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PROGRAMA DE DOCTORADO EN RECURSOS Y TECNOLOGÍAS AGRÍCOLAS

TESIS DOCTORAL

Análisis de la variabilidad genética del género *Capsicum* frente a estreses abióticos para su uso como portainjertos. Estudio de los mecanismos fisiológicos de tolerancia, del comportamiento agronómico y de la calidad del fruto.

Presentada por:
Ramón Gisbert Mullor

Dirigida por:
Dr. Salvador Vicente López Galarza
Dra. Ángeles Calatayud Chover

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Per a Marc,

l’arrel de tot.

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RESUMEN

En la actualidad, los estreses abióticos son una de las principales limitaciones en la producción agraria y seguridad alimentaria. La búsqueda de estrategias para conseguir una mejor tolerancia a los estreses abióticos es un objetivo de gran interés a nivel mundial, especialmente frente al estrés supra-térmico teniendo en cuenta el escenario de calentamiento global en el cual nos encontramos. Un modo de sortear los estreses ambientales, bajo el prisma de un manejo integrado o ecológico del cultivo, es la utilización de plantas injertadas como estrategia de adaptación. En pimiento, cultivo de gran importancia económica en nuestro país, la menor utilización de la técnica del injerto es debida a la inexistencia de patrones con suficiente adaptabilidad a condiciones de estrés abiótico. En este sentido, el equipo de investigación mixto entre la Universitat Politècnica de València (UPV) y el Instituto Valenciano de Investigaciones Agrarias (IVIA), el cual engloba esta tesis doctoral, ha sido capaz de seleccionar diferentes accesiones e híbridos de pimiento, entre ellos el híbrido NIBER®, que utilizados como portainjertos han obtenido buenos resultados en término de rendimiento en condiciones de estrés hídrico y salino, y generar información respecto a los diferentes mecanismos fisiológicos y moleculares responsables de dicha tolerancia. Sin embargo, hasta la fecha, la información sigue siendo limitada en diferentes sentidos: no se ha estudiado en profundidad el efecto del injerto a las altas temperaturas; no se ha profundizado en el estudio de los mecanismos de tolerancia del portainjerto NIBER® frente al estrés hídrico causado por un riego deficitario controlado; y por último, ningún estudio se ha centrado en analizar los efectos del propio injerto sobre el rendimiento y calidad de los frutos de variedades tradicionales de pimiento. En este sentido, los objetivos que se han planteado en esta tesis doctoral han sido: I) evaluación fisiológica y agronómica de la tolerancia al estrés supra-térmico de accesiones de *Capsicum annuum* L. utilizadas como portainjertos; II) estudio de los mecanismos fisiológicos que subyacen en el comportamiento de portainjertos tolerantes a estrés supra-térmico; III) estudio de las bases del comportamiento agronómico y fisiológico de portainjertos de pimiento tolerantes a estrés hídrico en condiciones controladas de riego deficitario; IV) evaluación agronómica y de la calidad del fruto de variedades tradicionales de pimiento injertadas sobre patrones tolerantes a estreses abióticos.

En esta tesis doctoral, por un lado, hemos sido capaces de seleccionar accesiones de pimiento, especialmente A57, que utilizadas como portainjertos confieren a la variedad injertada cierto grado de tolerancia al estrés térmico por altas temperaturas. También hemos podido comprobar que estos portainjertos tolerantes permiten a la variedad generar respuestas adaptativas a los efectos negativos de este estrés mediante la modificación de su comportamiento fisiológico, disminuyendo la fuga de electrolitos y la acumulación de H_2O_2, y aumentando el contenido de clorofilas y

carotenoides en las hojas. Esta respuesta fisiológica observada en las hojas resulta en una mejor adaptación fisiológica en su fase reproductiva, mejorando el contenido de prolina en las anteras y la viabilidad de los granos de polen, incrementando con ello el cuajado de frutos y en consecuencia el rendimiento comercial. Por otro lado, hemos visto que la tolerancia al estrés hídrico conferida por el portainjerto NIBER® es consecuencia de una modificación fisiológica que permite mantener los niveles de fotosíntesis con una adecuada eficiencia en el uso del agua, resultando en un mayor rendimiento comercial. En este sentido, el uso de la estrategia de riego deficitario combinada con el uso de portainjertos tolerantes al estrés hídrico y con un mayor desarrollo radical, como es el caso del NIBER®, puede considerarse útil para reducir el consumo de agua, amortiguando los efectos negativos del estrés sobre el rendimiento, debido principalmente a la menor incidencia de necrosis apical de los frutos (blossom-end rot o BER). Por último, hemos sido capaces de constatar que el uso de portainjertos tolerantes a estreses abióticos, como es NIBER®, en variedades tradicionales de pimiento podría ser una técnica útil para mejorar el rendimiento y la calidad comercial de este cultivo, principalmente al disminuir la incidencia de BER, sin afectar negativamente la calidad intrínseca del fruto.

ABSTRACT

Currently, abiotic stresses are one of the main limitations in agricultural production and food security. The search for strategies to achieve a better tolerance to abiotic stresses is an objective of great interest worldwide, especially in the face of supra-thermal stress, considering the global warming scenario in which we find ourselves. One way to overcome environmental stresses, under the prism of an integrated or ecological management of the crop, is the use of grafted plants as an adaptation strategy. In pepper, a crop of great economic importance in our country, the lesser use of the grafting technique is due to the lack of rootstocks with sufficient adaptability to abiotic stress conditions. In this sense, the mixed research team between Universitat Politècnica de València (UPV) and Instituto Valenciano de Investigaciones Agrarias (IVIA), which includes this doctoral thesis, has been able to select different pepper accessions and hybrids, including the NIBER® hybrid, which used as rootstocks have obtained good results in terms of yield under conditions of water and saline stress, and generate information regarding the different physiological and molecular mechanisms responsible for said tolerance. However, to date, the information remains limited in different ways: the effect of grafting at high temperatures has not been studied in depth; the study of the tolerance mechanisms of the NIBER® rootstock against water stress caused by controlled deficit irrigation has not been studied in depth; and finally, no study has focused on analyzing the effects of the graft itself on the yield and quality of the fruits of traditional pepper varieties. In this sense, the objectives that have been set in this doctoral thesis have been: I) physiological and agronomic evaluation of the tolerance to supra-thermal stress of accessions of *Capsicum annuum* L. used as rootstocks; II) study of the physiological mechanisms that underlie the behavior of rootstocks tolerant to supra-thermal stress; III) study of the bases of the agronomic and physiological behavior of pepper rootstocks tolerant to water stress under controlled conditions of deficit irrigation; IV) agronomic and fruit quality evaluation of traditional pepper varieties grafted on rootstocks tolerant to abiotic stresses.

In this doctoral thesis, on the one hand, we have been able to select pepper accessions, especially A57, which, when used as rootstocks, give the grafted variety a certain degree of tolerance to thermal stress from high temperatures. We have also been able to verify that these tolerant rootstocks allow the variety to generate adaptive responses to the negative effects of this stress by modifying its physiological behavior, decreasing electrolyte leakage and H_2O_2 accumulation, and increasing the content of chlorophylls and carotenoids in the leaves. This physiological response observed in the leaves results in a better physiological adaptation in its reproductive phase, improving the proline content in the anthers and the viability of pollen grains, thereby increasing fruit set and consequently

commercial yield. On the other hand, the tolerance to water stress conferred by the NIBER® rootstock is the consequence of a physiological modification that allows maintaining photosynthesis levels with an adequate water use efficiency, resulting in a higher commercial yield. In this sense, the use of the deficit irrigation strategy combined with the use of tolerant rootstocks to water stress and with greater root development, as is the case of NIBER®, can be considered useful to reduce water consumption, cushioning the negative stress effects on yield, mainly due to the lower incidence of blossom-end rot (BER). Finally, we have been able to verify that the use of tolerant rootstocks to abiotic stresses, such as NIBER®, in traditional pepper varieties could be a useful technique to improve the yield and commercial quality of this crop, mainly by reducing the incidence of BER, without negatively affecting the intrinsic fruit quality.

RESUM

En l'actualitat, els estressos abiòtics són una de les principals limitacions en la producció agrària i seguretat alimentària. La cerca d'estratègies per a aconseguir una millor tolerància als estressos abiòtics és un objectiu de gran interés a nivell mundial, especialment davant de l'estrés supra-tèrmic tenint en compte l'escenari de calfament global en el qual ens trobem. Una manera de sortejar els estressos ambientals, sota el prisma d'un maneig integrat o ecològic del cultiu, és la utilització de plantes empeltades com a estratègia d'adaptació. En pimentó, cultiu de gran importància econòmica al nostre país, la menor utilització de la tècnica de l'empelt és deguda a la inexistència de patrons amb suficient adaptabilitat a condicions d'estrés abiòtic. En aquest sentit, l'equip d'investigació mixt entre la Universitat Politècnica de València (UPV) i l'Institut Valencià d'Investigacions Agràries (IVIA), el qual engloba aquesta tesi doctoral, ha sigut capaç de seleccionar diferents accessions e híbrids de pimentó, entre ells l'híbrid NIBER®, que utilitzats com portaempelts han obtingut bons resultats en terme de rendiment en condicions d'estrés hídric i salí, i generar informació respecte als diferents mecanismes fisiològics i moleculars responsables d'aquesta tolerància. No obstant això, fins hui, la informació continua sent limitada en diferents sentits: no s'ha estudiat en profunditat l'efecte de l'empelt a les altes temperatures; no s'ha aprofundit en l'estudi dels mecanismes de tolerància del portaempelt NIBER® enfront de l'estrés hídric causat per un reg deficitari controlat; i finalment, cap estudi s'ha centrat en estudiar els efectes del propi empelt sobre el rendiment i qualitat dels fruits de varietats tradicionals de pimentó. En aquest sentit, els objectius que s'han plantejat en aquesta tesi doctoral han sigut: I) avaluació fisiològica i agronòmica de la tolerància a l'estrés supra-tèrmic d'accessions de *Capsicum annuum* L. utilitzades com portaempelts; II) estudi dels mecanismes fisiològics que subjauen en el comportament de portaempelts tolerants a estrés supra-tèrmic; III) estudi de les bases del comportament agronòmic i fisiològic de portaempelts de pimentó tolerants a estrés hídric en condicions controlades de reg deficitari; IV) avaluació agronòmica i de la qualitat del fruit de varietats tradicionals de pimentó empeltades sobre patrons tolerants a estressos abiòtics.

En aquesta tesi doctoral, d'una banda, hem sigut capaços de seleccionar accessions de pimentó, especialment A57, que utilitzades com portaempelts confereixen a la varietat empeltada un cert grau de tolerància a l'estrés tèrmic per altes temperatures. També hem pogut comprovar que aquests portaempelts tolerants permeten a la varietat generar respostes adaptatives als efectes negatius d'aquest estrés mitjançant la modificació del seu comportament fisiològic, disminuint la fugida d'electròlits i l'acumulació de H_2O_2, i augmentant el contingut de clorofil·les i carotenoides en les fulles. Aquesta resposta fisiològica observada

en les fulles resulta en una millor adaptació fisiològica en la seua fase reproductiva, millorant el contingut de prolina en les anteres i la viabilitat dels grans de pol·len, incrementant amb això el quallat de fruits i en conseqüència el rendiment comercial. D'altra banda, la tolerància a l'estrés hídric conferida pel portaempelt NIBER® és conseqüència d'una modificació fisiològica que permet mantenir els nivells de fotosíntesi amb una adequada eficiència en l'ús de l'aigua, resultant en un major rendiment comercial. En aquest sentit, l'ús de l'estratègia de reg deficitari combinada amb l'ús de portaempelts tolerants a l'estrés hídric i amb un major desenvolupament radical, com és el cas del NIBER®, pot considerar-se útil per a reduir el consum d'aigua, esmorteint els efectes negatius de l’estrés sobre el rendiment, degut principalment a la menor incidència de BER. Finalment, hem sigut capaços de constatar que l'ús de portaempelts tolerants a estressos abiòtics, com és NIBER®, en varietats tradicionals de pimentó podria ser una tècnica útil per a millorar el rendiment i la qualitat comercial d'aquest cultiu, principalment en disminuir la incidència de necrosi apical del fruit (blossom-end rot o BER), sense afectar negativament la qualitat intrínseca del fruit.

Índice

Capítulo 1

Introducción

1.1. Aspectos generales del cultivo del pimiento

1.1.1. Taxonomía y descripción botánica

El pimiento, pertenece al género *Capsicum*, que a su vez forma parte de la familia Solanaceae, constituida por más de 90 géneros y 2500 especies, incluyendo otros cultivos hortícolas de gran importancia comercial como tomate, berenjena y patata (Tripodi y Kumar, 2019).

El género *Capsicum*, originario de América tropical y subtropical, incluye más de 30 especies, cinco de las cuales (*C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. y *C. pubescens* Ruiz & Pavón) han sido domesticadas y cultivadas para el consumo humano (Carrizo García et al., 2016). De todas ellas, *C. annuum* L. es la especie económicamente más importante y la más cultivada en la actualidad.

Por lo que respecta a las características botánicas de la especie *C. annuum* L., son plantas anuales herbáceas provistas de un sistema radical pivotante y profundo, con un elevado número de raíces adventicias. El tallo, de porte erecto, presenta un crecimiento dicotómico, aunque en algunos casos presenta tricotomía en la primera diversificación del tallo. Las hojas son simples y enteras, presentando una forma más o menos lanceolada y un pecíolo largo. Las flores son hermafroditas y aparecen solitarias en cada nudo. El cáliz está formado por 5-8 sépalos y la corola por 5-8 pétalos soldados por la base. El androceo está constituido por un cono estaminal de 5-8 estambres. El gineceo está constituido de 2-4 carpelos soldados y consta de un ovario, un estilo y un estigma. La fructificación es una baya hueca semi-cartilaginosa y deprimida que en la madurez vira de color, y presenta un eje compuesto por un tejido placentario, en el que se disponen las semillas. Entre sus variedades, el fruto del pimiento, presenta una gran diversidad en tamaño, forma y color, que permite clasificarlas en diferentes grupos o tipos varietales (Pochard, 1966).

1.1.2. Importancia económica del cultivo del pimiento

La producción y el área cultivada de pimiento a nivel mundial ha aumentado sustancialmente en los últimos 30 años (1990-2020), pasando de 12,8 a 40,3 millones de toneladas cosechadas y de 2,8 a 3,7 millones de hectáreas cultivadas, destacando que aproximadamente el 90% de la producción son pimientos destinados al consumo en fresco (FAOSTAT, 2020).

Los principales países productores de pimiento para consumo en fresco en el año 2020 se muestran en la Tabla 1. Hay que destacar que la producción de China representa alrededor del 40% de la producción mundial. España ocupó la quinta posición con una producción de 1,47 millones de toneladas (FAOSTAT, 2020).

En lo que se refiere al territorio nacional, en la última década (2009-2019), tanto la superficie destinada a pimiento como el rendimiento se han visto incrementados en un 12,1% y 34,3% respectivamente (MAPA, 2020). En el año 2019, en España se dedicaron al cultivo del pimiento 21.229 ha, de las cuales, el 99,3% eran regadío y el 68,2% en superficie protegida. Por comunidades autónomas, Andalucía (82,3%), en especial la provincia de Almería (76,7%) y la Región de Murcia (8,6%), son las que presentan mayor superficie bajo ambiente protegido del territorio nacional.

El cultivo del pimiento en España es muy importante en la balanza económica comercial con 853,58 millones de kg exportados con un valor de 1.158,85 millones de euros (FEPEX, 2020), lo que indica la importancia y la necesidad de adaptar su cultivo a la nueva situación climática global y a las exigencias del mercado vinculadas con las empresas y los consumidores.

Tabla 1. Datos de producción de pimiento para consumo en fresco en toneladas (t x 10^3) de los mayores países productores a nivel mundial (FAOSTAT, 2020).

País	**Producción**
	t x 10^3
China	16.650,9
México	2.818,4
Indonesia	2.772,6
Turquía	2.636,9
España	1.472,9
Egipto	1.055,6
Nigeria	762,2
Argelia	717,7
EE. UU.	715,2
Países Bajos	430,0

1.1.3. Propiedades organolépticas y nutraceúticas del fruto de pimiento

El fruto del pimiento se encuentra en la categoría de alimentos funcionales por sus características organolépticas, nutraceúticas y su alta capacidad antioxidante, ya que es rico no solo en ácido ascórbico, carotenoides y fenoles, sino también en capsaicinoides, xantofilas y flavonoides. Los niveles de dichos compuestos pueden variar según el genotipo y la madurez, y están influenciados por las condiciones de cultivo (Chávez-Mendoza et al., 2015; Howard et al., 2000; Martínez-Ispizua et al., 2021). También se ha observado que las condiciones climáticas y de almacenamiento, así como las prácticas de procesamiento pueden afectar a los niveles de dichos compuestos (Jayaprakasha et al., 2012).

Los carotenoides son generalmente los principales fitoquímicos que se encuentran en las variedades de pimiento, que aportan un alto valor comercial a estas frutas en términos de características de sabor, color y propiedades antioxidantes, entre otras bioactividades. Los capsaicinoides se encuentran principalmente en los pimientos picantes y son responsables de la acritud de estas variedades. Los pimientos también son ricos en compuestos fenólicos, principalmente flavonoides y derivados del ácido fenólico, y vitaminas A y C, y distintos minerales, incluidos el hierro, el calcio y el manganeso, que contribuyen en gran medida a la dieta humana (Pandey y Rizvi, 2009). Todo ello hace del pimiento un alimento claramente funcional (Zhuang et al., 2012).

1.1.4. Principales fisiopatías del fruto de pimiento

1.1.4.1. Blossom-end rot (BER)

La necrosis apical del fruto, comúnmente conocido como blossom-end rot (BER) es una fisiopatía muy común en los frutos de algunos cultivos hortícolas, entre ellos los frutos de pimiento. La presencia de BER es la causa de una deficiencia de calcio en los tejidos de la zona distal del fruto. Su presencia se acentúa cuando las plantas de pimiento son expuestas a algún tipo de estrés abiótico, sobre todo estrés hídrico y salino. En estas condiciones, el transporte de calcio a los órganos con baja transpiración, entre ellos los frutos, puede verse afectado, aunque se ha suguerido que puede no ser debido al contenido total de calcio de la fruta, sino una partición y distribución anormal de calcio dentro del fruto lo que conduce a una deficiencia localizada y por lo tanto la aparición de BER (Saure, 2001, 2014). Los síntomas de esta fisiopatía están asociados a la perdida de solutos celulares, plasmólisis celular y ruptura de la membrana (López-Serrano et al., 2022; Mayorga-Gómez et al., 2020). La sintomatología del BER se caracteriza en primer lugar porque la superficie del fruto en la parte distal exhibe síntomas de reblandecimiento y aguado, y finalmente este tejido se decolora y se necrosa. Esta fisiopatía causa abundantes pérdidas en el rendimiento del cultivo, produciendo frutos no aptos para la comercialización o de destrío.

1.1.4.2. Rajado o micro rajado del fruto

Esta fisiopatía reduce el rendimiento comercial del cultivo del pimiento, pero no es tan común como BER. Se caracteriza por la presencia de grietas que generalmente se extienden a través de la pared del fruto hacia el área del lóculo debido a la contracción y expansión repetitiva en el crecimiento que debilita la cutícula del mismo (Yao et al., 2000). La incidencia se ve afectada principalmente por características varietales y algunos factores ambientales como fluctuaciones en la humedad relativa (Moreshet et al., 1999).

1.2. Principales problemas del cultivo del pimiento

La producción agrícola ha aumentado notablemente en los últimos tiempos con el fin de cubrir la demanda de alimentos de la población mundial. Sin embargo, este hecho viene acompañado por una parte, por una rápida expansión de los estreses bióticos, debido principalmente al fenómeno de la globalización, y por otra parte, a una mayor incidencia de estreses abióticos, debido principalmente a los problemas de escasez y baja calidad del agua de riego y las fluctuaciones de las temperaturas, principalmente por el aumento de temperatura como consecuencia del calentamiento global en el cual nos encontramos debido al fenómeno de cambio climático (IPCC, 2022). Ante esta situación, el riesgo de sufrir estreses bióticos y/o abióticos en los cultivos ha aumentado, resultando en una amenaza para la producción y la calidad de las cosechas.

1.2.1. Estreses bióticos

El control de los estreses bióticos sobre los cultivos son sin duda los de mayor importancia para asegurar una buena rentabilidad. De hecho, los mayores esfuerzos de los productores y de las empresas obtentoras de nuevos cultivares se centran en resolver este tipo de estreses (Onaga et al., 2016).

Entre los principales agentes causantes de estreses bióticos en el cultivo del pimiento podemos encontrar hongos, virus, bacterias, nematodos, insectos, arañas, etc. Entre todos ellos, los problemas más comunes son los causados por los hongos como *Phytophthora capsici*, *Verticillium dahliae*, *Rhizoctonia solani*, *Fusarium* spp. y *Leveillula taurica*; los virus como Tobacco Mosaic Virus (TMV), Potato Virus Y (PVY), Tomato Spotted Wilt Virus (TSWV) y Pepper Mild Mottle Virus (PMMV); nematodos principalmente *Meloidogyne incognita*; y diferentes insectos y arañas como pulgones (*Myzus persicae* y *Aphis gossypii*), mosca blanca (*Bemisia tabaci*), trips (*Frankliniella occidentalis*), ácaros (*Tetranychus urticae* y *Polyphagotarsonemus latus*), y larvas de lepidópteros, orugas o gusanos (*Spodoptera exigua* y *Spodoptera littoralis*).

Con el objetivo de afrontar la presencia de dichos estreses en el cultivo, los agricultores disponen de diferentes herramientas, entre las que destacan el control integrado mediante la lucha química racional, el control biológico, la mejora genética y prácticas preventivas y culturales como la rotación de cultivos o la solarización del suelo. Hay que destacar que, en los últimos años, gracias al trabajo realizado por diferentes casas obtentoras de semillas e institutos de investigación, se han desarrollado variedades y portainjertos comerciales tolerantes a enfermedades causadas principalmente por hongos del suelo (Attavar y Miles, 2021; Gisbert et al., 2010; Louws et al., 2010; Morra y Bilotto, 2015) y virus (Kang et al., 2010; Kenyon et al., 2014; Palloix et al., 2009).

1.2.2. Estreses abióticos

En la actualidad, los estreses abióticos son una de las principales limitaciones en la producción y seguridad alimentaria (Hatfield y Prueger, 2015; IPCC, 2022). Esta situación se agrava, como se ha comentado, debido a los cambios drásticos y rápidos en el clima global (Fahad et al., 2017) que pueden acarrear problemas de disponibilidad de agua en cantidad y calidad suficientes y aumentos de temperatura en las zonas Mediterráneas (IPCC, 2022).

1.2.2.1. Estrés térmico por altas temperaturas

En la península ibérica, sobre todo en algunas zonas del litoral mediterráneo, los cultivos se están viendo afectados por algunos de los estreses abióticos comentados anteriormente, entre ellos el estrés térmico por altas temperaturas (Moriondo et al., 2016). En gran parte debido al ciclo de cultivo empleado donde, en el caso del pimiento, las etapas fenológicas más importantes para el desarrollo del mismo, como pueden ser la floración y el cuajado, coinciden en gran medida con los meses de verano.

En España, el pimiento dulce para mercado en fresco se produce principalmente en tres ciclos: 1) ciclo clásico de Almería, de julio/agosto a febrero, y después una cosecha de primavera-verano, normalmente de cucurbitáceas; 2) ciclo extendido, adelantando siembras a mayo/junio y final de cosecha en abril/mayo; y 3) ciclo típico de Murcia, sur de Alicante y en algunos casos de Almería, entre noviembre/diciembre hasta junio/julio/agosto. Estos tres ciclos permiten tener una producción de pimiento durante todo el año. Los cultivos se desarrollan principalmente en invernaderos de plástico caracterizados por una tecnología limitada y con un control climático restringido. Con estas infraestructuras se produce una mala ventilación natural, lo que dificulta el control de las altas temperaturas en primavera y verano (García-Ruiz et al., 2018), produciéndose temperaturas no óptimas que afectan al desarrollo normal del cultivo. El aumento de las temperaturas es un escenario difícil de abordar para los agricultores del litoral mediterráneo debido a las escasas herramientas que poseen para disminuir la temperatura de los invernaderos. Las soluciones que se están llevando a cabo son, por una parte un buen manejo de la ventilación, que al ser el accionamiento de las ventanas mayoritariamente manual, todavía dificulta más el control de la temperatura (García-Ruiz et al., 2018; Parra et al., 2004), y por otra parte, reducir la intensidad de radiación solar mediante el encalado de las cubiertas del invernadero, aunque este hecho va acompañado de una reducción de la radiación fotosintéticamente activa (PAR) y por tanto de una disminución de las condiciones óptimas para el desarrollo del cultivo.

En términos generales, un estrés supra-térmico provoca en los cultivos numerosos cambios morfológicos, fisiológicos, bioquímicos y moleculares, afectando negativamente al crecimiento y la producción de los mismos. La exposición prolongada a altas temperaturas produce en las plantas una desnaturalización de las proteínas con la consiguiente pérdida de sus funciones, la inactivación de enzimas en cloroplastos y mitocondrias, pérdida de integridad y permeabilidad de las membranas y la producción de compuestos tóxicos, como las especies reactivas de oxígeno (ROS) (Wahid et al., 2007; Wang et al., 2003). Por otro lado, a nivel subcelular, las altas temperaturas producen modificaciones importantes en los tilacoides, afectando a la actividad del fotosistema II y dificultando el transporte de electrones (Porch y Hall, 2013). En consecuencia, el aparato fotosintético se ve afectado disminuyendo la fotosíntesis, aumentando la fotorrespiración y limitando el crecimiento y desarrollo de las plantas (Wise et al., 2004).

En el cultivo del pimiento, el desarrollo óptimo se produce con temperaturas medias de 21-23°C durante el crecimiento vegetativo y de 21°C durante el desarrollo del fruto, con una diferencia de 7-9°C entre la temperatura diurna y nocturna, por debajo de 15°C su desarrollo se ve mermado y a partir de 32°C el rendimiento puede verse afectado por falta de cuajado y aborto floral. Con temperaturas extremas (>40°C) pueden aparecer quemaduras en las hojas, tallos y frutos (*sunscald*) (Yasuor y Wien, 2020).

Las altas temperaturas en el cultivo del pimiento pueden causar grandes pérdidas en el rendimiento debido a la falta de cuajado de frutos, causado en gran medida por los efectos sobre la ontogenia de los granos de polen. Existen diferentes estudios en los que se han observado y corroborado los efectos de las altas temperaturas en los gametofitos masculinos del pimiento, que son más sensibles que los femeninos, ya no solo en su desarrollo y formación, sino también en las posteriores fases de germinación y crecimiento del tubo polínico (Aloni et al., 2001; Erickson y Markhart, 2002; Lin et al., 2022; Mercado et al., 1997; Reddy y Kakani, 2007; Yamazaki y Hosokawa, 2019). Los frutos cuajados en altas temperaturas pueden ver afectado su desarrollo, formando frutos de reducido peso y tamaño. Este hecho está íntimamente relacionado con la eficacia de la polinización y posterior fecundación de los óvulos, que condiciona el número de semillas por fruto, que está relacionado con el tamaño y peso del mismo (Marcelis y Baan Hofman-Eijer, 1997; Pagamas y Nawata, 2008).

1.2.2.2. Estrés hídrico

El estrés hídrico es uno de los principales factores limitantes para la producción de cultivos de hortalizas. El agua es cada vez más escasa, en cantidad y calidad, en todo el mundo, lo que afecta gravemente a la producción agrícola, especialmente en zonas áridas y semiáridas (Chai et al., 2015; Mancosu et al., 2015). Las previsiones indican que el cambio climático afectará gravemente al sector agrícola, aumentando la temperatura global y la evapotranspiración (ET), al mismo tiempo que se reducirán las precipitaciones con una distribución y patrón anormales, lo que en consecuencia incrementará la demanda de agua (IPCC, 2022; Kahil et al., 2015). Se espera que los episodios de sequía existentes se intensifiquen, particularmente en regiones donde la escasez de agua ya es una preocupación, como en la región mediterránea (Iglesias y

Garrote, 2015). Teniendo en cuenta estas predicciones y que la agricultura representa casi el 70% de todas las extracciones de agua, hasta el 95% en algunos países en desarrollo (AQUASTAT, 2020), es necesario plantear alternativas en la producción agrícola para reducir drásticamente el consumo de agua en la agricultura.

De forma generalizada, los efectos de un estrés hídrico abarcan desde los niveles morfológicos y fisiológicos hasta los moleculares, siendo evidentes en todas las etapas fenológicas del desarrollo de las plantas. El estrés hídrico en las plantas conduce a la generación de ROS, causando daño oxidativo y deteriorando las funciones normales de las células debido a la alteración de diferentes enzimas y a la limitación de la asimilación de CO_2 causada principalmente por un cierre estomático (Farooq et al., 2009; Ghadirnezhad Shiade et al., 2022). En este sentido, al verse reducida la tasa transpirativa de la planta por un estrés hídrico, la concentración de nutrientes puede reducirse debido a la restricción sufrida en la absorción y traslocación de los mismos (Sánchez-Rodríguez et al., 2013, 2014). Todo ello se resume en efectos sobre el desarrollo óptimo de las plantas, afectando a la producción de biomasa y desarrollo de los frutos.

En lo que respecta al cultivo del pimiento, en términos generales, éste se clasifica como un cultivo de altas exigencias hídricas, que pueden estimarse en alrededor de 5.000-7.000 $m^3 \cdot ha^{-1}$ año (Abdelkhalik et al., 2020; González-Dugo et al., 2007; Sezen et al., 2019). Sin embargo, el total de agua utilizada y la frecuencia de los riegos dependen de muchos factores, como es el tipo de suelo, ciclo de cultivo, condiciones de cultivo, al aire libre o protegido, etc.

El efecto del estrés hídrico en el cultivo del pimiento, al igual que en otros cultivos, puede variar dependiendo de la severidad y la duración del estrés, así como del momento en el que se presenta. El estrés hídrico impuesto en algunas etapas críticas de crecimiento, floración y cuajado, puede tener efectos a partir de los cuales la recuperación del rendimiento completo puede no ser posible (Yasuor y Wien, 2020).

En las últimas décadas, con el objetivo de minimizar el gasto de agua de riego y aumentar la eficiencia del uso de la misma, se está implementando con éxito en algunos cultivos la técnica del riego deficitario controlado. Esta técnica consiste en aplicar un volumen de riego total por debajo de la dosis requerida por la evapotranspiración de cultivo, solo en aquellas fases del cultivo en que la demanda hídrica por parte de la planta no es muy elevada (Fereres y Soriano, 2007). En general, la técnica de riego deficitario puede conllevar pérdidas importantes en los rendimientos de los cultivos hortícolas (Du et al., 2017; Fereres y Soriano, 2007). En el caso del pimiento, el uso de la técnica de riego deficitario controlado ha mantenido o reducido los rendimientos en comparación a las producciones obtenidas con riego normal dependiendo del momento de aplicación (Abdelkhalik et al., 2020; González-Dugo et al., 2007; Sezen et al., 2019).

Los mecanismos de tolerancia al estrés hídrico son muy complejos e implican una serie de procesos fisiológicos y bioquímicos a nivel de células, tejidos, órganos e inclusive de toda la planta. Entre ellos, podemos destacar el cierre estomático para reducir la perdida de agua por parte de la planta, el aumento de absorción de agua por un mayor volumen explorado de suelo debido a un sistema radical más grande y profundo, la acumulación de osmolitos y la síntesis de osmoprotectores (Ghadirnezhad Shiade et al., 2022). A pesar de que los avances en la investigación de los mecanismos de tolerancia frente al estrés hídrico no cesan, las herramientas que disponen los agricultores son escasas, entre las que cabe destacar el riego deficitario controlado ya comentado y el uso de bioestimulantes, cuya efectividad varía mucho entre cultivos y severidad del estrés (Colla et al., 2015; Rai et al., 2021).

1.2.2.3. Otros estreses abióticos importantes en el cultivo del pimiento

La salinidad es un problema frecuente que afecta a la producción de cultivos en todo el mundo (20% de las tierras cultivadas del mundo y el 33% de las tierras de regadío). Este proceso puede verse acentuado por el cambio climático, el aumento del uso de agua de baja calidad en el riego y la baja disponibilidad de este recurso debido al incremento de superficie regada asociado a la agricultura intensiva. La salinidad excesiva del suelo reduce la productividad de muchos cultivos agrícolas, incluida la mayoría de las hortalizas, que son particularmente sensibles a lo largo de la ontogenia de la planta. El umbral de salinidad de la mayoría de los cultivos de hortalizas es bajo (entre 1 y 2,5 dS m^{-1} en extractos de suelo saturados), en el caso del cultivo del pimiento, el óptimo se encuentra alrededor de 1,5 dS m^{-1} y se considera un cultivo moderadamente sensible a la salinidad (Machado y Serralheiro, 2017). En términos generales, los efectos de la salinidad en las plantas son el resultado tanto en un primer momento del estrés hídrico -debido a un mayor potencial osmótico en el suelo en comparación con los tejidos vegetales-, como a largo plazo por un efecto tóxico causado por la entrada de iones, principalmente Na^+ y Cl^-, en los tejidos vegetales (Munns y Tester, 2008; Tuteja, 2007). El resultado de estos efectos es una amplia gama de cambios fisiológicos, metabólicos y genómicos que provocan alteraciones en la fotosíntesis, la partición de carbohidratos, la respiración, el aumento de la producción de ROS y una absorción desequilibrada de otros nutrientes (Hu y Schmidhalter, 2005; Parida y Das, 2005). En general, estos cambios fisiológicos inducidos por la salinidad conllevan a una disminución del crecimiento y rendimiento del cultivo del pimiento (De Pascale et al., 2003; Rubio et al., 2009).

Por otro lado, también se han descrito y estudiado otro tipo de estreses abióticos como la toxicidad por acumulación de metales pesados (Desoky et al., 2019;

Lidiková et al., 2021), o el desequilibrio nutricional de las plantas de pimiento por falta de los principales nutrientes como son el nitrógeno (N), potasio (K) o fósforo (P) (Medina-Lara et al., 2008; Urrea-López et al., 2014).

1.3. Técnica del injerto. Herramienta para hacer frente estreses abióticos

Se han realizado numerosas aproximaciones para mejorar la tolerancia de los cultivos a los estreses tanto desde la mejora genética tradicional como desde la transformación genética, aunque los éxitos comerciales se han visto a menudo limitados por la complejidad de caracteres poligenéticos implicados en la tolerancia al estrés abiótico (Cuartero et al., 2006; Kim et al., 2014). La búsqueda de estrategias para conseguir una mejor resistencia a los estreses abióticos es un objetivo de gran interés a nivel mundial teniendo en cuenta además el escenario del cambio climático, que provocan tanto olas de calor como disminuciones en la precipitación y aumentos de la salinidad del agua de riego y del suelo, principalmente en las áreas Mediterráneas (IPCC, 2022).

La obtención de variedades tolerantes a estreses abióticos resulta menos interesante para las empresas obtentoras que la búsqueda de tolerancia a estreses bióticos, mucho más importantes por su mayor incidencia en el rendimiento económico de los cultivos hortícolas y también por la mayor urgencia en su resolución. En este sentido, la técnica del injerto hortícola ha resultado ser una estrategia con gran potencial para hacer frente de forma efectiva, eficiente y sostenible a los estreses bióticos, y más recientemente a los estreses abióticos.

Actualmente, el uso del injerto en hortícolas (Solanáceas y Cucurbitáceas) está generalizado en todo el mundo y se emplea cada vez más a escala comercial para mejorar la producción principalmente en sandía, melón, tomate, berenjena y pepino. Asia es el continente con mayor superficie cultivada de hortalizas injertadas, ya que sólo Corea del Sur y Japón produjeron 700.000 millones de plántulas injertadas en 2009. En los países europeos, España encabeza la lista con el mayor número de plántulas injertadas (Lee et al., 2010), donde casi el 100% de la sandía y casi el 90% del tomate de invernadero es producido por plantas injertadas.

La eficacia del injerto para conferir tolerancia a los cultivos hortícolas contra los estresores abióticos se ha atribuido a varios rasgos mejorados de las plantas injertadas relacionados con las características del patrón: un sistema radical más vigoroso, una mejor absorción de agua y nutrientes que permite una mayor eficiencia fotosintética y relaciones hídricas, una activación del sistema de defensa antioxidante, una mayor señalización hormonal y un movimiento a larga distancia de ARNm y proteínas que modulan y alivian la percepción del estrés abiótico en la variedad (Albacete et al., 2015; Han et al., 2022; Kumar et al., 2017; Schwarz et al., 2010).

Los principales estreses abióticos que definen el clima Mediterráneo son: salinidad, estrés hídrico y altas temperaturas. A tal respecto existen diferentes estudios que han demostrado que algunos patrones son capaces de aumentar la eficiencia en el uso de agua y nutrientes (Cantero-Navarro et al., 2016; Colla et al., 2011; Martínez-Andújar et al., 2016, 2017; Savvas et al., 2010) y que permiten mitigar las pérdidas de rendimiento del cultivo. Bajo condiciones salinas, se ha visto que el patrón puede incrementar el vigor, el rendimiento y mejorar la tolerancia de la variedad en tomate, melón, pepino y calabaza (Estañ et al., 2005; Huang et al., 2010; Orsini et al., 2013; Zhu et al., 2008). Respecto al estrés hídrico, se han descrito mecanismos para explicar la tolerancia de las plantas injertadas (Kumar et al., 2017). La relación entre el desarrollo del sistema radical del patrón y la tolerancia al estrés hídrico, debido a una mayor capacidad de absorción de agua y nutrientes y una mayor eficiencia en el uso del agua, han sido estudiados en numerosas especies de interés agrícola obteniendo notables resultados (López-Marín et al., 2017; Poudyala et al., 2015; Rouphael et al., 2008; Sánchez-Rodríguez et al., 2014; Savvas et al., 2010). También se han demostrado diferentes mecanismos de resistencia al estrés hídrico que confiere el portainjerto a la parte aérea de la planta en diferentes cultivos, a través de la señalización hormonal (Liu et al., 2016), el ajuste osmótico (Penella et al., 2014), la activación del sistema antioxidante (Sánchez-Rodríguez et al., 2016) y por lo tanto, la mejora de la eficiencia en el aparato fotosintético y la formación de biomasa.

La tolerancia a las altas temperaturas en plantas injertadas se ha estudiado principalmente en plantas de pepino y tomate. El pepino injertado sobre *Momordica charantia* L. en condiciones de estrés térmico mostró una sobre-expresión de genes relacionados con la fotosíntesis asociados a las proteínas Rubisco y OEE1 (Xu et al., 2018) que permiten mantener la fotosíntesis. Por otro lado, también en pepino injertado sobre *Luffa cylindrica* (L.) M. Roem. ha demostrado tolerancia al choque térmico (Li et al., 2014) mejorando el rendimiento de la planta principalmente a través de la vitalidad de la raíz, la eliminación de ROS y la acumulación de proteínas de choque térmico (HSP). En el caso del tomate, se ha demostrado que los efectos del estrés están atenuados en una variedad injertada sobre un patrón tolerante, en relación a los observados en plantas sin injertar que presentan una mayor acumulación de ROS, indicando un mayor estrés oxidativo y un mayor contenido de fenoles totales (Rivero et al., 2003).

La técnica del injerto está contribuyendo a una agricultura sostenible por reducir la aplicación de productos químicos en la desinfección de suelos, proporcionar una mejor resiliencia de las plantas frente al cambio climático y permitir injertar sobre un patrón tolerante la variedad deseada por sus características productivas o de calidad. Estos beneficios van a conducir en un futuro ya cercano a la expansión del injerto en el cultivo del pimiento (Leal-Fernández et al., 2013).

1.3.1. Uso del injerto en el cultivo del pimiento

A pesar de las bondades que se han descrito en los últimos años sobre las ventajas de la utilización de planta injertada frente a los estreses abióticos, esta se está utilizando principalmente en tomate, berenjena, melón y sandía. El uso de portainjerto en pimiento es todavía limitado (Lee et al., 2010), debido a que existen pocos patrones comerciales disponibles en el mercado y estos suelen ser sensibles a los estreses abióticos. Además, el injerto en pimiento puede presentar el problema de incompatibilidad en la unión patrón/variedad en algunas combinaciones incluso siendo ambas plantas de la misma especie. Sin embargo, su uso, al menos en España, ha crecido notablemente en la última década, pasando de unos 10.000 injertos a 30.0000 injertos de pimientos anuales, como afirman las principales empresas de injertos de Almería, y seguramente aumentará en los próximos años (López-Galarza, S., comunicación personal).

Debido a los pocos estudios realizados hasta la fecha en el uso de la técnica del injerto para hacer frente a estreses abióticos en el cultivo del pimiento, hace poco más de una década comenzó una línea de investigación entre el Instituto Valenciano de Investigaciones Agrarias (IVIA) y la Universitat Politècnica de València (UPV) a través de diferentes proyectos competitivos de investigación, en la cual esta englobada esta tesis doctoral. Fruto del trabajo realizado en los últimos años, se han obtenido resultados relevantes relacionados con el uso del injerto en pimiento para la tolerancia a estreses abióticos. Entre ellos cabe destacar los siguientes:

1. Se han seleccionado accesiones de diferentes especies del género *Capsicum* para evaluar su adaptación agronómica al estrés hídrico y salino, y se ha confirmado que algunos genotipos utilizados como portainjertos presentan tolerancia frente a dichos estreses abióticos.
2. Estas accesiones seleccionadas proporcionan, bajo condiciones de estrés hídrico y salino, rendimientos superiores a la variedad sin injertar, principalmente por la disminución del número de frutos afectados por la fisiopatía BER (López-Serrano et al., 2017; Penella et al., 2013, 2014).
3. A partir de las accesiones seleccionadas como tolerantes a los estreses hídrico y salino, se han realizado hibridaciones (mediante mejora genética clásica) y se han obtenido híbridos, entre ellos el portainjerto NIBER®. Los resultados de producción demostraron que las plantas de pimiento injertadas con el patrón NIBER® incrementaron la producción en condiciones de estrés salino respecto a la variedad sin injertar (López-Serrano et al., 2020).

4. Los portainjertos de *Capsicum* tolerantes seleccionados utilizan varias estrategias para afrontar la escasez de agua y el estrés salino: mejor ajuste osmótico; restricción del transporte de Cl^- a las hojas, compartimentalización de Na^+ en raíces y hojas y reducción del potencial osmótico al aumentar principalmente la síntesis de prolina; menor contenido en ROS; reducción de las concentraciones de ácido abscísico (ABA) en hojas bajo estrés salino, conduciendo a una mayor apertura estomática, y por lo tanto, una mayor producción de biomasa (López-Serrano et al., 2019, 2020; Penella et al., 2014, 2015, 2016, 2017).

Por otro lado, en el cultivo del pimiento también se han realizado ensayos para observar la influencia del injerto frente el estrés térmico, obteniendo buenos rendimientos y mayor biomasa en plantas injertadas (Palada y Wu, 2008). En un estudio en el que se evaluaron distintos portainjertos sobre una variedad de pimiento comercial, sometidos a distintas condiciones de radiación y temperatura, uno de dichos portainjertos, aumentó el área foliar y la biomasa fresca y seca de las hojas respecto a las plantas control no injertadas. Asimismo, las plantas injertadas sobre algunos portainjertos aumentaron el rendimiento de los frutos comerciales, en comparación con las plantas no injertadas (López-Marín et al., 2013). Otros autores, como Aidoo et al. (2017), han corroborado posteriormente el efecto del injerto frente a las altas temperaturas, obteniendo un aumento de biomasa aérea fresca y seca, así como una mayor tasa de desarrollo radicular respecto a plantas de pimiento no injertadas.

1.4. Objetivos de la tesis

En las últimas décadas se han corroborado las bondades de la técnica del injerto hortícola para mejorar el rendimiento y calidad comercial de diferentes cultivos hortícolas en condiciones de estrés abiótico (Kumar et al., 2017; Kyriacou et al., 2017; Lee et al., 2010; Schwarz et al., 2010). En este sentido, el equipo de investigación mixto entre la Universitat Politècnica de València (UPV) y el Instituto Valenciano de Investigaciones Agrarias (IVIA), el cual engloba esta tesis doctoral, ha sido capaz de seleccionar diferentes accesiones y híbridos de pimiento, entre ellos el híbrido NIBER®, que utilizados como portainjertos han obtenido buenos resultados en condiciones de estrés hídrico y salino, además de generar información respecto a los diferentes mecanismos fisiológicos, moleculares y genéticos responsables de dicha tolerancia (Calatayud et al., 2020; López-Serrano et al., 2017, 2019, 2020, 2022; Penella et al., 2013, 2014, 2015, 2016, 2017). Fruto de todo este conocimiento generado respecto a la tolerancia a los estreses abióticos mediante el uso de patrones tolerantes en pimiento, surgió la primera hipótesis de la presente tesis doctoral, sobre si el uso de la técnica del injerto en pimiento sería capaz de soslayar los efectos de las altas temperaturas, que tantas implicaciones negativas están teniendo en los últimops años.

Por otra parte, también se ha pretendido mejorar la producción de variedades tradicionales de pimiento, aumentando la diversidad en este cultivo, mediante el uso de patrones adaptados a las condiciones mediterráneas, valorando asímismo la calidad del fruto al utilizar esta estrategia.

Tomando como referencia todas las aportaciones realizadas hasta el momento, los objetivos principales de esta tesis doctoral han sido:

1. Evaluar fisiológica y agronómicamente la tolerancia al estrés supra-térmico de accesiones de *Capsicum annuum* L. utilizadas como portainjertos.
2. Estudiar los mecanismos fisiológicos que subyacen en el comportamiento de portainjertos tolerantes a estrés supra-térmico.
3. Estudiar las bases del comportamiento agronómico y fisiológico de portainjertos de pimiento tolerantes a estrés hídrico en condiciones controladas de riego deficitario.
4. Evaluar el comportamiento agronómico y la calidad del fruto de variedades tradicionales de pimiento injertadas sobre patrones tolerantes a estreses abióticos.

Los resultados obtenidos como consecuencia de los objetivos 1 y 2 se han publicado en dos artículos:

Gisbert-Mullor, R., Padilla, Y. G., Martínez-Cuenca, M. R., López-Galarza, S., y Calatayud, Á. (2021). Suitable rootstocks can alleviate the effects of heat stress on pepper plants. *Scientia Horticulturae*, *290*, 110529. Cápitulo 2.

Gisbert-Mullor, R., Padilla, Y. G., Calatayud, Á., y López-Galarza, S. (2023). Rootstock-mediated physiological and fruit set responses in pepper under heat stress. *Scientia Horticulturae*, *309*, 111699. Cápitulo 3.

Los resultados obtenidos respecto al objetivo 3 han sido publicados en los siguientes artículos:

Gisbert-Mullor, R., Pascual-Seva, N., Martínez-Gimeno, M. A., López-Serrano, L., Badal Marín, E., Pérez-Pérez, J. G., Bonet, L., Padilla, Y. G., Calatayud, Á., Pascual, B., y López-Galarza, S. (2020). Grafting onto an appropriate rootstock reduces the impact on yield and quality of controlled deficit irrigated pepper crops. Agronomy, 10(10), 1529. Capítulo 4.

Gisbert-Mullor, R., Martín-García, R., Bažon Zidarić, I., Pascual-Seva, N., Pascual, B., Padilla, Y. G., Calatayud, Á., y López-Galarza, S. (2023). A

Water Stress–Tolerant Pepper Rootstock Improves the Behavior of Pepper Plants under Deficit Irrigation through Root Biomass Distribution and Physiological Adaptation. Horticulturae, 9(3), 362. Cápitulo 5.

Finalmente, los resultados del objetivo 4 han sido publicados en el siguiente artículo:

Gisbert-Mullor, R., Ceccanti, C., Gara Padilla, Y., López-Galarza, S., Calatayud, Á., Conte, G., y Guidi, L. (2020). Effect of grafting on the production, physico-chemical characteristics and nutritional quality of fruit from pepper landraces. Antioxidants, 9(6), 501. Cápitulo 6.

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Capítulo 2

Suitable rootstocks can alleviate the effects of heat stress on pepper plants

Ramón Gisbert-Mullor[a], Yaiza Gara Padilla[b], Mary-Rus Martínez-Cuenca[b],
Salvador López-Galarza[a] and Ángeles Calatayud[b]

[a] Departamento de Producción Vegetal, CVER, Universitat Politècnica de València, Camí de Vera s/n, 46022 Valencia, Spain
[b] Instituto Valenciano de Investigaciones Agrarias, CV-315, Km 10,7, Moncada, 46113 Valencia, Spain

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2.1. Abstract

In this study, different pepper rootstocks are tested for their ability to overcome heat stress situations. This work aims to evaluate: (i) the physiological mechanisms that occur during long heat stress periods (7 days) under controlled conditions in a pepper variety grafted onto accessions; (ii) the heat stress behaviour of these grafted pepper plants under greenhouse conditions in terms of marketable yields. For this purpose, plants of Lamuyo- type sweet pepper 'Herminio F1' (VA), grafted onto six accessions (VA/A25, VA/A31, VA/A34, VA/A52, VA/ A57, VA/A6), and a self-grafted variety (VA/VA) were grown under controlled conditions in growth chambers (28/24 °C, day/night temperatures and 38/24 °C for control and heat stress, respectively) and under greenhouse conditions (38/24 °C). For the controlled conditions, relative growth rate, leaf area, electrolyte leakage, chlorophyll *a* fluorescence and heat shock proteins were determined. For the greenhouse conditions, fresh and dry weigh, electrolyte leakage and fruit yield were determined. Our results confirmed that grafting a pepper cultivar onto appropriate rootstocks such as A6, A25 and A57 can overcome the negative effects of heat stress conditions with a higher relative growth rate, leaf area and Fv/Fm, and lower electrolyte leakage under the controlled conditions, and with higher marketable yields under the greenhouse conditions.

Keywords:
Chlorophyll a fluorescence; electrolyte
leakage; grafting; heat shock proteins;
relative growth rate; thermal stress

2.2. Introduction

Sweet pepper displays a marked response to heat, the optimal temperature ranging between 20 °C and 30 °C. Above 32 °C, the temperature can cause serious problems in pollination and fertilization resulting in fruit drop (Erickson and Markhart, 2002; Guo et al., 2014). However, different lines of chilli pepper from *C. chacoense, C. bacatum, C. frutescens* to *C. annuum* have been identified as heat tolerant compared with some sweet peppers based on its cumulative temperature response index (Barchenger et al., 2019; Palada and Wu, 2008). Aloni et al. (1994) associated the susceptibility to high temperature of two *C. annuum* cultivars (sweet pepper and paprika) to light intensity and ethylene production. Although, the response to heat stress is totally dependent to their genetic background (Usman et al., 2014) and needs to be evaluated for each variety and heat stress conditions.

In mild winter climates with warm springs and hot summers, the cropping season of sweet pepper crops is actually intended to extend over the 12 months of the year, normally comprising one of these three cycles: 1) planting in July/August and crop-ending in February; 2) an extended cycle, from May/June to April/May of the following year; 3) and a cycle starting in November/December to harvest during June-July-August. Depending on each cycle, flowering and fruit set normally start two months after planting and last until the end of the cycle unless during the coldest and shortest daylength months of the season, at least December and January. During these cycles but particularly in the extended one, sweet pepper crops, normally in greenhouses, may have to withstand high temperatures, sometimes above 35 °C, which negatively impacts growth and yields, as well as pepper fruit quality (López-Marín et al., 2013). This situation can be aggravated by the global warming scenario as temperatures grow increasingly higher worldwide, particularly in the Mediterranean Basin (IPCC, 2018), which produces huge quantities of peppers and other vegetables.

Grafting is currently an effective alternative to relatively slow breeding programmes (Schwarz et al., 2010) to obtain varieties that adapt to abiotic stresses (Penella et al., 2017; Schwarz et al., 2010). It has been shown that using appropriate pepper rootstocks is an effective, feasible and sustainable strategy mainly against water and saline stress (Gisbert-Mullor et al., 2020; López-Marín et al., 2017; López-Serrano et al., 2017). Nevertheless, very few studies have screened rootstocks for heat stress with sweet pepper (Aidoo et al., 2017; López-Marín et al., 2013; Palada and Wu, 2008), and some have pointed out that grafting is a technique capable of reducing the negative effects of high temperatures on pepper plants.

One of the most sensitive plant cell components is the membrane, and high temperature increases its fluidity and ion-permeability due to protein denaturation, accompanied by metabolism inactivation (Ayenan et al., 2019; Hansen et al., 1994). Ion leakage has been considered a bio-marker to heat tolerance. In different crops, tolerant plants to high temperatures display less membrane permeability than non-tolerant plants (Ayenan et al., 2019; Camejo et al., 2006, 2005; De Silva and Asaeda, 2017; Gulen and Eris, 2004; Hu et al., 2010; Xu et al., 2017). Hence grafting is a good tool to reduce electrolyte leakage. In fact, tomato grafted onto eggplant has been found to reduce membrane permeability under prolonged and controlled heat stress conditions (Abdelmageed and Gruda, 2009).

Heat damage affects not only membranes fluidity, but also chloroplast (and mitochondria) activities, among other metabolic processes. In fact, photosynthesis is particularly sensitive to heat stress which induces its decrease. This decline in photosynthesis is related to an increased fluidity on thylakoid membranes (Biswal et al., 2011), which disrupts the photochemical reaction in the thylakoid lamellae and also the carbon metabolism in the stroma (Hu et al., 2020), and as a consequence an increase in the photorespiration rate occurs (Long et al., 2004). Limited photosynthesis causes a drop in photoassimilates restricting plant growth and ultimately affecting yields (Fahad et al., 2017; Taiz and Zeiger, 2015).

In grafted plants, rootstocks can influence the adaptive capacity of scion to photosynthesis under heat stress (Schwarz et al., 2010; Xu et al., 2018) as photosynthetic capacity can be dependent on the vitality of roots. Roots from rootstocks are usually larger and more vigorous, being capable of absorbing water and nutrients more efficiently than roots of the scion, which could alleviate substantially photosynthesis inhibition (Colla et al., 2008; Lee et al., 2010; López-Marín et al., 2013). In addition, the signalling compounds going through root-to-shoot like hormones, nutrients, genes, transcription factors and miRNA can alter scion perception responses to heat stress (Li et al., 2014a; Xu et al., 2018). However, how tolerant rootstocks regulate the photosynthesis processes under heat stress remains unknown (Li et al., 2016). Different plant combinations (scion/rootstock) have resulted successful in terms to improve photosynthetic apparatus protection in heat stress situations. Pepper plants grafted onto some rootstocks showed better maximum quantum yield of PSII, Fv/Fm, in contrast to ungrafted plans (López-Marín et al., 2013). Cucumber plants grafted onto *Momordica* have enhanced chlorophyll content, Fv/Fm and net photosynthesis compared to self-grafted plants (Tao et al., 2020; Xu et al., 2018). Moreover, grafting cucumber onto heat-tolerant *Luffa* rootstock alleviates heat-induced photosynthesis inhibition and oxidative stress (Li et al., 2016).

Plants activate stress-responsive mechanisms to minimise the harmful effects of heat stress, such as antioxidant activities, osmoprotection, hormonal signals, metabolites synthesis or induction of heat shock proteins (HSPs). Different studies have also demonstrated that heat stress promotes the accumulation of HSPs, and these proteins are considered to be master players for inducing tolerance. HSPs act as molecular chaperones, and are induced under heat conditions to protect cellular proteins against irreversible heat damage (Barua et al., 2003; Boston et al., 1996; Li et al., 2014a). HSPs comprise five major families of HSPs based on their approximate molecular weights, such as HSP100, HSP90, HSP70, HSP60, and small HSP (sHSP) (Gupta et al., 2010; Kotak et al., 2007; Wang et al., 2004). Some studies have revealed an increase in HSPs in grafted plants under heat stress. The HSP70 protein has been significantly induced in cucumber grafted onto *Luffa* at an earlier stage (12-48 h) of heat treatment (Li et al., 2016, 2014a). Compared with HSP70 and HSP90, sHSPs are apparently more limited and ATP-independent, they bind to non-native proteins ranging from peptides to big proteins with high efficiency, preventing irreversible aggregation of these proteins (Haslbeck et al., 2019). However, there are no studies in pepper grafted plants about the conservation of HSPs or their effects over time (days) to confer tolerance in heat stress situations.

For all these reasons, in order to adapt the pepper crop to protected cultivation systems in the global warming scenario, it seems important to select rootstocks capable of conferring the pepper scion the ability to face the problems caused by high temperatures in the hottest part of the season by screening pepper genotypes in these situations.

Consequently, our work objectives were to evaluate: (i) in controlled conditions in growth chambers, the physiological mechanisms that occur during heat stress periods (7 days) in the *C. annuum* cultivar “Herminio” grafted onto different *C. annuum* accessions; (ii) under greenhouse conditions, the heat stress tolerance during long periods of these grafted pepper plants in terms of marketable yields.

2.3. Materials and methods

Six *Capsicum annuum* L. genotypes were used as rootstocks together with the scion cultivar “Herminio F1” (Syngenta) (*Capsicum annuum*, Lamuyo type, B2 type of Pochard (1966) classification) (VA). Accessions, with their country of origin in brackets, were: A25 (United States), A31 (United States), A34 (Spain), A52 (United States), A57 (Israel), A6 (Mexico). These accessions were selected according to previous testing experiments under heat stress and control conditions leading to identify both tolerant and sensitive accessions (unpublished data). All the genotypes employed in the present study belong to the COMAV Institute collection (Universitat Politècnica de València, Valencia, Spain). Two experiments were done to meet the objectives.

2.3.1. Experiment 1: Physiological behaviour of pepper plants under the control and heat stress conditions of growth chambers

The variety grafted onto the six accessions (VA/A25, VA/A31, VA/ A34, VA/A52, VA/A57, VA/A6), the ungrafted variety (VA) and self- grafted variety (VA/VA) were evaluated under heat stress and control conditions.

The seeds of the variety and accessions were sown in 104-cell polystyrene trays in a fine structure peat substrate (80% white and 20% black, pH 5.7) (Gebr. Brill, Germany), on 27 December 2018. The graft was performed on 1 February 2019 by the tube-grafting method (Penella et al., 2014). On 4 March 2019, plants were transferred to 0.5-litre pots filled with the same peat substrate, and eight plants for each graft combination were randomly arranged in both growth chambers (control and heat stress conditions) for 7 days, each individual plant being the experimental unit. The climatic chambers conditions were 28/24 °C, day/night temperatures and 38/24 °C for control and heat stress, respectively, with a 16-hour photoperiod of 450±50 µmol m^{-2} s^{-1} PPFD (photosynthetic photon flux density).

Plants were fertigated by the ebb and flow method once at the beginning of the experiment and after that using a capillary mat, with a nutrient solution containing (in mmol L^{-1}): 6.6 NO_3^-; 0.5 $H_2PO_4^-$; 0.5 SO_4^{2-}; 0.5 NH_4^+; 3.0 K^+; 1.5 Ca^{2+}, 0.8 Mg^{2+} and in µmol L^{-1}: 15 Fe^{3+}, 6 Zn^{2+}, 12 Mn^{2+}, 30 B^{3+}, 0.8 Cu^{2+} and 0.5 Mo^{6+}. The electrical conductivity (EC) and pH of this nutrient solution was 0.8 dS m^{-1} and 6.5, respectively.

After 7 days under both climate conditions, relative growth rate, leaf area, electrolyte leakage, chlorophyll *a* fluorescence and heat shock proteins were determined.

The relative growth rate (RGR) was calculated by the following formula:

$$RGR = \frac{\ln W_2 - \ln W_1}{\Delta t}$$

where W_1 and W_2 were the total fresh biomass on day 0 (first day in growth chambers) and day 7, respectively, and Δt was 7 days. Before carrying out each weight, the substrate was saturated by submerging the tray in the nutrient solution during 24 hours and afterwards leaving it to drain for 6 hours to avoid variances between W_1 and W_2 in relation to the weight of the substrate. The RGR was measured for the eight plants of each genotype combination and thermal conditions.

The leaf area of four plants for each combination and growth chamber was determined using an area meter (model LI-3100C; Li-Cor, Lincoln, NE, USA).

To determine electrolyte leakage (EL), 18 discs of freshly cut leaves from the eight plants of each genotype combination and thermal condition were obtained with a hole-puncher (1.4 cm in diameter) and divided into three groups. The six discs from each group were placed in 50 mL flasks together with 20 mL of distilled water. The EL in the solution was calculated from the EC measures taken at 0 h (C_1) and after 2 h (C_2) at room temperature with a conductivity meter (Model Seven Easy Mettler Toledo, Mettler-Toledo AG, Switzerland). Total conductivity (C_3) was obtained after keeping flasks frozen (-40 °C) for 24 h. The results were expressed as a percentage of total conductivity.

$$EL(\%) = \frac{C_2 - C_1}{C_3 - C_1} \cdot 100$$

Chlorophyll *a* fluorescence analyses were done to evaluate the damage degree of the PSII reactions. The maximum quantum yield of PSII (Fv/Fm; where Fv = Fm – Fo) was measured on leaves after 30-minute dark adaptation with a portable pulse amplitude modulation fluorometer (PAM-2100; Walz, Effeltrich, Germany). The minimum fluorescence signal for the 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 10.000 μmol photon m^{-2} s^{-1} enabled maximum fluorescence (Fm) estimations to be made. Chlorophyll fluorescence parameters were measured for the eight plants of each genotype combination and thermal condition.

To measure HSP, frozen leaf samples (after 7 days in climatic chambers) were previously grounded with liquid nitrogen in a mortar until a fine powder formed. Total RNA was extracted from 100 mg of sample with the Rneasy Plant Mini Kit (Qiagen, USA) and was treated with the Rnase-Free Dnase Set (Qiagen, USA) to remove the remaining genomic DNA, following the manufacturer’s instructions. RNA concentration and purity were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) and the samples showing a proper concentration and suitable A260/A280 and A260/A230 absorption ratios were used. All the RNA samples were diluted to the same concentration before reverse transcription, in which cDNA was generated using the PrimeScript™ Reagent Kit (Perfect Real Time) (Takara, Japan).

Three primer pairs for the (HSP) genes were selected for quantitative real-time PCR (qRT-PCR) (Table 1): ubiquitin binding protein gene (CaUBI-3) as well as glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), which were used as the reference genes (Bin et al., 2012; Wan et al., 2011). The qRT-PCR was performed by the StepOne-Plus Real-Time PCR System (Applied Biosystems, USA) and SYBR® Premix Ex Taq™ II (Takara, Japan). Expression levels were calculated by the relative standard curve procedure using three independent biological replicates which were, in turn, technically replicated 3 times. These values were normalised with the geometric mean of the two reference genes and standardized in relation to the variety values under the control conditions. HSPs were measured in three plants for each genotype combination and thermal condition.

Table 1. The primer sequences of the target HSP genes.

Gene name	Gene ID	Primer sequence	References
CaHsp70- 13	CA00g89640	5’ ACTTTCTACCTCAGGCGACA 3’ (F)	Guo et al. (2016)
		5’ CATAACTCTTCAAACTTGGCTC 3’ (R)	
CaHsp3-Q	CA03g21390	5’ CTCGATGTCTCCCCTTTCGG 3’ (F)	Li et al. (2015)
		5’ TGATGCCCTGTTCCTTCCTG 3’ (R)	
CaHsp22.7	CA06g20260	5’ AATGTTTCCACAAGAGGCTGATCC 3’ (F)	Self- designed
		5’ CCTCCGTCTTCATCCCTGGTAT 3’ (R)	

F: Forward Primer; R: Reverse Primer.

2.3.2. Experiment 2: Agronomic evaluation of the pepper grafted plants under heat stress conditions in a greenhouse

The scion (cv. “Herminio”) grafted onto the six accessions (VA/A25, VA/A31, VA/A34, VA/A52, VA/A57, VA/A6), the ungrafted variety (VA) and the self-grafted variety (VA/VA) were evaluated under greenhouse conditions in soilless cultivation.

On 16 February 2019, the seeds of the cultivar and accessions were sown in 104-cell polystyrene trays in a fine structure peat substrate (80% white and 20% black, pH 5.7) (Gebr. Brill, Germany). The graft was performed on 27 March 2019. On 15 April 2019, plants were transplanted in 6-litre pots, in a medium structure peat substrate (100% white, pH 5.7) (Gebr. Brill, Germany), and placed in a Venlo-type glasshouse at a density of 2.5 plants m^{-2}. Pots were drip-irrigated, using anti-drain Netafim® drippers of 4 L h^{-1}, with a nutrient solution containing (in mmol L^{-1}): 14.0 NO_3^-; 1.5 $H_2PO_4^-$; 2.4 SO_4^{2-}; 0.5 HCO_3^-; 1.6 Cl^-; 1.2 NH_4^+; 6.0 K^+; 5.0 Ca^{2+}, 2.5 Mg^{2+}; 0.2 Na^+, and in µmol L^{-1}:

15 Fe^{3+}, 6 Zn^{2+}, 12 Mn^{2+}, 30 B^{3+}, 0.8 Cu^{2+} and 0.5 Mo^{6+}. The EC and pH of this nutrient solution were 2.2 dS m^{-1} and 6.5, respectively. The volume of the solution was controlled by the number of irrigations, which varied according to accumulated radiation. In addition, an attempt was made to maintain drainage between 15% and 20% of the total irrigation volume.

The heat stress conditions (day/night set point temperatures of 38/ 24 °C) began on 25 May 2019, 40 days after planting, when flower buds were observed in the second node. The set fruits in the first node, if any, were removed. The temperature, relative humidity and accumulate radiation values were recorded during the experiment using S8TH sensor (Oratge Instruments®, Valencia, Spain) with data logger MSIP801 (BSG Ingenieros, Valencia, Spain) and are presented in Fig. 1.

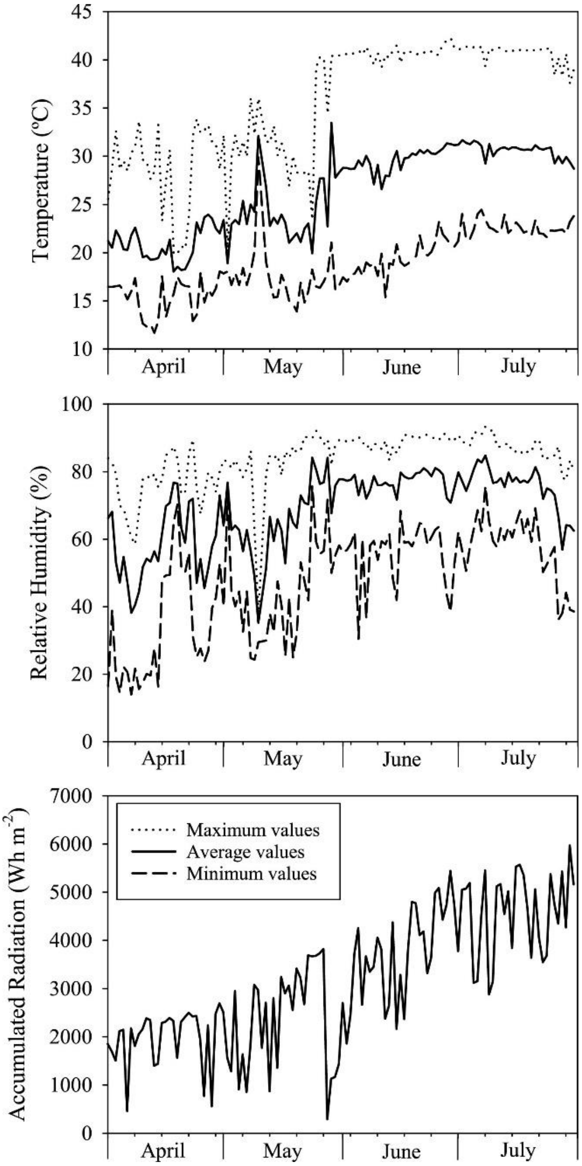

Fig. 1. Temperature (°C), relative humidity (%) and accumulated radiation (Wh m^{-2}) values inside the greenhouse.

The layout took a completely randomised design, based on our previous experience in this greenhouse, with three replications for each genotype combination and 10 plants per replication.

Fresh weight per plant was measured weighing leaves and stems which were later exposed to dry heat at 70 °C for 72h in a laboratory oven to measure dry weight.

EL was measured as it is described in the Experiment 1 (2.1. section).

Harvests were staggered between the end of June and the end of July and consisted in one harvest per week, matching five harvest rounds. Marketable and non-marketable production were evaluated following the criteria described by European Regulations (Official Journal of the European Union, 2011). First, fruits were partitioned into two categories: «Extra» Class and Class I (together hereafter they are referred to as marketable yield; MY). The fruit which, due to their defects (Blossom End Rot (BER), cracking, sunscald) did not reach these categories, are referred to as non-marketable yield (NMY). Then, fruit yield was measured as the weight of the fruits per plant (g/plant), number of fruits per plant and average marketable fruit weight (g/fruit) for all the plants of each genotype combination and replication.

2.3.3. Statistical analysis of data

For both experiments, the results for the different parameters were evaluated by an analysis of variance (ANOVA) using the Statgraphics Centurion XVII software (Statistical Graphics Corporation 2014). The RGR and EL data were *arcsin*-transformed before the analysis. Means were compared by the Fisher's least significance difference (LSD test) at $P \leq 0.05$. Stepwise multiple regression analysis for RGR as dependent variable and Fo, Fm, Fv/Fm, leaf area and EL as independent variables was performed as well as correlation analyses in Experiment 1 between the abovementioned parameters using the previously cited Statgraphics software.

2.4. Results

2.4.1. Experiment 1: Physiological behaviour of pepper plants under the control and heat stress conditions in growth chambers

2.4.1.1. Relative growth rate

The RGR was, on average for the genotypes, lower under heat stress, with a 28.1% reduction compared to the plants under the control conditions ($P \leq 0.01$; Table 2).

The interaction between the thermal conditions (TC) and genotypes (G) was also statistically significant ($P \leq 0.01$; Table 2). The RGR of VA, VA/VA, VA/A31, VA/A34 and VA/A52 was negatively affected by heat stress, whereas the VA/A25, VA/A57 and VA/A6 combinations were not affected by stressing temperatures (Fig. 2A). The RGR of the ungrafted cultivar (VA) under the control conditions was much higher than those of the grafted plants, but sharply dropped under stress conditions (Fig. 2A). The RGR of the self-grafted cultivar (VA/VA) under stress was less affected than that of VA, but was also negatively affected by stress. Furthermore, the RGR of VA/A25 and VA/A6 was higher than that of VA under stress, but similar to the VA/VA plants under heat stress.

2.4.1.2. Leaf area

Similarly to the RGR, the leaf area was higher on average in all the plants under the control conditions, with a 34.5% increase in relation to the plants under stress ($P \leq 0.01$; Table 2).

The interaction between the TC and G was also statistically significant, but explained very little about variation ($P \leq 0.05$; Table 2). The reduction in leaf area in the ungrafted variety (VA) under the stress conditions (46% in relation to its control) was much higher than in the grafted plants, which were 23% on average, including the self-grafted (VA/VA) one. Under the heat conditions, all the grafted combinations underwent significant reduction in the leaf area compared to their controls (Fig. 2B). The leaf area of the VA/A25 combination under heat stress was statistically higher than those of VA and VA/VA (Fig. 2B).

Table 2. Analysis of variance (ANOVA) of parameters relative growth rate (RGR), leaf area, electrolyte leakage (EL), Fv/Fm, Fo and Fm. % of the Sum of Squares for the factor thermal condition (TC) and genotype (G) as well as their interaction (TC*G).

ANOVA (*df*)	% Sum of Squares											
	RGR		Leaf area		EL		Fv/Fm		Fo		Fm	
TC (1)	33.1	**	51.6	**	19.8	**	23.2	**	7.5	**	3.6	*
G (7)	11.6	**	21.1	**	55.5	**	12.1	**	6.3	n.s.	21.1	**
TC*G (7)	14.7	**	6.7	*	12.3	**	8.2	*	4.6	n.s.	12.4	**
Residuals	40.6		20.6		12.4		56.5		81.7		62.9	
Residuals df	112		45		32		112		112		112	
Standard Dev. (+)	1.19		11.5		0.99		0.05		58.2		133	

*,** indicates significant differences at P ≤ 0.05 and P ≤ 0.01, respectively. n.s. denotes no significant differences. (+) Calculated as the square root of the residual mean square. *df*: degrees of freedom.

2.4.1.3. Electrolyte leakage

The EL value was higher under heat stress, with an 18.4% increase compared to the plants under the control conditions (P ≤ 0.01; Table 2).

The interaction between the TC and genotypes G was statistically significant (P ≤ 0.01; Table 2). The EL of the plants of the ungrafted cultivar (VA) under the stress conditions was much higher than for the other combinations (Fig. 2C). Compared to VA, the EL in the self-grafted cultivar (VA/VA) was 28.5% lower under the stress conditions and 17.7% lower under the control conditions.

Of all the studied combinations, VA/A25, VA/A31, VA/A57 and VA/ A6 showed no significant differences in EL between the stress and control conditions. Moreover, the EL in VA/A57 and VA/A25 lowered compared to VA/VA under stress, and was similar under the control conditions. The EL in VA/A57 under the heat stress conditions was less than in the VA grafted onto the other rootstocks, except for VA/A25 (Fig. 2C). Finally, VA/A31 and VA/A34 had a higher EL than VA/VA under both conditions (Fig. 2C).

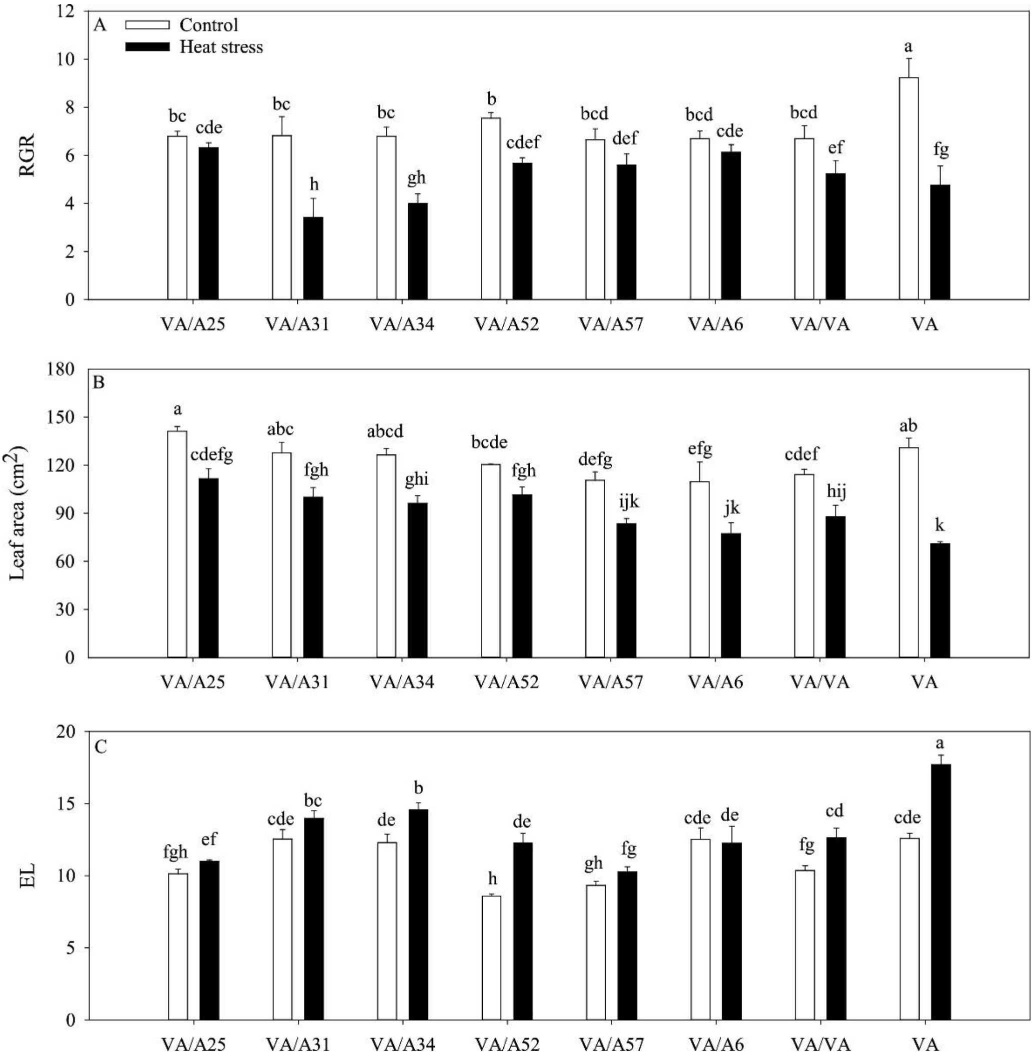

Fig. 2. Relative growth rate (RGR) (A), leaf area (B) and electrolyte leakage (EL) (C) in pepper genotypes under heat stress and the control conditions. Different letters indicate significant differences at $P \leq 0.05$ (Fisher's LSD test). Data are the mean of eight replicates for the RGR, four replicates for leaf area and three replicates for EL. Error bars represent standard error.

2.4.1.4. Chlorophyll a fluorescence

The maximum quantum yield of chlorophyll *a* fluorescence (Fv/Fm) was significantly lower in all the combinations subjected to heat stress, with an 8% reduction on average compared to those of the control conditions ($P \leq 0.01$; Table 2).

The interaction between the TC and G was also statistically significant ($P \leq 0.05$; Table 2). It is highlighted that the Fv/Fm under the control conditions was similar for all the G, but significant differences appeared under heat stress, where VA was much lower under stress conditions than in the other genotypes, except for VA/A34 and

VA/A6 (Fig. 3A). Significant differences between the control and stress conditions also appeared. The Fv/Fm in the VA and VA/VA, VA/A6, VA/A34 and VA/A31 combinations were significantly higher in the control than under stress, whereas no significant differences were observed in VA/ A25, VA/A52 and VA/A57 among thermal conditions (Fig. 3A). In the self-grafted cultivar (VA/VA), Fv/Fm was 10.4% higher in stress compared to the ungrafted VA.

The minimum chlorophyll fluorescence (Fo) was, on average, lower in all the G under the control conditions, with a 12% reduction compared to the plants under the heat stress conditions ($P \leq 0.01$; Table 2). The interaction between the TC and G was not statistically significant (Fig. 3B)

The maximum chlorophyll fluorescence (Fm) was lower in all the plants subjected to heat stress, with a reduction of 6.1% on average compared to the plants under the control conditions ($P \leq 0.05$; Table 2). The interaction between the TC and G was also statistically significant ($P \leq 0.01$; Table 2). In VA/A6, Fm was significantly lower under stress than under the control conditions, whereas the contrary took place in VA/ A57, which was higher under stress than under its control. With the other combinations, Fm was not significantly different between both the stress and control conditions (Fig. 3C).

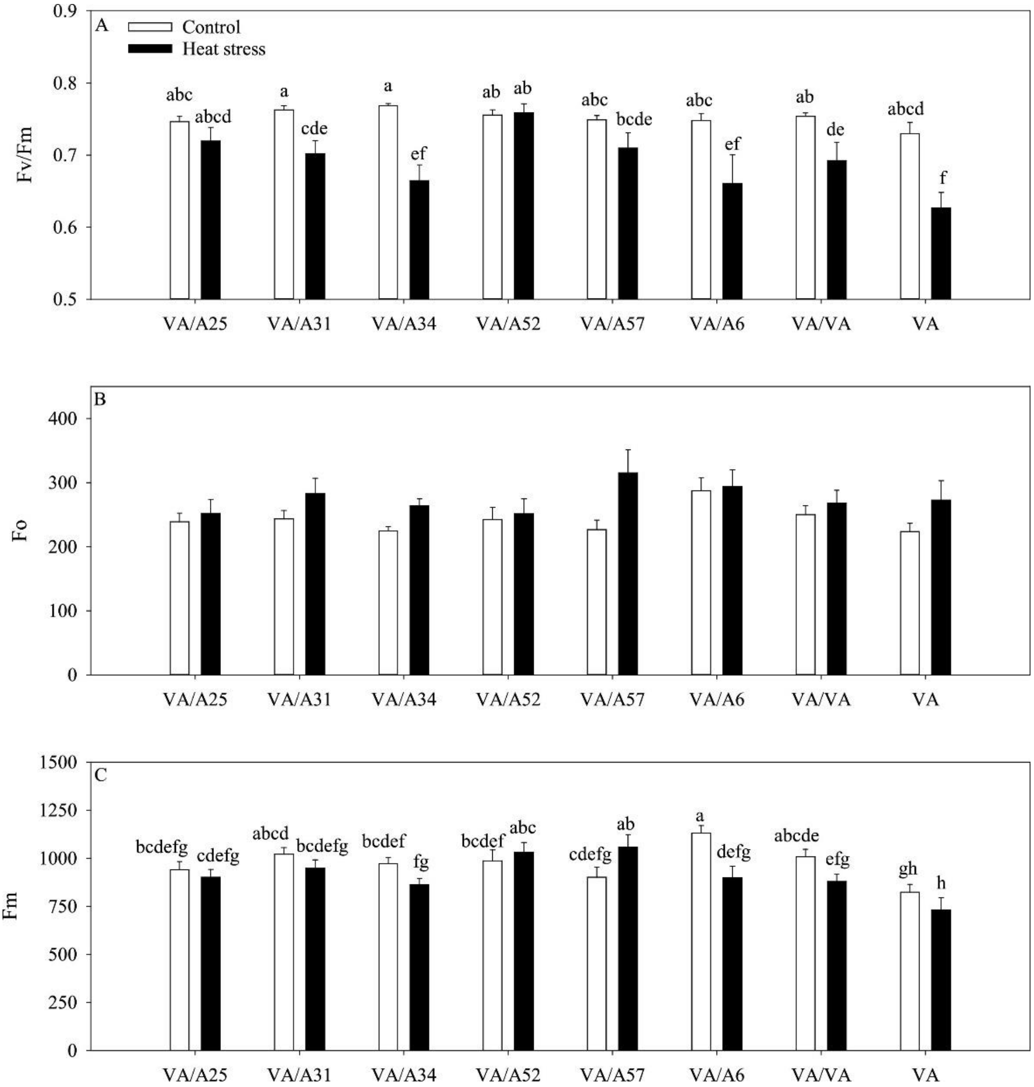

Fig. 3. Fv/Fm (A), Fo (B) and Fm (C) chlorophyll fluorescence parameters in pepper genotypes under heat stress and the control conditions. Different letters indicate significant differences at $P \leq 0.05$ (Fisher's LSD test). Data are the mean of eight replicates. Error bars represent standard error.

2.4.1.5. Heat Shock Proteins

To analyze whether HSPs contributed to the rootstock-induced thermo-tolerance of pepper plants, we compared the responses of two sHSP (HSP25.8 and HSP22.7) and one HSP70 (70-13) to heat stress between plant combinations. The qRT-PCR analysis indicated that the increased abundance of the transcripts of three HSP caused by heat stress was greater than under the control conditions after 7 days in climatic chambers for both grafted and ungrafted plants (Table 3).

Table 3 Analysis of variance (ANOVA) of the expression levels for HSP70-13, HSP25.8 and HSP22.7. % of the Sum of Squares for the factor thermal condition (TC) and genotype (G) as well as their interaction (TC*G).

ANOVA (*df*)	% Sum of Squares					
	HSP70-13		HSP25.8		HSP22.7	
TC (1)	38.6	**	16.7	**	31.6	**
G (7)	42.6	**	41.2	**	27.9	**
TC*G (7)	7.7	*	41.0	**	28.1	**
Residuals (32)	11.1		1.1		12.3	
Standard Dev. (+)	0.67		0.36		0.44	

*,** indicates significant differences at P ≤ 0.05 and P ≤ 0.01, respectively. n.s. denotes no significant differences. (+) Calculated as the square root of the residual mean square. *df*: degrees of freedom.

The interaction between the TC and G was statistically significant for the three analysed HSPs, with P ≤ 0.05 for HSP70-13 and P ≤ 0.01 for sHSPs (Table 3).

The expression levels in HSP70 (70-13) were lower than the values obtained in sHSP (Fig. 4A). Under heat stress, the highest values went to VA, VA/VA and VA/A6. Under the control conditions, the values for the grafted plants were lower than in VA and with significant differences.

The levels in HSP25.8 and HSP22.7 were higher in VA/A25, followed by VA/A31 and VA/A34 (Fig. 4B, C) for both sHSPs.

2.4.1.6. Multiple regression analysis

A stepwise multiple regression analysis was performed to predict RGR from the variables Fo, Fm, Fv/Fm, Leaf area and EL. The variables Fm, Leaf area and EL, statistically significantly predicted RGR, with an F (3, 95) = 9.24, p < 0.0001, and $R^2 =$ 0.43, according to the model: RGR = 0,000646957 + 0.0000129391*Fm + 0.00003678*Leaf area – 0.116089*EL.

2.4.2. Experiment 2: Agronomic evaluation of pepper grafted plants under the heat stress conditions in greenhouses

In the greenhouse experiment, we compared the ungrafted variety and the self-grafted to the variety grafted onto all the studied accessions under the heat stress conditions. Fresh weight of aerial part didn’t show significant differences between genotypes, however dry weight and EL showed differences between genotypes with P ≤ 0.05 and P ≤ 0.01, respectively (Table 4). DW was lower in VA/A6 compared to the other genotypes. For EL, highest values belong to VA and VA/VA, followed by VA/A25 and VA/A34, being the lowest values from VA/A52, VA/A6, VA/A57 and VA/A31.

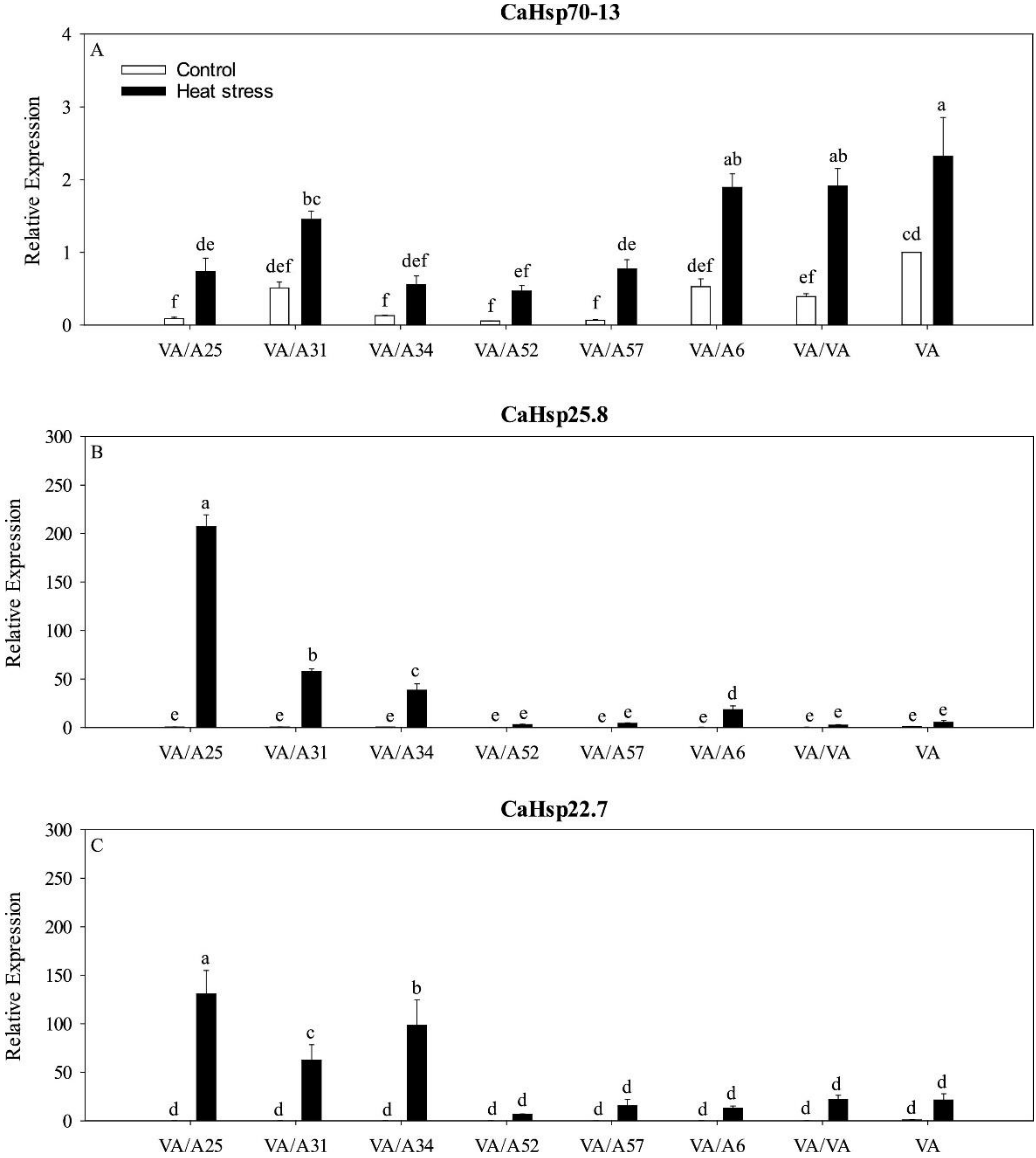

Fig. 4. Relative expression for CaHsp70-13 (A), CaHsp25.8 (B) and CaHsp22.7 (C) in pepper genotypes under heat stress and the control conditions. Different letters indicate significant differences at P ≤ 0.05 (Fisher's LSD test). Data are the mean of three replicates. Error bars represent standard error.

The marketable fruit number per plant of combinations VA/A57 and VA/A6 were higher than those of the cultivar (VA), the self-grafted cultivar (VA/VA) and the other combinations (P ≤ 0.01; Fig. 5A). The MY of VA/A57 was significantly higher than those of VA and VA/VA (Fig. 5B). The MYs of VA/A25 and VA/A6 were also high, but not significantly different from that of VA/A57, but were also significantly different to those of VA and VA/VA.

No significant differences between the different grafted genotypes and the ungrafted cultivar were observed for the number of non- marketable fruits per plant, non-marketable yield and average marketable fruit weight ($P \leq 0.05$; Fig. 5C, D, E).

Table 4 Analysis of variance (ANOVA) for fresh weight (FW), dry weight (DW) and electrolyte leakage (EL) from greenhouse experiment expressed as mean values by Genotype (G) and % of the sum of squares. Different letters in each column indicate significant differences at $P \leq 0.05$ using the LSD test.

Genotype (G)	FW (g plant^{-1})	DW (g plant^{-1})		EL (%)	
VA/A25	919	193.5	a	8.89	bc
VA/A31	1184	166.2	a	7.00	c
VA/A34	1235	185.8	a	8.16	bc
VA/A52	944	177.1	a	7.62	c
VA/A57	988	167.4	a	7.10	c
VA/A6	985	121.4	b	7.11	c
VA/VA	1390	178.0	a	10.66	ab
VA	1261	202.5	a	11.72	a
ANOVA (df)		% Sum of Squares			
G (7)	64.07	80.88	*	50.89	**
Residuals	35.93	19.12		49.13	
Standard Dev. (+)	174	15.8		1.89	

* and ** indicate significant differences at $P \leq 0.05$ or $P \leq 0.01$ respectively, using the LSD test. (+) Calculated as the square root of the residual sum of squares. df degrees of freedom.

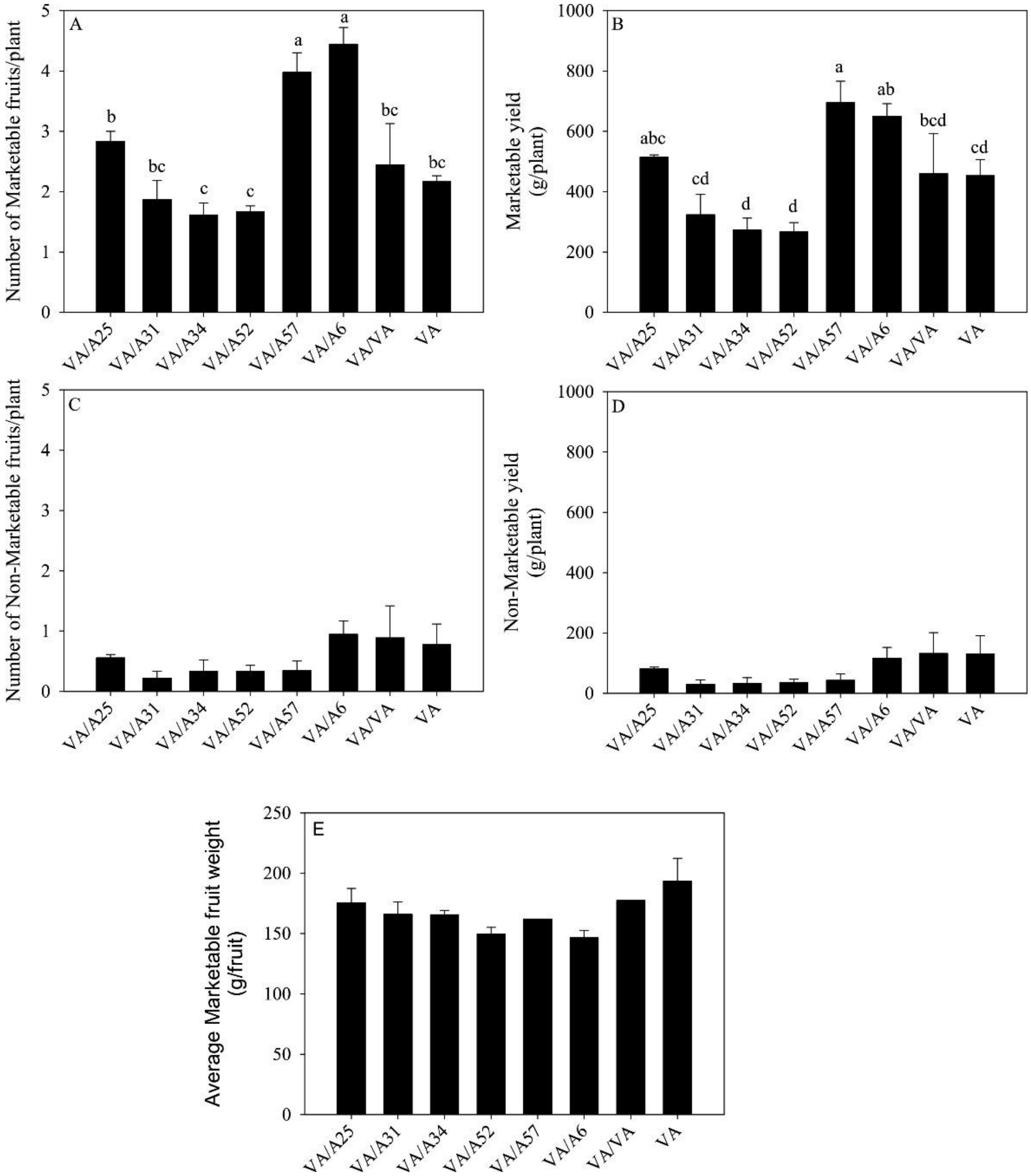

Fig. 5. Number of marketable fruits per plant (A), marketable yield (g/plant) (B), number of non-marketable fruits per plant (C) and non-marketable yield (g/plant) (D) and average marketable fruit weight (g/fruit) (E) in pepper genotypes under heat stress conditions. Different letters indicate significant differences at P ≤ 0.05 (LSD test). Data are the mean of three replicates. Error bars represent standard error.

2.5. Discussion

It is well-known that heat stress causes deleterious effects on growth. Temperature levels above 35 °C have been found to limit *Solanaceae* cultivation (Schwarz et al., 2010). These high temperatures as either heat shock or prolonged high temperatures dramatically affect growth, although studies of physiological responses to prolonged warming are rare (Wang et al., 2020).

In our experiments, we analysed the effect of high temperatures (38 °C, 10 °C above the control) for 7 days, which are usual conditions in the Mediterranean Region or hot climates in greenhouse crops (López-Marín et al., 2013) to simulate a heat wave. Our results showed both the negative effect of high temperatures on pepper plants and the possibility of tolerating heat stress using appropriate rootstocks.

One of the most important effects of high temperature on pepper crops from the economic-agronomy point of view is reduced growth and yields. The present study found that the highest RGR in pepper plant seedlings was observed in the genotypes grown under the control conditions, and the RGR was dependent on the accessions used as a rootstock. The pepper cultivar grafted onto accessions A25, A57 and A6 maintained plant growth, with no significant differences between heat stress and the control treatment. However, the cultivar grafted onto accessions A31, A34, A52, as well as the self-grafted cultivar and the cultivar itself, displayed reduced growth. These results suggest that RGR is dependent on the adaptability of roots to aerial temperature. Similar effects have been made by Rivero et al. (2003), who also observed that the dry weight of tomato plants grown at 35 °C lowered more in non-grafted plants than grafted plants. Li et al. (2014b) observed better shoot growth in cucumber grafted onto tolerant luffa at 36 °C than in ungrafted cucumber. However, in our experiments, the conservation into RGRs in VA/A25, VA/A57 and VA/A6 at high temperature was unable to preserve the leaf area. We observed reduced leaf areas in all the plant combinations under high temperature. This result could indicate that these grafted plants increased leaf thickness that acted as an adjustment strategy to acclimate at high temperature (Shu et al., 2016; Wahid et al., 2007) and to sustain the RGR.

The reduction in the RGR by high temperature is related to physiological and metabolic changes in plant cells. One of the most sensitive physiological alterations to heat stress is increased membrane fluidity due to protein denaturation and increased levels of unsaturated fatty acids with effects on plasma membrane stability (Hu et al., 2020; Wang et al., 2019). The amount of EL associated with membrane thermostability has been successfully employed to evaluate heat stress in several crops (Nadeem et al., 2018), including pepper (Wang et al., 2019), where heat-sensitive genotypes underwent greater membrane injury than heat-tolerant ones (Li et al., 2015). Some studies found low EL in tomato-grafted plants under heat stress conditions compared to ungrafted plants (Abdelhafeez et al., 1975; Abdelmageed and Gruda, 2009). Our study at growth chambers observed no significant differences in EL between the plants under heat stress and the control in the VA/A25, VA/A57, VA/A6 and VA/A31 combinations. What is more, under the stress conditions the EL values in VA/A25 and VA/A57 were lower than in the VA and VA/VA ones. This agrees with the RGR values of VA/A25 and VA/A57. Similar results were obtained in greenhouse conditions, where EL values were higher for VA and VA/VA.

The diminished membrane stability under heat stress could lead to increased thylakoid membrane fluidity (Prasad et al., 2008). Photosynthesis is one of the most sensitive processes to abiotic stresses via alterations to the photosynthetic apparatus (Zhou et al., 2015), and photosystem II (PSII) is regarded a sensitive and heat-labile component (Čajánek et al., 1998; Mathur et al., 2011). In our experiments, Fv/Fm under heat stress was lower than for the control conditions, which demonstrates that heat stress affects Fv/Fm. This ratio lowered in combinations VA/A31, VA/A34, VA/A6, VA/VA and VA under heat stress compared to their controls. A downfall of Fv/Fm may be the result of a drop in the rate constant of PSII that leads to a rise in Fo, whereas an increase in non-radiative energy dissipation leads to Fo and Fm to lower (Guidi et al., 2019; Kitajima and Butler, 1975). Fo remained unchanged in all the treatments and plant combinations, which indicates that heat stress did not induce modifications either at the antenna pigment level or in the excitation trapping efficiency at the active centres of PSII (Calatayud and Barreno, 2001). Fm behaviour was more erratic and dependent on plant combinations. Fm showed significant differences between heat stress and the control in VA/A6 and VA/A57, of which the last one showed an increased Fm vs. its control which could be due to an enhanced electron transport rate as Fv/Fm was unmodified.

The Fv/Fm ratio has been used as an early indicator of heat stress (Poudyal et al., 2018; Tsai et al., 2019; Zhou et al., 2015) and it allowed screening plants to heat stress by validating a negative correlation between high Fv/Fm values and the heat injury index in fruit set or the RGR (Poudyal et al., 2018). These results could indicate that Fv/Fm under our heat stress conditions could not be used as an earlier indicator of heat stress because the drop in Fv/Fm observed in some plant combinations could be due to a non-photochemical increase (Calatayud and Barreno, 2001; Wang et al., 2020) and it was not possible to separate heat damage from dynamic photoinhibition to preserve PSII.

In order to determine which physiological index contributed most to RGR under heat stress in growth chambers the regression analysis was analysed. EL is the physiological index that most contributed to explain the RGR variation. In fact, the most sensitive component under heat stress is the plasma membrane and it is the primary sites of injury driving to increase in EL (Wise et al., 2004).

Protein biosynthesis motivates plant growth and development as an essential biological process (Hu et al., 2020; Li et al., 2018; Shalgi et al., 2013). HSPs have evolved in plants as chaperon proteins to prevent protein denaturation and aggregation, and represent an essential role played under heat stress (Barua et al., 2003; Hu et al., 2020). It is well established that plant exposure to heat shock increases HSP accumulation, but very few studies have examined HSP accumulation over many consecutive days (Wang et al., 2020), which often occurs during heat waves under natural conditions. In this study, we analysed the relative expression of HSP70 and two small HSPs, sHSP25.8 and sHSP22.7, in grafted and ungrafted pepper plants under the control and high temperature for 7 days. The expression of the three HSPs was higher under heat stress compared to the control conditions. These results indicate that high temperature up-regulates the expression of HSPs, which agrees with Li et al. (2014b) for cucumber grafted plants and Wang et al. (2020) for *Arabidopsis*. However, relative HSP expression levels differ depending on HSPs and plants combinations. Our results showed that HSP70 expression increased significantly in relation to the control in all the plants, except for VA/A34 and VA/A52. Higher HSP70 expression has been detected in leaves of cucumber grafted onto luffa after 12 h, 36 h or 25 days (Li et al., 2016, 2014a, 2014b) at high temperature, but not in roots (rootstock) as HSP70 was not present in the xylem (Li et al. 2014a). The leaves of our VA plant displayed the highest HSP70 expression. This indicates that aerial parts induced HSP70 synthesis but it can't be discarded that stress signal comes from root to shoot (Li et al., 2014a) that can modulate the response of HSP70 synthesis in leaves similarly to the grafted plants with a low HSP70 expression. However, different heat temperature acclimation may induce distinct heat response pathways or other HSP70 or HPSs can act in heat response after 7 days.

We observed differential expression behaviour between sHSPs and HSP70. The sHSPs expression levels were higher than in HSP70 and were up-regulated by heat stress in all the plants. The accumulation of sHSPs was greater in VA/A25, and also in VA/A31 and VA/A34, but VA/ A6 only showed significant differences in sHSP25.8 comparing heat stress and control conditions. Unlike HSP70, no expression was observed in sHSPs in VA or VA/VA at high temperature, which indicates different plant combinations affect the expression of sHSPs that can be modulated by rootstocks with varying degrees of sHSPs synthesis. To date, as we are unaware of any research studies on sHSPs in pepper grafted plants, we herein present the first evidence that sHSPs were up-regulated by 7 day heat stress period.

In fact, the accumulation of HSPs and sHSPs plays a key role in both the heat stress response and acquired thermo-tolerance in plants (Wang et al., 2020; Zhou et al., 2011). Nevertheless, the mechanism by which the protective effects of HSPs on plant cells can be achieved is attributed to the chaperone machinery network, in which different HSPs/chaperones act cooperatively in connection with other signal and metabolic processes (Wang et al., 2004). Furthermore, distinct heat response pathways for thermo-tolerance or thermo-acclimation can be induced depending on the applied heat regime. Nevertheless, after the analysis of HSPs, as we were unable to distinguish if rootstocks' different degrees of tolerance correlated positively with fruit yield or the RGR, additional studies will be necessary to understand the complex network of HSPs.

Regarding the results obtained in experiment 2, the low yields were the result of both the severe heat stress suffered by plants and stressing temperatures, which reached 40 °C and strongly affected the flowering and fruit set processes (Erickson and Markhart, 2002; Yamazaki and Hosokawa, 2019), as well as the employed short crop cycle. However, the obtained low yields confirmed the observations made in the controlled experiment, where we observed that some rootstocks, A6, A25, and particularly A57, could better tolerate heat stress than the cultivar itself or the self-grafted cultivar according to the measured physiological parameters.

Apart from all these observations, it is interesting to point out that Penella et al. (2016) and López-Serrano et al. (2019) observed how the A25 accession used as a rootstock displayed higher photosynthesis and biomass than the ungrafted cultivar under saline or water stress. In the present study, we confirmed that the VA/A25 combination had higher RGR, leaf area and Fv/Fm values and lower EL than the ungrafted cultivar under the heat stress conditions. These results may indicate that, despite heat stress affecting aerial plant parts more, heat stress tolerance may be mediated by radical plant parts. Nevertheless, more studies are needed to confirm this hypothesis.

2.6. Conclusions

By way of conclusion, we observed how some genotypes can confer a certain degree of heat stress tolerance when used as rootstocks for a pepper cultivar by maintaining EL, which finally maintained the Fv/Fm ratio and resulted in a higher RGR and bigger fruit yields compared to the ungrafted cultivar. Accession A57 performed the best of all those we tested, and clearly its advantages are due to its genetic performance and not to a significant effect of the graft itself.

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Capítulo 3

Rootstock-mediated physiological and fruit set responses in pepper under heat stress

Ramón Gisbert-Mullor[a], Yaiza Gara Padilla[b], Ángeles Calatayud[b], Salvador López-Galarza[a]

[a] Departamento de Producción Vegetal,
CVER, Universitat Politècnica de València,
Camí de Vera s/n, 46022 Valencia, Spain
[b] Instituto Valenciano de Investigaciones
Agrarias, CV-315, Km 10,7, Moncada,
46113 Valencia, Spain

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3.1. Abstract

An increase in high temperature causes major losses in pepper yields, especially in greenhouses when extending the cropping season to late spring or summer in mild climate areas. Grafting has been identified as a possible tool to cope with this abiotic stress. The objective of this study was to analyze the heat stress impact on a sweet pepper variety grafted onto rootstocks with diverse heat stress tolerances to evaluate high-temperature effects on the leaf metabolism, pollen traits and fruit set. To do so, under two greenhouses conditions (28/22°C and 38/22°C for control and heat stress, respectively), we compared the variety grafted onto two rootstocks (VA/A57 and VA/A55, tolerant and nontolerant, respectively), and used varieties ungrafted (VA) and self-grafted (VA/VA) as controls. VA/A57 obtained the lowest electrolyte leakage, non-disturbed chlorophyll and carotenoids concentration values, increased ascorbic acid and phenols concentrations, and no hydrogen peroxide accumulation. These findings indicate better predisposition to overcome heat stress than other plant combinations. Such physiological responses in leaves conferred by the tolerant rootstock coincided with the highest proline concentration in anthers, and better pollen germination and fruit set compared to the other graft combinations. We conclude that grafting peppers onto a heat stress-tolerant rootstock, such as A57, could overcome negative high-temperature effects better than an ungrafted variety. Moreover, the better physiological performance noted in vegetative parts conferred by a heat stress-tolerant rootstock would also lead to better performance in the reproductive development phase. All this indicates that using tolerant rootstocks in pepper could be an interesting method to alleviate heat stress effects on this crop.

Keywords:
Capsicum annuum; graft; pollen; high temperature; antioxidants

3.2. Introduction

High temperatures that affect crops are relatively frequent in protected cultivation, particularly in warm regions, when extending the cropping season to late spring or summer. This situation could be aggravated in the near future as a consequence of climate change because the average temperature is expected to rise in different regions, especially on the Mediterranean coast (IPCC, 2018). This area produces vast quantities of vegetables, particularly sweet pepper (MAPA, 2021), an economically important crop in protected cultivation that is sensitive to heat stress (HS). In such a scenario, it has been observed that temperatures above 32°C can cause serious problems for sweet pepper plants because they affect plant metabolism, growth, pollination and fertilization (Erickson and Markhart, 2002). The translocation of photosynthesis assimilates to developing flowers depends on the leaf metabolism, which is also affected by HS. High-temperature stress affects crop yield as a consequence of a series of complex morphological, physiological, biochemical and molecular changes (Wang et al., 2003), which range from diminished photosynthesis activity, a higher respiration rate, oxidative damage, effects on ion uptake-movement, membrane fluidity and ion leakage, to stunted growth and cell death (Wahid et al., 2007). All these effects end up causing significant crop productivity losses in many species (Lobell and Gourdji, 2012; Zhao et al., 2017).

These limiting temperatures are normally exceeded in the situations described above. They coincide with the flowering phase and, thus, affect fruit set (Yamazaki and Hosokawa, 2019) and fruit drop and, consequently, fruit yield and quality (López-Marín et al., 2013).

Reduced fruit set is caused partly by damage to gametophytes, which results for the male gametophytes in poor pollen viability, decreased pollen germination, limited pollen tube growth and reduced stigma receptivity, and for the female gametophytes in reduced fertilization and ovule viability, limited embryogenesis and increased ovule abortion (Nadeem et al., 2018).

Pollen plays a crucial function in the fruit set and is considered more HS-sensitive than both vegetative tissues and the female gametophyte in *Solanaceae* species (Reddy and Kakani, 2007). This sensitivity to HS varies during pollen development, and is more sensitive in early development stages (anther wall development, microsporogenesis and microgametogenesis) (Raja et al., 2019). Pollen viability as a key trait for detecting HS-tolerance has been studied in many crops, such as cotton (Kakani et al., 2005), wheat and rice (Mesihovic et al., 2016) and tomato (Ayenan et al., 2019), and also in *Capsicum* species (Reddy and Kakani, 2007; Yamazaki and Hosokawa, 2019). Erickson and Markhart (2002) concluded that HS affects the pollen grains development in bell peppers during the final tetrad formation period to tetrad dissolution, and results in poorer pollen viability. This reduction effectively reduces fruit set and fruit size.

Metabolic studies into anthers and pollen have shown that a higher level of specific metabolites, such as flavonols, proline and polyamines, are associated to increased pollen viability (Falasca et al., 2010; Mattioli et al., 2012; Shan et al., 2020) due to their antioxidant capacity which, thus, protects them from the ROS generation caused by high temperatures (Xie et al., 2022). In particular, proline has been directly correlated to HS-tolerance, and is also the most abundant amino acid in anthers and pollen (Santiago and Sharkey, 2019). Accumulated proline is considered a key factor for pollen viability because it is used as an energy source to enhance pollen tube growth after germination (Biancucci et al., 2015). In general, proline levels in pollen decline with exposure to high-temperature stress, which is the case of tomato (Din et al., 2015) and rice (Tang et al., 2008). In hot pepper, Fang et al. (2016) proved that proline content was higher in fertile anthers compared to sterile ones. However as far as we know, there are no references to the behavior of proline pollen levels under HS in sweet pepper.

In the same way, carbohydrates are necessary for pollen development and germination, like osmoprotection and source of energy. The main soluble sugars in mature pollen are fructose, glucose, sucrose and starch (Paupière et al., 2014), and simple sugars are used for pollen grain development (Pacini, 1996). In pepper, pollen sugar metabolism is affected by suprathermal stress, mainly because diminished invertase activity increases sucrose and starch concentrations (Aloni et al., 2001). In tomato plants under HS, Firon et al. (2006) report that the ability of mature pollen grains of heat-tolerant cultivars to accumulate sugars was greater than in non tolerant cultivars, which affects the number of fruit per plant and seeds per fruit.

Other biocompounds, such as hormonal network, polyamines, lipids or other metabolic compounds, play a role in different aspects toward successful reproductive processes and can be modified under HS with negative responses for fruit set (Madhavi Reddy et al., 2016; Paupière et al., 2014).

Consequently, plant survival under HS depends on the plant's ability to perceive the temperature stimulus and to generate the appropriate response to cope with this stress by means of appropriate morphological, biochemical and signaling changes (Madhavi Reddy et al., 2016). Grafting is a special method of adapting plants to tolerate environmental stress (Schwarz et al., 2010). The grafting technique has been successfully used so that horticultural crops can cope with different abiotic stresses (Schwarz et al., 2010). However, there are very few studies on the use of rootstocks to cope with high temperatures in pepper (Aidoo et al., 2017; López-Marín et al., 2013; Palada and Wu, 2008), and none of these have studied the effects of using rootstocks on leaf physiology and fruit set under high-temperature conditions in detail.

Some pepper accessions have been recently identified as heat-tolerant in a study carried out by our work team (Gisbert-Mullor et al., 2021). Under climate chamber conditions, we observed how some accessions were able to confer a certain degree of tolerance to HS when using them as rootstocks and maintaining membrane permeability, which ultimately maintained the chlorophyll fluorescence parameter Fv/Fm ratio and resulted in a higher relative growth rate. We also found higher fruit yields in these grafted plants compared to the ungrafted cultivar under greenhouse conditions, which was related to the number of harvested fruits. However, we did not clearly identify the causes of fruit set degree, particularly if rootstocks were able to affect some aspects related to this important yield component (Gisbert-Mullor et al., 2021).

Consequently, the objective of this work was to study the effect of high temperature on fruit set in pepper and how it can be improved by using tolerant rootstocks. For this proposal, we analyzed the HS impact on a sweet pepper variety grafted onto rootstocks with diverse HS tolerances to evaluate whether: a) the leaf metabolism in different grafted plant combinations has a differential response to heat stress; b) these responses can be involved in fruit set.

3.3. Materials and methods

3.3.1. Plant material and growth conditions

The pepper variety 'Herminio F1' (*Capsicum annuum* L., Lamuyo type (Syngenta), B2 type of Pochard (1996) classification), was grafted onto two *Capsicum annuum* L. accessions selected from previous studies (Gisbert-Mullor et al., 2021): A57 (origin Israel, considered to be HS-tolerant) and A55 (origin Algeria, considered to be HS-sensitive), respectively named VA/A57 and VA/A55. Both accessions belong to the COMAV Institute collection (Universitat Politècnica de València, UPV, Valencia, Spain). The ungrafted variety (VA) and the self-grafted variety (VA/VA) were used as controls. The four plant groups were evaluated under both HS and control (C) conditions.

Experiments were conducted in two consecutive years (2020 and 2021) at the UPV Valencia, Spain.

Sowing took place on February 1, 2020, and March 3, 2021, in 104-cell polystyrene trays in fine structure peat substrate (80% white and 20% black, pH 5.7) (Gebr. Brill, Germany). On March 16, 2020, and April 12, 2021, plants were grafted by the tube-grafting method (Penella et al., 2014).

Seedlings were transplanted on April 1, 2020, and April 29, 2021, in 6-liter pots in medium structure peat substrate (100% white, pH 5.7) (Gebr. Brill, Germany), and placed in two Venlo-type glasshouses (C and HS conditions) at a density of two plants m^{-2}. Pots were drip-irrigated using antidrain Netafim® drippers of 4 L h^{-1}, with nutrient solution containing (in mmol L^{-1}) 14.0 NO_3^-; 1.5 $H_2PO_4^-$; 2.4 SO_4^{2-}; 0.5 HCO_3^-; 1.6 Cl^-; 1.2 NH_4^+; 6.0 K^+; 5.0 Ca^{2+}; 2.5 Mg^{2+}; 0.2 Na^+, and in µmol L^{-1}: 15 Fe^{3+}; 6 Zn^{2+}; 12 Mn^{2+}; 30 B^{3+}; 0.8 Cu^{2+} and 0.5 Mo^{6+} with electrical conductivity and pH of 2.2 dS m^{-1} and 6.5, respectively. The number of irrigations, which varied according to the accumulated radiation, controlled the volume of solution applied per pot. Drainage was maintained between 15% and 20% of the total irrigation volume.

The temperature treatments were 28/22°C and 38/22°C (day/night) for the control (C) and HS conditions, respectively. The HS conditions began on June 19, 2020, and May 26, 2021, and continued until July 15, 2020, and July 21, 2021. Before this, all the plants were subjected to the C conditions. The temperature and relative humidity for both greenhouses were recorded during the experiments using an S8TH sensor (Oratge Instruments®, Valencia, Spain) with data logger MSIP801 (BSG Ingenieros, Valencia, Spain) (Fig. 1S).

Experiments were laid out to be completely randomized with four repetitions per group of plants and per treatment. Each repetition consisted of 10 plants.

3.3.2. Experiment 1 (2020): rootstocks' influence on pepper physiological aspects under heat stress

For the physiological leaf analysis, all the measurements were taken on July 15, 2020, after 35 DAT (days after the heat-stress treatment). Electrolyte leakage (EL), chlorophyll and carotenoids concentration, ascorbate metabolism, total phenolic content and hydrogen peroxide quantification were determined. The analysis was carried out with 10 leaves per replicate (one leaf per plant; the third or fourth leaf from the apex), which were previously frozen with liquid nitrogen and stored at −80°C. Samples were ground by a mixer mill (MM400, Retsch, Hann, Germany) with liquid nitrogen to prevent melting. Fresh leaves were used for EL.

Fruit set was analyzed by tagging at least 20 flowers per plant. The fruit weighing around 25 g were considered set. The flower-tagging process began on 10 DAT to ensure that the tagged flowers developed under stress conditions. Fruit set was calculated as the number of set fruit, divided by the total number of sampled flowers, expressed as a percentage.

3.3.2.1. Electrolyte leakage

To analyze EL, 10 discs (1.4 cm in diameter) of freshly cut leaves were extracted from the 10 plants of each replicate with a perforator and were introduced in 50 mL matrass together with 20 mL of deionized water. Electrical conductivity measurements were taken at 0 h (EC1), at 2 h (EC2) and after leaving the matrass frozen (-40°C) for 48 h (EC3) with a conductivity meter (Model Seven Easy Mettler Toledo, Mettler-Toledo AG, Switzerland). E.L. was expressed as a percentage.

$$EL(\%) = \left(\frac{EC2 - EC1}{EC3 - EC1}\right) * 100$$

3.3.2.2. Chlorophyll and carotenoids concentration

Total chlorophyll (Chl a+b) and carotenoid (Car) contents were measured by spectrophotometry as indicated by Porra et al. (1989) with minor changes. First, 0.1 g fresh weigh (FW) of each sample was extracted in 2.5 mL of 100% (v/v) pure acetone and then centrifuged at 2000 rpm for 5 min. The supernatant was separated from the precipitate and its absorption was measured using a spectrophotometer (Lambda 25 UV/VIS, Perkin Elmer, Waltham, MA, USA) at 662nm, 645nm and 470nm, using 100% acetone for the blank. Concentration for Chl a+b and Car were expressed in µg g^{-1} FW and were calculated according to these equations:

$$Chl_{a+b} = 7.05 * Abs_{662} + 18.09 * Abs_{645} (\mu g\, mL^{-1})$$

$$Car = [(1000 * Abs_{470}) - (1.90 * Chl_a) - (63.14 * Chl_b)]/214\ (\mu g\, mL^{-1})$$

3.3.2.3. Ascorbate metabolism

Ascorbic acid (AsA), dehydroascorbate (DHA) and total ascorbic acid (AsAt, AsA+DHA) were quantified in parallel as described by Kampfenkel et al. (1995) with slight modifications. First, 0.4 g FW sample were extracted in 80% (w/v) trichloroacetic acid (TCA) centrifuged for 5 min at 15000 *g* and 4°C. Then two different determinations were followed (one for AsA and the other for AsAt): 50 µL of the extract supernatant were added to 150 µL of 0.2M phosphate buffer (pH = 7.4) and 50 µL of distilled water in the ascorbate tubes. In the case of total ascorbic acid tubes, the 50 µL of the extract supernatant were put together with 50 µL of 10mM dithiothreitol (DTT) and 100 µL of 0.2M phosphate buffer (pH = 7.4) and then introduced in a water bath at 42°C for 15 min. After the water bath, 50 µL of 0.5% (w/v) N-ethylmaleimide (NEM) were added only in the total ascorbic acid tubes which were then incubated for 1 min at room temperature. Afterwards, 250 µL of 10% (w/v) TCA, 200 µL of 42% (v/v) H_3PO_4, 200 µL of 4% (w/v) 2,2'-dipyridyl and 100 µL of 3% (w/v) $FeCl_3$ were added to AsA and total ascorbic acid tubes to be incubated in a water bath for 40 min at 42°C. Absorbance at 525 nm was measured for both determinations. The DHA concentration was calculated as: [AsAt] – [AsA]. AsA and DHA concentration were expressed in µg g^{-1} FW.

3.3.2.4. Total phenolic content

Total phenolic content determination was performed as described by Koç et al. (2010) with some variations. First of all, 0.1 g FW of the sample was extracted in 1.5 mL of 80% (v/v) methanol and placed in an ultrasound bath for 30 minutes at 25°C. Extracts were centrifuged for 10 min at 10000 g and 4°C, and 20 µL of the resulting supernatant were diluted with 80 µL of 80% (v/v) methanol. Afterward, the diluted extract was incubated with 0.7 mL of 1:10 diluted Folin-Ciocalteu solution (Sigma-Aldrich®) for 5 minutes in the dark. Then 0.7 mL of 6% (w/v) of Na_2CO_3 were added and the solution was incubated for 1 h in the dark at room temperature. Absorbance at 765 nm was recorded, using gallic acid (GA) as a standard. Total phenolic content was expressed as mg GA g^{-1} FW.

3.3.2.5. Hydrogen peroxide quantification

Hydrogen peroxide (H_2O_2) was quantified as described in Velikova et al. (2000) with minor adaptations. 0.25 g FW of the sample was extracted in 2 mL of 0.1% (w/v) TCA to be centrifuged at 10000 *g* and 4°C for 8 min. Afterward, 400 µL of the resulting extract were incubated with 600 µL of 0.1% (w/v) TCA, 0.5 mL of 100 mM potassium phosphate buffer (pH = 7) and 2 mL of 1M KI in the dark for 1 h at room temperature. Absorbance at 390 nm was measured, and the absorbance values were interpolated on a standard curve performed with 100 mM H_2O_2. Values were expressed as nmol H_2O_2 g^{-1} FW.

3.3.3. Experiment 2 (2021): rootstocks' influence on pepper fruit set components under heat stress

Based on experience from previous experiments and to avoid fruit competition within the plant to fruit set, plants were pruned to four stems by cutting back the second branch at each node after evidencing fruit set of the flower at the node. In this way, it was also easier to access flowers to collect pollen or anther samples for subsequent analyses.

To analyze fruit set, at least six reproductive nodes of each stem were analyzed, which totaled 24 flowers per plant. The flower-tagging process began on 10 DAT and the fruit weighing around 25 g were considered set. The average number of seeds per fruit was analyzed in one fruit per node, which resulted in six fruit per plant.

3.3.3.1. *In vitro* germination of the pollen grain percentage (%)

To determine the *in vitro* germination of the pollen grain percentage (% GP), the pollen of each flower on the day of anthesis was evenly distributed in the germination medium proposed by Reddy and Kakani (2007).

The pollen grain was incubated for 24 h at 25°C, % GP was determined in 10 fields of each Petri dish using a Leica DM750 microscope (Leica Microsystems, Wetzlar, Germany) at 10 x magnification. Pollen grain was considered germinated when pollen tube length exceeded the diameter of grain.

To calculate % GP, a minimum of 20 flowers were collected from each repetition and treatment. % GP was calculated as the number of germinated pollen grains divided by the total number of sampled pollen grains, and expressed as a percentage.

3.3.3.2. Primary metabolites analysis from anthers

One day before anthesis, a minimum of 10 flowers of each repetition and treatment were harvested. Anthers were immediately separated, frozen with liquid nitrogen and stored at −80°C. Samples were lyophilized before the analysis.

A primary metabolite analysis (proline, glycine, fructose, glucose, sucrose) was analyzed at the Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) by the Metabolomics Platform. First 10 mg of lyophilized anther tissue were ground with liquid nitrogen and extracted in 1400 µL 100% methanol and 60 µL of the internal standard (0.2 mg ribitol in 1 mL of water). The blend was extracted for 15 min at 70°C. The extract was centrifuged for 10 minutes at 14000 rpms, and the supernatant was transferred to a glass vial. Then 750 µl of $CHCl_3$ and 1500 µL of water were added. The mixture was vortexed for 15 seconds and centrifuged for 15 minutes at 14000 rpms. Next 150 µL aliquots of the methanol/ water supernatant were speed-dried for 3 h.

For derivatization purposes, dry residues were redissolved in 40 µL of 20 mg/mL methoxyamine hydrochloride in pyridine and incubated for 90 min at 37°C. Then 70 µL of MSTFA (N-methyl-N-[trimethylsilyl]trifluoroacetamide) and 6 µL of a mixture (3.7% [w/v] mix of fatty acid methyl esters ranging from 8 to 24 C) were added and samples were incubated for 30 minutes at 37°C.

Sample volumes of 2 µL were introduced in the split and splitless modes in a 6890 N gas chromatograph (Agilent Technologies Inc. Santa Clara, CA, USA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI, USA). Gas chromatography was carried out in a BPX35 (30 m × 0.32 mm × 0.25 µm) column (SGE Analytical Science Pty Ltd., Australia) with helium as the carrier gas at a constant flow rate of 2 mL/min. The liner was set at 250°C. The oven program was 85°C for 2 min with an 8°C/min ramp until 360°C. Mass spectra were collected at 6.25 spectra s^{-1} within the 35–900 *m/z* range with 70 eV ionization energy. Chromatograms and mass spectra were analyzed using the CHROMATOF program (LECO, St. Joseph, MI, USA). For absolute quantification purposes, the results were compared to a standard curve performed with commercial pure compounds using ribitol as an internal standard. All the metabolites were expressed as µg g^{-1} FW.

3.3.3.3. Biomass production

The aerial plant biomass in fresh (FW) and dry weight (DW) was measured at the end of the experiment (58 DAT). Fresh biomass was dried at 70°C during 72 h in a laboratory oven to determine DW. To correctly analyze the influence of the thermal condition on the dry biomass of each graft combinations, this parameter was analyzed as the percentage DW loss under the HS conditions compared to the C conditions. Aerial biomass was determined in three plants per repetition and treatment.

3.3.4. Statistical Analysis

The data for all the parameters were evaluated using two-way ANOVA analysis via Statgraphics Centurion XVII. Graft combinations (G) and thermal conditions (TC) were employed as the factors of the analysis. For the parameter number of seeds per fruit, the statistical analysis was performed using the inverse transformation of data, and the percentage data were arcsin-transformed before analyzing. Means (n=4) were compared by Fisher's least significance difference (LSD test) at P ≤ 0.05.

3.4. Results

3.4.1. Experiment 1: rootstocks' influence on pepper physiological aspects under heat stress

3.4.1.1. Electrolyte leakage

As compared to control, HS caused a significant increase (65.7%) of EL in all the graft combinations ($P \leq 0.01$; Fig. 1). No significant differences in EL were observed between graft combinations on average for temperature conditions. However, a statistically significant ($P \leq 0.01$; Fig. 1) TC x G interaction was detected: the differences in EL between the HS and C conditions were lower in the plants grafted onto the A57 rootstock (26.4%) than in the other plant combinations (66.4% for VA/A55, 80.4% for VA/VA and 99.6% for VA).

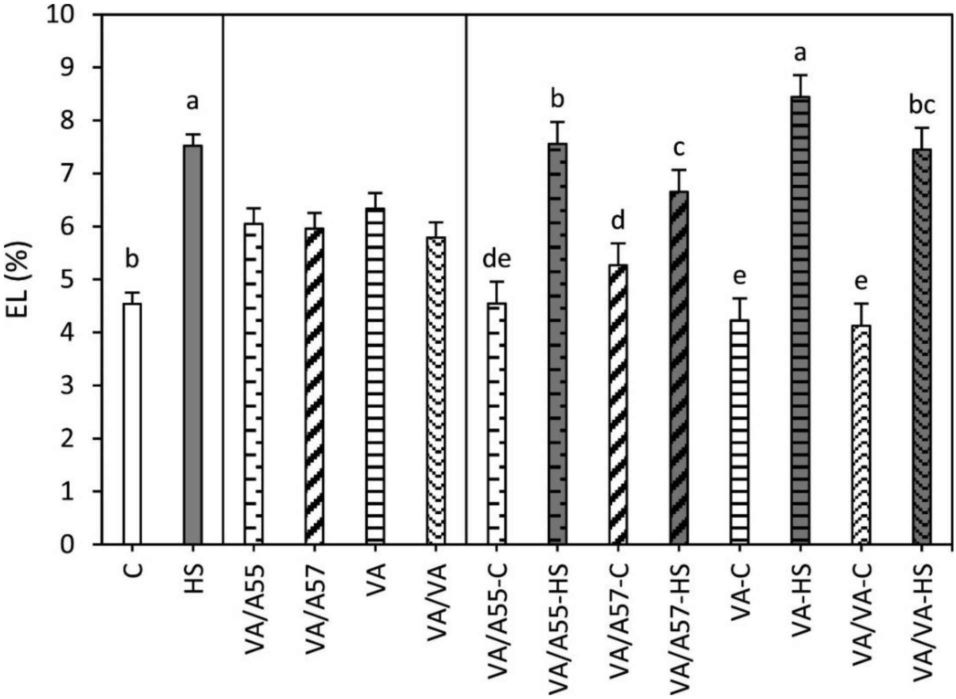

Fig. 1. Electrolyte leakage (EL) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

3.4.1.2. Chlorophyll and carotenoids concentration

The total chlorophyll (Chl a+b) and carotenoids (Car) contents presented a similar pattern, and were not affected by TC (Fig. 2 A,B). However for both thermal conditions, rootstocks A57 and A55 had a higher content of both parameters than VA and VA/VA ($P \leq 0.01$; Fig. 2 A,B). For both parameters, the TC x G interactions were also statistically significant ($P \leq 0.01$; Fig. 2 A,B). Under HS, for the variety grafted onto rootstock A57 and the ungrafted variety, chlorophyll and carotenoids contents significantly increased compared to their controls, but significantly lowered in VA/A55. No significant differences were observed in VA/VA.

3.4.1.3. Ascorbate and dehydroascorbate concentration

AsA and DHA contents were significantly higher under HS, on average for graft combinations, with a 5.4% and 182.3% increase, respectively, compared to the C conditions ($P \leq 0.05$; Fig. 3 A). On average for both thermal conditions, VA showed the highest AsA and DHA contents, but no significant differences with those of VA/A55 and VA/VA were found, respectively ($P \leq 0.05$; Fig. 3 A). Nevertheless, a statistically significant TC x G interaction ($P \leq 0.05$; Fig. 3 A) appeared for AsA and DHA contents. For AsA content, VA/A57 and VA had higher values under HS than under C, VA/A55 showed no differences, and VA/VA had higher AsA content under the C than the HS conditions. VA had the highest DHA content under HS among all the graft combinations. All the graft combinations had higher DHA content under the HS than the C conditions, except VA/VA.

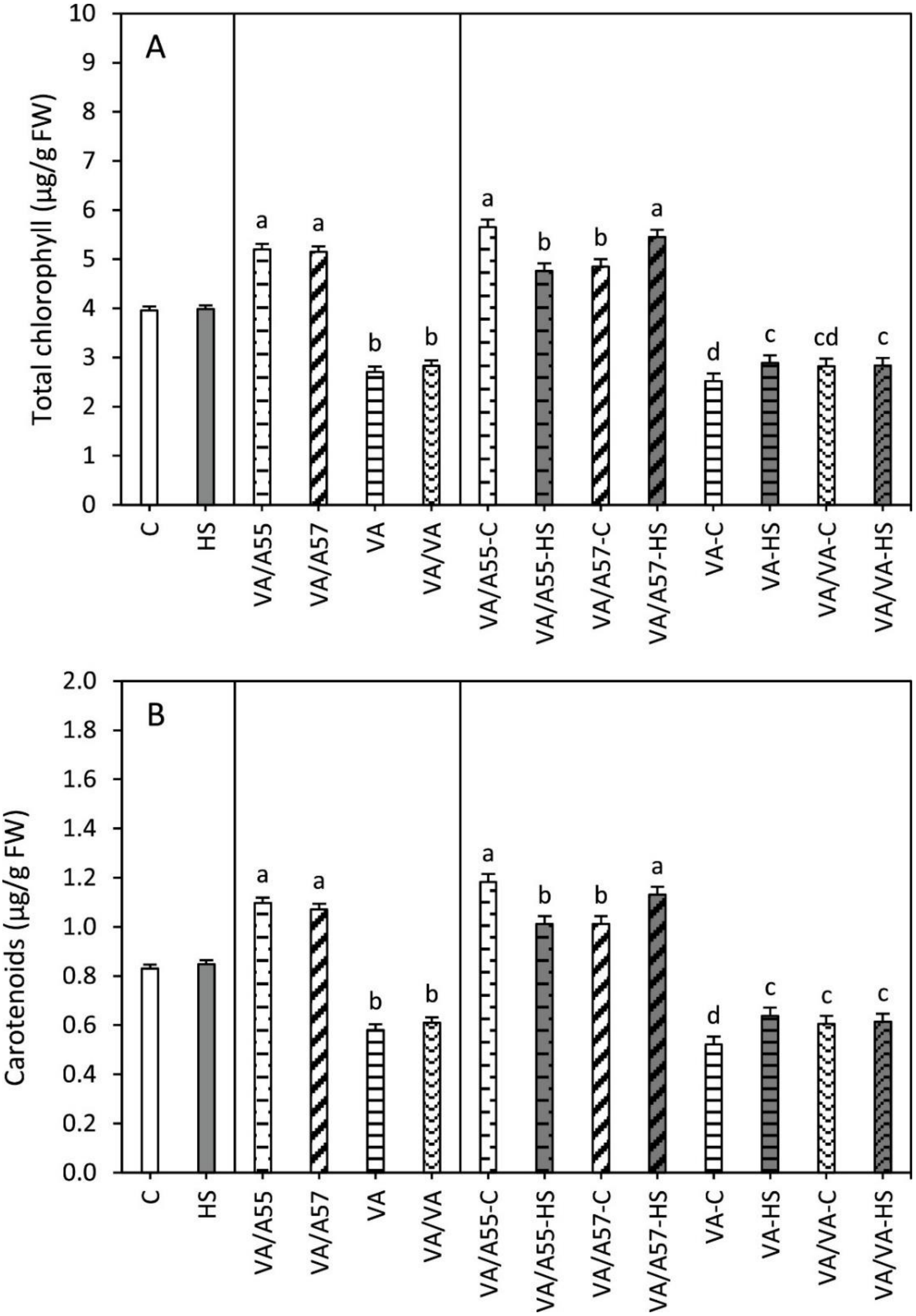

Fig. 2. Total chlorophyll (A) and carotenoids (B) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test (P ≤ 0.05). Error bars represent LSD.

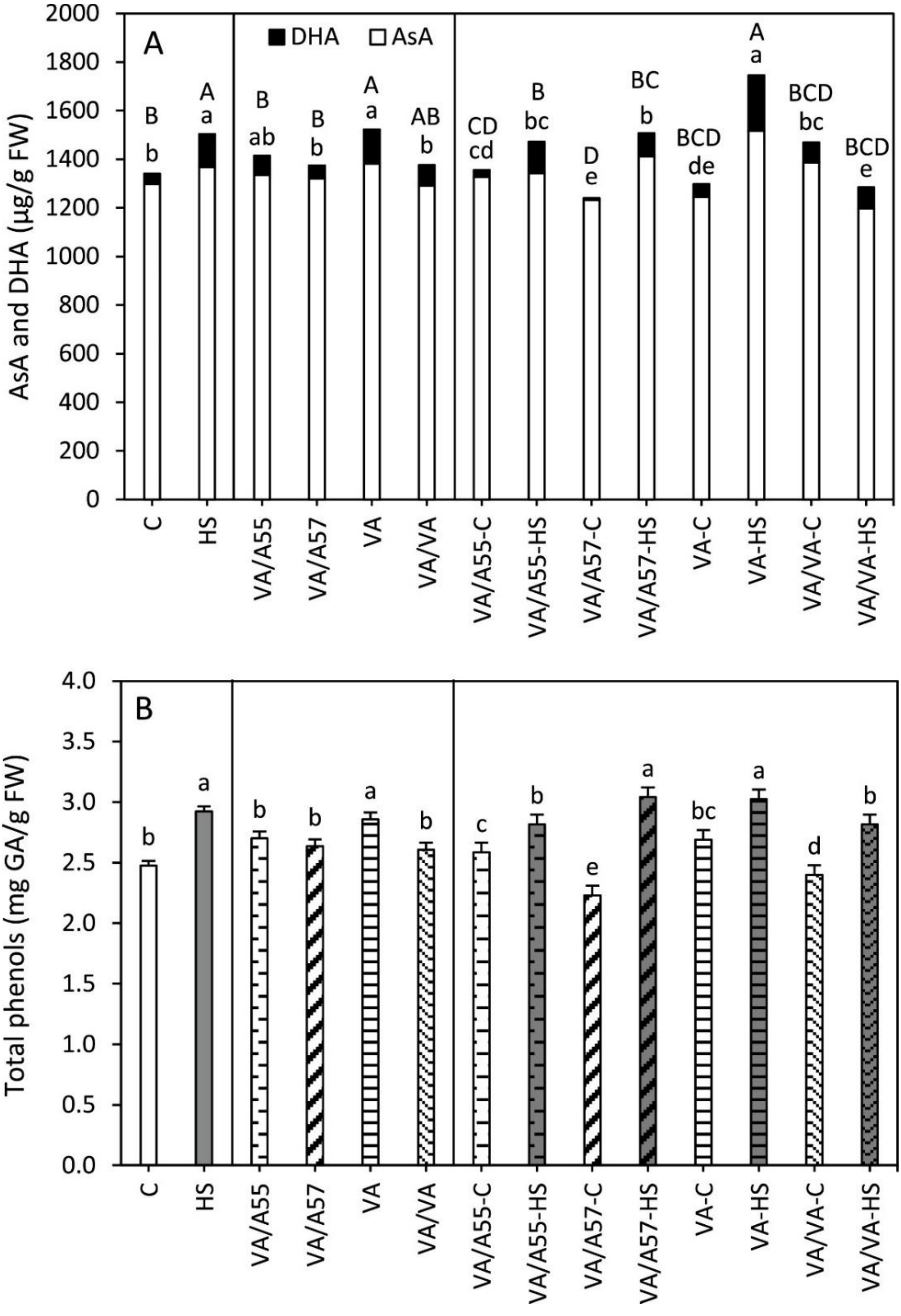

Fig. 3. Concentration of the different forms of ascorbate (AsA and DHA) (A) and total phenols (B) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test (P ≤ 0.05), for the parameter AsA lowercase letters and for DHA capital letters. Error bars represent LSD.

3.4.1.4. Total phenolic content

Total phenolic content was also higher under HS, on average for graft combinations, with an 18.1% increase compared to the plants under the C conditions ($P \leq 0.01$; Fig. 3 B), and VA had the highest content on average for both thermal conditions ($P \leq 0.01$; Fig. 3 B). However, the TC x G interaction was statistically significant ($P \leq 0.01$; Fig. 3 B) because, although all the graft combinations had a higher total phenols content under HS, the differences in the total phenols between the HS and C conditions were bigger in the plants grafted onto A57 (36.4%) than for the other plant combinations (9.0% for VA/A55, 17.4% for VA/VA and 12.4% for VA).

3.4.1.5. Hydrogen peroxide quantification

Hydrogen peroxide content (H_2O_2) globally increased to 52.9% in the heat-stressed plants compared to the control ones, and VA and VA/VA had a higher H_2O_2 content on average for the thermal conditions than VA/A57 and VA/A55 ($P \leq 0.01$; Fig. 4). The TC x G interaction was also statistically significant ($P \leq 0.01$; Fig. 4). While VA/A55, VA and VA/VA had significantly increased H_2O_2 content under HS compared to C, this parameter was not significantly modified in the VA/A57 plants under HS.

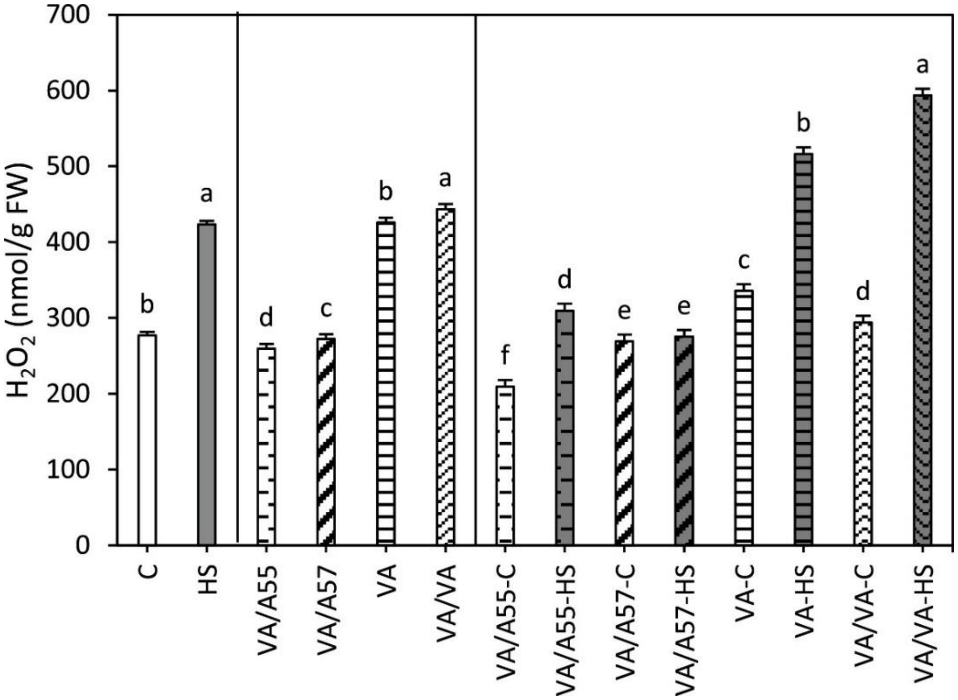

Fig. 4. Hydrogen peroxide contend (H2O2) in leaves of the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test ($P \leq 0.05$). Error bars represent LSD.

3.4.1.6. Fruit set percentage

Fruit set (%) was strongly affected by HS, with a reduction of 71.8% compared to the C condition on average for graft combinations, and VA/A57 was the rootstock that conferred the highest fruit set for both the HS and C conditions. Nevertheless, a statistically significant TC x G interaction (P ≤ 0.01; Fig. 5) was detected, in which the reduction in fruit set between the HS and C conditions was less in the plants grafted onto the rootstock A57 (57.7% reduction) than for the ungrafted cultivar (VA) (67.9% reduction), and was even lower compared to VA/A55 and VA/VA (84.2% and 80.2% reduction, respectively).

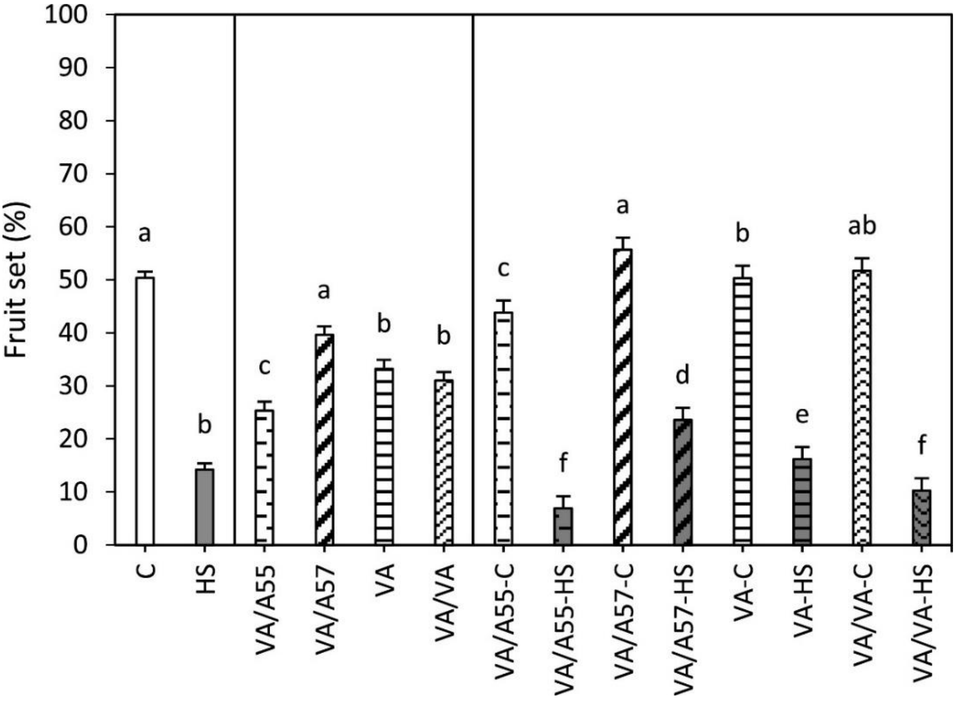

Fig. 5. Fruit set percentage for Experiment 1 in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test (P ≤ 0.05). Error bars represent LSD.

3.4.2. Experiment 2: rootstocks' influence on pepper fruit set components under heat stress

3.4.2.1. *In vitro* germination of pollen grain percentage

GP (%) was significantly lower under the HS conditions, on average for graft combinations, with a 30.8% reduction compared to the C conditions (P ≤ 0.01; Fig. 6). For both thermal conditions, VA/A57 had the highest percentage, similarly to VA/VA, and VA had the lowest germination percentage (P ≤ 0.01; Fig. 6). However, a statistically significant TC x G interaction (P ≤ 0.01; Fig. 6) was detected, since the reduction in % GP between the HS and C conditions was lower in the plants grafted onto rootstocks A57 and A55 (24.7% and 23.4% reduction, respectively) than in VA and VA/VA (36.9% and 37.5% reduction, respectively).

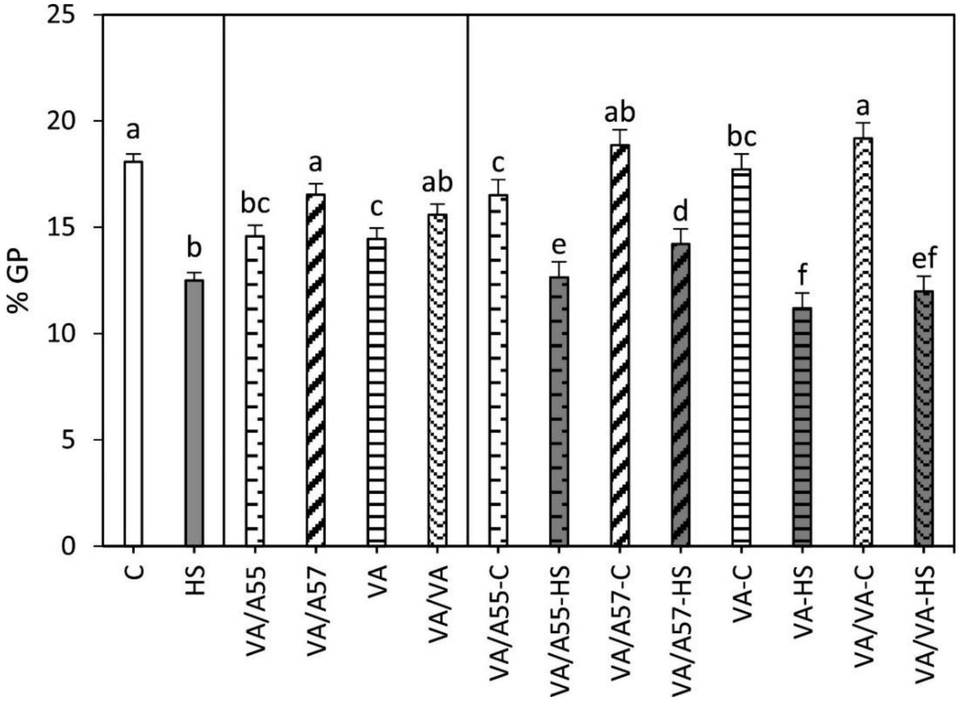

Fig. 6. In vitro germination of pollen grains (% GP) in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test (P ≤ 0.05). Error bars represent LSD.

3.4.2.2. Primary metabolites analysis from anthers

For the primary metabolites in anthers, the plants under the HS conditions presented reductions of 30.8% in proline, 8.2% in fructose, 10.7% in glucose and 46.5% in sucrose compared to the plants under the C conditions. No significant differences between temperature conditions were detected for glycine content (Table 1). Only proline showed significant differences between graft combinations on average for thermal conditions, and rootstock A57 had the highest content (P ≤ 0.01; Table 1). The TC x G interaction for this parameter was also statistically significant (P ≤ 0.01; Table 1). Similarly to % GP, all the graft combinations had higher proline content under C than HS but, in this case, although VA/A57 had the highest content under the HS conditions, the reduction in proline content between HS and C was also lower in the plants grafted onto A57 (31.5% reduction) than in VA, VA/VA and VA/A55 (34.5%, 54.7% and 42.2% reduction, respectively).

Table 1. Analysis of variance (ANOVA) for Proline, Glycine, Fructose, Glucose and Sucrose (μg g^{-1}FW) expressed as mean values by Thermal Condition (TC), Graft combination (G) and interaction (TC x G). % of the sum of squares are expressed.

	Proline		Glycine		Fructose		Glucose		Sucrose	
Thermal condition (TC)										
Control	73.51	a	0.576		110.3	a	73.31	a	45.35	a
Heat stress	43.57	b	0.576		101.3	b	65.47	b	24.26	b
Graft combination (G)										
A55	58.69	b	0.567		107.5		71.51		32.50	
A57	64.67	a	0.586		106.2		69.60		39.86	
VA	57.14	bc	0.562		108.1		72.21		31.92	
VA/VA	53.66	c	0.590		101.4		64.23		34.96	
TCxG										
A55-C	74.37	ab	0.579		110.9		76.90		41.51	
A57-C	76.77	a	0.591		111.2		70.91		51.63	
VA-C	69.03	b	0.557		110.4		75.85		41.21	
VA/VA-C	73.87	ab	0.578		108.8		69.58		47.06	
A55-HS	43.02	d	0.555		104.2		66.11		23.48	
A57-HS	52.58	c	0.581		101.2		68.29		28.09	
VA-HS	45.25	d	0.568		105.9		68.58		22.63	
VA/VA-HS	33.44	e	0.601		94.1		58.89		22.86	
ANOVA (*df*)	% Sum of Squares									
TC (1)	83.27	**	0.00	n.s.	19.02	*	22.53	**	69.77	**
G (3)	5.89	**	20.66	n.s.	6.52	n.s.	14.33	n.s.	6.15	n.s.
TC*G (3)	4,25	**	12.93	n.s.	3.45	n.s.	4.06	n.s.	1.23	n.s.
Residuals (24)	6,59		66.41		71.01		59.08		22.85	
Standard Desv. (+)	4,86		0.024		10.0		7.33		6.97	

*: P ≤ 0.05. **: P ≤ 0.01. n.s. not significant. (+) Obtained as the square root of the residual mean square. *df*: degrees of freedom.

3.4.2.3. Fruit set percentage

Similar to experiment 1, the fruit set percentage was also affected by HS (P ≤ 0.01), with a 25.2% reduction compared to the plants under the C conditions (Fig. 7 A), and for both thermal conditions. VA/A55 had the lowest fruit set percentage among the plant combinations (P ≤ 0.01; Fig. 7 A). The TC x G interaction was also statistically significant (P ≤ 0.01; Fig. 7 A) but, in this experiment, the plants grafted onto rootstock A57 were not affected by the HS conditions, while reductions of 40.5%, 29.5% and 23.5% in fruit set were obtained for the plants of VA/A55, VA and VA/VA, respectively.

3.4.2.4. Number of seeds per fruit

The fruit developed under the HS conditions, on average for graft combinations, had a smaller (59.5%) number of seeds compared to the fruit under the C conditions (P ≤ 0.01; Fig. 7 B). For both thermal conditions, VA/A57 and VA/VA had the larger number of seeds ($P \leq 0.01$; Fig. 7 B). The interaction between both factors was also statistically significant and can be explained because, despite the fact that all the graft combinations showed similar values in this parameter under the C conditions, under HS both VA/A57 and VA/VA obtained higher values than VA and VA/A55, and the latter gave the smallest number of seeds ($P \leq 0.01$; Fig. 7 B).

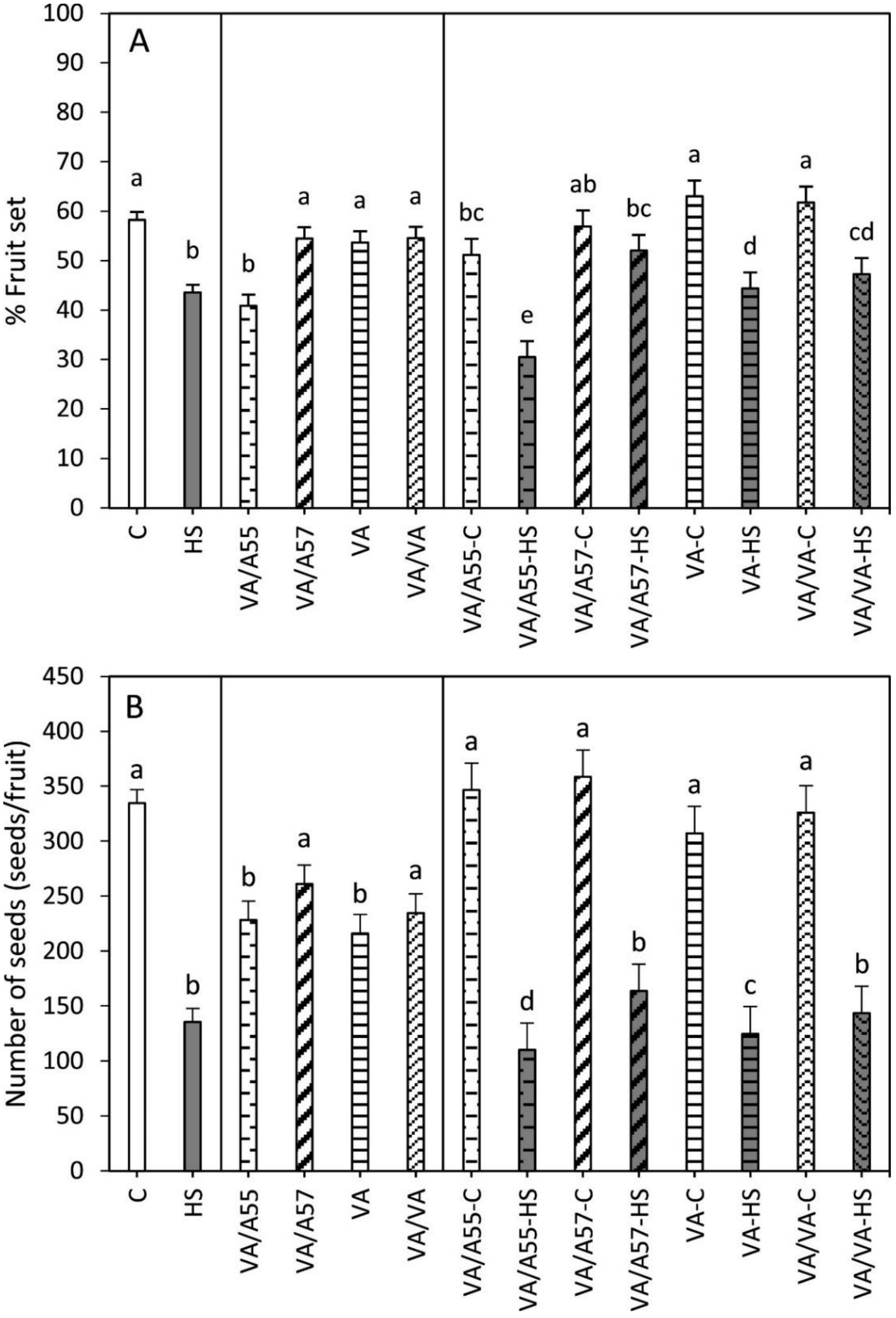

Fig. 7. Fruit set percentage for Experiment 2 (A) and number of seeds per fruit (B) in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively) under heat stress (HS) or control conditions (C). Bars with different letters are statistically different according with LSD test (P ≤ 0.05). Error bars represent LSD.

3.4.2.5. Biomass production

For the DW percentage under HS *versus* C, the plants grafted onto rootstock A57 displayed less loss for the DW percentage than other graft combinations (P ≤ 0.01; Fig. 8). No significant differences between thermal conditions were found in the other graft combinations.

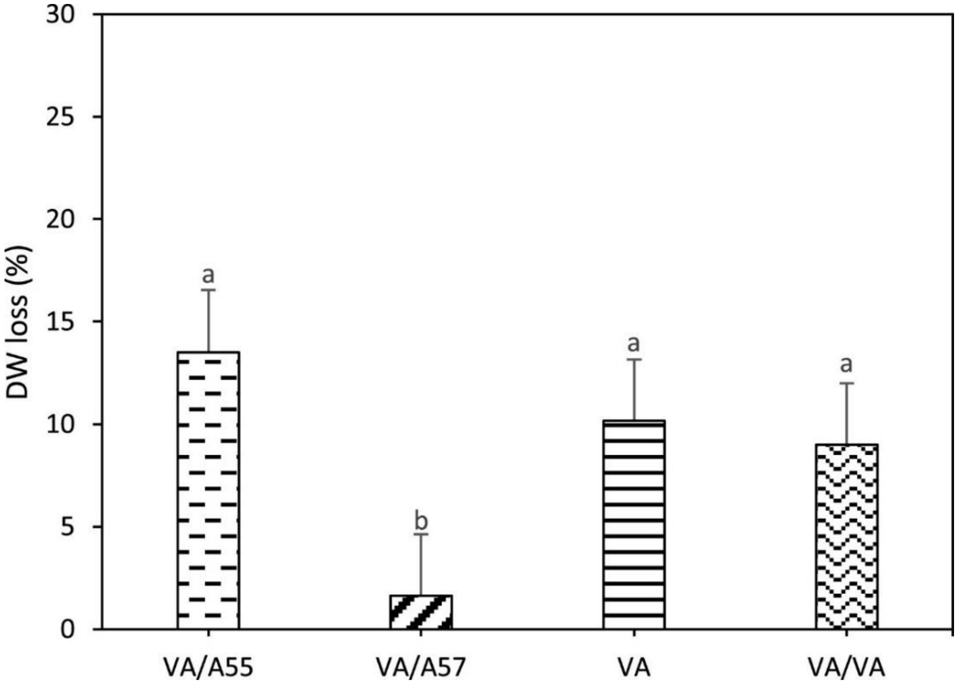

Fig. 8. Percentage dry weight loss under heat stress conditions compared to control conditions in the ungrafted plants (VA) and VA grafted onto A57, A55 or self-grafted (VA/A57, VA/A55 and VA/VA, respectively). Bars with different letters are statistically different according with LSD test (P ≤ 0.05). Error bars represent LSD.

3.5. Discussion

Plants' heat tolerance is decisive for both crop growth and productivity (Wei et al., 2019). In line with this, grafting plants onto tolerant rootstocks can provide efficient mechanisms for plants to overcome high temperature stress, as far as our knowledge, there is no research about the mechanisms underlying the tolerance of pepper grafted plants to such stress (Gisbert-Mullor et al., 2021; López-Marín et al., 2013; Palada and Wu, 2008).

In this study, we encountered differential physiological responses of pepper grafted plants in VA/A55 (sensitive rootstock), VA/A57 (tolerant rootstock) and VA/VA (self-grafted), and also in VA (the ungrafted variety) induced by HS. These responses are characterized in VA/A57 by the lowest EL, non-disturbed Chl and Car concentrations, increased AsA and phenols concentrations, and non-H_2O_2 accumulation under the HS conditions. These physiological responses conferred by rootstock A57 coincided with the highest proline concentration in anthers, and better pollen germination and fruit set compared to the other plant combinations.

One of the most sensitive physiological alterations under HS is increased membrane permeability because this affects its structure and functions (Nadeem et al., 2018; Wahid et al., 2007). EL values are an indicator of membrane injury and have been used to estimate membrane thermostability (Xu et al., 2006). According to our results, EL significantly depended on rootstock genotypes and treatments: the lowest EL values of all the plant combinations and the minor rise in relation to its controls were found in plants VA/A57. Other grafted plants, such as cucumber grafted onto *Momordica* or tomato grafted onto tomato or eggplant, have shown greater membrane stability compared to self-grafted plants or ungrafted plants under HS (Abdelhafeez et al., 1975; Abdelmageed and Gruda, 2009; Wei et al., 2019). Therefore, EL has been used as a screening test for HS tolerance (ElBasyoni et al., 2017; Nadeem et al., 2018). The EL parameter is principally influenced by the ability to balance reactive oxygen species (ROS) given that HS induces ROS generation and accumulation like H_2O_2 (Airaki et al., 2012; Xu et al., 2018). In this way, we found a positive correlation coefficient between the H_2O_2 concentration and EL ($r= 0.785$, $P< 0.05$), which indicates that the increase in EL was partially due to increased H_2O_2 production. However, the H_2O_2 concentration in VA/A57 under HS did not significantly differ from its control, which agrees with its lowest EL values. This effect could be partly due to better redox homeostasis in the VA/A57 plants supported by primary metabolites, such as carotenoids and chlorophyll accumulation, to avoid photooxidation in photosystems (Leverenz et al., 2015) and to achieve non-disturbed Fv/Fm values *versus* its control (Gisbert-Mullor et al., 2021). The same effects have been observed in cucumber grafted onto *Momordica* (Wei et al., 2019; Xu et al., 2018) at high temperature. The VA plants also had increased Chl and

Car concentrations under HS compared to their control, but the photosynthetic pigments of the plants grafted onto the sensitive rootstock A55 decreased. Loss of chlorophylls and carotenoids concentration could be a consequence of the heat-induced damage to the thylakoid membrane by, thus, disrupting membrane permeability, increasing chlorophylase enzyme activity, blocking energy to reaction centers of photosystems, decreasing photosynthesis, among other causes (Cornic, 2000; Ghai et al., 2016; Komayama et al., 2007; Wahid et al., 2007).

Other plant metabolites like phenols and ascorbate are involved in HS tolerance and also play an important function in maintaining redox homeostasis (Chaudhary et al., 2020). Phenols, such as secondary metabolites, are an important group of compounds that are essential for plant acclimatization and survival under different environmental conditions (Fraser and Chapple, 2011; Zandalinas et al., 2017). Their accumulation has been mentioned as a possible mechanism of tolerance to abiotic stresses (Hichem et al., 2009; Oh et al., 2009). However, contradictory results have been described in grafted plants. Rivero et al. (2003) observed greater accumulation for total phenols in tomato plants subjected to HS, and of these, lower accumulation in grafted plants compared to ungrafted ones. These authors concluded that grafted plants appeared more tolerant to HS. However, no significant differences have been observed in phenols content in fruit when pepper has been grafted onto different rootstocks (López-Marín et al., 2013). With our experimental conditions, the observed increase in phenols depended on heat treatment and rootstock genotype, with a significant increase for both VA and VA/VA57. Nevertheless, the differences in total phenols between the HS and C conditions were bigger in VA/VA57. Based on the VA/VA57 response, and by displaying the lowest EL values and the highest Chls and Car contents, it would seem that the increase in phenols could be associated with a HS tolerance factor.

AsA is considered a powerful antioxidant *per se* and an essential master compound in the AsA-glutathione cycle to scavenge ROS (Foyer and Noctor, 2005). Under our experimental conditions, the amount of AsAt increased in all the plant combinations in response to HS, and the AsA/AsAt ratio sharply dropped, which could indicate that a high AsA oxidation rate occurred in DHA (data not shown). The major increase in AsAt took place for the VA plants, followed by VA/A57. However, the higher DHA concentration observed in VA could indicate lesser AsA regeneration from DHA.

In vegetative parts, an efficient mechanism of HS tolerance plays a vital role in the formation of successful reproductive organs and, thus, positively affects final yield (Asseng et al., 2002; Wollenweber et al., 2003). We analyzed different reproductive parameters to confirm that VA/A57, which was best predisposed to overcome HS based on the physiological parameter results found in leaves, could exhibit the most suitable reproductive attributes. In most plant species, male gametophytes are more sensitive to high temperature than female gametophytes (Djanaguiraman et al., 2018). We analyzed a post-pollination event (pollen germination), primary metabolites in anthers, fruit set and number of seeds, which are all traits for thermo-tolerance studies in plants (Paupière et al., 2014). In pepper, high temperature during flowering impairs pollen germination, pollen tube growth and fertilization and, consequently, both flower abscission and fruit set lower (Aloni et al., 2001; Erickson and Markhart, 2002; Usman et al., 1999). Temperatures higher than 32°C provoke pollen abnormalities in pepper, such as shrunken and empty pollen grains with no appreciable exine (Erickson and Markhart, 2002). Under our experimental greenhouse conditions, HS reduced pollen germination despite this trait being rootstock-dependent with greater pollen germination when the variety was grafted onto A57. Reduced pollen vitality at high temperature has been described because insufficient carbohydrates are supplied from the tapetum and other anther tissues to pollen (Sato et al., 2000) in association with tapetum degeneration or malfunction (Mercado et al., 1997), as well as diminished invertase activity, which produce the hydrolysis from sucrose to hexoses and can be used by pollen (Aloni et al., 2001), among other aspects (Paupière et al., 2014). To support this idea, the amounts of fructose, glucose and sucrose in anthers according to our results revealed a significant difference between C and HS treatments, with a lower sugar concentration at high temperatures. Nevertheless, this was not rootstock-dependent, which could indicate that other factors are implicated in pollen germination because A57 displayed greater pollen viability, but not a higher sugar concentration.

Apart from sugars, proline is, among other essential metabolites (Mattioli et al., 2018, 2012; Sato et al., 2006; Xie et al., 2022), considered a key factor for pollen germination, and amino acid in the male reproductive part in the mature stage is more abundant and represents 60% of free amino acids (Paupière et al., 2014; Sangwan, 1978). Proline can act as a solute protectant during pollen development (Zhang et al., 1982) by protecting pollen grains from the desiccation that high temperature provokes, and by providing nutritive substances for pollen development (Fang et al., 2016). In tomato, Sato et al. (2006) reported decreased proline at moderately high temperature. Under HS, we observed a significant decrease in anthers' proline, but the plants grafted onto genotype A57 had the highest proline concentration under both the C and HS conditions. Observations made about the role of proline in pollen viability and development have been confirmed in a proline-deficient mutant of *Arabidopsis thaliana* (Paupière et al., 2014), and the interruption of proline synthesis leads to abortion and sterility during gametophyte development (Biancucci et al., 2015; Mattioli et al., 2018). In pepper, HS-tolerant genotypes have shown a higher proline concentration than susceptible ones (Saha et al., 2010), which confirms the master role of proline in pollen germination.

Favorable fruit set depends on several reproductive processes, including pollen germination and tube growth (Dahal et al., 2006; Madhavi Reddy et al., 2016). High temperature during pollen and/or fruit development in pepper has resulted in decreased fruit set and a smaller number of seeds per fruit (Aloni et al., 2001). In pepper, flower abortion occurs when day/night temperatures are higher than 34/21°C (Rylski and Spigelman, 1982), a situation that normally occurs in the Mediterranean Region in low-technology greenhouses. Under our experimental conditions, with temperatures of 38/22°C (day/night), fruit set significantly decreased in both experiments (2020 and 2021) at different magnitudes, and the VA/A57 plant combination was less affected by high temperature in fruit set terms, although no significant difference in fruit set was found in experiment 2 compared to its control.

In addition, significant correlations ($P < 0.05$) between fruit set and different parameters, such as number of seeds ($r=0.771$), pollen germination ($r=0.787$) and proline and sucrose concentrations in anthers ($r=0.718$ and $r=0.756$, respectively), were quantified and indicate the reliance of successful fruit set and pollen state.

As an integrated approach between both the experiments carried out in this study, and as we are aware that, because both experiments were run in different years, the parameters' correlations between them could not be established, we deduced that the physiological advantages observed in VA/A57, such as lower EL, higher Chls and Car contents, higher AsAt and also greater fruit set in Experiment 1, could be associated with the greater dry biomass maintenance and reproductive traits observed in Experiment 2 under HS. This connection might be based on the greater translocation capacity of the metabolites from photosynthesizing leaves to developing flowers (Aloni et al., 1991). In this way, the traits associated with the vegetative and/or reproductive stage could be useful for screening tolerant plants under HS.

3.6. Conclusions

By way of conclusion, pepper plant growth and development under high-temperature conditions depended on plants' ability to perceive stress and to generate tolerant responses, such as tissue integrity or accumulation of protective metabolites, and grafting peppers onto a HS-tolerant rootstock, such as A57, could overcome the negative high temperature effects better than an ungrafted variety.

We also indicate that the better physiological performance noted in vegetative parts conferred by a HS-tolerant rootstock would also seem to result in better performance in the reproductive phase because fruit set, which is one of the most important yield components in fruiting vegetables, improved. This scenario is associated with better pollen germination and proline content in pollen grains.

3.7. Supplementary materials

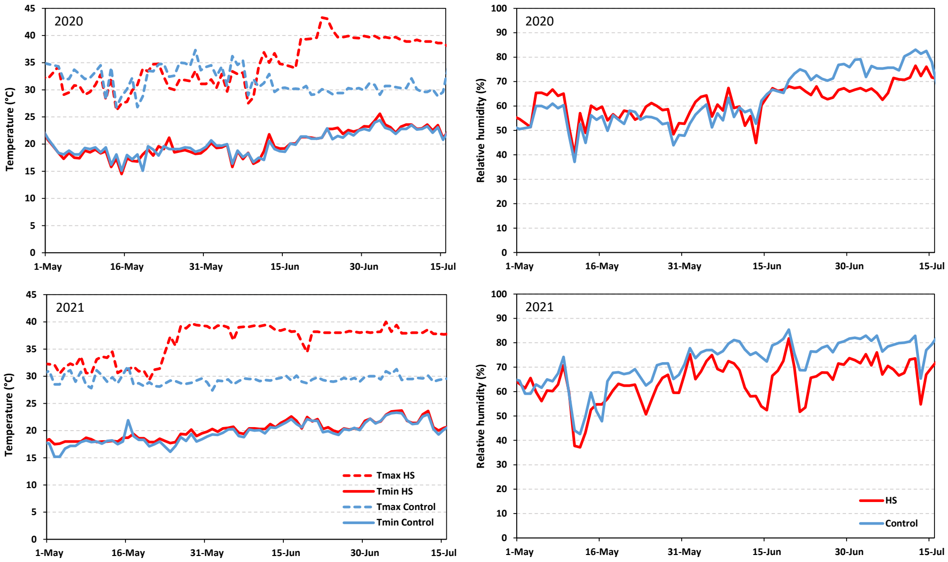

Fig. 1S. Temperature (°C) and relative humidity (%) values inside both greenhouses, heat stress (HS) and control conditions.

3.8. References

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Capítulo 4

Grafting onto an Appropriate Rootstock Reduces the Impact on Yield and Quality of Controlled Deficit Irrigated Pepper Crops

Ramón Gisbert-Mullor [1], Nuria Pascual-Seva [1], María Amparo Martínez-Gimeno [2], Lidia López-Serrano [2], Eduardo Badal Marín [2], Juan Gabriel Pérez-Pérez [2], Luis Bonet [2], Yaiza Gara Padilla [2], Ángeles Calatayud [2], Bernardo Pascual [1] and Salvador López-Galarza [1]

[1] Departamento de Producción Vegetal,
CVER, Universitat Politècnica de València,
Camí de Vera s/n, 46022 Valencia, Spain
[2] Instituto Valenciano de Investigaciones
Agrarias, CV-315, Km 10,7, Moncada,
46113 Valencia, Spain

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4.1. Abstract

In this study, hybrid pepper rootstock NIBER® is tested for its ability to overcome water stress situations under soil conditions. The impact of deficit irrigation (DI) on yield and fruit quality, irrigation water use efficiency is evaluated, and consequently, the agronomic impact of employing water-stress tolerant rootstock is compared to ungrafted pepper plants. For this purpose, plants of the California-type sweet pepper 'Maestral F1' grafted onto NIBER® underwent a sustained DI regime during seasons 2018 and 2019 and were compared to their respective controls. Plants were drip-fertirrigated, and volumetric soil water content was continuously monitored by capacitance sensors. Gas exchange and leaf water potential measurements were taken early in the morning and midday 58, 79, and 114 days after transplanting. Plant and fruit dry biomass, marketable quality, blossom-end rot incidence and harvest index were also determined. For consecutive years, our results confirmed that grafting a pepper cultivar onto an appropriate rootstock (NIBER® in this case) as part of a DI strategy can overcome the negative effects of sustained water stress conditions. The plant biomass production and fruit yields of grafted plants were less affected by DI due to less sensitivity to water stress. This can be attributed to a less marked reduction in shoot dry weight in the grafted plants, which allowed greater whole photosynthesis by maintaining sink activity compared to ungrafted plants.

Keywords:
Grafting; *Capsicum annum* L.; sustained deficit irrigation; drought stress; abiotic stress; volumetric soil water content; gas exchange; leaf water potential; biomass

Abbreviations used:

4.2. Introduction

Sweet pepper (*Capsicum annuum* L.) is an important vegetable crop around the world, with production at approximately 36 Mt obtained from 2 million ha. Currently, the leading sweet pepperproducing countries are China, Mexico, and Turkey, while Spain, Italy, and Romania are the main producers in Europe [1].

Drought stress is an important limiting factor for vegetable crop production. Water is becoming increasingly scarce worldwide, which seriously affects agricultural production, especially in arid and semiarid areas [2,3], due to increased global temperature and evapotranspiration (ET) and less precipitation, which would consequently increase water demands [4–6]. Already existing drought conditions are expected to worsen, particularly in regions where water scarcity is a concern, such as the Mediterranean Region [7]. The Mediterranean climate presents mild winter temperatures and long hot, dry summers, with interannual and seasonal-depending variable precipitation, which make irrigation essential for crop production [8–10].

Globally speaking, agriculture is the largest freshwater consumer and represents approximately 68% of water use [11]. By 2050, the world population is expected to be 9 billion people, which will involve a 60% increase in agricultural production and a 15% increase in water withdrawal [12], which would increase competition for water resources by urban and industrial users. To mitigate these water shortage effects, researchers are attempting to increase water productivity by different approaches [10,13–15]. Nowadays, it is even more important to maximize crop water productivity rather than crop yield per unit area [16].

Drought severity and duration affect yields in many ways by reducing plant growth, disturbing plant water relations, and affecting water-use efficiency. Due to different physiological processes, some parameters like leaf water potential, stomatal resistance, and transpiration rate, strongly influence plant-water relations. These, in turn, affect mineral nutrition, photosynthesis, and oxidative damage [17]. As these parameters depend on the cultivars and environment in which they are grown to a large extent, irrigation management should be adapted to each variety and environmental condition.

One important tool to reduce irrigation water use in agriculture is deficit irrigation (DI), which consists of applying water below full crop-water requirements. Fereres and Soriano [18] reviewed several cases about successful DI use for different crops, and stated that DI can not only increase water productivity, but also farmers' profits. However, DI may also mean major yield reductions [18], and therefore, crop responses to water deficit must be evaluated to achieve efficient water use, while obtaining adequate yields [3].

Another tool to cope with yield reductions related to water stress in some vegetables is to employ grafting technology. Grafting enables plants to overcome biotic and abiotic stresses, including water stress [19–21]. This fact has been particularly studied in tomato [22] and melon [23]. In recent years, several studies have also been conducted on pepper. Some rootstocks can confer pepper cultivars tolerance to water restrictions [24] as they can maintain stomatal conductance, and consequently, the photosynthetic rate under these conditions [25,26]. This water-stress tolerance leads to comparable yields to those of fully irrigated ungrafted plants [27], due to osmotic adjustment via, i.e., proline accumulation and/or the protective role of this substance [28]. More recently, López-Serrano et al. [29] stated that water stress severity in pepper plants was alleviated by using tolerant accessions as rootstocks, capable of partially opening stomata, preserving the relative water content, and consequently, both lowering oxidative stress and diminishing lipid peroxidation. These accessions were inserted in a classic breeding program to obtain more uniform hybrids in terms of germination, growth, and highest vigor to be used as rootstocks under water stress conditions, one of them is NIBER®.

Nevertheless, and as far as we know, no studies have been conducted on water restrictions using these rootstocks in pepper crops under greenhouse conditions, monitoring water availability for plants.

Therefore, it would be advisable to study the response of sweet pepper cultivars grafted onto drought-tolerant rootstocks to DI. Hence, the objective of this study was to evaluate the impact of DI on the yield and fruit quality of a green pepper cultivar using NIBER®, an F1 hybrid, as a rootstock, on irrigation water use efficiency, and consequently, on the agronomic impact that can lead to better profits with sweet pepper crops.

4.3. Materials and methods

4.3.1. Experimental Site

The experiment was conducted during two consecutive growing seasons (GS; 2018 and 2019) at the Instituto Valenciano de Investigaciones Agrarias (IVIA) Research Institute in Moncada (Valencia, Spain; latitude: 39.589517, longitude: –0.395550, elevation: 37 m). The climate is the Mediterranean, distinguished by warm wet winters and hot, dry summers. Temperature and relative humidity values along the experimental seasons are presented in Figure S1. Trials were conducted in an unheated plastic greenhouse (30 m long × 7.5 m wide) that was E-W oriented, and passively ventilated by opening side panels and roof vents. The soil composition within a 20-centimeter depth was sandy clay loam (68% sand, 11% clay, 21% silt) and contained 0.61% organic matter, 0.051% total N, 8 mg kg^{-1} of P, 301 mg kg^{-1} of K, and 2.87 meq 100 g^{-1} of assimilable Mg. Soil electrical conductivity (1:5) was 0.29 dS m^{-1}, and pH was 8.1. Irrigation was applied with a drip system with one single line per plant row and one emitter every 0.2 m with a 2.5 L h^{-1} discharge rate. Irrigation water had an electrical conductivity of 0.3 dS m^{-1}, 16.5 mg L^{-1} of NO_3^- content, very low concentrations of other macro- and micronutrients, and a pH of 8.1. Nutrients were applied during both crop seasons through the irrigation system according to the following nutritional plan and in line with Maroto [30]: 170 kg ha^{-1} of N; 40 kg ha^{-1} of P_2O_5; 200 kg ha^{-1} of K_2O.

4.3.2. Plant Material

Sweet pepper 'Maestral F1' (California-type Rijk Zwaan, The Netherlands) grafted onto rootstock F1 NIBER® (V/N) (obtained from the Universitat Politècnica de València (UPV)-IVIA, Spain) and ungrafted (V) plants were used in the experiments.

Sowing took place on 22 January 2018 and 25 January 2019, in 104-cell polystyrene trays on peat moss-based substrate (70% blonde, 30% dark), which is recommended for vegetable seedbeds (Pindstrup Mosebrug S.A.E., Sotopalacios, Spain). Trays were maintained in a Venlo-type greenhouse. On 1 March 2018 and 5 March 2019, plants were grafted by the tube-grafting method [27].

Seedlings were transplanted on 27 March 2018 and 2 April 2019 inside the plastic greenhouse in raised beds spaced 1.10 m apart with one plant row per ridge and 0.5 m spacing between plants within rows (1.8 plants m^{-2}). The raised part of the raised bed was 0.2 m wide, 6 m long, and 0.10 m high. Plants were horizontally supported by two nylon guide cords laid parallel to both sides of the plant line, as described by Maroto [30], and no pruning was performed.

The experiments were laid out to be complete randomized with three replicates. Each replicate consisted of 13 plants.

4.3.3. Irrigation Strategies

Two irrigation strategies (IS) were adopted: Irrigation with 100% and 50% of the estimated crop water requirements (control (C) and DI, respectively). Irrigation during the first four weeks after plantation was applied to assure the correct plant establishment. Irrigation management consisted of fixed 30-minute irrigation events, applied as often as necessary, depending on the VSWC and irrigation strategy. Crop irrigation water requirements were obtained by estimating crop evapotranspiration (ET_c) by the K_c–ET_o method [31]. The reference evapotranspiration (ET_o; in mm day^{-1}) was calculated by the calibrated radiation method, which is often used in the greenhouse industry in SE Spain [32]. This method requires daily solar radiation outside greenhouses and cladding greenhouse transmissivity (σ, in %). Solar radiation was obtained from an agroclimatic weather station located near the greenhouse, while transmissivity was calculated by using the radiation data collected before the crop period with a CMP3 pyranometer (Kipp and Zonen, Delft, The Netherlands), whose value was 43% during both seasons. The crop coefficient (K_c) values were those determined by Orgaz et al. [33] for sweet pepper. Initially, K_c took a value of 0.2 and rose to 1.4 at harvest.

The applied irrigation water, and its distribution according to growth stages, are presented in Table 1. For the environmental conditions during the experimental period, the ET_o calculated from outdoor solar radiation was 261.4 mm in 2018 and 265.1 mm in 2019. When applying the proposed K_c, the estimated water requirements were 230.5 mm in 2018 and 228.4 mm in 2019. The total applied water amount in the control strategy was 216.3 mm and 206.8 mm, respectively (Table 1), which means that the irrigation enforced in the control strategy was around 94% and 91% of the ET_c value in 2018 and 2019, respectively. The DI (V-DI and V/N-DI) received 58% and 56% of the control doses (V-C and V/N-C) in 2018 and 2019, respectively.

Table 1. Crop evapotranspiration (ET_c) and irrigation water applied in the different growth stages for the plants under the control and deficit irrigation conditions during 2018 and 2019.

Growth stages	Duration (days)	ET_c (mm)	Irrigation water applied (mm)	
			Control	Deficit Irrigation
2018				
Vegetative growth	34	22.1	25.7	21.9
Fruit development	29	45.0	54.0	27.0
Harvesting period	49	163.4	136.6	76.5
Total	112	230.5	216.3	125.4
2019				
Vegetative growth	34	19.7	20.2	22.6
Fruit development	43	82.9	76.2	33.3
Harvesting period	38	125.8	110.4	60.5
Total	115	228.4	206.8	116.4

4.3.4. Soil Moisture

The volumetric soil water content (VSWC; $m^3\ m^{-3}$) was continuously monitored by ECH_2O EC-5 capacitance sensors connected to an Em50 data logger using the ECH_2O Utility software (Decagon Devices, Inc., Pullman, WA, USA). VSWC values were used directly because factory calibration provides ± 3% accuracy for mineral soils. One sensor per replicate was placed below the dripline at a 20-centimeter depth, where the maximum root density was located and was equidistant between two adjacent emitters. The VSWC was measured and stored every 30 min, and its variation was used to determine in situ field capacity (FC) [34,35]. The permanent wilting point (PWP) was determined by using Richards Plates (Set for pF-determination; Eijkelkamp, Giesbeek, The Netherlands) [36]. To compare VSWC between irrigation strategies and growing seasons, their values are presented as available water content (AWC; %) before each irrigation event, and were determined as reported by Fernández et al. [37]:

$$AWC = (1 - [(VSWC_{FC} - VSWC_a) / (VSWC_{FC} - VSWC_{PWP})]) \times 100, \tag{1}$$

considering the subscripts a, FC and PWP correspond to the actual (before irrigation), field capacity, and permanent wilting point soil water content, respectively. When the $VSWC_a$ was greater than the $VSWC_{FC}$, the last one was considered, given that AWC cannot be greater than 100%. The values for the VSWC at FC and PWP were determined for the different GS and IS and are shown in Table 2.

Table 2. Volumetric soil water content (m3 m−3) at field capacity (FC) and permanent wilting point (PWP) for each growing season (2018 and 2019), irrigation strategy (control (C) and deficit irrigation (DI)) and plant type (ungrafted (V) and grafted (V/N)).

	Plant type	2018		2019	
		FC	PWP	FC	PWP
Control	V-C	0.19	0.08	0.24	0.13
	V/N-C	0.17	0.08	0.23	0.13
Deficit irrigation	V-DI	0.23	0.08	0.19	0.13
	V/N-DI	0.22	0.08	0.19	0.13

4.3.5. Physiological Measurements

Physiological measurements were taken during GS 2019. The gas exchange measurements were taken early in the morning (9.30 am to 10.30 am GMT) and at midday (1 pm to 2 pm) with three plants per replicate (9 per treatment) after 58, 79, and 114 days after transplanting (DAT). The net CO_2 assimilation rate (A_N, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), and transpiration rate (E_{leaf}, mmol H_2O m^{-2} s^{-1}) were determined on fully expanded leaves (3rd–4th leaf from the apex) in the steady-state under saturating light conditions (1000 µmol m^{-2} s^{-1}) and with 400 ppm CO_2 by an LI6400 infrared gas analyzer (LI-COR, Nebraska, USA) at 24 ± 2 °C and 65 ± 10% relative humidity. The average values of leaf vapor pressure deficit (considering 58, 79, and 114 DAT) were 1.68 ± 0.22 kPa and 2.50 ± 0.25 kPa at early morning and midday, respectively. Parameters A_N/g_s and A_N/E_{leaf} were calculated as intrinsic water efficiency and instantaneous water use efficiency, respectively.

The predawn (5.00 am to 6.00 am) and midday leaf water potential (1 pm to 2 pm) ($\Psi_{predawn}$ and Ψ_{leaf}, respectively) were determined by a Schölander-type pressure chamber (model 3000; Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Determinations were made on fully expanded leaves in an identical physiological state as that used in the gas exchange measurements of three plants per replicate (9 per treatment) after 58, 79, and 114 DAT.

Plant conductance of the hydraulic water flow from root systems to leaves (whole-plant hydraulic conductivity, Kh_{plant}) was calculated by analogy to Ohm's law [38]:

$$Kh_{plant} = E_{leaf\text{-}md} / (\Psi_{soil} - \Psi_{leaf}) \quad (2)$$

where $E_{leaf\text{-}md}$ is the maximum transpiration rate in the leaf measure in LICOR-6400 from 1 pm to 2 pm by assuming that $\Psi_{soil} = \Psi_{predawn}$. Kh_{plant} was measured at 58, 79, and 114 DAT.

4.3.6. Biomass and Fruit Yield

Harvests consisted of four passes between mid-June and the end of July in both years. The commercial production quality was evaluated following the criteria described by the European Regulations [39], insofar as the yield was partitioned into three categories: «Extra» Class, Class I (together hereafter referred to as marketable yield; MY) and Nonmarketable, the fruits which, given their defects, mainly due to blossom-end rot (BER), did not match the former categories.

Biomass was analyzed at the end of crop cycles. The aboveground plant part was partitioned into two parts and separately analyzed: Vegetative, including shoots with all their leaves (hereafter referred to as shoot dry weight (SDW)), and fruit (including all the fruit of all the passes of the harvests). The sum of both terms was the total dry weight (TDW). Each part was dried at 65 °C in a forced-air oven (Oven 100-800, Memmert, Germany) until constant weight and weighed on an analytical scale, which measures dry weights. The harvest index (HI) was determined as the ratio of the total yield to the total aboveground biomass (TDW) on a dry mass basis [37].

4.3.7. Statistical Analysis

The biomass and fruit yield data were subjected to an ANOVA analysis, including the three factors: IS, plant type (PT), and GS. The physiological parameters were separately analyzed for each measurement time (58, 79, and 114 DAT) as a multiway ANOVA (Statgraphics Centurion for Windows, Statistical Graphics Corp.). With these parameters, after verifying the significance of the interaction for each variable (data are not shown), a one-way ANOVA was performed by joining the plant combination and treatment for each studied time. Means were compared by the Fisher's least significance difference (LSD test) at $p < 0.05$. No significant differences were found among the replicates for each measured parameter.

4.4. Results

4.4.1. Soil Moisture

Figure 1 presents the weekly average values for the available water content (AWC) before each irrigation event. For both growing seasons (GS) and irrigation strategies (IS), AWC increased before the differential irrigation strategies started (25 and 24 April in 2018 and 2019, respectively), reaching values of 100% of the AWC in 2018, as a consequence of plants being over irrigated to assure their establishment. After this day, during both GS the soil moisture in the deficit irrigation (DI) treatment decreased to constant values at the end of the cycle [40% in 2018 and 26.5% (on average for both plants types (PT)) in 2019], which was lower than those reached in the control treatment. In 2018, this drop in AWC was more marked for V/N-DI than for V-DI. From early June, soil moisture also decreased in the control treatment in the two PT and during both GS. It is noteworthy that in 2019, some problems were encountered with the irrigation system, which involved an unplanned lack of irrigation for two days. Consequently, the AWC in the control treatments dropped below 50%, and rose to previous levels when irrigation was reestablished. It is also noteworthy that the standard error was generally lower for DI than for the control plants as soil moisture remained at constant values.

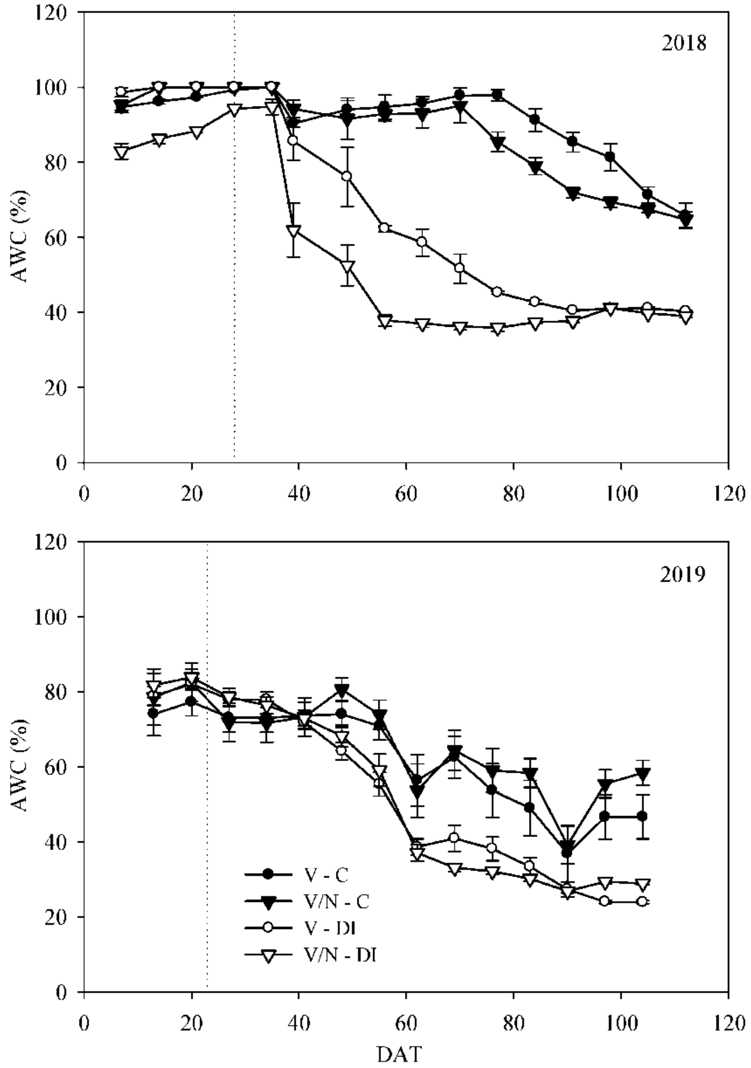

Figure 1. Weekly average soil moisture (expressed as a percentage of the available water content, AWC) during each growing season (2018 and 2019) for plant type, grafted (V/N) and ungrafted (V), and for both irrigation strategies: Control (C) and deficit irrigation (DI). DAT are days after transplanting. The vertical line represents the start of irrigation treatments.

4.4.2. Plant Water Relations

The predawn leaf water potential ($\Psi_{predawn}$) values (Figure 2A) after 58 DAT were not significantly different between the control and DI treatments. At 79 DAT and at the end of the experiment (114 DAT), the V-C and V/N-C values were higher (with no significant differences between them) compared to V/N-DI and V-DI, of which the latter had the lowest $\Psi_{predawn}$ values.

The midday leaf water potential Ψ_{leaf} values progressively lowered during the experiment as plant size enlarged in all the IS (Figure 2B). The application of the DI strategy significantly decreased Ψ_{leaf} in the DI plants, but only at the end of the experiment (114 DAT), with no differences appearing between the grafted and ungrafted plants.

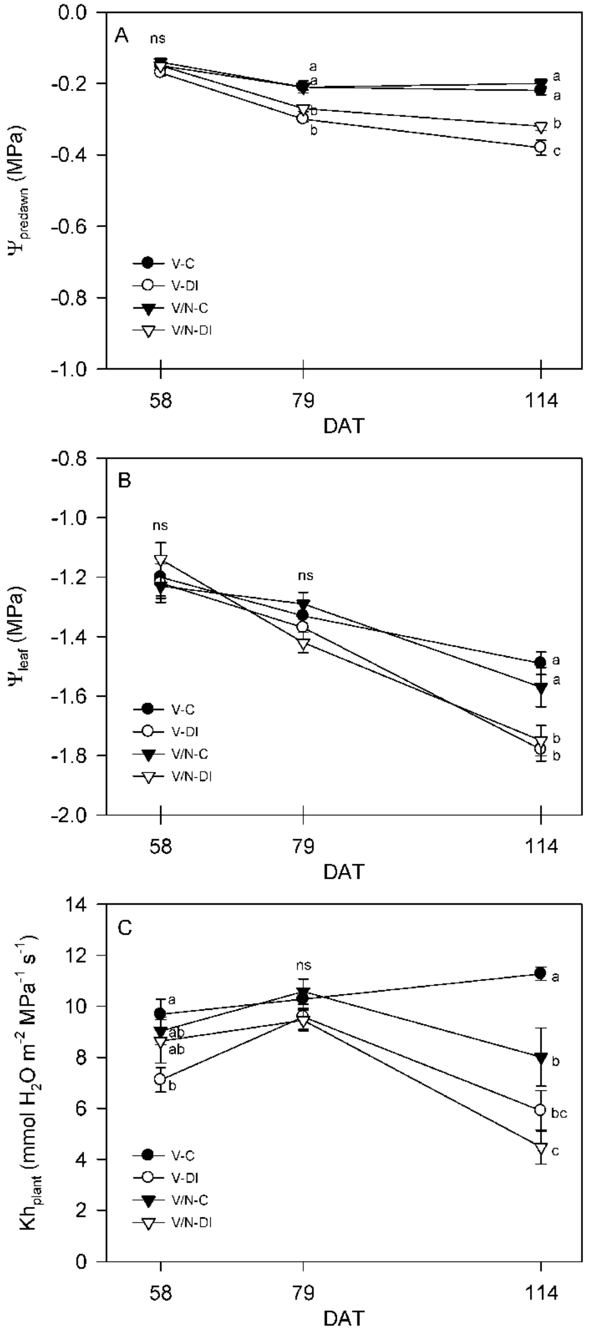

Figure 2. Predawn leaf water potential ($\Psi_{predawn}$) (A), midday leaf water potential (Ψ_{leaf}) (B), and whole plant hydraulic conductivity (Kh_{plant}) (C) in the ungrafted pepper plants (cultivar 'Maestral', V) and the plants grafted onto NIBER® (V/N) grown under well-irrigated (C) and deficit-irrigated (DI) conditions. Measurements were taken on 58, 79, and 114 days after transplanting (DAT). Data are the mean values for n = 9 ± SE. For each studied time, different letters indicate differences at $p \leq 0.05$ (LSD test). n.s. indicate no significant difference.

Whole plant hydraulic conductivity (Kh_{plant}) (Figure 2C) was not constant during the experiment. At 58 DAT, values ranged between 7 and 10 mmol H_2O m^{-2} MPa^{-1} s^{-1}, and the lowest values were for the V-DI plants, and the highest ones were measured for the V-C plants, whereas the grafted plants had intermediate values. At 79 DAT, no significant differences between treatments were observed for all the Kh_{plant} values. However, at the end of the experiment (114 DAT), differences between treatments were found. When comparing only the control treatment plants, the V plants had higher Kh_{plant} than the V/N plants. The DI treatment decreased Kh_{plant} (46% lower) in the V and V/N groups of the plants compared to the control, with the lowest values for the V/N-DI plants.

4.4.3. Photosynthetic Parameters

The net CO_2 assimilation rate (AN) measured early in the morning (Figure 3A) showed no significant differences between plants and treatments during the crop cycle. Nevertheless, the AN values obtained at midday (Figure 3B) were grouped for the significant differences in the control and DI plants at 58 DAT and 79 DAT. The lowest values were for the DI plants at both these times. At 114 DAT, the maximal AN was observed in the V-C plants followed by V/N-C, V-DI, and V/N-DI with the lowest values.

The transpiration rate (Eleaf) early in the morning (Figure 3C) increased from 58 DAT to 79 DAT, but with no significant differences between treatments at both DATs. At the end of the experiment, the DI strategy lowered the Eleaf values in the V and V/N plants compared to the control strategy. At midday, the Eleaf values of the control plants were higher than the values obtained early in the morning (Figure 3C,D). The measurements taken at 58 DAT and 79 DAT did not show any changes in the Eleaf of the V-C and V/N-C plants, but the Eleaf values were higher in the V-C plants than in the V/N-C plants at the end of the experiment. The application of the DI strategy led to equal Eleaf values for both the V and V/N plants at 58 DAT (Figure 3D). At 79 DAT, the DI strategy only reduced Eleaf in the V-DI plants. At the end of the experiment, the differences in Eleaf between IS were more evident, with the highest values for V-C and the lowest ones for V/N-DI.

Stomatal conductance (g_s) was measured early in the morning (Figure 3E) and only showed significant differences between IS at the end of the experiment, with similar reductions in the V and V/N plants compared to the control. The influence of IS on g_s was more evident at midday (Figure 3F). Although the measurements taken at 58 DAT and 79 DAT did not indicate any changes in g_s of the V-C and V/N-C plants, g_s was higher in the V-C than the V/N-C plants at the end of the experiment. At 58 DAT, the reduction in g_s accomplished by the DI strategy was equal for the V and V/N plants (Figure 3F). At 79 DAT, the DI strategy only reduced g_s in the V-DI plants. At the end of the experiment, the differences in g_s between IS were more evident, with the highest values for V-C and lowest ones for V/N-DI.

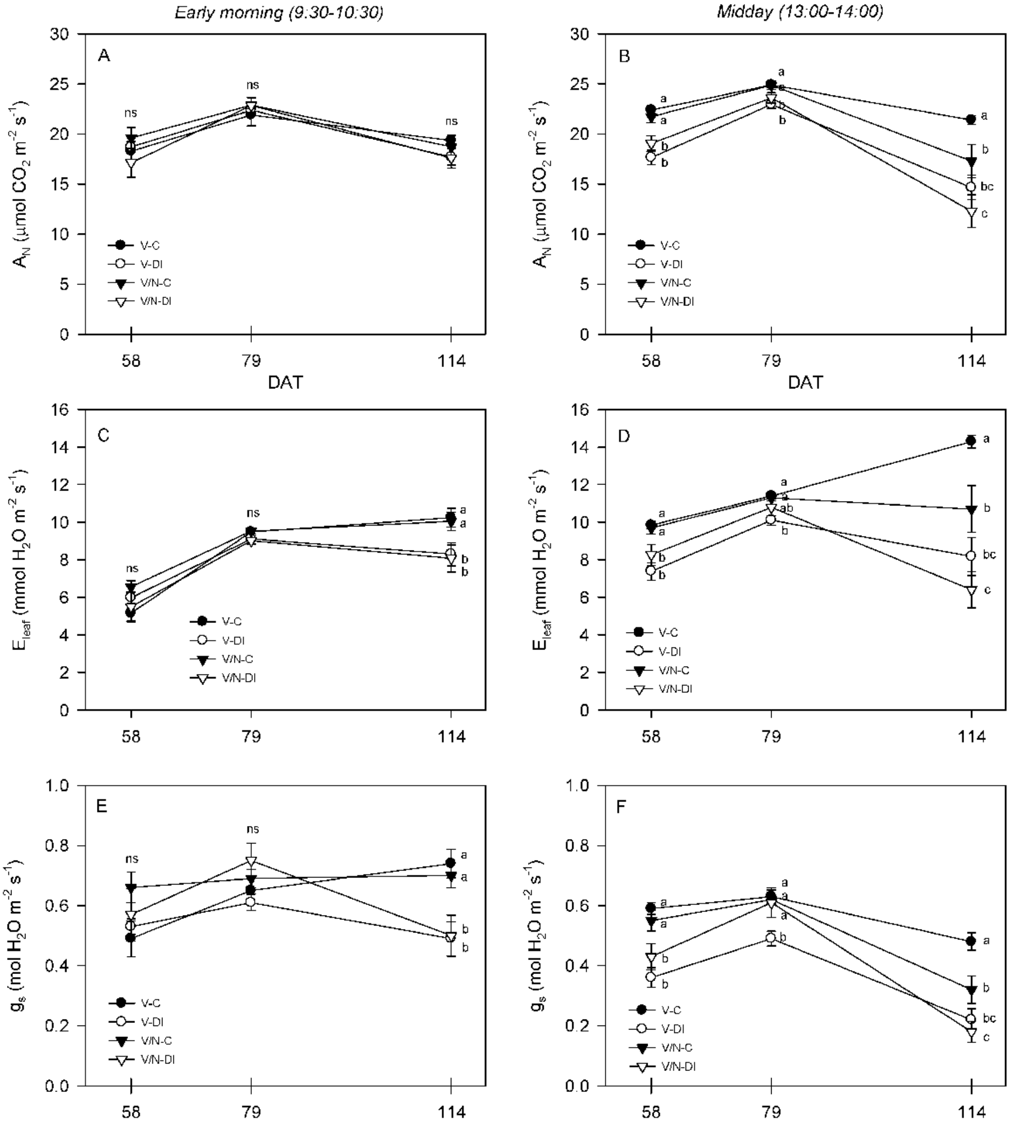

Figure 3. Net CO2 assimilation rate (AN) (A,B), leaf transpiration rate (Eleaf) (C,D), and stomatal conductance (g_s) (E,F) measured at two representative day times (early morning, midday) in the ungrafted pepper plants (cultivar 'Maestral', V) and the plants grafted onto NIBER® (V/N) grown under well-irrigated (C) and deficit-irrigated (DI) conditions. Measurements were taken on 58, 79, and 114 days after transplanting (DAT). Data are the mean values for n = 9 ± SE. For each studied time, different letters indicate differences at $p \leq 0.05$ (LSD test). n.s. indicate no significant difference.

Instantaneous water use efficiency (AN/Eleaf) progressively decreased early in the morning for all the plant combinations during the experiment, with minimum values at 114 DAT (Figure 4A). There were no significant differences between IS at 58 DAT and 79 DAT, whereas AN/Eleaf increased in the DI plants (V and V/N) at 114 DAT compared to the control. A similar response occurred at midday (Figure 4B). Regarding intrinsic water use efficiency (AN/g_s), a different shape behavior was observed during the experiment to the values measured early in the morning (Figure 4C) and at midday (Figure 4D). Early in the morning (Figure 4C), it was only at the end of the experiment when the AN/g_s values exhibited significant differences, with the highest values for the DI plants (V-DI and V/N-DI). From 79 DAT to 114 DAT, the AN/g_s values obtained at midday significantly increased (Figure 4D), where the DI plants obtained the maximum values compared to the control plants (V and V/N).

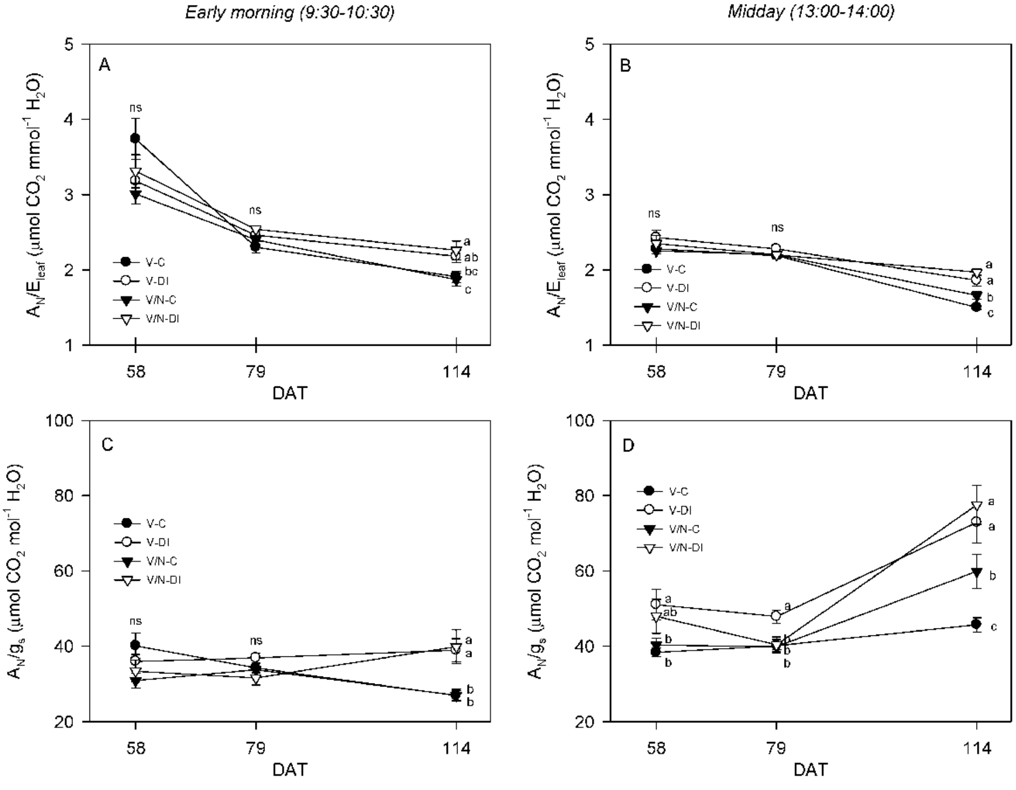

Figure 4. Instantaneous water use efficiency (AN/Eleaf) (A,B) and intrinsic water use efficiency (AN/g_s) (C,D) measured at two representative day times (early morning, midday) in the ungrafted pepper plants (cultivar 'Maestral', V) and the plants grafted onto NIBER® (V/N) grown under well-irrigated (C) and deficit-irrigated (DI) conditions. Measurements were taken on 58, 79, and 114 days after transplanting (DAT). Data are the mean values for n = 9 ± SE. For each studied time, different letters indicate differences at $p \leq 0.05$ (LSD test). n.s. indicate no significant difference.

4.4.4. Plant Biomass and Fruit Yield

Shoot dry weight (SDW) was significantly affected by IS ($p \leq 0.01$; Table 3), with a 20% decrease for the stressed plants versus the control ones, but the IS × PT interaction for this parameter was statistically significant ($p \leq 0.05$; Table 3) as the differences in SDW between DI and C were small (down to 50%) in the V/N plants in relation to V (Figure 5). Total dry weight (TDW) was also significantly affected by IS ($p \leq 0.01$; Table 3), with an average 22% decrease for the DI plants in relation to the control for both seasons. No significant IS × PT interaction was detected. The harvest index (HI) was affected by neither any analyzed factor nor their interaction for any season.

Table 3. Effect of irrigation strategy, plant type, and growing season on the shoot dry weight (SDW), total dry weight (TDW), and harvest index (HI).

	SDW ($g\ plant^{-1}$)		**TDW ($g\ plant^{-1}$)**		**HI (-)**	
Irrigation strategy (IS)						
C	176.3	a	311.7	a	0.437	
DI	140.5	b	240.8	b	0.416	
Plant type (PT)						
V	158.0		270.8		0.418	
V/N	158.8		281.7		0.434	
Growing season (GS)						
2018	157.8		272.5		0.421	
2019	158.9		280.0		0.432	
ANOVA (*df*)	**% Sum of the squares**					
IS (1)	69.85	**	81.01	**	13.18	n.s.
PT (1)	0.03	n.s.	1.89	n.s.	7.61	n.s.
GS (1)	0.06	n.s.	0.91	n.s.	3.56	n.s.
IS*PT (1)	6.51	*	1.89	n.s.	0.00	n.s.
IS*GS (1)	3.57	n.s.	0.55	n.s.	9.30	n.s.
PT*GS (1)	2.74	n.s.	2.52	n.s.	9.30	n.s.
IS*PT*GS (1)	3.01	n.s.	1.89	n.s.	0.19	n.s.
Residuals (16)	14.22		9.33		0.53	
Std. Dev.(+)	9.9		14.7		0.026	

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * indicate significance at P ≤ 0.01 and P ≤ 0.05, respectively, n.s. no significant difference. (+) Standard deviation, calculated as the square root of the residual sum of squares. *df* degrees of freedom.

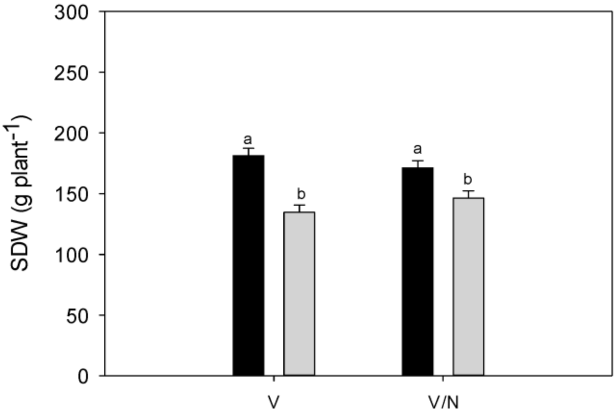

Figure 5. Shoot dry weight (SDW, g plant^{-1}) for the deficit irrigation plants (gray bars) and the control plants (black bars) in the ungrafted pepper plants (V) and the cultivar grafted onto NIBER® (V/N). Data are the mean values for n = 6. In each plant combination, different letters indicate significant differences at $p \leq 0.05$. Error bars represent the LSD value of the interaction.

The marketable yield (MY) and «Extra» class yield were also significantly affected by IS ($p \leq 0.01$; Table 4) and by the IS × PT interaction ($p \leq 0.05$; Table 4). Deficit irrigation reduced yields in relation to the control, down to 24% and 31% for the MY and «Extra» class yield, respectively. In both cases for the DI plants, higher yields were maintained in the V/N plants compared to V (Figure 6). Class I yield was affected by neither any analyzed factor nor their interaction.

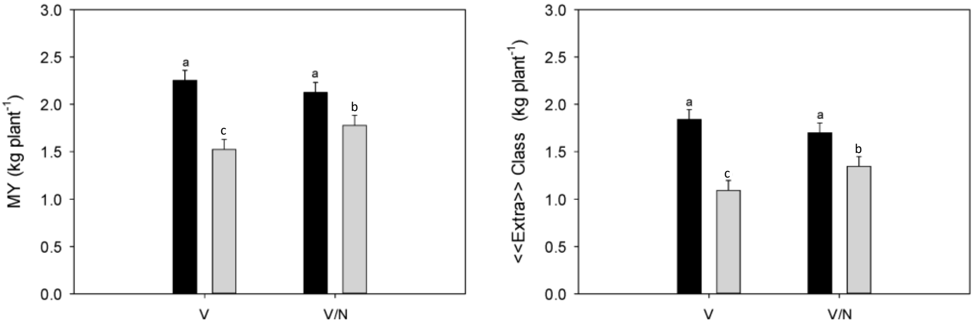

Figure 6. Marketable yield (MY, kg plant^{-1}) and «Extra» Class yield («Extra» Class, kg plant^{-1}) for the deficit irrigation plants (gray bars) and the control plants (black bars) in the ungrafted pepper plants (V) and the cultivar grafted onto NIBER® (V/N). Data are the mean values for n = 6. In each plant combination, different letters indicate significant differences at $p \leq 0.05$. Error bars represent the LSD value of the interaction.

Table 4. Effect of irrigation strategy, plant type, and growing season on marketable yield (MY), «Extra» Class yield («Extra» Class), and Class I yield (Class I).

	MY (kg plant^{-1})		**«Extra» Class (kg plant^{-1})**		**Class I (kg plant^{-1})**	
Irrigation strategy (IS)						
C	2.189	a	1.770	a	0.421	
DI	1.652	b	1.219	b	0.432	
Plant type (PT)						
V	1.889		1.466		0.425	
V/N	1.952		1.523		0.428	
Growing season (GS)						
2018	1.906		1.484		0.423	
2019	1.935		1.505		0.430	
ANOVA (*df*)	**% Sum of the squares**					
IS (1)	66.03	**	66.19	**	0.04	n.s.
PT (1)	0.89	n.s.	0.69	n.s.	0.74	n.s.
GS (1)	0.19	n.s.	0.09	n.s.	0.36	n.s.
IS*PT (1)	8.32	*	8.52	*	0.22	n.s.
IS*GS (1)	3.62	n.s.	4.10	n.s.	20.93	n.s.
PT*GS (1)	0.00	n.s.	0.67	n.s.	0.99	n.s.
IS*PT*GS (1)	2.48	n.s.	2.75	n.s.	0.53	n.s.
Residuals (16)	18.45		16.98		76.19	n.s.
Std. Dev. $^{(+)}$	0.174		0.171		0.067	n.s.

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * indicate significance at P ≤ 0.01 and P ≤ 0.05 respectively, n.s. no significant difference. $^{(+)}$ Standard deviation, calculated as the square root of the residual sum of squares. *df* degrees of freedom.

The «Extra» class number of fruits per plant and average fruit weight were only affected by IS (Table 5) as the DI values lowered by 25% and 8%, respectively, in relation to the control. As previously mentioned, in 2018, no fruits were affected by BER, and were not affect by any analyzed factor in 2019 (Table S1).

Table 5. Effect of irrigation strategy, plant type, and growing season on fruit number of «Extra» Class (No. «Extra» Class) and average fruit weight of «Extra» Class (AFW «Extra» Class).

	No. «Extra» Class (fruits plant^{-1})	AFW «Extra» Class (g fruit^{-1})
Irrigation strategy (IS)		
C	8.16 a	218.5 a
DI	6.06 b	201.0 b
Plant type (PT)		
V	7.11	204.6
V/N	7.11	214.9
Growing season (GS)		
2018	6.91	213.7
2019	7.31	205.7
ANOVA (*df*)	**% Sum of squares**	
IS (1)	50.43 **	26.15 *
PT (1)	0.00 n.s.	9.07 n.s.
GS (1)	1.80 n.s.	5.41 n.s.
IS*PT (1)	8.06 n.s.	0.96 n.s.
IS*GS (1)	5.45 n.s.	0.04 n.s.
PT*GS (1)	0.08 n.s.	2.73 n.s.
IS*PT*GS (1)	4.26 n.s.	0.14 n.s.
Residuals (16)	29.93	55.49
Std. Dev. (+)	0.99	15.6

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * indicate significance at P ≤ 0.01 and P ≤ 0.05 respectively, n.s. no significant difference. (+) Standard deviation, calculated as the square root of the residual sum of squares. *df* degrees of freedom.

4.5. Discussion

For two consecutive years, our results demonstrated that grafting a pepper cultivar onto the new hybrid rootstock, NIBER®, can overcome the negative effects of sustained water stress in biomass and yield terms.

The water applied during both GS was similar because the meteorological conditions were comparable that, in turn, led to equivalent water requirements. Harvesting is, as usual, the longest growth stage, and it presents the maximum daily water requirements. This means having to apply the largest amount of water, which represents 58% of the total amount.

For both GS and IS, AWC maintained close to 100% from planting to the establishment, which indicates that the VSWC came close to FC. The reduction of AWC after the establishment was more marked for V/N-DI than for V-DI, in 2018, and was probably related to the fact that water uptake was greater in the grafted plants. Afterward, the AWC in the DI strategies dropped to 40% and 50% in 2018 and 2019, respectively. These values were lower than 55%, the value established by Fernández et al. [37] as the threshold AWC, below which pepper ETc lowers in response to soil water deficit. Therefore, it can be stated that DI plants were water-stressed, and as the AWC for both plant types was similar during each GS, the stress level was similar for both PT. So, it can be hypothesized that the higher yield obtained in V/N in relation to V could be due to the grafted plants' better response to water stress conditions.

In fact, DI reduced both shoot and total dry weight by an average 20–22% for the DI plants (V/NDI and V-DI) in relation to the control for both seasons, which is a similar decrease to that observed by López-Marín et al. [24], and comes close to the reduced plant yields reported by several studies for pepper crops [40,41] and tomato [42]. What is more, the NIBER® rootstock had a beneficial effect on MY and «Extra» Class by maintaining a bigger yield than the ungrafted plants under stress conditions. These results, together with those previously obtained with water stress tolerant rootstocks [29], confirm our hypothesis on NIBER® water stress tolerance, now under field conditions, and monitoring soil water content.

Fereres and Soriano [18] pointed out that when water stress increases in severity terms at a sustained DI, with biomass production losses over 40%, the HI can be affected in many crops. However, in the present study, the HI was not affected by either IS or PT, which indicates that yield was lowered by DI to a similar extent as vegetative biomass. Similar results have been obtained by Abdelkhalik et al. [41], who observed differences for the HI between treatments, but only when sever DI was applied, and these authors did not observe any differences for a moderate deficit.

The reduction in SDW between the deficit and control irrigation regimes in the grafted plants was much less marked than in the ungrafted plants, around 56% less. This indicates the ability of some rootstocks to overcome the effects of water stress, as previously found by López-Marín et al. [24]. According to Lee et al. [43], the root system of selected rootstocks is usually much larger and more vigorous than that of cultivars, which allows them to more efficiently absorb water and nutrients compared to ungrafted plants. The NIBER® rootstock has shown better root development in several experiments (Reference [44] and unpublished data). As stated before, water content monitoring in the soil in our experiments affirms the better response of the NIBER® rootstock to water stress in biomass formation and yield terms.

We also tested the physiological processes associated with grafting and DI by focusing on water and photosynthetic relations to explain the obtained agronomic results. Under well-irrigated conditions, plant water status ($\Psi_{predawn}$ and Ψ_{leaf}) was apparently similar between the V/N and V plants. Similar results have been reported in other studies with mini-watermelon, where the leaf water potential in both ungrafted and grafted plants showed comparable values upon optimal irrigation [23]. However, the use of pepper plants grafted onto a drought-tolerant rootstock had a significant influence on plant water transport capacity compared to the ungrafted plants. The hydraulic conductance of the entire plant in the control treatment under high atmospheric demand conditions (at noon) was lower in V/N-C, and was accompanied by lower g_s values. Changes in hydraulic properties and stomatal behavior enable grafted plants to better regulate the water used by the plant by reducing the water lost by transpiration, which improves leaf water use efficiency (AN/Eleaf and AN/g_s). This can be attributed to the differences in the root system's water absorption capacity, as found in grafted melon plants [45].

We observed that DI strongly influenced the plant water status both in the grafted and ungrafted plants, especially at the end of the experiment (114 DAT), which coincides with the fruit ripening stage, and a similar plant water stress level was obtained (based on Ψleaf ≈ –1.8 MPa). The DI effects were also reflected in lower Khplant, which means that plant water transport capacity diminished compared to well-irrigated plants, and was similarly affected in the V and V/N plants. Changes in plant water relations affected g_s in both plant groups, with maximum stomatal regulation at midday. Stomatal limitation partially reduced the plant's water use, but also lowered the CO2 assimilation rate similarly in both the V and V/N plants. In both plant groups, water stress had less impact on photosynthesis than in g_s and Eleaf, which indicates that stomatal closure occurred earlier than CO2 fixation [26,46], and consequently and similarly enhanced water use efficiency (AN/Eleaf and AN/g_s).

The physiological alterations generated by DI significantly reduced yield and biomass production in both plant groups (V and V/N). This reduction has been associated mainly with changes in all the photosynthetic parameters and water relations, as observed in different crops like mini-watermelon [23], tomato [47] or pepper [24,27,29] when comparing grafted vs. ungrafted plants. Nevertheless, the highest fruit yields and SDW under DI were recorded in the grafted plants (V/NDI) compared to the V-DI plants, despite no significant differences being observed between them from the physiological point of view. The greater SDW reduction in the V-DI plants (25.7%) versus V/N-DI (14.5%), could lead to a more marked decrease in the intercepted radiation in the V-DI plants and could, thus, reduce the whole canopy photosynthesis in relation to V/N-DI. Although root biomass was not monitored in this experiment, SDW decreased in V-DI plants, and this could be explained by differences in biomass partitioning between the aerial part and roots. On the other hand, the capacity to maintain sink activity in the vegetative V/N-DI parts could be an adaptive advantage that leads to more shoot growth and bigger yields through regulating source-sink relations [24] with DI. Other mechanisms not associated to maintenance photosynthetic activity have been described to explain the better biomass and yield performance in grafted plants under DI as osmotic adjustment [48], modulation of antioxidant defenses [49] or hormonal signaling [50].

4.6. Conclusions

These results confirm that using appropriate rootstocks in pepper crops is a good strategy to better tolerate sustained DI, which promotes water stress in pepper, compared to ungrafted plants. This is a consequence of an adaptation morphology and physiology being capable of maintaining photosynthesis levels, and consequently, biomass production. They also confirm that hybrid NIBER® is an interesting rootstock for this purpose.

4.7. Supplementary materials

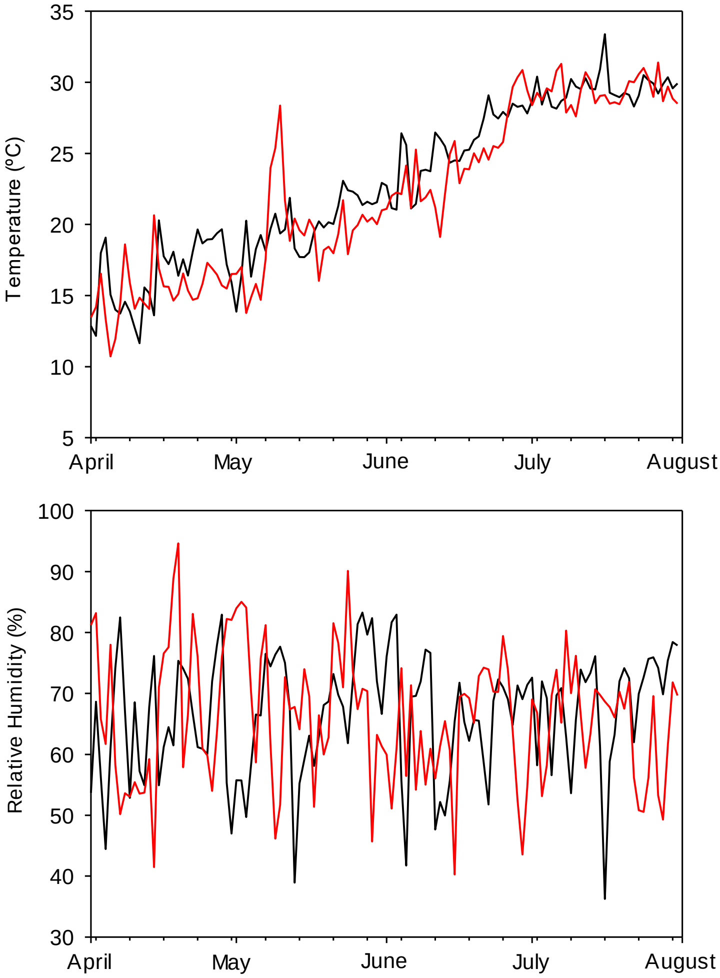

Figure S1. Temperature (°C) and relative humidity (%) values for 2018 (black line) and 2019 (red line).

Table S1. Effect of irrigation strategy and plant type on BER yield (BER) and the fruit number of BER (No. BER).

	BER ($kg\ plant^{-1}$)		No. BER ($fruit\ plant^{-1}$)	
Irrigation strategy (IS)				
C	0.017		0.137	
DI	0.034		0.394	
Plant type (PT)				
V	0.040		0.408	
V/N	0.012		0.123	
ANOVA (*df*)	% Sum of the squares			
IS (1)	6.14	n.s.	10.32	n.s.
PT (1)	16.41	n.s.	12.66	n.s.
IS*PT (1)	0.00	n.s.	0.70	n.s.
Residuals (8)	77.45		76.32	
Std. Dev. (+)	0.037		0.429	

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * indicate significance at P ≤ 0.01 and P ≤ 0.05 respectively, n.s. no significant difference. (+) Standard deviation, calculated as the square root of the residual sum of squares. *df* degrees of freedom.

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Capítulo 5

A Water Stress–Tolerant Pepper Rootstock Improves the Behavior of Pepper Plants under Deficit Irrigation through Root Biomass Distribution and Physiological Adaptation

Ramón Gisbert-Mullor [1], Rodrigo Martín-García [1], Iva Bažon Zidarić [2], Nuria Pascual-Seva [1], Bernardo Pascual [1], Yaiza Gara Padilla [3], Ángeles Calatayud [3] and Salvador López-Galarza [1]

[1] Departamento de Producción Vegetal, CVER, Universitat Politècnica de València, Camí de Vera s/n, 46022 Valencia, Spain
[2] Institute of Agriculture and Tourism, Karla Huguesa 8, 52440 Poreč, Croatia
[3] Instituto Valenciano de Investigaciones Agrarias, CV-315, Km 10,7, Moncada, 46113 Valencia, Spain

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5.1. Abstract

The use of rootstocks tolerant to water stress in pepper crops is a complementary technique for saving irrigation water without affecting yields by means of particular rootstock physiological traits, which changes the scion's perception stress. The present study aimed to analyze the morphological and physiological adaptation of the 'Cuerno' pepper cultivar grafted onto tolerant rootstock NIBER® subjected to capacitance sensor-based deficit irrigation. The stomatal conductance, relative water content and leaf water potential parameters were used to confirm the degree of crop stress. Leaf dry weight and root volume were higher in the grafted plants under the control irrigation and stress treatment conditions. Total fresh root biomass and root volume percentage of grafted plants under water stress were 24% and 33% higher, respectively, than the ungrafted plants. The grafted plants subjected to both water stress and control conditions had a higher marketable production than the ungrafted plants. The higher yields obtained using tolerant rootstocks were explained by the reduced blossom-end rot incidence.

Keywords:
Capsicum annuum, grafting, production,
root density, blossom-end rot

5.2. Introduction

Water scarcity for agricultural production remains a challenge, one aggravated by the continuous increase in today's agricultural demands and climate change considerations. If we consider the fact that agriculture represents almost 70% of all water extractions, and up to 95% in some developing countries [1], it is necessary to contemplate farming production alternatives to drastically reduce water use in agriculture without negatively affecting yields or product quality.

In order to minimize irrigation water use and to increase its efficiency, some woody crops have been successfully grown in recent decades by implementing the deficit irrigation (DI) technique [2]. However, herbaceous crops' sensitivity to water stress is much greater, and the success of this technique depends on species to a great extent. One of the reasons explaining this phenomenon is the poor root depth growth capacity of some horticultural crops under DI conditions, owing partly to greater root biomass accumulation with effective water/nutrient uptake on the surface soil horizon [3]. With peppers, most of the studied cases that have applied the controlled DI technique do not report the production levels obtained under normal irrigation conditions. However, differences in yield have been observed depending on the time when the DI is applied, the stress severity and the duration [4,5,6].

Water stress imposed in some critical pepper growth stages, mainly flowering and fruit set, can have long-term effects that may make completely recovered yield impossible [7]. In addition, water stress could also lead to higher incidence of blossom-end rot (BER) in pepper crops.

Grafts on water stress–tolerant rootstocks can confer drought tolerance up to a certain point and has been proven in cucumber [8], eggplant [8], tomato [9] and watermelon [10]. In peppers, different studies confirm the existence of rootstocks capable of developing physiological mechanisms that confer water stress tolerance [11,12]. In a study that used the water stress–tolerant hybrid rootstock NIBER® and irrigated at 50% of crop evapotranspiration (ETc) [13], this hybrid increased plants' water use efficiency, accounted for by the grafted plants' higher dry biomass and commercial yields compared with ungrafted plants. All this may be attributed to greater root development. Nonetheless, these studies did not investigate the effect of the root system's exploring capacity on the greater adaptation of the water stress–tolerant rootstock. To do so, and under more controlled conditions than those indicated in the previous work [13], the present study centers on determining up to what point a water stress–tolerant rootstock is able to maintain its production capacity under stress conditions caused by DI and to analyze, in turn, the capacity of root exploration, volume and weight to relate it to the differences in the yields or fruit quality that might be found.

5.3. Materials and methods

5.3.1. Growth Conditions

The experiment was carried out from February to June 2022 in the Venlo-type greenhouses belonging to the Universitat Politècnica de València, Spain. Growth took place in 25-L cylindrical containers using washed silica sand of distinct granulometry, with a 10% thickness index and a 2.28 uniformity coefficient [1].

Containers were drip-irrigated using three 4 L/h Netafim® (Netafim Ltd., Tel Aviv, Israel) anti-drain drippers per container with a nutritional solution containing (in mmol/L): 14.0 NO_3^-; 1.5 $H_2PO_4^-$; 2.4 SO_4^{2-}; 0.5 HCO_3^-; 1.6 Cl^-; 1.2 NH_4^+; 6.0 K^+; 5.0 Ca^{2+}; 2.5 Mg^{2+}; 0.2 Na^+; (in µmol L^{-1}): 15 Fe^{3+}; 6 Zn^{2+}; 12 Mn^{2+}; 30 B^{3+}; 0.8 Cu^{2+} and 0.5 Mo^{6+}. The electrical conductivity (EC) and pH of this nutritional solution were respectively 2.2 dS m^{-1} and 6.5. The values of the temperature, relative humidity and accumulated solar radiation are shown in Figure S1.

5.3.2. Plant Material

The traditional 'Cuerno' pepper cultivar was used and grafted onto rootstock F1 NIBER® (Universitat Politècnica de València and Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain) (GRA) or ungrafted (UGR). Seeding took place on 2 December 2021, on 104-cell polystyrene trays on a peat-based substrate (70% white peat; 30% black peat). Grafting was done on 24 December using the tube method [14]. Transplanting was performed on 1 February 2022. The experiment finished on 11 June 2022.

5.3.3. Irrigation Management and Control

Volumetric soil water content (VSWC; $m^3\ m^{-3}$) was continuously monitored by TEROS 10 capacitance sensors connected to a ZL6 data logger using the ZENTRA Cloud (METER Group AG, München, Germany). Two sensors per container were placed at 15-cm and 25-cm soil depths, equidistant between two adjacent emitters. The sensor located at the 25-cm soil depth was used to monitor the water drained below the root zone. VSWC was measured and stored every 15 min, and its variation was employed to determine in situ field capacity (FC) (defined as the amount of water held in soil after excess water has drained away and the rate at which the downward water movement materially decreases [2], which coincided with VSWC when its change came close to zero over time [3]). Irrigation was managed based on the VSWC, expressed as the percentage of FC to reduce sensor calibration importance. Irrigation management consisted of maintaining VSWC at 90% and 50% of FC in the control (CON) and deficit irrigation (DI) treatments, respectively, by varying the number of daily irrigations based on accumulated solar radiation, and by also ensuring 20% drainage to avoid salinity problems. VSWC was measured (in triplicate) in each of the four combinations.

The water stress treatment began on 5 April 2022, when the flowers of third nodes were at anthesis, and continued until the end of the experiment. Before the stress treatment commenced, all the fruits set were eliminated, and all the plants were irrigated in the same way as under the CON conditions.

5.3.4. Physiological Parameters

Stomatal conductance, relative water content and the leaf water potential were measured to confirm the plant water stress level. Stomatal conductance (gs, mol H_2O m^{-2} s^{-1}) was determined as reported in [13] in fully extended leaves (3rd and 4th leaves from the apex) 31, 45 and 59 days after DI treatment (DAT) started between 12:00 h and 14:00 h. For this purpose, the “LI-COR 600” porometer (LI-COR, Nebraska, St, Lincoln, NE, USA) was used. Relative water content (RWC) was measured 28, 42 and 56 DAT in leaves and was determined by weighing leaves before and after a 24-h rehydration process for which distilled water was employed to respectively obtain fresh weight (FW) and turgent weight (TW). To obtain dry weight (DW), leaves were dried at 65 °C for 72 h before being weighed. RWC was determined as RWC (%) = (FW − DW)/(TW − DW) × 100 [5]. The predawn water potential (05:00 h to 06:00 h) and the midday water potential (13:00 h to 14:00 h) ($\Psi_{predawn}$ and Ψ_{leaf}, respectively) were determined, following the methodology applied in [13] using a Schölander-type pressure chamber (model 3000; Soil Moisture Equipment Corp., Santa Bárbara, CA, USA). Determinations were made on fully extended leaves of an identical physiological status as that employed for the previous measurements and after 36, 50 and 63 DAT.

5.3.5. Production Parameters

Marketable and non-marketable yields, and both fruit weight and number, were determined. Non-marketable yield consisted of fruit affected by BER. Harvesting was spread out between early May and early June.

5.3.6. Biomass Parameters

The aerial and root biomass parameters were measured at the end of the experiment (67 DAT).

Fresh leaves and stems were weighed before being exposed to dry heat (for 72 h at 70 °C) in a laboratory oven. Then, dry weight (DW) was recorded.

Root biomass and root volume were measured by dividing the substrate into three vertical layers (corresponding to the 0–10, 10–20 and 20–30 cm depths), obtaining the roots from each layer and carefully washing them with distilled water. Root volume (mL) was obtained through the displaced water volume. Finally, the fresh weight (FW) and the DW of roots were obtained in the same way as used for the aerial part.

5.3.7. Experimental Design and Statistical Analysis

The experiment consisted of a two-factor randomized block design, where factors were water stress (WS) with two levels CON and DI, and grafting (G) with two levels UGR and GRA, formed by four repetitions (*n* = 16) of five plants. For all the parameters, measurements were taken in all the plants to obtain the mean of the five measurements per repetition. The results obtained for the different parameters were evaluated by an analysis of variance (ANOVA) using the Statgraphics Centurion XVII software (Statgraphics Technologies Inc., Virginia, USA). Means were compared by Fisher's least significant difference (LSD) test at $p \leq 0.05$.

5.4. Results

5.4.1. Irrigation Management

With the irrigation strategies applied, the average 100% volumetric water content of the substrate was maintained in CON, which was 60% in the DI strategy, compared to FC (Figure 1). The quantity of water used to irrigate the CON plants was 57% higher than that administered to the DI plants (CON: 256.2 L/plant; DI: 145.8 L/plant).

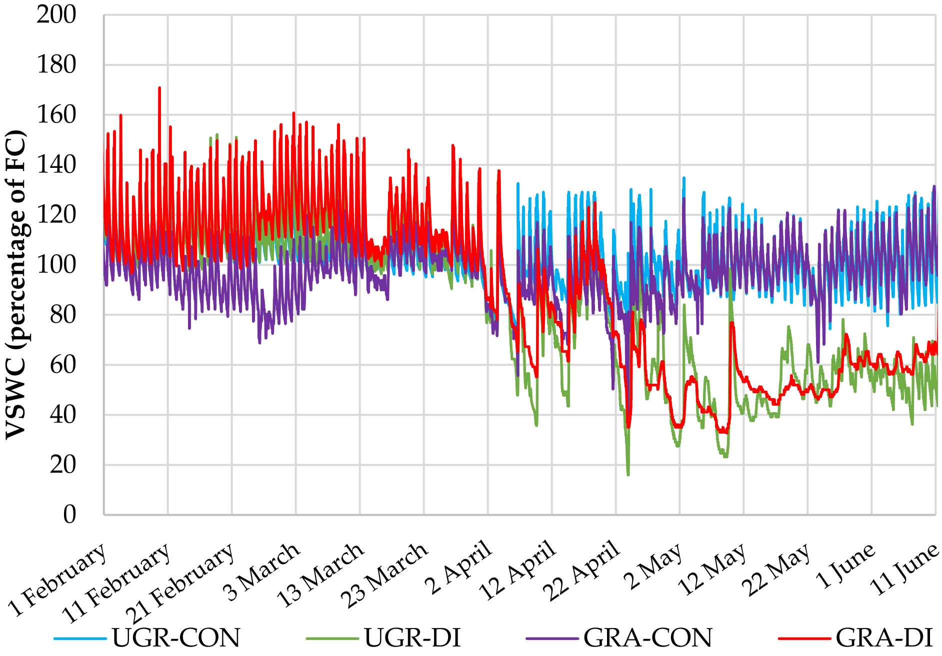

Figure 1. Volumetric soil water content (VSWC) expressed as a percentage of field capacity (FC) for the substrate corresponding to the grafting factor, grafted (GRA) and ungrafted (UGR), and for both water stress levels: control (CON) and deficit irrigation (DI).

5.4.2. Physiological Parameters

No significant interactions between the two factors (G × WS) were detected (data not shown) for both gs and RWC parameters. No significant differences for the grafting factor were noted. However, for the water stress effect, the stomatal conductance parameter and RWC (except for 28 DAT) were higher in the plants under the CON conditions than those under the DI conditions, as shown in Figure 2.

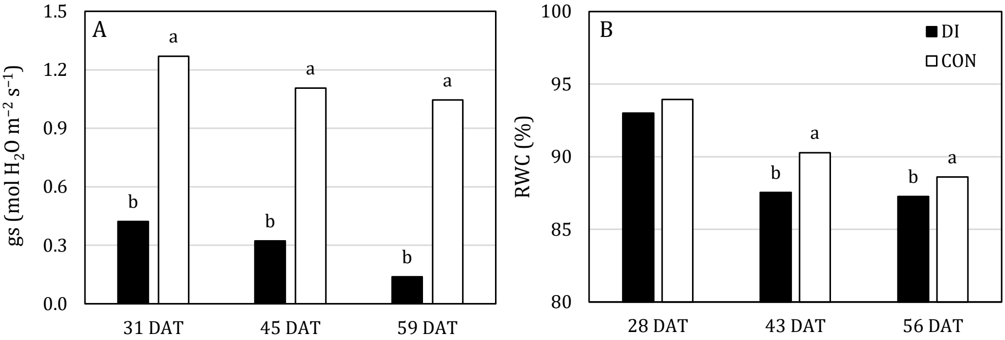

Figure 2. Stomatal conductance (gs) (**A**) and relative water content (RWC) (**B**) for both water stress levels: control (CON) and deficit irrigation (DI). Measurements were taken on 31, 45, and 59 DAT for gs and 28, 43 and 56 DAT for RWC. Different letters indicate significant differences at $p \leq 0.05$ (Fisher's LSD test). Data are the mean of grafted and ungrafted plants (four replicates each).

For both $\Psi_{predawn}$ and Ψ_{leaf} (Table 1), the results indicated significant differences in WS at all the sampling times (except for $\Psi_{predawn}$ at 36 DAT). The plants under the DI conditions had more negative values than those under the CON conditions. Moreover, significant differences appeared among the grafting factor for $\Psi_{predawn}$ at sampling times of 36 and 50 DAT, and for Ψ_{leaf} on 36 DAT, when more negative water potential values were obtained in the UGR plants (Table 1). No significant interactions appeared between factors.

Table 1. Effect of grafting and water stress on the predawn leaf water potential ($\Psi_{predawn}$) and the midday leaf water potential (Ψ_{leaf}). Measurements were taken on 36, 50, and 63 days after treatment (DAT).

	Leaf water potential (MPa)					
	36 DAT		50 DAT		63 DAT	
	$\Psi_{predawn}$	Ψ_{leaf}	$\Psi_{predawn}$	Ψ_{leaf}	$\Psi_{predawn}$	Ψ_{leaf}
Grafting (G)						
UGR	-0.426 a	-1.618 a	-0.398 a	-1.410	-0.531	-1.139
GRA	-0.348 b	-1.463 b	-0.333 b	-1.317	-0.493	-1.205
Water stress (WS)						
DI	-0.405	-1.639 a	-0.406 a	-1.444 a	-0.696 a	-1.276 a
CON	-0.369	-1.441 b	-0.324 b	-1.283 b	-0.328 b	-1.068 b
G ^{x} WS						
UGR-DI	-0.453	-1.698	-0.438	-1.479	-0.710	-1.233
UGR-CON	-0.400	-1.538	-0.358	-1.342	-0.353	-1.045
GRA-DI	-0.358	-1.580	-0.375	-1.408	-0.683	-1.320
GRA-CON	-0.338	-1.345	-0.290	-1.225	-0.303	-1.090

ANOVA (df)	% Sum of squares											
G (1)	46.50	**	24.34	**	18.99	*	10.69	n.s	0.89	n.s.	5.95	n.s
WS (1)	9.85	n.s	39.52	**	30.59	*	31.29	*	80.96	**	59.12	**
G ^{x} WS (1)	1.98	n.s	1.42	n.s	0.03	n.s	0.64	n.s	0.08	n.s	0.61	n.s
Residuals (12)	41.66		34.71		50.39		57.39		18.07		34.31	
Std. Dev. $^{(+)}$	0.043		0.107		0.061		0.125		0.101		0.092	

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * denote significance at P ≤ 0.01 and P ≤ 0.05, respectively. n.s. no significant difference. (+) Standard deviation, calculated as the square root of the residual mean square. df degrees of freedom.

5.4.3. Production Parameters

For marketable yield (Table 2), significant differences appeared in WS ($p < 0.01$), and the worst results were obtained for the plants under the DI conditions, with a lower fruit number, lighter weight per plant and lighter weight per fruit (Table 2).

For both fruit number and weight per plant, differences were found in grafting factor ($p < 0.01$), with higher GRA values on average for the two WS levels (Table 2).

For the percentage of fruit with BER, significant differences were obtained for both WS levels ($p < 0.01$), and the percentage was higher for DI. On average, for both WS levels, the GRA plants had a lower BER percentage and a lower fruit number with BER than the UGR plants (Table 2).

Table 2. Effect of grafting and water stress on marketable yield (fruit/plant, kg/plant and g/fruit) and blossom end rot (BER) (fruit/plant and %).

	Marketable yield			BER	
	(fruit/plant)	(kg/plant)	(g/fruit)	(fruit/plant)	(%)
Grafting (G)					
UGR	21.00 b	1.550 b	71.87	21.50 a	54.59 a
GRA	29.00 a	2.152 a	72.83	13.00 b	34.81 b
Water stress (WS)					
DI	11.25 b	0.779 b	69.26 b	19.75	62.09 a
CON	38.75 a	2.923 a	75.44 a	14.75	27.31 b
G^{x}WS					
UGR-DI	8.25	0.555	68.28	25.25	74.93
UGR-CON	33.75	2.544	75.47	17.75	34.25
GRA-DI	14.25	1.003	70.24	14.25	49.26
GRA-CON	43.75	3.301	75.41	11.75	20.36

ANOVA (df)	% Sum of squares				
G (1)	7.10 **	6.65 **	1.14 n.s	29.64 *	17.96 **
WS (1)	83.89 **	84.18 **	47.75 **	10.26 n.s	55.57 **
G^{x}WS (1)	0.44 n.s	0.44 n.s	1.29 n.s	2.56 n.s	1.59 n.s
Residuals (12)	8.57	8.73	49.83	57.54	24.88
Std. Dev. $^{(+)}$	5.07	0.398	3.65	6.84	13.44

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * denote significance at P ≤ 0.01 and P ≤ 0.05, respectively. n.s. no significant difference. (+) Standard deviation, calculated as the square root of the residual mean square. df degrees of freedom.

5.4.4. Biomass Parameters

For both fresh and dry biomasses of stems and leaves, significant differences in WS were found, and DI had a lower biomass. For the aerial part, it was only for the dry biomass in leaves where significant differences were observed in G, and the DW for the GRA plants was heavier than UGR ones (Table 3).

The GRA plants had higher fresh root biomass, DW biomass, and root volume quantities than the ungrafted plants, on average, for WS ($p < 0.01$). On average, for G, significant differences in WS appeared, with higher (fresh and dry) biomass results, and also for root volume in the plants under the CON conditions (Table 3). For fresh and dry biomasses, significant differences were observed in the interaction, and the GRA-CON plants obtained the highest result. Regarding fresh root biomass, under DI conditions, the plants grafted onto NIBER® developed a higher fresh biomass than the ungrafted ones, and similar GRA-DI values to UGR-CON were obtained for fresh and dry biomass (Table 3).

Table 3. Effect of grafting and water stress on the fresh weight (FW) and dry weight (DW) of leaves, stems and roots, and roots volume.

	Leaves		Stems		Roots		
	FW (g)	DW (g)	FW (g)	DW (g)	FW (g)	DW (g)	Volume (mL)
Grafting (G)							
UGR	766.5	112.3 b	859.9	173.9	305.0 b	33.26 b	394.5 b
GRA	715.9	119.7 a	786.8	181.6	393.2 a	40.58 a	517.8 a
Water stress (WS)							
DI	588.5 b	91.5 b	663.8 b	146.8 b	296.2 b	32.15 b	389.3 b
CON	893.9 a	140.4 a	983.0 a	208.7 a	402.0 a	41.69 a	523.1 a
G×WS							
UGR-DI	660.9	88.4	658.9	147.3	264.4 c	30.96 b	334.0
UGR-CON	872.2	136.2	1061	200.6	345.6 b	35.56 b	455.0
GRA-DI	516.2	94.7	668.7	146.4	328.0 b	33.34 b	444.5
GRA-CON	915.6	144.6	905.0	216.9	458.4 a	47.83 a	591.1
ANOVA (df)	% Sum of squares						
G (1)	1.22 n.s	2.17 **	1.56 n.s	1.33 n.s	36.72 **	25.56 **	37.69 **
WS (1)	44.48 **	95.82 **	29.69 *	85.83 **	52.92 **	43.41 **	44.37 **
G×WS (1)	4.22 n.s	0.04 n.s	2.00 n.s	1.68 n.s	2.85 *	11.64 *	0.41 n.s.
Residuals (12)	50.07	1.96	66.75	11.16	7.51	19.39	17.53
Std. Dev. $^{(+)}$	187.06	4.04	276.35	12.89	23.02	3.68	48.56

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * denote significance at P ≤ 0.01 and P ≤ 0.05, respectively. n.s. no significant difference. (+) Standard deviation, calculated as the square root of the residual mean square. df degrees of freedom.

Table 4 displays the spatial distribution of the dry biomass for roots per WS level and employed plant combination. Significantly greater root accumulation took place in the first 10 cm for all the studied combinations. It is worth noting that in these first 10 cm, the plants grafted onto NIBER® under the DI conditions had a higher biomass and bigger volume than the ungrafted plants under normal conditions.

Table 4. Distribution of dry biomass and root volume for grafting (GRA and UGR) and water stress factors (CON and DI).

	DW (g)			Volume (mL)		
	0-10 cm	10-20 cm	20-30 cm	0-10 cm	10-20 cm	20-30 cm
Grafting (G)						
UGR	21.81 b	5.565	5.892 a	226.1 b	69.19	99.23 a
GRA	31.70 a	4.820	4.067 b	373.8 a	72.31	71.71 b
Water stress (WS)						
DI	23.41 b	4.615 b	4.123 b	265.4 b	57.68 b	66.18 b
CON	30.09 a	5.769 a	5.835 a	334.5 a	83.83 a	104.76 a
G×WS						
UGR-DI	21.22 b	5.132	4.609	211.2	55.85	66.95 b
UGR-CON	22.39 b	5.997	7.175	240.9	82.53	131.50 a
GRA-DI	25.61 b	4.099	3.637	319.6	59.50	65.40 b
GRA-CON	37.79 a	5.541	4.496	428.0	85.13	78.03 b
ANOVA (df)	% Sum of squares					
G (1)	44.12 **	11.80 n.s.	33.10 **	62.64 **	0.74 n.s.	20.11 **
WS (1)	20.10 **	28.28 *	29.14 **	13.68 *	52.09 **	39.56 **
G×WS (1)	13.67 *	1.77 n.s	7.24 n.s	4.44 n.s	0.02 n.s.	17.91 **
Residuals (12)	22.11	58.15	30.52	19.24	47.14	22.42
Std. Dev. (+)	4.04	0.955	1.012	47.3	14.36	16.77

The mean values followed by the different lowercase letters in each column indicate significant differences at P ≤ 0.05 using the LSD test. ** and * denote significance at P ≤ 0.01 and P ≤ 0.05, respectively. n.s. no significant difference. (+) Standard deviation, calculated as the square root of the residual mean square. df degrees of freedom.

5.5. Discussion

The plants grown under the DI conditions certainly faced a stress situation, as demonstrated, on one hand, by the follow-up of the substrate's volumetric water content to adjust it to target levels, where the water stress applied in this experiment was maintained for a long enough period of time for plants to display the consequences of such stress. On the other hand, this was demonstrated by the measured physiological parameters, the stomatal conductance, the leaf water potential and the RWC, which are well known to be affected by drought stress [15,16]. The differences in RWC in leaves were the first evidence toward this conclusion, because higher RWC was observed in the leaves of the properly irrigated plants than in those under the DI condition. RWC in leaves is a measurement of plants' water status in relation to their water content. It partly estimates the degree of water stress [17]. In addition, the results of this experiment also showed that the plants that faced stress obtained lower stomatal conductance values for all the taken measurements. Leaf water potential, both at predawn and midday, were also clearly lower in the plants subjected to the deficit irrigation treatment.

The agronomic results obtained in this experiment demonstrated that grafting the traditional cv. Cuerno onto the tolerant NIBER® rootstock increased yield under both the CON and DI conditions. Under the DI conditions, however, the NIBER® rootstock was not equal to the ungrafted plants under the CON conditions because the generated stress was likely important and grossly reduced yield, which was more marked than that observed in previous experiments using the same rootstock conducted in soil conditions [13].

It can be stated that the degree of water stress to which the crop was submitted in order to maintain the 60% volumetric content in FC terms led to soil moisture that was too low for DI irrigation under these conditions because, when comparing the obtained yield between the two treatments, the CON plants obtained a 73% higher yield than the DI plants. Notwithstanding this, the loss in yield was less than that obtained when grafting the cv. Cuerno onto the NIBER® rootstock, whose marketable fruit production grew for both the CON and DI situations compared to the ungrafted plant yield.

Indeed, grafted plants subjected to water stress are capable of generating a 1.8-fold higher marketable mean yield compared to the production of ungrafted plants in the same situation. In CON situations, the grafted plants increased production by 23% compared to the ungrafted plants.

The differential behavior in yield between the two plant combinations could be associated with a robust root system in plants using NIBER® as rootstock. In fact, the whole biomass and root volume were higher in the plants grafted onto NIBER® than in the ungrafted ones under both optimal and stress irrigation conditions. These results are a consequence of the higher percentages of NIBER roots in the 0–10 cm layer, which normally sees a higher accumulation of pepper roots in high-frequency irrigation, as has been observed by [18] in drip-irrigated pepper and tomato crops; in our experiment, this accounted for 72%, on average, DW for both DI and CON conditions. Nevertheless, the total fresh biomass for roots in the grafted plants under stress conditions was 23% higher than in the ungrafted ones, and this percentage was higher (26%) for the root volume.

The higher root biomass and volume could be explained by the direct effect of a greater photosynthesis capacity in rootstocks tolerant to abiotic stresses. This has been frequently reported elsewhere as associated with a strong root system, which contributes to a higher water and nutrient uptake [11,12,14,19,20].

In any case, the better marketable yields noted when NIBER® was used as rootstock can be explained by lower BER incidence and, therefore, a greater marketable fruit number, and not by heavier mean fruit weight, as this parameter barely presented any variation between grafted and ungrafted plants. The lower BER incidence would have given way to better marketable fruit yields, which were similar in this case. From our experiment, the results showed that the BER percentage in the grafted plants was 36% lower than in the ungrafted plants.

This disorder has been related by some authors with high oxidative processes caused by the production of reactive oxygen species (ROS) [21]. Nevertheless, other authors related this disorder to scarce Ca translocation to organs and tissues with low or null transpiration, which results in this element being scarcely available locally [22].

In previous experiments, we found a better antioxidant capacity of NIBER®, which could explain the present and past observations of its BER tolerance [23]. On the other hand, some authors have stated that the rootstock can enable more water and ions to be transported to aerial parts, as observed by Roufhael et al. [24] in grafted mini-watermelon crops and Lee et al. [8] in different horticultural crops. Kyriacou et al. [25] suggested that lower BER incidence in pepper, observed in distal fruit zones, can be attributed to the improvement made by the rootstock in nutrient uptake/transport terms, Ca in this case, to apical fruit cells toward greater cell wall integrity. In our case, the obtained predawn water potential values would agree with the milder BER effects when using the NIBER® rootstock, because less-negative potentials denote higher water content in the grafted plants under both the DI and CON conditions. This demonstrates that the nighttime root pressure in the plants grafted onto NIBER® is higher and thus facilitates non-transpiring organs and fruit to acquire Ca [26,27]. This fact could be justified by the higher root volume found in the grafted plants, which would permit higher water uptake and intake through root pressure for the same soil water potentials [28].

5.6. Conclusions

To conclude, the DI in which water stress-tolerant rootstocks like NIBER® are employed can be considered helpful for lowering water use by cushioning effects on yield. However, more studies should be conducted to better adjust the irrigation reduction to values better tolerated by pepper plants. Even under extreme conditions like those herein applied, crop yield increased under both the regular irrigation and water stress conditions because of reduced BER incidence, and this could be due to the consequence of obtaining higher root mass and volume in the shallower root layer.

5.7. Supplementary materials

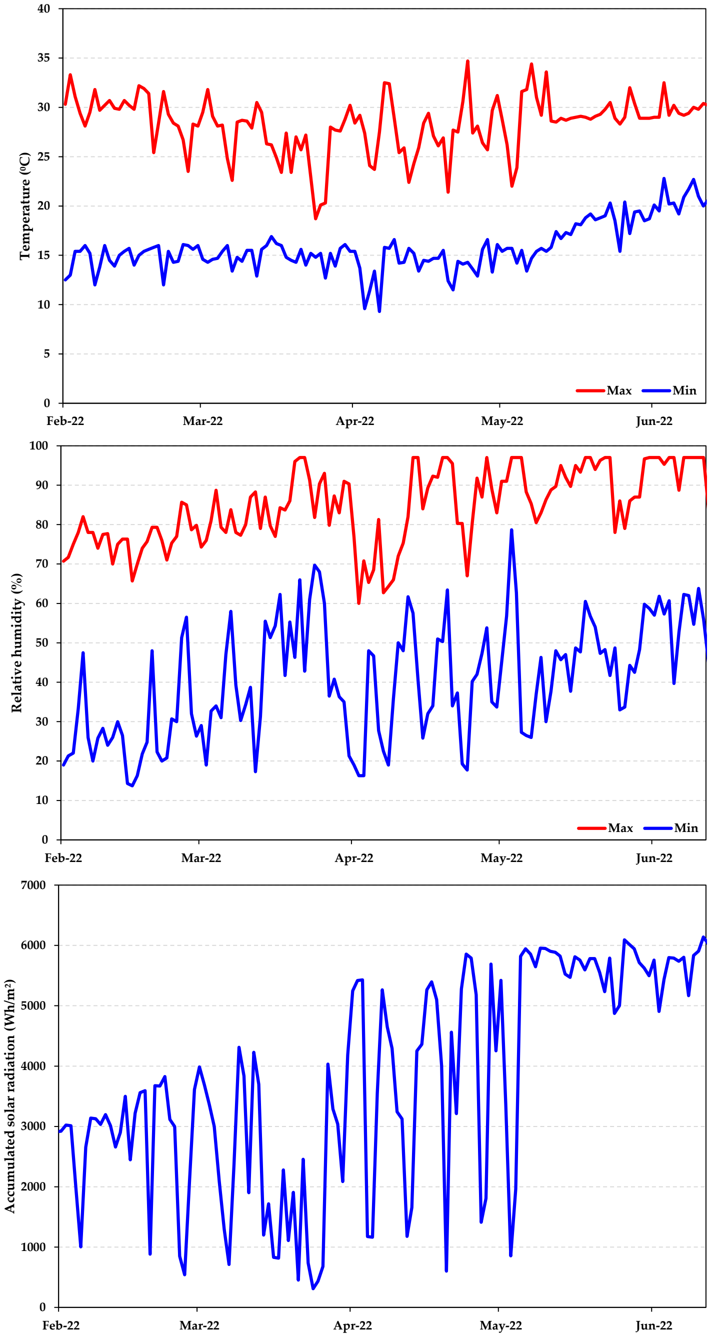

Figure 1. Temperature (°C), relative humidity (%) and accumulated solar radiation (Wh/m^2) values.

5.8. References

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Capítulo 6

Effect of Grafting on the Production, Physico-Chemical Characteristics and Nutritional Quality of Fruit from Pepper Landraces

Ramón Gisbert-Mullor [1], Costanza Ceccanti [2], Yaiza Gara Padilla [3], Salvador López-Galarza [1], Ángeles Calatayud [3], Giuseppe Conte [2,4] and Lucia Guidi [2,4]

[1] Departamento de Producción Vegetal, CVER, Universitat Politècnica de València, Camí de Vera s/n, 46022 Valencia, Spain
[2] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
[3] Instituto Valenciano de Investigaciones Agrarias, Centro de Citricultura y Producción Vegetal, Departamento de Horticultura, CV-315, Km 10,7, Moncada, 46113 Valencia, Spain
[4] Interdepartmental Research Center Nutrafood "Nutraceuticals and Food for Health", University of Pisa, 56124 Pisa, Italy

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6.1. Abstract

Grafting is a widely utilized agronomical technique to improve yield, disease resistance, and quality of fruit and vegetables. This work aims to assess the effect of grafting and fruit ripening on the production, physico-chemical characteristics, and nutritional quality of fruit from Spanish local pepper landraces. Landraces "Cuerno," "Sueca," and "Valencia" were used as scions, and "NIBER®" as the rootstock. Two ripening stages of the fruits were sampled: green and red. Grafting improved the yield and marketable quality and did not negatively influence the physico-chemical and nutritional characteristics of the fruit. It was noteworthy that the bioactive compound contents and antioxidant capacity were more related to maturity stage and genotype, and red fruit had a higher antioxidant capacity than green fruit. However, in all the scions, grafting significantly enhanced lycopene content in both red and green fruit. Another important effect of grafting was the volatile compound composition evidenced by discriminant analyses, which was characterized for the first time in the fruit of these landraces. The rootstock and scion combination could be a way to improve not only the production, but also the fruit quality of peppers.

Keywords:
Antioxidant activity; ascorbic acid; *Capsicum annuum*; carotenoids; lycopene; phenolics; scion; VOCs

6.2. Introduction

Sweet pepper (*Capsicum annuum* L.) is one of the most important vegetable crops grown in the world that covers 1.99 million hectares (ha) of crop-growing surface area [1]. It is of great cultural and economic importance because of its multiples uses and phenotypic diversity [2,3].

In the past century, modern breeding developed good-performance hybrid cultivars that are normally more productive, more resistant to disease and pests, and more uniform in germination, growth, and highest vigor terms [4,5]. This resulted in genetic erosion, the declining heterogeneity of the organoleptic characteristics of pepper fruits, and the replacement of using local pepper varieties [6,7]. Spain, similar to other countries in the Mediterranean Region, is characterized by the considerable versatility of agro-climatic regions, which promotes a wide range of different phenotypes [8]. Thus, Spain is considered a secondary center of diversity for peppers, especially for *C. annuum,* which was brought mainly from Mexico immediately after the discovery of America [9].

In addition, the genetic uniformity of cultivated pepper landraces enhances the incidence of biotic and abiotic stresses [6,8,10], with lower yields that contrast with the high yield needs to cover increased demand. From this point of view, grafting may provide an eco-friendly technology to increase yields in landraces and reinforce tolerance to biotic and abiotic stresses [10]. Based on its vigor, the rootstock has been utilized in both open field and protected cultivations [10], and grafting is used mainly with the Cucurbitaceae and Solanaceae families, which encompass the most important crops such as tomato, eggplant, cucumber, watermelon, melon, and pepper [11]. However, among these vegetables, grafting is a less common practice in peppers, probably because commercial rootstocks provide modest profits [12,13]. Several scientific reports have reported the effects of rootstock on pepper fruit quality, such as morphometric and yield characteristics [14,15], sweetness/acidity [16,17], or levels of functional compounds [18,19]. Another peculiarity of pepper is its aroma [20], which has become a quality parameter for consumers [21,22].

Evidently, quality is a set of attributes that determine consumer choice, and can be divided into the external fruit aspect, and also into nutritional and nutraceutical characteristics. These last properties have become increasingly important for consumers given the positive relation between fruits and vegetables and human health [23,24]. Many studies report how a healthy diet rich in fruit and vegetables (functional food) can help to delay senescence processes and also reduce the risk of pathologies including cancer and cardiovascular diseases [23]. The characteristic of functional food is its richness in antioxidant compounds and that it is capable of scavenging reactive oxygen species (ROS) that are the basis of cellular oxidative stress [25]. For this reason, consumers today demand fruits and vegetables with higher sensory and nutraceutical values that are sometimes more characteristic in landraces [26].

Sweet pepper fruit falls into the category of functional foods because it is rich not only in ascorbic acid, carotene, and phenols, but also capsaicinoids, xanthophylls, and flavonoids. It is thus characterized by its high antioxidant capacity [27,28]. The amount of those phytochemicals in peppers depends on many factors, including rootstock. However, opposite results about the influence of grafting on the amount of phytochemicals in pepper can be found in the literature [10,19,29,30].

To the best of our knowledge, no studies on the role and response of grafting on pepper landraces in fruit quality and productivity terms have been conducted. In this context, the present work focuses on the effect of grafting on pepper landraces through the characterization of the fruit productivity and quality of three typical sweet pepper landraces from Valencia (Spain) and by considering the effect of the fruit maturity stage as these landraces are consumed in both green and red fruit, depending on the final destination of production. To determine fruit quality, not only physico-chemical characteristics (dry matter, titratable acidity, color, and volatiles), but also nutraceutical aspects (phenols, vitamin C, pigments, and antioxidant capacity) were assayed to determine the "whole" fruit quality. The rootstock we utilized was "NIBER®," an F1 hybrid that we obtained in a classic breeding program. It has been demonstrated as being tolerant to abiotic stress [31] with higher yields (range of 32–80%) compared to ungrafted plants or other commercial pepper rootstocks [32] tested in Mediterranean conditions. As scions, we utilized "Cuerno," "Sueca," and "Valencia," three representative landraces of pepper morphologies in the Mediterranean Region from the Instituto Valenciano de Investigaciones Agrarias' (IVIA, Spain) traditional germplasm collection.

6.3. Materials and methods

6.3.1. Plant Material

Three pepper landraces from Valencia (Spain), namely “Cuerno,” “Valencia,” and “Sueca,” were grafted onto rootstock F1 NIBER®, obtained from a collaboration agreement reached between the Universitat Politècnica de València (UPV, Spain) and the IVIA. The ungrafted “Cuerno” (CU), “Valencia” (VU), and “Sueca” (SU) plants were used as the control plants. These landraces were selected based on fruit morphology representing the three main typologies in landraces pepper shape in Valencia fruits from “Cuerno” are elongated with very slightly marked shoulders; “Valencia” fruits are blocky with four shoulders, “Sueca” fruits are triangular shaped with three locules.

Seeds were sown on March 20, 2019, in 104-hole seed trays filled with enriched substrate for germination. After 2 months of sowing, plants were grafted by the tube-grafting method [33]. Two weeks after grafting, seedlings were transplanted in the field. The fruit from the ungrafted “Cuerno” plants (CU), “Valencia” (VU), “Sueca” plants (SU), and from the plants grafted onto NIBER, “Cuerno” (CG), “Valencia” (VG), and “Sueca” (SG) were used.

6.3.2. Soil-Field Experiment

The experiment was conducted from June to September in Moncada (Valencia, Spain; Latitude: 39.58951793357715, Longitude: −0.3955507278442383) in the IVIA’s experimental field. Soil was sandy clay loam (clay: 21.2%; silt: 11.8%; sand: 67%). Organic matter was 0.61%, pH 7.8, at 25 °C and EC 1:5 at 25 °C: 0.289 dS m^{-1}.

Plants were grown in the open air in single rows placed 150 cm apart with 40 cm between each plant. The experiment was laid out according to a complete randomized block design with three replicates. Each replicate consisted in 15 plants. Plant irrigation met 100% crop evapotranspiration (ETc), as described in Penella et al. [33] by means of a drip system. Nutrients were applied through the irrigation system at a rate (kg ha^{-1}) of 200 N, 50 P_2O_5, 250 K_2O, 110 CaO, and 35 MgO, as recommended by Maroto [34].

The average range of minimum and maximum temperatures during the experiment was 15–28 °C for June, 19–32 °C for July, 19–32 °C for August and 18–29 °C for September [35].

6.3.3. Fruit Yield and Quality Assessment

Fruits were harvested from 15 plants per replication from the end of July to mid-September in relation to fruit maturation. Quality commercial production was evaluated according to commercial practices, as were the measured fruits with physiological disorders, mainly blossom-end rot (BER).

Nine randomized fruits (3 per replication of plant material) were selected in July (green fruits) and mid-September (red fruits) to measure fruit quality, which included: physico-chemical characteristics (percentage dry weight, pulp thickness, color index determination, titratable acidity, volatile compounds) and nutraceutical characteristics (total phenol, lycopene, total chlorophyll, carotenoids and ascorbic acid content, antioxidant capacity).

6.3.4. Fruit Dry Material and Pulp Thickness

To determine the percentage of dry weight in green and red fruit, the fresh weight of samples (FW) was recorded. Dry weight (DW) was established after drying samples at 60 °C for 72 h in a laboratory oven. The percentage of DW (% DW) was defined as: (DW/FW) × 100. Pulp thickness was measured on three sides of each fruit in the equatorial area.

6.3.5. Fruit Color Index Determination

For the color index determination, the L (0–100, black to white), a (±red/green) and b (±yellow/blue) Hunter parameters of the color system were measured by a chromameter (Konica Minolta CM-700d). The color index was determined after making three determinations around the equatorial plane of fruit. Color intensity (Chroma) and hue angle (H) were calculated by the following equations: Chroma = $((a)^2 + (b)^2)^{1/2}$ and Hue angle = tg^{-1} (b/a) [36].

6.3.6. Titratable Acidity

Titratable acidity (TA) was determined by potentiometric titration with 0.1 M NaOH (Merck Co.) up to pH 8.1 using 10 mL of juice. Citric acid (Merck Co.) was used as a reference for the TA (% citric acid) calculations. The pHs of fruit juice were determined with a pH meter (HANNA HI 2212).

6.3.7. Total Phenolic Analysis and Antioxidant Capacity Measurements

The phenolic content was analyzed according to Dewanto et al. [37]. Briefly, 1 g of each sample was homogenized with a mortar in 4.0 mL 80% (v/v) methanol and centrifuged at 10,000 *g* for 15 min at 4 °C. Total phenolic content was determined by the Folin-Ciocalteau colorimetric method based on the procedure of Singleton and Rossi [38]. A 10 µL aliquot of the supernatant was mixed with 115 µL of distilled water, 125 µL of Folin-Ciocalteau reagent (Merck Co., Kenilworth, NJ, USA), and 1.25 mL of $NaHCO_3$ (7%) (Sigma-Aldrich, Co., St. Louis, MO, USA). The absorption (Abs) of the solution was measured at 760 nm in a spectrophotometer (Ultrospec 2100 Pro, GE Healthcare Ltd., Chalfont St Giles, Buckinghamshire, UK). Each measurement was compared with a standard curve of gallic acid (Sigma-Aldrich, Co., St. Louis, MO, USA) and total phenols were expressed as mg gallic acid equivalent (GA) g^{-1} FW, on the basis of a standard calibration curve ($Abs_{760} = 0.01146 \mu g\ mL^{-1} - 0.01515$; $R^2 = 0.932$).

Antioxidant capacity was measured with the method reported by Brand–Williams et al. [39]. Briefly, 10 µL of phenolic extract were added to 990 µL of a solution containing 3.12×10^{-5} M of 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, Co., St. Louis, MO, USA) in methanol (99.9% purity). The drop in absorbance at 515 nm was measured against a blank solution (with no extract) after a 30-min reaction time at room temperature (optimized for the highest antioxidant concentrations in the extract) in a spectrophotometer (Ultrospec 2100 Pro). Each measurement was compared with a standard curve of trolox (Sigma-Aldrich, Co., St. Louis, MO, USA) solution and the results were expressed as mg trolox equivalent (TE) g^{-1} FW, on the basis of a standard calibration curve ($Abs_{515} = 3.5887$ µg $mL^{-1} + 0.1412$; $R^2 = 0.984$).

6.3.8. Ascorbic Acid Concentration

Ascorbic acid content was spectrophotometrically determined as described by Kampfenkel et al. [40]. Briefly, 0.3 g of each sample was homogenized in 1 mL 6% trichloroacetic acid (TCA, Sigma-Aldrich, Co., St. Louis, MO, USA) and centrifuged at 10,000 *g* for 10 min. The supernatant was immediately used to analyze ascorbate. The absorption of the solution was measured at 525 nm in a spectrophotometer (Ultrospec 2100 Pro). Ascorbic acid was expressed as mg g^{-1} FW, on the basis of a standard calibration curve ($Abs_{525} = 0.07889$ µg $mL^{-1} + 0.0097$; $R^2 = 0.994$).

6.3.9. Chlorophyll and Carotenoids Concentration

Chlorophyll (Chl) (a and b) and carotenoid (Car) content were determined spectrophotometrically as described by Porra et al. [41]. Briefly, 0.3 g of each sample was added to 1.5 mL acetone 80% (v/v) (Scharlab Co.) and centrifuged at 7000 *g* for 10 min. The supernatant was used for the analysis. The absorption of the solution was measured at 663 nm, 648 nm, and 470 nm in a spectrophotometer (Ultrospec 2100 Pro). Chlorophyll (a and b) and carotenoid content of the extracts was calculated by the following equations:

$$\text{Chl } a = 12.25 \times Abs_{663} - 2.55 \times Abs_{648}\ (\mu g\ mL^{-1}), \quad (1)$$

$$\text{Chl } b = 20.31 \times Abs_{648} - 4.91 \times Abs_{663}\ (\mu g\ mL^{-1}), \quad (2)$$

$$\text{Car} = [(1000 \times Abs_{470} - 1.82 \times \text{Chl } a) - (85.02 \times \text{Chl } b)]/198\ (\mu g\ mL^{-1}). \quad (3)$$

Chlorophylls and carotenoids were expressed as µg g^{-1} FW.

6.3.10. Lycopene Concentration

The lycopene in pepper fruit was extracted using a hexane:ethanol:acetone (2:1:1; v:v:v) (Sigma-Aldrich Co., St. Louis, MO, USA) mixture following the method of Adejo et al. [42]. A powdered sample (0.001 g) was dissolved in 1 mL of distilled water and vortexed in a water bath at 30 °C for 1 h. Then 8.0 mL of hexane, ethanol, and acetone were added, capped, and vortexed again, followed by incubation in a dark cupboard for 60 min. Next, 1 mL of distilled water was added to each sample and revortexed before being left to stand and separate into phases. Care was taken to ensure that any formed bubbles had completely disappeared. The cuvette was rinsed with the upper layer of one of the blank samples before using more fresh blank samples to zero the spectrophotometer at 503 nm. The absorbance of the upper layers of the lycopene samples were read at the same wavelength of 503 nm in a spectrophotometer (Ultrospec 2100 Pro). The lycopene content of extracts was expressed as mg g^{-1} FW. Lycopene levels of the extracts were then calculated using:

$$\text{Lycopene (mg kg}^{-1}\text{ fresh wt)} = (A_{537} \times 8 \times 0.55)/(0.10 \times 172)$$

where 537 g $mole^{-1}$ is the molecular weight of lycopene, 8 mL is the volume of mixed solvent, 0.55 is the volume ratio of the upper layer to the mixed solvents, 0.10 g is the weight of pepper fruit added, and 172 mM^{-1} is the extinction coefficient for lycopene in hexane.

6.3.11. Volatiles Organic Compound Analysis

The volatile organic compounds (VOCs) were determined by solid-phase microextration-gas chromatography-mass-spectrometry (SPME-GC/MS) [43]. VOCs were extracted from 2 g of finely minced fruit samples and put inside a 20-mL glass vial, closed with an aluminum cap provided with PTFE-septum. Samples were conditioned at 60 °C for 10 min. VOCs were collected using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS) Stable Flex SPME fiber(50/30 µm; 2-cm long)/Supelco, Bellefonte, PA, USA). The SPME fiber was exposed to headspace for 30 min in a water bath at 60 °C. Before each analysis, the SPME fiber was conditioned for 30 min at 270 °C in the GC injector. The fiber was inserted into the injector of a single quadrupole GC/MS (TRAcE GC/MSm Thermo-Finningan, Waltham, MA, USA) set at 250 °C for 3 min in the splitless mode to keep the fiber in the injector for 30 min to achieve complete fiber desorption. The GC program conditions were the same as those reported by Povolo et al. [44]. GC was coupled with a Varian CP-WAX-52 capillary column (60 m × 0.32 mm; coating thickness 0.5 µm). The transfer-line and ion source were both set at 250 °C. The filament emission current was 70 eV. A mass range from 35 to 270 m/z was scanned at a rate of 1.6 amu/s. Acquisition was carried out by electron impact in the Full Scan (TIC) mode, and three replicates were run per fruit sample. VOCs were identified in three different ways: comparing with the mass spectra of the Wiley library (version 11/2008); injecting the authentic standard (2-methyl-3-heptanone); calculating LRI and matching with the reported indices [44,45]. Data were expressed as the relative abundance (%) of total VOCs.

6.3.12. Statistical Analysis

The results for all parameters, except VOCs, were subjected to a two-way ANOVA analysis using Statgraphics Centurion XVII (Statistical Graphics Corporation 2014) with treatment. Landraces were employed as factors of the analyses. Each ripening (green and red fruit) was separately analyzed. The percentage data were arcsin-transformed before analyzing. The least significant difference (LSD) at a 0.05-probability level was used as the mean separation test.

For VOCs, the statistical analysis was performed by the JMP software (SAS Institute Inc., Cary, NC, USA). The determination of ripening and the landrace effect on VOC composition was made by the following linear model:

$$y_{ijk} = \mu + R_i + V_j + R_i \times V_j e_{ijk},$$

where y_{ijz} = dependent variables; R_i = fixed effect of the *i*th ripening level (green; red); V_j = fixed effect of the *j*th pen (2 levels); ε_{ijk} = random residual.

To comply with ANOVA assumptions, data were tested for normality by the Anderson–Darling test ($p < 0.05$).

Multiple comparisons among treatments were performed by Fisher's least significant difference (LSD) at $p \leq 0.05$.

A multivariate statistical analysis was performed to analyze the VOCs profile by three complementary techniques to discriminate six groups (CG, VG, SG, CU, VU, SU): stepwise discriminant analysis (SDA); canonical discriminant analysis (CDA); discriminant analysis (DA) [46]. Analyses were run separately for the green and red fruit because the significant difference in the VOC profile between both two ripening levels would have made the grafting effect less evident.

The minimum number of VOCs able to discriminate the six groups was obtained by the SDA, a statistical technique specifically considered to select the number of variables that better separate groups. Subsequently, CDA derives a set of new variables, called canonical functions (CAN), which are linear combinations of the original interval variables, as reported in the following equation:

$$CAN = d_1X_1 + d_2X_2 + ... + d_nX_n,$$

where d_i are the canonical coefficients (CC) that indicate the contribution of each variable in composing CAN, and X_i are the scores of the n original variables.

CAN summarizes between-groups variation by highlighting their differences. In general, if k groups are involved in the study, k−1 CAN is extracted. Efficient separation between groups was calculated by the Mahalanobis distance and the corresponding

Hotelling's T-square test [47]. The Hotelling's T-square test extends the Student's *t*-test to the multivariate domain [48].

The ability of CAN to assign each sample to the six groups was calculated as the percent of correct assignment using DA [49]. The centroids of the six groups are calculated and, for each sample, the distances from the six centroids are evaluated. One sample is assigned to one of the six groups based on the shortest distance from the six groups' centroids [49].

6.4. Results

6.4.1. Fruit Yield

Grafting had a significant effect on total and marketable yields compared to the values recorded in the ungrafted plants for all landraces (Figure 1A). The higher fruit yield was attributable to a significant increase in the number of fruits per plant, which significantly increased because of the grafting on all the landraces (Figure 1B). In addition, grafting decreased the amount of BER fruit (Figure 1C), parameters for which the grafting and landrace interaction was also significant. Non-marketable yield decreased in all the grafted landraces compared to the ungrafted landraces (−27% in “Cuerno,” −61% in “Sueca,” and −46% in “Valencia”—data not shown), as did the yield of the BER fruit (−28% in “Cuerno,” −71% in both “Sueca” and “Valencia”).

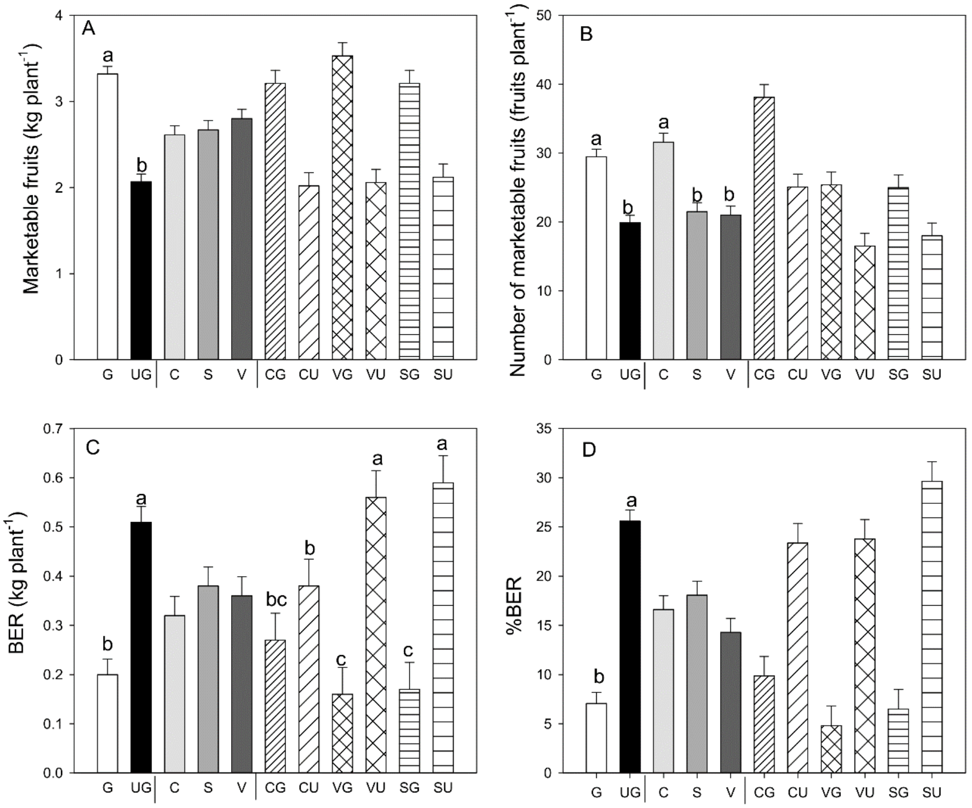

Figure 1. Marketable fruit yield (A), number of marketable fruits (B), production (C) and percentage (D) of fruit affected by blossom-end rot (BER) in the three pepper landraces “Cuerno” (C), “Valencia” (V), and “Sueca” (S) grafted (G) or ungrafted (U) on rootstock F1 NIBER®. Values are the mean ± SE of 15 plants per replicate (3 replicates) per landrace. Means were subjected to a two-way ANOVA with grafting and landrace as sources of variability. Different letters for the factors grafting and landrace, or their interaction, indicate significant differences at $p < 0.05$ using the LSD test. No letter indicates the non-significance of the *F* ratio.

6.4.2. Fruit Physico-Chemical Characteristics

The percentage of DW was not affected by either grafted fruits /or landraces (Table 1). No differences in the grafting technique were found for the parameters H and C of the green fruit, whereas the H parameter significantly changed in relation to grafting or landrace in the red fruit, with no interaction between the two variability factors (Table 1). In particular, the H parameter was higher in the red fruit from the ungrafted plants and was significantly higher in the fruit from Valencia vs. “Cuerno” and “Sueca.” Moreover, no significant interaction between grafting and landrace was found in the red fruit for these parameters.

As a general rule, grafted plants produce green and red fruit with significantly higher titratable acidity than ungrafted plants (Figure 2A,B). Titratable acidity was similar in the green fruit produced by the three landraces, while this parameter was significantly higher in the red fruit produced by “Valencia” and “Sueca” compared to “Cuerno.” No interaction was found for both the green and red fruit between grafting and genotype (Figure 2A,B). It was noteworthy that titratable acidity was higher in the red fruit than in the green fruit, and pH values were lower ($p \leq 0.05$; Student’s t-test). In the green fruit, pH values were lower in those produced by the grafted than the ungrafted plants (Figure 2C), and significant differences appeared among landraces. In the green fruit produced by the “Sueca” landrace, pH values were lower than those from “Cuerno” and “Valencia.” In the red fruit, lower pH values were recorded in the fruits from “Valencia” (Figure 2C,D). For pH values, a significant grafting and landrace interaction was found only for the green fruit. The lowest pH value was detected in the green fruit from the grafted “Valencia” plants, while the higher value went to the fruits from the same ungrafted landrace and the ungrafted “Cuerno” landrace (Figure 2C).

Table 1. Dry weight and color indices in the green and red fruits produced by the three pepper landraces “Cuerno” (C), “Valencia” (V), and “Sueca” (S) grafted (G) or ungrafted (U) on rootstock F1 NIBER®. Values are the mean of three fruits per replicate (3 replicates) per landrace. Means were subjected to a two-way ANOVA with grafting and landrace as sources of variability. Different letters for the factors grafting and landrace, or their interaction, indicate significant differences at $p < 0.05$ using the LSD test. No letter indicates the non-significance of the *F* ratio. ns: $p > 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$.

	Dry weight (%)				**Hue angle (H)**				**Chroma (C)**			
	Green		**Red**		**Green**		**Red**		**Green**		**Red**	
Grafting (G)												
Grafted (G)	6.45		9.12	a	110.9		31.3	b	21.7		32.0	
Ungrafted (U)	6.31		8.58	b	111.7		33.9	a	21.9		30.0	
Landrace (L)												
“Cuerno” C)	6.46		8.17	b	112.1		30.0	b	22.7		32.1	
“Sueca” (S)	6.44		9.97	a	110.4		30.4	b	22.0		29.8	
“Valencia” (V)	6.24		8.41	b	111.5		37.4	a	20.8		31.1	
G*L												
CG	6.66		8.09	d	111.1		30.3		22.7		31.2	
SG	6.43		10.4	a	110.0		29.2		21.0		29.0	
VG	6.26		8.89	c	111.7		34.3		21.4		35.8	
CU	6.26		8.26	d	113.2		29.8		22.6		33.0	
SU	6.45		9.55	b	110.7		31.6		23.0		30.6	
VU	6.22		7.92	d	111.3		40.5		20.2		26.5	
ANOVA (*df*)	% Sum of the squares											
G (1)	3.59	n.s.	8.76	**	4.80	n.s.	9.42	*	0.11	n.s.	5.00	n.s.
L (2)	7.03	n.s.	74.9	**	16.2	n.s.	59.9	**	6.63	n.s.	4.29	n.s.
G*L (2)	6.60	n.s.	7.57	*	7.83	n.s.	9.65	n.s.	5.03	n.s.	33.0	n.s.
Residuals (12)	82.8		8.76		71.2		21.0		88.2		57.8	
SD. (+)	0.41		0.33		1.87		2.46		3.42		4.21	

(+) Calculated as the square root of the residual sum of squares. *df*: degrees of freedom; SD: standard deviation.

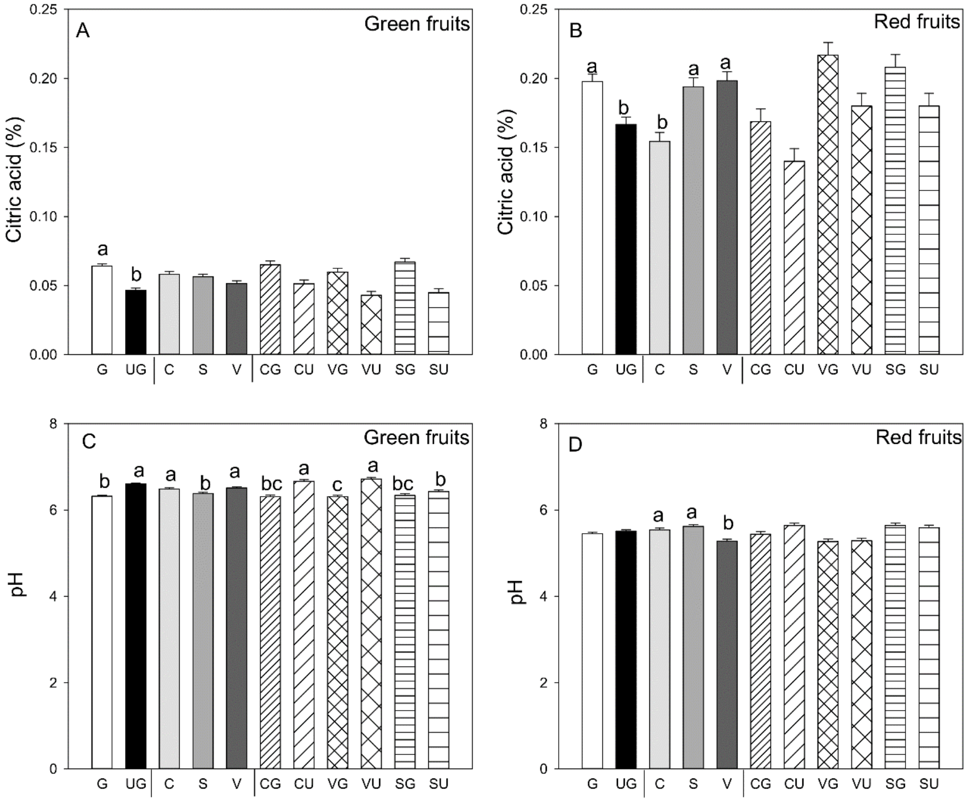

Figure 2. Titratable acidity (A,B) and pH values of pepper juice (C,D) in the green (A,C) and red (B,D) fruit produced by pepper landraces "Cuerno" (C), "Valencia" (V), and "Sueca" (S) grafted (G) or ungrafted (U) onto rootstock F1 NIBER®. Values are the mean ± SE of three fruits per replicate (3 replicates) per landrace. Means were subjected to a two-way ANOVA with grafting and landrace as sources of variability. Different letters for the factors grafting and landrace, or their interaction, indicate significant differences at $p < 0.05$ using the LSD test. No letter indicates the non-significance of the *F* ratio.

6.4.3. Nutraceutical Compounds and Antioxidant Capacity

Among the phytochemical compounds found in pepper fruit, phenols are of particular interest for their ability to scavenge free radicals. Independently of grafting and landrace, phenols concentration was significantly higher in the red than the green fruit ($p \leq 0.05$; Student's t-test) and it changed differently in both red and green fruit (Figure 3A). Indeed, grafting had a negative effect on phenols concentration in both colored fruit. In the green ones, and among landraces, those produced by "Valencia" had the highest value of these compounds (Figure 3B). In the red fruit, the landrace factor was not significant (Figure 3). However, the grafting and landrace interaction was significant and evidenced some differences: higher phenols concentration values for the fruit from all the ungrafted landraces and the grafted "Valencia" (Figure 3B).

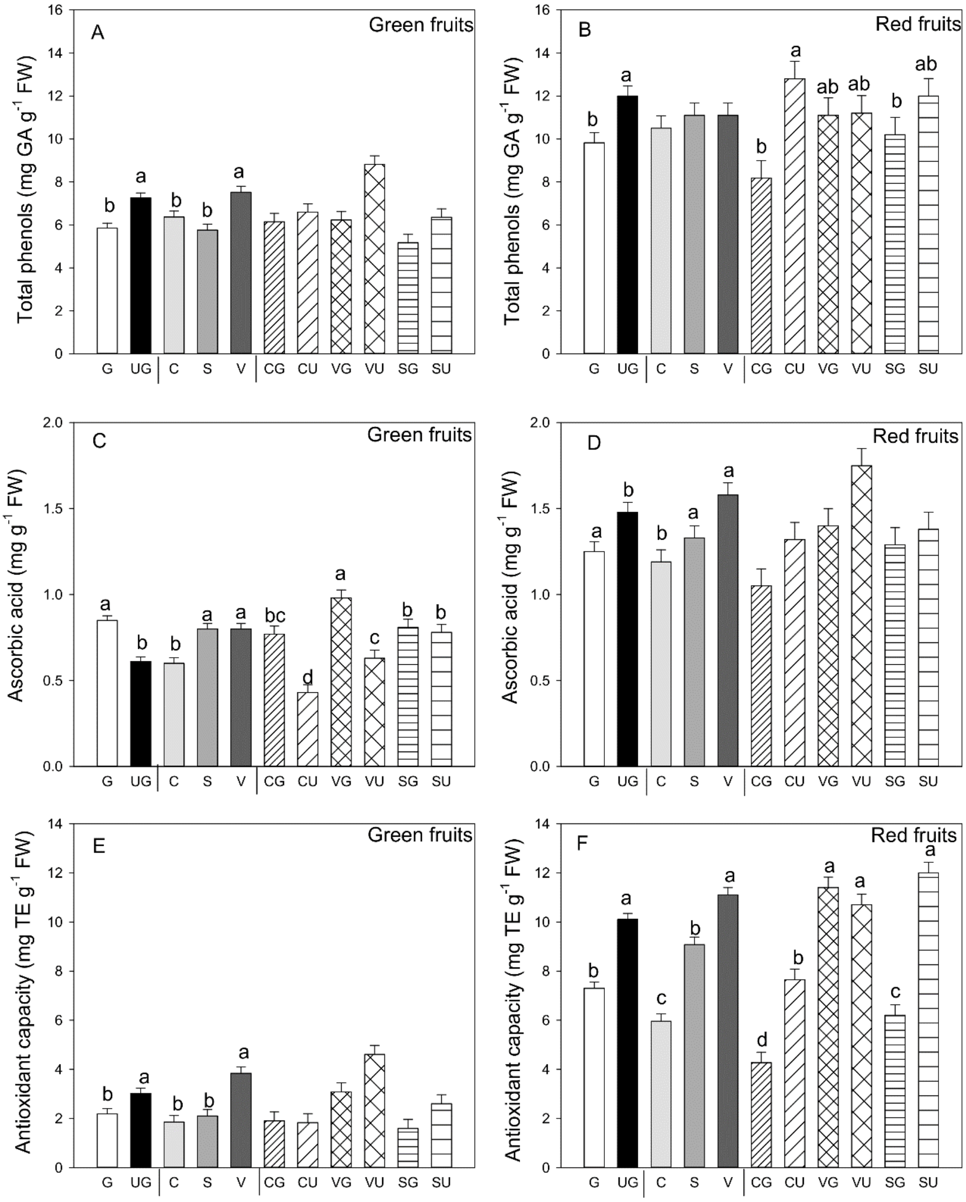

Figure 3. Total phenols (A,B), ascorbic acid amount (C,D) and antioxidant capacity (E,F) in the green (A,C,E) and red (B,D,F) fruit produced by the three pepper landraces "Cuerno" (C), "Valencia" (V), and "Sueca" (S) grafted (G) or ungrafted (U) on rootstock F1 NIBER®. Values are the mean ± SE of three fruits per replicate (3 replicates) per landrace. Means were subjected to a two-way ANOVA with grafting and landrace as sources of variability. Different letters for factors grafting and landrace, or their interaction, indicate significant differences at $p < 0.05$ using the LSD test. No letter indicates the non-significance of the *F* ratio.

Figure 3B,D show the ascorbic acid content in both the green and red fruit of the grafted and ungrafted pepper landraces. In the green fruit, grafting and landrace, and their interaction, were significant. Even for this antioxidant metabolite, it was evident in the green fruit that grafting enhanced content, which was not observed in the red fruit (Figure 3C,D). In both green and red fruit, ascorbic acid content was higher in the fruits of “Sueca” and “Valencia” and lower in those of “Cuerno.” For the green fruit, the landrace that produced fruit with the highest ascorbic acid content was the grafted “Valencia,” landrace, in which grafting enhanced ascorbic acid content, and also in the other landraces (Figure 3C).

Conversely, grafting negatively impacted the ascorbic acid content in the red fruit. Between landraces “Sueca” and “Valencia,” once again fruits were produced with higher ascorbic acid content (Figure 3D). No significant interaction between variability factors was found for ascorbic acid in the red fruit.

Antioxidant capacity was determined by the DPPH assay and was higher in the red fruit compared to the green ones ($p \leq 0.001$, Student’s *t*-test). Grafting generally reduced this parameter in both colored fruit (Figure 3E,F). The highest antioxidant capacity values were found in both the green (3.84 mg TE g^{-1} FW) and red (11.1 mg TE g^{-1} FW) fruit produced by the “Valencia” plants, followed by “Sueca,” whereas the lowest antioxidant capacity was recorded in fruit from “Cuerno.” A significant grafting and landrace interaction was observed only for the red fruit, which became evident as the highest antioxidant capacity was reported in the fruits produced by grafted and ungrafted “Valencia” plants, and by the ungrafted “Sueca” plants (Figure 3F). The lowest antioxidant capacity values were detected in the fruits from the grafted “Cuerno” plants.

The most evident result was the lower Chl a and b content in the red vs. the green fruit ($p \leq 0.001$; Student’s *t*-test) (Figure 4A,B). In the green fruit, this concentration was not influenced by grafting, but was strongly impacted by landrace, with the fruit from “Cuerno” presenting the highest content, followed by “Valencia” and finally by “Sueca.” The interaction between the two variability factors was evidenced as the highest Chl a and b content was detected in the ungrafted “Cuerno” fruit, followed by the grafted “Cuerno,” “Valencia,” and ungrafted “Valencia” (Figure 4A). In the red fruit, no variability factor induced significant differences in Chl a and b content (Figure 4B). Not even did carotenoid content change in the green and red fruit because of grafting, but marked differences were found among landraces: the green fruits produced by “Cuerno” and “Valencia” had a higher carotenoid content, with both “Cuerno” together with “Sueca” in the red fruit (Figure 4C,D).

As expected, the lycopene concentration was higher in the red versus the green fruit. The mean value, independently of grafting and genotype, was 2.78 mg g^{-1} FW compared to one of 16.39 mg g^{-1} FW in the red fruit ($p \leq 0.001$, Student’s *t*-test; Figure

4E,F). Although in lycopene content the green fruit was low, significant differences were found for grafting and landrace, but not for their interaction (Figure 4E). Grafting generally improved lycopene content (4.3- and 1.4-fold higher in the green and red fruit from the grafted vs. ungrafted plants, respectively), and the landraces that produced the fruit with the highest lycopene content in both fruits was grafted “Cuerno” (Figure 4E,F). For the red fruit, the grafting and landrace interaction was significant. The analysis underlined that grafted “Cuerno” produced the red fruit with the highest lycopene content, followed by grafted “Sueca” (Figure 4F). both grafted and ungrafted “Valencia” were the landraces that produced red fruit with the lowest lycopene content.

To understand the contribution of the different phytochemicals in both colored peppers, a correlation analysis was carried out between the different compounds and antioxidant capacity. In the green fruit from the ungrafted or grafted plants, a positive correlation appeared only between antioxidant capacity and phenols, even when the grafting technique was not considered (Table 2). The correlation found for the red fruit between phenols and antioxidant capacity was significant, but only for the grafted plants. For the red fruit, the correlation was significant when grafting was not considered. Chlorophyll and carotenoid content did not correlate with antioxidant capacity in the green and red fruit, whereas lycopene content correlated inversely with antioxidant capacity in the green fruit when grafting was not contemplated, and in the red fruit (Table 2). The correlations were also determined for each landrace, independently of the grafting technique, in both colored peppers (Table 3). In the “Cuerno” fruit, a significant correlation was found only in the red fruit between phenols and antioxidant capacity. Once again chlorophyll and carotenoid contents in the green and red fruit did not correlate with antioxidant capacity in any landraces, whereas lycopene was negatively related.

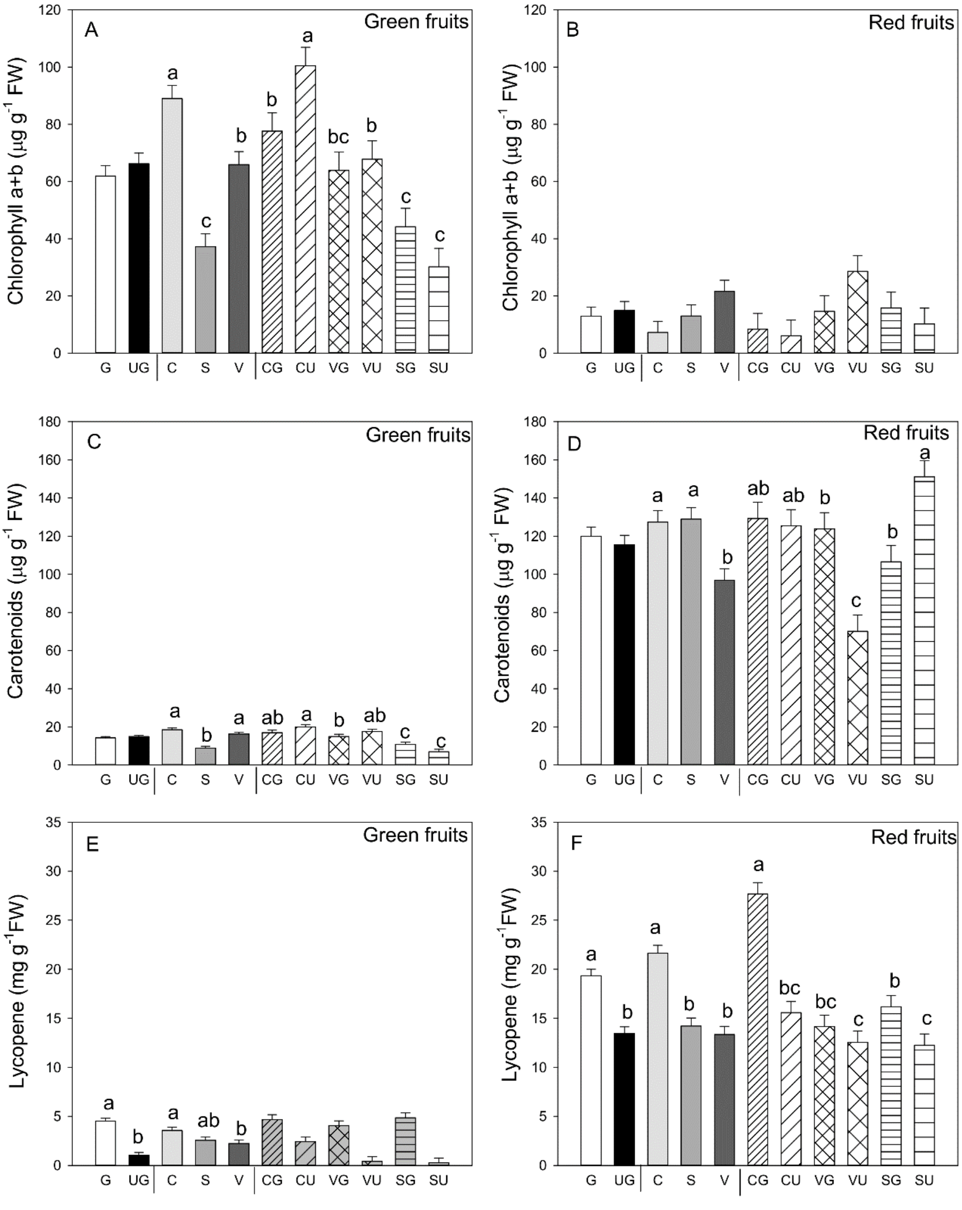

Figure 4. Chlorophyll a and b (A,B), carotenoid (C,D) and lycopene amount (E,F) in the green (A,C,E) and red (B,D,F) fruit produced by the three pepper landraces “Cuerno” (C), “Valencia” (V), and “Sueca” (S) grafted (G) or ungrafted (U) onto rootstock F1 NIBER®. Values were the mean ± SE of three fruit per replicate (3 replicates) per landrace. Means were subjected to a two-way ANOVA with grafting and landrace as sources of variability. Different letters for factors grafting and landrace, or their interaction, indicate significant differences at $p < 0.05$ using the LSD test. No letter indicates the non-significance of the *F* ratio.

Table 2. Correlation coefficients and their significance determined between each phytochemical and antioxidant capacity produced by the green and red fruit produced by the grafted (G) or ungrafted (UG) landraces on rootstock F1 NIBER®. Correlation coefficient r is reported when the correlation was significant. ns: $p > 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Phytochemicals	**GREEN UG PLANTS**	**GREEN G PLANTS**	**GREEN FRUITS**	**RED UG PLANTS**	**RED G PLANTS**	**RED FRUITS**
Phenols	** r = 0.85	** r = 0.77	*** r = 0.83	ns	* r = 0.71	* r = 0.53
Total chlorophylls	ns	ns	ns	ns	ns	ns
Carotenoids	ns	ns	ns	ns	ns	ns
Lycopene	ns	ns	* r = −0.57	* r = 0.68	** r = −0.78	*** r = 0.81
Ascorbic acid	ns	ns	ns	ns	* r = 0.65	** r = 0.56

Table 3. Correlation coefficients and their significance determined between each phytochemical and antioxidant capacity produced by the green and red fruit produced by the three landraces. Correlation coefficient r is reported when the correlation was significant. ns: $p > 0.05$; *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Phytochemicals	**GREEN CUERNO**	**GREEN VALENCIA**	**GREEN SUECA**	**RED CUERNO**	**RED VALENCIA**	**RED SUECA**
Phenols	ns	ns	*** r = 0.96	** r = 0.91	ns	ns
Total chlorophylls	ns	ns	ns	ns	ns	ns
Carotenoids	ns	** r = 0.85	ns	ns	ns	** r = 0.85
Lycopene	ns	* r = −0.83	ns	** r = 0.97	ns	ns
Ascorbic acid	ns	ns	ns	ns	ns	ns

6.4.4. Volatile Compounds

The pepper volatile profile was characterized by 51 compounds, identified in line with both mass spectra and linear retention index (Table 4). VOCs were classified according to their corresponding chemical class and relative distributions and were included in the following nine groups: organic acids, alcohols, aldehydes, alkanes, ketones, terpenes, esters, aromatic hydrocarbons, and furans. The nine compounds belonging to the different and fewer representative classes were grouped as “miscellaneous components.” The largest portion of VOCs present in peppers was represented by terpenes (42%), followed by aldehydes (16%), esters (11%), and alcohols (6%). Ripening level was the factor that most influenced the VOC profile. As reported in Table 3, most VOCs were significantly higher in the green fruit, except for hexanoic acid, 2-ethylhexanoic acid, decanoic acid, n-dodecane, 3-methyltridecane, 4-methyl 2-pentanone, cis-tagetone, beta-trans-ocimene, acetic acid ethyl ester, n octhyl formate, o-xylene, mesithylene, N-methylpyrrole or 1-methylpyrrole and carbon disulfide, which showed a non-significant difference. On the contrary, only a few VOCs showed significant differences in the three landraces: “Cuerno” had a higher level of ethanol and a lower level of β-linalool and 5-5 methyl 1,3 dithian-2-one, while “Valencia” had a lower level of nonenal, α-santalene, salycilic acid and ethyl ester, and higher 2-heptanone, cyclobutene, and furan 2,3-dihydro-4-methyl contents.

Table 4. Effect of ripening and landrace on the volatile profile of peppers.

	Ripening level		**SEM**	***P* value**	**Landrace**			**SEM**	***P* value**
	Green	**Red**			**“Cuerno”**	**“Sueca”**	**“Valencia”**		
acids									
hexanoic acid	0.40	0.23	0.09	ns	0.54	0.15	0.26	0.11	ns
2-ethylhexanoic acid	0.40	0.25	0.10	ns	0.46	0.12	0.37	0.12	ns
n-dodecanoic acid	1.33	0.59	0.18	**	1.34	0.79	0.74	0.22	ns
octanoic acid	1.17	0.54	0.12	***	0.97	0.66	0.95	0.14	ns
nonanoic acid	3.17	1.69	0.37	**	2.99	1.58	2.71	0.45	ns
decanoic acid	0.23	0.21	0.07	ns	0.22	0.14	0.30	0.09	ns
alcohols									
ethanol	2.43	0.31	0.32	***	2.64^{A}	1.03^{B}	0.43^{B}	0.39	**
2-octen-1-ol (E)	6.10	0.41	0.48	***	3.02	3.38	3.37	0.59	ns
cis -3 nonel -1- ol	7.42	0.08	1.44	***	3.73	6.09	1.43	1.76	ns
aldehydes									
acetic aldheyde	0.56	0.04	0.05	***	0.36	0.29	0.25	0.06	ns
pentanal	0.25	0.09	0.04	*	0.09	0.27	0.15	0.05	ns
hexanal	2.39	0.79	0.22	***	1.37	1.78	1.61	0.27	ns
(Z)-4-heptenal	2.20	0.28	0.17	***	1.06	1.32	1.35	0.21	ns
nonanal	2.15	0.59	0.19	***	1.76	1.15	1.19	0.24	ns
nonenal	24.30	0.72	4.41	***	18.05^{a}	17.47^{a}	2.01^{b}	5.40	*
nonadien 2-(trans)-6-(CIS)-al	7.84	2.09	1.27	**	6.41	5.14	3.34	1.56	ns
(2E,4E)-2,4-decadienal	1.49	0.07	0.12	***	0.83	0.76	0.75	0.15	ns
alkanes									
2-2, dimethyldecane	0.86	0.23	0.11	***	0.41	0.70	0.53	0.13	ns
n-dodecane	0.54	0.39	0.14	ns	0.28	0.54	0.57	0.17	ns
3-methyltridecane	0.13	0.01	0.06	ns	0.00	0.19	0.02	0.07	ns
1-cyclopropylpentane	1.18	0.27	0.11	***	0.71	0.72	0.76	0.13	ns
ketones									
1-penten-3-one	1.23	0.59	0.16	**	0.89	1.14	0.71	0.20	ns
4-methyl 2-pentanone	0.03	0.01	0.01	ns	0.01	0.00	0.05	0.02	ns
2-heptanone	0.70	0.37	0.10	*	0.31^{b}	0.50^{b}	0.80^{a}	0.12	*
cis-tagetone	3.23	0.23	2.00	ns	5.15	0.04	0.00	2.78	ns
5,5-dimethyl-1,3-dithian-2-one	6.70	2.21	0.68	**	1.52^{b}	5.66^{a}	6.20^{a}	0.83	***
terpenes									
2-propenyldiene-1-cyclobutene	0.04	0.79	0.11	***	0.07^{B}	0.45^{B}	0.73^{A}	0.14	**
3-carene	75.05	0.13	12.61	***	26.45	39.96	46.35	15.45	ns
beta-trans-ocimene	0.11	0.05	0.05	ns	0.05	0.12	0.07	0.06	ns
7-methyl-1-octene	0.60	0.09	0.07	***	0.44	0.28	0.31	0.09	ns
Allo-ocimene	1.66	0.03	0.28	***	0.55	0.92	1.06	0.34	ns
copaene	11.95	2.69	2.10	*	7.93	10.18	3.86	2.57	ns
cyclosativene	1.46	0.48	0.25	*	1.12	1.22	0.57	0.31	ns
β-linalool	5.70	1.50	0.62	***	1.44^{b}	3.79^{a}	5.55^{a}	0.76	**
α-santalene	0.62	0.05	0.11	***	0.57^{a}	0.35^{a}	0.09^{b}	0.13	*
α-Bergamotene	9.53	0.57	1.69	***	7.74	5.08	2.34	2.06	ns
β-farnesene	2.28	0.39	0.34	***	1.99	1.16	0.86	0.42	ns
esters									
	0.34	0.16	0.07	ns	0.40	0.20	0.14	0.09	ns
n-octyl formate	0.00	0.04	0.02	ns	0.05	0.00	0.01	0.03	ns
methyl salicylate	24.80	4.83	4.28	**	12.50	21.41	10.54	5.24	ns
ethyl salicylate	1.69	0.09	0.33	**	1.72^{a}	0.93^{a}	0.02^{b}	0.41	*
ethyl hexadecanoate	0.50	0.07	0.11	**	0.49	0.15	0.23	0.13	ns
aromatic hydrocarbons									
o-xylene	0.03	0.01	0.01	ns	0.01	0.02	0.03	0.01	ns
mesithylene	0.05	0.11	0.03	ns	0.05	0.13	0.06	0.04	ns
2,4,6-trimethylanisole	1.92	0.09	0.28	***	0.80	1.48	0.74	0.34	ns
furans	0.29	0.12	0.04	**	0.16	0.22	0.24	0.04	ns
furan,2,3-dihydro-4-methyl	0.70	0.37	0.10	*	0.31^{b}	0.50^{b}	0.80^{a}	0.12	*
2-pentylfuran	3.19	0.32	0.39	***	1.24	2.02	2.00	0.48	ns

	Ripening level		SEM	*P* value	Landrace			SEM	*P* value
	Green	Red			"Cuerno"	"Sueca"	"Valencia"		
miscellaneous component									
nitrogen oxide	1.00	0.34	0.14	**	0.51	0.69	0.81	0.17	ns
N-methylpyrrole	0.40	0.32	0.07	ns	0.38	0.41	0.29	0.09	ns
2-Isobutyl-3-methoxypyrazine	37.11	3.07	3.15	***	18.84	23.01	18.42	3.86	ns
carbon disulfide	0.41	0.42	0.12	ns	0.42	0.49	0.35	0.12	ns

^{a,b} Means with different letters within the same row are statistically different (*P* <0.05). ^{A,B} Means with different letters within the same row are statistically different (*P* <0.01). SEM = Standard error, * = 0.01 < *P*-value ≤ 0.05; ** = *P*-value ≤ 0.01; *** = *P*-value ≤ 0.001.

A multivariate discriminant analysis was performed to further investigate whether a VOC's signature discriminated the grafted from the ungrafted peppers. Of the 51 VOCs initially detected in peppers, and for both ripening levels, 10 VOCs were retained at the end of the SDA ($p < 0.001$): 4-methyl-2,3-dihydrofuran, allo-ocimene, nonanal, 2,4,6-trimethylanisole-, cyclosativene, methyl salicylate, octanoic acid, nonanoic acid, ethyl hexadecanoate for the green fruit and carbon disulfide, ethanol, pentanal, 2-propyldiene-1-cyclobutene, 2-heptanone, 7-methyl-1-octene, 2-Isobutyl-3-methoxypyrazine, beta-linalool, 5,5-dimethyl-1,3-dithian-2-one, salicylic acid methyl ester for the red fruit. The selected variables had a high discriminant power, with R^2 ranging from 0.57 to 0.30.

The CDA was applied to the selected variables which gave significant ($p < 0.01$) new variables called canonical variables (two canonical variables for the green fruit and one for the red fruit). For the green fruit, the first two variables accounted for 98% of total variability (Table 5), which thus indicates that the multivariate structure of the VOC could be well represented by only the first two canonical variables. This was confirmed by the scatter plot of canonical_1 × canonical_2 (Figure 5), which allowed the segregation of the six groups. Instead, for the red fruit, only the first canonical variable represented the multivariate structure by explaining 96% of variance (Table 5). Figure 6 demonstrates the discrimination effect of this canonical variable. For the green fruit, the canonical_1 variable markedly separated landraces. This separation was particularly evident for the grafted landraces ("Cuerno" scored higher than "Valencia," while "Sueca" was intermediate between both). The ungrafted ones were almost indistinguishable according to canonical_1, with a short, but significant, distance between them (Figure 5). The original variables, which accounted mostly for this discrimination, were allo-ocimene and cyclosativene (Table 5). The first was associated more with "Valencia", while the second one was associated with "Cuerno" as they showed a high negative and positive correlation with canonical_1. On the contrary, canonical_2 markedly separated the grafted plants from the ungrafted ones (Figure 6). The original variables that accounted mostly for this discrimination were4-methyl-2,3-dihydrofuran, N-methylpyrrole, allo-ocimene, 2,4,6-trimethylanisole, cyclosativene, methyl salicylate, octanoic acid and ethyl hexadecanoate (Table 5). All these VOCs correlated positively with canonical_2, which demonstrates a close relation with the grafted plants.

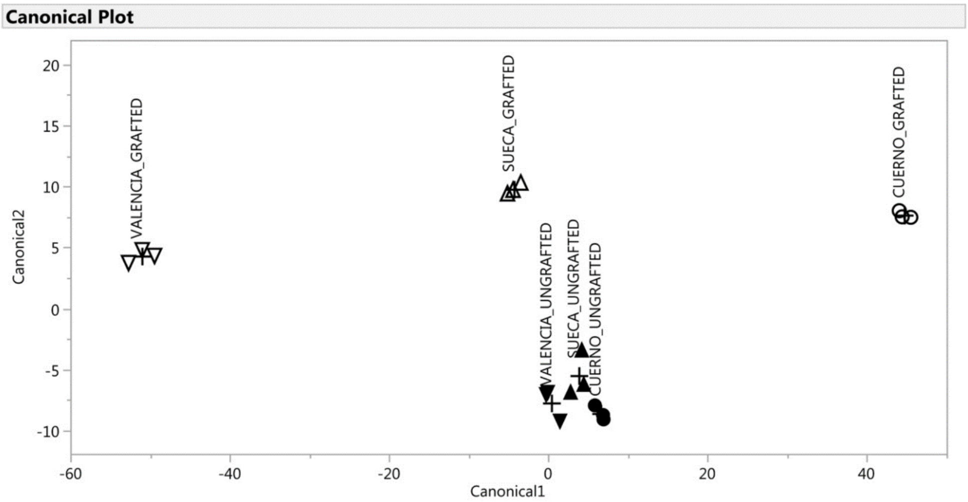

Figure 5. Discrimination of the VOCs of the green fruit based on the canonical discriminant analysis. ▲: SUECA_ungrafted; Δ: SUECA_grafted; ▼: "Valencia"_ungrafted; ∇: "Valencia"_grafted; ●: "Cuerno"_ungrafted; ○: "Cuerno"_grafted.

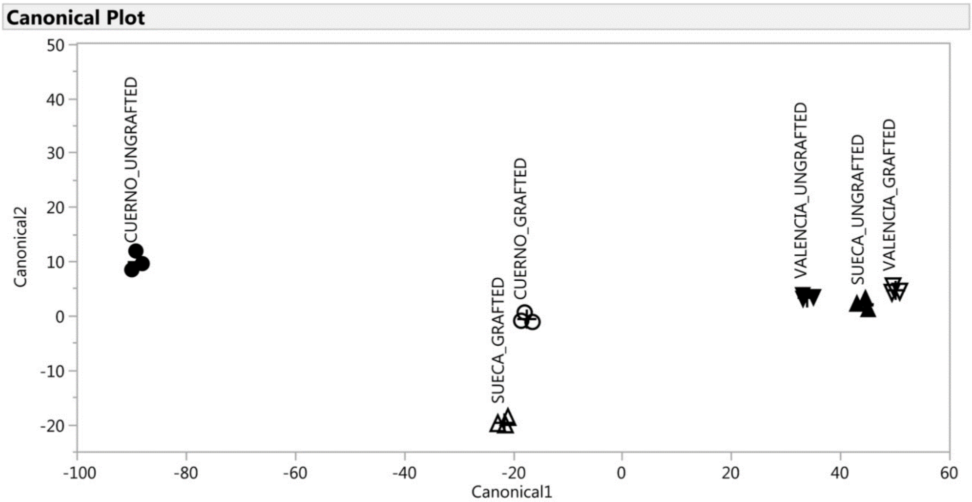

Figure 6. Discrimination of the VOCs of the red fruit based on the canonical discriminant analysis. ▲: SUECA_ungrafted; Δ: SUECA_grafted; ▼: "Valencia"_ungrafted; ∇: "Valencia"_grafted; ●: "Cuerno"_ungrafted; ○: Cuerno_grafte

Table 5. Correlations between the total canonical structure and the original variables.

	Canonical_1	**Canonical_2**
Green Peppers		
4-methyl-2,3-dihydrofuran	−0.09	0.84
N-methylpyrrole	0.18	0.54
allo-ocimene	−0.40	0.47
nonanal	0.27	0.34
2,4,6-trimethylanisole	−0.03	0.64
cyclosativene	0.49	0.80
methyl salicylate	0.19	0.54
octanoic acid	0.04	0.57
nonanoic acid	0.23	0.32
ethyl hexadecanoate	0.26	0.45
Variance explained (%)	*91.59*	*6.59*
Cumulative variance (%)	*91.59*	*98.18*
Red peppers		
carbon disulfide	−0.083	
ethanol	−0.739	
pentanal	−0.155	
2-propenyldiene-1-cyclobutene	0.789	
2-heptanone	0.429	
7-methyl,1-octene	0.270	
2-Isobutyl-3-methoxypyrazine	0.392	
beta-linalool	0.501	
5,5-dimethyl-1,3-dithian-2-one	0.392	
salicylic acid methyl ester	0.506	
Variance explained (%)	*96,14*	

The correlation of VOCs associated to the canonical variables are shown in bold.

For the red fruit, the first canonical could discriminate the six groups, which demonstrates that it was able to explain the effect of landrace and grafted effect (Figure 6). Finally, the DA classified each observation (peppers) to the correct group for both green and red fruit with 100% accuracy.

6.5. Discussion

Use of grafting is a good tool to improve vegetable production in areas where biotic-abiotic stresses can seriously determine reductions in not only crop yield, but also in production quality [50,51,52].

In this way, most research aims to assess the rootstock and scion interaction and the effect that this interaction has on both agronomic performance and stress resistance [10,14,53]. However, in recent years, many works report the effect of grafting on the production of vegetable quality, particularly the physico-chemical, nutritional and nutraceutical properties of the fruit produced by grafted plants [10,18,29,30]. Fruit quality is a set of attributes that determine consumer choice, which can be divided into external fruit aspect, nutritional and nutraceutical characteristics [54]. These last properties have been assumed to be of particular importance for consumers given the positive relations between fruit/vegetables and human health [55,56]. Many studies report that a healthy diet rich in fruit and vegetables, named functional food, helps to delay senescence processes in humans, but to also reduce the risk of important pathologies including cancer and cardiovascular diseases [56,57,58]. The important characteristic of functional food is its richness in antioxidant compounds, capable of scavenging ROS based on cellular oxidative stress [25].

Sweet pepper fruit falls in the category of functional foods because it is rich in ascorbic acid, carotene, phenols, and also in capsaicinoids, xanthophylls, and flavonoids and is, for this reason, characterized by high antioxidant capacity [27,28]. However, the amount of phytochemicals in peppers depends on many factors, including rootstock, although literature reports opposite results about the influence of grafting on the amount of phytochemicals in peppers.

Our study evidenced for all three landraces that grafting positively influenced marketable yield, whereas no differences were found among the three landraces. The higher yield in the sweet peppers from the grafted plants has already been demonstrated [14,59] and is attributable to an increase in the average number of fruit per plant. This increase can be related to the more vigorous root system of rootstock NIBER® that, in turn, induces an increase in water and nutrient uptake [31,32]. In addition, non-marketable yields and fruit with BER markedly dropped because grafting further increased production due to fewer losses. The minor incidence of fruit with BER symptoms caused by the grafted plants could be attributed to the rootstock better resisting abiotic stress, as previously reported in this species by Johkan et al. [60], Sanchez–Rodriguez et al. [61] and Penella et al. [13,33].

In addition to increased fruit production, the physico-chemical characteristics remained the same, or even improved, for the studied rootstock. Fruit dry matter was similar in the green fruit, or increased in the red fruit, of the three different pepper landraces grafted onto NIBER® compared to the fruit from the ungrafted plants, whereas no changes were recorded for pulp thickness. Other authors have found similar results [19], showing that an increment in dry matter could be attributed to the higher uptake and transport of mineral elements [62,63].

The perception of pepper fruit sourness is related to titratable acidity. Grafting also increased titratable acidity in both red and green fruit, which is an important factor for fruit quality if we consider the role played by acids in the flavor of fresh pepper products.

Much interest in pepper fruit quality has been shown as regards to phytochemicals, including ascorbic acid, lycopene, and phenols. However, the effect of grafting on nutraceutical aspects of pepper fruit is related to landraces, but also to the fruit maturation stage. First of all, phenolic compounds significantly decreased in both the green and red fruit obtained from the grafted plants, which has already been reported by other authors in this species and in other vegetables [18,64,65]. Phenol compounds were higher in the red than green fruits at a mean concentration of about 10.9 and 6.5 mg g^{-1} FW, respectively. These values are higher than those reported by Zhuang et al. [28] in red fruit, but are similar to those found by Blanco–Ríos et al. [66] and Chavez–Mendoza et al. [18]. Finally, the most relevant result for phenol compounds was the higher content found in fruit in relation to the ripeness state with red fruit containing significantly more phenols than green fruit, as previously reported by Lee et al. [67] and Chavez–Mendoza et al. [30].

The other important nutraceutical molecule in peppers is vitamin C, with fresh peppers being an excellent source of this compound. A different behavior was detected in both green and red fruit. The green ones produced by the grafted plants had a higher ascorbic acid content, which significantly lowered in the red ones. The ascorbic acid results reported in the literature also differ. Sanchez–Torres et al. [19] found in peppers that the amount of ascorbic acid changed depending on landraces more than on grafting, or even on both the scion/rootstock combination, as reported by López–Marín et al. [65], and a significant increase was observed by Chavez–Mendoza et al. [18]. This increase seems to be connected to the connection between scion and rootstock, which induces a good flow of water and minerals to thus improve the photosynthetic process, as reported by San Bautista et al. [68] in melon.

In our study, vitamin C was closely related to the genotype. Indeed “Sueca” and “Valencia” produced green and red fruit with higher vitamin C contents. Another interesting result, and one similar to that recorded for phenol compounds, was the amount of ascorbic acid recorded in the red fruit. Regardless of the grafting technique, vitamin C was much higher in red than in the green fruit, evidencing the important of the maturity stage.

The drop in vitamin C in the red fruit from the grafted plants was, however, compensated by the marked increase of lycopene observed in fruit from the grafted plants. It is well-known that ascorbic acid is a sensitive compound to heat treatment, unlike lycopene. Lycopene is a strong antioxidant [69] and its concentration was higher in the red than the green fruit and grafting positively impacted its concentration on both fruit. Another major difference was that related to the genotype with “Cuerno” being the landrace that, depending on grafting, produced fruit with a high lycopene content. Previous works have reported that lycopene content depends on genotype more than on grafting [70]. Obviously, red fruit contained 4- to 5-fold more lycopene than green ones, with similar values to those found by Chavez–Mendoza et al. [30]. The results obtained about lycopene were relevant when considering that lycopene is apparently the most efficient quencher of singlet oxygen and free radicals among carotenoids [71]. In addition, it does not convert into vitamin A (as β-carotene does) and is utilized entirely as an antioxidant. It is also well-known that lycopene is not sensitive to heat treatment as, for example, ascorbic acid is, and it remains in peppers even after cooking.

In a general way, total chlorophyll content was lower in the red than in the green fruit and concomitantly increased carotenoid. This obviously took place when considering the pepper fruit maturity stage. Clearly, in the red fruit, the conversion of chloroplast into chromoplast is related to both chlorophyll degradation and the increase in carotenoid biosynthesis, as already in pepper formerly by other authors [72,73].

Grafting led to no changes in chlorophyll and carotenoid contents, despite the significant interaction found for the total chlorophyll amount in the green fruit and the carotenoid levels in the red fruit. The highest total chlorophyll level was recorded in the green fruit produced by the ungrafted “Cuerno” compared to the other thesis, as with the fruit produced by the grafted plants of this landrace. Conversely, for “Sueca” and “Valencia”, no differences were observed in the total chlorophyll between the fruit produced by the grafted and ungrafted plants.

Antioxidant capacity was negatively influenced by grafting, and was higher in both colored peppers produced by the ungrafted plants, whereas a strong influence of genotype was recorded with landrace “Valencia”, which produced fruit with the highest antioxidant capacity (both green and red). These results evidenced that grafting did not improve fruit antioxidant capacity as reported by other authors [74] in tomato cultivar Cecilia F1, grafted onto He-man and Spirit. Our results contrast with those reported by

Chavez–Mendoza et al. [18] in fruit from grafted bell peppers. What our results evidence is that red fruit, independently of landrace or grafting, had a higher antioxidant capacity compared to green ones in accordance with the data reported by Blanco–Ríos et al. [66] in the red fruit of cultivar "Mazurca."

The correlation analysis revealed that antioxidant capacity was related only to the phenols compound in the green fruit of all the grafted landraces, but another picture appeared for the red fruit. In fact, a significant correlation between antioxidant capacity and phenols was found only for the grafted plants, but a significant contribution to antioxidant capacity was made in the red fruitby ascorbic acid. In the red fruits, an inverse correlation between antioxidant capacity and lycopene was noted as reported by Chavez–Mendoza et al. [30].

The analytical technique to analyze VOCs identified 51 different compounds. The terpenoid class includes a wide diversity of compounds with significant differences between fruit stage maturity and landraces. Most terpenoids are mono- and sesquiterpenes, of which 3-carene and β-linaool are mainly responsible for sweetness and fresh flowery aroma, and are typical in the fruit of peppers [75] and other fruit, vegetables and food aromas [76]. These compounds were significantly present in the green fruit, with no differences among landraces for 3-carene, but a higher concentration was observed in "Valencia" and "Sueca" compared to "Cuerno." Among sesquiterpenes, large amounts of copaene, α-bergamotene, and β-farnesene appear. β-farnesene is involved in sweet pepper fruit flavor andappeared in large amounts in the green fruit, but with no differences among landraces.

Even aldehydes, ketone, alcohols, and acids were recorded in pepper fruit and, once again, with the highest level in the green fruit as compared to the red ones. Within acids, nonanoic acid is prevalent, nonenal was the prevalent aldehyde and 2-octen-1-ol (E) and *cis* -3 nonel -1- ol were the prevalent alcohols. Many of these compounds derive from lipids such as linolenic and linoleic acid through the action of the lipoxygenase pathway, as reported by Luning et al. [77]. No differences among landraces were found for acids, but differences were noted for ethanol (higher in "Cuerno") among alcohols, for aldehyde nonenal (higher in "Cuerno" and "Sueca"), and also for ketones 2-heptanone (higher in "Valencia") and 5,5-dimethyl-1,3-dithian-2-one, which was higher in "Cuerno" and "Sueca". As formerly reported, numerous esters are found in pepper fruit [78] that typically improve fruity flavor and floral aroma in different *Capsicum* species. Methyl-salicylate is the most abundant one in green pepper fruit, and is a compound with a strong mint odor that abounds in pepper fruit [78], and is a distinctive flavor-active volatile [79].

Among furans and aromatic hydrocarbons, 2-pentylfuran, and 2,4,6-trimethylanisole are found in larger amounts in green fruit. The first furanic compound is abundant in bell pepper fruits.

Finally, in miscellaneous compounds 2-isobutyl-3-methoxypyrazine, a potent bell pepper odorant [77], was abundant in the green fruit with no differences among landraces.

A multivariate statistical analysis was performed to make an in-depth evaluation of the differences attributable to grafting and/or landraces. For the green fruit, the CDA can be explained by two canonical variables that explained 98% of the total variability in the dataset obtained from the analysis. The loading plots from the green fruit were composed of the axes of canonical_1 with canonical_2. The grafted “Cuerno” was evidently separate from other samples by canonical_1 on the score plot, and the compound that accounted for discrimination was cyclosativene, while the discrimination of the grafted “Valencia” was related to allo-ocimene. Cyclosativene is a tetracyclic sesquiterpene whose antioxidant capacity mitigates oxidative injuries in the neurodegenerative disorders field [80]. Allo-ocimene is a monoterpen that occurs in citrus and many other essential oils, and is characterized by a harsh terpene-like and somewhat citrusy character that is also typical in bell peppers [81]. The ungrafted landraces were indistinguishable and spared by the grafted plants according to canonical_2. As shown in Table 4, the graft involved a higher content of 4-methyl-2,3-dihydrofuran, N-methylpyrrole, 2,4,6-trimethylanisole, methyl salicylate, octanoic acid, ethyl hexadecanoate, cyclosativene and allo-ocimene.

For the red fruit, only the first canonical_1 variable explained 96% of variability (Table 4). According to this discrimination, three groups were observed, characterized by groups of peppers with a short, but significant, distance among them: “Valencia” (grafted and ungrafted) and “Sueca” ungrafted obtained the highest canonical scores; the ungrafted “Cuerno” obtained negative scores; “Cuerno” and “Sueca” had intermediate score values. Unlike the green fruit, a different response of the three landraces to grafting was observed as regards the profile of VOCs. As Figure 6 depicts, “Sueca” and “Valencia” were very close when ungrafted, while the graft, “Sueca” showed a similar profile to “Cuerno”. The VOCs most associated with ”Valencia” (grafted and ungrafted) and “Sueca” were 2-propyldiene-1-cyclobutene, 2-heptanone, beta-linalool, and methyl salicylate, while ethanol characterized mainly “Cuerno” ungrafted.

6.6. Conclusions

The findings reported herein support our hypothesis that grafting could be an interesting technique to improve the yield and marketable quality, mainly BER incidence, of pepper landraces without negatively affecting intrinsic fruit quality. These results also provide useful information about the pepper grafting effect on bioactive compounds and antioxidant capacity, with variation being much more related to maturity stage and genotype. The red fruit displayed higher antioxidant capacity than the green fruit, but grafting improved only lycopene content in both red and green fruit.

The volatile fraction of peppers was composed of different chemical classes, which were linked to and influenced by the graft as the discriminant analysis demonstrated. This study may also represent a useful approach to characterize the effect of graft on plant metabolism. This effect was more evident in the green fruit than in the red ones.

6.7. References

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Capítulo 7

Discusión General

7.1. Uso de la técnica del injerto para hacer frente al estrés térmico

Los objetivos planteados en este apartado de la tesis doctoral han sido: investigar si el uso de un portainjerto adecuado puede conferir a la variedad de pimiento cultivada tolerancia al estrés supra-térmico, y si eso fuera así estudiar qué mecanismos subyacen en esta tolerancia. Objetivos basados por una parte en la necesidad de mejorar el comportamiento productivo y la calidad del pimiento cultivado en invernadero, en áreas templado-cálidas como la mediterránea y en ciclos productivos que contemplen los meses de primavera tardía, verano y otoño temprano, en el escenario de aumento global de temperaturas. Los objetivos se han planteado avalados por que los equipos de investigación que desarrollan el proyecto en el que se enmarca esta tesis doctoral, han validado previamente que ciertos portainjertos son capaces de conferir tolerancias a la planta frente a otros estreses abióticos, principalmente hídrico y salino, habiendo sido capaces de desarrollar un híbrido (F1) de *Capsicum anuum*, NIBER®, capaz de mejorar los rendimientos de la variedad injertada en estas condiciones.

Siguiendo con la misma línea de investigación, teniendo en cuenta toda la experiencia y conocimiento de la técnica generada en este sentido, y sabiendo que hasta el momento no existe ningún portainjerto comercial de pimiento que pueda conferir tolerancia a la variedad para el estrés supra-térmico, los trabajos realizados se iniciaron con una selección previa de un número reducido de accesiones dentro del género *Capsicum* (Gisbert-Mullor, 2018), a través de la identificación de los genotipos con mayores probabilidades de tolerancia debido a sus características, principalmente por su zona origen, la evaluación agronómica y fisiológica del material vegetal, hasta seleccionar aquellas accesiones que podrían ser consideradas como tolerantes. Y todo ello bajo la hipótesis de que los mecanismos de tolerancia de ciertos portainjertos a los estreses abióticos, percibidos por el sistema radical, podrían ser similares para los estreses percibidos por la parte aérea de las plantas, como es el caso del estrés térmico por altas temperaturas.

Como ya apuntaron algunos autores en el cultivo del tomate (Ayenan et al., 2019), existen parámetros fisiológicos sensibles al estrés térmico que permiten seleccionar accesiones tolerantes a dicho estrés. Tanto Fv/Fm como EL fueron utilizados como biomarcadores de selección para esta tolerancia. Además, se implementaron otros parámetros con respuesta específica para el estrés supra-térmico, como el número de inflorescencias, la viabilidad del polen y el cuajado del fruto (Xu et al., 2017; Zhou et al., 2015), altamente relacionados con el rendimiento (Saeed et al., 2007) en estas condiciones.

Como consecuencia de ello y de lo observado en los estudios previos (Gisbert-Mullor, 2018), se seleccionaron las 6 accesiones con mejor tolerancia al estrés supra-térmico, todas ellas de la especie *Capsicum annuum* L. y se compararon con la variedad sin injertar e injertada sobre ella misma tanto en condiciones controladas de cámara climática como en condiciones de invernadero. De los resultados obtenidos en este estudio, cabe destacar que A57, fue la única accesión que no mostró diferencias significativas entre condiciones para los parámetros fisiológicos descritos como sensibles en condiciones de estrés supra-térmico, como RGR, EL y Fv/Fm, además de obtener mejores resultados que la variedad sin injertar en condiciones de estrés en el experimento en invernadero. Se estudió también, en condiciones de cámara climática, la respuesta de las HSPs por ser bioindicadoras del estrés térmico, en concreto se analizó la expresión relativa de HSP70 y dos sHSPs, sHSP25.8 y sHSP22.7. A pesar de saber que la acumulación de HSP y sHSP juega un papel clave en la respuesta al estrés térmico y en la termotolerancia de las plantas (Sun et al., 2002; Wang et al., 2020), no se pudo observar ninguna correlación positiva entre la expresión de estas proteínas y el rendimiento o RGR, y por lo tanto sería necesario realizar más estudios en este sentido para tratar de comprender la compleja red de las HSPs.

Como fue discutido en el primero de los artículos que forma parte de la tesis doctoral, en plántulas de pimiento se observó el mayor RGR en los genotipos cultivados bajo condiciones de control, y en ambas condiciones el RGR varió dependiendo de la accesión utilizada como portainjerto. Estos resultados sugieren que los efectos de las altas temperaturas sobre el RGR podrían ser explicados por la adaptabilidad de las raíces a la temperatura del aire. Rivero et al. (2003) también observaron que el peso seco de las plantas de tomate cultivadas a 35 °C fue menor en las plantas no injertadas que en las plantas injertadas. En la misma línea, Li et al. (2014) constataron un mayor crecimiento de los brotes de pepino injertado sobre luffa a 36 °C que en pepino sin injertar.

La reducción del RGR por alta temperatura es la consecuencia de cambios fisiológicos y metabólicos en las células vegetales. De hecho, la disminución de la estabilidad de las membranas bajo estrés por calor podría conducir a una mayor fluidez de las mismas (Prasad et al., 2008). Por otra parte, la fotosíntesis es uno de los procesos más sensibles al estrés abiótico a través de alteraciones en el aparato fotosintético (Zhou et al., 2015), y el fotosistema II (PSII) se considera un componente sensible y termolábil (Čajánek et al., 1998; Mathur et al., 2011). En nuestros experimentos, la relación Fv/Fm en condiciones de estrés por calor fue menor y el parámetro EL fue mayor que en las condiciones control, lo que demuestra que el estrés por calor afecta a estos parámetros. Sin embargo, el valor de ambos parámetros fue dependiente del portainjerto utilizado, como han constatado también Xu et al. (2017) y Poudyal et al. (2018) en tomate.

En el primer experimento (Capítulo 2), se observó que el mayor rendimiento de la variedad injertada sobre la accesión A57 era debido a un mayor número de frutos y no a un mayor peso de los mismos. En este sentido, es bien conocido que el estrés por altas temperaturas afecta principalmente a la formación y desarrollo de los gametofitos masculinos (Chaturvedi et al., 2021). El polen es sensible a las altas temperaturas a lo largo de su desarrollo, ya que afecta a la cantidad y la morfología de los granos, la arquitectura de las paredes celulares y al metabolismo del mismo (Hedhly, 2011; Zinn et al., 2010), aunque en el género *Capsicum* se ha observado que la sensibilidad del polen a las altas temperaturas varía entre especies (Reddy y Kakani, 2007).

En este sentido se planteó un doble experimento con el objetivo de profundizar en el efecto que ejerce el uso de portainjertos sobre el cuajado de frutos en condiciones de estrés supra-térmico.

La primera parte se centró en estudiar la respuesta del metabolismo de la hoja analizando diversos parámetros fisiológicos que pudieran verse afectados por el estrés térmico. Pudimos observar que la variedad injertada sobre A57 en condiciones de estrés térmico obtuvo una menor EL, una mayor concentración de Chl y Car y una menor acumulación de H_2O_2 en hojas, además de un mayor porcentaje de cuajado de frutos que la variedad sin injertar. A la vista de estos resultados y tras confirmar que las diferencias de rendimiento eran debidas al porcentaje de cuajado o número de frutos, la segunda parte del experimento se centró en estudiar diferentes parámetros relacionados directamente con el cuajado del fruto. De los resultados obtenidos cabe destacar que cuando la variedad fue injertada sobre A57 en condiciones de estrés, aumentó el contenido de prolina en las anteras, mejoró la germinación de los granos de polen y el número de semillas por fruto fue mayor, viéndose finalmente mejorado el porcentaje de cuajado de frutos respecto a la variedad sin injertar.

A pesar de que el estrés térmico por altas temperaturas afecta directamente a la parte aérea de la planta, observamos que el sistema radical, y por lo tanto el injerto sobre accesiones tolerantes, pueden paliar los efectos de dicho estrés. Como ya comentamos en el segundo artículo correspondiente a este apartado, el parámetro EL está influenciado por el contenido de las especies reactivas de oxígeno (ROS) dado que el estrés por calor induce la generación y acumulación de las mismas, como el H_2O_2 (Airaki et al., 2012; Xu et al., 2018). De esta forma, encontramos una correlación positiva entre la concentración de H_2O_2 y EL ($r= 0,785$, $P< 0,05$), lo que indicó que el aumento de EL se debió en parte a una mayor producción de H_2O_2. De hecho, la concentración de H_2O_2 en VA/A57 bajo estrés térmico no difirió significativamente de su control, lo que estuvo en concordancia con sus valores más bajos de EL. Este efecto podría deberse en parte a una mejor homeostasis redox en las plantas VA/A57 respaldada por metabolitos primarios, como la acumulación de carotenoides y clorofila, para evitar la fotooxidación en los fotosistemas (Leverenz et al., 2015) y lograr valores de Fv/Fm sin diferencias respecto a su control (Gisbert-Mullor et al., 2021). Los mismos efectos se han observado en pepino injertado en *Momordica* sometido a alta temperatura (Wei et al., 2019; Xu et al., 2018).

Un eficiente mecanismo de tolerancia al estrés térmico por altas temperaturas en las partes vegetativas juega un papel vital en la formación adecuada de órganos reproductivos, afectando positivamente al rendimiento final (Asseng et al., 2002; Wollenweber et al., 2003). En este sentido, fuimos capaces de corroborar que la combinación VA/A57, que era la mejor predispuesta a tolerar las altas temperaturas de acuerdo con los resultados de los parámetros fisiológicos observados en las hojas, exhibió también los más adecuados atributos reproductivos. De hecho, se encontraron correlaciones significativas ($P< 0.05$) entre cuajado de frutos y diferentes parámetros, como número de semillas ($r=0.771$), germinación de polen ($r=0.787$) y concentraciones de prolina y sacarosa en anteras ($r=0.718$ y $r=0.756$, respectivamente), confirmando la relación evidente entre el cuajado del fruto y el estado del polen.

El análisis de los resultados obtenidos de los diferentes experimentos realizados ha permitido seleccionar la accesión A57 como portainjerto tolerante al estrés por altas temperaturas. De hecho, de los artículos que conforman los capítulos 2 y 3, a pesar de corresponder a experimentos distintos, en conjunto se deduce que el portainjerto A57 es capaz de conferir a la variedad mayor tolerancia al estrés supra-térmico a través de un elevado número de parámetros fisiológicos, como EL, RGR, Fv/Fm, contenido de clorofilas y carotenoides en hojas, acumulación de H_2O_2 en hojas, contenido de prolina en las anteras, viabilidad del polen, relacionados directa y positivamente con esta tolerancia y que conducen a incrementar el rendimiento comercial.

En consecuencia, hemos utilizado esta accesión como parental para generar nuevos híbridos potencialmente tolerantes al estrés por altas temperaturas, y en un experimento preliminar, hemos observado que uno de los híbridos ha obtenido un 77% más de rendimiento comercial (kg por planta) que la variedad sin injertar (datos no mostrados) en condiciones de estrés. A la espera de confirmar estos resultados preliminares, cabe destacar que el uso de portainjertos híbridos resultaría una técnica eficaz para paliar los efectos de las altas temperaturas en el cultivo del pimiento, pudiendo mejorar tanto el rendimiento comercial como el económico de una manera sostenible con el medioambiente.

7.2. Uso de la técnica del injerto para hacer frente al estrés hídrico

El objetivo planteado en este segundo apartado de la tesis doctoral ha sido ampliar los conocimientos sobre los mecanismos fisiológicos que modulan la respuesta a la tolerancia del portainjerto híbrido NIBER® en condiciones de estrés hídrico.

Fruto de las investigaciones previas realizadas por el equipo de investigación en el cual se integra esta tesis doctoral se obtuvo el híbrido NIBER®, tolerante al estrés hídrico y salino. En este híbrido se ha estudiado con detalle los mecanismos fisiológicos responsables de la tolerancia al estrés salino (López-Serrano et al., 2020), pero no sus respuestas adaptativas frente al estrés hídrico.

Con esta necesidad de ampliar el conocimiento en este sentido se planteó el objetivo de evaluar el impacto del riego deficitario, en condiciones muy controladas, sobre el rendimiento, la calidad del fruto y la eficiencia del uso del agua de riego de una variedad híbrida de pimiento injertada sobre NIBER® y la variedad sin injertar.

Durante dos años consecutivos, nuestros resultados demostraron que al injertar una variedad de pimiento sobre el portainjerto NIBER® se superan los efectos negativos del estrés hídrico sostenido en términos de biomasa y rendimiento. Además, este portainjerto tuvo un efecto beneficioso sobre rendimiento comercial «Extra» –principal componente de los resultados económicos del cultivo–, al mantener un rendimiento mayor que las plantas sin injertar en condiciones de estrés.

Se evaluaron también los procesos fisiológicos asociados con el injerto y el riego deficitario, centrándose en el uso eficiente del agua y las relaciones fotosintéticas para explicar los resultados obtenidos en producción. En este sentido, en condiciones control el estado hídrico de la planta evaluados por el potencial hídrico ($\Psi_{predawn}$ y Ψ_{leaf}) fue similar entre las plantas injertadas y no injertadas. Estos resultados ya fueron constatados también en mini-sandía, donde el potencial hídrico de la hoja en plantas injertadas y no injertadas mostró valores similares aplicando un riego óptimo (Rouphael et al., 2008). Sin embargo, el uso de plantas de pimiento injertadas sobre NIBER® tuvo una influencia significativa en la capacidad de transporte de agua de la planta en comparación con las plantas sin injertar. La conductancia hidráulica de toda la planta en el tratamiento control en condiciones de alta demanda atmosférica (al mediodía) fue menor en las plantas injertadas, y estuvo acompañada de menores valores de g_s, indicando mayor cierre estomático. Los cambios en las propiedades hidráulicas y el comportamiento estomático permiten que las plantas injertadas regulen mejor el agua utilizada por la planta al reducir la pérdida por transpiración. Finalmente, estos resultados confirman nuestra hipótesis de partida sobre la tolerancia esperable de NIBER® al estrés hídrico en condiciones de campo controlando el contenido de agua del suelo.

Además, se demostró que el híbrido NIBER® utilizado como portainjerto fue capaz de mejorar la eficiencia en el uso del agua, produciendo una mayor biomasa y rendimientos comerciales en comparación con las plantas sin injertar en condiciones de riego deficitario. Esto se podría atribuir a las diferencias en la capacidad de absorción de agua y nutrientes del sistema radical, como se ha observado en plantas de melón injertadas (Agele y Cohen, 2009). Sin embargo, en los dos estudios pertenecientes al Capítulo 4 no se investigó el efecto de la capacidad exploradora del sistema radical sobre la mayor adaptación del portainjerto tolerante al estrés hídrico.

Para ello, y en condiciones más controladas que las utilizadas en los experimentos anteriores, el objetivo del segundo experimento se centró en corroborar hasta qué punto un portainjerto tolerante al estrés hídrico es capaz de mantener su capacidad productiva en condiciones de estrés por riego deficitario, y al mismo tiempo estudiar la capacidad de exploración de raíces, en términos de volumen y peso, para relacionarlo con las diferencias de rendimiento y calidad de frutos que se pudieran observar. Para ello, el experimento se realizó en contenedores de 25 litros con sustrato a base de arena de sílice para facilitar las labores de evaluación de la biomasa radical y distribución de las raíces.

Los resultados agronómicos obtenidos en este experimento demostraron que la variedad tradicional Cuerno cuando se injertó sobre el portainjerto NIBER® aumentó el rendimiento comercial tanto en condiciones control como en condiciones de riego deficitario, 23% y 45% respectivamente, en comparación a las plantas sin injertar. Hay que destacar que la pérdida de rendimiento que sufrieron las plantas en condiciones de riego deficitario fue muy elevada, debido a que el estrés generado fue muy intenso, incluso más marcado que el observado anteriormente en los experimentos realizados en condiciones de suelo, utilizando el mismo portainjerto.

Como ya hemos comentado, estas diferencias en términos de rendimiento comercial entre plantas injertadas y no injertadas podrían estar asociadas al sistema radical más vigoroso del híbrido NIBER®. De hecho, en este experimento fuimos capaces de demostrar que la biomasa y el volumen de raíces fueron mayores en las plantas injertadas sobre NIBER® que en las no injertadas, tanto en condiciones de riego óptimas como en condiciones de estrés hídrico. Este aumento de biomasa radical podría explicarse por un efecto directo sobre una mayor capacidad fotosintética que normalmente confieren los portainjertos que toleran estreses abióticos, lo que ha sido frecuentemente reportado en otros estudios asociado con un sistema radical robusto que contribuye a una mayor absorción de agua y nutrientes (López-Serrano et al., 2019, 2017; Penella et al., 2017, 2016, 2014).

Por otro lado, los mayores rendimientos obtenidos en las plantas injertadas con NIBER® fueron debidos a una menor incidencia de BER y no a un mayor número de frutos o peso medio de los frutos comerciales. Aunque la causa de esta fisiopatía no está del todo dilucidada, este trastorno ha sido relacionado por algunos autores con el estrés oxidativo causado por la producción de ROS bajo condiciones estresantes (Saure, 2014), mientras que otros autores relacionaron la incidencia de BER con la escasa translocación de Ca a órganos y tejidos con baja o nula transpiración, lo que resultaría en una deficiencia local de este elemento (de Freitas et al., 2011). En experimentos previos en condiciones de estrés salino (López-Serrano et al., 2020), el híbrido NIBER® mostró una mayor capacidad antioxidante que la variedad sin injertar, y fruto de los resultados de este estudio del Capítulo 5 hemos podido corroborar que las plantas injertadas sobre este portainjerto son capaces de desarrollar un sistema radical más potente, con mayor biomasa y volumen radical, lo que facilitaría la absorción de agua y nutrientes, entre ellos el Ca, como ya han observado algunos autores en diferentes cultivos (Lee et al., 2010; Rouphael et al., 2008). En este sentido, Kyriacou et al. (2017) sugirieron que la menor incidencia de BER en pimiento, observada en las zonas distales del fruto, se puede atribuir a la mejora realizada por el portainjerto en términos de absorción/transporte de nutrientes a las células apicales del fruto mejorando la integridad de la pared celular.

Por lo tanto, con todos los resultados obtenidos de este apartado, podemos asegurar que el uso del portainjerto NIBER® puede considerarse como una herramienta útil para reducir el consumo de agua amortiguando los efectos del estrés hídrico sobre el rendimiento comercial del cultivo, principalmente reduciendo la incidencia de BER, atribuido al mayor desarrollo del sistema radical que le facilita la absorción de agua y nutrientes, permitiéndole mejorar la eficiencia en el uso del agua.

7.3. Efecto del injerto sobre el rendimiento y calidad de variedades tradicionales

En este último apartado de la tesis doctoral, el objetivo principal ha sido el estudio agronómico y de la calidad de los frutos de pimiento de diferentes variedades tradicionales injertadas sobre el portainjerto híbrido NIBER®. En los capítulos anteriores hemos sido capaces de corroborar el adecuado comportamiento, en términos de rendimiento, de variedades híbridas y tradicionales cuando son injertadas sobre el portainjerto NIBER® en condiciones de riego deficitario. Sin embargo, hasta la fecha, ningún estudio se había centrado en estudiar los efectos del injerto sobre el rendimiento y calidad de los frutos de variedades tradicionales de pimiento.

En este contexto se planteó un experimento para evaluar el efecto del portainjerto NIBER® sobre la producción y calidad del fruto en tres variedades tradicionales de la Comunidad Valenciana. Para determinar la calidad de la fruta, se tuvo en cuenta el estado de madurez (verde y rojo) y se ensayaron no solo las características fisicoquímicas (materia seca, acidez titulable, color y volátiles), sino también aspectos nutracéuticos (fenoles, vitamina C, pigmentos y capacidad antioxidante).

De los resultados obtenidos, podemos observar que, para las tres variedades, el injerto influyó positivamente en el rendimiento comercial, mientras que no se encontraron diferencias entre las tres variedades locales. Esta mejora del rendimiento comercial en las plantas injertadas ya fue observada previamente por diferentes autores (Aidoo et al., 2017; Leal-Fernández et al., 2013) y es atribuible a un aumento en el número promedio de frutos por planta. En nuestro caso, este aumento pudo estar relacionado con el sistema radical más vigoroso del patrón NIBER ® que, a su vez, induce un aumento en la absorción de agua y nutrientes, como ya hemos comentado en apartados anteriores. De hecho, en este estudio, las diferencias de rendimiento también se deben atribuir a que las plantas injertadas sobre NIBER® presentaron una menor afección de BER en los frutos. Estos resultados coinciden y son corroborados por un estudio posterior que ya se ha discutido anteriormente.

Por otro lado, las características fisicoquímicas del fruto de las plantas injertadas, o bien no presentaron diferencias significativas respecto a las no injertadas, o mejoraron en algunos parámetros, como la materia seca y la acidez titulable. Para la materia seca, cabe destacar que en el caso de los frutos verdes no hubo diferencias entre injertadas y no injertadas, pero en el caso de los frutos rojos aumentó en las tres variedades injertadas sobre NIBER®. Otros autores han encontrado resultados similares, mostrando que un incremento en la materia seca podría atribuirse a la mayor absorción y transporte de elementos minerales (Leonardi et al., 2006; Pulgar et al., 2000).

Las características nutracéuticas de la fruta de pimiento estuvieron más relacionadas con las variedades locales y el estado de maduración de la fruta que con el efecto del propio injerto. El contenido de compuestos fenólicos fue significativamente mayor en los frutos rojos que en los verdes, como constataron Lee et al. (1995) y Chávez-Mendoza et al. (2015). Respecto el contenido de vitamina C, los frutos verdes de las plantas injertadas mostraron un mayor contenido que los frutos de las plantas sin injertar, pero para el caso de los frutos rojos se observó un comportamiento diferente, el contenido fue mayor en los frutos de las plantas sin injertar. Por otro lado, respecto al contenido de pigmentos fotosintéticos, no se observaron diferencias entre frutos de plantas injertadas y no injertadas, pero evidentemente los frutos verdes obtuvieron un mayor contenido de clorofilas totales y un menor contenido de carotenoides que los frutos rojos. En los frutos rojos, la conversión de cloroplasto en cromoplasto está relacionada tanto con la degradación de la clorofila como con el aumento de la biosíntesis de carotenoides, como ya se ha observado en pimiento anteriormente por otros autores (Marín et al., 2004; Mínguez-Mosquera y Hornero-Méndez, 1993). Por lo que respecta al análisis de licopeno, su concentración fue mayor en la fruta roja que en la verde y el injerto tuvo una respuesta positiva en su concentración en ambos estados de maduración. Obviamente, los frutos rojos contenían de 4 a 5 veces más licopeno que los verdes, con valores similares a los encontrados por Chávez-Mendoza et al. (2015). Los resultados obtenidos sobre el licopeno fueron relevantes si se considera que este compuesto es el inhibidor más eficiente de diferentes radicales libres entre los carotenoides, además, no se convierte en vitamina A (como lo hace el β-caroteno) y se utiliza completamente como antioxidante (Edge et al., 1997). Por último, la capacidad antioxidante se vio negativamente influenciada por el injerto, y fue mayor en los pimientos en ambos estados de madurez producidos por las plantas sin injertar. Estos resultados evidenciaron que el injerto no mejoró la capacidad antioxidante de la fruta tal y como demostraron Qaryouti et al. (2007) en tomate. Lo que nuestros resultados evidencian es que los frutos rojos, independientemente de la variedad o del injerto, tenían una mayor capacidad antioxidante en comparación con los verdes de acuerdo con los datos reportados por (Blanco-Ríos et al., 2013).

Respecto al contenido en compuestos volátiles (COV), se identificaron 51 compuestos diferentes, entre ellos ácidos orgánicos, alcoholes, aldehídos, alcanos, cetonas, terpenos, ésteres, hidrocarburos aromáticos y furanos. Cabe destacar que el nivel de maduración del fruto fue el factor que más influyó, la mayoría de los COV fueron significativamente más altos en los frutos verdes. Se realizó un análisis discriminante multivariado para evaluar las diferencias atribuibles al injerto y/o variedades tradicionales. Fruto de este análisis fuimos capaces de separar las distintas variedades tanto injertadas como no injertadas en los dos estados de maduración, aunque esta separación fue más evidente en los frutos verdes que en los rojos.

Por todo ello, los resultados obtenidos de este estudio respaldan nuestra hipótesis de que el injerto podría ser una técnica interesante para mejorar el rendimiento y la calidad comercial de las variedades tradicionales de pimiento, principalmente reduciendo la incidencia de BER, sin afectar negativamente la calidad intrínseca de la fruta, ofreciéndoles la oportunidad de competir en un mercado cada vez más exigente. Estos resultados también brindan información útil sobre el efecto del injerto de pimiento sobre los compuestos bioactivos y la capacidad antioxidante, con una variación más relacionada con la etapa de madurez y el genotipo. Este estudio también puede representar un enfoque útil para caracterizar el efecto del injerto en el metabolismo de la planta.

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Capítulo 8

Conclusión general

Como resultado de todo el trabajo realizado en esta tesis doctoral, las conclusiones para los diferentes apartados son las siguientes:

Estrés térmico por altas temperaturas:

- Se han seleccionado accesiones de pimiento, especialmente A57, que utilizadas como portainjertos confieren a la variedad injertada cierto grado de tolerancia al estrés térmico por altas temperaturas.
- El uso de un portainjerto tolerante al estrés por altas temperaturas permite a la variedad generar respuestas a los efectos negativos de este estrés mediante la modificación de su comportamiento fisiológico.
- Esta respuesta fisiológica al estrés conferida por el portainjerto tolerante observada en las hojas, resulta en una mejor adaptación en su fase reproductiva, incrementando con ello el cuajado de frutos y en consecuencia el rendimiento comercial.

Estrés hídrico:

- La tolerancia al estrés hídrico de una planta injertada es consecuencia de una modificación fisiológica conferida por un portainjerto tolerante, que permite mantener los niveles de fotosíntesis con una adecuada eficiencia en el uso del agua, resultando en un mayor rendimiento comercial.
- El uso de la estrategia de riego deficitario combinada con el uso de portainjertos tolerantes al estrés hídrico, como es el caso del NIBER®, puede considerarse útil para reducir el consumo de agua, amortiguando los efectos sobre el rendimiento. Este hecho puede atribuirse a un mayor desarrollo del sistema radical.
- Utilizando el portainjerto de pimiento NIBER®, el rendimiento del cultivo se incrementa tanto en condiciones normales de riego como de estrés hídrico debido principalmente a la menor incidencia de BER.

Rendimiento y calidad del fruto:

- El uso de portainjertos tolerantes a estreses abióticos en variedades tradicionales de pimiento, como es NIBER®, podría ser una técnica útil para mejorar el rendimiento y la calidad comercial de este cultivo, principalmente al disminuir la incidencia de BER, sin afectar negativamente la calidad intrínseca del fruto.