Screening Pepper Genotypes To Obtain Tolerant Rootstocks To Salt And Water Stress:

Physiological And Agronomical Responses Of The Grafted Plants

Doctoranda
Consuelo Penella Casañ

Directores
Ángeles Calatayud Chover
Sergio González Nebauer
Alberto San Bautista Primo
Salvador Vicente López Galarza

Noviembre 2015

Programa de Doctorado en Recursos y Tecnologías Agrarias

Pepper is a vegetable of extraordinary economic and social importance in our country. Unfortunately, the persistent exploitation of the land, the monoculture and the intensification of production processes, lead to the development of soil diseases. This coupled with the abiotic stress, mainly the salinity of waters and soil, suboptimal temperatures and water stress, can induce the appearance of physiological disorders in peppers as the Blossom-end rot (BER) and cracking or cracked, induce plant senescence and decrease not only production, but also the quality of the product. Salinity and water shortages are two among the biggest environmental problems that crops have to face in the Mediterranean area. A way to overcome the stresses under the prism of an ecological or integrated crop management, is the use of grafted plants as adaptation strategy. Although there has been remarkable progress in this technique (mainly in tomato, melon, watermelon), in the cultivation of pepper use remains rare. In this Doctoral thesis several pepper genotypes have been selected through different physiological parameters which indicate tolerance to salt and water stress. Commercial cultivars were grafted onto the selected genotypes and were grown under water stress, salinity and control conditions studying several physiological, agronomic responses and the interaction rootstock/scion. The results obtained concluded that genotypes selected and used as rootstocks improved commercial varieties to salt and water stress tolerance, both in terms of performance (commercial production) compared to other commercial characters and variety without grafting. Different physiological mechanisms explain the tolerance to stress, such as the ability to maintain the water potential through an osmotic adjustment, stimulation of the antioxidant system, exclusion

or retention of toxic ions (Na^+ and Cl^-) in saline in the roots and the maintenance of photosynthesis which allows to maintain the metabolic functions of grafted plants and production.

El pimiento es una hortaliza de extraordinaria importancia económica y social en nuestro país. Lamentablemente, la persistente explotación del suelo, el monocultivo y la intensificación de los procesos de producción, conducen al desarrollo de enfermedades del suelo. Esto unido a los estreses abióticos, principalmente la salinidad de las aguas y del suelo, temperaturas subóptimas y estrés hídrico, puede inducir la aparición de fisiopatias en el pimiento como el Blossom-end rot (BER) y cracking o rajado, inducir senescencia vegetal y disminuir no solo la producción, sino también la calidad del producto.

La salinidad y la escasez de agua son unos los mayores problemas medio ambientales a los que tienen que hacer frente los cultivos en el área Mediterránea. Un modo de sortear los estreses bajo el prisma de un manejo integrado o ecológico del cultivo, es la utilización de plantas injertadas como estrategia de adaptación. Aunque se ha producido un notable avance en esta técnica (principalmente en tomate, melón, sandía), en el cultivo del pimiento su utilización es poco frecuente aun. En esta Tesis Doctoral se han seleccionado mediante parámetros fisiológicos diferentes genotipos de pimiento tolerantes al estrés salino e hídrico. Los genotipos seleccionados fueron validados como patrones tolerantes a condiciones de estrés hídrico y salino injertados sobre una variedad comercial mediante el estudio de las respuestas fisiológicas, agronómicas y de la interacción patrón/variedad en ambas condiciones de estrés.

De los resultados obtenidos se concluye que los genotipos seleccionados y utilizados como patrones mejoraron la tolerancia de las variedades comerciales a la salinidad, en términos de rendimiento (producción comercial) de frutos comparando con otros patrones comerciales y la variedad sin injertar. Diferentes mecanismos fisiológicos explican la tolerancia al estrés, como la

capacidad de mantener el potencial hídrico mediante un ajuste osmótico, estimulación del sistema antioxidante, exclusión o retención de los iones tóxicos salinos (Na^+ y Cl^-) en las raíces y el mantenimiento de la fotosíntesis que permite mantener las funciones metabólicas de las plantas injertadas y la producción.

El pimentó és una hortalissa d'extraordinària importància econòmica i social al nostre país. Lamentablement, la persistent explotació del sòl, el monocultiu i la intensificació dels processos de producció, conduïxen al desenrotllament de malalties del sòl. Açò unit als estressos abiòtics, principalment la salinitat de les aigües i del sòl, temperatures subòptimes i estrés hídric, pot induir l'aparició de fisiopaties en el pimentó com el Blossom-end rot (BER) i cracking, induir senescència vegetal i disminuir no sols la producció, sinó també la qualitat del producte.

La salinitat i l'escassetat d'aigua són uns els majors problemes mitjà ambientals als que han de fer front els cultius en l'àrea Mediterrània. Una manera de sortejar els estressos davall el prisma d'un maneig integrat o ecològic del cultiu, és la utilització de plantes empeltades com a estratègia d'adaptació. Encara que s'ha produït un notable avanç en esta técnica (principalment en tomaca, meló, meló d'alger), en el cultiu del pimentó la seua utilització és poc freqüent. En esta Tesi Doctoral s'han seleccionat per mitjà de paràmetres fisiològics diferents genotips de pimentó tolerants a l'estrés salí i hídric. Els genotips seleccionats van ser validats com a patrons tolerants a condicions d'estrés hídric i salí empeltats sobre una varietat comercial per mitjà de l'estudi de les respostes fisiològiques, agronòmiques i de la interacció patrón/variedad en ambdós condicions d'estrés.

Dels resultats obtinguts es conclou que els genotips seleccionats i utilitzats com a patrons van millorar la tolerància de les varietats comercials a la salinitat, tant en termes de rendiment (producció comercial) de fruits comparant amb altres patrons comercials i la varietat sense empeltar. Diferents mecanismes fisiològics expliquen la tolerància a l'estrés, com la capacitat de mantindre el potencial hídric per mitjà d'un ajust osmòtic, estimulació del sistema antioxidant,

exclusió o retenció dels ions tòxics salins (Na^+ i Cl^-) en les arrels i el manteniment de la fotosíntesi que permet mantindre les funcions metabòliques de les plantes empeltades i la producció.

“AGRAÏMENTS” “AGRADECIMIENTOS” “RINGRAZIAMENTI”

Aún recuerdo como siendo una niña, a la sección de informativos que más atención le prestaba era la titulada como “el camp” i que acababa siempre con el precio de les “fruites i hortalisses” i con la frase “dades facilitades per el Institut Valencià de Investigacions Agraries”…mucho ha llovido desde entonces.

Jamás pensé que acertara tanto en el camino elegido, y es que la vida de vez en cuando te brinda experiencias maravillosas que superan con creces los malos tragos. Una de esas experiencias maravillosas para mí ha sido esta tesis, la cual ha sido “inusualmente especial”, tanto en resultados como en momentos vividos. Empezando porque tengo 4 directores. Creo que es la primera en la historia de la UPV, pero los 4 han sido muy importantes en este proceso.

Alberto, gràcies per la teua paciència i saber estar.

Salvador, sense el teu caracter, bon humor i experiència açò habría sigut altra cosa.

Sergio, preocupación y disposición han sido un ejemplo. Gracias por todos los consejos.

Gracias por haber confiado en mí desde el principio, vuestra sinceridad y por qué no, las risas que nos hemos pegado en algún que otro congreso.

Y la cuarta, para mí la “Dancing-Queen”, “jefa-mamá” o “mi jefa”,…No tengo palabras… Tot va canviar quan vaig rebre la teua telefonada…una beca en el IVIA? Casi caic rodant escales avall de l’emoció. I es que, en estos cuatre anys hem passat de tot…”risas y lágrimas”, però resumint, m’ho he passat de pel.lícula! Treballadora, saber fer bona ciència, no donar res per fet, càlidessa, “marota”, amiga, confident, afán de superació, lluitadora, transparent,…i més que

generosa. Clar exemple a seguir…espere haver estat a la altura. Qui te coneix estic segura que estarà d'acord amb mí. No tindré prou paraules d'agraïment mai.

Agradecer a mi equipo de "Horticultura" el haberse portado conmigo tan bien. Nacho, Pepe, Javi y Lidia, sois afortunados de formar parte de este departamento, disfrutarlo!

A mis chicas del "comedor", qué lástima no haberlo tenido antes. Quedarse a hacer "horas extras" nunca fue tan divertido. Millones de gracias a: Belén, Ana, Carmencita, Mary Rus, Amparito, Marian, Mª Carmen, …, y a Isa por ayudarnos en las tareas del laboratorio, gracias a todas!

Esta tesis ha sido también inusualmente viajera. Desde Holanda hasta Estados Unidos, pasando por Italia, y parando en un montón de congresos, ¡Qué buenas experiencias! Sin duda, ha sido gracias a las personas que me habéis facilitado la vida y me habéis hecho sentir como en casa, a pesar de que la tenía a cientos de kilómetros. Gracias a Elisa y Juan Carlos por vuestra oportunidad y paciencia. E grazie mille a Lucia e Marco. Suprattutto amici. Io vi porterò sempre nel mio cuore. E aspetto che … a dopo!

Muchos estudiantes habéis aportado vuestro granito de arena. Estas líneas de agradecimiento son para vosotros: Neus, Giulia, Giovanni, Nigel, Giacomo, Vicent, Marisa, Jordi i Enrique.

Gracias a ANECOOP, Cooperativa Surinver y Cooperativa La Protectora de "el Perelló", por permitir realizar en vuestras instalaciones los experimentos de campo. En especial estoy muy agradecida a Pepe, Nando y Enrique, por su disponibilidad y ganas de trabajar. Os recordaré siempre.

Moltes gràcies papis per estar sempre ahí! Tinc molta sort de tindre-vos. Vos vuic molt!! I gràcies a tota la meua familia que es preocupa per mi.

“Al club de las seis”, Patri, Texu, Isa, Ana, Cris… (y la sexta soy yo), sin vosotras todo sería muy aburrido, y a mis otras chicas, Lidia, Noe, Fany, Elvira, Silvia y Ana Belén. Gracias por estar siempre ahí. No podieu faltar en este escrit els meus xics “d’agronoms”, amb els que tants bons moments hem passat, Cris, Josep, Anna i Tono. Per gent com vosaltres tornaría a estudiar agrònoms altra vegada…o no?

Para ir acabando ya, Néstor. Pese a todo, sigues ahí. Gracias por confiar más en mí que yo en mí misma. Cuando me han flaqueado las fuerzas, tú me has dado impulso. Cuándo he estado mal, tu hombro era el primero en llegar. Cuando había que celebrar alegrías, tú también has sido el primero. La vida así se lleva mejor, espero corresponderte como mereces.

En último lugar, quiero agradecer al INIA su fantástica labor y la oportunidad de disfrutar de una de sus becas FPI-INIA, así como las becas de estancias en el extranjero, sin las cuales, esta tesis doctoral no hubiera sido lo mismo.

I moltes gràcies a tú per estar llegint esta tesi doctoral!

¡Y muchas gracias a ti por estar leyendo esta tesis doctoral!

E grazie mille a te per leggere questa doctoral tesi!

And thank you for being reading this doctoral thesis!

Xelo Penella

Res no m'agrada tant
com enramar-me d'oli cru
el pimentó torrat, tallat, tallat en tires.
Conte llavors, distret, **raone** amb l'oli cru, amb
els productes de la terra.

M'agrada molt el pimentó torrat,
més no massa torrat, que el desgracia,
sinó amb aquella carn molla que té
en llevar-li la crosta socarrada.

L'expose dins el plat en tongades incitants,
l'enrame d'oli cru amb un **pessic de sal**
i suque molt de pa,
com fan els pobres,
en l'oli, que té **sal** i ha pres una sabor del
pimentó torrat.

Després, en un pessic
del dit gros i el dit índex, amb un tros de pa,
agafe un tros de pimentó, l'enlaire àvidament,
eucarísticament,

me'l mire en l'aire.
de vegades arribe a l'èxtasi, a l'orgasme.
Cloc els ulls i me'l fot.
(Vicent Andrés Estellés)

Index

CHAPTER 3. EVALUATION FOR SALT STRESS TOLERANCE OF PEPPER GENOTYPES TO BE USED AS ROOTSTOCKS

CHAPTER 4. ROOTSTOCK ALLEVIATES PEG-INDUCED WATER STRESS IN GRAFTED PEPPER SEEDLINGS: PHYSIOLOGICAL RESPONSES

CHAPTER 5. SOME ROOTSTOCKS IMPROVE PEPPER TOLERANCE TO MILD SALINITY THROUGH IONIC REGULATION

CHAPTER 1
General Introduction

1.1. INTRODUCTION

Peppers, chiles, chillies, capsicum, or whatever their name, are a versatile crop that form part of most people's daily diet, particularly in some areas. Capsicum plants are essentially a crop of tropics, but it grows better in hotter regions (De, 2003). The fruit is consumed as a fresh, processed or dehydrated vegetable, and sometimes as a spice. Because of its great versatility, pepper consumption is increasing, but also because it may be an important source of pro-vitamin A (carotene), E (α-tocopherol), and particularly vitamin C (ascorbic acid), one of its most important attributes. Mature pepper fruits are also rich in carotenoids, compounds with antioxidant and anti-carcinogenic capacity. Furthermore, both immature and mature fruits contain a high content of phenolics, in particular the flavonoids for which antioxidant and other bioactive properties have been reported (Hervert-Hernández et al., 2010; Mateos et al., 2013; Rodríguez-Burruezo et al., 2009), and many essential nutrients for world populations.

Based on some organoleptic features and culinary purposes, pepper fruits are usually classified into two types. The term bell pepper refers to a non-pungent, chunky sweet pepper type, whereas chilli pepper generally refers to pungent chilli fruits (De, 2003). As a general rule, non-pungent pepper types are the most popular in the northern hemisphere, while the more pungent chilli peppers are widely consumed in the tropics and subtropic areas (Cichewicz and Thorpe, 1996).

1.2. ECONOMIC IMPORTANCE OF *Capsicum* Spp.

Peppers are grown in most countries in the world, with 1.93 million of ha of cultivated area. The world pepper production as a spice and vegetables has increased in the last 20 years (FAO, 2013) from more than 12 million tonnes in 1993 to more than 31 million of tonnes in 2013. China is the largest producer with almost 16 million tonnes, followed by Mexico (2.3 million tonnes), Turkey (2.2 million tonnes) and Indonesia (1.8 million tonnes). Spain is the fifth most important producer with almost 1 million tonnes and 18,100 ha cultivated in front of the USA (FAO, 2013) (Fig. 1).

Spanish peppers involve about 55% of the south Europe production (about, 200.000 ha, MAGRAMA, 2014), concentrated in the Mediterranean region. Andalusia and Murcia produce about 70% of the Spanish pepper production, with the Valencian Community ranking fifth among Spanish regional producers (MAGRAMA, 2012) (Fig. 2).

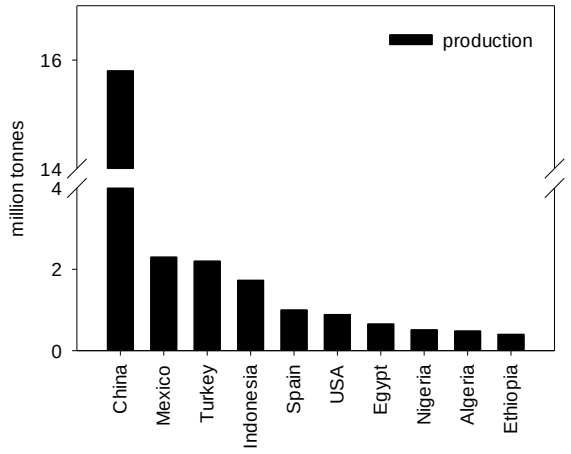


Figure 1. World production of chillies and peppers by country (million tonnes) (FAO 2013)

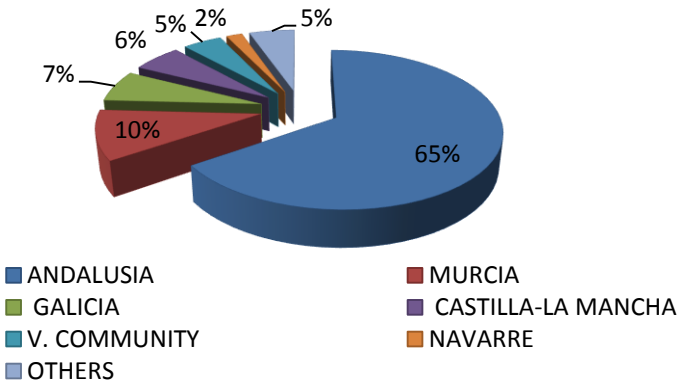


Figure 2. Pepper production rates by Spanish regions (MAGRAMA, 2012)

1.3. HISTORICAL AND BOTANICAL PERSPECTIVES

The *Solanaceae* family is a complex comprised of at least 98 genera and as many as 2,716 species, including *Capsicum* species (Hunziker, 2001; Olmstead et al., 1999). Other important crops belongs to this family: tomato, eggplant, potato and tobacco. The word "*Capsicum*" comes from a Greek-based derivate of the Latin term "*Kapto*", meaning "to bite", and is a certain reference to heat or pungency. *Capsaicin*, a volatile molecule, is a very stable molecule and is responsible for the pungency commonly associated with some peppers (Heiser and Pickersgill, 1969). However, other pepper species are non-pungent due to a single mutation, which causes loss of ability to produce capsaicinoids.

The genus *Capsicum* has been known in the central hemisphere and South America since the beginning of civilisation, and has probably evolved from an

ancestral form in the Bolivia-Peru area. It has formed a part of the human diet since about 7500 BC (MacNeish, 1964). Peppers were unknown in Europe, Asia and Africa prior to Christopher Columbus landing in the Americas. On his voyage, he encountered a plant whose fruit mimicked the pungency of black pepper, *Piper nigrum* L. The genus *Capsicum*, which is commonly known as "capsicum", "pepper", "bell pepper", "red chile", "chilli pepper", "tabasco", "paprika", "cayenne", etc., comprises as many as 40 species, approximately. The phenotypic variation in fruit shapes, sizes, colours and plant habits is vast (Bosland and Votava, 2003). *Capsicum* species, with few very exceptions, are diploid (2n=24, infrequently 2n=26) and have similar karyotypes (Lippert et al., 1966; Moscone et al., 1993). The morphological differences between wild and cultivated chillies are easily discerned. On the one hand, all wild forms of chillies have small, red, berry-like fruits whose colours and sizes attract birds. On the other hand, the five major cultivated or half-cultivated species of *Capsicum* are *Capsicum annuum* L., *Capsicum chinense* Jacq., *Capsicum frutescens* L., *Capsicum baccatum* L. (*C*. *var*. *pendulum*) and *Capsicum pubescens* R & P (De, 2003; Macrae, 1993; Russo, 2012). *C.annuum*, *C. frutescens* and *C. chinense* form a closely linked group, also called "*annuum* Complex" (Nuez et al., 1996) which, for several authors, are not differentiated species.

1.3.1. *C. annuum* L.

Of all the domesticated species of *Capsicum*, *C. annuum* is the most widely cultivated and is economically the most important one today. We can find these species as fresh, processed or dried (Andrews, 1995), and as bell varieties: NuMex, Jalapeño, red pepper, Serrano, and many others (Fig. 3).

It is a suffrutescent or herbaceous, short-lived perennial (cultivated as annual) of up to 1 m in height, cultivated from the sea level up to an altitude of 2,100 m. Leaves oblong, glabrous; flowers solitary, rarely in pairs, pure white to bluish white, very rarely violet; berries green, maturing into yellow, orange to red shading into brown or purple, pendent, rarely erect, extremely variable in size (up to 20 cm long and 10 cm in diameter), shape and pungency, sometimes lobed, seeds white or cream to yellow (Bosland and Votava, 2003).

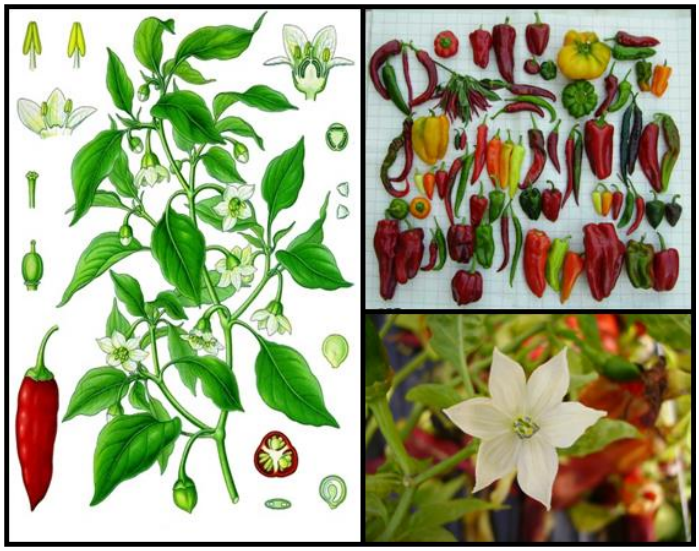


Figure 3. Scheme showing the typical morphology of *C. annuum* plants (Köhler's, 1887). On the right, an example of the diversity of fruits in C. annuum (top) and flower detail (bottom) (Courtesy of A. Rodríguez-Burruezo).

1.3.2. *C. Chinense* Jacq.

Capsicum chinense includes cultivars Habanero, Scotch bonnet, rocotillo, and chili blanco types, and is the dominant domesticated pepper of the Amazonas area. These cultivars are characterised as a small stout shrub of up to 1.5 m tall, glabrous and puberulent with two flowers, or more, at a node. Flowers are pendant (rarely erect) and have a prominent constriction between the base of the calyx and pedicel. The corolla is dull white (greenish white), spreading to recurved. Anthers are blue to violet, rarely yellow. The style and stigma are rarely exerted more than 1 mm. Fruits, of many different colours, contain seeds that are cream to yellow (D'Arcy and Eshbaugh, 1974; Russo, 2012) (Fig. 4). The name *C. chinense* is an anomaly in that no *Capsicum* peppers were ever native to China.

Figure 4. Flower detail and constriction between the base of the calyx and pedice,l and several fruits of *C. chinense* (Courtesy of A. Rodríguez-Burruezo).

1.3.3. *C. frutescens* L.

Capsicum frutescens contains cultivars of the tabasco, malegueta, African birdseye, piri-piri and Thai pepper types. These species are the source of Tabasco sauce, once the most famous hot sauce worldwide.

It is a small shrub, or tree-like shrub, that grows up to 2 m tall. It can be herbaceous to woody. Plants range from glabrous to pubescent, and are mostly puberulent. Typically, two flowers or more are present per node. Flowers lack a prominent constriction between the base of the calyx and pedicel. Calyx teeth are absent. The corolla is greenish white and spreading to recurved. Anthers are blue to violet, rarely yellow. The style and stigma are exerted 1.5 mm, or more, beyond the anthers. Immature fruit is green without dark pigmentation, while mature fruit is red, or very rarely orange, erect, and deciduous. Seeds are cream to yellow (Bosland and Votava, 2003)(Fig. 5).

Figure 5. Flower detail and and several fruits of *C. frutescens* (Courtesy of A. Rodríguez-Burruezo)

1.3.4. *C. baccatum* L. (C. var pendulum)

Capsicum baccatum var. *pendulum*, meaning berry-like, is known as aji, aji Amarillo, cuerno de oro, or cumbia, (Eshbaugh). It is one of the commonest domesticated peppers in Peru. It is also popular in Bolivia, Paraguay, North Argentina and Brazil. This lowland South American species has cream-colored flowers with paired gold or green markings. Typically, fruit are elongated with cream-colored seeds. This species is very hot and its various cultivars offer distinct flavours (Fig. 6).

Figure 6. Flower detail and several fruits of *C. baccatum* (Courtesy of A. Rodríguez-Burruezo)

1.3.5. *C. pubescens* R & P

Capsicum pubescens, the rocoto, locoto, Chile manzana, and others, is morphologically, and genetically, distinct from all the other domesticated peppers. It has large rotate purple or white flowers, typically with five to eight lobes. Fruits contain dark brown or black seeds, which are unique among domesticated peppers. It is found throughout the mid-elevation Andes at

between 1,500 m and 3,000 m. *C. pubescens* has large rugose pubescent leaves. It can be very large, and grows horizontally over the ground or on supporting vegetation, and its length can be in excess of 18 m. Stems often have mixed green and purplish pigments, which confer them a striped appearance (Fig. 7). Its consumption is very limited (De, 2003).

Figure 7. Flower detail, fruits and seeds of *C. baccatum* (middle and left) (Courtesy of A. Rodríguez-Burruezo). General appearance of flowers and fruits (right)

1.4. AGROCLIMATIC REQUIREMENTS

Peppers are well adapted to hot climates. The optimum temperature for seed germination is 25-30 ºC. For growth and fruit quality, areas with temperatures ranging from 21 ºC to 29 ºC are needed (Nonnecke, 1989). When temperature falls below 15 ºC or exceeds 32 ºC, growth is usually retarded, blossom end rot (BER), fruit-set ceases can appear, and yields lower (Knott and Deanon, 1967).

Mainly commercial pepper varieties need well-drained, friable sandy loam soil with pH between 6.5 and 7.5 being optimum for production. Salt content in soil and the irrigation water should be low. A salinity resistance threshold of 1.5 dS m^{-1} has been reported, below which no effect on growth occurs, and a 14% decrease in biomass production for every additional 1dS m^{-1} has been observed (Maas). Thresholds that range from 0 to 2 dS m^{-1} and slopes of salinity response curves that range from 8% to 15% have been reported for greenhouse peppers (Chartzoulakis and Klapaki, 2000; Navarro et al., 2003). Added organic matter will increase water holding capacity and supply nutrients and minerals. Peppers require high frequent soil fertility early in the growing cycle to supplement nitrogen. Lack or excess water induces flower abortion or further BER of fruits (Maroto and Borrego, 2008).

1.5. MAIN ENVIRONMENTAL PROBLEMS TO CULTIVATE PEPPER PLANTS

In the face of a changing climate, global food security demands increasing agricultural production on finite arable land without increasing water use (Davis et al., 2015). With a population increase predicted at around 9 billion by 2050, the World Food Summit on Food Security (2009) sets a target of a 70% increase in global food production. Environmental stresses represent the most limiting conditions for horticultural productivity and plant exploitation worldwide (Nilwik, 1981; Schwarz et al., 2010a). The most limiting factors among them are water availability, temperature, salinity, light, metal ion concentration and pathogens.

Many diseases and disorders can interfere with pepper production and quality, which may have a biotic (living) and abiotic (non-living) origin.

1.5.1. Biotic stresses

Capsicum plants can suffer attacks by different pathogens. The most troublesome and most important diseases and pests are: fungal diseases such as *Phytophthora capsici* (Fig. 8A-B), *Verticillium dahliae*, *Rhizoctonia solani*, *Fusarium* spp., bacteria such as *Xanthomonas campestris*, and powdery mildew (*Leveillula taurica* and *Oidiopsis taurica*), viruses (Fig. 8C) such as Beet Curly Top Virus (BCTV), Tomato Spotted Wilt Virus (TSWV), Pepper Mottle Virus (PMV), several Mosaic Virus (AMV), (CMV), (TMV), nematodes mainly *Meloidogyne incognita,* and several insects (Fig. 8 D-I) such as mites, thrips, aphids and termites.

Figure 8. (A) general view of a pepper field infected by *Phytophthora capsici*, (courtesy of Juan José Tuset, (B) Detail of roots infested with *P. capsici*, (C) Virus, (Courtesy of J.I. Marsal) (D) general view of a pepper field with mites; leaf discoloration and defoliation, (E) Bud parasitised by aphids (courtesy of A. Miguel), (F) Flower detail with thrips (courtesy of J.I. Marsal), (G) Pepper bitten by some insect, (H) Stem affected by termites, (I) Detail of leaf with aphids (courtesy of J. I. Marsal)

Biotic stresses can cause physiological changes in pepper plants, like electrolyte leakage, ion-flux change, hypersensitive cell death, and activation of defensive responses (Lee and Choi, 2013), which can result in diminished yield

and quality. Some of the most dangerous biotic factors are soil diseases, mainly in intensive farming, where limited crop rotations can lead to a build-up of soil-borne pathogens. The main injures to roots caused by these soil pathogens are smaller foliar size, and thin weak stems, wilt, depressed flowering, poorer fruit quality and shorter plant life spans (Rivero et al., 2003). The first symptoms are usually visible in leaves when the roots of the plant are completely infected. The only feasible option for the farmer is to take preventive measures, which involves soil treatments for the following crop season. Since soil fumigation with methyl bromide (MB) is forbidden, other alternatives must be adopted (Batchelor and Miller, 2008). Fumigants are used, but large amounts can be applied which may lead to phytotoxicity (Giannakou et al., 2002; Noe, 1998). Furthermore, the long-term use of fumigants may create changes in the microfauna of soil, which not always favours cultivated plants (Hague and Gowen, 1987). Steam treatment effectively kills pathogens and is not toxic, but it is not economically feasible everywhere because it requires the appropriate machinery for steaming, as well as water and fuel (FAO 2015). Soil solarisation is frequently used in countries with warm climates (Gordh and McKirdy, 2013). However, soil must be covered for 4-6 weeks during a hot period to stop vegetable production. Another alternative is biological control, in which organisms are selected on the basis of their ability to control diseases, but they can be used for aerial plagues.

Plant biotic resistance is another possibility. To improve the tolerance of crops, numerous attempts by traditional breeding programmes have been made. Although commercial success has been very limited due to the complexity of the trait, commercial cultivars can be found with some tolerance. At present, major efforts have being made for the genetic transformation of plants to improve their tolerance (Borsani et al., 2003). Although some increased

tolerance to pathogens has been reported in transgenic peppers (Arthikala et al., 2014; Watson and Preedy, 2015), given poor public acceptance of genetic engineering means in plants, other approaches to achieve resistance must be currently considered (Estañ et al., 2005).

One way of avoiding or reducing loss in production is to graft sensitive plants onto robust rootstocks. Several *Capsicum* rootstocks, including commercial cultivars, breed lines and wild accessions, can confer appropriate tolerance or resistance to *Phytophtora*, *Verticillium*, *Fusarium*, CMV, nematodes, etc. (Kokalis-Burelle et al., 2009; Morra and Bilotto, 2006; Oka et al., 2004; Yamakawa, 1982).

1.5.2. Abiotic stresses

During the growth cycle of peppers, like other plants, many unfavourable environmental conditions may occur, such as salinity, drought, extreme temperatures, moisture, light, mineral deficiencies or toxicities, pH and pollutants, which can all diminish plant yield (Ashraf, 2004; Foolad, 1996; Munns et al., 2006; Russo, 2012). Nearly 82% of the potential yield of crops is lost every year due to abiotic stress, and the amount of available productive arable lands continues to decrease worldwide, forcing farming to move to areas where the abiotic stress potential is higher (Hirt and Shinozaki, 2004).

In the Mediterranean region, one of the most important abiotic stresses are salinity, which is usually present in both soil and water, and water scarcity (**1.4.2.1.** and **1.4.2.2.**), but it is very difficult improve these environmental conditions through crop management.

Other abiotic stresses are: low temperature, which affects pepper vegetative development and reproduction by disturbing the function of the flower female organs and the number of viable pollen grains per flower (Polowick and Sawhney, 1985; Pressman et al., 2006; Shaked et al., 2004); high temperature and radiation promote stunted growth, a smaller photosynthetic rate, increased respiration, and lower water and ion uptake (Nilwik, 1981; Schwarz et al., 2010b). Therefore, the use of different shading screens is thought to be an alternative to overcome these problems (Ilahy et al., 2013; López-Marín et al., 2013). In the same way, heating is used to avoid chilling and frost injury, and cooling is employed to avoid high air temperatures (Russo, 2012).

1.5.2.1. Drought stress

Water scarcity is considered a key threat for the 21st century (UNESCO, 2012). Plants are often subjected to periods of soil and atmospheric water deficit during their life cycle. Only about 15% of the world's agricultural land is irrigated, but these irrigated lands account for almost half the global food production (Tilman et al., 2002). Drought, along with salinity, is one of the most important causes of low yields worldwide (Bodner et al., 2015). Adapted cultivars can improve the synchronisation between crop water demand and soil supply. For these reasons we need to know plant responses to water scarcity, which are complex and involve deleterious and/or adaptive changes (Chaves et al., 2002).

As soil dries, its matric potential becomes more negative (Taíz and Zeiger, 2010). Plants can continue to absorb water only as long as their water potential (Ψ_w) is lower (more negative) than that of soil. The water potential is the total of

both the solute potential (Ψ_s) and the turgor potential (Ψ_p); thus: $\Psi_w = \Psi_s + \Psi_p$ (Kramer and Boyer, 1995). In this way, one of the important pathways to enhance water stress tolerance is through osmotic adjustment, which maintains the leaf turgor required for stomatal opening, and to thus sustain photosynthesis and growth (Huang et al., 2010; Nio et al., 2011). Plants accumulate various types of compatible solutes, such as sugars, proline, glycinebetaine or potassium (Morgan, 1992; Munns et al., 1979; Nio et al., 2011), to reduce the osmotic potential and to be able to absorb water. In short, the accumulation of solutes by cells is a process by which the water potential can lower without an accompanying reduction in turgor or decrease in cell volume.

One of the prompt responses under drought stress is stomatal closure and reduced transpiration rates, which lower the water potential of plant tissues. Consequently, photosynthesis diminishes, mediated by decreased CO_2 availability caused by either: a) diffusion limitations through the stomata and/or mesophyll (Flexas et al., 2007), called stomatal effects; or b) an alteration in the CO_2 fixation reactions mediated by a diminished Rubisco activity, called non-stomatal (Lawlor and Cornic, 2002). Under water stress, as energy accumulates in plants, which consume less light energy through photosynthetic carbon fixation, the generation of reactive oxygen species (ROS) increases (Asada, 2006; Smirnoff, 1993). Accumulation of sorbitol, mannitol and proline, and the formation of radical scavenging compounds, e.g. ascorbate, glutathione and α-tocopherol, can help plants to cope with water stress (Rout and Das, 2013; Yordanov et al., 2003). These compounds can play a dual role as the non-enzymatic antioxidants that plants need to counteract the inhibitory metabolic effects of ROS generated under water stress (Gill and Tuteja, 2010), and also in the stabilisation of enzymes and proteins, and the protection of membrane integrity (Patade et al., 2012). Besides these physiological responses, plants

also undergo morphological changes (Vassileva et al., 2012), like stunted growth and, consequently, smaller yields.

Generally, pepper plants are sensitive to hydric deficit due to a big leaf area and higher stomata conductance (Campos et al., 2014; Delfine et al., 2000; Gonzalez-Dugo et al., 2010). In the pepper production industry, drought imposes huge reductions in crop yield and quality, with significant economic loss of up to 70% (Delfine et al., 2000; Fernández et al., 2005; Pascale et al., 2003). The two most critical moisture stress stages in peppers are the initial establishment of transplanted plants and the stage prior to blossoming (Bosland and Votava, 2003). Thus reduced yields and smaller fruits are frequently recorded under moisture stress conditions and, moreover, this scenario limits the water applied to peppers during the rapid growth period to reduce the final yield (Beese et al., 1982).

1.5.2.2. Salinity

Salinity can be disastrous because it causes many direct and indirect harmful effects. It inhibits seed germination, induces physiological dysfunctions and often kills non-halophyte plants, even at low concentrations, and limits agricultural development (Bartels and Sunkar, 2005; Shannon, 1997). Salinisation transforms fertile and productive land into barren land, and often leads to loss of habitat and loss of biodiversity (Ghassemi et al., 1995). Accumulation of salt in excessive amounts in cultivated soils is a common problem, especially under irrigated conditions, which threatens food production globally (Aktas et al., 2006a; Bohnert and Jensen, 1996; Zeng et al., 2003). The indiscriminate use of large quantities of chemical fertilisers and the

overexploitation of aquifers have dramatically multiplied the surface area affected by salinity (Rivero et al., 2003). Currently, one third of all irrigated lands in the world is affected by salinity to a greater or lesser degree (Pasternak, 1987), which reduces yields.

Salt stress has two components that negatively affect plant growth: the osmotic component and the ionic component. A heavy salt concentration lowers the water potential in soil and induces water stress in plants. This is known as the osmotic component of salinity. Accumulation of certain toxic ions represents the ionic component (Greenway and Munns, 1980).

The relative degree of each salt effect caused by different salinity levels and its consequences on crop production are not clearly understood (Pascale et al., 2003). Saline soils induced by protected culture are complex and can include high concentrations of K^+, Na^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, NO_3^- and Cl^- , which differ from the saline soils induced by seawater, in which NaCl is the most soluble and widespread salt (Huang et al., 2010; Luo et al., 2005). High concentrations of Na^+ reduce the uptake of Ca^{2+} and K^+, and this provokes reduced stomatal conductance, which results in a lower CO_2 concentration and, consequently, photosynthesis lowers. A high Cl^- concentration causes chlorophyll degradation and reduces the actual quantum yield of PSII electron transport (Mitra, 2015).

Salinity can also cause membrane destabilisation (Hasegawa et al., 2000), nutrient imbalance (Munns, 1993), and irreversible damage to plant cells and tissues (Meyer and Boyer, 1981). It is commonly accepted that growth inhibition by salt stress is associated with alterations in the hydric relationships within the plant, caused by osmotic effects with specific ionic consequences.

Salt tolerance mechanisms are:

- Salt exclusion. Plants can limit salt accumulation in their tissues by inhibiting root uptake. Some strategies to restrict salt transport into sensitive organs or tissues have also evolved (Munns, 2002a). Plants' ability to regulate the uptake and transport of salts is dependent on the following mechanisms: selectivity of uptake by root cells; preferential loading of K+ rather than Na+ into the xylem by stele cells; removal of salts from the xylem in upper root parts, the stem and leaf sheaths, based on the exchange of K+ and Na+
- Salt excretion: halophytes frequently take anatomical structures, like salt glands and salt bladders, designed to eliminate excess salt ions from the plant into its environment.
- Intracellular ion compartmentation. Sequestration of salts or ions into leaf and/or shoot vacuoles is a typical attribute of dicotyledonous halophytes. This accumulation is dependent on vacuolar H^+-translocating transporters and tonoplast Na+/H+ antiporters, which are induced by saline environments (Barkla and Pantoja, 1996). An immediate effect of salt stress is cell alkalinisation, linked with Na+/H+ antiporters activity of tonoplast vesicles (Hasegawa et al., 2000). In this case, potassium ions and different types of compatible organic solutes, such as soluble sugar and proline, accumulate in the cytoplasm to prevent dehydration and to maintain the osmotic and ionic balance between these two compartments (Munns and Tester, 2008), and to stabilise sub-cellular structures, such as membranes and proteins (Ashraf and Foolad, 2007; Huang et al., 2010).

In tolerant salt plants, it has been observed after initial loss of cellular turgor that plants can induce an osmotic adjustment to the decrease the external water potential by compartmentalising toxic ions in the vacuole and by synthesising compatible solutes in the cytoplasm (Hasegawa et al., 2000).

Among vegetables, pepper (mainly *C. annuum*) is very susceptible to salt stress. Negative effects on yield result from disturbances in membrane permeability, ion imbalance, water channel activity, stomatal conductance, and reduced total photosynthesis which modifies the carbon balance required to maintain growth and productivity (Kurunc et al. 2011, Piñero et al., 2014, Aktas et al., 2006b; Carvajal et al., 1999).

1.6. MAIN DISORDERS RELATED TO ABIOTIC STRESS IN PEPPER PLANTS

1.6.1. Blossom end rot

Blossom end rot (BER) is a serious disorder that commonly affects peppers grown under various environmental stresses. Symptoms are associated with the membrane leakage of cell solutes, cell plasmolysis, and membrane breakdown (de Freitas et al., 2012; Ho and White, 2005; Saure, 2001). Subsequently, the fruit surface exhibits water-soaked symptoms, and the tissue at the distal portion of the fruit becomes discoloured and necrotic. BER enhances fruit softening and causes premature ripening, which results in small-sized fruits (Aktas et al., 2005) (Fig. 9). In the internal fruit tissue, BER develops in the necrotic region of the parenchymal tissue, which surrounds young seeds, and in the distal placenta (Adams and Ho, 1992; Ho and White, 2005). The

predominant view of the cause of BER is that calcium translocation to the fruit tip is inadequate for the rapid fruit expansion that occurs under conditions which favour rapid fruit growth, such as high temperature and bright light, so that cell integrity is impaired with the consequent tissue disintegration (Turhan et al., 2006). Since Ca^{2+} is thought to play a central role, BER is termed a "calcium-related disorder" (Ho et al., 1993). BER incidence is related to environmental factors, such as high salinity, water scarcity, high temperature and ammonia nutrition, which contribute to Ca^{2+} deficiency (Aktas et al., 2005; Saure, 2014; Taylor et al., 2004).

Figure 9. General view of pepper fruits affected by BER (right) and detail of necrotic tissue

However, a close relationship between calcium levels and BER cannot be always demonstrated (Saure, 2001). Lantos (2007) showed that applying calcium did not necessarily reduce the yield losses caused by calcium deficiency.

The influence of stress on BER occurring in pepper is based in part on an increased NAD(P)H oxidase (an oxygen radicals-generating enzyme) activity, and on increased ROS production, such as superoxide radicals, hydroxyl radicals and singlet oxygen (O_2) in the fruit apoplast (Aktas et al., 2003, 2005; Mestre et al., 2012; Turhan et al., 2006). ROS are known to trigger cell death, which is characterised by a progressive loss of membrane integrity to result in cytoplasm swelling and in the release of cellular constituents (Van Breusegem and Dat, 2006), including loss of Ca^{2+} ions, which may explain the lower Ca^{2+} concentrations mainly in the apoplast (de Freitas et al., 2012).

A certain amount of stress, caused by either a single or an interaction of several environmental factors, like high relative humidity, pathogenic stem diseases, and dry or saline soils, may have a negative effect on calcium uptake (Marcelis and Ho, 1999). However, it does not always result in a corresponding degree of BER (Saure, 2001).

Mainly two kinds of phytohormones appear to interfere, especially with BER affection, and in opposite directions: bioactive gibberellins (GAs) and abscisic acid (ABA). An antagonism action between Ca^{2+} and vegetative growth has already been observed by Lyon et al., (1942). Low Ca^{2+} in the nutrient medium has been reported to result in the most extensive root systems, and indicates high GA activity. In this case, a low supply of Ca^{2+} may have caused the high BER incidence more indirectly via increased GA activity (de Freitas et al., 2012).

As an antagonist to GAs, ABA is known to reduce plant susceptibility to stress; e.g. by promoting the transport of Ca^{2+} to fruits. It has been recently demonstrated that applying ABA to highly stressed tomato plants alleviates BER symptoms (Tonetto de Freitas et al., 2014).

From a practical point of view, GA-signalling can be reduced; e.g. by root restriction (Bar-Tal and Pressman, 1996; Karni et al., 2000), by applying growth-retarding chemicals and by ABA (Lurie et al., 1996; Saito et al., 2004; Wui and Takano, 1995).

In short, BER development requires several steps: stress increases ROS production; ROS causes lipid peroxidation with increased leakiness of membranes, which leads to rapid vacuolation of parenchyma cells and loss of ions, including water-soluble apoplastic Ca^{2+}. Moreover, this situation is aggravated when plants are grown vigorously, when GAs levels are high and when ABA is low. These are typical BER symptoms (Saure, 2014). Accordingly, final Ca^{2+} deficiency can be considered only as a result, but not the cause, of BER.

In order to control BER solutions, it is necessary to reduce susceptibility to stress and alleviate stress severity through the:

- proper selection of suited production sites. Yet it is not always possible, and moreover, environmental conditions are unpredictable,
- improved management practices, like shading or applying calcium fruit sprays. However, there is not enough evidence to recommend their use in managing BER; or spraying ABA, which it is not available as a commercial solution (side effects and no commercial formulation), and by:
- breeding and selecting stress-resistant cultivars. Unfortunately programme are slow and it is difficult obtain a variety which collects commercial fruit attributes and a robust radicular system,
- robust rootstocks have induced increased production in horticultural crops, which has increased the leaf area in grafted tomato plants

(Albacete et al., 2009), and has maintained greater net CO_2 assimilation in grafted cucumber plants (Colla et al., 2010a; Davis et al., 2008), and has also shown a vigorous root system that increased the absorption of water and minerals in pepper grafted plants (Leal-Fernández et al., 2013). In this way, grafting susceptible plants onto robust rootstocks in order to reduce their susceptibility to stress can reduce the fruits affected by BER, maintain water uptake, contribute to better plant nutrition and, consequently, calcium deficiency may diminish (Giuffrida et al., 2013; King et al., 2010; Mándoki and Pénzes, 2012).

1.6.2. Fruit cracking

This is another common physiological disorder that reduces the marketable fruit yield, but this not such a commercial serious problem as BER. In cracked fruits, cracks usually extend through the wall into the locule area due to repeated shrinkage and expansion weakens the fruit cuticule (Yaoi et al., 2000). Incidence is affected by environmental factors (Moresehet et al., 1999), and mainly by varietal characteristics (San Bautista et al., 2011). Several studies have demonstrated the importance of the environment in cuticle cracking development, like low night vapour pressure deficit (Ehret et al., 1993), relative humidity (Johnson and Knavel, 1990) and temperature (Aloni et al., 1998). Fruits with wider expansion-shrinkage amplitude are usually associated with severe cracking symptoms. The water status of fruit is a key factor to determine fruit cracking severity (Russo, 2012). In this way, some solutions can be those that minimise changes in the water status of fruits. In this sense, the same strategies adopted to combat BER can be adopted. However, maintaining a

consistent optimised growing environment is the best way to prevent fruit cracks from developing.

1.6.3. Other disorders

Although peppers are grown mainly under protected cultivation, and the Mediterranean climate offers plentiful irradiation and mild temperature ranges, temperatures and irradiation in winter are suboptimal for pepper crops causing improper ovary development, malformation of flowers and unviable pollen production. At low temperature, parthenorcarpic, misshapen and malformed fruits are produced (Fig. 10) (Rylski et al., 1994). Another common disorder is flower drop. Flower buds, open flowers and immature pod drop are caused by a variety of conditions, like heat stress, insufficient water, and excessive or deficient nutrient levels, which have been reported as casual agents. Leaf rolling and chlorosis caused by CO_2 enrichment in greenhouses is unique to greenhouse peppers (Aloni and Karni, 2002).

Sunscald occurs on the side of fruits exposed to direct sunlight. It first appears as a wrinkled area, which can be soft and lighter in colour than the surrounding tissue. In peppers, this area collapses and turns white. Sunscald primarily affects fruits, but leaves and stems can also be injured. Fruits close to maturity are more sensitive to sunscald injury than immature fruits. Symptoms are similar in appearance to those of BER, but are consistently associated with exposure to direct sunlight (Macrae, 1993).

The use of adequate rootstocks through grafting has provided an alternative strategy to avoid or reduce loss of production caused by excess radiation and

suboptimal temperatures (López-Marín et al., 2013; Rivero et al., 2003; Schwarz et al., 2010b).

Figure 10. Deformed fruits in pepper fields and detail of stunted peppers (left and middle), and a crack in a pepper fruit due to abiotic stresses

Improving managing techniques, such as shading, cooling and heating, may help preventing these disorders from appearing. Looking for less sensitive varieties to the disorders or grafting onto tolerant rootstocks could be an alternative to obtain healthy large foliage, a useful feature to minimise changes in the water balance and to improve mineral absorption by plants.

1.7. COPING WITH ABIOTIC CONSTRAINTS

The impact of climate variability and unpredicted climate change on agricultural productivity is likely to be a major constraint to achieve increased food production. This makes the development of crop genotypes that are resilient to ambient stresses a major strategy for food security. Innovations in crop improvement are needed (Henry, 2014). They are carried out by making tremendous efforts, particularly by breeding companies with traditional breeding programmes. However, commercial success has been very limited due to the complexity of the trait and because practical selection tools are lacking, such as genetic markers that have made these tasks slow inefficient processes to date (Ashraf and Foolad, 2007; Flowers, 2004; Schwarz et al., 2010b). It is very difficult to combine suitable commercial fruit characteristics (high production and quality) with resistance to environmental factors, particularly when traditional varieties are grown for their quality and adaptation traits, as they are usually very sensitive to stress (Finckh, 2008; Lammerts van Bueren et al., 2011).

More recently, major efforts are being made to achieve genetic transformation (Borsani et al., 2003; Cuartero et al., 2006; Martinez-Rodriguez et al., 2008). The transfer of a single gene or a few genes has led to claims of improvement in abiotic stress tolerance (Chinnusamy et al., 2005; Kim et al., 2014; Mickelbart et al., 2015). However, the nature of genetically complex mechanisms of abiotic stress tolerance, and potential detrimental side effects, make this task extremely difficult (Flowers, 2004; Wang et al., 2003). Lack of public acceptance of genetic engineering means that searching for other strategies to generate improved tolerances to abiotic stresses in plants is a priority (Estañ et al., 2005; Munns, 2002b).

One environtal-friendly technique for avoiding or reducing loss in commercial yields caused by abiotic stress conditions is to graft susceptible commercial cultivars onto rootstocks capable of reducing the negative effect of external stress on shoots (Colla et al., 2010b; Rivero et al., 2003; Sánchez-Rodríguez et al., 2014; Savvas et al., 2010; Schwarz et al., 2010b). The use of grafted plants is an eco-friendly strategy that allows plants to overcome both soil-borne diseases and environmental stress(King et al., 2010; Penella et al., 2013; Schwarz et al., 2010b).

1.8. GRAFTING

Grafting is defined as the natural or deliberate fusion of plant parts to establish vascular continuity among them (Pina and Errea, 2005), and the resulting genetically composite organism functions as a single plant (Mudge and Janick, 2009). The term scion refers to the shoot piece that comes from a donor plant and which will be the canopy of the grafted plant. The term rootstock refers to the plant that receives and fuses with the scion and functions as the root system of the grafted plant.

Although grafting of vegetables is an ancient practice, grafting did not become common practice in herbaceous vegetables and ornamentals until the 20th century (Lee, 1994; Lee et al., 2010). The cultivation of grafted horticultural plants began in Korea and Japan at the end of 1920, with the grafting of watermelon plants to squash rootstocks (Yamakawa, 1982). Since then, the use of this technique in watermelon, cucumber, melon, tomato, eggplant, pepper and ornamental cactus has been exponentially increased. Moreover, grafting is being used for not typical fruit vegetables, as artichoke (Temperini et al., 2013;

Trinchera et al., 2013). The advantages of vegetable grafting are attributed principally to resistance of rootstocks to soil-borne diseases (fungus, bacterial wilt and nematodes), but also to increased vigour and stress tolerance. The problems associated with banning methylbromide for soil fumigation have increased vegetable grafting in Europe and the United States in the last few years.

Cleft, approach, micro- and tube grafting are techniques that can reliably join pepper scions with compatible rootstocks, and the same applies to eggplant and tomato (Miguel et al., 2007). Lately, the most popular type is the tube-grafting method, which consists in cutting the growing rootstock tip at a 45º angle below the cotyledons, attaching it to the scion, which has been preciously cut at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip (Fig. 11).

Figure 11. Pepper seedling grafted by the tube-grating method

Commercial varieties are not usually selected to cope with abiotic stress. So grafting onto robust rootstocks can be an interesting method to cope with these problems.

1.8.1. Grafting to cope with salt stress

Grafting plants onto tolerant rootstocks is one of several approaches that cushion the impact of salinity (Chartzoulakis and Klapaki 2000) and is a common agronomic practice in tomato and melon. Several studies have been conducted in these species to elucidate the mechanisms involved in increased salinity tolerance of grafted plants. This increased tolerance of grafted plants is generally associated with their capacity to exclude or retain and/or accumulate toxic ions, Na^+ and Cl^- in rootstock roots, which thus limits their transport to leaves rather than through either the synthesis of osmotically active metabolites or the induction of antioxidant systems (Estañ et al. 2005; Zhu et al. 2008; Edelstein et al. 2011). Other authors have indicated that the influence of rootstock on a scion's salt tolerance is due to the more efficient control of stomatal functions (changes in stomatal regulation and water relations), which suggests that the grafting incision may alter the hormonal signalling between roots and shoots (Aloni et al., 2010). In other cases, such raised tolerance has been explained by the re-establishment of ionic homeostasis (Martinez-Rodriguez et al. 2008). Nevertheless, the mechanism of resistance against salinity in grafted plants displays great complexity in association with specific rootstock/scion interactions (Ferreira-Silva et al., 2010; Zhu et al., 2008), and can vary among species. As far as we know, very few studies of this type have been conducted in pepper to elucidate whether or not the salt tolerance conferred by rootstocks is also due to exclusion and/or retention mechanisms, as in tomato or melon, given their better capacity to alleviate the toxic effects of salts or other processes; e.g., maintenance of water relations or enhanced antioxidant capacity.

1.8.2. Grafting to overcome water stress

A new perspective to improve resistance to water stress is the use of tolerant accessions as rootstocks for a desirable commercial cultivar. The interactions among the graft, vegetable plants and water stress have been mostly studied in melon, cucumber (Rouphael et al., 2012) and tomato (Nilsen et al., 2014; Sánchez-Rodríguez et al., 2013) by focusing on the growth effects of grafting, and on its physiological effects, mainly on hydric relations and photosynthesis traits. Grafted plants usually show an increased uptake of water and minerals compared with self-rooted plants as a result of the vigorous root system used as the rootstock (Martínez-Ballesta et al., 2010; Ruiz et al., 2006; Sánchez-Rodríguez et al., 2013). Greater SOD and CAT activities, higher levels of proline accumulation and lower levels of lipid peroxidation have been found in tobacco scions grafted onto drought-tolerant rootstocks (Liu et al., 2014). Tomato grafted onto a drought-tolerant line has shown not only reduced growth, but also water conservation and increased photosynthetic rates under mild drought conditions (Nilsen et al., 2014). However, there have been no reports on the physiological alterations of pepper after grafting and exposure to water stress. It would be interesting to find scion/rootstock combinations capable of higher water absorption capacity and Water Use Efficiency (WUE), which would result in the major capacity of photosynthesis maintenance, with acceptable stomatal conductance and an adequate hydric plant balance to maintain growth under suboptimal water conditions in order to finally obtain increased yields.

1.8.3. Grafting compatibility and incompatibility

During graft union formation, many researchers have observed callus proliferation from both the rootstock and scion, callus bridge formation, differentiation of new vascular tissue from callus cells and secondary xylem and phloem production (Hartmann et al., 2002). Poor or incorrect callus formation between the rootstock and scion could lead to defoliation, stunted scion growth and low survival of grafted plants (Johkan et al., 2009; Kawaguchi et al., 2008), which would thus reduce the water flow to shoots as decreased hydraulic conductance (Martínez-Ballesta et al., 2010).

There is no precise definition of "graft compatibility", which generally means accomplishing a successful graft union, as well as extended survival and proper functioning of the rootstock-scion composite (Goldschmidt, 2014). Graft incompatibility may be defined as failure to form a successful graft union. Lack of/drop in the number of, differentiated vascular bundles, or the dysfunction of differentiated vascular bundles at the graft union, has been reported to inhibit the transport of nutrients to scion (Breen and Muraoka, 1975; Breen, 1975; Parkinson et al., 1987).

The major causes implicated in graft incompatibility in solanaceous crops are anatomical and/or biochemical (Deloire and Hébant, 1982; Ives et al., 2012; Kawaguchi et al., 2008).

Peppers have been described as being compatible only with other *Capsicum*, unlike other *Solanaceae* species like tomato or eggplant, which can be grafted onto different species within their family (Deloire and Hébant, 1982; Ives et al., 2012; Kawaguchi et al., 2008; Miguel et al., 2007).

Characterisation of incompatibility is not a simple process because graft combinations can initially unite with apparent success, but then gradually develop incompatibility symptoms with time, due to either a failure at the union or the development of abnormal growth patterns (Kawaguchi et al., 2008). The earliest methods used to predict graft incompatibility relied on external symptoms, such as a swollen union, death or decline in vegetative growth and scion vigour, and with marked differences in the growth of both the scion and rootstock (Mudge and Janick, 2009). Nonetheless, the fact that these methods are unreliable has resulted in the development and use of several standardised laboratory methods, such as:

- **Electrophoresis test** to look for cambial peroxidase banding (chestnut, oak and maple) (Zarrouk et al., 2010). Peroxidases produce specific lignins. These compounds have to be similar for both the scion and stock for the graft to be a long-term success.
- **Measurements of breaking weight** (Lindsay et al., 1974) by applying mechanical strength under greenhouse conditions and noticing that compatible grafts can rapidly support the scion, which incompatible grafts cannot achieve.
- **Measurements of electrical resistance** (Yang et al., 1992). Based on the characterisation of differentiating and non-differentiating callus tissues by the external electrical potential.
- ***In vitro* grafting techniques** (Errea et al., 2001; Pina et al., 2009, 2012). A rapid reliable system for studying physiological and molecular processes, but under artificial conditions.
    - **Micrografts**
    - ***In vitro* callus fusion**, undifferentiated tissue from graft partners

- **Histological studies.** Stain tissues at the graft union, examined microscopically.
    - **Optical and fluorescence microscopy** (Ives et al., 2012)
    - **Electron microscopy**
    - **Confocal microscopy**
- **X-ray tomography** as a method to study the 3D structure of the graft interface of grapevines (Milien et al., 2012)

However, all these methods are invasive (destructive), slow and/or most of them have been devised for woody plants. As interesting and closest method can be found in the literature is:

- **Chlorophyll fluorescence imaging (CFI)** (Calatayud et al., 2013). CFI methods are based on the hypothesis that grafting causes stress in plants: mechanical wounding in scions and rootstocks result in localised cell death, loss of water and solute, and disruption of the vascular system. Activating repairing mechanisms places high metabolic demand on the grafting area: supplying carbon skeletons, synthesis of new molecules or increased antioxidant enzyme activity. Many of these processes can be supported by photosynthetic activity. Changes in photosynthesis are associated with variations in fluorescence parameters. The use of images for monitoring florescence parameters allows us to detect alterations, or not, in grafted plants, and has proven an intuitive, quick and non-invasive method for providing details and spatial and temporal heterogeneity information. CFI has been used successfully to predict compatibility in melon graft plants, where the Fv/Fm ratio, which has been associated with the maximal quantum yield of PSII, resulted a sensitive chlorophyll fluorescence parameter

capable of distinguishing compatible and incompatible melon-grafted plants. These advantages of CFI have not yet been tested with other species.

1.9. THESIS OBJECTIVES

Grafting is an eco-friendly technique widely used in tomato, melon or eggplant. Yet it is less exploited in peppers, basically because because rootstock genotypes that are simultaneously tolerant to biotic or abiotic stresses and that, in addition, can improve commercial yields to amortise extra cost incurred by grafting are lacking.

The primary reason for grafting pepper is to increase plant vigour, uniformity and disease tolerance, but very few commercial pepper rootstocks are available because attention has been mainly paid to biotic stresses, and only high value pepper transplants used for protected cultivations are produced as grafted plants (Lee and Oda, 2010; Lee et al., 2010; Oka et al., 2004; Santos and Goto, 2004).

However, the incidence of abiotic stress is very high, an increase in the global climate change is forecast, and salinity and water stress are commonly found in areas where peppers are grown. Doing several screenings to find *Capsicum* plants tolerant to abiotic stress are necessary for them to be used as rootstocks.

To select appropriate rootstocks, searching for resistances in wild pepper types is crucial to amplify genetic diversity (Naegele et al., 2014). Currently, wild species of pepper and plant lines have not been phenotypically characterised

as rootstocks, especially under salt and water stress, and to be finally validated in terms of productivity and quality parameters.

Very little is known about physiological responses in pepper-grafted plants and about their behaviour when they are subject to water deficit and salinity, which hinders the identification of tolerant plants. Knowing how grafting alleviates abiotic stress (physiological markers) is essential for performing more phenotypical screenings of different rootstock/scion combinations.

To achieve this general objective, this thesis was divided into the following specific objectives:

- Screening among *Capsicum* spp. accessions from germplasm banks to find pepper plants that tolerate salinity and water deficit to be used as rootstocks.

- Studying the compatibility between *Capsicum* spp. accessions and pepper commercial varieties using chlorophyll fluorescence imaging (CFI) as a new non-invasive method for their identification and early compatibility prediction compared with conventional microscopy methods.

- Testing the agronomic value in grafted plants using a selected tolerant rootstock under field conditions (salt and water stress).

- Studying the physiological and biochemical responses to salt and water stress of grafted pepper plants in order to know how tolerance to abiotic stresses improves through the robust rootstocks that correlate with agronomical responses.

1.10. REFERENCES

Adams, P., and Ho, L. C. (1992). The susceptibility of modern tomato cultivars to Blossom-End Rot in relation to salinity. J. Hortic. Sci. Biotechnol. 67, 827–840.

Aktas, H., Abak, K., and Cakmak, I. (2006a). Genotypic variation in the response of pepper to salinity. Sci. Hortic. (Amsterdam). 110, 260–266.

Aktas, H., Abak, K., and Cakmak, I. (2006b). Genotypic variation in the response of pepper to salinity. Sci. Hortic. (Amsterdam). 110, 260–266.

Aktas, H., Karni, L., Aloni, B., and Bar-Tal, a. (2003). Physiological and biochemical mechanisms leading to blossom-end rot in greenhouse-grown peppers, irrigated with saline solution. Acta Hortic. 609, 81–88.

Aktas, H., Karni, L., Chang, D. C., Turhan, E., Bar-Tal, A., and Aloni, B. (2005). The suppression of salinity-associated oxygen radicals production, in pepper (Capsicum annuum) fruit, by manganese, zinc and calcium in relation to its sensitivity to blossom-end rot. Physiol. Plant. 123, 67–74.

Albacete, A., Martínez-Andújar, C., Ghanem, M. E., Acosta, M., Sánchez-Bravo, J., Asins, M. J., et al. (2009). Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescence, and increased leaf area and crop productivity in salinized tomato. Plant. Cell Environ. 32, 928–38.

Aloni, B., and Karni, L. (2002). Effects Of CO2 enrichment on yield, carbohydrate accumulation and changes in the activity of antioxidative enzymes in bell pepper (Capsicum annuum L.). J. Hortic. Sci. Biotechnol. 77, 534–540.

Aloni, B., Karni, L., Rylski, I., and Cohen, Y. (1998). Cuticular cracking in pepper fruit. I. Effect of night temperature and humidity. J. Hortic. Sci. Biotechnol. 73, 743–749.

Andrews, J. (1995). Peppers: the domesticated Capsicums. Texas, US: University of Texas Press. 302 pp.

Arthikala, M.-K., Sánchez-López, R., Nava, N., Santana, O., Cárdenas, L., and Quinto, C. (2014). RbohB, a Phaseolus vulgaris NADPH oxidase gene, enhances symbiosome number, bacteroid size, and nitrogen fixation in nodules and impairs mycorrhizal colonization. New Phytol. 202, 886–900.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol. 141, 391–6.

Ashraf, M. (2004). Genetic Variation for Salinity Tolerance in Spring Wheat. Hereditas 120, 99–104.

Ashraf, M., and Foolad, M. R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 59, 206–216.

Barkla, B. J., and Pantoja, O. (1996). PHYSIOLOGY OF ION TRANSPORT ACROSS THE TONOPLAST OF HIGHER PLANTS. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 159–184.

Bar-Tal, A., and Pressman, E. (1996). Root retriction and potassium and calcium solution concentrations affect dry-matter production, cation uptake, and blossom-end rot in greenhouse tomato. J. Am. Soc. Hortic. Sci. 121, 649–655.

Bartels, D., and Sunkar, R. (2005). Drought and Salt Tolerance in Plants. CRC. Crit. Rev. Plant Sci. 24, 23–58.

Batchelor, T. A., and Miller, M. K. (2008). The role of the Montreal Protocol in reducing quarantine and preshipment uses of methyl bromide. in Proceedings of the 8th International Conference on Controlled Atmosphere and Fumigation in Stored Products (Chengdu), 88–93.

Beese, F., Horton, R., and Wierenga, P. J. (1982). Growth and Yield Response of Chile Pepper to Trickle Irrigation1. Agron. J. 74, 556. Bodner, G., Nakhforoosh, A., and Kaul, H.-P. (2015). Management of crop water under drought: a review. Agron. Sustain. Dev. 35, 401–442.

Bohnert, H. J., and Jensen, R. G. (1996). Strategies for engineering water-stress tolerance in plants. Trends Biotechnol. 14, 89–97.

Borsani, O., Valpuesta, V., and Botella, M. a (2003). Developing salt tolerant plants in a new century : a molecular biology. Plant Cell 10, 101–115.

Bosland, P. W., and Votava, E. J. (2003). Peppers: vegetable and spice capsicums. , ed. null. 183 pp.

Breen, P. J. (1975). Effect of peach/plum graft incompatibility on seasonal carbohydrate changes. J. Am. Soc. Hortic. Sci. 100, 253–259.

Breen, P. J., and Muraoka, T. (1975). Seasonal nutrient levels and peach/plum incompatibility. J. Am. Soc. Hortic. Sci. 100, 339–342.

Van Breusegem, F., and Dat, J. F. (2006). Reactive oxygen species in plant cell death. Plant Physiol. 141, 384–90.

Calatayud, Á., San Bautista, A., Pascual, B., Maroto, J. V., and López-Galarza, S. (2013). Use of chlorophyll fluorescence imaging as diagnostic technique to predict compatibility in melon graft. Sci. Hortic. (Amsterdam). 149, 13–18.

Campos, H., Trejo, C., Peña-Valdivia, C. B., García-Nava, R., Conde-Martínez, F. V., and Cruz-Ortega, M. R. (2014). Stomatal and non-stomatal limitations of bell pepper (Capsicum annuum L.) plants under water stress and re-watering: Delayed restoration of photosynthesis during recovery. Environ. Exp. Bot. 98, 56–64.

Carvajal, M., Martínez, V., and Alcaraz, C. F. (1999). Physiological function of water channels as affected by salinity in roots of paprika pepper. Physiol. Plant. 105, 95–101.

Chartzoulakis, K., and Klapaki, G. (2000). Response of two greenhouse pepper hybrids to NaCl salinity during different growth stages. Sci. Hortic. (Amsterdam). 86, 247–260.

Chaves, M. M., Pereira, J. S., Maroco, J., Rodrigues, M. L., Ricardo, C. P. P., Osorio, M. L., et al. (2002). How plants cope with water stress in the field. Photosynthesis and growth. Ann. Bot. 89, 907–916.

Chinnusamy, V., Jagendorf, A., and Zhu, J.-K. (2005). Understanding and Improving Salt Tolerance in Plants ´. Crop Sci. 45, 437–448.

Cichewicz, R. H., and Thorpe, P. A. (1996). The antimicrobial properties of chile peppers (Capsicum species) and their uses in Mayan medicine. J. Ethnopharmacol. 52, 61–70.

Colla, G., Rouphael, Y., Leonardi, C., and Bie, Z. (2010a). Role of grafting in vegetable crops grown under saline conditions. Sci. Hortic. (Amsterdam). 127, 147–155.

Colla, G., Rouphael, Y., Leonardi, C., and Bie, Z. (2010b). Role of grafting in vegetable crops grown under saline conditions. Sci. Hortic. (Amsterdam). 127, 147–155.

Cuartero, J., Bolarín, M. C., Asíns, M. J., and Moreno, V. (2006). Increasing salt tolerance in the tomato. J. Exp. Bot. 57, 1045–1058.

D'Arcy, W. G., and Eshbaugh, W. H. (1974). New world peppers (Capsicum-Solanaceae) north of Colombia: a resume. Baileya 19, 93–105.

Davis, A. R., Perkins-Veazie, P., Sakata, Y., López-Galarza, S., Maroto, J. V., Lee, S.-G., et al. (2008). Cucurbit Grafting. CRC. Crit. Rev. Plant Sci. 27, 50–74.

Davis, K. F., Rulli, M. C., and D'Odorico, P. (2015). The global land rush and climate change. Earth's Futur (in press).

De, A. K. (2003). Capsicum: The genus Capsicum. London: CRC Press.

Delfine, S., Alvino, A., Loreto, F., Centritto, M., and Santarelli, G. (2000). Effects of water stress on the yield and photosynthesis of field-grown sweet pepper (Capsicum annuum L.). Proc. Third Int. Symp. Irrig. Hortic. Crop. Vols 1 2, 223–229 942.

Deloire, A., and Hébant, C. (1982). Peroxidase activity and lignification at the interface between stock and scion of compatible and incompatible grafts of Capsicum on Lycopersicum. Ann. Bot. 49, 887–891.

Ehret, G., Kiemle, C., Renger, W., and Simmet, G. (1993). Airborne remote sensing of tropospheric water vapor with a near-infrared differential absorption lidar system. Appl. Opt. 32, 4534–51.

Errea, P., Garay, L., and Marín, J. A. (2001). Early detection of graft incompatibility in apricot (Prunus armeniaca) using in vitro techniques. Physiol. Plant. 112, 135–141.

Eshbaugh, W. H. A byosystematic and evolutionary study of Capsicum baccatum. Brittonia 22, 31–43.

Estañ, M. T., Martinez-Rodriguez, M. M., Perez-Alfocea, F., Flowers, T. J., and Bolarin, M. C. (2005). Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. J. Exp. Bot. 56, 703–712.

FAO (Food and Agriculture Organization of the United Nations). (2013). FAO Statistical Programme of Work. http://faostat3.fao.org/browse/Q/*/E

Fernández, M. D., Gallardo, M., Orgaz, F., Thompson, F., and Fereres, E. (2005). Water use and production of a greenhouse pepper crop under optimum and limited water supply. J. Hortic. Sci. Biotechnol. 80, 87–96.

Ferreira-Silva, S. L., Silva, E. N., Carvalho, F. E. L., de Lima, C. S., Alves, F. A. L., and Silveira, J. A. G. (2010). Physiological alterations modulated by rootstock and scion combination in cashew under salinity. Sci. Hortic. (Amsterdam). 127, 39–45.

Finckh, M. R. (2008). Sustainable disease management in a European context.. D. B. Collinge, L. Munk, and B. M. Cooke Dordrecht: Springer Netherlands.

Flexas, J., Diaz-Espejo, A., Galmés, J., Kaldenhoff, R., Medrano, H., and Ribas-Carbo, M. (2007). Rapid variations of mesophyll conductance in response to changes in CO2 concentration around leaves. Plant. Cell Environ. 30, 1284–98.

Flowers, T. J. (2004). Improving crop salt tolerance. J. Exp. Bot. 55, 307–319.

Foolad, M. R. (1996). Genetic analysis of salt tolerance during vegetative growth in tomato, Lycopersicon esculentum Mill. Plant Breed. 115, 245–250.

De Freitas, S. T., Jiang, C. Z., and Mitcham, E. J. (2012). Mechanisms Involved in Calcium Deficiency Development in Tomato Fruit in Response to Gibberellins. J. Plant Growth Regul. 31, 221–234.

Ghassemi, E., Jakeman, A., and HA, N. (1995). Salinization of land and water resources, CABI. Wallingford Oxon. 260 pp.

Giannakou, I. O., Sidiropoulos, A., and Prophetou-Athanasiadou, D. (2002). Chemical alternatives to methyl bromide for the control of root-knot nematodes in greenhouses. Pest Manag. Sci. 58, 290–296.

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930.

Giuffrida, F., Cassaniti, C., and Leonardi, C. (2013). The influence of rootstock on growth and ion concentrations in pepper (*Capsicum annuum* L.) under saline conditions. J. Hortic. Sci. Biotechnol. 88, 110–116.

Goldschmidt, E. E. (2014). Plant grafting: new mechanisms, evolutionary implications. Front. Plant Sci. 5, 727-738.

Gonzalez-Dugo, V., Durand, J.-L., and Gastal, F. (2010). Water deficit and nitrogen nutrition of crops. A review. Agron. Sustain. Dev. 30, 529–544.

Gordh, G., and McKirdy, S. (2013). The Handbook of Plant Biosecurity: Principles and Practices for the Identification, Containment and Control of Organisms that Threaten Agriculture and the Environment Globally. Springer Science & Business Media. 560 pp.

Greenway, H., and Munns, R. (1980). Mechanisms of Salt Tolerance in Nonhalophytes. Annu. Rev. Plant Physiol. 31, 149–190.

Hague, N. G. M., and Gowen, S. R. (1987). “Chemical control of nematodes,” in Principles and practice of nematode control in crops, eds. R. H. Brown and B. R. Kerry (Academic Press), 131–178.

Hartmann, H., Kester, D., Davies, F., and Geneve, R. (2002). Plant propagation. Principes and practices. seventh. Upper Saddle River, NJ: Prentice Hall.

Hasegawa, P. M., Bressan, R., Zhu, J.-K., and Bonhert, H. J. (2000). Plant cellular and molecular responses to high salinity. Annu. Rev. Plant. 51, 463–499.

Heiser, C. B., and Pickersgill, B. (1969). Names for the cultivated Capsicum species. Taxon 18, 277–283.

Henry, R. J. (2014). Genomics strategies for germplasm characterization and the development of climate resilient crops. Front. Plant Sci. 5, 68-78.

Hervert-Hernández, D., Sáyago-Ayerdi, S. G., and Goñi, I. (2010). Bioactive compounds of four hot pepper varieties (Capsicum annuum L.), antioxidant capacity, and intestinal bioaccessibility. J. Agric. Food Chem. 58, 3399–3406.

Hirt, H., and Shinozaki, K. (2004). Plant Responses to Abiotic Stress. Springer Science & Business Media. 380pp.

Ho, L. C., Belda, R., Brown, M., Andrews, J., and Adams, P. (1993). Uptake and Transport of Calcium and the Possible Causes of Blossom-end Rot in Tomato. J. Exp. Bot. 44, 509–518.

Ho, L. C., and White, P. J. (2005). A cellular hypothesis for the induction of blossom-end rot in tomato fruit. Ann. Bot. 95, 571–581.

Huang, Y., Bie, Z., He, S., Hua, B., Zhen, A., and Liu, Z. (2010). Improving cucumber tolerance to major nutrients induced salinity by grafting onto Cucurbita ficifolia. Environ. Exp. Bot. 69, 32–38.

Hunziker, A. T. (2001). Genera Solanacearum. The Genera of Solanaceae illustrated, arranged according to a new system. , ed. G. Verlag Liechtenstein: Ruggel.

Ilahy, R., Thouraya, R., Tlili, I., and Jebari, H. (2013). Effect of Different Shading Levels on Growth and Yield Parameters of a Hot Pepper (*Capsicum annuum* L.) Cultivar 'Beldi' Grown in Tunisia. Food 7, 31–35.

Ives, L., Brathwaite, R., Barclay, G., Isaac, W. A., and Connor, C. B. (2012). Graft Compatibility of Scotch Bonnet. J. Agri. Sci. Tec. 2, 81–92.

Johkan, M., Mitukuri, K., Yamasaki, S., Mori, G., and Oda, M. (2009). Causes of defoliation and low survival rate of grafted sweet pepper plants. Sci. Hortic. (Amsterdam). 119, 103–107.

Johnson, D., and Knavel, D. E. (1990). Inheritance of Cracking and Scarring in Pepper Fruit. Agriculture 115, 172–175.

Karni, L., Aloni, B., Bar-Tal, A., Moreshet, S., Keinan, M., and Yao, C. (2000). The effect of root restriction on the incidence of blossom-end rot in bell pepper (*Capsicum annuum* L.). J. Hortic. Sci. Biotechnol. 75, 364–369.

Kawaguchi, M., Taji, A., Backhouse, D., Oda, M., and Science, R. (2008). Anatomy and physiology of graft incompatibility in solanaceous plants. J. Hortic. Sci. Biotechnol. 83, 581–588.

Kim, H., Lee, K., Hwang, H., Bhatnagar, N., Kim, D.-Y., Yoon, I. S., et al. (2014). Overexpression of PYL5 in rice enhances drought tolerance, inhibits growth, and modulates gene expression. J. Exp. Bot. 65, 453–64.

King, S. R., Davis, A. R., Zhang, X., and Crosby, K. (2010). Genetics, breeding and selection of rootstocks for solanaceae and cucurbitaceae. Sci. Hortic. (Amsterdam). 127, 106–111.

Knott, J. E., and Deanon, J. R. (1967). “Eggplant, tomato and pepper vegetable production in southeast asia,” in (Los Banos, Laguna, Philippines: University of Philippines: los Banos Press), 99–109.

Köhler’s (1887). Medizinal-Pflanzen in naturgetreuen.

Kokalis-Burelle, N., Bausher, M. G., and Rosskopf, E. N. (2009). Greenhouse evaluation of Capsicum rootstocks for management of Meloidogyne incognita on grafted bell pepper. Nematropica 39, 121–132.

Kramer, P. J., and Boyer, J. S. (1995). Water Relations of Plants and Soils. Academic Press. 495 pp.

Kurunc, A., Unlukara, A., and Cemek, B. (2011). Salinity and drought affect yield response of bell pepper similarly. Acta Agric. Scand. Sect. B — Soil Plant Sci. 61, 514–522.

Lammerts van Bueren, E. T., Jones, S. S., Tamm, L., Murphy, K. M., Myers, J. R., Leifert, C., et al. (2011). The need to breed crop varieties suitable for organic farming, using wheat, tomato and broccoli as examples: A review. NJAS - Wageningen J. Life Sci. 58, 193–205.

Lantos, F. (2007). A kalcium növényélettani szerepének, jelentő- ségének vizsgálata a paprikatermesztésben, hiánytüneteinek visszaszorítása növénynemesítői eljárásokkal. [Study of the physiological role of calcium in paprika (Capsicum annuum) production]. Agrár- és Vidékfejlesztési Szeml 2, 125–129.

Lawlor, D. W., and Cornic, G. (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant, Cell Environ. 25, 275–294.

Leal-Fernández, C., Godoy-Hernández, H., Núñez-Colín, C. A., Anaya-López, J. L., Villalobos-Reyes, S., and Castellanos, J. Z. (2013). Morphological response and fruit yield of sweet pepper (Capsicum annuum L.) grafted onto different commercial rootstocks. Biol. Agric. Hortic. 29, 1–11.

Lee, J. M. (1994). Grafting Methods , and Benefits. HortScience 29, 235–239.

Lee, J. M., Kubota, C., Tsao, S. J., Bie, Z., Echevarria, P. H., Morra, L., et al. (2010). Current status of vegetable grafting: Diffusion, grafting techniques, automation. Sci. Hortic. (Amsterdam). 127, 93–105.

Lee, J.-M., and Oda, M. (2010). “Grafting of Herbaceous Vegetable and Ornamental Crops,” in Horticultural Reviews (John Wiley & Sons, Inc.), 61–124.

Lee, S., and Choi, D. (2013). Comparative transcriptome analysis of pepper (Capsicum annuum) revealed common regulons in multiple stress conditions and hormone treatments. Plant Cell Rep. 32, 1351–1359.

Lippert, L. F., Smith, P. G., and Bergh, B. O. (1966). Cytogenetics of the vegetable crops. garden pepper,capsicum sp. Bot. Rev. 32, 24–55.

Liu, J., Li, J., Su, X., and Xia, Z. (2014). Grafting improves drought tolerance by regulating antioxidant enzyme activities and stress-responsive gene expression in tobacco. Environ. Exp. Bot. 107, 173–179.

López-Marín, J., González, A., Pérez-Alfocea, F., Egea-Gilabert, C., and Fernández, J. A. (2013). Grafting is an efficient alternative to shading screens to alleviate thermal stress in greenhouse-grown sweet pepper. Sci. Hortic. (Amsterdam). 149, 39–46.

Luo, Q., Yu, B., and Liu, Y. (2005). Differential sensitivity to chloride and sodium ions in seedlings of Glycine max and G . soja under NaCl stress. 162, 1003-1012.

Lurie, S., Handros, A., Fallik, E., and Shapira, R. (1996). Reversible Inhibition of Tomato Fruit Gene Expression at High Temperature (Effects on Tomato Fruit Ripening). Plant Physiol. 110, 1207–1214.

Lyon, C. B., Beeson, K. C., and Barrentine, M. (1942). Macro-element nutrition of the tomato plant as correlated with fruitfulness and occurence of Blossom-End Rot. Bot. Gazzete 103, 651–667.

Maas, E. V. (1976).“Crop salt tolerance,” in Agriccultural salinity assessment and management. ASCE manuals and reports on engineering practices no 71, ed. K. K. Tanji (New York: American Societay Civil Engineers. ASCE).

MacNeish, R. S. (1964). “The food-gathering and incipient agriculture stafe of prehistoric middle america,” in Natural environments and early cultures, ed. R. Wauchope (Austin: University of Texas Press), 413–426.

Macrae, R. (1993). Encyclopedia of food science, food technology and nutrition (Peppers and Chilies). Academic Press.

MAGRAMA (Ministerio de Agricultura, Alimentación y Medio Ambiente) (2014). Avance Anuario Estadística. http://www.magrama.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/#para1

MAGRAMA (Ministerio de Agricultura, Alimentación y Medio Ambiente) (2012). Hortalizas de fruto pimiento: Análisis provincial de superficie, rendimiento y producción. http://www.magrama.gob.es/estadistica/pags/anuario/2013/AE_2013_13_06_28_02.pdf

Mándoki, Z., and Pénzes, B. (2012). Effects of Using Chemical-Free Root-Knot Nematode (Meloidogyne Incognita) Control Methods on the Occurrence of Blossom-End Rot in Pepper. J. Plant Prot. Res. 52, 337–341.

Marcelis, L. F. M., and Ho, L. C. (1999). Blossom-end rot in relation to growth rate and calcium content in fruits of sweet pepper (*Capsicum annuum* L.). J. Exp. Bot. 50, 357–363.

Maroto, J. V., and Borrego, J. V. M. i (2008). Elementos de horticultura general. Mundi-Prensa. 452 pp.

Martínez-Ballesta, M. C., Alcaraz-López, C., Muries, B., Mota-Cadenas, C., and Carvajal, M. (2010). Physiological aspects of rootstock–scion interactions. Sci. Hortic. (Amsterdam). 127, 112–118.

Martinez-Rodriguez, M. M., Esta??, M. T., Moyano, E., Garcia-Abellan, J. O., Flores, F. B., Campos, J. F., et al. (2008). The effectiveness of grafting to improve salt tolerance in tomato when an “excluder” genotype is used as scion. Environ. Exp. Bot. 63, 392–401.

Mateos, R. M., Jiménez, A., Román, P., Romojaro, F., Bacarizo, S., Leterrier, M., et al. (2013). Antioxidant systems from pepper (Capsicum annuum L.): Involvement in the response to temperature changes in ripe fruits. Int. J. Mol. Sci. 14, 9556–9580.

Mestre, T. C., Garcia-Sanchez, F., Rubio, F., Martinez, V., and Rivero, R. M. (2012). Glutathione homeostasis as an important and novel factor controlling blossom-end rot development in calcium-deficient tomato fruits. J. Plant Physiol. 169, 1719–1727.

Meyer, R. F., and Boyer, J. S. (1981). Osmoregulation, Solute Distribution, and Growth in Soybean Seedlings Having Low Water Potentials. Planta 151, 482–489.

Mickelbart, M. V., Hasegawa, P. M., and Bailey-Serres, J. (2015). Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. Nat. Rev. Genet. 16, 237–251.

Miguel, A., De la Torre, F., Baixauli, C., Maroto, J. V, Jordá, M. C., López, M. M., et al. (2007). Injerto de hortalizas. Valencia, Spain: Ministerio de Agricultura, Pesca y Alimentación y Fundación Rural Caja. 360pp.

Milien, M., Renault-Spilmont, A.-S., Cookson, S. J., Sarrazin, A., and Verdeil, J.-L. (2012). Visualization of the 3D structure of the graft union of grapevine using X-ray tomography. Sci. Hortic. (Amsterdam). 144, 130–140.

Mitra, G. N. (2015). Regulation of Nutrient Uptake by Plants: A Biochemical and Molecular Approach. 301pp.

Morgan (1992). Osmotic Components and Properties Associated With Genotypic Differences in Osmoregulation in Wheat. Aust. J. Plant Physiol. 19, 67.

Morra, L., and Bilotto, M. (2006). Evaluation of new rootstocks for resistance to soil-borne pathogens and productive behaviour of pepper (Capsicum annuum L.). J. Hortic. Sci. Biotechnol. 81, 518–524.

Moscone, E. A., Lambrou, M., Hunziker, A. T., and Ehrendorfer, F. (1993). C-banded karyotypes in Capsicum (Solanaceae). Plant Syst. Evol. 186, 213–229.

Mudge, K., and Janick, J. (2009). A history of grafting. Rev. Vol. 35 35, 437–494.

Munns, R. (1993). Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. Plant Cell Environ. 16, 15–24.

Munns, R. (2002a). “Avenues for increasing salt tolerance of crops,” in Plant Nutrition, 370–371.

Munns, R. (2002b). Comparative physiology of salt and water stress. Plant cell Environ. 25, 239–250.

Munns, R., Brady, C., and Barlow, E. (1979). Solute Accumulation in the Apex and Leaves of Wheat During Water Stress. Aust. J. Plant Physiol. 6, 379.

Munns, R., James, R. A., and Läuchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. J. Exp. Bot. 57, 1025–43.

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681.

Naegele, R. P., Boyle, S., Quesada-Ocampo, L. M., and Hausbeck, M. K. (2014). Genetic diversity, population structure, and resistance to Phytophthora capsici of a worldwide collection of eggplant germplasm. PLoS One 9, e95930.

Navarro, J. M., Garrido, C., Martínez, V., and Carvajal, M. (2003). Water relations and xylem transport of nutrients in pepper plants grown under two different salts stress regimes. Plant Growth Regul. 41, 237–245.

Nilsen, E. T., Freeman, J., Grene, R., and Tokuhisa, J. (2014). A Rootstock Provides Water Conservation for a Grafted Commercial Tomato (Solanum lycopersicum L.) Line in Response to Mild-Drought Conditions: A Focus on Vegetative Growth and Photosynthetic Parameters. PLoS One 9, e115380.

Nilwik, H. J. M. (1981). Growth Analysis of Sweet Pepper (Capsicum annuum L.): 1. The Influence of Irradiance and Temperature under Glasshouse Conditions in Winter. Ann. Bot. 48, 129–136.

Nio, S. A., Cawthray, G. R., Wade, L. J., and Colmer, T. D. (2011). Pattern of solutes accumulated during leaf osmotic adjustment as related to duration of water deficit for wheat at the reproductive stage. Plant Physiol. Biochem. PPB Société française Physiol. végétale 49, 1126–37.

Noe, J. P. (1998). “Crop-and nematode-management systems,” in Plant nematode interactions, eds. K. R. Barker, G. A. Perderson, and G. L. Windham (Madison: ASA, CSSA, SSSA Press).

Nonnecke, I. L. (1989). Vegetable Production. AVI Book. 661pp.

Nuez, F., Gil, R., and Costa, J. (1996). El cultivo de pimientos, chiles y ajies. Mundi-Prensa. 607pp.

Oka, Y., Offenbach, R., and Pivonia, S. (2004). Pepper Rootstock Graft Compatibility and Response to Meloidogyne javanica and M. incognita. J. Nematol. 36, 137–41.

Olmstead, R. G., Sweere, J. A., Spangler, R. E., Bohs, L., and Palmer, J. D. (1999). "Phylogeny and provisional classification of the Solanaceae based on chloroplast DNA," in Solanaceae IV: Advances in biology and utilization, eds. M. Nee, D. E. Symon, R. N. Lester, and J. P. Jessop (New, UK: Royal Botanic Gardens), 111–137.

Parkinson, M., Jeffree, C. E., and Yeoman, M. M. (1987). Incompatibility in cultured explant-grafts between members of Solanaceae. New Phytol. 107, 489–498.

Pascale, S. De, Ruggiero, C., and Barbieri, G. (2003). Physiological Responses of Pepper to Salinity and Drought. J. Am. Sociol. Hortic. Sci. 128, 48–54.

Pasternak, D. (1987). Salt Tolerance and Crop Production-A Comprehensive Approach. Annu. Rev. Phytopathol. 25, 271–291.

Patade, V. Y., Bhargava, S., and Suprasanna, P. (2012). Halopriming mediated salt and iso-osmotic PEG stress tolerance and, gene expression profiling in sugarcane (Saccharum officinarum L.). Mol. Biol. Rep. 39, 9563–9572.

Penella, C., Nebauer, S. G., Lopéz-Galarza, S., SanBautista, A., Gorbe, E., and Calatayud, A. (2013). Evaluation for salt stress tolerance of pepper genotypes to be used as rootstocks. J. Food, Agric. Environ. 11, 1101–1107.

Pina, A., and Errea, P. (2005). A review of new advances in mechanism of graft compatibility- incompatibility. Sci. Hortic. 106, 1–11.

Pina, A., Errea, P., and Martens, H. J. (2012). Graft union formation and cell-to-cell communication via plasmodesmata in compatible and incompatible stem unions of Prunus spp. Sci. Hortic. 143, 144–150.

Pina, A., Errea, P., Schulz, A., and Martens, H. J. (2009). Cell-to-cell transport through plasmodesmata in tree callus cultures. Tree Physiol. 29, 809–818.

Piñero, M. C., Houdusse, F., Garcia-Mina, J. M., Garnica, M., and Del Amor, F. M. (2014). Regulation of hormonal responses of sweet pepper as affected by salinity and elevated CO2 concentration. Physiol. Plant. 151, 375–89.

Polowick, P. L., and Sawhney, V. K. (1985). Temperature effects on male fertility and flower and fruit development in Capsicum annuum L. Sci. Hortic. 25, 117–127.

Pressman, E., Shaked, R., and Firon, N. (2006). Exposing pepper plants to high day temperatures prevents the adverse low night temperature symptoms. Physiol. Plant. 126, 618–626.

Rivero, R., Ruiz, J., and Romero, L. (2003). Role of grafting in horticultural plants under stress conditions. J. Food. Agri. Sci. 1, 70–74.

Rodríguez-Burruezo, A., Prohens, J., Raigón, M. D., and Nuez, F. (2009). Variation for bioactive compounds in ají (Capsicum baccatum L.) and rocoto (C. pubescens R. & P.) and implications for breeding. Euphytica 170, 169–181.

Rouphael, Y., Cardarelli, M., Rea, E., and Colla, G. (2012). Improving melon and cucumber photosynthetic activity, mineral composition, and growth performance under salinity stress by grafting onto Cucurbita hybrid rootstocks. Photosynthetica 50, 180–188. Rout, G. R., and Das, A. B. (2013). Molecular Stress Physiology of Plants. Springer Science & Business Media. 440 pp.

Ruiz, J. M., Rios, J. J., Rosales, M. A., Rivero, R. M., and Romero, L. (2006). Grafting between tobacco plants to enhance salinity tolerance. J. Plant Physiol. 163, 1229–1237.

Russo, V. (2012). Peppers: botany, production and uses. London: CAB International. 280 pp.

Rylski, I., Aloni, B., Karni, L., and Zaidman, Z. (1994). Fruit development and fruit quality under different environmental conditions in tomato and pepper crops. Acta Hortic. 366, 45–56.

Saito, S., Hirai, N., Matsumoto, C., Ohigashi, H., Ohta, D., Sakata, K., et al. (2004). Arabidopsis CYP707As encode (+)-abscisic acid 8'-hydroxylase, a key enzyme in the oxidative catabolism of abscisic acid. Plant Physiol. 134, 1439–49.

San Bautista, A., Calatayud, A., Nebauer, S. G., Pascual, B., Maroto, J. V., and López-Galarza, S. (2011). Effects of simple and double grafting melon plants on mineral absorption, photosynthesis, biomass and yield. Sci. Hortic. (Amsterdam). 130, 575–580.

Sánchez-Rodríguez, E., Leyva, R., Constán-Aguilar, C., Romero, L., and Ruiz, J. M. (2014). How does grafting affect the ionome of cherry tomato plants under water stress? Soil Sci. Plant Nutr. 60, 145–155.

Sánchez-Rodríguez, E., Romero, L., and Ruiz, J. M. (2013). Role of Grafting in Resistance to Water Stress in Tomato Plants: Ammonia Production and Assimilation. J. Plant Growth Regul. 32, 831–842.

Santos, H. S., and Goto, R. (2004). Enxertia em plantas de pimentão no controle da murcha de fitóftora em ambiente protegido. Hortic. Bras. 22, 45–49.

Saure, M. C. (2001). Blossom-end rot of tomato (Lycopersicon esculentum Mill.) — a calcium- or a stress-related disorder? Sci. Hortic. (Amsterdam). 90, 193–208.

Saure, M. C. (2014). Why calcium deficiency is not the cause of blossom-end rot in tomato and pepper fruit - a reappraisal. Sci. Hortic. (Amsterdam). 174, 151–154.

Savvas, D., Colla, G., Rouphael, Y., and Schwarz, D. (2010). Amelioration of heavy metal and nutrient stress in fruit vegetables by grafting. Sci. Hortic. (Amsterdam). 127, 156–161.

Schwarz, D., Rouphael, Y., Colla, G., and Venema, J. H. (2010a). Grafting as a tool to improve tolerance of vegetables to abiotic stresses: Thermal

stress, water stress and organic pollutants. Sci. Hortic. (Amsterdam). 127, 162–171.

Schwarz, D., Rouphael, Y., Colla, G., and Venema, J. H. (2010b). Grafting as a tool to improve tolerance of vegetables to abiotic stresses: Thermal stress, water stress and organic pollutants. Sci. Hortic. (Amsterdam). 127, 162–171.

Shaked, R., Rosenfeld, K., and Pressman, E. (2004). The effect of low night temperatures on carbohydrates metabolism in developing pollen grains of pepper in relation to their number and functioning. Sci. Hortic. (Amsterdam). 102, 29–36.

Shannon, M. C. (1997). Adaptation of plants to salinity. Adv. Agron. 60, 75–120.

SMIRNOFF, N. (1993). The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol. 125, 27–58.

Taíz and Zeiger (2010). Stress Physiology. Fifth edition. Sinoauer Associates. 676 pp.

Taylor, M. D., Locascio, S. J., and Alligood, M. R. (2004). Blossom-end rot incidence of tomato as affected by irrigation quantity, calcium source, and reduced potassium. HortScience 39, 1110–1115.

Temperini, O., Calabrese, N., Temperini, A., Rouphael, Y., Tesi, R., Lenzi, A., et al. (2013). Grafting artichoke onto cardoon rootstocks: Graft compatibility, yield and Verticillium wilt incidence. Sci. Hortic. (Amsterdam). 149, 22–27.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. Nature 418, 671–7.

Tonetto de Freitas, S., McElrone, A. J., Shackel, K. A., and Mitcham, E. J. (2014). Calcium partitioning and allocation and blossom-end rot development in tomato plants in response to whole-plant and fruit-specific abscisic acid treatments. J. Exp. Bot. 65, 235–47.

Trinchera, A., Pandozy, G., Rinaldi, S., Crinò, P., Temperini, O., and Rea, E. (2013). Graft union formation in artichoke grafting onto wild and cultivated cardoon: an anatomical study. J. Plant Physiol. 170, 1569–78.

Turhan, E., Aktas, H., Deventurero, G., Karni, L., Bar-Tal, a, and Aloni, B. (2006). Blossom-end rot is associated with impairment of sugar metabolism and growth of pepper (Capsicum annuum L.) fruits. J. Hortic. Sci. Biotechnol. 81, 921–927.

Vassileva, V., Demirevska, K., Simova-Stoilova, L., Petrova, T., Tsenov, N., and Feller, U. (2012). Long-Term Field Drought Affects Leaf Protein Pattern and Chloroplast Ultrastructure of Winter Wheat in a Cultivar-Specific Manner. J. Agron. Crop Sci. 198, 104–117.

Wang, W., Vinocur, B., and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. Planta 218, 1–14.

Watson, R. R., and Preedy, V. R. (2015). Genetically Modified Organisms in Food: Production, Safety, Regulation and Public Health. Elsevier Science. 516 pp.

Wui, M., and Takano, T. (1995). Effect of temperature and concentration of nutrient solution during the stage of the fruit development on the incidence of blossom-end rot in fruits of tomato, Lycopersicon escultentum L. Environ. Control Biol. 33, 7–9.

Yamakawa, K. (1982). Use of rootstocks in Solanaceous fruit-vegetable production in Japan. JARQ-Japan Agric. Res. Q. 15, 175–179.

Yaoi, C., Moreshet, S., Aloni, B., and Karni, L. (2000). Effect of water stress and climatic factors on the diurnal fluctuation in diameter of bell peppers. J. Hortic. Sci. Biotechnol. 75, 6–11.

Yordanov, I., Velikova, V., and Tsonev, T. (2003). Plant Responses To Drought and Stress Tolerance. Bulg. J. Plant Physiol 38, 187–206.

Zarrouk, O., Testillano, P. S., Risueño, M. C., Moreno, M. Á., and Gogorcena, Y. (2010). Changes in cell/tissue organization and peroxidase activity as markers for early detection of graft incompatibility in peach/plum combinations. J. Am. Soc. Hort. Sci. 135, 9–17

Zeng, L., Lesch, S. M., and Grieve, C. M. (2003). Rice growth and yield respond to changes in water depth and salinity stress. Agric. Water Manag. 59, 67–75.

Zhu, J., Bie, Z., Huang, Y., and Han, X. (2008). Effect of grafting on the growth and ion concentrations of cucumber seedlings under NaCl stress. Soil Sci. Plant Nutr. 54, 895–902.

CHAPTER 2
Evaluation Of Some Pepper Genotypes As Rootstocks In Water Stress Conditions

Consuelo Penella[1], Sergio G. Nebauer[2], Salvador Lopéz-Galarza[2], Alberto SanBautista[2], Adrián Rodríguez-Burruezo[3], Ángeles Calatayud[1]

[1]*Instituto Valenciano de Investigaciones Agrarias (IVIA). Departamento de Horticultura. Ctra. Moncada-Naquera km. 4.5. 46113-Moncada, Valencia, Spain.*

[2]*Universitat Politècnica de València. Departamento de Producción Vegetal. Camino de Vera 14, 46020 Valencia, Spain.*

[3]*Instituto de Conservación y Mejora de la Agrodiversidad Valenciana (COMAV). Ciudad Politécnica de la Innovación. Universitat Politècnica de València. Camino de Vera s/n, 46020 Valencia, Spain.*

Horticultural Science, 41:192-200 (2014)

2.1. ABSTRACT

Water stress is a major environmental factor that limits crop production and so it is worthwhile developing crop varieties that can produce higher yields despite water scarcity. Increasing pepper tolerance to water stress by grafting onto robust rootstocks could be an optimal and environmentally friendly approach. Our work evaluated the behavior of 18 pepper genotypes during vegetative and reproductive stages under water stress in order to select tolerant genotypes as rootstocks for pepper cultivation. The pepper tolerance screening was based on photosynthetic parameters. The genotypes 'Atlante', 'C-40', 'Serrano', 'PI-152225', 'ECU-973', 'BOL-58' and 'NuMex Conquistador' were found to be the most tolerant as they maintained net photosynthetic rates under water stress. The selected genotypes were validated in terms of productivity as rootstocks on a pepper cultivar under severe water stress. Plants grafted onto 'Atlante', 'PI-152225' and 'ECU-973' showed significantly less yield reduction caused by water stress than ungrafted cultivars.

Keywords: Capsicum annuum; chlorophyll fluorescence; graft; photosynthesis; yield

2.2. INTRODUCTION

Bell pepper (Capsicum annuum L.) is one of the most important crops in the world (Villa-Castonera et al. 2003) and it is one of the most susceptible to water stress, mainly because it has a large transpiring leaf surface and a high stomatal conductance of water vapor (Alvino et al. 1994; Delfine et al. 2002). In the pepper production industry, drought imposes huge reductions in crop yield and quality, with significant economic losses of up to 70% (Delfine et al. 2002; De Pascale et al. 2003; Fernandez et al. 2005). Irrigation is essential for pepper production as these plants are particularly sensitive to moisture stress during flowering and fruit setting (Bosland, Votava 2000). Reduced yields and smaller fruits are frequently recorded under conditions of water stress; and according to Beese et al. (1982) limiting the water applied to peppers during a period of rapid growth reduces the final yield.

Conventional methods for detecting water stress tolerance in plants are laborious and destructive – these methods include water and osmotic potential (Bajji et al. 2000), relative water content (González et al. 2008), leaf mass per area ratio (Yadollahi et al. 2011), proline and antioxidant system measurements (Anjum et al. 2012). The development of non-destructive and rapid technologies – such as leaf gas exchange or chlorophyll (Chl) a fluorescence – provide information about photosynthesis during the plant life cycle. Photosynthesis was found to be an informative indicator for the study of water stress effects because of its extreme sensitivity to environmental stress (Massacci et al. 2008). For this reason, these technologies have been used to analyze variations in the response to water stress in different species (Barnaby et al. 2013; Flexas et al. 2004a; Munns et al. 2010; Xu et al. 2013). The main effect of water stress is the reduction in carbon fixation associated with stomatal closure and the subsequent increase in resistance to CO2 diffusion in the leaves

(Kaiser 1987). This effect results in a decrease in the rate of leaf photosynthesis and photochemical Chl a fluorescence parameters (Calatayud et al. 2006; Lu, Zhang 1998). Moreover, the decrease in carbohydrate synthesis reduces plant growth and, therefore, has a great impact on crop yield (Stuart et al. 2011). The need to find pepper plants resistant to water stress has led to several studies and approaches to increase yields and improve quality (Karam et al 2009; Schwarz et al. 2010). Grafting can be an adaptation strategy in integrated or organic agricultural production systems that enables plants to overcome soil borne diseases and environmental stresses (Colla et al. 2010; King et al. 2010; San Bautista et al. 2011). Grafting could enable plant breeders to combine desired shoot characteristics with root features that provide tolerance to water stress (Colla et al. 2010). The cultivation of grafted plants has expanded widely (mainly in tomato, melon and watermelon) (Lee et al. 2010); but this practice is still limited in peppers (King et al. 2010; Miguel et al. 2007) and little information exists regarding water stress tolerant pepper rootstocks.

In this work, we hypothesize that drought-tolerant plants will improve the response of the scion to water stress when used as rootstocks, and thus confer greater tolerance and increased yields when compared to ungrafted plants. We evaluated the performance of 18 pepper genotypes during vegetative and reproductive stages under water stress in order to select tolerant genotypes as rootstocks for pepper cultivation. The pepper tolerance screening was based on photosynthetic parameters that enable measurements for comparing the behavior of the same plant in two phenological states (given that the plants could show varying sensitivity to water stress during the cycle). For practical reasons, a good rootstock must be tolerant to water stress during all states of development. The selected genotypes were validated as rootstocks on a pepper cultivar in terms of a productivity parameter under severe water stress.

2.3. MATERIALS AND METHODS

2.3.1. Experiment 1: Screening pepper genotypes to be used as rootstocks under water stress conditions during vegetative and reproductive stages

Many different genotypes were used in this study and a numerical code for each cultivar is indicated in brackets. The commercial rootstock cultivars used in this study were: 'Atlante' (Ramiro Arnedo (1)); 'C40' (Ramiro Arnedo (2)); 'Tresor' (Nunhems (3)); the accessions of Capsicum annuum 'Serrano Criollo de Morelos-334' (4); 'Serrano' (5); 'Pasilla Bajío' (6); 'Pimiento de Bola' (7); 'Piquillo de Lodosa' (8); 'Guindilla' (9); 'Habanero' (10); and 'NuMex Conquistador' (17); the accessions of Capsicum chinense Jacq. 'PI-152225' (11); ECU-973 (12) and the accessions of Capsicum baccatum L. var. pendulum 'BOL-134' (13) and 'BOL-58' (14); the accessions of Capsicum pubescens R.&P. 'BOL 60 amarillo' (15) and 'BOL 60 rojo' (16); and the accession of Capsicum frutescens L. 'BOL-144' (18). All of these accessions belong to the collection of the COMAV Institute (Universitat Politècnica de València, Valencia, Spain). Seeds were germinated in moistened perlite under greenhouse conditions at 28±2ºC and 80% relative humidity. The seedlings with eight mature leaves were transferred on 15 January 2011 to 15 L pots (containing a dust substrate as coir) in a heated polyethylene greenhouse at the Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain). Plants were drip–irrigated with Hoagland's No.2 nutrient solution contained (all in mM): 14 NO_3^-, 1.0 $H_2PO_4^-$, 2.0 SO_4^{2-}, 1.0 NH_4^+, 16.0 K^+, 4.0 Ca^{2+} and 2.0 Mg^{2+}. Micronutrients were also provided (all in μM): 15 Fe^{2+}, 10 Mn^{2+}, 5 Zn^{2+}, 30 B^{3+}, 0.75 Cu^{2+} and 0.6 Mo^{6+} (Maynard, Hochmuth 2007). The EC of the nutrient solution was 1.9 dS m-1 and

pH 6.1. The greenhouse conditions in this period were 16-22 ºC and 50-70 % of relative humidity.

After 15 days in the pots, 16 plants were divided in two groups (eight plants each) for control and water deficit treatments. Water deficit treatment was initiated by reducing the volume of irrigation water to 60% of the control, the latter being based on estimations of the weekly crop evapotranspiration (ETc). The volume of each irrigation and the number of irrigations were scheduled to maintain drainage at between 10% and 20% (depending on solar radiation).

Eight plants per cultivar were used in each treatment with a density of 4.1 plant m^2. Plants were grown in pots for five months. The environmental parameter ranges in the greenhouse were: temperature (21-24ºC); relative humidity (52-72%); and solar radiation (750-1150 μmol m^{-2} s^{-1}).

Net CO_2 fixation rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance of water vapor (gs, mol H_2O m^{-2} s^{-1}) and substomatal CO_2 concentration (Ci, μmol CO_2 mol^{-1} (air)) were measured at steady-state under conditions of saturating light (1200 μmol m^{-2} s^{-1}) and 400 ppm CO_2 with a LI-6400 (LI-COR, Nebraska, USA). To evaluate the presence of chronic photoinhibitory processes, the maximum quantum yield of PSII (F_v/F_m: $(F_m-F_o)/F_m$) was measured on leaves after 30 minutes of dark adaptation using a portable pulse amplitude modulation fluorometer (MINI PAM, Walz, Effeltrich, Germany). The background fluorescence signal for dark adapted leaves (F_o) was determined with a 0.5 μmol photon m^{-2} s^{-1} measuring light at a frequency of 600 Hz. The application of a saturating flash of 10,000 μmol photon m^{-2} s^{-1} enabled estimations of the maximum fluorescence (F_m).

Gas exchange and fluorescence measurements (n= 8 per treatment) were performed on the third or fourth leaf from the shoot apex. Measurements were performed at two months (T1, vegetative stage) and five months (T2, reproductive stage) after starting the water deficit treatment.

At the end of experiment (T2), Chl *a* fluorescence imaging under water stress was measured in one genotype ('ECU-97', code 12) tolerant to water stress and another genotype sensitive to water stress ('Piquillo de Lodosa', code 8) based on the photosynthesis rate measurements. Chlorophyll *a* fluorescence imaging was used to provide more detailed information on the spatial heterogeneity of photosynthetic activity under water stress in two genotypes that differ in their photosynthetic rate behavior. Six different plants were used for each genotype and measurements were performed at the third or fourth leaf from the apex with an Imaging-PAM fluorometer (Walz, Effeltrich, Germany). Pepper leaves were darkened for 10 minutes prior to the Fv/Fm measure. Actinic illumination (204 μmol photons m^{-2} s^{-1}) was then turned on and saturating pulses were applied at 20 s intervals for 5 min in order to determine the maximum fluorescence (F'_m), and the Chl fluorescence yield during the actinic illumination (F_s). The quantum efficiency of PSII photochemistry, ϕ_{PSII}, was calculated according to Genty et al. (1989) using the formula: $(F'_m-F_s)/F'_m$. The coefficient of photochemical quenching, q_p, is a measurement of the fraction of open centers calculated as $(F'_m-F_s)/(F'_m-F'_o)$ (Schreiber 1986). Calculation of quenching due to the non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, according to the equation $NPQ= (F_m-F'_m)/F'_m$ (Bilger, Björkman 1991). The measured value of NPQ was divided by four (NPQ/4) for the display of values < 1.000. Images of the fluorescence parameters were displayed by means of a false color code ranging from 0.00 (black) to 1.00 (purple). A single representative leaf is presented in Figure 2. The three small circles in each image are the AOI (areas of interest) and are accompanied by a

small red box displaying the averaged values of the selected fluorescence parameters within this AOI. Three AOI were selected in the central part of the leaf (see Fig. 2). For more details about Chl *a* fluorescence measurements see Calatayud et al. (2006).

Data was analyzed using ANOVA type III and means were compared using Fisher's least significance difference (LSD) test at $P \leq 0.05$ (Statgraphics Centurion for Windows, Statistical Graphics Corp.).

2.3.2. Experiment 2: Yield responses to water stress conditions of the commercial cv. 'Verset' grafted onto the selected genotypes of Experiment 1

The experiment was performed during 2012 in a sweet pepper producing area in Alicante (Spain) and the cultivar 'Verset' F1 was used as scion (California type; Rijk Zwaan, The Netherlands). The genotypes 1, 2, 5, 11, 12, 14 and 17 (all selected as tolerant in Experiment 1), and genotype 3 (a commercial rootstock used by growers and selected as sensitive) were used as rootstocks. Ungrafted 'Verset' plants were used as controls. Pepper seeds were sown in a series of steps to obtain the appropriate diameter for grafting. The graft was performed in the middle of February using the tube grafting method (cutting the growing tip of the rootstock at a 45º angle below the cotyledons, attaching the scion, previously cut at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip). The plants were transplanted to 104-cell trays. They were maintained in a chamber with relative humidity above 95% and air temperature around 28-29º C for a 4-6 day period. The grafted plants were then

removed from the humidity chamber and placed in a greenhouse until transplanted.

The water stress treatment plants were irrigated to satisfy 50% of the evapotranspiration (ETc) by modifying the number of irrigations and maintaining the volume constant during each irrigation, while the irrigation control plants received 100% of ETc.

The seedlings were transplanted on 23 April at a density of 2.1 plants m^2 in a loam soil to a polyethylene greenhouse that featured a randomized block design with three replicates, each consisting of 25 seedlings with 8-10 mature leaves. The electrical conductivity of the irrigation water was 1.03 dS m^{-1}. Fertilizers were applied at a rate of 200 kg ha^{-1} N, 50 kg ha^{-1} P_2O_5, 250 kg ha^{-1} K_2O, 110 kg ha^{-1} CaO, and 35 kg ha^{-1} MgO, as recommended by Maroto (2005).

Harvest was staggered from the beginning of July to the end of September. The marketable fruits were counted and weighed for each genotype and treatment.

Data were subjected to ANOVA type III and means were compared using Fisher's least significance difference (LSD) test at $P \leq 0.05$ (Statgraphics Centurion for Windows, Statistical Graphics Corp.).

2.4. RESULTS AND DISCUSSION

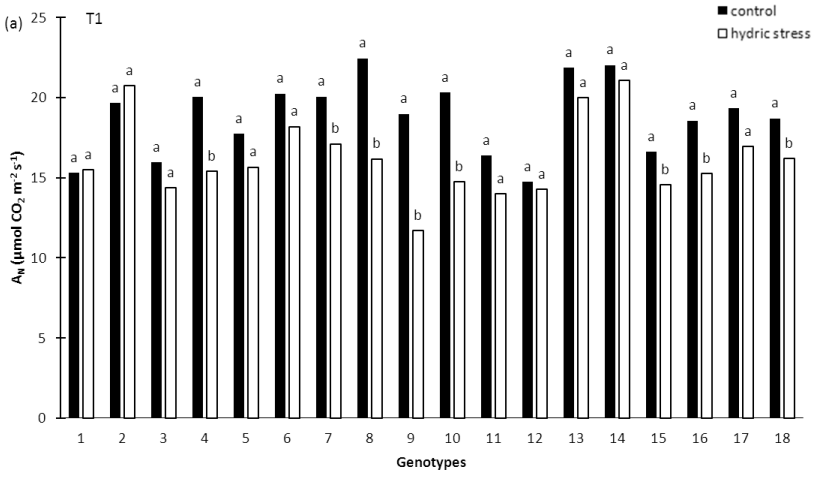
2.4.1.Experiment 1

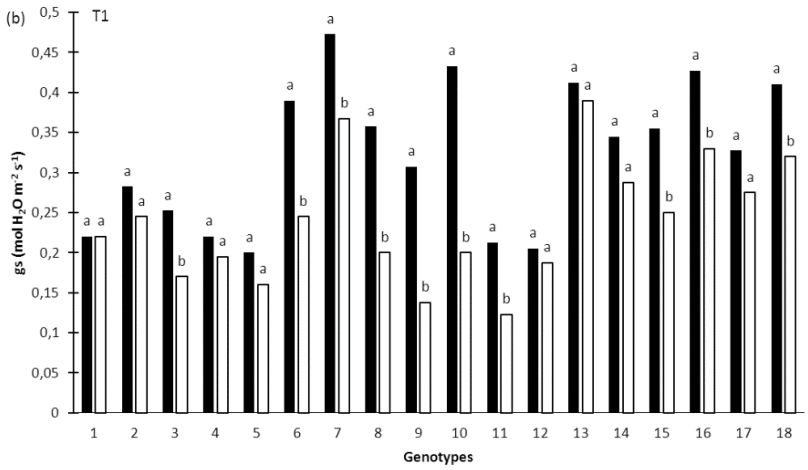
In this work, we evaluated 18 pepper genotypes under water stress conditions in a greenhouse. Photosynthesis measurements were used as a quick and sensitive method that could help identify plants tolerant to water stress (Barnaby et al. 2013; Chaves et al. 2003; Jones 2007; Munns et al. 2010; Xu et al. 2013). Screening for specific tolerance traits in a controlled greenhouse environment is often necessary to reduce the complexity of interactions between genetic and environmental effects on plants. Since tolerance to abiotic stress has been described as a phenomenon specific to the developmental stage (Ashraf 2004), it has been evaluated at different stages in the present study.

One of the earliest responses to water stress is a decrease in stomatal aperture (Chaves et al. 2009; Munns, Tester 2008). This abiotic stress may restrict net photosynthesis either due to diffusional limitation in CO_2 supply arising from a partial closure and/or mesophyll conductance restriction; or by impairing the CO_2 fixation reactions (Niu et al. 2010). In our results, photosynthesis, and stomatal conductance were negatively affected by water stress in vegetative (Fig. 1A, B) and reproductive stages (Fig 1C, D) in some genotypes. A logarithmic correlation between net CO_2 photosynthetic rate and stomatal conductance was observed (A_N = 6.14ln g_s + 25.6; R^2 = 0.68). Moreover, stomatal conductance was related to substomatal CO_2 concentration (C_i = 75.5ln g_s + 365; R^2 = 0.84). These relations indicate that lowered g_s values are responsible for the diminishing intercellular CO_2 concentration, so suggesting stomatal constraints. Only genotypes 1, 5, 12, 14 and 17 maintained A_N and g_s

parameters with values that did not significantly differ during growth in comparison to the controls (Fig. 1). Genotype 11 at T1 (Fig. 1B) and genotype 2 at T2 (Fig. 1C) showed significant differences for g_s parameters with respect to their controls without an effect on A_N. This can be explained by the fact that only very critically low levels of g_s, described as lower than 0.1 mol H_2O $m^{-2}s^{-1}$ (Flexas et al. 2004b), in these genotypes affect photosynthesis. Since limitation by CO_2 was the main factor responsible for the decrease in net photosynthetic carbon uptake rates (Chaves and Oliveira, 2004), we have selected A_N as the indicator parameter for plant tolerance to stress. In this context, the net photosynthesis rates of the genotypes 'Atlante' (1), 'C-40' (2), 'Serrano' (5), 'PI-152225' (11), 'ECU-973' (12), 'BOL-58' (14) and 'NuMex Conquistador' (17) were unaffected by water stress. No differences were observed when compared with their controls in the measured periods (Fig. 1).

The Chl *a* fluorescence parameter F_v/F_m is the maximum quantum yield of PSII photochemistry and is frequently used as an indicator of damaged photoinhibition. In our study, F_v/F_m measured at T1 and T2 did not show significant differences between control and stress treatments (data not shown). Other studies have shown little or no effect on F_v/F_m (Lee et al. 2004; Naumann et al. 2007; Niu et al. 2010;) even when leaf growth and gas exchange were reduced.


(a)
T1
control
hydric stress
A_N (µmol CO_2 m^{-2} s^{-1})
Genotypes


(b)
T1
gs (mol H_2O m^{-2} s^{-1})
Genotypes

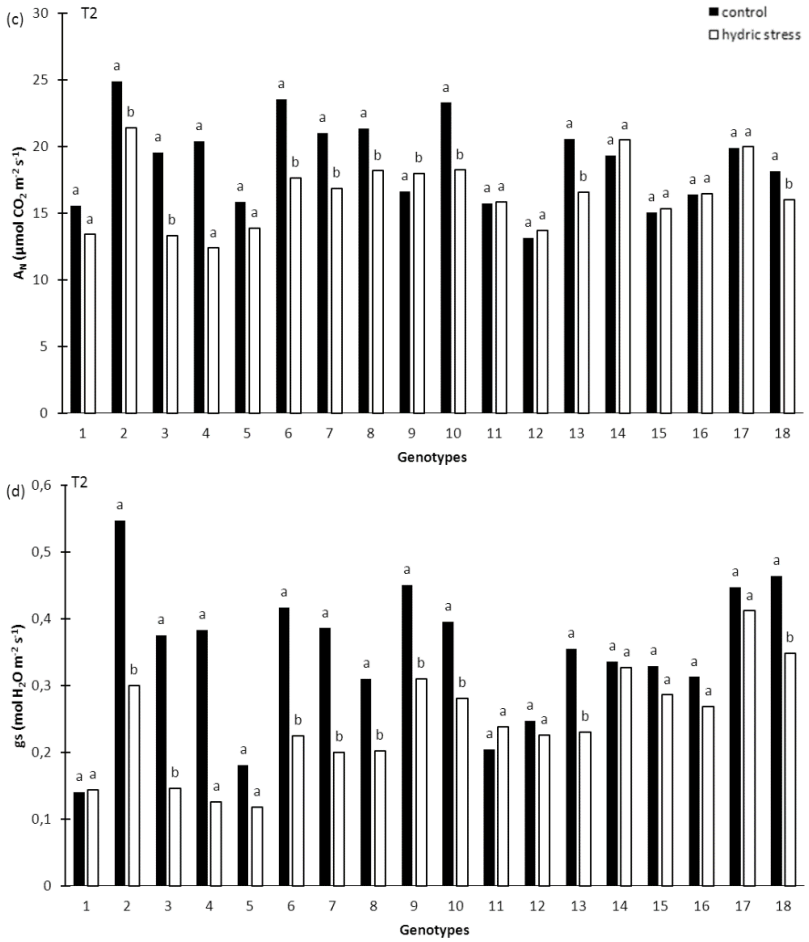


Figure 1. Gas exchange parameters in pepper genotypes measured after (a, b) 2 (T1, vegetative stage) and (c, d) 5 months (T2, reproductive stage) in the control (100% of ETc) and water stress (60% of ETc). A_N – assimilation rate of CO2 fixation; g_s – stomatal conductance to water vapour; values are means of 8 samples; for comparison of means, analysis of variance (ANOVA) followed by the least significant difference (LSD) test were performed and calculated at P≤ 0.05 confidence level; values followed by different letters (on the top of the bars) indicate significant differences between control and water stress treatment

Figure 2 shows Chl *a* fluorescence imaging of F_v/F_m after dark adaptation and Chl *a* fluorescence parameters at steady-state kinetics for a single representative leaf in both stress tolerant (12) and stress sensitive genotypes (8) at T2 under water stress. When both genotypes were compared, the ratio F_v/F_m (0.746 and 0.725 mean values for three AOI for genotype 12 and 8, respectively) and the parameter q_P (0.791 and 0.746 mean values, respectively) were unaffected. This indicates that the photochemistry of PSII and its ability to reduce the primary acceptor electron Q_A was also unaltered by water stress. The ϕ_{PSII} related to the quantum yield of non-cyclic electron transport at any given light intensity (Genty et al. 1989) decreased in genotype 8 (0.412 in Fig. 2) with respect to genotype 12 (0.536 in Fig. 2). Since the q_P parameter was unaffected, the decrease in the rate of non-cyclic electron transport may be caused by factors beyond the Q_A acceptor. Considering the adverse effects of water stress on the electron transport rate, the decrease of photosynthesis could be partially responsible for a decreased availability of ATP and reduced power in genotype 8. However, the possibility of damaged Calvin cycle enzymes after six months of water stress must also be considered (Calatayud et al. 2004; Guidi et al. 2001). The Chl a fluorescence image in ϕ_{PSII} showed the heterogeneous distribution of light utilization and photosynthetic activity over the leaf surface in the genotype 8. The ϕ_{PSII} values in genotype 8 were lower in the upper-left leaf part (0.392) compared to values in the middle of the leaf (0.422).

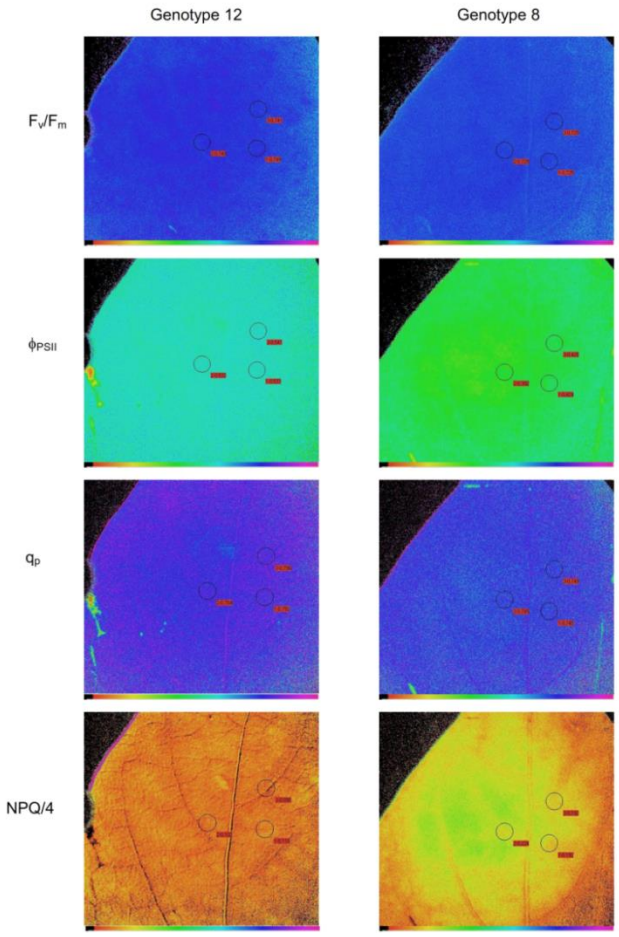


Figure 2. Chlorophyll fluorescence images of Fv/Fm, ϕ_{PSII}, q_P and NPQ/4 at steady-state with actinic illumination of 204 μmol photons m^{-2} s^{-1} at the end of water stress period (6 months) in tolerant (12) and sensitive (8) genotypes in term of photosynthesis rate. The false colour code depicted at the bottom of each image ranges from 0.000 (black) to 1.000 (pink). Images are of a single leaf representative. The three small circles in each image are the AOI – and are accompanied by a little red box displaying the averaged values of the selected fluorescence parameters within this AOI. AOI are defined by the user using PAM software

The heterogeneity of images suggests that pigment composition and concentration, water potential, and stomatal function differ in cells between different regions of the leaf, contributing to spatial differences in photochemical activity under water stress. A decrease in photosynthetic quantum conversion (ϕ_{PSII}) favored the development of non-photochemical quenching (NPQ) in genotype 8 (0.203) compared with genotype 12 (0.103). The NPQ constitutes an important protective response that could dissipate excitation energy in a light-harvesting antenna complex (Müller et al. 2001) and avoid photoinhibition damage (Calatayud et al. 2006) as indicated by the unchanged F_v/F_m ratios. An increase of NPQ on the left of the leaf (0.224) (heterogeneity) in genotype 8 was associated with a decrease of ϕ_{PSII} in this area.

2.4.2. Experiment 2

A significant interaction between genotype x and the irrigation schedule was found in marketable yields ($P \leq 0.01$) (Fig. 3). In general terms, under severe water stress, the grafted cultivar 'Verset' achieved higher marketable yields when compared with ungrafted plants (Fig. 3).

Grafted plants usually show an increased uptake of water and minerals when compared with ungrafted plants as a consequence of a vigorous root system in the rootstock (Martínez-Ballesta et al. 2010). These favorable effects could be due to a correct callus connection between rootstock and scion. A low or incorrect callus formation could lead to defoliation, reduction of scion growth, and a low survival of grafted plants (Martínez-Ballesta et al. 2010). In our results, although genotype 5 appeared to be tolerant in terms of photosynthesis rate (Experiment 1), it provided lower fruit yields when used as rootstock by the

grafted cultivar in control conditions. Furthermore, we observed that plants grafted onto genotype 5 showed a lower growth (1m mean height) than other grafted plants (2m mean height) and its stem diameter at the graft union was approximately three-fold greater than those observed in other plant combinations. These responses are characteristic of graft incompatibility and are due to a poor connection of vascular bundles between rootstock and scion (Oda et al. 1996). Moreover, similar results were obtained for this genotype under saline conditions (Penella et al. 2013).

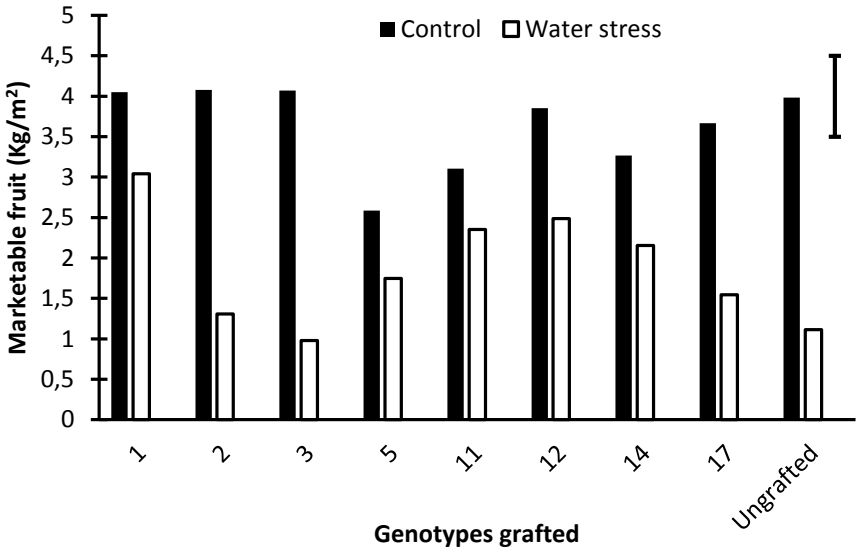


Figure 3. Interaction between genotype x irrigation for marketable fruits (kg/m^2) of cultivar 'Verset' ungrafted or grafted onto genotypes 1, 2, 3, 5, 11, 12, 14 and 17 under control (100% of ETc) or water stress (50% of ETc). Values are mean of n=75 plants. Vertical bar indicates treatment differences by LSD at $P \leq 0.05$

The tolerance to water stress of the rootstocks could result in yield increases in the scion (Sanchez-Rodriguez et al. 2013). Under severe water stress, the grafted plants of our selected tolerant genotypes showed higher marketable yields (mainly in genotypes 1, 11 and 12) than ungrafted plants. In contrast, grafting did not increase yield in control conditions. The main reason to graft is to enhance tolerances to abiotic and biotic stresses – as conferred by robust rootstocks (Lee et al. 2010). Genotype 3 was identified as sensitive to water stress in terms of photosynthesis rate and showed lower yields under water stress conditions in the field. The behavior of this sensitive genotype in terms of A_N during the vegetative and reproductive stages in Experiment 1 was in accordance with the yield decrease in the field under severe water stress when genotype 3 was used as rootstock.

2.5. CONCLUSIONS

Our results confirm that several of the accesions (11, 12, 14 and 17) selected for water stress in this work have shown comparable yields to the commercial rootstocks (1, 2 and 3) under irrigation, and in water stress conditions the yields of the rootstocks 11 and 12 were comparable only to those of the commercial rootstock 'Atlante' (1).

Nevertheless, improvements in management should be made to obtain higher yields of these accessions under stressed and non-stressed conditions, and/or to achieve greater water use efficiency to compensate for the extra cost of grafting. In addition, these results suggest that photosynthesis rate measurements could be considered a useful parameter to screen large

collections of genotypes to drought tolerance before using them as rootstocks with satisfactory yields despite water stress.

2.6. REFERENCES

Alvino A., Centritto M., De Lorenzi. F., 1994. Photosynthesis response of sunlit and shade pepper (Capsicum annuum) leaves at different positions in the canopy under two water regimes. Australia Journal Plant Physiology, 21:377–391.

Ashraf M., 2004. Some important physiological selection criteria for salt tolerance in plants. Flora 199: 361-376.

Anjum S.A., Farooq M., Xie X.Y., Liu, X.J., Ijaz, M.F., 2012. Antioxidant defense system and proline accumulation enables hot pepper to perform better under drought. Scientia Horticulturae, 140: 66-73.

Bajji M., Lutts S., Kinet J.M., 2000. Physiological changes after exposure to and recovery from polyethylene glycol-induced water deficit in roots and leaves of durum wheat (Triticum durum Desf.) cultivars differing in drought resistance. Journal of Plant Physiology, 157: 100-108.

Barnaby J.Y., Kim M., Bauchan G., Bunce J., Reddy V., Sicher R.C., 2013. Drought responses of foliar metabolites in three maize hybrids differing in water stress tolerance. PLOS one 8: e77145.

Beese F., Horton R., Wierenga.,P.J., 1982. Growth and yield response of chilli pepper to trickle irrigation. Agronomy Journal, 74: 556-561.

Bilger W., Björkman O., 1991. Temperature dependence of violaxanthin deepoxidation and non-photochemical fluorescence quenching in intact leaves of Hedera canariensis and Malva parviflora L. Planta, 184:226-234.

Bosland P.W., Votava E.J., 2000. Peppers: vegetable and spice capsicums. Crop Production Science in Horticulture Series. In: Atherton J., Rees A. (eds.). CABI Publishing, New York: 204.

Calatayud A., Iglesias D.J., Talón M., Barreno E., 2004. Response of spinach leaves (Spinacia oleracea L.) to ozone measured by gas exchange, chlorophyll a fluorescence, antioxidant systems and lipid peroxidation. Photosynthetica, 42:23-29.

Calatayud A., Roca D., Martínez P.F., 2006. Spatial-temporal variations in rose leaves under water stress conditions studied by chlorophyll fluorescence imaging. Plant Physiology and Biochemistry, 44: 564-573.

Chaves M.M., Flexas J., Pinheiro C., 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annals of Botany, 103:551-560.

Chaves M.M., Oliveira M.M.. 2004. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. Journal of Experimental Botany, 55:2365-2384.

Colla G., Rouphael Y., Leonardi C., Bie Z., 2010. Role of grafting in vegetable crops grown under saline conditions. Scientia Horticulturae, 127:147-155.

Delfine S., Tognetti R., Loreto F., Alvino A., 2002. Physiological and growth responses to water stress in field-grown bell pepper (Capsicum annuum L.). Journal of Horticulture Science and Biotechnology, 77: 697–704.

De Pascale S., Ruggiero, C., Barbieri G., 2003. Physiological responses of pepper to salinity and drought. Journal of the American Society and Horticultural Science, 128: 48-54.

Fernández M.D., Gallardo M., Bonachela S., Orgaz F., Thompson R.B., Fereres E., 2005. Water use and production of a greenhouse pepper crop under optimum and limited water supply. Journal of Horticulture Science and Biotechnology, 80: 87-96.

Flexas J., Bota J., Cifre J., Escalona J.M., Galmés J., Gulías J., Lefi E.K., Martínez-Cañellas F., Moreno M.T., Ribas-Carbó Mi., Riera D., Sampol B., Medrano H., 2004a. Understanding down-regulation of photosynthesis under water stress: future prospects and searching for physiological tools irrigation management. Annual Applied Biology, 144: 273-283.

Flexas J., Bota J., Loreto F., Cornic G., Sharkey T.D., 2004b. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biology, 6:269-279.

Genty B., Briantais J.M., Baker. N.R., 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta, 990:87-92.

González A., Martín I., Eyerbe L., 2008. Yield and osmotic adjustment capacity of barley under terminal water-stress conditions. Journal of Agronomy and Crop Science, 194: 81-91.

Guidi L., Nali C., Lorenzini G., Filippi F., Soldatini G.F., 2001. Effect of chronic ozone fumigation on the photosynthetic process of poplar clones showing different sensitivity. Environmental Pollution, 113:245–254.

Kaiser W.M., 1987. Effect of water deficit on photosynthetic capacity. Physiologia Plantarum, 71:142-149.

Karam F., Masaad R., Bachour R., Rhayem C., Rouphael Y., 2009. Water and radiation use efficiency in drip-irrigated pepper (Capsicum annuum L.): Response to full and deficit irrigation regimes. European Journal of Horticultural, 74: 79-85.

King S.R., Davis A.R., Zhang X., Crosby K., 2010. Genetics, breeding and selection of rootstocks for Solanaceae and Cucurbitaceae. Scientia Horticulturae, 127:106-111.

Lee J.M., Kubota C., Tsao S.J., Biel Z., Hoyos Echevaria P., Morra L., Oda M., 2010. Current status of vegetable grafting: diffusion, grafting techniques, automation. Scientia Horticulturae, 127:93-105.

Lee H.Y., Jun S.S., Hong Y.N., 2004. Increased photoinihibition in dehydrated leaves of hot pepper (Capsicum annuum L.) is not accompanied by an incremental loss of functional PSII. Journal Plant Biology, 47:83-91.

Lu C., Zhang J., 1998. Effects of water stress on photosynthesis, chlorophyll fluorescence and photoinhibition in wheat plants. Australian Journal of Plant Physiology, 25:883-892.

Maroto J.V., 2005. Horticultura herbacea (Herbaceous horticulture) 5th Ed. Mundi-Prensa. Madrid. Spain: 465.

Martínez-Ballesta M., Alcaraz-López C., Muries B., Mota-Cadenas C., Carvajal M., 2010. Physiological aspects of rootstock-scion interactions. Scientia Horticulturae, 127:112-118.

Massacci A., Nabiev S.M., Pietrosant L., Nematov S.K., Chernikova T.N., Thor K., Leipner J., 2008. Response of the photosynthetic apparatus of cotton (Gossypium hirsutum) to the onset of drought stress under field conditions studied by gas exchange analysis and chlorophyll fluorescence imaging. Plant Physiology and Biochemistry, 46:189-195.

Maynard D.N., Hochmuth G.J., 2007. Knott's handbook for vegetable growers. John Wiley & Sons, Inc. New York.

Miguel A., De la Torre F., Baixauli C., Maroto J.V., Jordá M.C., López M.M., García-Jiménez J., 2007. Injerto en hortícolas (Graft in vegetables). Ministerio de Agricultura, Pesca y Alimentación and Fundación Ruralcaja. Valencia. Spain.

Müller P., Li X.P., Niyogi K.K., 2001. Non-photochemical quenching: a response to excess light energy. Plant Physiology, 125:1558-1566.

Munns R., Tester M., 2008. Mechanisms of salinity tolerance. Annual Review Plant Biology, 59: 651-681.

Munns R., James R.A., Sirault X.R.R., Furbank R.T., Jones H.G., 2010. New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. Journal Experimental Botany, 61: 3499-3507.

Naumann J.C., Young D.R., Anderson J.E., 2007. Linking leaf chlorophyll fluorescence properties to physiological responses for detection of salt and drought stress in coastal plant species. Physiologia Plantarum, 131:422-433.

Niu G., Rodriguez D.S., Crosby K., Leskovar D., Jifon J., 2010. Rapid screening for relative salt tolerance among chile pepper genotypes. HortScience, 45: 1192-1195.

Oda M., Nagata M., Tsuji K., Sasaki H., 1996. Effects of Scarlet eggplant rootstock on growth, yield, and sugar content of grafted tomato fruits. Journal of the Japanese Society for Horticultural Science, 65: 531-536.

Penella C., Nebauer S.G., López-Galarza S., SanBautista A., Gorbe E., Calatayud A., 2013. Evaluation for salt tolerance of pepper genotypes to be used as rootstocks. Journal of Food, Agriculture and Environment, 11: 1101-1107.

San Bautista A., Calatayud A., Nebauer S.G., Pascual B., Maroto J.V., López-Galarza S., 2011. Effects of simple and double grafting melon plants on mineral absorption, photosynthesis, biomass and yield. Scientia Horticulturae, 130:575-580.

Sánchez-Rodríguez E., Romero L., Ruiz J.M., 2013. Role of Grafting in Resistance to Water Stress in Tomato Plants: Ammonia Production and Assimilation. Journal of Plant Growth Regulation, 32:831–842.

Schreiber U., Bilger W., Schliwa U., 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynthesis Research, 10: 51-62.

Schwarz D., Rouphael Y., Colla G., Venema J.H., 2010. Grafting as a tool to improve tolerance of vegetables to abiotic stresses: Thermal stress, water stress and organic pollutants. Scientia Horticulturae, 127: 162-171.

Stuart L., Emel D., Bala A.A., Hikmet B., 2011. The drought response displayed by a DRE-binding protein from Triticum dicoccoides. Plant Physiology and Biochemistry, 49:346-351.

Villa-Castorena M., Ulery A.L., Catalán-Valencia E.A, Remmenga M.D., 2003. Salinity and nitrogen rate effects on the growth and yield of Chile pepper plants. Soil Science Society of American Journal, 67:1781-1789.

Xu L., Yu J., Han L., Huang B., 2013. Photosynthetic enzyme activities and gene expression associated with drought tolerance and post-drought recovery in Kentucky bluegrass. Environmental and Experimental Botany, 89: 28-35.

Yadollahi A., Arzani K., Ebadi A., Wirthensohn M., Karimi S., 2011. The response of different almond genotypes to moderate and severe water stress in order to screen for drought tolerance. Scientia Horticulturae, 129: 403-413.

CHAPTER 3
Evaluation For Salt Stress Tolerance Of Pepper Genotypes To Be Used As Rootstocks

Consuelo Penella[1], Sergio G. Nebauer[2], Salvador Lopéz-Galarza[2], Alberto SanBautista[2], Elisa Gorbe[3], Ángeles Calatayud[1]

[1]*Instituto Valenciano de Investigaciones Agrarias (IVIA). Departamento de Horticultura. Ctra. Moncada-Naquera km. 4.5. 46113-Moncada, Valencia, Spain.*

[2]*Universitat Politècnica de València. Departamento de Producción Vegetal. Camino de Vera 14, 46020 Valencia, Spain.*

[3] *Wageningen UR Greenhouse Horticulture, Droevendaalsesteeg 1 6708 PB Wageningen, The Netherlands; and Horticultural Supply Chains, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, the Netherlands.*

Journal of Food, Agriculture & Envirnoment, 11:1101-1107 (2013)

3.1. ABSTRACT

Salinity is a major environmental constraint on crop productivity and grafting can be a sustainable strategy to enhance plant tolerance under adverse growth conditions. Screening different graft combinations under field conditions can be a slow and expensive processes. In this study, plants of 18 genotypes of *Capsicum* spp. were evaluated during 5 months to select salt tolerant plants to be used as rootstocks in greenhouse under controlled conditions. Their net photosynthetic rate was used as a rapid and sensitive methodology for screening their tolerance to salt stress conditions. The germination potential of some genotypes was also tested under different salinity conditions to see if it would be useful to accelerate the screening process. According to photosynthesis rate, the commercial rootstock 'Tresor' and the genotypes 'Serrano' (*C. annuum*), 'ECU-973' (*C. chinense*) and 'BOL-58' (*C. baccatum*) were the most tolerant during this period. Nevertheless the evaluation of pepper genotypes for salinity tolerance based on the germination performance and chlorophyll fluorescence parameter Fv/Fm ratio were not a good indicators of the sensitivity along plant ontogeny. Finally, the selected genotypes as salt-tolerant were validated under field conditions as rootstocks of two interesting pepper cultivars, concluding that using the rootstocks selected by the net photosynthetic rate improved the salt tolerance of the scion in terms of marketable yields and fruit quality

Key words: germination, graft, pepper, photosynthesis, vegetable production

3.2. INTRODUCTION

Peppers (*Capsicum* spp.) are economically and socially important crops in the world. Countries in Mediterranean basin produce around 5.242.450 Tn in a 234022 Ha. Unfortunately, the continuous soil exploitation, the monoculture, and/or intensive agricultural practices have led to the increase of soil salinity[1] and soil-borne diseases, which results in loss of yield and fruit quality[2].

The use of grafted plants is a strategy that allows plants to overcome soil-borne diseases and environmental stresses[3-5]. Their use is becoming more appreciated due to the current trend towards a green and sustainable agriculture. The cultivation of grafted plants has greatly expanded mainly in tomato and watermelon, but this practice is still limited in peppers[4,6].

Several *Capsicum* rootstocks, including commercial cultivars, breed lines and wild accessions, can give appropriate tolerance or resistance to *Phytophtora, Verticilium, Fusarium,* CMV, etc[7,8]., but there is little information about their tolerance to abiotic stresses.

Abiotic stresses can result in plant senescence, decreasing both yield and product quality[9]. Soil and water salinity are a serious problem in Mediterranean areas where summer crops as pepper are often inevitable irrigated with saline water. Salt tend to accumulate in soil because of the high evaporative demand is associated to an insufficient leaching ions[10]. In addition, this situation increases the risk of physiological disorders in pepper fruits, particularly in bell peppers, such as blossom-end rot (BER) and cracking. Damages caused by salinity are responsible for high economic losses worldwide[11]. The grafting technique could enable plant breeders to combine desired shoot characteristics with root features that give tolerance to salinity stress[3].

Photosynthesis, together with cell growth, are the primary processes affected by salinity[12,13]. The effects on photosynthesis performance may be due to stomatal and non-stomatal limitations[14]. The response of photosynthesis to salinity conditions varies depending on plant ontogeny, among different species and also within the same species[15]. Indeed, several studies have demonstrated that the evolution of stress tolerance at various stages of development differs among cultivars of a given species[16]. Therefore, specific stages throughout the plant ontogeny, such as germination, vegetative and reproductive stages should be evaluated during the screening period to obtain tolerant plants.

Our aim was to evaluate the behavior of 18 pepper genotypes under salinity conditions in order to select tolerant plants to be used as rootstocks for pepper cultivation. The screening was based on photosynthetic parameters. Furthermore, tolerant genotypes selected from the screening process were evaluated for their potential of germination under salt conditions. Finally, the selected genotypes were validated as rootstocks of two pepper cultivars in terms of productivity and quality parameters.

3.3. MATERIALS AND METHODS

3.3.1. Experiment 1: Screening pepper genotypes under salinity conditions to be used as rootstocks.

3.3.1.1.Plant material and growing conditions

The commercial rootstocks cultivars ‘Atlante’ (Ramiro Arnedo (1)), ‘C40’ (Ramiro Arnedo (2)), ‘Tresor’ (Nunhems (3)); the genotypes of *Capsicum annuum* L. ‘Serrano Criollo de Morelos’ (4), ‘Serrano’ (5), ‘Pasilla Bajío’ (6), ‘Pimiento de Bola’ (7), ‘Piquillo de Lodosa’ (8), ‘Guindilla’ (9), and ‘Numex

Conquistador' (17); the genotypes of *Capsicum chinense* Jacq. 'PI-152225' (11), ECU-973 (12) and 'Morro de vaca' (10); the genotypes of *Capsicum baccatum* L. var. *pendulum* 'BOL-134' (13) and 'BOL-58' (14); the genotypes of *Capsicum pubescens* R.&P. 'BOL 60 amarillo' (15) and 'BOL 60 rojo' (16) and the accession of *Capsicum frutescens* L. 'BOL-144' (18) were used in this study. A numerical code for every cultivar is indicated in brackets. All the genotypes used for the present study belong to the collection of the COMAV institute (Universitat Politécnica de Valencia, Valencia, Spain). Seeds were germinated in moistened perlite at 28 ºC under greenhouse conditions. The seedlings were transferred to 15 L pots containing coconut coir fiber in a heated polyethylene greenhouse on 15th January 2011 in the Instituto Valenciano de Investigaciones Agrarias (Valencia, Spain). Plants were irrigated with Hoagland's No.2 nutrient solution[17].

After 15 days in the pots, plants were divided in two groups for control and saline treatments. Salinity treatment was initiated by adding NaCl (40mM) to the irrigation solution to reach an EC of 5 dS m^{-1}. The EC of the nutrient solution in the control treatment was 1 dS m^{-1}. Drip irrigation was supplied based on estimations of the weekly crop evapotranspiration (ETc), even though the saline solution was allowed to drain freely from the pots and the control drainage was controlled from 10% to 20% depending on solar radiation.

Eight plants per cultivar were used in each treatment. Plants were grown for 5 months and were kept free from insects and diseases using greenhouse standard management procedures.

3.3.1.2. Leaf gas exchange and chlorophyll a fluorescence

Net CO_2 fixation rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}) and substomatal CO_2 concentration (Ci, μmol CO_2 mol^{-1} (air)) were measured at steady-state conditions of saturating light (1200 μmol m^{-2} s^{-1}). Light curves were previously performed (data not shown) and A_N was saturated at 900 μmol m^{-2} s^{-1} determine after light response curve. The CO_2 concentration was 400 ppm and flow air rate was 500 μmol s^{-1} with a LI-6400 (LI-COR, Nebraska, USA).

To evaluate the presence of chronic photoinhibitory processes, the maximum quantum yield of PSII (Fv/Fm=(Fm-Fo)/Fm) was measured on leaves after 30 minutes of dark adaptation using a portable pulse amplitude modulation fluorometer (MINI PAM, Walz, Effeltrich, Germany). The background fluorescence signal for dark adapted leaves (Fo) was determined with a 0.5 μmol photon m^{-2} s^{-1} measuring light at a frequency of 600 Hz. The application of a saturating flash of 10000 μmol photon m^{-2} s^{-1} enabled estimations of the maximum fluorescence (Fm).

Measurements were performed twice 2 months (T1) and 5 months (T2,) after starting the salinity treatment. During the gas exchange and Fv/Fm measurements the environmental greenhouse ranges were: temperature 21-23ºC; relative humidity 65-70%; and solar radiation 800-1200 μmol m^{-2} s^{-1} from 9:00 am to 11:00 am (GMT) in sunny days. Eight plants per treatment were measured on the third or fourth fully expanded leaf from the shoot apex.

Data were analyzed by ANOVA and means were compared using Fisher`s least significance difference (LSD) test at $p<0.05$ (Statgraphics Plus 5.1 for Windows, Statistical Graphics Corp.).

3.3.2. Experiment 2: Potential of germination under salt conditions of the tolerant genotypes selected from experiment 1

From the results of Experiment 1, four genotypes with the highest tolerance to salinity stress (3, 5, 12 and 14) and a sensitive genotype (8) were selected. Seeds of these genotypes were sterilized with 1.5% sodium hypoclorite solution for 7 min, rinsed with sterile distilled water several times, and placed in closed Petri dishes (Ø 9 cm) under aseptic conditions on a Murashige and Skoog culture medium (Sigma-Aldrich) containing 0 (M1), 15 (M2), 40 (M3), 60 (M4) or 100 (M5) mM NaCl for a germination test under salinity conditions. The pH was adjusted to 5.7. Each treatment experiment (genotype x salinity concentration) consisted in four separated replicates of 100 seeds. Seeds were allowed to germinate in a phytotron (Sanyo MLR-350H) at 25ºC, 85% RH and 16 h irradiance (PAR: 45 μmol m^{-2} s^{-1}). The number of germinated seeds was recorded daily using radicle extrusion (≥ 2mm long) as criterion.

Germination data of each replicate were fitted to the logistic function[18] $G = A[1 \pm exp(\beta - kt)]^{-1}$, defined as a special form of the Richards function, where G = cumulative germination (%); t = germination time (days); and A, β and k are function parameters, being A the maximum germination percentage (asymptote when $t \rightarrow \infty$), k is a "rate parameter" and β places the curve in relation to the time axis, without any biological significance. In addition, derived quantities with biological significance were also calculated, as the time at the inflexion point to reach 50% of final germination percentage ($Gt\ 50 = \beta/k$, *days*), and mean relative cumulative germination rate ($k/2$, $days^{-1}$).Variables (A, β/k and $k/2$) were

analysed by ANOVA (Statgraphics Plus 5.1 for Windows, Statistical Graphics Corp.). Percentage data were arcsin transformed before analysis. Mean separations were performed with the LSD test at $P<0.05$.

3.3.3. Experiment 3: Grafting of two cultivars onto four selected genotypes of experiment 1

The experiment was performed during 2012 in a sweet pepper producing area in Valencia, Spain, using 'Adige' F1 (Lamuyo type; Sakata Seeds, Japan) and 'Lipari' F1 (Italian type; Clause Spain) cultivars, grafted onto four salinity tolerant genotypes 3, 5, 12 and 14, according to the results obtained in Expt. 1. Ungrafted 'Adige' and 'Lipari' plants were used as controls. Grafted and ungrafted plants were transplanted on the 21th of March at a density of 2.1 plants m^{-2} in a sandy soil (pH=8.0; $EC_{(1/5)}$=1.2 dS m^{-1}; Sand= 76%), under polyethylene greenhouse. The electrical conductivity and pH of the irrigation water were 3.5 dS m^{-1} and 7.60, respectively with 88 meq l^{-1} of Na^{+} and 111 meq l^{-1} of Cl^{-}. Fertilizers were applied at a rate (kg m^{-1}) of 200 N, 50 P_2O_5, 250 K_2O, 110 CaO and 35 MgO[19].

The tube grafting method was used, by cutting the growing tip of the rootstock at a 45º angle below cotyledons, attaching subsequently the scion, previously cut at a 45º angle above cotyledons and fixing rootstock and scion with a clip.

Harvest was performed from the end of May until the end of July. Fruits were graded in two classes: marketable and unmarketable fruits. The latter fruits were mainly (>95%) affected by BER.

A randomized complete block design was performed with three replicates, each consisting of 25 plants. Data were subjected to ANOVA and means were

compared using Fisher's least significance difference (LSD) test at $P<0.05$ (Statgraphics Plus 5.1 for Windows, Statistical Graphics Corp.).

3.4. RESULTS

3.4.1.Effect of stress conditions on photosynthetic parameters

The pepper genotypes grown under control conditions in this study differed significantly ($P<0.05$) among themselves in the net CO_2 fixation rate (Fig. 1). The genotypes 2, 4, 6, 7, 8, 10, 13 and 14 showed the highest photosynthetic rates, with values near or above 20 μmol CO_2 m^{-2} s^{-1}. In contrast, genotypes 1, 3, 11 and 12 showed the lowest rates.

In March (T1), after 2 months of salinity conditions, the genotypes 2, 3, 5, 12, 13 and 14 maintained the photosynthetic rate and stomatal conductance under salt conditions, when compared to controls (Fig. 1A and B). In genotypes 6 and 11, stomata closed by the effect of salinity, although A_N remained unaffected. Net photosynthesis rate and stomatal conductance decreased due to salt stress in the remaining genotypes (Fig. 1A and B).

After 5 months of treatment (T2), the net photosynthetic rate in genotypes 1, 3, 5, 12 and 14 did not differ from controls under salinity conditions (Fig. 1C). However, only genotype 1 maintained the stomatal conductance (Fig. 1D) in response to salt conditions without significant differences respect to control. Nevertheless, comparing the g_s value of the 18 genotypes in the control treatment, genotype 1 had the lowest.

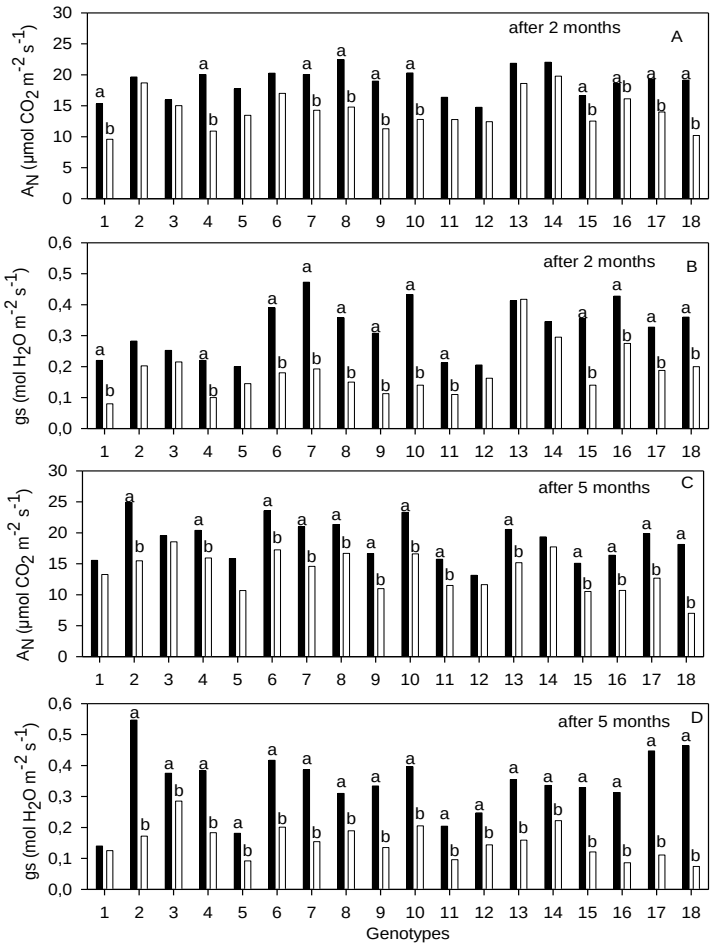


Figure 1. Leaf gas exchange parameters in pepper genotypes measured after 2 (T1) and 5 months (T2) in the control (1dSm^{-1}, ■) and irrigated with water salinity (5 dSm^{-1}, □). A_N net photosynthesis at T1 (A) and T2 (C) and stomatal conductance to water vapour (g_s) at T1 (B) and T2 (D). Values are means of 8 samples. For comparison of means, analysis of variance (ANOVA) followed by the least significance differences (LSD) test calculated at $P<0.05$, was performed. Values for each genotype followed by different letter indicate significant differences. Non letter indicates non-significant difference for each genotype

Along T1 and T2 a logarithmic correlation between net photosynthetic rate and stomatal conductance was observed (A_N = 6.62 ln g_s + 26.4; R^2 = 0.80). In addition, stomatal conductance was also related to substomatal CO_2 concentration (C_i = 67.6 ln g_s + 355; R^2 = 0.86).

Fv/Fm ratio did not changed along the experiment neither among genotypes nor treatments (data not show).

In summary, the net photosynthetic rate of the genotypes 'Tresor' (3), 'Serrano' (5), 'ECU-973' (12) and 'BOL-58' (14) was not affected by salinity conditions and consistently in both measurements.

3.4.2. Seed germination test

The selected salt-tolerant genotypes in Experiment 1 (3, 5, 12 and 14) and the genotype 8 (sensitive to salinity) were used to test the germination potential under salinity conditions.

The ANOVA of the logistic function parameters A, *β/k* and *k*/2 of our studies are shown in Table 1. The interaction "Genotype x Salinity" was significant (*P*<0.01). The coefficients of determination (R^2) for curves ranged from 0.90 to 0.989 and F ratio values of the statistical model were significant (*P*<0.01). The source of variation (*P*<0.01) for *β/k* was 79.79 of total sum of square appeared for genotypes.

Table 1. Analysis of variance of parameters A(%), β/k (days) and k/2 (days-1), obtained by curve-fitting to logistic function for seeds from different genotypes and salt concentrations.

	Percentage of the total sum of square+		
Source	A	β/K	*k*/2
Genotypes	11.970**	79.798**	48.972**
Salt concentration	19.911**	9.252**	8.918**
Genotypes x Salt	28.612*	6.807**	18.850**
Error	39.507	4.143	23.260
Standard deviation ++	3.066	0.649	0.761

*,** indicated significant at p≤0.05 and p≤0.01, respectively.
+ For each component, the sum of squares is given as a percentage of the total.
++ Calculated as the square root of the nutrient between the absolute value of the residual sum of squares (using the units indicate) and the degrees of freedom of the error.

The fitted curves corresponding to the average values of each variance source (genotype and NaCl concentration) are shown in Fig. 2. High values (>80%) of final germination were reached in most of combinations under salinity conditions. The seeds with higher A values had also a higher germination rate. The seeds of the genotypes 8 and 12 showed the slower germination rates (Fig. 2), requiring higher periods to reach 50% of final germination. By contrast, the genotype 5 showed the highest values of maximum germination percentages, requiring the shortest periods to reach 50% of final germination (Fig. 2).

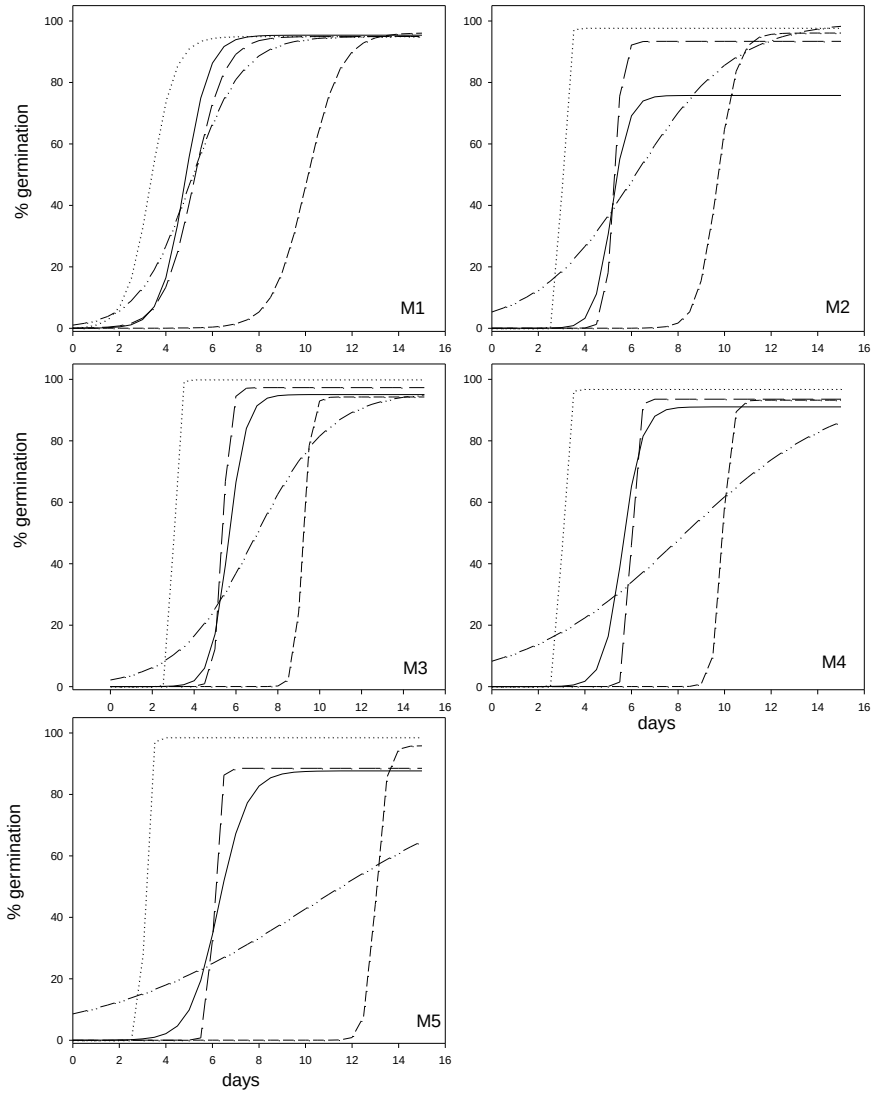


Figure 2. Logistic models (G = A[1 ± exp (β – kt)] -1) fitted to cumulative pepper seed germination curves of the genotypes: 3 (—), 5 (·····), 8 (– –), 12 (–··–··) and 14 (— —) during 16 days under different salinity conditions: M1 (control), M2 (15mM NaCl), M3 (40mM NaCl), M4 (60mM NaCl) and M5 (100mM NaCl).

3.4.3. Field experiment

In general terms and for both cultivars ‘Adige’ and ‘Lipari’, genotype 3 and followed by genotype 14, gave the best response when used as rootstocks (Table 2). Furthermore, in all grafted plants the efficiency was higher compared with ungrafted plants (except in genotype 12 grafted onto ‘Lipari’). This was reflected by a higher marketable yield and lower unmarketable yield due to BER. However, total yield was similar for ‘Adige’ in all grafted or ungrafted plants. No differences were found between marketable yield of ungrafted plants and grafted plants onto genotypes 5 and 12 (Table 2).

In ‘Lipari’, marketable yield were lower when 5 and 12 genotypes were used, mainly for 12. But the highest losses in the unmarketable fruits was achieved in ungrafted plants with significant differences respect to grafted plants (Table 2).

The percentage of fruits affected by BER was lower in ‘Lipari’ than in ‘Adige’. The fruit number and the mean fruit weight of commercial fruits were similar in all ‘Lipari’ combinations but not in ‘Adige’ plants, where a lower number heavier fruits were obtained when using the rootstock 12.

Table 2. Production parameters: yield (kg m^{-2}), fruit number per m^2 and mean fruit weight (g $fruit^{-1}$) of marketable and unmarketable yield, percentage of the number of unmarketable yield (%) and total yield (kg m^{-2})

Cultivar	Rootstock genotype	Marketable yield			Unmarketable yield			Total yield
		kg m^{-2}	Fruit number m^{-2}	g $fruit^{-1}$	kg m^{-2}	%	Fruit number m^{-2}	kg m^{-2}
Adige	3	3.4 a	26.3 a	129.5 b	1.5 b	30.3 c	8.5 b	4.9
	5	1.5 bc	15.2 bc	98.8 b	3.6 a	72.2 a	25.8 a	5.1
	12	1.8 bc	10.0 c	187.8 a	2.2 b	56.4 ab	19.8 a	3.9
	14	2.5 ab	19.9 ab	125.8 b	1.9 b	40.1 bc	7.6 b	4.4
	ungrafted	1.4 c	12.1 bc	112.9 b	3.2 a	70.7 a	17.7 a	4.6
	Significance (F values)	P<0.05	P<0.05	P<0.01	P<0.01	P<0.01	P<0.01	NS
Lipari	3	4.1 a	14.3	289.6	1.5 c	26.3 c	2.1 b	5.6 a
	5	3.1 bc	14.3	220.4	1.7 b	36.4 ab	24.3 a	4.8 ab
	12	2.6 c	13.0	206.7	1.3 c	34.0 b	21.2 a	3.9 b
	14	3.9 ab	15.2	257.2	0.8 d	17.0 d	7.2 b	4.7 ab
	ungrafted	3.1 bc	11.8	267.4	2.1 a	41.3 a	13.5 ab	5.3 a
	Significance (F values)	P<0.05	NS	NS	P<0.01	P<0.01	P<0.05	P<0.05

Values are mean of n=50 plants of cultivar “Adige” and “Lipari” grafted or not onto genotype 3, 5, 12 and 14. Different letters in each column indicate significant differences at P<0.05 using the LSD test. NS – not significant.

3.5. DISCUSSION

The productivity of several commercial pepper crops (mainly bell peppers) is limited by salinity stress in many areas of the world. The screening of salt-tolerant pepper has been developed mainly in genotypes with poor commercial value[20,21]. A new perspective for screening genotypes is their use as rootstocks to improve the tolerance of a desirable cultivar to abiotic stresses. With this aim we tested 18 pepper genotypes grown under salinity conditions in a greenhouse in the Mediterranean area.

Salinity can affect photosynthesis as a result of ion imbalance, ion toxicity and osmotic stress in plants[14,22]. A limitation of CO_2 supply due to partial stomatal closure has been described as an early response to salt stress[21]. In this work, stomatal conductance and photosynthesis were negatively affected by salinity in T1 and T2 periods in some genotypes. The low substomatal CO_2 concentration under stomata closure suggested stomatal constraints to photosynthesis. Stomatal conductance decreased in salinity conditions in genotypes 3, 5, 12 and 14 at T2 but not at T1, although A_N did not show significant differences along the experiment in these genotypes when compared to controls. This can be explained by the fact that only very critically low levels of g_s in these genotypes affected photosynthesis, which is in agreement with[5,23].

The reduction in photosynthesis rate can also be due to alterations in leaf photochemistry[14]. The leaf chlorophyll fluorescence ratio, Fv/Fm is a classic parameter reflecting the whole PSII function, and its decrease is associated with PSII damage or photoinhibition under environmental stresses[24]. In our experiment, the Fv/Fm measured both periods did not show significant differences between control and stress treatments, implying that PSII activity was not affected by salt stress. Other studies have shown little or no effect on Fv/Fm[21,25-27] even when leaf growth and gas exchange were reduced. The decrease of stomatal conductance and the photosynthesis rate, leaving PSII unaffected suggested a highly resistant PSII activity under stress conditions[26,28] and/or that the limitation of photosynthesis by reduction of Rubisco activity does not occur until this stress is highly severe[14,29]. Based on fluorescence and gas exchange parameters, our results suggested that diffusional restriction is the main factor that limits photosynthesis in sensitive pepper genotypes in our salinity conditions.

Since the limitation by CO_2 was the main factor responsible for the decrease in the net photosynthetic carbon uptake rate[30], we selected the A_N as the indicator parameter of sensitivity or tolerance with regard to salinity stress.

We observed some differences in the photosynthetic performance between T1 and T2 in some pepper genotypes. Genotypes 2, 6, 11 and 13 showed a decrease in A_N at T2 but not at T1 under salinity condition, and only genotype 1 did not show significant differences at T2 but it did at T1. These results may indicate that the genotypes after a period of salinity exposure were more sensitive under our growth conditions. A longer exposure to salinity conditions, which may cause ion accumulation in the leaves[31] or alterations in osmotic adjustment[32] and can be the cause of higher photosynthetic decrease.

Since many crops show different sensitiveness at different stages of their ontogeny, others may have a similar response among them. In that case, determining the response of the seeds in terms of the germination performance under salinity stress conditions would be useful to accelerate the screening process.

The sensitivity or tolerance to salinity during the germination stage is species-dependent; many crop are vulnerable to stress during seed germination[33], while others are relatively tolerant[34]. Salt stress can reduce germination either by limiting water absorption by the seed[35], by affecting the mobilisation of stored reserves[36], by directly affecting the structural organization or synthesis of proteins in germinated embryos[37] or by intake of toxic ions, which may affect metabolic activities[38]. In our study, in general terms, the maximum germination rate decreased and/or the seeds required a longer period to reach the 50% of the final germination percentage as NaCl increased in the media. However the magnitude of this response varied among genotypes. Those that germinated rapidly at low stress conditions also germinated properly at high stress levels.

The same effect was demonstrated[33] in different *Lycopersicum* accessions under salinity. The genotype 5 showed the highest germination rate even at 100mM NaCl and it had higher photosynthetic rate under salinity conditions at T1 and T2, On the other hand, genotype 12 showed a lower maximum germination rate and required a longer period to reach the 50% of final germination percentage, but it had a higher photosynthesis rate under salinity stress at T1 and T2. This indicates that this genotype is more sensitive to salinity stress during germination or requires more time to germinate. Finally, genotype 8 takes longer to germinate, even in the control treatment, compared to other genotypes, at the end the germination percentage was high but it was sensitive at T1 and T2.

The observed differences in the response to salinity-tolerance in the genotypes during germination phase were not representative of salinity tolerance of these potential rootstocks during T1 and T2. As a consequence, our results indicate that the screening of pepper genotypes for salinity tolerance based only in the germination performance is not a good indicator of their sensitivity in the adult plant-stage. Nevertheless, the selection of a desirable rootstock only for the germination phase is not limiting factor because from an agronomic point of view grafting is done in commercial nurseries, where seeds are germinated in optimal conditions, i.e. no saline water and substrates, due to the high cost of the seeds.

The screening of salt tolerant genotypes has been used for the introduction of salt-tolerant crops[20,21], although these crops often have poor yield and low quality fruits. Grafting is a well established technique for crop production under salinity conditions[3,39]. The increase of salt tolerance observed in grafted plant is due to the use of salt tolerant rootstocks, although plants grafted onto different rootstocks respond more or less differently to salinity[40]. Several authors (see

review[3]) have reported an increase in growth and fruit yield in grafted plants under salinity conditions, mainly in tomato[40,41], watermelon[42,43] or eggplants[44,45], but there are few studies about the effect of grafting of pepper plants under salinity conditions. Our proposal is the use of select salt tolerant pepper genotypes as rootstocks. Pepper is classified as moderately sensitive to salt stress but its response is cultivar-dependent[21,46]. The graft of interesting but salt-sensitive pepper cultivars ('Adige' and 'Lipari') onto our salt tolerant genotypes (3, 5, 12, 14) may provide the capacity for inducing salt tolerance on them. In general terms, our results indicate that the selected tolerant genotypes induced a better response to the scions when used as rootstocks in comparison with ungrafted plants. The grafted pepper plants had, in general higher marketable yields compared with the ungrafted cultivars when cultured in high saline water and soil. The marketable yield with regard to the total fruit yield was relatively low in ungrafted 'Adige' plants (30%) and in 'Adige' plants grafted on genotype 5 (29%) compared with those grafted on 3 (70%), 14 (57%) and 12 (45%). The genotype 5 resulted in lower marketable yields when used as rootstock of both cultivars. This genotype seems to have affinity problems, as the same has been observed in other experiment under water stress conditions[47] and this fact could explained the lower marketable fruit yields.

More experiments need to be carried out in this direction to understand the interactions rootstock-scion in these selected genotypes.

The occurrence of BER was the main cause of the unmarketability of the fruits in both tested cultivars. Pepper has been described as very susceptible to BER[48]. This disorder has been associated to a local deficiency of calcium[49]. The high incidence of BER in our experiment could be due to the high salinity both in the nutrient solution and soil, combined with the climatic conditions of spring-summer, with high temperatures and high leaf transpiration rates as has been

observed[50] in pepper, and this can diminish the calcium partitioned to the fruits. In our study, tolerant rootstocks to saline stress significantly decreased the percentage of BER in the fruits respect to ungrafted plants, although it was high by our salinity conditions, this is an important conclusion from this study, as this disorder adversely affect marketable yields and as it can diminish used tolerant rootstocks. On the other hand, the occurrence of BER not only depended on the rootstock but also on the scion used since 'Lipari' had lower percentage of BER than 'Adige'. Therefore, the salt tolerance of the grafted plants is a combination of the salt tolerances of both scion and rootstock[39].

3.6. CONCLUSIONS

The photosynthetic rate A_N is a useful sensitive parameter for the selection of salt tolerant genotypes and it is related to the plant production, what has been validated in terms of yield and fruit quality under salinity conditions. The use of salt-tolerant rootstocks has been proved as an excellent and sustainable strategy to improve the salt tolerance of pepper plants, even though the level of improvement depends on the sensitivity of the scion. Wild pepper genotypes used as rootstocks are interesting as a source of tolerance to salinity stress. Nevertheless, further studies are needed to search the best scion/rootstock combinations in order to optimize the crop value.

3.7. REFERENCES

[1]Rozema, J. and Flowers, T. 2008. Crops for a salinized world. Science. **322**:1478-1480.

[2]Lacasa, A., Guerrero, M.A., Guirao, P. and Ros, C. 2002. Alternatives to methyl bromide in sweet pepper crops in Spain. In: Batchelor, T. et al. (eds.). Proceedings of the International Conference Alternatives to Methyl Bromide. The remaining challenges. European Comission, Brussels, Belgium. p. 172-177.

[3]Colla, G., Rouphael, Y., Leonardi, C. and Bie, Z. 2010. Role of grafting in vegetable crops grown under saline conditions. Sci. Hortic. **127**:147-155.

[4]King, S.R., Davis, A.R., Zhang, X. and Crosby, K. 2010. Genetics, breeding and selection of rootstocks for Solanaceae and Cucurbitaceae. Sci. Hortic. **127**:106-111.

[5]San Bautista, A., Calatayud, A., Nebauer, S.G., Pascual, B. ,Maroto, J.V. and López-Galarza, S. 2011 Effects of simple and double grafting melon plants on mineral absorption, photosynthesis, biomass and yield. Sci. Hortic **130**:575-580.

[6]Miguel, A., De la Torre, F., Baixauli, C., Maroto, J.V., Jordá, M.C., López, M.M. and García-Jiménez, J. 2007. Injerto de hortalizas. Ministerio de Agricultura, Pesca y Alimentación y Fundación Ruralcaja. Valencia. Spain, 63p.

[7]Morra, L. 2004. Grafting in vegetable crops. In: International Workshop. The productions in the greenhouse after the Era of the Methyl Bromide. p. 147-154.

[8]Kokalis-Burelle, N., Bausher, M.G. and Rosskopf, E.N. 2009. Greenhouse evaluation of Capsicum rootstocks for management of Meloidogyne Incognita on grafted bell pepper. Nematropica. **39**:121-132.

[9]López-Marín, J., González, A., Pérez-Alfocea, F., Egea-Gilabert, C. and Fernández, J.A. 2013. Grafting is an efficient alternative to shading screens to alleviate thermal stress in greenhouse-grown sweet pepper. Sci. Hortic.**149**: 39-46.

[10]De Pascale, S., Ruggiero, C., Barbieri, G. and Maggio, A. 2003. Physiological responses of pepper to salinity and drought. J. Am. Soc. Hort. Sci. **128**: 48-54.

[11]Rai M. K., Kalia, R.K., Singh, R., Gangola, M.P. and Dhawan, A.K. 2011. Developing stress tolerant plants through in vitro selection-An overview of recent progress. Env. Exp. Bot. **71**:89-98.

[12]Munns, R., Jame, R.A. and Läuchi, A. 2006. Approaches to increasing the salt tolerance of wheat and other cereal. J. Exp. Bot. **57**:1025-1043.

[13]Parida, A.K. and Das, A.B. 2005. Salt tolerance and salinity effects on plants: a review. Eco. Environ. Saf. **60**:324-349.

[14]Chaves, M.M., Flexas, J. and Pinheiro, C. 2009., Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann. Bot. **103**:551-560.

[15]Chartzoulakis, K. and Klapaki, G. 2000, Response of two greenhouse pepper hybrids to NaCl salinity during different growth stages. Sci. Hortic. **86**:247-260.

[16]Almansouri, M., Kinet J.M. and Lutts, S. 2001. Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). Plant Soil. **231**:243-254.

[17]Maynard, D.N. and Hochmuth, G.J. 1997. Knott's handbook for vegetable growers. John Wiley & Sons, Inc. New York, 78p.

[18]Torres, M. and Frutos G. 1989. Analysis of germination curves of aged fennel seeds by mathematical-models. Env. Exp. Bot. **29**:409-415.

[19]Maroto, J.V. 2002. Horticultura Herbácea Especial. 5ª Edición. Ed. MundiPrensa. Madrid, Spain. p. 465.

[20]Aktas, H., Abak, K. and Cakmak, I. 2006. Genotypic variation in the response of pepper to salinity. Sci. Hortic. **110**:260-266.

[21]Niu, G., Rodriguez, D.S., Crosby, K., Leskovar, D. and Jifon, J. 2010. Rapid screening for relative salt tolerance among Chile pepper genotypes. HortSci. **45**:1192-1195.

[22]Munns, R. and Tester, M. 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. **59**:651-681.

[23]Flexas, J., Bota, J., Loreto, F., Cornic, G. and Sharkey T.D. 2004 Diffusive and metabolic limitations to photosynthesis under drought and salinity in C_3 plants. Plant Biol. **6**:269-279.

[24]Baker, N. R. 2008. Chlorophyll fluorescence: A probe of photosynthesis in vivo. Annu. Rev. Plant Biol. **59**: 89-113.

[25]Naumann, J.C., Young, D.R. and Anderson J.E. 2007. Linking leaf chlorophyll fluorescence properties to physiological responses for detection of salt and drought stress in coastal plant species. Physiol. Plant. **131**:422-433.

[26]Lee, H.Y., Jun, S.S. and Hong, Y.N. 2004. Increased photoinihibition in dehydrated leaves of hot pepper (*Capsicum annuum* L.) is not accompanied by an incremental loss of functional PSII. J. Plant Biol. **47**:83-91.

[27]Yan, K., Chen, P., Shao, H., Zhao, S., Zhang, L., Xu, G. and Sun J. 2012. Responses of photosynthesis and photosystem II to higher temperature and salt stress in sorghum. J. Agron. Crop Sci. **198**:218-226.

[28]Baker, N.R. and Rosenqvist, E. 2004. Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. J. Exp. Bot. **55**:1607-1621.

[29]Bota, J., Medrano H. and Flexas J. 2004. Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress?. New Phytol. **162**:671-681.

[30]Chaves, M.M. and Oliverira, M.M. 2004. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J. Exp. Bot. **55**:2365-2384.

[31]Greenway, H. and Munns, R. 1990. Mechanisms of salt tolerance in non-halophytes. An. Rev. Plant Phisiol. and Plant Mol. Biol. **31**:149-190.

[32]Silva, C., Martínez, V. and Carvajal, M. 2008. Osmotic versus toxic effects of NaCl on pepper plants. Biol. Plant. **52**: 72-79.

[33]Foolad, M.R. and Lin, G.Y. 1997. Genetic potencial for salt tolerance during germination in Lycopersicon species. HortSci. **32**:296-300.

[34]Murillo-Amador, B., López-Aguilar R., Kaya, C., Larrinaga-Mayoral, J. and Flores-Hernández, A. 2002. Comparative effects of NaCl and Polyethylene Glycol on germination, emergence and seedling growth of cowpea. J. Agron. Crop Sci. **188**:235-247.

[35]Dodd, G.L. and Donovan, L.A. 1999. Water potencial and ionic effects on germination and seedling growth of two cold desert shrubs. Am. J. Bot. **86**:1146-1153.

[36]Bouaziz, A. and Hicks, D.R. 1990. Consumption of wheat seed reserves during germination and early growth as affected by soil-water potential. Plant Soil. **128**:161-165.

[37]Ramagopal, S. 1990. Inhibition of seed-germination by salt and its subsequent effect on embryonic protein-synthesis in barley. J. Plant Physiol. **136**:621-625.

[38]Smith, P.T. and Comb, B.G. 1991. Physiological and enzymatic activity of pepper seeds (*Capsicum annuum*) during priming. Physiol. Plant. **82**:71-78.

[39]Huang, Y., Bie, Z., Liu, P., Niu, M., Zhen, A., Liu, Z., Lei, B., Gu, D., Lu, C. and Wang B. 2013. Reciprocal grafting between cucumber and pumpkin demonstrates the roles of the rootstock in the determination of cucumber salt tolerance and sodium accumulation. Sci. Hortic. **149**:47-54.

[40]Estañ, M.T., Martinez-Rodriguez, M.M., Perez-Alfocea, F., Flowers, T.J. and Bolarin M.C. 2005. Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. J. Exp. Bot. **56**:703-712.

[41]Santa-Cruz, A., Martinez-Rodriguez, M.M., Perez-Alfocea, F., Romero-Aranda, R. and Bolarin, M.C. 2002. The rootstocks effect on the tomato salinity response depends on the shoot genotype. Plant Sci. **162**:825-831.

[42]Goreta, S., Bucevic-Popovic, V., Selak, G.V., Pavela-Vanric, M. and Perica S. 2008. Vegetative growth, superoxide dismutase activity and ion concentration of salt-stressed watermelon as influenced by rootstock. J. Agri. Sci. **146**:695-704.

[43]Yetisir, H. and Uygur U. 2010. Responses of grafted watermelon onto different gourd species to salinity stress. J. Plant Nutr. **33**:315-327.

[44]Liu, Z.I., Zhu, Y.L., Wei, G.P., Yang, L.F., Zhang, G.W. and Hu C.M. 2007. Metabolism of ascorbic acid and glutathione in leaves of grafted eggplant seedling under NaCl stress. Acta Bot. Boreal Occident Sin. **27**:1795-1800.

[45]Wei, G.P., Zhu, Y.L., Liu, Z.L., Yang L.F. and Zhang G.W. 2007. Growth and ionic distribution of grafted eggplant seedling with NaCl stress. Acta Bot. Boreal Occident Sin. **27**:1172-1178.

[46]Navarro, J.M., Garrido, C., Carvajal, M. and Martinez, V. 2002. Yield and fruit quality of pepper plants under sulphate and chloride salinity. J. Hort. Sci. Biotech. **77**:52-57.

[47]Calatayud, A., Penella, C., Marsal, J.I., Bonet, L,. Nebauer, S.G. ,San Bautista A. and Lopéz-Galarza, S. 2013. Empleo del injerto en pimiento como mejora frente a la escasez de agua. Agricola Vergel 366:212-216.

[48]Bruce, R.R., Chesness, J.L., Keisling, T.C., Pallas, J.E., Smittle, D.A., Stansell, J.R. and Thomas A.W. 1980. Irrigation of crops in the southeastern United States: Principles and practices. USDA., Science and Education Administration. Southern Region. Agricultural Reviews and Manuals ARM-S, p.9.

[49]Marcelis, L.F.M. and Ho, L.C. 1999. Blossom-end rot in relation to growth rate and calcium content in fruits of sweet pepper (*Capsicum annuum* L.). J. Exp. Bot. **50**:357-363.

[50]Navarro, J.M., Garrido, C., Flores, P. and Martinez V. 2010. The effect of salinity on yield and fruit quality of pepper grown in perlite, Sp. J. Ag. Res. 8:142-150.

CHAPTER 4
Rootstock Alleviates PEG-Induced Water Stress In Grafted Pepper Seedlings: Physiological Responses

Consuelo Penella[1], Sergio G. Nebauer[2], Alberto SanBautista[2], Salvador Lopéz-Galarza[2], Ángeles Calatayud[1]

[1]*Instituto Valenciano de Investigaciones Agrarias (IVIA). Departamento de Horticultura. Ctra. Moncada-Naquera km. 4.5. 46113-Moncada, Valencia, Spain.*

[2]*Universitat Politècnica de València. Departamento de Producción Vegetal. Camino de Vera 14, 46020 Valencia, Spain.*

Journal Plant Physiology, 171:842-851 (2014)

4.1. ABSTRACT

Recent studies have shown that tolerance to abiotic stress, including water stress, is improved by grafting. In a previous work, we took advantage of the natural variability of *Capsicum* spp and selected accessions tolerant and sensitive to water stress as rootstocks. The behaviour of commercial cultivar 'Verset' seedlings grafted onto the selected rootstocks at two levels of water stress provoked by adding 3.5 and 7% PEG (polyethylene glycol) was examined during 14 days. The objective was to identify the physiological traits responsible for the tolerance provided by the rootstock in order to determine if the tolerance is based on the maintenance of the water relations under water stress or through the activation of protective mechanisms. To achieve this goal, various physiological parameters were measured, including: water relations; proline accumulation; gas exchange; chlorophyll fluorescence; nitrate reductase activity; and antioxidant capacity. Our results indicate that the effect of water stress on the measured parameters depends on the duration and intensity of the stress level, as well as the rootstock used. Under control conditions (0% PEG) all plant combinations showed similar values for all measured parameters. In general terms, PEG provoked a strong decrease in the gas exchange parameters in the cultivar grafted onto the sensitive accessions, as also observed in the ungrafted plants. This effect was related to a lower relative water content in the plants, provoked by an inefficient osmotic adjustment that was dependent on reduced proline accumulation. At the end of the experiment, chronic photoinhibition was observed in these plants. However, the plants grafted onto the tolerant rootstocks, despite the reduction in photosynthetic rate, maintained the protective capacity of the photosynthetic machinery mediated by osmotic adjustment (based on a higher proline content). In addition, water

stress limited uptake and further NO_3^- transfer to the leaves. An increased nitrate reductase activity in the roots was observed, mainly in plants grafted onto the sensitive rootstocks, as well as the ungrafted plants, and this was associated with the lessened flux to the leaves. This study suggests that PEG-induced water stress can be partially alleviated by using tolerant accessions as rootstocks.

***Key words*:** graft; osmotic potential; pepper; photosynthesis; water stress

4.2. INTRODUCTION

Pepper is one of the most important cultivated crops in the Mediterranean climate, where water shortage is a major problem limiting productivity. An improvement of plant yield under drought is one of the main scientific and economic challenges in these areas. Plants exposed to water stress may have different types of response: susceptibility, resistance mediated by avoidance, or tolerance. Water stress plant tolerance involves biochemical, physiological, and morphological mechanisms that enable plants to function during periods with decreased water availability (Nio et al., 2011) and prevent or alleviate damage. One of the important pathways to enhance water stress tolerance is through osmotic adjustment (OA), which maintains the leaf turgor necessary for stomatal opening and thus sustains photosynthesis and growth (Huang et al., 2010; Nio et al., 2011). Various types of compatible solutes accumulate: such as sugars, proline, gycinebetaine, or potassium (Munns et al., 1979; Morgan, 1992; Nio et al., 2011). These compounds can be added to a list of non-enzymatic antioxidants that plants need to counteract the inhibitory metabolic effects of reactive oxygen species (ROS) provoked by stress (Gill and Tuteja, 2010). They also play a role in the stabilisation of enzymes and proteins, as well as in the protection of membrane integrity (Patade et al., 2012).

Photosynthesis is extremely sensitive to water stress. The effects of water stress can be direct: such as decreased CO_2 availability caused by diffusion limitations through the stomata and/or the mesophyll (Flexas et al., 2007); or by alteration in CO_2 fixation reactions (Lawlor and Cornic, 2002). Photosynthetic responses to water stress are complex since they involve the interplay of limitations taking place at different parts of the plant (Chaves et al., 2009). Alterations in the photosynthetic process can provoke alteration in the uptake

and translocation of mineral nutrients (Calatayud et al., 2008). Nitrate reductase (NR) is a key enzyme responsible for nitrogen (N) assimilation and is connected with carbon metabolism (Masclaux-Daubresse et al., 2010): N assimilation requires NADH to drive NR, as well as carbon skeletons derived from photosynthesis for synthesis of aminoacids (Yousfi et al., 2012). A large fraction of leaf N is allocated to the photosynthesis apparatus. NR activity has been reported to decrease under water stress (Foyer et al., 1998); but the effect on grafted pepper has not been previously studied.

Mechanisms for plant adaptation and survival to water stress have been favoured by natural selection. Taking advantage of drought-resistant accessions is an important gateway for obtaining tolerant crops (although in pepper these accessions have a poor commercial value). A new perspective to improve resistance to water stress is the use of these tolerant accessions as rootstocks for a desirable commercial cultivar. Grafting has become a valid strategy to increase tolerance in plants under several abiotic stresses (Huang et al., 2010; Martínez-Ballesta et al., 2010; Colla et al., 2010). The interactions between graft, vegetable plants, and water stress have been mostly studied in tomato (Sánchez-Rodríguez et al., 2013) and melon (Rouphael et al., 2008); and there are no reports on physiological alterations of pepper after grafting and exposure to water stress. Water scarcity is a major problem in arid and semi-arid regions and limited information exists regarding water stress tolerance in pepper grafted plants using accessions as rootstock. Our study offers promising results that could improve the understanding of several physiological mechanisms involved in scion and pepper rootstock interaction under water stress conditions.

In previous experiments we selected four accessions: two that were resistant and two that were sensitive to water stress (Calatayud et al., 2011). The aim of the present work is to study the responses to water stress of a commercial

pepper cultivar grafted onto these rootstocks in order to identify the physiological traits responsible for the tolerance to this stress. Furthermore, we want to assess if this tolerance is based on the ability to maintain the water relations under low water availability little water is available; or through the activation of protective mechanisms in the scion – and if these effects depend on intensity of the water stress. For this purpose, several physiological parameters were determined, including: photosynthesis; chlorophyll (Chl) fluorescence; lipid peroxidation levels; relative water content (RWC); proline concentration; osmotic potential; and NR activity. We present evidence that grafting plants onto appropriate (tolerant) rootstocks is a good tool against water stress mediated by an efficient osmotic adjustment. Furthermore, these physiological parameters could be useful for screening processes when selecting tolerant plants.

4.3. MATERIALS AND METHODS

4.3.1. Plant material and greenhouse conditions

Based on previous studies (Calatayud et al., 2011), the drought tolerant accessions 'ECU-973' of *Capsicum chinense* Jacq. (code 12) and 'BOL-58' of *Capsicum baccatum* L. var. *pendulum* (code 14), and the water stress susceptible accessions 'Piquillo de Lodosa' (code 8) and 'Serrano' of *Capsicum annuum* L. (code 5) were chosen as rootstocks in this study. The pepper cultivar 'Verset' (California type; Rijk Zwaan) was grafted onto these four pepper accessions. The pepper seeds were sown on 1 December 2011 in 100-cell polystyrene trays filled with peat-based substrate and kept under a Venlo-type glasshouse. The plants were transplanted to 54-cell trays. The graft was

performed on 12 February using the tube grafting method (cutting the growing tip of the rootstock at a 45º angle below the cotyledons, attaching the scion, previously cut at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip). Ungrafted 'Verset' plants were used as controls.

One month after grafting, the plants were placed in 5 L polyethylene pots covered with aluminium sheets (the root system having been previously washed clean of substrate). Pots were filled with a nutrient solution containing (in mmol L^{-1}): 12.3 NO_3^-; 1.02 $H_2PO_4^-$; 2.45 SO_4^{2-}; 3.24 Cl^-; 5.05 K^+; 4.23 Ca^{2+}, 2.55 Mg^{2+} and micronutrients (15.8 µM Fe^{2+}, 10.3 µM Mn^{2+}, 4.2 µM Zn^{2+}, 43.5 µM B^{5+}, 1.4 µM Cu^{2+}) that had been artificially aerated. The electrical conductivity and pH of this nutrient solution was 2.1 dS m^{-1} and 6.5, respectively. Nutrient solution was added daily to compensate for absorption. After 7 days of seedling acclimation to the pots, PEG 8000 (Sigma Co) was dissolved in a nutrient solution for inducing osmotic stress at 3.5% and 7% PEG. The osmotic potential of the solutions, measured with a vapour osmometer (Digital osmometer, Wescor, Logan, USA), were -0.35 and -0.77 MPa respectively. Nutrient solution (0% PEG) was approximately -0.05 MPa due to the presence of the nutrient salt.

The treatments were defined by three PEG levels (0%, 3.5%, and 7%) and four plant combinations (the cultivar 'Verset' grafted onto rootstock accessions 5, 8, 12 and 14). The grafted combinations (rootstock/cultivar) were labelled as: 5/cultivar, 8/cultivar, 12/cultivar and 14/cultivar. The ungrafted cultivar was used as control. The layout was completely randomised with three replications for each combination and six plants per replication.

All physiological measurements were performed at 7 (T1) and 14 (T2) days after PEG addition on a fully expanded mature leaf (third or fourth leaf from the shoot apex).

During the culture, plants were grown in a Venlo-type greenhouse under natural light conditions (610-870 □mol m^{-2} s^{-1}) and temperature ranges were 21-24 ºC; and relative humidity was 52-72%.

4.3.2. Water relations

The osmotic potential of leaf sap (Ψ_s in MPa) was measured using an osmometer (Digital osmometer, Wescor, Logan, USA). Two independent determinations were performed on each replicate and plant combination, obtained from 6 plants per treatment and combination.

The leaves were tightly wrapped in aluminium foil, frozen at -70 ºC, and stored in liquid nitrogen. After thawing, sap was collected from syringes at 25 ºC and placed in the osmometer (Rodríguez-Gamir et al., 2010). Osmolyte content (mmol kg^{-1}) was converted to MPa using the Van't Hoff equation. The osmotic adjustment (OA) was determined as the difference between the osmotic potential of the leaves at full turgor for control plants and the stressed plants (Garcia-Sanchez et al., 2007). Full turgor was achieved by rehydrating the leaves with distilled water in darkness for 24 h.

Six other similar leaves from two independent plants of each plant combination, PEG treatment, and replicate were collected to determine the (RWC) as (FW-DW)/(TW-DW) x 100 where FW is fresh weight, DW is dry weight, and TW is turgid weight.

4.3.3. Proline determination

Proline content was determined as described by Bates et al. (1973). Leaf pepper tissue (0.05 g) was ground in 3% sulfosalicylic acid, the homogenate

was filtered, and 0.75 mL glacial acetic acid, and 0.75 mL ninhydrin reagent (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6N phosphoric acid) were added to an aliquot of the filtrate. The reaction mixture was boiled for 1 hour, and readings were taken at a wavelength of 520 nm in a spectrophotometer. Three independent determinations were performed in three different extracts, obtained from 18 plants per treatment and combination (one leaf per plant or 500 mg (FW) of roots, and six plants per extract).

4.3.4. Photosynthetic activity and chlorophyll fluorescence

CO_2 fixation rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and substomatal CO_2 concentration (C_i, μmol CO_2 mol^{-1} air) were measured at steady-state while maintaining the plants at 1000 μmol m^{-2} s^{-1} during 10-15 min and 400 ppm CO_2 with a LI-6400 (LI-COR, Nebraska, USA). Light curves were previously performed (data not shown) and A_N was saturated at 900 μmol m^{-2} s^{-1}. Current fluorescence yield (Fs) and the maximum light adapted fluorescence (Fm') were determined with the LI-6400 in the presence of an actinic illumination of 1000 μmol photons m^{-2} s^{-1}, and photochemical PSII efficiency (ϕ_{PSII}) was computed as the quotient (Fm' – Fs)/Fm' (Genty et al., 1989).

To evaluate the presence of chronic photoinhibitory processes, the variable fluorescence ratio Fv/Fo= Fm-Fo/Fo (Babani and Lichtenthaler, 1996) was measured on leaves after 15 minutes in darkness using a portable pulse amplitude modulation fluorometer (PAM-2100, Walz, Effeltrich, Germany). The background fluorescence signal for dark adapted leaves (Fo) was determined with a 0.5 μmol photon m^{-2} s^{-1} measuring light at a frequency of 600 Hz. The

application of a saturating flash of 10000 μmol photon m^{-2} s^{-1} enabled estimations of the maximum fluorescence (Fm).

Gas exchange and fluorescence determinations were performed from 9:00 am to 11:00 am (GMT). One measurement per plant was performed, and ten different plants were used (n=10) for each PEG treatment and plant combination.

4.3.5. Nitrate reductase activity

Nitrate reductase activity (EC 1.6.6.1) was determined *in vivo* following the methods described by Hageman and Hucklesby (1971) and Jaworki (1971). Discs of 1 cm diameter in mature fresh leaves, or pieces of 1 cm in roots, were punched out. Samples (200 mg) were suspended in a glass vial containing 10 mL of 100 mM potassium phosphate buffer (pH 7.5), 1% (v/v) *n*-propanol and 100 mM KNO_3. The glass vial was subjected to vacuum infiltration three times in order to induce anaerobic conditions in the incubation medium. Plant samples were incubated in a water bath at 30 ºC for 60 min in the dark and placed in a boiling water bath for 5 min to stop enzymatic reaction. Nitrite released from plant material was determined colourimetrically at 540 nm (spectrophotometer PerkinElmer, Lambda 25) by adding 0.02% (w/v) N-Naphthylethylenediamine and 1% sulphanilamide. A standard curve with KNO_2 was prepared to calculate the amount of NO_2 contained in the samples (Calatayud et al., 2008). Sampling and replicates were used as described for proline determination.

4.3.6. Lipid peroxidation

Lipid peroxidation was estimated through malondialdehyde (MDA) determinations using thiobarbituric acid reaction, according to the protocol reported by Heath and Parker (1968)**,** and modified in Dhindsa et al. (1981). The non-specific background absorbance reading at 600 nm was subtracted from specific absorbance reading at 532 nm. Sampling and replicates used as described for proline determination.

4.3.7. Statistical analyses

The results were subjected to multifactor variance analysis (Statgraphics Centurion for Windows, Statistical Graphics Corp.). The effect of the genotype and stress level was estimated and significant interactions (genotype x stress level) were observed for all the analysed parameters. The mean comparisons were performed using Fisher's least significance difference (LSD) test at $P < 0.05$.

4.4. RESULTS

4.4.1. Plant water status

Seedling under control conditions maintained RWC leaf values above 90% during the experiment (Fig. 1). The presence of PEG in the nutrient solution reduced the RWC of the leaves (Fig. 1). At T1 this effect was more dramatically observed at 7% PEG, and the ungrafted cultivar was the most sensitive (37%; Fig. 1A). The 12/cultivar and 14/cultivar plants were less affected (70% and 68%, respectively; $P < 0.05$). After 14 days (T2) RWC fell, even at 3.5% PEG (Fig. 1B). The ungrafted plants, as well as the 5/cultivar and 8/cultivar plants

had lower RWC values at 80% ($P < 0.05$). These genotypes showed the lowest reductions at 7% PEG (Fig. 1B), and the ungrafted plants had the lowest RWC values (35%), followed by the 5/cultivar and 8/cultivar plants ($P < 0.05$). The 12/cultivar and 14/cultivar plants maintained RWC values near 90% under 3.5% PEG without significant differences with respect to their controls and between 63%-65% at 7% PEG, respectively ($P < 0.05$).

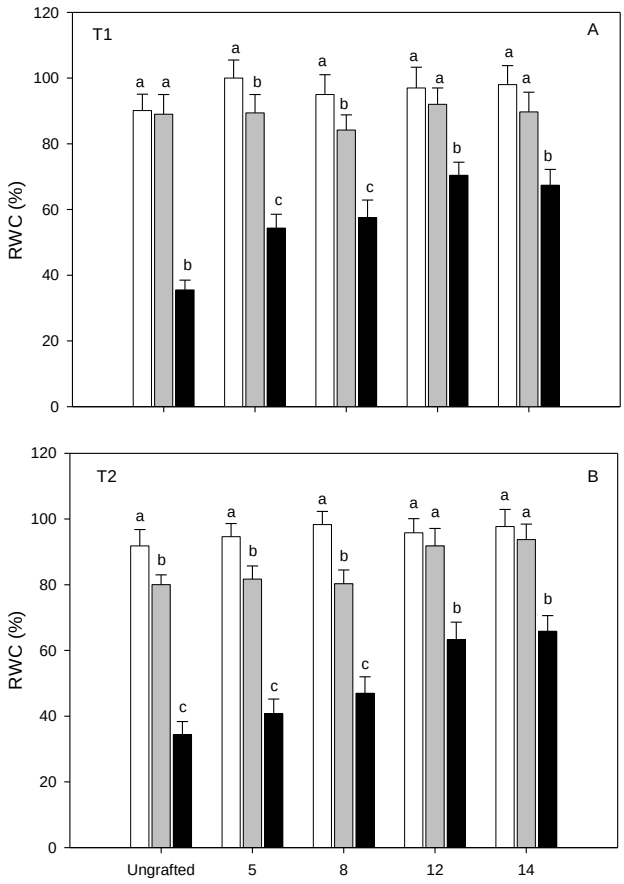


Figure 1. Effect of PEG addition at 0% (□), 3.5% (▒) and 7% (■) on relative leaf water content (RWC %) during 7 day (A) and 14 day exposure (B) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14. Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at $P<0.05$ (LSD test).

4.4.2. Leaf osmotic potential

Leaf osmotic potential values at T1 and T2 are shown in Fig. 2. The Ψ_s remained unchanged in control conditions during the experimental period, with values near -1 MPa. The osmotic potential decreased in relation to time exposure and PEG concentration. At 3.5% PEG, the 14/cultivar plants showed the largest decreases ($P < 0.05$) in Ψ_s at T1 and T2 (Fig. 2A,B). This effect was also observed at T1 in the ungrafted plants and in the 12/cultivar plants at T2. At higher PEG concentrations, the 12/cultivar and 14/cultivar plants showed the lowest Ψ_s values during the experiment ($P < 0.05$). Furthermore, the 5/cultivar and 8/cultivar as well as the ungrafted plants showed significant but less intense decreases (Fig. 2).

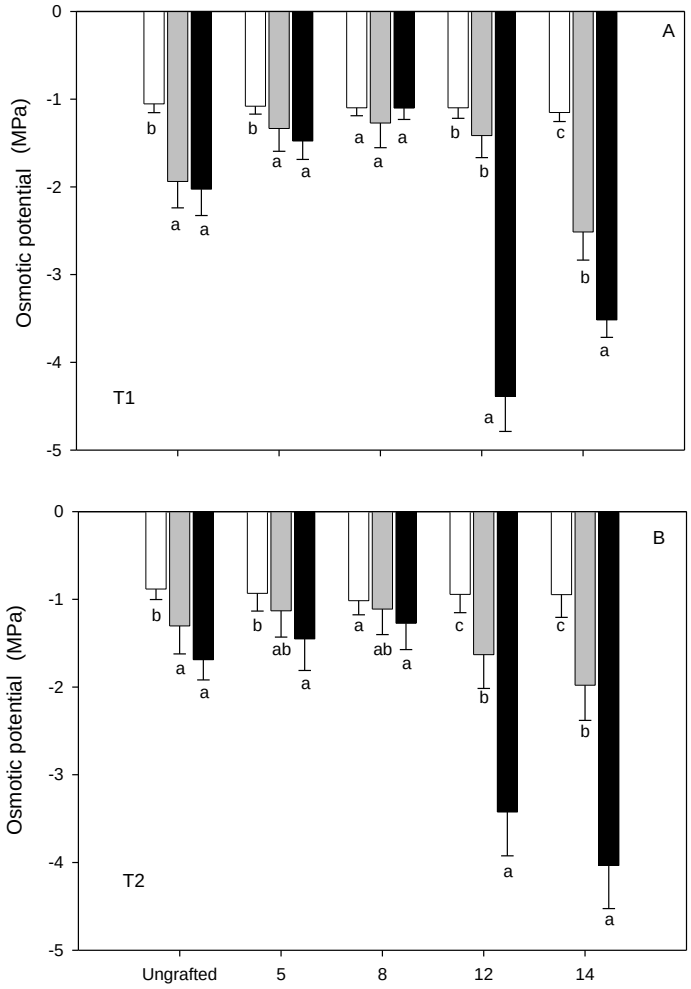


Figure 2. Leaf osmotic potential (MPa) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% ( ), 3.5% ( ) and 7% ( ) during 7 day (A) and 14 day exposure (B). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at $P<0.05$ (LSD test).

Osmotic adjustment was observed at T1 in ungrafted plants and in 14/cultivar plants at 3.5% PEG, and in 12/cultivar and 14/cultivar plants at 7% PEG (Table 1). After 14 days, the highest OA was induced in the 12/cultivar and 14/cultivar plants at both PEG concentrations (Table 1).

Table 1. Osmotic adjustment (MPa) in the grafted pepper plants (cultivar 'Verset') onto the pepper accessions 5, 8, 12 and 14. Ungrafted 'Verset' plants were used as controls. Determinations were performed after 7 (T1) and 14 (T2) days under water stress conditions by PEG addition (3.5% and 7%). Each value is the mean of six independent determinations.

		Cultivar	5	8	12	14
T1	3.5% PEG	0.81*	0.12	0.25	0.27	1.17*
	7% PEG	0.07	-0.30	-0.41	2.12*	1.38*
T2	3.5% PEG	0.23	0.04	-0.09	0.61*	1.25*
	7% PEG	0.06	-0.27	-0.41	0.98*	1.71*

Significant differences in relation to controls (0% PEG and full turgor) (P<0.05) are indicated by asterisks

4.4.3. Accumulation of proline

Proline accumulation was induced in pepper seedlings by drought and PEG exposure (Fig. 3). No effect of stress level was observed in the accumulation of proline. At T1 (Fig. 3A) a slight increase (P < 0.05) was observed in all genotypes irrespective of the PEG concentration in the culture medium, except for 12/cultivar and 14/cultivar plants where the proline concentration decreased with respect to the controls. Proline levels increased after 14 days (T2) (Fig. 3B)

of water stress treatment. Two to three-fold increases were observed in the cultivar and 5/cultivar and 8/cultivar plants. The maximum increase was found for 12/cultivar and 14/cultivar plants ($P < 0.05$), with rises from 12 mg/ g DW at 0% PEG to 32 and 49 mg/ g DW under 7% PEG conditions, respectively.

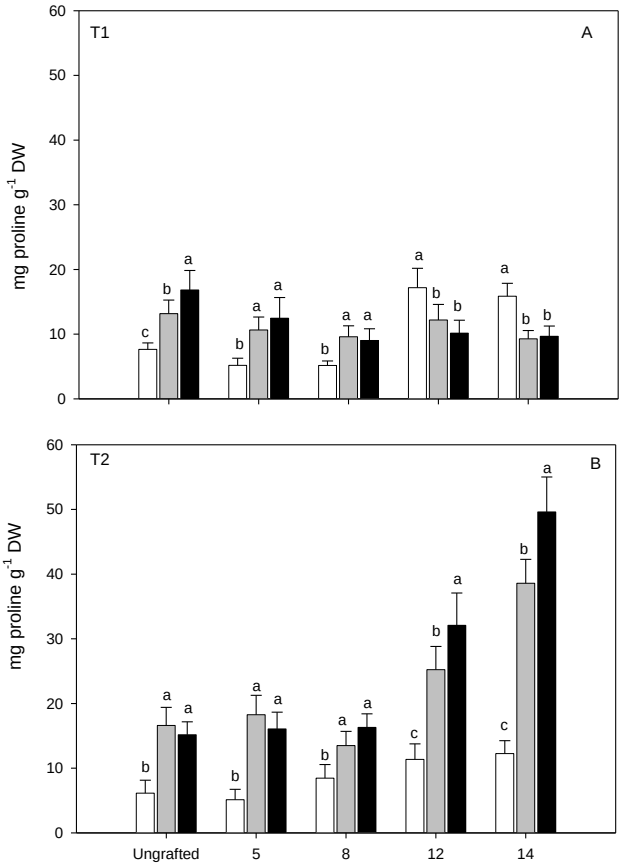


Figure 3. Changes in proline concentration (mg proline /g DW) from ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0%( ), 3.5% ( ) and 7% ( ) during 7 day (A) and 14 day exposure (B). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at P<0.05 (LSD test).

4.4.4. Photosynthetic parameters

PEG provoked a significant reduction in the photosynthetic rate (Fig. 4A, B), stomatal conductance (Fig. 4C,D), and photochemical PSII efficiency (Fig. 4E,F) in the studied pepper genotypes.

At T1 the A_N progressively diminished with the drought stress level in the ungrafted plants and 5/cultivar plants (Fig. 4A). In the 8/cultivar and 14/cultivar plants no significant effect of 3.5% PEG was observed; and in the 12/cultivar plant, the photosynthetic rate fell at 3.5% PEG; but did not fall further at 7% PEG. In the ungrafted plants, the photosynthetic rate reached null values at T2 in the 7% PEG media (Fig. 4B). At this concentration, the 12/cultivar and 14/cultivar plants showed smaller reductions ($P < 0.05$) in the photosynthetic rate. No effect for PEG concentration was observed in the grafted plants at T2 (Fig. 4B).

Differences in the stomatal conductance to drought were observed among genotypes (Fig. 4C,D). At T1, the ungrafted plants, 5/cultivar, and 8/cultivar plants maintained higher stomatal openings at 3.5% PEG when compared to 12/cultivar and 14/cultivar plants ($P < 0.05$). In addition, g_s fell to values near zero at 7% PEG in these genotypes. By contrast, stomata closed to values near 0.1 mol m^{-2} s^{-1} in 12/cultivar and 14/cultivar plants, irrespective of the stress level (Fig. 4C), and did not change at T2 (Fig. 4D). Stomatal conductance was also strongly reduced in the ungrafted, 5/cultivar, and 8/cultivar plants at T2.

Substomatal CO_2 concentration (Ci) decreased with stomatal closure in all grafted plants (data not shown). In contrast in the ungrafted cultivar, Ci increased ($P < 0.05$) at low stomatal conductances under water stress.

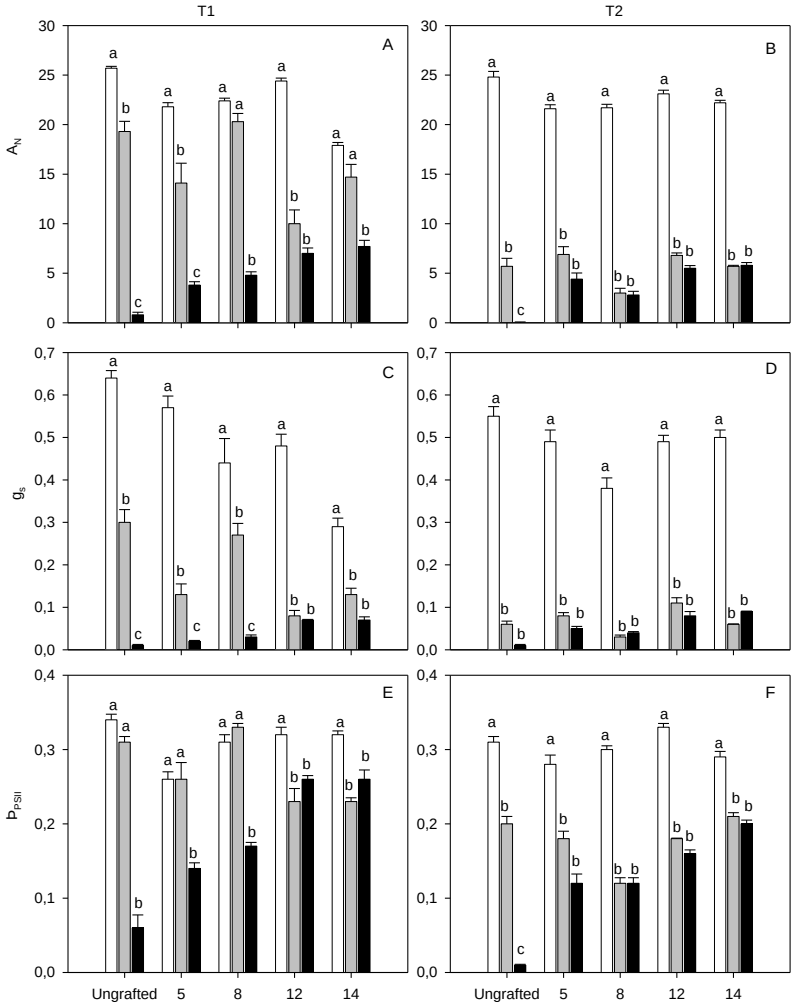


Fig. 4. Net CO_2 assimilation rate (A_N; μmol CO_2 m^{-2} s^{-1}) (A, B); leaf stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) (C, D) and actual quantum efficiency of PSII (ϕPSII) (E, F) in ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% ( ), 3.5% ( ) and 7% ( )during 7 day (A, C, D) and 14 day exposure (B, D, F). Dates are mean values ± SE for n= 10. Within each plant combination different letters indicate significant differences at *P*<0.05 (LSD test).

No effect for 3.5% PEG on the ϕ_{PSII} was observed at T1 in the ungrafted, 5/cultivar, and 8/cultivar plants (Fig. 4E). By contrast, this parameter fell by more than 55% of the control values at 7% PEG in these genotypes. In 12/cultivar and 14/cultivar plants, the reduction provoked by PEG ranged from 75 to 81% of control values at T1, irrespective of the stress level. At T2, the response of the photochemical PSII efficiency was similar to that observed for the photosynthetic rate (Fig. 4B).

Similar Fv/Fo values were observed for all genotypes under control conditions (Fig. 5A,B). No changes were produced at T1 by 3.5% PEG, except for the 8/cultivar plants (where Fv/Fo increased with respect to its control). However, at 7% PEG, Fv/Fo fell in the ungrafted plants (32% of control value) and, to a lesser extent in the 5/cultivar and 8/cultivar plants (Fig. 5A). At T2, the decrease in Fv/Fo increased with the stress level (Fig. 5B). The ungrafted plants showed the lowest values, being zero at 7% PEG; while 12/cultivar and 14/cultivar plants showed the smallest reduction ($P < 0.05$) in Fv/Fo at 7% PEG (Fig. 5B).

4.4.5. Changes in nitrate reductase activity

Differing responses of NR activity to drought were observed in leaves and roots (Fig. 6). NR activity increased in roots (Fig. 6B,D) in all the water stress treatments when compared to control conditions – the highest values ($P < 0.05$) being for ungrafted plants, 5/cultivar, and 8/cultivar plants at 7% PEG and T2 (Fig. 6D). By contrast, water stress decreased NR activity in the leaves, and the lowest value ($P < 0.05$) was observed for ungrafted plants at 7% PEG followed by 5/cultivar and 8/cultivar plants (Fig. 6A, C). In the leaves, after 7 and 14 days

of severe water stress, 12/cultivar and 14/cultivar plants showed the highest NR activity levels – while the lowest values were observed in the ungrafted plants.

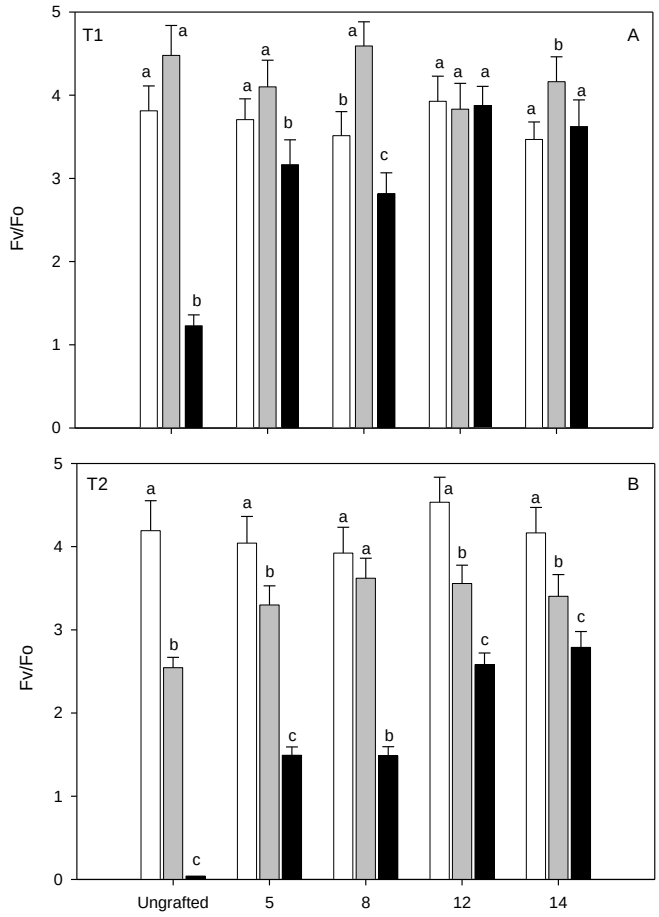


Figure 5. Variations in dark-adapted Fv/Fo ratio in leaves of ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during 7 day (A) and 14 day exposure (B). Dates are mean values ± SE for n= 10. Within each plant combination different letters indicate significant differences at *P*<0.05 (LSD test).

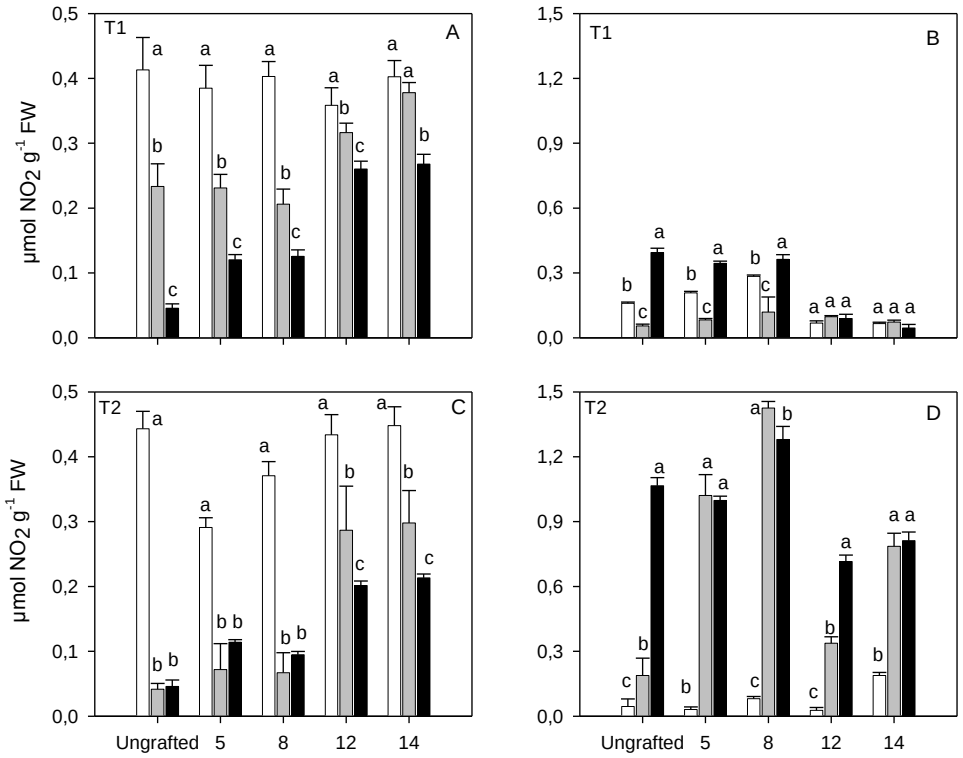


Figure 6. Nitrate reductase activity (µmol NO_2 g^{-1} FW) in leaf (A, C) and roots (B, D) of ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% (□), 3.5% (▒) and 7% (■) during 7 day (A, B) and 14 day exposure (C, D). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at *P*<0.05 (LSD test).

4.4.6. Lipid peroxidation

Lipid peroxidation in pepper leaves increased with time and PEG levels (Fig. 7). At T1 MDA content increased with higher PEG levels (Fig. 7A) in all plants. The

increase was highest in the ungrafted plants. After 14 days of exposure, lipid peroxidation increased significantly at 7% PEG in all plants and 12/cultivar and 14/cultivar plants at 3.5%. It is noteworthy that no further MDA accumulation was produced in these genotypes at 7%, whereas MDA accumulated to higher levels in 5/cultivar, 8/cultivar, and ungrafted plants (Fig. 7B).

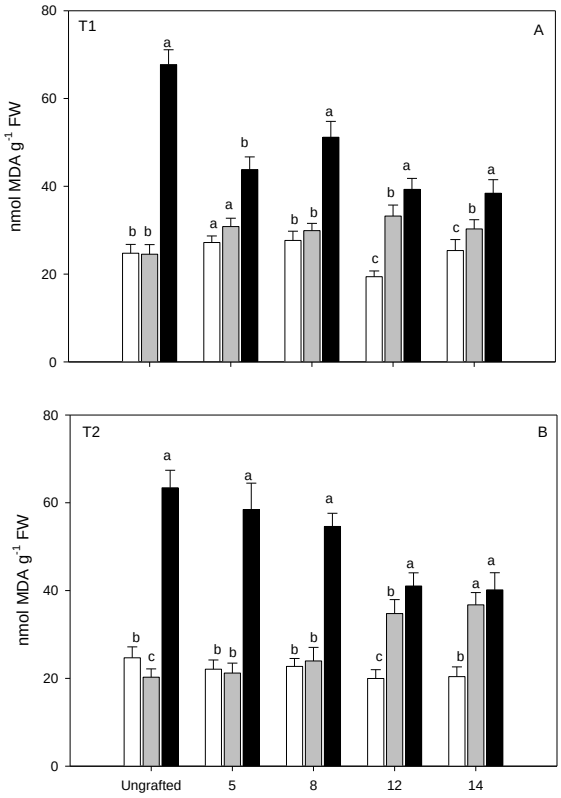


Figure 7. Leaf malondialdehyde content (nmol MDA g^{-1} FW) in leaves of ungrafted pepper plants (cultivar 'Verset') and cultivar grafted onto accessions 5, 8, 12 and 14 after PEG addition at 0% ( ), 3.5% ( ) and 7% ( ) during 7 day (A) and 14 day exposure (C). Dates are mean values ± SE for n= 6. Within each plant combination different letters indicate significant differences at *P*<0.05 (LSD test).

4.5. DISCUSSION

Water stress induced by PEG led to significant changes in physiologic parameters in pepper seedlings. The effect depended on the duration and the intensity of the stress level. Moreover, consistent differences were observed between susceptible (5 and 8) and tolerant accessions (12 and 14) when used as rootstocks, although such differences vanished in the absence of water stress. The following discussion aims to establish which physiological processes could explain the different responses among grafted plants, including tolerant and sensitive accessions such as rootstocks and ungrafted plants.

Water status in a plant is highly sensitive to water stress and therefore is dominant in determining plant responses to stress. Leaf RWC decreased under water stress, but its effects were significantly dramatic only under the 7% PEG treatment. The highest RWC values (62-67%) were observed in the 12/cultivar and 14/cultivar plants after 14 days, when compared with ungrafted plant values (34%) ($P < 0.05$). Similarly, the leaves of tomato plants grafted onto *Solanum mammosum* – (with a greater ability for passive water uptake) maintained higher leaf water potential than self-grafted plants – despite greater water loss through transpiration under water stress conditions (Weng, 2000).

An alteration in the relationship between RWC and ψ_s was found. In this sense, the leaf ψ_s was lowest in 12/cultivar and 14/cultivar plants, compared with 5/cultivar, 8/cultivar, and ungrafted plants; although the RWC values at 3.5% PEG in T1 and T2 remained unchanged. This can be explained by the fact that the relationship between ψ_s and RWC is not unique (Acevedo et al., 1979), and other factors such as the rate of transpiration, stomatal aperture, or development of the root system can modulate this relation (Weng, 2000).

Nevertheless, decreases in ψ_s may have contributed to the ability of these accessions (12 and 14) to uptake more water from the nutrient solution and could have minimised the harmful effects of water stress (Nio et al., 2011; Ming et al., 2012). Significant correlations were demonstrated between ψ_s and the tolerance to drought in different crops, i.e. PEG-tolerant chilli pepper clones (Santos-Díaz and Ochoa-Alejo, 1994); tomato PEG-adapted cell lines (Handa et al., 1982); or barley after 36 days without irrigation (González et al., 2008).

Although the decrease in ψ_s could be a consequence of a reduction in the water content of tissues, active osmotic adjustment was observed in the studied genotypes, and mainly in the plants grafted onto the tolerant genotypes (12 and 14). The osmotic adjustment may have involved the accumulation of a range of osmotically active molecules, including organic compounds such as sugars, free aminoacids, glycinebetaine, soluble proteins, and organic acids (Chaves et al., 2003); and with macronutrients such as inorganic components (Patakas et al., 2002). Free proline is considered an important osmoprotectant and accumulation following salt, drought, and heavy metal exposure is well documented (Gill and Tuteja, 2010). In our work, a strong correlation between ψ_s decrease and proline content increase was observed at T2 (ψ_s = -0.752 [proline] - 0.205; r^2 = 0. 87; $P < 0.05$) for all plant combinations and treatments; and at T1 for 5/cultivar, 8/cultivar, and ungrafted plants (ψ_s = -0.087 [proline] - 0.540; r^2 = 0. 79; $P < 0.05$). Nevertheless, the decrease at T1 in ψ_s was not related to the increase in proline in the 12/cultivar and 14/cultivar plants (ψ_s = 0.318 [proline] - 6.288; r^2 = 0.62; $P < 0.05$). At this earlier period, other components such as glycinebetaine, carbohydrates, aminoacids, and macronutrients could have contributed to reducing the osmotic potential (Munns et al., 1979; Morgan, 1992; Navarro et al., 2003) in these plant combinations. Similar time-dependent behaviour was reported in wheat (Nio et al., 2011), where K^+ was mainly involved in the osmotic responses to water stress during

earlier periods; whereas proline was mainly accumulated after long exposures. Alternatively, pepper plants (12 and 14) could have used the mineral components of the nutrient solution to produce the decrease in osmotic potential, such as described for sugarcane cells (Patade et al., 2012) during the first seven days of water stress.

The osmotic adjustment, mainly through the increase in proline content, and related to the duration and severity of the water stress, helped the 12/cultivar and 14/cultivar plants maintain tissue water status and avoid drought-induced damage. Similar results were obtained by Anjum et al. (2012) in pepper plants.

Moreover, osmolyte proline accumulation was proposed to act as a protein stabilizer, a metal quelator, an inhibitor of lipid peroxidation, and a scavenger of radical oxygen species (ROS) under salt, drought, and metal stress (Gill and Tuteja, 2010). Production of these species at higher levels may damage cellular membrane and other biologically vital components such as chlorophylls, DNA, proteins, and lipids (Blokhina et al., 2003). Lipid peroxidation is considered to be one of the most damaging processes as its decreases membrane fluidity; increases the leakiness of the membranes, and inactivates receptors, enzymes, and ion channels. The final product of lipid peroxidation is MDA – which is used as an index of oxidative membrane damage (Calatayud et al., 2002; Ozkur et al., 2009). In our work, improvement in proline accumulation under water stress helped maintain osmotic potential; and may also be involved in protection against oxidative damage as indicated by lower levels of MDA in the 12/cultivar and 14/cultivar plants (mainly at the end of the experiment under 7% PEG). These results indicate that these genotypes when used as rootstocks provide protection to the scion. By contrast, the ungrafted plants and 5/cultivar and 8/cultivar plants showed less capacity to retain water in their cells: a minor

decrease of $\psi_{s,}$ was associated with a minor increase in proline concentration, and as a consequence, a higher level of lipid peroxidation.

The oxidative stress provoked by water stress had a direct effect on proper PSII function. The Fv/Fo parameter, a sensitive Chl fluorescence ratio is related to the maximum quantum yield of PSII photochemistry (Babani and Lichtenthaler, 1996). A decline in Fv/Fo indicates a disturbance or damage of the photosynthetic apparatus, and has been frequently used as an indicator of photoinhibition (Calatayud et al., 2004). A decrease in the Fv/Fo ratio occurs under water stress, and the most dramatic decrease occurred in ungrafted plants at T2 under 7% PEG, where the values were zero. According to our observations (see above), the Fv/Fo ratio suggested a higher resistance for 12/cultivar and 14/cultivar plants to water stress. The decrease in Fv/Fo in ungrafted plants, 5/cultivar, and 8/cultivar plants may be as a result of an increase in protective non-radiative energy dissipation associated with a regulated decrease in photochemistry – described as down-regulation and/or chronic photodamage of the PSII centres (Genty et al., 1989; Osmond, 1994). The Fv/Fo ratio seems a robust parameter, and several authors have concluded that PSII photochemistry cannot be impaired by relatively severe water stress; although A_N and gs can decrease significantly (Lawlor and Tezara, 2009). In our experiment, all plant combinations, regardless of the Fv/Fo values, showed a significant decrease in the net carbon gain, due in part to stomatal closure that restricts water losses. The decrease in the rate of photosynthesis may be due to the chronic water stress effect of metabolic inhibition, or the down-regulation of photosynthesis as described by Chaves et al. (2003) and Cornic (2000). Distinguishing between these alternatives is difficult (Flexas et al., 2004). Acclimation to water stress requires responses that enable essential reactions of primary metabolism to continue for the plant to tolerate water deficit (Foyer et al., 1998). The ability to maintain the functionally, or protective capacity of the

photosynthetic machinery under water stress, is of major importance for drought tolerance in pepper plants (del Amor et al., 2010). Our results indicate that rootstocks 12 and 14 provide the variety with the ability to maintain water relations and protective mechanisms that enable the maintenance of a residual photosynthetic rate (on 'stand-by'). The robust behaviour of the cultivar 'Verset' grafted onto accessions 12 and 14 was in accordance with our previous results in field conditions where water availability was reduced by 50% compared to the control treatment (Calatayud et al., 2013). In this experiment, pepper cultivar grafted onto these genotypes showed higher marketable fruit production when compared with ungrafted plants and 'Verset' grafted onto 5 and 8 (Calatayud et al., 2013).

Maintenance of tissue water status helps the plants to avoid the dehydration and protects the carboxylation and other enzymes from inactivation and denaturation (Anjum et al., 2012). By contrast, a strong decrease in the photosynthetic rate in 5/cultivar, 8/cultivar plants, and ungrafted plants, along with a decrease in RWC (a weak osmotic adjustment), and a decrease in Fv/Fo was observed under water stress. In the absence of protective mechanisms, an increase in oxidative damage was produced (measured as lipid peroxidation) and chronic photoinhibition of metabolic machinery limiting photosynthesis. The degree of oxidative stress has been described as being closely associated with the resistance/susceptibility of a genotype to water stress (Mittler, 2002; Anjum et al., 2012).

At the whole plant level, water scarcity induces complex changes in C and N metabolism resulting from modifications in the availability of nutrients (Foyer, 1998; Imsande and Touraine, 1994). In addition to the discussed changes in carbon assimilation, water stress may restrain nitrate acquisition by the roots, as well as restrict the ability of plants to assimilate and reduce nitrogen (Yousfi

et al., 2012; Kocheva et al., 2007). In most herbaceous plants, NR activity takes place predominantly in the leaves (Scheurwater et al., 2002; Reda et al., 2011). In our results under control conditions, where the plants have free access to nutrients, NR activity was higher in leaves than in roots in all plant combinations at T1 and T2. The reduction of NO_3^- in the leaves may provide the advantage of enabling the direct use of excess reductants produced by photosynthesis (Pate, 1983). In our work, the predominant site of NO_3^- reduction (leaves or roots) was dependent on the water stress intensity and time of exposure. NR activity in leaves decreased considerably in all plant combinations under drought, but especially in ungrafted plants, as well as 5/cultivar and 8/cultivar plants. However, since NR activity was calculated on a FW basis, and PEG treatment affected the RWC of the leaves, the absolute value of NR activity could be overestimated in these treatments. The utilisation of nitrate in the leaves is governed by CO_2 fixation (Larsson et al., 1989). In our results, a decrease in NR activity in the leaves can be linked to a decline in the rate of photosynthesis due to stomatal closure, according to Fresneau et al. (2007); or due to a decrease in the NO_3^- transport from root to leaves due to loss of turgor and lower transpiration flow (Sharma and Dubey, 2005; Yousfi et al., 2012). Water stress would limit the uptake and further the transfer of NO_3^- to upper plant parts (Yousfi et al., 2012), and subsequently, a part of the nitrate uptake could be reduced in the roots. Observed differences in NR activity may depend on PEG concentration, time exposure, and plant combinations. After 7 days under 3.5% PEG with moderate photosynthesis inhibition, NR activity was located mainly in the leaves. This could be interpreted as that the rate of carbon fixation was not a limiting factor for NO_3^- reduction (Larsson et al., 1989). When the water stress was severe (7% PEG), or when the time exposure with PEG was longer (14 days), photosynthetic activity was compromised, and under this extreme situation the behaviour between rootstocks differed. Sensitive genotypes (5 and 8) with lower NR activity in the leaves showed low levels of photosynthetic

activity, i.e. when internal CO_2 concentration was reduced due to stomatal closure (Fresneau et al., 2007) and greater root NR activity (irrespective of PEG concentration). Tolerant rootstocks (12 and 14) showed increased root NR activity at only T2 in 7% PEG, although to a lesser extent. This could be because the remaining water transpiration flux (highest E values) enables reductions through the NO_3^- transport to the leaves. The significant increase in root NR activity may indicate that nitrate flux to roots was not restricted by water stress and that active NO_3^- reduction occurs in the roots, possibly due a minor transpiration flux to leaves.

Considering the overall results of this study, we can conclude that the response of commercial pepper cultivar to water stress can be improved by grafting when using appropriate accessions as rootstocks. It seems that grafting methods could be a useful tool for increasing resistance to water stress. Under these experimental conditions, accessions 12 and 14 grafted onto cultivar, alleviate the water stress effect. This effect may be attributed to enhanced osmotic adjustment because of active proline accumulation (as reflected by the lower reduction in RWC) which may protect leaves from excessive dehydration caused by damaged photosynthesis systems. In addition, the methods used in this work appear to be suitable for testing the water stress resistance of pepper rootstocks.

4.6. REFERENCES

Del Amor FM, Cuadra-Crespo P, Walker DJ, Cámara JM, Madrid R. Effect of foliar application of antitranspirant on photosynthesis and water relations of pepper plants under different levels of CO_2 and water stress. J Plant Physiol. 2010; 167:1232–38.

Anjum SA, Farooq M, Xie X, Liu X, Ijaz MF. Antioxidant defense system and proline accumulation enables hot pepper to perform better under drought. Sci Hortic 2012; 140:66–73.

Babani F, Lichtenthaler HK. Light-induced and age-dependent development of chloroplasts in etiolated barley leaves as visualized by determination of photosynthetic pigments, CO_2 assimilation rates and different kinds of chlorophyll fluorescence ratios. J Plant Physiol 1996; 148:555–66.

Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil 1973; 39:205–7.

Blokhina O, Virolainen E, Fagerstedt K V. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot. 2003;91:179–94.

Calatayud A, Alvarado JW, Barreno E. Differences in ozone sensitivuty in three varieties of cabbage (*Brassica oleracea* L.) in the rural Mediterranean are. J Plant Physiol 2002; 159: 863-68.

Calatayud A, Iglesias DJ, Talón M, Barreno E. Response of spinach leaves (*Spinacia oleracea* L.) to ozone measured by gas exchange, chlorophyll *a* fluorescence, antioxidant systems, and lipid peroxidation. Photosynthetica 2004; 42:23–9.

Calatayud A, Gorbe E, Roca D, Martínez PF. Effect of two nutrient solution temperatures on nitrate uptake, nitrate reductase activity, NH_4^+ concentration and chlorophyll a fluorescence in rose plants. Environ Exp Bot 2008; 64:65–74.

Calatayud A, San Bautista A, López-Galarza S, Bonet L, Buesa I, Nebauer SG. Screening for salt and water stress tolerance in pepper based on photosynthesis parameters.2011. International symposium on vegetables grafting. Book of abstracts. p 43.

Calatayud A, Penella C, Marsal JI, Bonet L, Nebauer SG, San Bautista A, López-Galarza S. Empleo del injerto en pimiento como mejora frente a la escasez de agua. Agrícola Vergel 2013; 336:212-216.

Chaves MM, Flexas J, Pinheiro C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot. 2009; 103:551–60.

Chaves MM, Maroco JP, Pereira JS. Understanding plant responses to drought - from genes to the whole plant. Funct Plant Biol 2003; 30:239–64.

Colla G, Rouphael Y, Leonardi C, Bie Z. Role of grafting in vegetable crops grown under saline conditions. Sci Hortic 2010; 127:147–55.

Cornic G. Drought stress inhibits photosynthesis by decreasing stomatal aperture – not by affecting ATP synthesis. Trends Plant Sci 2000; 5:187–8.

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA. Leaf Senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot. 1981; 32:93–101.

Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biol 2004; 6:269–79.

Flexas J, Diaz-Espejo A, Galmés J, Kaldenhoff R, Medrano H, Ribas-Carbo M. Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. Plant Cell Environ. 2007; 30:1284–98.

Foyer CH. Drought-induced effects on nitrate reductase activity and mRNA and on the coordination of nitrogen and carbon metabolism in maize leaves. Plant Physiol 1998;117:283–92.

Fresneau C, Ghashghaie J, Cornic G. Drought effect on nitrate reductase and sucrose-phosphate synthase activities in wheat (*Triticum durum* L.): role of leaf internal CO_2. J Exp Bot 2007; 58:2983–92.

Garcia-Sanchez F, Syvertsen JP, Gimeno V, Botia P, Perez-Perez JG. Responses to flooding and drought stress by two citrus rootstocks seedling with different water-use efficiency. Physiol Plant 2007; 130: 532-42.

Genty B, Briantais JM, Baker NR. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 1989; 990:87–92.

Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 2010; 48:909–30.

González A, Martín I, Ayerbe L. Yield and Osmotic Adjustment Capacity of Barley Under Terminal Water-Stress Conditions. J Agron Crop Sci 2008; 194:81–91.

Hageman RH, Hucklesby DP. Nitrate reductase from higher plants. Methods Enzymol 1971; 23:491–503.

Handa AK, Bressan RA, Handa S, Hasegawa PM. Characteristics of cultured tomato cells after prolonged exposure to medium containing polyethylene glycol. Plant Physiol. 1982;69:514–21.

Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 1968; 125:189–98.

Huang Y, Bie Z, He S, Hua B, Zhen A, Liu Z. Improving cucumber tolerance to major nutrients induced salinity by grafting onto Cucurbita ficifolia. Environ Exp Bot. 2010; 69:32–8.

Imsande J, Touraine B. N demand and the regulation of nitrate uptake. Plant Physiol 1994; 105:3–7.

Jaworki EG. Nitrate reductase assays in intact plant tissue. Biochem Biophys Res Commun 1971; 43:1274-79.

Kocheva KV, Georgiev GI, Vunkova-Radeva RV. Contribution of mineral nutrition to the response of barley seedlings to polyethylene glycol–induced mild water stress. J Plant Nutr Soil Sci 2007; 170:392–7.

Larsson M, Larsson CM, Whitford PN, Clarkson DT. Influence of osmotic stress on nitrate reductase activity in wheat (*Triticum aestivum* L.) and the role of abscisic acid. J Exp Bot 1989; 40:1265–71.

Lawlor DW, Cornic G. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant, Cell Environ 2002; 25:275–94.

Lawlor DW, Tezara W. Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. Ann Bot 2009;103:561–79.

Martínez-Ballesta MC, Alcaraz-López C, Muries B, Mota-Cadenas C, Carvajal M. Physiological aspects of rootstock–scion interactions. Sci Hort 2010; 127:112–8.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot 2010; 105:1141–57.

Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 2002; 7:405–10.

Ming DF, Pei ZF, Naeem MS, Gong HJ, Zhou WJ. Silicon alleviates PEG-induced water-deficit stress in upland rice seedlings by enhancing osmotic adjustment. J Agron Crop Sci 2012; 198:14–26.

Morgan J. Osmotic components and properties associated with genotypic differences in osmoregulation in wheat. Aust J Plant Physiol 1992; 19:67-76.

Munns R, Brady C, Barlow E. Solute accumulation in the apex and leaves of wheat during water stress. Aust J Plant Physiol. 1979; 6:379-89.

Navarro JM, Garrido C, Martínez V, Carvajal M. Water relations and xylem transport of nutrients in pepper plants grown under two different salts stress regimes. Plant Growth Regul 2003; 41:237–45.

Nio SA, Cawthray GR, Wade LJ, Colmer TD. Pattern of solutes accumulated during leaf osmotic adjustment as related to duration of water deficit for wheat at the reproductive stage. Plant Physiol Biochem 2011; 49:1126–37.

Osmond CB. What is photoinhibition? Some insights from comparisons of shade and sun plants. In: Baker NR, Bowyer JR , editors. Photoinhibition of Photosynthesis: from molecular mechanisms to field. Oxford: BioScientific Publishers, 1994. pp 1-24.

Ozkur O, Ozdemir F, Bor M, Turkan I. Physiochemical and antioxidant responses of the perennial xerophyte *Capparis ovata* Desf. to drought. Environ Exp Bot 2009; 66:487–92.

Patade VY, Bhargava S, Suprasanna P. Effects of NaCl and iso-psmotic PEG stress on growth, osmolytes accumulation and antioxidant defense in cultured sugarcane cells. Plant Cell Organ Cult 2012; 108: 279-86.

Patakas A, Nikolaou N, Zioziou E, Radoglou P, Noitsakis B. The role of organic solute and ion accumulation in osmotic adjustment in drought stressed grapevines. Plant Sci 2002; 163:361–7.

Pate JS. Patterns of nitrogen metabolism in higher plants and their ecological significance. In: Lee JA, McNeill S, Rorison IH, editors. Nitrogen as an ecological factor. Oxford: Blackwell Scientific Publishing , 1983. pp 225-55.

Reda M, Migocka M, Kłobus G. Effect of short-term salinity on the nitrate reductase activity in cucumber roots. Plant Sci 2011; 180:783–8.

Rodríguez-Gamir J, Intrigliolo DS, Primo-Millo E, Forner-Giner MA. Relationships between xylem anatomy, root hydraulic conductivity, leaf/root ratio and transpiration in citrus trees on different rootstocks. Physiol Plant 2010; 139:159–69.

Rouphael Y, Cardarelli M, Rea E, Colla G. Grafting of cucumber as a means to minimize copper toxicity. Environ Exp Bot 2008; 63:49-58.

Sánchez-Rodríguez E, Romero L, Ruiz JM. Role of grafting in resistance to water stress in tomato plants: ammonia production and assimilation. J Plant Growth Regul 2013; 1–12.

Santos-Diaz MS, Ochoa-Alejo N. Effect of water-stress on growth, osmotic potential and solute accumulation in cell-cultures from chili-pepper (a mesophyte) and creosote bush (a xerophyte). Plant Sci 1994; 96:21–9.

Scheurwater I, Koren M, Lambers H, Atkin OK. The contribution of roots and shoots to whole plant nitrate reduction in fast- and slow-growing grass species. J Exp Bot 2002; 53:1635–42.

Sharma P, Dubey RS. Modulation of nitrate reductase activity in riceseedling under aluminium toxicity and water stress: role of osmolytes as enzyme protectant. J Plant Physiol 2005; 162: 854-64.

Weng JH. The role of active and passive water uptake in maintaing leaf water status and photosynthesis in tomato under water deficit. Plant Prod Sci 2000; 3: 296-98.

Yousfi S, Serret MD, Márquez AJ, Voltas J, Araus JL. Combined use of $\delta^{13}C$, δ18O and δ15N tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit. New Phytol 2012; 194:230–44.

CHAPTER 5
Some Rootstocks Improve Pepper Tolerance To Mild Salinity Through Ionic Regulation

Consuelo Penella[1], Sergio G. Nebauer[2], Ana Quiñones[1], Salvador Lopéz-Galarza[2], Alberto SanBautista[2], Ángeles Calatayud[1]

[1]*Instituto Valenciano de Investigaciones Agrarias (IVIA). Centro de citricultura y producción vegetal. Ctra. Moncada-Naquera km. 4.5. 46113-Moncada, Valencia, Spain.*

[2]*Universitat Politècnica de València. Departamento de Producción Vegetal. Camino de Vera 14, 46020 Valencia, Spain.*

Plant Science, 230:12-22 (2015)

5.1. ABSTRACT

Grafting has been proposed as an interesting strategy that improves the responses of crops under salinity. In pepper, we reported increased fruit yield of the commercial 'Adige' cultivar under salinity when grafted onto accessions *Capsicum chinense* Jacq. 'ECU-973' (12) and *Capsicum baccatum* L. var. *pendulum* 'BOL-58' (14), whereas no effect was observed when grafted onto accession *Capsicum annuum* L var. 'Serrano' (5). We also analysed the physiological and biochemical mechanisms related to the tolerance conferred by these rootstocks. Responses to salinity (40mM NaCl) were studied in the different plant combinations for 30 days by determining water relations, mineral content, proline accumulation, photosynthetic parameters, nitrate reductase activity and antioxidant capacity. Higher salt tolerance was achieved when the 'Adige' cultivar was grafted onto the 12 genotype, which allowed not only lower Na^+ and Cl^- accumulation in the scion, but also ion selectivity maintenance, particularly Na^+/K^+ discrimination. These traits led to a minor negative impact on photosynthesis, nitrate reductase activity and lipid peroxidation in grafted scion leaves. This work suggests that using tolerant pepper rootstocks that maintain the scion's ion homeostasis is a promising strategy to provide salinity tolerance and can consequently improve crop yield.

***Key words**:* Graft; NaCl; Ions; Pepper; Photosynthesis; Yield, Water relations

5.2. INTRODUCTION

Sweet pepper is one of the most important vegetable crops in arid and semiarid regions with salinity problems, and is considered sensitive to salinity [1, 2], even though salt tolerance can vary between pepper genotypes [3]. Maas [4] reported a salinity resistance threshold of 1.5 dS m^{-1}, below which no effect on growth and a 14% decrease in biomass production for every additional 1 dS m^{-1} were observed. Thresholds ranging from 0 to 2 dS m^{-1} and slopes of salinity response curves ranging from 8% to 15% have been reported for greenhouse peppers [5, 6]. By way of example, the use of irrigation water of 4.4 dS m^{-1} [7] resulted in reductions of 46% in pepper dry biomass and of 25% in marketable pepper fruits. In pepper plants, the negative effects of salinity on yield have been mainly described as a result of increased salt in leaves, which can lead to salt toxicity and may result in reduced total photosynthesis, which modifies the carbon balance required to maintain growth [2]. The results of the salt ions responsible for such inhibition in pepper plants are controversial. Accordingly, Na^+ or Cl^- can lead to inhibition [8, 9], an increase in Na^+ accumulation in leaves can be responsible [10], or increased Cl^- may be the cause of disturbance in the plant [5].

Grafting plants onto tolerant rootstocks is one of several approaches that can cushion the impact of salinity [11] and is a common agronomic practice in tomato and melon. Several studies have been conducted in these species to elucidate the mechanisms involved in increased salinity tolerance of grafted plants. This increased tolerance of grafted plants is generally associated with their capacity to exclude or retain and/or accumulate toxic ions, Na^+ and Cl^- in rootstock roots, thus limiting their transport to leaves rather than through the synthesis of osmotically active metabolites or the induction of antioxidant systems [12-14]. Other authors have indicated that influence of rootstock on the

salt tolerance of the scion is due to a more efficient control of stomatal functions (changes in stomatal regulation and water relations), which indicate that the grafting incision may alter hormonal signalling between roots and shoots [15]. In other cases, this raised tolerance has been explained by the re-establishment of ionic homeostasis [16].

Nevertheless, the mechanism of resistance against salinity in grafted plants displays great complexity in association with specific rootstock/scion interactions [17,18], and can vary among species. As far as we know, very few studies of this type have been conducted in pepper to elucidate whether or not salt tolerance conferred by rootstocks is also due to exclusion and/or retention mechanisms, as in tomato or melon given their better capacity to alleviate the toxic effects of salts or other processes; e.g., maintenance or water relations or antioxidant capacity. Guifrida et al. [19] found that stunted growth due to salinity was attenuated in pepper-grafted plants when compared to non-grafted plants associated primarily with reduced uptake of salt ions and, therefore, with a lower concentration of these ions in the grafted plants instead of maintaining leaf turgor by osmotic adjustments.

In previous experiments we selected three pepper accessions with different degrees of salinity tolerance [20] under mild salt stress. In this study, we used these accessions as rootstocks and we identified different behaviours in response to salinity for fruit yield. In order to identify the reason for this disparity, the second step was to study the physiological responses to salinity stress involved in increased tolerance of some pepper-grafted plants and to test the hypothesis that tolerance might be related to the role of rootstocks in altering the stress perception by the scion. To fulfil these objectives, we discussed differences in pepper-grafted plants adaptation mechanisms in response to mild salt stress by comparing some physiological parameters: photosynthesis; lipid

peroxidation levels; relative water content (RWC); proline concentration; osmotic potential (Ψ_S); ions concentration; nitrate reductase activity (NR). We present evidence that grafting plants onto appropriate (tolerant) rootstocks is a good tool against salinity stress, which is mediated mainly by reducing ionic toxicity to the scion, and it improves yield.

5.3. MATERIAL AND METHODS

5.3.1. Plant material

Based on previous studies, we selected three pepper accessions (wild types) with a different salinity tolerance [20]: 'ECU-973' of *Capsicum chinense* Jacq. (code 12) as being tolerant; 'BOL-58' of *Capsicum baccatum* L. var. *pendulum* (code 14) as being moderately tolerant; and 'Serrano' of *Capsicum annuum* L. (code 5) as being less tolerant. These accessions were chosen as rootstocks and the pepper cultivar 'Adige' (Lamuyo type, Sakata Seeds, Japan) was grafted onto these three pepper accessions in this study. The pepper seeds for grafting were sown on 1 December in 100-cell polystyrene trays filled with peat-based substrate and kept in a Venlo-type glasshouse. The graft was performed on 12 February using the tube-grafting method (cutting the growing tip of the rootstock at a 45º angle below the cotyledons, attaching to the scion, previously cut at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip). The grafted combinations (cultivar/rootstock) were labelled A/5, A/12 and A/14. Ungrafted 'Adige' plants were sown 2 weeks later to obtain plants with a similar biomass to grafted plants at the time of transplantation (10-12 true leaves).

5.3.2. Soil-field experiment

One month after grafting and for 2 consecutive years, grafted and ungrafted plants were transplanted in a sweet pepper-producing area in Valencia (east Spain) with salinity problems in soil and water. Plant density was 2.1 plants m^{-2} in sandy soil (pH=8.0; EC_{es} as saturated past was 6.64 dS m^{-1}; Sand= 76%) in polyethylene greenhouses. The electrical conductivity and pH of the irrigation water were 4.5 dS m^{-1} and 7.60, respectively, with 32 meq l^{-1} of Na^+ and 41 meq l^{-1} of Cl. Fertilisers were applied at a rate of 200 UF N, 50 UF P_2O_5, 250 UF K_2O, 110 UF CaO and 35 UF MgO [20]. A randomised complete block design was used with three replicates, each consisting of 25 plants/year. Fruit was harvested from the end of May to the end of July and marketable fruits were weighed.

5.3.3. Hydroponic greenhouse experiment

One month after grafting, the root system of the plants was washed to clean the substrate and plants were placed in 5 L polyethylene pots covered with aluminium sheets. Pots were filled with a standard nutrient solution for pepper [21]. The electrical conductivity (EC) and pH of this nutrient solution was 1.7 dS m^{-1} and 6.5, respectively. Nutrient solution was added daily to compensate for uptake. After 7 days of leaving seedling plants to acclimatise to pots, salinity treatment was initiated by adding NaCl (40mM) to the nutrient solution to reach an EC of 5.2 dS m^{-1} NaCl, similarly to that used in the soil-field experiment.

Treatments were defined by two salinity levels (0 and 40mM NaCl) and four plant combinations: the cultivar 'Adige' grafted onto rootstock accessions 5, 12 and 14, and ungrafted 'Adige' plants were used as the controls. The layout was completely randomised with three replications per combination and six plants per replication.

During the culture, plants were grown in a Venlo-type greenhouse under natural light conditions (610-870 □mol m^{-2} s^{-1}), temperature ranges were 21-24ºC, and relative humidity was 52-72%.

All the physiological measurements were taken on 14 (T1) and 28 (T2) days after NaCl addition on fully expanded mature leaves (third or fourth leaf from the shoot apex).

5.3.4. Water relations

The osmotic potential of leaf sap (Ψ_s in MPa) was measured with an osmometer (Digital osmometer, Wescor, Logan, USA). Two independent determinations were made on each replicate and plant combination, obtained from six plants per treatment and combination at T1 and T2.

Leaves were tightly wrapped in aluminium foil, frozen in liquid nitrogen and stored at -80ºC. After thawing, sap was collected from syringes at 25ºC and placed in the osmometer. Osmolyte content (mmol kg^{-1}) was converted into MPa using the Van't Hoff equation [22].

Six other similar leaves from two independent plants of each plant combination, salinity treatment and replicate were collected to determine the (RWC) as (FW-

DW)/(TW-DW) x 100, where FW is fresh weight, DW is dry weight, and TW is turgid weight [22].

5.3.5. Ion analysis

The leaves and roots collected at T1 and T2 for n ≥ 5 samples of each treatment and plant combination were dried at 70ºC for 4 days. Dried samples were digested in a mixture at 70% of HNO_3-$HClO_3$ (2:1). Macronutrients (K^+, Ca^{2+}, Mg^{2+} and Na^+) were measured by ICP emission spectrometry (iCAP 6000, Thermo Scientific. Cambridge, United Kingdom).

The chloride concentration (Cl^-) in the dry plant material was extracted with 0.1N HNO_3 in 10% (v/v) acetic acid and was determined by potentiometric tritation with $AgNO_3$ in a chloride analyzer (Sherwood, MKII 926). The results were expressed as mg g^{-1} DW.

5.3.6. Proline determination

Proline content (mg g^{-1} DW) was determined as described by [23]. Leaf pepper tissue (0.05 g) was ground in 3% sulphosalicylic acid, the homogenate was filtered, and 0.75 mL of glacial acetic acid and 0.75 mL of ninhydrin reagent (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6N phosphoric acid) were added to an aliquot of the filtrate. The reaction mixture was boiled for 1 h, and readings were taken at a wavelength of 520 nm in a spectrophotometer. Three independent determinations were made in three different extracts

obtained from 18 plants per treatment and combination (one leaf per plant, and six plants per extract).

5.3.7. Photosynthetic activity and chlorophyll fluorescence

The CO_2 fixation rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and substomatal CO_2 concentration (C_i, μmol CO_2 mol^{-1} air) were measured in the steady state while maintaining plants at 1,000 μmol m^{-2} s^{-1} for 10-15 min and 400 ppm CO_2 with a LI-6400 (LI-COR, Nebraska, USA). Light curves were previously performed (data not shown) and A_N was saturated at 900 μmol m^{-2} s^{-1}. The gas exchange and fluorescence determinations were made from 9 am to 11 am (GMT). One measurement per plant was taken, and ten different plants were used (n=10) for each treatment (control and salinity stress) and plant combination.

5.3.8. Nitrate reductase activity

Nitrate reductase activity (EC 1.6.6.1) in leaves was determined *in vivo* following the methods described by [24,25]. Discs, 1 cm in diameter, were punched out of mature fresh leaves. Samples (200 mg) were suspended in a glass vial containing 10 mL of 100 mM potassium phosphate buffer (pH 7.5), 1% (v/v) *n*-propanol and 100 mM KNO_3. The glass vial was subjected 3 times to vacuum infiltration in order to induce anaerobic conditions in the incubation

medium. Plant samples were incubated in a water bath at 30ºC for 60 min in the dark and were placed in a boiling water bath for 5 min to stop the enzymatic reaction. The nitrite released from the plant material was determined colorimetrically at 540 nm (spectrophotometer PerkinElmer, Lambda 25) by adding 0.02% (w/v) N-naphthylethylenediamine and 1% sulphanilamide. A standard curve with KNO_2 was prepared to calculate the amount of NO_2 that the samples contained. Sampling and replicates were used as described for proline determination.

5.3.9. Lipid peroxidation

Lipid peroxidation in leaves was estimated through malondialdehyde (MDA) determinations using the thiobarbituric acid reaction following the protocol reported by [26]**,** and modified in [27]. The non-specific background absorbance reading at 600 nm was subtracted from the specific absorbance reading at 532 nm. The sampling and replicates used were those described for proline determination.

5.3.10. Statistical analyses

The results were subjected to a multifactor variance analysis (Statgraphics Centurion for Windows, Statistical Graphics Corp.). The effect of the genotype and salt stress level was estimated and significant interactions (genotype x stress level) were observed for some analysed parameters. Only the significance for the comparisons made among stress levels for each plant

combination is shown in the figures. The significance of the comparisons made among genotypes is indicated in the text. Mean comparisons were made using Fisher's least significance difference (LSD) test at $P < 0.05$.

The data obtained in some measurement parameters were subjected to linear regression and analyses to identify the relationships between the physiological parameters.

5.4. RESULTS

5.4.1. Fruit yield

No differences ($P<0.05$) in fruit yield were observed between the study years (data not shown), thus the average data are presented (Table 1). Accession 12, followed by 14, gave the best response in marketable fruit yield when used as rootstocks (Table 1).

Table 1. Marketable fruit yield under water and soil salinity conditions. Values are the mean of n=50 plants and standard error of the cultivar 'Adige' grafted or not onto genotypes 5, 12 and 14 for 2 years. Different letters in each column indicate significant differences at *P*<0.05 using the LSD test

Plant combination	kg plant^{-1}
Ungrafted	0.560 ± 0.014 c
A/5	0.640 ± 0.056 bc
A/12	0.960 ± 0.028 a
A/14	0.840 ± 0.169 ab

5.4.2. Water relations

Plant water relations were assessed by the determination of RWC and Ψ_s (Figs. 1 and 2). No changes in RWC were observed in the experiment in any plant combination, except for ungrafted plants (Fig. 1A, B), where RWC diminished ($P < 0.05$) after salt treatment.

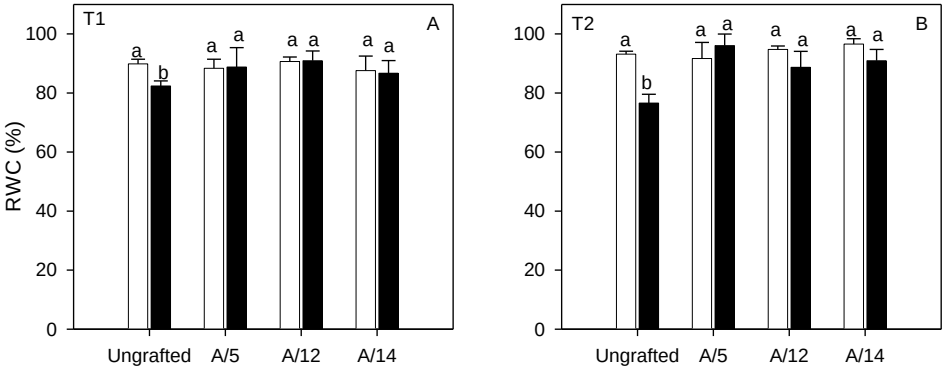


Figure 1. Effect of NaCl addition at 0 mM (□) and 40mM (■) on relative leaf water content (RWC %) for exposures of 14 days (A) and 28 days (B) in ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14. Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test)

The Ψ_s of all the plant combinations significantly reduced ($P < 0.05$) under salinity at T1 and T2 (Fig. 2). At T1, no significant interaction was found. At T2,

differences between treatments were greater in ungrafted and A/5 than in A/12 and A/14 (P< 0.05).

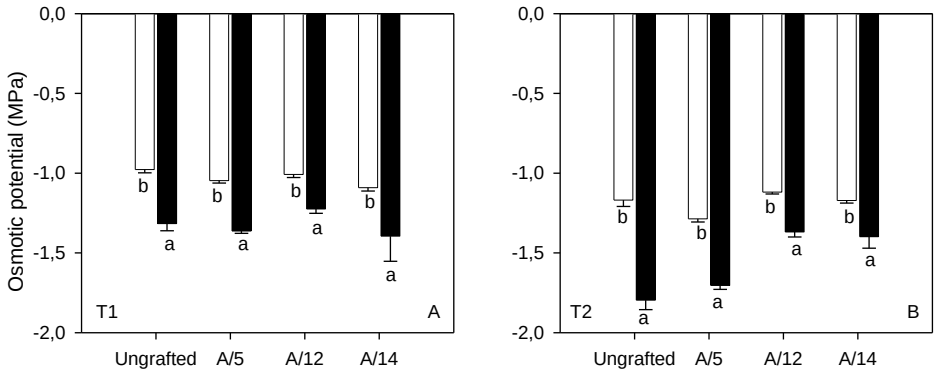


Figure 2. Leaf osmotic potential (MPa) in ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12, and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test)

5.4.3. Ion partitioning

The Na^+ concentration in leaves and roots increased under NaCl (Fig. 3A) in all the plant combinations. The Na^+ concentration in leaves was higher in ungrafted and A/5 plants (Fig. 3A) if compared with A/12 and A/14 (P<0.05) at T1 and T2 under salinity. In general terms, the Na^+ concentration in the roots under salinity was higher than in leaves (Fig. 3B), with a lower concentration found in A/12 and A/14.

Chloride content was approximately 4 times higher than Na^+ in leaves. The Cl^- concentration in leaves (Fig. 3C) increased with a higher NaCl concentration and time exposure, but this incident did not occur in roots (Fig. 3D) and in none of the plant combinations. Ungrafted and A/5 obtained the highest Cl^- levels in leaves, whereas A/12 and A/14 plants showed a greater accumulation in roots ($P<0.05$) (Fig. 3D).

In general terms, a consistent K^+ content reduction trend was observed in leaves at T1 under saline conditions in all the plant combinations (Fig. 3E). This decrease occurred at T2 only in ungrafted and A/5 plants, but not in A/12 and A/14, where no significant differences in the K^+ levels were found if compared with their controls (Fig. 3E). In roots, a marked increase in K^+ content was observed in A/12 at T1 (Fig 3F). In contrast, the K^+ concentration at T2 did not change in A/5 and A/14 under salinity (Fig. 3F).

The Na^+/K^+ ratio increased significantly depending on salt application and the exposure time in the ungrafted and A/5 leaves (Fig. 3G). The lower values ($P<0.05$) in leaves were observed for A/12 and A/14. In the root compartment (Fig. 3H) under salt treatment at T1, the Na^+/K^+ values increased in ungrafted and A/5. At T2, the Na^+/K^+ ratio in roots lowered under salt conditions if compared to the values obtained at T1 in these plant combinations due to a sharp drop in the Na^+ content in roots at T2.

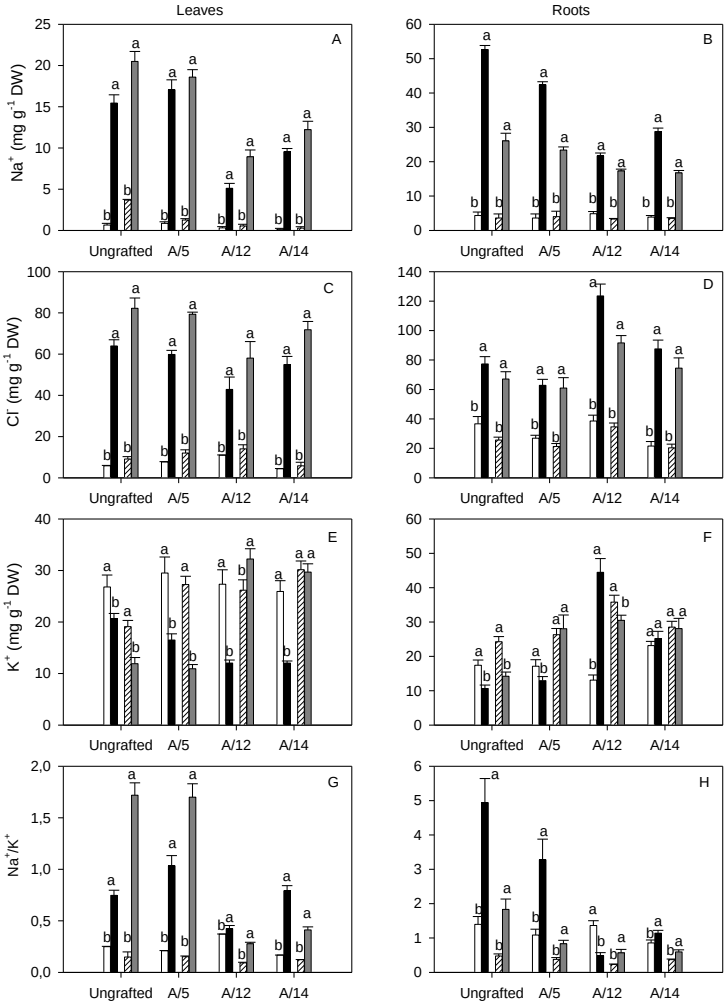


Figure 3. Concentrations of Na^+ (A, B), Cl^- (C, D), K^+ (E, F) in mg g-1 DW and the Na^+/K^+ ratio (G, H) in the leaves and roots of ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12, and 14 after addition of NaCl at 0mM and 40mM for exposures of 14 days (,) and 28 days (,), respectively. Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

The Ca^{2+} (Fig. 4A) and Mg^{2+} levels (Fig. 4C) were similar in leaves for the tandem ungrafted and A/5 plants, with reduced plant exposure to NaCl (Fig. 4). In A/12 and A/14, the Ca^{2+} and Mg^{2+} concentrations in leaves showed minor variations between the control and treated samples (Fig. 4 A, C). In roots, the Mg^{2+} levels (Fig. 4D) lowered in all the plant combinations with time, while the Ca^{2+} levels lowered in A/5 at T1 and T2, but increased in ungrafted, A/12 and A/14 at T2 (Fig. 4B).

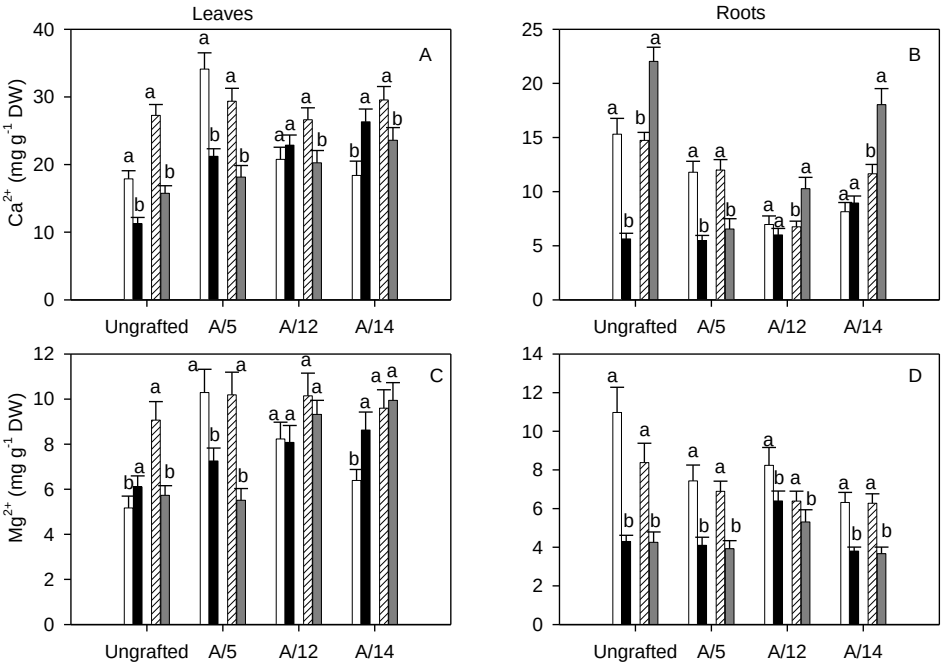


Figure 4. Ionic concentration of Ca^{2+} and Mg^{2+} in the leaves (A, C) and roots (B, D) in mg g^{-1} DW of ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12, and 14 after addition of NaCl at 0mM and 40mM for exposures of 14 days (□, ■) and 28 days (▨, ▬), respectively. Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

5.4.4. Proline content in leaves

Under the control conditions, no significant differences were found in the proline leaf content between plant combinations with time. Salinity gave rise to increased leaf proline content ($P<0.05$). This increase was similar for all the plants at T1 (Fig. 5A). At T2 (Fig. 5B) under 40mM NaCl, proline content substantially increased in ungrafted and A/5 if compared with their control values, but not in 12/cultivar and 14/cultivar ($P< 0.05$), which showed similar values to T1.

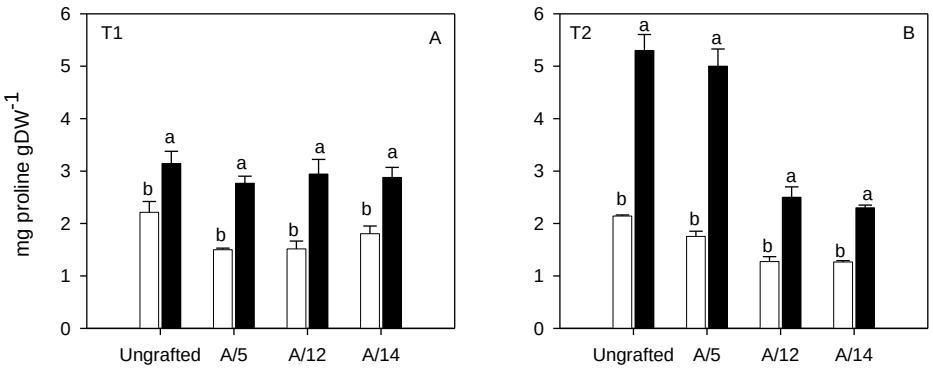


Figure 5. Changes in the proline concentration (mg proline g^{-1}DW) from ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test)

5.4.5. Gas exchange parameters

As shown in Figure 6, the A_N (Fig. 6A, B) and g_s (Fig. 6C, D) of the grafted plants did not differ from those of the ungrafted plants under the control conditions. The photosynthesis rate significantly lowered in all the plants ($P<0.05$) in response to salt stress, except 12/cultivar at T2, when the A_N values did not significantly differ from those of the control (Fig. 6B).

A decrease in g_s under salt treatment was observed in all the plants (Fig. 6C, D). Significant differences were found for the ungrafted, A/5 and A/14 plants if compared to 12/A at T1 and T2. A minor decrease, but with a significant difference compared to its control, was noted for 12/cultivar.

Instantaneous carboxylation efficiency, estimated by the A_N/C_i ratio (Fig. 6E, F), reduced in ungrafted, A/5 and A/14 at T1 and T2. Interestingly at T2, minor differences were seen in the A_N/C_i values in A/12, followed by A/14, if compared to their controls, but no significant differences were observed between them.

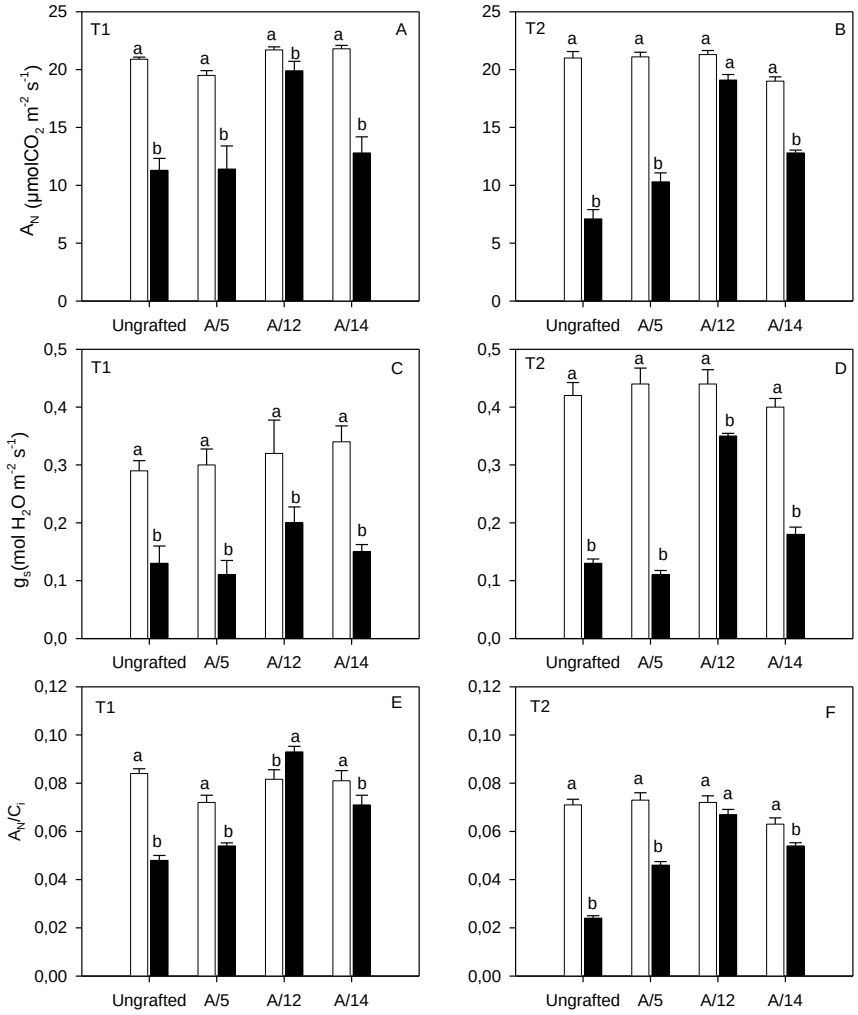


Fig 6. The Net CO_2 assimilation rate (A_N; µmol CO_2 m^{-2} s^{-1}) (A, B); leaf stomatal conductance (g_s; mol H_2O m^{-2} s^{-1}) (C, D) and instantaneous carboxylation efficiency (A_N/C_i; E, F) in ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A, C, E) and 28 days (B, D, F). Dates are mean values±SE for n=10. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test).

5.4.6. Nitrate reductase activity in leaves

Salt stress resulted in diminished NR activity in leaves after 14 (Fig. 7A) and 28 (Fig 7B) days of mild NaCl treatment. Under salinity, the greatest NR activity at T1 and T2 was seen for A/12 plants, with significant differences ($P< 0.05$) if compared to ungrafted and A/5. Nevertheless, the inhibition percentages due to salt application at T2 were not associated with the NR control values: 74% for ungrafted, 50% for 5/cultivar, 22% for 12/cultivar and 32% for 14/cultivar (Fig. 7B).

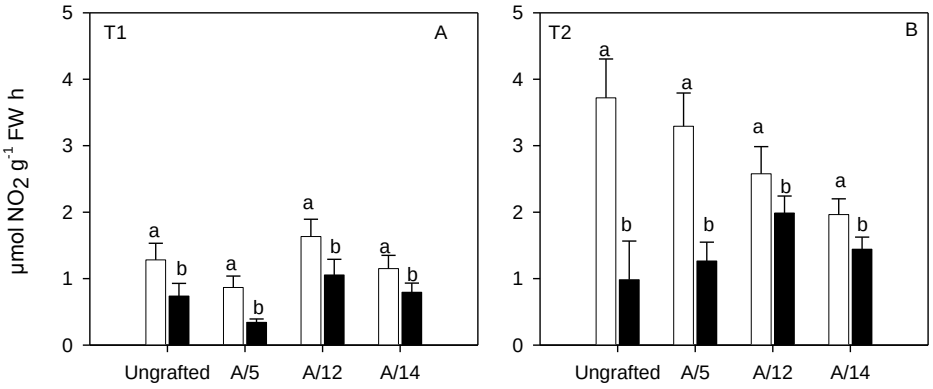


Figure 7. Nitrate reductase activity (µmol NO_2 g^{-1} FW h) in the leaves of ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test)

5.4.7. Lipid peroxidation

At T1 (Fig. 8A), MDA content increased and significant differences were observed only in the ungrafted plants. After 28 days of salt exposure, lipid peroxidation increased significantly in the ungrafted and 5/cultivar plants ($P<0.05$). It is noteworthy that no further MDA accumulation occurred in any of the plant combinations (Fig. 8B).

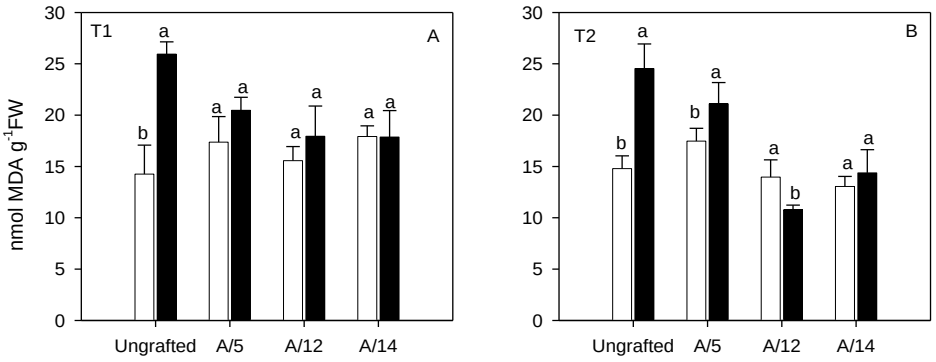


Fig 8. Leaf malondialdehyde (MDA) content (nmol MDA g^{-1}FW) in the leaves of ungrafted pepper plants (cultivar 'Adige') and the cultivar grafted onto accessions 5, 12 and 14 after addition of NaCl at 0mM (□) and 40mM (■) for exposures of 14 days (A) and 28 days (B). Dates are mean values±SE for n=6. In each plant combination, different letters indicate significant differences at $P < 0.05$ (LSD test)

5.4.6. Relationship between osmotic potential, ions and proline concentrations and photosynthesis in leaves

Regression analyses were performed with the physiological study parameters to identify the dependence relations among them. Only the significant linear

relations that help to understand the tolerance mechanisms to salinity (Na^+, Cl^- and K^+ concentration and proline level *vs.* osmotic potential and A_N, and the relations between MDA concentrations and salt ions and A_N) are shown in Table 2.

At T1 and T2, the data gave an inverse linear relationship among Ψ_S and Na^+, Cl^- and proline, but a positive correlation with the K^+ level in leaves. Proline was the parameter that obtained the steepest slope values to modify Ψ_S. A positive linear correlation among the MDA concentrations in leaves *vs.* Cl^- and Na^+ was observed with strong dependence for the last of them. The A_N level showed an inverse linear relation with MDA.

A_N at T1 correlated negatively with the Na^+, Cl^- and proline concentrations, but not significantly only for the last parameter ($P > 0.05$). The regression analysis indicated inhibition of A_N with greater dependency of Na^+ and Cl^-. Nevertheless at T2, A_N lowered, which was due mainly to an increased proline concentration. Although A_N showed a positive dependency with the K^+ levels, no significant influence was found, not even at T1 and T2.

Table 2. Linear regression and statistical analysis between mineral ions concentration (mg g-1 DW) in the leaves of the cultivar "Adige" ungrafted and grafted onto different pepper genotypes (5, 12 and 14), and proline (mg g^{-1} DW), as related to osmotic potential (Ψ_s s in MPa), CO_2 fixation rate (A_N, μmol CO_2 m^{-2} s^{-1}) and malondialdehyde (nmol MDA g^{-1} FW).

Salt treatment time	Regression equations	*P**	R^2
T1	ψ_S= -0.021[Na^+] -1.05	0.0035	0.782
	ψ_S= -0.006 [Cl^-] – 0.99	0.0003	0.898
	ψ_S= 0.02 [K^+] – 1.61	0.008	0.716
	ψ_S= -0.22 [Proline] – 0.64	0.0229	0.616
	A_N = -0.641 [Na^+] + 21.35	0.0002	0.919
	A_N = -0.1616 [Cl^-] + 22.47	0.0017	0.828
	A_N = 0.642 [K^+] + 2.48	0.1333 ns	0.701
	A_N = -7.856 [Proline] + 37.28	0.0548 ns	0.593
	MDA = 0.401[Na^+] + 15.96	0.0182	0.633
	MDA = 0.099[Cl^-] + 15.31	0.0352	0.549
	MDA = -0.716[A_N] + 28.73	0.0352	0.551
T2	ψ_S= -0.029 [Na^+] -1.13	0.0001	0.923
	ψ_S= -0.0065 [Cl^-] – 1.105	0.0031	0.792
	ψ_S= 0.021 [K^+] – 1.87	0.0514 ns	0.495
	ψ_S= -0.143 [Proline] – 1.02	0.0332	0.986
	A_N = -0.647 [Na^+] + 21.81	0.0004	0.897
	A_N = -0.144 [Cl^-] + 22.46	0.0032	0.788
	A_N = 0.537 [K^+] + 4.88	0.0794 ns	0.635
	A_N = -2.943[Proline] + 24.38	0.0018	0.910
	MDA = 0.3898[Na^+] + 13.04	0.053	0.4848
	MDA = 0.068[Cl^-] + 13.43	0.1921 ns	0.2646
	MDA = -0.619[A_N] + 26.46	0.0283	0.579

5.5. DISCUSSION

We demonstrated that the behaviour, based on fruit yield, of the 'Adige' pepper cultivar to moderate salt stress can be improved when grafted onto robust rootstocks. This result was related to the performance of some physiological parameters when ungrafted and grafted plants were compared. Nevertheless, the effect of the graft itself as a barrier to restrict the transport of toxic salt ions from roots to leaves cannot be ruled out since the cultivar was not grafted onto its own roots. The best salt acclimation was obtained when accession 12 was used as a rootstock (A/12), based on not only yield, but also on the minor negative effects caused by salt treatment on photosynthesis, NR activity and lipid peroxidation. Furthermore, some favourable physiological characteristics for salt acclimation, such as higher K^+ Ca^{2+} and Mg^{2+} levels in leaves and a lower Na^+/K^+ ratio, were seen in this plant combination. The latter parameter has been demonstrated as a good indicator of salt tolerance [28].

Salt tolerance in plants is usually associated with the ability to restrict the uptake and/or transport of saline ions from roots to leaves and their compartmentalisation [29]. In this study, more Cl^- was withheld in the roots of rootstocks in A/12 and A/14, and less Cl^- was transported to their leaves if compared with the ungrafted and A/5 plants under NaCl stress ($P<0.05$). This suggests either maximised Cl^- retrieval to the rootstock or a retention mechanism in the roots of these plant combinations. Unlike Cl^-, rootstocks 12 and 14 showed a reduced Na^+ net uptake, consequently their leaves gave a lower Na^+ concentration value if compared with the others ($P<0.05$). Two mechanisms can explain the lower Na^+ concentration in roots: firstly, as suggested by Aktas et al. [3], in salt-tolerant pepper genotypes, a plasma membrane Na^+/H^+ antiporter protein is activated in root cells upon NaCl

exposure to extrude Na+ from roots into the growth medium. This mechanism has been reported in different grafted plants, such as melon [30–32], tomato [29,33,34], watermelon [35] and cucumber [36]. Alternatively, the root system of rootstocks 12 and 14 might be able to control Na^+ influx and to allow minor entry from medium to roots, as reported for pumpkin roots [14].

Regarding concentration; leaf Cl^- accumulation exceeded that of Na^+ in all the plant combinations. This is in accordance with the results obtained by Navarro et al. [37] and Chartzoulakis and Klapaki [5] in the 'Orlando' variety and the 'Sonar' pepper variety, respectively. The higher Cl^- concentration, if compared to Na^+ (mainly in roots), can be linked to a higher passive uptake root component and a very feebly active Cl^- uptake system [38]. However, it is unknown whether some rootstocks are capable of regulating the transport of Na^+ or/and Cl^- to leaves [39]. Based on our results, the capacity to regulate Na^+ and Cl^- uptake and transport could be linked to the ability of vigour rootstocks (comparing A/5 *vs.* A/12 and A/14), which indicates that the involved physiological and biochemical mechanisms operate at the rootstock level, as observed in grafted melon plants [13] or cucumber plants [14]. Nevertheless, the lower foliar Na^+ and Cl^- content observed in the tolerant grafted plants (A/12 and A/14) can also be associated with the stimulated growth and development of the shoot, which led to the dilution of toxic ions.

Regulation of ion homeostasis and selectivity, particularly Na^+/K^+ discrimination, is closely linked to the lower Na^+ concentration and its relation to salt tolerance [40]. Given the similar physico-chemical structure between Na^+ and K^+, a high Na^+ concentration in the external solution can lower the K^+ level in the tissues of many plants species [41]. In our study, the Na^+/K^+ ratio in leaves of the ungrafted and A/5 pepper plants under salinity was significantly higher ($P< 0.05$) than those of the plants grafted onto rootstocks 12 and 14, and the latter is able

to select, use and transport K^+ to leaves, as in many vegetable-grafted plants exhibiting salinity tolerance; e.g., tomato [12], melon [42] or cucumber [17,36]. However, the direct relation between K^+ homeostasis and salinity tolerance has not been well-established [11]. In some species, Na^+ can be balanced by a higher K^+ concentration [43], while in other plants, tolerance is due to the capacity of roots to maintain K^+ transport in the xylem, as in tomato-grafted plants [44]. Accordingly, a different behaviour for K^+ accumulation in leaves was observed in A/12 and A/14 under the salt conditions. The significant increase in the K^+ concentration in leaves from T1 to T2 could be related with long-term developed tolerance to salt mediated through the major K^+ transport in these grafted plants.

Despite the negative effect on plant growth derived from its toxic effect, accumulation of ions under salinity can help maintain the turgor pressure of plants [37,45]. In addition, different osmolytes can be involved in the reduction of ψ_S, including organic compounds such as sugars, free amino acids, glycinebetaine, soluble proteins, proline and organic acids [46–48], and/or macronutrients such as inorganic components [49]. According to our results, a strong negative correlation between the reduction in leaf ψ_S and salt ions content for all the plant combinations was observed in the experiment. The linear regressions equations showed that Na^+ and Cl^- display a different response on ψ_S. The lower osmotic potential seems to be achieved mainly by Na^+ and, to a lesser extent by Cl^-. This can be explained by a more marked change in Na^+ accumulation if compared to Cl^- between the ungrafted and A/5 *vs.* A/12 and A/14 plants, rather than by the absolute concentration of both ions. The reduced osmotic potential assigned to Na^+ was consistent with pepper plants [50], and salt-tolerant species such as *Centarurea ragusina* [51], *Atriplex nummularia* [52] or *Aster tripolium* [53]. The contribution of K^+, Mg^{2+} and Ca^{2+} to

ψ_s under the salinity conditions in our study was more relevant in the A/12 and A/14 plants at T2, where K^+, Mg^{2+} and Ca^{2+} represented 30-35% of the total ions if compared to 15% in the ungrafted and A/5 plants.

The adjustment of the osmotic potential through inorganic ion uptake supposes a much lower energy cost than that conferred by the organic molecules synthesised in the cell [54]. However in order to reduce ψ_s, ungrafted and A/5 plants required proline synthesis to produce sufficient osmotics under salt stress conditions. As a result, the reduction in ψ_s strongly related to proline accumulation in these plant combinations (r^2= 0.95), but more weakly so in A/12 and A/15 (r^2 = 0. 36). Although high proline levels or other compatible solutes may protect plants by scavenging the oxygen-free radicals caused by salt stress [11,36.55,56], the amounts observed in the ungrafted and A/5 plants were related with the greater salt sensitivity of these genotypes, as reported for other species such as wheat [57], barley [58], *Centaurea ragusina* [51] or rice [59].

Plants respond to lower water availability under salinity by reducing their leaf transpiration, stomatal conductance, and by adjusting their root water uptake [60,61]. Under prolonged periods of exposure to salt, root conductivity can be partially recovered, mainly through the accumulation of compatible solutes and/or ions in roots. These responses should be involved in the maintenance of the relative water content in the leaves of grafted pepper genotypes in the experiment. Despite the reduction of the leaf osmotic potential and stomatal conductance described in the ungrafted plants, no root conductivity recovery should occur in the experiment since RWC was significantly lower under salinity. According to this relation, a reduction in either the functionality or the amount of aquaporins has been reported to occur in pepper plants under salinity [37,62].

In this experiment, the Na^+ and Cl^- concentrations did not provoke salt toxicity symptoms in our pepper plants, and only minor leaf chlorosis was noted and small necrotic areas were observed mainly in ungrafted plants. These results agree with the higher lipid peroxidation levels reported in ungrafted plants when compared with grafted plants. Lower MDA concentrations were found in A/12 plants, followed by A/14. Nevertheless, gas exchange parameters were affected after a 2-week salt exposure and extended to T2. Excessive Na^+ and Cl^- accumulation is harmful and may disrupt the integrity of the photosynthetic apparatus [31]. In line with this, a positive dependence between MDA and the salt ion concentration and a negative relation with the photosynthesis values were established. Reduced photosynthetic capacity can be related to higher leaf Na^+ or Cl^- concentrations [29,35,63,64]. In our experiments, the highly significant correlation found between A_N and Na^+ and Cl^- foliar concentrations suggested that both ions can be involved in reduced photosynthesis, although the regression analyses indicated a predominant inhibition effect by Na+. This effect can be linked to the concentration level in leaves and/or a major toxic power to promote inhibition. In contrast to the reductions observed in the other plant combinations, maintenance of A_N in the A/12 plants can be attributed, at least in part, to increased K^+ levels or to other beneficial macronutrients, such as Ca^{2+} and Mg^{2+}, which contribute to better regulate stomata regulation under salinity [41]. Notwithstanding, g_s significantly lowered under mild salt stress in all the plant combinations and for the time exposures, which corroborates a previous finding that g_s are very sensitive to salt [55,65]. In addition, the diminished instantaneous carboxylation efficiency (A_N/C_i) noted at T1 and T2 in the ungrafted, A/5 and A/14 plants suggests that salt stress affects photosynthesis by metabolic limitations, probably in association with reduced Rubisco carboxylase activity [66]. In contrast, stomatal limitations to

photosynthesis should occur in A/12 at T1 and T2 since no changes in A_N/Ci were observed under salinity [67].

There is evidence that photosynthesis regulates nitrate reduction by modulating NR activity [68,69], which agrees with the results presented herein, which indicate that salt application diminishes A_N and NR activity. The most tolerant rootstock (A/12) in A_N terms exhibited lower NR inhibition if compared with the others. A drop in NR by salt can be due to: reduced nitrate transport to leaves, mainly because of nitrate/chloride competition [70]; inactivation of NO_3^- transporters by toxic effects of salt ions [71]; the disruption of root membrane integrity [62]; diminished NO_3^- transport from roots to leaves due to a lower transpiration flow [22] and, consequently, low NO_3^- loading into the root xylem, which affects NR activity [72]. Accordingly, and in accordance with the results obtained, the more marked decrease noted in NR activity (ungrafted and A/5 plants) in leaves can be associated with higher Cl^- and Na^+ accumulations and/or lower carbon fixation rates.

In conclusion, the greater salt tolerance of grafted plants shown in yield, mainly the A/12 (and A/14) combinations, can be attributed to their ability to restrict Cl^- transport to leaves and to diminished Na^+ loading in roots and leaves, thus favouring K^+ (Ca^{2+} and Mg^{2+}) uptake and allowing a smaller osmotic potential with a lower energy cost. These traits led to a minor inhibitory effect on photosynthesis and NR activity, which favourably affected fruit yield when compared with the A/5 and ungrafted plants. Nonetheless, although ionic and water homeostasis are crucial parameters in salt tolerance, the maintenance of shoot vigour and leaf function are vitally important, as described in other species, such as tomato [44, 73] or melon [30]. The relative contribution of both groups of processes to induced tolerance to salinity in grafted pepper plants needs to be further assessed. An effect of the grafting procedure *per se* cannot

be simply ruled out, and self-grafted plants should also be assayed under salinity conditions. Knowledge of the physiological and biochemical processes that promote salt stress tolerance can improve our understanding of not only the mechanisms involved in scion and rootstock interactions, but also of the selection of robust rootstocks to be used under field salinity conditions.

5.6. REFERENCES

[1] A. Kurunc, A. Unlukara, B. Cemek, Salinity and drought affect yield response of bell pepper similarly, Acta Agric. Scand. Sect. B — Soil Plant Sci. 61 (2011) 514–522.

[2] M.C. Piñero, F. Houdusse, J.M. Garcia-Mina, M. Garnica, F.M. del Amor, Regulation of hormonal responses of sweet pepper as affected by salinity and elevated CO2 concentration, Physiol. Plant. 151 (2014) 375-389.

[3] H. Aktas, K. Abak, I. Cakmak, Genotypic variation in the response of pepper to salinity, Sci. Hortic. 110 (2006) 260–266.

[4] E.V. Maas, Crop salt tolerance, KK Tanji (ed). Agricultural salinity assessment and management. ASCE manuals and reports on engineering practices no 71. Amer. Soc. Civil Eng. New York.

[5] K. Chartzoulakis, G. Klapaki, Response of two greenhouse pepper hybrids to NaCl salinity during different growth stages, Sci. Hortic. 86 (2000) 247–260.

[6] J.M. Navarro, C. Garrido, M. Carvajal, V. Martinez, Yield and fruit quality of pepper plants under sulphate and chlorode salinity, J. Hortic. Sci. Biotech. 77 (2002) 52-57.

[7] S. De Pascale, C. Ruggiero, G. Barbieri. Physiological responses of pepper to salinity and drought, J. Amer. Soc. Hort. Sci. 128 (2003) 48-54.

[8] P.C. Bethke, M.C. Drew, Stomatal and nonstomatal compoments to inhibition of photosynthesis in leaves of *Capsicum annuum* during progressive exposure ot NaCl salinity, Plant Physiol. 99 (1992) 219-226.

[9] A. Korkmaz, R. Sirikçi, F. Kocaçinar, Ö. Deger, A.R. Demirkirian. Alleviation of salt-induced adverse effects in pepper seedling by seed application of gycinebetaine, Sci. Hortic. 148 (2012) 197-205.

[10] R. Azuma, N. Ito, N. Nakayama, R. Suwa, N.T. Nguyen, J.A. Larrinaga-Mayoral, M. Esaka, H. Fujiyama, H. Saneoka, Fruits are more sensitive to salinity than leaves and stems in pepper plants (Capsicum annuum L.), Sci. Hortic 125 (2010) 171-178.

[11] G. Colla, Y. Rouphael, C. Leonardi, Z. Bie, Role of grafting in vegetable crops grown under saline conditions, Sci. Hortic. 127 (2010) 147–155.

[12] M.T. Estañ, M.M. Martinez-Rodriguez, F. Perez-Alfocea, T.J. Flowers, M.C. Bolarin, Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot., J. Exp. Bot. 56 (2005) 703–712.

[13] M. Edelstein, Z. Plaut, M. Ben-Hur, Sodium and chloride exclusion and retention by non-grafted and grafted melon and Cucurbita plants, J. Exp. Bot. 62 (2011) 177–184.

[14] Y. Huang, Z. Bie, P. Liu, M. Niu, A. Zhen, Z. Liu, et al., Reciprocal grafting between cucumber and pumpkin demonstrates the roles of the rootstock in the determination of cucumber salt tolerance and sodium accumulation, Sci. Hortic. 149 (2013) 47–54.

[15] B. Aloni, R. Cohen, L. Karni, H. Aktas, M. Edelstein, Hormonal signaling in rootstock-scion interactions, Sci. Hortic. 127 (2010) 119-126.

[16] M.M. Martinez-Rodriguez, M.T. Estañ, E. Moyano, J.O. Garcia-Abellan, F.B. Flores, J.F. Campos, et al., The effectiveness of grafting to improve salt tolerance in tomato when an “excluder” genotype is used as scion, Environ. Exp. Bot. 63 (2008) 392–401.

[17] J. Zhu, Z. Bie, Y. Huang, X. Han, Effect of grafting on the growth and ion concentrations of cucumber seedlings under NaCl stress, Soil Sci. Plant Nutr. 54 (2008) 895–902.

[18] S.L. Ferreira-Silva, E.N. Silva, F.E.L. Carvalho, C.S. de Lima, F.A.L. Alves, J.A.G. Silveira, Physiological alterations modulated by rootstock and

scion combination in cashew under salinity, Sci. Hortic. 127 (2010) 39–45.

[19] F. Giuffrida, C. Cassaniti, C. Leonardi, The influence of rootstock on growth and ion concentrations in pepper (*Capsicum annuum* L.) under saline conditions, J. Hortic. Sci. Biotechnol. 88 (2013) 110–116.

[20] C. Penella, S.G. Nebauer, S. Lopéz-galarza, A. Sanbautista, E. Gorbe, Evaluation for salt stress tolerance of pepper genotypes to be used as rootstocks, J. Food, Agric. Environ. 11 (2013) 1101–1107.

[21] C. Sonneveld, N. Straver, R. Donnan, Nutrient solutions for vegetables and flowers grown in water or substrates, Naaldiwijk, The Netherlands: Proefstation voor tuinbouw onder glas te Naaldiwjk. 10° ed, 1994.

[22] C. Penella, S.G. Nebauer, A.S. Bautista, S. López-Galarza, Á. Calatayud, Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: Physiological responses, J. Plant Physiol. 171 (2014) 842–851.

[23] L.S. Bates, R.P. Waldren, I.D. Teare, Rapid determination of free proline for water-stress studies, Plant Soil. 39 (1973) 205–207.

[24] R.H. Hageman, D.P. Hucklesby, Nitrate reductase from higher plants, Methods Enzymol. 23 (1971) 491–503.

[25] E.G. Jaworski, Nitrate reductase assay in intact plant tissues., Biochem. Biophys. Res. Commun. 43 (1971) 1274–1279.

[26] R.L. Heath, L. Packer, Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation, Arch. Biochem. Biophys. 125 (1968) 189–198.

[27] R.S. Dhindsa, P. Plumb-Dhindsa, T.A. Thorpe, Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase, J. Exp. Bot. 32 (1981) 93–101.

[28] R. Munns, M. Tester, Mechanisms of salinity tolerance, Annu. Rev. Plant Biol. 59 (2008) 651–681.

[29] N. Fernández-García, V. Martínez, M. Carvajal, Effect of salinity on growth, mineral composition, and water relations of grafted tomato plants, J. Plant Nutr. Soil Sci. 167 (2004) 616–622.

[30] L. Romero, A. Belakbir, L. Ragala, J.M. Ruiz, Response of plant yield and leaf pigments to saline conditions: effectiveness of different rootstocks in melon plant (*Cucumis melo* L.), Soil Sci. Plant Nutr. 43 (1997) 855–862.

[31] Y. Rouphael, M. Cardarelli, E. Rea, G. Colla, Improving melon and cucumber photosynthetic activity, mineral composition, and growth performance under salinity stress by grafting onto Cucurbita hybrid rootstocks, Photosynthetica. 50 (2012) 180–188.

[32] M. Edelstein, M. Ben-Hur, R. Cohen, Y. Burger, I. Ravina, Boron and salinity effects on grafted and non-grafted melon plants, Plant Soil. 269 (2005) 273–284.

[33] G. Chen, X. Fu, S. Herman Lips, M. Sagi, Control of plant growth resides in the shoot, and not in the root, in reciprocal grafts of flacca and wild-type tomato (*Lysopersicon esculentum*), in the presence and absence of salinity stress, Plant Soil. 256 (2003) 205–215.

[34] D. Savvas, A. Savva, G. Ntatsi, A. Ropokis, I. Karapanos, A. Krumbein, et al., Effects of three commercial rootstocks on mineral nutrition, fruit yield, and quality of salinized tomato, J. Plant Nutr. Soil Sci. 174 (2011) 154–162.

[35] G. Colla, Y. Roupahel, Effect of salinity on yield, fruit quality, leaf gas exchange, and mineral composition of grafted watermelon plants, HortScience. 41 (2006) 622–627.

[36] Y. Huang, Z. Bie, S. He, B. Hua, A. Zhen, Z. Liu, Improving cucumber tolerance to major nutrients induced salinity by grafting onto Cucurbita ficifolia, Environ. Exp. Bot. 69 (2010) 32–38.

[37] J.M. Navarro, C. Garrido, V. Martínez, M. Carvajal, Water relations and xylem transport of nutrients in pepper plants grown under two

[38] A. Altman, K. MendeL, Characteristics of the uptake mechanism of chloride ions in excised roots of a woody plant (Citrus), Physiol. Plant. 29 (1973) 157–162.

[39] G. Colla, C.M.C. Suarez, M. Cardarelli, Y. Rouphael, Improving Nitrogen Use Efficiency in Melon by Grafting, Hortscience. 45 (2010) 559–565.

[40] K.M. Volkmar, Y. Hu, S. H., Physiological responses of plants to salinity: a review, Can. J. Plant Sci. 78 (1997) 19–27.

[41] Y. Hu, U. Schmidhalter, Drought and salinity: A comparison of their effects on mineral nutrition of plants, J. Plant Nutr. Soil Sci. 168 (2005) 541–549.

[42] F. Orsini, R. Sanoubar, G.B. Oztekin, N. Kappel, M. Tepecik, C. Quacquarelli, et al., Improved stomatal regulation and ion partitioning boosts salt tolerance in grafted melon, Funct. Plant Biol. 40 (2013) 628–636.

[43] M.A. Hajibagheri, A.R. Yeo, T.J. Flowers, J.C. Collins, Salinity resistance in *Zea mays*: fluxes of potassium, sodium and chloride, cytoplasmic concentrations and microsomal membrane lipids, Plant, Cell Environ. 12 (1989) 753–757.

[44] A. Albacete, C. Martínez-Andújar, M.E. Ghanem, M. Acosta, J. Sánchez-Bravo, M.J. Asins, et al., Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescence, and increased leaf area and crop productivity in salinized tomato., Plant. Cell Environ. 32 (2009) 928–38.

[45] A. Blum, R. Munns, J.B. Passioura, N.C. Turner, R.E. Sharp, J.S. Boyer, et al., Genetically engineered plants resistant to soil drying and salt stress: How to interpret osmotic relations?, Plant Physiol. 110 (1996) 1051–1053.

[46] R. Munns, C. Brady, E. Barlow, Solute accumulation in the apex and leaves of wheat during water stress, Aust. J. Plant Physiol. 6 (1979) 379-389.

[47] J. Morgan, Osmotic components and properties associated with genotypic differences in osmoregulation in wheat, Aust. J. Plant Physiol. 19 (1992) 67-76.

[48] S.A. Nio, G.R. Cawthray, L.J. Wade, T.D. Colmer, Pattern of solutes accumulated during leaf osmotic adjustment as related to duration of water deficit for wheat at the reproductive stage., Plant Physiol. Biochem. 49 (2011) 1126–1137.

[49] A. Patakas, N. Nikolaou, E. Zioziou, K. Radoglou, B. Noitsakis, The role of organic solute and ion accumulation in osmotic adjustment in drought-stressed grapevines, Plant Sci. 163 (2002) 361–367.

[50] M.C. Martínez-Ballesta, V. Martínez, M. Carvajal, Aquaporin functionality in relation to H^+-ATPase activity in root cells of *Capsicum annuum* grown under salinity, Physiol. Plant. 117 (2003) 413–420.

[51] S. Radić, P. Peharec Štefanić, H. Lepeduš, V. Roje, B. Pevalek-Kozlina, Salt tolerance of *Centaurea ragusina* L. is associated with efficient osmotic adjustment and increased antioxidative capacity, Environ. Exp. Bot. 87 (2013) 39–48.

[52] J.A.G. Silveira, S.A.M. Araújoa, J.P.M.S. Lima, R.A. Viégas, Roots and leaves display contrasting osmotic adjustment mechanisms in response to NaCl-salinity in *Atriplex nummularia*, Environ. Exp. Bot. 66 (2009) 1–8.

[53] A. Ueda, M. Kanechi, Y. Uno, N. Inagaki, Photosynthetic limitations of a halophyte sea aster (*Aster tripolium* L) under water stress and NaCl stress, J. Plant Res. 116 (2003) 65–70.

[54] R. Munns, Comparative physiology of salt and water stress, Plant Cell Environ. 25 (2002) 239-250.

[55] Y. He, Z. Zhu, J. Yang, X. Ni, B. Zhu, Grafting increases the salt tolerance of tomato by improvement of photosynthesis and enhancement of antioxidant enzymes activity, Environ. Exp. Bot. 66 (2009) 270–278.

[56] J.M. Ruiz, J.J. Ríos, M.A. Rosales, R.M. Rivero, L. Romero, Grafting between tobacco plants to enhance salinity tolerance, J. Plant Physiol. 163 (2006) 1229–1237.

[57] T.D. Colmer, R. Munns, T.J. Flowers, Improving salt tolerance of wheat and barley: future prospects, Aust. J. Exp. Agric. 45 (2005) 1425–1443.

[58] Z. Chen, T.A. Cuin, M. Zhou, A. Twomey, B.P. Naidu, S. Shabala, Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance, J. Exp. Bot. 58 (2007) 4245–4255.

[59] S. Lutts, G. Guerrier, Peroxidase activities of two rice cultivars differing in salinity tolerance as affected by proline and NaCl, Biol. Plant. 37 (1995) 577–586.

[60] A. Poljakoff-Mayber, Morphological and anatomical changes in plants as a response to salinity stress, A Poljakoff-Mayber and J Gale (eds). Plants in saline environments. Ecological Series 15, Springer-Verlag, Berlin, Germany, 1975.

[61] M. Calvo-Polanco, B. Sánchez-Romera, R. Aroca, Mild salt stress conditions induce different responses in root hydraulic conductivity of Phaseolus vulgaris over-time, PLoS One. 9 (2014). 3e90631.

[62] M. Carvajal, V. Martínez, C.F. Alcaraz, Physiological function of water channels as affected by salinity in roots of paprika pepper, Physiol. Plant. 105 (1999) 95–101.

[63] R.R, Walker, D.H. Blackmore, Q. Sung, Carbon dioxide assimilation and foliar ion concentration in leaves of lemon (*Citrus limon* L.) trees irrigated with NaCl or Na_2SO_4. Aust. J. Plant Physiol. 20 (1993) 173-175.

[64] M.C. Martínez-Ballesta, V. Martínez, M. Carvajal, Osmotic adjustment, water relations and gas exchange in pepper plants grown under NaCl or KCl, Environ. Exp. Bot. 52 (2004) 161–174.

[65] Q. Jiang, D. Roche, T.A. Monaco, D. Hole, Stomatal conductance is a key parameter to assess limitations to photosynthesis and growth potential in barley genotypes, Plant Biol. 8 (2006) 515–521.

[66] E.N. da Silva, R.V. Ribeiro, S.L. Ferreira-Silva, R.A. Viégas RA, J.A.G. Silveira, Salt stress induced damages on the photosynthesis of physic nut young plants, Sci. Agric. 68 (2011) 62-68.

[67] J. Flexas, J. Bota, F. Loreto, G. Cornic, T.D. Sharkey, Diffusive and metabolic limitations to photosynthesis under drought and salinity in C) plants, Plant Biol. 6 (2004) 269–79.

[68] W.M. Kaiser, E. Brendle-Behnisch, Rapid Modulation of Spinach Leaf Nitrate Reductase Activity by Photosynthesis: I. Modulation *in vivo* by CO_2 Availability, Plant Physiol. 96 (1991) 363–367.

[69] S. Yousfi, M.D. Serret, A.J. Márquez, J. Voltas, J.L. Araus, Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit, New Phytol. 194 (2012) 230–44.

[70] G.K. Abd-El-Baki, F. Siefritz, H.M. Man, H. Weiner, R. Kaldenhoff, W.M. Kaiser, Nitrate reductase in *Zea mays* L. under salinity, Plant, Cell Environ. 23 (2000) 515–521.

[71] H. Lin, S.S. Sandra, K.S. Schumaker, Salt sensitivity and the activities of H-ATPase in cotton seedlings, Crop Sci. 37 (1997) 190-197.

[72] M. Debouba, H. Maâroufi-Dghimi, A. Suzuki, M.H. Ghorbel, H. Gouia, Changes in growth and activity of enzymes involved in nitrate reduction and ammonium assimilation in tomato seedlings in response to NaCl stress, Ann. Bot. 99 (2007) 1143–1151.

[73] M.E. Balibrea, J. Dell'Amico, M.C. Bolarín, F. Pérez-Alfocea. Carbon partitioning and sucrose metabolism in tomatoplants growing under salinity. Physiol. Plantarum 110 (2000) 503-511.

CHAPTER 6
Can a robust rootstock of pepper improve a scion's salt tolerance?

6.1. ABSTRACT

The performance of a wild-type salt-tolerant pepper (Capsicum annuum L.) accession (A25) as a rootstock was assessed. A greenhouse experiment was conducted to determine growth, mineral partitioning, gas exchange and chlorophyll a fluorescence parameters, antioxidant systems and proline content in a commercial pepper cultivar Adige when grafted onto A25 plants (A/A25) and ungrafted plants (A) under salinity conditions (80 mM NaCl) for 14 days. Salt stress induced significantly stunted growth of A plants (-40.6% of leaf DW) compared to the control conditions, while no alterations were observed in A/A25 at the end of the experiment. Accumulation of Na^+ and Cl^- in leaves and roots was similar in either grafted or ungrafted plants. Conversely, SOD, CAT and APX activity and the non-photochemical quenching coefficient significantly increased under high salinity in A plants. Despite the activation of protective mechanisms, A plants showed severely reduced photosynthetic CO2 assimilation (-45.6% of AN390) and substantial buildup of MDA by-product, which suggests they are unable to counteract salt-triggered damage. In contrast, A/A25 plants did not show alterations in photosynthesis under salinity and MDA levels only increased slightly. Our results underline that, as grafted plants, A/A25 showed higher salt tolerance, likely due to ion compartmentalization and proline accumulation. Under 80 mM of NaCl, the A/A25 plants grown in the field also yielded a larger amount of marketable fruit (+75%) and showed lower Blossom end Root incidence (-31%).

Keywords: Capsicum annuum; Grafting; NaCl stress; Growth; Photosynthesis; Antioxidant system

6.2. INTRODUCTION

One of the most important challenges in agriculture is knowledge of crop responses under global change conditions since the induced alterations in agricultural ecosystems will affect plant metabolism and productivity. These effects on plants will differ for each region depending on current climatic conditions. In the Mediterranean region, predictive models of climate change indicate a combination of reduced rainfall and increased temperature that will further impose higher evapotranspiration loss and increased water stress problems for many crop species (Chartzoulakis and Psarras, 2005; Majumder, 2015). The reduced availability of high-quality irrigation water will also increase the use of saline water, thus further aggravating already existing water and soil salinity problems (Jensen et al., 2014). The term “salinity” implies high concentration of salts in soil and/or water, and NaCl constitutes the predominant part of this salinity (Türkan and Demiral, 2009). Nowadays, about 7% of the world`s land area and 20% of irrigated land are affected by salinity (Ferreira-Silva et al., 2010). Salinity affects plant performance through the development of osmotic stress and disruption of ion homeostasis (Munns and Tester, 2008; Shabala and Munns, 2002; Penella et al., 2015). In general terms, effects of salinity on plants are the result of both water stress (due to a higher osmotic potential in soil as compared to plant tissues) and a toxic effect caused by the influx of Na^+ and Cl^- ions into plant tissues (Tuteja, 2007; Munns and Tester, 2008). The final result of these effects is a wide range of physiological, metabolic and genomic changes that provoke alterations in photosynthesis, carbohydrate partition, respiration, increased reactive oxygen species (ROS) production, and an unbalanced uptake of other nutrients (Parida and Das, 2005, Hu and Schmidhalter, 2005; Chaves et al., 2009). Overall, the physiological changes induced by salinity correspond to diminished plant growth and yields.

In spite of these deleterious effects, plants present different degrees of tolerance to salinity, conferred by biochemical pathways, which can alleviate the negative effect of salt toxicity; amongst them: (I) retention and acquisition of water mediated by the biosynthesis of osmotically-active metabolites (mainly proline, glycine-betaine or sugars) (Singh et al., 2014); (II) maintenance of ion homeostasis, which minimizes the perturbation of toxic effect of Na^+ and Cl^- into plant tissue and/or favors compartmentalization in vacuoles (Rivero et al., 2014; Razzaghi et al., 2015); (III) induction of antioxidant systems (Ashraf et al., 2012; Hu et al., 2012; Wang et al., 2012; Fini et al., 2014); (IV) over production of hormones, mainly abscisic acid (Krasenski and Jonak, 2012; Yoshida et al., 2014); (V) synthesis of specific stress-associated molecules such as heat-shock proteins (Wang et al., 2004; Krasenski and Jonak, 2012; Perez-Salamò et al., 2014); or (VI) late embryogenesis abundant proteins (Parida and Das, 2005, Radíc et al., 2013). In summary, a plant's response to salinity results from many different morpho-anatomical, biochemical and physiological adaptations, which has led to long lists of plants being established: from sensitive, moderately-tolerant to tolerant species (Ashraf and Wu, 1994; Shannon and Grieve, 1998; Munns, 2002, Grieve et al., 2012). Therefore, salt tolerance is dependent not only on plant species, but sometimes different genotypes that belong to the same species can also have a different degree of salt tolerance (Shabala and Munns, 2012). Modifications of salt-sensitive crops, based mostly on the manipulation of genes that protect and maintain the function and structure of cellular components under salinity stress represent an important goal in the last few decades (Golldack et al., 2011; Peleg et al., 2011). However, the nature of the genetically-complex mechanisms of salinity stress tolerance and lack of public acceptance of genetic transformation mean that other approaches have to be considered in an attempt to obtain salt-tolerant genotypes.

A promising perspective to improve resistance to salinity, recently utilized also for herbaceous species, is the use of grafting of commercial cultivars onto robust rootstocks. Pepper is one of the most important crops in Spain which is usually classified as a salt-sensitive species (Kurunc et al. 2011; del Amor and Cuadra-Crespo, 2011), even though Aktas et al. (2006) observed that salt tolerance can vary amongst pepper genotypes. Given the poor genetic basis of cultivated pepper accessions, the screening of wild pepper species has been performed in previous works to assess naturally-occurring genetic variation to salinity in order to select salt-tolerant rootstocks (Penella et al., 2014). Tomato and melon are the commonest species in which the grafting practice has been efficiently applied to obtain salt-tolerant morphs (Estañ et al., 2005, Edelstein et al., 2011, Orsini et al., 2013). It has been demonstrated that tolerance in a grafted plant corresponds to the capacity for exclusion and/or retention of toxic ions Na^+ and Cl^- in rootstock roots, which limits their transport to leaves rather than the synthesis of osmotically active metabolites (Estañ et al., 2005, Edelstein et al., 2011, Orsini et al., 2013).

In a previous work, a wild-type pepper accession (code A25) with high tolerance to salinity was selected. This rootstock was the most tolerant one to salinity compared to other rootstocks in terms of yield (unpublished data). The aim of the present work was to characterize the performance of Adige, a commercial pepper cultivar (Penella et al., 2014) sensitive to salinity stress when grafted onto a robust salt-tolerant rootstock, such as A25 (A/A25 plants). In particular, stomatal responses, antioxidant systems and proline accumulation were investigated to elucidate the salt tolerance-based mechanisms found in A/A25. For this purpose, A (ungrafted) pepper plants versus A/A25 plants, subjected to mild salt stress (80 mM NaCl) for 14 days, were compared in the open air and in the greenhouse.

6.3. MATERIAL AND METHODS

6.3.1. Plant material

Based on previous studies, a pepper accession of Capsicum annuum L. from the COMAV Genebank at the UPV university (Valencia, east Spain) was selected, which was tolerant to salinity (code A25). This accession was chosen to be used as a rootstock and pepper cultivar 'Adige' (A) (Lamuyo type, Sakata Seeds, Japan) was the scion. Seeds of A25 were sown in 96-hole seed trays filled with an enriched substrate for germination. After 2 months, A-plants were grafted onto A25 (A/A25). The graft was performed by the tube-grafting method (Penella et al., 2015). The ungrafted 'Adige' (A) plants were sown 2 weeks later to obtain plants with a similar biomass to that of the grafted plants at the time of transplantation (10-12 true leaves). The plants obtained by the aforementioned procedure were utilized for both field and greenhouse experiments.

6.3.2. Soil -field experiment

A preliminary experiment was conducted in spring/early summer 2013 in a field with soil with a moderate salt concentration (pH=8.0; EC as saturated past was 6.64 dS m^{-1}; Sand= 76%). The electrical conductivity and pH of the irrigation water were 7.5 dS m^{-1} and 7.60, respectively, with 57.5 meq l^{-1} of Na^{+} and 71.2 meq l^{-1} of Cl^{-}. Plant density was 2.5 plants m^{-2} in sandy soil (in polyethylene greenhouses). Fertilizers were applied at a rate of 200 UF N, 50 UF P_2O_5, 250 UF K_2O, 110 UF CaO and 35 UF MgO. A randomized complete block design was used with three replicates, each consisting of 25 plants. There was no significant difference among replicates in production. Ripe fruits were harvested from the

end of May to the end of July, and marketable and unmarketable fruits, mainly due to blossom end rot (BER), were weighed.

6.3.3. Greenhouse experiment

Seeds were sown on January 29th (2014) and the grafting for A/A25 performed on March 29th. After 3 weeks of acclimation, 30 plants of each combination (A and A/A25) were separated into two groups: controls (C) and NaCl-treated plants (+NaCl). For salt treatment, 80 mM of NaCl were added to a half-strength Hoagland's solution (pH 6.5±0.1; EC 8.0 dS m^{-1}). Both groups were watered daily with excess half-strength Hoagland's solution (pH 6.5±0.1; EC 1.1 dS m^{-1}) to minimize salt accumulation in the substrate for the 14 d that the experiment lasted. Potted plants were grown under greenhouse conditions at the facilities provided by the University of Pisa (Pisa, Italy). Temperatures ranged between 8.7 °C and 22.9 °C during the day**,** and remained above 12 °C at night**.** Relative humidity (RH) was between 37.7% and 96.3%, with daily maximum photosynthetically active radiation (PAR) levels within the greenhouse range of 850-1530 µmol m^{-2} s^{-1} (directly provided by sunlight).

All the physiological measurements were taken on fully expanded mature leaves (third or fourth leaf from the shoot apex) at the end of the salt treatment period. Two independent physiological determinations were made on each replicate and plant combination, obtained from six plants per treatment and combination.

6.3.4. Biomass and ion determination

Plants were harvested after 14 d of treatment. Leaves and roots were separated and their fresh weight (FW) was recorded. For dry weight (DW) determinations, leaves and roots were dried at 70 °C for 72 h in a laboratory oven and then weighed. Leaves and roots were milled and digested with concentrated HNO_3. Na^+ and K^+ were measured with an atomic absorption spectrophotometer (Ultrospec 2100, Pharmacia). Chloride analysis was performed on the water extracts of dry materials. The sample (250 mg DW) was incubated in water at 60 °C for 30 min. Following centrifugation, the supernatant was collected and Cl^- was determined in an ion cromatograph (DX-100 ion chromatograph Dionex™, Thermo Scientific).

6.3.5. Water potential and relative water content

The leaf water potential at pre-dawn (Ψ_w) and the relative water content (RWC) were measured on the leaves sampled before dawn by a standard methodology (Guidi et al., 2008).

6.3.6. Gas exchange and PSII photochemistry measurements

The net CO_2 assimilation rate, stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) in the saturating light (A_{N390}, i.e. at 800±28 μmol quanta $m^{-2}s^{-2}$ and 390 μmol CO_2 mol^{-1}) determinations were taken on fully expanded leaves

(3rd- 4th leaf from the apex) at room temperature (RT) and 75% RH with a portable Li-COR 6400 (Li-Cor Inc.) infrared gas analyzer. In the same leaves, the response of light-saturated CO_2 assimilation to variable internal CO_2 concentrations (A/C_i curves) was measured as reported in Guidi et al. (2008). From the A/C_i curves, the following photosynthetic parameters were calculated according to Long and Bernacchi (2003): the apparent maximum carboxylation rate of ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco), V_{cmax}, the maximum rate of the electron transport (J_{max}), which is equivalent to the ribulose-1,5-*bis*P (RuBP) regeneration rate, and use of triose-P (TPU).

The chlorophyll *a* fluorescence parameters were estimated from the measurements taken on the dark- (for 30 min) and light-adapted leaves (about 800 µmol $m^{-2}s^{-1}$) by IMAGING-PAM (Walz, Effeltrich, Germany). The maximum quantum efficiency of PSII was calculated as $F_v/F_m = (F_m - F_0/F_m)$. The operating quantum efficiency of PSII photochemistry, Φ_{PSII}, was calculated as $(F'_m - F')/F'_m$. The electron transport rate was calculated as ETR= 0.5 x Φ_{PSII} x PAR x 0.84 µequivalents m^{-2} s^{-1}. The photochemical quenching (q_P) factor was determined as $(F'_m - F')/(F'_m - F'_0)$. Non photochemical quenching (NPQ) was expressed as $F_m/F'_m - 1$, where F'_m was maximal fluorescence during a saturating flash of light of about 8000 µmol m^{-2} s^{-1}, and F'_0 was the minimal fluorescence estimated by the approach of Oxborough and Baker (1997) $F_0' = F_0/(F_v/F_m + F_0/F_m')$.

6.3.7. Leaf lipid peroxidation

Leaf lipid peroxidation was estimated with the malondialdehyde (MDA) concentration measurements taken by the thiobarbituric acid reaction, as reported in Penella et al. (2015).

6.3.8. Antioxidant enzymes

Antioxidant enzyme activities were measured in the fresh leaf material extracted with 1 mL of 100 mM potassium phosphate buffer (pH 7.0) that contained ethylenediamine tetra-acetic acid (EDTA). The extract was then centrifuged at 11000 x g at 4 °C for 15 min, and the supernatant was used for all the enzyme assays, while the protein determinations were performed with the Protein Assay Kit II (Bio Rad).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured at 560 nm, based on the inhibition of nitroblue tetrazolium (NBT) reduction by SOD (Beyer and Fridovich, 1987). One unit of SOD was defined as the enzymatic amount required to reduce the NBT reduction state by 50%. Catalase (CAT; EC 1.11.1.6) activity was measured at 270 nm by determining the rate of conversion of H_2O_2 into O_2 and water, as described by Cakmak and Marschner (1992). Catalase activity was expressed as µmol H_2O_2 per mg protein and per minute. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined following the H_2O_2-dependent oxidation of ascorbate (AsA) at 265 nm in a reaction mixture composed of 50 µM AsA, 90 µM H_2O_2, 50-100 µg proteins and 0.1 M phosphate buffer (pH 6.4) (Nakano and Asada, 1981). APX activity was corrected by subtracting the non-enzymatic H_2O_2-dependent ASA oxidation and H_2O_2-non-dependent ASA oxidation. APX activity was expressed as µmol AsA per mg protein and per minute.

6.3.9. Proline

Proline content was determined according to the method of Bates et al. (1973) with some minor modifications. Plant material (100 mg FW) was ground in an ice-cold mortar with 2 mL of 3% sulfosalicylic acid. Homogenates were centrifuged for 30 min at 10,000 x*g* at 4 °C. The supernatant was filtered through 0.2 µm Minisart® SRT 15 aseptic filters and 1 mL of the filtrate was mixed with equal volumes of glacial acetic acid and of ninhydrin reagent (1.25 g ninhydrin, 30 mL of glacial acetic acid, 20 mL 6 M H3PO4), and was incubated for 1 h at 100 °C. The reaction was stopped by placing test tubes in ice-cold water. Samples were vigorously mixed with 2 mL toluene. After 20 min, the light absorption of the toluene phase was estimated at 520 nm, with toluene used for a blank. The proline concentration was determined with a standard curve and calculated on a FW basis.

6.3.10. Tocopherol and β-carotene determination

The amount of α-tocopherol and ß-carotene was determined by HPLC according to Döring et al. (2014). Thirty mg of leaves were homogenized in 3 mL of 100% HPLC-grade methanol and incubated overnight at 4 °C in the dark. The supernatant was filtered through 0.2 µm Minisart® SRT 15 aseptic filters and immediately analyzed. The analysis was performed at RT with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length) and methanol/ethylacetate (68/32, v/v) was used as the mobile phase (flow rate 1 mL min^{-1}). α-tocopherol and ß-carotene were detected at 280 nm and 445 nm, respectively. Pure authentic standards were used to quantify the α-tocopherol and ß-carotene content of each sample.

6.3.11. Ascorbic acid content

Total ascorbate (AsAt), dehydroascorbate (DHA) and reduced ascorbate (AsA) were determined as described by Degl'Innocenti et al. (2005), based on the method of Kampfenkel et al. (1995). The ratio between AsA and AsA total (AsA/AsAt) was reported.

6.3.12. Statistical analysis

The experiment was completely randomized and the results were subjected to a two-way ANOVA (Statgraphics Centurion for Windows, Statistical Graphics Corp.) with salt treatment and plant type as the variability factors. The data of marketable fruits and the percentage (angularly transformed) of the fruits affected by BER were subjected to a one-way ANOVA with plant type as the variability factor. Means (n=6; ± SE) were compared using Fisher's least significance difference (LSD) test at $P < 0.05$.

6.4. RESULTS

6.4.1. Fruit yield

Adige grafted onto accession A25 (A/A25) gave the best response in marketable fruit yield associated with the lowest percentage of BER with significant differences with A plants (Table 1).

Table 1. Marketable fruit yield and percentage of fruit affected by Blossom end Root (BER) under water and soil salinity conditions. Values are the mean of 50 replicates *per* cultivar Adige ungrafted (A) or grafted onto the A25 genotype (A/A25). Different letters in each column indicate significant differences at P<0.05 using the LSD test, following a one-way ANOVA test with plant type as the variability factor.

Graft combination	Marketable yield (kg plant^{-1})	BER (%)
A	1.84 b	49a
A/A25	3.23 a	18b

6.4.2. Ion partitioning

After 14 days of culture in the greenhouse, Na^+ (Fig. 1A, D) and Cl^- (Fig. 1B, E) increased in both roots and shoots under salinity (80 mM NaCl) in both plant types. The Cl^- concentration was higher in leaves (Fig. 1E) than in roots (Fig. 1B) (3.3 *vs.* 6.1 mg g^{-1} DW, respectively; P<0.01), while no differences were observed in Na^+ content (1.5 *vs*. 1.7 mg g^{-1} DW in roots and leaves, respectively; P<0.01). The K^+/Na^+ ratio was higher in leaves than in roots (4-fold; P<0.001), and was significantly lower in both plant organs when salinity was applied (Fig. 1 C, F).

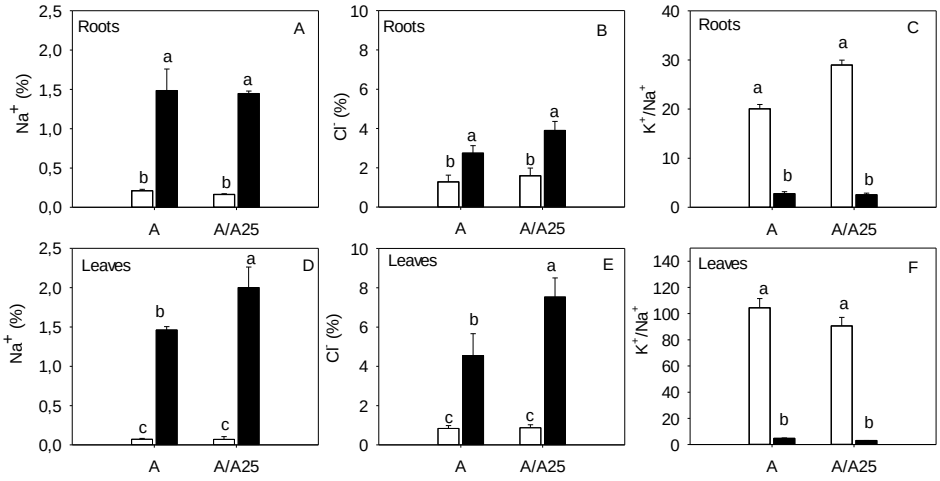


Figure 1. Mineral content (on a DW basis) in the roots and leaves of the control (white bars) and salt-treated plants (black bars) of pepper cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25). Means (n=6; ± SE) with different letters being significantly different at 0.05 according to a two-way ANOVA, with salt treatment and plant type as the variability factors.

6.4.3. Water potential

Leaf water potential (ψ_w) significantly decreased following NaCl treatment in both genotypes, and reached values of -0.22 and -0.32 MPa in A and A/A25, respectively (Fig. 2). However, no differences between the control and stressed plants in RWC were observed (Fig. 2, inside).

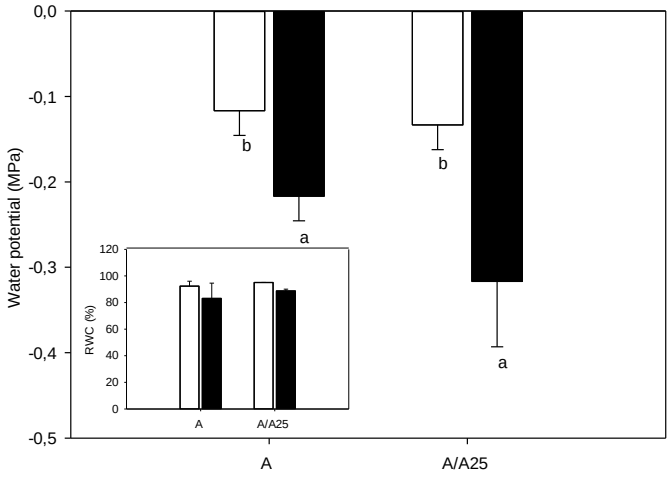


Figure 2. Water potential and RWC (inside) of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Means (n=6 ± SE) with different letters are significantly different at 0.05 according to a two-way ANOVA with salt treatment and plant type as the variability factors. Absence of letters (inside box) indicates that the F ratio was not significant.

6.4.4. Gas exchange and chlorophyll fluorescence parameters

At ambient atmospheric CO_2 concentrations, salinity significantly lowered the net assimilation rate at light saturation (A_{N390}), but only in A plants, whereas no differences were observed in A/A25 between controls and salt-treated individuals (Table 2). The intercellular CO2 concentration (C_i) lowered in the salt-treated leaves of A/A25, but no differences were observed in A. Stomatal conductance (g_s) decreased significantly in both plant combinations (Table 2). The effects of NaCl treatment on V_{cmax} and J_{max} were pronounced in A plants (with a significant

difference compared to its control), whereas no effects were detected in A/A25. Interestingly these two parameters were higher in A/A25 compared to A plants under control. Likewise, no effects on TPU were observed following salt stress in A/A25 and, once again, a significant reduction in the ungrafted A plants was observed (Table 2).

Table 2. Gas exchange parameters of cultivar Adige ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions. Plants maintained in optimal nutrient solution represent the controls. The CO_2 assimilation rate at 390 µmol mol^{-1} CO_2 (µmol CO_2 mol^{-1}) (A_{N390}), the intercellular CO_2 concentration (µmol CO_2 mol^{-1}) (C_i) and stomatal conductance to water vapor (mol H_2O m^{-2} s^{-1}) (g_s) were determined from the response curve of the CO_2 photoassimilation *versus* light intensities. The apparent maximum carboxylation rate of Rubisco (V_{cmax}, µmol CO_2 $m^{-2}s^{-1}$), the maximum rate of electron transport (J_{max}, µmol e^- m^{-2} s^{-1}), which is the equivalent to the RuBP regeneration rate, and the use of triose-P (TPU; µ Pi $m^{-2}s^{-1}$) were determined from response curve of CO_2 photoassimilation *vs.* C_i. Values are the mean of four replicates *per* genotype. Different letters in each column indicate significant differences at $P<0.05$ using the LSD test, following a two-way ANOVA test with NaCl treatment and plant type as the variability factors.

Graft combination	Treatment	A_{N390}	C_i	g_s	V_{cmax}	J_{max}	TPU
A	control	6.91 b	221.0 a	0.092 b	64.5 b	71.5 b	4.75 a
	NaCl	3.76 c	210.5 ab	0.035 c	31.0 c	44.0 c	2.40 b
A/A25	control	9.45 a	214.0 ab	0.135 a	124.0 a	103.5 a	5.55 a
	NaCl	8.18 ab	179.0 b	0.082 b	137.0 a	99.5 a	4.60 a

The maximum PSII quantum yield of primary photochemistry (F_v/F_m) did not change in both the genotypes following salinity stress, but showed values typical of healthy leaves (Björkman and Demmig, 1987) (*data not shown*). The ETRs for each plant combination subjected, or not, to salinity were plotted according to PAR (Fig. 3A, B). When PAR fell within the 0-200 µmol m^{-2} s^{-1} range in both plant types, the light-response curves of the ETR for the pepper-stressed plants closely overlapped that of the controls. Yet when PAR was above 200 µmol m^{-2} s^{-1}, in A-stressed plants, a significant separation of the light-response curves of ETR occurred (Fig. 3A). In A/A25 plants, the curves for control and salt did not show significant differences due to PAR (Fig. 3B).

The decrease in ETR in A salted plants was mainly caused by the substantial rise in NPQ (Fig. 3C). In A/A25 no differences in the NPQ values between the controls and treated plants were detected (Fig. 3D). The q_P coefficient remained unchanged in A/A25 under salt stress (Fig. 3F), and lowered in A-stressed plants (Fig. 3E).

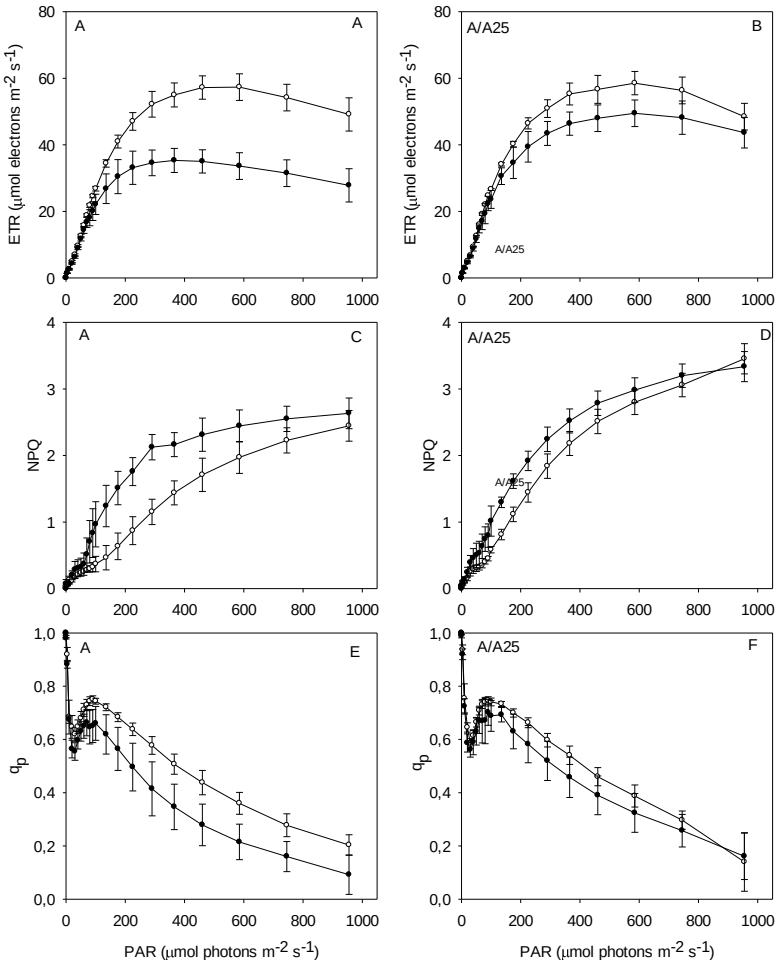


Figure 3. Electron transport rate (ETR), non-photochemical quenching (NPQ) and photochemical quenching coefficient (q_P) in response to photosynthetic active radiation (PAR) in cultivar Adige, ungrafted (A) or grafted onto the A25 accession (A/A25) under salinity conditions (closed circles). The plants maintained in optimal nutrient solution represent controls (open circles). Values are the mean of 6± SE replicates per plant combination.

6.4.5. Antioxidant enzymes

SOD activity increased significantly in both genotypes following salinity (Fig. 4A), but the rise in A/A25 was even more pronounced. In A plants, CAT activity increased significantly following salinity conditions (Fig. 4B), whereas no changes in APX activity were recorded (Fig. 4C). A different behavior was observed in A/A25 plants, in which salt stress did not induce changes in CAT activity, but significantly reduced APX activity (Fig. 4B, C).

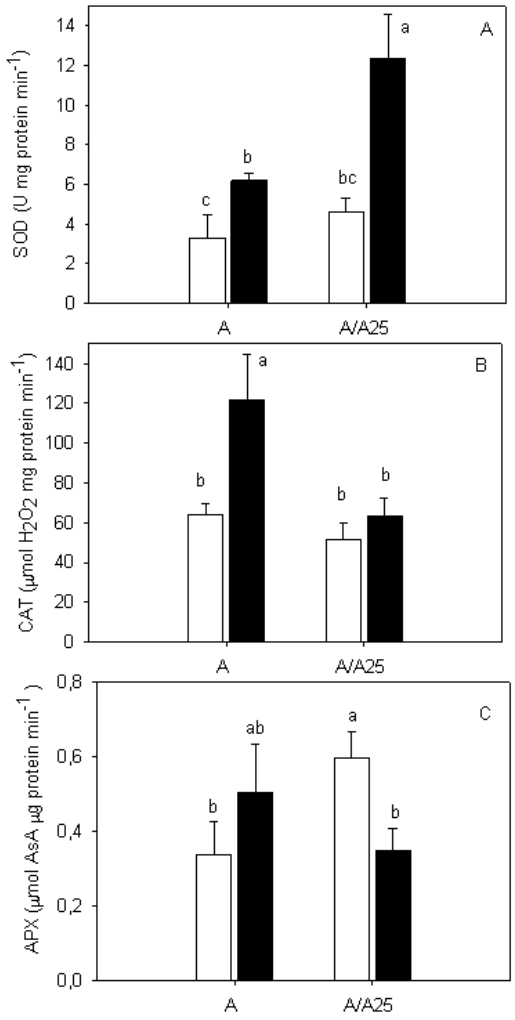


Figure 4. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activity in leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Means (*n*=6; ± SE) with different letters are significantly different at 0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors.

6.4.6. Effect of salt treatment on lipid peroxidation, α-tocopherol, β-carotene, ascorbate and proline

NaCl treatments led to a significant rise in the levels of the MDA by-products content in both kinds of pepper plants (Fig. 5A), but this increase was higher for A plants under salt stress. The α-tocopherol concentration (Fig. 5B) was also affected by NaCl treatment in A plants, whose a significant reduction was detected, but no differences were found between controls and treated plants for A/A25 (Fig. 5B). Another important antioxidant in chloroplast is β-carotene, which did not change in all plants following salt stress (Fig. 5C), even though a smaller amount was found in A/A25 compared to A plants (Fig. 5C). Finally, total AsA significantly increased in A plants under salinity conditions (+256% as compared to the controls). The decrease in the AsA/AsA total ratio in A salt-stressed leaves (from 0.85 to 0.52 in controls) indicated that a large amount of AsA was oxidized into DHA (Fig. 5D). In A/A25 plants, a significant increase in the AsA/AsA total ratio was reported following the salinity treatment (Fig. 5D).

Proline content sharply increased, but only in A/A25 plants following NaCl stress, whereas no changes in A plants were observed (Fig. 6A).

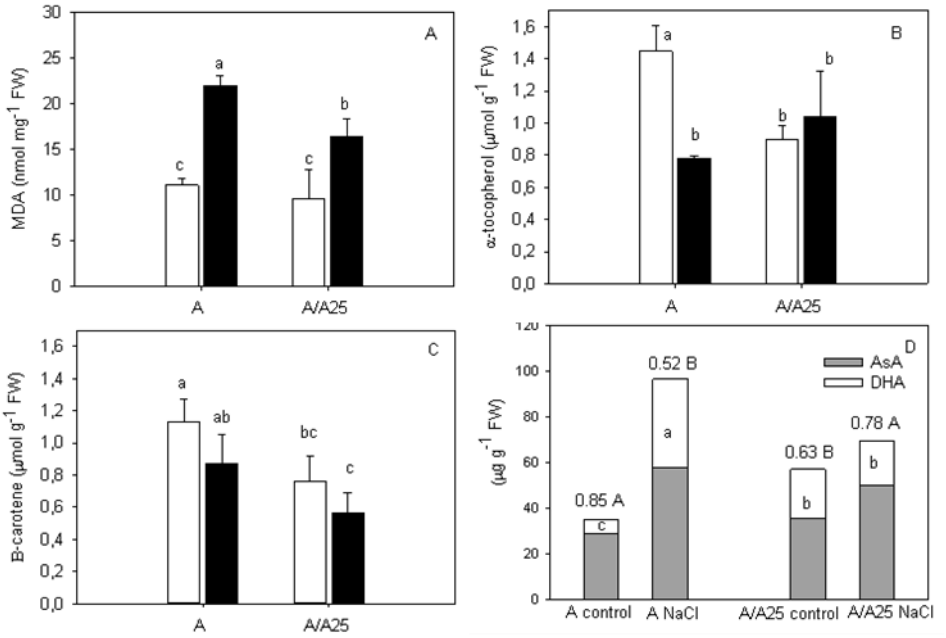


Figure 5. Malondialdehyde by-products (MDA), α-tocopherol, β-carotene and ascorbic acid in the leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 accession (A/A25) under salinity conditions (black bar). Control is represented by white bars. In graph D, different forms of ascorbate are reported. The numbers above the bars indicate the AsA/AsA total ratio and capital letters indicate the difference. Means (*n*=6 ±SE) with different letters are significantly different at 0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors.

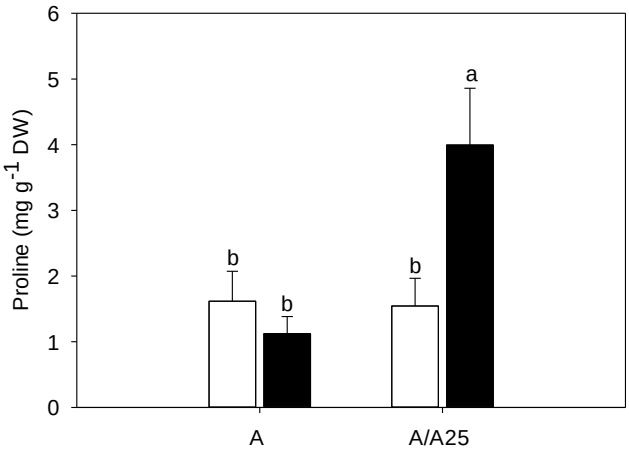


Figure 6. Proline content in the leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Each value represents the mean of 6 samples ± SE. Means with different letters are significantly different at P≤0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors.

6.4.7. Biomass

A/A25 plants developed a bigger root system than A plants (Fig. 7). No significant effect of salinity was noted on root FW and DW between the same plant types (Fig. 7A, C). The root FW/DW ratio did not change in both genotypes (Fig. 7E). A sharper drop in shoot biomass (leaf FW and DW) occurred as a consequence of salinity stress in A plants, but no changes in A/A25 were observed (Fig. 7B, D).On the contrary in A/A25, the FW/DW leaves ratio significantly lowered, but only in A/A25 (Fig. 7F)

.

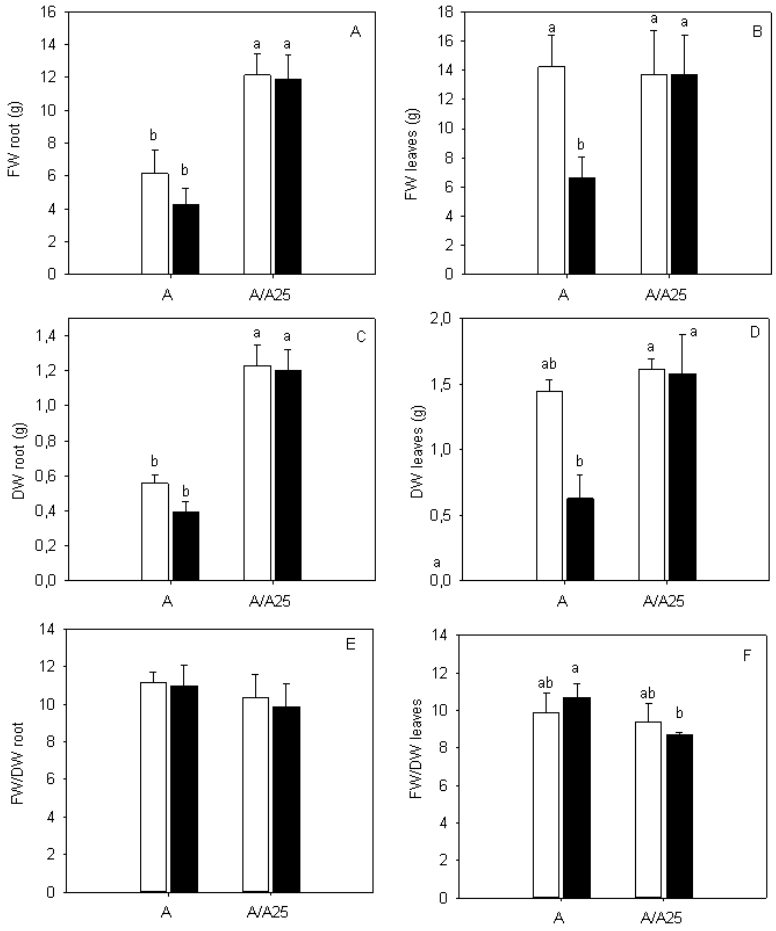


Figure 7. FW and DW, and their ratio for the root and leaves of cultivar Adige, ungrafted (A) or grafted onto the A25 genotype (A/A25) under salinity conditions (black bar). Control is represented by white bars. Each value represents the mean of 6 samples ± SE. Means with different letters are significantly different at 0.05 according to the two-way ANOVA, with salt treatment and plant type as the variability factors. Absence of letters indicates that the F ratio of the interaction is not significant.

6.5. DISCUSSION

Under salinity stress, reduced plant growth is induced by different biochemical, physiological and molecular alterations (Munns, 2002; Krasensky and Jonak, 2012). The selection of salt tolerant accessions to be used as rootstocks could be a promising approach to ameliorate the negative effects of salinity on pepper productivity (Penella et al., 2015). In the present study, we demonstrated that peppers grafted onto the accession A25 were less sensitive to salt stress compared to their ungrafted counterparts. The lower salt sensitivity exhibited by A/A25 was clearly demonstrated by the lack of negative effects on plant growth, increased marketable yield and the fewer BER symptoms appearing. The ameliorative effect of grafting on plant's growth under salinity conditions fully agrees with other findings in tomato and melon (Santa-Cruz et al., 2002; Estañ et al., 2005; Martinez-Rodriguez et al., 2008; He et al., 2009).

In a plant, accumulation of Na^+ and Cl^- in roots, and mainly in leaves, is the main deleterious consequence of salt exposure, which can inhibit different metabolic functions (Munns, 2002; Shabala and Munns, 2012). The mechanisms by which grafting improves salt tolerance can be numerous. For example, it has been reported that the positive effect induced by using a salt-tolerant rootstock in citrus is related to the rootstock's ability to exclude chloride, the main toxic ion in this species (Fernandez-Ballester et al., 2003; Moya et al., 2002). Similarly, rootstocks of *Vitis* spp. also vastly differ in their ability to exclude Cl^-, and also in their salinity tolerance given that the main cause of salt-induced damage in grapevines is related to Cl^- accumulation (Stevens et al., 1996; Fisarakis et al., 2001; Gibberd et al., 2003). In contrast, sodium has been reported to be the main ion responsible for NaCl toxicity in pepper (Penella et al., 2015) and tomato (Fernandez-Garcia et al., 2004). Martinez-Rodríguez et al. (2008) reported the effectiveness of grafting to enhance fruit yield in tomato and provided evidence

that the positive effect induced by rootstocks is related to the re-establishment of ionic homeostasis.

In the present study, both the A and A/A25 salt-treated plants exhibited similar Na^+ and Cl^- levels in both leaves and roots, and presented a lower K^+/Na^+ ratio compared with their controls. Hence the ability to restrict the uptake and/or transport of ions from roots to leaves was similar in A and A/A25. This means that the avoidance/reduction of salt uptake was not the factor that conferred salt tolerance to A/A25 plants, a similar finding to that already reported by He et al. (2009) in tomato-grafted plants. This notion is further confirmed as pepper-grafted plants accumulated even more ions (both Na^+ and Cl^-) under salinity in leaves than the ungrafted ones. The drop in the K^+/Na^+ ratio was attributable to the higher Na^+ content as plants did not lose their K^+ uptake ability.

According to Munns biphasic model (Munns and Tester, 2008), salt tolerance can be improved by reducing the negative osmotic effects on growth and/or maintaining leaf-root growth and functions for longer by diluting toxic ions (Balibrea et al., 2000; Yeo, 2007). Maintenance of shoot and root vigor is dependent mainly on photosynthetic capacity and gas exchange attributes (Duarte et al., 2014; Penella et al., 2015). Photosynthetic activity remained unchanged in A/A25 plants under salt conditions compared to their controls and, therefore, also in the supply of photosynthates to plants, as confirmed by the absence of reduced plant growth. Conversely, the leaf CO_2 assimilation rate sharply dropped in the salt stressed A plants compared to both controls and A/A25 plants. Salt stress has been reported to reduce CO_2 assimilation through different mechanisms: (I) decreased stomatal conductance (Chaves et al., 2009; Shabala and Munns, 2012); (II) reduced mesophyll conductance to CO_2 (Flexas et al., 2004); (III) impaired Rubisco activity (Galmes et al., 2013). Stomatal closure is certainly one of the main responses of plants under salinity to minimize

water loss (Aroca et al., 2012; Shabala and Munns, 2012). Stomatal conductance decreased under salt treatment in both the A and A/A25 plants, which could be one of the reasons for their unchanged RWC values, and this suggests a typical conservative strategy (Tardieu and Simonneau, 1998; García-Sánchez et al., 2010; Sade et al., 2012). Notably in the grafted plants, the CO_2 assimilation rate did not change in relation to g_s reduction under salinity conditions. In contrast, in A plants the sharp reduction in g_s induced a marked decrease in A_{N390} (about -45%), to suggest that mesophyll limitations also occurred, as confirmed by the unchanged C_i. Accumulation of intercellular CO_2 was also likely attributable to the marked reduction in the V_{cmax} as observed in A plants. Other authors have reported that carboxylation efficiency under stress conditions is limited by the amount, activity and kinetics of Rubisco, as well as by an effect on CO_2 diffusion limitation (Carmo-Silva and Salvucci, 2012; Koyro et al., 2013). The A/C_i curves also showed a significant decrease in J_{max} in A salt-treated plants and TPU, according to the Farquhar model (Farquhar et al., 1980), whereas no alterations were observed in grafted plants. These results suggest that carboxylation efficiency, ribulose-1,5-*bis*phosphate regeneration and triose-phosphate utilization were maintained in A/A25, whereas these processes were severely unpaired in A. The TPU rate has been proposed to at least provide an indication of the feedback between growth and CO_2 assimilation (Wullschleger, 1993). The sharp drop in A_{N390} in the A salt-treated plants related to the limitation in TPU can be considered one of the main reasons for reduced growth (Long and Bernacchi, 2003; von Caemmerer, 2000; Sharkey et al., 2007).

The stomatal and biochemical limitations imposed on photosynthesis in A plants submitted to the salt treatment were likely accompanied by a lowered ATP and NADPH consumption rate for CO_2 assimilation, which would imply a lower down-regulated ETR rate (Baker and Rosenqvist, 2004). A progressive drop in ETR can be compensated by increased thermal dissipation (NPQ) (Medrano et al.,

2002). Accordingly, NPQ increased once A plants were subjected to salinity, even though they underwent higher excitation pressure on PSII and more reaction centers were closed, as evidenced by an over-reduction of Q_A (Calatayud and Barreno, 2001; Guidi and Calatayud, 2014; Kalaji et al., 2014). This is particularly evident at high light (800-1000 µmol photons m^{-2} s^{-1}) when salt stress can accelerate photodamage to the reaction center of PSII (Nishiyama and Murata, 2014). Even though the actual PSII efficiency was compromised, the dissipation mechanisms were able to preserve PSII to irreversible damage, and the F_v/F_m values remained unchanged. Conversely, the chlorophyll fluorescence parameters in the A/A25 salt-treated plants confirmed that no alterations occurred in the biochemical and photochemical chloroplast processes, as previously revealed by gas exchange analyses. These results coincide with previous findings, which highlight that the use of tolerant rootstock improves the photosynthesis performance of the scion under salinity conditions (Moya et al., 2002; Massai et al., 2004; He et al., 2009; Penella et al., 2015).

Although the marked accumulation of toxic ions occurred in the A/A25 plants subjected to salinity, no effects were detected in photosynthesis and, consequently, the antioxidant systems were further activated in these plants, except SOD activity. Conversely, the activity of the most important enzymes involved in removing and/or scavenging ROS (SOD, CAT and APX) was significantly stimulated in the A plants under salinity. The activities of these enzymes and/or antioxidant molecules have long since been described as being actively involved in response to several abiotic stresses, including salt toxicity in both grafted and ungrafted plants (López-Gómez et al., 2007; He et al., 2009; Sanchez-Rodríguez et al., 2012; Shaheen et al. 2013). In this context, it is assumed that the simultaneous involvement of different antioxidant components is necessary to obtain an increase (and/or a faster response) in plant defenses

when plants face high salinity (Jaleel et al., 2009). However in the A plants, the antioxidant system did not efficiently sustain ROS scavenging in relation to salinity-triggered ROS production, as demonstrated by the marked increase in the MDA by-product levels.

AsA is a key metabolite that plays key roles in plant stress and is the most important H_2O_2-reducing compound, which acts together with glutathione in the ascorbate-glutathione cycle (Foyer and Noctor, 2011; Foyer and Shigeoka, 2011). Total AsA increased significantly (about 44% compared to the controls) in the A plants under salinity, and the AsA/(DHA+AsA) ratio also sharply dropped, which indicates that a high AsA oxidation rate occurred. No differences were observed in the total AsA and AsA/(DHA+AsA) ratio in the A/A25 plants under salt stress compared to their controls. Ascorbate is also essential for α-tocopherol regeneration (Szarka et al., 2012), a lipophilic antioxidant and an indispensable protector of plant membranes (Mène-Saffrané and DellaPenna, 2010; Das and Roychoudhury, 2014). Despite the increase in the amount of DHA found in A plants under salinity, oxidation of AsA was not sufficient to efficiently sustain the α-tocopherol regeneration rate given that this compound decreased in these plants. The inability to efficiently sustain α-tocopherol regeneration can further increase membrane lipid peroxidation, as revealed by the dramatic increase in the MDA by-products level in A plants under stress.

Accumulation of osmolytes, such as proline, is a well-known adaptive mechanism in plants against salt stress conditions (Ashraf and Foolad, 2007; Szabados and Savouré, 2010). Several studies have attributed a dual role to proline: compatible osmolyte and antioxidant compound (Szabados and Savouré, 2010). It has been previously reported that under salt stress proline can contribute by stabilizing many functional units, such as Complex II, in the electron transport chain and key enzymes, such as Rubisco (Ashraf et al., 2008). In A/A25 leaves, proline content

increased 2.6-fold in the presence of NaCl excess, compared to a non-significant increase noted in A leaves.

Our results generally suggest that A/A25 plants were tolerant to the salt concentration adopted in this experiment given the adjustments made in the physiological processes. In fact, stomatal closure preserves excess water loss, as evidenced by the maintenance of RWC. At the same time, the reduction of g_s was not limiting for CO_2 assimilation, which did not change in A/A25 plants under salinity. However, these plants accumulated in plant tissues a high concentration of toxic ions, i.e., Cl^- and Na^+ (even higher than in A plants under stress). Three mechanisms are available to plant cells for preventing excessive Na^+ accumulation in the cytosol to: (I) restrict Na^+ by selective ion uptake; (II) store Na^+ in vacuoles; (III) export Na^+ back to the growth medium or to the apoplastic space (Zu, 2001). It has been reported in the euhalophyte *Salicornia europaea* that a high Na^+ concentration in the shoots did not lead to a reduced plant growth and photosynthesis, which implies that the mechanism adopted by this species is to store Na^+ in the vacuoles (Lv et al., 2012). The fact that no effects were observed at the physiological and biochemical levels in A/A25 plants suggests that these plants under saline conditions could either restrict excess salts in vacuoles or compartmentalize ions in different (other?) tissues, where they are less harmful (Zhu, 2003). However, the high salt ions concentration in the apoplastic space of leaves can occur and lead to the partial dehydration of cells, and turgor loss, or can damage the plasma membrane surface (Speer and Kaiser, 1991; Volkmar et al., 1998). Accumulation of toxic ions in the apoplast can enhance NADPH-oxidase activity, the main producer of signal transduction-associated ROS in cells during these processes (Mittler et al., 2004), and can lead to anion superoxide production (Rejeb et al., 2015). This toxic compound spontaneously dismutes to oxygen and hydrogen peroxide, but the uncatalyzed

dismutation reaction rate is second-order compared to the initial superoxide concentration (Buchanan et al., 2002). In contrast, the catalyzed dismutation reaction by SOD is of the first-order. For this reason, SOD activity, which increased in A/A25 plants under salinity, quickly led to H_2O_2 production: this could be the beginning of a reaction cascade in response to salt stress (Miller et al., 2011; Rejeb et al., 2015). According to this concept, Bose et al. (2013) reported that the intrinsically higher SOD levels in halophytes are required for the rapid induction of the H_2O_2 'signature', and to trigger a cascade of adaptive (genetic and physiological) responses. Recently, several research works have indicated that proline accumulation occurs in stressed plants and can be mediated by signaling molecules, including H_2O_2 (e.g. Zhu, 2002; Zhang et al., 2008; Yang et al., 2009; Wen et al., 2013). In this context, it may be speculated that proline is the key metabolite by which A/A25 plants tolerated the salinity conditions imposed in the present experiments. Conversely, it would seem that the main effect may be related to the robustness of the rootstock A25. Other mechanisms not contemplated herein could be implicated in the resilience of A/A25.

In conclusion, the grafting technique can be considered a valid strategy for ameliorating the salt tolerance of pepper. The larger amount of marketable fruits and the lower BER incidence in the A/A25 plants under salt is the best demonstration of A25 rootstock's validity under high salinity.

6.6. REFERENCES

Aktas, H., Abak, K., Cakmak, I., 2006. Genotypic variation in the response of pepper to salinity. Sci. Hort. 110, 260-266.

Aroca, R., Porcel, R., Ruiz-Lozano, J.M., 2012. Regulation of root water uptake under abiotic stress conditions. J. Exp. Bot. 63, 43-57.

Ashraf, M.A., Ashraf, M., Shahbaz, M., 2012. Growth stage-based modulation in antioxidant defense system and proline accumulation in two hexaploid wheat (Triticum aestivum L.) cultivars differing in salinity tolerance. Flora 207, 388-397.

Ashraf, M., Athar, H.R., Harris, P.J.C., Kwon T.R., 2008. Some prospective strategies for improving crop salt tolerance. Adv. Agronomy 97, 45-110.

Ashraf, M., Foolad, M.R., 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Env. Exp. Bot. 59, 206-216.

Ashraf, M., Wu, L., 1994. Breeding for salinity tolerance in plants. Crit. Rev. Plant Sci. 13, 17-42.

Baker, N.R., Rosenqvist, E., 2004. Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. J. Exp. Bot. 55, 1607-1621.

Balibrea, M.E., Dell'Amico, J., Bolarín, M.A., Pérez-Alfocea, F., 2000. Carbon partitioning and sucrose metabolism in tomato plants growing under salinity. Physiol. Plant. 110, 503-511.

Bates, L.S., Waldren, R.P., Teare, J.D., 1973. Rapid determination of free proline for water stress studies. Plant Soil 39, 205-207.

Beyer, W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. Anal. Bioch. 161, 559-566.

Björkman, O., Demmig, B., 1987. Photon yield of O2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta 170, 489-504.

Bose, J., Rodrigo-Moreno, A., Shabala, S., 2014. ROS homeostasis in halophytes in the context of salinity stress tolerance. J. Exp. Bot. 65, 1241-1257.

Buchanan, B., Gruissem, W., Jones, R., 2002. Biochemistry and Molecular Biology of Plants. John Wiley & Sons, pp. 1408.

Cakmak, I., Marschner, H. (1992). Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol. 98, 1222-1227.

Calatayud, A., Barreno, E., 2001. Chlorophyll a fluorescence, antioxidant enzymes and lipid peroxidation in tomato in response to ozone and benomyl. Environ. Pollut. 115, 283-289.

Carmo-Silva, A.E., Salvucci, M.E., 2012. The temperature response of CO2 assimilation, photochemical activities and Rubisco activation in Camelina sativa, a potential bioenergy crop with limited capacity for acclimation to heat stress. Planta 236, 1433-1445.

Chartzoulakis, K., Psarras, G., 2005. Global change effects on crop photosynthesis and production in Mediterranean: the case of Crete, Greece. Agricult., Ecosys. Environ. 106, 147–157

Chaves, M.M., Flexas, J., Pinheiro, C., 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann. Bot. 103, 551-560.

Das, K., Roychoudhury, A., 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers Environ. Sci. 2, article 53.

Degl'Innocenti, E., Guidi ,L., Pardossi, A., Tognoni, F., 2005. Biochemical study of leaf browning in minimally processed leaves of lettuce. J. Agricult. Food Chem. 53, 9980-9984.

del Amor, F.M., Cuadra-Crespo, P., 2011. Plant growth-promoting bacteria as a tool to improve salinity tolerance in sweet pepper. Funct. Plant Biol. 39, 82-90.

Döring, A.S., Pellegrini, E., Campanella, A., Trivellini, A., Gennai, C., Petersen, M., Nali, C., Lorenzini, G., 2014 How sensitive is Melissa officinalis to realistic ozone concentration. Plant Physiol. Biochem. 74, 156-164.

Duarte, B., Sleimi, N., Caçador, I., 2014. Biophysical and biochemical constraints imposed by salt stress: learning from halophytes. Frontiers in Plant Sci., 5, article 746

Edelstein, M., Plaut, Z., Ben-Hur, M., 2011. Sodium and chloride exclusion and retention by non-grafted and grafted melon and Cucurbita plants. J. Exp. Bot. 62, 177-184.

Estañ, M.T., Martinez-Rodriguez ,M.M., Perez-Alfocea, F., Flowers T.J., Bolarin, M.C., 2005. Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. J. Exp. Bot. 56, 703-712.

Farquhar, G.D., von Caemmerer, S., Berry, J.A., 1980. A biochemical model of photosynthetic CO2 assimilation in leaves of C3 species. Planta 149, 78-90.

Fernandez-Ballester, G., Garcia-Sanchez, F., Cerda, A., Martinez, V., 2003. Tolerance of citrus rootstock seedlings to saline stress based on the ability to regulate ion uptake and transport. Trees Physiol. 23, 265-271.

Ferreira-Silva, S.L., Silva, E.N., Carvalho, F.E.L., de Lima, C.S., Alves, F.A.L., Silveira, J.A.G., 2010. Physiological alterations modulated by rootstock and scion combination in cashew under salinity. Sci. Hort. 127, 39-45.

Fini, A., Guidi L., Giordano, C., Baratto, M.C., Ferrini, F., Brunetti, C., Calamai, L., Tattini, M., 2014. Salinity stress constrains photosynthesis in Fraxinus ornus more when growing in partial shading than in full sunlight: consequences for the antioxidant defense system. Ann. Bot. 114, 525-538.

Fisarakis, I., Chartzoulakis, K., Stabrakas, D., 2001. Response of Sultana vines (V. vinifera L.) on six rootstocks to NaCl salinity exposure and recovery. Agricul. Water Management 51, 13-27.

Flexas, J., Bota, J., Loreto, F., Cornic, G., Sharkey, T.D., 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biol. 6, 269-279.

Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 155, 2-18.

Foyer, C.H., Shigeoka, S., 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol. 155, 93-100.

Galmés, J., Aranjuelo, I., Medrano, H., Flexas, J., 2013. Variation in Rubisco content and activity under variable climatic factors. Photosyn. Res. 117, 73-90.

García-Sánchez, F., Rubio, F., Martinez ,V., 2010. Abiotic stresses: salinity and drought. In: A. Gonzalez-Fontes, A. Garate, I. Bonilla (Eds.). Agricultural Sciences: Topics in Modern Agriculture, Studium Press, USA.

Gibberd, M.R., Walke,r R.R., Condon, A,G., 2003. Whole-plant transpiration efficiency of Sultana grapevine grown under saline conditions is increased through the use of a Cl−-excluding rootstock. Funct. Plant Biol. 30, 643-652.

Golldack, D., Lüking, I., Yang, O., 2011. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant Cell Rep. 30, 1383-1391.

Grieve, C., Grattan, S., Maas, E., 2012. Plant salt tolerance. In: Wallender, W.W., Tanji, K.K. (Eds.). Agricultural Salinity Assessment and Management: Second Edition. American Society of Civil Engineers, pp. 405-459.

Guidi, L., Calatayud, A., 2014. Non-invasive tools to estimate stress-induced changes in photosynthetic performance in plants inhabiting Mediterranean areas. Environ. Exp. Bot. 103, 42-52.

Guidi, L., Degl'Innocenti, E., Remorini, D., Massai, R., Tattini, M., 2008. Intercation of water stress and solar irradiance on the physiology and biochemistry of Ligustrum vulgare. Tree Physiol. 28, 873-883.

He, Y., Zhu, Z., Yang, J., Ni, X., Zhu, B., 2009. Grafting increases the salt tolerance of tomato by improvement of photosynthesis and enhancement of antioxidant enzymes activity. Environ. Exp. Bot. 66, 270-278.

Hu, L., Li, H., Pang, H., Fu, J., 2012. Responses of antioxidant gene, protein and enzymes to salinity stress in two genotypes of perennial ryegrass (Lolium perenne) differing in salt tolerance. J. Plant Physiol. 169, 146-156.

Hu, Y. Schmidhalter, U., 2005. Drought and salinity: A comparison of their effects on mineral nutrition of plants. J. Plant Nut. Soil Sci. 168, 541-549.

Jaleel, C.A., Manivannan, P., Wahid, A., Farooq, M., Al-Juburir, H.J., Somasundaram, R.,. Panneerselvam, R., 2009. Drought stress in plants: a review on morphological characteristics and pigments composition. Int. J. Agric. Biol. 11, 100-105.

Jensen, C.R., Ørum, J.E., Pedersen, S.M., Andersen, M.N., Plauborg, F., Liu, F., Jacobsen, S.E., 2014. A short overview of measures for securing water resources for irrigated crop production. J. Agron. Crop Sci. 200, 333-343.

Kalaji, H.M., Schansker, G., Ladle ,R.J., Goltsev, V., Bos,a K., Allakhverdiev, S.I., Bresti,c M., Bussotti, F., Calatayud, A., Dąbrowsk,i P., Elsheery, N.I., Ferroni, L., Guidi, L., Hogewoning, S.W., Jajoo, A., Misra, A.M., Nebauer ,S.G., Pancaldi S., Penella, C., Poli, D., Pollastrini, M., Romanowska-Duda, Z.B., Rutkowska, B., Serôdio, J., Suresh, K., Szulc, W., Tambussi, E., Yanniccari, M., Zivcak, M., 2014. Frequently asked questions about in vivo chlorophyll fluorescence: practical ssues. Photo. Res. 122, 121-158.

Kampfenkel, K., Van Montagu M., Inzè, D., 1995. Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal. Biochem. 225, 165-167.

Koyro, H.W, Hussain, T., Huchzermeyer, B., Khan, M.A., 2013. Photosynthetic and growth responses of a perennial halophytic grass Panicum turgidum to increasing NaCl concentrations. Environ. Exp. Bot. 91, 22-29.

Krasensky, J., Jonak, C., 2012. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J.Exp. Bot. 63, 1593-1608.

Kurunc, A., Unlukara, A., Cemek, B., 2011. Salinity and drought affect yield response of bell pepper similarly. Acta Agricul. Scandinavica, Section B-Soil Plant Sci. 61, 514-522.

Long ,S.P., Bernacchi, C.J., 2003. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. J. Exp. Bot. 54, 2393-2401.

López-Gómez, E., San Juan, M.A., Diaz-Vivancos, P., Beneyto, J.M., García-Legaz, M.F., Hernández, J.A., 2007. Effect of rootstocks grafting and boron on the antioxidant systems and salinity tolerance of loquat plants (Eriobotrya japonica Lindl.). Environ. Exp. Bot. 60, 151-158.

Lv, S., Jiang, P., Chen, X., Fan, P., Wang, X., Li, Y., 2012. Multiple compartmentalization of sodium conferred salt tolerance in Salicornia europaea. Plant Physiol. Biochem. 51, 47-52.

Majumder, M., 2015. Impact of urbanization on water shortage in face of climatic aberrations. Springer. ISBN: 978-981-4560-72-6.

Martinez-Rodriguez, M.M., Estañ, M.T., Moyano, E., Garcia-Abellan, J.O., Flores, F.B., Campos, J.F., Al-Azzawi, M.J., Flowers, T.J., Bolarín, M.C. 2008. The effectiveness of grafting to improve salt tolerance in tomato when an 'excluder' genotype is used as scion. Environ. Exp. Bot. 63, 392-401.

Massai, R., Remorini, D., Tattini, M., 2004. Gas exchange, water relations and osmotic adjustment in two scion/rootstock combinations of Prunus under various salinity concentrations. Plant Soil 259, 153-162.

Medrano, H., Escalona, J.M., Bota, J., Gulías, J., Flexas, J., 2002. Regulation of photosynthesis of C3 plants in response to progressive drought: stomatal conductance as a reference parameter. Ann. Bot. 89, 895-905.

Mène-Saffrané, L., DellaPenna, D., 2010. Biosynthesis, regulation and functions of tocochromanols in plants. Plant Physiol. Biochem. 48, 301-309.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R., 2011. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 33, 453-467.

Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490-498.

Moya, J.L., Gomez-Cadenas, A., Primo-Millo, E., Talon, M., 2003. Chloride absorption in salt-sensitive Carrizo citrange and salt-tolerant Cleopatra mandarin citrus rootstocks is linked to water use. J. Exp. Bot. 54, 825-833.

Moya, J.L., Tadeo, F.R., Gómez-Cadenas, A., Primo-Millo, E., Talón, M., 2002. Transmissible salt tolerance traits identified through reciprocal grafts between sensitive Carrizo and tolerant Cleopatra citrus genotypes. J. Plant Physiol. 159, 991-998.

Munns R., 2002. Comparative physiology of salt and water stress. Plant, Cell Environ 25 239–250.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Ann. Rev. Plant Biol. 59, 651-681.

Nakano, Y., Asada, K., 1981,). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867-880.

Nishiyama, Y., Murata, N., 2014. Revised scheme for the mechanism of photoinhibition and its application to enhance the abiotic stress tolerance of the photosynthetic machinery. Appl. Micro. Biotech. 98, 8777-8796.

Orsini, F., Sanouba,r R., Oztekin, G.B., Kappel, N., Tepecik, M., Quacquarelli, C., Tuzel, Y., Bon,a S., Gianquinto, G., 2013. Improved stomatal regulation and ion partitioning boots salt tolerance in grafted melon. Funct. Plant Biol. 40, 628-636.

Oxborough, K., Baker, N.R., 1997. Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of qP and Fv′/Fm′ without measuring F0′. Photo. Res. 54, 135-142.

Parida, A.K., Das, A.B., 2005,). Salt tolerance and salinity effects on plants: a review. Ecotoxic. Environ. Safety 60, 324-349.

Peleg, Z., Apse, M.P., Blumwald, E., 2011. Engineering salinity and water-stress tolerance in crop plants: getting closer to the field. In: Turkan, I. (Ed.), Plant Responses to drought and Salinity stress: Developments in a Post-Genomic Era. Academic Press, Vol 5, pp. 405-443.

Penella, C., Nebauer, S.G., Quiñones, A., San Bautista, A., López-Galarza, S., Calatayud, A., 2015. Some rootstocks improve pepper tolerance to mild salinity through ionic regulation. Plant Sci. 230, 12-22.

Penella, C., Nebauer, S.G., San Bautista, A., López-Galarza, S., Calatayud, A., 2014. Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: Physiological responses. J. Plant Physiol. 171, 842-851.

Pérez-Salamó, I., Papdi, C., Rigó, G., Zsigmond, L., Vilela, B., Lumbreras, V., Nagy, I., Horváth, B., Domoki, M., Darula, Z., Medzihradszky, K., Bögre, L., Koncz, C., Szabados, L., 2014. The heat shock factor A4A confers salt tolerance and is regulated by oxidative stress and the mitogen-activated protein kinases MPK3 and MPK6. Plant Physiol. 165, 319-334.

Radić, S., Štefanić, P.P., Lepeduš, H., Roje, V., Pevalek-Kozlina, B. (2013). Salt tolerance of Centaurea ragusina L. is associated with efficient osmotic adjustment and increased antioxidative capacity. Environ. Exp. Bot. 87, 39-48.

Razzaghi, F., Jacobsen, S.E., Jensen, C.R., Andersen, M.N., 2015. Ionic and photosynthetic homeostasis in quinoa challenged by salinity and drought – mechanisms of tolerance. Funct. Plant Biol. 42, 136-148.

Rejeb, K.B., Benzarti, M. Debez, A., Bailly, C., Savouré, A., Abdelly, C., 2015. NADPH oxidase-dependent H2O2 production is required for salt-induced antioxidant defense in Arabidopsis thaliana. J. Plant Physiol. 174, 5-15.

Rivero, R.M., Mestre, T.C., Mittler, R., Rubio, F., Garcia-Sanchez, F., Martinez V., 2014. The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. Plant, Cell Environ. 37, 1059-1073.

Sade, N., Gebremedhin, A., Moshelion, M., 2012. Risk-taking plants. Anisohydric behavior as a stress-resistance trait. Plant Signal. Behavior 7, 767-770.

Sánchez-Rodríguez, E., Rubio-Wilhelmi M.M, Blasco, B., Leyva, R., Romero, L., Ruiz, J.M., 2012. Antioxidant response resides in the shoot in reciprocal grafts of drought-tolerant and drought-sensitive cultivars in tomato under water stress. Plant Sci. 188-189: 89-96.

Santa-Cruz, A., Martinez-Rodriguez, M.M., Perez-Alfocea, F., Romero-Aranda, R., Bolarin, M.C., 2002. The rootstock effect on the tomato salinity response depends on the shoot genotype. Plant Sci. 162, 825-831.

Shabala S., Munns R. (2012). Salinity Stress: physiological constraints and adaptative mechanisms. In: Plant Stress Physiology, Shabala S., Ed. Pp. 59-93. CAB International.

Shaheen, S., Naseer, S., Ashrafa, M., Akram, N.A., 2013. Salt stress affects water relations, photosynthesis, and oxidative defense mechanisms in Solanum melongena L. J. Plant Interac. 8, 85-96.

Shannon, M.C., Grieve, C.M., 1998. Tolerance of vegetable crops to salinity. Sci. Hortic. 78, 5-38.

Sharkey, T.D., Bernacchi, C.J., Farquhar, G.D., Singsaas, E.L., 2007,). Fitting photosynthetic carbon dioxide response curves for C3 leaves. Plant Cell Environ. 30, 1035-1040.

Singh, M., Kumar, J., Singh, V.P., Prasad, S.M., 2014. Proline and salinity tolerance in plants. Biochemistry & Pharmacology, 3: 1000e170

Speer, M., Kaiser, W.M., 1991. Ion relations of symplastic and apoplastic space in leaves from Spinacia oleracea L. and Pisum sativum L. under salinity. Plant Physiol. 97, 990-997.

Stevens, R.M., Harvey, G., Davies, G., 1996. Separating the effects of foliar and root salt uptake on growth and mineral composition of four grapevine cultivars on their own roots and Ramsey rootstocks. J. Am. Soc. Hortic. Sci.121, 569-575.

Szabados, L., Savouré, A., 2010. Proline: a multifunctional amino acid. Trends Plant Sci.15, 89-97.

Szarka, A., Tomasskovics, B., Bánhegyi, G., 2012. The ascorbate-glutathione-α-tocopherol triad in abiotic stress response. Inter. J. Mol. Sci. 13, 4458-4483.

Tardieu, F., Simonneau, T., 1998. Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modelling isohydric and anisohydric behaviours. J. Exp. Bot. 49, 419-432.

Türkan, I., Demiral, T., 2009. Recent developments in understanding salinity tolerance. Environ.Exp. Bot. 67, 2-9.

Tuteja, N., 2007. Mechanisms of high salinity tolerance in plants. Meth. Enzymol. 428, 419-438.

Volkmar, K. M., Hu, Y., Steppuhn, H., 1998. Physiological responses of plants to salinity: a review. Can. J. Plant Sci. 78, 19-27.

Von Caemmerer, S., 2000. Biochemical models of leaf photosynthesis. Techniques in Plant Sciences; no. 2. Csiro Publishing.

Wang, W., Vinocur, B., Shoseyov, O., Altman, A., 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9, 244-252.

Wang, Y., Li L., Cui, W., Xu, S., Shen, W., Wang, R., 2012. Hydrogen sulfide enhances alfalfa (Medicago sativa) tolerance against salinity during seed germination by nitric oxide pathway. Plant Soil 351, 107-119.

Wen, J.-F., Gong, M., Liu, Y., Hu, J.L., Deng, M.H., 2013. Effect of hydrogen peroxide on growth and activity of some enzymes involved in proline metabolism of sweet corn seedlings under copper stress. Sci. Hort. 164, 366-371.

Wullschleger, S.D., 1993. Biochemical limitations to carbon assimilation in C3 plants. A retrospective analysis of the A/Ci curves from 109 species. J. Exp. Bot. 44, 907-920.

Yang, S.L., Lan, S.S., Gong, M., 2009. Hydrogen peroxide-induced proline and metabolic pathway of its accumulation in maize seedlings. J. Plant Physiol. 166, 1694-1699.

Yeo, A.,2007. Salinity. In: Yeo A., Flowers, T. (Eds.) Plant Solute Transport, Blackwell Publishing Ltd, Oxford, UK.

Yoshida, T., Mogami, J., Yamaguchi-Shinozaki, K., 2014. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Curr. Op. Plant Biol. 21, 133-139.

Zhang, L.P., Mehta, S.K., Liu, Z.P., Yang, Z.M., 2008. Copper-induced proline synthesis is associated with nitric oxide generation in Chlamydomonas reinhardtii. Plant Cell Physiol. 49, 411-419.

Zhu J.K. (2002). Salt and drought stress signal transduction in plants. Ann. Rev. Plant Biol. 53, 247-273.

Zhu, J.K., 2003. Regulation of ion homeostasis under salt stress. Curr. Op. Plant Biol. 6, 441-445.

CHAPTER 7
Could we anticipate the incompatibility phenomena in pepper grated plants? Chlorophyll fluorescence imaging reflects histological studies

7.1. ABSTRACT

The cellular events that led to a successful grafting in plants require the development of functional vascular connections. Until now, graft union development has been studied by destructive methods like anatomic and histological studies. We proposed a quick and non-invasive method to estimate (in)-compatibilities in pepper plants through the variations in chlorophyll fluorescence images (CFI). To validate this method we compared CFI values with histological studies in order to demonstrate if CFI can reflect the morphological and anatomical development at the graft interface between both graft partners in pepper. To reach this objective we used the commercial pepper cultivar 'Adige' and different *Capsicum* spp. accessions typified with different compatibility degrees in terms of yield and quality in previous works performed by this research group and different graft combinations with known graft compatibility as controls: eggplant grafted on *S. torvum* and pepper homografts (high compatibility), pepper grafted on *S. torvum* and pepper grafted on tomato like incompatibles. Many repair mechanisms at the graft area can be supported by photosynthetic activity; an increase in photochemical processes can help to facilitate the graft union. The best graft union showed a higher Fv/Fm values associated with higher values of photosynthetic induction processes (Φ_{PSII} and qP) as well as with vascular regeneration across the graft interface. The results showed that CFI monitoring changes in photosynthesis ways reflect histological behaviour measurements in grafted pepper plants. CFI can be used to evaluate graft compatibility at early stages of development as a prediction method of studying (in) compatibility in grafted plants; anticipating quickly future incompatibility problems in the field.

Keywords: callus cells, chlorophyll fluorescence imaging, graft (in)-compatibility, pepper, photochemical quenching, vascular connections

7.2. INTRODUCTION

Grafting can be defined as the natural or deliberate fusion of plant parts so that vascular continuity is established between them and the resulting genetically composite organism functions as a single plant (Mudge and Janick, 2009). Grafting is a technique that has been widely used for centuries in woody plants. Nowadays, this technique is being greatly expanding in vegetables plants particularly in Solanaceae and Cucurbitaceae families, to reduce pathogens infections (Biles et al., 1989; Padgett and Morrison, 1990) or to increase resistance to abiotic stresses, such as drought (Penella et al., 2014a,b,c; Sánchez-Rodríguez et al., 2013), salinity (Orsini et al., 2013; Penella et al. 2015), or heavy metals (Savvas et al., 2010). This is also used to enhance nutrient uptake (Ruiz et al., 1997) or to increase yields and fruit quality (Penella et al., 2013; Rouphael et al., 2010).

During the graft union formation between rootstock and scion, many researchers have observed callus proliferation (from both the rootstock and the scion), callus bridge formation, differentiation of cambium tissue from callus cells and the production of secondary xylem and phloem (Aloni et al., 2010; Hartmann et al., 2002; Pina and Errea, 2005). A low or incorrect callus formation between the rootstock and scion could lead to defoliation, reduction of scion growth and low survival of grafted plants (Johkan et al., 2009; Kawaguchi et al., 2008) reducing water flow to shoots (decreased hydraulic conductance) (Martínez-Ballesta et al., 2010).

There is no precise definition of graft compatibility and generally means the establishment of a successful graft union as well as extended survival and proper functioning of the composite rootstock-scion (Goldschmidt, 2014). Graft incompatibility may be defined as failure to form a successful graft union. A lack

of, or decrease in number of differentiated vascular bundles, or the dysfunction of differentiated vascular bundles at the graft union has been reported to inhibit transport of nutrients to scion (Schöning and Kollmann, 1997; Wang and Kollmann, 1996). Characterization of incompatibility is not a simple process because graft combinations can initially unite with apparent success, but gradually develop incompatibility symptoms with time, due to either alimited and/or not fully functional vascular reconnection between scion and rootstock at the graft interface which causes the subsequent failure of the graft union (Errea et al., 1994, 2001) or the development of abnormal growth patterns (Kawaguchi et al., 2008).

The major causes implicated in graft incompatibility in Solanaceous crops are anatomical and/or biochemical (Deloire and Hébant, 1982; Ives et al., 2012).

Pepper (Capsicum annuum) is grown in most countries of the world, with 1.93 million of ha cultivated area and is one of the most important crops in Mediterranean area. Grafted pepper plants are used to cope with biotic and abiotic stresses. Peppers have been described as compatible only with other Capsicum species but not with all of them. In this sense, Otsuka (1957) reported that tomato/pepper or pepper/tomato graft combinations were completely incompatible because plant growth was severely suppressed, in contrast with other Solanaceae species like tomato or eggplant, which are able to be grafted onto some different species within their family (Ives et al. 2012, Deloire et al., 1982, Kawaguchi et al. 2008; Miguel et al., 2007).

The first methods used to predict graft incompatibility relied on external symptoms such as swollen union, death or decline in vegetative growth and vigour of the scion, and marked differences in growth of both scion and rootstock (Otsuka, 1957). Afterwards, physiological and anatomical methods for

the diagnosis of graft (in)-compatibility have been developed, such as the measurement of peroxidase and catalase concentrations as the enzymes implicated in graft development (Fernandez-Garcia et al., 2004); the hormone levels (Yin et al., 2012); ROS production (Irisarri et al., 2015); accumulation of sugars (Kawaguchi et al. 2008), hydraulic root conductivity (Clearwater et al., 2004) or histological measurements (Pina et al., 2012). However, all these methods are invasive (destructive), slow and/or most of them are thought to woody plants.

The use of X-ray tomography to visualize the 3D structure of the graft union (Milien et al., 2012) is a non-destructive method to evaluate internal structure in the graft area, but the potential impact of the ionizing effects of the X-rays on the living tissue can be negative, as it has been demonstrated in a growth inhibited Arabidopsis seedling (Dhondt et al., 2010) and consequently has to be considered.

Another non-destructive method without effects on the plant tissues and on the subsequent development of the plant is the use of the chlorophyll fluorescence imaging (CFI). CFI has been used to predict compatibility in melon-grafted plants (Calatayud et al., 2013). The method of CFI is based on the hypothesis that grafting causes stress in plants: mechanical wounding in scion and rootstocks result in localized cell deaths, loss of water and solute, and disruption of the vascular system. The activation repair mechanisms requires a high metabolic demand of the plant in the grafting area, as it needs to supply carbon skeletons, synthesis of new molecules or increasing antioxidant enzymes activity. As many of these processes can be supported by photosynthetic activity and changes in photosynthesis are associated to variations in fluorescence parameters, the use of images for monitoring

florescence parameters will allow visualize possible alterations in grafted plants. This could be an intuitive, quick and non-invasive method providing detailed information on spatial and temporal heterogeneity. The potential of having a rapid and non-destructive method to diagnose compatibility in grafted plants could be of great economic importance in the seedling production industry.

As mentioned before, CFI has been used with success to predict compatibility in melon graft plants, where Fv/Fm ratio, associated with maximal quantum yield of PSII, was identified as a sensitive chlorophyll fluorescence parameter useful to distinguish compatible from incompatible graft unions. Nevertheless, CFI`s advantages have not been tested with other plant species yet.

The aim of this work was to evaluate the potential of CFI to predict compatibility/incompatibility in different pepper plant combinations using positive controls (pepper grafted onto pepper) and negative controls (tomato/pepper and eggplant/pepper), connecting values of CFI parameters to histological studies in order to demonstrate if CFI can reflect the morphological and anatomical development at the graft interface between both graft partners in pepper.

In order to establish this correlation, the commercial pepper cultivar ‘Adige’ and the different Capsicum sp accessions typified with different compatibility degrees in terms of yield and quality in previous works performed by this research group (Penella et al. 2013, Penella et al. 2014a, c, Penella et al. 2015). All the tested accessions were highly or moderately resistant to salt or drought stress (Penella et al. 2013; 2014a,b,c; 2015). In this study, we also used different graft combinations with known graft compatibility as controls: eggplant grafted on S. torvum and pepper homografts (high compatibility), pepper grafted on S. torvum and pepper grafted on tomato like incompatibilities.

7.3. MATERIAL AND METHODS

7.3.1. Plant materials and grafting plants

In this study, a total of nine combinations of plants were evaluated for graft compatibility. Cultivar "Adige" *Capsicum annuum* L. (Lamuyo type; Sakata) (code A), was grafted onto the accessions of *C. annuum* L. (code A25 and code A5), *Capsicum chinense* Jacq. (code C12), *Capsicum baccatum* L. var. *pendulum* (code B14) used in previous studies on physiological and agronomical responses that showed different compatibility degree (Penella et al., 2013, 2014a, c, 2015). In addition, A cultivar was grafted onto commercial rootstocks *Solanum torvum* Sw. "Torvum vigor" (Ramiro Arnedo) (code ST) and onto *L. esculentum* x *L. hirsutum* "Beaufort" (De Ruiter Seeds) (code TOM) described in the bibliography as incompatible (Kawaguchi et al., 2008). Besides tomato var. Gordal (Mascarell seeds) was grafted on ST (ST/TOM), this combination has been described as moderately incompatible (Kawaguchi et al., 2008). *Solanum melongena* L. eggplant "Cristal" (semillas Fitó) (code EGG) was also grafted onto ST (ST/EGG) and self-grafted plants of 'Adige' (A/A) were used as positive controls (Table 1).

Plants were sown on 15th January 2014 in 104-cell polystyrene trays filled with peat-based substrate and kept under a Venlo-type glasshouse. The plants were transplanted to 54-cell trays. The different graft combinations were performed on 21 March using the tube grafting method (cutting the growing tip of the rootstock at a 45º angle above the cotyledons, and fixing the rootstock and scion with a clip) (Penella et al., 2013).

Table 1. Plant combinations and their code used in histological and chlorophyll fluorescence measurements

Rootstock (code)	Scion	Graft plant
C. annuum L. var. Adige (A)	Adige (A)	A/A
C. annuum (A25)	Pepper var. Adige (A)	A25/A
C. annuum (A5)	Pepper var. Adige (A)	A5/A
C. *baccatum* (B14)	Pepper var. Adige (A)	B14/A
C. *chinense* (C12)	Pepper var. Adige (A)	C12/A
S. torvum (ST)	Eggplant var. Cristal (EGG)	ST/EGG
S. torvum (ST)	Pepper var. Adige (A)	ST/A
S. torvum (ST)	Tomato var. Gordal (TOM)	ST/TOM
Tomato Beaufort (BEU)	Adige (A)	BEU/A

7.3.2. Light microscopy

The graft interfaces were fixed 30 days after grafting (DAG) in 3% glutaraldehyde in 50 mM Sorensen buffer (28.5% KH_2PO4 50 mM and 71.5 % Na_2HPO4 mM.) at pH 7.2 for 2 h. After that, plant material was washed four times during 15 min in the same buffer. After infiltration in LR white resin:ethanol (1:2 v/v, 1:1 v/v, 2:1 v/v) for 60 min per stage, the specimens were embedded in historesin LR white overnight (London Resin Co., Woking, Surrey,

UK) at 4 ºC according to Tadeo et al. (1997), and transversally sectioned at 2 µm using glass knives in a Leica RM 2165 Rotary Microtome (Leica Instruments, Heidelberg, Germany). The sections were stained in 0.05% toluidine blue 0 (Cl 52040, Merck, Darmstad, Germany) (O'Brien and McCully, 1981), desiccated and mounted in Eukitt Mounting Medium 15322 (Electron Microscopy Sciences, Hatfield, PA, USA). Representative sections of three tissue samples per plant from ten plants were viewed under a Leitz Ortholux II fluorescence microscope (Leitz, Wetzal, Germany) operating in an optical mode and the images were captured with a Leica DC300 camera.

7.3.3. Chlorophyll fluorescence imaging

CFI measurements of grafted plants were performed 30 DAG from 15-20 plants per combination at 2 cm above and below the graft interface and the graft interface using an imaging-PAM fluorometer (Walz, Effeltrich, Germany). All plants were placed in the dark for 10 min prior to measurement. Images and values of minimum Chl fluorescence yield in the dark-adapted state, Fo, were determined using light pulses at low frequency (1 Hz). Maximum fluorescence Fm was determined by applying a blue saturation pulse (10 Hz). The maximum quantum yield of PSII photochemistry (Fv/Fm ratio) was determined as Fm-Fo/Fm and images were captured. Actinic illumination (260 µmol m^{-2} s^{-1}) was then switched on and saturating pulses were applied at 20 s intervals for 5 min to determine F`m and Chl fluorescence during actinic illumination (Fs). The actual quantum efficiency of PSII photochemistry ($\phi_{PSII}$= (F`m-Fs)/F`m) (Genty et al., 1989), photochemical quenching (qP= (F`m-Fs)/(F`m-F`o) (Schreiber et al., 1986) and the non-photochemical quenching (NPQ= F`m-Fs/F`m) (Bilger

and Björkman, 1991) were calculated. The value of F`o was estimated using the approximation of Oxborough and Baker (1997), F`o = Fo/(Fv/Fm + Fo/F`m). The PAM-software selects areas in the fluorescence image for each plant. Three areas in stem of the plants (graft area, the rootstock and the scion) were selected. Fluorescence parameter values of all pixels within each area were averaged. Each value in the tables is the mean of the corresponding area of all samples (obtained from 15-20 different plants). Figure 2 shows the images of only a single plant (representative plant). Further information on CFI measurements can be obtained from (Calatayud et al., 2008, 2013) .

7.3.4. Statistical analysis

One-way ANOVA was performed (Statgraphics Centurion XVI for Windows, Statistical Graphics Corp.) to compare the means of the fluorescence parameters. Mean separations were performed when significant differences were found using the least significance difference at $P<0.05$.

7.4. RESULTS AND DISCUSSION

7.4.1. Histological evaluation of scion/rootstock interactions

Table 1 summarizes the plant codes used for histological and CFI studies. Pepper homo-grafting (A/A) and the use of the intra-specific grafts (rootstock and scion belonging to the same botanical species) rootstocks B14, C12 and

A25 showed a higher yield (Penella et al., 2014a, c; Penella 2015). Whereas, the intra-specific combination ‘Adige’ grafted onto the rootstock A5 (A5/A) had a lower growth than other grafted plants (A/B14, A/C12 and A/A25) and its stem diameter at the graft union was approximately three-fold greatest and provided lower fruit yields (Penella et al., 2013, 2014a).

The cellular events that led to a successful graft union include adhesion of the two graft partners, callus cell proliferation at the graft interface and cross-bridge formation of the vascular bundle to establish a functional vascular connection (reviewed by (Aloni et al., 2010; Goldschmidt, 2014; Mudge and Janick, 2009; Pina and Errea, 2005). Nevertheless, incomplete or non-functional vascular connections impede the vital upward and downward whole plant transfer routesm, which might result in a dieback of the graft. By 30 DAG, a well developed vascular graft union was observed in the pepper homografts (A/A) and intraspecific heterografts eggplant grafted on *Solanum torvum* (ST/EGG) (Fig. 1A and 1B) and ‘Adige’ grafted in the pepper rootstock accessions A25 and C12 (Fig. 1C, D). In these combinations, most of the necrotic layer was absorbed at this stage and group of small callus cells are clustering resembling symplastic domains which is a prerequisite to begin more vascular differentiation (Pina et al., 2009). Higher levels of vascular differentiation were observed in the combination A25/A (Fig. 1C) than in the combination C12/A (Fig 1. 1D). In all combinations, cluster of callus cells were associated with the cut ends of the xylem from which them were derived and filled the graft interface. ‘Adige’ grafted on rootstock accession B14/A showed a high cellular activity at the graft interface and callus cells bridging the two graft partners (Fig. 1E). Some developing tracheid elements were observed but not completely new xylem and phloem formation was displayed across the graft union 30 DAG. Similar anatomical results were obtained when grafted tomato onto *Solanum*

torvum (ST/TOM) (more distantly taxonomic species) (Fig. 1F), indicating that the compatibility behaviour of both graft combinations (B14/A and ST/TOM) was similar, moderately compatible, as reported by Kawaguchi et al. 2008 for ST/TOM.

A stronger level of graft incompatibility was observed when pepper cv. 'Adige' was grafted onto rootstock accession A5 (A5/A). In this case, histological examination provided clear evidence of discontinuous xylem elements in the graft union as well as large areas of unbroken necrotic lines along the wounded edges of the rootstock and the scion (Fig. 1G). This result was consistent with the anatomy of the severely incompatible union tomato/pepper (BEU/A) (Fig 1H). In addition, 'Adige' grafted on *Solanum torvum* (ST/A) produced weak unions, characterized by limited fusion between both graft partners (Fig. 1I) and the presence of cells enriched with green material inside the vacuoles similar to phenolic compounds, that are involved in the incompatibility reaction inhibiting division, development and differentiation into new tissues during the graft union formation (Errea, 1998; Hudina et al., 2014; Pina et al., 2012).

In these three combinations A5/A, BEU/A and ST/A, the rootstock and scion tissue produced new vascular elements as well, but these did not cross the scion/rootstock border and therefore no graft union was formed. In incompatible heterografts between Arabidopsis grafted on tomato rootstock, it was reported that the remaining necrotic layer that developed at the graft interface seemed to inhibit the differentiation of vascular tissue across the graft union, either directly or indirectly, and thus prevented full vascular graft union formation between the two plants, since neither vascular bridge nor full graft union was visible (Flaishman et al., 2008). Other studies also reported the presence of narrow and irregular xylem elements in incompatible tomato/pepper heterografts (Ives et al., 2012, Kawaguchi et al., 2008).

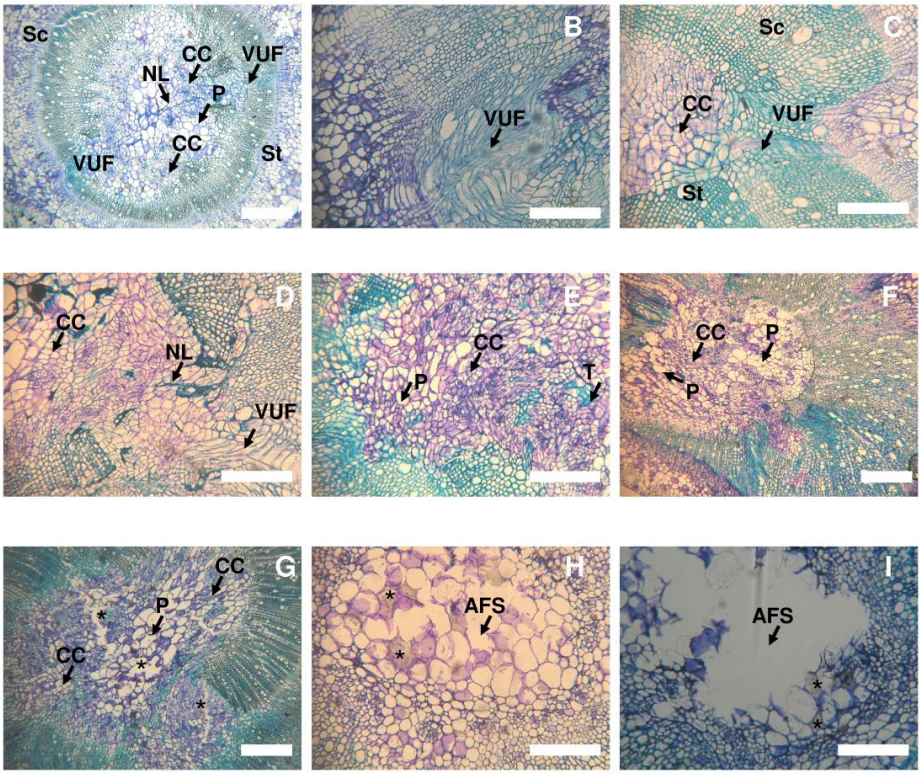


Figure 1. Transversal sections of different graft combinations 30 days after grafting. A) 'Adige' grafted on 'Adige', (A/A). B) Eggplant grafted on '*Solanum torvum'*, ST/EGG. C) 'Adige' grafted on the rootstock accession 'A25', A25/A. D) 'Adige' grafted on the rootstock accession 'C12', C12/A. E) 'Adige' grafted on the rootstock accession 'B14', B14/A. F) Tomate grafted on '*Solanum torvum'*, ST/TOM. G) 'Adige' grafted on the rootstock accession A5, A5/A. Asterisks (*) show limited fusion between both graft partners. H) 'Adige' grafted on the tomato rootstock Beaufort, BEU/A. Asterisks represent phenols stained green into the vacuoles. I) 'Adige' grafted on *Solanum torvum*, ST/A. Bars= 200 µm (A, F, G) and 400 µm (B, C, D, E, H and I). Abbreviations: VUF: vascular union formation, T: traqueid elements; St: stock, Sc: Scion; NL: necrotic layer; CC: cluster of callus cells; P: pith cells; AFS: air filled space

7.4.2. Chlorophyll fluorescence imaging in grafted plants

The same grafted plants combinations (Table 1) used for histological evaluation were analysed by CFI.

In table 2, the mean values of Fv/Fm ratio for rootstock, scion and graft area of the nine plant combinations are shown. The Fv/Fm is one of the most common fluorescence parameter, as it is an indicator of plant stress (Rolfe and Scholes, 2010) and reflects the maximal efficiency of excitation capture of dark-adapted plants and is correlated with the number of functional PSII reaction centers (Oquist and Chow, 1992). Attending to Fv/Fm values in the rootstock area, four groups of plants can be distinguished according to ANOVA analyse: A/A, A25/A, B14/A, C12/A, ST/TOM and ST/EGG showed the higher Fv/Fm values, A5/A with intermediate value, followed of the combination BEU/A and with lower Fv/Fm value ST/A. In compatible tomato grafted plants observations of the structure of graft union showed formation of xylem and phloem vessels through the graft union 8 days after grafting (Fernández-García et al., 2004). But narrow and irregular connections were observed in graft union between incompatible graft plants as tomato/pepper or pepper/tomato 3 weeks after grafting (Kawaguchi et al., 2008). CFI measurements were performed at 30 days after grafting, therefore the anatomical symptoms associated with graft (in)-compatibility has been already internally manifested. The lower Fv/Fm ratio in rootstocks areas have been measured in incompatible heterografted plants BEU/A and ST/A. As reported by the histological study, a weak graft connection occurs in these plants combinations, in such a way that it is expected that the translocation of assimilate from scion to the rootstock result in higher carbohydrate concentration in the scion part and lower concentration in the rootstocks (Kawaguchi et al., 2008). A limited assimilate supply to the rootstocks could reduce the size of root system and decreased metabolic

activity increasing damage to the photosynthetic apparatus and decreasing Fv/Fm in rootstock area. Likewise, the Fv/Fm values in the graft area followed the same tendency showed by the rootstock area, but the values underwent an important decrease for the incompatible grafts ST/A and BEU/A. It is probably a consequence of the weak connection between rootstocks (*S. torvum* and tomato) and the scion in the graft area since similar results were obtained in less compatible melon grafted plants (Calatayud et al., 2013). A low or incorrect callus formation lead to a bad vascular connection at the rootstock-scion graft interface affecting water and nutrient translocation that can alter the photosynthesis behaviour in the graft zone (Martínez-Ballesta et al., 2010). For this reason, Fv/Fm values decreased to a greater extent compared with rootstocks values. These insufficient connections of vascular bundles were reflected in the scion part with lowest Fv/Fm values in ST/A and BEU/A (Table 2). Fv/Fm images of representative's samples (Fig. 2) allowed visualize the rootstock, graft and the scion areas, indicating that the technique is able to display large areas of graft zones. These results are in agreement with the observations reported in melon grafted plants (Calatayud et al., 2013). The observation of color changes (ranging from black (0.000) to pink (1.000) revealed spatial changes in the Fv/Fm images. In A/A, A25/A, B14/A, C12/A, ST/TOM and ST/EGG different intensities of blue colors were observed associated with higher values of Fv/Fm. In A/A5 a black line was observed across graft area-scion indicating a null Fv/Fm values. A dramatic change in colors from blue-green and brown of Fv/Fm in ST/A and BEU/A were observed, that correspond with lower Fv/Fm values. It should be noted that the scion area in ST/A and BEU/A showed the colors green and brown associated with lowest Fv/Fm values.

Table 2. . Fv/Fm values for different plants combinations in the areas of the rootstock, graft zone and scion

Plant combination	Fv/Fm rootstock	Fv/Fm graft area	Fv/Fm scion
A/A	0.760a	0.746a	0.760a
A25/A	0.781a	0.774a	0.779a
B14/A	0.791a	0.770a	0.774a
C12/A	0.807a	0.753a	0.757a
A5/A	0.754ab	0.723b	0.709b
ST/EGG	0.782a	0.770a	0.760a
ST/A	0.675c	0.233d	0.306d
ST/TOM	0.788a	0.769a	0.757a
BEU/A	0.713b	0.633c	0.453c

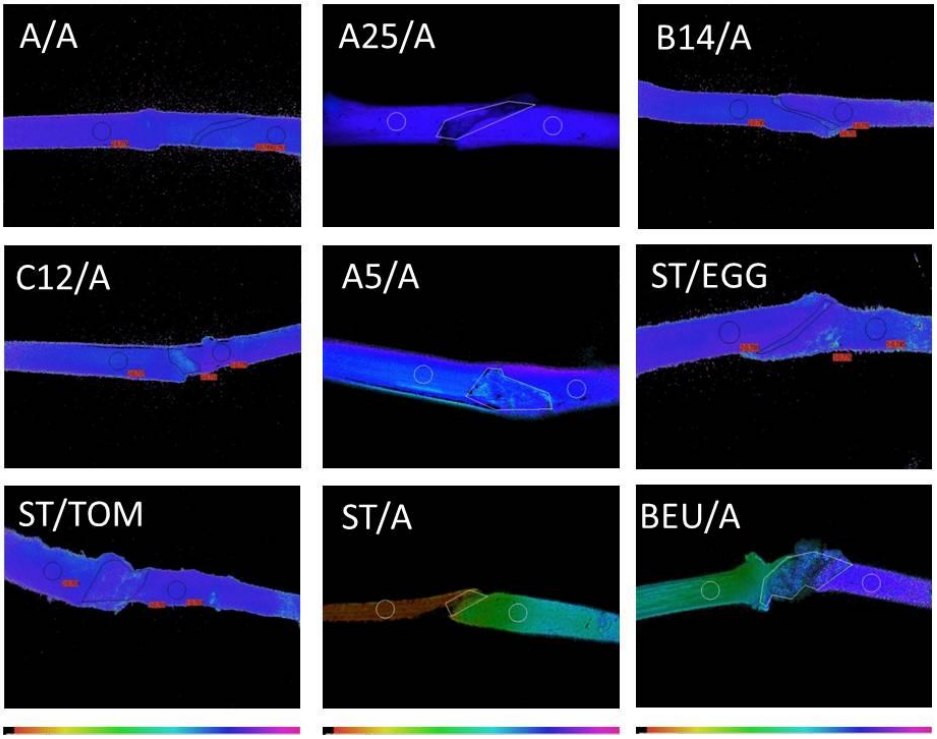


Figure 2. Chlorophyll fluorescence images of Fv/Fm after dark-adapted 30 days after grafting in different plant combinations: 'Adige' grafted on Adige, (A/A). 'Adige' grafted on the rootstock accession A25, A25/A. 'Adige' grafted on the rootstock accession B14, B14/A. 'Adige' grafted on the rootstock accession C12, C12/A. 'Adige' grafted on the rootstock accession A5, A5/A. Eggplant grafted on *Solanum torvum*, ST/EGG. Tomato grafted on *Solanum torvum*, ST/TOM. 'Adige' grafted on *Solanum torvum*, ST/A. 'Adige' grafted on the tomato rootstock 'Beaufort', BEU/A. The false colour code depicted at the bottom of each image ranges from 0.000 (black) to 1.000 (pink). Images were taken from a single plant.

When Fv/Fm values were compared at the scion from the different graft unions, the decrease in incompatible unions were more marked. Four categories could also be well definited: compatible plants (A/A, A25/A, B14/A, C12/A, ST/TOM and ST/EGG), moderate compatible A5/A and incompatible TOM/A and strong incompatible ST/A. If a weak graft connection occurs in A5/A, TOM/A and ST/A, the probability of nutrient uptake reaching the scion decrease, leading to alteration of PSII photochemistry (Calatayud et al., 2013). In order to study the cause of this noticeable decline in Fv/Fm at the scion area, we analysed their photochemical and non-photochemical processes (Table 3). Photochemical statistical analysis allowed differentiating four groups: A/A, A25/A and ST/EGG with higher values of Φ_{PSII} and q_P; B14/A, C12/A and ST/TOM with moderate decrease of photochemical processes; A5/A with considerable decrease and the last group with the plant combinations ST/A and BEU/A with the lowest photochemical values. The decrease in Fv/Fm for the graft combinations A5/A, BEU/A and ST/A (Table 2) could be the result of an increase in protective non-radiative energy dissipation, photodamage of PSII centres or both (Osmond, 1994). Inasmuch as NPQ is believed to indicate the capacity for photoprotective process (Osmond, 1994), the decline in Fv/Fm ratio was attributable to PSII stress, because NPQ was adversely affected in scion areas for the three plant combinations (Table 3). In severely damaged tissues resulted in a decreased in NPQ values (Berger et al., 2007). In addition, the lower qP levels (Table 3) in these plant combinations compared with compatible union indicate that the capacity for reoxidizing Q_A decrease, increased excitation pressure on PSII and contributed to the closure of PSII reaction centres. Closed PSII centres may cause an increase in Q_A pool fully reduced and deny the possibility of electron transport to PSI and beyond (Seaton and Walker, 1990). According with this result, the Φ_{PSII}, correlated with the quantum yield of non-cyclic electron transport (Genty et al., 1989), and was markedly reduced mainly in ST/A and

BEU/A (Table 3). This decrease in the photochemical processes seems reasonable to attribute that the ATP and NADPH might be considered reduced in these plant combinations. This reflect that a low or incorrect callus formation (Fig. 1) affected vascular connection in the rootstock/scion interface and may determine a decrease in water and nutrient translocation (Martínez-Ballesta et al., 2010) affecting photosynthesis performance limiting the availability of assimilate for plant growth.

A higher Φ_{PSII} and q_P in scion area in compatible and moderate compatibility grafted plants A/A, A25/A, B14/A, C12/A, ST/TOM and ST/EGG could be related with a greater supply of carbohydrates (Quilliam et al., 2006), development of defence reactions (Guidi et al., 2007) or associated to sink metabolism in this area surrounding of wound provoked by grafted (Calatayud et al., 2013). This increase in photochemical process can feed the new connections formation at the graft interface. Associated with an electron flow stimulated (Φ_{PSII}), NPQ increased as a protection mechanism in these plant combinations (Berger et al., 2007).

7.4.3. Connecting values of CFI parameters to histological studie

CFI and histological observations allowed distinguishing different compatibility behaviour in our nine combinations through its fluorescence parameters and the anatomical changes. Plant combinations with higher Fv/Fm values associated with higher values of photosynthetic induction processes (Φ_{PSII} and qP) were comparable to highest cellular activity and vascular regeneration across the graft interface indicating a higher compatibility. Evidences of this high

relationship between CFI and anatomical observations can be a source metabolism in the vicinity of the graft area (scion). These cells with a greater capacity for carbohydrate synthesis would contain higher concentrations of Calvin cycle allowing a rapid induction of $\Phi_{PSII.}$ The graft area can act as a sink of carbohydrates for restoring the adhesion of both graft partners, incite callus cell proliferation and bring with success the functional vascular connection.

In general terms, under histological point of view and CFI values, three plants combinations groups could be set up: A/A, ST/EGG and A25/A with highest compatibility, expressed as higher Φ_{PSII} and qP and high cellular activity. ST/TOM and B14/A can be definite as moderate compatibility with intermedia values of CFI and not completely new xylem and phloem formation. C12/A displayed a better compatibility than B14/A under histological studies but moderate compatibility in terms of CFI values. The third group was the incompatible plants combinations, A5/A, BEU/A and ST/A with a lowest Φ_{PSII} and qP and discontinuous xylem elements and unbroken necrotic lines.

Anatomical and CFI observations provided clear evidences that the graft combination A25/A showed the highest vascular regeneration across the graft interface. Therefore, A25 is highly recommended for grafting pepper cultivars in this salt and hydric tolerant rootstock, that facilitate the free movement of water and solutes across the graft interface.

7.5. CONCLUSIONS

CFI provided information on graft stage and represents a quick and non-invasive technique for screening (in)-compatibility union in vegetables. The

main interest of CFI methods is associated with the image that permits large areas of graft zones to be viewed. One practical advantage of CFI is that not requires sample preparation, is not destructive or invasive. In addition, CFI allows evaluate the graft union development on the same graft plant overtime, and anticipate the incompatibility problems between new tested rootstock/scion combinations. CFI monitoring changes in photosynthesis ways reflect histological behaviour measurements in grafted pepper plants. However, CFI does not replace systematically classical histology in terms of understanding morphological and anatomical developments at the graft interface.

7.6. REFERENCES

Aloni, B., Cohen, R., Karni, L., Aktas, H., and Edelstein, M. (2010). Hormonal signaling in rootstock-scion interactions. *Sci. Hortic*. 127, 119–126.

Berger, S., Benediktyová, Z., Matous, K., Bonfig, K., Mueller, M. J., Nedbal, L., and Roistsch, T. (2007). Visualization of dynamics of plant-pathogen interaction by novel combination of chlorophyll fluorescence imaging and statistical analysis: differential effects of virulent and avirulent strains of *P. syringae* and of oxylipins on *A. thaliana*. *J. Exp. Bot.* 58, 797–806. doi:10.1093/jxb/erl208.

Biles, C., Martyn, R., and Wilson, H. (1989). Isoenzymes and general proteins from various watermelon cultivars and tissue types. *HortSci.* 24, 810–812.

Bilger, W., and Björkman, O. (1991). Temperature dependence of violaxanthin de-epoxidation and non-photochemical fluorescence quenching in intact leaves of *Gossypium hirsutum* L. and *Malva parviflora* L. *Planta* 184, 226–234. doi:10.1007/BF01102422.

Calatayud, Á., Gorbe, E., Roca, D., and Martínez, P. F. (2008). Effect of two nutrient solution temperatures on nitrate uptake, nitrate reductase activity, NH4+ concentration and chlorophyll a fluorescence in rose plants. *Environ. Exp. Bot.* 64, 65–74. doi:http://dx.doi.org/10.1016/j.envexpbot.2008.02.003.

Calatayud, Á., San Bautista, A., Pascual, B., Maroto, J. V., and López-Galarza, S. (2013). Use of chlorophyll fluorescence imaging as diagnostic technique to predict compatibility in melon graft. *Sci. Hortic*. 149, 13–18. doi:10.1016/j.scienta.2012.04.019.

Clearwater, M. J., Lowe, R. G., Hofstee, B. J., Barclay, C., Mandemaker, A. J., and Blattmann, P. (2004). Hydraulic conductance and rootstock effects in grafted vines of kiwifruit. *J. Exp. Bot*. 55, 1371–82. doi:10.1093/jxb/erh137.

Deloire, A., and Hébant, C. (1982). Peroxidase activity and lignification at the interface between stock and scion of compatible and incompatible grafts of Capsicum on Lycopersicum. Ann. Bot. 49, 887–891.

Dhondt, S., Vanhaeren, H., Van Loo, D., Cnudde, V., and Inzé, D. (2010). Plant structure visualization by high-resolution X-ray computed tomography. *Trends Plant Sci.* 15, 419–22. doi:10.1016/j.tplants.2010.05.002.

Errea, P. (1998). Implications of phenolic compounds in graft incompatibility in fruit tree species. Sci. Hortic. 74, 195–205. doi:10.1016/S0304-4238(98)00087-9.

Errea, P., Felipe, A., and Herrero, M. (1994). Graft establishment between compatible and incompatible Prunus spp. *J. Exp. Bot.* 45, 393–401. doi:10.1093/jxb/45.3.393.

Errea, P., Garay, L., and Marín, J. A. (2001). Early detection of graft incompatibility in apricot (*Prunus armeniaca*) using in vitro techniques. *Physiol. Plant.* 112, 135–141.

Fernández-García, N., Carvajal, M., and Olmos, E. (2004). Graft union formation in tomato plants: peroxidase and catalase involvement. *Ann. Bot*. 93, 53–60. doi:10.1093/aob/mch014.

Flaishman, M. a., Loginovsky, K., Golobowich, S., and Lev-Yadun, S. (2008). *Arabidopsis thaliana* as a model system for graft union development in

homografts and heterografts. *J. Plant Growth Regul*. 27, 231–239. doi:10.1007/s00344-008-9050-y.

Genty, B., Briantais, J.-M., and Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990, 87–92. doi:http://dx.doi.org/10.1016/S0304-4165(89)80016-9.

Goldschmidt, E. E. (2014). Plant grafting: new mechanisms, evolutionary implications. *Front. Plant Sci*. 5, 727. doi:10.3389/fpls.2014.00727.

Guidi, L., Mori, S., Degl'Innocenti, E., and Pecchia, S. (2007). Effects of ozone exposure or fungal pathogen on white lupin leaves as determined by imaging of chlorophyll a fluorescence. *Plant Physiol. Biochem*. 45, 851–857. doi:http://dx.doi.org/10.1016/j.plaphy.2007.07.001.

Hartmann, H., Kester, D., Davies, F., and Geneve, R. (2002). Plant propagation. Principles and Practices. Seven Edition. Upper Saddle River, NJ: Prentice Hall.

Hudina, M., Orazem, P., Jakopic, J., and Stampar, F. (2014). The phenolic content and its involvement in the graft incompatibility process of various pear rootstocks (*Pyrus communis* L.). *J. Plant Physiol*. 171, 76–84. doi:10.1016/j.jplph.2013.10.022.

Irisarri, P., Binczycki, P., Errea, P., Martens, H. J., and Pina, A. (2015). Oxidative stress associated with rootstock-scion interactions in pear/quince combinations during early stages of graft development. *J. Plant Physiol.* 176, 25–35. doi:10.1016/j.jplph.2014.10.015.

Ives, L., Brathwaite, R., Barclay, G., Isaac, W. A., and Connor, C. B. (2012). Graft Compatibility of Scotch Bonnet (*Capsicum chinense* Jacq) with selected salt-tolerant Solanaceous. *J. Agric. Sci. Tech*. 2, 81–92.

Johkan, M., Mitukuri, K., Yamasaki, S., Mori, G., and Oda, M. (2009). Causes of defoliation and low survival rate of grafted sweet pepper plants. *Sci.Hortic*. 119, 103–107. doi:10.1016/j.scienta.2008.07.015.

Kawaguchi, M., Taji, A., Backhouse, D., and Oda, M. (2008). Anatomy and physiology of graft incompatibility in solanaceous plants. *J. Hortic. Sci. Biotechnol.* 83, 581–588.

Martínez-Ballesta, M. C., Alcaraz-López, C., Muries, B., Mota-Cadenas, C., and Carvajal, M. (2010). Physiological aspects of rootstock–scion interactions. *Sci. Hortic.* 127, 112–118. doi:10.1016/j.scienta.2010.08.002.

Miguel, A., De la Torre, F., Baixauli, C., Maroto, J. V., Jordá, M. C., López, M. M. and García-Jiménez, J. (2007). Injerto de hortalizas. Ministerio de Agricultura, Pesca y Alimentación y Fundación Ruralcaja. Valencia. Spain, 63 p.p.

Milien, M., Renault-Spilmont, A.-S., Cookson, S. J., Sarrazin, A., and Verdeil, J.L. (2012). Visualization of the 3D structure of the graft union of grapevine using X-ray tomography. *Sci. Hortic*. 144, 130–140. doi:10.1016/j.scienta.2012.06.045.

Mudge K., Janick J., Scofield S., and Goldschmidt E. E. (2009). A history of grafting. *Hortic. Rev*. doi:10.1002/9780470593776.ch9.

O'Brien, T., and McCully, M. (1981). The study of plant structure: principles and selected methods. Termarcarphi Pty. Ltd., Melbourne.

Oquist, G., and Chow, W. S. (1992). On the relationship between the quantum yield of Photosystem II electron transport, as determined by chlorophyll fluorescence and the quantum yield of CO_2-dependent O_2 evolution. *Photosynth. Res*. 33, 51–62. doi:10.1007/BF00032982.

Orsini, F., Sanoubar, R., Oztekin, G. B., Kappel, N., Tepecik, M., Quacquarelli, C., Tuzel Y., Bona, S., and Gianquinto G. (2013). Improved stomatal regulation and ion partitioning boosts salt tolerance in grafted melon. *Funct. Plant Biol.* 40, 628–636. doi:10.1071/FP12350.

Osmond, C. B. (1994). "What is photoinhibition? Some insights from comparisons of shade and sun plants" in *Photoinhibition of Photosynthesis*, ed N.R. Baker and J.R. Bowyer, (Bios Scientific Publishers, Oxford), 1-24.

Otsuka, K. (1957). Nutriophysiological study on grafted plants. Effects of rootstocks and scions on the growth and mineral accumulation in Solanacearum. *Jpn. J. Soil Sci. Plant Nutr.* 28: 285-289.

Oxborough, K., and Baker, N. R. (1997). An instrument capable of imaging chlorophyll *a* fluorescence from intact leaves at very low irradiance and at

cellular and subcellular levels of organization. Plant. Cell Environ. 20, 1473–1483. doi:10.1046/j.1365-3040.1997.d01-42.x.

Padgett, M., and Morrison, J. C. (1990). Changes in grape berry exudates during fruit development and their effect on mycelial growth of Botrytis cinerea. J. Am. Soc. Hortic. Sci. 115, 269–273.

Penella, C., Nebauer, S. G., Lopéz-Galarza, S., SanBautista, A., Gorbe, E., and Calatayud, A. (2013). Evaluation for salt stress tolerance of pepper genotypes to be used as rootstocks. J. Food, Agric. Environ. 11, 1101–1107.

Penella, C., Nebauer, S. G., López-Galarza, S., Bautista, A. S., Rodríguez-Burruezo, A., and Calatayud, A. (2014a). Evaluation of some pepper genotypes as rootstocks in water stress conditions. Hort. Sci. 41, 192–200.

Penella, C., Nebauer, S. G., Bautista, A. S., López-Galarza, S., and Calatayud, Á. (2014b). Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: Physiological responses. J. Plant Physiol. 171, 842–851. doi:10.1016/j.jplph.2014.01.013.

Penella, C., Nebauer, S. G., Bautista, A. S., López-Galarza, S., and Calatayud, Á. (2014c). Improving water stress resistance of pepper through grafting. Abstract retrieved from Abstract in Innovation in vegetable grafting for sustainability. 2nd COST action FA1204 congress, Carcavelos (Portugal), 44.

Penella, C., Nebauer, S. G., Quiñones, A., San Bautista, A., López-Galarza, S., and Calatayud, A. (2015). Some rootstocks improve pepper tolerance to mild salinity through ionic regulation. Plant Sci. 230, 12–22. doi:10.1016/j.plantsci.2014.10.007.

Pina, A., and Errea, P. (2005). A review of new advances in mechanism of graft compatibility-incompatibility. Sci. Hortic. 106, 1–11. doi:10.1016/j.scienta.2005.04.003.

Pina, A., Errea, P., and Martens, H. J. (2012). Graft union formation and cell-to-cell communication via plasmodesmata in compatible and incompatible stem unions of Prunus spp. Sci. Hortic. 143, 144–150. doi:10.1016/j.scienta.2012.06.017.

Pina, A., Errea, P., Schulz, A., and Martens, H. J. (2009). Cell-to-cell transport through plasmodesmata in tree callus cultures. Tree Physiol. 29, 809–818. doi:10.1093/treephys/tpp025.

Quilliam, R.S., Swarbrick, P.J., Scholes, J.D., and Rolfe, S.A. (2006) Imaging photosynthesis in wounded leaves of Arabidopsis thaliana. J. Exp. Bot. 57, 55-69. doi: 10.1093/jxb/erj039.

Rolfe, S. A., and Scholes, J. D. (2010). Chlorophyll fluorescence imaging of plant-pathogen interactions. Protoplasma 247, 163–75. doi:10.1007/s00709-010-0203-z.

Rouphael, Y., Schwarz, D., Krumbein, A., and Colla, G. (2010). Impact of grafting on product quality of fruit vegetables. Sci. Hortic. 127, 172–179. doi:10.1016/j.scienta.2010.09.001.

Ruiz, J. M., Belakbir, a., López-Cantarero, I., and Romero, L. (1997). Leaf-macronutrient content and yield in grafted melon plants. A model to evaluate the influence of rootstock genotype. Sci. Hortic. 71, 227–234. doi:10.1016/S0304-4238(97)00106-4.

Sánchez-Rodríguez, E., Romero, L., and Ruiz, J. M. (2013). Role of Grafting in Resistance to Water Stress in Tomato Plants: Ammonia Production and Assimilation. J. Plant Growth Regul. 32, 831-842 doi:10.1007/s00344-013-9348-2.

Savvas, D., Colla, G., Rouphael, Y., and Schwarz, D. (2010). Amelioration of heavy metal and nutrient stress in fruit vegetables by grafting. Sci. Hortic. 127, 156–161. doi:10.1016/j.scienta.2010.09.011.

Schöning, U., and Kollmann, R. (1997). Phloem translocation in regenerating in vitro- heterografts of different compatibility. J. Exp. Bot. 48, 289–295.

Schreiber, U., Schliwa, U., and Bilger, W. (1986). Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth. Res. 10, 51–62. doi:10.1007/BF00024185.

Seaton, G. G. R., and Walker, D. A. (1990). Chlorophyll Fluorescence as a Measure of Photosynthetic Carbon Assimilation. Proc. R. Soc. B Biol. Sci. 242, 29–35. doi:10.1098/rspb.1990.0099.

Tadeo, F. R., Gómez-Cadenas, A., Ben-Cheikh, W., Primo-Millo, E., and Talón, M. (1997). Gibberellin-ethylene interaction controls radial expansion in citrus roots. Planta 202, 370–378. doi:10.1007/s004250050139

Wang, Y., and Kollmann, R. (1996). Vascular Differentiation in the Graft Union of in-vitro Grafts with Different Compatibility. — Structural and Functional Aspects. J. Plant Physiol. 147, 521–533. doi:10.1016/S0176-1617(96)80041-1.

Yin, H., Yan, B., Sun, J., Jia, P., Zhang, Z., Yan, X., et al. (2012). Graft-union development: a delicate process that involves cell-cell communication between scion and stock for local auxin accumulation. J. Exp. Bot. 63, 4219–32. doi:10.1093/jxb/ers109

CHAPTER 8
General Discussion

8.1. GENERAL DISCUSSION

The productivity of several commercial pepper crops (mainly bell peppers) is limited by salinity stress and water scarcity in many areas of the world (Campos et al., 2014; Delfine et al., 2000; Kurunc et al., 2011). Grafting has been proposed as an interesting strategy that improves yields and quality of many crops under these stresses (Colla et al., 2010a; Lee and Oda, 2010; Schwarz et al., 2010). However, at the moment no commercial pepper rootstocks can confer abiotic stress tolerances to the scion grafted onto them. In fact, most studies performed on pepper grafted plants have been focused on both obtaining resistances to biotic stresses and obtaining yield increments, but few works have been done to study the effect of grafting on salt and water stress in pepper plants, despite it is known that grafted plants show tolerances to abiotic stresses and improve yields in other crops like tomato (Djidonou et al., 2013; Estañ et al., 2005; Sánchez-Rodríguez et al., 2013) or melon (Colla et al., 2010b; Edelstein et al., 2011; Orsini et al., 2013).

Therefore, the main objective of this PhD has been the understanding of mechanisms of tolerance to salt and water stresses of pepper accessions in order to use those tolerant as rootstock of pepper scions. The study cover different aspects: the acquisition of germplasm; the screening based on biomass and photosynthetic parameters; the agronomic tests of the selected accessions used as rootstocks in terms of vigor, yields and fruit quality; the characterization of the affinity/compatibility observed between some genotypes and the physiological and biochemical basis of these tolerances.

In this study accessions from the five major cultivated species of *Capsicum* (*C. annuum*, *C. chinense*, *C. baccatum*, *C. frutescens* and *C. pubescens*) have been studied and compared to commercially available rootstocks.

A new perspective for screening genotypes tolerant to abiotic stresses has been performed. It is known that photosynthesis is normally reduced when plants are under any case of stress (Bota et al., 2004; Chaves et al., 2002; Mittler, 2006). In our results, photosynthesis and stomatal conductance have been found as good indicator to distinguish between tolerant and sensitive genotypes to both, water and salt stress. This observation was confirmed by their biomass, as the latest is the most well-known effect of these stresses (Delfine et al., 2000; Nebauer et al., 2013; Perica et al., 2008), considering that a decline of A_N can be considered one of the main reason of growth reduction (Flexas et al., 2004; James et al., 2008; Long and Bernacchi, 2003). In fact, in our experiments grafted plants onto tolerant accessions an absence of negative effects on plant growth was realized, correlated with a maintenance of A_N. The ameliorative effect of grafting on plant's growth is in agreement with other findings in tomato and melon (Estañ et al., 2005; He et al., 2009; Martinez-Rodriguez et al., 2008; Santa-Cruz et al., 2002).

Some accessions belonging to *C. annuum*, *C. chinense* and *C. baccatum* have shown superiority against water scarcity and salinity. For these reasons, experiments were continued with these species. Moreover, some accessions behaved as tolerant to both stresses, ranking theirself as the most interesting plant material for further research. Few studies can be found in which a rootstock can tolerate both water scarcity and salinity, and mainly are done in woody plants, such as grapevine (Walker et al. 2002; Meggio et al. 2014) and citrus (García-Sánchez et al., 2007; Rewald et al., 2012; Syvertsen et al., 1988), finding only some in tomato (Albacete et al., 2015). None of the studied

accessions belong *C. frutescens* and *C. pubescens* shown tolerances to both water and salt stress screening experiments, being discarded in the next experiments.

Pepper yields of several pepper cultivars of different commercial types (“Adige”, Lamuyo type, “Lipari”, Italian type and “Verset”, California type) were generally increased when grafted onto the best genotypes selected (A25, C12 and B14). The occurrence of BER was the main cause of the unmarketability of the fruits in the tested cultivars. It should be noted that this increase is mainly due to a declining production of fruits affected by BER. In other words, total production of fruits generally was not increase but rather commercial fruits rate was higher because minor production losses were achieved. Moreover, the occurrence of BER not only depended on the rootstock but also on the scion used. By contrast, grafting did not increase yield in control conditions when C12 and B14 accessions were used, but A25 allowed a marketable yield increase under control conditions of 37%, and 118% under salt conditions compared with ungrafted plants in the same conditions.

Since many crops show different sensitiveness at different stages of their ontogeny, others may have a similar response among them. In that case, determining the response of the seeds in terms of the germination performance under salinity stress conditions would be useful to accelerate the screening process. The sensitivity or tolerance to salinity during the germination stage is species-dependent; many crops are vulnerable to stress during seed germination (Foolad and Lin, 1997), while others are relatively tolerant (Murillo-Amador et al., 2002). For these reasons, the selected genotypes were further studied under salt stress during the germination phase. In contrast to the observed photosynthesis behavior, during the vegetative and reproductive stage, germination rates observed were not representative of a higher salinity

tolerance, and for this reason they can not be used as a tolerance indicator for these stresses in pepper plants.

The Chl a fluorescence parameter F_v/F_m is the maximum quantum yield of PSII photochemistry and is frequently used as an indicator of damage photoinhibition (Gorbe and Calatayud, 2012; Guidi and Calatayud, 2014; Kalaji et al., 2014). However, in our screening studies F_v/F_m did not result as sensitive as other photosynthetic parameters. Changes were only observed in photosynthetic quantum conversion (Φ_{PSII}) and non-photochemical quenching (NPQ) noted in sensitive genotypes. A decrease in Φ_{PSII} favored the development of NPQ in sensitive genotypes compared to the tolerant accessions. The NPQ constitutes an important protective response that could dissipate excitation energy in light-harvesting antenna complex (Muller et al., 2001) and avoid photoinhibition damage (Calatayud et al., 2006) as indicated by the unchanged F_v/F_m ratios. Similar results were found in other species such as *Gossipum hirsutum* (Masacci et al., 2008) under water stress, *Acacia floribunda* (Sommerville et al., 2010), and rose (Calatayud et al., 2008).

For obtaining a desirable responses tackling abiotic stress, is necessary that scion and rootstock show a good compatibility. From screening experiments, some accessions like A5 were selected as valid genotypes to be used as rootstocks. Surprisingly, agronomic experiments revealed worst results than expected. For this reason we decide to study the physiological basis of graft compatibility as a previous step to evaluate the behavior of the rootstocks in the field, and also to understand the physiological response of grafted pepper plants to abiotic stresses. In addition, detecting the compatibility grade in advance will permit to apply the grafting technique more efficiently, with lower cost, ensuring better performance as grafted plants.

To tackle these different histological observations like callus formation, new cambium and vascular connections, as well as chlorophyll fluorescence parameters like F_v/F_m, Φ_{PSII}, NPQ, and q_p were evaluated in the different graft combinations with different compatibility degrees. As expected, the higher level of graft incompatibility was confirmed when pepper was grafted onto *S. torvum* and "Beaufort" (tomato), followed by A5, despite of its promising results obtained in the screening but related with its incompatible response in the agronomical tests. In this case, histological examination provided clear evidence of discontinuous xylem elements in the graft union as well as large areas of unbroken necrotic lines along the wounded edges of the rootstock and the scion. These observations were correlated with lower Fv/Fm, Φ_{PSII}, NPQ, and q_p values. By contrast, anatomical observations and CFI values provided clear evidences that the graft combinations A/A and A/A25 showed the highest vascular regeneration across the graft interface, whereas the accessions B14 and C12 were moderately compatible with higher CFI parameters and agronomical and physiological good responses under abiotic stress.

In conclusion, our results showed that CFI reflects the anatomical performance of the graft status and can be a useful and non-destructive technique for the assessment of graft compatibility in pepper grafted plants.

Finally, physiological and biochemical basis of the tolerances of pepper grafted plants was studied. Thus, rootstocks and scions under water stress and salinity were deeply studied. In our experiments, a commercial pepper cultivar grafted onto the tolerant selected rootstocks was used. In this way, we identified the physiological traits responsible for the tolerance and also differences in pepper grafted plants adaptation mechanisms in responses to salt and water stresses were analyzed. The plant tolerance might be related to the role of rootstock in altering the stress perception by the scion.

At a similar osmotic pressure of the nutritive solution, provoked by NaCl or PEG pepper plants grafted plants onto the accessions C12 and B14 rootstocks activated tolerance mechanisms based on ion exclusion or retention under salinity, whereas osmotic adjustment based on proline accumulation was performed under water stress. Several studies have attributed a dual role to proline: compatible osmolyte and antioxidant compound (Hayat et al., 2012; Jaleel et al., 2007). Free proline is considered an important osmoprotectant and accumulation following salt, drought, and heavy metal exposure is well documented (Gill and Tuteja, 2010).

Decreases in Ψ_s may have contributed to the ability of the tolerant accessions to uptake more water from the soil or the nutrient solution and could have minimized the harmful effects of water and salt stresses (Ming et al., 2012; Nio et al., 2011). Although the decrease in Ψ_s could be a consequence of a reduction in the water content of tissues, active osmotic adjustment was observed mainly in the plants grafted onto the selected tolerant genotypes. The osmotic adjustment may have involved the accumulation of a range of osmotically active molecules, including organic compounds such as sugars, free amino acids, glycinebetaine, soluble proteins, organic acids and proline (Chaves et al., 2003), and with macronutrients such as inorganic components (Patakas et al., 2002). In our works, a strong correlation between Ψ_s decrease and proline content increase was observed in plants grafted onto the selected genotypes.

The maintenance of scion homeostasis under salinity was achieved through the restriction of Cl^- transport to leaves and to diminished Na^+ loading in roots and leaves, thus favoring K^+ uptake. Nonetheless, although ionic and water homeostasis are crucial parameters in abiotic stress tolerance, the maintenance of shoot vigor and leaf function are vitally important. In this way, plants grafted

onto A25 accession accumulated high concentration of toxic ions in plant tissues. According to Munns' biphasic model (Munns and Tester, 2008), salt tolerance can be improved by reducing the negative osmotic effects on growth and/or maintaining leaf-root growth and functions for longer to dilute toxic ions (HajibagherI et al. 1989; Silva et al. 2008; Radić et al. 2013). In this combination, the absence of negative effects at physiological and biochemical level indicates that under saline conditions these plants either restrict the excess salts in the vacuole or compartmentalize the ions in different tissues where they are less harmful, as have also identify by other authors in tomato grafted plants (He et al., 2009). As a further confirm, grafted plants onto A25 under salinity accumulated in leaves even more ions (both Na^+ and Cl^-) than the ungrafted one. The reduction of K^+/Na^+ ratio was attributable to the higher Na^+ content given that plants did not lose their K^+ uptake ability. According to our results, a strong negative correlation between the reduction in leaf Ψs and salt ions content was observed.

Under both stresses, a minor negative impact on photosynthesis (mainly A_N and g_s), nitrate reductase activity and lipid peroxidation were observed on scion leaves grafted onto C12, B14 and A25 rootstocks the later only under NaCl addition.

We speculate that under a stress stimulus proline represents the key metabolite by which plants could face water scarcity and salinity conditions. Proline content was increased in the presence of NaCl in plants grafted onto A25 compared to non-significant increment in leaves of ungrafted or and also was increased in plants grafted onto C12 and B14 accessions under PEG conditions, compared to plants grafted onto A5 and A8 sensitive rootstocks. We didn't observe this increase under NaCl conditions in C12 and B14 plant combinations. Perhaps in

that experiments, the level of induced stress was not strong enough for these tolerant plants to synthetize proline, or plants could maintain their metabolic processes through ion retention or exclusion. Summarizing, improvement in proline accumulation under water and salt stresses helped maintaining osmotic potential and may also be involved in protection against oxidative damage as indicated by lower levels of MDA in plants grafted onto the selected accessions.

Better maintenance of the photosynthesis rate and a better regulate stomata regulation under stress conditions were noted when plants were grafted onto the selected genotypes.

There is an evidence that photosynthesis regulates nitrate reduction by modulating NR activity (Kaiser and Spill, 1991; Yousfi et al., 2012), which agrees with the results presented in this doctoral thesis. The most tolerant rootstocks in A_N terms exhibited lower NR inhibition under water and salt stress, if compared with the plants grafted onto sensitive genotypes and ungrafted.

These studies represent the first body of experimental evidence which demonstrates as the grafting technique is a valid grafting technique as a valid strategy for facing water and salt stress of pepper. From agronomic studies we have demonstrated that this resilience is conferred when a robust rootstock is selected, through changes in the scion behavior when grown under abiotic stresses. Moreover, as it was previously known, the physiological and biochemical strategies that plants develop to cope with stresses are diverse, such as retention or ion selection, compartmentalization of toxic ions in the vacuole, or proline synthesis among others. At the end, all processes lead to higher pepper yields in grafted plants under abiotic stressed environments. Specifically, from our agronomic, physiological and compatibility experiments, the genotype “A25”, a *C. annuum* accession, has shown promising characteristics to be a robust rootstock for its tolerances to water and salt

stresses, improving the results obtained by commercial rootstocks in both conditions. This priceless plant material could be further improved by breeding programs, being an excellent “starting” material.

8.2. REFERENCES

Albacete, A., Andújar, C., Dodd, I., Giuffrida, F., Hichri, I., Lutts, S., et al. (2015). Rootstock-mediated variation in tomato vegetative growth under drought, salinity and soil impedance stresses. in *Acta Horticulturae* (International Society for Horticultural Science (ISHS), Leuven, Belgium), 141–146.

Bota, J., Stasyk, O., Flexas, J., and Medrano, H. (2004). Effect of water stress on partitioning of 14C-labelled photosynthates in Vitis vinifera. *Funct. Plant Biol.* 31, 697–708. Available at: http://dx.doi.org/10.1071/FP03262.

Calatayud, a., Iglesias, D. J., Talón, M., and Barreno, E. (2006). Effects of long-term ozone exposure on citrus: Chlorophyll a fluorescence and gas exchange. *Photosynthetica* 44, 548–554. doi:10.1007/s11099-006-0070-1.

Calatayud, Á., Gorbe, E., Roca, D., and Martínez, P. F. (2008). Effect of two nutrient solution temperatures on nitrate uptake, nitrate reductase activity, NH4+ concentration and chlorophyll a fluorescence in rose plants. *Environ. Exp. Bot.* 64, 65–74.

Campos, H., Trejo, C., Peña-Valdivia, C. B., García-Nava, R., Conde-Martínez, F. V., and Cruz-Ortega, M. R. (2014). Stomatal and non-stomatal limitations of bell pepper (Capsicum annuum L.) plants under water stress and re-watering: Delayed restoration of photosynthesis during recovery. *Environ. Exp. Bot.* 98, 56–64.

Chaves, M. M., Maroco, J. P., and Pereira, J. S. (2003). Understanding plant responses to drought — from genes to the whole plant. *Funct. Plant Biol.* 30, 239–264. doi:10.1071/FP02076.

Chaves, M. M., Pereira, J. S., Maroco, J., Rodrigues, M. L., Ricardo, C. P. P., Osorio, M. L., et al. (2002). How plants cope with water stress in the field. Photosynthesis and growth. *Ann. Bot.* 89, 907–916.

Colla, G., Rouphael, Y., Leonardi, C., and Bie, Z. (2010a). Role of grafting in vegetable crops grown under saline conditions. *Sci. Hortic. (Amsterdam).* 127, 147–155. doi:10.1016/j.scienta.2010.08.004.

Colla, G., Suarez, C. M. C., Cardarelli, M., and Rouphael, Y. (2010b). Improving Nitrogen Use Efficiency in Melon by Grafting. *HORTSCIENCE* 45, 559–565.

Delfine, S., Alvino, A., Loreto, F., Centritto, M., and Santarelli, G. (2000). Effects of water stress on the yield and photosynthesis of field-grown sweet pepper (Capsicum annuum L.). *Proc. Third Int. Symp. Irrig. Hortic. Crop. Vols 1 2*, 223–229 942.

Djidonou, D., Zhao, X., Simonne, E. H., Koch, K. E., and Erickson, J. E. (2013). Yield, Water-, and Nitrogen-use Efficiency in Field-grown, Grafted Tomatoes. *HortScience* 48, 485–492.

Edelstein, M., Plaut, Z., and Ben-Hur, M. (2011). Sodium and chloride exclusion and retention by non-grafted and grafted melon and Cucurbita plants. *J. Exp. Bot.* 62, 177–184. doi:10.1093/jxb/erq255.

Estañ, M. T., Martinez-Rodriguez, M. M., Perez-Alfocea, F., Flowers, T. J., and Bolarin, M. C. (2005). Grafting raises the salt tolerance of tomato through limiting the transport of sodium and chloride to the shoot. *J. Exp. Bot.* 56, 703–712. doi:10.1093/jxb/eri027.

Flexas, J., Bota, J., Loreto, F., Cornic, G., and Sharkey, T. D. (2004). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C(3) plants. *Plant Biol.* 6, 269–79. doi:10.1055/s-2004-820867.

Foolad, M. R., and Lin, G. Y. (1997). Genetic potential for salt tolerance during germination in lycopersicon species. *HortScience* 32, 296–300.

García-Sánchez, F., Syvertsen, J. P., Gimeno, V., Botía, P., and Perez-Perez, J. G. (2007). Responses to flooding and drought stress by two citrus rootstock seedlings with different water-use efficiency. *Physiol. Plant.* 130, 532–542. doi:10.1111/j.1399-3054.2007.00925.x.

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909–930.

Gorbe, E., and Calatayud, A. (2012). Applications of chlorophyll fluorescence imaging technique in horticultural research: A review. *Sci. Hortic. (Amsterdam).* 138, 24–35. doi:10.1016/j.scienta.2012.02.002.

Guidi, L., and Calatayud, A. (2014). Non-invasive tools to estimate stress-induced changes in photosynthetic performance in plants inhabiting Mediterranean areas. *Environ. Exp. Bot.* 103, 42–52. doi:10.1016/j.envexpbot.2013.12.007.

HAJIBAGHERI, M. A., YEO, A. R., FLOWERS, T. J., and COLLINS, J. C. (1989). Salinity resistance in Zea mays: fluxes of potassium, sodium and chloride, cytoplasmic concentrations and microsomal membrane lipids. *Plant, Cell Environ.* 12, 753–757. doi:10.1111/j.1365-3040.1989.tb01635.x.

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., and Ahmad, A. (2012). Role of proline under changing environments: A review. *Plant Signal. Behav.* 7, 1456–66. doi:10.4161/psb.21949.

He, Y., Zhu, Z., Yang, J., Ni, X., and Zhu, B. (2009). Grafting increases the salt tolerance of tomato by improvement of photosynthesis and enhancement of antioxidant enzymes activity. *Environ. Exp. Bot.* 66, 270–278. doi:10.1016/j.envexpbot.2009.02.007.

Jaleel, C. A., Manivannan, P., Lakshmanan, G. M. A., Sridharan, R., and Panneerselvam, R. (2007). NaCl as a physiological modulator of proline metabolism and antioxidant potential in Phyllanthus amarus. *Comptes Rendus - Biol.* 330, 806–813. doi:10.1016/j.crvi.2007.08.009.

James, R. A., Von Caemmerer, S., Condon, A. G. T., Zwart, A. B., and Munns, R. (2008). Genetic variation in tolerance to the osmotic stress component of salinity stress in durum wheat. *Funct. Plant Biol.* 35, 111–123.

Kaiser, W. M., and Spill, D. (1991). Rapid Modulation of Spinach Leaf Nitrate Reductase by Photosynthesis : II. In Vitro Modulation by ATP and AMP. *Plant Physiol.* 96, 368–375. doi:10.1104/pp.96.2.368.

Kalaji, H. M., Schansker, G., Ladle, R. J., Goltsev, V., Bosa, K., Allakhverdiev, S. I., et al. (2014). Frequently asked questions about in vivo chlorophyll fluorescence: practical issues. *Photosynth. Res.*, 121–158. doi:10.1007/s11120-014-0024-6.

Kurunc, A., Unlukara, A., and Cemek, B. (2011). Salinity and drought affect yield response of bell pepper similarly. *Acta Agric. Scand. Sect. B — Soil Plant Sci.* 61, 514–522. doi:10.1080/09064710.2010.513691.

Lee, J.-M., and Oda, M. (2010). "Grafting of Herbaceous Vegetable and Ornamental Crops," in *Horticultural Reviews* (John Wiley & Sons, Inc.), 61–124. doi:10.1002/9780470650851.ch2.

Long, S. P., and Bernacchi, C. J. (2003). Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. *J. Exp. Bot.* 54, 2393–2401. doi:10.1093/jxb/erg262.

Martinez-Rodriguez, M. M., Esta??, M. T., Moyano, E., Garcia-Abellan, J. O., Flores, F. B., Campos, J. F., et al. (2008). The effectiveness of grafting to improve salt tolerance in tomato when an "excluder" genotype is used as scion. *Environ. Exp. Bot.* 63, 392–401.

Meggio, F., Prinsi, B., Negri, A. S., Simone Di Lorenzo, G., Lucchini, G., Pitacco, A., et al. (2014). Biochemical and physiological responses of two grapevine rootstock genotypes to drought and salt treatments. *Aust. J. Grape Wine Res.* 20, 310–323. doi:10.1111/ajgw.12071.

Ming, D. F., Pei, Z. F., Naeem, M. S., Gong, H. J., and Zhou, W. J. (2012). Silicon Alleviates PEG-Induced Water-Deficit Stress in Upland Rice Seedlings by Enhancing Osmotic Adjustment. *J. Agron. Crop Sci.* 198, 14–26. doi:10.1111/j.1439-037X.2011.00486.x.

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. *Trends Plant Sci.* 11, 15–9. doi:10.1016/j.tplants.2005.11.002.

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681.

Murillo-Amador, B., Lopez-Aguilar, R., Kaya, C., Larrinaga-Mayoral, J., and Flores-Hernandez, A. (2002). Comparative Effects of NaCl and Polyethylene Glycol on Germination, Emergence and Seedling Growth of Cowpea. *J. Agron. Crop Sci.* 188, 235–247. doi:10.1046/j.1439-037X.2002.00563.x.

Nebauer, S. G., Sánchez, M., Martínez, L., Lluch, Y., Renau-Morata, B., and Molina, R. V. (2013). Differences in photosynthetic performance and its correlation with growth among tomato cultivars in response to different salts. *Plant Physiol. Biochem.* 63, 61–9.

Nio, S. A., Cawthray, G. R., Wade, L. J., and Colmer, T. D. (2011). Pattern of solutes accumulated during leaf osmotic adjustment as related to duration of water deficit for wheat at the reproductive stage. *Plant Physiol. Biochem. PPB Société française Physiol. végétale* 49, 1126–37. doi:10.1016/j.plaphy.2011.05.011.

Orsini, F., Sanoubar, R., Oztekin, G. B., Kappel, N., Tepecik, M., Quacquarelli, C., et al. (2013). Improved stomatal regulation and ion partitioning boosts salt tolerance in grafted melon. *Funct. Plant Biol.* 40, 628–636. doi:10.1071/FP12350.

Patakas, A., Nikolaou, N., Zioziou, E., Radoglou, K., and Noitsakis, B. (2002). The role of organic solute and ion accumulation in osmotic adjustment in drought-stressed grapevines. *Plant Sci.* 163, 361–367. doi:10.1016/S0168-9452(02)00140-1.

Perica, S., Goreta, S., and Selak, G. V. (2008). Growth, biomass allocation and leaf ion concentration of seven olive (Olea europaea L.) cultivars under increased salinity. *Sci. Hortic. (Amsterdam).* 117, 123–129. doi:10.1016/j.scienta.2008.03.020.

Radić, S., Peharec Štefanić, P., Lepeduš, H., Roje, V., and Pevalek-Kozlina, B. (2013). Salt tolerance of Centaurea ragusina L. is associated with

efficient osmotic adjustment and increased antioxidative capacity. *Environ. Exp. Bot.* 87, 39–48. doi:10.1016/j.envexpbot.2012.11.002.

Rewald, B., Raveh, E., Gendler, T., Ephrath, J. E., and Rachmilevitch, S. (2012). Phenotypic plasticity and water flux rates of Citrus root orders under salinity. *J. Exp. Bot.* 63, 2717–27. doi:10.1093/jxb/err457.

Sánchez-Rodríguez, E., Romero, L., and Ruiz, J. M. (2013). Role of Grafting in Resistance to Water Stress in Tomato Plants: Ammonia Production and Assimilation. *J. Plant Growth Regul.* 32, 831–842. doi:10.1007/s00344-013-9348-2.

Santa-Cruz, A., Martinez-Rodriguez, M. M., Perez-Alfocea, F., Romero-Aranda, R., and Bolarin, M. C. (2002). The rootstock effect on the tomato salinity response depends on the shoot genotype. *Plant Sci.* 162, 825–831. doi:10.1016/S0168-9452(02)00030-4.

Schwarz, D., Rouphael, Y., Colla, G., and Venema, J. H. (2010). Grafting as a tool to improve tolerance of vegetables to abiotic stresses: Thermal stress, water stress and organic pollutants. *Sci. Hortic. (Amsterdam).* 127, 162–171. doi:10.1016/j.scienta.2010.09.016.

Silva, C., Martínez, V., and Carvajal, M. (2008). Osmotic versus toxic effects of NaCl on pepper plants. *Biol. Plant.* 52, 72–79. doi:10.1007/s10535-008-0010-y.

Syvertsen, J., Lloyd, J., and Kriedemann, P. (1988). Salinity and drought stress effects on foliar ion concentration, water relations, and photosynthetic characteristics of orchard citrus. *Aust. J. Agric. Res.* 39, 619. doi:10.1071/AR9880619.

Walker, R. R., Blackmore, D. H., Clingeleffer, P. R., and Correll, R. L. (2002). Rootstock effects on salt tolerance of irrigated field-grown grapevines (Vitis vinifera L. cv. Sultana).: 1. Yield and vigour inter-relationships. *Aust. J. Grape Wine Res.* 8, 3–14. doi:10.1111/j.1755-0238.2002.tb00206.x.

Yousfi, S., Serret, M. D., Márquez, A. J., Voltas, J., and Araus, J. L. (2012). Combined use of $\delta^{13}C$, δ18O and δ15N tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit. *New Phytol.* 194, 230–44. doi:10.1111/j.1469-8137.2011.04036.x.

Final Conclusions

FINAL CONCLUSIONS

From all the studies performed in this PhD in order to understand the mechanisms of tolerance to salt and water stresses of pepper accessions to use them as rootstocks of pepper scions, we can conclude that,

- Photosynthesis rate measurements could be considered a useful parameter to screen large collections of pepper accessions to drought and salinity tolerance
- Satisfactory yields were obtained when our selected genotypes by the photosynthetic rate were used as rootstocks in both water and salt stresses, confirming that some wild *Capsicum* genotypes used as rootstocks are interesting as a source of tolerance to abiotic stress
- The selected accessions provide yields comparable or superior to commercial rootstocks commonly used in pepper crops mainly due to a fruits affected by BER decline
- Chlorophyll Fluorescence Imaging (CFI) is a good tool to evaluate scion/rootstock compatibility degree, reflecting the anatomical performance of the graft status, been a promising technique, fast and non-invasive, to early predict compatibility in different combinations and in a large number of plants
- The selected tolerant *Capsicum* rootstocks use several strategies to face water scarcity and salt stress: enhancing osmotic adjustment through proline accumulation; restricting Cl^- transport to leaves and Na+ loading in roots and leaves, thus favoring K^+ (Ca^{2+} and Mg^{2+}) uptake and allowing a decrease in the osmotic potential, increasing SOD synthesis and decreasing of ROS synthesis. Consequently, minor inhibitory effect on photosynthesis and NR

activity, and minor amount of MDA are shown when tolerant rootstocks were used in grafted plants.

- As a summary, there are accessions of *Capsicum* capable of bearing moderate both salt and drought conditions through different mechanisms, and confer these tolerances to pepper cultivars via grafting, when good compatibility exists, that can be measured with CFI.