**Universidad de Granada**

**Facultad de Ciencias**

**Departamento de Fisiología Vegetal**


# Efecto de la técnica agrícola del injerto en las respuestas fisiológicas de resistencia ante un estrés hídrico moderado en plantas de tomate cherry

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Memoria de Tesis Doctoral presentada por la licenciada en Biología
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Granada a Enero de 2013

Director de la Tesis

Fdo. Juan Manuel Ruiz Sáez

Doctoranda

Fdo. Eva Sánchez Rodríguez

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*A mis padres*

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*La ciencia es respecto del alma lo que es la luz respecto de los ojos, y si las raíces son amargas, los frutos son muy dulces.*

*Aristóteles (384 AC-322 AC) Filósofo griego.*

# Índice

## INDICE GENERAL

# Resumen

Debido a la disponibilidad limitada de zonas de cultivo, la alta demanda de vegetales fuera de temporada, y las prácticas agrícolas intensivas que conllevan a una rotación de cultivos muy limitada, los vegetales son a menudo cultivados bajo condiciones desfavorables que inducen estrés. Para hacer frente a estos problemas, se intentan desarrollar por parte de las empresas de reproducción cultivos más tolerantes a estréses abióticos. Sin embargo, debido a la necesidad de seleccionar marcadores genéticos, resulta un proceso demasiado costoso y lento. Por ello, el uso de injertos se ha convertido en una herramienta rápida alternativa a la metodología convencional, y que permite incrementar la tolerancia de los vegetales a diversos estreses ambientales. De hecho, el uso de vegetales injertados ha sido muy popular en los últimos años. Sin embargo, son pocos los trabajos que se han centrado en estudiar su efecto bajo condiciones de estrés hídrico, a pesar de que se sabe que las plantas injertadas muestran un incremento en la absorción de agua y nutrientes en comparación con las plantas sin injertar. Por ello, los objetivos de nuestro trabajo fueron:

1. Evaluar el papel de injerto en la resistencia al estrés hídrico puesto que se trata de una técnica agronómica alternativa, rápida, eficaz, de bajo coste y muy disponible para la agricultura.

2. Analizar si la resistencia es debido bien a propiedades de la base radicular resistente o bien a propiedades de la parte aérea resistente.

Para llevar a cabo estos objetivos, previamente hemos seleccionado un cultivar de tomate cherry con mayor tolerancia a un estrés hídrico moderado, cv. Zarina, y otro que presenta una mayor sensibilidad a este estrés, cv. Josefina. Realizamos injertos recíprocos entre ambos cultivares, así como autoinjertos que nos servirán de control de la propia técnica. Los tratamientos aplicados consistieron en un control (100% de la capacidad de campo) y un tratamiento de estrés hídrico moderado (50% capacidad de campo). Nos centramos en el estudio de la respuesta antioxidante, el metabolismo fenólico, metabolismo de las poliaminas y de la prolina, así como los cambios en el ionoma en plantas de tomate cherry injertadas bajo estos tratamientos. Este diseño experimental nos permite comprobar que respuestas están más determinadas por la parte aérea tolerante y cuáles están más influenciadas por la parte radicular tolerante dentro de las plantas injertadas.

***- Respuesta antioxidante en injertos recíprocos entre cultivares de tomate tolerante y sensible al estrés hídrico.***

Recientemente las plantas injertadas han sido utilizadas para inducir resistencia a diferentes estreses abióticos. En nuestro trabajo, utilizamos cultivares de tomate que difieren en su tolerancia al estrés hídrico (Zarina tolerante y Josefina sensible) para obtener plantas injertadas que crecieron bajo estrés hídrico moderado, con el objetivo de comprobar el papel del parte aérea y el parte radicular en la producción de biomasa foliar y la respuesta antioxidante. Se determinaron algunos indicadores de estrés y la actividad de

las enzimas relacionadas con la respuesta antioxidante. Nuestros resultados mostraron que cuando la parte aérea corresponde al cultivar tolerante Zarina, los cambios en las actividades de las enzimas antioxidantes fueron más largos y consistentes. Sin embargo, cuando la parte aérea corresponde al genotipo sensible, Josefina, la respuesta antioxidante es más limitada y el estrés oxidativo fue evidente. Estos resultados reflejan que la técnica del injerto usando al cultivar Zarina como parte aérea podría ser una herramienta útil y efectiva para mejorar la respuesta antioxidante en tomate bajo estrés hídrico, ayudando a mantener una biomasa mayor bajo este estrés.

***- Metabolismo fenólico en plantas de tomate injertadas y no injertadas bajo la influencia de estrés hídrico.***

El uso de injertos con una parte radicular capaz de paliar los efectos del estrés hídrico puede ser una solución para reducir las pérdidas de producción. Para responder al estrés, las plantas pueden inducir el metabolismo de los fenilpropanoides. El objetivo de este capítulo fue determinar la respuesta de los injertos recíprocos realizados entre un cultivar tolerante, Zarina, y otro más sensible, Josefina. El análisis de la vía fenilpropanoide se realizó enzimática y metabólicamente. Se determinaron las actividades DAHP sintasa, shikimato deshidrogenasa, fenilalanina amonio liasa, cinamato 4-hidrolasa, y 4-coumarato CoA ligasa, así como los metabolitos característicos de esta vía mediante HPLC-MS. Las plantas *JosxZar* incrementan la concentración de compuestos fenólicos tras el estrés hídrico. Esto podría estar correlacionado con un

aumento en la actividad de las enzimas de síntesis, así como un descenso de aquellas relacionadas con la degradación de fenoles. Por tanto deducimos que el metabolismo fenólico está más influenciado por la parte aérea, y por tanto, la capacidad para inducir tolerancia por parte de la parte radicular depende a su vez del genotipo del parte aérea.

***- Papel de los injertos en la resistencia al estrés hídrico en plantas de tomate: producción de amonio y asimilación.***

El estrés hídrico puede restringir la habilidad de las plantas para reducir y asimilar el nitrógeno a través de la inhibición de las enzimas implicadas en su metabolismo. Por otro lado, las plantas producen cantidades significativas de amonio a través de la reducción de nitrato y de la fotorrespiración, y debe ser rápidamente asimilado a compuestos nitrogenados no tóxicos. El objetivo de este capítulo fue determinar la respuesta en la reducción de nitrógeno y asimilación de amonio bajo condiciones de déficit hídrico en injertos recíprocos realizados entre un cultivar de tomate tolerante al estrés hídrico, Zarina, y otro sensible, Josefina. Nuestros resultados mostraron que cuando el cv. Zarina (tolerante) fue empleado como parte radicular (*ZarxJos*), estas plantas presentaron una mejora en la absorción y asimilación de nitrato, provocando un crecimiento favorable bajo estrés hídrico. Por otro lado, cuando el cv. Zarina es utilizado como parte aérea (*JosxZar*), estas plantas injertadas mostraron un incremento en el ciclo de fotorrespiración, el cual genera aminoácidos y

proteínas, y podría explicar su mejor crecimiento bajo las condiciones de estrés. En conclusión, los injertos que mejoran la absorción de nitrógeno o la fotorrespiración, incrementan la fotoasimilación de nitrato en plantas de tomate mejorando el crecimiento bajo déficit hídrico.

***- Interacciones entre el metabolismo de la prolina y las poliaminas en plantas de tomate injertadas bajo condiciones de estrés hídrico.***

En este capítulo estudiamos los cambios inducidos en los niveles de prolina y poliaminas y en las enzimas relacionadas con sus metabolismos, en respuesta a un estrés hídrico moderado utilizando plantas injertadas de dos cultivares que difieren en su respuesta a este estrés. Observamos que las interacciones entre las vías de prolina y poliaminas son competitivas. Los cultivares con mayor grado de estrés (Josefina y *ZarxJos*) presentaron acumulación de prolina bajo las condiciones de déficit hídrico, lo cual podría ser un síntoma de estrés. Mientras Zarina y *JosxZar* (más tolerantes al estrés hídrico) mostraron un aumento en la degradación de prolina asociado a una mejora en la biosíntesis de poliaminas. Así, el uso del cv. Zarina como parte aérea en plantas injertadas podría determinar una mejor resistencia frente al estrés hídrico asociado con una elevada biosíntesis de poliaminas.

***- Efecto del injerto en el ionoma de plantas de tomate cherry bajo condiciones de estrés hídrico.***

Generalmente, la absorción de nutrientes por la raíz y su transporte a la parte aérea disminuyen con la sequía debido una restricción de la tasa de transpiración, lo cual podría contribuir a una limitación de crecimiento bajo déficit hídrico. Además, la respuesta de los vegetales injertados a condiciones de estrés debido al estado nutricional puede ser diferente al que presentan las plantas sin injertar, dependiendo principalmente del genotipo de la parte radicular. El objetivo de este capítulo fue determinar la respuesta al estrés hídrico de injertos recíprocos realizados entre el cv. Zarina, y el cv. Josefina, examinando la absorción y concentración de nutrientes. Se determinó el contenido total y los flujos de absorción de los macro y micronutrientes. Nuestros resultados mostraron que el uso del cultivar tolerante Zarina como parte radicular (*ZarxJos*) mejoraba el ionoma de estas plantas, con un incremento en la concentración y absorción de nitrógeno, fósforo y potasio, así como de hierro y cobre bajo condiciones de estrés hídrico. Es conocido que un sistema radicular vigoroso es capaz de absorber nutrientes de forma más eficiente, y en nuestro caso se ha demostrado que tanto el cv. Zarina como el injerto *ZarxJos* desarrollaron un mejor sistema radicular bajo estrés hídrico. Estos resultados confirman la hipótesis de que las plantas injertadas en una parte radicular vigorosa pueden mejorar la nutrición mineral y la absorción de nutrientes con respecto a las plantas sin injertar, especialmente bajo condiciones de estrés hídrico. Esto podría relacionarse con una mayor biomasa de estas plantas bajo las condiciones de estrés estudiadas.

Por tanto las conclusiones que derivan de los trabajos especificados serían:

1. La utilización en plantas injertadas del cv. Zarina tolerante al estrés hídrico como parte aérea y el cv. Josefina sensible al estrés hídrico como base radicular (*JosxZar*) determina: cambios en la actividad de las enzimas antioxidantes bajo condiciones de estrés hídrico: incrementan las actividades SOD, CAT y las enzimas del ciclo de Halliwell-Asada, mientras que no se aprecian cambios en la actividad LOX. Así, estas plantas no muestran estrés, ya que la concentración foliar de $O_2^{-\cdot}$ y $H_2O_2$ no cambia con respecto a las condiciones control. Por tanto, el uso del cultivar Zarina como parte aérea en los injertos puede ser una técnica útil y efectiva para mejorar la respuesta antioxidante frente al estrés hídrico en plantas de tomate. A su vez, el metabolismo fenólico parece estar más influenciado por la parte aérea en las plantas injertadas, ya que sólo la utilización como parte aérea del cv. Zarina incrementa la síntesis y acumulación de fenoles en las plantas de tomate bajo estrés hídrico. Este mecanismo puede servir como respuesta frente al estrés generado, mejorando en crecimiento de estas plantas bajo déficit hídrico. Por otro lado, las interacciones entre la parte radicular y la parte aérea tienen fuertes efectos en las respuestas del metabolismo de N bajo condiciones de estrés hídrico. El uso del cv. Zarina como parte aérea activa el ciclo de la fotorrespiración, generando aminoácidos y proteínas. Por tanto nuestros resultados demuestran una compleja interacción entre el metabolismo fotorrespiratorio y la asimilación de $NO_3^-$, y que la utilización de la técnica del

injerto puede mejorar la respuesta bajo condiciones de estrés hídrico moderado. Bajo condiciones de estrés hídrico, los metabolismos de las poliaminas y de la prolina parecen ser competitivos, ya que tanto el cv. Zarina como el injerto *JosxZar* muestran una elevada degradación de prolina asociada a una mejora en la síntesis de poliaminas. La base radicular Zarina en el injerto *ZarxJos* no provoca en la parte aérea sensible Josefina la inducción de estos procesos fisiológicos, lo que nos sugiere con los resultados comentados anteriormente, que en el injerto estas respuestas se deben al genotipo de la parte aérea.

2. Sin embargo, cuándo la parte aérea corresponde al genotipo sensible Josefina (*ZarxJos*), la actividad de las enzimas antioxidantes es limitada y el estrés oxidativo es más evidente bajo las condiciones de estrés hídrico. Por otro lado, el uso del cv. Zarina como parte radicular (*ZarxJos*) mejora la absorción y asimilación de $NO_3^-$ en las plantas estresadas. Así mismo, el uso del cultivar tolerante como parte radicular (*ZarxJos*) provoca una mejora del ionoma en las plantas injertadas bajo condiciones de estrés hídrico, de forma que incrementa la absorción y concentración de N, P y K, así como de Fe y Cu, probablemente debido a su mayor vigor radicular. Estos resultados confirman la hipótesis de que en plantas injertadas con un parte radicular vigoroso puede mejorar la absorción de agua y nutrientes con respecto a plantas sin injertar, especialmente bajo condiciones de estrés hídrico.

# *Capítulo 1*

## *Interés general y Objetivos*

Debido a la disponibilidad limitada de zonas de cultivo, la alta demanda de vegetales fuera de temporada, y las prácticas agrícolas intensivas que conllevan una rotación de cultivos muy limitada, los vegetales son a menudo cultivados bajo condiciones desfavorables que inducen estrés. Entre estas condiciones desfavorables, el estrés hídrico es el factor ambiental más adverso que afecta al crecimiento y productividad de los cultivos. Las pérdidas de cosecha debidas al estrés hídrico probablemente exceden a las provocadas por la combinación del resto de estreses. Por todo ello, la sequía tiene un profundo impacto en la agricultura y en los sistemas ecológicos, de ahí que la capacidad de las plantas para soportar este estrés sea de gran importancia desde el punto de vista económico. De este modo, en la actualidad y con el fin de mejorar la productividad agrícola, es necesaria la obtención de cultivos con altas producciones bajo condiciones de estrés hídrico.

Para hacer frente a estas condiciones desfavorables es fundamental el desarrollo de cultivos más tolerantes a estos estreses. Sin embargo, debido a la carencia de buenas herramientas de selección como marcadores genéticos, es un proceso muy lento y costoso. Por ello, hoy día los injertos se han convertido en una herramienta rápida y eficaz para obtener cultivos hortícolas con mayor grado de tolerancia frente a diferentes estreses ambientales. En el pasado, la utilización de cultivos injertados se centró en limitar los efectos de los patógenos del suelo, pero su uso se ha extendido en los últimos años. Así,

los injertos pueden inducir resistencia frente a altas y bajas temperaturas, mejorar la absorción de nutrientes, incrementar la síntesis de hormonas endógenas, limitar el efecto negativo de metales pesados, mejorar la eficiencia en el uso del agua e incrementar la resistencia a salinidad. Sin embargo, son pocos los trabajos que se han centrado en estudiar su efecto bajo condiciones de estrés hídrico, a pesar de que se sabe que las plantas injertadas muestran un incremento en la absorción de agua y nutrientes en comparación con las plantas sin injertar.

Previamente al trabajo realizado en esta Tesis Doctoral, se desarrolló un estudio preliminar bajo condiciones de estrés hídrico moderado con 5 cultivares comerciales de tomate cherry, con el objetivo de determinar la resistencia genotípica de estos cultivares frente a este estrés y seleccionar los cultivares más tolerantes y sensibles este estrés. Observamos que el cv. Zarina mantenía el crecimiento bajo condiciones de estrés hídrico con respecto a las condiciones control, por lo que lo definimos como tolerante a este estrés. Mientras, el cv. Josefina mostró la mayor reducción en su tasa de crecimiento tras la aplicación del estrés hídrico moderado, considerándolo como el cv. más sensible. Así, nuestro posterior objetivo fue estudiar qué procesos fisiológicos intervienen en esta mejor tolerancia al déficit hídrico en estos dos cultivares de tomate cherry que difieren en su crecimiento bajo condiciones de estrés hídrico modero. Para ello, analizamos actividades enzimáticas y concentración de

compuestos involucrados en procesos fisiológicos tan importantes como el metabolismo oxidativo, fotorrespiración, metabolismo del nitrógeno y metabolismo de la prolina.

Los resultados más destacados de estos procesos se describen brevemente a continuación (para más detalle ver publicaciones del Anexo I):

(i) Las plantas responden al estrés hídrico cerrando sus estomas, lo cual reduce la disponibilidad de $CO_2$ para la fotosíntesis, provocando un aumento de la producción de especies reactivas de oxígeno (ROS). Por ello, los mecanismos que reducen este estrés oxidativo pueden jugar un papel importante en la tolerancia frente al déficit hídrico. El cv. Zarina mantuvo la concentración de ROS y peroxidación lipídica a niveles de las plantas bien irrigadas, lo cuál pudo ser debido a un aumento de la actividad catalasa (CAT) bajo condiciones de estrés, así como una activación de las enzimas implicadas en el ciclo de Halliwell-Asada, procesos que están involucrados en la detoxificación de ROS. Sin embargo, en el cv. Josefina no se observó incremento en la actividad de estos procesos, lo cual conllevó a un incremento en las ROS con el déficit hídrico.

(ii) Bajo condiciones de estrés hídrico se ha comprobado que la absorción de nitrógeno (N) disminuye, atribuido a una menor tasa de transpiración y

transporte de N desde la raíz a la parte aérea. A su vez, para proteger al aparato fotosintético contra la fotoinhibición, el exceso de fotones puede ser consumido por la fotorrespiración, proceso que genera grandes cantidades de amonio. El cv. Zarina, tolerante a este estrés, incrementó la actividad de las principales enzimas que intervienen en la fotorrespiración, junto con un aumento en la asimilación de nitrato y del amonio resultante. Esto se tradujo en mayores concentraciones de N así como en aminoácidos y proteínas. En el cultivar susceptible, cv. Josefina, disminuyó la asimilación de nitrato a la vez que no se estimuló la fotorrespiración.

(iii) Bajo condiciones de un estrés hídrico moderado otra de las respuestas comúnmente inducidas es la producción y/o acumulación de los llamados osmolitos compatibles, como es el caso de la prolina. Se le han atribuido otras funciones como estabilizar estructuras sub-celulares como membranas y proteínas, eliminar radicales libres y actuar como buffer celular del potencial redox bajo estrés. El estrés hídrico provocó un aumento en la concentración de prolina en el cultivar más susceptible, mientras que en el cv. Zarina, más tolerante a este estrés, no mostró diferencias con respecto al control. Las enzimas implicadas en la síntesis de prolina se vieron incrementadas. La actividad prolina deshidrogenasa (PDH), encargada de su degradación, disminuyó de en el cultivar susceptible; sin embargo, en el cv. Zarina aumentó de forma significativa bajo condiciones de estrés.

En definitiva, nuestros resultados muestran que una elevada actividad de las enzimas detoxificadoras de ROS, junto con un aumento en la asimilación de N y fotorrespiración son procesos fundamentales en la respuesta frente al estrés hídrico; mientras que la degradación de prolina parece ser más importante que su acumulación como respuesta de tolerancia.

Una vez seleccionados dos cultivares de tomate cherry que difieren en su grado de tolerancia frente a un estrés hídrico moderado, cv. Zarina y cv. Josefina, realizamos injertos recíprocos entre ellos, así como autoinjertos para comprobar el efecto del propio injerto, y fueron sometidos a dos tratamientos: control (100% de la capacidad de campo) y estrés hídrico moderado (50% de la capacidad de campo). Por lo tanto, los objetivos generales de esta Tesis Doctoral fueron:

1. Evaluar el papel de injerto en la resistencia al estrés hídrico puesto que se trata de una técnica agronómica alternativa, rápida, eficaz, de bajo coste y muy disponible para la agricultura.
2. Analizar si la resistencia es debido bien a propiedades de la base radicular resistente o bien a propiedades de la parte aérea resistente. Para lo cual se estudian en este trabajo procesos fisiológicos esenciales

en la resistencia al estrés hídrico como : respuesta antioxidante, metabolismo fenólico, fotorrespiración y asimilación de amonio, metabolismo de la prolina y las poliaminas, y la absorción y concentración de nutrientes.

# *Capítulo 2*

## *Introducción*

## 2.1. Estrés hídrico en plantas

### *2.1.1. Introducción*

Las plantas están sujetas a varios estreses ambientales que afectan de forma adversa a su crecimiento, metabolismo y producción. De forma general, los estreses pueden clasificarse en bióticos (insectos, bacterias, hongos y virus) y abióticos (luz, temperatura, disponibilidad de agua, nutrientes y estructura del suelo) (Lichtenthaler 1998). Entre estos últimos, la sequía es el factor ambiental con un efecto más adverso en el crecimiento y productividad de los cultivos. Las pérdidas en la producción agrícola debidas al estrés hídrico probablemente exceden a las pérdidas ocasionadas por todas las otras causas combinadas (Reddy *et al.* 2004). Las plantas experimentan estrés hídrico cuando el suministro de agua a las raíces es limitado o cuando la tasa de transpiración es muy alta. Estas dos condiciones coinciden bajo climas áridos y semiáridos (Chaves *et al.* 2003). Así, en la región Mediterránea, los ciclos vegetativo y reproductivo principalmente en los cultivos de verano están afectados por episodios recurrentes de estrés hídrico. Se sabe que la sequía tiene un profundo impacto en los sistemas agrícolas y ecológicos, de ahí que la capacidad de las plantas para soportar este estrés sea de gran importancia económica (Shao *et al.* 2008). Sin embargo, aunque los efectos generales del estrés hídrico en el crecimiento de las plantas son conocidos, los efectos primarios del déficit hídrico a nivel bioquímico y fisiológico han sido menos estudiados (Zhu 2002; Skirycz *&* Inzé 2010; Xu *et al*. 2010). Por lo tanto, la

necesidad de nuevas alternativas para una agricultura sostenible, como plantas más tolerantes a la sequía, proporcionaría una solución práctica para aliviar el problema de la limitación de agua (Xoconostle-Cázares *et al.* 2010). Por ello, en la actualidad y con el fin de mejorar la productividad agrícola, es necesario conseguir cultivos con altas producciones bajo estreses ambientales desfavorables.

### *2.1.2. Efecto en el crecimiento y desarrollo de las plantas*

El crecimiento y desarrollo de las plantas depende de la división celular y su diferenciación, y estos procesos están afectados por el déficit hídrico (Skirycz *&* Inzé 2010). El crecimiento es uno de los procesos fisiológicos más sensibles al estrés hídrico debido a la reducción de la presión de turgor. La expansión celular sólo ocurre cuando la presión de turgor es mayor que el umbral de producción de pared celular (Shao *et al.* 2008). El estrés hídrico suprime la expansión y crecimiento celular debido a la baja presión de turgor, ya que bajo este tipo de estrés se reduce el contenido hídrico y el potencial hídrico total (Karthikeyan *et al*. 2007). La reducción del crecimiento bajo condiciones de estrés hídrico ha sido bien caracterizada en diversos tipos de plantas, entre las cuales se encuentran el pimiento (Delfine *et al*. 2002), la patata (Ierna *&* Mauromicale 2006) y el tomate (Park *et al*. 2005) (Figura 1A).

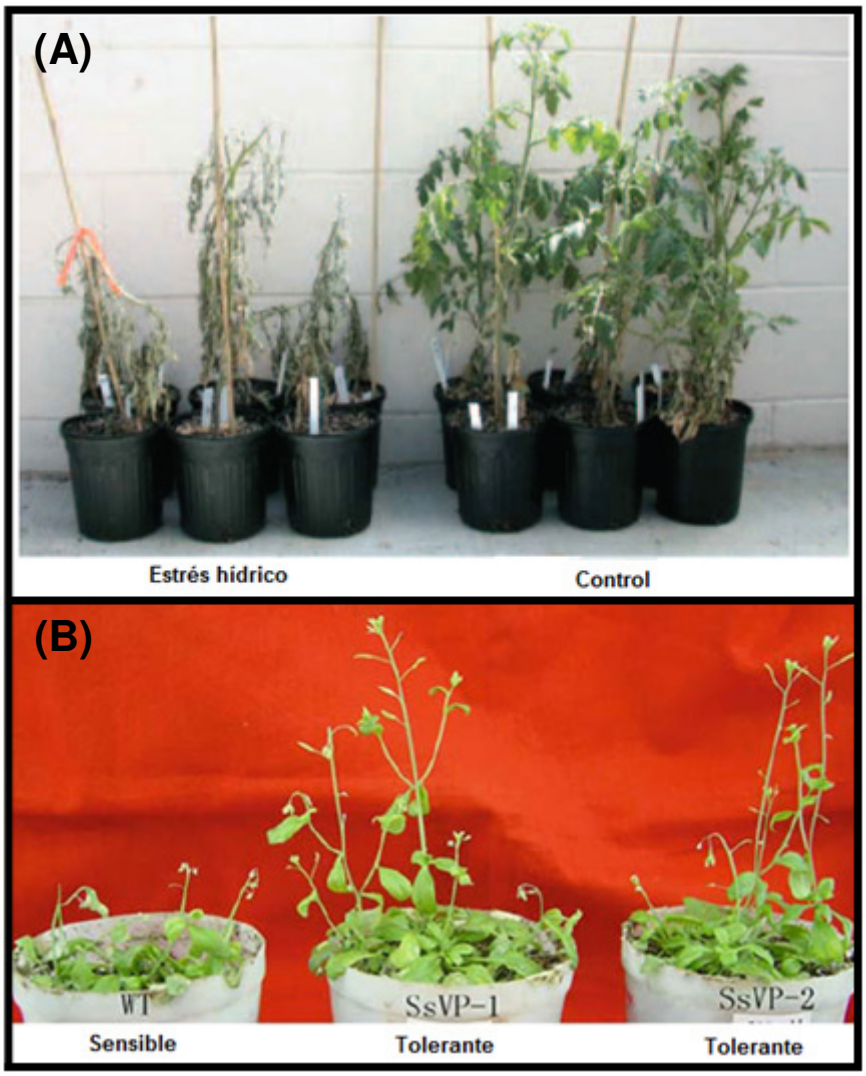


**Imagen 1:** (A) Plantas de tomate sometidas a estrés hídrico (Park *et al.* 2005). (B) Síntomas visuales de plantas de *Arabidopsis thaliana* sometidas a estrés hídrico. Obsérvese las diferencias entre genotipos sensibles y tolerantes (Guo *et al.* 2006).

Por otro lado, se ha comprobado que la reducción de crecimiento foliar resulta más sensible que el crecimiento radicular en situaciones de estrés hídrico. La reducción de la expansión foliar puede resultar beneficiosa para las plantas bajo condiciones de déficit hídrico, permitiendo que una menor área foliar

resulte expuesta y así reducir la pérdida de agua por transpiración (Mahajan *&* Tuteja 2005). Sin embargo, la disminución del área foliar puede ser una importante causa de pérdidas en la producción de los cultivos, ya que se reduce la tasa fotosintética (Takahashi *&* Murata 2008). Reddy *et al.* (2003) observan que una reducción del peso de la planta y del área foliar bajo estrés hídrico podría estar asociado con un descenso del alargamiento celular y un aumento de la senescencia en plantas de cacahuete.

Por el contrario, el crecimiento de la raíz puede mejorar con el déficit hídrico para facilitar la capacidad del sistema radicular de extraer agua de las capas más profundas del suelo (Mahajan *&* Tuteja 2005). Se ha observado un aumento en la longitud y densidad radicular en plantas de arroz crecidas bajo condiciones de déficit hídrico, lo cual se ha interpretado como un mecanismo de defensa frente a este estrés (Ji *et al.* 2012). Sin embargo, en otros estudios se ha visto que el estrés por sequía disminuye la longitud de la raíz en plantas de *Albizzia* (Nanjo *et al.* 1999). Resultados similares se observan en plantas de algodón, donde el peso seco de la raíz disminuye cuando se somete a un estrés hídrico moderado o severo (Duruoha *et al*. 2008).

La disminución del crecimiento puede ser considerada como un mecanismo para preservar carbohidratos para el metabolismo, prolongando el suministro de energía, y así permitir una mejor recuperación tras el periodo de estrés

(Bartels *&* Sunkar 2005). Se ha comprobado que la reducción del crecimiento ocurre rápidamente tras el estrés hídrico, independientemente de la tasa fotosintética y del estatus de carbono de la planta (Skirycz *et al.* 2010), argumentando que esta reducción del crecimiento no es simplemente un efecto secundario de la limitación de recursos, sino una importante respuesta adaptativa e incluso de tolerancia frente a este estrés. Aunque la disminución del crecimiento incrementa la tasa de supervivencia, durante episodios de estrés moderado cuando la supervivencia no se ve comprometida, puede ser visto como contraproducente ya que provoca pérdidas de rendimiento innecesarias y consecuencias importantes para la agricultura (Skirycz *&* Inzé 2010). Así, Fazeli *et al*. (2007) concluyen que el cv. Yekta de plantas de sésamo es más resistente al déficit hídrico que el cv. Darab basándose, entre otros factores, en una menor disminución de la biomasa. Por lo tanto, nuevas variedades de cultivos en los que el crecimiento se vea menos afectado durante los períodos esporádicos de estrés moderado sería muy ventajoso para la productividad de la planta, especialmente en regiones áridas y semiáridas como la cuenca Mediterránea.

### *2.1.3. Respuestas de resistencia al estrés hídrico*

Como organismos sésiles, las plantas se ven obligadas a desarrollar diversos mecanismos a nivel fisiológico, bioquímico y molecular que les permitan adaptarse y sobrevivir bajo condiciones de sequía (Figura 1). La respuesta de

la planta a su vez, dependerá tanto de su "estrategia" inherente como de la duración y severidad del periodo de estrés hídrico (Cruz de Carvalho 2008). Una de las adaptaciones más extendidas consiste en acumular agua para retrasar o escapar del estrés. De esta forma, algunas plantas son capaces de mantener sus funciones biológicas a bajos potenciales hídricos, aunque con un desarrollo limitado (Ramanjulu *&* Bartels 2002). Este es el caso de plantas tolerantes a la sequía, como la planta de la resurrección, que disminuye sus funciones biológicas bajo condiciones de estrés, las cuales son restauradas cuando el potencial hídrico incrementa (Bartels 2005).

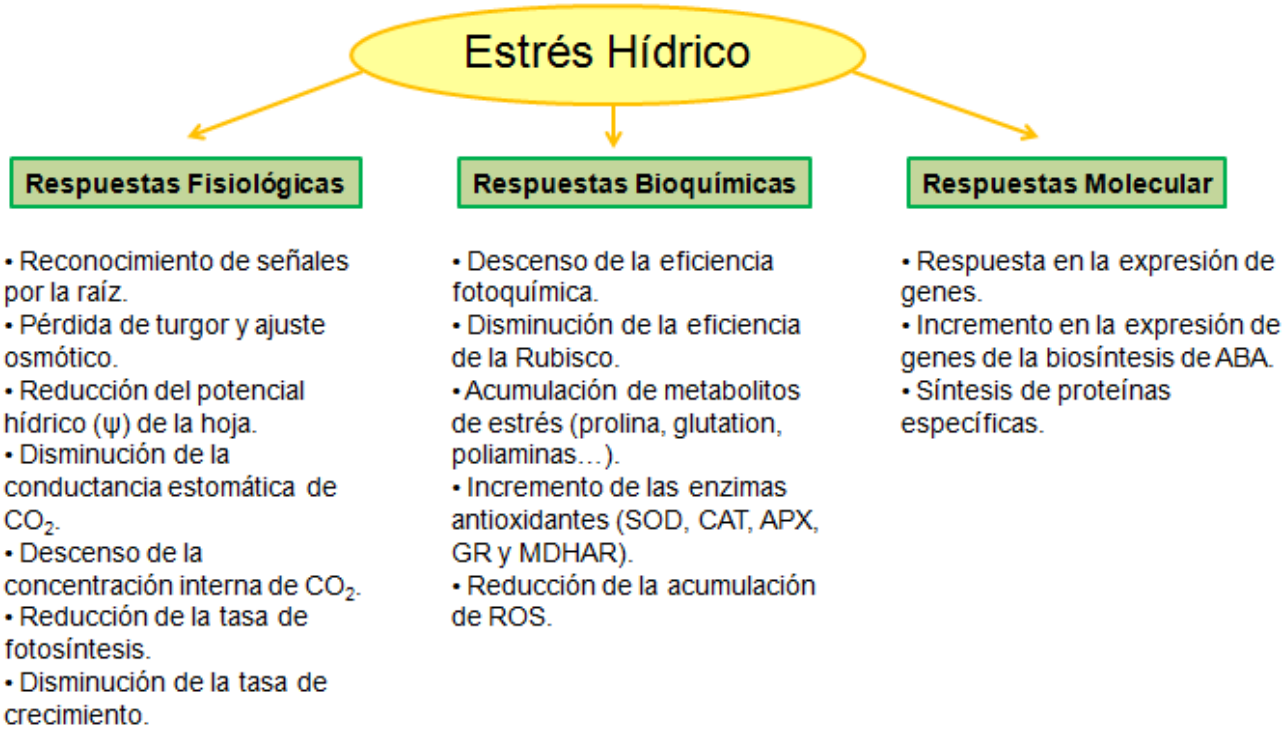


**Figura 1:** Respuestas fisiológicas y moleculares de las plantas frente al estrés hídrico (modificado de Shao *et al.* 2008).

Otra de las estrategias para limitar las pérdidas de agua consiste en promover el cierre estomático mediado por ácido abscísico (ABA), reduciendo así la elevada pérdida de agua por transpiración (Blum 1996). Sin embargo, esta adaptación disminuye la fotosíntesis y reduce la disponibilidad de $CO_2$, resultando en un desequilibrio entre la generación y la utilización de electrones que conlleva a una sobreproducción de especies reactivas de oxígeno (ROS) (Figura 2).

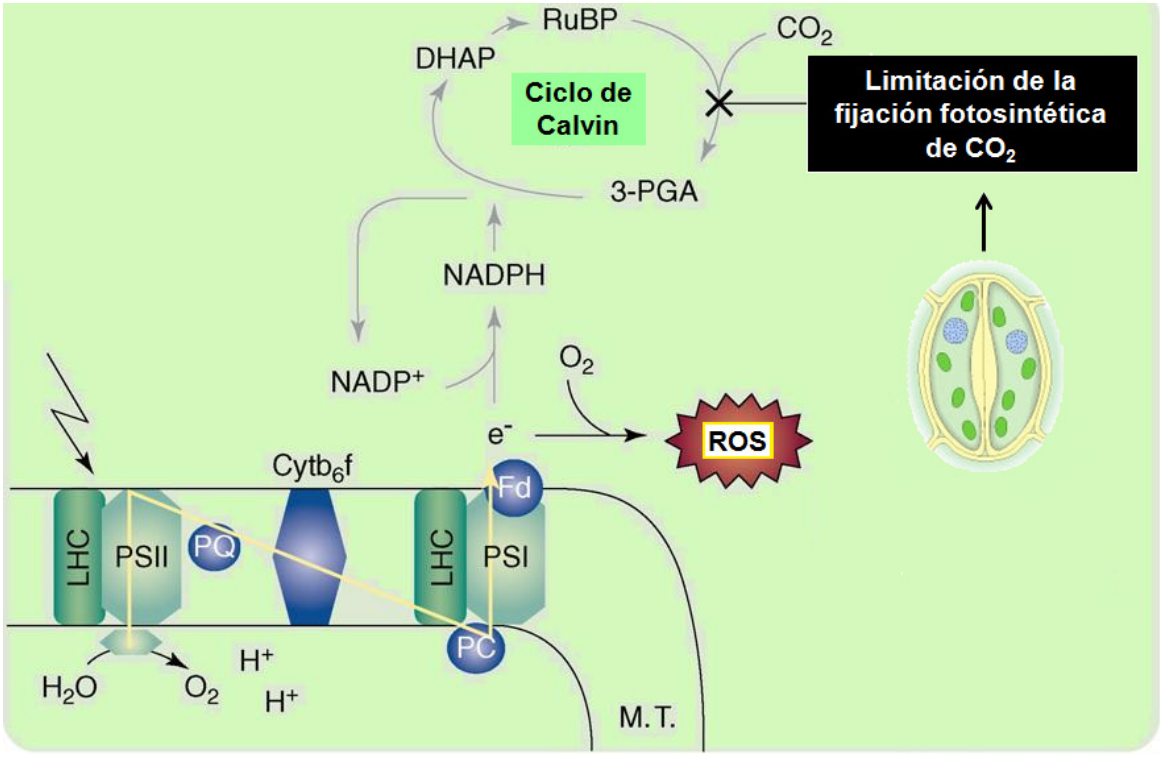


**Figura 2:** Alteración de la fotosíntesis y generación de ROS por el estrés hídrico (modificado de Takahashi *&* Murata 2008).

Estos cambios fotoquímicos en los cloroplastos resultan en la disipación del exceso de energía en el fotosistema II (PSII) a través de la generación de ROS

como el anión superóxido ($O_2^-$), el oxígeno en estado de singlete ($^1O_2$), el peróxido de hidrógeno ($H_2O_2$) y el radical hidroxilo ($OH^\cdot$), las cuales son potencialmente peligrosas bajo condiciones de déficit hídrico (Peltzer *et al*. 2002). Esto se debe a que las ROS libres tienen efectos adversos sobre las estructuras biológicas, incluyendo daño al ADN, oxidación de aminoácidos y proteínas, y peroxidación de lípidos (Asada 1999; Johnson *et al*. 2003). Para evitar el daño, las plantas disponen de mecanismos de detoxificación de ROS, que pueden dividirse en sistemas enzimáticos constituidos por las enzimas superóxido dismutasa (SOD), catalasa (CAT), ascorbato peroxidasa (APX), glutation reductasa (GR), deshidroascorbato reductasa (DHAR) y monodeshidroascorbato reductasa (MDHAR), y sistemas no enzimáticos formados por compuestos antioxidantes como fenoles (flavonoides, antocianinas, carotenoides…), ácido ascórbico (AsA) y glutation (GSH) (Reddy *et al*. 2004) (Figura 3). Por ello, la tolerancia al estrés hídrico se ha relacionado con un eficiente proceso antioxidante (Kranner *et al.* 2002; Montero-Tavera *et al.* 2008). Se ha comprobado que los niveles de estas enzimas antioxidantes se mantienen más elevados durante estrés por sequía y por calor en un cultivar tolerante al estrés hídrico de *Poa pratensis* en comparación con un cultivar susceptible (Wang *&* Huang 2004). De acuerdo con estos datos, en plantas de maíz aclimatadas a la sequía se ha observado que un aumento en la actividad de las enzimas CAT, SOD y peroxidasa confiere tolerancia al estrés oxidativo (Selote *&* Khanna-Chopra 2006). Asímismo, Cia *et al.* (2012) demuestran que una mejora en la actividad de las enzimas CAT y APX podría tener un papel

protector en cultivares tolerantes al estrés hídrico de caña de azúcar. Por tanto, queda demostrado que éste es un proceso básico para seleccionar plantas tolerantes a un estrés hídrico.

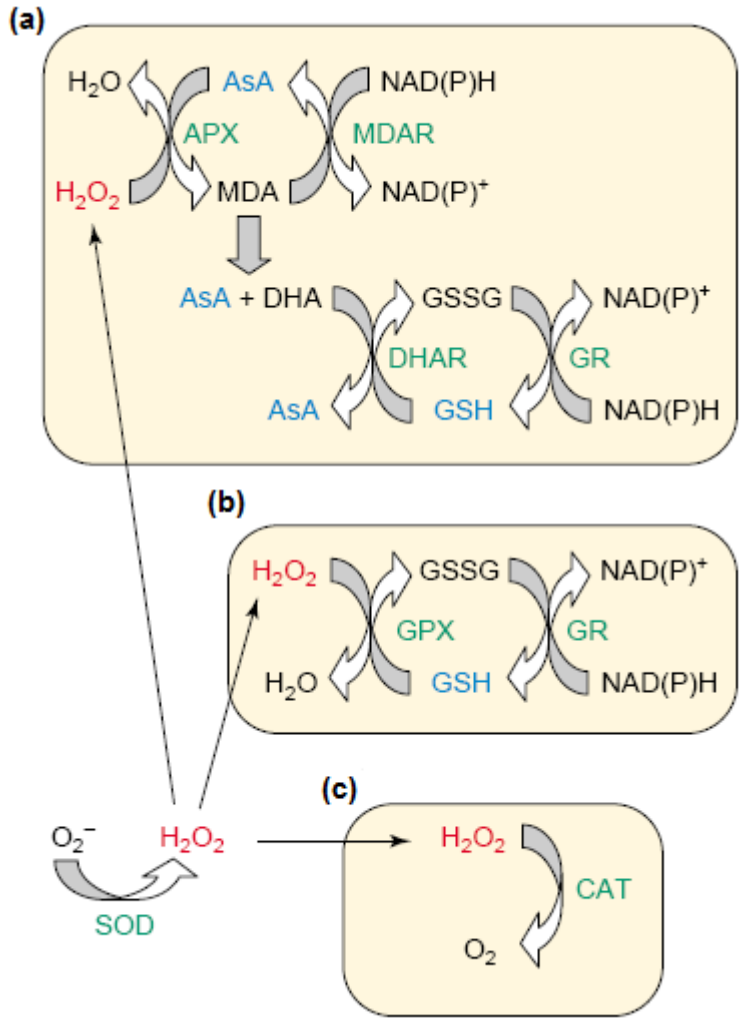


**Figura 3:** Sistemas antioxidantes de las plantas. (a) Ciclo ascorbato-glutation. (b) Ciclo glutation peroxidasa (GPX). (c) Catalasa (CAT). ROS en rojo, antioxidantes en azul y enzimas detoxificadoras en verde. Abreviaturas: ascorbato peroxidasa (APX), dehidroascorbato (DHA), DHA reductasa (DHAR), glutation reductasa (GR), glutation oxidado (GSSG), monodehidroascorbato (MDA), MDA reductasa (MDAR), superóxido dismutasa (SOD) (Modificado de Mittler 2002).

Como consecuencia de la limitación de la fotosíntesis bajo condiciones de estrés hídrico, disminuye el uso de electrones dando lugar a fotoinhibición (Roland *et al.* 2006). Para proteger al aparato fotosintético, este exceso de fotones puede ser utilizado por la fotorrespiración en plantas $C_3$. Esta vía metabólica tiene lugar en el cloroplasto, peroxisoma y mitocondria, y es consecuencia de la oxigenación de ribulosa-1,5-bifosfato (RuBP) catalizada por la RuBP carboxilasa/oxigenasa (Rubisco), la cual genera una molécula de glicerato-3-fosfato (3-PGA) y una de glicolato-2-fostafo (2-PG). Este 2-PG es hidrolizado por la enzima fosfoglicolato fosfatasa a glicolato, el cual es transportado al peroxisoma y oxidado a glioxilato por la glicolato oxidasa (GO). El glioxilato es transaminado a glicina por una reacción catalizada por la enzima glutamato:glioxilato aminotransferasa (GGAT), y es transportado a la mitocondria. Posteriormente, la glicina es transformada a serina por la acción de las enzimas glicina descarboxilasa e hidrometiltransferasa. La serina formada en la mitocondria es transportada al peroxisoma, donde es transformada por la serina:glioxilato aminotransferasa (SGAT) a hidroxipiruvato, que es reducido a glicerato por la enzima hidroxipiruvato reductasa (HR). Finalmente, el glicerato se mueve al cloroplasto, donde es fosforilado por la glicerato kinasa, dando lugar a una molécula de 3-PGA, que entra el ciclo de Calvin (Wingler *et al*. 2000) (Figura 4).

Aunque este proceso produce $H_2O_2$, puede resultar una vía beneficiosa para las plantas bajo déficit hídrico cuando la tasa de carboxilación de la RuBP se reduce (Noctor *et al.* 2002). En primer lugar, es una ruta alternativa de disipación de energía, la cual podría provocar fotoinhibición del aparato fotosintético (Smirnoff 1993). En segundo lugar, desplaza la producción de $H_2O_2$ a los peroxisomas en lugar de los cloroplastos, donde la producción del radical hidroxilo se ve favorecida y donde las enzimas del ciclo de Calvin son dianas de inhibición por $H_2O_2$ (Noctor *et al.* 2002). Finalmente, en la fotorrespiración se produce glicina, la cual es precursora de glutatión, un metabolito del ciclo ascorbato-glutation, proporcionando así una protección adicional frente al estrés oxidativo generado en condiciones de sequía (Wingler *et al.* 2000) (Figura 4). De acuerdo con la hipótesis de considerar la fotorrespiración como un proceso protector frente a condiciones de estrés, se ha observado una mayor actividad Rubisco en cultivares tolerantes a la sequía de girasol y trigo bajo condiciones de déficit hídrico (Pancović *et al.* 1999; Demirevska *et al.* 2008). Carmo-Silva *et al*. (2012) observan en plantas de algodón que una disminución en la actividad de la Rubisco limita la fotosíntesis bajo condiciones de estrés hídrico y estrés térmico. Asímismo, se ha comprobado cómo algunos cultivares de arroz sensibles al estrés hídrico muestran una baja capacidad de asimilar carbono debido a un descenso de actividad de la Rubisco, demostrando así la importancia de este proceso en la respuesta frente a este tipo de estrés (Ji *et al.* 2012).

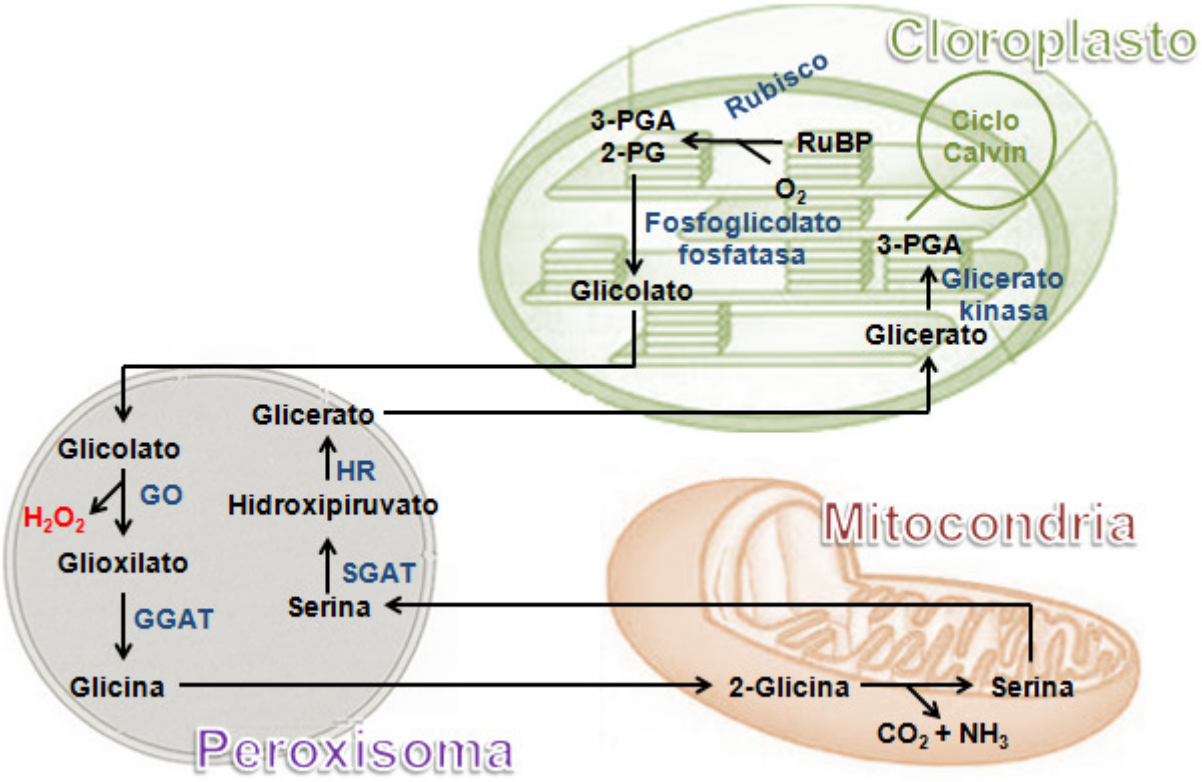


**Figura 4:** Fotorrespiración en plantas superiores. Abreviaturas: glicerato-3-fosfato (3-PGA), glicolato-2-fosfato (2-PG), glicolato oxidasa (GO), glutamato:glioxilato aminotransferasa (GGAT), hidroxipiruvato reductasa (HR), serina:glioxilato aminotransferasa (SGAT).

Los compuestos fenólicos, incluyendo fenoles simples (ácidos fenólicos y derivados) y polifenoles (flavonoides y compuesots poliméricos) también juegan un papel importante en la detoxificación de radicales libres (Ksouri *et al*. 2007). Estos compuestos ayudan a detoxificar las ROS porque neutralizan los radicales antes de que lleguen a dañar las células, y por tanto son importantes para las plantas bajos condiciones adversas (Løvdal *et al.* 2010). Su capacidad para actuar como antioxidantes depende de los potenciales de reducción de sus radicales y la accesibilidad de los radicales. Así, una respuesta común al

déficit hídrico es incrementar la síntesis de compuestos fenólicos (Parida *et al.* 2004). Se ha encontrado un aumento considerable en los niveles de compuestos fenólicos tras estreses bióticos y abióticos, tales como heridas, sequía, toxicidad de metales, y la deficiencia de nutrientes (Winkel-Shirley 2002). Hichem *et al.* (2009) demuestran que un cultivar de maíz tolerante al estrés salino presenta una elevada acumulación de polifenoles. Bajo estrés hídrico se ha observado un incremento en el contenido fenólico de plantas de lechuga (Oh *et al.* 2010). Sin embargo, poco se sabe acerca de como el estrés hídrico puede afectar a la concentración de fenoles en hojas en relación a la tolerancia a la sequía y la intensidad de ésta (Tattini *et al.* 2004).

Finalmente, la acumulación de compuestos u osmolitos, conocida como "ajuste osmótico" u "osmorregulación", se ha propuesto a menudo como una solución para contrarrestar el efecto negativo del déficit hídrico en la producción de los cultivos. Esta acumulación de osmolitos en las células resulta en un descenso del potencial osmótico celular, manteniendo así la absorción de agua y la presión de turgor, lo cual puede contribuir a mantener procesos fisiológicos como la apertura estomática, fotosíntesis y expansión celular (Blum 1996, Serraj *&* Sinclair 2002). Los solutos que se acumulan durante el ajuste osmótico incluyen iones como $K^+$, $Na^+$ y $Cl^-$, o solutos orgánicos que incluyen compuestos ricos en nitrógeno, como prolina y otros aminoácidos, poliaminas y amonios cuaternarios (Tamura *et al.* 2003).

La acumulación de prolina es un importante indicador de tolerancia al estrés hídrico en plantas superiores (Tanner 2008). Además de su papel como osmolito en el ajuste osmótico, la prolina contribuye a estabilizar las estructuras subcelulares, elimina radicales libres, y amortigua el potencial redox bajo condiciones de estrés (Hare *&* Cress 1997; Vendruscolo *et al.* 2007). La acumulación de prolina bajo condiciones de estrés genera a menudo una gran controversia en base a si una alta acumulación de prolina confiere tolerancia a través de un efecto directo beneficioso, o bien si es un marcador de la sensibilidad y por tanto es un indicador del daño causado por este estrés. En relación al estrés hídrico, Parida *et al.* (2008) indican que una inducción de los niveles de prolina podría estar relacionada con la tolerancia a la sequía en plantas de algodón. Algunos autores utilizan esta acumulación de prolina como criterio de selección de tolerancia a la sequía en plantas de tabaco (Vanrensburg *et al.* 1993). Por el contrario, Bansal & Nagarajan (1996) detectan incremento de los niveles de prolina en hojas de diez cultivares de patata. Los cultivares más susceptibles a la sequía muestran altos niveles de prolina, mientras que las variedades tolerantes acumulan menor cantidad. La misma tendencia se observa también en cultivares de mandioca, donde el cultivar tolerante muestra menores niveles de prolina bajo condiciones de estrés hídrico (Sunderasan & Sudhakaran, 1996).

Entre los compuestos orgánicos relacionados con la osmorregulación también encontramos a las poliaminas (espermidina, espermina y putrescina), que son compuestos policatiónicos que están implicadas en gran variedad de procesos fisiológicos, como el crecimiento y el desarrollo de las plantas. Efectivamente, Farooq *et al.* (2010) demuestran que la aplicación exógena de espermina mejoraba el estatus hídrico en plantas de arroz bajo estrés hídrico. Resultados similares se demuestran también en plantas de soja, donde su aplicación exógena mejora el crecimiento tanto del tallo como de la raíz bajo condiciones de déficit hídrico (Amooaghaie 2011). Asímismo, un incremento en los niveles endógenos de espermidina se ha relacionado con una mejor tolerancia al déficit hídrico en plantas de guisante (Nayyar & Chander, 2004). Por todo ello, la acumulación tanto de prolina como de poliaminas es considerada como un mecanismo de tolerancia frente a sequía en plantas (Kavi Kishor *et al.* 1995; Zhou *&* Yu 2010).

Sin embargo, aunque los compuestos orgánicos son los mayores constituyentes de la osmorregulación en las plantas durante el déficit hídrico, los iones inorgánicos como el $K^+$ también pueden contribuir al ajuste osmótico (Roberts 1998). De hecho, la síntesis y acumulación de solutos orgánicos consume más energía que la absorción de iones inorgánicos (Premachandra *et al.* 1989). Otros osmolitos que se acumulan en respuesta al estrés son azúcares como sacarosa, polioles, azúcares-alcoholes (pinitol, sorbitol,

manitol, etc.) y oligosacáridos (Mahajan *&* Tuteja 2005). Así, en trigo, los cultivares con alta concentración de $K^+$, prolina y azúcares solubles se muestran más resistentes al estrés hídrico (Wang *&* Li 2000; Verbruggen *&* Hermans 2008).

## 2.2. Papel de los injertos en las plantas hortícolas

### *2.2.1. Generalidades*

Debido a la disponibilidad limitada de zonas de cultivo, la alta demanda de vegetales fuera de temporada, y las prácticas agrícolas intensivas que conllevan a una rotación de cultivos muy limitada, los vegetales son a menudo cultivados bajo condiciones desfavorables que inducen estrés (Savvas *et al.* 2010). Para hacer frente a estos problemas, se intentan desarrollar por parte de las empresas de reproducción cultivos más tolerantes a estréses abióticos. Sin embargo, debido a la necesidad de seleccionar marcadores genéticos, resulta un proceso demasiado costoso y lento. Por ello, el uso de injertos se ha convertido en una herramienta rápida alternativa a la metodología convencional, y que permite incrementar la tolerancia de los vegetales a diversos estreses ambientales (Flores *et al.* 2010). De hecho, el uso de vegetales injertados ha sido muy popular en los últimos años (Lee *&* Oda 2003). Actualmente, el uso de plantas hortícolas de la familia Solanáceas, incluyendo un número importante de cultivos anuales como el tomate, la

berenjena y el pimiento, y Cucurbitáceas, como el melón, calabacín y sandía, se ha incrementado recientemente para obtener resistencia a enfermedades edáficas (Bletsos *et al.* 2003; Davis *et al.* 2008), así como para mejorar la tolerancia a estreses abióticos como salinidad, altas y bajas temperaturas, humedad (Ahn *et al.* 1999; Rivero *et al.* 2003a,b; Estañ *et al.* 2005; Venema *et al.* 2008; Addelmageed *&* Gruda 2009), mejorar la absorción de agua y nutrientes (Santa-Cruz *et al.* 2002), extender la duración del tiempo de cosecha (Lee 1994), y mejorar la calidad de los frutos (Fernández-García *et al.* 2004a,b; Colla *et al.* 2006).

El proceso de realización de injertos incluye: (1) elección de las especies o cultivares de la parte aérea y la parte radicular, (2) creación de la unión del injerto mediante manipulación física, (3) sanación de la unión, y (4) aclimatación de la planta injertada (Lee *et al.* 2010). Los métodos de realización del injerto varían en función del tipo de cultivo, la experiencia y preferencia del agricultor, la maquinaría disponible, el número de injertos, así como el propósito del propio injerto (Figura 5). Por lo general, los agricultores con menos experiencia y a menor escala prefieren la técnica “por aproximación” (Figura 5C), mientras que profesionales con mayor experiencia y a gran escala escogen la técnica “de empalme” (Figura 5D-J).

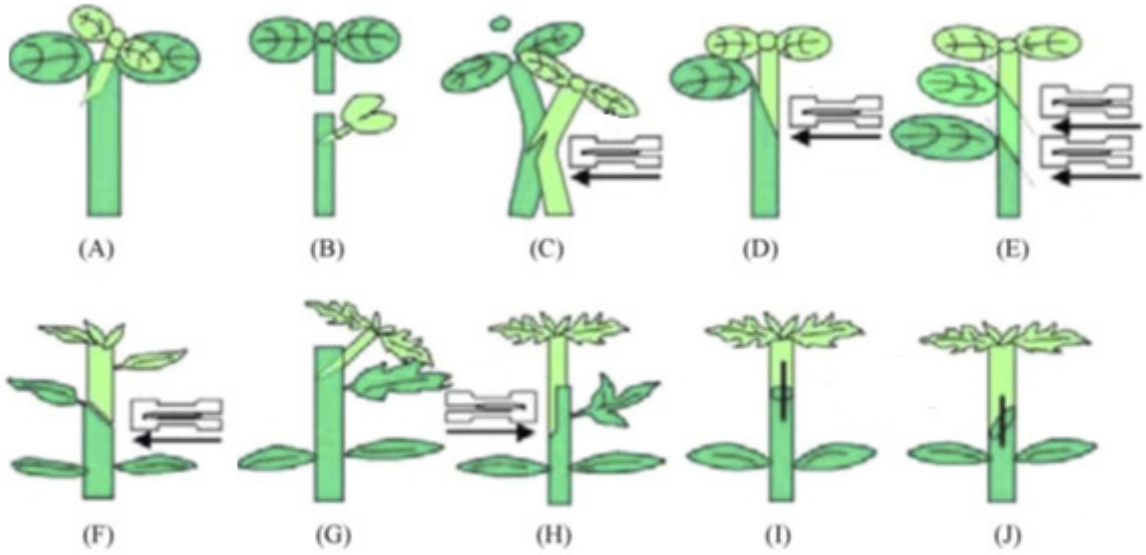


**Figura 5:** Principales métodos para la realización de injertos en solanáceas y cucurbitáceas: (A, B) por inserción; (C) por aproximación; (D, E, F) de empalme; (F, H) por hendidura; (I, J) por clavija. (Modificado de Lee *et al.* 2010).

El éxito del injerto depende a su vez de la compatibilidad de la unión del injerto en términos de la rápida formación de las conexiones entre el parte aérea y el parte radicular, y la rápida renovación del crecimiento de la raíz y la parte aérea (Cohen *et al.* 2007). En plantas injertadas, la regeneración vascular es restablecida por procesos complejos, que incluyen diferenciación estructural del tejido parenquimático a ambos lados de la unión del injerto. Así, Hartmann *et al.* (2002) observaron la secuencia de eventos en la formación de la unión del injerto: (1) el corte del tejido de la parte aérea con capacidad de actividad meristemática es colocado en contacto con un corte similar del tejido de la parte radicular, de forma que las regiones cambiales de ambos estén próximas

para que se forme una interconexión a través del puente calloso. Una vez que los dos componentes del injerto, parte aérea y parte radicular, están en contacto, proliferan nuevas células parenquimatosas, produciendo un tejido calloso que se entremezcla y entrelaza, rellenando los espacios entre los dos componentes y conectando la parte aérea y la parte radicular; (2) nuevas células cambiales se diferencian, formando una conexión continua cambial entre parte aérea y parte radicular; (3) en el último paso del proceso de establecimiento del injerto, la capa cambial formada en el puente calloso comienza a formar los nuevos tejido vasculares.

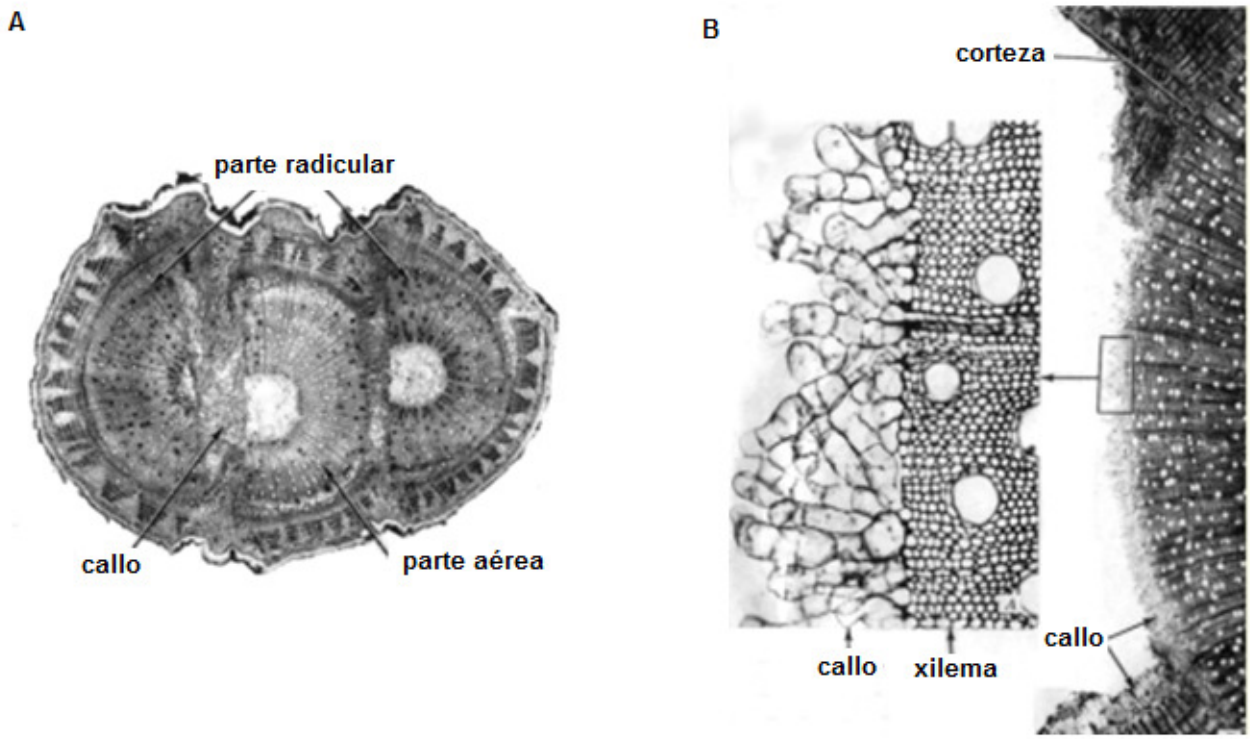


**Imagen 2:** (A) Corte transversal de un injerto que muestra la importancia del desarrollo del callo en la unión del injerto. (B) Producción del xilema indiferenciado por parte del callo. (Modificado de Hartmann *et al.* 2002).

La producción de un nuevo xilema y floema establece la conexión vascular entre la parte aérea y la parte radicular, permitiendo el paso de agua y nutrientes entre ambas partes (Imagen 2). Una vez realizado el injerto, una apropiada aclimatación es fundamental para la supervivencia de la planta. La aclimatación implica una sanación de la superficie de corte y una consolidación para la supervivencia en campo o invernadero. Además, se ha comprobado que el mantenimiento de una elevada humedad en la realización del injerto es crítica para la producción adecuada de éste (Lee *&* Oda 2003).

En la formación de los injertos por tanto, se ve afectada la morfología del sistema vascular. La formación del puente calloso entre la parte aérea y la parte radicular y la diferenciación del nuevo tejido vascular, junto con la producción del xilema y floema secundarios, son cruciales para una adecuada interacción parte aérea-parte radicular (Hartmann *et al.* 2002). Kawaguchi *et al.* (2008) observaron que la inhibición del crecimiento y la alta mortalidad en injertos entre tomate y pimiento era debida a discontinuidades en los haces vasculares en la unión del injerto. Asimismo, tras el ensamblaje de las células de la parte radicular y la parte aérea en el injerto, la diferenciación y lignificación del xilema son necesarias. Algunos autores han sugerido que las peroxidasas juegan un importante papel en el proceso de lignificación (Whetten *et al.* 1998; Quiroga *et al.* 2000). La observación de la estructura de unión en el

injerto muestra que la formación del xilema y del floema ocurre 8 días después de la realización del injerto en plantas de tomate (Fernández-García *et al.* 2004a). De este modo, la medida de la conductancia hídrica indica que la unión del injerto es completamente funcional a los 8 días, coincidiendo con un incremento de las actividades peroxidasa y catalasa, sugiriendo que estas enzimas están implicadas en el desarrollo del injerto y son necesarias para una óptima comunicación entre el parte aérea y el parte radicular.

### *2.2.2. Características fisiológicas de las plantas injertadas*

#### *2.2.2.1. Crecimiento y efecto hormonal*

El crecimiento de las plantas está influenciado por varios factores (condiciones ambientales, estado nutricional y actividad hormonal) relacionados con diferentes procesos fisiológicos. El desarrollo de una adecuada estructura del sistema radicular se ha relacionado con una mejora en el crecimiento en plantas de melón injertadas en especies *Cucurbita* (Bletsos 2005). De acuerdo con estos resultados, Yetisir *et al.* (2007) observan que plantas injertadas de melón presentan un mayor número de hojas y peso seco en comparación con las plantas sin injertar.

Por otro lado, el crecimiento de las plantas depende a su vez de la actividad fotosintética, la cual puede verse afectada por los injertos. De hecho, los resultados obtenidos en plantas de calabacín y de tomate injertadas sugieren

que la parte aérea tiene efecto en la función estomática, produciendo una elevada tasa de fijación de $CO_2$ y una menor resistencia estomática que las plantas no injertadas (Rouphael *et al.* 2008; He *et al.* 2009). Más recientemente se ha sugerido que la restricción del área foliar podría resultar de la inhibición de la fotosíntesis, lo cual disminuye la disponibilidad de fotoasimilados para el crecimiento de la hoja. Esto ha sido observado en plantas de sandía no injertadas comparadas con plantas injertadas en la parte radicular de calabaza, las cuales muestran una mayor producción de la parte aérea que es debida probablemente a su capacidad de mantener una elevada tasa de asimilación de $CO_2$ (Colla *et al.* 2010).

Otra hipótesis que se ha postulado es que algunas señales originadas en la raíz debido al injerto, como ABA y citoquininas, pueden proteger la fotosíntesis en las hojas de la parte aérea (Etahadnia *et al*. 2008). Sin embargo un incremento de la concentración de ABA en el xilema puede inducir un cierre estomático, disminuyendo la fijación de $CO_2$ e incrementado la generación de ROS. De acuerdo con esta idea, un incremento en la actividad de las enzimas antioxidantes ha sido observada en plantas de tomate injertadas (Rivero *et al.* 2003b). El bajo nivel de ROS en plantas injertadas, probablemente debido a las citoquininas que activan enzimas detoxificadoras de ROS (Pogany *et al.* 2003), es una de las posibles razones que pueden disminuir la fotoinhibición (Zhou *et al.* 2009).

Como hemos visto, las hormonas de las plantas son importantes factores endógenos que regulan todos los aspectos del desarrollo vegetativo y reproductivo, y juegan un papel fundamental en la comunicación raíz-parte aérea. De acuerdo con este concepto, las auxinas son producidas en los ápices de los brotes y son translocadas a la raíz, donde afectan a su desarrollo, morfología y funcionalidad (Aloni *et al.* 2010). Asimismo, las auxinas también afectan a la producción y actividad de las citoquininas, las cuales son producidas en la raíz y translocadas a la parte aérea donde controlan importantes procesos del desarrollo como el crecimiento y la productividad (Jones 1986; Albacete *et al.* 2008). Bangerth (1994) demostró que existe un bucle de realimentación en el que una disminución en el flujo de auxinas del tallo estimula la síntesis y la exportación de citoquininas de la raíz. Por todo ello, la realización de injertos podría tener efectos en el balance hormonal de la planta, afectando a procesos importantes del desarrollo. Efectivamente, Sorce *et al.* (2002) han demostrado en melocotón, que las plantas no injertadas presentaban un balance equilibrado auxinas/citoquininas, mientras que en las plantas injertadas este balance se alteraba. Por lo tanto, las propiedades vigorizantes de la parte radicular inducen una alta tasa de crecimiento en la parte aérea, posiblemente debido a un incremento del suministro de citoquininas a la parte aérea y un descenso de auxinas. Van Hooijdonk *et al.* (2010) han mostrado que las partes radiculares con diferente vigor pueden

modificar la arquitectura de la parte aérea tras el injerto. Así, una base radicular con poco vigor y de pequeño tamaño reduce la longitud del tallo y el número de nudos de la parte aérea comparado con una base radicular más vigorosa. En este caso se ha propuesto que la parte radicular de menos vigor reduce el transporte de auxinas a la raíz y por tanto disminuye la cantidad de citoquininas producidas en la raíz y transportadas a la parte aérea. Por otro lado, numerosos estudios han demostrado que los injertos contribuyen de forma significativa a la producción y floración bajo diferentes condiciones de cultivo, debido principalmente al efecto hormonal. Así, Stevens *&* Weswood (1984) observan que tras injertar plantas de cerezo con diferentes bases radiculares se aprecian diferencias significativas en el porcentaje de cuajado de frutos así como en la producción final. Además, las bases radiculares que proporcionaban un mayor porcentaje de cuajado y mayor producción mostraban un nivel más alto de citoquininas en sus haces vasculares. Asimismo, se ha demostrado que en plantas de sandía injertadas la formación de flores femeninas es más temprana que en plantas sin injertar (Sakata *et al.* 2007).

#### *2.2.2.2. Injertos y estrés abiótico*

Una de las posibles aplicaciones de los injertos en la producción de vegetales comerciales es mitigar el daño causado por diferentes estreses abióticos. En este sentido, tanto la estructura radicular como la eficiencia de absorción están

determinadas por la parte radicular, por ello es razonable asumir que las plantas injertadas con diferentes bases radiculares pueden presentar diferentes habilidades en la toma de nutrientes (Savvas *et al.* 2010). De hecho, varios trabajos demuestran que los injertos pueden limitar la toxicidad por metales pesados o nutrientes (Edelstein *et al.* 2005; Rouphael *et al.* 2008; Arao *et al.* 2008). Las pérdidas en producción causadas por la salinidad también pueden ser mitigadas en plantas injertadas utilizando bases radiculares capaces de reducir el daño inducido por la salinidad en la parte aérea (Colla *et al.* 2010). Se han propuesto varias explicaciones para este proceso: (1) elevada acumulación de prolina y azúcares en hoja (Ruiz et al. 2005); (2) alta capacidad antioxidante en hoja (López-Gómez et al. 2007); (3) baja acumulación de $Na^+$ y/o $Cl^-$ en hojas (Fernández-García *et al.* 2004c; Estañ *et al.* 2005; Zhu *et al.* 2008). De acuerdo con estos datos, Liu *et al.* (2007) observan un mejor crecimiento de la parte aérea en plantas de berenjena injertadas bajo condiciones de estrés salino, en comparación con plantas sin injertar, en las cuales se producía una inhibición en la elongación de los tallos.

Bajo estrés térmico, bien por bajas o por altas temperaturas, los injertos también pueden mejorar la respuesta de las plantas a este estrés. El estrés térmico puede provocar desórdenes fisiológicos en las plantas hortícolas, limitando su crecimiento y producción, y dependiendo de su intensidad y duración puede conllevar una disfunción irreversible, muerte celular y finalmente la muerte de la planta (Allen & Ort 2001). Plantas injertadas de

calabacín utilizando como parte radicular una especie de calabaza, con una temperatura óptima de crecimiento más baja que el calabacín, permite un buen crecimiento de las plantas de calabacín bajo condiciones de temperatura subóptima (Zhou *et al.* 2007). Resultados similares se han obtenido en plantas de tomate, donde una correcta elección de la base radicular permite un óptimo crecimiento de la parte aérea bajo condiciones de estrés térmico (Rivero *et al.* 2003a,b; Venema *et al.* 2008). Por tanto, en el caso del estrés térmico, la capacidad de la parte radicular para aliviar el efecto negativo en la parte aérea de las altas o bajas temperaturas de la zona radicular dependerá de cómo se vea afectado su crecimiento y funcionalidad, de la interacción entre la parte radicular y la parte aérea, así como de la duración e intensidad del estrés.

Una de las vías para reducir las pérdidas de producción producidas por el déficit hídrico y mejorar el estado hídrico de los cultivos es la utilización de injertos (García-Sánchez *et al.* 2007; Satisha *et al.* 2007). Rouphael *et al.* (2008) demostraron que las plantas de minisandía injertadas mostraban una producción un 60% mayor que las plantas sin injertar bajo condiciones de estrés hídrico. Resultados similares se han obtenido con plantas injertadas de kiwi y de vid, demostrando que los cultivares resistentes al estrés hídricos y utilizados como base radicular, mejoran la producción de la parte aérea bajo condiciones de estrés hídrico (Clearwater *et al.* 2004). La mayor producción observada en las plantas injertadas podría ser debida a una mejora en la absorción de agua y nutrientes, así como una mejor asimilación de $CO_2$. Un

experimento utilizando injertos con mutantes de tomate deficientes en ABA demuestra que el cierre estomático puede ocurrir independientemente del estado hídrico de la hoja, sugiriendo que es la raíz la que produce una señal que controla la conductancia estomática (Holbrook *et al.* 2002). Por tanto una correcta selección de la parte aérea y radicular en plantas injertadas podría mejorar el crecimiento y la producción de las plantas hortícolas bajo condiciones de estrés hídrico.

Las plantas almacenan minerales y otros nutrientes en diferentes órganos, como la raíz, el tallo, las hojas y/o los frutos. Estos órganos tienen una considerable influencia en la absorción y translocación de los nutrientes en la planta y juegan un papel importante en procesos fisiológicos como el crecimiento, la transducción de señales y el desarrollo (Wang *et al.* 2006; Flowers *&* Colmer 2008). En plantas injertadas, la influencia de la parte radicular en el contenido mineral de la parte aérea ha sido atribuido a características físicas del sistema radicular, como el desarrollo lateral y vertical de la raíz, lo cual mejora la absorción de agua y nutrientes (Heo 1991; Jang 1992). Sin embargo, en árboles frutales injertados, no se han encontrado efectos de la parte radicular en el contenido mineral (Chaplin *&* Westwood 1980), y en algunos casos, se ha observado una mayor influencia de la parte aérea. Tagliavani *et al.* (1993) sugieren que tanto el vigor de la parte aérea como del sistema radicular tienen un importante papel en la absorción y

translocación de nutrientes. A su vez, el contenido de macro y micronutrientes depende del propio elemento y de las condiciones ambientales, por lo que el efecto de la parte aérea y la parte radicular puede variar. La influencia del injerto en el contenido de nitrógeno (N) en plantas de melón determinó que el genotipo de la parte radicular tenía más importancia que la parte aérea (Ruiz *et al.* 1997). Por tanto, si la acumulación de nitrato ($NO_3^-$) está determinada por la combinación parte radicular-parte aérea, la actividad nitrato reductasa (NR) también se ve afectada por el injerto. De esta forma las características de la parte radicular en plantas injertadas incrementan la absorción, transporte y acumulación de $NO_3^-$ en la parte aérea, y consecuentemente se estimula la NR y la asimilación de $NO_3^-$, disminuyendo la concentración foliar de $NO_3^-$ en comparación con las plantas no injertadas (Ruiz & Romero 1999). Resultados similares fueron obtenidos por Pulgar *et al.* (2000) en plantas de sandía, donde los niveles de $NO_3^-$ y amonio ($NH_4^+$) fueron más bajos en las hojas de las plantas injertadas, indicando que la parte radicular mejora la eficiencia de la NR favoreciendo la integración del $NO_3^-$ a aminoácidos y proteínas. Otros autores, sin embargo, no encuentran diferencias significativas en el estatus de N con la realización de injertos, concluyendo que el contenido de N depende de las condiciones ambientales en las cuales se desarrolle la planta (Colla *et al.* 2010).

Por otro lado, se ha demostrado que la parte radicular puede mejorar algunas características morfológicas y fisiológicas debido a un incremento de la

absorción de fósforo (P) y su translocación a las hojas de la parte aérea (Ruiz *et al.* 1996). Así, las concentraciones de P en las hojas y tallos de plantas de calabacín se vieron afectadas por la combinación parte radicular-parte aérea, siendo los valores de las plantas injertadas más elevados que las plantas sin injertar (Rouphael *et al.* 2008). Sin embargo, otros autores demuestran que no hay diferencias significativas en la concentración de P en plantas de calabacín injertadas en dos tipos diferentes de base radicular (Huang *et al.* 2010). Todos estos resultados indican que las características morfológicas de la raíz no son el único factor que puede afectar a la absorción y translocación de P en plantas, y que el genotipo de la parte aérea también tiene que ser tenido en cuenta.

En el caso del calcio ($Ca^{2+}$) y el magnesio ($Mg^{2+}$), se ha observado que su contenido está influenciado por la parte radicular, pero en general, no hay efecto de la parte aérea en su absorción. Esto puede deducirse de trabajos como el de Ruiz *et al.* (1997), donde observan que en plantas de melón injertadas las concentraciones de $Ca^{2+}$ son más bajas que en plantas no injertadas, mientras que el $Mg^{2+}$ sufre un comportamiento opuesto. Sin embargo, los niveles de ambos minerales disminuyen cuando las plantas de tomate son injertadas en plantas de pimiento y viceversa, con respecto a las plantas sin injertar (Kawaguchi *et al.* 2008). Para el sodio ($Na^{+}$) y el potasio ($K^{+}$), no se encontraron diferencias significativas en su contenido en plantas de tomate injertadas y no injertadas (Santa-Cruz *et al.* 2002; He *et al.* 2009);

mientras que otros autores muestran incrementos significativos de $K^+$ y cambios no significativos en la concentración de $Na^+$ (Fernández-García *et al.* 2004c; Martínez-Rodríguez *et al.* 2008).

En conclusión, la conexión parte aérea-parte radicular es fundamental para un óptimo crecimiento, y absorción y transporte de agua y nutrientes. Una deficiencia en nutrientes minerales y agua podría causar una supresión del crecimiento de la parte aérea y bajas concentraciones de carbohidratos en la raíz, lo cual conllevará una disminución del crecimiento de la raíz, provocando un descenso en la absorción de agua y nutrientes. En otras palabras, una alteración fisiológica inducida por una unión vascular discontinua del injerto puede provocar inhibición del crecimiento debido a una comunicación restringida entre la parte radicular y la parte aérea. Por lo tanto, además de la compatibilidad y la correcta combinación de la parte aérea y la parte radicular para el objetivo previsto (resistencia al estrés), se debe tener cuidado al realizar el injerto para lograr una óptima conexión entre la parte aérea y la parte radicular.

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# *Capítulo 3*

## *Antioxidant response resides in the shoot in reciprocal grafts of drought-tolerant and drought-sensitive cultivars in tomato under water stress*

***Plant Science (2012)188, 89-96***

**ABSTRACT**

Recently grafted plants have been used to induce resistance to different abiotic stresses. In our work, grafted plants of tomato cultivars differing in water stress tolerance (Zarina and Josefina) were grown under moderate stress, to test the roles of roots and shoots in production of foliar biomass and antioxidant response. Stress indicators and activities of selected enzymes related to antioxidant response were determined. Our results showed that when shoots are of the drought tolerant genotype Zarina, the changes in antioxidant enzyme activities were large and consistent. However, when shoots are of the drought-sensitive genotype Josefina, the antioxidant enzyme activities were more limited and the oxidative stress was evident. These results reflect that the technique of grafting using Zarina as scion can be useful and effective for improving the antioxidant response in tomato under water stress.

**Keywords:**Grafting, water stress, Halliwell-Asada cycle, antioxidant response, tomato.

**Abbreviations:** APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, glutathione; LOX, lipooxygenase; LRWC, leaf relative water

content; MDHAR, monodehydroascorbate reductase; RGR, relative growth rate; ROS, reactive oxygen species; SOD, superoxide dismutase.

## INTRODUCTION

Many crops are cultivated in areas where the climatic conditions are not always ideal and where precipitations may periodically be below optimal levels. The predicted increase of dry days per year for many areas of the globe will further exacerbate the problem, especially in arid and semiarid zones of the Mediterranean Basin (Luterbacher *et al.* 2006). Under various stresses such as drought and chilling, $CO_2$ assimilation is suppressed, leading to increases in excess excitation energy and electron flux to $O_2$ (Zhou *et al.* 2009), leading to photo-oxidative stress by overproduction of reactive oxygen species (ROS) (Pinheiro *et al.* 2004). Due to their chemical properties, ROS are highly reactive and can damage proteins, chlorophylls, membrane lipids and nucleic acids (Blokhina *et al.* 2003). To prevent or alleviate these damages, plants posses a complex antioxidant system to detoxify ROS, including low-molecular mass antioxidants as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and enzymes involved in the ascorbate-glutathione cycle (Halliwell-Asada cycle) (Figure 1) (Foyer *&* Halliwell 1976). The activities of these antioxidant enzymes are increased in response to several abiotic stresses such as drought (Jaleel *et al.* 2007), salinity (Manivannan *et al.* 2008), ozone (Puckette *et al.* 2007), and temperature (Ali *et al.* 2005). In this context, it is

believed that a simultaneous increase in several components of the antioxidative defense system would be necessary in order to obtain an increase in the plant protective mechanisms (Jaleel *et al.* 2009).

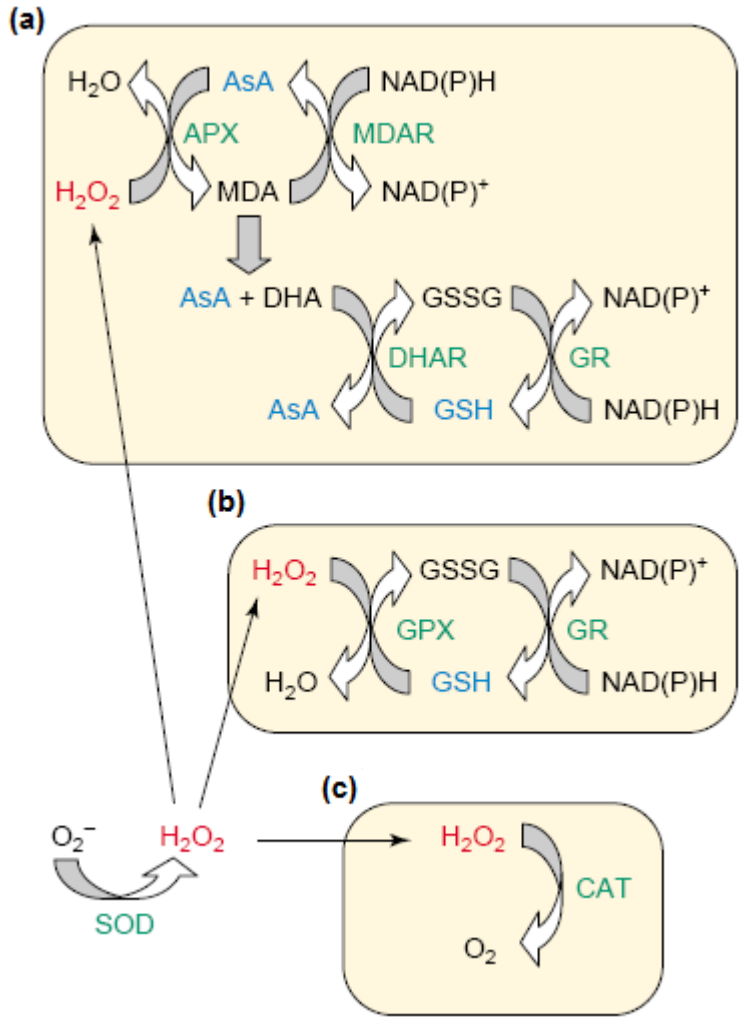


**Figure 1.** Antioxidant systems in plants. (a) Halliwell-Asada cycle. (b) Glutation peroxidase cycle (GPX). (c) Catalase (CAT). ROS en red, antioxidants in blue and scavengers enzymes in green. Abbreviations: ascorbate peroxidase (APX), dehydroascorbate (DHA), DHA reductase (DHAR), glutation reductase (GR), glutation oxidized (GSSG), monodehydroascorbate (MDA), MDA reductase (MDAR), superoxide dismutase (SOD) (Modified of Mittler 2002).

Cultivation of grafted plants began in late 1920, primarily to counteract or diminish attacks by soil pathogens such as *Fusarium oxysporum* (Yamakawa 1983). However, the current applications of grafting involve virtually all fields of plant physiology. For example, grafted plants have been used recently to induce resistance to low and high temperatures (Rivero *et al.* 2003; Zhou *et al.* 2009), enhance nutrient uptake (Ahmedi *et al.* 2007), induce resistance against heavy metal toxicity (Rouphael *et al.* 2008) and increase synthesis of endogenous hormones (Dong *et al.* 2008). Currently, most fruit crops and many horticultural species are grown as scion-rootstock combinations. Although this strategy increases the work required by breeders (selection for rootstock, scion and the combination), it may allow obtain desired features. For instance, salt tolerance may be conferred by a suitable rootstock, while retaining the excellent fruit yield and quality traits of the scion (Estañ *et al.* 2005; Martínez-Rodríguez *et al.* 2008). Romero *et al.* (1997) observed that root characteristics such as exclusion of $Cl^-$ ion and/or decrease in $Cl^-$ absorption are of primary importance in determining salt tolerance in melon plants; whereas Chen *et al.* (2003) showed that the shoot tolerance due to its ABA level rather than that of the root in tomato under salinity stress. Santa-Cruz *et al.* (2002) suggested that both shoot and root characteristics played important roles in salt tolerance. However, information on how drought tolerance and ROS metabolism in leaves are influenced by rootstock-scion combinations is still scarce.

Tomato is one of the most important vegetable crops worldwide and also one of the most water demanding (Peet 2005). Therefore, adoption of deficit irrigation strategies may result in significant savings of irrigation water. In a previous work, we selected a drought tolerant (cv. Zarina) and a sensitive one (cv. Josefina) using 5 commercial tomato cultivars, and analysed certain biochemical indicators in these genotypes (Sánchez-Rodríguez *et al.* 2010). The aim of the present work was to evaluate the usefulness of grafting as a tool for increasing water stress resistance in tomato plants. We studied the response to moderate water stress of different combinations scion-rootstock in grafted, self-grafted and ungrafted tomato plants to test the viability and efficiency of this grafting technique in terms of production and antioxidant response.

## MATERIALS AND METHODS

### *Plant material and treatments*

Two tomato (*Lycopersicon esculentum* Mill) cultivars, Zarina and Josefina, were used as scion and rootstock (Figure 2). The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm x 3 cm x 10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Grafting was performed when seedlings has developed 3-4 true leaves. In the vermiculite trays used for germination, the seedlings were cut over the

cotyledons, using the shoot as scion and the remaining plant part as rootstock. Grafts were made inmediately after cutting the plants and grafting clips were used to adhere the graft union. Self-grafted plants were included as controls. After grafting, seedlings were covered with a transparent plastic lid to maintain a high humidity level and to facilitate graft formation and were left in the shade for 24 h. The plastic was opened slightly every day to allow reduction in relative humidity and it was removed 6 days after grafting. Afterwards, ungrafted and grafted plants were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with relative humidity of 50±10%, at 25ºC/15ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) 350 µmol $m^{-2}s^{-1}$ (measured with an SB quantum 190 sensor, LI-COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. Throughout the experiment, the plants were grown with a complete nutrient solution containing: 4 mM $KNO_3$, 3 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 6 mM $KH_2PO_4$, 1 mM $NaH_2PO_4$, 2 µM $MnCl_2$, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4$, 0,1 µM $Na_2MoO_4$, 5 µM Fe-EDDHA, and 50 µM $H_3BO_3$ (Sánchez-Rodríguez *et al.* 2010).

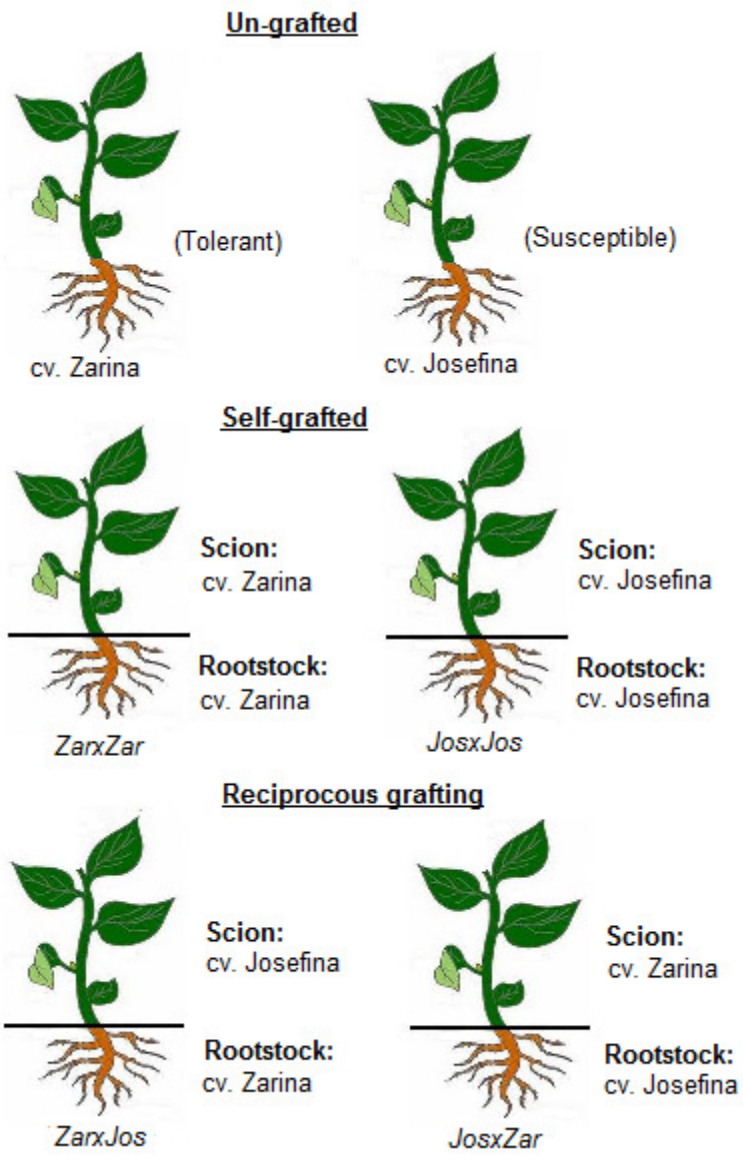


**Figure 2.** Outline of the grafting design.

The water-stress treatments began 45 days after germination and were maintained during 22 days. The control treatment received 100% field capacity (FC) irrigation, whereas moderate water stress corresponded to 50 % field capacity. The experimental design was a randomized complete block with 12

treatments (Zarina ungrafted, Josefina ungrafted, Zarina self-grafted, Josefina self-grafted, *JosxZar* and *ZarxJos* well-watered 100% FC and water stress 50%) (Figure 2) arranged in individual pots with six plants per treatment (one plant per pot) and three replications each. All plants were at the late vegetative stage (not flowering) when harvested. Leaves fully expanded (excluding petioles) and roots were harvested, frozen immediately in liquid $N_2$, and kept at -80ºC until used.

*Relative-Growth-Rate (RGR) and Leaf Relative Water Content (LRWC)*

All plants were at the late vegetative stage when harvested. Leaves fully expanded (excluding petioles) and roots were harvested, frozen immediately in liquid $N_2$, and kept at -80ºC until used. To determine the relative growth rate (RGR), leaves and roots from three plants per cultivar were sampled on day 45 after germination, immediately before starting the water-stress treatment (Ti). The leaves and roots were liophilized, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 67 days after germination (22 days of treatments, Tf). The relative growth rate was calculated from the increase in DW at the beginning and at the end of the water-stress treatment, using the equation RGR = (ln DWf – ln DWi)/(Tf – Ti) where T is the time and the subscripts denote the final and initial sampling (i.e. days 0 and 22, respectively, after the water-stress treatment) (Bellaloui *&* Brown 1998).

Leaf relative water content (LRWC) was measured following the method of Barrs and Weatherley (1962).

*Photosynthetic pigments*

Total chlorophyll and carotenoid were extracted in methanol and centrifugated at 5000 g for 5 min. Thereafter, the absorbance of the supernatant was measured at 664, 648, and 470 nm. The chlorophyll a (Chl a) and chlorophyll b (Chl b) and carotenoids were estimated by using the equation of Lichtenthaler (1987).

*Concentrations of malondialdehyde, $O_2^{\cdot -}$ and $H_2O_2$ in leaf extracts*

For the MDA assay, leaves were homogenized with 5 mL of 50 mM solution containing 0.07 % $NaH_2PO.2H_2O$ and 1.6 % $Na_2HPO_4 \cdot 12\ H_2O$ and centrifuged at 20,000 g for 25 min in a refrigerated centrifuge. For measurement of MDA concentration, method of Heath and Packer (1968) was used. Results were expressed as nmol $g^{-1}$ DW.

Determination of $O_2^{\cdot -}$ in leaf extracts was based on the ability to reduce nitro blue tetrazolium (NBT) (Kubis 2008). Optical density was measured at a

wavelength of 580 nm and the $O_2^{\cdot -}$ concentration was expressed as $A_{580}$ $g^{-1}$ DW.

The $H_2O_2$ concentration in leaf extracts was colorimetrically measured as described by Mukherje and Choudhuri (1983). Leaf samples were extracted with cold acetone to determine the $H_2O_2$ levels. The intensity of yellow colour of the supernatant was measured at 415 nm. The result of $H_2O_2$ concentration was expressed as µmol $g^{-1}$ DW.

*Ascorbate assay*

The extraction and quantification of total AsA, reduced AsA, and dehydroascorbate (DHA) in leaf extracts followed the method of Law et al. (1992), modified from Okamura (1980). This method is based on the reduction of $Fe^{3+}$ to $Fe^{2+}$ by AsA in acid solution. Leaves material were homogenized in liquid $N_2$ with metaphosphoric acid at 5 % (w/v) and centrifuged at 4ºC for 15 min. Absorbance was measured at 525 nm against a standard AsA curve that followed the same procedure as above. The results were used to quantify the total AsA concentration, while the reduced AsA was quantified in the same way as the previous procedure, replacing 0.1 mL of DTT with 0.1 mL of distilled $H_2O$. Finally, the DHA concentration was deduced from the difference between total AsA and reduced AsA. The result of ascorbate forms were expressed as mg $g^{-1}$ DW.

*Gluthathione assay*

Reduced GSH, oxidized GSH (GSSG), and total GSH (reduced GSH+GSSG) were the recycling assay initially described by Tietze (1969) and modified by Noctor and Foyer (1998). GSSG and total GSH were assayed in the same extract. A standard curve was developed by preparing solutions of 0.002–0.0001 g $mL^{-1}$ of GSH in 60 mL of metaphosphoric acid (pH 2.8) containing 1 mM of ethylenediaminetetraacetic acid (EDTA), diluting 1:2,000 with of $Na_2PO_4$ 50mM, and analyzing in the same manner as for the extracts. GSH levels were estimated as the difference between total GSH and GSSG.

*Antioxidant enzymatic activities in leaf extracts*

SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), according to the methods of Giannopolitis and Ries (1977) with some modifications (Yu *et al.* 1998). Leaves material were homogenized in liquid $N_2$ with buffer Heppes-HCl 50 mM pH 7.6 and centrifuged at 4ºC for 10 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

CAT (EC 1.11.1.6) activity was determined by following the consumption of $H_2O_2$ at 240 nm for 5 min (Nakano *&* Asada 1981). Leaves material were homogenized in liquid $N_2$ with buffer Heppes-HCl 25 mM pH 7.8 and centrifuged at 4ºC for 15 min. The reaction mixture contained 25 mM Tris-acetate buffer (pH 7.0), 0.8 mM Na-EDTA and 20 mM $H_2O_2$, and enzyme assay was performed at 25 ºC.

The activitives of the enzymes APX (EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.1) were assayed in leaf extracts following Rao *et al.* (1996). APX activity was determined by registering the absorbance change at 290 nm. GR activity was measured after monitoring the oxidation of NADPH at 340 nm for 3 min.

Dehydroascorbate reductase activity (DHAR; EC 1.8.5.1) in leaf extracts was measured at 265 nm for 3 min following the change in absorbance resulting from the formation of AsA (Nakano *&* Asada 1981). In addition, the enzyme monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) was assayed by registering the change in absorbance of the samples at a wavelength of 340 nm (Foyer *et al.* 1989).

LOX activity in leaf extracts was measured according to Minguez- Mosquera *et al.* (1993), using 50 mM K-phosphate buffer (pH 6.0) for extraction.

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

*Statistical analysis*

The data were analyzed by a simple variance analysis (ANOVA) and differences between the means were compared by Fisher´s least-significant difference test (LSD).

## RESULTS

*Relative-Growth-Rate (RGR) and Leaf Relative Water Content (LRWC)*

The total biomass diminished significantly in the cv. Josefina as well as in *JosxJos* under water-stress conditions. The cv. Zarina did not appear to be affected by the stress, while its self-graft had a negative effect on total biomass, with a reduction of 41% with respect to control. In terms of reciprocal grafts, none presented a reduction of total biomass under water-stress conditions, values being higher in the graft *ZarxJos* in both treatments, well-watered and water stress (Table 1).

In cv. Josefina and its self-graft, the total RGR declined under stress conditions. In contrast, the cv. Zarina was not significantly affected, although a 30% loss was found for *ZarxZar* with respect to the well-watered treatment. In the grafts *JosxZar* and *ZarxJos*, no differences were observed under stress conditions. In terms of biomass, the graft *ZarxJos* maintained the highest levels of total RGR (Table 1).

Both in cv. Josefina as well as *JosxJos*, the root RGR fell with the water-deficit treatment. The cv. Zarina increased its RGR to 88% under stress conditions, while its self-graft was not visibly affected. The reciprocal grafts behaved in the contrary way, with root RGR in *JosxZar* decreasing 21% under stress, and *ZarxJos* increasing 20% (Table 1).

LRWC is a reliable indicator of the capacity of the plant to re-establish its water balance after a water-deficit situation, as well as the capacity to tolerate this stress. Only cv. Josefina, its self-graft, and *JosxZar* showed a significant decline in LRWC with the stress applied, while the rest were not appreciably affected (Table 1).

**Table 1:** Influence of moderate water stress on dry weight, RGR and LRWC in ungrafted, grafted and self-grafted tomato plants.

| Rootstock x Scion/ Treatment | Total Biomass (g DW) | Total RGR (mg/g/day) | Radicular RGR (mg/g/day) | LRWC (%) |
|---|---|---|---|---|
| Zar ungrafted | | | | |
| Well-watered | 7.06±0.73 | 62.34±5.03 | 38.56±3.60 | 89.97±1.0 |
| Water stress | 6.15±0.53 | 58.14±4.53 | 72.49±3.91* | 89.83±5.11 |
| $LSD_{0.05}$ | 2.52 | 18.81 | 14.77 | 4.47 |
| *ZarxZar* | | | | |
| Well-watered | 8.91±0.70 | 81.03±3.66 | 46.03±0.52 | 87.53±3.12 |
| Water stress | 6.20±0.37* | 56.58±3.23* | 59.66±3.44* | 90.02±1.77 |
| $LSD_{0.05}$ | 2.20 | 13.56 | 9.67 | 9.98 |
| Jos ungrafted | | | | |
| Well-watered | 10.59±0.42 | 70.36±1.84 | 37.91±5.80 | 90.31±0.18 |
| Water stress | 5.82±0.18* | 43.18±1.38* | 29.19±0.21* | 79.80±1.44* |
| $LSD_{0.05}$ | 1.27 | 6.40 | 6.12 | 4.03 |
| *JosxJos* | | | | |
| Well-watered | 10.49±1.08 | 58.42±4.88 | 23.20±0.86 | 88.56±0.45 |
| Water stress | 7.08±0.04* | 41.08±0.31* | 15.97±4.73* | 83.68±1.22* |
| $LSD_{0.05}$ | 3.02 | 13.59 | 3.35 | 3.63 |
| *JosxZar* | | | | |
| Well-watered | 6.41±0.98 | 51.30±7.64 | 26.01±6.14 | 92.59±4.89 |
| Water stress | 4.94±0.71 | 39.59±7.12 | 20.60±6.27* | 76.48±3.75* |
| $LSD_{0.05}$ | 3.36 | 19.02 | 4.39 | 7.14 |
| *ZarxJos* | | | | |
| Well-watered | 10.13±1.93 | 59.60±8.10 | 54.39±0.30 | 94.63±2.12 |
| Water stress | 7.62±0.45 | 41.61±3.19 | 65.54±4.22* | 92.85±0.01 |
| $LSD_{0.05}$ | 4.50 | 24.16 | 10.07 | 5.89 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05) Asterik (*) indicates significant difference with controls groups (well-watered)

### *Phoosynthetic pigments, $O_2^{\cdot-}$ and $H_2O_2$ concentration and lipid peroxidation*

In Zarina ungrafted and reciprocal grafts (*JosxZar* and *ZarxJos* combinations), no differences were observed under stress conditions for Chl a, Chl b and carotenoids. Conversely, a significant decrease under water stress conditions in the concentration of these pigments were found in the cv. Josefina and selfgrafts (*ZarxZar* and *JosxJos*) (Table 2).

ROS concentration is a good indicator of tissue oxidative stress. In cv. Zarina and its self-graft, water stress did not increase significantly $O_2^{-\cdot}$ (Figure 3A) and $H_2O_2$ concentration (Figure 3C). Conversely, a significant increase in the concentration of both compounds was found in the cv. Josefina and in the combination *JosxJos*. Considering the reciprocal grafts, only the combination *ZarxJos* showed an increase in the $O_2^{-\cdot}$ and $H_2O_2$ concentrations (Figure 3A and 3C), whereas no significant differences were observed in the combination *JosxZar* (Figure 3 and 3C).

**Table 2:** Influence of moderate water stress on photosynthetic pigments in ungrafted, grafted and self-grafted tomato plants.

| Rootstock x Scion/ Treatment | Chl a (µg $g^{-1}$FW) | Chl b (µg $g^{-1}$FW) | Carotenoids (µg $g^{-1}$FW) |
|---|---|---|---|
| Zar ungrafted | | | |
| Well-watered | 58.7±6.0 | 33.1±3.6 | 65.5±4.8 |
| Water stress | 44.8±3.7 | 31.2±2.2 | 54.9±2.7 |
| $LSD_{0.05}$ | 15.1 | 9.1 | 11.7 |
| *ZarxZar* | | | |
| Well-watered | 106.8±13.9 | 62.3±9.5 | 63.3±5.2 |
| Water stress | 51.0±7.9* | 30.9±9.2* | 38.4±2.7* |
| $LSD_{0.05}$ | 14.5 | 14.6 | 12.5 |
| Jos ungrafted | | | |
| Well-watered | 97.8±18.7 | 75.1±11.5 | 75.2±3.1 |
| Water stress | 60.6±5.2* | 39.8±3.3* | 55.0±5.1* |
| $LSD_{0.05}$ | 14.2 | 15.5 | 12.2 |
| *JosxJos* | | | |
| Well-watered | 146.2±25.8 | 93.1±17.4 | 62.4±3.7 |
| Water stress | 72.0±8.5* | 52.2±5.1* | 35.0±9.2* |
| $LSD_{0.05}$ | 27.7 | 28.8 | 21.1 |
| *JosxZar* | | | |
| Well-watered | 81.0±9.6 | 54.8±4.7 | 63.4±5.0 |
| Water stress | 73.1±8.2 | 42.5±3.8 | 67.2±0.6 |
| $LSD_{0.05}$ | 24.00 | 12.9 | 10.9 |
| *ZarxJos* | | | |
| Well-watered | 91.4±11.6 | 54.1±11.7 | 65.6±1.7 |
| Water stress | 99.1±13.3 | 55.9±6.6 | 65.4±4.0 |
| $LSD_{0.05}$ | 13.9 | 28.5 | 9.3 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05). Asterisk (*) indicates significant difference with controls groups (well-watered).

Both SOD and CAT are antioxidant enzymes. A large increase in the activitives of both enzymes was observed in cv. Zarina and the combination *ZarxZar* under water stress, while cv. Josefina and its self-graft registered no increase in SOD activity and a decrease in CAT activity, respectively (Figure 3B and 3D). Considering the reciprocal grafts, only *JosxZar* showed higher SOD and CAT activities with respect to control conditions (Figure 3B and 3D).

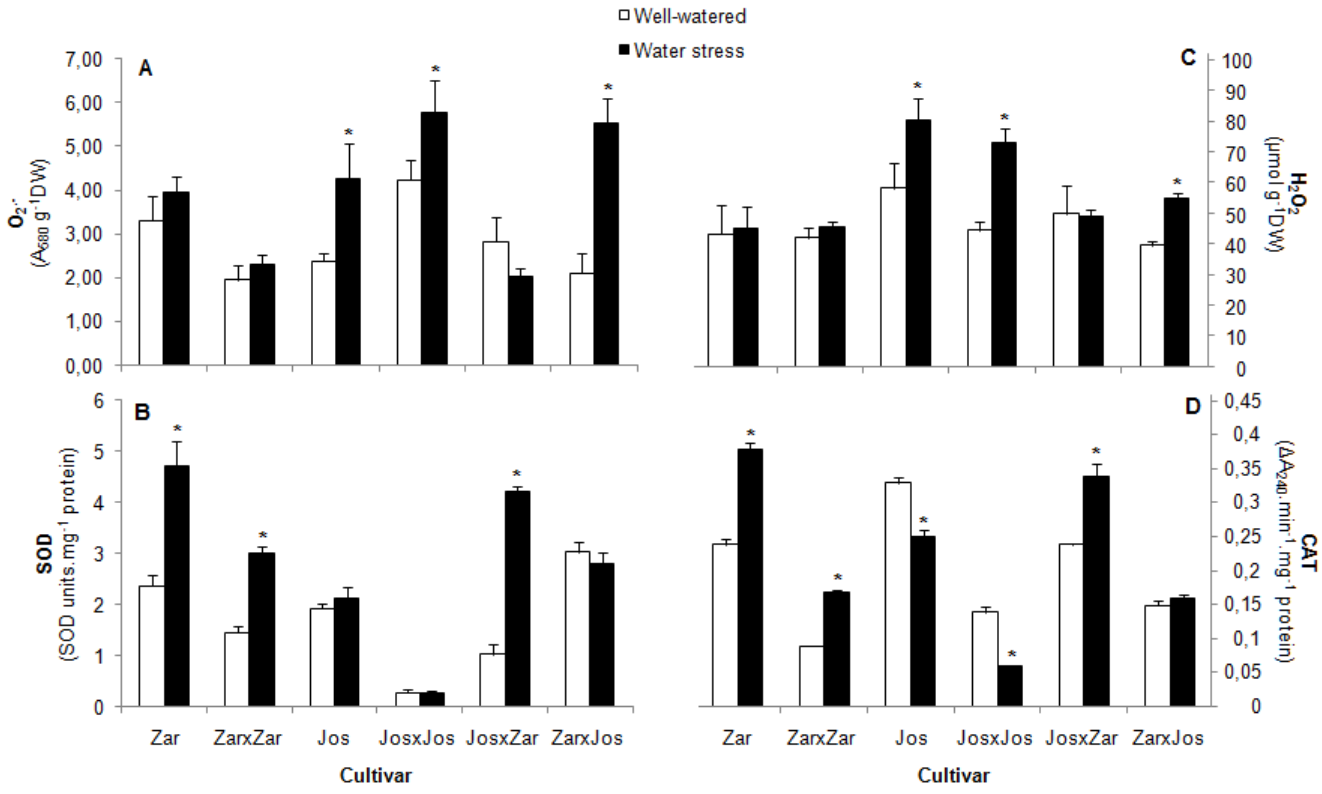


**Figure 3.** Influence of moderate water stress on $O_2^{\cdot-}$ (A), SOD activity (B), $H_2O_2$ (C) and CAT activity (D) in leaf extracts of ungrafted, grafted and self-grafted tomato plants. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

MDA concentration, a measure of lipid peroxidation, and LOX activity were also measured in leaf extracts. MDA concentration was increased significantly in cv. Josefina and in the combinations *JosxJos* and *ZarxJos* under water stress conditions (Figure 4A). Conversely, cv. Zarina, and the combinations *ZarxZar* and *JosxZar* showed a significant decrease in MDA concentration (Figure 4A). LOX activity only increased in cv. Josefina, *JosxJos* and *ZarxJos* under stress conditions (Figure 4B).

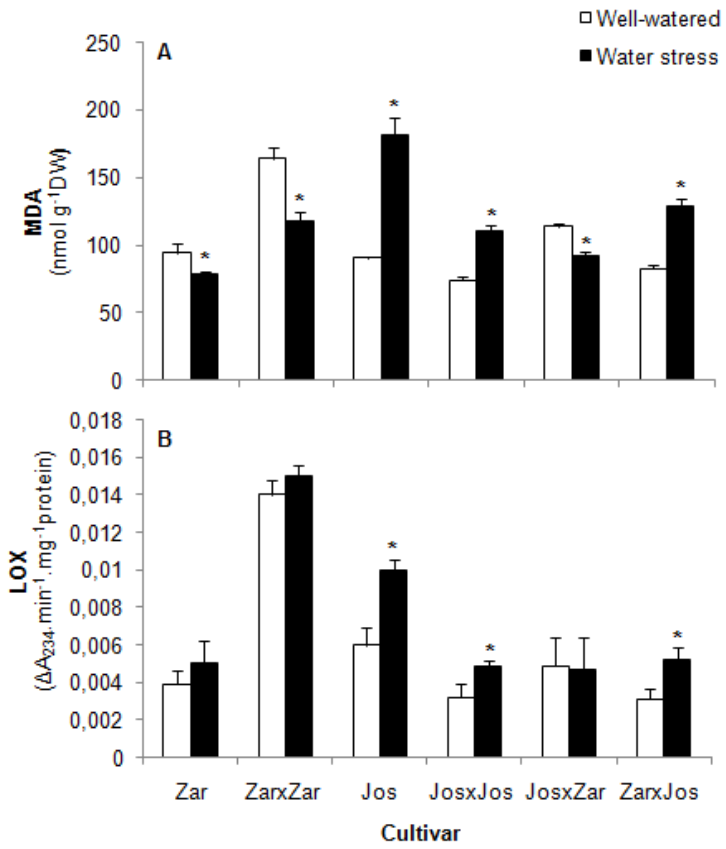


**Figure 4.** Influence of moderate water stress on MDA (A) and LOX activity (B) in leaf extracts of ungrafted, grafted and self-grafted tomato plants. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

*Ascorbate and gluthathione forms and Halliwell-Asada cycle*

To complete the examination of the non-enzymatic antioxidant activity, we analysed the concentration of the different AsA forms in leaves (Table 3). No significant differences in the total AsA leaf concentration were observed in cv. Zarina and its self-graft, cv. Josefina and *ZarxJos* under water stress.

**Table 3:** Influence of moderate water stress on AsA forms in ungrafted, grafted and self-grafted tomato plants.

| Rootstock x Scion/ Treatment | Reduced AsA (mg $g^{-1}$DW) | DHA (mg $g^{-1}$DW) | Total AsA (mg $g^{-1}$DW) | AsA red/DHA |
|---|---|---|---|---|
| Zar ungrafted | | | | |
| Well-watered | 103.2±3.6 | 26.7±2.3 | 147.0±2.5 | 4.0±0.6 |
| Water stress | 137.8±9.9* | 23.1±1.8 | 159.4±13.0 | 6.5±1.0* |
| $LSD_{0.05}$ | 22.5 | 6.4 | 18.3 | 1.6 |
| *ZarxZar* | | | | |
| Well-watered | 101.4±4.1 | 90.7±2.2 | 187.9±8.4 | 1.9±0.5 |
| Water stress | 128.8±9.9* | 55.7±6.3* | 164.6±4.6 | 2.5±0.3 |
| $LSD_{0.05}$ | 22.9 | 14.9 | 34.3 | 1.4 |
| Jos ungrafted | | | | |
| Well-watered | 161.6±4.9 | 48.8±0.4 | 165.3±9.9 | 3.6±0.1 |
| Water stress | 122.5±10.4* | 37.3±7.4* | 146.0±16.9 | 4.0±0.6 |
| $LSD_{0.05}$ | 24.4 | 5.7 | 41.6 | 1.7 |
| *JosxJos* | | | | |
| Well-watered | 100.5±8.8 | 52.1±12.5 | 163.9±12.4 | 2.6±0.5 |
| Water stress | 99.9±4.8 | 29.3±2.8* | 125.3±2.6* | 3.8±0.5 |
| $LSD_{0.05}$ | 11.3 | 17.2 | 26.9 | 1.5 |
| *JosxZar* | | | | |
| Well-watered | 118.4±13.2 | 56.8±4.3 | 171.5±13.7 | 2.0±1.1 |
| Water stress | 222.4±2.8* | 53.1±8.9 | 255.6±17.0* | 3.5±1.5* |
| $LSD_{0.05}$ | 28.7 | 21.0 | 46.4 | 0.9 |
| *ZarxJos* | | | | |
| Well-watered | 89.9±1.9 | 63.3±5.5 | 144.9±3.2 | 1.4±0.1 |
| Water stress | 102.6±11.6 | 41.3±2.7* | 153.2±8.5 | 2.3±0.2 |
| $LSD_{0.05}$ | 25.0 | 13.0 | 19.3 | 0.9 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05). Asterisk (*) indicates significant difference with controls groups (well-watered).

However, in *JosxJos* the total AsA concentration decreased and in *JosxZar* there was an increase of 49% with respect to control conditions. In cv. Zarina and its self-graft, water stress increased reduced AsA by 33 and 27%, respectively, wheras in cv. Josefina there was a decrease in reduced AsA with respect to control conditions.

**Table 4:** Influence of moderate water stress on GSH forms in ungrafted, grafted and self-grafted tomato plants.

| Rootstock x Scion/ Treatment | Reduced GSH (µg $g^{-1}$DW) | GSSG (µg $g^{-1}$DW) | Total GSH (µg $g^{-1}$DW) | GSH red/GSSG |
|---|---|---|---|---|
| Zar ungrafted | | | | |
| Well-watered | 3.40±0.70 | 1.68±0.35 | 5.09±0.35 | 2.33±0.75 |
| Water stress | 3.81±0.67 | 1.71±0.33 | 5.52±0.63 | 2.52±0.84 |
| $LSD_{0.05}$ | 0.71 | 1.36 | 1.01 | 1.15 |
| *ZarxZar* | | | | |
| Well-watered | 2.15±0.47 | 1.53±0.90 | 3.68±0.47 | 2.89±1.26 |
| Water stress | 2.87±0.70 | 1.71±0.57 | 4.58±0.19 | 2.98±0.97 |
| $LSD_{0.05}$ | 1.35 | 0.87 | 1.43 | 0.50 |
| Jos ungrafted | | | | |
| Well-watered | 3.09±0.22 | 2.10±0.10 | 5.19±0.26 | 1.47±0.11 |
| Water stress | 2.90±0.31 | 1.47±0.16* | 4.07±0.15* | 2.08±0.50 |
| $LSD_{0.05}$ | 1.07 | 0.54 | 0.84 | 1.44 |
| *JosxJos* | | | | |
| Well-watered | 2.76±0.76 | 2.85±0.86 | 5.62±0.13 | 1.34±0.66 |
| Water stress | 3.07±0.22 | 2.41±0.04 | 5.49±0.26 | 1.27±0.06 |
| $LSD_{0.05}$ | 1.21 | 0.59 | 0.83 | 0.85 |
| *JosxZar* | | | | |
| Well-watered | 1.52±0.40 | 2.84±0.53 | 4.57±0.41 | 0.69±0.29 |
| Water stress | 2.14±0.73* | 2.17±0.34 | 4.31±0.41 | 1.38±0.62* |
| $LSD_{0.05}$ | 0.42 | 1.76 | 1.61 | 0.62 |
| *ZarxJos* | | | | |
| Well-watered | 2.43±0.32 | 3.00±0.28 | 5.14±0.27 | 0.73±0.17 |
| Water stress | 1.80±0.06 | 2.25±0.37 | 3.39±0.41* | 0.89±0.15 |
| $LSD_{0.05}$ | 0.89 | 1.29 | 1.37 | 0.64 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05). Asterisk (*) indicates significant difference with controls groups (well-watered).

No significant differences in the total GSH leaf concentration were observed in cv. Zarina and its self-graft under water stress (Table 4). However, in cv. Josefina and *ZarxJos* the total GSH concentration decreased with respect to control conditions. Among the reciprocal grafts, only *JosxZar* showed increases in reduced GSH under water stress conditions. GSSG concentrations decreased only in Josefina. These changes led to significant increasesin the GSH red/GSSG ratio in the *JosxZar* (Table 4).

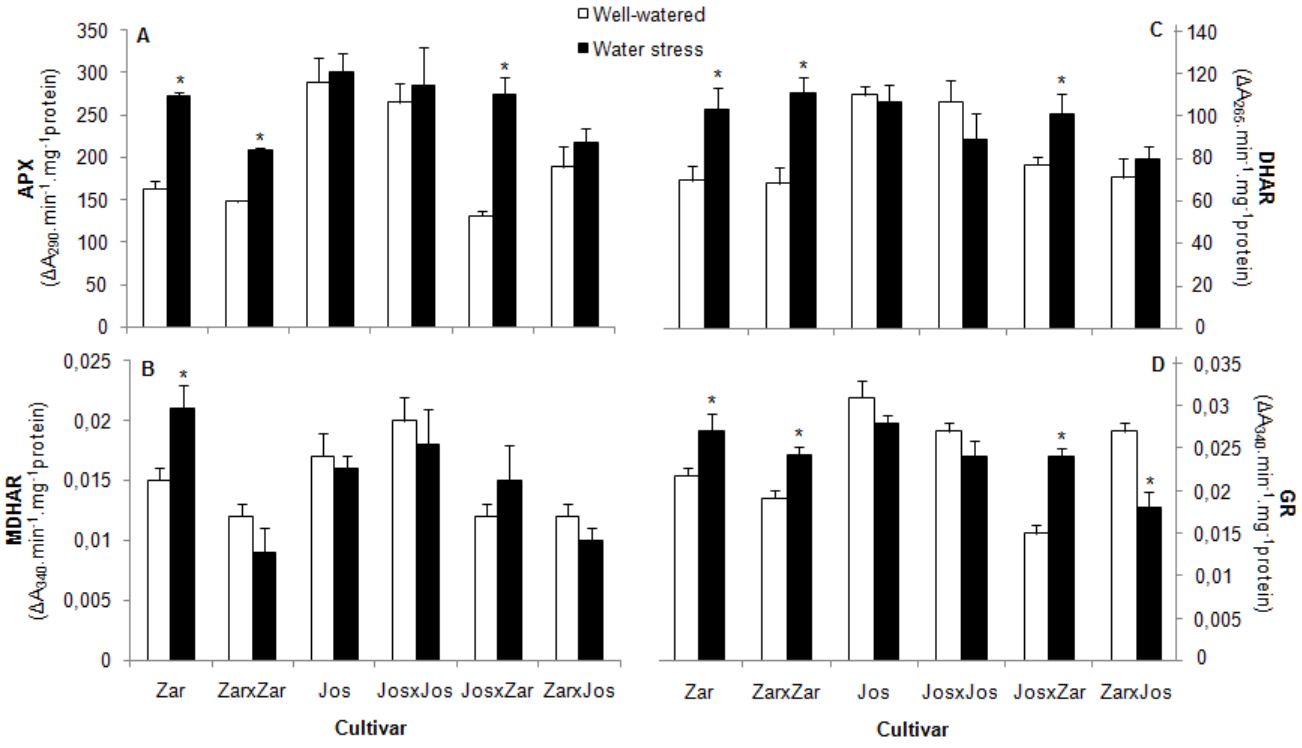


**Figure 5.** Influence of moderate water stress on APX activity (A), MDHAR activity (B), DHAR activity (C) and GR activity (D) in leaf extracts ungrafted, grafted and self-grafted tomato plants. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

With regard to the enzymes involved in the Halliwell-Asada cycle, an increases in activities of APX, MDHAR, DHAR and GR in leaf extracts (Figure 5) was found in cv. Zarina, its self-graft and *JosxZar* under water stress. In cv. Josefina and its self-grafting, not differences significant were observed in the enzymes involved in the Halliwell-Asada cycle.

## DISCUSSION

Growth reduction under water-stress conditions has been well characterized in plants such as pepper, potato, and tomato (Delfine *et al.* 2002; Ierna *et al.* 2006; García *et al.* 2007). In our study, the lower loss of biomass and the greatest RGR was found in cv. Zarina, which is defined as most tolerant; while cv. Josefina is defined as drought-sensitive cultivar (Sánchez-Rodríguez *et al.* 2010). Our results showed that reciprocal grafting (*JosxZar* and *ZarxJos* combinations) maintained growth under water stress, but *ZarxJos* was greater (Table 1). The grafts *ZarxZar* and *JosxJos* showed decreases in total biomass and RGR under stress conditions; however, the total biomass was greater in the selfgrafts with respect to the cvs. Zarina and Josefina ungrafted (Table 1). These results are consistent with a recent report (Alan *et al.* 2007), who observed that the graft itself had a positive effect on growth, due possibly to a hormonal effect. Since the root systems of genotypes selected for being used as rootstocks are usually much larger and more vigorous, they can absorb

water and nutrients much more efficiently as compared to non-grafted plants (Lee *et al*. 2010). During drought stress, the plant water relations play a key role in the activation and/or modulation of antioxidant defense mechanism (Menconi *et al.* 1995). Drought-induced reduction in leaf pigments are considered to be a typical oxidative stress indicators which might be attributed to pigment photo-oxidation, chlorophyll degradation and/or chlorophyll synthesis deficiency (Ahmed *et al.* 2009). Reduction in chlorophyll concentration is identified as a drought response mechanism in order to minimize the light absorption by chloroplasts (Pastenes *et al.* 2005). According to these authors, our results showed a decrease on chlorophyll content and carotenoids in Josefina ungrafted and selfgrafs, whereas in reciprocal grafts (*JosxZar* and *ZarxJos* combinations), the values were higher. Abdelmageed *et al.* (2009) showed that the chlorophyll content was significantly higher in grafted tomato plants under heat stress conditions.

Plants subjected to water stress tend to overproduce ROS in different plant tissues (Pinheiro *et al.* 2004), and in response the enzyme SOD constitutes the first line of cellular defense, detoxifying $O_2^{-\cdot}$ radicals and giving rise to $H_2O_2$ production. Some studies indicate that water stress can induce SOD activity by an overproduction of ROS under such conditions (Khanna-Chopra *&* Selote 2007; Hameed *et al.* 2011). Figure 3 shows that SOD activity was higher in cv. Zarina, its self-graft and *JosxZar* under water stress conditions, which could

explain the less $O_2^{-\cdot}$ concentration in these cultivars. So, we might assume that the ROS production under water stress conditions remained lower when we used cv. Zarina as shoot in grafted plants. To test the above hypothesis, we determined the concentration of leaf $H_2O_2$, since this is the first compound resulting from the detoxification of the $O_2^{-\cdot}$ radical by SOD (Jaleel *et al*. 2009). Our results show that the $H_2O_2$ concentration had a behavior inverse to that SOD activity (Figure 3), this could be due either to lower ROS production or to more efficient detoxification of this compound owing to the activity CAT (Jaleel *et al.* 2009). Really, the cultivar and grafted plants with lower $H_2O_2$ show higher CAT activity. So, Figure 3 reflect that in cv. Zarina and *JosxZa*r grafted plants the SOD and CAT activities were significantly higher than in rest of cultivars, and therefore we deduce that in *JosxZar* the detoxification of ROS by these enzymes is somewhat more efficient than *JosxJos* and *ZarxZar* (Figure 3). Willenkens *et al.* (1997) demonstrated that an $H_2O_2$ accumulation in the different tissues of a plant could result in reduced biomass. In fact, we previously shows lower total biomass cv. Josefina and self-graft (Sánchez-Rodríguez *et al.* 2011). As indicated above, the accumulation of foliar $H_2O_2$ is lower in cv. Zarina and reciprocal grafting (Figure 3), and thus it be expected that the reduction in foliar biomass would be lower. In this sense, the use of grafted plants implies an advantage with respect to non-grafted plants. These data agree with the results of Rivero *et al.* (2003) who reported a larger biomass in grafted tomato plants under thermal stress.

It was observed that water deficit raised the $H_2O_2$ concentration and the quantity of MDA in wheat plants (Esfendiari *et al.* 2007). In this sense, low concentrations in $H_2O_2$ and MDA have been associated with water-stress tolerance in pea plants and wheat (Moran *et al*. 1994; Sairam *et al.* 2000). Our results show MDA concentrations in cv. Zarina, its self-graft and *JosxZar* did not increase under water stress (Figure 4), which could be associated with not increase $H_2O_2$ concentration (Figure 3). It was proved that singlet oxygen and superoxide anions can be formed during the LOX-catalysed oxidation of fatty acids (Lynch *&* Thompson 1984). Increased LOX activities are interpreted as reasons for an increased lipid peroxidation under stress conditions (Aziz *&* Larher 1998). The higher LOX activity is correlationed with higher MDA concentration in cv. Josefina, its self-graft and *ZarxJos*.

Halliwell-Asada cycle constitutes an important detoxification pathway for dissipation of $H_2O_2$ and other reactive oxygen radicals in chloroplasts (Sgherri *et al.* 2003). It is assumed that the increased activities of the enzymes of ascorbate/glutathione pathway, especially that of APX confer general resistant to array of environmental stresses (de Gara *et al.* 2000). Our result showed that in cv. Zarina, *ZarxZar* and *JosxZar* plants the activities of APX, DHAR and GR were higher than other plants under water stress (Figure 5), and therefore we deduce that in these plants the detoxification of $H_2O_2$ by these enzymes is somewhat more efficient. The higher DHAR and GR activities in *JosxZar* may

contribute to higher AsA contents (Table 3). These results agree with He *et al.* (2009) in tomato plants under salinity stress, where they observed higher DHAR and GR activities and AsA content in grafted plants. So, this indicates that in this combination grafted plants (*JosxZar*) the ascorbate/glutathione detoxification system functions better under water stress, detoxifying greater amounts of $H_2O_2$ and thereby preventing this compound from reaching high toxicity levels by accumulating in plant tissues. On the other hand, high ratios of reduced AsA/DHA and reduced GSH/GSSG are essential to eliminate ROS in cells (Nayyar *&* Gupta 2006). In agreement with this, cv. Zarina and *JosxZar* again registered the highest differences for these ratios, and therefore appear to present a greater capacity to eliminate ROS. In this way, grafted plants achieve greater tolerance to water stress apparently by developing a better antioxidant system, which in turn leads to better overall plant development.

In summary, when shoots are of the drought tolerant genotype Zarina (Zarina ungrafted and *JosxZar*), the changes in antioxidant enzyme activities were large and consistent under moderate water stress: increases of SOD, CAT, and Halliwell-Asada cycle occured, with not changes in LOX. These plants did not show stress, since leaf $O_2^{-\cdot}$ and $H_2O_2$ concentration not change, and reduced AsA increased, and decreases in MDA and DHA occurred. However, when shoots are of the drought-sensitive genotype Josefina (Josefina ungrafted and *ZarxJos*), the antioxidant enzyme activities were more limited and the oxidative

stress was evident under water stress conditions. In general, selfgrafted plants showed a similar behavior that its cultivars ungrafted. These results reflect that the technique of grafting using Zarina as scion can be useful and effective for improving the antioxidant response in tomato under water stress.

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# *Capítulo 4*

## *Phenolic metabolism in grafted versus non-grafted cherry tomatoes under the influence of water stress*

***Journal Agricultural Food Chemistry***

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**ABSTRACT**

Use of grafts using rootstocks capable of palliating the effects of water stress can be a possible solution to reduce yield losses. For response to stress, plants can induce the metabolism of phenylpropanoids. The aim of the present work is to determine the response of reciprocal grafts made between one tolerant cultivar, Zarina, and a more sensitive cultivar, Josefina. The analysis of the phenylpropanoids pathway was carried out both enzymatically and metabolically. DAHP synthase, shikimate dehydrogenase, phenylalanine ammonium lyase, cinnamate 4-hydroxylase, and 4-coumarate CoA ligase activities were determined and characteristic metabolites from the pathway were measured by means of HPLC-MS. Growth in the grafts *JosxZar* and *ZarxJos* was not appreciably affected by stress. *JosxZar* increased the concentration of phenolic compounds after water stress. This could be correlated with the greater activity of synthesis enzymes and as well as with a decrease in phenol-degrading enzymes. Phenolic metabolism is more influenced by the aerial part, and therefore we conclude that the capacity of inducing tolerance in rootstocks depends on the genotype of the shoot.

**Key words:** *Solanum lycopersicum,* quercetin, kaempferol, HPLC, phenolic compounds, shikimate pathway, grafting.

**Abbreviations:** 4CL, 4-coumarate coenzyme A ligase; C4H, cinnamate 4-hydroxylase; DAHPS, 3-deoxy-7-phosphoheptulonate synthase; GPX, guaiacol peroxidase; LRWC, leaf relative water content; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase; SKDH, shikimate dehydrogenase.

## INTRODUCTION

Water has become a scarce resource in many regions of the world, especially in arid and semiarid zones of the Mediterranean Basin. Increased competition for water between agriculture, industry, and urban consumption brings into focus the need to improve irrigation practices in commercial plant production (Schwarz *et al.* 2010). One possible solution to reduce yield losses and improve crop growth under water-deficit conditions involves the use of grafts using rootstocks capable of palliating the effects of this stress in the shoot (Schwarz *et al.* 2010). Zones that produce grafted horticultural produces of great economic importance, including annuals of the family Solanaceae such as tomato, eggplant, and pepper, have in recent years begun to use plants that are resistant to soil-borne diseases (Bletsos *et al.* 2003), tolerant to environmental stress such as salinity and extreme temperatures (Rivero *et al.* 2003; Estañ *et al.* 2005), more efficient in water and nutrient uptake (Santa-Cruz *et al.* 2002), and better fruit quality (Fernández-García *et al.* 2004). Today grafts are

considered a rapid alternative to conventional reproduction, with the aim of boosting tolerance against environmental stress in crops (Flores *et al.* 2010).

Plants have developed diverse mechanisms to combat damage caused by water stress. One biochemical factor involved in the response to stress is the metabolism of phenylpropanoids (Evrenosoğlu *et al.* 2010). Plant resistance to biotic and abiotic stress is often regulated by the metabolism of phenolic compounds, so that greater activity of related enzymes and the accumulation of phenolic compounds has been correlated with resistance to these types of stress (Evrenosoğlu *et al.* 2010). These compounds are generally synthesised by the shikimate pathway, using intermediates of carbohydrate metabolism (Figure 1) (Dixon *&* Paiva 1995). The pathway begins with 3-deoxy-7-phosphoheptulonate synthase (DAHPS, EC 2.5.1.54), which is the key enzyme controlling the carbon flow towards phenolic metabolism. Another important enzyme in the pathway is phenylalanine ammonia-lyase (PAL, EC 4.3.1.24), which catalyses the non-oxidative deamination of L-phenylalanine to form *trans*-cinnamic acid. This reaction is the first step in the biosynthesis of a great number of secondary products derived from phenylpropanoid in plants such as flavonoids and isoflavonoids, coumarins, lignins, hydroxycinnamic acid esters, and phenolic compounds (Jones 1984). In response to different types of environmental stress, increases have been found in PAL activity and in other enzymes of the phenylpropanoid pathway in tomato and lettuce plants (Rivero *et al.* 2003; Oh *et al.* 2009). On the other way, some important differences were detected for the contents of the phenolic compounds during the growing periods

such as kaempferol accumulates in the leaves of grafted and non-grafted watermelon plants and rootstocks (Evrenosoğlu *et al.* 2010).

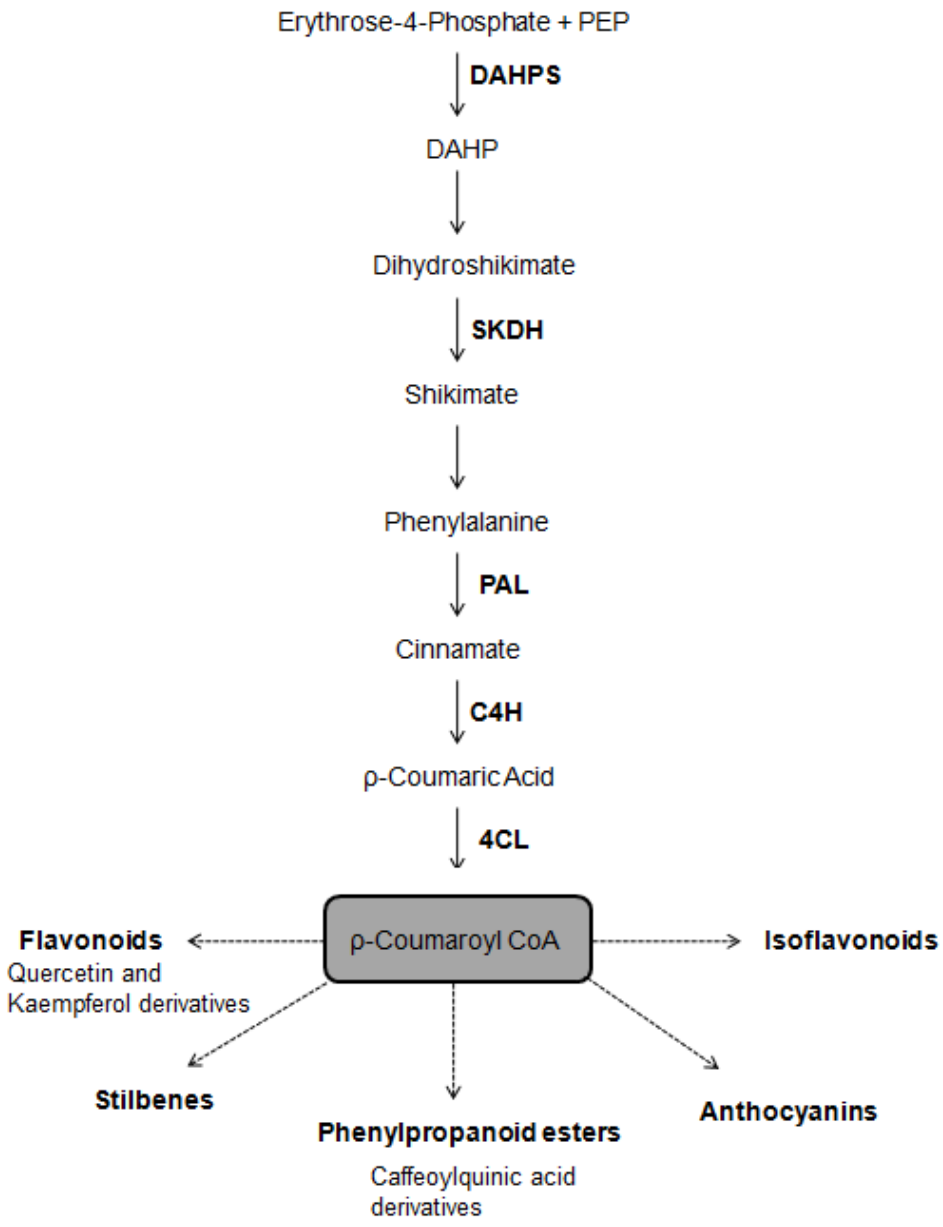


**Figure 1.** Simplifies scheme for flavonoid and phenylpropanoid synthesis in tomato. The steps are catalyzed by deoxyarabino-heptulosonato 7-fosfato synthase (DAHPS), shikimate dehydrogenase (SKDH), phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL).

Given that tomato is one of the most important crops worldwide, and that its production is concentrated in semiarid regions, where water stress is frequent, it is of great interest to ascertain whether grafting is a valid strategy to improve water-stress tolerance in this plant. In preliminary studies, we have observed that the cv. Zarina shows better water-stress tolerance than cv. Josefina, which is more drought sensitive (Sánchez-Rodríguez *et al.* 2010). In this light, the aim of the present work is to determine the response of reciprocal grafts made between one tolerant cultivar, Zarina, and a more sensitive cultivar, Josefina, to moderate water stress, examining phenolic metabolism and the accumulation of its metabolites.

## MATERIALS AND METHODS

### *Plant material and treatments*

Two tomato (*Lycopersicon esculentum* Mill) cultivars, Zarina and Josefina, were used as scion and rootstock (Figure 2). The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm x 3 cm x 10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Grafting was performed when seedlings has developed 3-4 true leaves. In the vermiculite trays used for germination, the seedlings were cut over the cotyledons, using the shoot as scion and the remaining plant part as rootstock. Grafts were made immediately after cutting the plants and grafting clips were

used to adhere the graft union. Self-grafted plants were included as controls. After grafting, seedlings were covered with a transparent plastic lid to maintain a high humidity level and to facilitate graft formation and were left in the dark for 24h. The plastic was opened slightly every day to allow reduction in relative humidity and it was removed 6 days after grafting. Afterwards, ungrafted and grafted plants were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with relative humidity of 50±10%, at 25 ºC/15 ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol/$m^2$/s (LI-COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. The complete nutrient solution used to grow the plant during the experiment was the same as described in a recent study (Sánchez-Rodríguez *et al.* 2010). The water-stress treatments began 45 days after germination and maintained 22 days. The control treatment received 100% field capacity (FC) irrigation, whereas moderate water stress corresponded to 50% FC. The experimental design was a randomized complete block with 12 treatments (Zarina ungrafted, Josefina ungrafted, Zarina self-grafted, Josefina self-grafted, *JosxZar* and *ZarxJos* well-watered 100% FC and water stress 50% FC) (Figure 2) arranged in individual pots with six plants per treatment (one plant per pot) and three replications each.

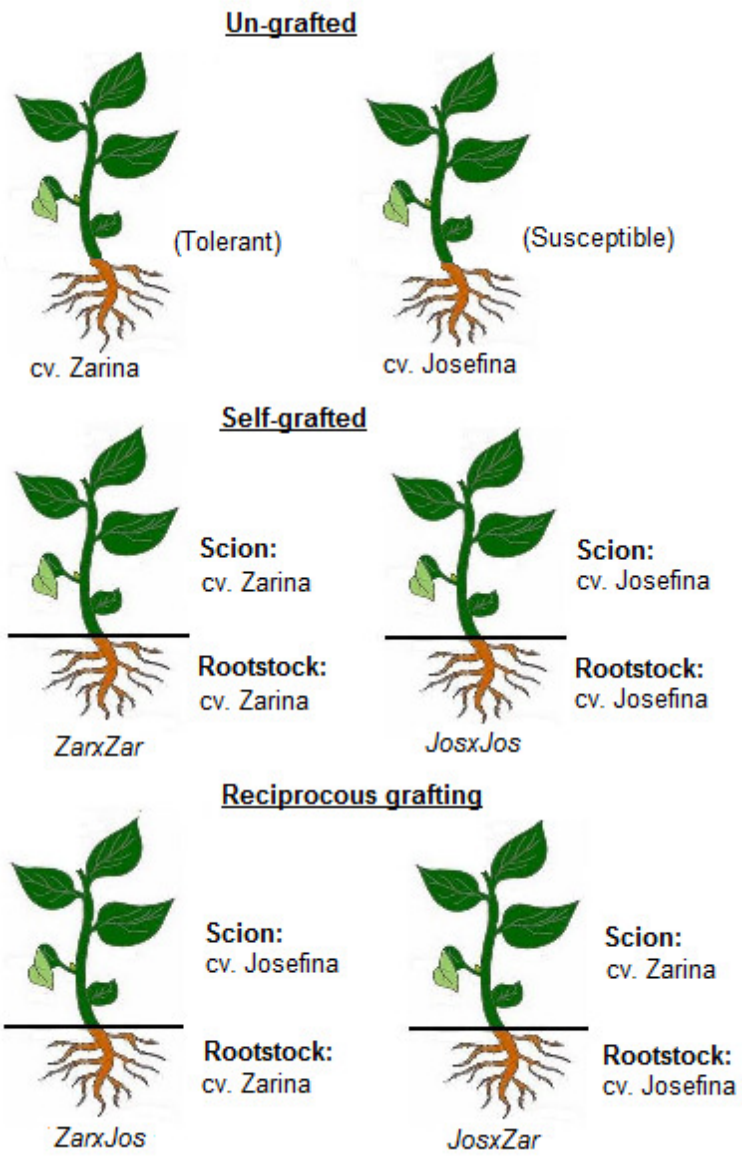


**Figure 2.** Outline of the grafting design.

*Relative-Growth-Rate (RGR) and Leaf Relative Water Content (LRWC)*

All plants were at the late vegetative stage when harvested. Leaves fully expanded (excluding petioles) and roots were harvested, frozen immediately in

liquid $N_2$, and kept at -80ºC until used. To determine the relative growth rate (RGR), leaves and roots from three plants per cultivar were sampled on day 45 after germination, immediately before starting the water-stress treatment (Ti). The leaves and roots were liophilized, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 67 days after germination (22 days of treatments, Tf). The relative growth rate was calculated from the increase in DW at the beginning and at the end of the water-stress treatment, using the equation RGR = (ln DWf – ln DWi)/(Tf – Ti) where T is the time and the subscripts denote the final and initial sampling (i.e. days 0 and 22, respectively, after the water-stress treatment) (Bellaloui *&* Brown 1998).

Leaf relative water content (LRWC) was measured following the method of Barrs *&* Weatherley (1962).

*Analysis of phenolic compounds by HPLC/UV-PAD/ESI-MS$^n$*

For the identificaiton and characterization of phenolics, 0.1 g of lyophilised leaves was extracted with 1 mL of water/methanol (1:1) by sonication for 1 h, followed by overnight maceration and another sonication period (1 h). The resulting extract was centrifuged and filtered through a 0.45 µm PVDF membrane.

Chromatographic analyses were carried out on a Phenomenex reverse-phase column 250 mm x 4.6 mm i.d., 5 µm, Li-Chrospher 100 RP-18, with a 4 mm x 4 mm i.d. guard column of the same material (Luna, Phenomenex). The mobile phase consisted of two solvents: water/acetic acid (1%) (A) and acetonitrile (B), starting with 5% B and using a gradient to obtain 50% at 30 min and 80% at 37 min. The flow rate was 1 mL/min and the injection volume 20 µL. Spectroscopic data from all peaks were accumulated in the range of 200-400 nm, and chromatograms were recorded at 280, 320, and 360 nm. The HPLC/UV-PAD/ESI-$MS^n$ analyses were carried out with an Agilent HPLC equipped with a PAD and mass spectrometer in series (Agilent Technologies, Waldbronn, Germany).

The mass spectrometer was an ion trap mass analyzer equipped with an electrospray ionization interface. The ionization conditions were adjusted at 350 ºC and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from *m/z* 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were adquired in the negative ionization mode. $MS^n$ was carried out on the most abundant fragment ion observed in the first-generation mass spectrum.

The identification of the peaks was obtained analyzing the extracted-ion chromatograms of the ion current at *m/z* values corresponding to the $[M-H]^-$ ions of the individual investigated compounds, as well as their fragmentation. Quantification of the identified analytes was performed by HPLC-PDA detection using the external standard method with calibration graphs, as a function of concentration based on peak area, detected at the wavelength corresponding to their maximum absorbance.

*Preparation of enzyme extract for assay*

For determination of 3-deoxy-7-phosphoheptulonate synthase (DAHPS, DS-Mn, DS-Co, EC 2.5.1.54) and phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) activities, whole fresh leaf was homogenized in 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifugated at 15,000 *g* for 15 min at 4 ºC. The supernatant was passed through a Sephadex G-25 column (24 x 100 mm) previously equilibrated with the same buffer.

For determination of shikimate dehydrogenase (SKDH, EC 1.1.1.25) and polyphenol oxidase (PPO, EC 1.10.3.2) activities, whole fresh leaf was homogenized in 50 mM potassium phosphate buffer (pH 7.0). Homogenates were centrifuged at 15,000 *g* for 15 min at 4 ºC.

For determination of cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) fresh sample of leaf was homogenized in 200 mM potassium phosphate buffer (pH 7.5) containing 2 mM of 2-mercaptoethanol. Homogenates were centrifuged at 10,000 *g* for 15 min at 4º C.

For determination of 4-coumarate coenzyme A ligase (4CL, EC 6.2.1.12) the extract buffer was 0.05 M Tris-HCl (pH 8.8) containing 14 mM mercaptoethanol and 30% glycerol. Homogenates were centrifuged at 10,000 *g* for 15 min at 4º C.

For determination of guaicol peroxidase (GPX, EC 1.11.1.9) the extract buffer was 50 mM Tris-HCl (pH 7.5) containing 5 mM mercaptoethanol, 2 mM DTT (dithiothreitol), 0.5 mM PMSF and 2 mM EDTA-Na. Homogenates were centrifuged at 16,500 *g* for 30 min at 4 ºC.

*Enzyme assay*

DAHPS activity was assayed using modified method (Morris *et al.* 1989). The reaction mixture for DS-Mn was 50 mM K-Epps buffer (pH 8.0), 0.5 mM DTT, 0.5 mM $MnCl_2$, 3 mM PEP (phosphoenol-pyruvate) and 0.6 mM E4P (erythrose-4-phosphate). The reaction mixture for DS-Co was 50 mM K-Epps buffer (pH 8.6), 10 mM $MgCl_2$, 3 mM PEP, and 3 mM E4P. DS-Mn and DS-Co reaction

were initiated by the addition of enzyme extract to reaction mixture, followed by incubation for 30 min for DS-Mn and 20 min for DS-Co at 37ºC. The reaction was terminated by adding of 25% trichloroacetic acid to the reaction mixture. For controls, 25% trichloroacetic acid was added to the mixture prior to start of the reaction. After centrifuging (10,000 *g*, 15 min, 4 ºC), supernatant was collected, to which 25 mM $NaIO_4$ containing 0.125 N $H_2SO_4$ was added. After incubation for 30 min at 37ºC 2%$NaAsO_2$ containing 0.5 N HCl and thiobarbituric acid were added, followed by incubation for 10 min at 100 ºC. The absorbance at 280 nm was then measured.

PAL activity was measured by a modified method (Tanaka *et al.* 1974). The reaction mixture was 0.4 mL of 100 mM Tris-HCl buffer (pH 8.8), 0.2 mL of 40 mM phenylalanine and 0.2 mL of enzyme extract. The reaction mixture was incubated for 30 min at 37 ºC, and the reaction was terminated by adding of 25% trichloroacetic acid. In the control of PAL assay, the same amount of phenylalanine was added after termination. To remove precipitated protein, the assay mixture was centrifuged at 10,000 *g* for 15 min at 4 ºC, and the absorbance of the supernatant was measured at 280 nm relative to the control.

SKDH activity was assayed in 0.1 M Tris-HCl buffer (pH 9). Reaction mixture contained 1.45 mL of 2 mM shikimic acid, 1.45 mL of 0.5 mM NADP and 0.1 mL

of supernatant. Increase of absorbance due to reduction of NADP was read over 1 min at 340 nm (Ali *et al.* 2006).

PPO assay was performed in mixture containing 2.85 ml of 50 mM potassium phosphate buffer (pH 7.0), 50 µL of 60 mM catechol and 0.1 mL of supernatant. Increase in absorbance was read over 2 min at 390 nm (Aquino-Bolaños *&* Mercado-Silva 2004).

C4H activity was assayed by using the method described previously with slight modification (Lamb *&* Rubery 1975). The extract was added to 4.8 mL of reaction buffer (50 mM phosphate buffer containing 2 mM of 2-mercaptoethanol, 2 mM *trans*-cinnamic acid, and 0.5 mM NADPH), which was incubated for 1 h at 37 ℃. The reaction was stopped with 6 M of HCl and readjusted to pH 11 with 6 M of NaOH, and then, absorbance value of the sample was measured at 280 nm.

The activity 4CL was determined with the spectrophotometric method, using caffeic acid as the preferred phenolic substrate (Knoblock *et al.* 1975). The reaction mixture was 5 µM p-coumaric acid, 50 µM ATP, 1 mM CoA-SH, 15 mM $Mg_2SO_4$. The reaction mixture was incubated at 40 ℃ for 10 min and then the absorbance was measured at 333 nm.

GPX activity was determined by following the previously reported method (Kalir *et al.* 1984).

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

*Statistical analysis*

Data compiled were submitted to an analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple-range test ($P>0.05$).

## RESULTS

*Relative-Growth-Rate (RGR) and Leaf Relative Water Content (LRWC)*

The total biomass diminished significantly in the cv. Josefina as well as in *JosxJos* under water-stress conditions. The cv. Zarina did not appear to be affected by the stress, while its self-graft had a negative effect on total biomass, with a reduction of 41% with respect to control. In terms of reciprocal grafts, none presented a reduction of total biomass under water-stress conditions, values being higher in the graft *ZarxJos* in both treatments, well-watered and water stress (Table 1). In cv. Josefina and its self-graft, the total RGR declined

under stress conditions. In contrast, the cv. Zarina was not significantly affected, although a 30% loss was found for *ZarxZar* with respect to the well-watered treatment. In the grafts *JosxZar* and *ZarxJos*, no differences were observed under stress conditions. In terms of biomass, the graft *ZarxJos* maintained the highest levels of total RGR (Table 1).

**Table 1:** Influence of moderate water stress on dry weight, RGR and LRWC in ungrafted, grafted and self-grafted tomato plants.

| Rootstock x Scion/ Treatment | Total Biomass (g DW) | Total RGR (mg/g/day) | Radicular RGR (mg/g/day) | LRWC (%) |
|---|---|---|---|---|
| Zar ungrafted | | | | |
| Well-watered | 7.06±0.73 a | 62.34±5.03 a | 38.56±3.60 b | 89.97±1.00 a |
| Water stress | 6.15±0.53 a | 58.14±4.53 a | 72.49±3.91 a | 89.83±5.11 a |
| $LSD_{0.05}$ | 2.52 | 18.81 | 14.77 | 4.47 |
| p-value† | ns | ns | ** | ns |
| *ZarxZar* | | | | |
| Well-watered | 8.91±0.70 a | 81.03±3.66 a | 46.03±0.52 b | 87.53±3.12 a |
| Water stress | 6.20±0.37 b | 56.58±3.23 b | 59.66±3.44 a | 90.02±1.77 a |
| $LSD_{0.05}$ | 2.20 | 13.56 | 9.67 | 9.98 |
| p-value | * | *** | * | ns |
| Jos ungrafted | | | | |
| Well-watered | 10.59±0.42 a | 70.36±1.84 a | 37.91±5.80 a | 90.31±0.18 a |
| Water stress | 5.82±0.18 b | 43.18±1.38 b | 29.19±0.21 b | 79.80±1.44 b |
| $LSD_{0.05}$ | 1.27 | 6.40 | 6.12 | 4.03 |
| p-value | ** | ** | * | ** |
| *JosxJos* | | | | |
| Well-watered | 10.49±1.08 a | 58.42±4.88 a | 23.20±0.86 a | 88.56±0.45 a |
| Water stress | 7.08±0.04 b | 41.08±0.31 b | 15.97±4.73 b | 83.68±1.22 b |
| $LSD_{0.05}$ | 3.02 | 13.59 | 3.35 | 3.63 |
| p-value | * | * | ** | * |
| *JosxZar* | | | | |
| Well-watered | 6.41±0.98 a | 51.30±7.64 a | 26.01±6.14 a | 92.59±4.89 a |
| Water stress | 4.94±0.71 a | 39.59±7.12 a | 20.60±6.27 b | 76.48±3.75 b |
| $LSD_{0.05}$ | 3.36 | 19.02 | 4.39 | 7.14 |
| p-value | ns | ns | * | ** |
| *ZarxJos* | | | | |
| Well-watered | 10.13±1.93 a | 59.60±8.10 a | 54.39±0.30 b | 94.63±2.12 a |
| Water stress | 7.62±0.45 a | 41.61±3.19 a | 65.54±4.22 a | 92.85±0.01 a |
| $LSD_{0.05}$ | 4.50 | 24.16 | 10.07 | 5.89 |
| p-value | ns | ns | * | ns |

Values are mean ± S.E. (n=9). Means followed by the same leter in each cultivar do not differ significantly.

Both in cv. Josefina as well as *JosxJos*, the root RGR fell with the water-deficit treatment. The cv. Zarina increased its RGR to 88% under stress conditions, while its self-graft was not visibly affected. The reciprocal grafts behaved in the contrary way, with root RGR in *JosxZar* decreasing 21% under stress, and *ZarxJos* increasing 20% (Table 1).

LRWC is a reliable indicator of the capacity of the plant to re-establish its water balance after a water-deficit situation, as well as the capacity to tolerate this stress. Only cv. Josefina, its self-graft, and *JosxZar* showed a significant decline in LRWC with the stress applied, while the rest were not appreciably affected (Table 1).

*Phenolic compounds*

The phenolic qualities and quantities of tomato upon environmental stress and modulated by the genetic variability are of great interest from the point of view of the tomato plant health and the quality of the fresh produce since tomato is one of the most important vegetables worldwide (Slimestad *&* Verheul 2009). The total content in phenolic acids increased significantly under water-stress conditions in the cv. Zarina, its self-graft and in *JosxZar*, while in the cv. Josefina, a decline of 30% was registered with respect to its control (Figure 3A).

The total flavonoids increased in concentration only in cv. Zarina and *ZarxZar*, while cv. Josefina showed a decrease under stress conditions (Figure 3B).

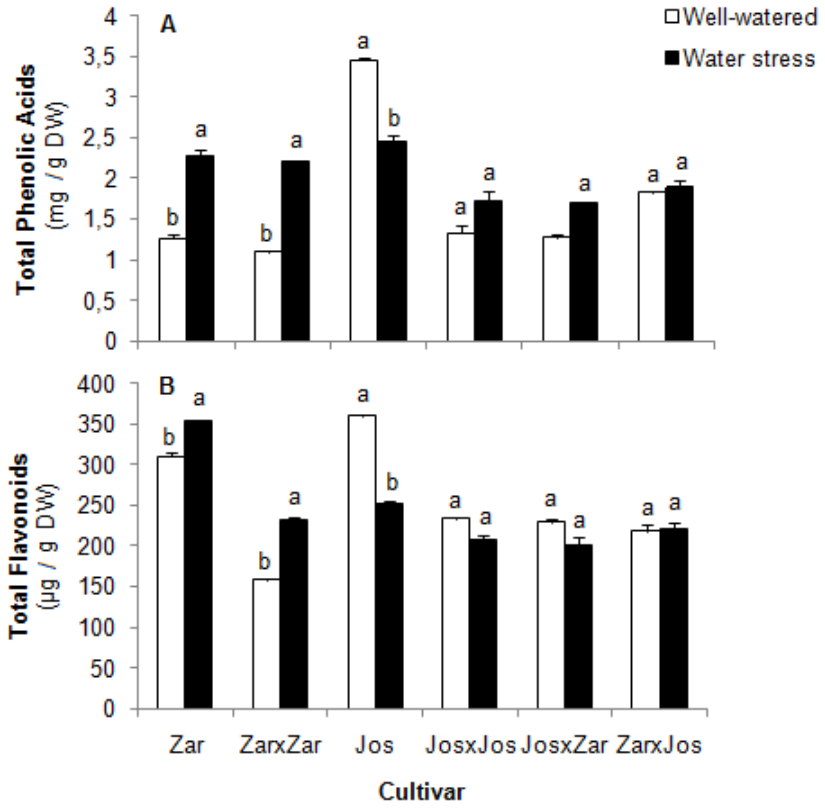


**Figure 3.** Influence of moderate water stress on phenolics acids (A) and flavonoids total (B) in ungrafted, grafted and self-grafted tomato plants. Columns are mean ± S.E. (*n = 9*). Means followed by the same letter in each cultivar do not differ significantly.

The hydromethanolic extracts of tomato leaves showed major peaks of phenolic metabolites that have been previosuly reported in literature and were characterized by HPLC-PDA-ESI-MS/MS (negative ionization mode) as presented in the Figure 2, besides other compounds present in small amounts.

Two caffeoylquinic acids were characterized (compound 1 and 2) (Figure 4) by means of -$MS^2$ of their deprotonated molecular ion (*m/z* 353) giving a base peak at *m/z* 191. In the compound **1** a relative intense ion at *m/z* 179 is also observed, whereas in **2** this ion is weak or undetectable, and according to previous report (Mabry *et al.* 1970) can be labeled as 3-caffeoylquinic acid (**1**) and 5-caffeoylquinic acid (**2**), respectively. Additionally, we also detected a di-caffeoylquinic acid ($[M-H]^-$, *m/z* 515) (**7**) (Figure 4).

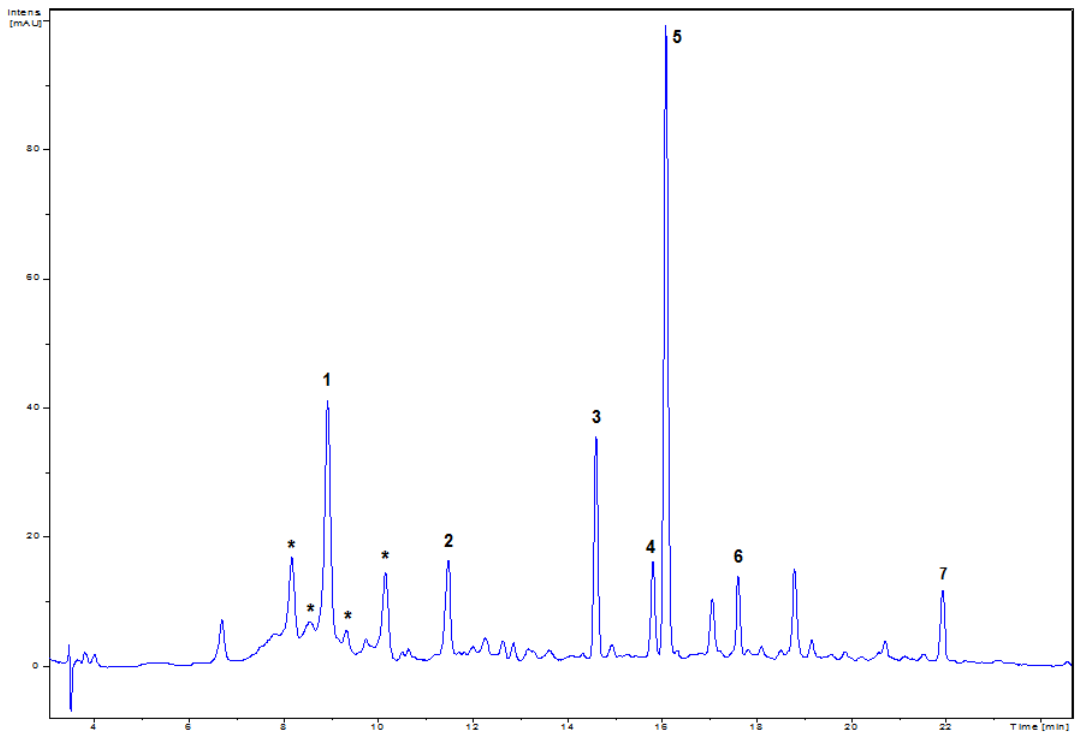


**Figure 4.** HPLC chromatogram (280 ± 10 nm) of extract of leaf of tomato revealing the presence of 1-7 peaks. (**1**) 3-caffeoylquinic acid ; (**2**) 5-caffeoylquinic acid ; (**3**) quercetin 3-apiosyl rutinoside; (**4**) kaempferol 3-apiosyl rutinoside; (**5**) quercetin 3-rutinoside (rutin); (**6**) kaempferol 3-rutinoside; (**7**) di-caffeoylquinic acid , (*) CQA derivatives.

With respect to the flavonoids, in the UV chromatogram of the tomato leaf hydroalcoholic extracts (Figure 3) we have detected four compounds (**3**-**6**). The compound **5** present in all samples showed UV spectra of quercetin derivative (Mabry *et al.* 1970) and deprotonated molecular ion at *m/z* 609. Its $MS^2$ fragmentation gave the unique peak at *m/z* 301 corresponding to the deprotonated ion of its aglycone, as well as absence of intermediate ions, indicating a interglycosidic linkage rhamnosyl (1→6) glucoside, (rutinoside) (Cuyckens *et al.* 2001). All these data indicate that this compound is quercetin-3-*O*-rutinoside (rutin), widely distributed in tomato cultivars (Slimestad *&* Verheul 2009) (Figure 5).

The other quercetin-derivative (**3**) present in the samples showed in -MS a deprotonated molecular ion 132 Da more intense than in **5** (*m/z* 741) which could indicate that the compound is a pentosyl derivative of **5**. The $MS^2$ fragmentation of this compound is similar to the observed for quercetin-3-*O*-(2´´-pentosyl-6´´-rhamnosyl) glucoside characterized in tomato (Ferreres *et al.* 2010) of which structure was also described in tomato (Slimestad *&* Verheul 2009) as quercetin-3-*O*-(2´´-apiosyl-6´´-rhamnosyl) glucoside (quercetin-3-*O*-(2´´-apiosyl) rutinoside) (Figure 5).

The compounds **4** and **6** shows UV spectra of kaempferol derivatives (Mabry *et al.* 1970) and their deprotonated molecular ions are 16 amu lower than in **3** and

**5** respectively (*m/z* 725 and 593). In this sense, their MS fragmentations are similar to those of **3** and **5**, and only diferentiated in the ion of the deprotonated aglycone, and it would be tentatively labeled as kaempferol-3-*O*-(6´´-rhamnosyl) glucoside (kaempferol-3-*O*-rutinoside) (**6**) identified in different works on tomato (Moco *et al.* 2007; Gómez-Romero *et al.* 2010) and the kaempferol-3-*O*-(2´´-apiosyl-6´´-rhamnosyl)glucosid(kaempferol-3-*O*-(2´´-apiosyl)rutinoside) (**4**) by comparison with the previously described data for the quercetin derivatives. Recent studies (Moco *et al.* 2007; Slimestad *&* Verheul 2009) described in tomato a kaempferol rutinoside pentoside that should coincide with our compound **4**.

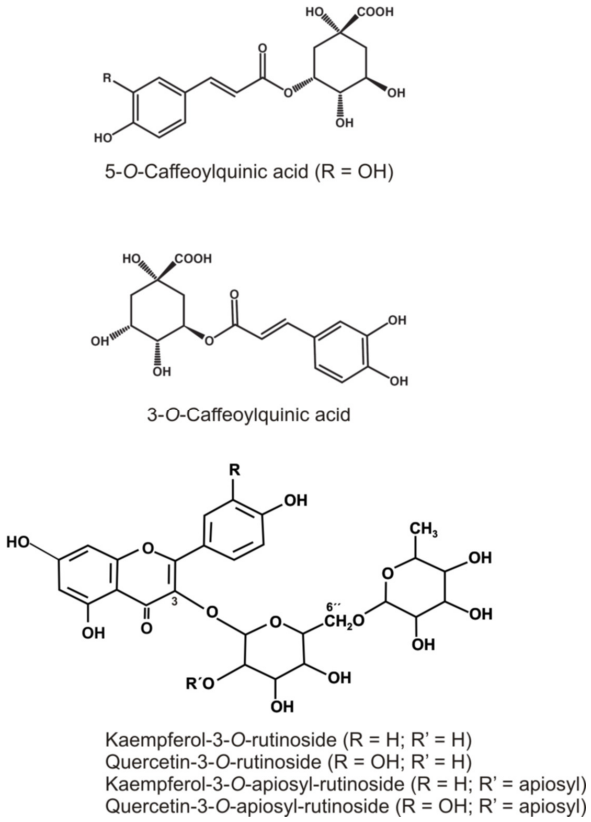


**Figure 5.** Structure of phenolic acids and flavonoids (**1-7**).

The quantities were determined for the derivates of caffeolylquinic acid and flavonols (quercetin and kaempferol glycosyl derivatives) present in the leaves in order to determine the possible alteration of the levels of these metabolites as a consequence of the water stress applied. Both cv. Zarina as well as *ZarxZar* registered a significant increase under water-deficit conditions in practically all the hydroxycinnamic acids and derivatives found (Table 2). In contrast, in the cv. Josefina under water-stress conditions these metabolites diminished with respect to their control (Table 2). In *JosxJos* the quantity of 5-caffeoylquinic acid declined, while the quantity of di-caffeoylquinic acid increased (Table 2). The graft *JosxZar* showed a general increase in the compounds studied, while, in *ZarxJos*, only the quantity of di-caffeoylquinic acid increased under stress (Table 2). With respect to flavonoids and derivatives, the cv. Zarina registered a significant rise in the compounds quercetin 3-apiosyl rutinoside and kaempferol 3-apiosyl rutinoside under the stress conditions applied (Table 2). However, its self-graft showed an increase in all the flavonoids found. Both the cv. Josefina as well as its self-graft, under water stress, showed a reduction in the quantity of quercetin 3-rutinoside and kaempferol 3-rutinoside with respect to their control (Table 2). In the reciprocal grafts, no changes were found in the quantities of flavonoids and derivatives with water stress (Table 2).

**Table 2:** Quantities of phenolic compounds in ungrafted, grafted and self-grafted tomato plants. [1] CQA: caffeoylquinic acid; Querc: quercetin; Kaemp: kaempferol; rut: rutinoside; api: apiosyl.

| Compounds[1] | Hydroxycinnamic acids and derivatives | | | | Flavonoids and glycosides | | | |
|---|---|---|---|---|---|---|---|---|
| Cultivar/Water treatment | 3-CQA (µg/g DW) | 5-CQA (µg/g DW) | di-CQA (µg/g DW) | CQA-derivaties (mg/g DW) | Querc-3-api-rut (µg/g DW) | Querc-3-rut (µg/g DW) | Kaemp-3-api-rut (µg/g DW) | Kaemp-3-rut (µg/g DW) |
| Zar ungrafted | | | | | | | | |
| Well-watered | 172.0± 11.5 b | 326.2±27.5 a | 156.8±10.1 b | 0.61±0.09 b | 41.6±3.6 b | 234.6±13.2 a | 16.4±1.9 b | 17.4±1.7 a |
| Water stress | 251.7± 5.7 a | 383.0±17.3 a | 436.0±26.6 a | 1.20±0.11 a | 61.6±3.9 a | 241.3±9.0 a | 32.0±1.4 a | 17.8±0.9 a |
| $LSD_{0.05}$ | 35.9 | 90.2 | 79.1 | 0.41 | 14.9 | 44.58 | 6.6 | 5.5 |
| p-value[†] | ** | ns | *** | * | * | ns | ** | ns |
| *ZarxZar* | | | | | | | | |
| Well-watered | 188.5±17.1 b | 93.0±7.4 b | 189.3±10.0 b | 0.63±0.06 b | 29.8±0.4 b | 115.3±1.9 b | 8.5±0.2 b | 5.1±0.1 b |
| Water stress | 330.7±14.8 a | 244.6±9.8 a | 341.9±6.0 a | 1.30±0.05 a | 41.7±1.7 a | 165.9±4.9 a | 13.4±0.6 a | 9.8±0.4 a |
| $LSD_{0.05}$ | 62.9 | 34.1 | 32.5 | 0.23 | 5.0 | 14.7 | 1.7 | 1.1 |
| p-value | *** | *** | ** | * | * | * | * | ** |
| Jos ungrafted | | | | | | | | |
| Well-watered | 525.7±12.5 a | 494.2±2.9 a | 447.6±12.8 a | 1.99±0.04 a | 42.2±1.1 a | 189.1±2.7 a | 12.9±0.4 a | 11.2±3.8 a |
| Water stress | 447.5±12.3 b | 258.4±1.4 b | 331.7±46.3 b | 1.42±0.01 b | 53.4±1.7 a | 172.1±2.7 b | 16.4±1.4 a | 10.8±0.1 a |
| $LSD_{0.05}$ | 48.8 | 9.1 | 77.5 | 0.12 | 15.6 | 12.7 | 4.3 | 13.6 |
| p-value | * | *** | * | * | ns | ** | ns | ns |
| *JosxJos* | | | | | | | | |
| Well-watered | 192.1±5.1 a | 154.1±23.3 a | 248.5±5.5 b | 0.89±0.09 a | 36.3±0.3 a | 171.5±1.4 a | 14.3±0.1 a | 11.2±0.2 a |
| Water stress | 172.5±5.9 a | 84.8±2.7 b | 735.0±13.2 a | 0.74±0.01 a | 36.2±0.9 a | 144.3±2.0 b | 14.8±0.5 a | 10.9±0.4 a |
| $LSD_{0.05}$ | 21.9 | 65.3 | 39.8 | 0.26 | 2.8 | 6.9 | 1.5 | 1.3 |
| p-value | ns | ** | *** | ns | ns | * | ns | ns |
| *JosxZar* | | | | | | | | |
| Well-watered | 226.3±9.1 b | 126.9±3.6 a | 198.5±23.3 b | 0.82±0.05 b | 34.2±1.7 a | 170.4±4.4 a | 13.1±0.4 a | 12.1±0.7 a |
| Water stress | 262.3±7.9 a | 142.1±5.1 a | 292.2±9.2 a | 1.02±0.03 a | 36.2±0.3 a | 159.0±4.2 a | 14.6±0.8 a | 11.9±0.2 a |
| $LSD_{0.05}$ | 33.5 | 17.5 | 74.7 | 0.18 | 4.9 | 16.9 | 2.5 | 2.3 |
| p-value | * | ns | ** | * | ns | ns | ns | ns |
| *ZarxJos* | | | | | | | | |
| Well-watered | 317.1±9.5 a | 225.5±4.3 a | 210.4±2.1 b | 1.08±0.03 a | 47.6±1.1 a | 156.1±1.8 a | 14.7±0.8 a | 10.2±0.2 a |
| Water stress | 284.8±17.2 a | 201.0±11.2 a | 348.1±15.5 a | 1.08±0.06 a | 41.6±2.5 a | 138.0±7.6 a | 12.2±0.3 a | 9.6±0.7 a |
| $LSD_{0.05}$ | 54.8 | 33.3 | 43.4 | 0.20 | 7.7 | 21.9 | 2.6 | 2.1 |
| p-value | ns | ns | ** | ns | ns | ns | ns | ns |

*Activities of flavonoid and phenylpropanoid synthesis and degradation-related enzymes*

To study the synthesis of phenolic compounds, we analysed the activity of the main enzymes in the shikimate pathway. The activity of the enzymes 3-deoxy-7-phosphoheptulonate synthase (DAHP, DS-Mn, DS-Co, EC 4.1.2.15), shikimate dehydrogenase (SKDH, EC 1.1.1.25), phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) and 4-coumarate coenzyme A ligase (4CL, EC 6.2.1.12) significantly increased in the cv. Zarina, as well as in its self-graft with respect to its control, except for DAHP-DS Mn activity in Zarina self-grafted plants (Figure 6). In contrast, both the cv. Josefina as well as its self-graft diminished in the activity of these enzymes under moderate water stress, except for C4H activity (Figure 6). The graft *JosxZar* increased only in the activity of the enzymes DAHP DS-Co, PAL, and C4H with respect to control, whereas the rest of the enzymes studied did not register changes in activity under stress (Figure 6). Finally, *ZarxJos* showed no alterations in activity in any of the enzymes of the cycle under the treatment applied (Figure 6).

In terms of the enzymes in charge of the degradation of phenols, polyphenol oxidase (PPO, EC 1.10.3.2) and guaicol peroxidase (GPX, EC 1.11.1.7), the cv. Zarina showed a decline in the activity of both enzymes (Figure 7), while PPO activity fell only in *ZarxZar* under water-stress conditions (Figure 7). Only the

graft *JosxJos* presented an increase in the activity of these enzymes with respect to its control (Figure 7). However, activity in cv. Josefina and the reciprocal grafts was not affected by the stress (Figure 7).

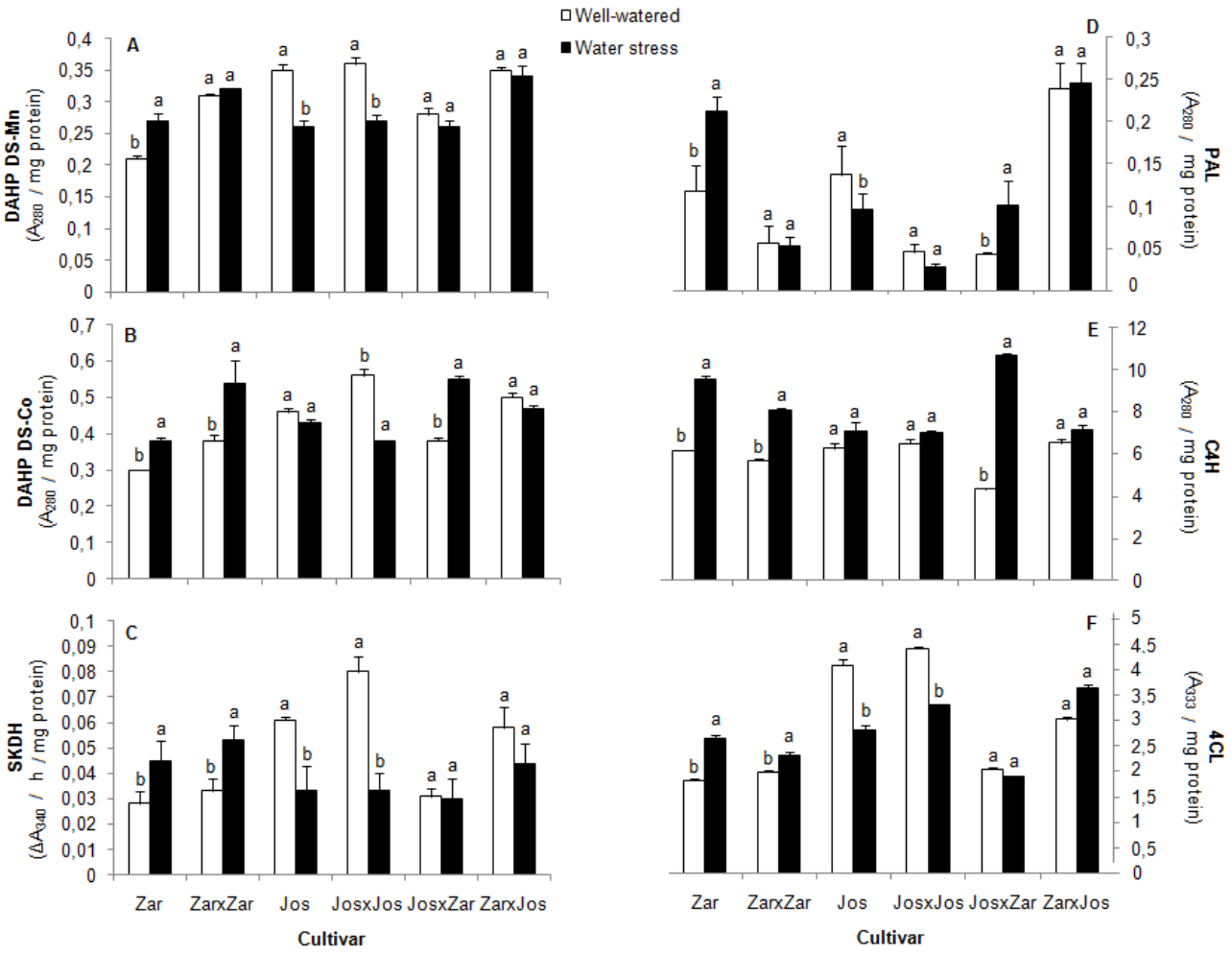


**Figure 6.** Influence of moderate water stress on flavonoid and phenylpropanoid synthesis-related enzymes activity in ungrafted, grafted and self-grafted tomato plants. Columns are mean ± S.E. (*n = 9*). Means followed by the same letter in each cultivar do not differ significantly.

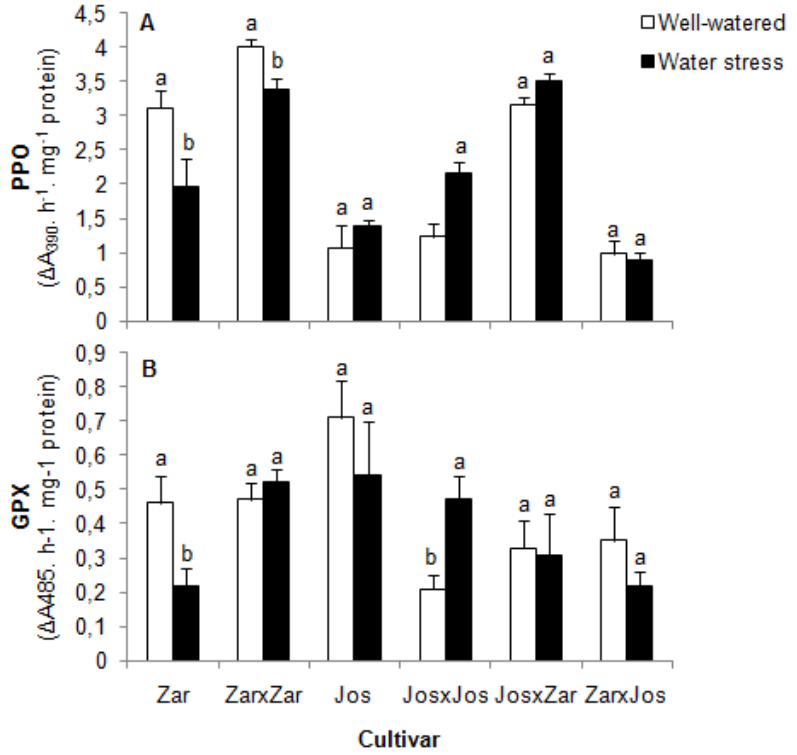


**Figure 7.** Influence of moderate water stress on flavonoid and phenylpropanoid degradation-related enzymes activity in ungrafted, grafted and self-grafted tomato plants. Columns are mean ± S.E. (*n = 9*). Means followed by the same letter in each cultivar do not differ significantly.

## DISCUSSION

Environmental stress exerts the most limiting conditions for horticultural productivity. Thus, it has been amply demonstrated that water stress can limit growth (Shao *et al.* 2008), and thus the capacity of plants to tolerate this stress is of crucial economic importance. A current method of adapting plants to environmental stress is grafting (Lee *&* Oda 2003). Our results show neither the total biomass nor the total RGR were affected with the cv. Zarina, while the cv. Josefina declined in growth under stress conditions (Table 1). These results

coincide with those found in our previous work (Sánchez-Rodríguez *et al.* 2010). The grafts *ZarxZar* and *JosxJos* showed decreases in these parameters under stress conditions; however, the total biomass was greater in the self-grafts with respect to the cvs. Zarina and Josefina (Table 1). These results are consistent with a recent report (Alan *et al.* 2007), who observed that the graft itself had a positive effect on growth, due possibly to a hormonal effect. Growth in the grafts *JosxZar* and *ZarxJos* was not appreciably affected by stress (Table 1).

LRWC is considered a reliable indicator of the plant's capacity to return to a favourable state after water deficit and also of its capacity to tolerate this stress (Sánchez-Rodríguez *et al.* 2010). The cv. Zarina, its self-graft and *ZarxJos* maintained the LRWC under stress conditions, which could be associated with greater growth of the root system (Table 1). Thus, the graft *ZarxJos* registered an increase in the root RGR under stress conditions, this possibly being associated with better LRWC. These results confirm on the one hand that the use of the tolerant cultivar (Zarina) as rootstock transmits resistance to the water-stress-sensitive shoot as in the case of cv. Josefina. Also, the graft *JosxZar* did not lose biomass or total RGR under stress conditions, but it proved less effective than *ZarxJos*, as a decline in LRWC was noted, possibly due to the low root RGR.

In response to water stress, plants can accumulate a broad range of antioxidants, including phenolic compounds (Keles *&* Öncel 2002). Our results

show that in the cv. Zarina, its self-graft and *JosxZar* increased the concentration of phenolic compounds after water stress (Table 2). This could be correlated with the greater activity of synthesis enzymes (Figure 6) as well as with a decrease in phenol-degrading enzymes (Figure 7). Prior studies have demonstrated that an increase in the phenolic concentration, as well as in the enzyme PAL, could be correlated with better drought resistance (Hura *et al.* 2008; Oh *et al.* 2010). In this case, the shoot of Zarina had a stronger influence on phenolic metabolism than did the rootstock. In turn, a greater phenolic content was related to improved growth in grafted plants (Alan *et al.* 2007; Demirsoy *&* Macit 2007), these coincides with our results.

The individual analysis of the phenolic compounds indicated that the cv. Zarina and its self-graft increased the concentration of most of the hydroxycinnamic acids and derivatives, this increase being more pronounced in di-caffeoylquinic acid. Also, the content in the flavonoids studied was found to be higher (Table 2). In contrast, cv. Josefina and its self-graft showed a contrary trend, with a significant decline in most of these compounds under water stress (Table 2). In both self-grafts, the quantity of each compound identified was relatively lower than that registered for its respective ungrafted cultivar. These results coincide with those recent reported in watermelon plants (Evrenosoğlu *et al.* 2010), since we found that the grafted cultivars maintained lower concentrations of kaempfeol than did the cultivars used as ungrafted rootstocks. In the reciprocal grafts, only *JosxZar* increased in hydroxycinnamic acids studied under water stress (Table 2). This appears to indicate that the use of the cv. Zarina as a

shoot is important in providing a greater phenolic content in the leaf, while its use as a rootstock did not substantially improve the phenolic metabolism or the accumulation of phenolic metabolites.

Our results are important from the standpoint of improving water-stress resistance in the cultivation of cherry tomato by the use of grafting. The self-grafts displayed a behaviour very similar to the ungrafted cultivars but it was the graft itself that prompted the fall in phenolic content. This could be explained by low transfer efficiency of phenolic compounds from the rootstock to the scion. In addition, the use of the water-stress-tolerant cv. Zarina as a rootstock improves the growth of the cv. Josefina under moderate water deficit. This may be due to better root RGR and the maintenance of the LRWC, although other processes could be involved, and more studies are needed to clarify this point. Besides, the use of the cv. Zarina as a shoot for grafted plants maintains growth under stress conditions, which might be explained by the higher phenolic metabolism, although other processes could also be involved. However, this graft reflects a certain stress with the decline in LRWC under water stress, due possibly to the decrease in root RGR. These results could indicate that phenolic metabolism is more influenced by the aerial part, and therefore we conclude that the capacity of inducing tolerance in rootstocks depends on the genotype of the shoot.

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# *Capítulo 5*

## *Interactions between proline and polyamines pathways in tomato grafted plants under water stress conditions*

***Physiologia Plantarum (2012) (Under review)***

**ABSTRACT**

The changes induced in the levels and related-enzymes of proline (Pro) and polyamines (PAs) in response to moderate water stress were studied using grafting between two cultivars with difference drought-response. We observed that interactions between the pathways of Pro and PAs are competitive. The cultivars more drought-stressed (Josefina ungrafted and *ZarxJos*) presented a Pro accumulation under water stress conditions; while Zarina ungrafted and *JosxZar* (more drought-tolerant) showed a higher Pro degradation associated with an enhanced of PAs biosynthesis. So, the used of Zarina like scion in grafted plants could determine a higher drought resistance associated with higher biosynthesis of PAs, and enhance in its degradations, which presented a rise of spermidine and decrease of putrescine.

**Keywords:** proline, polyamines, water stress, tomato.

**Abbreviations:** ADC, arginine descarboxylase; DAO, diamine oxidase; GDH, glutamate dehydrogenase; OAT, ornithine-δ-aminotransferase; ODC, ornithine descarboxylase; PAO, polyamine oxidase; P5CS, $\Delta^1$-pyrroline-5-carboxylate synthetase; PDH, proline dehydrogenase; Pro, proline; Put, putrescine; Spd, spermidine, SPDS, spermidine synthase; Spm, spermine.

## INTRODUCTION

Water deficit is a severe environmental factor and the greatest limitation of crop productivity, exerting a clear effect on plant growth (Rampino *et al.* 2006). Plants undergo drought stress when the water supply to the root is scarce or when the transpiration rate is very high. These two conditions often coincide in arid or semi-arid climates. The response of plants to drought consists of numerous processes that should function in coordination to alleviate cell hyperosmolarity and ionic imbalance (Parida *et al.* 2008). One response commonly induced under water deficit is the production and/or accumulation of the so-called compatible osmolytes. The accumulation of these compounds during prolonged water stress can help stabilize the tertiary structure of proteins in dehydrated cells (Kavi Kishor *et al.* 2005). Proline (Pro) is a compatible osmolyte displaying osmoregulatory action and also a capability of preventing enzyme inactivation and the damage to cell membranes and organelles. Moreover, Pro controls gene expression encoding stress proteins; it is a source of nitrogen, carbon, and reducing compounds (for example, NAD/NADH) and also manifest antioxidant action (Kuznetsov *&* Shevyakova, 1999). However, Pro accumulation under stress conditions often generates great controversy as to whether high proline accumulation confers tolerance through a direct beneficial effect, or whether it is a marker of the sensitivity and therefore is an indicator of the damage caused by this stress. Some authors use this proline accumulation as a selection criterion for drought tolerance in

tobacco plants (Vanrensburg *et al.* 1993). On the contrary, Schafleitner *et al.* (2007) detected in potato plants, that the rise in the Pro concentration was more pronounced in the cultivar most susceptible to this type of stress. Also, Juan *et al.* (2005) found in tomato plants that the cultivars most tolerant shown less Pro accumulation than the cultivars most susceptible under salinity stress.

Another class of molecules, polyamines (PAs), also play an important role in the control of cell metabolism. PAs are low molecular and aliphatic nitrogen organic cation, putrescine (Put), spermidine (Spd) and spermine (Spm) are the main PAs found in plants, and occur as free molecular bases (free forms), or are often conjugated to small molecules like phenolic acids (conjugated forms) and to various macromolecules like proteins (bound forms) (Groppa *&* Benavides 2008). Many researchers have shown the relationships of PAs metabolism (including changes in forms, contents, biosynthetic and catabolic enzymes of PAs) with plant responses to various abiotic stress conditions (Bae *et al.* 2008; Radyukina *et al.* 2009; Zhang *et al.* 2009). So, PAs may stabilize the membranes, scavenge free-radical, modulate the activities of certain ion channels and control many aspects of DNA, RNA and protein turnover under drought stress (Groppa *&* Benavides, 2008; Alcázar *et al.* 2010). Accordingly, Spd significantly increased in water stressed chickpea plants (Nayyar *&* Chander 2004). The reduced levels of PAs in soybean, especially Put and Spd, were related to higher stress injury and decreased water content (Nayyar *et al.* 2005).

Glutamate is a common precursor in the biosynthesis of Pro and PAs (Figure 1) (Alcázar *et al.* 2010). Glutamate is conversed into Pro in two reactions, catalysed by glutamate dehydrogenase (GDH) and $\Delta^1$-pyrroline-5-carboxylate synthesase (P5CS) (Raymond *&* Smirnoff, 2002). Another precursor of Pro synthesis is ornithine, which is transaminated to P5C by ornithine-δ-aminotransferase (OAT) (Verbruggen *&* Hermans, 2008). On the other hand, the metabolism and accumulation of Pro depends also on its degradation, which is catalysed by the enzyme proline dehydrogenase (PDH) (Raymond *&* Smirnoff, 2002). In the case of PAs, glutamate is more a distant precursor conversed firstly into ornithine or arginine, which in turn serve as substrates for two enzymes, ornithine descarboxylase (ODC) and arginine descarboxylase (ADC), respectively. The following addition of aminopropyl groups to Put in two reactions catalyzed by Spd synthase (SPDS) and Spm synthase (SPMS) leads to the formation of Spd and Spm, respectively (Groppa *&* Benavides, 2008). Free PAs level in plant cell depends not only on their synthesis but also on their transport, degradation and conjugation. Put degradation is catalyzed by diamine oxidase (DAO), whereas Spd and Spm are oxized by a polyamine oxidase (PAO) (Groppa *&* Benavides, 2008). Therefore, high constitutive or stress-induced level of PAs in plants would prevent active Pro accumulation and vice versa. Larher *et al.* (1998) showed that, in response to osmotic stress, rapeseed leaves accumulated Pro, and treatment with Spm suppressed this accumulation. However, opposite results were also published, and Ozturk *&* Demir (2003) showed that plant treatment with PAs stimulated Pro accumulation in spinach leaves. Thus, it is not known to what extent

changes in pathways linked to PAs metabolism can be involved in control of metabolic responses related to osmotic adjustment in higher plants, such as that responsible for Pro accumulation under water stress conditions.

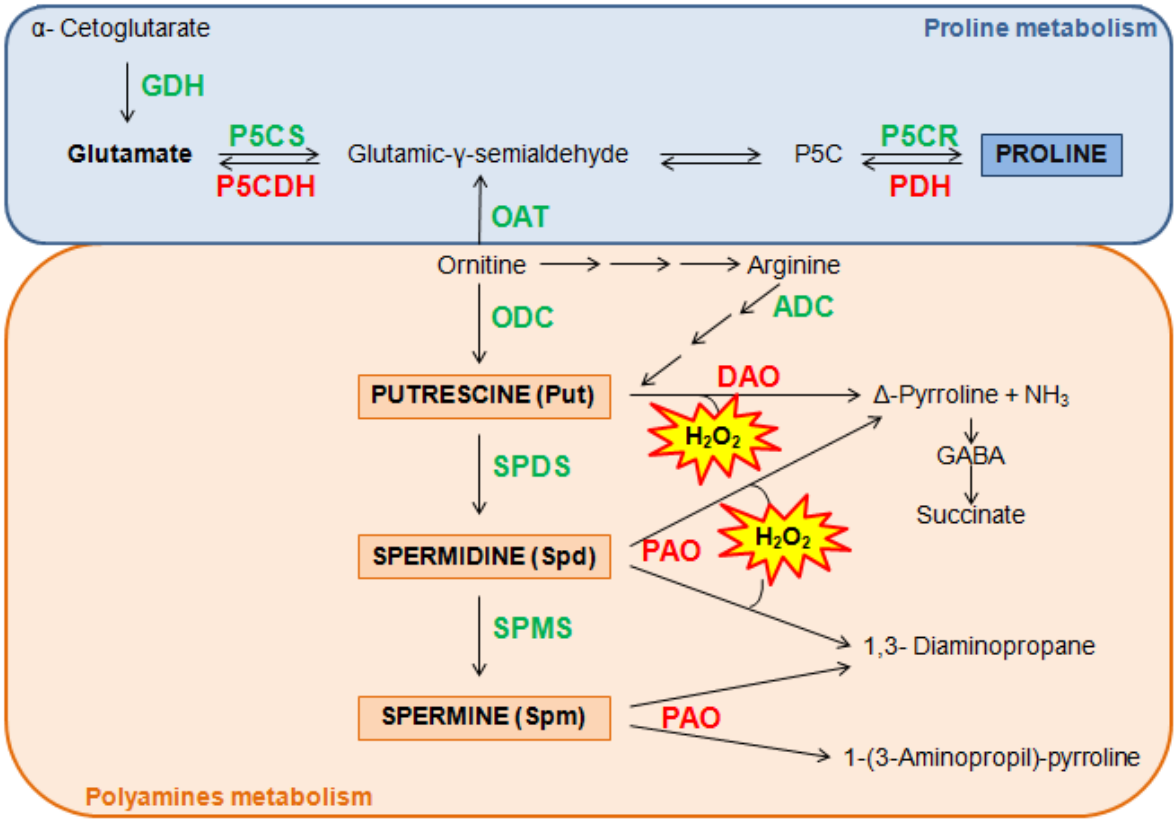


**Figure 1.** A schematic representation of proline and polyamines metabolisms.

In horticultural production, grafting is a useful technique to increase yield, avoid disease, alter habit and improve stress tolerance (Shaterian *et al*. 2005). Also, grafting can affect PAs and Pro metabolism plants. So, Miklos *et al.* (2006) reported that grafting affected the Put level of grapevine leaves and the effect depends on the rootstock genotypes. Besides, grafting in tobacco plants resulted in greater Pro and this is reflected in better biomass under salinity

conditions (Ruiz *et al.* 2006). However, the type of interaction between metabolic pathways of two classes of low-molecular protector compounds, Pro and PAs, especially under water stress conditions and in grafting plants, it remained unclear. Thus, the objetives of this experiment were to investigate the effects of moderate water stress on Pro and PAs contents and their metabolisms, and the possible roles of rootstocks/scion combinations in these processes. We studied the changes in the levels of Pro and PAs, and possible links between both pathways in the shoots of reciprocal grafts made between one drought tolerant cultivar, Zarina, and a more drought sensitive cultivar, Josefina, under moderate water stress.

## MATERIAL AND METHODS

### *Plant material and treatments*

Two tomato (*Lycopersicon esculentum* Mill) cultivars, Zarina and Josefina, were used as scion and rootstock (Figure 2). The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm x 3 cm x 10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Grafting was performed when seedlings has developed 3-4 true leaves. In the vermiculite trays used for germination, the seedlings were cut over the cotyledons, using the shoot as scion and the reamining plant part as rootstock. Grafts were made inmediately after cutting the plants and grafting clips were used to adhere the graft union. Self-grafted plants were included as controls.

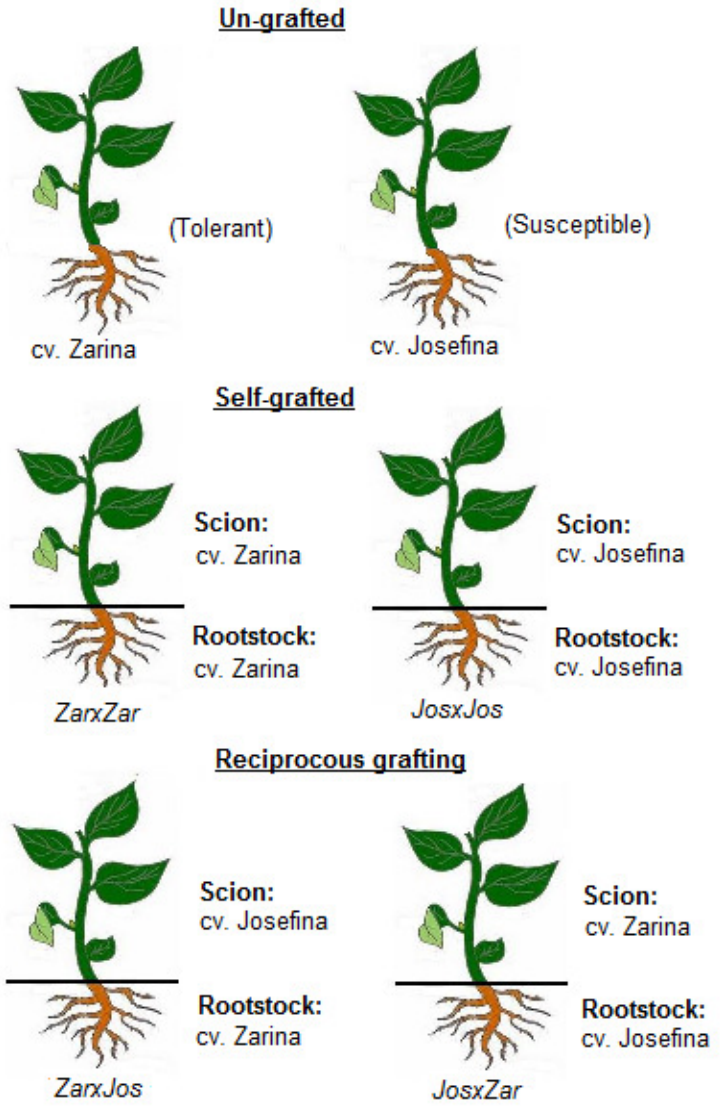


**Figure 2.** Outline of the grafting design.

After grafting, seedlings were covered with a transparent plastic lid to maintain a high humidity level and to facilitate graft formation and were left in the dark for 24h. The plastic was opened slightly every day to allow reduction in relative humidity and it was removed 6 days after grafting. Afterwards, ungrafted and grafted plants were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled

conditions with relative humidity of 50±10%, at 25ºC/15ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol $m^{-2}s^{-1}$ (LI-COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. The complete nutrient solution used to grow the plant during the experiment was the same as described in a recent study (Sánchez-Rodríguez *et al.* 2010).

The water-stress treatments began 45 days after germination and maintained 22 days. The control treatment received 100% field capacity (FC) irrigation, whereas moderate water stress corresponded to 50 % FC. The experimental design was a randomized complete block with 12 treatments (Zarina ungrafted, Josefina ungrafted, Zarina self-grafted, Josefina self-grafted, *JosxZar* and *ZarxJos* well-watered 100% FC and water stress 50% FC) arranged in individual pots with six plants per treatment (one plant per pot) and three replications each.

*Enzyme extractions and assay for proline metabolism*

$\Delta^1$-pyrroline-5-carboxylate synthetase (P5CS, E.C. 2.7.2.11/1.2.1.41) extraction was carried out according to Sumithra *et al.* (2006). Leaves were homogenized with extraction buffer containing 100 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride

(PMSF) and then centrifugated at 10 000 *g* for 15 min. The supernatant was used for enzyme assays. P5CS activity was measured as describe by Charest *&* Phan (1990). The reaction mixture contained: 100 mM Tris-HCl (pH 7.2), 25 mM $MgCl_2$, 75 mM sodium glutamate, 5 mM ATP and the enzyme extract. The reaction was initiated by the addition of 0.4 mM NADPH. The activity was measured as the rate of consumption of NADPH monitored by decreased in absorbance at 340 nm.

For ornithine-δ-aminotransferase (OAT, E.C. 2.6.1.13) and proline dehydrogenase (PDH, E.C. 1.5.99.8) extraction, leaves were homogenized in 100 mM potassium-phosphate buffer (pH 7.8). The homogenate was filtered and centrifuged at 12000 *g* for 20 min (4 ºC). OAT was assayed according to Charest *&* Phan (1990) in 0.2 M Tris-KOH buffer (pH 8.0) containing 5 mM ornithine, 10 mM α-ketoglutarate and 0.25 mM NADH. The decrease in absorbance of NADH was monitored at 340 nm for 1 min after initiating the reaction with the addition the enzyme extract.

PDH activity was assayed by the reduction of $NAD^+$ at 340 nm (Charest *&* Phan, 1990). The reaction mixture contained 0.15 M $Na_2CO_3$-HCl buffer (pH 10.3) containing 2.67 mM L-proline and 10 mM $NAD^+$.

Glutamate dehydrogenase (GDH, EC1.4.1.2-4) was assayed by measuring the decrease in the absorbance due to consumption of NADH at 340 nm

(Konomori *et al*. 1972). The reaction mixture contained 0.2 M Tris-HCl buffer (pH 8.0), 1.5 M $NH_4Cl$, 0.5 M α-ketoglutaric acid, 3 mM NADH and 0.2 mL of enzyme extract.

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine serum albumin as the standard.

*Proline content*

For the determination of the free-proline concentration, leaves were homogenized in 5 mL of ethanol at 96%. The insoluble fraction of the extract was washed with 5 mL of ethanol at 70%. The extract was centrifuged at 3500 *g* for 10 min and the supernatant was preserved 4ºC for the proline determination (Irigoyen *et al*. 1992). An aliquot of this supernatant was taken and, after adding reactive ninhydrin acid reagent (ninhydrin, phosphoric acid 6 M, glacial acetic acid 60%) and glacial acetic acid at 99%, was placed in a bath at 100ºC. After 45 min, the tubes were cooled and 5 mL of benzene were added, and the absorbance of the organic phase was measured. The result of proline concentration was expressed as µg $g^{-1}$ dry weight (DW).

*Enzyme extractions and assay for polyamines metabolism*

ADC and ODC activities were determined according to Birecka *et al.* (1985), with some modifications. Plant material (0.7 g) was homogenized in 50 mM

potassium-phosphate buffer (pH 6.3) containing 5 mM EDTA, 0.1 mM PMSF, 40 µM pyridoxal phosphate (PLP), 5 mM DTT, 20 mM ascorbic acid, and 0.1% polyvinylpyrrolidone. The homogenate was centrifuged at 12000 *g* for 40 min at 4℃ and the supernatant was dialyzed at 4℃, against 1 mL of 10 mM potassium phosphate buffer (pH 6.3) containing 0.05 mM PLP, 1 mM DTT, 0.1 mM EDTA for 24 h in darkness. The dialyzed extract was used for enzyme assay. The ADC (or ODC) reaction mixtures contained 100 mM Tris–HCl buffer (pH 7.5), 40 µM PLP, 5 mM DTT, 5 mM EDTA, 40 mM L-arginine (or ornithine for ODC determination) and the dialyzed enzyme extract for ADC (or ODC) determination. The reaction mixture was incubated at 37℃ for 60 min, and centrifuged at 3000 *g* for 10 min; 0.5 mL of the supernatant was mixed with 1 mL of 2 mM NaOH, then were benzoylated in accordance with the method of Aziz *&* Larher (1995). HPLC conditions were the same as at the measurement of polyamines.

The assay mixture for spermidine synthase (SPDS) contained 0.1 M Tris-HCl buffer, pH 8.0, 30 µM putrescine, 25 µM dcSAM, 20 µM adenine and the enzyme solution in a total volume of 0.2 mL. After incubation at 37ºC for 30 min, the reaction was stopped by adding 20 µM of 1.2 M perchloric acid and centrifuged (Yonn *et al.* 2000). For the detection of Spd after enzymatic reaction, HPLC conditions were the same as at the measurement of polyamines.

Diamine oxidase (DAO) and polyamine oxidase (PAO) activities were determined by measuring the generation of $H_2O_2$, a product of the oxidation of polyamines, as described by Su *et al.* (2005), with some modifications. Plant material (0.7 g) was homogenized in 100 mM potassium-phosphate buffer (pH 6.5). The homogenate was centrifuged at 10000 *g* for 20 min at 4℃. The supernatant was used for enzyme assay. The reaction mixture contained 2.5 mL of potassium-phosphate buffer (100 mM, pH 6.5), 0.2 mL of 4-aminoantipyrine/N,N-dimethylaniline reaction solutions, 0.1 mL of horseradish peroxidase (250 U/mL), and 0.2 mL of the enzyme extract. The reaction was initiated by the addition of 0.15 mL of 20 mM Put for DAO determination and 20 mM Spd +Spm for PAO determination. 0.001 absorbance unit of the change in the optical density at 555 nm per min was considered one unit of enzyme activity.

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine serum albumin (BSA) as the standard.

*Polyamine forms content*

Plant material (0.7 g) was homogenized in 1 mL of 6% (v/v) cold perchloric acid (PCA), kept on ice for 1 h, and then centrifuged at 21000 *g* for 30 min. The pellet was extracted twice with 1 mL of 5% PCA and recentrifuged. The three supernatants were pooled and used to determine the levels of free and

PS conjugated PAs, whereas the pellet was used to determine the levels of bound PAs. The pellet was resuspended in 5% PCA and hydrolyzed for 24 h at 110℃ after being mixed with 12 N HCl (1:1, v/v). The hydrolyzates were filtered, dried at 70℃, and then resuspended in 1 mL of 5% PCA for analysis of bound PAs. For conjugated PAs, 1 mL of the supernatant were mixed with 1 mL of 12 N HCl and hydrolyzed under the conditions described above. The supernatant, hydrolyzed supernatant, and the pellet were benzoylated in accordance with the method of Aziz *&* Larher (1995). The benzoyl derivatives were separated and analyzed by a HPLC (Agilent 1100 system, United States). 10 µL of acetonitrile (ACN) solution of benzoyl polyamines was injected into a 5 µm particle size $C_{18}$ reverse phase column. Column temperature was maintained at 30℃. Samples were eluted from the column with 40% ACN at a flow rate of 1 mL/min. PAs peaks were detected with a UV detector at 254 nm. 1,6-hexanediamine was used as an internal standard.

*Statistical Analysis*

Data compiled were submitted to an analysis of variance (ANOVA), and differences between the means were compared by Duncan's multiple-range test ($p > 0.05$).

## RESULTS

### *Proline metabolism*

The enzymes related with biosynthesis and degradation of Pro are shown in Table 1. The GDH activity registered an increase under stress conditions in Josefina ungrafted, *JosxJos* and *ZarxJos*; however, the activity of this enzyme in the rest of cultivars did not augment with respect to well-watered conditions (Table 1). Josefina ungrafted, *JosxJos* and *ZarxJos* increased in P5CS activity after water stress, while the rest of cultivars appeared not to be affected (Table 1). Meanwhile, OAT activity fell 48, 68 and 56% in Zarina ungrafted, *ZarxZar* and *JosxZar*, respectively; while the rest of the cultivars remained unaffected after the moderate water-stress treatment (Table 1). Also, the activity of PDH, the main enzyme in Pro catabolism, diminished significantly in Josefina ungrafted and *ZarxJos*, but augmented 100% in Zarina ungrafted and *JosxZar* (Table 1). The Pro concentration increased in all the cultivars after the water-stress treatment, except for the cv. Zarina and *JosxZar*, where the Pro levels remained stable with respect to control (Figure 3).

**Table 1:** Influence of moderate water stress on GDH, P5CS, OAT and PDH in ungrafted, grafted and self-grafted tomato plants.

| Rootstock x Scion/ Treatment | GDH ($\Delta A_{340}.min^{-1}.mg^{-1}$ protein) | P5CS ($\Delta A_{340}.min^{-1}.mg^{-1}$ protein) | OAT ($\Delta A_{340}.min^{-1}.mg^{-1}$ protein) | PDH ($\Delta A_{340}.min^{-1}.mg^{-1}$ protein) |
|---|---|---|---|---|
| Zar ungrafted | | | | |
| Well-watered | 0.84±0.07 | 0.53±0.07 | 0.82±0.22 | 0.20±0.02 |
| Water stress | 0.76±0.09 | 0.36±0.05 | 0.44±0.09* | 0.42±0.01* |
| $LSD_{0.05}$ | 0.25 | 0.19 | 0.21 | 0.06 |
| *ZarxZar* | | | | |
| Well-watered | 1.29±0.11 | 0.46±0.02 | 0.75±0.07 | 0.17±0.01 |
| Water stress | 1.01±0.11 | 0.36±0.07 | 0.24±0.06* | 0.12±0.01 |
| $LSD_{0.05}$ | 0.32 | 0.15 | 0.21 | 0.03 |
| Jos ungrafted | | | | |
| Well-watered | 0.68±0.11 | 0.12±0.04 | 0.93±0.15 | 0.48±0.15 |
| Water stress | 1.52±0.11* | 0.23±0.04* | 0.79±0.07 | 0.12±0.02* |
| $LSD_{0.05}$ | 0.23 | 0.09 | 0.36 | 0.32 |
| *JosxJos* | | | | |
| Well-watered | 0.56±0.07 | 0.05±0.01 | 0.44±0.09 | 0.16±0.02 |
| Water stress | 0.96±0.07* | 0.17±0.04* | 0.35±0.08 | 0.13±0.01 |
| $LSD_{0.05}$ | 0.21 | 0.08 | 0.26 | 0.03 |
| *JosxZar* | | | | |
| Well-watered | 0.68±0.05 | 0.16±0.01 | 1.09±0.15 | 0.17±0.02 |
| Water stress | 0.81±0.08 | 0.22±0.05 | 0.47±0.10* | 0.36±0.06* |
| $LSD_{0.05}$ | 0.21 | 0.13 | 0.29 | 0.14 |
| *ZarxJos* | | | | |
| Well-watered | 0.43±0.05 | 0.17±0.05 | 0.82±0.21 | 0.66±0.13 |
| Water stress | 0.76±0.04* | 0.35±0.01* | 0.83±0.22 | 0.15±0.02* |
| $LSD_{0.05}$ | 0.14 | 0.11 | 0.16 | 0.29 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05). Asterisk (*) indicates significant difference with controls groups (well-watered).

*Polyamines metabolism*

The enzymes related to PAs biosynthesis are shown in Figure 4. The ODC activity registered a decreased under stress conditions in Josefina ungrafted and its selfgraft; however, the activity of this enzyme in the rest of cultivars did not augment with respect to well-watered conditions (Figure 4A). Zarina ungrafted, *JosxZar* and *ZarxJos* increased in ADC activity after water stress, while the rest of cultivars appeared not to be affected (Figure 4B). Meanwhile,

SPDS activity enhanced in Zarina ungrafted and *JosxZar*; while in Josefina ungrafted and *ZarxJos* decreased a 65 and 61%, respectively (Figure 4C).

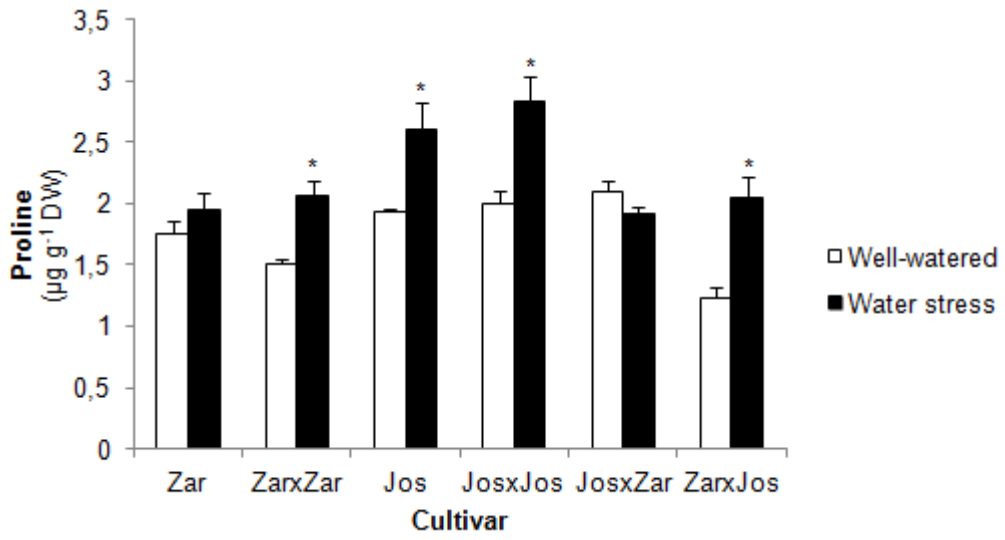


**Figure 3.** Proline content in ungrafted, grafted and self-grafted tomato plants well-watered and subjected to moderate water stress. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

The enzymes related to PAs degradation are shown in Figure 5. Zarina ungrafted and *ZarxZar* increased in DAO activity after water stress, while the rest of cultivars appeared not to be affected (Figure 5A). Moreover, the PAO activity enhanced en Zarina ungrafted, *ZarxZar* and *JosxZar* (Figure 5B).

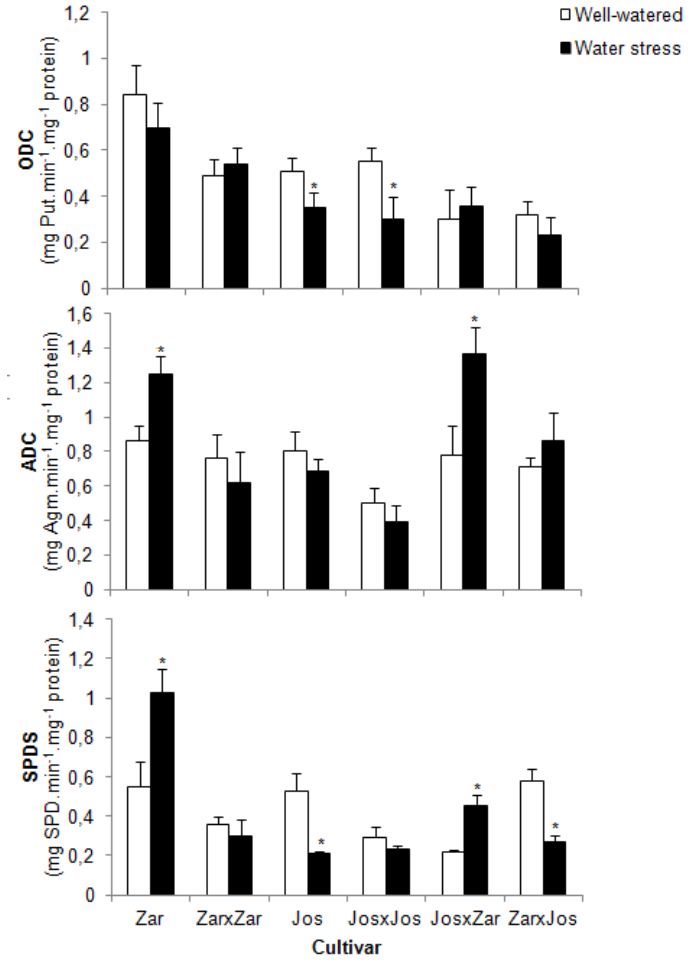


**Figure 4.** Response of proline biosynthesis related-enzymes in ungrafted, grafted and self-grafted tomato plants well-watered and subjected to moderate water stress. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

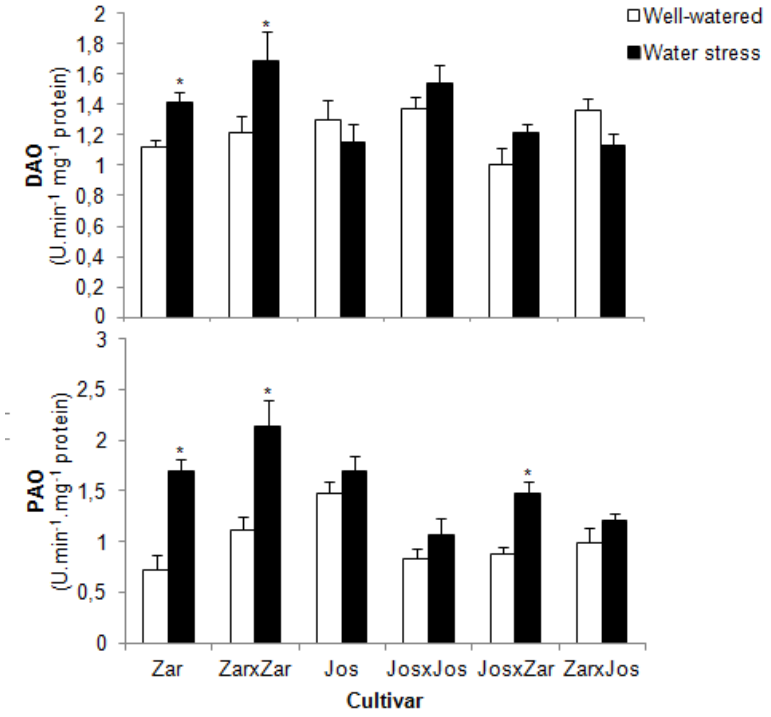


**Figure 5.** Response of proline degradation related-enzymes in ungrafted, grafted and self-grafted tomato plants well-watered and subjected to moderate water stress. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

The free, conjugated and bound PAs content are shown in Table 2. The free Put content disminished in Zarina ungrafted, *JosxZar* and *ZarxJos*, while in Josefina and its selfgraft registered an increase (Table 2). The free Spd was found to be the most abundant free PAs in leaves of tomato cherry, and only the cv. Zarina and *JosxZar* showed an increase under water stress conditions (Table 2). With the water stress treatment, the Spm content decreased in Zarina, *JosxZar* and *ZarxJos* when compared with well-watered conditions. With regard to the total free PAs, only Zarina ungrafted showed a rise under

water stress (Figure 5A). Total conjugated PAs presented an enhanced in *ZarxZar*, and a decreased in Josefina ungrafted and ZarxJos, while not significant differences were observed in the rest (Figure 6B). Zarina ungrafted, *ZarxZar* and *ZarxJos* showed a decrease in bound PAs content (Figure 6C). The total content of free, conjugated and bound PAs (each including Put, Spd and Spm) under water stress decreased in Josefina ungrafted and *ZarxJos*, while in the rest of cultivars the total PAs level remained stable with respect to control (Figure 6D).

**Table 2:** Influence of moderate water stress on free polyamines content in ungrafted, grafted and self-grafted tomato plants.

| | **Free PAs** (mg $g^{-1}$DW) | | |
|---|---|---|---|
| Rootstock x Scion/ Treat. | Put | Spd | Spm |
| Zar ungrafted | | | |
| Well-watered | 0.04±0.01 | 3.62±0.31 | 0.18±0.02 |
| Water stress | 0.02±0.00* | 4.99±0.24* | 0.06±0.09* |
| $LSD_{0.05}$ | 0.01 | 1.10 | 0.09 |
| *ZarxZar* | | | |
| Well-watered | 0.01±0.00 | 4.25±0.99 | 0.13±0.01 |
| Water stress | 0.02±0.00 | 4.11±0.86 | 0.11±0.06 |
| $LSD_{0.05}$ | 0.01 | 3.88 | 0.04 |
| Jos ungrafted | | | |
| Well-watered | 0.01±0.00 | 4.12±0.65 | 0.92±0.24 |
| Water stress | 0.05±0.01* | 4.10±1.04 | 0.67±0.09 |
| $LSD_{0.05}$ | 0.02 | 0.41 | 0.27 |
| *JosxJos* | | | |
| Well-watered | 0.02±0.01 | 4.29±0.35 | 0.83±0.27 |
| Water stress | 0.06±0.01* | 4.70±0.57 | 0.80±0.19 |
| $LSD_{0.05}$ | 0.01 | 1.07 | 0.17 |
| *JosxZar* | | | |
| Well-watered | 0.04±0.01 | 3.81±0.69 | 0.71±0.08 |
| Water stress | 0.01±0.00* | 4.68±0.65* | 0.58±0.09* |
| $LSD_{0.05}$ | 0.01 | 0.65 | 0.09 |
| *ZarxJos* | | | |
| Well-watered | 0.05±0.02 | 4.85±0.70 | 0.89±0.19 |
| Water stress | 0.03±0.01* | 4.30±0.86 | 0.52±0.13* |
| $LSD_{0.05}$ | 0.01 | 1.09 | 0.19 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05). Asterisk (*) indicates significant difference with controls groups (well-watered).
[1] nd (not detected)

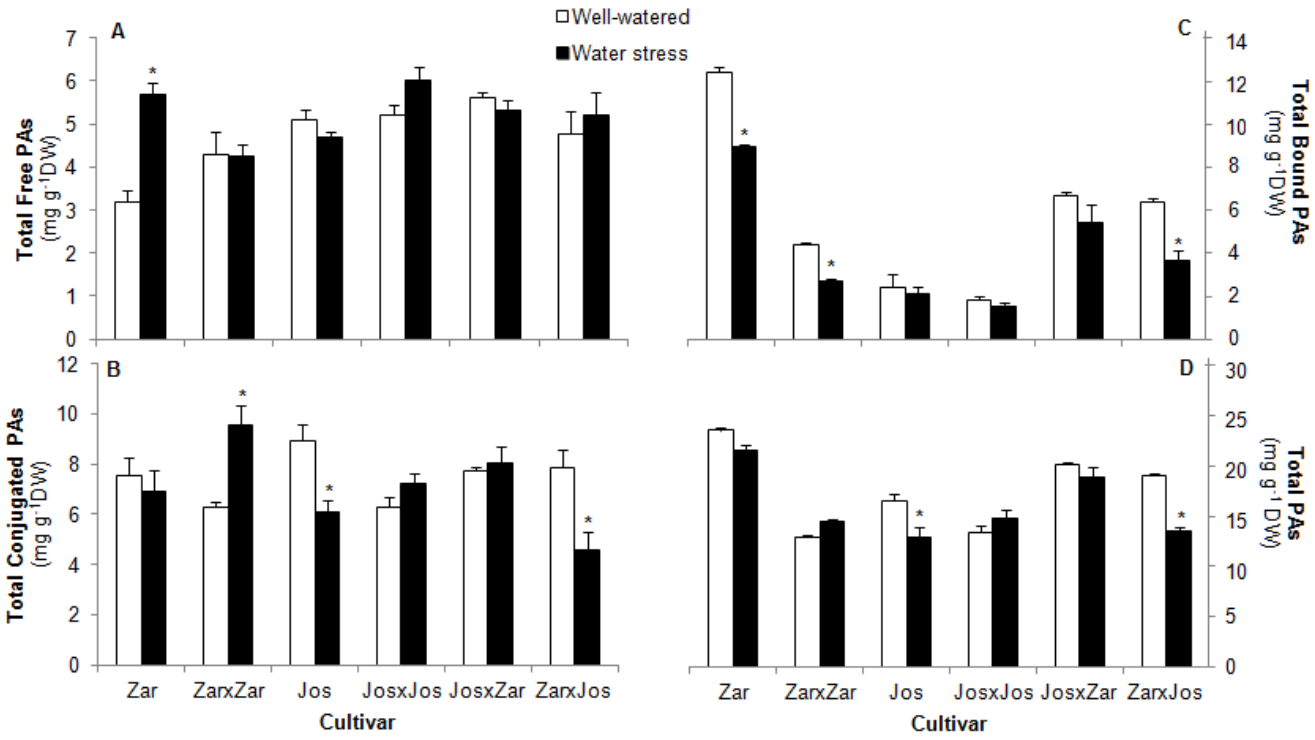


**Figure 6.** Free, conjugated, bound and total polyamines content in ungrafted, grafted and self-grafted tomato plants well-watered and subjected to moderate water stress. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

## DISCUSSION

To study proline biosynthesis, we analysed the enzymes GDH, P5CS, and OAT. The GDH activity is important under conditions of low water potential of the leaf because glutamate accumulates at low photosynthetic potential (Lawlor *&* Cornic 2002). Besides, in plants under water stress, proline is synthesised preferably from glutamate by the enzyme P5CS (Delauney *&* Verma 1993). In our study, we found that Jos ungrafted (drought-sensible),

*JosxJos* and *ZarxJos* showed higher values for the activity of both enzymes under water stress conditions (Table 1). In turn, the cv. Zarina (drought-tolerant), its self-graft and *JosxZar* did not present significant changes in the activity of these enzymes under stress conditions (Table 1). The relative contribution of the ornithine pathway to proline synthesis is little known, so we analysed the activity of OAT, an enzyme that synthesises Pro from ornithine. Our data reveal that, as opposed to what occurs with GDH and P5CS, OAT activity presents no significant changes in the most stressed cultivars (Josefina ungrafted, *JosxJos* and *ZarxJos*) (Table 1). However, the cv. Zarina and *ZarxZar* showed a decline in OAT activity (Table 1). So, although some studied report increased OAT activity under water-stress or saline conditions (Hervieu *et al.* 1995; Roosens *et al.* 2002), many other studies indicate that under these conditions the increased proline is due primarily to the activity of enzymes of the glutamate pathway (Roosens *et al.* 1999; Parida *et al.* 2008). Pro metabolism and accumulation also depend on their degradation, which is catalysed by the mitochondrial enzyme PDH (Raymond *&* Smirnoff 2002). The PDH activity diminishes significantly in Josefina ungrafted and *ZarxJos* (Table 1), which registered greater GDH and P5CS activity (Table 1), demonstrating a low Pro-degradation rate during the water stress in those cultivars with the highest degree of stress oxidative, observed in previous work (Sánchez-Rodríguez *et al.* 2012). These data agree with other authors who contend that PDH activity also determines the proline accumulation (Ruiz *et al*. 2005; Parida *et al.* 2008). However, the cv. Zarina and *JosxZar* presents a significant rise in PDH activity under stress conditions (Table 1). These data are

consistent with findings by Rosales *et al.* (2007) and López-Carrión *et al.* (2008) in tomato fruits and Chinese cabbage plants under stress conditions, respectivately. Pro accumulation is normally considered to be a stress-tolerance mechanism in plants (Kavi Kishor *et al.* 1995). Thus, Hien *et al.* (2003) found higher Pro in the rice cultivar most tolerant to water deficit. Similarly, Parida *et al.* (2008) observed greater Pro accumulation in the tolerant cotton cultivar with respect to the cultivar more susceptible to drought. However, our results in the present study show a Pro accumulation under drought conditions in the tomato cultivars most affected by stress (Josefina ungrafted, selfgrafted and *ZarxJos*) (Sánchez-Rodríguez *et al.* 2012), while cv. Zarina and *JosxZar* (more drought-tolerant) presented no increase in its concentration (Figure 3). The increase in PDH activity, together with the maintenance of the activity of biosynthesis enzymes (Table 1), could explain the fact that the Pro concentration does not increase in Zarina and *JosxZar* after the stress treatment. These results agree with those reported by Schafleitner *et al.* (2007) in potato plants, where the rise in the proline concentration was more pronounced in the cultivar most susceptible to this type of stress. Also, Juan *et al.* (2005) found in tomato plants that the cultivars most tolerant shown less Pro accumulation than the cultivars most susceptible under salinity stress. In this respect, Laboren (1995) indicated that Pro accumulation in the leaves of drought-stressed plants is associated with a constant loss in N-transport capacity, reflecting that the accumulation of this amino acid is a clear symptom of the response to the deterioration by water deficit and not an adaptive characteristic for survival.

There are many studies which have demostrated the involvement of PAs metabolism (biosynthesis, catabolism and regulation) in the plant response to abiotic stress, most remarkably on drought stress (Bae *et al.* 2008; Radyukina *et al.* 2009; Zhang *et al.* 2009). In plants, there are two alternative pathways leading to Put formation: descarboxylation of either arginine or ornithine by ADC or ADC, respectively (Groppa *&* Benavides, 2008). However, recent reviews reported that ADC pathway predominates in response to environmental stres (Bassard *et al.* 2010). Effectively, our results showed that more drought-tolerant cultivars (Zarina ungrafted and *JosxZar*) (Sánchez-Rodríguez *et al.* 2012) presented an enhanced of ADC and SPDS activities under water stress conditions (Figure 4B and 4C). These data agree with other authors who found that an increase in ADC and SPDS activities are correlated with a drought-tolerance in rice and *Arabidopsis* plants (Kasukabe *et al.* 2004; Capell *et al.* 2004, Alcázar *et al.* 2010). Free PAs level in plant cell depends not only on their synthesis but also on their transport, degradation and conjugation. Put degradation is catalyzed by diamine oxidase (DAO), whereas Spd and Spm are oxized by a polyamine oxidase (PAO) (Groppa *&* Benavides, 2008). Similar results were found for DAO and PAO activities, where Zarina ungrafted, its self-graft and *JosxZar* showed an increase under water stress conditions (Figure 5). It is known that activation of PAO and DAO leading to production of 1,3-diaminopropane (DAP), and this was important in tolerating osmotic stress in rape (Aziz *et al.* 1997). Also, in drought tolerant grapevine an increase in PAO activity was observed as compared to sensitive

genotype (Toumi *et al.* 2010). Besides, it have been demostrated that $H_2O_2$ produced by PAs catabolism may promote activation of anti-oxidative defence responses in certain stress conditions (Wimalasekera *et al.* 2011). In a previous work, we found an enhanced in antioxidant response in Zarina and *JosxZar* under water stress conditions (Sánchez-Rodríguez *et al.* 2012), which could be related with an increase in PAO and DAO activities (Figure 5).

In our present study, we simultaneously focused on the changes in contents of endogenous free, total conjugated and total bound PAs and their total in ungrafted and grafted plants during water stress. With regard to free PAs, Josefina ungrafted and *JosxJos* showed an increase in Put content under water stress conditions (Table 2). In some plants, an increase in Put content leads depolarization of membranes, loss of turgor and causes formation of necrotic spots (Bouchereau *et al.* 1999). So, the salt sensitivity in rice was associated with excessive accumulation of Put in the shoot system (Krishnamurthy *&* Bhagwat 1989). These results, could explain that cv. Josefina was more drought-sensible. However, Zarina ungrafted and *JosxZar* showed less accumulation of Put and an increase in Spd under water stress conditions (Table 2). It was found that an induction of PAs (Spd or Spm) and not Put accumulation, may confers stress tolerance (Bouchereau *et al.* 1999). Similar results were found in vetiver grass, where the decrease of free Put, and the increase of Spd and Spm free might be the typical responses to moderate water deficit (Zhou *&* Yu, 2010). The above results could indicate that when free Put level is low, free Spm or Spd contents increase (Zarina

ungrafted and *JosxZar*), suggesting that Put declines because increase its degradation (Figure 5), or it is converted into Spd and/or Spm (Figure 4). So, we suggest that both catabolism of Put and Spd/Spm synthesis, can be affected by water stress and rootstock/scion combinations, and may determinine the amounts of free PAs reaching the shoots. The content of total conjugated and bound PAs showed a similar trends (Figure 6B and 6C), however, it have been reported that both conjugated and bound PAs play a minor role in the defence against drought (Yang *et al.* 2007). In cv. Zarina, total bound PAs were higher than free PAs. Bound PAs may act as storage forms of PAs from which the free bases may be released during growth, or these conjugated may be transportated as and when required (Martin-Tanguy 1997). Besides, as PAs are essential for cellular growth, deregulation of PAs homeostasis may negatively affect cell proliferation and lead to cell death (Takao *et al.* 2006). Our results showed a homeostais of total PAs in all cultivars, except Josefina ungrafted and *ZarxJos* (Figure 6D). Changes in total PAs accumulation can be used as a criterion of stress sensitive (Katiyar *&* Dubey, 1990); and effectively, these cultivars were most stressed under water stress conditions (Sánchez-Rodríguez *et al.* 2012).

In turn of the regulation of Pro and PAs contents under stress conditions, it should be primarily noted that some researchs suppot the idea that interactions between the pathways of Pro and PAs are competitive and determined by the availability of a common precursor, glutamate (Thompson 1980). Therefore, high constitutive or stress-induced level of PAs in plants

would prevent active Pro accumulation and vice versa. Larher *et al.* (1998) showed that, in response to osmotic stress, rapessed leaves accumulated Pro, and treatment with Spm suppressed this accumulation. Under drought conditions, transgenic soybean plants with elevated constitutive synthesis of Pro accumulated more Pro and less Spm that wild-type plants (Simon *et al.* 2006). Effectively, our work showed that in the cultivars which presented Pro accumulation (self-grafts, Josefina ungrafted and *ZarxJos*) (Figure 3), not presented an enhanced in PAs biosynthesis. However, Zarina ungrafted and *JosxZar*, showed a higher PAs biosynthesis (Figure 4), associated to induction of Spd and not Put accumulation (Table 2); while the content of Pro did not change under water stress conditions (Figure 3). In these cultivars, the Pro degradation was higher under water stress (Table 1), thus supporting the idea that Pro degradation may serve as a source of precursors for PAs synthesis under stress conditions (Cvikrová *et al.* 2012).

In conclusion, the present study has shown that under water stress conditions, the Pro and PAs metabolisms were competitive. The cultivars more stressed (Josefina ungrafted and *ZarxJos*) presented a Pro accumulation under water stress conditions; while Zarina ungrafted and *JosxZar* (more drought tolerant) showed a higher Pro degradation associated with a enhanced of PAs biosynthesis. So, the used of Zarina like scion in grafted plants could determine a higher drought resistance associated with higher biosynthesis of PAs, and enhance in its degradations, which presented a rise of Spd and decrease of Put.

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# *Capítulo 6*

## *Role of grafting in resistance to water stress in tomato plants: ammonia production and assimilation*

*Plant Biology (2012) (Under review)*

**ABSTRACT**

Generally, drought depresses nutrient uptake by the root and transport to the shoot due to a restricted transpiration rate, which may contribute to growth limitation under water deprivation. Moreover, water stress may also restrict the ability of plants to reduce and assimilate nitrogen through the inhibition of enzymes implicated in nitrogen metabolism. Plants produce significant quantities of $NH_4^+$ through the reduction of $NO_3^-$ and photorespiration, it must be rapidly assimilated into non-toxic organic nitrogen compounds. The aim of the present work is to determine the response of reciprocal grafts made between one tolerant cultivar, Zarina, and a more sensitive cultivar, Josefina, to nitrogen reduction and ammonium assimilation under water stress conditions. Our results show that when Zarina (tolerant cultivar) was employment as rootstock grafted with Josefina (*ZarxJos*), this plants showed an improved N uptake and $NO_3^-$ assimilation, it triggered a favorable physiological and growth response to water stress. On the other hand, when Zarina was employment as scion (*JosxZar*), this grafted plants showed an increase in photorespiration cycle, which may generate amino acids and proteins, and could explain their better growth under stress conditions. In conclusion, grafting that improve N uptake or improve photorespiration, increase leaf $NO_3^-$ photoassimilation in water stress experiment on tomato.

**Keywords:** Ammonium assimilation, Nitrate reduction, GS/GOGAT cycle, Photorespiration, Grafting.

**Abbreviations:** AAT, aspartate aminotransferase; GDH, glutamate dehydrogenase; GGAT, glutamate:glyoxylate aminotransferase; GO, glyoxylate oxidase; GOGAT, glutamate synthase; GS, glutamine synthetase; HR, hydroxypyruvate reductase; $NH_4^+$, ammonium; NiR, nitrite reductase; $NO_2^-$, nitrite; $NO_3^-$, nitrate; NR, nitrate reductase; 2-PG, glycolate-2-phosphate; 3-PGA, glycerate-3-phosphate; RuBP, ribulose-1,5-biphosphate; SGAT, serine:glyoxylate aminotransferase.

## INTRODUCTION

Water is crucial for plant growth (Boyer 1982), such increases in the prevalence of drought will have important impacts on the productivity of agricultural (Passioura 2007). Generally, drought depresses nutrient uptake by the root and transport to the shoot due to a restricted transpiration rate, affecting active transport and membrane permeability (Hsiao 1973; Kramer *&* Boyer 1995). Drought-dependent nitrogen deficiency may contribute to growth limitation under water deprivation (Heckathorn *et al.* 1997). Many researchers have shown a directly proportional relationship between nitrate ($NO_3^-$) and yield, and also between yield and foliar nitrogen (N) content (Kim *et al.* 2011; Li *&* Lascano

2011; Naudin *et al.* 2010). For this reason, the crops that maintain high N content and productivity under water stress are indispensable.

Nitrate is the main nitrogen source in agricultural soils. However, the reduced nitrogen form available to plants for assimilation into amino acids and proteins is ammonium ($NH_4^+$) (Miflin *&* Habash 2002). The reduction of $NO_3^-$ to nitrite ($NO_2^-$) catalyzed by nitrate reductase (NR) is considered the limiting step in N assimilation (Figure 1). In turn, $NO_2^-$ is reduced by nitrite reductase (NiR) to form $NH_4^+$. Drought affects different steps of nitrogen metabolism, namely ion uptake, nitrogen assimilation, and amino acid and protein synthesis. Water stress may also restrict the ability of plants to reduce and assimilate nitrogen through the inhibition of enzymes implicated in nitrogen metabolism, such as NR. Therefore NR, the first enzyme in the pathway of nitrate assimilation, has proved to be one of the enzymes that exhibit declining activity in water-stressed leaves of several species (Fresneau *et al.* 2007; Robredo *et al.* 2011). Such, an increase in the NR activity leads to a corresponding increase in the potential for nitrate reduction and confers a greater capacity for general aminoacid synthesis, protein synthesis or total nitrogen (Singh *&* Usha 2003).

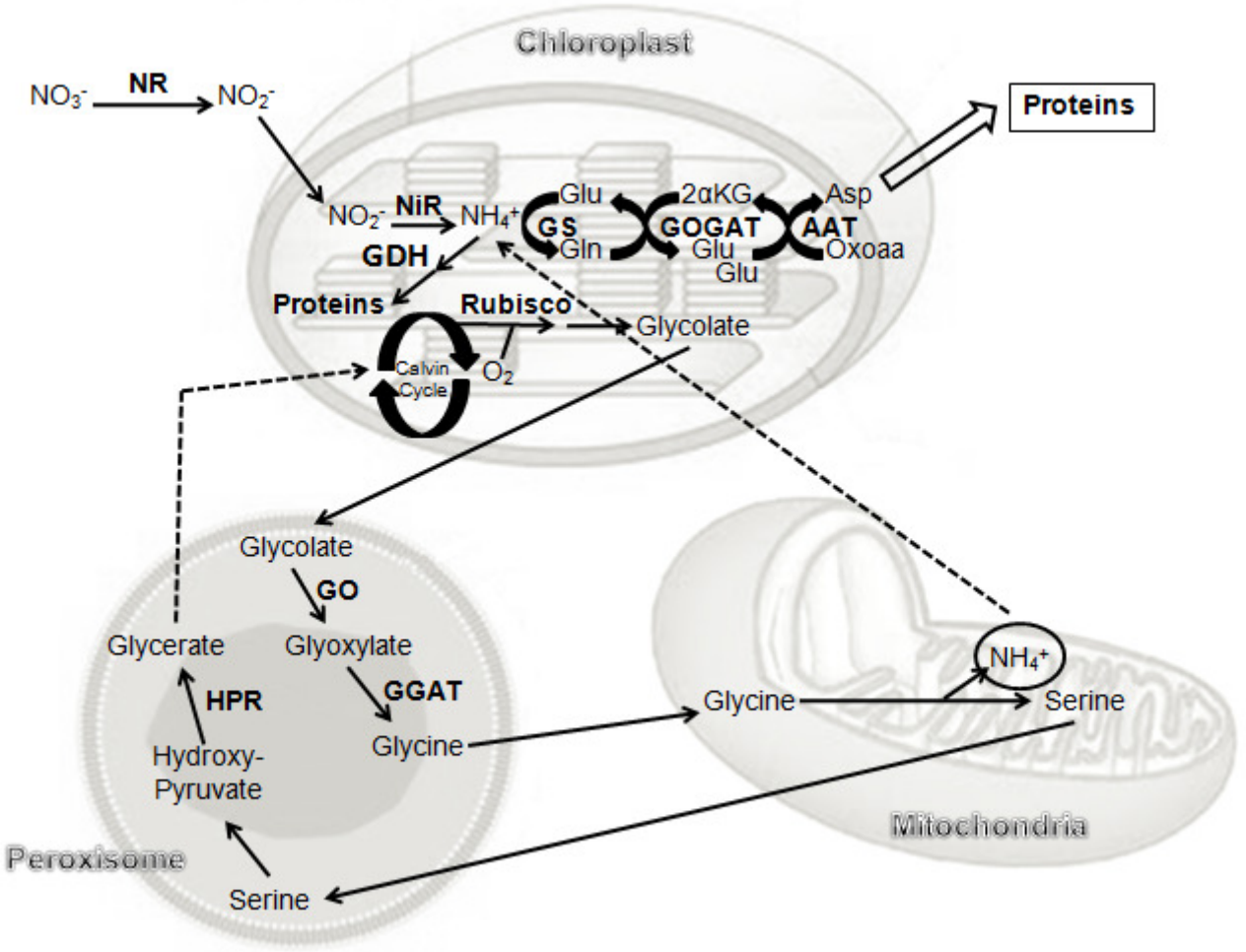


**Figure 1.** A schematic representation of the nitrate reduction, photorespiratory cycle and ammonium assimilation.

Another effect known of water stress in plants is a closure of stomata to avoid further loss of water through transpiration (Lawlor 1995). This could provoke photoinhibition by diminishing the use of electrons by photosynthesis (Roland *et al.* 2006). As protection of the photosynthetic apparatus against such damage, this excess of photons can be used by photorespiration in C3 plants. Thus, this process has been viewed as a wasteful process, a vestige of the high $CO_2$

atmospheres under which plants evolved (Wingler *et al.* 2000). At best, according to current thought, photorespiration may mitigate photoinhibition under high light and drought stress (Wingler *et al.* 2000; Kozaki *&* Takeba 1996), or may generate amino acids such as glycine for other metabolic pathways (Noctor *et al.* 1999). Photorespiration takes place in the chloroplasts, peroxisomes, and mitochondria, a consequence of the oxygenation of ribulose-1,5-biphosphate (RuBP) catalyzed by RuBP carboxylase/oxygenase (Rubisco), which generates one molecule of glycerate-3-phosphate (3-PGA) and one of glycolate-2-phosphate (2-PG) (Figure 1). This 2-PG is hydrolysed by phosphoglycolate phosphatase to glycolate, which is transported to peroxisome and oxidized to glyoxylate by glyoxylate oxidase (GO). Glyoxylate is transaminated to glycine by the reaction catalysed by glutamate: glyoxylate aminotransferase (GGAT), and is transported to the mitochondria. Subsequently, glycine is transformed into serine by the action of the enzymes glycine decarboxylase and hydromethyltransferase. The serine formed in the mitochondria is transported to peroxisome, where it is transformed by serine: glyoxylate aminotransferase (SGAT) to hydroxypyruvate, which is reduced to glycerate by hydroxypyruvate reductase (HR). Finally, glycerate moves to the chloroplast, where it is phosphorylated by glycerate kinase, giving rise to a molecule of 3-PGA, which enters the Calvin cycle (Wingler *et al.* 2000).

Plants produce significant quantities of $NH_4^+$ through the reduction of $NO_3^-$ and photorespiration in the step from glycine to serine (Figure 1). In fact,

photorespiration can produce 20- fold more $NH_4^+$ than that generated by $NO_3^-$ reduction and is considered the largest source of this cation, especially in C3 plants (Hirel *&* Lea 2001). The $NH_4^+$ is toxic to plants, causing proton extrusion associated with ammonium uptake, cytosolic pH disturbances, uncoupling of photophosphorylation, etc (Kronzucker *et al.* 2001). Therefore, it must be rapidly assimilated into non-toxic organic nitrogen compounds. This assimilation occurred via glutamine synthetase (GS) and glutamate synthase (GOGAT) (Figure 1). Nitrogen is the incorporated into aspartate and other amides and amino acids by aspartate aminotransferase (AAT). Alternatively, glutamate dehydrogenase (GDH) can also catalyze $NH_4^+$ incorporation into glutamate by reductive amination of 2-oxoglutarate (Cammaerts *&* Jacobs 1985). The function of the alternative GDH pathway remains obscure and is proposed to play a complementary role under adverse environment (Lu *et al.* 2005). Wang *et al.* (2007) reported that the GS/GOGAT cycle does not play a major role in $NH_4^+$ assimilation under salinity stress in wheat plants. In cucumber plants under nitrate stress, GS/GOGAT cycle decreased possibly due to low water potential and $NH_3$ toxicity (Yang *et al.* 2010). Besides, other author shown that resistance to water stress was increased to improve the activity in key enzymes of N metabolism (Xu *&* Zhou 2006; Sánchez-Rodríguez *et al.* 2011a).

Grafting is a horticultural technology, practiced for many years and in many parts of the world, in order to overcome many abiotic stress (Abdelmageed *&* Gruda 2009; Ahn *et al.* 1999; Estañ *et al.* 2005; Venema *et al.* 2008; Rivero *et*

*al.* 2003). Grafted plants usually show increased uptake of water and minerals compared with self-rooted plants, as a consequence of the vigorous root system used as rootstock (Ruiz *et al.* 2006; Santa-Cruz *et al.* 2002). So, Ruiz *et al.* (1997) concluded that N content was influenced more by the rootstock genotype than the scion in melon plants. The utilization of certain rootstocks has been found to stimulate the NR activity and nitrogen metabolism in roses, melon and tobacco plants (Agbaria *et al.* 1996; Pulgar *et al.* 2000; Ruiz *et al.* 2006). The characteristics of the rootstocks could result in increased absorption, upward transport and accumulation of $NO_3^-$ in the scion, thereby stimulating NR and $NO_3^-$ assimilation (Martínez-Ballesta *et al.* 2010). However, little is known about the effect of grafting on the activity of enzymes involved in $NH_4^+$ assimilation.

The practical and horticultural aspects of grafting technology have been described in several reviews (Lee *&* Oda 2003; Martínez-Ballesta *et al.* 2010), but less had been compiled about the physiological implications of the rootstock-scion interactions as a barrier for the translocation of water and nutrients, or the effect of the rootstock-scion connection on N metabolism of the grafted plants. In a previous work, we selected the most drought tolerant (cv. Zarina) and sensitive (cv. Josefina) among 5 commercial tomato cultivars (Sánchez-Rodríguez *et al.* 2010), and observed that the cv. Zarina presented an improvement in N metabolism under water-stress conditions (Sánchez-Rodríguez *et al.* 2011a). Therefore, the aim of the present work was to examine the ways in which the grafting affect to enzymes involved in N metabolism in

respond to moderate water stress associated with photorespiration as a mechanism to generate $NH_4^+$, in order to determine the involvement of grafting in this process under stress conditions. We study the response to moderate water stress with different combinations scion-rootstock in grafted, self-grafted and ungrafted tomato plants using cv. Zarina and cv. Josefina to test the viability and efficiency of this grafting technique in terms of N metabolism.

## Materials and methods

### *Plant material and treatments*

Two tomato (*Solanum lycopersicum*, *Lycopersicon esculentum* Mill) cultivars, Zarina and Josefina, were used as scion and rootstock (Figure 2). The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm x 3 cm x 10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Grafting was performed when seedlings has developed 3-4 true leaves. In the vermiculite trays used for germination, the seedlings were cut over the cotyledons, using the shoot as scion and the reamining plant part as rootstock. Grafts were made inmediately after cutting the plants and grafting clips were used to adhere the graft union. Self-grafted plants were included as controls. After grafting, seedlings were covered with a transparent plastic lid to maintain a high humidity level and to facilitate graft formation and were left in the shade for 24 h. The plastic was opened slightly every day to

allow reduction in relative humidity and it was removed 6 days after grafting. Afterwards, ungrafted and grafted plants were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with relative humidity of 50±10%, at 25ºC/15ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol $m^{-2}s^{-1}$ (measured with an SB quantum 190 sensor, LI-COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. Throughout the experiment, the plants were grown in a complete nutrient solution containing (Sánchez-Rodríguez *et al.* 2010). The water-stress treatments began 45 days after germination and maintained 22 days. The control treatment received 100% field capacity (FC) irrigation, whereas moderate water stress corresponded to 50 % field capacity. The experimental design was a randomized complete block with 12 treatments (Zarina ungrafted, Josefina ungrafted, Zarina self-grafted, Josefina self-grafted, *JosxZar* and *ZarxJos* well-watered 100% FC and water stress 50%) (Figure 2) arranged in individual pots with six plants per treatment (one plant per pot) and three replications each.

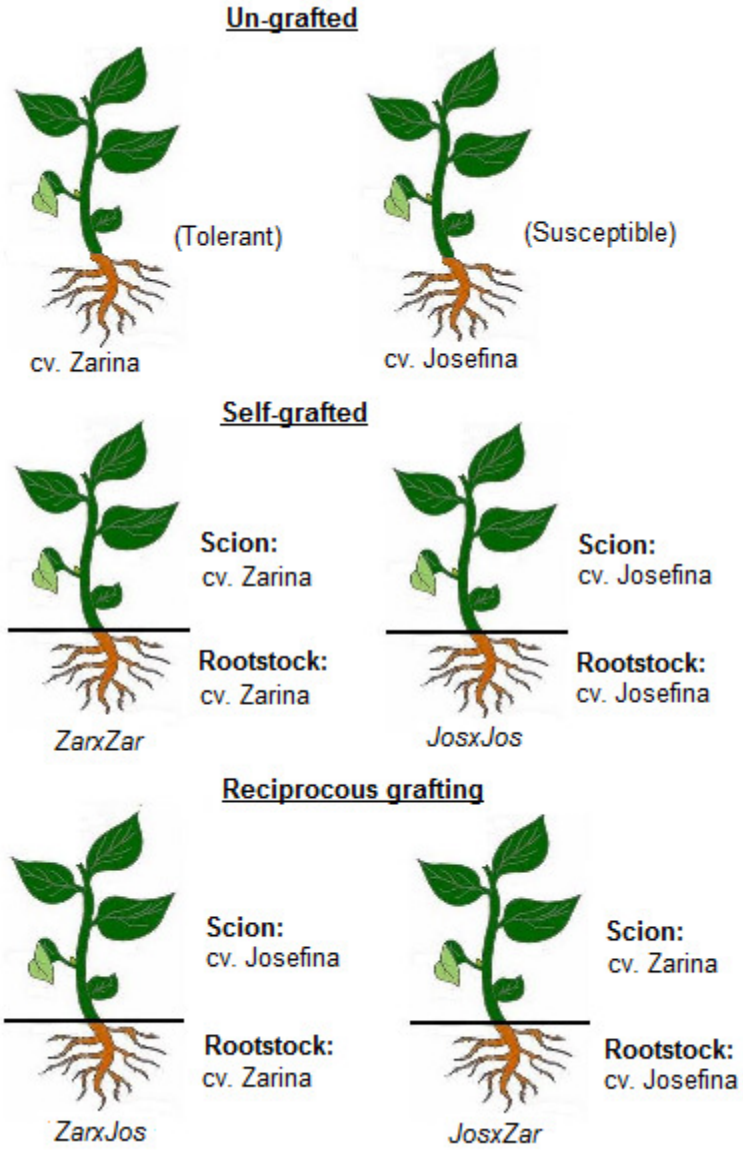


**Figure 2.** Outline of the grafting design.

*Plant sampling*

All plants were at the late vegetative stage when harvested. Leaves (excluding petioles) were sampled on day 67 after germination. The plant material was

rinsed three times in distilled water after disinfection with 1% non-ionic detergent and then blotted on filter paper. A part of the plant material was used for the assay of fresh weight (FW), amino acids, proteins, and of NR, NiR, GS, GOGAT, AAT, Rubisco, GO, GGAT, HR and GDH enzymatic activities. The rest of the plant material was lyophilised and used to determine $NO_3^-$, $NH_4^+$ and organic and total reduce N and total N.

*Analysis of N forms, soluble protein and free amino acid concentration*

$NO_3^-$ was analysed from an aqueous extraction of 0.2 g of DW in 10 mL of Millipore-filtered water. A 100 µL aliquot was taken for $NO_3^-$ determination and added to 10% (w/v) salicylic acid in sulphuric acid at 96%, measuring the $NO_3^-$ concentration by spectrophotometry as performed by Cataldo *et al.*, (1975). $NH_4^+$ was analyzed from an aqueous extraction and was determined by using the colorimetric method described by Krom (1980).

For the total reduced-N determination, a sample of 0.1 g DW was digested with sulphuric acid and $H_2O_2$ (Wolf 1982). After dilution with deionized water, a 1mL aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 µM sodium phosphate and 5.4%, w/v, sodium hydroxide), 15%/0.03% (w/v) sodium silicate/sodium nitroprusside and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37 ºC for 15 min, and

total reduced N was measured by spectrophotometry according to the method of Baethgen and Alley (1989). Total N concentration was assumed to represent the sum of total reduced N and $NO_3^-$.

Amino acids and proteins were determined by homogenization of 0.5 g FW in 50 mM cold $KH_2PO_4$ buffer at pH 7 and centrifugation at 12000 g for 15 min. The resulting supernatant was used for the determination of total amino acids by the ninhydrin method (Yemm *&* Cocking 1955). Soluble proteins were measured with Bradford reagent (Bradford 1976).

*Nucleotide analysis*

Pyridine nucleotides were extracted from liquid N-frozen leaves material in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA (for $NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12,000 g for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 100 mM bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method (Matsumura *&* Miyachi 1980) with some modification (Gibon *&* Larher 1997).

*Enzyme extractions and assays*

Leaves were ground in a mortar at 4ºC in 50 mM buffer $KH_2PO_4$ (pH 7.5) containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 30000 g for 20 min. The resulting extract (cytosol and organelle fractions) was used to measure enzyme activity of NR, NiR, GOGAT and GDH. The extraction medium was optimised for these enzyme activities so that these could be extracted together according to the same method (Groat *&* Vance 1981; Kaiser *&* Lewis 1984; Lillo 1984; Singh *&* Srivastava 1986).

The NR assay followed the methodology of Kaiser *&* Lewis (1984).The $NO_2^-$ formed was colorimetrically determined at 540 nm after azocoupling with sulphanilamide and naphthylethylenediamine dihydroclhoride according to the method of Hageman *&* Hucklesby (1971).

NiR activity was defined by the disappearance of $NO_2^-$ from the reaction medium (Lillo 1984). After incubation at 30ºC for 30 min, the $NO_2^-$ content was determined colorimetrically as above.

GOGAT activity was assayed spectrophotometrically at 30ºC by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat *&* Vance (1981) and Singh *&* Srivastava (1986), always within 2 h of extraction. Two controls, without ketoglutarate and glutamine, respectively, were used to correct for

endogenous NADH oxidation. The decrease in absorbance was recorded for 5 min.

GDH activity was assayed by monitoring the oxidation of NADH at 340 nm essentially as indicated by Groat *&* Vance (1981) and Sing *&* Srivastava (1986). The reaction mixture consisted of 50 mM buffer $KH_2PO_4$ (pH 7.5) with 200 mM $NH_4^+$ sulphate, 0.15 mM NADH, 2.5 mM 2-oxoglutarate and enzyme extract. Two controls, without ketoglutarate and $NH_4^+$ sulphate, respectively, were used to correct for endogenous NADH oxidation. The decrease in absorbance was recorded for 3 min.

GS was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser *&* Lewis (1984). Leaves were ground in a mortar at 0 ºC in 50 ml of maleic acid- KOH buffer (pH 6.8), containing 100 mM sucrose, 2% (v/v) β- mercaptoethanol and 20% (v/v) ethylene glycol. The homogenate was centrifuged at 30000 g for 20 min. The resulting extract was used to measure enzyme activity of GS .The reaction mixture used in the GS assay was composed of 100 mM $KH_2PO_4$ buffer (pH 7.5) with 4 mM EDTA, 1000 mM L-sodium glutamate, 450 mM $MgSO_4 \cdot 7H_2O$, 300 mM hydroxylamine, 100 mM ATP and enzyme extract. Two controls were prepared, one without glutamine and the other without hydroxylamine. After incubation at 28ºC for 30 min, the

formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride (Wallsgrove *et al.* 1979).

AAT activity was assayed spectrophotometrically at 340 nm using the method published by Gonzalez *et al.* (1995). AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 8), 4 mM $MgCl_2$, 10 mM aspartic acid and enzyme extract. The decrease in absorbance was recorded for 3 min.

Rubisco activity was measured spectrophotometrically by coupling 3-phosphogliceric acid formation with NADH oxidation at 25 ºC according to Lilley *&* Walker (1974) with some modification (Nakano *et al.* 2000). The total activity was assayed after crude extract was activated in a 0.1 mL activation mixture containing 33 mM tris-HCl (pH 7.5), 0.67 mM EDTA, 33 mM $MgCl_2$, 10 mM $NaHCO_3$ for 15 min. Initial rubisco activity measurements were carried out in a 0.1 mL reaction medium containing 5 mM Hepes-NaOH (pH 8.0), 1 mM $NaHCO_3$, 2 mM $MgCl_2$, 0.25 mM DTT, 0.1 mM EDTA,1 U glyceraldehyde 3-phosphate dehydrogenase, 0.5 mM ATP, 0.015 mM NADH, 0.5 mM phosphocreatine, 0.06 mM RuBP and 10 µL extract. The change in absorbance at 340 nm was monitored.

For the GO determination, fresh leaf tissue (0.25 g) was ground in a chilled mortar with PVPP and 1 ml of 50 mM Tris-HCl buffer (pH 7.8) with 0.01% triton

X-100 and 5 mm 1,4-dithioerythritol (DTT). The homogenate was centrifuged at 30000 g for 20 min. The supernatant was decanted and immediately used for the enzyme assay. GO was assayed as described by Feierabend *&* Beeevers (1972) with modifications. A volume of assay mixture containing 50 mM Tris-HCl buffer (pH 7.8), 0.009% triton X-100, 3.3 mM phenylhydrazine HCl (pH 6.8), 50 µL of plant extract and 5 mM glycolic acid (neutralized to pH 7 with KOH) was used to start the reaction. GO activity was determined by following the formation of glyoxylate phenylhydrazone at 324 nm for 2 min after an initial lag phase of 1 min.

For determination of GGAT and HR, leaves were ground in a chilled mortar in 100 mM Tris-HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20000 g for 10 min. The resulting extract was used to measure enzyme activity. The extraction medium was optimised for the enzyme activities such that they could be extracted together using the same method (Hoder *&* Rej 1983).

GGAT activity was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH. The reaction was assayed in a mixture containing 100 mM Tris-HCl (pH 7.3), 20 mM glutamate, 1 mM glyoxylate, 0.18 mM NADH, 0.11 mM pyridoxal-5-phosphate, 83 mM $NH_4Cl$ and 0.3 U GDH in a final volume of 0.6 mL (Igarashi *et al.* 2006).

HR assay was performed with 100 mM Tris-HCl (pH 7.3), 5 mM hydroxypyruvate and 0.18 mM NADH. Activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Hoder *&* Rej 1983).

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

*Statistical analysis*

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics 6.1 programme. Means were compared by Fisher's least-significant differences (LSD).

**RESULTS**

*$NH_4^+$ production: reduction of $NO_3^-$ and photorespiration*

Nitrate levels showed an increase significant of 75% in ungrafted Zarina under water stress conditions, however, in cv. Josefina a decrease was observed (Table 1). In self-grafting, *ZarxZar* and *JosxJos*, not significant differences was observed between well-watered and water stress. In reciprocal grafting, only

*ZarxJos* showed stronger increase in nitrate concentration under stress conditions, while in *JosxZar* not significant differences was observed (Table 1). The results of NR assays reflected significant differences in cv. Zarina and its self-graft, where higher increase were observed under water stress conditions (Table 1). Besides, Josefina ungrafted and *JosxJos* showed a decrease NR activity with respect well-watered conditions (Table 1). In reciprocal grafting, as with nitrate, NR activity increased only *ZarxJos*. On the other hand, for NiR activity, not significant differences were observed in any cases (Table 1). In the case of $NH_4^+$ concentration, ungrafted Zarina showed a decreased with respect to control conditions; however in *ZarxZar* an increased was observed under water stress (Table 1). In the reciprocal grafting, not significant differences were observed under stress conditions (Table 1).

With regard to the photorespiration process, both the initial activity as well as the total Rubisco only showed differences significant in ungrafted Zarina and *JosxZar*, which presented an increased in Rubisco activity under water stress conditions (Figure 3A and 3B).

**Table 1:** Influence of moderate water stress on response of $NO_3^-$ reduction and $NH_4^+$ concentration in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | $NO_3^-$ | NR | NiR | $NH_4^+$ |
|---|---|---|---|---|
| Zar ungrafted | | | | |
| Well-watered | 10.89±0.09 | 1.01±0.15 | 6.57±0.65 | 1.12±0.01 |
| Water stress | 19.09±0.09* | 2.29±0.30* | 5.71±1.33 | 0.44±0.01* |
| $LSD_{0.05}$ | 4.18 | 0.72 | 2.16 | 0.02 |
| *ZarxZar* | | | | |
| Well-watered | 10.09±0.14 | 0.88±0.04 | 5.72±0.79 | 0.49±0.00 |
| Water stress | 9.75±0.11 | 1.89±0.13* | 4.22±0.49 | 0.72±0.01* |
| $LSD_{0.05}$ | 2.38 | 0.31 | 1.99 | 0.04 |
| Jos ungrafted | | | | |
| Well-watered | 30.62±0.14 | 4.28±0.46 | 3.32±0.37 | 0.34±0.01 |
| Water stress | 20.50±0.14* | 2.87±0.14* | 4.20±0.91 | 0.67±0.01* |
| $LSD_{0.05}$ | 5.11 | 1.04 | 2.08 | 0.02 |
| *JosxJos* | | | | |
| Well-watered | 20.81±0.09 | 2.00±0.07 | 2.71±0.51 | 0.42±0.01 |
| Water stress | 21.01±0.09 | 1.17±0.22* | 3.12±0.54 | 0.86±0.01* |
| $LSD_{0.05}$ | 6.28 | 0.50 | 1.59 | 0.03 |
| *JosxZar* | | | | |
| Well-watered | 20.73±0.09 | 2.59±0.14 | 3.42±0.34 | 0.67±0.01 |
| Water stress | 20.75±0.18 | 1.93±0.36 | 4.26±0.78 | 0.66±0.01 |
| $LSD_{0.05}$ | 4.13 | 0.82 | 1.82 | 0.02 |
| *ZarxJos* | | | | |
| Well-watered | 20.60±0.13 | 2.01±0.22 | 5.46±0.73 | 0.61±0.02 |
| Water stress | 29.93±0.06* | 3.14±0.27* | 4.69±0.49 | 0.63±0.02 |
| $LSD_{0.05}$ | 6.22 | 0.75 | 1.87 | 0.06 |

$NO_3^-$ and $NH_4^+$ were expressed as mg $g^{-1}$ DW; Nitrate reductase (NR) was expressed as mM $NO_2^-$ $h^{-1}$ mg $prot^{-1}$; Nitrite reductase (NiR) was expressed as mM $NO_2^-$ $h^{-1}$mg $prot^{-1}$.
Asterisk (*) indicates significant difference with controls groups (well-watered).

The activity of enzymes that complete the cycle of photorespiration, GO, GGAT and HPR, showed a general increased under water stress conditions in ungrafted Zarina, *ZarxZar* and *JosxZar* (Table 2). However in cv. Josefina, its self-graft and *ZarxJos*, a general decreased were observed with respect to well-watered (Table 2). With respect to the different forms of pyridine dinucleotides, our results showed a decrease in NADH in Josefina ungrafted, *JosxJos* and *ZarxJos* under water stress (Table 3), while in *JosxZar* presented an increase of 32% in the $NADP^+$ concentration under water stress with respect well-watered

plants. Zarina ungrafted and *JosxZar* showed an increase in $NADP^+$ under water stress (Table 3). In NADH/NAD ratio, only *JosxZar* showed an significant increase of 43% under water stress, while in Josefina ungrafted, its self-graft and *ZarxJos* declined with respect control conditions (Table 3).

**Table 2:** Influence of moderate water stress on some photorespiration enzymes in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | GO | GGAT | HR |
|---|---|---|---|
| Zar ungrafted | | | |
| Well-watered | 1.96±0.12 | 0.20±0.07 | 2.45±0.11 |
| Water stress | 4.59±0.75* | 0.32±0.08* | 3.26±0.22* |
| $LSD_{0.05}$ | 1.62 | 0.10 | 0.43 |
| *ZarxZar* | | | |
| Well-watered | 1.28±0.22 | 0.29±0.04 | 0.63±0.16 |
| Water stress | 3.79±0.27* | 0.59±0.15* | 0.98±0.05* |
| $LSD_{0.05}$ | 0.74 | 0.14 | 0.28 |
| Jos ungrafted | | | |
| Well-watered | 7.77±0.70 | 1.10±0.57 | 3.24±0.22 |
| Water stress | 2.52±0.03* | 0.17±0.03* | 0.68±0.01* |
| $LSD_{0.05}$ | 1.48 | 0.22 | 0.46 |
| *JosxJos* | | | |
| Well-watered | 7.68±0.97 | 0.37±0.09 | 1.98±0.44 |
| Water stress | 6.13±0.36 | 0.06±0.01* | 0.13±0.01* |
| $LSD_{0.05}$ | 2.21 | 0.19 | 0.94 |
| *JosxZar* | | | |
| Well-watered | 1.67±0.17 | 0.18±0.04 | 1.72±0.06 |
| Water stress | 6.71±0.40* | 0.59±0.19* | 1.68±0.17 |
| $LSD_{0.05}$ | 0.93 | 0.11 | 0.39 |
| *ZarxJos* | | | |
| Well-watered | 4.52±0.40 | 0.64±0.12 | 3.00±0.26 |
| Water stress | 0.93±0.12* | 0.14±0.03* | 2.95±0.14 |
| $LSD_{0.05}$ | 0.89 | 0.26 | 0.64 |

Glycolate oxidase (GO), glutamate: glyioxylate aminotransferase (GGAT) and hydroxypyruvate reductase (HR) activities were expressed as $\Delta A\ h^{-1}$ mg $prot^{-1}$.
Asterisk (*) indicates significant difference with controls groups (well-watered).

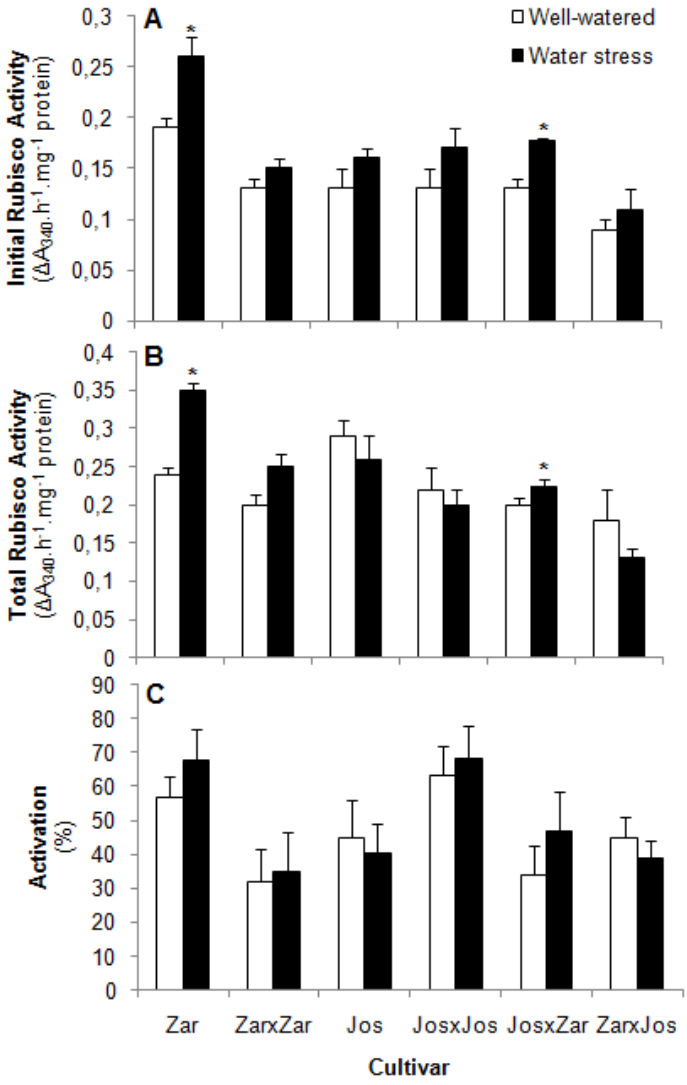


**Figure 3.** Response of Rubisco activity in ungrafted, grafted and self-grafted tomato plants well-watered and subjected to moderate water stress. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

*Incorporation of $NH_4^+$ and assimilation products*

The enzymes of the GS/GOGAT cycle were increased under water stress conditions in Zarina ungrafted and in reciprocal grafting (*JosxZar* and *ZarxJos*) (Table 4). However, in cv. Josefina not significant differences was observed, while self-grafting showed a decrease in GS and GOGAT activities under water stress conditions (Table 4).

**Table 3:** Influence of moderate water stress on pyridine dinucleotides concentration in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | $NAD^+$ | NADH | $NADP^+$ | NADPH | NADH/NAD |
|---|---|---|---|---|---|
| Zar ungrafted | | | | | |
| Well-watered | 2.22±0.12 | 3.54±0.25 | 1.62±0.08 | 2.24±0.15 | 1.57±0.09 |
| Water stress | 1.95±0.11 | 4.01±0.26 | 2.32±0.10* | 2.62±0.11 | 2.01±0.14 |
| $LSD_{0.05}$ | 0.32 | 0.52 | 0.24 | 0.54 | 0.55 |
| *ZarxZar* | | | | | |
| Well-watered | 2.00±0.12 | 2.44±0.14 | 1.86±0.18 | 1.85±0.12 | 1.26±0.04 |
| Water stress | 1.85±0.11 | 2.01±0.05 | 2.06±0.17 | 1.84±0.06 | 1.11±0.02 |
| $LSD_{0.05}$ | 0.42 | 0.62 | 0.43 | 0.48 | 0.36 |
| Jos ungrafted | | | | | |
| Well-watered | 0.79±0.11 | 2.49±0.15 | 2.54±0.21 | 1.98±0.11 | 3.14±0.19 |
| Water stress | 1.58±0.14* | 1.21±0.13* | 2.15±0.31 | 2.15±0.22 | 0.89±0.12* |
| $LSD_{0.05}$ | 0.58 | 0.45 | 0.55 | 0.46 | 0.41 |
| *JosxJos* | | | | | |
| Well-watered | 1.01±0.14 | 2.65±0.09 | 2.16±0.15 | 1.78±0.08 | 2.64±0.14 |
| Water stress | 1.68±0.11* | 1.59±0.10* | 2.11±0.10 | 1.98±0.15 | 0.99±0.08* |
| $LSD_{0.05}$ | 0.34 | 0.54 | 0.26 | 0.35 | 0.26 |
| *JosxZar* | | | | | |
| Well-watered | 1.98±0.08 | 3.01±0.11 | 2.15±0.14 | 1.86±0.10 | 1.50±0.09 |
| Water stress | 1.89±0.05 | 3.98±0.12* | 2.62±0.20* | 1.97±0.20 | 2.15±0.11* |
| $LSD_{0.05}$ | 0.25 | 0.61 | 0.40 | 0.26 | 0.28 |
| *ZarxJos* | | | | | |
| Well-watered | 1.95±0.14 | 2.54±0.14 | 1.99±0.11 | 1.97±0.11 | 1.34±0.15 |
| Water stress | 2.04±0.11 | 2.01±0.09* | 2.09±0.08 | 1.98±0.09 | 0.97±0.04* |
| $LSD_{0.05}$ | 0.24 | 0.34 | 0.22 | 0.29 | 0.24 |

$NAD(P)^+$ and NAD(P)H were expresed as $\mu M\ g^{-1}DW$.
Asterisk (*) indicates significant difference with controls groups (well-watered).

With regard AAT activity, cv. Zarina, *JosxZar* and *ZarxJos* showed an increase of 47, 22 and 67%, respectively. Besides, not significant differences were observed in other graft combination (Table 4). Finally, by contrast, the GDH activity increased significantly only in cv. Josefina, *JosxJos* and *ZarxZar*, while in the rest not differences were observed (Figure 4).

**Table 4:** Influence of moderate water stress on enzymes responsible for $NH_4^+$ assimilation in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | GS | GOGAT | AAT |
|---|---|---|---|
| Zar ungrafted | | | |
| Well-watered | 0.17±0.01 | 1.34±0.26 | 1.87±0.40 |
| Water stress | 0.25±0.01* | 2.18±0.35* | 2.75±0.33* |
| $LSD_{0.05}$ | 0.03 | 0.62 | 0.80 |
| *ZarxZar* | | | |
| Well-watered | 0.22±0.02 | 1.13±0.29 | 7.52±1.46 |
| Water stress | 0.17±0.01* | 0.37±0.04* | 5.17±0.44 |
| $LSD_{0.05}$ | 0.04 | 0.62 | 2.24 |
| Jos ungrafted | | | |
| Well-watered | 0.13±0.01 | 0.89±0.11 | 5.74±0.37 |
| Water stress | 0.18±0.02 | 0.85±0.17 | 5.92±0.56 |
| $LSD_{0.05}$ | 0.05 | 0.14 | 1.42 |
| *JosxJos* | | | |
| Well-watered | 0.15±0.01 | 1.32±0.41 | 3.62±1.10 |
| Water stress | 0.11±0.01* | 0.40±0.07* | 0.89±0.05 |
| $LSD_{0.05}$ | 0.02 | 0.88 | 2.34 |
| *JosxZar* | | | |
| Well-watered | 0.18±0.01 | 1.18±0.08 | 4.19±0.61 |
| Water stress | 0.26±0.02* | 1.84±0.52* | 5.11±0.53* |
| $LSD_{0.05}$ | 0.05 | 0.12 | 0.71 |
| *ZarxJos* | | | |
| Well-watered | 0.14±0.01 | 0.91±0.28 | 3.43±0.53 |
| Water stress | 0.24±0.01* | 1.61±0.21* | 5.73±0.82* |
| $LSD_{0.05}$ | 0.02 | 0.55 | 2.08 |

Glutamine sintetase (GS), glutamate sintase (GOGAT) and aspartate aminotransferase (AAT) activities were expressed as $\Delta A\ h^{-1}$ mg $prot^{-1}$.
Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05). Asterisk (*) indicates significant difference with controls groups (well-watered).

Reduced N was increased in Zarina ungrafted and *ZarxZar* under water stress conditions (Figure 5A). However, a significant decrease was observed in cv. Josefina and its self-graft, while reciprocal grafting not showed significant difference with respect well-watered conditions (Figure 5A). With regard total N, only cv. Zarina and *ZarxJos* showed an increase under stress conditions (Figure 5B). The soluble amino acids not significant differences were observed in diferent combination grafting (Figure 5C). Also, the soluble proteins increased in the cv. Zarina and reciprocal grafting, while in the rest the values were not affected or fell after water stress (Figure 5D).

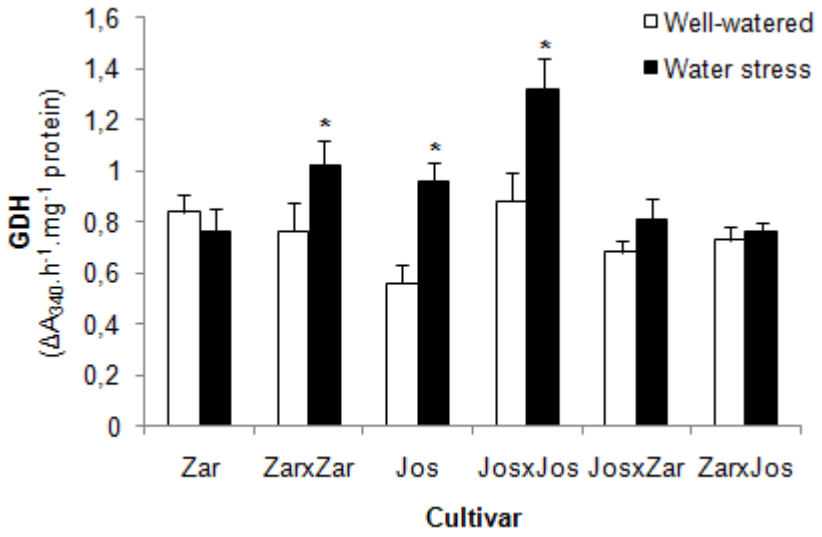


**Figure 4.** Response of glutamate deshydrogenase (GDH) in ungrafted, grafted and self-grafted tomato plants well-watered and subjected to moderate water stress. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

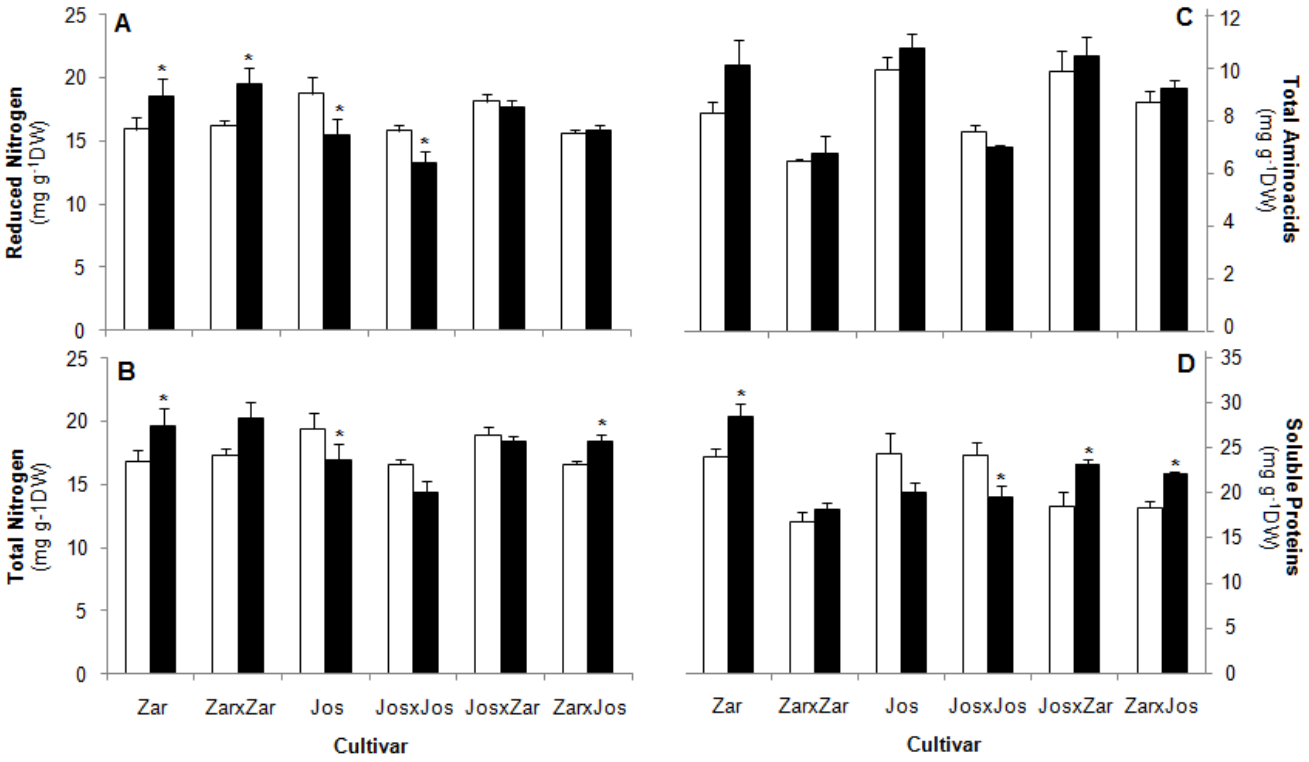


**Figure 5.** Response in foliar concentration of organic (A), total N (B), total aminoacid (C) and soluble proteins (D) in ungrafted, grafted and self-grafted tomato plants well-watered and subjected to moderate water stress. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

## Discussion

Generally, drought can depressed nutrient uptake by the root and transport to the shoot due to a restricted transpiration rate (Hsiao 1973; Kramer *&* Boyer 1995). However, water and nutrient uptake could be increased in grafted plants as a result of the enhancement of vigour by the rootstock root system and its effects on plant yield (Ruiz *et al.* 1997). Indeed, in our previous work we studied

the effects of graft in uptake fluxes, and found that the use of cv. Zarina as rootstock (*ZarxJos*) improves the $NO_3^-$ uptake flux under stress conditions (Sánchez-Rodríguez *et al.* 2011b). According to these data, our result shown an increase in $NO_3^-$ concentration and NR activity under water stress only in cv. Zarina ungrafted and *ZarxJos* (Zarina used as rootstock) (Table 1). The characteristics of the rootstocks could result in increased absorption, upward transport and accumulation of $NO_3^-$ in the scion, thereby stimulating NR and $NO_3^-$ assimilation. Similar results were obtained by Ruiz *&* Romero (1999) in melon plants, NR activity and $NO_3^-$ accumulation were conditioned significantly by the scion-rootstock interaction and by rootstock genotype, whereas the scion genotype did not show any such effect. Many researchers have shown a directly proportional relationship between $NO_3^-$ and yield (Kim *et al.* 2011; Li *&* Lascano 2011; Naudin *et al.* 2010). Also, Ruiz *et al.* (1997, 2001, 2006) have shown the essential role $NO_3^-$ assimilation in the increase in yield. Our result showed that cv. Zarina, *JosxZar* and *ZarxJos* presented greater biomass and relative growth rate (RGR) associated with high leaf relative water content (LRWC) under water deficit conditions, indicating that these cultivars are more tolerant to this growth situation (Sánchez-Rodríguez *et al.* 2011c).

It has been estimated that the production of $NH_4^+$ by photorespiration is much greater than the primary assimilation of $NH_4^+$ resulting from nitrate reduction (Wingler *et al.* 2000). Our results shown that only cv. Zarina, its selfgraft and

*JosxZar* (cv. Zarina like scion) presented an increased in initial and total Rubisco activity under water stress (Figure 3) and in the activities of the enzymes GO, GGAT and HPR (Table 3). These results are agree with Ferreira-Silva (2010), where observed that the higher stability shown by Rubisco in cashew BRS/BRS grafted plants could indicate that this combination can be more resistant under salinity. Moreover, it was found that the grafted bitter melon seedlings possessed a higher Rubisco activity than ungrafted seedlings under flooding stress (Liao *&* Lin 1996). On the other hand, photorespiration serves as an important redox mechanism that increases the cytosolic NADH/NAD ratio (Backhausen *et al.* 1994). In *JosxZar*, which increases the photorespiration, we observed an increase in NADH concentration and the ratio NADH/NAD, while in Zarina ungrafted and *ZarxZar* not significant difference were observed (Table 3). Because the first step of $NO_3^-$ assimilation occurs in the cytosol and uses NADH, this may explain why we observed $NO_3^-$ assimilation to be greater when photorespiration was highest in cv. Zarina ungrafted and its selfgraft. However, in *JosxZar,* photorespiration is high although not enhanced nitrate uptake by the root and $NO_3^-$ assimilation is therefore lower.

Besides, the reassimilation of $NH_4^+$ produced by the photorespiratory nitrogen cycle is essential for maintaining nitrogen status (Wingler *et al.* 2000). In higher plants, $NH_4^+$ is mainly assimilated through the concerted action of GS and

GOGAT. Alternatively, GDH can also catalyze $NH_4^+$ incorporation into glutamate by reductive amination of 2-oxoglutarate (Cammaerts *&* Jacobs 1985). Regarding water stress, several authors have shown that the decline in GS activity is correlated this stress (Robredo *et al.* 2011), and here, drought stress provoked a marked decrease in GS activity in cv. Josefina and self-grafting (Table 4). The GS/GOGAT cycle only was increase under stress conditions in cv. Zarina ungrafted, *JosxZar* and *ZarxJos* (Table 4). In *ZarxJos* increase of ammonium assimilation could result from increased $NO_3^-$ assimilation; whereas in *JosxZar*, the $NH_4^+$ in water-stressed plant might mainly result from increased photorespiration. These results are agree with Masclaux-Daubresse *et al.* (2006) in tobacco plants, and provide strong evidence that the GS/GOGAT cycle is the primary route of ammonium assimilation and the GDH plays a minor role. In fact, GDH activity only showed an increased in Josefina ungrafted, its self-graft and *ZarxZar* under water stress (Figure 4). In this sense, it has been demonstrated that $NH_4^+$ might be a signal responsible for the induction of the GDH activity (Ferrario-Mery *et al.* 2002). Thus, the increased $NH_4^+$ under water stress observed in Josefina ungrafted, its self-graft and *ZarxZar* (Table 1) might be responsible for the higher GDH activity (Figure 4), which has been previously shown in wheat seedlings exposed to salinity (Wang *et al.*, 2007). Moreover, the $NH_4^+$ is toxic to plants and this might result in decreased biomass in these cultivars (Sánchez-Rodríguez *et al.* 2011c). Besides, it has been demonstrated that the combined action of GS and GOGAT is the principal pathway for assimilating ammonia, and the amination activity of

GDH functions only when the GS/GOGAT cycle pathway is inhibited under stress conditions (Oaks 1995) such as salinity (Skopelitis *et al.* 2006) or drought (Mena-Petite *et al.*,2006). Finally, the glutamate and glutamine generated in the GS/GOGAT cycle are allocated to the synthesis of aspartate and asparagine, produced in the reactions catalyzed by AAT and asparagine synthetase (Hodges 2002). Our results showed an increase in AAT activity under water stress in cv. Zarina, *JosxZar* and *ZarxJos* (Table 4). This could be related to the increase in the GS/GOGAT cycle in these cultivar and grafting (Table 4).

The result of the incorporation of $NH_4^+$ can be quantified by the analysis of reduced N, which is generally the product of N assimilation and is formed mainly by amino acids and proteins. The total N, the result of the sum of the total reduced N and the $NO_3^-$, is considered a critical parameter to determine the nutritional state of plants (Ruiz *&* Romero 1999). The greater efficiency in $NO_3^-$ reduction and $NH_4^+$ reassimilation in Zarina ungrafted, *JosxZar* and *ZarxJos* plants was confirmed by the results for protein and total N, which were higher under water stress (Figure 5). Ruiz *et al.* (2006) showed similar results in grafted tobacco plants, where observed higher amino acids, protein and total N in all grafted plants with respect to non-grafted plants. These results confirm those previously found by other authors, where they observe that an increase in the NR activity leads to a corresponding increase in the potential for $NO_3^-$

reduction and confers a greater capacity for general amino acid synthesis, protein synthesis or total N assimilation (Singh *&* Usha 2003).

The increase on N metabolism displayed by the *ZarxJos* and *JosxZar* combinations associated with other physiological factors, such as maintenance of LRWC in *ZarxJos* or higher photorespiration in *JosxZar*, strongly suggest that these combinations are the most capable at coping with drought moderate stress. In addition, the results demonstrate that interactions between the rootstock and scion may exert a strong effect on the N metabolism responses of tomato plants under water stress. In this study, when Zarina (tolerant cultivar) was employment as rootstock grafted with Josefina (*ZarxJos*), these plants showed an improved N uptake and $NO_3^-$ assimilation (Table 1). On the other hand, when Zarina was employment as scion (*JosxZar*), this grafted plants showed an increase in photorespiration cycle (Table 2), which may generate amino acids and proteins (Figure 5C and 5D). Our study offers promising results that could improve the understanding of some physiological mechanism involved with scion and rootstock interaction under water stress conditions. However, further studies are needed to better elucidate some biochemical, molecular and genetic traits that might exert control on N metabolism associated with drought resistance in grafted plants. Such these traits could be utilized in plant breeding programs by means of the selection of improved genotypes of rootstock and scion using molecular marker assisted techniques.

In conclusion, grafting that improve $NO_3^-$ photoassimilation or improve photorespiration. Consequently, complex interaction between photorespiratory metabolism and $NO_3^-$ assimilation may be more important than previously recognized in plant leaves.

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# *Capítulo 7*

## *How does grafting affect to ionome on cherry tomato plants under water stress?*

***Scientia Horticulturae (2012) (Under review)***

**ABSTRACT**

The response of grafted vegetables to stress conditions owing to the nutrient status may be different than that of self-rooted plants, depending mainly on the rootstock genotype. The aim of the present work is to determine the response of reciprocal grafts made between one tolerant cultivar, Zarina, and a more sensitive cultivar, Josefina, to moderate water stress, examining uptake and concentration of nutrient. The tomato cultivars Zarina (drought-tolerant) and Josefina (drought-sensible) were reciprocal grafted and selfgrafted, and ungrafted were used as control. The total content and uptake fluxes of macro- and micronutrients were determined in leaves. Our results show that the use of drought-tolerant cv. Zarina like rootstocks (*ZarxJos*) showed improve ionome, with increases in nitrogen (N), phosphorous (P) and potassium (K) concentration and uptake fluxes, and an increase in iron (Fe) and copper (Cu) concentration and uptake under water stress. Besides, the vigorous root system of rootstocks is often capable of absorbing plant nutrients more efficently than scion root, and we have showed that cv. Zarina and *ZarxJos* develop a better radicular system. This result confirms the hypothesis that grafted plants on vigorous rootstocks can improve mineral nutrition and nutrient uptake with respect to ungrafted plants, especially under water stress conditions.

**Keywords:** mineral nutrition, ionome, grafting, uptake fluxes, tomato.

**Abbreviations:** B, boron; Ca, calcium; Cl, chlorine; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; N, nitrogen; P, phosphorous; S, sulfur; Zn, zinc.

## INTRODUCTION

The study of the mineral composition of a plant and the changes in this composition in response to physiological and environmental stimuli, the plant's developmental state, and genetic modifications, has been recently defined as ionome (Salt *et al.* 2008). Therefore, the ionome can provide information on the functional state of an organism under different growth conditions. Due to the limited availability of arable land, the high demand for off-season vegetables and the intensive farming practices with limited crop rotations, vegetables are often cultivated under unfavourable conditions which induce stress (Savvas *et al.* 2010). These conditions include dry environments, because water is an economically scarce resource in many areas of the world, especially in arid and semiarid regions such as the Mediterranean basin. Water stress causes various physiological disorders leading to severe crop loss. One environmentally-friendly technique for avoiding or reducing losses in production caused by abiotic stress conditions in high-yielding genotypes belonging to Solanaceae and Cucurbitaceae families would be to graft them onto rootstocks capable of reducting the negative effect of external stress on the shoot (Savvas *et al.* 2010). The cultivated area of grafted Solanaceae, including a number of

important annual fruit-crop plants such as tomato, eggplant and pepper, has increased in recent years in order to obtain resistance to soil-borne diseases (Bletsos *et al.* 2003; Davis *et al.* 2008), tolerance against abiotic stresses such as salinity, wet soils and high and low temperatures (Abdelmageed *&* Gruda, 2009; Ahn *et al.* 1999; Estañ *et al.* 2005; Venema *et al.* 2008; Rivero *et al.* 2003a,b) and to improve fruit quality (Colla *et al.* 2006; Fernández-García *et al.* 2004a,b).

Although the water absorption and nutrient uptake are independent processes in the root, the need for available water for growth and nutrient transport makes them intimately related (Viets 1972). Generally, drought reduces not only nutrient uptake by the root but also nutrient transport from the root to the shoot due to a restricted transpiration rate, depressed active transport, and reduced membrane permeability. The overall result is the uptake power of the plant is disminished (Kramer *&* Boyer 1995). During formation of the graft union, the vascular connection in the rootstock-scion interface may determine water and nutrient translocation, affecting other physiological traits (Oda *et al.* 2005; Johkan *et al.* 2009). However, water and nutrient uptake could be increased in grafted plants as result of the enhancement of vigour by the rootstock root system and its effects on plant yield (Ruiz *et al.* 1997). The influence of the rootstock on the mineral content in aerial plant parts was attributed to physical characteristics of the root system, such as lateral and vertical development, which resulted in enhanced uptake of water and minerals, this being one of the

main motives for the widespread use of grafted rootstocks (Heo 1991). However, in grafted fruit trees, no effect of the rootstock on the leaf mineral content was found and, in this case, more influence of the scion on leaf nutrient content was observed (Seki *et al.* 2008). Tagliavani *et al.* (1993) suggested that vigour of both the scion and root system had an important role in the uptake and translocation of nutrients in grafted fruit trees, while, in cucumber grafted plants changes of rootstock had an influence on the leaf content of certain essential minerals under salinity (Huang *et al.* 2010). Therefore, the contents of macro- and micronutrients are affected by the rootstock and scion characteristics, but, depending on the element and environmental conditions, the effect of the rootstock and/or scion may change.

Given that tomato is one of the most important crops worldwide, and that its production is concentrated in semiarid regions, where water stress is frequent, it is of great interest to ascertain whether grafting is a valid strategy to improve water-stress tolerance and its ionome in this plant. In preliminary studies, we have observed that the cv. Zarina shows better water-stress tolerance and better mineral content than cv. Josefina, which is more drought sensitive (Sánchez-Rodríguez *et al.* 2010a,b). In this light, the aim of the present work is to determine the response of reciprocal grafts made between one tolerant cultivar, Zarina, and a more sensitive cultivar, Josefina, to moderate water stress, examining uptake and concentration of nutrient.

## MATERIAL AND METHODS

### *Plant material and growth conditions*

Two tomato (*Solanum lycopersicum*) cultivars, Zarina and Josefina, were used as scion and rootstock (Figure 1). The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm x 3 cm x 10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Grafting was performed when seedlings has developed 3-4 true leaves. In the vermiculite trays used for germination, the seedlings were cut over the cotyledons, using the shoot as scion and the reamining plant part as rootstock. Grafts were made inmediately after cutting the plants and grafting clips were used to adhere the graft union. Self-grafted plants were included as controls. After grafting, seedlings were covered with a transparent plastic lid to maintain a high humidity level and to facilitate graft formation and were left in the shade for 24 h. The plastic was opened slightly every day to allow reduction in relative humidity and it was removed 6 days after grafting. Afterwards, ungrafted and grafted plants were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with relative humidity of 50±10%, at 25ºC/15ºC (day/night), and a 16h/8h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 µmol $m^{-2}s^{-1}$ (measured with an SB quantum 190 sensor, LI-COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter,

and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. Throughout the experiment, the plants were grown in a complete nutrient solution (Sánchez-Rodríguez et al., 2010a). The water-stress treatments began 45 days after germination and maintained 22 days. The control treatment received 100% field capacity (FC) irrigation, whereas moderate water stress corresponded to 50 % field capacity. The experimental design was a randomized complete block with 12 treatments (Zarina ungrafted, Josefina ungrafted, Zarina self-grafted, Josefina self-grafted, *JosxZar* and *ZarxJos* well-watered 100% FC and water stress 50%) (Figure 1) arranged in individual pots with six plants per treatment (one plant per pot) and three replications each.

*Root biomass and metabolic efficiency*

All plants were at the late vegetative stage when harvested. Leaves fully expanded (excluding petioles) and roots were harvested, frozen immediately in liquid $N_2$, and kept at -80ºC until used. The leaves and roots were dried in a forced-air oven at 70ºC for 24 h, and the dry weight (DW) was recorded as grams per plant.

Metabolic activity in the root tips was measured by the reduction in an electron acceptor 2,3,5-triphenyltetrazolium chloride (TTC) following the method of Steponkus (1971).

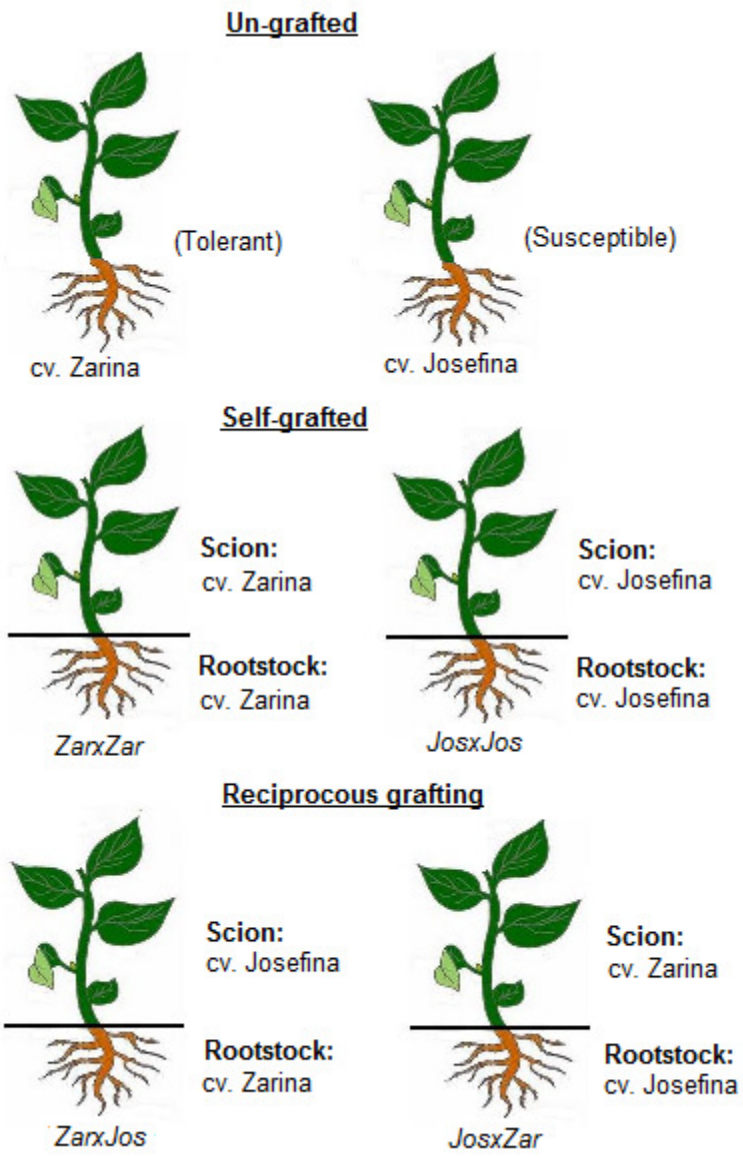


**Figure 1.** Outline of the grafting design.

*Determination of mineral nutrients*

The nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), boron (B), and copper (Cu), were

mineralized by wet digestion, following Wolf (1982). For this, 0.2 g of dry roots and leaves were ground and mineralized with $H_2SO_4$ 12 N and $H_2O_2$, 30% P free, at a temperature of 275 to 300ºC. From the resulting mineralization, and after the addition of 20 mL of deionized $H_2O$, the mineral nutrients were determined as described above.

The reduced N concentration was determined by colorimetry based on the Berthelot reaction, with slight modifications (Krom 1980).

The total P concentration was determined using the colorimetric nitrovanadomolybdate method of Hogue et al., 1970 while the total K concentration was analyzed by flame photometry (Lachica *et al.* 1973).

The total Mg and Ca concentration were quantified by atomic absorption spectrophotometry (Hocking and Pate 1977), as were the micronutrients, Fe, Mn, and Zn. The total B concentration was determined by the colorimetric method of azomethine-H (Wolf 1974). Reduced S was extracted by mineralization with nitric/perchloric acid. For this, a quantity of 0.2 g of cherry tomato ground dry was digested with a mixture of $HNO_3/HClO_4$ (v/v) and $H_2O_2$ at 30%. The reduced S was determined on an aliquot of the mineralization using $BaSO_4$ in suspension by means of a surfactant agent such as gum arabic,

all this against a standard curve of $K_2SO_4$ and a turbidmetric reading at 435 nm (Novozamsky *&* Vaneck 1977).

The Cl, $NO_3^-$, $PO_4^{3-}$, $SO_4^{2-}$, soluble K, Mg and Ca concentrations in roots and leaves were determined from an aqueous extraction following the method of Cataldo *et al.* (1975) with slight modifications. The Cl concentration was determined by the method of Diatloff *&* Rengel (2001), based on the displacement of biocyanate by chloride, which in the presence of $Fe^{3+}$ forms the highly colored complex ferric thiocyanate. The determination of $NO_3^-$ was based on a colorimetric reaction formed by the bonding with salicylic acid in a basic medium (Cataldo *et al*. 1975). The total N concentration was determined as the sum of the reduced N and $NO_3^-$ concentrations. The $PO_4^{3-}$ was determined following the method of Hogue *et al.* (1970). The $SO_4^{2-}$ was determined following the method of Novozamsky *&* Vaneck (1977). The total S corresponded to the sum of the concentrations of reduced S and $SO_4^{2-}$. Soluble Mg and Ca were quantified by atomic absorption spectrophotometry (Hocking *&* Pate 1977), while the soluble K concentration was analyzed by flame photometry (Lachica *et al.* 1973).

The soluble Fe, Mn, Cu and Zn concentrations were determined from an HCl 1M extraction following the method of Cataldo *et al.* (1975) with slight

modifications. These nutrients were quantified by atomic absorption spectrophotometry (Hocking *&* Pate 1977)

*Determination of uptake fluxes*

Over the period under study, determination of nutrients uptake fluxes were calculated from the RGR, the dry weight (DW), the nutrient total concentrations, and soluble nutrient concentration contents of leaves (l) and roots (r) as folllows (Kruse *et al*. 2007):

$$(\text{Total Nutrient})_r = RGR*DW_r*[\text{Total Nutrient}]_r \quad (1)$$

$$(\text{Total Nutrient})_l = RGR*DW_l*[\text{Nutrient}]_l \quad (2)$$

$$(\text{Reduced Nutrient})_r = RGR*DW_r*[\text{Total nutrient-Soluble nutrient}]_r \quad (3)$$

$$(\text{Reduced Nutrient})_l = RGR*DW_l*[\text{ Total nutrient-Soluble nutrient}]_l \quad (4)$$

The following fluxes were determined from experimental data:

$$J^{Upt}\text{Nutrient} = (\text{Total Nutrient})_r+(\text{ Total Nutrient})_l+(\text{Reduced Nutrient})_r+(\text{Reduced Nutrient})_l \quad (5)$$

*Statistical analysis*

The data compiled were subjected to a simple ANOVA at 95% confidence. A two-tail ANOVA was applied to ascertain wheter the cultivar and treatment applied significantly affected the results, and the means were compared by Fisher´s least-significant differences (LSD).

## RESULTS

*Root biomass and metabolic efficiency*

The cv. Zarina augmented its radicular biomass to 28% under stress conditions. In cv. Josefina the root biomass disminished with the water-deficit treatment, while *JosxJos* was not visibly affected. The reciprocal grafts behaved different way, with radicular biomass in *JosxZar* was not affected under stress, and *ZarxJos* increasing 24% (Figure 2).

In the root metabolic efficiency, cv. Zarina and its self-graft increased to 63 and 74% under stress conditions. On the contrary, in cv. Josefina as well as *JosxJos*, the radicular efficiency declined under water stress (Figure 2). In the reciprocal grafts, *JosxZar* decreased to 20% with respect control conditions, whereas *ZarxJos* showed a rise under water stress (Figure 2).

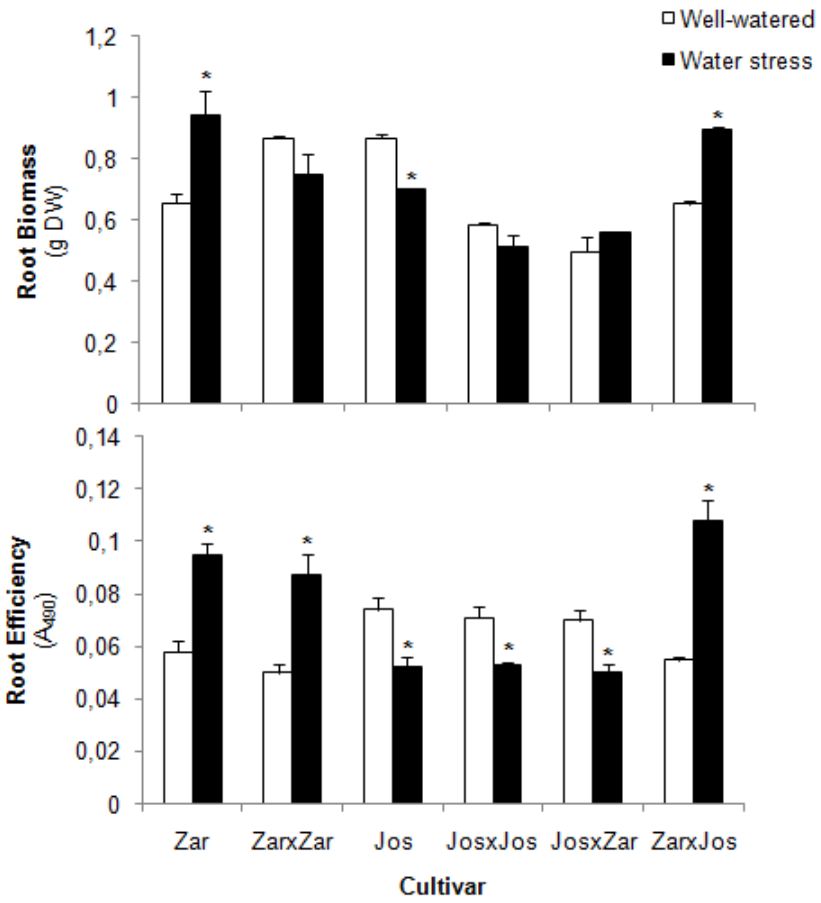


**Figure 2.** Influence of moderate water stress on root biomass and root efficiency in ungrafted, grafted and self-grafted tomato plants. Columns are mean ± S.E. (*n = 9*) and differences between means were compared by Fisher´s least-significant difference test (LSD; P = 0.05). Asterisk (*) indicates significant difference with control groups.

*Mineral Nutrient Accumulation in Leaves*

Mineral macronutrients concentrations (N, P, S, Ca, Mg, and K) in cherry tomato leaves for the reciprocal grafting studied are presented in Table 1. Both cv. Zarina ungrafted and its self-grafting under water stress registered a 16 and

20% increase with respect to well-watered conditions in N concentration, respectively. On the contrary, cv. Josefina ungrafted and *JosxJos* lowered the N concentration under water stress. In the reciprocal grafting, only *ZarxJos* presented an increase of 27% in N concentration under stress conditions. Identical results were found in P concentrations. Meanwhile, water deficit generally lowered (cv. Zarina, *ZarxZar*, *JosxJos* and *ZarxJos*) or maintained (cv. Josefina and *JosxZar*) the S concentration. No significant increases were detected in the Ca concentration under stress in comparison to well-watered plants. The Mg concentration was maintained in cv. Zarina, cv. Josefina and their self-grafting; howeve under water stressr, in *JosxZar* lowered a 27% and in *ZarxJos* increased a 41%, wich respect to well-watered conditions. Zarina and *ZarxJos* were the only plants to present a higher K concentration under water deficit, whereas the rest of the cultivars or grafted plants showed no differences or even concentrations declined in these elements under water-stress conditions (Table 1).

The concentrations of mineral micronutrients (Fe, Cu, Mn, Zn, Cl, and B) in leaves are reflected in Table 2. For Fe concentration, only cv. Zarina, its self-grafting and *ZarxJos* showed an increase of 13, 69 and 81% in water stress with respect to well-waterd, respectively. Similar results were found to Cu, where cv. Zarina and *ZarxJos* increased its concentration under water stress. Generally, Mn, Zn nor B concentrations lowered or maintained under water

stress in all plants, especially in c. Josefina ungrafted. However, in the case of Cl, Zarina was the only cultivar to present a higher concentration under water deficit, whereas *JosxJos* and *JosxZar* showed a decline (Table 2).

**Table 1:** Influence of moderate water stress on foliar concentration of macronutrients (mg $g^{-1}$ DW) in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | N | P | S | Ca | Mg | K |
|---|---|---|---|---|---|---|
| Zar ungrafted | | | | | | |
| Well-watered | 15.9±0.9 | 3.3±0.3 | 31.7±0.9 | 13.1±0.2 | 2.3±0.0 | 20.7±0.2 |
| Water stress | 18.5±1.4*[a] | 5.1±0.2* | 21.8±2.8* | 11.9±0.2 | 2.4±0.1 | 26.6±1.0* |
| $LSD_{0.05}$ | 2.6 | 0.9 | 8.19 | 1.9 | 0.2 | 2.9 |
| *ZarxZar* | | | | | | |
| Well-watered | 16.2±0.3 | 2.7±0.3 | 30.3±0.7 | 16.7±2.4 | 1.8±0.1 | 28.4±5.4 |
| Water stress | 19.4±1.2* | 4.4±0.3* | 24.3±2.2* | 13.2±0.7 | 2.1±0.1 | 27.9±2.2 |
| $LSD_{0.05}$ | 2.8 | 1.0 | 4.4 | 7.0 | 0.5 | 6.3 |
| Jos ungrafted | | | | | | |
| Well-watered | 18.7±1.2 | 5.4±2.8 | 28.4±0.6 | 13.4±0.9 | 2.3±0.0 | 21.8±1.9 |
| Water stress | 14.4±1.3* | 2.8±0.3* | 28.3±0.8 | 12.3±0.1 | 2.5±0.1 | 18.5±0.7 |
| $LSD_{0.05}$ | 2.9 | 1.1 | 3.1 | 2.6 | 0.4 | 5.7 |
| *JosxJos* | | | | | | |
| Well-watered | 15.8±0.4 | 4.1±0.1 | 33.1±0.8 | 13.9±1.4 | 2.0±0.0 | 23.7±0.8 |
| Water stress | 13.2±0.9* | 3.3±0.1* | 27.8±1.3* | 13.3±1.6 | 2.1±0.0 | 17.1±1.4* |
| $LSD_{0.05}$ | 2.2 | 0.5 | 4.4 | 2.1 | 0.1 | 4.5 |
| *JosxZar* | | | | | | |
| Well-watered | 18.1±0.6 | 4.2±0.1 | 19.0±3.1 | 10.7±0.1 | 2.9±0.1 | 30.3±0.4 |
| Water stress | 17.7±0.4 | 3.8±0.3 | 29.1±2.1 | 11.2±0.3 | 2.1±0.2* | 23.5±0.6* |
| $LSD_{0.05}$ | 1.6 | 0.6 | 10.5 | 1.0 | 0.4 | 2.1 |
| *ZarxJos* | | | | | | |
| Well-watered | 15.6±0.3 | 3.9±0.4 | 33.1±0.8 | 16.1±0.9 | 1.7±0.0 | 11.4±0.5 |
| Water stress | 19.8±0.4* | 5.8±0.4* | 25.8±0.6* | 14.2±0.6 | 2.4±0.1* | 16.8±1.6* |
| $LSD_{0.05}$ | 1.1 | 1.3 | 2.9 | 3.1 | 0.2 | 2.7 |
| | | | Analysis of Variance | | | |
| Cultivars (C) | ***[b] | ** | *** | *** | *** | *** |
| Treatments (T) | ns | ns | ** | *** | ** | *** |
| CxT | * | * | *** | * | *** | *** |
| LSD | 1.82 | 1.75 | 1.65 | 1.06 | 0.10 | 1.94 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05).
[a] Asterisk (*) indicates significant difference with controls groups (well-watered)
[b] The levels of significance were represented by p>0.05: ns (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***)

**Table 2:** Influence of moderate water stress on foliar concentration of micronutrient (μg $g^{-1}$ DW) in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | Fe | Cu | Mn | Zn | B | Cl (mg $g^{-1}$ DW) |
|---|---|---|---|---|---|---|
| Zar ungrafted | | | | | | |
| Well-watered | 271±28.7 | 98±11.1 | 597±73.7 | 35.0±5.9 | 132±15.6 | 20.9±0.3 |
| Water stress | 306±77.9 *[a] | 134±2.4* | 654±61.3 | 32.5±1.4 | 149±3.1 | 22.7±0.4* |
| $LSD_{0.05}$ | 25.6 | 31.5 | 166.1 | 7.1 | 33.8 | 1.1 |
| *ZarxZar* | | | | | | |
| Well-watered | 202±9.0 | 100±12.9 | 789±81.7 | 24.6±2.6 | 140±6.8 | 21.3±0.3 |
| Water stress | 341±41.5 * | 125±9.1 | 831±75.5 | 18.9±0.5 | 142±6.8 | 20.5±0.2 |
| $LSD_{0.05}$ | 78.0 | 44.1 | 76.1 | 7.5 | 20.5 | 0.9 |
| Jos ungrafted | | | | | | |
| Well-watered | 281±21.6 | 89±7.2 | 579±88.4 | 15.7±3.4 | 155±3.2 | 17.4±0.3 |
| Water stress | 230±21.0 | 66±7.6* | 380±19.1* | 8.9±2.2* | 105±6.4* | 17.2±0.1 |
| $LSD_{0.05}$ | 83.8 | 15.3 | 68.2 | 1.2 | 15.2 | 0.8 |
| *JosxJos* | | | | | | |
| Well-watered | 191±19.9 | 92±15.4 | 645±79.8 | 16.8±2.0 | 136±7.8 | 18.3±0.1 |
| Water stress | 248±15.5 | 117±22.2 | 634±56.1 | 7.0±3.1* | 154±5.5 | 16.2±0.4* |
| $LSD_{0.05}$ | 70.2 | 25.2 | 36.6 | 1.2 | 20.3 | 0.8 |
| *JosxZar* | | | | | | |
| Well-watered | 236±7.1 | 92±11.4 | 592±61.7 | 50.6±0.8 | 156±2.3 | 16.3±0.1 |
| Water stress | 277±6.3 | 105±8.9 | 668±87.9 | 21.1±2.4* | 139±1.8* | 15.4±0.2* |
| $LSD_{0.05}$ | 86.4 | 40.3 | 98.9 | 7.2 | 6.4 | 0.7 |
| *ZarxJos* | | | | | | |
| Well-watered | 240±31.6 | 75±6.8 | 830±64.9 | 11.7±1.8 | 161±6.8 | 18.2±0.1 |
| Water stress | 435±62.1 * | 117±13.2* | 780±21.3* | 20.2±6.7 | 161±4.7 | 18.3±0.6 |
| $LSD_{0.05}$ | 53.6 | 41.4 | 68.6 | 9.3 | 17.7 | 1.4 |
| | | | Analysis of Variance | | | |
| Cultivars (C) | ***[b] | *** | *** | *** | *** | *** |
| Treatments(T) | ns | *** | ns | *** | ns | *** |
| CxT | *** | ns | ns | *** | *** | ** |
| LSD | 35.34 | 12.51 | 130.79 | 3.27 | 13.69 | 0.64 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05).
[a] Asterisk (*) indicates significant difference with controls groups (well-watered)
[b] The levels of significance were represented by p>0.05: ns (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***)

### *Uptake Fluxes in Mineral Nutrient*

The data for macronutrient uptake by the grafting tomato plants studied are presented in Table 3. Both in N and the P, uptake diminished in cv. Josefina

and its self-grafitng with respect to control, whereas in leaves of cv. Zarina, *ZarxZar* and *ZarxJos*, the transport increased under stress conditions. In the case of S and Ca, generally all cultivars and grafting registered a decline in the uptake fluxes (Table 3). Finally, no significant differences in uptake of Mg and K in cv. Zarina, its self-grafting, cv. Josefina and *ZarxJos* were found under stress conditions, while in the rest of the grafting values sharply fell (Table 3).

**Table 3:** Influence of moderate water stress on uptake fluxes of macronutrients (mg plant$^{-1}$day$^{-1}$) in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | N | P | S | Ca | Mg | K |
|---|---|---|---|---|---|---|
| Zar ungrafted | | | | | | |
| Well-watered | 140±8.2 | 95±8.6 | 105±11.7 | 24.3±2.3 | 11.1±1.6 | 48.8±5.2 |
| Water stress | 178±12.5*[a] | 126±14.3* | 40±1.6* | 12.3±1.0* | 14.7±0.3 | 37.2±1.5 |
| $LSD_{0.05}$ | 31.5 | 16.4 | 32.9 | 7.1 | 4.5 | 15.1 |
| *ZarxZar* | | | | | | |
| Well-watered | 223±48.1 | 105±19.6 | 144±17.4 | 40.7±2.9 | 8.5±0.6 | 73.8±7.3 |
| Water stress | 320±33.7* | 169±17.1* | 50±9.3* | 16.2±2.4* | 6.6±1.3 | 63.8±6.1 |
| $LSD_{0.05}$ | 63.1 | 52.4 | 54.9 | 10.6 | 2.2 | 15.8 |
| Jos ungrafted | | | | | | |
| Well-watered | 389±81.9 | 268±5.4 | 104±1.4 | 13.3±1.0 | 10.7±1.1 | 45.4±4.0 |
| Water stress | 177±11.3* | 86±6.1* | 96±3.5 | 11.1±0.5 | 12.8±0.3 | 36.2±0.6 |
| $LSD_{0.05}$ | 29.8 | 22.6 | 10.5 | 3.2 | 3.3 | 11.4 |
| *JosxJos* | | | | | | |
| Well-watered | 300±53.8 | 151±19.0 | 101±13.6 | 24.4±4.2 | 11.1±1.5 | 42.2±5.2 |
| Water stress | 148±6.2* | 69±0.2* | 47±0.7* | 12.2±1.1* | 4.4±0.2* | 18.5±1.0* |
| $LSD_{0.05}$ | 50.3 | 52.8 | 7.7 | 2.0 | 2.2 | 14.8 |
| *JosxZar* | | | | | | |
| Well-watered | 115±31.5 | 171±7.6 | 118±28.8 | 14.8±4.4 | 10.2±2.9 | 37.5±11.5 |
| Water stress | 147±44.9 | 166±13.6 | 40±4.4* | 10.7±2.4 | 4.7±0.6* | 19.9±4.4* |
| $LSD_{0.05}$ | 52.4 | 23.0 | 51.1 | 13.9 | 1.2 | 4.4 |
| *ZarxJos* | | | | | | |
| Well-watered | 119±9.5 | 72±21.4 | 44±10.6 | 31.6±8.6 | 5.1±1.4 | 33.9±9.1 |
| Water stress | 221±55.6* | 80±18.5 | 21±7.5* | 12.5±1.4* | 5.3±1.3 | 25.5±1.7 |
| $LSD_{0.05}$ | 39.4 | 8.8 | 6.2 | 14.2 | 1.3 | 15.8 |
| | | | Analysis of Variance | | | |
| Cultivars (C) | ***[b] | *** | *** | *** | *** | *** |
| Treatments (T) | *** | *** | *** | *** | *** | *** |
| CxT | *** | *** | *** | *** | *** | * |
| LSD | 44.30 | 18.47 | 12.00 | 3.43 | 1.32 | 7.31 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05).
[a] Asterisk (*) indicates significant difference with controls groups (well-watered)

**Table 4:** Influence of moderate water stress on uptake fluxes of micronutrients (mg plant$^{-1}$ day$^{-1}$) in ungrafted, grafted and self-grafted tomato plants.

| Cultivar/Water Treatment | Fe | Cu | Mn | Zn | B | Cl (mg g$^{-1}$ DW) |
|---|---|---|---|---|---|---|
| Zar ungrafted | | | | | | |
| Well-watered | 4.7±0.5 | 2.2±0.2 | 1.4±0.2 | 0.15±0.01 | 4.5±0.6 | 47.2±4.1 |
| Water stress | 8.1±1.6*[a] | 2.7±0.1 | 1.0±0.0 | 0.08±0.01* | 3.8±0.4 | 81.0±8.1* |
| $LSD_{0.05}$ | 2.7 | 0.8 | 0.5 | 0.05 | 2.0 | 25.4 |
| *ZarxZar* | | | | | | |
| Well-watered | 6.2±0.8 | 3.8±0.3 | 3.2±0.1 | 0.40±0.03 | 9.1±0.9 | 121.1±12.1 |
| Water stress | 9.4±1.7* | 3.3±0.4 | 1.4±0.2* | 0.13±0.02* | 7.5±0.8 | 52.3±9.6* |
| $LSD_{0.05}$ | 2.4 | 1.5 | 0.8 | 0.13 | 3.6 | 42.9 |
| Jos ungrafted | | | | | | |
| Well-watered | 7.9±0.9 | 3.0±0.3 | 1.7±0.1 | 0.19±0.02 | 8.2±0.4 | 82.4±3.9 |
| Water stress | 3.9±0.3* | 1.8±0.2* | 0.7±0.1* | 0.09±0.01* | 3.5±0.3* | 34.6±2.2* |
| $LSD_{0.05}$ | 1.8 | 1.0 | 0.4 | 0.06 | 1.3 | 12.6 |
| *JosxJos* | | | | | | |
| Well-watered | 6.3±0.7 | 2.7±0.1 | 1.9±0.2 | 0.18±0.02 | 6.4±0.7 | 72.0±9.2 |
| Water stress | 3.9±0.3* | 1.9±0.2* | 0.8±0.1* | 0.08±0.01* | 3.7±0.1* | 36.1±0.5* |
| $LSD_{0.05}$ | 2.3 | 0.7 | 0.7 | 0.06 | 2.1 | 15.7 |
| *JosxZar* | | | | | | |
| Well-watered | 4.5±0.5 | 1.5±0.4 | 1.5±0.5 | 0.15±0.04 | 5.0±1.5 | 51.4±15.4 |
| Water stress | 9.1±2.3* | 1.6±0.3 | 0.8±0.1 | 0.10±0.02 | 3.4±0.8 | 37.3±8.8* |
| $LSD_{0.05}$ | 2.6 | 1.5 | 1.6 | 0.14 | 2.8 | 9.3 |
| *ZarxJos* | | | | | | |
| Well-watered | 4.4±2.6 | 2.5±0.7 | 1.4±0.2 | 0.13±0.03 | 7.3±1.9 | 52.3±3.4 |
| Water stress | 7.3±1.0* | 2.9±0.2 | 0.8±0.1* | 0.09±0.01 | 3.2±0.3 | 68.8±17.2 |
| $LSD_{0.05}$ | 2.7 | 1.1 | 0.3 | 0.11 | 5.4 | 18.8 |
| | | | Analysis of Variance | | | |
| Cultivars (C) | *[b] | *** | *** | *** | *** | *** |
| Treatments (T) | *** | *** | *** | *** | *** | *** |
| CxT | ns | *** | *** | *** | *** | *** |
| LSD | 1.34 | 0.35 | 0.22 | 0.02 | 0.90 | 9.30 |

Values are mean ± S.E. (n=9) and differences between means were compared by Fisher´s least-significance test (LSD; P = 0.05). Asterisk (*) indicates significant difference with controls groups (well-watered).

[a] Asterisk (*) indicates significant difference with controls groups (well-watered)

[b] The levels of significance were represented by p>0.05: ns (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***)

For the micronutrients (Table 4), cv. Zarina significantly increased in uptake of Fe and Cl under stress, whereas *ZarxZar*, *JosxZar* and *ZarxJos* only showed a increase in Fe uptake fluxes. For the rest of the micronutrients, all the cultivars and grafting registered a general decline in transport under stress with respect to control conditions (Table 4).

## DISCUSSION

The foliar N concentrations have been correlated positively with the yield. So, the higher nitrogen uptake efficiency of some graft combinations can minimize or even eliminate yield losses owing to marginal soil fertility (Simonne *et al.* 2010). However, under water-stress conditions, it has been showed that N uptake diminishes in soy and rice plants (Tanguilig *et al.* 1987) and wheat (Hu *et al.* 2006). Agree with these results, our data reflect a decline in the N concentration associated with worst uptake under stress conditions only in cv. Josefina susceptible and its self-grafting (Tables 1 and 3). On the other hand, our results show a higher N concentration and uptake in cv. Zarina tolerant, its self-grafting and *ZarxJos* under water stress (Tables 1 and 3). These results agree with Ruiz *et al.* (1997), who tested the effects of two different rootstocks on the leaf macronutrient contents of melon plants, and concluded that, in general, N was influenced more by the rootstock genotype than by scion. In addition to the rootstock-scion interaction, the N content depends on the environmental conditions in which plants develop.

P uptake can be reduced by grafting, depending mainly on the genotype of the rootstock (Kawaguchi *et al.* 2008). We found that P concentration and its uptake disminushed in cv. Josefina and *JosxJos* under stress conditions. The low N and P concentrations observed could be ascribed to the smaller root system (Figure 2). In contrast, higher P concentrations in the leaves of grafted plants, or

higher translocation rates from root to shoot, in comparation with non-grafted plants heve been reported by Leonardi *&* Giuffrida (2006) for eggplant grafted. Similar results were found for cucumber, watermelon and melon grafted (Rouphael *et al.* 2008; Colla *et al.* 2010; Salehi *et al*. 2010). We have found higher P concentration and its uptake fluxe (Table 1 and 3) under water stress in cv. Zarina, *ZarxZar* and *ZarxJos*. Due to the low mobility of P, a more vigorous root system in these plants (Figure 2) could increase active P uptake by the plants. Phosphorous nutritional status is very critical for photosynthesis; carbon partitioning and energy transfer of plants and the energy required for ion uptake is supplied by an energy rich coenzyme, pricipally ATP (Maathuis 2009). So, increases in N and P concentration and uptake have been considered as a tolerance mechanism to salinity stress in potato and strawberry plants (Khalifa *et al.* 2000; Kaya *et al.* 2001) and in tomato plants under water stress (Sánchez-Rodríguez *et al.* 2010b). In our work, this increased concentration and uptake of N and S could be considered as a tolerance mechanism too, as well as the use of cv. Zarina like rootstocks improves growth of cv. Josefina in grafted plants *ZarxJos* (Sánchez-Rodríguez *et al.* 2011a).

Sulphur intervenes in the production of glutation and forming part of the sulpholipids that are essential for stabilization of photosynthetic compounds (Maathuis 2009). In our work, all cultivars and grafting plants showed a lower S concentration and uptake under stress conditions with respect to well-watered

plants (Tables 1 and 3), which was probably due to the ion antagonism between $H_2PO_4^-$ and $SO_4^{2-}$ in the nutrient solutions (Guo *et al.* 2004).

Enhanced Ca uptake due to grafting and higher Ca translocation rates are important in fruiting Solanaceae (Savvas *et al.* 2010). Fernández-García *et al.* (2004c) found a significant increase in leaf Ca concentrations when the tomato cultivars 'Fanny' and 'Goldmar' were grafted onto the tomato rootstock hybrid 'AR-9704'. Similarly, Leonardi *&* Giuffrida (2006) found significant increase in the leaf Ca concentrations of tomato and eggplant grafted plants. Besides, the impact of grafting on Mg uptake depends largely on the rootstock genotype. Some tomato rootstocks such as 'He-Man' may decrease the leaf Mg concentration (Savvas *et al.* 2009). However, other rootstocks seem to increase significantly the Mg uptake in graft eggplants and mini-watermelon (Leonardi *&* Giuffrida 2006; Rouphael *et al.* 2008). Our results showed no differences significant in Ca and Mg concentrations in all cultivars and grafting plant, except *JosxZar* and *ZarxJos* with a decrease and an increase in Mg concentration, respectively (Tables 1 and 3). These results agree with Colla *et al.* (2010), who observed no differences in Ca content, but the concentration of Mg in leaves was influenced significantly by the grafting combination in grafted watermelon.

Enhancement of K uptake due grafting has been also reported by some authors, specifically Leonardi *&* Giuffrida (2006) for eggplants, Qi *et al.* (2006) for melon grafted, and Rouphael *et al.* (2008) for mini-watermelon. Our data

reflect an increase in the K concentration under stress conditions only in cv. Zarina and *ZarxJos* (Table 1). Several authors hold that a greater K accumulation improves stomatal resistance, benefiting drought tolerance (Sinha 1978; Kafkafi *&* Xu, 1999), which could explain why cv. Zarina is more tolerant to water stress than cv. Josefina. The use of cv. Zarina and rootstock in grafted plants *ZarxJos* increases the leaf K concentration, which could facilitate the adjustment of water stress in these plants.

Micronutrients are essential for plant growth and they are involved in virtually all metabolic and cellular functions, like energy metabolism, primary and secundary metabolism, cell protection, gene regulation, hormone perception, signal transduction and reproduction (Hansch *&* Mendel, 2009). In most cases, grafting of fruit vegetables either decreases or has no effect on the uptake of micronutrients (Edelstein *et al.* 2005; Rouphael *et al.* 2008; Savvas *et al.* 2009). However, some rootstocks increase the efficiency of grafted plants to take up and translocated Fe and/or Mn to the shoot, in comparison with the corresponding self-rooted cultivars (Rivero *et al.* 2004; Colla *et al*. 2010; Huang *et al.* 2010). Our data in relation to micronutrient concentrations and uptake fluxes in leaves showed in general no significant differences or decrease under water stress, except in the case of Fe, Cu and Cl, which rose in the cv. Zarina; and, only Fe and Cu in *ZarxJos* (Table 2 and 4). Cu and Fe participates against oxidative stress binding proteins and enzymes (Hansch *&* Mendel, 2009), and this would coincide with the results reported in previous works (Sánchez-

Rodríguez *et al.* 2011b), demonstrating that cv. Zarina and *ZarxJos* presents more vigorous enzymatic antioxidant activity. In turn, a higher Cl concentration could be related to stronger stomatal resistance together with K in these same cultivars.

In conclusion, the use of drought-tolerant cv. Zarina like rootstocks (*ZarxJos*) showed improve ionome, with increases in N, P and K concentration and uptake fluxes, and an increase in Fe and Cu concentration and uptake under water stress. Besides, the vigorous root system of rootstocks is often capable of absorbing plant nutrients more efficently than scion root, and we have showed that cv. Zarina and *ZarxJos* develop a better radicular system. This result confirms the hypothesis that grafted plants on vigorous rootstocks can improve mineral nutrition and nutrient uptake with respect to ungrafted plants, especially under water stress conditions.

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# *Capítulo 8*

## *Discusión General*

La mayoría de los estudios realizados con injertos se han centrado en obtener resistencia a enfermedades, e incrementar la tolerancia frente a salinidad, altas y bajas temperaturas, toxicidad por metales, o bien mejorar la calidad de los frutos o extender el tiempo de postcosecha. Sin embargo, son pocos los trabajos que se han centrado en estudiar su efecto bajo condiciones de estrés hídrico, a pesar de que se sabe que las plantas injertadas muestran un incremento en la absorción de agua y nutrientes en comparación con las plantas sin injertar. Por ello, el objetivo general de esta Tesis Doctoral ha sido estudiar el efecto a nivel fisiológico del injerto recíproco entre dos cultivares de tomate cherry bajo déficit hídrico moderado, uno con mayor grado de resistencia a este estrés (cv. Zarina) y otro más sensible (cv. Josefina). A su vez, este estudio nos permite dilucidar que parte del injerto, la parte radicular o la parte aérea, es más relevante en cada una de las respuestas fisiológicas frente a este estrés hídrico moderado. Conocer estas respuestas permitirá aplicar la técnica de realización de injertos de forma más eficiente, mejorando el rendimiento de cultivos tan importantes como el tomate.

En el caso del injerto ***JosxZar***, cuando utilizamos el cultivar tolerante al estrés hídrico Zarina como parte aérea, podemos observar que bajo estrés hídrico mantiene el crecimiento foliar con respecto a las condiciones control, sin embargo, no incrementa su crecimiento radicular (Capítulo 3, Tabla 1), lo cual podría estar relacionado con una disminución en su eficiencia radicular

(Capítulo 7, Figura 2). Por tanto, podemos deducir que la utilización de una parte aérea con mayor tolerancia al estrés hídrico (cv. Zarina) no provoca un aumento en la capacidad de absorción y crecimiento de la raíz sensible (cv. Josefina), lo que se traduce en un descenso en el contenido hídrico foliar (Capítulo 3, Tabla 1). Este hecho, puede desencadenar en la planta un estrés oxidativo (Pinheiro *et al.* 2004), por lo que una rápida y eficaz respuesta antioxidante resulta esencial como mecanismo protector en la planta (Jaleel *et al.* 2009). Resultados previos realizados en nuestro grupo de investigación muestran que la parte aérea del cv. Zarina es capaz de activar el sistema antioxidante bajo condiciones de estrés hídrico moderado, lo cual le permite mantener los niveles de ROS y por tanto su crecimiento se mantiene (Sánchez-Rodríguez *et al*. 2010). Efectivamente, observamos que su utilización como parte aérea en el injerto *JosxZar* permite a la planta injertada mantener una mejor respuesta antioxidante, incrementando la actividad de enzimas detoxificadoras como SOD y CAT (Capítulo 3, Figura 3), así como aumentando los niveles de compuestos antioxidantes tan importantes como el ascorbato (AsA) y el glutation (GSH) (Capítulo 3, Tablas 3 y 4). En esta respuesta detoxificadora, también podemos incluir a los fenoles, ya que por su estructura química se consideran buenos antioxidantes (Parr *&* Bolwell 2000). Así, un aumento en el contenido de estos compuestos se ha relacionado con una mayor resistencia a diversos tipos de estrés (Evrenosoğlu *et al.* 2010). Al igual que ocurre con la respuesta antioxidante, la actividad de algunas de las enzimas que intervienen en la biosíntesis de fenoles se incrementan bajo

estrés hídrico en el injerto *JosxZar* (Capítulo 4, Figura 6), lo que conlleva una acumulación de compuestos fenólicos bajo estas condiciones (Capítulo 6, Figura 3). Recientemente, se ha sugerido que las peroxidasas, en presencia de fenoles y AsA, pueden actuar como un sistema detoxificador de $H_2O_2$ muy eficiente (Sgherri *et al*. 2003), lo cual puede correlacionarse con los datos que obtenemos en las plantas injertadas *JosxZar*. A su vez, un incremento en la acumulación de fenoles puede favorecer la protección del propio aparato fotosíntético (Hura *et al.* 2009), aunque son necesarios más estudios de la eficiencia fotosíntetica en estas plantas para confirmar esta teoría.

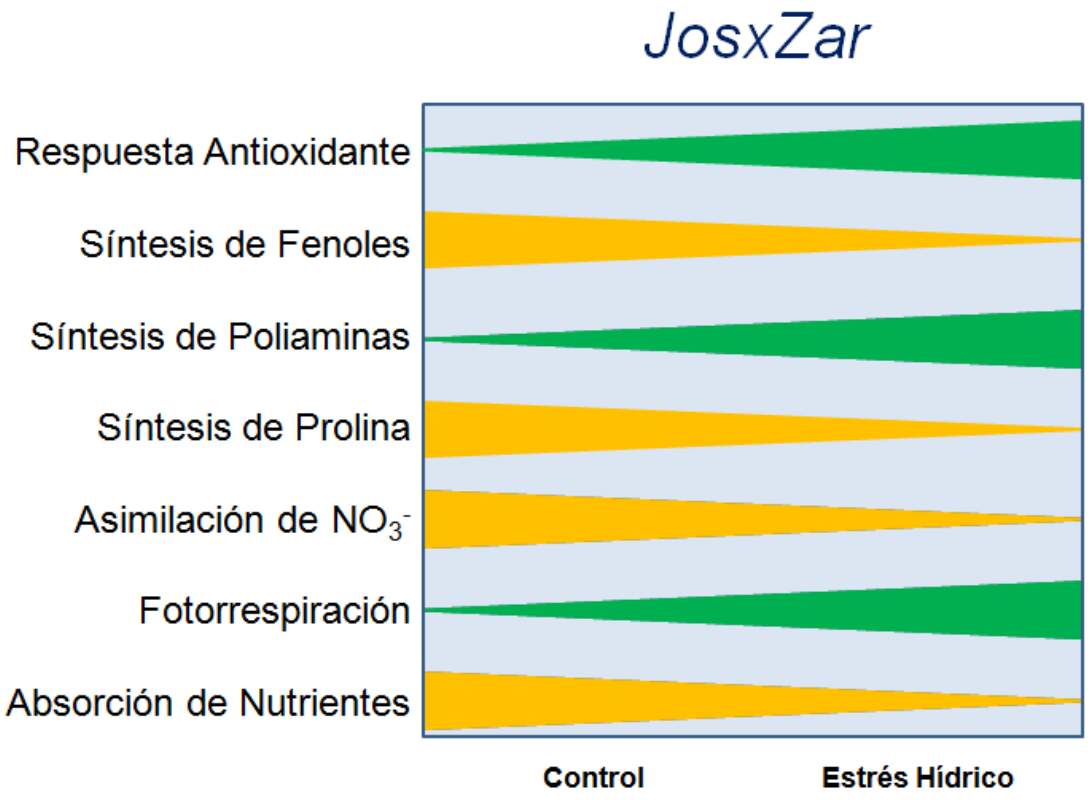
Por otro lado, se ha postulado que el $H_2O_2$ generado en el catabolismo de las PAs actuá como molécula señal promoviendo la activación de la respuesta antioxidante (Groppa *&* Benavides 2008). Este mecanismo podría estar ocurriendo en las plantas injertadas *JosxZar*, ya que observamos un incremento en la actividad de las enzimas encargadas de la degradación de PAs (Capítulo 5, Figura 5). Asimismo, las enzimas relacionadas con la biosíntesis de estos compuestos también se ven incrementadas bajo estrés hídrico, lo que se traduce en un aumento en la concentración de Spd (Capítulo 7, Tabla 2). Se ha relacionado el metabolismo de las PAs con el metabolismo fenilpropanoide, de forma que las PAs pueden derivarse a la biosíntesis de compuestos fenólicos como derivados del cafeíco y ferúlico (Bassard et al. 2010). Nuestros datos parecen corroborar esta teoría, ya que observamos un

aumento en los ácidos fenólicos derivados del cafeíco bajo condiciones de estrés hídrico (Capítulo 4, Tabla 2) asociado con una mayor disponibilidad de PAs (Capítulo 5, Tabla 2). Por otro lado, las PAs, especialmente la Spd, pueden actuar como moléculas antioxidantes debido a su actividad detoxificadora (Bors *et al.* 1989), y su capacidad para inhibir la peroxidación lipídica (Kitada *et al.* 1979). Así, este aumento que observamos en la concentración de Spd en las plantas *JosxZar* puede ayudar a la capacidad de respuesta antioxidante que se presenta bajo condiciones de estrés hídrico. Es importante señalar que la misma respuesta es observada en las plantas del cv. Zarina sin injertar. Por tanto, todos estos datos nos llevan a pensar que tanto la respuesta antioxidante como el metabolismo fenólico y el de PAs están más determinados por el cultivar utilizado como parte aérea, que por la parte radicular; de forma que el uso del cv. Zarina como parte aérea proporciona a las plantas injertadas una mayor capacidad de respuesta antioxidante frente a un estrés hídrico moderado. Esto se ve reflejado en un mantenimiento de la biomasa foliar en las plantas injertadas bajo estrés hídrico en comparación con las plantas sin injertar del cv. sensible Josefina (Capítulo 3, Tabla 1).

En las plantas injertadas *JosxZar* no se produce un incremento en la asimilación de N (Capítulo 6, Tabla 1); mientras que presenta un aumento en la actividad de las enzimas relacionadas con el proceso de fotorrespiración bajo condiciones de estrés hídrico (Capítulo 6, Figura 3 y Tabla 2). El aumento en la

fotorrespiración puede servir también como mecanismo fotoprotector que ayuda a eliminar el exceso de electrones. A su vez, este proceso genera elevadas cantidades de $NH_4^+$, que puede ser altamente tóxico para la célula (Hirel *&* Lea 2001). Sin embargo, en los injertos *JosxZar* no observamos acumulación de este catión bajo nuestras condiciones de estrés, ya que probablemente sea utilizado por el ciclo GS/GOGAT y la enzima aspartato aminotrasferasa (AAT) para la generación de aminoácidos (aas) (Capítulo 6, Tabla 4). En esta nueva generación de aas puede intervenir también la propia degradación de prolina, ya que se ha postulado que este proceso puede redirigirse a la síntesis de otros aminoácidos más esenciales como la glutamina y el glutamato, los cuales son donadores de aminoácidos y se transportan para la síntesis de nuevas proteínas (Rubio-Wilhelmi *et al.* 2012). Efectivamente, nuestros datos muestran como en las plantas *JosxZar* bajo condiciones de estrés hídrico se incrementa la degradación de prolina (Capítulo 5, Tabla 1). Asimismo, esta degradación puede generar NADH (Lehmann et al. 2010), mejorando el estado redox de la planta; tal y como observamos también en nuestros datos (Capítulo 6; Tabla 3). Por lo tanto, al igual que observamos para la respuesta antioxidante, la activación del metabolismo del N y la degradación de la prolina se encuentran más influenciados por la parte aérea del injerto, es decir, vienen determinados por la utilización del cv. Zarina como parte aérea; ya que la respuesta del cv. Zarina es muy similar al injerto *JosxZar*. La menor capacidad de absorción radicular observada en el injerto *JosxZar* (Capítulo 7, Figura 2) se traduce a su vez en que no observamos una mejora ni en la

absorción ni en la acumulación de macronutrientes y micronutrientes esenciales bajo condiciones de estrés (Capítulo 7, Tabla 1 y 3). En conclusión, el incremento en la respuesta antioxidante, fotorrespiracion y síntesis de poliaminas en las plantas injertadas *JosxZar* (Esquema 1) favorecen el crecimiento bajo condiciones de estrés hídrico, manteniendo la biomasa foliar a niveles similares a las plantas control.




**Esquema 1:** Esquema de las principales respuestas fisiológicas en las plantas injertadas *JosxZar* frente al estrés hídrico.

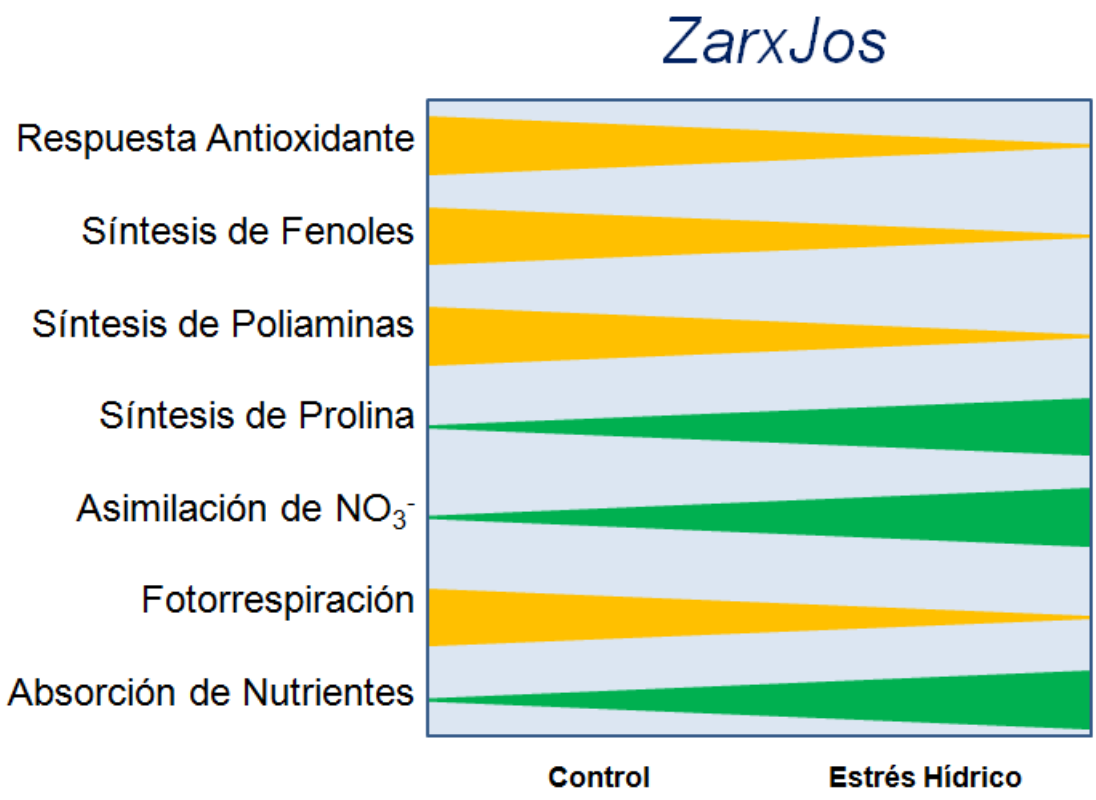
En las plantas injertadas ***ZarxJos***, utilizando el cv. Zarina resistente al estrés hídrico como parte radicular, observamos una respuesta diferente. En estas

plantas, bajo condiciones de estrés hídrico, muestran un incremento en la eficiencia radicular, lo cual permite mantener su LRWC y biomasa foliar (Capítulo 3, Tabla 1). A su vez, se mantiene el vigor e incluso aumenta con respecto a los cultivares sin injertar, lo cual puede resultar beneficioso en la producción de tomate bajo invernadero, ya que los agricultores prefieren plantas con mayor vigor, lo cual les permite reducir el número de plantas de tomate y disminuyendo así los costes (King *et al.* 2010). En este caso, por tanto, podemos deducir que la utilización de una parte radicular tolerante al estrés hídrico (cv. Zarina) permite mejorar el LRWC y la biomasa foliar de la parte aérea sensible a este estrés (cv. Josefina). Por el contrario, sin embargo, hemos observado que en las plantas *ZarxJos* no se incrementa la actividad de las principales enzimas antioxidantes bajo condiciones de estrés (Capítulo 3, Figuras 3 y 5). Esto se ve reflejado en un incremento de la concentración de ROS y de la peroxidación lipídica (Capítulo 3, Figuras 3 y 4). A su vez, en esta planta no se incrementan los principales antioxidantes como el GSH y el AsA (Capítulo 3, Tablas 3 y 4); y de forma similar, ni la concentración de fenoles ni en la actividad de las enzimas encargadas de su síntesis observamos respuesta (Capítulo 4, Figuras 3 y 6). Por tanto, en este caso, la parte radicular tolerante al estrés hídrico (cv. Zarina) no logra inducir una respuesta antioxidante en la parte aérea sensible (cv. Josefina) bajo estas condiciones de estrés, y refuerza la idea de que el metabolismo antioxidante es más dependiente de la parte aérea del injerto que de la propia parte radicular.

De forma similar, el metabolismo de las PAs también parece estar determinado por la parte aérea, ya que las plantas *ZarxJos* no presentan un incremento en la actividad de las enzimas de biosíntesis de estos compuestos (Capítulo 5, Figura 4), y por tanto observamos una disminución en la concentración de PAs totales con respecto al control (Capítulo 5, Figura 6). Efectivamente, el cv. Josefina muestra una respuesta similar en el metabolimo de las PAs bajo condiciones de estrés hídrico moderado que el injerto *ZarxJos*, lo que nos vuelve a demostrar la importancia de la parte aérea en este metabolismo; ya que la parte radicular Zarina no consigue estimular una respuesta en la parte aérea sensible.

El aumento en la absorción de N bajo condiciones de estrés hídrico mostrada en las plantas injertadas *ZarxJos* se ve traducido en un incremento en la asimilación de nitrato (Capítulo 6, Tabla 1); y por tanto de su posterior incorporación a aminoácidos y proteínas (Capítulo 6, Tabla 4 y Figura 5). Efectivamente, estas plantas muestran un aumento en la concentración del aminoácido prolina (Capítulo 5, Figura 3), ya que bajo condiciones de estrés hídrico se incrementa su biosíntesis y sin embargo, disminuye su degradación (Capítulo 5, Tabla 1). Este aumento en la concentración de prolina podría ser interpretado como un síntoma del estrés oxidativo que presentan estas plantas bajo condiciones de estrés hídrico. Estos datos corroboran nuestra idea de que

la degradación de prolina es más importante que su propia síntesis, ya que estimula la síntesis de poliaminas. Sin embargo, el proceso de fotorrespiración disminuye con el estrés hídrico en las plantas injertadas *ZarxJos*, lo que nos demuestra que este proceso está más determinado por la parte aérea del injerto. Otra de las consecuencias del aumento en la biomasa y eficiencia radiculares observadas en el injerto *ZarxJos* bajo estrés hídrico (Capítulo 7, Figura 2) es el incremento en la concentración de macronutrientes esenciales. Se ha demostrado que un sistema radicular más vigoroso que mejore la absorción de agua y nutrientes en la planta puede mejorar el crecimiento de la planta (Rouphael *et al.* 2008); lo cual sucede en nuestras plantas injertadas con el portainjetos de Zarina (Capítulo 3, Tabla 1). Por lo tanto esta mayor capacidad de absorción de nutrientes junto con el incremento en la asimilación de nitrato podrían tener un importante papel en el crecimiento de la parte aérea sensible en el injerto *ZarxJos*, explicando el mantenimiento de la biomasa foliar bajo las condiciones de estrés hídrico moderado (Esquema 2).




**Esquema 2:** Esquema de las principales respuestas fisiológicas en las plantas injertadas *ZarxJos* frente al estrés hídrico.

Por tanto podemos concluir que a la hora de realizar injertos con plantas de tomate, es importante la selección adecuada tanto de la parte radicular, como de la parte aérea. En este trabajo hemos demostrado que no sólo la parte radicular es importante en el injerto, sino que el genotipo de la parte aérea también tiene influencia en la respuesta fisiológica de la planta frente al estrés hídrico. También hemos demostrado que en plantas injertadas la mayor resistencia a un estrés hídrico en una parte aérea sensible, como en nuestro caso el cv. Josefina, se debe principalmente a un aumento de vigor, y por lo

tanto de la absorción de agua y nutrientes por la base radicular, lo que hace que este aspecto sea determinante en la elección de raíces adecuadas en los injertos frente a este tipo de estrés.

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# *Capítulo 9*

## *Conclusiones*

1. Las plantas injertadas consistentes en el cv. Josefina sensible al estrés hídrico como base radicular y el cv. Zarina tolerante como parte aérea (*JosxZar*) mejoran la respuesta a un estrés hídrico. Nuestros resultados confirman que estas plantas injertadas reducen la aparición de un estrés oxidativo bajo condiciones de déficit hídrico por inducción de los siguientes procesos fisiológicos dependientes de la parte aérea tolerante (cv. Zarina):

- Incremento de las actividades SOD, CAT y las enzimas del ciclo de Halliwell-Asada, mientras que no se aprecian cambios en la actividad LOX. De esta manera en estas plantas el uso del cultivar Zarina como parte aérea en los injertos puede ser una técnica útil y efectiva para mejorar la respuesta antioxidante frente al estrés hídrico en plantas de tomate.

- Activación de la síntesis y acumulación de fenoles en las plantas de tomate injertadas bajo estrés hídrico. Esto se ve reflejado en la acumulación principalmente de derivados del ácido cafeíco y en la activación de las enzimas PAL, DAHP y C4H.

- Inducción de la degradación de prolina asociada a una mejora en la síntesis de poliaminas. Estas plantas injertadas presentan una acumulación de espermidina debido a un aumento en la actividad SPDS, mientras que el incremento en la actividad PDH se refleja en la disminución de prolina.

- Incremento del proceso de fotorrespiración, reflejado en una mayor actividad de las enzimas Rubisco, GO y GGAT. Este mecanismo permite que estas plantas injertadas incrementen el ratio NADH/NAD.

  Por lo tanto, todos estos datos confirman que una base radicular sensible no impide la aparición de respuestas de tolerancia en una parte aérea resistente. Esto determina la viabilidad de la realización de estos injertos bajo estas condiciones de estrés hídrico moderado.

2. El uso del cultivar tolerante como base radicular en las plantas injertadas (*ZarxJos*) provoca un mejor crecimiento frente al estrés hídrico en la parte aérea sensible del cv. Josefina. En estas plantas injertadas la base radicular tolerante determina los siguientes mecanismos fisiológicos:

- Mejora del metabolismo del N, ya que el uso del cv. Zarina como parte radicular (*ZarxJos*) mejora la absorción y posterior asimilación de $NO_3^-$ en las plantas estresadas.
- Mejora del ionoma foliar en las plantas injertadas bajo condiciones de estrés hídrico, de forma que incrementa la absorción y concentración de N, P y K, así como de Fe y Cu, probablemente debido a su mayor vigor radicular. Estos resultados confirman la hipótesis de que en plantas injertadas con una base radicular vigorosa puede mejorar la absorción de agua y nutrientes con respecto a plantas sin injertar, especialmente bajo condiciones de estrés hídrico.

Finalmente nuestros datos sugieren que la base radicular tolerante (cv. Zarina) en plantas injertadas no induce la aparición de respuestas de resistencia a estrés en la parte aérea sensible del cv. Josefina. En definitiva, estos datos nos indican que la clave para seleccionar bases radiculares que mejoren la resistencia al estrés hídrico en la parte aérea sensible se debería basar en la vigorosidad de la raíz y en su eficiencia metabólica.

# *Anexo I*

## *Curriculum Vitae*

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2005-2006: Máster en Biotecnología. Escuela Superior de Enseñanza Abierta y Centro de Enseñanzas Virtuales. Universidad de Granada.

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2007-2012: Estudiante predoctoral en la Universidad de Granada, Facultad de Ciencias, Departamento de Fisiología Vegetal.

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2006-2007: Beca de Colaboración Investigación. Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada. Ministerio de Educación y Ciencia.

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## PARTICIPACIÓN EN CONGRESOS

**2008:** Comité Organizador como Vocal del XII Simposio Ibérico sobre Nutrición Mineral de las Plantas de Granada.

**2008:** XII Simposio Ibérico sobre Nutrición Mineral de las Plantas (Granada). Poster: “Eficacia en el uso de nitrógeno en plantas de tomate (*Solanum lycopersicum*) sometidas a estrés hídrico moderado”. Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Blasco B, Cervilla LM, Ríos JJ, Rosales MA, Romero L, Ruiz JM.

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**2010:** XVIII Congress of the Federation of European Societies of Plant Biology (FESPB) (Valencia) Póster: "The role of photorespiration during water stress in two tomato cultivars with contrasting drought tolerance". Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Blasco B, Leyva R, Melgarejo R, Romero L, Ruiz JM.

**2010:** XXVIII International Horticultural Congress (Lisboa) Póster: "Grafting between two cultivars of cherry tomato differing in their tolerance to drought: effect in nitrogen-use efficiency". Sánchez-Rodríguez E, Rubio-Wilhelmi MM, Blasco B, Leyva R, Romero L, Ruiz JM.

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# *Anexo II*

## *Publicaciones preliminares*

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# Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants

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ABSTRACT

Water stress strongly affects horticultural cultivars, reducing yield and fruit quality. Also the physiological functions of the plant are altered by this stress, due fundamentally to the formation of reactive oxygen species and water relationships. This study examines the response of five cherry tomato varieties to oxidative stress generated by moderate water deficit. Our results indicate that the cultivar Zarina is more tolerant to this stress, registering greater biomass and leaf relative water content (LRWC), associated with high antioxidant activity and low content in osmoprotective compounds. Also, we found a positive correlation of relative growth rate (RGR) total and foliar with LRWC, and a negative one with the parameters malondialdehyde (MDA), $H_2O_2$, test antioxidants, phenolic content, proline and quaternary ammoniums compounds (QAC), indicating the importance of lipid peroxidation as the determinant physiological process in selecting tomato plants tolerant to water stress.



## 1. Introduction

Plants, when subjected to environmental stress, undergo alterations in their growth, metabolism, and production. Among these, drought is the most adverse environmental factor regarding growth and productivity of cultivars. Losses in agricultural yield due to water stress probably exceed the losses inflicted by all other causes combined [1]. It is known that drought has a profound impact on agricultural and ecological systems, and thus the capacity of plants to withstand this stress is of great economic importance [2]. Therefore, at present, with the aim of improving agricultural yield within the earth's limited resources, it is necessary to develop crops able to give a high yield when growing in stressed environments.

Water stress influences plant growth in several ways. For example, shoot biomass significantly decreased in wheat under drought conditions [3]. In potato plants, stem length and dry weight diminished under water stress [4,5], and in tomato plant, shoot weight and total leaf area were lower than well-watered [6]. Also, water deficit diminishes leaf size, longevity, and number of leaves per plant [2].

The damage caused by water stress has two primary causes: first, the formation of reactive oxygen species (ROS) and, second, the alteration of water relationships within the plant. The extent to which plants can avoid or buffer these physiological processes determines the degree of resistance to water stress. Therefore the study of the metabolic and biochemical responses to water deficit is vital to present-day agriculture in order to select plants with high yield and stability under this type of stress [7].

Plants respond to water stress by producing abscisic acid (ABA), which stimulates the closure of the guard cells of the stomata to reduce water loss [8]. This process decreases $CO_2$ availability for photosynthesis, resulting in an imbalance between the generation and the use of electrons, provoking the overproduction of ROS. Free ROS attack biological structures, damaging DNA, prompting the oxidation of amino acids and proteins, and provoking lipid peroxidation [9,10]. To avoid such damage, plants have developed ROS-detoxification mechanisms that can be divided into enzymatic and non-enzymatic systems. The enzymatic systems comprise superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutation reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR). The non-enzymatic systems are composed of antioxidants such as phenols (flavonoids, anthocyanins, carotenoids, etc.), ascorbic acid (AsA) and glutation (GSH) [11]. Currently, there is clear evidence that many stress situations raise total foliar

*Abbreviations:* ABA, abscisic acid; APX, acorbate peroxidase; AsA, ascorbate; CAT, catalase; DHAR, dehydroascorbate reductase; GSH, glutation; GR, glutation reductase; LRWC, leaf relative water content; LOX, lipooxygenase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; QAC, quaternary ammoniums compounds; RGR, relative growth rate; ROS, reactive oxygen species; SOD, superoxidedismutase.

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antioxidant activity. Dhindsa and Matowe [12] demonstrated that water-stress-tolerant *Tortula ruralis* registered low lipid-peroxidation levels together with higher SOD and CAT activities. Also, Fazeli et al. [13] studied the behaviour of antioxidant enzymes under water stress in two sesame cultivars, observing that the SOD and CAT activities were higher in the most tolerant cultivar.

In plant cells, the components of the Halliwell–Asada cycle, which involves AsA, GSH, APX, MDHAR, and GR, represent the primary $H_2O_2$-detoxification mechanism. It has been confirmed that water-stressed *Hordeum* species show high GR and APX activities [14]. Furthermore, the activities of MDHAR, GR, and DHAR show a significant increased in rice plants subjected to water deficit [15]. Also, high GR and DHAR levels were found in lettuce leaves under drought [16]. Within the non-enzymatic systems, AsA is the main antioxidant synthesized in plant cells, which reacts chemically with $^1O_2$, $O_2^-$, OH and the radical thiol and which acts as a natural substrate for many plant peroxidases [9,17]. A high ratio of reduced AsA to oxidized AsA is essential to eliminate ROS in cells. It has been confirmed that these compounds increase in plants acclimated to drought stress [18]. Also, it has been concluded that the buffering capacity provided by AsA generates stress resistance in plants [19].

Finally, with respect to ROS detoxification under water-stress conditions, the role of phenols is also noteworthy. These compounds present two functions with the aim of preventing ROS formation. First, it has been verified that water deficit intensifies blue fluorescence provoked by the accumulation of phenolic compounds [20]. By radiation absorption, phenolic compounds transform highly destructive low-wavelength radiation (λ) into blue radiation of greater λ and therefore less destructive to leaf-cell structures, including the photosynthetic apparatus [21]. Also, Hura et al. [22,23] found a positive correlation between the emission of blue fluorescence and the total phenol content in *Triticale* plants subjected to water stress. These results were found also in maize, where the emission was greater in cultivars sensitive to water stress. Secondly, phenolic compounds also show an antioxidant action, which depends principally on the number and position of the hydroxyl groups and their structure [24]. Antioxidant activity has been demonstrated in flavonoids, mainly for their ability to sequester ROS, such as the anion superoxide, and the radicals hydroxyl and peroxyl [24].

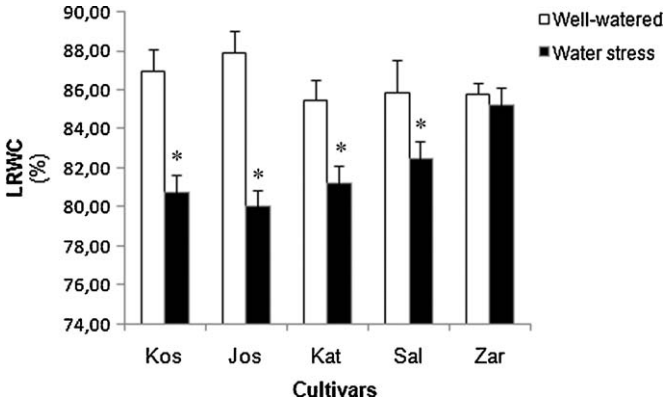


**Fig. 1.** Effect of moderate water stress on LRWC in leaves of five tomato cultivars: 'Kosaco', 'Josefina', 'Katalina', 'Salomé' and 'Zarina'. Columns are mean ± S.E. ($n = 9$) and differences between means were compared by Fisher's least-significant difference test (LSD; $P = 0.05$). *Significant difference with control groups (well-watered).

As indicated above, one of the main ways in which water deficit harms plants is by altering the water relationships under this type of stress. In this regard, water stress causes water loss within the plant and therefore a reduction in its relative content. In this sense, one of the most reliable and widely used indicators for defining both the sensitivity and/or the resistance to water stress in plants is leaf relative water content (LRWC) [25]. One of the most common strategies of plants for avoiding water stress is the accumulation of the so-called compatible solutes, also called osmoprotectors or osmolytes [26]. During osmotic stress, plant cells accumulate solutes to prevent water loss and re-establish cell turgour. The solutes that accumulate during osmotic adjustment include ions such as $K^+$, $Na^+$, and $Cl^-$, or organic solutes that include compounds that contain N, such as proline and other amino acids, polyamines, and QAC [27]. Proline accumulates in a great variety of plant species in response to stress such as drought, salinity, and extreme temperatures. Although its osmotolerant role in plants is not

**Table 1**
Dry weight and RGR in plants of 5 cultivars of cherry tomato plants well-watered and subjected to moderate water stress.

| Cultivar/water treatment | Total biomass (g DW) | Total RGR (mg g$^{-1}$ day$^{-1}$) | Foliar biomass (g DW) | Foliar RGR (mg g$^{-1}$ day$^{-1}$) |
|---|---|---|---|---|
| Kosaco | | | | |
| Well-watered | 12.83 ± 1.15 | 87.02 ± 1.22 | 11.76 ± 0.72 | 87.23 ± 1.25 |
| Water stress | 8.40 ± 0.57* | 68.15 ± 2.30* | 7.54 ± 0.91* | 64.74 ± 0.65* |
| $LSD_{0.05}$ | 3.58 | 8.02 | 3.25 | 1.54 |
| Josefina | | | | |
| Well-watered | 12.76 ± 1.46 | 84.21 ± 5.02 | 11.66 ± 0.57 | 82.05 ± 3.20 |
| Water stress | 7.63 ± 0.71* | 61.65 ± 0.32* | 6.77 ± 1.15* | 54.84 ± 0.21* |
| $LSD_{0.05}$ | 4.51 | 16.10 | 3.59 | 8.12 |
| Katalina | | | | |
| Well-watered | 13.29 ± 1.09 | 83.41 ± 0.51 | 11.85 ± 0.50 | 87.25 ± 0.62 |
| Water stress | 8.51 ± 0.57* | 63.95 ± 1.00* | 7.65 ± 0.43* | 64.38 ± 1.71* |
| $LSD_{0.05}$ | 3.44 | 5.15 | 1.84 | 5.10 |
| Salomé | | | | |
| Well-watered | 12.17 ± 0.64 | 100.21 ± 0.75 | 11.22 ± 0.87 | 100.09 ± 0.75 |
| Water stress | 7.51 ± 0.57* | 78.25 ± 0.51* | 6.77 ± 0.45 | 76.25 ± 0.91* |
| $LSD_{0.05}$ | 2.39 | 2.53 | 2.74 | 3.09 |
| Zarina | | | | |
| Well-watered | 11.38 ± 0.62 | 93.52 ± 8.11 | 10.87 ± 0.57 | 93.85 ± 2.33 |
| Water stress | 8.83 ± 0.68 | 79.23 ± 9.27 | 7.95 ± 0.63* | 80.28 ± 0.34* |
| $LSD_{0.05}$ | 2.58 | 14.01 | 2.38 | 6.87 |

Values are mean ± S.E. ($n = 9$) and differences between means were compared by Fisher's least-significance test (LSD; $P = 0.05$).
* Significant difference with controls groups (well-watered).

clear, under stress conditions, proline can act as a mediator of osmotic adjustment, stabilizer of subcellular structures, eliminator of free radicals, and as a buffer of redox potential [28–31]. However, despite this research, there is currently great controversy on the protective properties of proline accumulation. Hanson [32] concluded that proline accumulation is not an adaptive feature, but rather only a symptom of stress. In agreement with this contention, findings show that drought-tolerant wheat plants have a higher LRWC related to a lower proline concentration [25].

Given that tomato cultivation is concentrated in semi-arid zones, where water stress is frequent, it is important to ascertain its response to this stress as well as selecting cultivars that are more resistant and productive under these conditions. Although different drought-resistance strategies have been extensively studied, few studies have focused on the variation between cultivars with respect to tolerance characteristics or the avoidance of water stress. Therefore, the present work investigates whether the genotypic variability among 5 cherry tomato cultivars is related to the oxidative stress and examine how these responses are correlated with growth parameters under moderate water stress.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Five cherry tomato cultivars were used: Kosaco, Josefina, Katalina, Salome, and Zarina [*Solanum lycopersicum* L. (*Lycopersicon esculentum* Mill.)]. The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm × 3 cm × 10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Afterwards, the seedlings were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with relative humidity of 50 ± 10%, at 25 °C/15 °C (day/night), and a 16 h/8 h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 μmol $m^{-2}$ $s^{-1}$ (measured with an SB quantum 190 sensor, LI-COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. Throughout the experiment, the plants were grown in a complete nutrient solution containing: 4 mM $KNO_3$, 3 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 6 mM $KH_2PO_4$, 1 mM $NaH_2PO_4$, 2 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.25 μM $CuSO_4$, 0.1 μM $Na_2MoO_4$, 5 μM Fe-EDDHA, and 50 μM $H_3BO_3$. The nutrient solution (pH 5.8) was renewed every 3 days and the substrate was partially rinsed with distilled water to avoid nutrient accumulation. The water-stress treatments began 45 days after germination and maintained 22 days. The control treatment received 100% field capacity (FC) irrigation, whereas moderate water stress corresponded to 50% field capacity. The latter was selected to represent water-deficit stress on the basis of a preliminary experiment carried out on *S. lycopersicum* watered with 100, 75, 50 and 25% FC, showing that 50% irrigation procedure led to a significant decrease of growth. Independently of the procedure for watering (100 or 50% FC), plants received the same quantity of nutrients.

### 2.2. Sample of plants and determination of the relative growth rate (RGR) and leaf relative water content (LRWC)

All plants were at the late vegetative stage when harvested. Leaves fully expanded (excluding petioles) were harvested, frozen immediately in liquid $N_2$, and kept at −80 °C until used. To determine the relative leaf growth rate (RGR), leaves from three plants per cultivar were sampled on day 45 after germination, immediately before starting the water-stress treatment (Ti). The leaves were dried in a forced-air oven at 70 °C for 24 h, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 67 days after germination (22 days of treatments, Tf). The relative growth rate was calculated from the increase in leaf DW at the beginning and at the end of the water-stress treatment, using the equation RGR = (ln DWf − ln DWi)/(Tf − Ti) where *T* is the time and the subscripts denote the final and initial sampling (i.e. days 0 and 22, respectively, after the water-stress treatment) [33].

Leaf relative water content (LRWC) was measured following the method of Barrs and Weatherly [34].

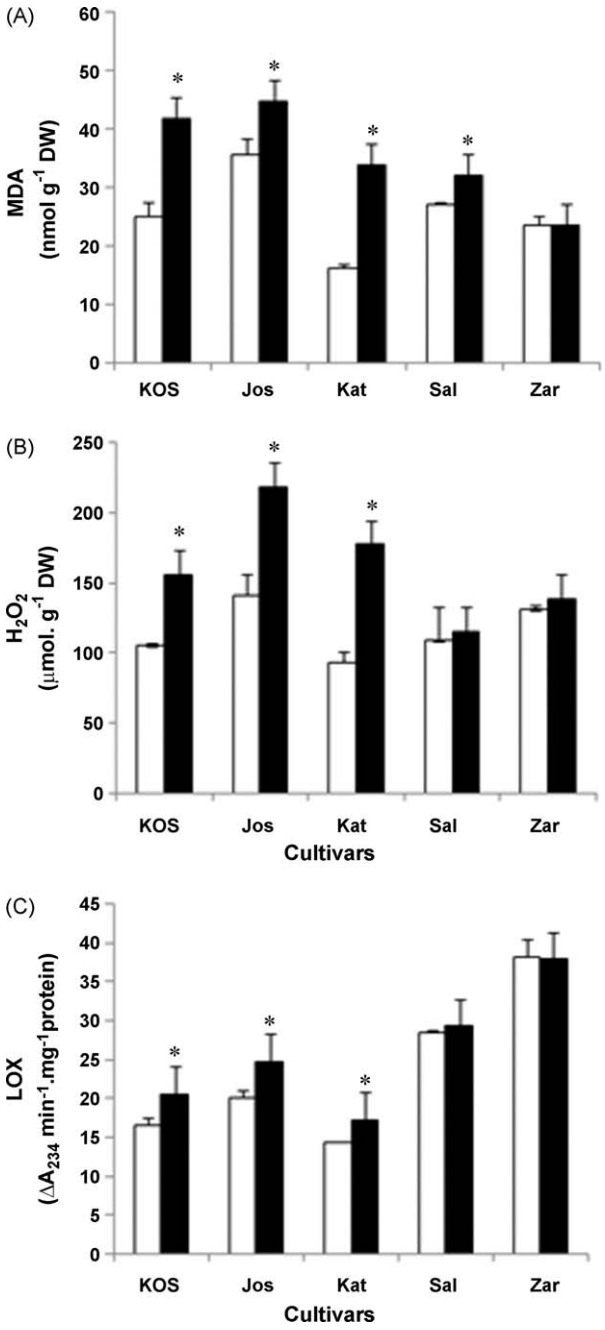


**Fig. 2.** Effect of moderate water stress on (A) MDA, (B) $H_2O_2$ concentration and (C) LOX activity in leaves of five tomato cultivars: 'Kosaco', 'Josefina', 'Katalina', 'Salomé' and 'Zarina'. Columns are mean ± S.E. ($n$ = 9) and differences between means were compared by Fisher's least-significant difference test (LSD; $P$ = 0.05). *Significant difference with control groups (well-watered).

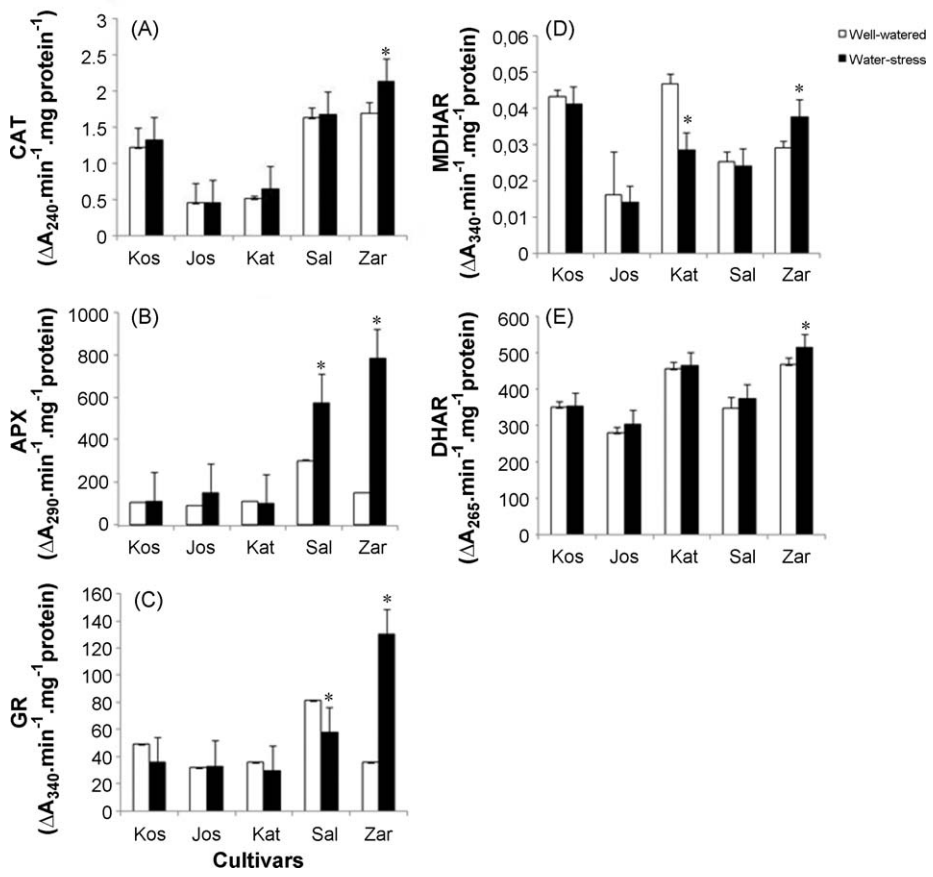


**Fig. 3.** Effect of moderate water stress on activities (A) CAT, (B) APX, (C) GR, (D) MDHAR and (E) DHAR in leaves of five tomato cultivars: 'Kosaco', 'Josefina', 'Katalina', 'Salomé' and 'Zarina'. Columns are mean ± S.E. ($n$ = 9) and differences between means were compared by Fisher's least-significant difference test (LSD; $P$ = 0.05). *Significant difference with control groups (well-watered).

**Table 2**
Concentration of reduced AsA, DHA, total AsA and the relationship of reduced AsA/DHA in the leaves of 5 cultivars of cherry tomato plants well-watered and subjected to moderate water stress.

| Cultivar/water treatment | Reduced AsA (mg g$^{-1}$ DW) | DHA (mg g$^{-1}$ DW) | Total AsA (mg g$^{-1}$ DW) | AsA red/DHA |
|---|---|---|---|---|
| Kosaco | | | | |
| Well-watered | 0.30 ± 0.023 | 0.087 ± 0.0012 | 2.03 ± 0.03 | 0.17 ± 0.017 |
| Water stress | 0.29 ± 0.015 | 0.068 ± 0.0023* | 2.70 ± 0.07* | 0.12 ± 0.006* |
| $LSD_{0.05}$ | 0.06 | 0.0080 | 0.23 | 0.05 |
| Josefina | | | | |
| Well-watered | 0.32 ± 0.035 | 0.084 ± 0.0050 | 1.57 ± 0.02 | 0.26 ± 0.035 |
| Water stress | 0.17 ± 0.006* | 0.061 ± 0.0003* | 1.71 ± 0.08* | 0.11 ± 0.005* |
| $LSD_{0.05}$ | 0.10 | 0.0161 | 0.13 | 0.10 |
| Katalina | | | | |
| Well-watered | 0.37 ± 0.026 | 0.083 ± 0.0005 | 1.63 ± 0.12 | 0.29 ± 0.004 |
| Water stress | 0.28 ± 0.018 | 0.063 ± 0.0010* | 1.93 ± 0.12* | 0.17 ± 0.023* |
| $LSD_{0.05}$ | 0.08 | 0.0051 | 0.28 | 0.06 |
| Salomé | | | | |
| Well-watered | 0.22 ± 0.019 | 0.100 ± 0.0007 | 1.41 ± 0.06 | 0.19 ± 0.017 |
| Water stress | 0.55 ± 0.004* | 0.078 ± 0.0005* | 2.58 ± 0.06* | 0.27 ± 0.011* |
| $LSD_{0.05}$ | 0.05 | 0.0025 | 0.25 | 0.05 |
| Zarina | | | | |
| Well-watered | 0.21 ± 0.004 | 0.093 ± 0.0081 | 2.22 ± 0.04 | 0.10 ± 0.004 |
| Water stress | 0.46 ± 0.017* | 0.079 ± 0.0092 | 2.37 ± 0.01 | 0.24 ± 0.011* |
| $LSD_{0.05}$ | 0.048 | 0.0140 | 0.15 | 0.03 |

Values are mean ± S.E. ($n$ = 9) and differences between means were compared by Fisher's least-significance test (LSD; $P$ = 0.05).
* Significant difference with controls groups (well-watered).

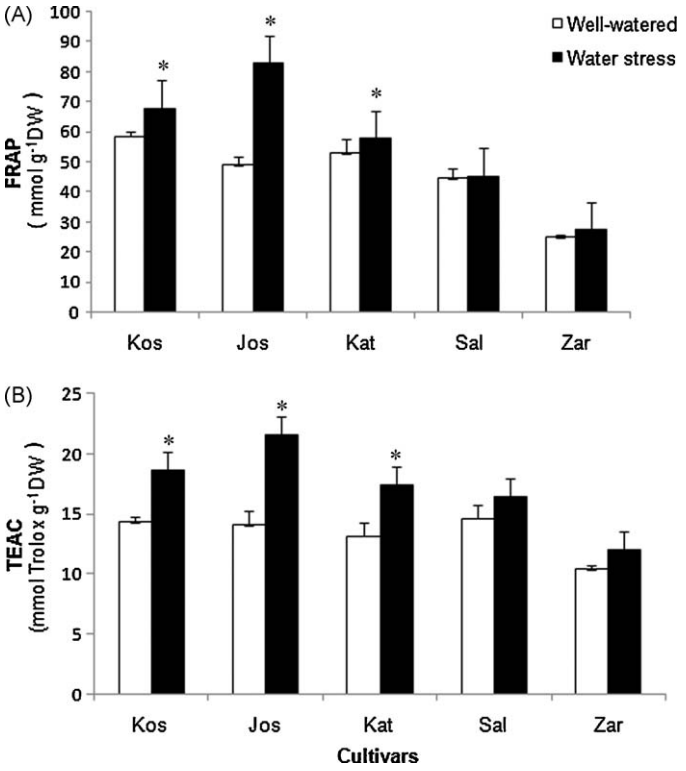


**Fig. 4.** Effect of moderate water stress on antioxidant test (A) FRAP and (B) TEAC in leaves of five tomato cultivars: 'Kosaco', 'Josefina', 'Katalina', 'Salomé' and 'Zarina'. Columns are mean ± S.E. ($n = 9$) and differences between means were compared by Fisher's least-significant difference test (LSD; $P = 0.05$). *Significant difference with control groups (well-watered).

### 2.3. Malondialdehyde concentration and $H_2O_2$

For the MDA assay, leaves were homogenized with 5 mL of 50 mM solution containing 0.07% $NaH_2PO_4{\cdot}2H_2O$ and 1.6% $Na_2HPO_4{\cdot}12H_2O$ and centrifuged at 20,000 × *g* for 25 min in a refrigerated centrifuge. For measurement of MDA concentration, 4 mL of 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added to a 1-mL aliquot of the supernatant. The mixture was heated at 95 °C for 30 min, quickly cooled in an ice bath and then centrifuged at 10,000 × *g* for 10 min. The absorbance of the supernatant was read at 532 and 600 nm. The concentration of MDA was calculated using the MDA extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ [35]. The result of MDA was expressed as nmol $g^{-1}$ DW.

The $H_2O_2$ content of leaf samples was colorimetrically measured as described by Mukherje and Choudhuri [36]. Leaf samples were extracted with cold acetone to determine the $H_2O_2$ levels. An aliquot (1 mL) of the extracted solution was mixed with 200 mL of 0.1% titanium dioxide at 20% (v/v) $H_2SO_4$ and the mixture was then centrifuged at 6000 × *g* for 15 min. The intensity of yellow colour of the supernatant was measured at 415 nm. The result of $H_2O_2$ concentration was expressed as μmol $g^{-1}$ DW.

### 2.4. Ascorbate assay

The extraction and quantification of total AsA, reduced AsA, and dehydroascorbate (DHA) followed the method of Law et al. [37] modified in turn from Okamura [38]. This method is based on the reduction of $Fe^{3+}$ to $Fe^{2+}$ by AsA in acid solution. Some 0.5 g of plant material were homogenized in liquid $N_2$ with 5 mL of metaphosphoric acid at 5% (w/v) and centrifuged at 4 °C for 15 min. Afterwards, 0.2 mL of supernatant was added to a test tube together with 0.5 mL of sodium phosphate buffer 150 mM (pH 7.5) and 0.1 mL of dithiothreitol (DTT) 10 mM. The mixture was stirred and incubated at room temperature in darkness for 10 min. Next, 0.1 mL of N-ethylmaleimide at 0.5% (w/v) was added together with 0.4 mL of orthophosphoric acid at 44% (v/v), 0.4 mL of 2,2′-bipyridyl at 4% (w/v) in ethanol at 70% and 0.2 mL of $FeCl_3$ at 3% (w/v). The resulting reaction mixture was stirred and incubated at 40 °C in darkness for 40 min. Finally, the absorbance was measured at 525 nm against a standard AsA curve that followed the same procedure as above. The results were used to quantify the total AsA concentration, while the reduced AsA was quantified in the same way as the previous procedure, replacing 0.1 mL of DTT with 0.1 mL of distilled $H_2O$. Finally, the DHA concentration was deduced from the difference between total AsA and reduced AsA. The result of ascorbate forms was expressed as mg $g^{-1}$ DW.

### 2.5. Antioxidant test

The FRAP assay was made with FRAP reagent, i.e. 1 mM TPTZ and 20 mM $FeCl_3$ in 0.25 M $CH_3COONa$, pH 3.6. An aliquot of 100 mL of extract (1 g per 10 mL in methanol) was added to 2 mL of FRAP reagent and mixed thoroughly. After the mixture was left at room temperature (20 °C) for 5 min, absorbance at 593 was measured. Calibration was against a standard curve (25–1600 mM $Fe^{3+}$) using freshly prepared ammonium ferrous sulphate [39]. The result of FRAP was expressed as mmol $g^{-1}$ DW.

The free radical scavenging capacity of extracts was determined as described by Re et al. [73] using 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonate) solution (ABTS) and 2,2′-azo-bis (2-methyl-propionamidine) dihydrochloride (AAPH), for production of the

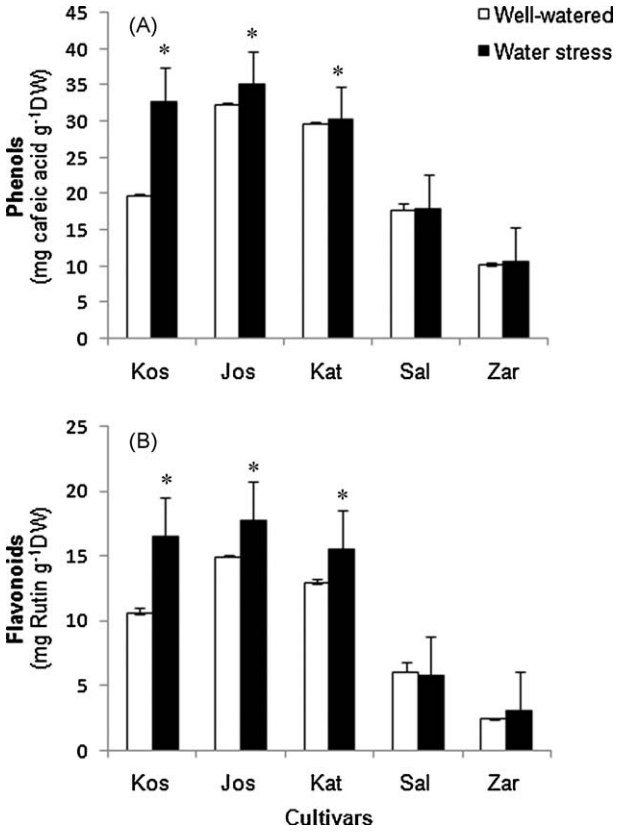


**Fig. 5.** Effect of moderate water stress on (A) phenolics and (B) flavonoids concentration in leaves of five tomato cultivars: 'Kosaco', 'Josefina', 'Katalina', 'Salomé' and 'Zarina'. Columns are mean ± S.E. ($n = 9$) and differences between means were compared by Fisher's least-significant difference test (LSD; $P = 0.05$). *Significant difference with control groups (well-watered).

ABTS radical ($ABTS^{\bullet -}$). The TEAC value of an extract represents the concentration of a Trolox solution that has the same antioxidant capacity as the extract. The result of TEAC was expressed as mmol Trolox $g^{-1}$ DW.

### 2.6. Antioxidant enzymatic activities

CAT (1.11.1.6) activity was determined by following the consumption of $H_2O_2$ at 240 nm for 5 min [40]. The reaction mixture (3 mL total volume) contained 25 mM Tris–acetate buffer (pH 7.0), 0.8 mM Na-EDTA and 20 mM $H_2O_2$, and enzyme assay was performed at 25 °C.

The enzymes APX (EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.1) were assayed following Rao et al. [41]. APX activity was determined by registering the absorbance change at 290 nm for 3 min of a reaction mixture (3.75 mL) containing 100 mM phosphate potassium buffer (pH 7.5), 0.5 mM AsA, 0.2 mM $H_2O_2$ and 0.75 mL enzyme extract. GR activity was measured after monitoring the oxidation of NADPH at 340 nm for 3 min in a reaction mixture (3.5 mL) containing 100 mM Tris–HCl (pH 7.8), 2 mM $Na_2$-EDTA, 0.2 mM NADPH, 0.5 mM GSSG and 0.75 mL enzyme extract.

Dehydroascorbate reductase activity (DHAR; EC 1.8.5.1) was measured at 265 nm for 3 min following the change in absorbance resulting from the formation of AsA [40]. The reaction mixture (3.1 mL) contained 25 mM phosphate sodium buffer (pH 7), 2.5 mM GSH, 0.4 mM DHA, and 0.1 mL enzyme extract. In addition, the enzyme monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) was assayed by registering the change in absorbance of the samples at a wavelength of 340 nm [42]. The reaction mixture (3.3 mL) contained 100 mM HEPES–HCl (pH 7.6) buffer, 2.5 mM AsA, 25 mM NADPH and 300 mL enzyme extract.

LOX activity was measured according to Minguez-Mosquera et al. [43], using 50 mM K-phosphate buffer (pH 6.0) for extraction.

The protein concentration of the extracts was determined according to the method of Bradford [44], using bovine-serum albumin as the standard.

### 2.7. Phenolic compounds

The phenols of the plant material were extracted with MeOH. Total phenolic content was assayed quantitatively by absorbance at 765 nm with Folin–Ciocalteau reagent [45]. The content in total flavonoids was measured by the colorimetric method of Kim et al. [46], with minor modifications after extraction with methanol in liquid N. The results of phenols and flavonoids concentration were expressed as mg cafeic acid $g^{-1}$ DW and mg Rutin $g^{-1}$ DW, respectively.

### 2.8. Proline concentration

For the determination of the free-proline concentration, leaves were homogenized in 5 mL of ethanol at 96%. The insoluble fraction of the extract was washed with 5 mL of ethanol at 70%. The extract was centrifuged at 3500 × *g* for 10 min and the supernatant was preserved 4 °C for the proline determination [47]. An aliquot of this supernatant was taken and, after adding reactive ninhydrin acid reagent (ninhydrin, phosphoric acid 6 M, glacial acetic acid 60%) and glacial acetic acid at 99%, was placed in a bath at 100 °C. After 45 min, the tubes were cooled and 5 mL of benzene were added, and the absorbance of the organic phase was measured. The result of proline concentration was expressed as μg $g^{-1}$ DW.

### 2.9. Quaternary ammoniums compounds

The total quaternary ammoniums were extracted from dry material in 10 mL of distilled water under a process of stirring in a vortex for 24 h. For the determination, the method used by Grive and Gratton [48] was followed. The samples were diluted with sulphuric acid 2N 1:1. Next, an aliquot was taken and a reaction mixture containing iodine and potassium iodide was added. The tubes were kept 16 h at 4 °C, after which they were centrifuged at 10,000 rpm for 15 min at 0 °C. The supernatant was discarded and 5 mL of 1,2-dichloroethanol was added to the residue and incubated 2 h. The reading was made at 365 nm against a standard betaine curve. The result of quaternary ammonium concentration was expressed as mg betaine $g^{-1}$ DW.

### 2.10. Statistical analysis

The data were analysed by a simple variance analysis (ANOVA) and differences between the means were compared by Fisher's least-significant difference test (LSD).

## 3. Results

### 3.1. Biomass and relative growth rate (RGR)

Plant growth was determined as the accumulation of total and foliar dry weight (DW) as well as total and foliar RGR. Among the five cultivars, the strongest influence of water stress was found in cv. Josefina, as it presented a decline of 40 and 28% with respect to control plants in total biomass and total RGR, respectively (Table 1). On the contrary, the cv. Zarina showed no significant

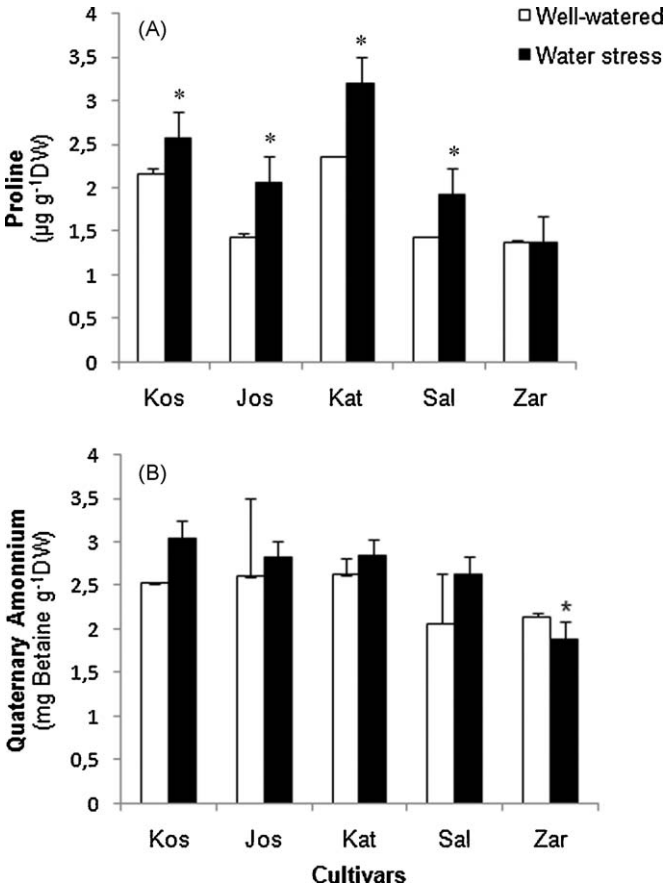


**Fig. 6.** Effect of moderate water stress on (A) proline and (B) quaternary ammonium concentration in leaves of five tomato cultivars: 'Kosaco', 'Josefina', 'Katalina', 'Salomé' and 'Zarina'. Columns are mean ± S.E. ($n$ = 9) and differences between means were compared by Fisher's least-significant difference test (LSD; $P$ = 0.05). *Significant difference with control groups (well-watered).

differences with respect to the well-watered treatment in these two growth parameters.

We found the same trend in the foliar biomass and foliar RGR, being cv. Josefina the one that presented the most pronounced decrease in these parameters under water stress (Table 1). In this case, we found significant differences in cv. Zarina, but with a decline of only 25% in foliar biomass and just 13% in foliar RGR with respect to control. These results imply a differential response to the moderate water-stress conditions in the five cultivars studied.

### 3.2. LRWC

After the water-stress treatment, except for Zarina, the others varieties showed a significant LRWC reduction as compared to their controls (Fig. 1).

### 3.3. Lipid peroxidation and $H_2O_2$ concentration

The MDA and $H_2O_2$ concentration was measured in leaves as an indicator of oxidative stress in plants. In our experiment, we found a significant increase in both parameters under stress conditions (Fig. 2A and B). The cv. Zarina presented no significant differences in either of the parameters with respect to control. In the other cultivars the increase was indeed significant, values being highest in Katalina and Josefina.

Drought significantly changed the specific activities of LOX (Fig. 2C). Significant enhancements in total LOX activity were observed in the leaves of cv. Kosaco, Josefina and Katalina under water stress, while no significant induction was observed in cv. Salome and Zarina.

### 3.4. CAT activity, forms of ascorbate and Halliwell–Asada cycle

During water stress, no significant rise in the CAT activity was noted in the cultivars, except in the cv. Zarina, where an increase of 26% was found with respect to the well-watered treatment (Fig. 3A). With regard to the enzymes of the Halliwell–Asada cycle (APX, MDHAR, DHAR, and GR), in general, a significant increase in activity was found in the cv. Zarina (Fig. 3B–E).

To complete the examination of the non-enzymatic antioxidant activity, we analysed the concentration of the different forms of AsA in leaves (Table 2). The total AsA increased in all the cultivars, except for Zarina, which did not differ with respect to control. The reduced AsA, however, augmented only in the cv. Salome and Zarina, with a rise of 145 and 112%, respectively. The DHA declined in all the cultivars, except for Zarina, where no significant differences were found between water-stressed and well-watered plants. In addition, increases of 42 and 140% were found in the reduced AsA/DHA coefficient in the cultivars Salome and Zarina, respectively.

### 3.5. Antioxidant test

The antioxidant activity of the leaves in the cultivars studied was analysed by the FRAP and TEAC tests. The results reflect a significant augment in the cultivars Kosaco, Josefina, and Katalina, this increase being greater in Josefina (Fig. 4A and B). On the contrary, in the cultivars Salome and Zarina no significant differences were found with respect to the control conditions.

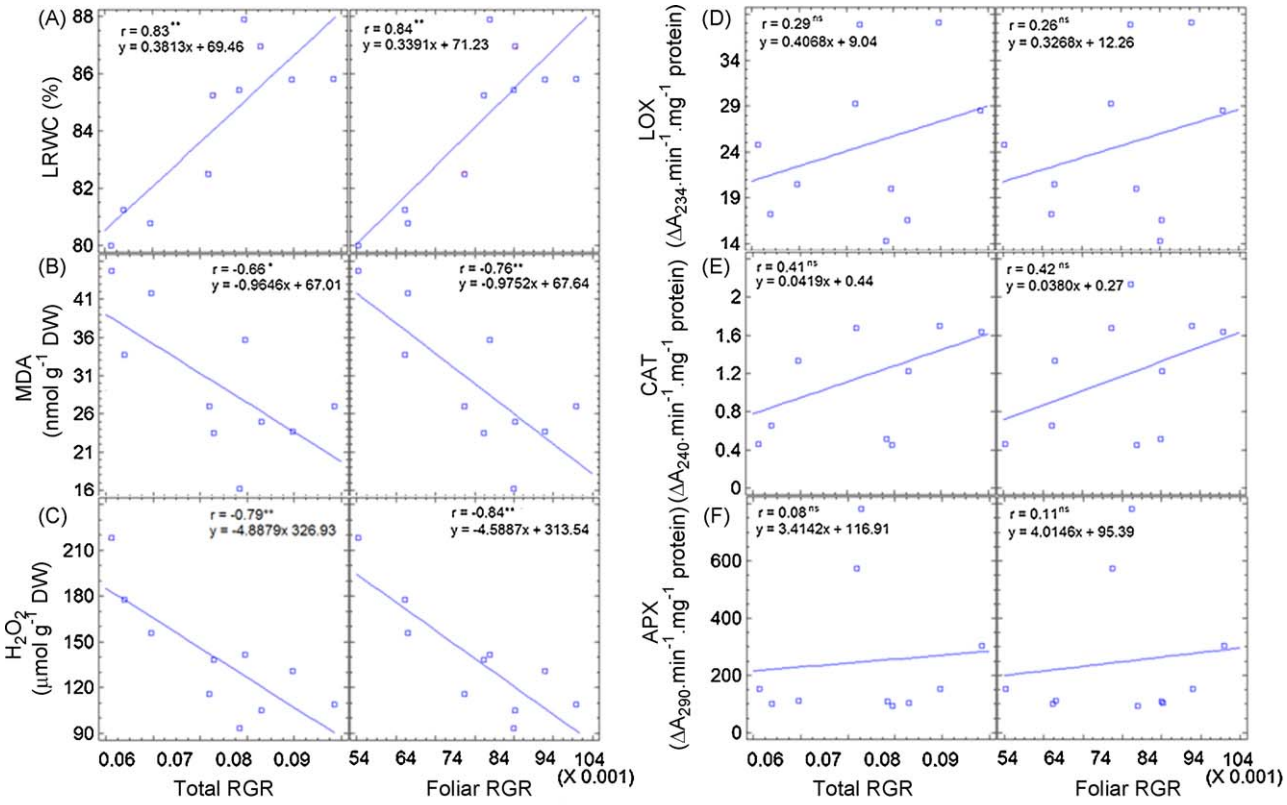


**Fig. 7.** (A–C) The relationship of RGR total and foliar with different physiological parameters. The water-stress treatments and the five genotypes assayed are included within each parameter. Each circle corresponds to the mean of nine replicates. The levels of significance were represented by $P > 0.05$: ns (not significant), $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

### 3.6. Phenolic compounds

Another metabolic process that has been related to the responses of water stress in plants involves phenolic compounds. Kosaco, Josefina and Katalina registered a significant increase under water stress in both phenolic content and flavonoids, while the other varieties showed no significant differences (Fig. 5A and B).

### 3.7. Osmoprotective compounds

To avoid water loss to stress, plants can accumulate osmolites [26]. To test for this response in cherry tomato plants submitted to water stress, we analysed the concentration of proline and QAC in leaves of the different varieties (Fig. 6A and B). In the case of proline, a significant rise in concentration was found under stress conditions in all the cultivars except for Zarina. This rise was highest in the case of the cultivars Josefina and Katalina (45 and 36%, respectively). In the case of the QAC, only the cv. Zarina declined in its concentration with respect to control.

## 4. Discussion

Growth reduction under water-stress conditions has been well characterized in plants such as pepper, potato, and tomato [5,49,50]. Also, Fazeli et al. [13] concluded that the sesame cv. Yekta was more resistant to water deficit than cv. Darab 14, based (among other factors) on the less severe loss of biomass. In our study, the less notable loss of biomass and the greatest RGR was found in Zarina. As conjectured by Fazeli et al. [13], this could be defined as the cultivar most tolerant to water deficit.

During drought stress, the plant water relations play a key role in the activation and/or modulation of antioxidant defense mechanism [51]. Hence, LRWC is considered a reliable indicator that reflects the water content in relation to maximum water content, therefore it indicates the level of hydration [52]. It has been demonstrated that water deficit diminishes LRWC in several species of plants, including chives, wheat and turfgrass [53–55]. Rampino et al. [25] identified genotypes tolerant and sensitive to water stress in based of RWC in wheat. In our study, the highest level of LRWC was found in the cv. Zarina and, as conjectured by Rampino et al. [25], this could be defined as the cultivar most tolerant to water deficit. Higher water-retaining ability during dehydration is an import strategy for acquiring resistance [54].

The MDA and $H_2O_2$ concentration was measured in leaves as an indicator of oxidative stress in plants. It was observed that water deficit raised the $H_2O_2$ concentration and the quantity of MDA in wheat plants [56]. In this sense, low concentrations in $H_2O_2$ and MDA have been associated with water-stress tolerance in pea plants and wheat [57,58]. In our experiment, we found a significant increase in both parameters under stress conditions, except in cv. Zarina. Mittler [59] suggested that the damage to cell membranes may be caused by high $H_2O_2$ levels, which could accelerate the Haber–Weiss reaction, increasing the formation and therefore prompting lipid peroxidation. Our data suggest that the low level peroxidation (denoted by the MDA concentration) in cv. Zarina was due to a lower accumulation of $H_2O_2$, thus indicating greater water-stress tolerance than in the rest of the cultivars studied.

It was proved that singlet oxygen and superoxide anions can be formed during the LOX-catalysed oxidation of fatty acids [60]. Increased LOX activities are interpreted as reasons for an increased lipid peroxidation under stress conditions [61]. Our results show increased activity in those cultivars that in turn presented higher MDA contents. The cv. Zarina did not present higher LOX activity, this perhaps related to a lower quantity of MDA and $H_2O_2$.

APX and CAT provide efficient $H_2O_2$ scavenging. Various responses of these enzymes to water stress have been reported. Decreased CAT activities under water stress have been observed in

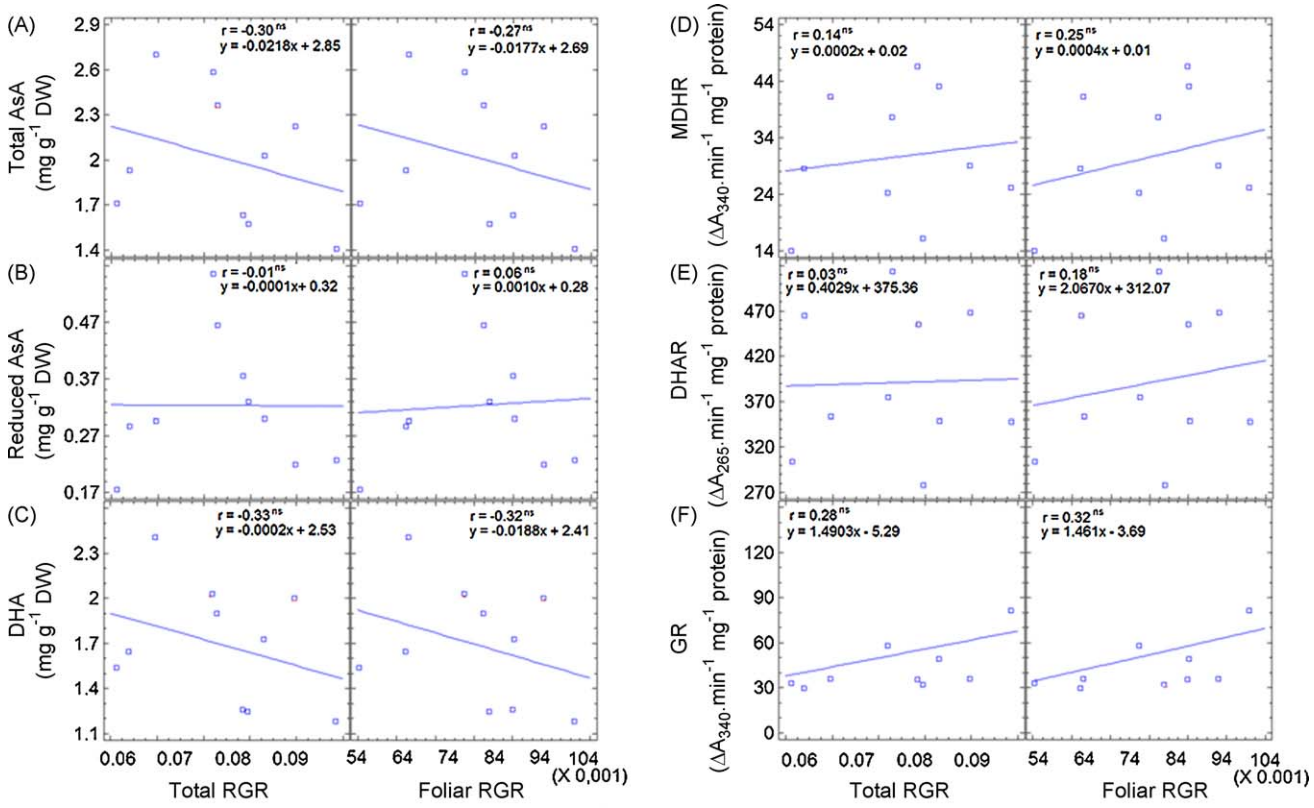


Fig. 7. (*Continued*)

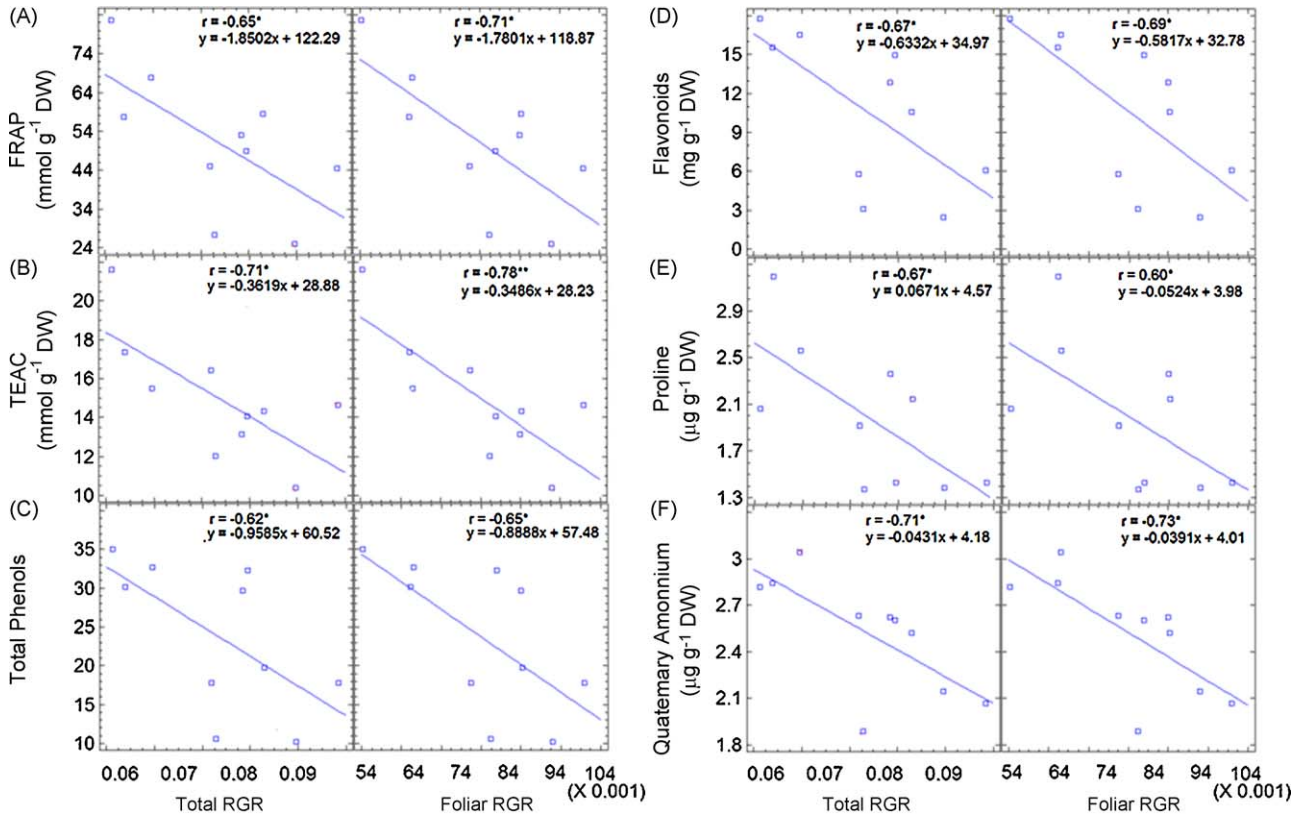


Fig. 7. (*Continued*).

sunflower, wheat and tomato [62,63,6]. Other authors demonstrated that high activity of these enzymes conferred tolerance to water deficit in several species of plants such as moss, *Poa pratensis* and maize [12,53,64]. In agreement with these results, we found that the joint activity of these enzymes in the cv. Zarina would have had an important role in preventing the formation of ROS, and therefore the appearance of excessive damage by oxidative stress, achieving better water-deficit tolerance.

The antioxidant levels of a plant are also a good indicator of the redox state, which is indispensable for the stress tolerance development. Previous studies demonstrated an increase in the AsA and GSH content in different plant species subjected to water deficit, such wheat and maize [65]. Also, higher increases in these compounds have been confirmed in plants acclimated to drought stress [18]. On the other hand, a high ratio of reduced AsA to DHA is essential to eliminate ROS in cells [66]. In agreement with this, Zarina and Salomé registered the highest values for this ratio, and therefore appear to present a greater capacity to eliminate ROS. The regeneration of oxidized AsA (MDHA or DHA) to reduced AsA is also a highly important process in the antioxidant response. This regeneration involves the enzymes MDHAR, DHAR, and GR, which are found in most cell compartments [67]. In our study, cv. Zarina had the highest values for these enzymes, while Josefina and Kosaco had the lowest. In previous studies, it was confirmed that water-stress-tolerant *Hordeum* species show high GR and APX activity [14]. In short, these results attribute cv. Zarina with the greatest activation of the Halliwell–Asada cycle, indicating a specific response of the plant to water deficit. This response would maintain the levels of reduced AsA, thereby facilitating the detoxification of ROS during the stress episode. In general, the capacity to detoxify ROS has been related to stress tolerance in some earlier studies [54].

Another metabolic process that has been related to the responses of water stress in plants is that of phenolic compounds. Phenols and their metabolism during this type of stress are utilized to prevent the formation of ROS [68]. On the contrary of what occurred with the enzymes and antioxidant compounds, in the case of the phenols, the highest values corresponded to the cultivars most sensitive to water deficit (Kosaco, Josefina, and Katalina). These data agree with those of Hura et al. [23], who reported an increase in phenols in maize plants subjected to water deficit. In a previous study, the same authors found that an increase in phenol content was more pronounced in genotypes sensitive to water stress [22]. Therefore, our results confirmed that the accumulation of phenols is not a tolerance mechanism, but can be used as a stress indicator, since its accumulation is found in the tomato cultivars most sensitive to water deficit.

In recent years, a number of tests have been developed for the general study of the antioxidant capacity of different plants extracts. These assays measured the ability to reduce pro-oxidant substances by using different radicals or ionic methyls as oxidants [69]. Some of the most widely used tests to measure the antioxidant capacity are the FRAP and TEAC test, which in our work behaved similarly to the different antioxidant compounds analysed (ascorbate and phenolic compounds).

To avoid water loss during stress, plants can accumulate osmolites [26]. To test for this response in cherry tomato plants subjected to water stress, we analysed the concentration of proline and QAC. Proline can have various functions under stress conditions, as a mediator of osmotic adjustment, a stabilizer of subcellular structures, an eliminator of free radicals, and a buffer of the redox potential and also it can be an important component of cell-wall proteins [28–31,70]. Fig. 6A shows that the cv. Zarina presented the lowest values of this amino acid, while the highest corresponded to Kosaco, Katalina, and Josefina, the cultivars most sensitive to water stress. These data disagree with those of authors who indicate that a higher proline accumulation results in greater water-stress tolerance [28]. However, our results support the

observation of Hanson [32] that proline is not an adaptive feature but only a stress symptom. In this sense, Rampino et al. [25] found that drought-tolerant wheat plants showed a higher RWC related to a lower proline concentration. QAC accumulate rapidly in plants in response to environmental stress, including salinity, drought, and low temperatures [71]. Our data indicate that the cv. Zarina presented the lowest concentration in QAC, while the highest values again corresponded to Kosaco, Katalina, and Josefina. Our data indicated that as in the case of proline and under our experimental conditions, the accumulation of QAC constituted a stress symptom. These results agree with those of Alian et al. [72] in tomato, where greater water-stress tolerance was not correlated with greater osmotic adjustment.

To determine the relationship between the different parameters indicative of oxidative stress and the foliar and total RGR growth parameters under moderate water stress, we studied the correlation coefficients. Our aim was to identify the most reliable indicators of water-stress resistance to be used as selection criteria for sensitive and tolerant cultivars. We found a positive correlation with LRWC, and a negative one with the parameters MDA, $H_2O_2$, test antioxidants, phenolic content, proline and ammoniums (Fig. 7A–C).

In conclusion, we found that, as in other cultivars, tomato presented genotypic differences in the response to oxidative stress, triggered by water deficit. The cv. Zarina presented a better RGR together with minor oxidative damage associated with a stronger response of antioxidant enzymes. Similarly, we conclude that drought tolerance does not always correlate with the osmotic potential of tissues, implying that the osmotic adjustment is not the only process that influences tolerance. Although more studies are needed, the cv. Zarina could be considered adequate in programmes to improve the development of more drought-tolerant plants. With respect to the correlation coefficients related to growth parameters, we found that under the water-stress conditions studied, there was a positive relationship between total and foliar RGR with LRWC and a negative one with MDA, $H_2O_2$, test antioxidants, phenolic content, proline, and ammoniums. Therefore, we propose these parameters as biochemical indicators for the selection of cultivars that are less susceptible to moderate water stress.

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REGULAR ARTICLE

# Study of the ionome and uptake fluxes in cherry tomato plants under moderate water stress conditions

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**Abstract** Nutritional imbalance under water-deficit conditions depresses plant growth by affecting nutrient uptake, transport, and distribution. The present work analyses the variations in the foliar concentrations of macro- and micronutrients as well as the transport of these nutrients in five cherry tomato cultivars under well-watered and moderately water-stressed conditions with the aim of establishing whether the ionome of the plants is related to the degree of sensitivity or tolerance to this type of stress. The results show a general reduction in growth together with a lower concentrations and uptake both of macro- as well as micronutrients in all the cultivars studied, except for cv. Zarina, which showed better growth and increased in concentrations and uptake nitrogen, phosphorus, magnesium, potassium, and chloride with respect to control plants. In conclusion, in this work, our results suggest that a better understanding of the role of the mineral elements in plant resistance to drought could improve fertilization in arid and semi-arid regions in order to increase the tolerance of plants grown under these conditions.

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**Keyword** Mineral nutrients · Ionome · Drought · Tomato

**Abbreviations**

| | |
|---|---|
| LRWC | Leaf relative water content |
| RGR | Relative growth rate |

## Introduction

The study of the mineral composition of a plant and the changes in this composition in response to physiological and environmental stimuli, the plant's developmental state, and genetic modifications, has been recently defined as ionome (Salt et al. 2008). Therefore, the ionome can provide information on the functional state of an organism under different growth conditions.

The increased frequency of dry periods in many regions of the world leads to droughts in cultivated lands. Water is generally considered to be one of the limiting factors that affect numerous metabolic, physiological, and biochemical processes involved in crop productivity (Turner and Kramer 1980). Although the water absorption and nutrient uptake are independent processes in the root, the need for available water for growth and nutrient transport makes them intimately related (Viets 1972). Generally, drought reduces not only nutrient uptake by the root but also nutrient transport from the root to the

shoot due to a restricted transpiration rate, depressed active transport, and reduced membrane permeability. The overall result is that the uptake power of the plant is diminished (Hsiao 1973; Kramer and Boyer 1995).

It is difficult to clarify the potential effect of water stress in mineral uptake and its accumulation in cultivated plants. Several studies have demonstrated the effect of the species and genotype in the concentrations of nutrients (Alam 1999) while other studies indicate that nutrient uptake falls when water stress intensifies in plants such as rice, corn, soy, and wheat (Tanguilig et al. 1987; Hu et al. 2006). In tomato plants, there is evidence that factors such as salinity and light intensity affect nutrient uptake (Fernández-García et al. 2004; Gent and Ma 1998). A better understanding of the role of mineral nutrients in the resistance of plants to drought could improve the application of fertilizers in arid and semi-arid zones, and in regions that undergo temporary drought (Hu and Schmidhalter 2005).

Given that the tomato cultivar is concentrated in semi-arid zones where water stress is frequent, it is important to ascertain how this type of stress affects the ionome of this plant. In addition, the flow of nutrients taken up can be a useful index of the effect of this type of stress in the plant. Therefore, the aim of the present work was to assess the affect of moderate water stress in terms of uptake and concentration of nutrients and identify any differences in this respect in the five cherry tomato genotypes studied.

## Material and methods

### Plant material and growth conditions

Five cherry tomato cultivars were used: Kosaco, Josefina, Katalina, Salomé, and Zarina [*Solanum lycopersicum* L. (*Lycopersicon esculentum* Mill.)]. The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm×3 cm×10 cm) in the nursery Semillero Saliplant S.L. (Carchuna, Granada). Afterwards, the seedlings were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with relative humidity 50±10%, at 25°C/15°C (day/night), and a 16 h/8 h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 $\mu mol^{-2}\ s^{-1}$ (measured with an SB quantum 190 sensor, LI—COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. Throughout the experiment, the plants were grown in a complete nutrient solution (Sánchez-Rodríguez et al. 2010). The water-stress treatments began 45 days after germination and maintained 22 days. The control treatment received 100% field capacity irrigation, whereas moderate water stress corresponded to 50% field capacity (FC). The latter was selected to represent water deficit stress on the basis of a preliminary experiment carried out on *S. lycopersicum* watered with 100, 75, 50 and 25% FC, showing that 50% irrigation procedure led to a significant decrease of growth. Independently of the procedure for watering (100 or 50% FC), plants received the same quantity of nutrients.

### Determination of mineral nutrients

The nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), boron (B), and copper (Cu), were mineralized by wet digestion, following Wolf (1982). For this, 0.2 g of dry roots and leaves were ground and mineralized with $H_2SO_4$ at 98% and $H_2O_2$ at 30%, at temperature of 275 to 300°C. From the resulting mineralization, and after the addition of 20 mL of deionized $H_2O$, the mineral nutrients were determined as described above.

The reduced N concentration was determined by colorimetry based on the Berthelot reaction, with slight modifications (Krom 1980).

The total P concentration was determined using the colorimetric nitrovanadomolybdate method of Hogue et al. 1970 while the total K concentration was analyzed by flame photometry (Lachica et al. 1973).

The total Mg and Ca concentration were quantified by atomic absorption spectrophotometry (Hocking and Pate 1977), as were the micronutrients, Fe, Mn, Cu and Zn. The total B concentration was determined by the colorimetric method of azomethine-H (Wolf 1974). Reduced S was extracted by mineralization with nitric/percholic acid. For this, a quantity of 0.2 g of cherry tomato ground dry was digested with a mixture of $HNO_3/HClO_4$ (v/v) and $H_2O_2$ at 30%. The reduced S was determined on an aliquot of the

mineralization using $BaSO_4$ in suspension by means of a surfactant agent such as gum arabic, all this against a standard curve of $K_2SO_4$ and a turbidmetric reading at 435 nm (Novozamsky and Vaneck 1977).

The Cl, $NO_3^-$, $PO_4^{3-}$, $SO_4^{2-}$, soluble K, Mg and Ca concentrations in roots and leaves were determined from an aqueous extraction following the method of Cataldo et al. (1975) with slight modifications. The Cl concentration was determined by the method of Diatloff and Rengel (2001), based on the displacement of biocyanate by chloride, which in the presence of $Fe^{3+}$ forms the highly colored complex ferric thiocyanate. The determination of $NO_3^-$ was based on a colorimetric reaction formed by the bonding with salicylic acid in a basic medium (Cataldo et al. 1975). The total N concentration was determined as the sum of the reduced N and $NO_3^-$ concentrations. The $PO_4^{3-}$ was determined following the method of Hogue et al. (1970). The $SO_4^{2-}$ was determined following the method of Novozamsky and Vaneck (1977). The total S corresponded to the sum of the concentrations of reduced S and $SO_4^{2-}$. Soluble Mg and Ca were quantified by atomic absorption spectrophotometry (Hocking and Pate 1977), while the soluble K concentration was analyzed by flame photometry (Lachica et al. 1973).

The soluble Fe, Mn, Cu and Zn concentrations were determined from an HCl 1 M extraction following the method of Cataldo et al. (1975) with slight modifications. These nutrients were quantified by atomic absorption spectrophotometry (Hocking and Pate 1977)

### Determination of uptake fluxes

Over the period under study, determination of nutrients uptake fluxes were calculated from the RGR, the fresh weight (FW), the nutrient total concentrations, and soluble nutrient concentration contents of leaves (l) and roots (r) as folllows (Kruse et al. 2007):

$$(\text{Total Nutrient})_r = \text{RGR}^*\text{FW}_r^*[\text{Nutrient}]_r \quad (1)$$

$$(\text{Total Nutrient})_l = \text{RGR}^*\text{FW}_l^*[\text{Nutrient}]_l \quad (2)$$

$$(\text{Reduced Nutrient})_r = \text{RGR}^*\text{FW}_r^*[\text{Total nutrient} - \text{Soluble nutrient}]_r \quad (3)$$

$$(\text{Reduced Nutrient})_l = \text{RGR}^*\text{FW}_l^*[\text{Total nutrient} - \text{Soluble nutrient}]_l \quad (4)$$

The following fluxes were determined from experimental data:

$$\begin{aligned} J^{\text{Upt}}\text{Nutrient} = {} & (\text{Total Nutrient})_r \\ & + (\text{Total Nutrient})_l \\ & + (\text{Reduced Nutrient})_r \\ & + (\text{Reduced Nutrient})_l \end{aligned} \quad (5)$$

### Statistical analysis

The data compiled were subjected to a simple ANOVA at 95% confidence. A two-tail ANOVA was applied to ascertain whether the cultivar and treatment applied significantly affected the results, and the means were compared by Fisher´s least-significant differences (LSD).

## Results

### Mineral nutrient accumulation in leaves

The concentration of mineral macronutrients (N, P, S, Ca, Mg, and K) in cherry tomato leaves for the five cultivars studied are presented in Table 1. All the cultivars presented a reduced N concentration under water-stress conditions, except for cv. Zarina, which registered a 27% increase with respect to well-watered conditions, and Katalina, which was no significant. Meanwhile, water deficit generally lowered or maintained the P concentration, except in cv. Zarina, which presented an increase of 177%. For S, concentrations fell in all three cultivars significantly, except in cv. Katalina. No significant increase were detected in the Ca concentration under stress in comparison to well-watered plants. As in the case of N and P, Zarina was the only cultivar to present an increase in the Mg and K concentration (16% and 7%, respectively), whereas the rest of the cultivars showed no differences or even concentrations declined in these elements under water-stress conditions (Table 1).

The concentrations of mineral micronutrients (Fe, Cu, Mn, Zn, Cl, and B) in leaves are reflected in

**Table 1** Foliar concentration of macronutrients (mg $g^{-1}$ DW) in plants of 5 cultivars of cherry tomato plants well-watered and subjected to moderate water stress

| Cultivar/water treatment | N | P | S | Ca | Mg | K |
|---|---|---|---|---|---|---|
| Kosaco | | | | | | |
| Well-watered | 20.9±2.7 | 7.4±0.7 | 35.1±1.5 | 16.8±1.3 | 2.2±0.1 | 21.8±1.3 |
| Water stress | 16.8±1.5*[a] | 7.7±0.8 | 30.9±1.3 | 15.0±0.2 | 2.1±0.7 | 19.4±0.9 |
| $LSD_{0.05}$ | 1.5 | 2.4 | 4.9 | 3.7 | 0.4 | 4.4 |
| Josefina | | | | | | |
| Well-watered | 18.3±3.2 | 7.2±0.9 | 35.1±0.6 | 14.4±0.3 | 1.9±0.3 | 17.6±1.5 |
| Water stress | 16.3±2.4* | 6.8±0.8 | 30.2±0.8* | 13.6±0.4 | 1.8±0.3 | 16.0±0.7 |
| $LSD_{0.05}$ | 2.1 | 2.6 | 2.7 | 1.4 | 0.1 | 4.8 |
| Katalina | | | | | | |
| Well-watered | 17.7±3.9 | 5.3±0.7 | 36.3±0.8 | 14.0±0.2 | 2.6±0.2 | 17.7±01 |
| Water stress | 17.4±4.1 | 3.6±0.1* | 37.1±0.6 | 14.1±0.2 | 1.7±0.3* | 16.9±0.2 |
| $LSD_{0.05}$ | 1.3 | 1.6 | 2.4 | 1.0 | 0.1 | 0.7 |
| Salomé | | | | | | |
| Well-watered | 19.4±5.4 | 4.6±0.3 | 38.3±0.8 | 14.1±0.2 | 2.3±0.5 | 19.4±0.7 |
| Water stress | 16.2±2.2* | 4.8±0.1 | 30.1±0.8* | 13.9±0.3 | 2.6±0.7 | 18.2±0.7 |
| $LSD_{0.05}$ | 2.1 | 0.7 | 3.1 | 1.1 | 0.2 | 2.8 |
| Zarina | | | | | | |
| Well-watered | 15.7±2.5 | 3.2±0.4 | 31.5±0.8 | 12.9±0.6 | 2.3±0.2 | 18.7±0.2 |
| Water stress | 20.0±3.1* | 8.9±0.2* | 25.5±1.5* | 12.6±0.2 | 2.7±0.5* | 20.1±0.4* |
| $LSD_{0.05}$ | 3.1 | 4.7 | 4.3 | 1.7 | 0.1 | 1.3 |
| | | | Analysis of variance | | | |
| Cultivars (C) | ns | ** | *** | *** | *** | *** |
| Treatments (T) | *[b] | * | ** | ns | * | ns |
| CxT | *** | ** | ns | ns | *** | ns |
| LSD | 14.08 | 0.18 | 20.01 | 1.11 | 0.12 | 8.94 |

Values are mean±S.E. ($n$=9) and differences between means were compared by Fisher´s least-significance test (LSD; $P$=0.05)

[a] Asterisk (*) indicates significant difference with controls groups (well-watered)

[b] The levels of significance were represented by $p>0.05$: ns (not significant), $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

Table 2. No significant differences were found for Fe in any of the cultivars under stress conditions, except for cv. Zarina. However, Cu increased in concentration in the cultivars Salomé and Zarina under water deficit by 44 and 38%, respectively. In all the cultivars the Mn concentration declined, although less in the case of cv. Zarina proportionally. Generally, neither Zn nor B registered significant differences under water-stress conditions with respect to well-watered plants, excepted in cv. Kosaco for Zn and B, and in cv. Katalina for B. However, in the case of Cl, Zarina was the only cultivar to present a higher concentration under water deficit (Table 2).

## Uptake fluxes in mineral nutrient

The data for macronutrient uptake by the cherry tomato plants studied are presented in Table 3. Both in N and the P, uptake diminished in all varieties with respect to control, except for cv. Zarina under stress conditions, where transport increased. This can be correlated with the greater concentration of these two nutrients in the leaf of Zarina under tress conditions. In the case of Ca and S, all the cultivars registered a decline, though this trend was less pronounced in Zarina. Finally, no significant differences in uptake of Mg and K in Zarina were found under stress conditions, while in the rest of the cultivars values sharply fell (Table 3).

For the micronutrients, cv. Zarina significantly increased in uptake of Fe and Cl under stress, by 18 and 24%, respectively. For the rest of the micronutrients, all the cultivars registered a general decline in transport under stress with respect to control conditions (Table 4).

**Table 2** Foliar concentration of micronutrient (μg $g^{-1}$ DW) in plants of 5 cultivars of cherry tomato plants well-watered and subjected to moderate water stress

| Cultivar/water treatment | Fe | Cu | Mn | Zn | Cl (mg $g^{-1}$ DW) | B |
|---|---|---|---|---|---|---|
| Kosaco | | | | | | |
| Well-watered | 242.2±4.7 | 300.2±7.3 | 343±41 | 26.1±1.7 | 25.5±0.3 | 125±2.8 |
| Water stress | 249.5±8.6 | 200.2±15.7* | 122±42* | 6.3±1.5* | 20.5±0.7* | 99±3.2* |
| $LSD_{0.05}$ | 27.4 | 48.1 | 165.6 | 6.5 | 1.6 | 9.0 |
| Josefina | | | | | | |
| Well-watered | 210.4±37.4 | 284.4±12.4 | 258±11 | 14.6±0.8 | 22.8±0.1 | 124±3.9 |
| Water stress | 194.3±9.3 | 136.2±1.7* | 124±14* | 16.7±7.8 | 21.2±0.6* | 127±4.6 |
| $LSD_{0.05}$ | 17.08 | 35.0 | 51.4 | 22.0 | 1.5 | 12.9 |
| Katalina | | | | | | |
| Well-watered | 281.0±56.6 | 144.3±14.0 | 572±53 | 10.5±0.5 | 20.2±0.1 | 123±3.7 |
| Water stress | 293.8±6.9 | 163.5±16.1 | 500±24* | 11.6±2.8 | 20.3±0.1 | 95±10.5* |
| $LSD_{0.05}$ | 18.4 | 59.3 | 42.8 | 8.1 | 0.3 | 23.7 |
| Salomé | | | | | | |
| Well-watered | 245.2±6.4 | 138.7±11.4 | 646±39 | 14.9±1.5 | 18.3±0.2 | 80.1±5.9 |
| Water stress | 268.4±11.3 | 199.6±7.3* | 530±55* | 19.0±3.0 | 16.9±0.1* | 88.9±6.8 |
| $LSD_{0.05}$ | 36.3 | 37.8 | 88.0 | 9.3 | 0.6 | 19.1 |
| Zarina | | | | | | |
| Well-watered | 174.1±2.3 | 352.4±9.9 | 231±14 | 21.0±0.2 | 19.2±0.5 | 75.9±6.6 |
| Water stress | 232.1±21.7*[a] | 488.7±14.5* | 172±3* | 28.5±3.0 | 21.0±0.6* | 77.2±4.5 |
| $LSD_{0.05}$ | 30.8 | 48.8 | 42.0 | 8.4 | 1.6 | 16.9 |
| | | | Analysis of variance | | | |
| Cultivars (C) | ns | *** | *** | ** | *** | *** |
| Treatments (T) | *[b] | ns | *** | ns | *** | ns |
| CxT | ns | *** | ns | ** | *** | *** |
| LSD | 48.8 | 24.7 | 72.7 | 6.5 | 0.8 | 11.3 |

Values are mean±S.E. ($n$=9) and differences between means were compared by Fisher´s least-significance test (LSD; $P$=0.05)

[a] Asterisk (*) indicates significant difference with controls groups (well-watered)

[b] The levels of significance were represented by $p>0.05$: ns (not significant), $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

## Discussion

In our previous work (Sánchez-Rodríguez et al. 2010), we determined that the cultivar Zarina presented a higher RGR and LRW under water-stress conditions, than the rest of the cultivars studied. Therefore, in agreement with other authors (Fazeli et al. 2007; Rampino et al. 2006; Selote and Khanna-Chopra 2006), this cultivar was defined as more tolerant to moderate water deficit.

Nitrogen is required by plants in large quantities and is essential in the biochemistry of non-enzymatic compounds such as coenzymes, photosynthetic pigments, secondary metabolites, and polyamines (Maathuis 2009). Under water-stress conditions, it has been showed that N uptake diminishes in soy and rice plants (Tanguilig et al. 1987), wheat (Hu et al. 2006), and beans (Zayed and Zeid 1997). The decline in N uptake can be attributed to a lower rate of transpiration and N transport from the root to the shoot (Alam 1999). Our results also show a lower N concentration and uptake under water deficit in all the cultivars, except for cv. Zarina, where values increased (Tables 1 and 3). Barnett and Naylor (1966) argued that the high N levels in crops subjected to water deficit were owed fundamentally to a rapid accumulation of free amino acids or proteins. In turn, this higher N concentration in the cv. Zarina could be related to the higher RGR in

**Table 3** Uptake fluxes of macronutrients (mg plant$^{-1}$ day$^{-1}$) in plants of 5 cultivars of cherry tomato plants well-watered and subjected to moderate water stress

| Cultivar/water treatment | N | P | S | Ca | Mg | K |
|---|---|---|---|---|---|---|
| Kosaco | | | | | | |
| Well-watered | 242.1±9.0 | 150.2±4.0 | 74.7±1.3 | 217.4±14.1 | 42.6±0.4 | 291.3±10.1 |
| Water stress | 111.2±7.6*[a] | 81.4±5.2* | 36.4±1.0* | 103.2±2.1* | 22.3±1.0* | 139.2±6.3* |
| $LSD_{0.05}$ | 32.7 | 18.4 | 4.7 | 39.7 | 3.1 | 33.2 |
| Josefina | | | | | | |
| Well-watered | 190.5±9.9 | 131.6±16.5 | 67.6±0.9 | 163.5±2.8 | 34.4±0.6 | 202.3±15.1 |
| Water stress | 89.4±1.6* | 65.4±2.7* | 31.7±1.0* | 79.1±1.7* | 16.5±0.2* | 98.3±4.7* |
| $LSD_{0.05}$ | 27.9 | 46.5 | 3.8 | 9.3 | 1.7 | 44.1 |
| Katalina | | | | | | |
| Well-watered | 218.6±2.1 | 124.7±10.9 | 80.1±1.3 | 173.2±2.9 | 55.1±0.6 | 247.5±0.8 |
| Water stress | 101.9±4.0* | 44.2±1.6* | 38.1±0.8* | 90.7±1.3* | 17.2±0.0* | 113.4±1.6* |
| $LSD_{0.05}$ | 12.5 | 30.8 | 4.3 | 9.1 | 1.7 | 5.0 |
| Salomé | | | | | | |
| Well-watered | 326.5±30.5 | 149.6±9.7 | 108.7±3.0 | 258.7±3.6 | 69.5±1.3 | 371.8±13.7 |
| Water stress | 119.7±4.7* | 70.4±0.8* | 43.5±0.3* | 111.2±2.5* | 33.4±0.4* | 149.7±4.4* |
| $LSD_{0.05}$ | 85.9 | 27.2 | 8.47 | 12.4 | 3.9 | 40.1 |
| Zarina | | | | | | |
| Well-watered | 181.6±16.5 | 100.4±11.2 | 76.6±3.0 | 182.6±6.4 | 43.3±0.5 | 231.6±2.7 |
| Water stress | 206.2±23.1* | 124.7±35.7* | 46.7±0.8* | 124.7±2.1* | 41.1±0.7 | 227.2±3.9 |
| $LSD_{0.05}$ | 18.9 | 20.0 | 8.8 | 18.8 | 3.7 | 13.2 |
| | | Analysis of variance | | | | |
| Cultivars (C) | ***[b] | ns | *** | *** | *** | *** |
| Treatments (T) | *** | *** | *** | *** | *** | *** |
| CxT | *** | ** | *** | *** | *** | *** |
| LSD | 29.7 | 29.1 | 3.4 | 11.2 | 1.4 | 16.5 |

Values are mean±S.E. ($n$=9) and differences between means were compared by Fisher´s least-significance test (LSD; $P$=0.05)

[a] Asterisk (*) indicates significant difference with controls groups (well-watered)

[b] The levels of significance were represented by $p>0.05$: ns (not significant), $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

this cultivar under stress conditions. Given that the N uptake showed genetic variation, the selection of more efficient lines in taking up N could be a good strategy in arid and semi-arid zones (Bänziger et al. 2002).

Phosphorus is essential for plant growth, cell-energy homeostasis (ATP), nucleic acid formation, and reversible protein phosphorylation (Maathuis 2009). Our data reflect an augment in the P concentration associated with better uptake under stress conditions only in cv. Zarina (Tables 1 and 3). The positive effects of P in the growth of plants under water stress have been attributed to increased water-use efficiency, stomal conductance (Brück et al. 2000), photosynthesis (Ackerson 1985), high cell-membrane stability, and water relationships (Sawwan et al. 2000).

Sulphur intervenes in the production of glutation, in addition to forming part of the sulpholipids that are essential for stabilization of photosynthetic compounds (Maathuis 2009). In the case of S, all the cultivars showed a lower concentration and uptake under stress conditions with respect to well-watered plants (Tables 1 and 3). Our results agree with those reported for wheat (Hu et al. 2006) and beans (Zayed and Zeid 1997).

Calcium has structural functions and acts as the second messenger (Maathuis 2009). No significant

**Table 4** Uptake fluxes of micronutrients (mg plant$^{-1}$ day$^{-1}$) in plants of 5 cultivars of cherry tomato plants well-watered and subjected to moderate water stress

| Cultivar/water treatment | Fe | Cu | Mn | Zn | Cl | B |
|---|---|---|---|---|---|---|
| Kosaco | | | | | | |
| Well-watered | 5.6±0.14 | 4.5±0.08 | 4.6±0.4 | 0.5±0.08 | 304.4±7.0 | 5.7±0.4 |
| Water stress | 2.7±0.19*[a] | 1.5±0.08* | 1.0±0.2* | 0.2±0.06* | 131.5±8.4* | 3.7±0.1* |
| $LSD_{0.05}$ | 0.68 | 0.33 | 1.3 | 0.03 | 30.4 | 1.3 |
| Josefina | | | | | | |
| Well-watered | 4.6±0.14 | 3.7±0.12 | 3.1±0.1 | 0.3±0.03 | 237.7±1.4 | 6.6±0.1 |
| Water stress | 2.0±0.30* | 0.9±0.01* | 0.7±0.0* | 0.2±0.01* | 114.6±6.9* | 3.4±0.2* |
| $LSD_{0.05}$ | 0.92 | 0.35 | 0.3 | 0.04 | 19.7 | 0.6 |
| Katalina | | | | | | |
| Well-watered | 5.0±0.39 | 2.3±0.13 | 9.3±0.6 | 0.4±0.01 | 248.4±3.6 | 7.9±0.2 |
| Water stress | 2.8±0.18* | 1.2±0.07* | 3.0±0.1* | 0.4±0.04 | 119.7±0.6* | 3.2±0.2* |
| $LSD_{0.05}$ | 1.20 | 0.42 | 1.7 | 0.13 | 10.2 | 1.0 |
| Salomé | | | | | | |
| Well-watered | 7.4±0.80 | 2.90±0.19 | 11.6±0.5 | 0.5±0.00 | 313.2±6.5 | 8.7±0.1 |
| Water stress | 3.7±0.47* | 2.7±0.08 | 4.2±0.4* | 0.2±0.01* | 126.1±2.0* | 3.9±0.1* |
| $LSD_{0.05}$ | 0.26 | 0.58 | 1.9 | 0.03 | 19.0 | 0.5 |
| Zarina | | | | | | |
| Well-watered | 3.8±0.09 | 5.5±0.11 | 3.5±0.1 | 0.4±0.00 | 195.6±8.4 | 5.8±0.2 |
| Water stress | 4.5±0.30* | 5.9±0.12 | 2.0±0.1* | 0.3±0.02* | 241.7±5.4* | 4.7±0.1* |
| $LSD_{0.05}$ | 0.69 | 0.45 | 0.4 | 0.06 | 27.8 | 0.7 |
| | | Analysis of variance | | | | |
| Cultivars (C) | ***[b] | *** | *** | *** | *** | *** |
| Treatments (T) | *** | *** | *** | *** | *** | *** |
| CxT | *** | *** | *** | *** | *** | *** |
| LSD | 0.7 | 0.2 | 0.7 | 0.3 | 0.1 | 0.4 |

Values are mean±S.E. ($n$=9) and differences between means were compared by Fisher´s least-significance test (LSD; $P$=0.05). Asterisk (*) indicates significant difference with controls groups (well-watered)

[a] Asterisk (*) indicates significant difference with controls groups (well-watered)

[b] The levels of significance were represented by $p>0.05$: ns (not significant), $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

differences were found in terms of Ca accumulation in leaves submitted to water deficit, although its uptake was significantly affected in all the cultivars studied (Tables 1 and 3). These data support the idea that although the uptake of Ca diminishes with the water deficit under drought conditions, its accumulation descends very slightly in comparison with P and K (Hu and Schmidhalter 2005).

Magnesium occupies a central position in the chlorophyll molecule and intervenes as an enzymatic cofactor, while K acts fundamentally as an osmotic mediator and helps maintain turgour (Hu and Schmidhalter 2005; Maathuis 2009). In our work, both elements showed a higher leaf concentration under stress conditions in cv. Zarina, while uptake was not significantly affected (Tables 1 and 3). Greater oxidative stress and the antioxidant response had been related to lower concentrations of Mg (Kumar Tawari et al. 2006). This could be explained partly by the fact that the cultivars that present a lower Mg concentration show reduced growth, perhaps due to oxidative stress, as we have demonstrated in previous works (Sánchez-Rodríguez et al. 2009). On the other hand, several authors hold that a greater K accumulation improves stomatal resistance, benefiting drought tolerance. This was supported by Sinha (1978) studying drought-tolerant wheat and by Kafkafi and Xu (1999), who demonstrated that leaves with greater K accumulation were more effective at closing the stomata under water deficit.

In short, these results in relation to the macronutrient concentrations show that the cv. Zarina could be considered more water-stress tolerant, presenting higher N, P, Mg, and K concentrations.

Micronutrients, though essential for plant growth, are required in lower concentrations than macronutrients (<0.01% of dry matter) (Williams and Salt 2009). Iron is involved in photosynthesis, mitochon-

drial respiration, N assimilation, and osmoprotection (Hänsch and Mendel 2009). Another element that also participates in photosynthesis and mitochondrial respiration is Cu, although it is also required in the protection against oxidative stress and cell-wall synthesis. Manganese acts primarily as an enzyme activator, while Zn is an important component of enzymes related to protein synthesis, energy production and maintenance of biomembrane integrity (Hänsch and Mendel 2009). Chloride is related to the balance of the electric charge as well as, with K, stomatal opening and closing. Meanwhile, B is involved in numerous processes such as protein synthesis, sugar transport, and respiration (Hänsch and Mendel 2009). In previous works made in wheat, it has been shown that the contents of some micronutrients diminish, whereas others, such as Zn, remain unaffected by water deficit (Hu et al. 2006).

Our data in relation to micronutrient concentrations in leaves showed in general no significant concentration differences under stress conditions, except in the case of Fe, Cu, and Cl, which significantly rose in the cv. Zarina (Table 2). In this cultivar, Fe and Cl uptake also augmented under stress conditions (Table 4). A greater Fe concentration could be associated with a higher quantity of chlorophylls, which would accelerate photosynthesis in cv. Zarina. In turn, a higher Cl concentration could be related to stronger stomatal resistance together with K in this same cultivar. As mentioned above, Cu participates against oxidative stress, and this would coincide with the results reported in previous works (Sánchez-Rodríguez et al. 2010), demonstrating that cv. Zarina presents more vigorous enzymatic antioxidant activity.

In general, our results for the cv. Zarina coincide with those reported by Premachandra et al. (1995), who observe that a drought-tolerant line of sorghum presented higher Mg, P, K, and Cl concentrations, which contribute to a better osmotic adjustment and therefore better water potential in the leaf as compared to a more susceptible line. Water stress commonly provokes water loss and thereby lowers its relative content. To avoid this, plants can accumulate compatible solids, including ions such as K, Na, and Cl (Tamura et al. 2003). In this sense, the cv. Zarina again proved to be the most tolerant, in such a way that its higher LRWC could be associated with a greater K and Cl accumulation.

In conclusion, the five cherry tomato cultivars studied show genotypic variability in nutrient uptake. The nutrient uptake and accumulation in general diminished in those cultivars that were more susceptible to moderate water stress, while the cultivar Zarina maintained adequate growth during stress together with a greater LRWC and, in turn, nutrient uptake was not affected by stress and even improved in the case of N, P, Mg, K, and Cl. The changes in the ionome found in this study suggest that these might be an important response against water stress, this requiring further research.

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# Ammonia production and assimilation: Its importance as a tolerance mechanism during moderate water deficit in tomato plants

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ABSTRACT

Nitrate assimilation diminishes under water stress. This can augment the photorespiratory rate as a protection mechanism, increasing the ammonium concentration, which must be rapidly assimilated. We therefore examined the effect of moderate water stress in photorespiration and N assimilation, as possible tolerance mechanisms in cherry tomato. Five cherry tomato cultivars with different degrees of water stress tolerance were submitted to two water treatments: well-watered (100% FC) and water stress (50% FC). In the susceptible cultivars, nitrate assimilation declined but without stimulating photorespiration. Zarina, a stress-tolerant cultivar, showed increased activity of the main enzymes involved in photorespiration, together with greater assimilation of nitrates and of the resulting ammonium. This translates as higher concentrations of N as well as amino acids and proteins. We characterize these mechanisms in the cv. Zarina (tolerant) as essential to water stress tolerance, acting on N metabolism as well as helping to maintain or augment biomass.



## Introduction

The growing frequency of dry periods in many regions of the world is imposing drought in cultivated areas. Generally, drought depresses nutrient uptake by the root and transport to the shoot due to a restricted transpiration rate, affecting active transport and membrane permeability. The overall result is lower uptake by the plant (Hsiao, 1973; Kramer and Boyer, 1995). Nitrogen (N), required in great quantities by plants, is essential in the biochemistry of non-enzyme compounds such as coenzymes, photosynthetic pigments, secondary metabolites, and polyamines (Maathuis, 2009). Under water stress conditions, N uptake diminishes in soybean and rice (Tanguilig et al., 1987), wheat (Hu et al., 2006) and bean (Zayed and Zeid, 1997). The loss in N uptake can be attributed to a lower transpiration rate and N transport from the root to the shoot (Alam, 1999). Although there are several forms of soil N, nitrate ($NO_3^-$) is the most bioavailable and best assimilated by plants (Lea and Azevedo, 2006). The reduction of $NO_3^-$ to $NO_2^-$ catalyzed by nitrate reductase (NR) is considered the limiting step in N assimilation. In turn, $NO_2^-$ is reduced by nitrite reductase (NiR) to form $NH_4^+$. Under water deficit, foliar NR activity reportedly diminishes in *Leymus chinensis* plants and wheat (Xu and Zhou, 2006; Fresneau et al., 2007), with this being attributed to a decline in the internal $CO_2$ concentration in the leaf and/or a fall in the nitrate supply, although in other studies with tomato undergoing low environmental moisture, this inhibition was not detected (Brewitz et al., 1996).

Another known consequence of moderate water stress is photosynthesis limitation, primarily from restriction of the intercellular $CO_2$ concentration due to stomatal closure (Cornic and Briantais, 1991; Quick et al., 1992). This could provoke photoinhibition by diminishing the use of electrons by photosynthesis (Roland et al., 2006). As protection of the photosynthetic apparatus against such damage, this excess of photons can be used by photorespiration in $C_3$ plants. Photorespiration takes place in the chloroplasts, peroxisomes, and mitochondria, a consequence of the oxygenation of ribulose-1,5-biphosphate (RuBP) catalyzed by RuBP carboxylase/oxygenase (Rubisco), which generates one molecule of glycerate-3-phosphate (3-PGA) and one of glycolate-2-phosphate (2-PG). This 2-PG is hydrolyzed by phosphoglycolate phosphatase to glycolate, which is transported to peroxisome and oxidized

*Abbreviations:* AAT, aspartate aminotransferase; GDH, glutamate dehydrogenase; GGAT, glutamate: glyoxylate aminotransferase; GO, glyoxylate oxidase; GOGAT, glutamate synthase; GS, glutamine synthetase; HR, hydroxypyruvate reductase; LRWC, leaf relative water content; N, nitrogen; $NH_4^+$, ammonia; $NO_2^-$, nitrite; $NO_3^-$, nitrate; NR, nitrate reductase; NiR, nitrite reductase; 2-PG, glycolate-2-phosphate; 3-PG, glycerate-3-phosphate; RGR, relative growth rate; ROS, reactive oxygen species; RUBP, ribulose-1,5-biphosphate; Rubisco, RUBP carboxylase/oxigenase; SGAT, serine: glyoxylate aminotransferase.

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to glyoxylate by glyoxylate oxidase (GO). Glyoxylate is transaminated to glycine by the reaction catalyzed by glutamate: glyoxylate aminotransferase (GGAT), and is transported to the mitochondria. Subsequently, glycine is transformed into serine by the action of the enzymes glycine decarboxylase and hydromethyltransferase. The serine formed in the mitochondria is transported to peroxisome, where it is transformed by serine: glioxylate aminotransferase (SGAT) to hydroxypyruvate, which is reduced to glycerate by hydroxypyruvate reductase (HR). Finally, glycerate moves to the chloroplast, where it is phosphorylated by glycerate kinase, giving rise to a molecule of 3-PGA, which enters the Calvin cycle (Wingler et al., 2000).

Osmond and Björkman (1972) proposed that photorespiration could be an important photoprotective mechanism, although there is great controversy in this regard. Some researchers have demonstrated that this process can protect the photosynthetic apparatus against photoinhibition (Park et al., 1996; Guan et al., 2004; Bai et al., 2008) and bolster protection against different types of stress (Shi-Wei et al., 2007). Other authors suggest that photorespiration plays no significant part in protection against photoinhibition (Meng et al., 1999; Nogués and Alegre, 2002). However, a suppression of photorespiration can harm plants, slowing the assimilation rate of $CO_2$ as well as growth, and altering chloroplast structure (Shi-Wei et al., 2007). Under water stress, $CO_2$ assimilation diminishes, resulting in a lower level of electron use by photosynthesis. This decline in $CO_2$ assimilation is caused mainly by stomal closure (Wingler et al., 2000).

Plants produce significant quantities of ammonium ($NH_4^+$) through the reduction of $NO_3^-$ and photorespiration in the step from glycine to serine. In fact, this process can produce 20-fold more $NH_4^+$ than that generated by $NO_3^-$ reduction and is considered the largest source of this cation, especially in $C_3$ plants (Hirel and Lea, 2001). At high concentrations, $NH_4^+$ is toxic for plant cells and should be rapidly assimilated to organic compounds (Linka and Weber, 2005). Here, other key enzymes associated with N metabolism intervene: glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). The GS/GOGAT cycle incorporates photorespiratory and non-respiratory $NH_4^+$, providing N for transport and for maintenance of the nitrogen status in the plant (Suzuki and Knaff, 2005), and it can thus be used as a marker of the N status (Kichey et al., 2006). With regard to GDH, a possible role is to be an adaptive enzyme susceptible to environmental variables (Stitt et al., 2002). The activity of these enzymes can be inhibited by moisture deficit (Sibout and Guerrier, 1998; Xu and Zhou, 2006).

To date, the effect of water stress on the enzymes related to N metabolism is poorly known. Our research group has designed an experimental model of five commercial cherry tomatoes which display different degrees of tolerance to moderate water stress (Sánchez-Rodríguez et al., 2010a) and which present genotypic differences in N uptake (Sánchez-Rodríguez et al., 2010b). The aim of the present work was to examine the ways in which the enzymes involved in N metabolism respond to moderate water stress associated with photorespiration as a mechanism to generate $NH_4^+$, in order to determine the involvement of this process under stress conditions.

## Materials and methods

### *Plant material and treatments*

Five cherry tomato cultivars were used: Kosaco, Josefina, Katalina, Salomé, and Zarina [*Solanum lycopersicum* L. (*Lycopersicon esculentum* Mill.)]. The seeds of these cultivars were germinated and grown for 30 days in a tray with wells (each well 3 cm × 3 cm × 10 cm) in the nursery Semillero Sali-plant S.L. (Carchuna, Granada). Afterwards, the seedlings were transferred to a cultivation chamber at the Plant Physiology Department of the University of Granada under controlled conditions with relative humidity 50 ± 10%, at 25 °C/15 °C (day/night), and a 16 h/8 h photoperiod with a PPFD (photosynthetic photon-flux density) of 350 μmol$^{-2}$ s$^{-1}$ (measured with an SB quantum sensor, LI—COR Inc., Lincoln, NE, USA). Under these conditions, the plants grew in individual pots (25 cm upper diameter, 17 cm lower diameter, and 25 cm high) of 8 L in volume and filled with a 1:1 perlite:vermiculite mixture. Throughout the experiment, the plants were grown in a complete nutrient solution (Sánchez-Rodríguez et al., 2010a). The water stress treatments began 45 days after germination and were maintained for 22 days. The control treatment received 100% field capacity irrigation, whereas moderate water stress corresponded to 50% field capacity (FC). Independent of the procedure for watering (100 or 50% FC), plants received the same quantity of nutrients. We used a randomized complete block design with 2 treatments, arranged in individual pots with six plants per treatment and three replications each. The experiment was repeated three times under the same conditions.

### *Plant sampling*

All plants were at the late vegetative stage when harvested. Leaves (excluding petioles) were sampled on day 67 after germination. The plant material was rinsed three times in distilled water after disinfection with 1% non-ionic detergent and then blotted on filter paper. A part of the plant material was used for the assay of fresh weight (FW), amino acids, proteins, and of NR, NiR, GS, GOGAT, aspartate aminotransferase (AAT), Rubisco, GO, GGAT, HR and GDH enzymatic activities. The rest of the plant material was lyophilized and used to determine $NO_3^-$, $NH_4^+$ and organic and total reduce N and total N.

### *Analysis of N forms, soluble protein and free amino acid concentration*

$NO_3^-$ was analyzed from an aqueous extraction of 0.2 g of DW in 10 mL of Millipore-filtered water. A 100 μL aliquot was taken for $NO_3^-$ determination and added to 10% (w/v) salicylic acid in sulphuric acid at 96%, measuring the $NO_3^-$ concentration by spectrophotometry, as performed by Cataldo et al. (1975). $NH_4^+$ was analyzed from an aqueous extraction and was determined by using the colorimetric method described by Krom (1980).

For the total reduced N determination, a sample of 0.1 g DW was digested with sulphuric acid and $H_2O_2$ (Wolf, 1982). After dilution with deionized water, a 1 mL aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 μM sodium phosphate and 5.4% (w/v) sodium hydroxide), 15%/0.03% (w/v) sodium silicate/sodium nitroprusside and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37 °C for 15 min, and total reduced N was measured by spectrophotometry according to the method of Baethgen and Alley (1989). Total N concentration was assumed to represent the sum of total reduced N and $NO_3^-$.

Amino acids and proteins were determined by homogenization of 0.5 g FW in 50 mM cold $KH_2PO_4$ buffer at pH 7 and centrifugation at 12,000 × *g* for 15 min. The resulting supernatant was used for the determination of total amino acids by the ninhydrin method (Yemm and Cocking, 1955). Soluble proteins were measured with Bradford G-250 reagent (Bradford, 1976).

### *Nucleotide analysis*

Pyridine nucleotides were extracted from liquid N-frozen leaf material in 1 mL of 100 mM NaOH (for NAD(P)H) or 5% TCA (for

$NAD(P)^+$). The extracts were boiled for 6 min, cooled on ice and centrifuged at 12,000 × *g* for 6 min. Samples were adjusted to pH 8.0 with HCl or NaOH and 100 mM bicine (pH 8.0). Nucleotides were quantified by the enzyme-cycling method (Matsumura and Miyachi, 1980) with some modification (Gibon and Larher, 1997).

*Enzyme extractions and assays*

Leaves were ground in a mortar at 0 °C in 50 mM buffer $KH_2PO_4$ (pH 7.5) containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT) and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 30,000 × *g* for 20 min. The resulting extract (cytosol and organelle fractions) was used to measure enzyme activity of NR, NiR, GOGAT and GDH. The extraction medium was optimized for these enzyme activities so that they could be extracted together according to the same method (Groat and Vance, 1981; Kaiser and Lewis, 1984; Lillo, 1984; Singh and Srivastava, 1986).

The NR assay followed the methodology of Kaiser and Lewis (1984). The $NO_2^-$ formed was colorimetrically determined at 540 nm after azocoupling with sulphanilamide and naphthylethylenediamine dihydroclhoride according to the method of Hageman and Hucklesby (1971).

NiR activity was defined by the disappearance of $NO_2^-$ from the reaction medium (Lillo, 1984). After incubation at 30 °C for 30 min, the $NO_2^-$ content was determined colorimetrically as above.

GOGAT activity was assayed spectrophotometrically at 30 °C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986), always within 2 h of extraction. Two controls, without ketoglutarate and glutamine, respectively, were used to correct for endogenous NADH oxidation. The decrease in absorbance was recorded for 5 min.

GDH activity was assayed by monitoring the oxidation of NADH at 340 nm essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986). The reaction mixture consisted of 50 mM buffer $KH_2PO_4$ (pH 7.5) with 200 mM $NH_4^+$ sulphate, 0.15 mM NADH, 2.5 mM 2-oxoglutarate and enzyme extract. Two controls, without ketoglutarate and $NH_4^+$ sulphate, respectively, were used to correct for endogenous NADH oxidation. The decrease in absorbance was recorded for 3 min.

GS was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis (1984). Leaves were ground in a mortar at 0 °C in 50 mL of maleic acid–KOH buffer (pH 6.8), containing 100 mM sucrose, 2% (v/v) β-mercaptoethanol and 20% (v/v) ethylene glycol. The homogenate was centrifuged at 30,000 × *g* for 20 min. The resulting extract was used to measure enzyme activity of GS. The reaction mixture used in the GS assay was composed of 100 mM $KH_2PO_4$ buffer (pH 7.5) with 4 mM EDTA, 1000 mM L-sodium glutamate, 450 mM $MgSO_4 \cdot 7H_2O$, 300 mM hydroxylamine, 100 mM ATP and enzyme extract. Two controls were prepared, one without glutamine and the other without hydroxylamine. After incubation at 28 °C for 30 min, the formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride (Wallsgrove et al., 1979).

AAT activity was assayed spectrophotometrically at 340 nm using the method published by Gonzalez et al. (1995). AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8), 4 mM $MgCl_2$, 10 mM aspartic acid and enzyme extract. The decrease in absorbance was recorded for 3 min.

Rubisco activity was measured spectrophotometrically by coupling 3-phosphogliceric acid formation with NADH oxidation at 25 °C according to Lilley and Walker (1974) with some modification (Nakano et al., 2000). The total activity was assayed after crude extract was activated in a 0.1 mL activation mixture containing 33 mM tris–HCl (pH 7.5), 0.67 mM EDTA, 33 mM $MgCl_2$, 10 mM $NaHCO_3$ for 15 min. Initial rubisco activity measurements were carried out in a 0.1 mL reaction medium containing 5 mM Hepes–NaOH (pH 8.0), 1 mM $NaHCO_3$, 2 mM $MgCl_2$, 0.25 mM DTT, 0.1 mM EDTA, 1 U glyceraldehyde 3-phosphate dehydrogenase, 0.5 mM ATP, 0.015 mM NADH, 0.5 mM phosphocreatine, 0.06 mM RuBP and 10 μL extract. The change in absorbance at 340 nm was monitored.

For the GO determination, fresh leaf tissue (0.25 g) was ground in a chilled mortar with PVPP and 1 mL of 50 mM Tris–HCl buffer (pH 7.8) with 0.01% triton X-100 and 5 mm 1,4-dithioerythritol (DTT). The homogenate was centrifuged at 30,000 × *g* for 20 min. The supernatant was decanted and immediately used for the enzyme assay. GO was assayed as described by Feierabend and Beevers (1972) with modifications. A volume of assay mixture containing 50 mM Tris–HCl buffer (pH 7.8), 0.009% triton X-100, 3.3 mM phenylhydrazine HCl (pH 6.8), 50 μL of plant extract and 5 mM glycolic acid (neutralized to pH 7 with KOH) was used to start the reaction. GO activity was determined by following the formation of glyoxylate phenylhydrazone at 324 nm for 2 min after an initial lag phase of 1 min.

For determination of GGAT and HR, leaves were ground in a chilled mortar in 100 mM Tris–HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20,000 × *g* for 10 min. The resulting extract was used to measure enzyme activity. The extraction medium was optimized for the enzyme activities such that they could be extracted together using the same method (Hoder and Rej, 1983).

GGAT activity was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH. The reaction was assayed in a mixture containing 100 mM Tris–HCl (pH 7.3), 20 mM glutamate, 1 mM glyoxylate, 0.18 mM NADH, 0.11 mM pyridoxal-5-phosphate, 83 mM $NH_4Cl$ and 0.3 U GDH in a final volume of 0.6 mL (Igarashi et al., 2006).

HR assay was performed with 100 mM Tris–HCl (pH 7.3), 5 mM hydroxypyruvate and 0.18 mM NADH. Activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Hoder and Rej, 1983).

The protein concentration of the extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

*Statistical analysis*

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics 6.1 program. Means were compared by Fisher's least-significant differences (LSD). The significance levels for both analyses were expressed as $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ or NS (not significant).

## Results

*$NH_4^+$ production: reduction of $NO_3^-$ and photorespiration*

Under moderate water stress conditions, the quantity of $NO_3^-$ diminished significantly in all the cherry tomato cultivars studied except in Zarina, which registered a 70% increase with respect to the well-watered treatment (Table 1). The NR activity diminished with water stress in the cultivars in which the $NO_3^-$ decreased, whereas in the cv. Zarina the NR activity significantly augmented under stress conditions (Table 1). However, the NiR activity was not inhibited in any cultivar under water stress (Table 1). With respect to the quantity of $NH_4^+$, the foliar concentration in all the water-stressed cultivars fell, except in Zarina, which showed an increase of 16% with respect to well-watered plants (Table 1).

**Table 1**
Response of $NO_3^-$ reduction and $NH_4^+$ concentration in plants of 5 cultivars of cherry tomato well-watered and subjected to moderate water stress.

| Cultivar/water treatment | $NO_3^-$ | NR | NiR | $NH_4^+$ |
|---|---|---|---|---|
| *Kosaco* | | | | |
| Well-watered | 47.88 ± 4.97 | 3.58 ± 0.08 | 4.39 ± 0.13 | 0.375 ± 0.017 |
| Water stress | 15.75 ± 4.90* | 2.34 ± 0.09* | 5.36 ± 0.06* | 0.270 ± 0.007* |
| $LSD_{0.05}$ | 4.81 | 0.27 | 0.32 | 0.040 |
| *Josefina* | | | | |
| Well-watered | 34.07 ± 1.30 | 7.21 ± 0.13 | 3.91 ± 0.01 | 0.358 ± 0.007 |
| Water stress | 22.81 ± 4.77* | 0.86 ± 0.03* | 4.37 ± 0.02* | 0.266 ± 0.010* |
| $LSD_{0.05}$ | 10.49 | 0.30 | 0.05 | 0.026 |
| *Katalina* | | | | |
| Well-watered | 29.49 ± 2.69 | 9.24 ± 0.13 | 3.90 ± 0.01 | 0.491 ± 0.008 |
| Water stress | 22.38 ± 2.83* | 4.49 ± 0.10* | 4.21 ± 0.04* | 0.272 ± 0.009* |
| $LSD_{0.05}$ | 6.29 | 0.34 | 0.10 | 0.027 |
| *Salomé* | | | | |
| Well-watered | 39.78 ± 3.81 | 10.93 ± 0.44 | 3.60 ± 0.27 | 0.260 ± 0.005 |
| Water stress | 22.39 ± 4.25* | 8.35 ± 0.11* | 3.98 ± 0.06* | 0.219 ± 0.002* |
| $LSD_{0.05}$ | 12.11 | 0.97 | 0.30 | 0.013 |
| *Zarina* | | | | |
| Well-watered | 13.94 ± 4.78 | 2.77 ± 0.07 | 3.32 ± 0.12 | 0.236 ± 0.004 |
| Water stress | 23.83 ± 4.19* | 3.76 ± 0.12 | 3.65 ± 0.11 | 0.274 ± 0.006* |
| $LSD_{0.05}$ | 9.49 | 0.31 | 0.34 | 0.017 |

$NO_3^-$ and $NH_4^+$ were expressed as mg $g^{-1}$ DW; nitrate reductase (NR) was expressed as mM $NO_2^-$ $h^{-1}$ mg $prot^{-1}$; nitrite reductase (NiR) was expressed as mM $NO_2^-$ $h^{-1}$ mg $prot^{-1}$.
* Asterisk indicates significant difference with controls groups (well-watered).

With regard to the photorespiration process, both the initial activity as well as the total Rubisco generally declined under a moderate water deficit in the cultivars Kosaco, Josefina, Katalina, and Salomé (Fig. 1A and B). On the contrary, activity of this enzyme in cv. Zarina increased with water stress (Fig. 1A and B). However, the percentage of activation was affected little after stress, with significant decreases being registered only for the cv. Kosaco (Fig. 1C). In general, in the cv. Kosaco, Josefina, Katalina, and Salomé, water stress either decreased or had no effect on the different forms of pyridine dinucleotides. On the contrary, Zarina showed an increase of 78% in the $NADP^+$ concentration under water stress, with no significant differences in the $NAD^+$, NADH, and NADPH concentrations in comparison to well-watered plants (Table 2).

In general, both GO and GGAT declined in activity after the stress was applied in all the cultivars except Zarina, which registered 31% and 61% greater activity, respectively (Table 3). On the contrary, the water stress hardly had an effect on the HR activity in all the cultivars studied, except for cv. Josefina, which diminished with respect to well-watered plants (Table 3).

### *Incorporation of $NH_4^+$ and assimilation products*

The enzymes of the GS/GOGAT cycle were affected little by the water stress, except for cv. Zarina, where both enzymes significantly increased under the water deficit (Table 4). With respect to this cycle, it also merits highlighting that the cv. Kosaco also underwent a fall in the GS/GOGAT activity under water-stress conditions (Table 4). Similarly, under moderate water stress, the AAT activity dipped in the cultivars Katalina and Salomé, while it was augmented 93% in cv. Zarina (Table 4). Finally, by contrast, the GDH activity increased significantly in all the cultivars under water deficit, except in Zarina, which was not significantly affected by the stress (Fig. 2).

Both the reduced as well as the total N concentrations declined in all the cultivars after the water stress, except in Zarina, in which values rose 27% and 51%, respectively, with respect to control (Fig. 3A and B). The soluble amino acids generally diminished significantly in the cultivars Kosaco, Josefina, Katalina, and Salomé, but not significantly in Zarina (Fig. 3C). Also, the soluble proteins

**Table 2**
Response of pyridine dinucleotides concentration in plants of 5 cultivars of cherry tomato well-watered and subjected to moderate water stress.

| Cultivar/water treatment | $NAD^+$ | NADH | $NADP^+$ | NADPH |
|---|---|---|---|---|
| *Kosaco* | | | | |
| Well-watered | 3.49 ± 0.38 | 1.34 ± 0.18 | 1.88 ± 0.13 | 3.01 ± 0.22 |
| Water stress | 3.18 ± 0.09 | 0.24 ± 0.12* | 1.18 ± 0.12* | 0.12 ± 0.05* |
| $LSD_{0.05}$ | 1.09 | 0.61 | 0.51 | 0.63 |
| *Josefina* | | | | |
| Well-watered | 0.83 ± 0.17 | 2.88 ± 0.19 | 1.99 ± 0.29 | 1.75 ± 0.14 |
| Water stress | 1.68 ± 0.17* | 0.82 ± 0.16* | 2.00 ± 0.06 | 1.82 ± 0.02 |
| $LSD_{0.05}$ | 0.69 | 0.72 | 0.84 | 0.41 |
| *Katalina* | | | | |
| Well-watered | 3.19 ± 0.25 | 2.20 ± 0.20 | 1.49 ± 0.09 | 2.30 ± 0.22 |
| Water stress | 3.03 ± 0.17 | 1.75 ±0.24* | 1.00 ± 0.09* | 1.91 ± 0.20* |
| $LSD_{0.05}$ | 0.85 | 0.39 | 0.36 | 0.30 |
| *Salomé* | | | | |
| Well-watered | 3.92 ± 0.28 | 3.88 ± 0.41 | 3.97 ± 0.40 | 2.94 ± 0.31 |
| Water stress | 3.59 ± 0.20 | 4.37 ± 0.30 | 2.20 ± 0.12* | 2.48 ± 0.10* |
| $LSD_{0.05}$ | 0.97 | 1.42 | 1.18 | 0.31 |
| *Zarina* | | | | |
| Well-watered | 2.28 ± 0.22 | 3.90 ± 0.38 | 1.35 ± 0.02 | 2.20 ± 0.23 |
| Water stress | 2.72 ± 0.26 | 4.46 ± 0.39 | 2.41 ± 0.10* | 2.38 ± 0.20 |
| $LSD_{0.05}$ | 0.97 | 1.51 | 0.15 | 0.86 |

$NAD(P)^+$ and NAD(P)H were expressed as μM $g^{-1}$ DW.
* Asterisk indicates significant difference with controls groups (well-watered).

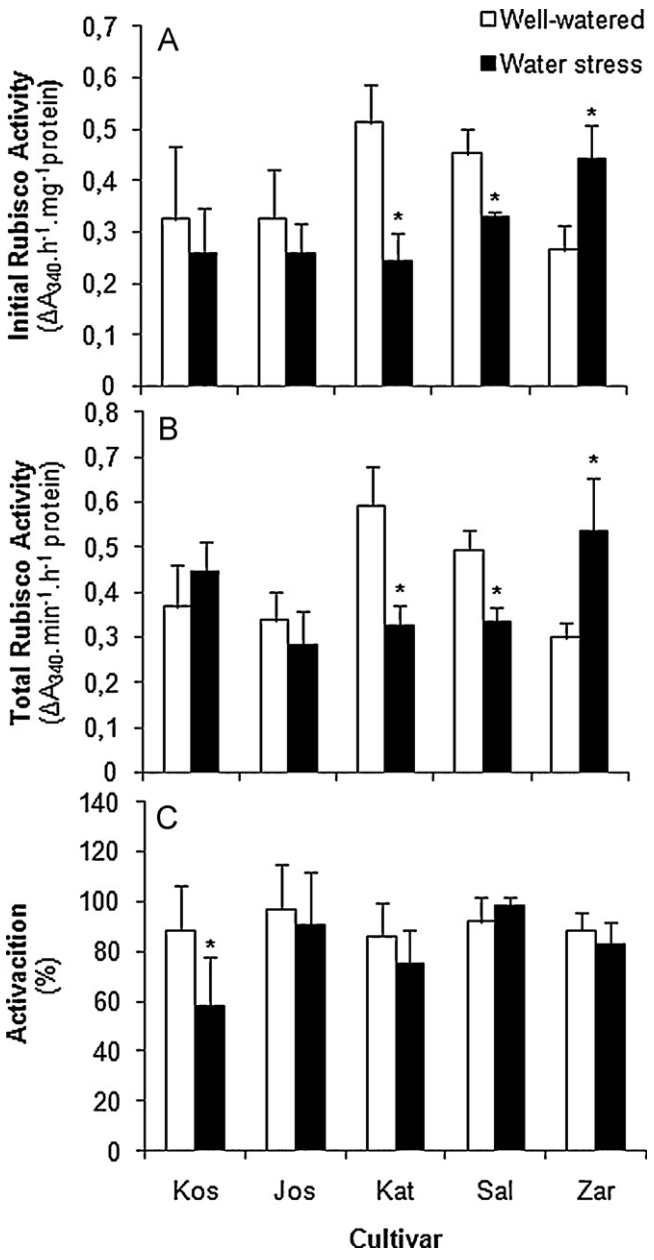


**Fig. 1.** Response of Rubisco activity in plants of 5 cultivars of cherry tomato well-watered and subjected to moderate water stress. Asterisk (*) indicates significant difference with control groups.

increased in the cv. Zarina, while in the rest, the values were not affected or fell after water stress (Fig. 3D).

## Discussion

In prior results reported by our research group, we found that cv. Zarina presented greater biomass and relative growth rate (RGR) associated with high leaf relative water content (LRWC) under water deficit conditions, indicating that this cultivar is more tolerant to this growth situation (Sánchez-Rodríguez et al., 2010a). Water stress can diminish the uptake of available N in the plant (Llorens et al., 2003). Our results confirm that the moderate water deficit decreased the $NO_3^-$ content in the cultivars Kosaco, Josefina, Katalina, and Salomé, associated in turn with a decline in the NR activity (Table 1). These results have been described previously in maize (Foyer et al., 1998), *L. chinensis* (Xu and Zhou, 2006) and

**Table 3**
Response of some photorespiration enzymes in plants of 5 cultivars of cherry tomato well-watered and subjected to moderate water stress.

| Cultivar/water treatment | GO | GGAT | HPR |
|---|---|---|---|
| *Kosaco* | | | |
| Well-watered | 2.11 ± 0.07 | 1.86 ± 0.54 | 5.91 ± 0.16 |
| Water stress | 1.50 ± 0.06* | 1.38 ± 0.45 | 5.98 ± 0.06 |
| $LSD_{0.05}$ | 0.22 | 1.96 | 0.38 |
| *Josefina* | | | |
| Well-watered | 2.19 ± 0.07 | 4.25 ± 0.91 | 5.28 ± 0.12 |
| Water stress | 1.70 ± 0.03* | 1.21 ± 0.18* | 4.57 ± 0.08* |
| $LSD_{0.05}$ | 0.17 | 2.58 | 0.32 |
| *Katalina* | | | |
| Well-watered | 3.00 ± 0.06 | 2.90 ± 0.40 | 5.94 ± 0.06 |
| Water stress | 2.16 ± 0.07* | 1.46 ± 0.34* | 6.15 ± 0.13 |
| $LSD_{0.05}$ | 0.22 | 1.17 | 0.33 |
| *Salomé* | | | |
| Well-watered | 1.84 ± 0.04 | 1.12 ± 0.11 | 4.57 ± 0.01 |
| Water stress | 1.66 ± 0.07 | 1.87 ± 0.47 | 4.74 ± 0.03 |
| $LSD_{0.05}$ | 0.18 | 1.35 | 0.28 |
| *Zarina* | | | |
| Well-watered | 1.93 ± 0.05 | 1.08 ± 0.16 | 5.21 ± 0.05 |
| Water stress | 2.56 ± 0.07* | 1.75 ± 0.04* | 5.37 ± 0.06 |
| $LSD_{0.05}$ | 0.19 | 0.46 | 0.17 |

Glycolate oxidase (GO), glutamate: glyoxylate aminotransferase (GGAT) and hydroxypyruvate reductase (HR) activities were expressed as $\Delta A\ h^{-1}$ mg $prot^{-1}$.
* Asterisk indicates significant difference with controls groups (well-watered).

wheat (Fresneau et al., 2007) subjected to water deficit, a result attributed to a decline in the internal concentration of leaf $CO_2$ and/or a decrease in the $NO_3^-$ supply. On the contrary, cv. Zarina maintained a high $NO_3^-$ concentration, together with greater NR activity (Table 1). This increase in the NR activity and therefore the reduction of $NO_3^-$ could improve the growth of the plant under stress conditions (Singh and Usha, 2003). By contrast, the NiR was not affected negatively by the stress (Table 1), possibly for being a constitutive enzyme that did not alter the degree of expression according to the $NO_2^-$ levels in the growth medium (Heldt, 2005). As occurred with $NO_3^-$ under water limitation, the quantity of $NH_4^+$ diminished in the cultivars studied, except in Zarina (Table 1), this perhaps is being associated with the greater reduction of $NO_3^-$ shown in this cultivar under water stress (Table 1). However, $NH_4^+$

**Table 4**
Response of enzymes responsible for $NH_4^+$ assimilation in plants of 5 cultivars of cherry tomato well-watered and subjected to moderate water stress.

| Cultivar/water treatment | GS | GOGAT | AAT |
|---|---|---|---|
| *Kosaco* | | | |
| Well-watered | 0.980 ± 0.023 | 0.204 ± 0.053 | 5.89 ± 0.45 |
| Water stress | 0.668 ± 0.018* | 0.107 ± 0.003* | 5.51 ± 0.23 |
| $LSD_{0.05}$ | 0.063 | 0.092 | 1.09 |
| *Josefina* | | | |
| Well-watered | 0.706 ± 0.051 | 0.372 ± 0.023 | 4.68 ± 0.24 |
| Water stress | 0.583 ± 0.029 | 0.433 ± 0.028 | 5.34 ± 0.39 |
| $LSD_{0.05}$ | 0.126 | 0.102 | 0.97 |
| *Katalina* | | | |
| Well-watered | 0.537 ± 0.015 | 0.209 ± 0.039 | 3.46 ±0.57 |
| Water stress | 0.527 ± 0.018 | 0.245 ± 0.097 | 4.67 ± 0.25* |
| $LSD_{0.05}$ | 0.052 | 0.059 | 0.32 |
| *Salomé* | | | |
| Well-watered | 0.487 ± 0.017 | 0.553 ± 0.024 | 4.24 ± 0.46 |
| Water stress | 0.314 ± 0.012* | 0.570 ± 0.019 | 2.85 ± 0.21* |
| $LSD_{0.05}$ | 0.044 | 0.057 | 1.08 |
| *Zarina* | | | |
| Well-watered | 0.333 ± 0.026 | 0.251 ± 0.056 | 2.01 ± 0.20 |
| Water stress | 0.453 ± 0.032* | 0.528 ± 0.064* | 3.85 ± 0.34* |
| $LSD_{0.05}$ | 0.08 | 0.089 | 0.85 |

Glutamine synthetase (GS), glutamate synthase (GOGAT) and aspartate aminotransferase (AAT) activities were expressed as $\Delta A\ h^{-1}$ mg $prot^{-1}$.
Values are mean ± S.E. ($n = 9$) and differences between means were compared by Fisher's least-significance test (LSD; $P = 0.05$).
* Asterisk indicates significant difference with controls groups (well-watered).

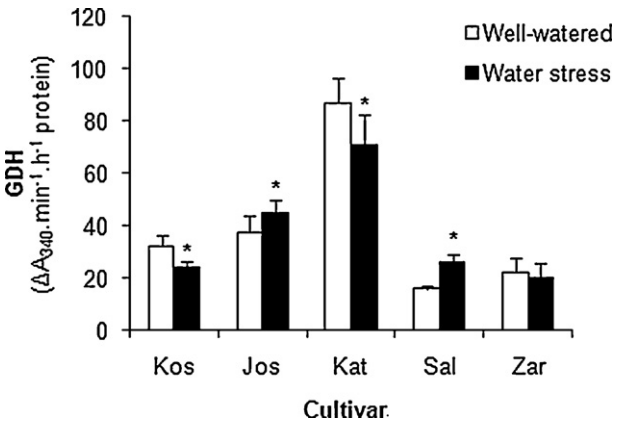


**Fig. 2.** Response of glutamate dehydrogenase (GDH) in plants of 5 cultivars of cherry tomato well-watered and subjected to moderate water stress. Asterisk (*) indicates significant difference with control groups.

originates not only from the reduction of $NO_3^-$, but is also generated when the photorespiration rate is high due to the oxidation of glycine (Hirel and Lea, 2001), a process that was activated under water stress conditions in our experiment.

There are data showing a decline in the quantity and activity of Rubisco in stressed plants (Majumdar et al., 1991; Parry et al., 2002), a decline that could be related to the general stimulation of senescence and/or oxidative damage. Our results show that the water deficit depressed Rubisco activity in most of the cultivars studied (Fig. 1), this having been noted previously in plants such as tobacco and soybean (Flexas et al., 2006). Some authors have associated the decline in this activity with a fall in LRWC (Lawlor and Cornic, 2002; Parry et al., 2002). This coincides with the results previously described in the plants used in the present study (Sánchez-Rodríguez et al., 2010a), where a drop in LRWC was found in the cultivars Kosaco, Josefina, Katalina, and Salomé. However, Zarina presented an improvement in Rubisco activity under water deficit, associated with a greater LRWC (Sánchez-Rodríguez et al., 2010a). These results agree with those reported previously in drought-tolerant sunflower and wheat (Pancović et al., 1999; Demirevska et al., 2008), showing greater Rubisco activity together with greater LRWC.

The result of the activation of Rubisco under water stress conditions in cv. Zarina could explain why this cultivar presented higher $NADP^+$ levels under stress conditions (Table 2). This situation could be due to a stimulation of the photorespiration process, which would generate $CO_2$ that would enter the Calvin cycle, where, though Rubisco, carboxylase would generate $NADP^+$. This would act as an acceptor of electrons, encouraging less formation of ROS within the cell under stress conditions. These findings correspond to previous results reported for the cv. Zarina (Sánchez-Rodríguez et al., 2010a). In turn, when the water deficit worsens, it blocks electron transport, inducing a deficit in the NADPH supply (Haupt-Herting and Fock, 2000). This does not occur in the cv. Zarina, which maintained constant NADPH levels under water deficit conditions (Table 2).

To understand of the above hypothesis, we analyzed some of the enzymes involved in photorespiration. The first enzyme involved, GO, acts in the peroxisomes to form glyoxylate. GGAT is responsible for the formation of glycine, an amino acid that is afterwards oxidized in the inner membrane of the mitochondria, producing $CO_2$, great quantities of $NH_4^+$, and serine. Finally, HR reduces hydroxypyruvate to glycerate (Wingler et al., 2000). Our results show that the activity of these enzymes are not affected or diminish under stress conditions in most of the cultivars studied: Kosaco, Josefina, Katalina, and Salomé (Table 3). As in our results, Wingler et al. (2000) observed that the activities of these enzymes are affected little by water stress. However, cv. Zarina showed a significant boost in the activity of the enzymes GO and GGAT under water-stress conditions. This could indicate that this cultivar activates the photorespiration process, which could be a response to this type of stress. Guan et al. (2004) demonstrated that there were genotypic differences in the activation of the photorespiration in grape cultivars, so that the cultivars with a higher photorespiratory rate maintained a better response to drought.

The $NH_4^+$ generated not only by the reduction of $NO_3^-$ but especially by photorespiration, must be rapidly assimilated due to its

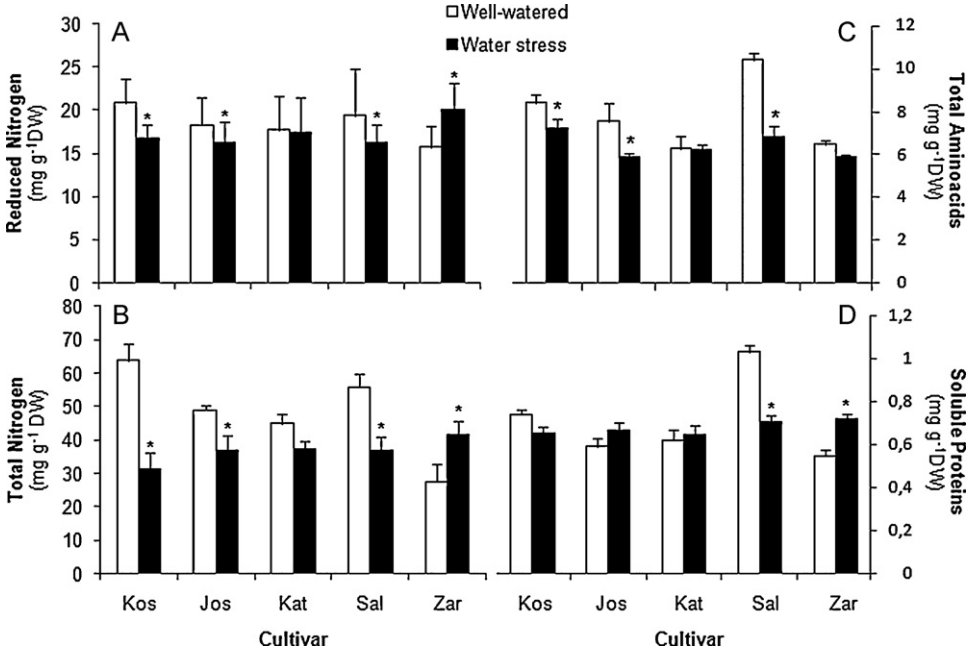


**Fig. 3.** Response in foliar concentration of organic (A), total N (B), total amino acid (C) and soluble proteins (D) in plants of 5 cultivars of cherry tomato well-watered and subjected to moderate water stress. Asterisk (*) indicates significant difference with control groups.

toxicity to the cell. This is performed mainly by the GS/GOGAT cycle (Suzuki and Knaff, 2005). In our results, only the cv. Zarina displayed increased activity of this cycle under stress conditions, being correlated with a higher $NH_4^+$ concentration (Table 1), caused both by greater $NO_3^-$ reduction (Table 1) and photorespiration (Table 3). By contrast, in the other cultivars studied, water stress lowered the $NH_4^+$ concentration (Table 1), given that there was less reduction of $NO_3^-$ (Table 1) and less photorespiration (Table 3), a circumstance that would explain the reduction of the GS/GOGAT cycle in these cultivars (Table 4). GDH is a very active enzyme in the presence of $NH_4^+$ and constitutes another mitochondrial pathway for this cation, given that it catalyzes the reversible reaction of α-cetoglutarate and $NH_4^+$ to glutamate (Frechilla et al., 2002). It has been demonstrated that GDH confers resistance to different types of stress (Cruz et al., 2006). In our work, we found that only in the cultivars sensitive to water stress was this enzymatic activity significantly spurred (Fig. 2). This may indicate that these cultivars, due to stress, try to produce more glutamate and eliminate $NH_4^+$ via GDH in order to raise the concentration of organic compounds and thereby minimize the harmful consequences of water stress, a situation that did not occur in the tolerant cultivar Zarina under these growth conditions (Fig. 2). Finally, the glutamate and glutamine generated in the GS/GOGAT cycle are allocated to the synthesis of aspartate and asparagine, produced in the reactions catalyzed by AAT and asparagine synthetase (Hodges, 2002). Various functions have been attributed to AAT. This enzyme has a central role in C and N metabolism, since it retains and releases oxo-acids that help to coordinate the N metabolism and amino acid synthesis with the availability of carbon skeletons of the Krebs cycle (Hodges, 2002). Our results show that only the cv. Zarina stimulated AAT activity significantly under stress conditions (Table 4). This could be related to the increase in the GS/GOGAT cycle in this cultivar (Table 4).

The result of the incorporation of $NH_4^+$ can be quantified by the analysis of reduced N, which is generally the product of N assimilation and is formed mainly by amino acids and proteins. The total N, the result of the sum of the total reduced N and the $NO_3^-$, is considered a critical parameter to determine the nutritional state of plants (Ruiz and Romero, 1999). Our results show that both the reduced N and the total N diminished under water stress in all the cultivars studied, except for Zarina (Fig. 3A and B). The reduction of these N forms under water-stress conditions has previously been demonstrated in wheat plants subjected to this type of stress (Sinclair et al., 2000). Also, the cv. Zarina presented a greater concentration of both N forms (Fig. 3A and B), which could improve the plant growth under water stress conditions (Singh and Usha, 2003). In general, nitrogenous compounds with high and low molecular weights (amino acids and proteins) are the main products of $NO_3^-$ assimilation (Barneix and Causin, 1996). Under water stress, the concentration in amino acids diminished in a generalized way in the cultivars sensitive to this kind of stress: Kosaco, Josefina, Katalina, and Salomé (Fig. 3C). This finding coincides with the data reported by Xu and Zhou (2006). However, the cv. Zarina maintained the levels of amino acids under stress conditions (Fig. 3C), at the same time as soluble proteins augmented (Fig. 3D). The content in soluble proteins may be used as an index of Rubisco protein, since the concentration of this protein in leaves reach 30–50% of the total soluble proteins (Long et al., 2006). This could explain the high Rubisco activity found in the cv. Zarina under water stress (Fig. 1A and B).

In conclusion, the present study indicates that moderate water stress negatively affects both $NH_4^+$ synthesis as well as assimilation in the more sensitive cultivars in our study (Kosaco, Josefina, Katalina, and Salomé). On the contrary, the cv. Zarina presented an improvement in N metabolism under water-stress conditions, as well as improvement in Rubisco activity, in the generation of $NADP^+$, and in the induction of photorespiration. Under our experimental conditions, we these mechanisms in cv. Zarina (drought tolerant) were essential in water-stress tolerance, N metabolism intervened in the maintenance or improvement of the biomass. Meanwhile, the stimulation of Rubisco activity and photorespiration in photoprotection helped in the use of excess electrons, thereby avoiding the appearance of excessive ROS, which are usually generated in this type of stress.

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