently affected by salinity and water stress conditions (Yousfi *et al.* 2012). Again, as for the other two isotopes, $\delta^{15}N$ failed to separate treatments in the second experiment. The relationships of $\delta^{13}C$ with $\delta^{18}O$ and $\delta^{15}N$ in the second experiment followed the same pattern as in the first but were far weaker, reflecting the fact that stress conditions were only imposed in the last part of the crop cycle.

$\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ reflect the environmental conditions prior to sampling

While in the first experiment $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ decreased compared with the $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ values, the opposite trend occurred in the second experiment. This contrasting pattern between the two experiments is in agreement with the opposing trends in evaporative conditions of these experiments during the days prior to harvesting, compared with the rest of the growing period. Thus, in the first experiment there was a decrease in temperature together with an increase in relative humidity, while in the second experiment the trend was opposite to this (Supporting Information Fig. S1). A reduction in the evaporative demand of the atmosphere will decrease the $\delta^{18}O$ of the current photoassimilates (Barbour & Farquhar 2000; Farquhar, Cernusak & Barnes 2007) and may contribute to keeping the stomata more open, therefore decreasing $\delta^{13}C_{wsf}$ (Farquhar, Ehleringer & Hubick 1989; Körner, Farquhar & Wong 1991). Moreover, absolute differences between *dm* and *wsf* were more evident for $\delta^{18}O$ than for $\delta^{13}C$, which agrees with the fact that $\delta^{18}O$ directly reflects the differences in transpiration, while the effect of evaporative conditions on $\delta^{13}C_{dm}$ is more indirect, being mediated through the influence of stomatal opening on *Ci/Ca* (Farquhar, Ehleringer & Hubick 1989; Farquhar, Cernusak & Barnes 2007).

In addition, the fact that in the second experiment stress was only imposed late during the plant cycle supports the observation that both the $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ were generally higher than the $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ of the same plants. Moreover, the relationships within each stress condition between *dm* and *wsf* signatures of each isotope were strong in the first experiment, which agrees with the fact stresses were

imposed early in the plant cycle. However, as expected, the second experiment exhibited few relationships within each growing condition between *dm* and *wsf* signatures of each isotope, probably because the stress treatments were shorter and imposed late during the plant cycle. Nevertheless, the better correlation of the gas exchange traits measured at the end of treatments with $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ in comparison to $\delta^{13}C_{dm}$ and $\delta^{18}O_{dm}$ (Supporting Information Fig. S4) supports the concept that the isotope signatures of the *wsf* better reflect the actual photosynthetic and transpirative conditions of the plant.

Overall, the above evidence supports the fact that both $\delta^{13}C_{wsf}$ and $\delta^{18}O_{wsf}$ record actual growing conditions. Thus the $\delta^{13}C_{wsf}$, representing newly produced assimilates (Brandes *et al.* 2007), consistently recorded the photosynthetic and transpirative conditions at the time that photoassimilate gas exchange measurements were performed (Cabrera *et al.* 2011). In the same sense as for the $\delta^{13}C_{dm}$, the leaf dry matter $\delta^{18}O$ integrates not only the evaporative conditions of the leaf when the gas exchange measurements were performed but also the whole evaporative period of the leaf (Cabrera *et al.* 2011). Newly assimilated organic matter (i.e. *wsf*) is assumed to carry not just the signature of the leaf water at the time when it was produced (Gessler *et al.* 2007b), but also to reflect the evaporative leaf water enrichment that existed in the leaves when gas photoassimilates were produced (Cabrera *et al.* 2011). In addition, a higher $\delta^{18}O_{wsf}$ compared with $\delta^{18}O_{dm}$ may also reflect the fact that leaf tissue contains secondary compounds that are often depleted in ^{18}O (Barbour 2007). However in our study, differences between the $\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$ of control plants did not support such a possibility. On the other hand, there is a risk that some degree of exchange of oxygen atoms between the solvent (water) and the assimilates occurs during the process of extracting the *wsf* (Marino & DeNiro 1987) However, while the $\delta^{18}O$ signature of the water used to extract the *wsf* was -6.5 ‰, much lower than that of the plant *dm* or *wsf*, in the second experiment the $\delta^{18}O_{wsf}$ was higher than $\delta^{18}O_{dm}$, which suggests that effects of the oxygen isotope signature of the water are minor.

The differences between $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ reflect the different N fraction analysed

The $\delta^{15}N_{wsf}$ also recorded recent growing conditions as inferred from the fact that correlations in both experiments between $\delta^{13}C_{wsf}$ and $\delta^{15}N_{wsf}$ were stronger than between $\delta^{13}C_{dm}$ and $\delta^{15}N_{dm}$. However $\delta^{15}N_{wsf}$ was consistently higher than $\delta^{15}N_{dm}$, regardless of the experiments and growing conditions, which suggests that differences in nitrogen containing compounds between *dm* and the *wsf* are involved. Proteins are generally ^{15}N-enriched relative to the $\delta^{15}N$ value of the total biomass (Werner & Schmidt 2002). However in our study, while precipitation of proteins should decrease the bulk $\delta^{15}N$ of the remaining fraction, the $\delta^{15}N_{wsf}$ (from which proteins were precipitated) was higher than $\delta^{15}N_{dm}$. Alternatively, secondary metabolism may be associated with specific isotope effects (Werner & Schmidt 2002). Thus aromatic and heteroaromatic compounds, which receive defined N-atoms directly from the amide-N of glutamine (Purich 1998; Zalkin & Smith 1998), are in general ^{15}N-enriched, but other products like chlorophyll, lipids, amino sugars and alkaloids are depleted in ^{15}N. The higher $\delta^{15}N$ of the N compounds in the *wsf* would be a consequence of the specific metabolic pathway that synthesises them (Werner & Schmidt 2002), together with photorespiration, which tends to cause a relative ^{15}N-enrichment in N-metabolism precursors (Tcherkez 2011).

Differences between $\delta^{15}N_{wsf}$ and $\delta^{15}N_{dm}$, were the smallest under the most severe stress and the highest under control conditions. This suggests that factors accounting for such differences change depending on the level of stress. This effect may be sustained by the specific nitrogen-containing compounds present in the *wsf* and their change in response to growing conditions. For example, a number of nitrogen-containing compounds accumulate in plants exposed to salinity (Munns & Tester 2008). These include amino acids, amides, imino acids, quaternary ammonium compounds and polyamides extracted in water (Mansour 2000) compared with the total nitrogen pool in the *dm*, which was mostly accounted by protein enzymes such as RuBisCO, structural proteins (Masclaux-Daubresse *et al.* 2010) and specific stress proteins (Mansour 2000). The soluble fraction may change dramatically as a result of growing conditions, since, for example, it is related to the increase in osmotic compatible compounds generated in response to stress (Munns & Tester 2008). In fact, the

percentage of N in *wsf* varied strongly (between 3-10%, data not shown) between treatments, being the highest for both experiments in the intermediate stress conditions.

A higher $\delta^{15}N_{dm}$ in the better conditions may be associated with a higher protein content (Werner & Schmidt 2002) and in fact, positive relationships across treatments between $\delta^{15}N_{dm}$ and shoot nitrogen content have been reported (Yousfi *et al.* 2010, 2012). However, the larger differences between $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$ under control compared with stress conditions suggest that water-soluble nitrogen-containing compounds other than proteins are contributing to the higher $\delta^{15}N_{dm}$ of plants in the absence of stress.

Comparative performance of isotopic signatures on dm and wsf identifying genotypic adaptation to stress

Under the long-term stress conditions of the first experiment, the $\delta^{13}C_{dm}$ was higher in the tolerant than the susceptible genotypes and plant biomass was positively correlated with $\delta^{13}C_{dm}$ under the intermediate stress (Yousfi, Serret & Araus 2009). However, our study showed that $\delta^{13}C_{wsf}$ performed better than $\delta^{13}C_{dm}$ in the sense that it separated tolerant from susceptible genotypes and correlated positively with biomass at the two stress conditions assayed. These results suggest an acclimation mechanism in tolerant genotypes consisting of stomatal closing, which may prevent water loss, together with salt uptake driven by the transpirative stream, together with a delayed senescence and thus a higher intrinsic photosynthetic performance than the susceptible genotypes. In agreement with lower g_s in the tolerant genotypes, differences in $\delta^{18}O_{wsf}$ between tolerant and susceptible genotypes were observed under severe stress with a similar trend under moderate salinity, with tolerant genotypes showing higher values. In support to the above possibility, Yousfi, Serret & Araus (2009) reported higher N content and K^+/Na^+ ratios in the subset of tolerant genotypes. However, $\delta^{18}O_{wsf}$ did not correlate with biomass under any growing conditions, and even the $\delta^{18}O_{dm}$ correlated negatively with biomass in the most severe treatment, which makes the conclusion on the mechanism involved in tolerance less clear.

Concerning the second experiment, tolerant genotypes (particularly RIL47) tended to show higher $\delta^{13}C$ (mostly on *dm*) and $\delta^{18}O$ (mostly on *wsf*) at the different salinity stresses compared with the parental genotypes, and positive relationships of biomass to both $\delta^{13}C$ and $\delta^{18}O$ were observed. The higher $\delta^{13}C$ and $\delta^{18}O$ of the tolerant genotypes suggests that a lower transpiration rate is involved. In that sense, Yousfi *et al.* (2010) reported a lower accumulation of Na in the tolerant genotypes of this study. In disagreement with our results, Munns & James (2003) suggest that screening for high g_s may be the most effective way of selecting genotypes that will grow fast in saline soil. However, in the two experiments of our study, plants were exposed to salinity for a relatively long period, which means that preventing the rise of toxicity triggered by accumulation of sodium may be a priority for the plants, rather than avoiding the osmotically induced water stress. In a more recent study on durum wheat (Yousfi *et al.* 2012), where the most tolerant genotypes were those exhibiting lower $\delta^{13}C$ and $\delta^{18}O$ together with a higher g_s, plants were exposed to salinity for a shorter period that our first experiment (about 3 compared with 9 weeks) or at a different plant stage than in the second experiment (tillering versus flowering-grain filling).

In the first experiment the $\delta^{15}N$ of tolerant plants was higher than in the susceptible ones within each of the stress conditions. In the case of $\delta^{13}C$, the $\delta^{15}N$ in the *wsf* performed better than in the *dm*. Thus, while $\delta^{15}N_{dm}$ was positively correlated with biomass under control and moderate stress conditions (Yousfi, Serret & Araus 2009), $\delta^{15}N_{wsf}$ and biomass also correlated positively in the most severe treatment. The differences in $\delta^{15}N$ between tolerant and susceptible plants suggest that genotypic tolerance to salinity is mediated through a higher N uptake and⁄or assimilation (Handley *et al.* 1997; Ellis, Forster & Gordon 2002; Yousfi *et al.* 2012). In agreement with this, tolerant genotypes consistently exhibit higher N content than susceptible ones (Youfi, Serret & Araus; Yousfi *et al.* 2010, 2012). Thus a higher susceptibility to abiotic stresses would produce a decrease in demand relative to a constant N supply, leading to stress-induced depletion in plant ^{15}N (Mariotti *et al.* 1982). Alternatively, a lower photorespiration does not appear to be involved since this process would go in the opposite direction decreasing $\delta^{15}N$ in N-metabolism precursors (Tcherkez 2011). Results from the second experiment had the same trend but it was less clear, with

tolerant genotypes exhibiting higher $\delta^{15}N$ under control as well as in some of the stress conditions. However, as for $\delta^{13}C$, no clear advantage was found between $\delta^{15}N_{dm}$ and $\delta^{15}N_{wsf}$, with only $\delta^{15}N_{dm}$ being (positively) correlated with biomass in the most severe stress.

In summary for the first experiment, where the stress was applied shortly after plant emergence and extended for more than two months until harvest, both $\delta^{13}C_{wsf}$ and $\delta^{15}N_{wsf}$ performed better than $\delta^{13}C_{dm}$ and $\delta^{15}N_{dm}$ in assessing genotypic performance, particularly under the most severe stress conditions, whereas $\delta^{18}O$ performed very poorly, regardless of whether *dm* or *wsf* were being analysed. For the second experiment, where stress was applied later in the plant cycle and extended for few weeks, trends were less clear, but overall the three isotopic signatures performed better when analysed in *dm* and satisfied the aim of identifying genotypic performance under the most stressful treatments.

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Supporting Tables and Figures

Table S1. Effect of growing conditions during the reproductive stage on the different stable isotopes analysed in the four genotypes of durum wheat (Cham, Lahn, RIL47, RIL85) from experiment 2. Data shown are means of the three replicates for each genotype. Means followed by different letters are significantly different (P<0.05) according to Tukey's HSD test. Levels of significance: ns, not significant; *, P < 0.05; **, P < 0.01. Abbreviations for variables and treatments as defined in Table 1.

Treatments	Variable	Cham	Lahn	RIL47	RIL85	*P*
FI	$\delta^{13}C_{dm}$ (‰)	-32.60 ^{a}	-32.06 ^{a}	-32.60 ^{a}	-32.28 ^{a}	ns
	$\delta^{13}C_{wsf}$ (‰)	-31.56 ^{a}	-31.83 ^{a}	-31.35 ^{a}	-32.11 ^{a}	ns
	$\delta^{15}N_{dm}$ (‰)	5.94 ^{a}	7.86 ^{a}	8.22 ^{a}	7.75 ^{a}	ns
	$\delta^{15}N_{wsf}$ (‰)	7.42 ^{a}	13.26 ^{b}	14.04 ^{b}	11.38 ^{ab}	*
	$\delta^{18}O_{dm}$ (‰)	29.67 ^{a}	29.97 ^{a}	30.06 ^{a}	30.19 ^{a}	ns
	$\delta^{18}O_{wsf}$ (‰)	30.04 ^{a}	30.50 ^{a}	31.04 ^{a}	30.01 ^{a}	ns
FI-12 dS m^{-1}	$\delta^{13}C_{dm}$ (‰)	-32.11 ^{b}	-32.04 ^{ab}	-33.09 ^{c}	-31.32 ^{ab}	ns
	$\delta^{13}C_{wsf}$ (‰)	-30.73 ^{ab}	-31.56 ^{a}	-29.81 ^{b}	-30.88 ^{ab}	*
	$\delta^{15}N_{dm}$ (‰)	2.83 ^{a}	3.65 ^{a}	6.10 ^{b}	4.05 ^{a}	ns
	$\delta^{15}N_{wsf}$ (‰)	7.68 ^{a}	7.63 ^{a}	12.34 ^{b}	6.51 ^{a}	**
	$\delta^{18}O_{dm}$ (‰)	30.79 ^{a}	30.16 ^{a}	30.85 ^{a}	30.87 ^{a}	ns
	$\delta^{18}O_{wsf}$ (‰)	32.57 ^{a}	32.19 ^{a}	33.21 ^{a}	34.67 ^{a}	ns
FI-17 dS m^{-1}	$\delta^{13}C_{dm}$ (‰)	-31.79 ^{a}	-31.69 ^{a}	-32.29 ^{b}	-31.56 ^{a}	**
	$\delta^{13}C_{wsf}$ (‰)	-30.79 ^{a}	-31.23 ^{a}	-30.74 ^{a}	-31.06 ^{a}	ns
	$\delta^{15}N_{dm}$ (‰)	2.74 ^{a}	3.57 ^{ab}	4.42 ^{b}	3.54 ^{ab}	ns
	$\delta^{15}N_{wsf}$ (‰)	6.11 ^{a}	7.72 ^{a}	7.71 ^{a}	6.92 ^{a}	ns
	$\delta^{18}O_{dm}$ (‰)	30.65 ^{a}	30.06 ^{a}	31.12 ^{a}	31.23 ^{a}	ns
	$\delta^{18}O_{wsf}$ (‰)	33.11 ^{a}	33.07 ^{a}	34.49 ^{b}	34.41 ^{b}	**
DI	$\delta^{13}C_{dm}$ (‰)	-32.34 ^{a}	-31.89 ^{a}	-32.41 ^{a}	-32.37 ^{a}	ns
	$\delta^{13}C_{wsf}$ (‰)	-31.43 ^{a}	-31.74 ^{a}	-31.69 ^{a}	-31.72 ^{a}	ns
	$\delta^{15}N_{dm}$ (‰)	4.82 ^{a}	6.96 ^{a}	7.39 ^{a}	6.09 ^{a}	ns
	$\delta^{15}N_{wsf}$ (‰)	11.14 ^{a}	12.66 ^{a}	13.47 ^{a}	12.75 ^{a}	ns
	$\delta^{18}O_{dm}$ (‰)	29.33 ^{a}	30.65 ^{ab}	31.50 ^{b}	30.25 ^{ab}	ns
	$\delta^{18}O_{wsf}$ (‰)	27.99 ^{a}	31.85 ^{bc}	34.32 ^{c}	30.81 ^{ab}	**
DI-12 dS m^{-1}	$\delta^{13}C_{dm}$ (‰)	-31.88 ^{ab}	-32.24 ^{b}	-32.41 ^{b}	-32.49 ^{b}	*
	$\delta^{13}C_{wsf}$ (‰)	-31.16 ^{a}	-31.69 ^{a}	-30.99 ^{a}	-31.81 ^{a}	ns
	$\delta^{15}N_{dm}$ (‰)	3.02 ^{a}	4.37 ^{a}	5.28 ^{a}	6.09 ^{a}	ns
	$\delta^{15}N_{wsf}$ (‰)	9.56 ^{a}	12.02 ^{a}	11.30 ^{a}	11.37 ^{a}	ns
	$\delta^{18}O_{dm}$ (‰)	30.30 ^{ab}	30.39 ^{ab}	30.93 ^{b}	29.79 ^{a}	*
	$\delta^{18}O_{wsf}$ (‰)	31.25 ^{a}	34.21 ^{b}	33.58 ^{ab}	31.90 ^{ab}	*
DI-17 dS m^{-1}	$\delta^{13}C_{dm}$ (‰)	-32.21 ^{b}	-31.84 ^{ab}	-31.99 ^{ab}	-31.50 ^{a}	*
	$\delta^{13}C_{wsf}$ (‰)	-31.16 ^{a}	-31.69 ^{a}	-30.99 ^{a}	-31.81 ^{a}	ns
	$\delta^{15}N_{dm}$ (‰)	1.13 ^{a}	3.47 ^{b}	4.35 ^{b}	4.71 ^{b}	**
	$\delta^{15}N_{wsf}$ (‰)	6.87 ^{a}	8.33 ^{a}	9.46 ^{a}	6.71 ^{a}	ns
	$\delta^{18}O_{dm}$ (‰)	30.49 ^{a}	30.48 ^{a}	31.53 ^{ab}	31.16 ^{a}	ns
	$\delta^{18}O_{wsf}$ (‰)	31.96 ^{a}	33.44 ^{ab}	35.06 ^{b}	32.93 ^{a}	*

Table S2. Correlation coefficients of the linear regressions between the carbon, nitrogen and oxygen isotope compositions of the dry matter and the water soluble fraction against plant biomass. Relationships were studied across the set of 4 durum wheat genotypes and three replications per genotype, within each of the six growing conditions assayed during the reproductive stage (experiment 2). Abbreviations for variables and treatments as defined in Tables 1 and 3. Levels of significance: ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

	FI	FI-12 dS m^{-1}	FI-17 Ds m^{-1}	DI	DI-12 dS m^{-1}	DI-17 dS m^{-1}
$\delta^{13}C_{dm}$	0.338 ^{ns}	0.066 ^{ns}	0.727***	0.353 ^{ns}	0.268 ^{ns}	0.622**
$\delta^{13}C_{wsf}$	0.498 ^{ns}	0.049 ^{ns}	0.069 ^{ns}	-0.096 ^{ns}	0.237 ^{ns}	-0.257 ^{ns}
$\delta^{15}N_{dm}$	-0.341 ^{ns}	0.284 ^{ns}	0.366 ^{ns}	-0.079 ^{ns}	-0.439 ^{ns}	0.557*
$\delta^{15}N_{wsf}$	-0.304 ^{ns}	0.274 ^{ns}	-0.100 ^{ns}	0.064 ^{ns}	-0.118 ^{ns}	0.507 ^{ns}
$\delta^{18}O_{dm}$	-0.347 ^{ns}	-0.150 ^{ns}	0.563*	0.387 ^{ns}	-0.238 ^{ns}	0.572*
$\delta^{18}O_{wsf}$	-0.439 ^{ns}	0.105 ^{ns}	0.426 ^{ns}	0.367 ^{ns}	0.359 ^{ns}	0.514*

Figure S1. Daily air temperatures for experiment 1 (A) and experiment 2 (B) and daily relative air humidities for experiment 1 (C) and experiment 2 (D).

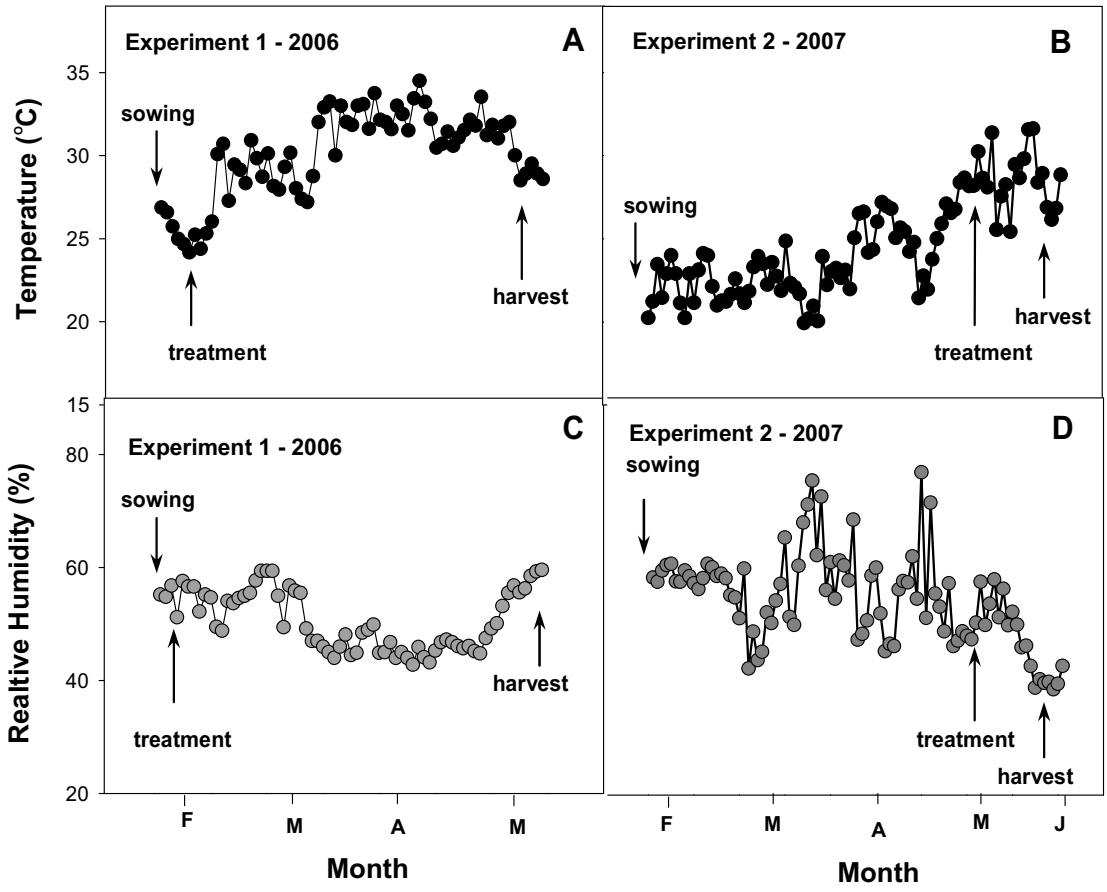


Figure S2. Cluster analysis of the three growing conditions assayed, using as variables (A) nitrogen isotope composition of the dry matter ($\delta^{15}N_{dm}$) and (B) nitrogen isotope composition of the water soluble fraction ($\delta^{15}N_{wsf}$) (B). Abbreviations for treatments are as in the legend of Fig. 1.

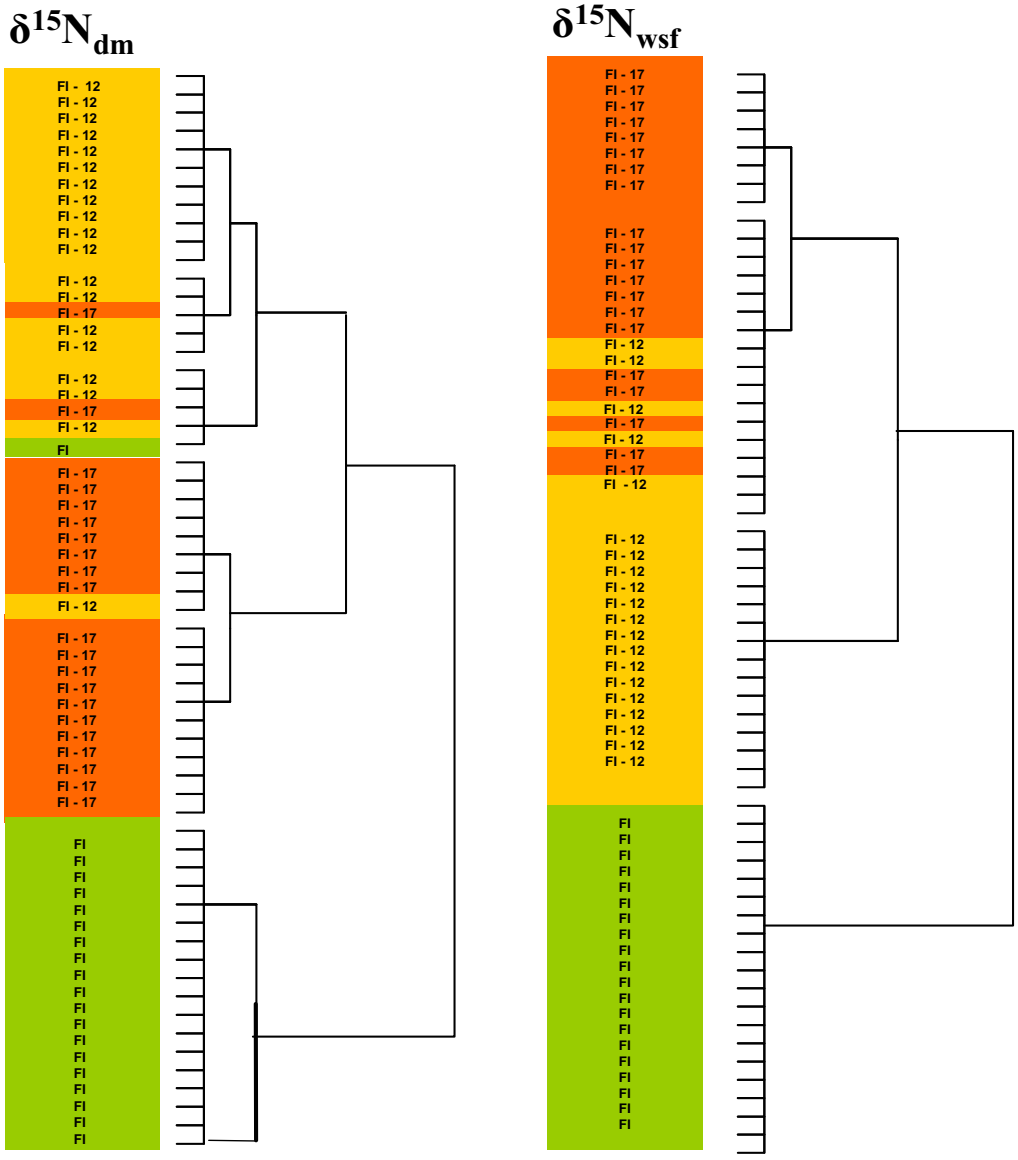


Figure S3. Cluster analysis of the three growing conditions assayed, using as variables (A) oxygen isotope composition of the dry matter ($\delta^{18}O_{dm}$) and (B) oxygen isotope composition of the water soluble fraction ($\delta^{18}O_{wsf}$). Abbreviations for treatments are as in the legend of Fig. 1.

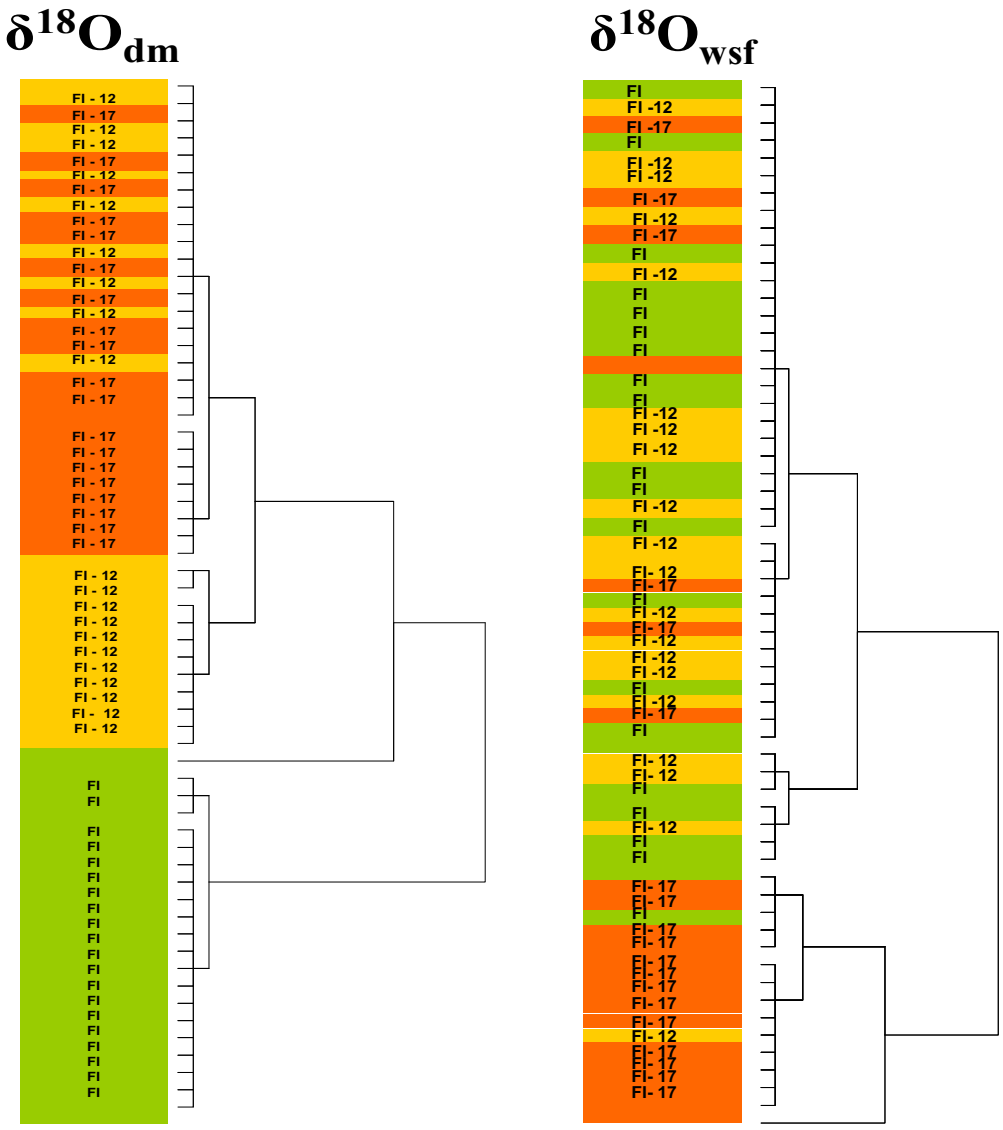
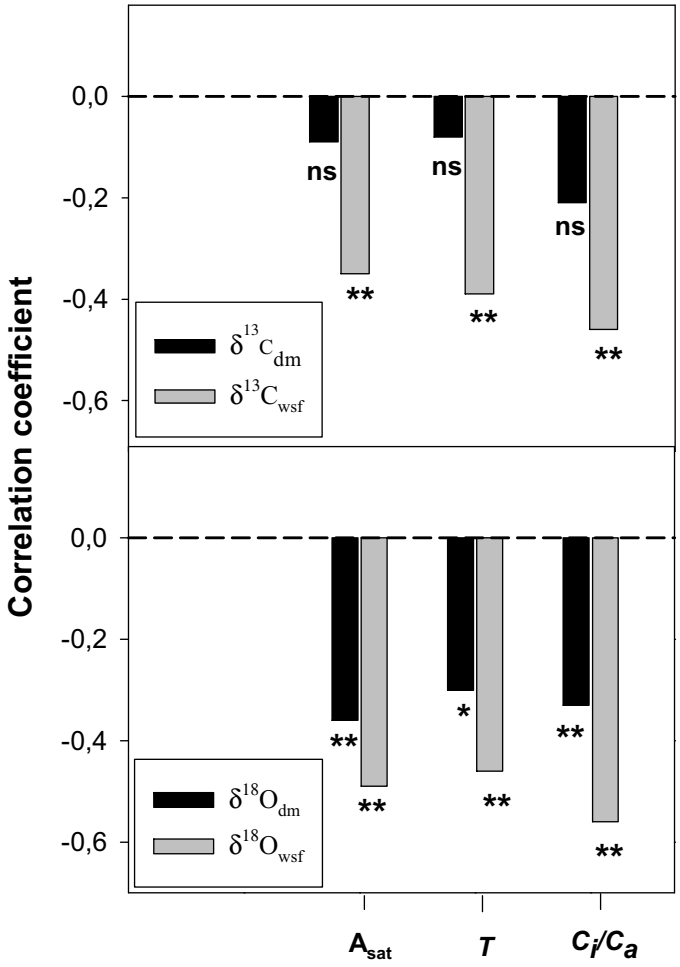


Figure S4. Correlation coefficients of the relationships across all treatments and individual plants measured between light-saturated net photosynthesis (A_{sat}), transpiration (T) and the intercellular versus the atmospheric CO_2 concentration (C_i/C_a) against (upper figure) carbon isotope composition in the total dry matter and the water soluble fraction ($\delta^{13}C_{dm}$ and $\delta^{13}C_{wsf}$) and (lower figure) oxygen isotope composition in the total dry matter and the water soluble fraction ($\delta^{18}O_{dm}$ and $\delta^{18}O_{wsf}$). Relationships of A_{sat} and T to the isotope signatures were fitted through second degree polynomic, whereas the relationships of C_i/C_a with the isotope signatures were lineal.

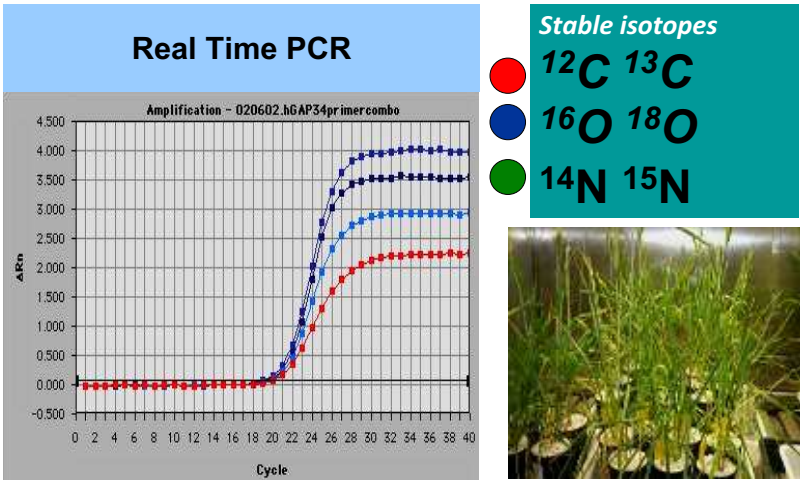


Capítulo 6

Gene expression and physiology of resistant and sensitive durum wheat genotypes during salinity and water stress

Yousfi, S., Serret, M.D., Márquez A.J., Betti M., Araus, J.L.

Artículo para enviar



Resumen

Explicar la relación entre la regulación génica y la expresión fenotípica sigue siendo una limitación en la mejora de plantas resistentes a los estreses abióticos. Los estudios que relacionan la expresión de los genes con los mecanismos fisiológicos responsables de la variabilidad natural genotípica al estrés salino y al estrés hídrico siguen siendo escasos. Dos genotipos susceptibles y tolerantes a la salinidad fueron cultivados bajo cinco combinaciones de salinidad y régimen de riego. Después un mes, se cosecharon las plántulas y se analizaron las tasas de transcripción de diferentes genes mediante PCRs en Tiempo Real (RT-PCR). Los genes estudiados incluyeron dos que codifican para factores de transcripción inducidos por la sequía (TaDREB1A y TaDREB2B), otros dos para la glutamina sintetasa citosólica y plastídica (GS1, GS2) y uno para el "antiporter" tonoplástico específico de Na^+/H^+ (NHX1). Además se analizaron los niveles de expresión de las enzimas GS1 y GS2 por Western blot. Modificaciones entre la expresión de los diferentes genes en plantas estresadas con respecto a las plantas control (completamente regadas con solución nutritiva normal) se relacionaron con las características fisiológicas de estas plantas reportadas en un estudio anterior: biomasa de la planta, isótopos estables del carbono, oxígeno y nitrógeno ($\delta^{13}C$, $\delta^{18}O$ y $\delta^{15}N$), parámetros de intercambio de gases, concentración de iones, contenido en N y la actividad enzimática de la nitrato reductasa (NR) y la glutamina sintetasa (GS). Se detectaron diferencias genotípicas en la expresión de todos los genes, excepto para el gen NHX1. Todas las características relacionadas con el metabolismo del nitrógeno se correlacionaron mejor que el resto de los caracteres fisiológicos con los genes de GS y TaDREB. Los resultados indican claramente que las respuestas genotípicas debidas a la salinidad se asociaron con el estado hídrico de la planta y el metabolismo del N. Sin embargo el nivel de expresión correlacionaba con el crecimiento y las características fisiológicas sólo en la salinidad alta.

Gene expression and physiological responses of resistant and sensitive durum wheat genotypes under salinity and water stress

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Summary

Elucidating the connections between gene regulation and phenotypic expression remains a bottleneck in breeding for abiotic stresses. Studies relating the gene expression with the physiological mechanisms responsible of the natural genotypic variability to salinity and water stress are still scarce. Two tolerant and two susceptible genotypes to salinity were grown under five combinations of salinity and irrigation regimes. After > 1 month plants shoots were harvested and the transcription rates of different genes were analysed by Real Time PCR (RT-PCR).The genes included encoded for two drought responsible transcription factors induced by dehydration (TaDREB1A and TaDREB2B), the cytosolic and plastidic glutamine synthethase (GS1, GS2) and the specific Na^+/H^+ antiporter NHX1. In addition the expression levels of the GS1 and GS2 enzymes were analyzed by Western blot. Modification in the expression of the different genes of stressed plants with regard to the control plants (fully irrigated with normal nutrient solution) were related with the physiological characteristics of these plants reported in a previous study: plant biomass, the stable carbon, oxygen and nitrogen isotopes compositions ($\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$), ion concentration, N content and nitrate reductase (NR) and glutamine synthetase (GS) activities. Genotypic differences in expression existed for all the genes except NHX1. All the nitrogen metabolism traits correlated better with GS and TaDREB genes than the other physiological traits. The results clearly indicate that genotypic responses in front to salinity were associated with water status and N metabolism. Nevertheless the rate of expression correlated with growth and physiological traits only at high salinity.

Key words**:** Gene expression, durum wheat (*Triticum turgidum* ssp. *durum*), salinity, water deficit, Real-time PCR, TaDREB, Glutamine synthetase, NHX1, nitrogen metabolism, $\delta^{13}C$, $\delta^{18}O$, $\delta^{15}N$.

Introduction

Salinity and drought are among the most damaging abiotic stresses afecting today's agriculture. Durum wheat is one of the most cultivated herbaceous crops in the south and east Mediterranean basin (http://www.fao.org/docrep/006/y4011e/y4011e04.htm) where both salinity and water deficit are the main constraints limiting productivity (Araus, 2004). Tolerance to these abiotic stresses is a complex phenomenon, comprising a number of physiological and biochemical mechanisms at both cellular and whole organism levels activated at different stages of plant development. Plant stress tolerance involves changes at whole-plant, tissue, cellular, physiological and molecular levels. Additional factors complicating the scenario are the intensity of these stresses (Munns, 2005; Munns & Tester, 2008) and the interactions between them (Yousfi *et al.,* 2010, 2012). Molecular responses to abiotic stress include perception, signal transduction, gene expression and ultimately metabolic changes in the plant thus providing stress tolerance (Agarwal *et al.,* 2006). One of the main cellular events occurring during stress conditions is extensive modification of gene expression resulting in a strict control of the different physiological and biochemical responses to stress (Rampino *et al.,* 2006). Plants perceive the stress signal and transmit it to the cellular machinery to activate different adaptive responses. In recent years, the expression and regulation of genes in plants have been not only uncovered but also proposed as a tool to improve crop response to stresses (Araus *et al.,* 2008 and references herein). Numerous studies have shown that the expression of a vast array of genes is induced or repressed (up or down regulated) by environmental stresses such as drought and salinity (Gao *et al.,* 2005; Agarwal *et al.,* 2006; Munns & Tester, 2008; Lata *et al.,* 2011b; Rowley & Mockler, 2011). These (and other) environmental stresses induce the expression of a variety of genes in many plant species (Xiong *et al.,* 2002; Shinozaki *et al.,* 2003; Bartels & Sunkar, 2005). Numerous stress-induced genes have been identified using microarray experiments (Kreps *et al.,* 2002; Seki *et al.,* 2002). The products of these genes are thought to promote stress tolerance and to regulate gene expression through signal transduction pathways (Xiong *et al.,* 2002; Shinozaki *et al.,* 2003).

To date the most studied genes are those that encode the expression of transcription factors which are master regulators that control gene clusters whose products contribute to protect and/or reduce the stress-induced cellular damage. A single transcription factor can control the expression of many target genes through specific binding of the transcription factor to the cis-acting element in the promoters of respective target genes (Agarwal & Jha, 2010). Within this category the DREBs (dehydration responsive element binding) proteins are major plant transcription factors that regulate the expression of many osmotic stress-inducible genes and play a critical role in improving the osmotic stress tolerance of plants (Liu *et al.,* 2000; Zhu, 2002; Shinozaki *et al.,* 2003 Mohsenzadeh *et al.,* 2009).

In fact many studies indicate that the DREB proteins are important transcription factors in regulating abiotic stress related genes and play a critical role in imparting stress endurance to plants. DREB factors have been extensively characterized and grouped into several molecular classes based on similarities in the function or amino acid sequence of the proteins they encode (Bohnert *et al.,* 1995). The first isolated cDNAs encoding DRE binding proteins, CBF1 (CRT binding factor1), DREB1A and DREB2A were identified through yeast one-hybrid screening from *Arabidopsis* (Stockinger *et al.*, 1997; Liu *et al.,* 1998). Since then, many DREBs have been isolated from various plants. In fact DREB1 and DREB2 are two main subgroups of DREB subfamily, involved in two different signal transduction pathways under cold and dehydration respectively. DREB1/DREB2-homologous genes have also been identified in various cereals and millet crops (Nakashima *et al.,* 2009; Lata *et al.,* 2011a, b). The expression of *Arabidopsis* DREB2A and its homolog DREB2B were stimulated by dehydration and high salinity, but not by cold and ABA (Liu *et al.,* 1998; Nakashima *et al.,* 2000).

In wheat, the TaDREB1A transcription factor was isolated from a drought-induced cDNA library of bread wheat; it was found to be induced by low temperature, ABA, salinity and drought (Shen *et al.,* 2003; Kurahashi *et al.,* 2009) and plays a most important role as transcription factors in wheat (Shen *et al.,* 2003; Thomashow *et al.,* 2001). Similarly another transcription factor TaDREB2B was found in bread wheat to be

induced in wheat plants under ABA, drought, salinity and cold stress (Egawa *et al.,* 2006). Currently, both TaDREB1A and TaDREB2B are widely used in studies aiming to the implementation of molecular breeding for better performance to abiotic stresses in wheat. Thus, Kurahashi *et al.* (2009) reported in bread wheat that the more tolerant accessions to dehydration tended to accumulate more TaDREB1 transcripts of the transcription factor genes more abundantly than the sensitive accessions. Moreover these results suggest that the genotypic differences in abiotic stress responses might be due to further upstream genetic factor(s) in the ABA-dependent signal pathway.

There is a large body of literature about the effect of salinity and water stress on photosynthetic and nitrogen metabolism of plants (Munns, 2005; Munns & Tester, 2008), including durum wheat (Yousfi *et al.,* 2009, 2010, 2012). In this species and concerning nitrogen metabolism, salinity and drought decrease nitrogen content (Yousfi *et al.,* 2009, 2010) as well as in the activities of key enzymes involved in nitrogen metabolism such as nitrate reductase and glutamine synthetase (Yousfi *et al.,* 2012). The effect of these stresses at the ecophysiological level have been also investigated, which include changes in the signatures of different stable isotopes related with the photosynthetic transpiratory and N metabolisms. We refer to the carbon, oxygen and nitrogen isotope compositions ($\delta^{13}C$, $\delta^{18}O$ and $\delta^{13}N$). However studies aiming to link gene expression with a physiological response are still scarce.

Glutamine synthetase (GS, EC 6.3.1.2) plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine (Miflin & Lea, 1977; Miflin & Habash, 2002). GS functions as the major assimilatory enzyme for ammonia produced from nitrate or ammonia nutrition. It also reassimilates ammonia released as a result of photorespiration and the breakdown of proteins and nitrogen transport compounds. Higher plants have two major types of GS – cytosolic (GS1) and plastic (GS2) isoforms – which perform nonoverlapping metabolic functions, with cytosolic GS assimilating ammonium from primary sources in roots, nodules and leaves and the chloroplastic GS holding a major role in ammonium assimilation within photorespiratory nitrogen cycling (Bernard & Habash, 2009).

GS activity is one of the best physiological markers describing plant N status, whatever its developmental stage and N nutrition (Kichey *et al.,* 2006; Bernard & Habash, 2009), also it was reported that GS activity is affected by water deficit (Bernard & Habash, 2009, Yousfi *et al.,* 2012) and salinity (Wang *et al.,* 2007; Carillo *et al.,* 2008, Yousfi *et al.,* 2012). GS1 appears to play a key role in plant growth and further leaf senescence (Bernard & Habash, 2009).

The enzyme is the product of multiple genes with complex promoters that ensure the expression of the genes in an organ- and tissue-specific manner and in response to a number of environmental variables affecting the nutritional status of the cell. Cytosolic glutamine synthetase (GS1) is usually encoded by three to five genes, and involved in generating glutamine for intercellular N transport (Edwards *et al.,* 1990; Brugière *et al.,* 1999; Tabuchi *et al.,* 2005; Bernard *et al.,* 2008). GS1 gene families are regulated in response to plant nitrogen status, as well as to environmental cues, such as nitrogen availability and abiotic stresses (Bernard *et al.,* 2009). Plastidic enzyme isoforms (GS2) is encoded by a smaller number of genes, often only one; although three subcopies have now been identified in hexaploid wheat (Bernard & Habash, 2009). The GS2 is expressed primarily in green leaves, where it reassimilates photorespiratory ammonia. Molecular studies have shown that transcription of the GS2 gene is positivley regulated by diverse factors such as light and photorespiration (Tingey *et al.,* 1988; Edwards & Coruzzi, 1989). Expression of GS genes in wheat is regulated with both GS2 and GS1 assimilating or recycling ammonia in leaves. However, during leaf senescence, expression of the cytosolic GS1 isozymes genes were the predominant forms (Bernard *et al.,* 2008).

Salinity tolerance comes from genes that limit the rate of salt uptake from the soil and the transport of salt throughout the plant, adjust the ionic and osmotic balance of cells in roots and shoots (Munns, 2005). In durum wheat the Na^+ exclusion trait may be relevant preventing leaf injury and enhancing yield at moderate salinity (Husain *et al*., 2003). However traits other than Na^+ exclusion are important at high salinity, where the osmotic effect of the NaCl outweighs its salt-specific effect on growth and yield. Species that cannot exclude 98% of the salt from the transpiration

stream (e.g. barley) must also be able to compartmentalize the salt in vacuoles, thereby protecting the cytoplasm from ion toxicity and avoiding buildup in the cell wall which would cause dehydration. In fact at the cellular level, compartmentalization of excessive Na^+ in the vacuole by the tonoplast Na^+/H^+ exchanger is considered central to salt tolerance (Rowley & Mockler, 2011).

If Na^+ and Cl^- are sequestered in the vacuole of the cell, K^+ and organic solutes should accumulate in the cytoplasm and organelles to balance the osmotic pressure of the Na^+ and Cl^- in the vacuole. Among the genes responsible for this are membrane proteins controlling Na^+ transport. Active ion transport occurs through symporters and antiporters that can transport ions against an electrochemical potential gradient. Transport is driven by the electrochemical potential difference of a coupled solute, usually H^+. The Na^+/H^+ antiporter catalyse the exchange of Na^+ for H^+ across membranes and have a variety of functions such as regulating cytoplasmic Ph, sodium levels and cell turgor (Serrano *et al.,* 1999). Molecular analyses in *Arabidopsis* led to the identification of a vacuolar NHX1, Na^+/H^+ antiporters upregulated at the genetic level in response to NaCl (Apse *et al.,* 1999; Gaxiola *et al.,* 1999). The NHX family of antiporters (Na^+/H^+ exchangers) are highly selective for Na^+ (Munns, 2005).

Whereas the list of cloned genes with likely relevance for controlling K^+ or Na^+ uptake by roots and transport within the plant, and which are candidates for overexpression studies is high, transformation experiments with the greatest successes have come from manipulating tonoplast Na^+ transport (Munns, 2005; Khan, 2011). Thus an antiporter cloned from *Arabidopsis*, *AtNHX1*, when overexpressed it increased salt tolerance in different species (Apse *et al*., 1999; Zhang & Blumwald, 2001; Zhang *et al*., 2001) including wheat (Xue *et al*., 2004). Overexpression of other NHX1 homologs have also increased salt and drought stress tolerance (e.g. Brini *et al.,* 2007; Chen et *al.,* 2007). Moreover overexpression of NHX1 has also resulted in enhanced plant tolerance to drought stress (Gaxiola *et al.,* 2001; Park *et al.,* 2005). Clearly the NHX family of Na^+/H^+ of antiporters are able to confer increased Na^+ tolerance across a wide range of plant species, and aside from being a single trait, may be even more relevant from a genetic engineering standpoint as sixth-generation soybean plants

expressing AtNHX1 proved to be just as resistant (Li *et al.,* 2010) to salt stress as the first generation transgenic plants, indicating this single trait change in heritable.

A research programme for increasing tolerance of wheat to salinity and drought should tackle the problem in a multi-disciplinary approach, integrating the physiological dissection of tolerance traits and the genetic and genomics tools, such as transcription factors of responsive stress gene. The usefulness of combining molecular and physilogical traits was proposed in this study to track differences in response to salinity and drought of wheat genotypes. Overall objective of this study was to characterize the response of four durum wheat genotypes with contrasting performances under salinity to different combinations of salinity and irrigation regimes using as molecular proves transcript levels of five target genes and associated them with several physilogical traits. In firtst, expression of candidate genes TaDREB1A, TaDREB2B, GS1, GS2 and NHX1 was studied in leaves of durum wheat genotypes growing under different conditions using the SYBR green-based real time PCR. In addition, various physiological criteria evaluated under salinity and deficit irrigation in our previous study (Yousfi *et al.,* 2012) were correlated with the induction ratio of each target genes studies here. These correlations were elaborated in order to test the relashionship of transcripts levels of differents responsive genes with a set of most important physilogical parameters as: shoot biomass, the isotopic signatures of C, O and N, ion accumulation, total N content and the activity of glutamine synthetase and nitrate reductase enzymes. Moreover we compared the Western blot of glutamine synthetase elaborated in leaves extract and the induction ratio of the GS1 and GS2 genes. Understanding the molecular basis of salt and drought stress signalling and their relashionships with physiological mechanisms as nitrogen metabolism and/or stable isotopes signatures in wheat may provide reliable information for screening genotypes more tolerant to these abiotic stresses, even that implications of both approaches, molecular and physiological together, in breeding programs are indeed currently absent or scarce.

However gene expression studies about salinity have deliver below expectations, perhaps because the treatments are often traumatic and unnatural

(Munns, 2005; Munns & Tester, 2008). In that sense our study assayed 5 different growing conditions, including moderate and severe salinity together with the combinations of salinity and water limitation

Material and Methods

Plant material and growth conditions

Four recombinant inbred lines (RILs) of durum wheat [*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.], here designated as RIL24, RIL30 (salt-susceptible) and RIL47, RIL85 (salt-tolerant), were chosen on the basis of their contrasting shoot biomass among a set of 112 RILs evaluated in a previous study for tolerance to continuous salinity during the vegetative stage (Yousfi *et al*., 2009). Plants were grown in a controlled chamber (Conviron E15, Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at the Experimental Fields of the University of Barcelona, for over one month. Experimental growing conditions were: 400 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 70% relative humidity, 25/15 °C day/night temperature and a 14-h photoperiod. Seeds were planted in 3 dm^3 pots containing perlite and watered to field capacity with complete Hoagland solution (Hoagland & Arnon, 1950). Subsequently, five different growth conditions were imposed: (i) FI or control (fully irrigated, 100 % of container capacity, with normal Hoagland solution, 1.8 dS m^{-1}), (ii) FI-12 (fully irrigated with saline Hoagland solution, 12 dS m^{-1}), (iii) FI-17 (fully irrigated with saline Hoagland solution, 17 dS m^{-1}), (iv) DI (deficit irrigated to 35 % of container capacity, with normal Hoagland solution) and (v) DI-12 (deficit irrigated to 35 % of container capacity with saline Hoagland solution, 12 dS m^{-1}). Water deficit was imposed progressively over one week by decreasing irrigation. Salinity treatment was also imposed by adding NaCl progressively to the nutrient solution, starting with a salt concentration of 4 dS m^{-1} to reach the final levels of 12 dS m^{-1} (~ 120 mM NaCl) or 17 dS m^{-1} (~ 170 mM NaCl).

Shoot biomass

At harvest the last fully expanded leaf blade of each plant were sampled and stored at -80ºC for analysis of enzyme activity and Real time PCR. The rest of the shoot was then

harvested; oven dried at 70ºC for 48 h, weighed and ground finely for isotopes stable and ion analyses.

Ion analysis

Ion analysis was performed in shoots by inductively coupled plasma emission spectrometry (L3200RL, Perkin Elmer, Rodgau, Germany) as described in Yousfi *et al.* (2012).

N concentration and stable isotope signatures

The total N conctent and the stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotope ratios in shoots were measured using an elemental analyzer (Flash 1112 EA; ThermoFinnigan, Germany) coupled with an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan, Germany), operating in continuous flow mode. Values were expressed in δ notation (Coplen, 2008): $\delta^{13}C = [(^{13}C/^{12}C)_{sample} / (^{13}C/^{12}C)_{standard}]) -1$. In addition, the $^{18}O/^{16}O$ ratios were determined in shoot samples of all plants tested. Analyses were carried at Iso-Analytical Limited (Crewe, Cheshire CW2 8UY, UK).

Enzyme activity determinations

Glutamine synthetase (GS; EC 6.3.1.2) and Nitrate reductase (NR; EC 1.6.6.1) activites were assayed according to reaction (Márquez *et al.,* 2005) and (Pajuelo *et al.,* 2002) respectively (Yousfi *et al.,* 2012).

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from 100 mg of wheat leaves material (fully expanded upper leaf of the main plant tiller) using Plant RNA Reagent (Invitrogen, California, USA), according the manufacturer's instruction. cDNA was synthesised using an oligo (dT_{18}) primer from total RNA samples that had been pre-treated with RNase-free DNase and purified through Qiagen Rneasy column (Qiagen, Australia).

Real time PCR

The transcript levels of wheat genes were quantified with real-time PCR with an ABI Prism 7700 sequence detection system (Applied Biosystems) using SYBR Green PCR

Master Mix (Applied Biosystems) according to the manufacturer's instructions. The wheat 18S gene used as reference was amplified in parallel with the target gene allowing gene expression normalization and providing quantification. The sequences and names of primer pairs used for real-time PCR are listed in Table 1. Detection of real time RT-PCR products was done by using the SYBRR Green Universal Master mix kit (Applied Biosystems) following the manufacturer's recommendations. PCR was performed in a reaction mixture containing 2 µl of cDNA sample, 0.6 µl of each forward and reverse primer, 10 µl of the SYBRR Green Master mix and 6.8 µl of PCR water. PCR cycling conditions comprised an initial cycle at 50 ºC for 2 min, one cycle at 95 ºC for 10 min, followed by 45 cycles at 95 ºC for 15 s and at 60 ºC for 1 min. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. cDNAs to be amplified (target and reference) were made with the same PCR master mix and within a single ABI Prism run. At the end of the PCR run, a melting curve was generated and analyzed with the Dissociation Curves Software version 1.0 (Applied Biosystems).

Table 1. Primer sequences of genes used in Real-Time PCR analyse

Gene	Accession	Origin	Primer sequence
GS1	DQ124209	Wheat	(F)AAG.GAC.GGC.GGG.TTC.AA (R)GCG.ATG.TGC.TCC.TTG.TGC.TT
GS2	DQ124212	Wheat	(F)GCC.CTC.GCT.GCC.AAG.AA (R)TCG.GCC.TTT.TCA.GGT.CCTT
TaDREB1A ***(EREBP/AP2)***	AF303376	Wheat	(F) CAC TCT CTT GGA TGG TAG TGT CG (R) AGT GCA AGA CAG AAG ACT TGG AG
TaDREB2B ***(EREBP/AP2)***	AB193608	Wheat	(F) CTC TGA AAC GAT CAG GCG ATG G (R) GTG TAT TCT CAG GTC CTC CTT TCC
NHX1	AY040245	Wheat	(F) CCA TCG TCG CCA TCA ACA T (R) GGT GGC CGA AGA CAA TGC
18S	M82356		(F) GGCC.GCT.CCT.AGC.CCT.AAT.TG (R)TGA.GCA.CTC.TAA.TTT.CTT.CAA.AGT.ACG

Western blot and electrophoresis

SDS-PAGE separation and transference to PVDF membrane of proteins from wheat leaves was performed according to Pajuelo et al. (1993). Western blots were performed using the ECL Plus Western blotting system (GE Healthcare) according to

the manufacturer instructions. The membranes were blocked for 15 min at RT in 1x TBST containing 10% of non-fat dry milk. Detection of GS was carried out using an antibody raised in rabbit against purified cytosolic GS from nodules of *Phaseolus vulgaris* at 1:1,000 dilution (Bennet & Cullimore, 1990). Anti-rabbit secondary antibodies labelled with peroxidase were obtained from GE Healthcare and used at 1:10,000 dilution. Detection of GS protein in the membranes was carried out with a quimioluminescence detection system (Fujifilm LAS 3000 mini, Fujifilm) and the correspondent bands were quantified using the Multigauge V 3.0 software (Fujifilm).

Statistical analysis

The Sequence Detection System software version 1.7 (Applied Biosystems) calculates the ΔR_n using the equation $\Delta R_n = (R_n^+) - (R_n^-)$, where R_n^+ is the fluorescence signal of the product at any given time and R_n^- is the fluorescence signal of the baseline emission during cycles 3 to 15. An arbitrary threshold was set at the midpoint of the log ΔR_n versus cycle number plot. The C_T value is defined as the cycle number at which the ΔR_n crosses this threshold. The threshold cycle (C_T) values of the triplicate PCRs were averaged and used for quantification of transcripts. The quantification of the relative transcript levels was performed using the comparative C_T method (Livak & Schmittgen, 2001). The transcript levels of the target genes were normalized against the 18S gene transcript levels as described in the ABI PRISM 7700 Sequence Detection System user bulletin (Applied Biosystems). The induction ratio (IR) was calculated as recommended by the manufacturer and corresponds to $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (C_T, \text{Target gene - CT, 18S})_{\text{stressed}} - (C_T, \text{Target gene - CT, 18S})_{\text{control}}$. Data were subjected to factorial ANOVA to test the effects of treatment (salinity, irrigation), genotype and their interaction for each target gene studied. Mean comparisons for genotype (tolerant vs susceptible) and treatments effects were performed using Tukey's honestly significant difference (HSD) test. In order to test the association between transcript levels and physiological traits, a relationship between the induction ratio (IR) of each target gene and the set of physiological parameters measured was constructed across genotypes. Data were analysed using SPSS statistical package (SPSS Inc., Chicago, IL, USA).

Results

Effect of genotype and growing condition on gene induction ratio

Treatment effect was only significant by ANOVA for GS2 and TaDREB1A, where stresses decreased the induction ratio in the first case and increased it in the second. Treatment effect for the other three genes were not significant due to the large variability among replicates even when GS1 and TaDREB2A tended to decrease and NHX1to increase as response to stress (Table 2). Genotypic effects were significant for all the five genes assayed, with the tolerant genotype RIL47 exhibiting higher values for four of the five genes while the susceptible genotypes RIL24 and RIL30 showed higher values than the other two genotypes for the gene TaDREB1A (Table 2).

Table 2. Genotype and treatment effects on induction ratio (IR) of five genes (GS1, GS2, TaDREB1A, TaDREB2B, NHX1) and four durum wheat genotypes (RIL47; RIL85; RIL24; RIL30) grown under different combinations of salinity and water supply and their combinations. FI: Full irrigation (i.e. control) with normal Hoagland solution; FI-12 dS m^{-1}: Full irrigation with Hoagland solution at 12 dSm^{-1}; FI-17 dSm^{-1}: Full irrigation with Hoagland solution at 17 dS m-1; DI: Deficit irrigation (35% pot capacity) with normal Hoagland solution; DI-12 dS m-1: Deficit irrigation with Hoagland solution at 12 dS m-1. Genotype values are the means of 20 measurements (5 treatments and 4 replications per treatment), while treatment values are the means of the 16 measurements (4 genotypes and 4 replications per genotype). Means followed by different letters are significantly different ($P < 0.05$) according to Tukey's HSD test. Significance: ns, not significant.

	Induction ratio (IR)				
	GS1	**GS2**	**TaDREB1A**	**TaDREB2B**	**NHX1**
Genotype					
Tolerant RIL47	1.51 ^{c}	1.35 ^{b}	2.38 ^{a}	1.08 ^{b}	6.46 ^{b}
Tolerant RIL 85	0.52 ^{ab}	0.41 ^{a}	1.96 ^{a}	0.50 ^{a}	0.62 ^{a}
Susceptible RIL24	0.30 ^{a}	0.58 ^{a}	5.97 ^{b}	0.51 ^{a}	2.26 ^{a}
Susceptible RIL 30	0.88 ^{b}	0.68 ^{a}	6.01 ^{b}	0.85 ^{ab}	1.69 ^{a}
Treatment					
FI	1.00 ^{a}	1.00 ^{b}	1.00 ^{a}	1.00 ^{a}	1.00 ^{a}
FI- 12 dS m^{-1}	1.04 ^{a}	0.91 ^{b}	5.24 ^{b}	0.82 ^{a}	3.51 ^{a}
FI-17 dS m^{-1}	0.87 ^{a}	0.41 ^{a}	2.66 ^{ab}	0.44 ^{a}	3.93 ^{a}
DI	0.56 ^{a}	0.32 ^{a}	2.06 ^{ab}	0.93 ^{a}	3.22 ^{a}
DI- 12 dS m^{-1}	0.52 ^{a}	0.92 ^{b}	9.21 ^{c}	0.52 ^{a}	2.26 ^{a}
Level of significance					
Genotype (G)	0.000	0.000	0.014	0.022	0.013
Treatment (T)	ns	0.001	0.000	ns	ns
G×T	0.012	0.046	0.000	0.001	ns

Genotype by environment interaction was significant for all the genes except NHX1 (Table 2), indicates that genotypes behave differently under the different stresses assayed. In that sense tolerant genotypes showed higher induction ratio for GS1 and GS2 in all the stress treatments except at FI-12 for the first case (Supporting Information, Fig. S1). Tolerant genotypes showed lower induction ratio at FI-12 and DI-12 for TaDREB1A and higher for TaDREB2B, respectively, whereas at FI-17 and DI tolerant genotypes exhibited higher induction ratio for TaDREB2B and no differences for TaDREB1A. Finally tolerant genotypes exhibited higher induction ratio for NHX1 for all the stress conditions except D1-12 where the pattern was the opposite.

Relationships between transcript levels and plant biomass.

Relationships between induction ratios and plant biomass across all the five growing conditions and all the individual plants measured were only assayed for the two genes (GS2 and TaDREB1A) which exhibited significant treatment effect in the ANOVA analysis (Table 2). However of these two genes only TaDREB1A showed a clear pattern of relationships between induction ratios and biomass across treatments and individual plants. Thus relationship was exponential negative (Fig. 1a) with a lack of response in biomass for induction ratios

Relationships between induction ratios for each of the 5 genes and biomass within each of the four stress conditions were further investigated. Induction ratios of GS1, GS2 and TaDREB2B genes correlated positively with biomass across individual plants only within one of the most severe treatments (FI-17), whereas no correlations existed for biomass and NHX1 (Fig. 2).

Relationships of transcript levels and stable C, O and N isotopes signatures

Of the two genes where induction ratio was significantly affected by treatments (GS2 and TaDREB1A) only the second showed a clear pattern of relationships across environments with stable isotopes; particularly with the nitrogen isotope composition ($\delta^{15}N$). As in the case of the relationship of TaDREB1A with biomass (Fig. 1a) relationship of TaDREB1A with $\delta^{15}N$ was exponential negative, with no further decrease in $\delta^{15}N$ above induction ratios between 5-10 (Fig. 1c).

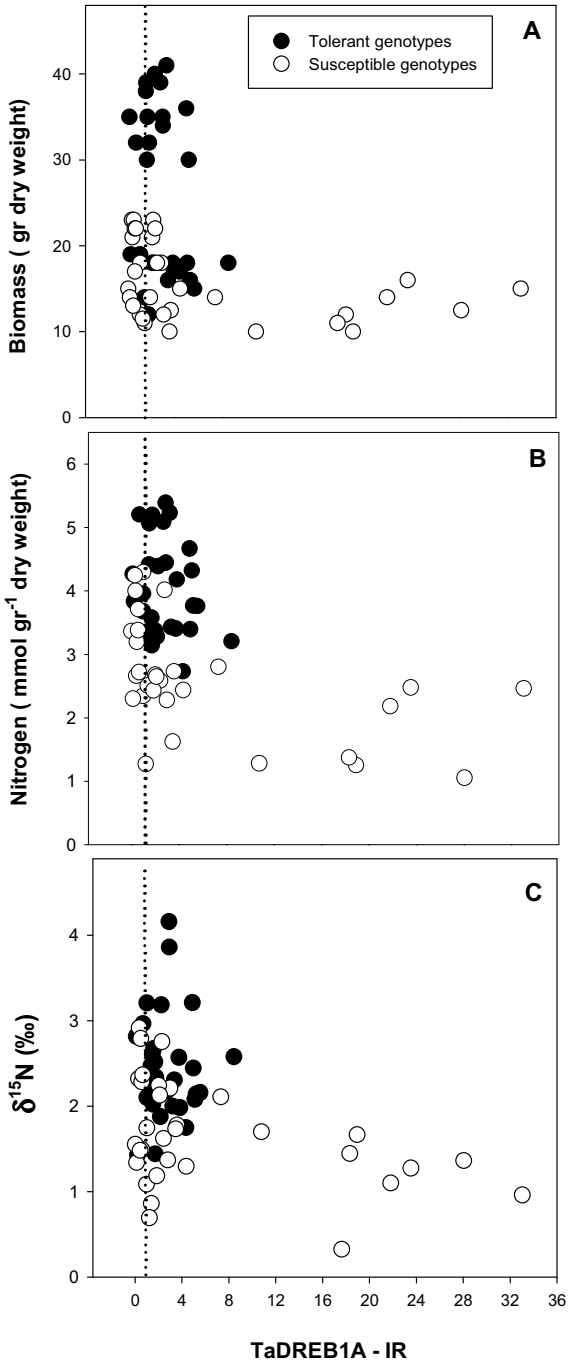

Figure 1. Relationships between the induction ratio of TaDREB1A and (A) shoot biomass at harvest, (B) nitrogen concentration and (C) nitrogen isotope composition ($\delta^{15}N$).

Induction ratios of GS1, GS2 and TaDREB2B genes correlated negatively with carbon isotope composition ($\delta^{13}C$) of individual plants at the same severe treatment as for biomass (FI-17), whereas TaDREB1A correlated positively with $\delta^{13}C$ at the mild stress FI-12 (Fig. 3). No correlation existed for any of the genes and growing conditions between induction ratios and the oxygen isotope composition ($\delta^{18}O$). The induction ratios for GS1, GS2, TaDREB1A and TaDREB2B genes were (positively) correlated with nitrogen isotope composition ($\delta^{15}N$) across individual plants only at FI-17, whereas no correlations were found for NHX1. Therefore from the three isotopes $\delta^{15}N$ was the (best performer) most homogeneous in terms of correlations with gen induction ratios and levels of expression NHX1 were the less related with isotopic signatures.

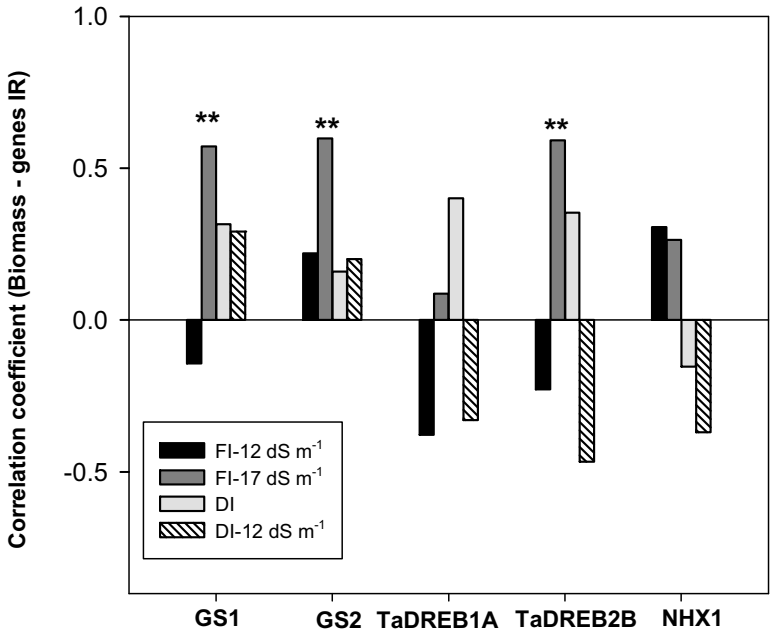

Figure 2. Correlation coefficients of the linear relationships, across plants within each of the stress conditions, between the rate of expression of each of the five genes (GS1, GS2, TaDREB1A, TaDREB2B, NHX1) and shoot biomass. Abbreviations of treatments as in Table 2.

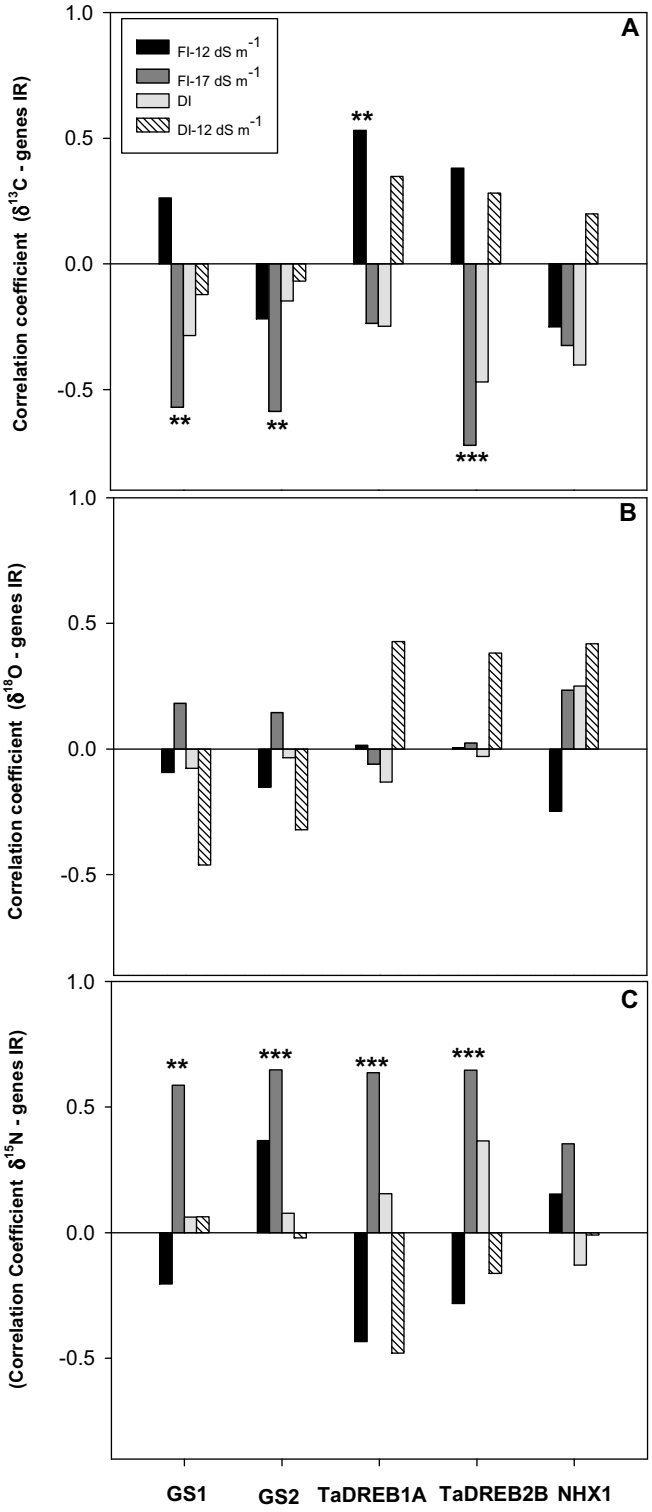

Figure 3. Correlation coefficients of the linear relationships, across plants within each of the stress conditions, between the rate of expression of each of the five genes (GS1, GS2, TaDREB1A, TaDREB2B, NHX1) and (A) carbon isotope composition ($\delta^{13}C$), (B) oxygen isotope composition ($\delta^{18}O$) and (C) nitrogen isotope composition ($\delta^{15}N$). Abbreviations of treatments as in Table 2.

Relationships between transcript levels and N metabolism traits

Again of the two genes where induction ratio was significantly affected by treatments (GS2 and TaDREB1A) only the second one showed a clear pattern of relationships across environments with N metabolism traits. Thus for example TaDREB1A related with N content in the same manner as for the biomass and $\delta^{15}N$: relationship was exponential negative, with no decrease in N beyond induction ratios between 5-10 (Fig. 1b).

Induction ratios of GS1, GS2 and TaDREB2B correlated positively with N content (N) at FI-17, whereas TaDREB1A correlated negatively with N at FI-12 and DI-12 and TaDREB2B also negatively with DI-12 (Fig. 4). Therefore correlations changed of sign depending on the stress conditions. By contrast the sign of the relationships were always the same (positive) when induction ratios were correlated against the activities of the Glutamine Synthase (GS) and the Nitrate Reductase (NR). Thus GS activity were positively correlated with induction ratios of GS1 (at FI-17 and DI), GS2 (FI-17), TaDREB1A (FI-17 and DI) and TaDREB2B (FI-17 and DI), whereas NR activity was correlated with induction ratios of GS1 (FI-17), GS2 (FI-17, DI-12), TaDREB2B (FI-17 and DI) and NHX1 (FI-17).

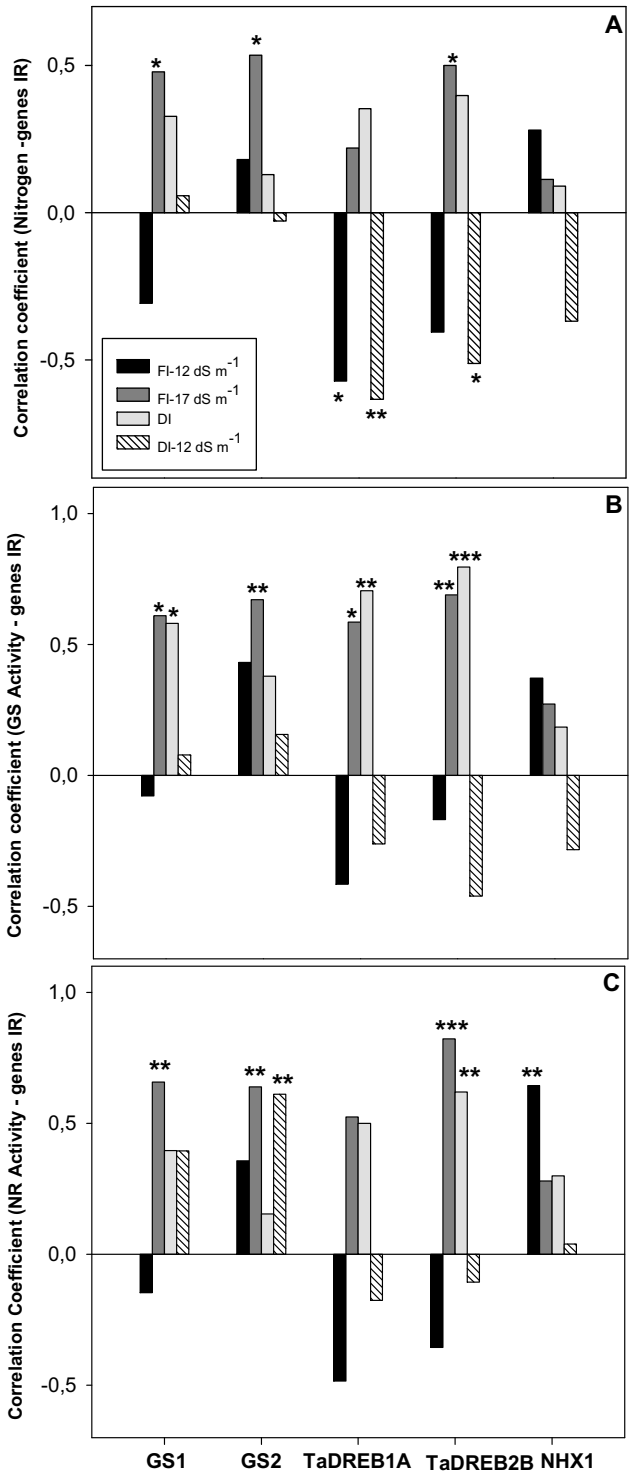

Figure 4. Correlation coefficients of the linear relationships, across plants within each of the stress conditions, between the rate of expression of each of the five genes (GS1, GS2, TaDREB1A, TaDREB2B, NHX1) and (A) nitrogen content (N), (B) glutamine synthetase activity (GS) and (C) nitrate reductase activity (NR). Abbreviations of treatments as in Table 2.

Levels of GS1 and GS2 enzymes

No matter the growing conditions GS1 was less abundant than GS2 enzyme (Fig. 5). In agreement with a better performance of tolerant genotypes the levels of GS1 and GS2 enzymes were higher in the tolerant compared with the susceptible genotypes an regardless of the growing conditions. However and except for Whereas the two most severe stresses (FI-17 and DI-12) decreased of the levels of both enzymes in the two susceptible genotypes (RIL24 and RIL30) no clear effect of the growing conditions was shown for the moderate stresses in the susceptible genotypes for any of the stresses in the tolerant ones.

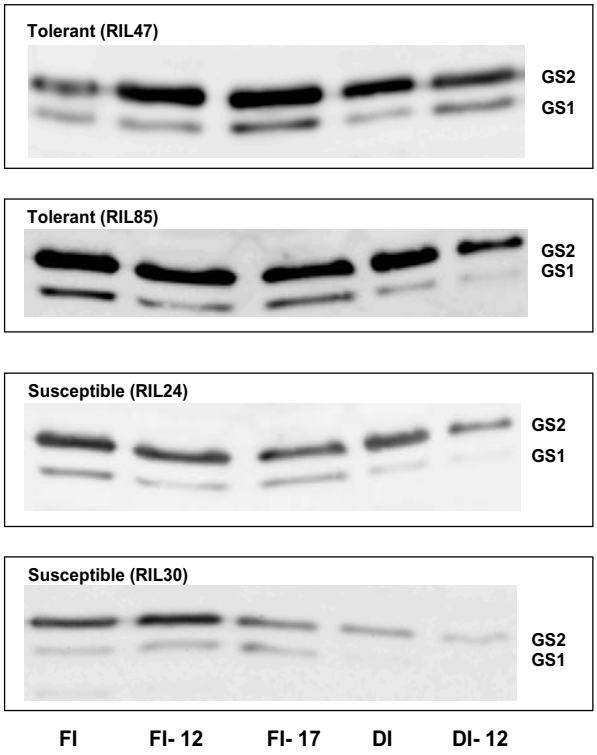

Figure 5. Western blot of the citosolic (GS1) and plastidic (GS2) glutamine synthetase of the two tolerant and two susceptible genotypes at each of the five different growing conditions. Abbreviations of treatments as in Table 2.

Discussion

Expression of DREB genes

DREB1 and DREB2 are two main subgroups of DREB subfamily, apparently involved in two different signal transduction pathways under cold and dehydration respectively (Lata *et al.,* 2011b). Thus DREB1 genes, including DREB1A, DREB1B, and DREB1C, are reported to be rapidly and transiently induced by cold, but not by dehydration and high-salinity stress (Liu *et al.,* 1998). By contrast, DREB2A and DREB2B genes are induced upon dehydration and high salinity, but not by cold stress (Liu *et al.,* 1998; Nakashima *et al.,* 2000, 2009; Agarwal *et al.,* 2006). However in our study with durum wheat TaDREB1A was induced and TaDREB2B down regulated by salinity and water stress. In that sense Pellegrineschi *et al.* (2004) report that stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delayed water stress symptoms. Moreover Egawa *et al.* (2006) and Kurahashi *et al.* (2009) reported in different wheat cultivars, that the TaDREB1 gene is induced not only by low temperature but also by salinity and drought. In the same sense transgenic *Arabidopsis* and rice plants overexpressing OsDREB1A also displayed tolerance to low temperatures, high salinity and drought (Dubouzet *et al.,* 2003; Ito *et al.,* 2006). However Shen *et al.* (2003) reported that TaDREB1 (A DREB1-type transcription factor isolated from wheat) was strongly induced by cold but it responded poorly to drought, salinity and ABA. Concerning TaDREB2, Egawa *et al.* (2006) reported that the expression of a DREB2 homolog isolated in bread wheat was activated (among other stresses) as response to drought and salinity. In the same sense transgenic *Arabidopsis* plants overexpressing *OsDREB2B* showed enhanced expression of DREB2A target genes and improved drought and heat-shock stress tolerance (Matsukura *et al.,* 2010).

Nemoto & Sasakuma (2002) report that most of the early salt-stress responding genes (WESR1–4) in bread wheat (*Triticum aestivum* L.) were induced by both osmotic stress and salinity. In our results transcription rate of TaDREB2B tended to decreased as response to stress. However rate of expression may change during the exposition to stress, either increasing followed by decreases or vice versa (Lata *et al.,* 2011b). For example Latini *et al.* (2007) report the isolation and characterization of a gene in

Triticum durum, namely TdDRF1, which belongs to the DREB gene family and produces three forms of transcripts with consistent differences among the three isoforms of TdDRF1 as response to water stress. Egawa et al. (2006) report in bread wheat that both DREB1 and DREB2 are induced by dehydration; therefore osmotic stress such as salinity and water stress may down regulate their expression.

Concerning genetic variability, in our study the tolerant genotypes exhibited at FI-12 and DI-12 lower transcription rates than susceptible ones for both TaDREB1A TaDREB2B, whereas the opposite occurred only for TaDREB2B at FI-17 and DI. However Kurahashi *et al.* (2009) reported in bread wheat a higher transcription rate of TaDREB1 as response to water stress. Nevertheless differences in the ploidy level between durum wheat (tetraploid) and bread wheat (hexaploid) may be also behind the differences in pattern of expression. Thus in a study where 17 synthetic hexaploid wheat lines, produced by crossing the durum wheat (i.e. tetraploid) wheat cultivar with 17 accessions of *Ae. tauschii*, showed that Allopolyploidization altered the expression levels of the stress-responsive genes in synthetic wheats (Kurahashi *et al.*, 2009).

Different genes exhibited different transcription rates as well as not the same pattern between tolerant and susceptible genotypes. Thus Hamid *et al.* (2011) working with *Atriplex halimus* conclude that DREB was regulated by the osmotic component but not by the ionic one of salt stress. It seemed that DREB was not involved in the regulation of sodium manipulating genes like **NHX1,** SOS1 or H^+-PPase. Moreover, DREB was the most upregulated gene under salt (fivefold) and drought (twofold) conditions, which reinforced the importance of this gene in A. halimus tolerance to stress. This agree with the differences we observed between treatments in the rate of transcription of TaDREB1A.

NHX1 expression and ion accumulation.

Shi *et al.,* (2002) demonstrated for the AtNHX1 transcript up-regulation following treatment with NaCl, KCl or ABA, as well as detecting strong expression in guard cells and root hairs, suggesting AtNHX1 plays a role in pH regulation and/ K^+ homeostasis

along with storing Na^+ in the enlarged vacuoles in root hair cells, respectively. Our results agree in the sense that the different salinity treatments increased the rate of expression of the NHX1 gene. Even the water stress upregulated the expression of this gene. In fact there have been many examples of increased salt tolerance (as well as drought tolerance) resulting from overexpression of the NHX family of Na^+/H^+ antiporters from various plant species, including wheat (Xue *et al.,* 2004) and other plants (Xu *et al,* 2009; Rowley & Mockler, 2011).

Xue *et al*., (2004) reported that when AtNHX1 was overexpressed in wheat in moderately saline soil, salinity tolerance increased in the best transgenic line. However Na^+ concentration in the shoot decreased which makes difficult to know how the antiporter was acting. In our study we did not found relationship between the level of expression of NHX1 and the biomass or even the concentration of Na^+ or K^+. In fact current understanding of the overall control of Na+ accumulation and of osmotic stress tolerance at the whole-plant level is limited (Munns & Tester, 2008).

In spite the fact expression of NHXI gene was higher in the tolerant genotypes no correlations were found between NHXI expression and biomass within each of the growing conditions. In that sense Husain *et al*., (2003) reported that in durum wheat the Na^+ exclusion trait may be relevant preventing leaf injury and enhancing yield at moderate salinity.

In wheat, correlation between the expression of genes encoding NHX1 antiporters in salt-tolerant cultivars and their salt tolerance was shown (Saqip *et al.,* 2005) suggesting that the higher expression of endogenous vacuolar Na^+/H^+ antiporters in roots and shoots of the salt-resistant wheat genotypes facilitated Na^+ exclusion from the cytosol, improving salt tolerance (Saqip *et al.,* 2005). However, it was reported that NHX1 proteins act not only as Na^+/H^+ antiporters under salinity but possess also K^+/H^+ exchange activity with similar efficiency (Venema *et al.,* 2002). The NHX1 gene is expressed in roots and leaves of wheat, and selectively transports Na^+ into the vacuole, as well as K^+ in nonsaline conditions (Apse *et al*., 2003; Munns, 2005).

Another factor explaining the lack of relationships between the expression of NNX1 and biomass is the fact that while these transporters are critical determinants of salt tolerance there is only little evidence linking them to salt stress perception (Horie *et al.,* 2009). This has been observed in Arabidopsis with a group of transporters, the Na^+/H^+ exchanger AtNHX1, and the close orthologues AtNHX2, 3, 4, 5 and 6 which are involved in compartmentalization of Na^+ to the vacuole (Horie *et al.,* 2009; Qiu *et al.,* 2004; Yokoi *et al.,* 2002).

GS genes and nitrogen metabolism

Expression of GS1 and GS2 genes were positively correlated with biomass, better nitrogen metabolism (higher N content and $\delta^{15}N$ and activities of NR and GS) water status (i.e. lower $\delta^{13}C$) but only at high salinity (FI-17). Bernard and Habash (2009) conclude that cytosolic GS genes, transcripts and proteins are responsive to both the plant N status and environmental cues. Overall, this suggests that GS may constitute a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and seed production.

During the vegetative stage, GS2 is the predominant isozyme in the leaf mesophyll cells, where it assimilates ammonia originating from nitrate reduction and photorespiration (Tobin & Yamaya, 2001). In our results with wheat at the vegetative stage the Western blot shows GS2 is more abundant than GS1 regardless of the growing condition even when the tolerant genotypes showed an increase in GS1 as response to high salinity. However our results doesn't agree with that reported in the sense that in response to drought or salt stress, the abundance of the GS2 polypeptide and its activity decline, whereas cytosolic GS tends to increase or maintain the same level in the leaves (Bauer *et al.,* 1997; Lutts *et al.,* 1999; Santos *et al.,* 2004; Martinelli *et al.,* 2007). Expression of GS genes in leaves in wheat was developmentally regulated, with both GS2 and GS1 assimilating or recycling ammonia in leaves. During leaf senescence the cytosolic isozymes GS1 was a predominant form, suggesting major roles in assimilating ammonia. A preliminary analysis of three different wheat genotypes showed that the ratio of leaf GS2 protein to GS1 protein was variable (Bernard *et al.,* 2008).

In fact the role of GS2 may be also important in plant growth. A recent study on bread wheat suggests that certain TaGS2 haplotypes may be valuable in breeding varieties with improved agronomic performance and N-use efficiency (Li *et al.,* 2011). In fact in our results GS2 also correlated positively with biomass, nitrogen metabolism and water status in a rather similar manner than for GS1 and mostly at high salinity (FI-17).

The response of cytosolic GS to abiotic and biotic stresses occurred mainly at the transcript abundance levels. However, it is unclear whether an increase in transcription occurs, or whether mRNA transcripts are more stable (Bernard & Habash, 2009). Moreover GS activity is also regulated post-translationally (Miflin & Habash, 2002). The complex regulation of cytosolic GS at the transcriptional to post-translational levels is key to the establishment of a specific physiological role for each isoenzyme (Bernard & Habash, 2009). This would justify in our results the lack of relationship across genotypes between the expression level for GS1 gene and either the activity of the GS enzyme (in three of the 5 growing conditions). In the same sense expression for GS2 gene was correlated with GS activity at FI-17. Moreover no clear relationships existed between the transcription rates of GS1 and GS2 and the amount of both enzymes determined by Western blot. The two growing conditions that showed positive genotypic correlations between expression rates of GS genes (GS1 and GS2) and biomass and nitrogen status were the only ones (FI-17 and DI) which exhibited a decrease in the expression rate compared with control conditions.

Transgenic poplar plants expressing a pine cytosolic GS gene show enhanced water stress resistance compared with controls (El-Khatib *et al.,* 2004). These results suggest that GS overexpression leads to an increased photorespiratory activity, thus providing a protective sink for electrons from photosynthetic reaction centres (although the photoprotective role of photorespiration remains controversial). Moreover the response of cytosolic GS to osmotic stress is related to the reassimilation of nitrogen from increased amino acid catabolism, and is necessary for the production of proline (Brugière *et al.,* 1999).

Cai *et al.* (2009) reported in rice that overexpression of cytosolic rice GS increased metabolic level, with plants showing higher total GS activities and soluble protein concentrations in leaves and higher total amino acids and total nitrogen content in the whole plant. However some of these transgenic plants exhibited higher sensitivity to salt, drought, and cold stress conditions, whereas the other two types of GS-overexpressed plants failed to show any significant changes for these stress conditions compared with wild-type plants (Cai *et al.,* 2009). These results contrast to those of previous studies: overexpressing GS2 in rice resulted in increased salt and cold tolerance (Hoshida *et al.,* 2000) and transgenic poplar plants overexpressing cytosolic GS1 were characterized by enhanced tolerance to water stress (Pascual *et al.,* 2008). Li *et al.* (2011) conclude that certain TaGS2 haplotypes may be valuable in breeding wheat varieties with improved agronomic performance and N-use efficiency.

Salt and water stresses can increase the proteolytic activity and decrease the protein synthesis in cells, which causes an accumulation of free amino acids. This, in turn, might reduce the need for ammonium incorporation to form amino acids and indirectly cause the excessive accumulation of ammonium. Thus, under stress conditions a higher transcriptional level in GS1 overexpressed plants may induce more ammonium absorption in roots, leading to a greater accumulation of ammonium, which may be toxic and induce cell senescence (Cai *et al.,* 2009). In fact in our results the relationship of GS1 (and GS2) with biomass and nitrogen metabolism was significant only at high salinity level (FI-17) and was also positive related with a better water status (lower $\delta^{13}C$).

GS plays a pivotal role in re-assimilation ofNH_4^+ in higher plants. The GS activity and the degree of proline accumulation were relatively lesser in salt susceptible cultivar than salt tolerant cultivars of foxtail millet (Veeranagamallaiah *et al.,* 2007). Moreover GS synthetase amount and activity increased as response to NaCl stress in both tolerant and susceptible cultivars of foxtail millet indicates in relation with proline biosynthesis (Veeranagamallaiah *et al.,* 2007). These authors report in five-day-old seedlings germinated in dark for 5 days an increase (using Western blotting) in the expression pattern (i.e. in the amount) of GS as response to salinity. In our results

under more real growing conditions salinity and water stress tended to decrease expression rate compare with control plants.

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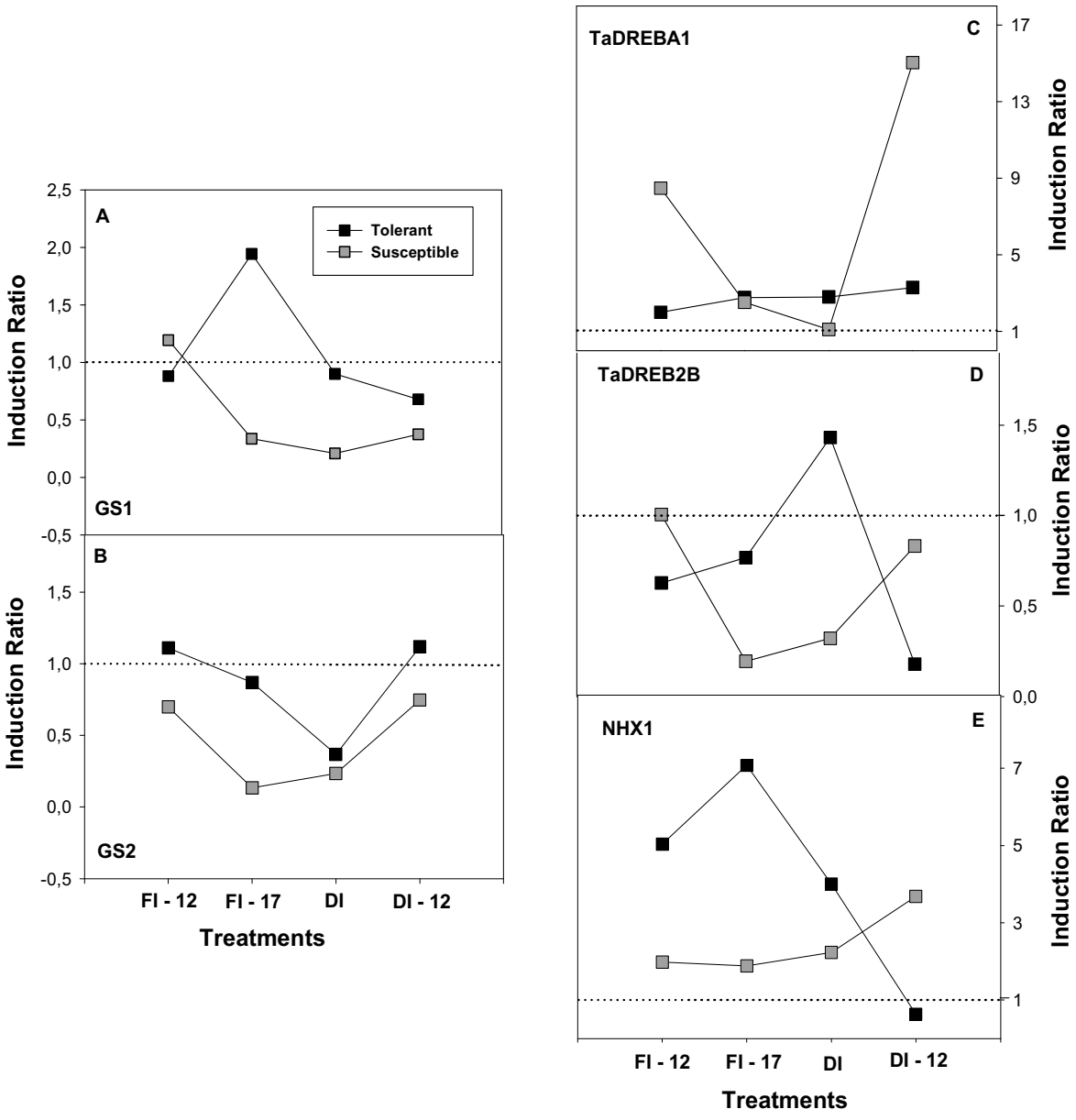
Supporting Information

Figure S1. Induction ratio values for each gene of the tolerant and the susceptible genotype for each of the four different stress conditions (FI-12, FI-17, DI, DI-12). The values shown are the means of two genotypes. Abbreviations of treatments as in Table 2.



DISCUSIÓN GENERAL

DISCUSIÓN

Es de importancia estratégica para la agricultura mediterránea desarrollar nuevas variedades de trigo duro con mayor producción potencial, junto a una mejor adaptación a las condiciones ambientales principales que limitan la productividad, tales como la salinidad. Según Munns en sus revisiones bibliográficas sobre el tema (2002, 2003, 2005, 2006, 2008) en los últimos 20 años los fisiólogos han dedicado grandes esfuerzos a recopilar información descriptiva acerca del efecto de la salinidad sobre las plantas, tratando de localizar caracteres que confieran tolerancia a la salinidad. Sin embargo, a pesar de la amplia información existente, todavía no existe un consenso generalizado en muchos puntos fundamentales a la hora de abordar un programa de mejora para condiciones salinas. A tal efecto es necesario perfeccionar los programas actuales con metodologías más eficientes a fin y efecto de poder elegir criterios multidisciplinarios y protocolos eficientes de fenotipeado, junto con un conocimiento más profundo de los mecanismos fisiológicos, metabólicos y moleculares que modulan las respuestas de las plantas a la salinidad. En esta Tesis tratamos de evaluar algunas respuestas de genotipos de trigo duro a la salinidad usando varios criterios de selección enmarcados en distintas disciplinas. En primer lugar se estudiaron características morfo-fisiológicas esenciales para la selección tales como la biomasa, el contenido en clorofila, la concentración de iones y los parámetros de intercambio de gases para obtener información sobre el comportamiento y el crecimiento de las plantas crecidas en condiciones salinas. Estos parámetros se complementaron con las composiciones de diferentes isótopos estables, específicamente la composición isotópica del carbono ($\delta^{13}C$), uno de los criterios más usados e informativos en los estudios de adaptación de trigo a la sequía, junto con isótopos menos estudiados, como son las composiciones isotópicas del oxígeno ($\delta^{18}O$) y del nitrógeno ($\delta^{15}N$) . Para profundizar al máximo en la información aportada por las firmas isotópicas, se estudió el uso combinado de los tres isótopos estables $\delta^{13}C$, $\delta^{18}O$, y $\delta^{15}N$ y sus relaciones con el metabolismo del nitrógeno; específicamente el contenido total de nitrógeno (N) y las actividades de glutamina sintetasa (GS) y la nitrato reductasa (NR), dos enzimas clave en los procesos de asimilación y captación

del nitrógeno en las plantas (Capítulo 3). También se han estudiado qué fracción de dichos isótopos (sea la materia seca total o la fracción soluble en agua, que reflejan la actividad fotosintética de la planta a largo o corto plazo, respectivamente) es la más adecuada evaluando la variabilidad genética y el efecto de diferentes niveles de estrés (Capítulo 4).

Muchos autores informan que los marcadores fisiológicos del estrés son efecto de la transducción de una señal genética a la hora de la percepción del estrés por las plantas. A tal efecto, en esta Tesis se analizó, por un lado la respuesta genética de plantas de trigo duro a la salinidad a través de la expresión de diferentes genes: incluyendo factores de transcripción que responden frente al estrés hídrico y salino, un gen relacionado con el acúmulo de sodio en la vacuola y genes asociados al metabolismo del nitrógeno (específicamente que codifican por diferentes GS) (Capítulo 6). Por otro lado, en el mismo capítulo se han estudiado las relaciones entre los niveles de transcripción de dichos genes frente a las respuestas fisiológicas de la planta, incluyendo la biomasa acumulada, las firmas isotópicas, el contenido total de nitrógeno (N), las actividades de glutamina sintetasa (GS) y nitrato reductasa (NR) y la concentración de iones, con el objetivo de evaluar el comportamiento genotípico de trigo duro frente a diferentes niveles de estrés.

1 - Salinidad y estrés hídrico: ¿Qué tienen en común?

Las respuestas de las plantas al estrés salino e hídrico tienen mucho en común. La salinidad reduce la capacidad de las plantas para absorber agua y esto da lugar de manera rápida a una disminución en la tasa de crecimiento debido a una serie de cambios metabólicos idénticos a los causados por el estrés hídrico. Munns y Tester (2008) confirmaron que las plantas responden a la salinidad en dos fases: una primera fase que es rápida y que provoca un estrés osmótico idéntico al estrés hídrico y una fase posterior más lenta, debida a la acumulación de Na^+ en las hojas y que se caracteriza como un estrés iónico o tóxico que provoca una aceleración de la senescencia de las hojas maduras. La salinidad afecta el estado hídrico de la planta

mediante su efecto sobre el potencial osmótico del agua del suelo. Un incremento de salinidad disminuye el potencial osmótico dando lugar a un estrés hídrico que a su vez afecta la conductancia estomática y la fotosíntesis (West et al., 1986; Yeo et al., 1985). Nuestros resultados muestran que tanto la salinidad como el estrés hídrico inducen una disminución de la fotosíntesis (A_{sat}) y la transpiración (T) a causa de limitaciones estomáticas (Tablas 3, 2, 1 y 1 en los Capítulos 1, 2, 3 y 4 respectivamente). Heuer y Plaut (1989) indicaron que la salinidad y el estrés hídrico afectan la difusión del CO_2 en las hojas debido a una disminución en la conductancia estomática. La disminución en el cociente C_i/C_a (indistintamente expresado como P_i/P_a) encontrada en nuestros resultados confirma la hipótesis de que las limitaciones en la actividad fotosintética en los tratamientos salinos son básicamente de índole estomático (el componente hídrico del estrés salino es el que está afectando la actividad fotosintética) más que causadas por una alteración intrínseca del aparato fotosintético. Aparte de la disminución provocada por la salinidad en las tasas de los parámetros fotosintéticos, se ha observado un aumento en composición isotópica del carbono ($\delta^{13}C$) (o una disminución en la discriminación, $\Delta^{13}C$) en todos los capítulos de la Tesis en experimentos bajo condiciones salinas. Una disminución de $\Delta^{13}C$ es una respuesta típica a un descenso del cociente C_i/C_a causada por una limitación estomática de la fotosíntesis que frecuentemente es debida a un estrés hídrico (Farquhar et al., 1982; Acevedo 1993; Araus et al., 1997; Araus et al., 2002). Los resultados obtenidos en los parámetros de intercambio de gases y en la $\delta^{13}C$ bajo nuestras condiciones experimentales nos permiten concluir que una gran parte del efecto de la salinidad sobre las plantas es resultado de un estrés hídrico causado por un efecto osmótico de la sal (disminuyendo el potencial hídrico) en el medio de crecimiento. Esto fue evidente en todos los experimentos de nuestra Tesis que en general implicaban exposiciones a la salinidad relativamente largas (ver más abajo). En este sentido, la reducción inicial en el crecimiento de las plantas bajo condiciones salinas es probablemente debida a las señales hormonales generadas por las raíces como respuesta a un efecto osmótico del estrés salino que causan un cierre estomático (Munns et al., 2006). Sin embargo, los efectos posteriores y específicos de la sal pueden tener un impacto sobre el crecimiento si la cantidad de la sal acumulada por la

planta llega a niveles excesivos. Según Munns (2008), la segunda fase del estrés salino o fase específica de la toxicidad separa claramente entre especies y genotipos que difieren en la capacidad de tolerancia a los suelos salinos. Los efectos específicos de la sal desarrollados con el tiempo en la planta, suponen un estrés adicional al estrés osmótico debido a la incapacidad de producir fotoasimilados, y dan lugar a dos categorías de plantas: "sensibles" y "tolerantes" a la sal. Los procesos que adaptan específicamente una planta a los suelos salinos implican la regulación de la absorción y de la compartimentación de la sal dentro de la planta, para retrasar al máximo el momento en que se acumula a niveles tóxicos en las hojas fotosintéticamente activas. De acuerdo con esto, indicamos que en los experimentos analizados en esta Tesis, las plantas se expusieron a la salinidad durante un período relativamente largo (2 meses en el experimento I, casi 3 semanas en el experimento II y aproximadamente un mes en el experimento III), lo que significa que la prevención de la aparición de la toxicidad provocada por la acumulación del sodio podría ser una prioridad para las plantas, en lugar de evitar la escasez de agua inducida osmóticamente por un déficit hídrico. Sin embargo, aunque categorizar las plantas como susceptibles o tolerantes a la salinidad en función de la respuesta frente al acúmulo de iones es un enfoque muy útil en ecología vegetal o para separar distintas especies en base a su comportamiento frente a la sal (Isla et al., 1998; Munns y James, 2003) los resultados de esta Tesis no apoyan el que sea un criterio adecuado para evaluar variabilidad genotípica en tolerancia a la salinidad dentro de una misma especie. Así en nuestros trabajos el acúmulo de iones (Na^+, K^+, Mg^{2+}, Ca^{2+} y sus cocientes) no llegaba a separar tan adecuadamente entre genotipos sensibles y tolerantes a la salinidad (Tablas 6 y S3 de los Capítulos 1 y 3, respectivamente) como los isótopos estables, aunque si fueron criterios adecuados para separar entre condiciones de cultivo (Tablas 4, 3 y 3 de los Capítulos 1, 2 y 3, respectivamente).

2 - ¿Cómo los isótopos estables pueden contribuir a una mejora más eficiente para tolerancia a la salinidad?

Durante los últimos años, ha aumento el interés por los estudios de la abundancia natural de los isótopos estables del carbono (expresado como composición -$\delta^{13}C$- o discriminación -$\Delta^{13}C$), oxígeno (como composición -$\delta^{18}O$- o enriquecimiento - $\Delta^{18}O$) y nitrógeno (como composición -$\delta^{15}N$- o enriquecimiento $\Delta^{15}N$) en fisiología de cultivos. Sin embargo, hasta la fecha, sólo los isótopos del carbono se han investigado ampliamente como herramientas de fenotipeado y se han propuesto para su aplicación en la mejora de plantas.

Los resultados de esta Tesis han demostrado que los isótopos estables del carbono, oxigeno y nitrógeno son criterios eficientes y específicos para evaluar las respuestas de genotipos de trigo duro a diferentes condiciones salinas. Munns y James (2003) sugirieron que la selección por una conductancia estomática (g_s) alta puede ser la manera más eficaz para la selección de genotipos que crecen rápidamente en suelos salinos. Sin embargo, nuestros resultados mostraron que la conductancia estomática (g_s) y otros parámetros de intercambio gaseoso no pueden diferenciar entre genotipos tolerantes y susceptibles a la sal (Tabla S1 del Capítulo 3). La razón estribaría en el carácter instantáneo de estas mediciones sujetas por lo tanto a variaciones en función de cambios en el nivel de estrés que sufre la planta o incluso ciclos diarios en las tasas de intercambio gaseoso (Araus et al., 2002). Por el contrario los isótopos estables de carbono tendrían un mejor comportamiento. Así el Capítulo 2 de esta Tesis muestra que la $\Delta^{13}C$ fue el primer parámetro escogido (por delante de g_s y otros parámetros de intercambio gaseoso) para explicar diferencias genotípicas en biomasa en salinidad moderada y severa (Tabla 4 del Capítulo 2). Además de la composición isotópica del carbono, la $\delta^{15}N$ (Capítulos 1, 3 y 5), así como la $\delta^{18}O$ (Capítulos 3, 5) nos permitieron detectar tanto diferencias entre tratamientos, como variabilidad genotípica en trigo duro asociada a la tolerancia y la susceptibilidad frente a la sal. Las firmas isotópicas en nuestro caso, eran mejores criterios que los parámetros más convencionales, tales como la concentración de iones o los parámetros de intercambio de gases que

separaron perfectamente entre tratamientos pero fueron inadecuados para evaluar diferencias genotípicas bajo determinadas condiciones de estrés salino (Capítulos 1, 2, 3).

2 - 1 ***La composición isotópica del carbono ($\delta^{13}C$): Papel importante en la selección de genotipos tolerantes a la sal***

Farquhar y Richards (1984) y Farquhar et al. (1989) indicaron que la composición isotópica del carbono ($\delta^{13}C$), con frecuencia expresada como la discriminación isotópica ($\Delta^{13}C$), medida en materia seca proporciona información sobre la eficiencia del uso de agua a largo plazo en plantas C_3. Tanto los dos niveles de salinidad estudiados (moderada y severa) como el estrés hídrico o la combinación de ambos provocaron un aumento en $\delta^{13}C$ (o disminución en $\Delta^{13}C$) en comparación con el control (Capítulos 1, 2, 3 y 4). Las condiciones que inducen el cierre de estomas, tales como la salinidad o el déficit hídrico restringen el suministro de CO_2 a los sitios de carboxilación, que luego aumentan la $\delta^{13}C$ (o disminuyen $\Delta^{13}C$) de la materia vegetal (Farquhar et al., 1989).

Según Araus et al. (2003) cuando el estrés hídrico moderado es el factor limitante del rendimiento en plantas de trigo duro, surge una correlación positiva entre $\Delta^{13}C$ y el rendimiento (o negativa entre $\delta^{13}C$ y rendimiento). En esta Tesis la $\delta^{13}C$ se correlacionaba negativamente con la biomasa y el contenido en nitrógeno (N) (Figura 3 del Capítulo 3), lo que significa que los genotipos tolerantes a la sal con mayor g_s y con menor $\delta^{13}C$ son los que tienen mayor producción de biomasa y mayor contenido en N. Sin embargo, la relación negativa observada entre $\Delta^{13}C$ y biomasa en los Capítulos 1 y 2 (Tabla 7 del Capítulo 1 y Figura 7 del Capítulo 2) sugiere que los genotipos con menor g_s (y por lo tanto una menor transpiración) fueron los más tolerantes a la salinidad. Esta discrepancia entre experimentos en el signo de las correlaciones entre firma isotópica del carbono y biomasa sugiere que el efecto de estrés hídrico causado por la salinidad puede interaccionar con un efecto de toxicidad asociada al acúmulo de iones y al desarrollo de la senescencia. En este sentido es

posible que para unas condiciones de salinidad determinadas los genotipos más tolerantes fueran aquellos que mantengan un menor flujo transpiratorio en orden a no favorecer el acúmulo de iones en las hojas a la vez que retrasen la senescencia de estas hojas. En los dos casos los genotipos tolerantes a la salinidad mostrarían una menor $\Delta^{13}C$ (o mayor $\delta^{13}C$) que los susceptibles. Tanto una menor conductancia estomática como una mayor actividad intrínseca fotosintética asociada a una senescencia retrasada podrían aumentar el C_i/C_a y en consecuencia disminuir $\Delta^{13}C$ (o aumentar la $\delta^{13}C$) (Farquhar et al., 1982, 1989; Araus et al., 1997; Brugnoli et al., 1988). De acuerdo con esto, estos mismos autores indican que en las plantas C_3 la discriminación (Δ) contra ^{13}C por la enzima carboxilación está asociada con la fotosíntesis a través del cociente C_i/C_a. Este índice refleja las magnitudes relativas de asimilación neta (A) y la conductancia estomática (g_s) que se relacionan respectivamente con la demanda y la disponibilidad del CO_2. Posiblemente las condiciones del experimento (duración del estrés, estado fenológico en los momentos de la aplicación del estrés y posterior cosecha) podrían estar implicados en estas divergencias. Este es un tema para futuros estudios.

En cualquier caso la firma isotópica del carbono puede ser un buen indicador para evaluar el comportamiento genotípico bajo condiciones salinas discriminando entre tolerantes y susceptibles (Tablas 6 y S1 de los Capítulos 1 y 3, respectivamente). Sin embargo, los parámetros de intercambio de gases y la concentración de iones (Tablas S1 y S3 del Capítulo 3; y datos no mostrados del Capítulo 2) analizados distinguían muy bien entre tratamientos pero no presentaban ninguna diferencia significativa entre genotipos tolerantes y susceptibles de trigo duro bajo condiciones salinas.

2 - 2 La composición isotópica del oxigeno ($\delta^{18}O$): ventajas e inconvenientes

Alternativamente a la composición isotópica del carbono, la composición isotópica del oxígeno medida en la biomasa de la planta ($\delta^{18}O$) parece reflejar la variación en: (i) la composición isotópica de la fuente del agua; (ii) enriquecimiento evaporativo en las

hojas debido a la transpiración; y (iii) fraccionamiento bioquímico durante la síntesis de la materia orgánica (Yakir, 1992; Farquhar y Lloyd, 1993). La firma isotópica del oxígeno se puede representar como enriquecimiento respecto a la fuente del agua utilizada por la planta ($\Delta^{18}O$) y de esta forma eliminamos el primero de los tres factores que pueden afectar esta firma isotópica. Sin embargo es frecuente expresar esta firma isotópica directamente como $\delta^{18}O$, debido a la dificultad de conocer con certeza la $\delta^{18}O$ del agua (o aguas de riego y lluvia) a lo largo del cultivo y por el hecho de que la $\delta^{18}O$ del agua sea la misma para todos los genotipos evaluados.

La salinidad sola o combinada con estrés hídrico aumenta la composición isotópica del oxigeno ($\delta^{18}O$) (Tabla 1 del Capítulo 3). Según nuestra información es el primer estudio de $\delta^{18}O$ en trigo duro bajo condiciones salinas ya que en general se han hecho pocos estudios sobre la composición isotópica del oxigeno. Para unas condiciones de demanda evaporativa constantes una alta $\delta^{18}O$ puede estar relacionada con una disminución en la g_s y una menor transpiración (Barbour, 2007; Farquhar et al., 2007). En conformidad con esta teoría, los genotipos susceptibles a la sal (Tabla 1 del Capítulo 3) presentaron una alta $\delta^{18}O$ y una baja g_s, mientras que para los genotipos tolerantes a la sal, la $\delta^{18}O$ era más baja y la g_s, más alta. Según varios autores (Barbour et al., 2000a, Barbour, 2007; Scheidegger et al., 2000; Cernusak et al., 2009) la $\Delta^{18}O$ (o la $\delta^{18}O$) puede ayudar en la separación de los efectos independientes de la fotosíntesis y la conductancia estomática sobre $\Delta^{13}C$ ($\delta^{13}C$) en plantas C_3 tales como el trigo. Respecto a esto, las relaciones entre las actividades enzimáticas de la nitrato reductasa (NR) y la glutamina sintetasa (GS) con $\delta^{18}O$ eran mucho más débiles que las observadas entre los dos enzimas y $\delta^{13}C$ confirmando el hecho de que $\delta^{18}O$ no está directamente asociada con la fotosíntesis como $\delta^{13}C$ (Figura 5 del Capítulo 3).

La $\delta^{18}O$ ha sido un buen criterio para la separación entre diferentes condiciones de crecimiento en nuestro estudio. Sin embargo, la correlación entre $\delta^{18}O$ y biomasa no ha tenido una tendencia clara (Figura 3 del Capítulo 3) excepto cuando quitamos los tratamientos más severos (salinidad alta y combinación de salinidad y déficit hídrico), ya que entonces la $\delta^{18}O$ se correlacionó fuertemente con la biomasa. (Figura 3 insertada del Capítulo 3). Por otra parte, no hubo separación entre genotipos

tolerantes y susceptibles a la sal en los mismos tratamientos severos (Tabla S1 del Capítulo 3). Este resultado de la $\delta^{18}O$ en condiciones de estrés severo nos hace pensar que puede ser efecto de su sensibilidad a las condiciones ambientales y a los cambios en g_s. De hecho muchos autores confirman que la $\delta^{18}O$ depende de determinados factores ambientales que afectan a la transpiración, tales como la humedad del aire y la temperatura (Barbour y Farquhar, 2000; Barbour et al., 2000a; Scheidegger et al., 2000; Helliker y Ehleringer, 2002). En este sentido y teniendo en cuenta la fuerte dependencia de la composición isotópica del oxigeno ($\delta^{18}O$) de la conductancia estomática (g_s), bajo nuestras condiciones de estrés severo las tasas de g_s han disminuido de manera extrema muy rápidamente lo que probablemente sea la causa de la incapacidad de detectar diferencias genotípicas en estas condiciones usando $\delta^{18}O$ como criterio de evaluación. Por contra el descenso de C_i/C_a asociado al cierre estomático puede ser menor por lo que quedaría margen para expresar las diferencias genotípicas en $\delta^{13}C$. Otros factores a considerar es la precisión analítica, mayor normalmente en los análisis de $\delta^{13}C$ que en los de $\delta^{18}O$.

2 - 3 La composición isotópica del nitrógeno ($\delta^{15}N$): una manera de trazar la importancia de la vía del nitrógeno en la adaptación y la selección genotípica

La variación natural en la composición isotópica del nitrógeno ($\delta^{15}N$) de las plantas se ha propuesto como un criterio útil para la selección, ya que está relacionada con el metabolismo del nitrógeno de la planta, a pesar de que no existe un conocimiento preciso de los mecanismos profundos que determinan la $\delta^{15}N$ en la planta (Handley et al., 1997; Robinson et al., 2000; Tcherkez, 2011). En todos los capítulos de la Tesis se ha observado una disminución en el contenido del nitrógeno (N) y en la $\delta^{15}N$ en condiciones salinas. Un descenso de la g_s como resultado del estrés salino, hídrico o la combinación de ambos da lugar a una menor pérdida de amoníaco y óxido nitroso, lo que podría ser una de las causas de la disminución en $\delta^{15}N$ en respuesta al estrés salino o hídrico comparado con las plantas controles (Farquhar et al., 1980; Smart y Bloom, 2001). La correlación positiva entre $\delta^{15}N$ y el cociente C_i/C_a (Figura S3 del Capítulo 2), así como la correlación entre $\delta^{15}N$ y la g_s y la transpiración (T) en los

tratamientos de riego completo (FI, FI-12 dSm^{-1} y FI-17 dSm^{-1}) se podrían explicar por qué el cociente C_i/C_a junto con la g_s y la *T* son parámetros que pueden relacionar la permeabilidad de la hoja con el estado funcional del metabolismo de N, incluyendo enzimas claves en la asimilación del nitrógeno como la nitrato reductasa (NR) y la glutamina sintetasa (Gs). De hecho considerando todas las condiciones de crecimiento conjuntamente la $\delta^{15}N$ estaba correlacionada negativamente con $\delta^{13}C$ en varios de nuestros experimentos (Capítulos 1, 2, 3).

Otros mecanismos aparte de la pérdida de N también podrían estar implicados en el descenso de la $\delta^{15}N$ de las hojas en respuesta a salinidad o sequía. Una alta concentración externa de N en relación a una demanda modesta inducida por la salinidad también daría lugar a una discriminación contra el ^{15}N y por lo tanto un menor $\delta^{15}N$ en las plantas (Mariotti et al., 1982). Recientemente, Tcherkez (2011) propuso un modelo que explica la variabilidad de la $\delta^{15}N$ en hojas en las que se demuestra una alta sensibilidad de la $\delta^{15}N$ a valores tanto de la fotorespiración (cuya importancia relativa aumenta por cualquier situación de estrés) como de la entrada de N en las plantas (reducida por una menor actividad de la NR). A tal efecto y teniendo en cuenta que la $\delta^{15}N$ refleja la capacidad de la planta para asimilar y usar nitrógeno, se obtuvo una correlación fuerte y positiva en los diferentes capítulos de la Tesis entre la composición isotópica del nitrógeno ($\delta^{15}N$) en la hoja y el contenido en nitrógeno (N) y la biomasa (Figura 3 del Capítulo 1 y 3, Figuras 5 y 2 de los Capítulos 2 y 4, respectivamente).

Evans (2001) indica que los diferentes mecanismos de captación del nitrógeno y sus vías de asimilación, así como el reciclaje del nitrógeno en las plantas pueden discriminar contra $\delta^{15}N$. Las plantas no dependen sólo de la $\delta^{15}N$ de las fuentes de N que empleen, sino también del equilibrio entre la actividad enzimática y la concentración externa de nitrógeno (Mariotti et al., 1982). De acuerdo con esto, en el Capítulo 3, la actividad enzimática de la NR y de la GS se correlacionaron fuerte y positivamente con la $\delta^{15}N$ (Figura 5 del Capítulo 3). Así que independientemente qué mecanismos que afectan la $\delta^{15}N$ de la planta, este criterio parece reflejar la capacidad

de la planta para usar nitrógeno disponible y los cambios provocados por la salinidad sobre la actividad enzimática. Nuestros resultados confirman esta hipótesis, de forma que la salinidad disminuye la actividad de la glutamina sintetasa y la nitrato reductasa causando una disminución en el contenido del nitrógeno (N) asimilado y en la composición isotópica del nitrógeno ($\delta^{15}N$) de la hoja (Tabla 2 del Capítulo 3).

Un resultado a priori paradójico se ha encontrado al comparar el efecto de los estreses salino e hídrico en los valores de $\delta^{15}N$ en hojas y raíces (Capítulo 3 y 4). Mientras como apuntamos arriba comparado con los valores en plantas control la $\delta^{15}N$ de las hojas disminuye en respuesta a la severidad del estrés, lo contrario se produce en las raíces, donde $\delta^{15}N$ aumenta en respuesta al estrés en comparación con el control. Por otra parte en condiciones control las hojas tenían una $\delta^{15}N$ mayor que las raíces. Esto podría estar relacionado con los procesos de fraccionamiento durante la asimilación por la nitrato NR de la raíz, de forma que el nitrato no asimilado por la NR y exportado vía xilema hacia las hojas estaría más enriquecido en ^{15}N respecto al N asimilado en las raíces (Evans, 2001; Tcherkez, 2011). Por lo tanto, el NO_3^- que no se reduce en las raíces y que se exporta a las hojas sería el responsable de que las hojas tengan una $\delta^{15}N$ mayor que las raíces (Evans et al., 1996). Sin embargo, las condiciones de estrés salino e hídrico, limitando el transporte xilemático de NO_3^- enriquecido en ^{15}N hacia la parte aérea (aunque posiblemente también la actividad de la NR en las hojas) podría causar una disminución en la $\delta^{15}N$ de las hojas y en consecuencia un aumento de $\delta^{15}N$ en las raíces en relación con las plantas control. Otro aspecto a considerar en la pauta de respuesta opuesta de $\delta^{15}N$ frente al estrés entre hojas y raíces podría tener que ver con el papel de la fotorespiración y reciclado del nitrógeno que tiene lugar en las hojas (Tcherkez, 2011).

Entre los resultados mas interesantes de esta Tesis están los obtenidos con la $\delta^{15}N$ como criterio de evaluación genotípica. El rango de variabilidad genotípica encontrado en este estudio usando $\delta^{15}N$ como criterio es muy alto y significativo en comparación con otros parámetros analizados. La $\delta^{15}N$ puede ser un indicador primordial para nuestras condiciones de crecimiento. Además de ser la primera

variable escogida en el "Stepwise" (Tabla 8 del Capítulo 1), $\delta^{15}N$ muestra una clara diferenciación entre los genotipos tolerantes y sensibles a la sal en todos los estreses estudiados. Los resultados del Capítulo 1 (Tabla 6), el Capítulo 3 (Figura 1 y Tabla S1) muestran la superioridad clara de la $\delta^{15}N$ como herramienta para el estudio de la variabilidad genotípica en condiciones salinas. Esto refuerza el potencial de este parámetro ($\delta^{15}N$) como un criterio para la selección de genotipos para tolerancia a la salinidad. La posibilidad de medir $\delta^{15}N$ en las primeras etapas del ciclo de la planta (Capítulos 1, 3 y 4) y tener una buena predicción de las diferencias en biomasa entre los genotipos fortalecería aún más la validez del potencial de $\delta^{15}N$ como criterio fisiológico de selección para tolerancia a la salinidad. La $\delta^{15}N$ en las plantas ha sido propuesta como un indicador de la variación genotípica que refleja el equilibrio entre la absorción y la asimilación del N (Handley et al., 1997, Robinson et al., 2000, Pritchard y Guy 2005). Por otra parte Ellis et al (2002) han usado $\delta^{15}N$ como criterio fenotípico en un estudio de mapeo molecular de una población de cebada expuesta a salinidad (Ellis et al., 2002) aunque este trabajo no aportaba una clara relación entre la $\delta^{15}N$ y la biomasa. Coque et al. (2006) en maíz, encontraron 10 QTLs para $\delta^{15}N$ que coincidieron con los QTLs implicados en la eficiencia del uso del nitrógeno. Todos estos trabajos anteriores refuerzan el potencial $\delta^{15}N$ como herramienta de selección en los programas de mejora, aunque es necesario profundizar en los mecanismos que determinan la $\delta^{15}N$ en la planta.

2 - 4 Uso combinado de la composición isotópica del ^{13}C, ^{18}O y ^{15}N: Un modelo para el estudio de la variabilidad genotípica a diferentes condiciones salinas

Comprender las relaciones entre $\delta^{13}C$, $\delta^{18}O$ y $\delta^{15}N$ y el crecimiento de las plantas sometidas a un amplio rango de estreses abióticos puede ayudar a diseñar estrategias de mejora más eficientes eligiendo en cada caso los criterios más adecuados para la selección. Sin embargo, hay pocos estudios (y en general contradictorios) que examinan las bases fisiológicas de $\delta^{18}O$ y $\delta^{15}N$ y sus potenciales en mejora. Lo mismo podríamos decir de las relaciones de $\delta^{13}C$ y $\delta^{18}O$ con el metabolismo del nitrógeno. Como se ha mencionado en el apartado anterior, $\delta^{15}N$ está relacionada con la actividad

enzimática de GS y NR (Figura 5 del Capítulo 3); además está relacionada con $\delta^{13}C$, lo que (como apuntamos más arriba) puede deberse al hecho de que $\delta^{13}C$ es un indicador de condiciones hídricas y fotosintéticas (es decir de disponibilidad de energía) que afectan la actividad de la NR. Nuestros resultados mostraron que la $\delta^{13}C$ y $\delta^{15}N$ se correlacionaban negativamente en las diferentes condiciones de crecimiento (Capítulo 3).

La asimilación de N requiere NADH para la NR y ATP para el funcionamiento de la GS, así como esqueletos de carbono derivados de la fotosíntesis para la síntesis de aminoácidos (Tcherkez, 2011). De acuerdo con esto, la actividad enzimática de la GS y la NR se correlacionó negativamente con $\delta^{13}C$ y $\delta^{18}O$ (Figura 5 del Capítulo 3). Una disminución de la actividad enzimática de la NR y la GS estaría asociada con un aumento en $\delta^{13}C$ por efecto del estrés, que a su vez puede ser interpretado como el resultado de una actividad fotosintética restringida por la disminución de g_s en condiciones de estrés. Se sabe que el fraccionamiento isotópico del $^{14}N/^{15}N$ se produce durante la asimilación del nitrato amónico por las plantas (Tcherkez y Farquhar, 2006). De esta manera, la $\delta^{15}N$ también se ve afectada por los cambios provocados en la actividad fotosintética debida a la salinidad y a los cambios producidos en la actividad enzimática. A tal efecto, se observó una correlación fuerte y positiva (señalada también en apartado anterior) entre la $\delta^{15}N$ y la actividad enzimática de la NR y la GS (Figura 5 del Capítulo 3). Estos resultados nos permiten deducir que una menor $\delta^{13}C$ en condiciones óptimas implica una mayor actividad enzimática y mayor $\delta^{15}N$. Lo contrario ocurre en condiciones de estrés salino e hídrico, ya que éstos provocan un aumento en $\delta^{13}C$ y un descenso de la actividad enzimática, el contenido de N y la $\delta^{15}N$.

En esta Tesis, el estudio del uso combinado de la $\delta^{13}C$, $\delta^{18}O$ y $\delta^{15}N$ (Capítulo 3) confirma la eficiencia de las firmas isotópicas para evaluar diferencias genotípicas tanto en crecimiento como en respuesta del metabolismo nitrogenado frente a la salinidad. El modelo conceptual propuesto en el Capítulo 3 (Figura 6) separa claramente entre las respuestas directas del crecimiento de la planta asociadas al estado hídrico (mediante la $\delta^{18}O$) y la fotosíntesis (mediante la $\delta^{13}C$) y las que corresponden a los efectos indirectos vinculados con el metabolismo del nitrógeno (a

través de las actividades de GS y NR junto con N y $\delta^{15}N$). Este modelo muestra claramente que existe un vínculo evidente entre la firma isotópica del carbono (indicador de actividad fotosintética) y el metabolismo de N (que afecta al balance energético de las enzimas clave a través de los flujos de fotorrespiración), lo que podría explicar porqué $\delta^{15}N$ y $\delta^{13}C$ se correlacionaron en todas las condiciones de crecimiento en este estudio. Un análisis de este modelo nos permite concluir que los isótopos relacionados con la actividad fotosintética/transpiratoria ($\delta^{13}C$ y $\delta^{18}O$) y el isótopo estable relacionado con el metabolismo del nitrógeno ($\delta^{15}N$) se complementan entre ellos para informarnos de una forma clara acerca de las vías que afectan la variabilidad genotípica de respuesta a diferentes tipos y niveles de estrés salino e hídrico. Además, este modelo (junto con el Path análisis) nos mostró el efecto directo e indirecto de cada isótopo estable sobre la actividad enzimática y el contenido en nitrógeno, dos marcadores fisiológicos principales en la variación de biomasa bajo condiciones de estrés. En resumen, podemos concluir que en nuestras condiciones experimentales, en los estreses moderados (tales como la salinidad moderada o el estrés hídrico) el crecimiento de la planta está asociado al metabolismo del N y la $\delta^{18}O$, mientras que en las condiciones de estrés severo (salinidad severa y combinación de salinidad y déficit hídrico) el metabolismo del N o la $\delta^{13}C$ parecen relevantes en las diferencias en biomasa.

3 - Estrés en estado vegetativo o reproductivo: ¿Qué isótopo estable y qué fracción son mejores indicadores?

La tolerancia a la salinidad de los cultivos puede variar en función de su etapa de crecimiento (Mass y Grieve, 1990). Zeng et al., (2002) encontraron que diferentes genotipos de arroz tolerantes a la sal presentaban respuestas diferentes según su etapa de crecimiento. Los resultados del Capítulo 5 de esta Tesis, compararon las respuestas de plantas de trigo duro sometidas a condiciones salinas en diferentes etapas de crecimiento: justo después de la germinación (experimento I) y durante la floración y el llenado del grano (experimento II) usando como criterios de evaluación la $\delta^{13}C$, $\delta^{18}O$ y $\delta^{15}N$ medidos en materia seca y en fracción soluble. Se sabe que los

isótopos estables cuando se analizan en materia seca (*ms*) integran a largo plazo la respuesta de las plantas a diferentes condiciones de crecimiento. Por contra ,los análisis de $\delta^{13}C$ y $\delta^{18}O$ medidos en fracción soluble (*fs*) de la materia orgánica reflejan los asimilados recién producidos por los órganos fotosintéticos e informan sobre el funcionamiento fotosintético ($\delta^{13}C$) y transpiratorio ($\delta^{18}O$) en el momento que las plantas son cosechadas (Brandes et al., 2007; Gessler et al., 2007; Cabrera et al., 2011). En este sentido, la $\delta^{15}N$ de la fracción soluble puede informar sobre el metabolismo actual del N en respuesta a condiciones de estrés.

Los isótopos estables analizados en el experimento I (especialmente $\delta^{15}N$ y $\delta^{13}C$) fueron mejores indicadores del estrés salino que en el experimento II y siguieron una pauta coherente tanto en la materia seca como en la fracción soluble. En cuanto al segundo experimento, en el que se aplicó el estrés en estadios más tardíos del desarrollo de las plantas durante unas pocas semanas, las tendencias de los isótopos fueron menos claras, pero en general las tres firmas isotópicas ($\delta^{13}C$, $\delta^{18}O$ y $\delta^{15}N$) se mostraron mejor en los análisis de *ms* y por tanto se pudo comparar el comportamiento genotípico en los tratamientos más severos. El interés de la selección de genotipos tolerantes a la sal en las primeras etapas del ciclo del cultivo (Experimento I) se debe a la posibilidad de preparar las polinizaciones en la época de floración bajo ciertas condiciones y así mejorar la cosecha. Esto es particularmente evidente para la salinidad, la cual en contraste con el estrés hídrico bajo condiciones mediterráneas, puede estar ya presente en la siembra, afectando así el crecimiento del cultivo y el vigor temprano, y por lo tanto limitar el rendimiento del grano en el ciclo del cultivo.

4.- Salinidad mediana y salinidad severa: ¿En cual de las dos funcionan mejor los isótopos para seleccionar genotipos tolerantes a la sal?

A pesar de que se han realizado numerosos trabajos para determinar el grado de tolerancia de distintos cultivos a la salinidad, la información sobre aspectos como el ambiente en el que debe efectuarse la selección, así como el nivel salino a emplear son

aun insuficientes (Richards et al., 1987). Los resultados de todos los capítulos de la Tesis indican que la salinidad moderada (FI-12 dSm^{-1}) más que la severa (FI-17 dSm^{-1}) fue la condición más adecuada para evaluar el comportamiento genotípico en condiciones salinas. Mass y Poss (1989) sugirieron que el mantenimiento de la salinidad en niveles bajos es una estrategia importante para evaluar el crecimiento de estos genotipos. En este sentido los niveles altos de salinidad pueden afectar de la misma manera a todos los genotipos impidiendo observar variabilidad genotípica. En el Capítulo 1 (Tabla 7) la $\delta^{15}N$ en materia seca fue el criterio que mejor se correlacionaba con las diferencias genotípicas en biomasa en salinidad moderada mientras que en la salinidad severa no hubo correlación. Por otro lado en el mismo Capítulo las diferencias genotípicas de la $\delta^{13}C$ en materia seca se dieron solo en la salinidad moderada. La $\delta^{18}O$ en el Capítulo 3 (Tabla S1) también apoya esta hipótesis de que los isótopos estables son mejores indicadores en ambientes de salinidad moderada. Así, $\delta^{18}O$ separó entre genotipos solo en FI-12 dSm^{-1}. Estos resultados son muy claros en los dos experimentos donde la salinidad se ha aplicado justo después de la germinación (Experimento I y III). Mientras que en el experimento II donde la salinidad se aplicó más tarde, no se dio una pauta clara para los tres isótopos analizados, pero se puede ver que existen correlaciones entre biomasa y isótopos en la salinidad severa (FI-17 dSm^{-1}) y en la combinación de salinidad severa y déficit hídrico (DI-17 dSm^{-1}). Este resultado se podría explicar por la duración del estrés (únicamente 2 semanas) mientras que los 2 meses anteriores las plantas crecieron en condiciones óptimas. En estas condiciones solamente un estrés severo pudo repercutir de manera clara limitando la biomasa final, mientras que el efecto de los estreses moderados fue menos evidente.

5 - Integrando expresión genética, adaptación genotípica, marcadores fisiológicos y metabolismo del nitrógeno

Establecer las relaciones que existen entre la expresión genética y el fenotipo siendo un tema fundamental en la mejora por estrés abiótico. Los estudios que relacionan la expresión de los genes con los mecanismos fisiológicos responsables de la variabilidad

natural genotípica en tolerancia al estrés salino e hídrico siguen siendo escasos. Agarwal et al. (2006) indican que las respuestas moleculares al estrés abiótico incluyen la percepción, la transducción de señales, la expresión génica y por último los cambios metabólicos y fisiológicos en la planta que confieren tolerancia al estrés. A tal efecto, nuestros resultados del capítulo 6 indican claramente que la expresión génica esta asociada con diferencias genotípicas para criterios fisiológicos tales como el estatus fotosintético-hídrico en la planta ($\delta^{13}C$), el metabolismo del nitrógeno (a través la $\delta^{15}N$, el contenido en nitrógeno y las actividades de la glutamina sintetasa (GS) y la nitrato reductasa (NR).

Los resultados de esta Tesis mostraron que los niveles de expresión de los genes de glutamina sintetasa (GS1 y GS2) y los factores de transcripción DREB (TaDREB1A y TaDREB2B) detectaron variabilidad genotípica (Tabla 2 del Capítulo 6). En general los genotipos tolerantes mostraron tasas de expresión mayores que los susceptibles, menos en el caso de la expresión del gen TaDREB1A.

Los resultados de la Tabla 2 (Capítulo 6) también mostraron que en plantas de trigo duro TaDREB1A fue inducida por la salinidad y estrés hídrico con niveles de transcripción más altos en comparación con el control, mientras que TaDREB2B fue menos inducida, con niveles de transcripción más bajos. En muchos estudios la inducción de los genes DREB1 y DREB2 tiene una respuesta contradictoria bajo diferentes condiciones de estrés. Egawa et al. (2006) y Kurahashi et al. (2009) indican que en diferentes plantas de trigo TaDREB1 se induce no solamente bajo condiciones de temperaturas bajas pero también en condiciones de salinidad y sequía, lo que coincide con nuestros resultados en este Capítulo. Sin embargo, resultados opuestos a los nuestros se presentaron por Liu et al. (1998) y Shen et al. (2003) que a su vez mostraron que los genes DREB1 se inducen de manera rápida por el frío, pero no por el estrés hídrico y la alta salinidad.

Nuestros resultados mostraron que todos los criterios del metabolismo del nitrógeno (contenido de N, $\delta^{15}N$ y las actividades de NR y GS) se correlacionaron mejor

y con una pauta coherente con los genes de glutamina sintetasa (GS1 y GS2) y los factores de transcripción (TaDREB1A y TaDREB2B) (Figura 3 y 4 del Capítulo 6). Bernard y Habash (2009) concluyen que las transcripciones de los genes de la GS citosólica, y las proteínas son sensibles tanto al estado del N en la planta como a las señales ambientales. En general, esto sugiere que la GS puede constituir un punto de regulación en el que las señales ambientales se integran y se traducen en una respuesta de la planta en términos de crecimiento. Por otro parte, pensamos que las correlaciones altas que existen entre los genes TaDREB y los criterios del metabolismo del nitrógeno son debidos a la especificad de los factores TaDREB al estrés osmótico (Kurahashi et al., 2009) que afecta el aparato fotosintético (fuente de energía para las enzimas clave del metabolismo del nitrógeno) que a su vez puede afectar el metabolismo del nitrógeno.

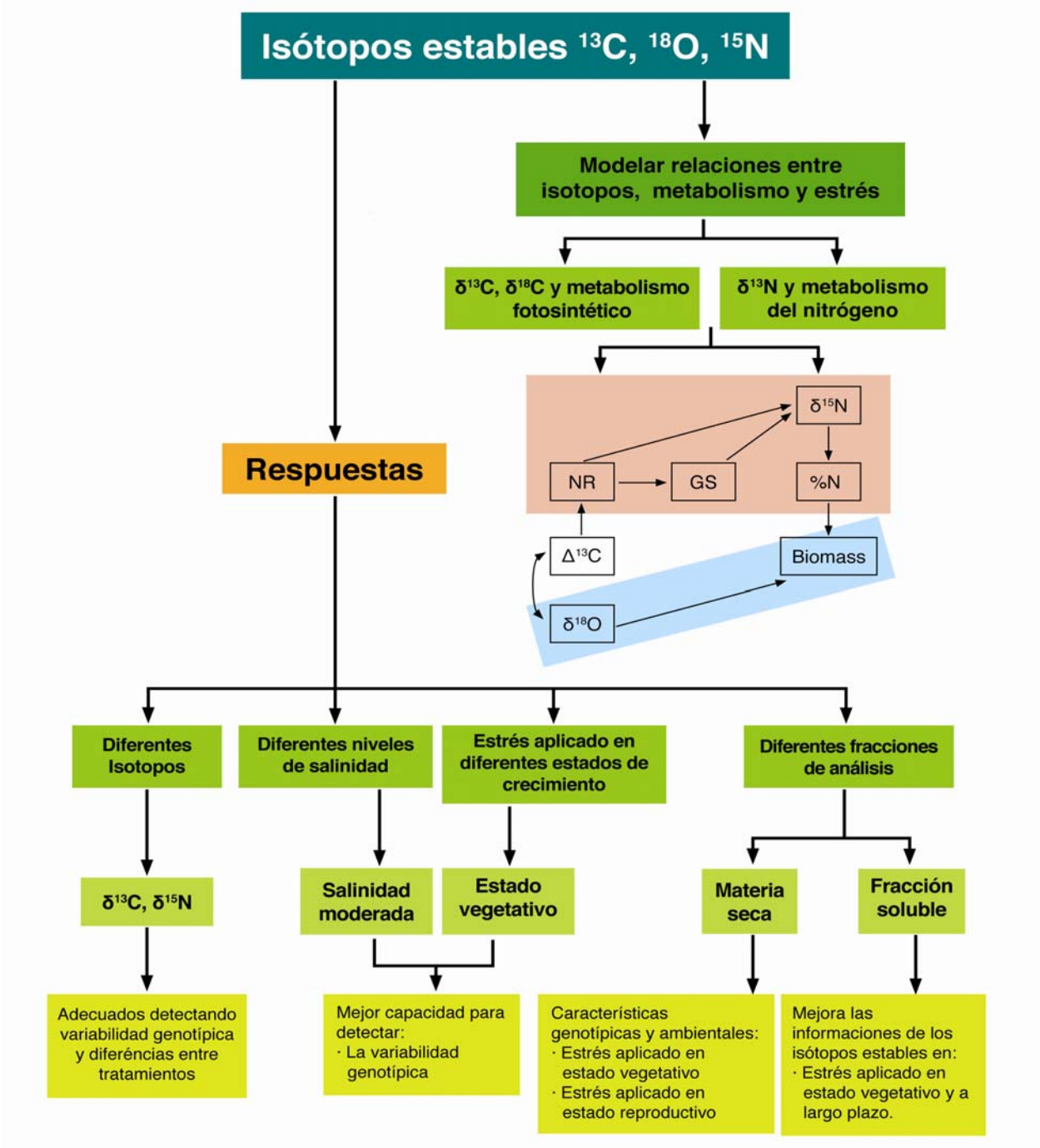
En nuestro estudio no hemos encontrado relación entre el nivel de expresión del gen NHX1 (Na^+/H^+ antiporters) y la biomasa, las concentraciones de Na^+ y K^+, los isótopos estables y la actividad de la GS (Figuras 2, 3 y 4 del Capítulo 6). A tal efecto, la expresión de NHX1 no parece estar implicada en las respuestas de la planta frente a distintas condiciones de estrés ni explicando la variabilidad genotípica. Según Munns y Tester (2008) el conocimiento global que relacionan los mecanismos de acumulación de Na^+ y de la tolerancia al estrés osmótico a nivel de toda la planta sigue siendo limitado.

En resumen, podemos concluir que estudio de la transcripción génica asociado a las firmas de los isótopos estables de carbono y nitrógeno ($\delta^{13}C$ y $\delta^{15}N$) y el metabolismo nitrogenado (contenido en nitrógeno y las actividades enzimáticas de la GS y la NR) tiene un alto potencial para profundizar aún más el conocimiento de los efectos que provoca la salinidad y el estrés hídrico sobre el mecanismo de tolerancia de la planta, y ayuda a comprender cómo esta serie de mecanismos fisiológicos y bioquímicos se activan a nivel molecular.

6 - Representación esquemática de los resultados de la Tesis

Esquema A

Esquema B


Isótopos estables ¹³C, ¹⁸O, ¹⁵N
Modelar relaciones entre isotopos, metabolismo y estrés
δ¹³C, δ¹⁸C y metabolismo fotosintético
δ¹³N y metabolismo del nitrógeno
Respuestas
δ¹⁵N
NR
GS
%N
Δ¹³C
Biomass
δ¹⁸O
Diferentes Isotopos
Diferentes niveles de salinidad
Estrés aplicado en diferentes estados de crecimiento
Diferentes fracciones de análisis
δ¹³C, δ¹⁵N
Salinidad moderada
Estado vegetativo
Materia seca
Fracción soluble
Adecuados detectando variabilidad genotípica y diferéncias entre tratamientos
Mejor capacidad para detectar:
· La variabilidad genotípica
Características genotípicas y ambientales:
· Estrés aplicado en estado vegetativo
· Estrés aplicado en estado reproductivo
Mejora las informaciones de los isótopos estables en:
· Estrés aplicado en estado vegetativo y a largo plazo.

Conclusiones

CONCLUSIONES

1- Los caracteres morfofisiológicos clásicamente asociados con la adaptación a la salinidad en trigo duro, como son el contenido en clorofila, concentración de iones y las tasas de intercambio gaseoso son efectivos para separar entre los diferentes tratamientos de salinidad; sin embargo no son criterios adecuados para detectar diferencias genotípicas.

2- Las composiciones isotópicas del carbono ($\delta^{13}C$) del oxígeno ($\delta^{18}O$) y del nitrógeno ($\delta^{15}N$) y el contenido en nitrógeno fueron criterios eficaces a la hora de discriminar entre condiciones de crecimiento.

3- La $\delta^{15}N$ fue mejor criterio que la $\delta^{13}C$ para seleccionar entre genotipos tolerantes y susceptibles a la salinidad, mientras que la $\delta^{18}O$ no fue adecuado para detectar variabilidad genotípica frente a salinidad.

4- Tanto $\delta^{15}N$ como $\delta^{13}C$ funcionaron mejor determinando variabilidad genotípica cuando el estrés salino se aplicó justo después de la germinación y por un periodo largo. Cuanto el estrés se aplicó en estadios más tardíos del desarrollo de las plantas y durante unas pocas semanas, la eficiencia de los isótopos detectando variabilidad genotípica disminuyó.

5- La salinidad moderada permite seleccionar genotipos de trigo duro tolerantes en épocas tempranas de crecimiento. La salinidad elevada o la combinación de salinidad y limitación de agua representan un nivel de estrés demasiado elevado que impidió la expresión de la variabilidad genética.

6- La $\delta^{15}N$ y $\delta^{13}C$ en la fracción hidrosoluble de las hojas detectaba la variabilidad genotípica mejor que la $\delta^{15}N$ y $\delta^{13}C$ de la materia seca, en plantas sometidas a la salinidad después de la germinación; incluso en condiciones de salinidad severa. Cuando la salinidad se aplicaba en plantas adultas la tendencia fue la opuesta.

7- La salinidad afecta la fotosíntesis de las plantas, básicamente a través de un cierre estomático, lo que se traduce en aumentos en $\delta^{13}C$ y $\delta^{18}O$. Esto se observó tanto en trigo duro como en los otros genotipos evaluados (tritordeum, triticale y cebada).

8- La salinidad e incluso el estrés hídrico también tienen un claro efecto en el metabolismo del nitrógeno, disminuyendo tanto las actividades de la GS y NR como el contenido de nitrógeno en la planta.

9- Los cambios en $\delta^{15}N$ en respuesta a la salinidad y sequía son reflejo del efecto de estos estreses sobre el metabolismo nitrogenado.

10-Tanto la actividad fotosintética como el estado hídrico (inferidos mediante $\delta^{13}C$ y $\delta^{18}O$) como el metabolismo del nitrógeno (a través de las actividades de GS y NR junto con N y $\delta^{15}N$) se complementan entre ellos para informarnos de una forma clara acerca de las vías que afectan la variabilidad genotípica de trigo duro frente a diferentes tipos y niveles de estrés salino e hídrico.

11- Los niveles de expresión de los genes de glutamina sintetasa y de factores de transcripción DREB detectaron variabilidad genotípica. En general los genotipos tolerantes mostraron tasas de expresión mayores que los susceptibles.

12- Por lo general los niveles de expresión de esos genes únicamente correlacionaron con la biomasa y los indicadores de fotosíntesis ($\delta^{13}C$) y el metabolismo nitrogenado (N y $\delta^{15}N$) en la salinidad más severa (FI-17).

13- La expresión del gen antiporter tonoplástico NHX1 no parece estar implicada en las respuestas de la planta frente a distintas condiciones de estrés ni explicando la variabilidad genotípica.

14- Únicamente TaDREB1A mostraba una pauta clara a través de las diferentes condiciones de crecimiento entre sus niveles de expresión y la biomasa de la planta y su metabolismo del nitrógeno.

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El riego inadecuado, a largo plazo y en condiciones de elevada demanda evapotranspirativa, junto con el empleo de aguas de mala calidad, un abonado excesivo y la falta de un drenaje adecuado inducen frecuentemente a la salinización de las tierras cultivables provocando un aumento importante en la superficie afectada por la salinidad.

La salinidad es un factor ambiental que limita de una manera remarcable la producción de los cultivos en muchos lugares del mundo, pero sobre todo en regiones áridas y semiáridas como las del mediterráneo. En estas condiciones, donde se cultiva frecuentemente el trigo duro, la mejora para la tolerancia a la salinidad en condiciones de riego deficitario puede ser una de las estrategias para aliviar este problema.

Esta Tesis demuestra que las composiciones isotópicas del carbono ($\delta^{13}C$), oxígeno ($\delta^{18}O$) y nitrógeno ($\delta^{15}N$), así como el contenido en nitrógeno en la materia seca son criterios potencialmente eficaces a la hora de discriminar entre condiciones de crecimiento y entre genotipos tolerantes o susceptibles a la sal. Asimismo, este trabajo refleja la importancia del metabolismo nitrogenado en la tolerancia a la salinidad. Además la Tesis desarrolla un modelo que relaciona la tolerancia genotípica a diferentes condiciones de salinidad y sequía con las firmas de los tres isótopos, junto con los intercambios fotosintéticos y transpiratorios y parámetros claves del metabolismo nitrogenado como son el contenido en nitrógeno y las actividades de la glutamina sintetasa y la nitrato reductasa. Finalmente se estudian las relaciones entre la expresión de genes potencialmente claves en la tolerancia a la salinidad y sequía y la variabilidad genotípica frente a distintas combinaciones de dichos estreses.

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