Programa de Doctorado en Bioingeniería

TESIS DOCTORAL

Caracterización de los factores abióticos que intervienen en la aparición de ciertas fisiopatías en el cultivo sin suelo

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Elche, 2022

La presente Tesis Doctoral, titulada *"Caracterización de los factores abióticos que intervienen en la aparición de ciertas fisiopatías en el cultivo sin suelo"*, se presenta bajo la modalidad de **tesis por compendio** de las siguientes **publicaciones**:

- **Virginia Birlanga,** José Ramón Acosta-Motos, José Manuel Pérez-Pérez (2021). Genotype-dependent tipburn severity during lettuce hydroponic culture is associated with altered nutrient leaf content. *Agronomy* **11,** 616. doi: 10.3390/agronomy11040616**.**

- **Virginia Birlanga,** José Ramón Acosta-Motos, José Manuel Pérez-Pérez (2022). Mitigation of calcium-related disorders in soilless production systems" *Agronomy* **12,** 644. doi: 10.3390/agronomy12030644**.**

El Dr. D. José Manuel Pérez Pérez, director de la tesis doctoral titulada **“Caracterización de los factores abióticos que intervienen en la aparición de ciertas fisiopatías en el cultivo sin suelo”**

INFORMA:

Que Dña. Virginia Birlanga Murcia ha realizado bajo mi supervisión el trabajo titulado **“Caracterización de los factores abióticos que intervienen en la aparición de ciertas fisiopatías en el cultivo sin suelo”** conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

Lo que firmo para los efectos oportunos, en Elche.

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a.C.	Antes de Cristo
ABA	Ácido abscísico
B^+	Catión boro
BER	*Blossom end rot* (podredumbre apical)
°C	Grados Celsius
Ca	Calcio
Ca $(NO_3)_2$	Nitrato cálcico
Ca^{2+}	Catión calcio
CAX1	*Cation exchanger 1 (*Intercambiador de cationes 1*)*
CEBAS-CSIC	Centro de Edafología y Biología Aplicada del Segura. Consejo Superior de Investigaciones Científicas
CHD	*Crisphead*
Cl^-	Anión cloruro
CO_2	Dióxido de carbono
Cu	Cobre
dap	*Days after planting (*días después de la plantación)
dch	Día del trasplante a cultivo hidropónico
DFT	*Deep floating techique* (técnica de piscinas flotantes)
DW	*Dry weight* (peso seco)
EE. UU	Estados Unidos
f	Número de apertura de la cámara
Fe	Hierro
FW	*Fresh weight* (peso fresco)
g	Gramos
G × A	Interacción genotipo-ambiente
GAs	Giberelinas
GHG	*Net greenhouse gas* (Gases de efecto invernadero netos)
GOAK	*Green oak* (hoja de roble verde)
h	Horas
ha	hectáreas
I	Maduración intermedia (hoja lechuga))
IPCC	*Intergovernmental panel on climate change* (Grupo Intergubernamental de Expertos sobre el Cambio Climático)
J	Juvenil (hoja lechuga)
K	Potasio
K^+	Catión potasio

Kcal	Kilocalorías
L	Litros
L.	Lactuca
LSD	*Least significant differences* (Diferencias mínimas significativas)
M	Madura (hoja lechuga)
M/J ratios	Ratio entre Hojas maduras y juveniles
Mg	Magnesio
ml	Mililitros
Mn	Manganeso
µg	Microgramo
mS/cm	Milisiemens por centímetro
N	Norte
Na	Sodio
Na^+	Catión sodio
NFT	*Nutrient film technique (*la técnica de la película nutriente*)*
NGST	*New growing system technique (*Nueva técnica de sistema de cultivo*)*
NO_3^-	Anión Nitrato
O_2	Oxígeno
P	Fósforo
PCR	*Polymerase Chain Reaction* (Reacción en Cadena de la Polimerasa)
PGPRs	*Plant growth-promoting rhizobacteria* (Rizobacterias promotoras del crecimiento vegetal)
PME	*Pectin methylesterases* (Pectinas metilesterasas)
PO_4^{2-}	Anión fosfato
QTL	*Quantitative trait loci* (loci de rasgos cuantitativos)
R:S	*Root-to-shoot ratio* (Relación raíz/brote)
RA	*Root Area* (área radicular)
Ref.	Referencia
RGB	*Red green blue (*rojo, verde, azul, sistema de composición de colores basado en la adición de los colores primarios de la luz)
RIL	*Recombinant inbred lines* (líneas endógamas recombinantes)
RIL	*Recombinan inbred line* (Línea de reproducción recombinada)
ROAK	*Red oak* (hoja de roble roja)
RSA	*Root system architecture (*arquitectura radicular*)*
RWC	*Root water content (*contenido de agua radicular)
S.L.U	Sociedad de Responsabilidad Limitada Unipersonal
SA	*Shoot area* (área foliar)

Se	Selenio
SEM	*Standard error of the mean* (error estándar de la media)
SWC	*Shoot water content(*contenido de agua en la parte aérea)
TB	*Tipburn* (necrosis del margen foliar)
TS	*Tipburn severity* (severidad de Tipburn)
V.B.	Virginia Birlanga
W	Vatios
W	*West* (oeste)
WC	*Water content* (contenido de agua)
Zn	Zinc

ABSTRACT

In the current scenario of human-induced climate change, extreme weather events are likely to affect agricultural production worldwide. Soilless production systems have recently emerged as a solution to optimise the use of natural resources, such as water and soil, and will therefore contribute to reducing the environmental impact of agriculture.

Nutritional imbalance due to adverse environmental factors, such as drought, high temperatures and salinity, could lead to calcium-related physiological disorders during plant growth, such as blossom end rot (BER) in fruits and tipburn (TB) in leaves, which are a serious problem in crop production.

The analysis of the agronomic, physiological and genetic factors that favour the induction of physiopathies such as BER in tomato and TB in lettuce is essential to understand the mechanisms involved in these degeneration processes and to be able to provide solutions, from the exogenous supply of nutrients at certain stages of the crop to the genetic improvement of the varieties to be used.

In this thesis, a low-cost closed hydroponic culture procedure has been devised to evaluate the growth of lettuce in a hydroponic culture. Growth rate, plant mass and rate of TB at different stages of the year were monitored by imaging and associated with their nutrient concentration. We studied 12 lettuce genotypes of crisphead and oak-leaf subtypes, which differed in their resistance to TB, during three growing seasons (autumn, winter and spring). We found interesting genotype × environment (G × E)

interactions for some of the traits studied during early growth. By analysing TB incidence and leaf nutrient content, we were able to identify several nutritional traits that were highly correlated with cultivar- and genotype-dependent TB.

Technification of processes, a digitalisation of systems and continuous improvement of genetic factors of varieties in agriculture will provide us with the right tools for an accurate use of natural resources required by agriculture in the coming years.

RESUMEN GLOBAL

En el actual escenario de cambio climático provocado por el ser humano, es probable que los fenómenos meteorológicos extremos afecten a la producción agrícola en todo el mundo. Los sistemas de producción sin suelo han surgido recientemente como solución para optimizar el uso de los recursos naturales, como el agua y el suelo, y por tanto contribuirán a reducir el impacto ambiental de la agricultura.

El desequilibrio nutricional debido a factores ambientales adversos, como la sequía, las altas temperaturas y la salinidad, podría producir trastornos fisiológicos relacionados con el calcio durante el crecimiento de las plantas, como la podredumbre del extremo de la flor o *blossom end rot* (BER) en los frutos y la quemadura de la punta o *tipburn* (TB) en las hojas, que constituyen un grave problema en la producción de cultivos.

El análisis de los factores agronómicos, fisiológicos y genéticos que favorecen la inducción de fisiopatías como la BER en el tomate y la TB en la lechuga es fundamental para entender los mecanismos que intervienen en estos procesos de degeneración y poder proporcionar soluciones desde su aporte exógeno de nutrientes en ciertas etapas del cultivo como en la mejora genética de las variedades a utilizar.

En esta Tesis se ha ideado un procedimiento de bajo coste de cultivo hidropónico cerrado, para así poder evaluar el crecimiento de lechugas en un cultivo hidropónico. Se monitoreó mediante imágenes la tasa de crecimiento, la masa vegetal y el grado de TB en diferentes etapas del año y

se asoció a su concentración nutricional. Estudiamos 12 genotipos de lechuga de los subtipos *crisphead* y hoja de roble, que diferían en su resistencia al TB, durante tres temporadas de cultivo (otoño, invierno y primavera). Encontramos interesantes interacciones genotipo × ambiente (G × A) para algunos de los rasgos estudiados durante el crecimiento temprano. Al analizar la incidencia de TB y el contenido de nutrientes en las hojas, pudimos identificar una serie de rasgos nutricionales que estaban altamente correlacionados con el TB dependiente del cultivar y del genotipo.

La tecnificación de los procesos, la digitalización de los sistemas y una mejora continua de los factores genéticos de las variedades nos proporcionarán las herramientas adecuadas para un uso preciso de los recursos naturales que requiere la agricultura durante los próximos años.

1. INTRODUCCIÓN GENERAL

1.1 Problemática de la agricultura en el contexto de cambio climático actual

El Grupo Intergubernamental de Expertos de Naciones Unidas sobre el Cambio Climático (IPCC por sus siglas en inglés) en el resumen para los responsables políticos del Grupo de Trabajo III en su Contribución del al Sexto Informe de Evaluación 2022 [1], coincide en su afirmación de que las actividades realizadas por el ser humano son la causa principal del calentamiento global que se ha observado desde mediados del siglo XX hasta la actualidad (Figura 1).

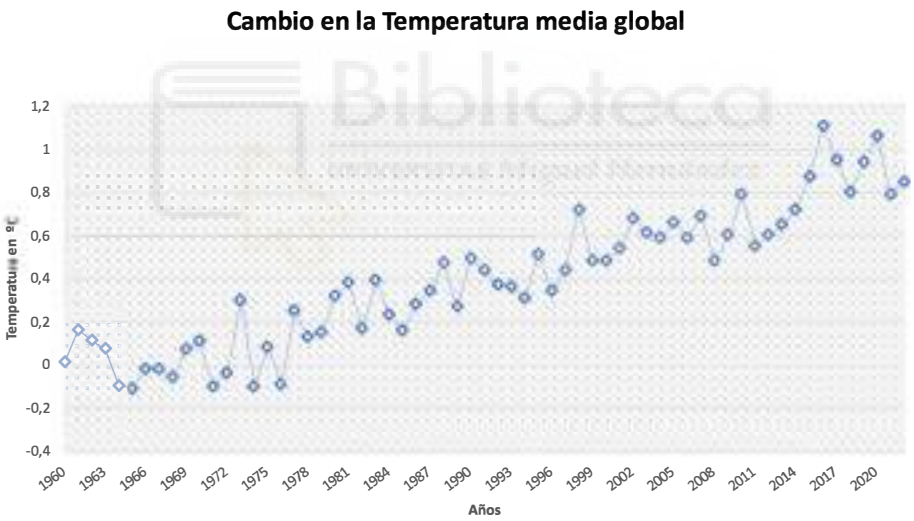

Figura 1. Calentamiento global últimos 60 años. Datos obtenidos de [2]

La implementación de nuevas estrategias para paliar los efectos del cambio climático se debe llevar a cabo de forma rápida y coordinada, debido al ritmo al que están aumentando los gases de efecto invernadero (GHG) en la atmósfera y el peligro que ello conlleva de que la temperatura global aumente más de 2 °C de media en la

próxima década [3]. En el último informe del IPCC se ha estimado que un 23 % del total de las emisiones de GHG generadas entre 2007 y 2016 provienen principalmente de la agricultura, la silvicultura y otros usos antropogénicos de los recursos terrestres. La preocupación por el cambio climático global y la búsqueda de medidas que permitan minimizar su impacto ambiental ha hecho que se pongan sobre la mesa nuevas estrategias de uso y consumo de los recursos naturales.

Debido a su situación geográfica y a sus condiciones climáticas de temperaturas elevadas y precipitaciones escasas, España, y específicamente las comunidades autónomas de la zona mediterránea, se encuentra entre las regiones más proclives a verse afectadas por los efectos del cambio climático global [4]. Se han desarrollado diversas estrategias para reducir el uso excesivo de los recursos naturales, evitar la acumulación de GHG en la atmosfera y proteger los suelos de la desertificación y la contaminación. Los suelos actúan tanto como una fuente como un sumidero de GHG y desempeñan un papel crucial en el intercambio de energía, agua y aerosoles con la atmósfera. Por tanto, la gestión sostenible de los suelos puede ayudar a atenuar los impactos negativos de los diversos factores de estrés ambiental, especialmente los que dependen del cambio climático, sobre los ecosistemas y las sociedades que se basan en la agricultura [3].

La tecnificación de los métodos de cultivo, así como la digitalización de las actividades agrícolas y la obtención de nuevas variedades que crezcan de forma más eficiente y se adapten mejor a las condiciones adversas, son los principales focos en los que los agricultores están poniendo su empeño en aplicar una agricultura sostenible. Este enfoque debe ser respetuoso con el medio ambiente y encontrar un equilibrio adecuado, considerando que las nuevas prácticas de cultivo van a permitir obtener mejores rendimientos y

aumentar los ingresos de los agricultores sin que se afecten negativamente otros indicadores de calidad medioambiental [5,6].

1.2 Sistemas de cultivo sin suelo: ventajas y limitaciones

El incremento de la población mundial y de la demanda de alimentos, la excesiva urbanización, la salinización y desertización de los suelos de cultivo debido a estreses ambientales relacionados con el cambio climático, han reducido la disponibilidad de suelos fértiles para su uso en agricultura. Por este motivo, muchos sectores agrícolas han decidido utilizar técnicas de cultivo sin suelo que les están permitiendo mantener un equilibrio adecuado entre la producción agrícola y la sostenibilidad del medio ambiente. Con el cultivo sin suelo se busca reducir el uso excesivo de agua y nutrientes, evitar la contaminación de suelos por lixiviación de nitratos y fosfatos que, finalmente, pueden provocar eutrofización y la contaminación de los acuíferos y las aguas superficiales [7–9].

La obtención de alimentos mediante el uso de sistemas de producción sin suelo permite aplicar una agricultura sostenible e incrementar la seguridad alimentaria [7,10]. La agricultura sin suelo contribuye también a mejorar el crecimiento de las plantas y producir alimentos de excelente calidad gracias a una gestión adecuada del sistema radicular de las plantas, ya que se ejerce un control más uniforme y preciso de las necesidades de agua y fertilizantes de las plantas durante las distintas etapas de su ciclo vital [8,11]. En la Tabla 1 se muestran las principales ventajas e inconvenientes que proporciona el uso del cultivo sin suelo.

Tabla 1. Ventajas e inconvenientes de los sistemas de cultivo sin suelo.

Ventajas	Inconvenientes
• Productos de calidad	• Inversión económica elevada de las instalaciones
• Reducción del desperdicio de agua	• Utilización de agua de calidad
• Control exhaustivo de plagas	• Enfermedades en la raíz por falta de sustrato
• Mejor control de la relación suelo/aire	• Mayor riesgo de problemas al mínimo cambio en el manejo de las condiciones ambientales
• Control de la zona radicular	• Posibilidad de contaminación de subsuelos en circuitos abiertos
• Mayor precocidad	• Cambios de temperatura pueden afectar de forma más directa a las raíces
• Mayor rendimiento de los cultivos	• Nivel de cualificación alto en el manejo de los cultivos
• Menor impacto ambiental que en un suelo convencional	• Posibilidad de contaminación de patógenos de una a planta a otra a través del agua
• Posibilidad de monitoreo a nivel fisiológico y productivo	
• Posibilidad de automatización	

1.3 Fisiopatías causadas por desequilibrios nutricionales

El uso de sistemas de cultivo sin suelo supone una elección especialmente válida en zonas donde hay una grave degradación del suelo y el suministro de agua está limitado. Como se ha comentado anteriormente, un elevado grado de especialización es necesario para una correcta selección de las variedades a plantar teniendo en cuenta

su sistema radicular y el manejo de la solución nutritiva, así como el control del entorno o ambiente donde van a crecer las plantas [10,11]. El control adecuado de los sistemas sin suelo proporciona mayores rendimientos y una mejor calidad en la producción (Figura 2).

Figura 2. Conjunto de factores que intervienen en la producción y rendimiento de las plantas. Datos obtenidos de [12].

El sistema radicular de las plantas es el encargado de proporcionar la suficiente concentración de nutrientes y agua al resto de órganos en desarrollo para un correcto crecimiento y diferenciación de la planta [12]. Las características de la solución nutritiva, la regulación fisiológica de la planta, así como el entorno donde se desarrolla el sistema radicular va a determinar la capacidad de las raíces de absorber la suficiente cantidad de nutrientes y de agua [13]. Además, estas deben de ser metabólicamente activas, de lo contrario, se producirán cambios en la capacidad de absorción y por lo tanto podrían aparecer desequilibrios nutricionales [12,14].

Los desequilibrios nutricionales y la falta de absorción de agua por parte de las raíces durante en el desarrollo de las plantas pueden desde detener el crecimiento o promover de manera incontrolada el crecimiento vegetativo, hasta la posibilidad de que ocurran abortos de flores y/o reducción de los rendimientos por una disminución en el número de frutos [15,16]. Si estos problemas se producen durante el desarrollo de los frutos y /o de las hojas estos pueden dar lugar a la aparición de fisiopatías como la podredumbre apical del fruto de tomate, peseta o *blossom end rot* (BER), y la necrosis del margen foliar en hojas de lechuga o *tipburn* (TB), provocando una merma de la calidad de la producción [17–21].

Hay muchos elementos que pueden desencadenar desequilibrios nutricionales. Factores como la salinidad del medio de cultivo, las altas temperaturas o fotoperiodos muy largos influyen en un correcto metabolismo y desarrollo de la planta en particular del sistema radicular. Se ha estudiado que en los sistemas de cultivo sin suelo con circuito cerrado pueden aparecer cierta salinidad en el medio, ya que las raíces no son capaces de absorber todos los nutrientes de la solución y, además, el agua que se aporta a la solución nutritiva contiene iones como Na^+ y Cl^- que son difíciles de absorber por las raíces de la planta. Todo ello conlleva una alta concentración de sales en el medio de cultivo, por lo que un control adecuado de las cantidades nutricionales aportadas a la planta en cada estado fisiológico es muy importante [22].

El estrés por temperaturas elevadas se produce cuando se produce un aumento de las temperaturas de al menos 10–15 °C por encima de la temperatura óptima de desarrollo, rebasando la denominada temperatura máxima crítica (Tabla 2) durante un largo periodo de tiempo, lo que puede desencadenar daños irreversibles en el desarrollo de las plantas [23].

Las altas temperaturas pueden provocar, dependiendo del estado de desarrollo de la planta, diversos problemas de crecimiento como son: (1) inhibir o ralentizar la germinación de la semilla [23], (2) provocar abortos de flores y frutos jóvenes [15,24], (3) favorecer una elevada transpiración en las hojas a través de los estomas lo que puede originar desequilibrios nutricionales por un excesivo y brusco transporte vía xilema de agua pero no así de algunos nutrientes, como el Ca^{2+}, hacia los órganos en desarrollo como los frutos y las hojas, ocasionando BER en tomates y pimientos o TB en lechugas o brasicáceas [25–28]. Por lo tanto, un adecuado manejo de las temperaturas en los invernaderos permitirá también un correcto desarrollo de las plantas y la limitación de los efectos negativos de las fisiopatías sobre el rendimiento y la calidad de los cultivos.

Tabla 2. Temperaturas máximas críticas. Datos obtenidos de [29] con algunas modificaciones.

Cultivo	Fase de desarrollo	Temperatura máxima crítica (°C)
Coliflor	Crecimiento de la cabeza	22
Lechuga	Crecimiento de la cabeza	28
Tomate	Dos semanas antes de antesis	29
Pimiento	Floración	32
Maíz dulce	3–4 semanas después de la floración	32
Aguacate	Floración y desarrollo del fruto	33
Calabaza	Floración	35

1.4 Generalidades del cultivo de lechuga

La lechuga (*Lactuca sativa*. L) es uno de los cultivos de hortalizas de hoja más importantes del mundo y se produce en climas moderados (temperatura media alrededor de 15 °C y precipitaciones de unos 500–1000 mm al año). Casi exclusivamente se utilizan para

consumo en fresco y hay ciertos mercados en auge que suelen utilizar sus hojas para hacer mezclas de ensaladas o alimentos de cuarta gama. La producción mundial en 2020 ascendió a 27.6 millones de toneladas siendo los principales países productores China con un 51.8 % de la producción total mundial, EE. UU. con un 15.9 %, e India y España, con un 4.1 % y un 3.5% respectivamente [30].

Figura 3. Producción de lechuga en España (miles de toneladas). Datos obtenidos de [28].

En España en los últimos años se ha cosechado alrededor de 34 000 ha y se han obtenido unas 969 000 toneladas de producción [30] siendo las principales comunidades españolas productoras de lechuga, la Región de Murcia (421 000 toneladas), Andalucía (391 900 toneladas) y un 23% restante que se reparten principalmente entre la Comunidad Valenciana, Castilla-La Mancha, Canarias, y Galicia (Figura 3).

La lechuga pertenece al género *Lactuca*, este género se encuentra dentro de la familia *Asteráceas* (Compuestas) que se compone de más de 1 300 géneros y 20 000 especies, de las cuales

muy pocas se cultivan [31,32]. Una de las principales características de esta familia es que presentan flores compuestas con múltiples inflorescencias [32]. Además de la lechuga, otras especies de interés agrícola en esta familia son la achicoria, la endibia, la escarola, el girasol, la alcachofa, la manzanilla o el cardo [31].

Figura 4. Distribución geográfica de las principales especies del género *Lactuca*. Datos obtenidos de [33], con algunas modificaciones.

El género *Lactuca* incluye al menos 100 especies que se distribuyen principalmente en la región mediterránea del hemisferio norte, siendo 17 de ellas nativas de Europa [32,33]. El género *Lactuca* se divide en siete subsecciones (*Lactuca*, subsección *Lactuca*, *Phoenixopus*, *Mulgedium*, *Lactucopsis*, *Tuberosae*, *Micranthae* y *Sororiae*) [32–35]. Según su origen geográfico, se distinguen cinco especies principales *L. serriola*, *L. salingna*, *L. virosa*, *L. altaica* y *L. sativa* (lechuga cultivada), esta última especie está presente principalmente en Europa (Figura 4;[36]).

Al menos tres de estas especies son sexualmente compatibles con la lechuga cultivada, *L. sativa* [33,35,37], lo que proporciona recursos genéticos importantes en la mejora de esta especie [35,38].

Muchos programas de mejora genética ponen el foco en estas especies ya que son fuentes genéticas muy interesantes que ayudan a incrementar notablemente la variabilidad de la especie en características de tipo tanto morfológicas como de resistencias a enfermedades o estreses o adaptabilidad a diversos ambientes [34,39,40].

Muchos sistemas de clasificación taxonómica de la subsección *Lactuca* se han propuesto mediante el uso de marcadores moleculares, morfológicos, genéticos y bioquímicos a lo largo de los años. Algunos ejemplos son las técnicas como los marcadores de proteínas [41,42], la utilización de mapeo de genes mediante marcadores polimórficos [35,43–46] o técnicas basadas en la reacción en cadena de la polimerasa (PCR por sus siglas en inglés) [47].

Dentro la especie *L. sativa* hay diversas subespecies cultivadas muy conocidas distinguidas por su morfología [46], como (1) Mantecosa o Trocadero (var. *capitata* L.), que se caracteriza porque sus hojas blandas forman cogollo, son lobuladas y comprenden gamas de colores desde verdes muy claritos a verdes oscuros que se cultivan principalmente en Inglaterra, Francia, Holanda y el oeste de Europa central [39]; (2) Iceberg y Batavia (var. *capitata* L.) cuyas hojas son crujientes y forman un cogollo y también pueden ser de una amplia gama de verdes, cultivándose en EE. UU. (principalmente el tipo Iceberg) y en Inglaterra, Francia, Holanda (principalmente el tipo Batavia) [39]; (3) Romanas (var. *longuifolia* L.), con sus hojas lobuladas y que crecen de forma alargada con un tallo principal grueso, cultivándose especialmente en áreas mediterráneas de Europa, este de Asia y norte de África [32,39]; (4) lechugas de corte, hoja de roble y Lollos (var. *crispa* L.*)* en este caso sus hojas no forman cogollo y crecen alrededor del tallo dejando un hueco en el centro en forma de roseta. Se cultivan principalmente en Italia, Francia y Republica Checa [41]. Su morfología es bastante heterogénea pudiendo ser desde hojas

lisas a muy rizadas y serradas. También cubre una amplia gama de colores que van desde rojos intensos a verdes muy claros [32]; (5) lechugas Espárrago (var. *asparagina*), sus hojas amargas crecen junto al tallo de forma vertical, y se suelen comer cocinadas. Muchos autores localizan esta morfología propia de las lechugas Espárrago en los ancestros de *Lactuca* originarios en Egipto [32] pero principalmente se cultiva en China [41]; (6) lechuga latina o grasa (var. *latin*) [48], sus hojas son parecidas a las hojas tipo mantecosa, no forman cabezas *per se*, sino que sus hojas crecen alrededor del tallo y se cultivan principalmente en la zona del mediterráneo [32].

El origen de la lechuga no se conoce con detalle, se puede distinguir diferentes orígenes según su genética y su geografía. Algunos autores indican que *L*. *serriola* y *L*. *sativa* pertenecen a una población híbrida y heterogénea, de la que *L. sativa* habría surgido como resultado de la selección antrópica, mientras que *L. serriola* habría surgido como adaptación a los diferentes ambientes donde crece de forma asilvestrada [32,33,49]. El origen geográfico atribuido a las especies de *Lactuca* es Egipto e Irán [49] donde se han encontrado jeroglíficos y pinturas de hace más de 6 500 años en las que aparecen plantas cuyas características morfológicas eran muy similares a la lechuga tipo romana actual [49]. Los egipcios utilizaban las hojas de estas plantas como forraje para el ganado y para extraer aceite de las semillas, entre otros usos [50,51]. Se conoce que de Egipto fue transportada a Grecia, ya que aparece reflejada en los escritos de Sócrates (450 a.C.), Aristóteles (356 a.C.), Teofrasto (322 a.C.) y Dioscórides (60 a.C.) y también es utilizada por los romanos por lo que rápidamente se extendió su cultivo por toda Europa [52]. Finalmente, llegó a América en 1494 de la mano de los españoles tras el descubrimiento de este continente [51].

1.5 Programa de mejora de lechuga en la empresa

La lechuga cultivada (*Lactuca sativa* L.) es una planta herbácea anual y autógama, contiene un numero de cromosomas diploide (2n=18), presenta inflorescencias de 20-25 flores tubulares en una sola bráctea, lo que visualmente hace parecer una sola flor grande, obteniendo por cada inflorescencia alrededor de 20-25– semillas [53]. Una de las particularidades de esta especie es su reproducción, ya que la maduración de las anteras y la posterior polinización por el polen durante el crecimiento de los pistilos ocurre antes de la apertura de la flor, estrategia reproductiva llamada cleistogamia [54]. Este sistema reproductivo hace que, en condiciones naturales, sus poblaciones silvestres y/o cultivadas sean líneas endogámicas [37,44,55]. El alto porcentaje de homocigosis proporciona uniformidad fenotípica y estabilidad genotípica en las líneas de lechuga utilizadas habitualmente en los programas de mejora genética [54].

Uno de los principales objetivos de cualquier programa de mejora es poder aportar características interesantes y deseables para el consumidor y el productor. Entre estas características se incluyen caracteres morfológicos, de color, resistencia a enfermedades, tolerancia a estreses abióticos como bióticos o aumentar el rendimiento del cultivo o la producción. Según estas premisas, el programa genético de la empresa Bayer (Monsanto Agricultura España, S.L.U.) en Murcia se basa en el desarrollo de nuevas variedades de lechugas con la combinación de los métodos de mejora convencionales asistidos por marcadores moleculares. La importancia de la utilización de los marcadores moleculares es poder combinar toda la información fenotípica de las poblaciones con su genotipo. También se trabaja con la introgresión en líneas elite de características procedentes de especies silvestres como *L. virosa*, *L. saligna* o *L. serriola*. Para ello se utiliza una estrategia basada en la introgresión de

los caracteres de interés en líneas élite mediante retrocruzamiento y selección mediante marcadores [54].

1.6 Objetivos de mi Tesis Doctoral

La limitación creciente de los recursos naturales, como la disponibilidad de agua de riego y nutrientes minerales, o la pérdida de suelos fértiles por cambios en el uso del suelo o su contaminación, exige el desarrollo de nuevas formas de cultivo que permitan un mejor aprovechamiento de los recursos disponibles y la reducción de los costes de producción. Es por ello que una mejor comprensión de la estructura y función del sistema radicular, es fundamental para la absorción de agua y nutrientes del suelo y, en consecuencia, para la mejora de la productividad y posterior rendimiento de los cultivos.

Una de las estrategias de mejora durante el desarrollo de nuevas variedades de lechuga y de tomate es la tolerancia a la necrosis del margen foliar en hoja de lechuga o *tipburn* (TB) y a la podredumbre apical en frutos del tomate, peseta o *blossom end rot* (BER). TB y BER son dos fisiopatías relacionadas con factores abióticos que pueden causar pérdidas notables en la producción de estos dos cultivos durante su crecimiento en condiciones de invernadero. Entender la respuesta de las plantas a situaciones de estrés y la contribución del sistema radicular a esta respuesta, nos proporcionará información relevante para el desarrollo de variedades tolerantes a estas fisiopatías.

En este contexto se enmarcan los dos trabajos científicos presentados en esta memoria y que constituyen mi Tesis Doctoral, encaminada a:

- Describir y revisar los principales factores que contribuyen al desarrollo de TB en lechuga y de BER en tomate.
- Proponer soluciones para reducir y/o paliar los problemas

relacionados con TB y BER en los sistemas de producción sin suelo.

- Desarrollar un sistema de cultivo hidropónico para el cultivo de lechuga en invernadero.
- Caracterizar el sistema radicular de lechuga durante su cultivo en hidroponía y cuantificar algunos parámetros de crecimiento.
- Caracterizar nutricionalmente los genotipos estudiados y su relación con el TB.

2. RESUMEN GLOBAL DE MATERIALES Y MÉTODOS

2.1 Sistema hidropónico para el cultivo de lechuga

Para este trabajo hemos diseñado un sistema de hidroponía estacionaria de raíz flotante en plántulas jóvenes de lechuga encaminado a la obtención de imágenes de su sistema radicular de forma periódica, que nos ha permitido llevar a cabo un seguimiento individualizado de las plántulas durante todo el proceso productivo (Figura 5A–C; véanse los materiales y métodos del segundo artículo en las páginas 65-67).

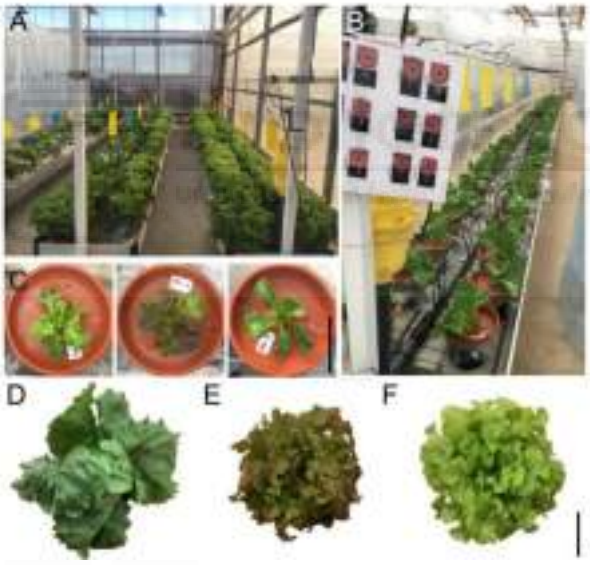

Figura 5. Sistema de cultivo hidropónico y cultivares de lechuga utilizados en este trabajo. (A-B) Ensayo en nuestros invernaderos con la técnica estacionaria de raíz flotante que se ha utilizado en este trabajo, tipología hoja de roble (A) e Iceberg (B). (C) Ejemplo de imágenes de la parte foliar en los distintos tipos de lechugas estudiados. (D-F) Detalle de la roseta en la lechuga de tipología Iceberg o *Crisphead* (CHD; D), de hoja de roble de tipo *Red Oak* (ROAK; E) o *Green Oak* (GOAK; F). Barras de escala: 10 cm.

El material genético utilizado en este estudio fue seleccionado de la colección de germoplasma del programa de mejora de lechuga de Monsanto Agricultura España S.L.U. (Murcia, España). Los genotipos se seleccionaron según los resultados de la evaluación de la tolerancia al TB realizada en la empresa entre los años 2016 a 2019. Para los experimentos se seleccionaron varios genotipos de las principales variedades de lechuga comercializadas en España [32].

- Lechuga Iceberg (*Lactuca sativa* subsp. *capitata* [L.]) también denominada *Crisphead* (CHD): se caracteriza por formar una cabeza con hojas generalmente verdes, gruesas y crujientes (Figura 5D). Tiene hojas redondas y muy apretadas siendo su textura crujiente y refrescante. Se llama Iceberg por su resistencia al frío. Esta variedad de lechuga es la menos nutritiva de todas y contiene una mayor concentración de azúcares (Tabla 3).
- Lechuga de hoja de roble (*Lactuca sativa* subsp. *crispa* [L.]) de color rojo (*Red Oak*; ROAK): se reconoce por sus hojas onduladas y sus tonalidades, que van de la verde a la púrpura, lo que le da un bonito color (Figura 5E). Morfológicamente muestra una presentación voluminosa por sus hojas rizadas, así como una textura tierna y ligeramente crujiente. Desde el punto de vista nutricional, esta variedad es rica en compuestos flavonoides, con capacidad antioxidante, y tiene un sabor ligeramente dulce. Cómo se muestra en la Tabla 3, este tipo de lechuga contiene mayor contenido en betacarotenos que el resto.
- Lechuga de hoja de roble de color verde (*Green Oak*; GOAK): no forma cogollo y tiene hojas lobuladas. Suele formar rosetas abiertas con hojas verdes separadas (Figura 5F), tiene una textura tierna y ligeramente crujiente, así como un sabor delicado y dulce. Tiene un contenido elevado en vitaminas A y C (Tabla 3).

Tabla 3. Valores nutricionales de los tres tipos de lechugas estudiados. Datos obtenidos de [55], con algunas modificaciones.

Composición nutricional (en 100 g de peso fresco)	CHD	GOAK	ROAK
Agua (g)	95.60	95.00	95.60
Energía (kcal)	14.00	15.00	13.00
Fibra (g)	1.20	1.30	0.90
Metabolismo primario (g)			
Proteínas	0.90	1.36	1.33
Lípidos totales	0.14	0.15	0.22
Hidratos de carbono	2.97	2.87	2.26
Azúcares	1.97	0.78	0.48
Glucosa	0.91	0.36	0.20
Fructosa	1.00	0.43	0.28
Sacarosa	0.05	0.00	0.00
Macronutrientes (mg)			
Potasio (K)	141.00	194.00	187.00
Calcio (Ca)	18.00	36.00	33.00
Fósforo (P)	20.00	29.00	28.00
Sodio (Na)	10.00	28.00	25.00
Magnesio (Mg)	7.00	13.00	12.00
Micronutrientes (mg, salvo que se indique lo contrario)			
Hierro (Fe)	0.41	0.86	1.20
Manganeso (Mn)	0.13	0.25	0.20
Zinc (Zn)	0.15	0.18	0.20
Cobre (Cu)	0.03	0.03	0.03
Selenio (Se, µg)	0.10	0.60	1.50

Tabla 3. Valores nutricionales de los tres tipos de lechugas estudiados. Datos obtenidos de [55], con algunas modificaciones (continuación).

Composición nutricional (en 100 g de peso fresco)	CHD	GOAK	ROAK
Vitaminas liposolubles (mg, salvo que se indique lo contrario)			
Vitamina A o retinol (µg)	25.00	370.00	375.00
Caroteno. beta (µg)	299.00	4440.00	4500.00
Caroteno. alfa (µg)	4.00	0.00	0.00
Luteína y zeaxantina (µg)	277.00	1730.00	1720.00
Vitamina E (alfa-tocoferol)	0.18	0.22	0.15
Tocoferol gama	0.09	0.41	0.24
Tocotrienol alfa	0.01	0.00	0.01
Vitamina K (filoquinona) (µg)	24.10	126.00	140.00
Vitaminas hidrosolubles (mg)			
Vitamina C (ácido ascórbico)	2.80	9.20	3.70
Tiamina o vitamina B1	0.04	0.07	0.06
Riboflavina o vitamina B2	0.03	0.08	0.08
Niacina o vitamina B3	0.12	0.38	0.32
Colina o vitamina B4	6.70	13.60	11.80
Ácido pantoténico o vitamina B5	0.09	0.13	0.14
Piridoxina o vitamina B6	0.04	0.09	0.10
Biotina o vitamina B8	0.1	0.2	0.2
Ácido fólico o vitamina B9 (µg)	29	38	36

Las semillas se sembraron en bandejas de 198 alveolos con un sustrato compuesto de 80 % de perlita y un 20 % de turba, y se regaron con agua hasta su saturación. A continuación, se incubaron durante 72 h en una cámara fría, a 10–12 °C y 70–75 % de humedad relativa, para sincronizar su germinación. Seguidamente, las bandejas se trasladaron al semillero, a 20 °C y condiciones de fotoperiodo (luz/oscuridad) de

12/12 h (otoño), 10/14 h (invierno) y 13/11 h (primavera). Cuando las plántulas presentaron 2 o 3 hojas expandidas, a los 25–30 días, se trasplantaron a un invernadero multitúnel para su cultivo hidropónico, con temperaturas medias de 17–21 °C durante el día y 8–12 °C durante la noche y con 10–13 h de luz y 14–11 h de oscuridad, respectivamente, dependiendo de la temporada de cultivo. Para evitar variaciones extremas de temperatura en el interior del invernadero, esta se controló mediante apertura y cierre de ventanas.

Para el cultivo hidropónico de lechuga se han utilizado macetas opacas y estancas de 3 L de capacidad que se sellaron por arriba con platos de plástico a los que se les hicieron dos orificios: uno central de unos 3 cm de diámetro para introducir la planta y otro lateral de 1 cm de diámetro para introducir el tubo de aireación. Las macetas se dispusieron en dos filas paralelas en 3 mesas de invernadero, utilizando la parte central de las mesas para la ubicación del tubo principal por donde se bombeaba el aire en las macetas mediante un compresor de aire (Astralpool, Ref. 06863) programado a una velocidad de descarga de 2.5 L/min y en ciclos de funcionamiento de 1 min cada 4 h. Las plantas se irrigaron con una solución nutritiva optimizada para cultivos de hoja basada en el trabajo de [56]. Los parámetros de esta disolución se calcularon para un depósito de 1500 L, la solución se extraía del depósito mediante una bomba de succión y con una manguera se añadía la solución nutritiva a las macetas una vez por semana. La conductividad eléctrica de la disolución en el momento de la preparación fue de 2.37 mS/cm y se mantuvo su pH entre 6 y 7 (véanse los materiales y métodos del segundo artículo en las páginas 65-67).

2.2 Análisis cuantitativo del crecimiento y del TB

Para llevar a cabo el análisis morfométrico del sistema radicular en las plántulas de lechuga cultivadas en hidroponía se ha desarrollado un procedimiento sencillo para la obtención de imágenes nítidas del sistema radicular en su totalidad. A partir de estas imágenes y mediante un proceso supervisado de análisis, hemos cuantificado algunos descriptores morfológicos de su crecimiento (véanse los materiales y métodos del segundo artículo en las páginas 65-67).

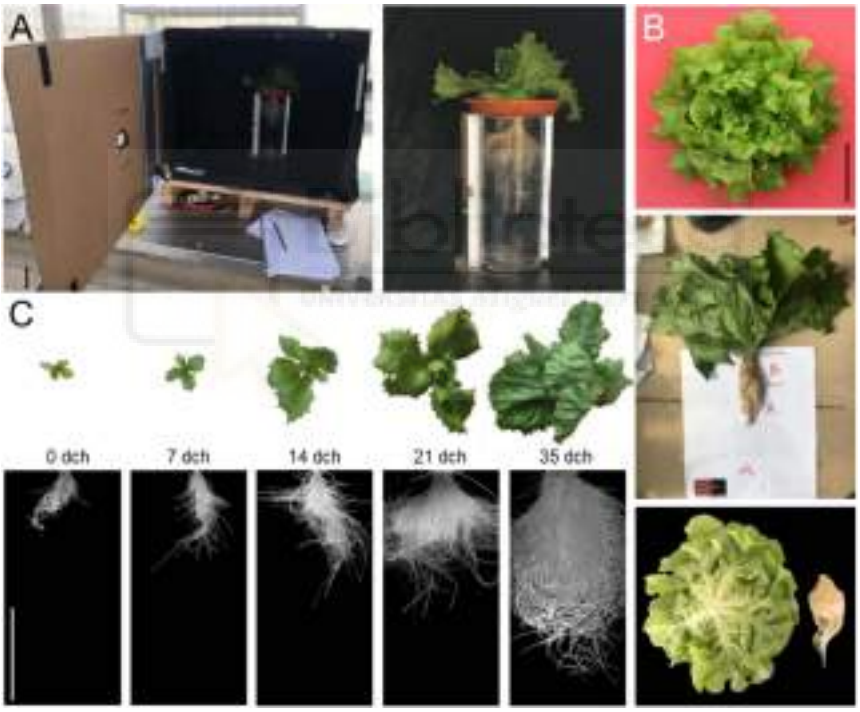

Figura 6. Procedimiento experimental utilizado para la toma de imágenes. (A) Banco de fotografía construido para la obtención de imágenes del sistema radicular. (B) Ejemplo de las imágenes obtenidas de la roseta vegetativa. (C) Imágenes del sistema radicular (abajo) y de la roseta vegetativa (arriba) que han sido procesadas para su análisis cuantitativo. Barras de escala: 10 cm.

Para la obtención de imágenes en condiciones homogéneas, se diseñó una caja opaca de cartón con estructura de madera de 50 × 40 × 70 cm. En la parte basal de la caja había una rejilla que permitía la iluminación desde abajo mediante un foco de 220 W de luz blanca. Sobre la rejilla se colocaba un jarrón cilíndrico de cristal, de 12 cm de diámetro y 28 cm de longitud, y el plato de plástico con las plántulas de lechuga sobre esta, con las raíces sumergidas en la disolución nutritiva (Figura 6A). Para la obtención de imágenes de la parte vegetativa de las plántulas, el plato de plástico se colocaba en una bandeja de color rojo con un hueco en el centro y rellena de solución nutritiva, lo que permitía que las raíces estuvieran en contacto con la disolución nutritiva en todo momento (Figura 6B). Las fotos se tomaron con la cámara de 12 megapíxeles, con una apertura de la cámara (también llamado f) de 2.2 en un teléfono móvil iPhone 6s.

Se han tomado fotos de la parte radicular y de la parte aérea desde el día del trasplante a cultivo hidropónico (0 dch) hasta la quinta semana de cultivo (35 dch). Las plántulas se muestrearon los días 0, 7, 14, 21, y 35 dch (Figura 6C). Al final del experimento se obtuvieron fotos de la parte radicular y de la parte aérea y se cosecharon por separado la parte aérea y la parte radicular de cada plántula. En la parte aérea, se midió el número de hojas de su roseta, la longitud del tallo principal y se obtuvo el preso fresco y seco, tanto de la parte aérea como de la radicular.

Para determinar la incidencia de TB en las plántulas estudiadas durante su muestreo, se ha establecido una escala cualitativa, valorada de 1 (sin síntomas) a 9 (síntomas agudos de TB). Para más detalle, véanse los materiales y métodos y la Figura S1 del segundo artículo en las páginas 65-67 y 84.

2.3 Análisis de nutrientes

Hemos medido la concentración de nutrientes minerales en las hojas de las plántulas en primavera. Para ello, se recogieron muestras a los 21 dch de hojas de la zona externa de la roseta (hojas más maduras), de la zona intermedia de la roseta (hojas intermedias), y de la zona más interna de la roseta (hojas más jóvenes) (Figura 7A). Se midió la longitud de cada hoja y se dividieron éstas en dos partes, correspondientes a la parte apical y a la parte basal de la hoja (Figura 7B). De cada muestra se tomaron fotos para el análisis del área y el perímetro foliar, y se midió el peso fresco y el peso seco. Las muestras se almacenaron envueltas en papel albal en un congelador a –80 °C. Las muestras se prepararon en primer lugar en el laboratorio de José Manuel Pérez Pérez de la universidad Miguel Hernández de Elche (UMH) para su posterior análisis en laboratorio de ionómica en el Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC). Cuando todas las muestras estuvieron recolectadas, se pesaron con una balanza de precisión (peso fresco) y se secaron durante 72 h en una estufa a 80 °C. A continuación, se pesaron de nuevo (peso seco) y se molieron en un mortero. Se introdujeron en tubos tipo *Eppendorf* de 1.5 mL (rotulados y pesados con anterioridad) y se volvieron a pesar.

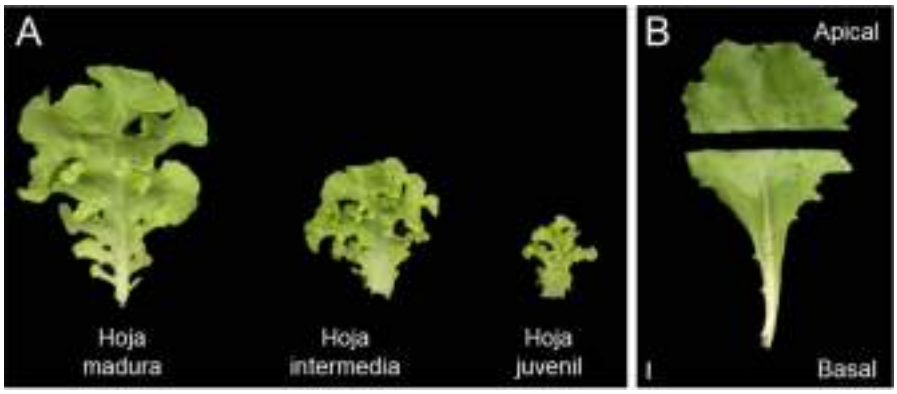

Figura 7. Muestras utilizadas para el análisis de nutrientes. (A) Tipología de las hojas utilizadas para el análisis de nutrientes. (B) Secciones en las que se han dividido las muestras de hojas maduras e intermedias. Barra de escala: 1 cm.

Una vez procesadas las muestras, se transportaron al laboratorio especializado en análisis de nutrientes que se encuentra en el CEBAS-CSIC en Murcia. Se utilizaron 2 mg de cada muestra para el análisis de nutrientes, según el procedimiento habitual utilizado en el laboratorio (véanse los materiales y métodos del segundo artículo en las páginas 65-67).

3. DISCUSIÓN

3.1 Sistemas de producción sin suelo

A partir de una revisión bibliográfica de las fuentes más recientes relacionadas con los nuevos sistemas de producción sin suelo, se concretaron que éstos surgen como una herramienta tecnológica que permite aprovechar al máximo los recursos limitados de agua, nutrientes y disponibilidad de área de cultivo. Una de las principales ventajas de este sistema de cultivo es el control preciso del componente ambiental dentro de los invernaderos y un manejo proporcionado de las necesidades nutricionales de las plantas en las distintas etapas de su ciclo vital [11].

Los sistemas de cultivo sin suelo pueden clasificarse en varios tipos en función del uso de la solución nutritiva o del estado físico del medio de crecimiento [57–59]. En consecuencia, distinguimos entre: (1) sistemas de circuito abierto si la solución nutritiva se desecha después de su uso, y (2) sistemas de circuito cerrado si la solución nutritiva se reformula después de su uso y se devuelve al sistema. La solución nutritiva se compone principalmente de agua, oxígeno y nutrientes esenciales para la planta [60]. Por otro lado, el sistema radicular puede crecer (*i*) directamente en el aire (cultivo aeropónico), (*ii*) en una solución nutritiva líquida (cultivo hidropónico y acuicultura cuando se combina con la producción piscícola), o (*iii*) en un sustrato sólido con solución nutritiva añadida (cultivo en sustrato inerte). Para más detalles, véanse el apartado 2 y las Figuras 1-4 del primer artículo en las páginas 48-52).

Para aprovechar los recursos ambientales y reducir los costes de producción, cada vez es más habitual el uso de sistemas cerrados, con el fin de reutilizar los desechos y/o productos derivados lo máximo posible, limitando la lixiviación de los nutrientes y la contaminación del suelo [61,62].

En esta Tesis doctoral se ha utilizado un sistema hidropónico estacionario de raíz flotante para evaluar el cultivo de lechuga en diferentes temporadas, ya que nos permitía evaluar el desarrollo radicular de forma individualizada, y reducir el consumo de agua y de solución nutritiva durante la realización de los experimentos.

3.2 Correlación con los factores que afectan al TB y el análisis cuantitativo del crecimiento de la lechuga

En los sistemas sin suelo es muy importante el manejo adecuado de la solución nutritiva debido a la falta de poder amortiguador y a la baja capacidad de intercambio catiónico del medio [60]. Factores como la temperatura, la radiación y la humedad relativa en el interior de los invernaderos favorecen la aparición de ciertas fisiopatías [18,25,63]. En estos casos, los principales órganos afectados son los frutos y las hojas. Dos ejemplos son el BER cuyo síntoma más destacable en tomate o pimiento es la podredumbre apical del fruto, y el TB que en cultivos de hoja como la lechuga causa la necrosis en el extremo de las hojas. BER y TB están causados generalmente por factores ambientales como un rápido aumento de las temperaturas, el estrés hídrico, salino o térmico que provocan un crecimiento acelerado de las plantas, induciendo a estas a una situación de estrés [64–66].

Como parte del trabajo en esta Tesis doctoral se evaluó el crecimiento radicular y foliar en un sistema hidropónico de cultivo en 12 genotipos de diferentes cultivares (4 por cada cultivar) de lechuga *Iceberg* (CHD), hoja de roble verde (GOAK) y hoja de roble roja (ROAK) en tres temporadas de cultivo (otoño, invierno y primavera). Este análisis proporcionó información relevante no solo a nivel de genotipo y cultivar sino de su interacción con el ambiente (interacción G × A), cuyos resultados fueron significativos en todos los cultivares

estudiados (véanse el apartado de resultados del segundo artículo en las páginas 68-78).

Las altas intensidades de luz y las temperaturas elevadas son factores ambientales que ocasionan un crecimiento acelerado de los frutos y de las hojas que pueden desencadenar la aparición de BER en tomate y TB en lechuga [63,67–72].

Como muestran los resultados de esta Tesis en el segundo artículo, encontramos que la mayor tasa de crecimiento de todos los cultivares de lechuga estudiados se produjo principalmente en primavera, donde las temperaturas fueron más altas que en otoño e invierno (véanse la Tabla S1 del segundo artículo en la página 90). Los resultados también mostraron que la incidencia de TB fue máxima en primavera, y mucho menor en invierno para la mayoría de los cultivares estudiados. Por tanto, el efecto combinado de una alta tasa de crecimiento y las elevadas temperaturas que se produjeron en primavera puede causar una reducción del suministro de nutrientes desde las raíces hacia las hojas en desarrollo, lo que resultaría en un aumento observado de la incidencia del TB en primavera (véanse el apartado de discusión del segundo artículo en las páginas 79-81).

Un factor importante en el sector agrícola y que se está viendo agravado debido al cambio climático son las sequías. Estas se definen como periodos en los que la cantidad de agua perdida por evapotranspiración a través de las hojas y el suelo es mayor que la cantidad de agua aportada por las precipitaciones y que pueden absorber las plantas por las raíces en contacto con el suelo. Las raíces junto con la disponibilidad de agua juegan un papel muy importante en la adaptabilidad a estos ambientes y diversos estudios demuestran que raíces más profundas son cruciales para una mayor tolerancia a la sequía en las plantas ya que son capaces de encontrar agua en zonas más profundas del suelo asegurando la disponibilidad de agua para la

planta [72–74].

En nuestro trabajo hemos podido comprobar que los genotipos CHD mostraron raíces más profundas en comparación con los de GOAK y ROAK. Por otra parte, se observaron que los sistemas radiculares de GOAK y ROAK tenían un peso mayor y eran más superficiales que los del cultivar CHD (véanse el apartado de discusión del segundo artículo en las páginas 79-81). Nuestros resultados podrían ayudar en la búsqueda y selección de variedades de lechuga con una alta tolerancia a la sequía, además de avanzar en la mejora genética de la lechuga frente al estrés y su adaptabilidad a las situaciones de cambio climático actual [75,76].

En nuestros resultados pudimos observar que la concentración de nutrientes totales en las hojas de CHD era menores que en los cultivares OAK (véanse el apartado de resultados del segundo artículo en las páginas 68-78). También los genotipos (C3 y C8) cuyos valores de nutrientes eran más bajos fueron los que mayores síntomas de TB mostraron, por lo que esto puede ser debido a un desequilibrio nutricional.

Además, se observó que estos genotipos también obtuvieron altas tasas de crecimiento coincidiendo con síntomas agudos de TB y también mostraron una menor concentración de calcio en sus hojas, esto apoya la hipótesis de que una alta tasa de crecimiento determina la cantidad de calcio en las hojas y que, en este caso, una baja disponibilidad de calcio está directamente relacionada con una alta incidencia de TB.

A nivel nutricional, nuestros resultados mostraron que los genotipos CHD obtuvieron una menor concentración de fósforo en las hojas frente a los genotipos OAK, lo que se correlaciona con un sistema radicular más superficial, que podría ser más eficiente en la absorción de fosfatos, ya que estos tienden a ser más abundantes en la zona

superficial del suelo [77].

3.3 BER, TB y posibles soluciones

Uno de los objetivos de la revisión bibliográfica que se ha realizado en esta Tesis es aportar soluciones para minimizar los diferentes trastornos fisiológicos, como el BER y el TB, cuya incidencia es probable que aumente en las próximas décadas debido al cambio climático.

Una de las posibles soluciones para mejorar el estado nutricional de las plantas es el uso de fertilizantes orgánicos, ya que se ha comprobado que en sistemas de producción de tomate puede mejorar el estado nutricional de las plantas, aumentar la producción de frutos y evitar problemas de BER [78,79]. También se ha observado que la aplicación exógena de calcio a las hojas de lechuga en momentos concretos de su desarrollo puede evitar problemas relacionados con los desequilibrios nutricionales como el TB [80].

Recientemente, se ha propuesto el uso de nanopartículas para mitigar los efectos de una fertilización excesiva, lo que podría conducir a una nutrición más precisa y a una reducción de los nutrientes aportados, tanto en los sistemas de cultivo convencionales como en los hidropónicos [10]. Algunas estrategias, como el uso de nanopartículas para la fertilización, podrían ayudar a suministrar nutrientes de forma muy precisa y localizada, especialmente en las etapas fisiológicas más críticas, como la fructificación, y así evitar los efectos causados por los desequilibrios nutricionales en ciertas fases del ciclo de crecimiento de la planta que son más sensibles a la aparición de BER y TB.

A lo largo de los años se ha estudiado el comportamiento de las fitohormonas en el sistema vascular de la planta y cómo pueden influir estas a lo largo del ciclo de cultivo. En el crecimiento del fruto se sabe que intervienen dos etapas: la división celular, influida por la

señalización de las auxinas, y la expansión celular, regulada conjuntamente por las auxinas y las giberelinas. La maduración del fruto se produce cuando los niveles de auxinas y giberelinas disminuyen con un aumento continuo de ácido abscísico (ABA) y etileno [81]. La aplicación de giberelinas en plantas de tomate se relacionó con el aumento de BER [20]. A raíz de este estudio se observó que la adición de un inhibidor de la biosíntesis de giberelinas en plantas de tomate redujo los síntomas de BER en los frutos [82]. La utilización de estos inhibidores podría ser una alternativa adecuada para disminuir los problemas de BER en la producción de tomate.

Hay que saber que una buena selección genética y/o varietal nos marcará principalmente el grado de TB y BER que van a presentar nuestros cultivos. Muchos estudios han destacado que la susceptibilidad a BER y TB es altamente dependiente del genotipo [18,72]. El uso de herramientas genómicas ha permitido la identificación de loci de rasgos cuantitativos o QTL (*quantitative trait loci*) para la incidencia de TB en varias poblaciones de líneas endógamas recombinantes o RIL (*recombinant inbred lines*) de lechuga y el posterior desarrollo de marcadores moleculares vinculados a estos QTL [83]. Estos estudios permitirán el desarrollo de marcadores moleculares adicionales y su utilización para incorporar los alelos de resistencia encontrados en este trabajo a los cultivares de lechuga CHD que son sensibles al TB [83,84].

Para poder acceder a un mayor control de los factores ambientales en un sistema de cultivo sin suelo es importante conocer las herramientas de las que se dispone actualmente para un control más exhaustivo de las condiciones ambientales, así como del control fisiológico preciso de los cultivos. El uso de prácticas de gestión inteligentes y su digitalización nos permiten controlar no solo el manejo del ambiente dentro del invernadero sino indicar los posibles estados críticos a nivel de la planta, su evapotranspiración, consumo

de agua y nutrientes e incluso la detección de patógenos.

Actualmente es posible automatizar un sistema de cultivo hidropónico utilizando sensores baratos que monitorizan y controlan parámetros ambientales como la intensidad de la luz, la humedad relativa, así como el pH, la conductividad eléctrica y la temperatura de la solución nutritiva [10,85,86]. En este sentido, Hasan et al. [87] utilizaron drones para detectar enfermedades en los tomates mediante el análisis de imágenes foliares. También el uso de sensores que miden procesos fisiológicos como la fotosíntesis, la transpiración y la conductancia estomática de las hojas ha permitido detectar y cuantificar el impacto del estrés por sequía en las plantas de tomate [88].

4. CONCLUSIONES Y PROYECCIÓN FUTURA

4.1 Conclusiones

- A partir de una revisión sistemática de los artículos publicados por otros autores, hemos resumido los factores abióticos y los mecanismos fisiológicos implicados en la necrosis foliar en hojas de lechuga y la podredumbre apical del fruto del tomate; ambas fisiopatías están relacionadas con la deficiente traslocación de calcio desde el sistema radicular hacia las hojas y el fruto respectivamente.

- Hemos valorado las ventajas e inconvenientes de las distintas modalidades de cultivo sin suelo, ya que permiten el uso de sensores remotos y su automatización para llevar a cabo el control preciso del equilibrio nutricional durante todo el ciclo de cultivo.

- Hemos propuesto la implementación de nuevas estrategias de mejora genética para el desarrollo de nuevos cultivares con niveles elevados de tolerancia frente a los factores ambientales relacionados con la necrosis foliar en hojas de lechuga y la podredumbre apical del fruto del tomate.

- Hemos diseñado un sistema de hidroponía estacionaria de raíz flotante en plántulas jóvenes de lechuga para llevar a cabo un seguimiento individualizado del crecimiento radicular y foliar de las plántulas durante todo el proceso productivo.

- Hemos evaluado el crecimiento radicular y foliar de 12 genotipos de diferentes cultivares de lechuga, CHD, GOAK y ROAK, en tres temporadas de cultivo, otoño, invierno y primavera, durante su cultivo en hidroponía.

- Los genotipos de lechuga CHD mostraron raíces más profundas en comparación con los GOAK y ROAK, lo que podría estar relacionado

con una mayor resistencia a la sequía de los genotipos CHD.

- Nuestros resultados sugieren que, en condiciones de hidroponía, un sistema radicular con raíces finas es más eficiente en la absorción de nutrientes, concretamente de fósforo, mientras que, en suelo, el mayor diámetro y longitud de la raíz favorecerá la exploración del sustrato y por tanto la búsqueda de agua y su absorción.

- El crecimiento de las plántulas de lechuga en condiciones de hidroponía fue mucho menor durante el invierno que en la primavera o el otoño. Tanto el peso fresco de las raíces como de los brotes dependen del tipo de cultivo y de la estación de crecimiento.

- Se ha observado que los genotipos CHD presentaban los niveles más bajos de nutrientes en sus hojas, mientras que en los genotipos GOAK, la concentración de nutrientes minerales era, en contraposición, muy elevados.

- Hemos encontrado una asociación estadísticamente significativa entre el contenido de nutrientes en las hojas y la incidencia de necrosis foliar en primavera, de manera que los genotipos con niveles más bajos de nutrientes totales en hojas, como C3 y C8, presentaron síntomas severos de necrosis foliar. Esta información puede ayudar a reducir los daños en la hoja mediante una gestión adecuada de la fertilización, especialmente de los cambios que se puedan producir en el calcio en los distintos órganos de la planta.

4.2 Proyección futura

En la presente Tesis Doctoral se ha desarrollado un protocolo de fenotipado simultáneo del sistema radicular y de la parte aérea en diversos genotipos de lechuga durante su crecimiento en hidroponía en invernadero. Nuestra configuración experimental nos permitirá evaluar diferentes genotipos de lechuga en soluciones nutritivas definidas para seleccionar genotipos tolerantes a la necrosis foliar y altamente productivos para su utilización en cultivo hidropónico. Además, se podría utilizar un sistema de evaluación de la necrosis foliar en condiciones controladas similar al descrito en [89], para confirmar que la necrosis foliar se produce por la alteración de la homeostasis de calcio, utilizando los genotipos más sensibles, C3, C8, G3 y R2, en medios de cultivo con distintas concentraciones de calcio.

Se han encontrado resultados interesantes acerca de la interacción G × A que merecen investigaciones adicionales. En referencia al peso fresco radicular, podríamos seleccionar los genotipos que triplicaron el peso fresco de su sistema radicular durante la temporada de primavera, como G3 y R5, para la identificación de los determinantes genéticos implicados en la variación en la arquitectura radicular de la lechuga de hoja de roble mediante la implementación de una estrategia de mapeo de QTL tras su cruzamiento con la variedad control, sensible a la necrosis foliar.

Para indagar sobre los mecanismos fisiológicos implicados en la necrosis foliar, resultaría de interés conocer los perfiles hormonales en muestras de hojas jóvenes de los cuatro genotipos de lechuga (G3, R2, G1 y R5) que difieren en la respuesta a la necrosis foliar. Por otro lado, sería interesante estudiar el transcriptoma en muestras de hojas jóvenes de estos mismos genotipos. La comparación de los genes de expresión diferencial entre los genotipos que presentan tolerancia (G1 y R5) o sensibilidad (G3 y R2) a la necrosis foliar nos permitirá identificar los genes específicos que participan en la respuesta de

tolerancia a esta fisiopatía. El análisis detallado de la función de estos genes a partir de la información pública depositada en las bases de datos nos permitirá determinar las rutas en las que participan y su eventual correlación con las posibles diferencias metabólicas y fenotípicas encontradas previamente.

Finalmente se procedería a realizar una la validación mediante retrotranscripción seguida de PCR cuantitativa de alguno de los genes identificados para su validación como biomarcadores. Todos estos resultados adicionales nos proporcionarán información que supondrá un avance significativo tanto a nivel de ciencia básica, al expandir el conocimiento de las bases genéticas y moleculares que regulan la respuesta a la necrosis foliar en respuesta al estrés nutricional, como aplicada, con el posible desarrollo de herramientas que puedan ser utilizadas para la mejora genética de otras Asteráceas de interés agronómico, como la endivia o la alcachofa, o en brasicáceas, como la col, el repollo, el brócoli o la coliflor.

5. BIBLIOGRAFÍA

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Review

Mitigation of Calcium-Related Disorders in Soilless Production Systems

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Abstract: In the current scenario of human-driven climate change, extreme weather events will likely affect agricultural production worldwide. Soilless production systems have recently arisen as a solution to optimize the use of natural resources, such as water and soil, and hence will contribute to reducing the environmental impact of agriculture. However, nutritional imbalance due to adverse environmental factors, such as drought, high temperatures, and salinity, might produce calcium-related physiological disorders during plant growth, such as blossom-end rot (BER) in fruits and tipburn (TB) in leaves, which are a serious problem in crop production. Here, we discuss the different agronomic, physiological, and genetic factors that favor the induction of BER in tomato and TB in lettuce and anticipate the use of an integration of breeding and technological approaches to alleviate nutritional disorders in soilless production systems.

Keywords: climate change; soilless agriculture; blossom-end rot; tipburn; calcium deficiency

Citation: Birlanga, V.; Acosta-Motos, J.R.; Pérez-Pérez, J.M. Mitigation of Calcium-Related Disorders in Soilless Production Systems. *Agronomy* **2022**, *12*, 644. https://doi.org/10.3390/agronomy12030644

Academic Editor: Leo Sabatino

Received: 2 February 2022
Accepted: 3 March 2022
Published: 6 March 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Climate Change and Agriculture

Scientists now agree that human activities are the main drivers of climate change [1]. Agriculture, forestry, and other land uses contribute approximately 13% of the carbon dioxide (CO_2), 44% of the methane, and 81% of the nitrogen oxide emissions, which together represent 23% of the net greenhouse gas (GHG) emissions [1]. However, only 29% of total anthropogenic CO_2 emissions during the 2007–2016 period were neutralized by Earth's natural responses; hence, it is expected that the global atmospheric CO_2 levels will further increase [2].

Human-driven climate change will enhance extreme weather events that negatively affect terrestrial ecosystems. As a result, there will be an increase in degradation and desertification in many regions of the planet, leading to a reduction in crop yields and consequently food security will be affected globally. Soil is both a source and a sink for GHGs and performs a crucial role in the exchange of energy, water, and aerosols between the soil surface and the atmosphere. Therefore, sustainable soil management can help mitigate the negative impacts of various environmental stressors, especially those dependent on climate change, on ecosystems, and societies [2]. Farmers are now implementing a set of agricultural practices to reduce the effects of climate change, through changes in tillage practices, the selection of crop species and cultivars that grow more efficiently and are better adapted to adverse conditions, as well as through the implementation of a more sustainable use of natural resources. Therefore, a proper balance must be found by considering the contributions of these new practices to produce better yields, increasing farmers' incomes and other environmental indicators [3,4].

The objective of this review is to provide farmers with a compendium of strategies, tools, and solutions to problems directly related to crop quality, such as blossom end rot (BER) on fruits and tipburn (TB) in leaves. In this study we will analyze the possible triggers of these physiological disorders by studying the environmental factors directly related to climate change. In addition, new production and managing strategies will be described for a more efficient use of resources that contribute to reducing the appearance of these symptoms, whether due to environmental or genetic factors or a combination of both.

2. Soilless Production Systems: Challenges and Solutions

A new model of industrial-scale agriculture, known as soilless agriculture, has emerged in recent years as a system that optimizes the use of natural resources, such as water and soil, and that allows for better environmental control due to its implementation indoors. Soilless agriculture contributes to better plant growth thanks to an adequate management of the root zone in terms of a more uniform and precise control of water and fertilizer needs. With this technique, it is possible to produce healthy vegetables of excellent quality [5,6].

Soilless agriculture not only improves the quality of agricultural products, but also contributes to the reduction of their environmental impact by ensuring a more efficient use of water and fertilizers, mainly nitrates and phosphates (NO_3^- and PO_4^{2-}), which can reach rivers and seas due to leaching by torrential rains, causing the contamination of surface waters by eutrophication [7]. The possibilities provided for helping reduce the environmental impact of agricultural systems include the reuse of industrial waste as a growing medium. Soilless cropping systems in which 50% of the drainage was recirculated, reduced NO_3^- and PO_4^{2-} emissions as compared to systems without drainage recovery [8,9]. At present, soilless farming has become consolidated as a suitable tool to optimize intensive crop production and reduce the use of non-renewable resources.

2.1. Soilless Cropping Systems

Soilless cropping systems can be classified into several types based on the use of the nutrient solution or the physical state of the root growth media (Table 1). Consequently, we distinguish between open-loop systems (Figure 1a), if the nutrient solution is discarded after use, or closed-loop systems (Figure 1b), if the nutrient solution is reformulated after use and returned to the system. The nutrient solution consists of water, oxygen (O_2), and all essential plant nutrients [10]. The root system could grow in the air (aeroponic cultivation), on the liquid nutrient solution (hydroponic cultivation), and on a solid substrate with added nutrient solution (substrate cultivation).

Table 1. Classification of soilless cropping systems.

Classification	Categories	Characteristics
Nutrient solution use	Open-loop systems	The used nutrient solution is discarded
	Closed-loop systems	Nutrient solution is reformulated and returned to the system
Physical state of root growth media	Gaseous (aeroponic cultivation)	Spray column system Schwalbach system Aero-Gro system
	Liquid (hydroponic cultivation)	Deep floated technique Nutrient film technique New growing system technique
	Liquid (aquaponic cultivation)	Nutrient solution is derived from waste from fish production
	Solid (substrate cultivation)	Directly in substrate Systems of cultivation in bags or containers Single unit culture systems

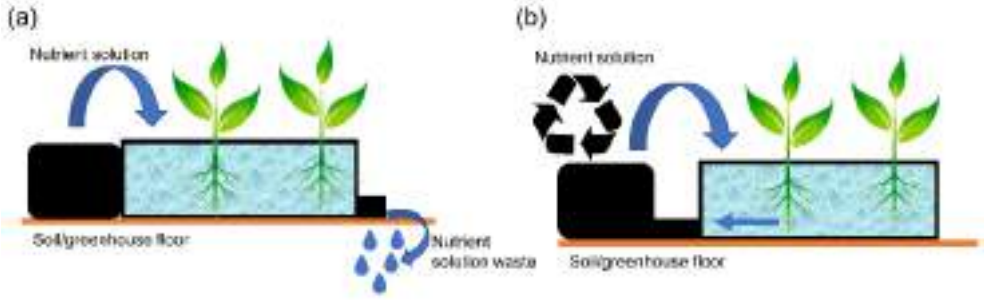

Figure 1. Soilless cropping systems as regards to nutrient solution uses. (**a**) A scheme of an open-loop system in which nutrient solution residues are not recycled, and (**b**) a scheme of a closed-loop system in which nutrient solution residues are reintroduced into the system.

Soilless cropping systems have been improved over time, showing many advantages as compared to conventional systems, because they avoid direct contact with the soil and therefore minimize the problems related to soil diseases [11].

In open-loop systems (Figure 1a), allowing an excess of nutrients and water to the plants compensates for irregular transpiration, prevents salt accumulation, and corrects nutritional imbalances. In these systems, however, a large amount of nutrients and water is drained away, thus increasing production costs and contaminating the surrounding environment [11]. In contrast, in closed-loop systems (Figure 1b) the drainage solution is collected onto a reservoir for additional treatments to reduce the risk of root-borne diseases and to reformulate the nutrient composition, which might then be used for other plots or reintroduced into the system.

2.1.1. Aeroponic Cultivation

For this type of cultivation, the roots are suspended in the air in dark chambers. The nutrient solution is normally sprayed onto the root system at scheduled intervals for optimal aeration [6].

Some of these systems are as follows:

- Spray column system: This consists of a cylindrical platform made of opaque polyvinyl chloride, with lateral perforations through which the plants are introduced. The nutrient solution is sprayed over the upper part of the roots to ensure a permanent contact with the nutrient solution while the lower part of the root is well aerated (Figure 2a).
- Schwalbach system: This consists of a growth chamber in which the roots grow in the air and are kept in complete darkness. The nutrient solution is sprayed at different distribution points located near the leaves to ensure optimal foliar application, after which it drains to the root, where the excess solution is recovered (Figure 2b).
- Aero-Gro system: The nutrient solution is injected onto the roots directly through finely separated droplets at low pressure, avoiding clogging problems in pipes and spray nozzles (Figure 2c).

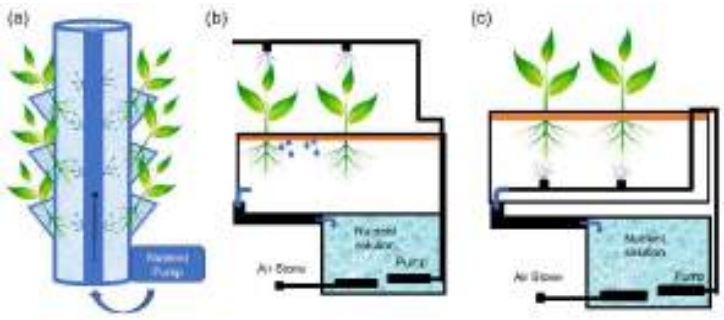

Figure 2. Aeroponic cultivation systems. (**a**) Spray column system, (**b**) Schwalbach system, and (**c**) Aero-Gro system.

2.1.2. Hydroponic Cultivation

As stated above, in hydroponic cultivation the roots are completely submerged in the nutrient solution without any solid substrate. It is very important for light not to reach the nutrient solution to avoid algal blooms, as this would result in low oxygen availability, and this may affect root growth, and consequently, result in reduced plant yield [11].

There are different types of hydroponic systems:

- Deep floating technique (DFT): It incorporates perforated polystyrene sheets as growing units that are placed on top of the tanks filled with the nutrient solution. The aerial part of the plants grows on these sheets with their roots submerged in the tank solution. These systems have an air pump that aerates the nutrient solution (Figure 3a).
- Nutrient film technique (NFT): This system is based on pumping a thin layer of nutrient solution onto the root system through constant flow. This is achieved by placing a small channel with a 1% slope to ensure that the nutritive solution reaches the roots by laminar flow. The excess solution drains into a collecting tank where the conductivity and pH values are restored and the nutrient solution can be pumped back to the top of the channel (Figure 3b).
- New growing system technique (NGST): This system is based on a channel formed by polyethylene bags located internally in three interconnected layers and wrapped by a layer of black polyethylene, which prevents direct contact of light with the root system. The entire system is suspended in the air and leveled to collect drainage at the end of the growing line. The irrigation system is in continuous operation and the drained solution reaches a tank where the nutrient levels are adjusted, heated, and pumped back into the system. The irrigation pipe is located close the root system to facilitate heating of the roots [11] (Figure 3c).

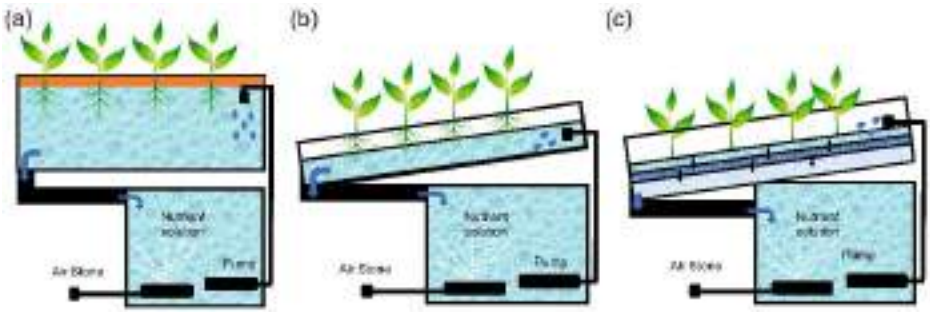

Figure 3. Hydroponic cultivation systems. (**a**) Deep floating technique (DFT) system, (**b**) nutrient film technique (NFT) system, and (**c**) new growing system technique (NGST).

2.1.3. Aquaponic Cultivation

The concept of aquaponics is based on integrating the industrial production of fish (aquaculture) with the cultivation of plants (horticulture), with the aim of establishing a nutritional balance between both species, in such a way that the use of resources (water and nutrients) is shared in the same production system [12,13]. It is based on the use of waste from the aquaculture production, totally or partially, as a nutrient solution for plant growth in a hydroponic cultivation system (Figure 4) [12–14].

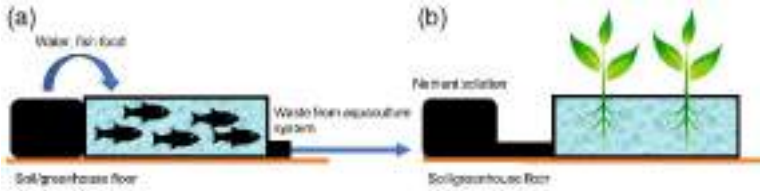

Figure 4. Aquaponic system consisting of an (**a**) aquaculture system for fish production, which is connected to a (**b**) hydroponic system used for crop production.

2.1.4. Cultivation in Organic and Inorganic Substrates

These systems are based on the use of different substrates that provide optimal oxygen and humidity conditions for the correct development of the plant. Organic substrates of natural origin such as peat, or substrates derived from by-products of agricultural activity, such as coconut fiber, cereal straw, or wood shavings, can be used. Inorganic substrates of natural origin with a high porosity, such as sand or volcanic gravel, can also be applied. In addition, inorganic substrates resulting from the industrial transformation such as rock wool, fiberglass, perlite, or vermiculite, are also frequently used [10]. Substrate cultivation systems are characterized by better aeration as compared to water cultivation systems, but at the same time, the flow of water must be continuous to achieve maximum production [11].

Three systems can be distinguished:

- Growing directly on substrate: These systems are delimited by a thick polyethylene mat that prevents the nutrient solution from leaking into the soil. The irrigation system utilized is drip irrigation, and the excess nutrient solution is sent to a tank where the appropriate adjustments will be made for reusing the nutrient solution (Figure 5a,b).
- Growing in bags or containers: The root volume is delimited by elongated two-color polyethylene bags closed at the ends and with two drainage holes filled with substrate. The plants will grow in these bags, the nutritive solution will be dripped in, and the excess solution will be channeled to a tank for further adjustment and reuse [11] (Figure 5c).
- Single unit culture systems: These systems were developed due to the need to control the transmission of fungal diseases in the continuous systems. In this case, the container is the basic cultivation unit and is placed parallel to the drip line. This allows for better control of individual plants, but this system in large-scale production could be prohibitively expensive (Figure 5d,e).

From an environmental and economic point of view, and with the aim of developing cultivation systems that are as sustainable as possible, the implementation of these new types of soilless cropping systems is increasingly widespread. The improvements in these new production systems allow better usage of the nutritive solution and the reuse of the substrates and other supplies, which will lead to a mitigation of the environmental impact of modern agricultural production [10,11].

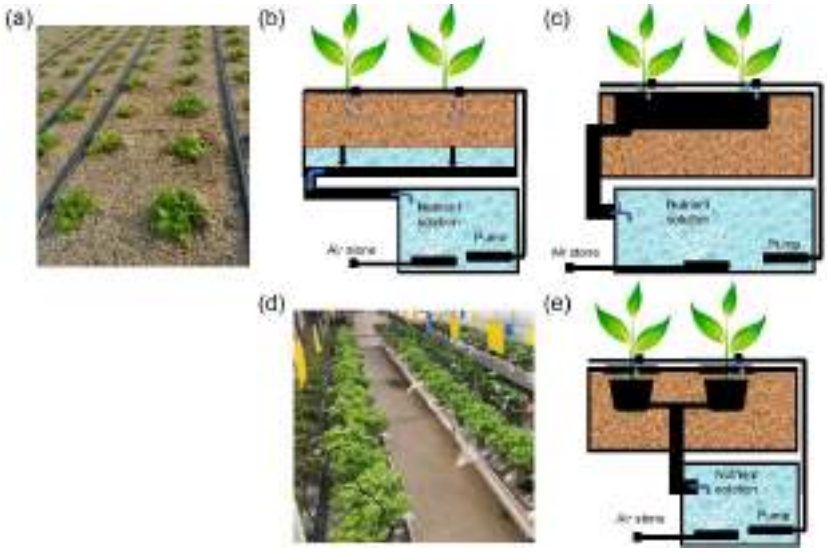

Figure 5. Substrate cultivation systems. (**a**) Lettuce plants growing in sand as an inert substrate, (**b**) a scheme of plants growing directly onto substrate, (**c**) a scheme of plants growing in containers, (**d**) lettuce plants growing in individual containers, and (**e**) a scheme of plants growing in individual containers.

2.2. Physiological Disorders in Soilless Cropping Systems

Various physiological disorders can arise in plants growing in these soilless cropping systems and are mainly caused by nutritional imbalance due to adverse environmental factors and not by the effect of the nutrients themselves [15,16]. Environmental stresses such as those related to temperature, irradiation, or relative humidity, favor the appearance of different physiological disorders [16–19]. The most common of these physiological disorders are BER in fruits such as tomatoes, peppers, squash, cucumbers, melons, etc., and TB, which causes necrosis of the leaf margins in leafy crops such as lettuce or cabbage. BER and TB are generally caused by environmental factors such as soil moisture fluctuations, salinity, and heat stress, among others [18,20]. In these cases, endogenous calcium (Ca^{2+}) content is reduced, and the rapidly growing tissues are mostly affected. Good management of greenhouse environmental conditions, the use of stress-tolerant varieties, and proper handling of the nutrient solution can alleviate these problems [10,21].

3. Physiological Disorders: Blossom-End Rot (BER) and Tipburn (TB)

Climate change is one of the main challenges facing the agricultural sector. Water quantity and quality will be mainly affected by rising temperatures in the coming decades [3,22]. The flexibility of plants to cope with these environmental stresses will depend on their adaptability, and the search for more tolerant genotypes will require the implementation of different strategies to avoid negative effects on plant growth and development.

Physiological disorders such as BER and TB are on the rise due to climate change, are often difficult to predict, and the challenge of controlling the onset of symptoms makes them a serious problem in crop production [15,23,24]. In the early 20th century, BER was believed to be caused by parasitic organisms, chemical toxicity, high transpiration, and lack of soil moisture [25]. However, from the middle of the 20th century, the appearance of BER in tomato, pepper, or watermelon and TB in leafy vegetables, was directly associated to mild Ca^{2+} deficiency in fruits and leaves, respectively [15,18].

Following the new soilless cropping techniques, where the producer provides the necessary nutrient levels that the plant requires at any time, the possibility that mild Ca^{2+}

deficiency alone is not the main cause of these physiological disorders is beginning to be assessed [18,26]. Several authors speculate on the cause–effect relationship of Ca^{2+} deficiency in both disorders, observing that on many occasions, fruits with these symptoms contained equal or higher concentrations of Ca^{2+} in their tissues [26]. Other studies indicated that either low levels or high levels of Ca^{2+} in the nutrient solution led to the appearance of BER in fruits of various species [27]. These results suggest that Ca^{2+} deficiency by itself may not be the causative of BER, but rather that a nutrient imbalance is involved in its appearance. Many authors, in their eagerness to predict and act on time against these problems, have centered their attention on the study of the main triggers of these physiological disorders. These studies distinguish different agronomic, physiological, and genetic factors that favor the induction of BER and TB [15,18,20,26,28]. In the following sections, we will succinctly describe all these factors and their relationships.

3.1. Abiotic Factors Influencing BER and TB

3.1.1. Drought

In agriculture, droughts are generally defined as the periods in which the water losses by transpiration through the leaves, and by evaporation through the soil exceed the amount of water input from precipitation and subsequent water uptake by the roots of the plants. The incidence and intensity of droughts have increased in some regions of the earth, and these are expected to rise in future climate change scenarios [29]. As plants require water for their metabolism, periods of drought can be fatal by reducing crop production to near-unproductive levels (or even causing crop death) or, at best, result in low yields and low-quality products. Depending on the decrease in the irrigation levels by droughts, lettuce and carrot yields are expected to be 25–30% lower than usual. Under these conditions, vegetables and fruits such as apples and pears will generally be sweeter, but smaller. For this reason, consumers and markets will likely have to change their expectations [24].

Studies have been carried out on lettuce and tomato with different irrigation regimes, and under field and greenhouse conditions, to assess commercial traits such as growth, crop maturity, and marketability. Several studies have indicated that deeper roots are key drivers of drought tolerance in plants [30,31]. In addition, a higher incidence of BER and TB has been associated with insufficient water uptake by roots [32–35]. In experiments with different cultivars of lettuce, it was found that TB occurred more frequently in iceberg lettuce than in butterhead lettuce, which is also more drought tolerant [34,36,37]. A similar situation was found in tomatoes, where cultivars with larger fruits suffered more BER symptoms than cultivars with smaller tomatoes [19]. Therefore, breeding new varieties with high drought tolerance is essential for developing vegetables that are better adapted to the consequences of projected climate change, such as BER and TB [36,38].

3.1.2. High Temperature

Global warming has led to the increase in the timing, intensity, and duration of heat-related impacts, such as heat waves [2]. In fact, several studies have shown that high temperatures can cause physiological, biochemical, and morphological changes in crops, which leads to inadequate plant development and consequently to yield losses [39–41]. In broccoli, heat can cause malformations, such as uneven heads with large flower buds, bracts on the heads, or soft heads [42]. On the other hand, it has been observed that flavonoid content and glucosinolate composition in the broccoli florets increased with higher temperatures [43].

High light intensities and high temperatures are environmental factors that cause accelerated photosynthesis and growth rates that can trigger BER in tomato and TB in lettuce [42,44–49]. Hence, BER is likely to occur in fruit tissues as the rapid growth rate increases exponentially, and Ca^{2+} supply to other parts of the plant is restricted by mass flow of free Ca^{2+} through the xylem [20]. It has been hypothesized that an increased demand for Ca^{2+} in rapidly growing tissues, such as occurs in fruits during cell growth,

might indirectly lead to BER when Ca^{2+} is limited [50]. In addition, it has been proposed that light and temperature influence Ca^{2+} absorption and distribution within the plant, thus limiting Ca^{2+} concentration within the fruit during heat stress [17]. Lettuce grown at high temperatures display an accelerated growth that causes little uniformity in the closure of the head and enhances early flowering of lateral stems, which causes bitterness in the leaves and enhances TB incidence [16,36,37,49,51].

3.1.3. Salinity

Salinity is one of the most recognizable factors that will be enhanced by climate change. In any climate change scenario, salinity occurs because of global warming, which causes ice melting at the poles and thus a rise in sea levels. This further causes coastal waters to come into contact with farmland and contaminate the soil with high levels of salts, which will directly affect the growth, quality, and yield of crops that are grown near the coastline. Saline soils encompass approximately 10% of the land surface, and 50% of irrigated land worldwide [52]. High salt levels in soil lead to aggravated dehydration of plant cells, ion toxicity, and oxidative stress, which can cause growth inhibition, damage at the molecular level, and even plant death [31,53]. In addition, soil salinity prevents nutrient uptake by the plant and alters the permeability of the plasma membrane, causing increased salt accumulation in some plant tissues [17,54]. In fact, the selective uptake of Ca^{2+} over Na^{+} is a suitable indicator of salinity stress [55]. Alam and co-authors [56] studied the response of 27 tomato genotypes to various salt treatments to determine their response. They observed that the seedlings from the saline treatments had higher concentrations of Na+ in the leaves, as well as greater root length, fresh and dry weight. It has been observed that in soils with a heterogeneous distribution of salts, the root system absorbs significantly more Ca^{2+} than Na^{+}, and this could be a critical factor that contributed to greater Ca^{2+}/Na^{+} in the fruit and, therefore, to a lower incidence of BER observed in tomato fruits grown in these soils [57]. These authors studied the effect of different saline irrigation regimes under different potential limits of the soil matrix on tomato crop yield and reported BER incidence. The effects of salinity stress on the growth of two types of lettuce under the NFT hydroponic system were analyzed, and it was observed that the amount of fresh and dry matter of the different lettuce types were significantly affected by salinity levels [58].

Water with a low salt content enhanced tomato quality, including fruit density, soluble solids, total acid, vitamin C, and sugar–acid ratio, and had a lower BER incidence than the other more saline treatments. High salinity levels led to a reduction in tomato yield, a decrease in leaf area index and chlorophyll content, together with the appearance of BER symptoms. All this evidence shows that tomato has a moderate salt tolerance index, and mild salinity levels improve osmotic regulation, increase adenosine triphosphatase enzyme activity, and stimulate crop growth [59] Additionally, mild salinity enhanced tomato sensory attributes due to increases in sugar, organic acid, and amino acid contents [59,60]. Inoculation of growth-promoting rhizobacteria in tomato plants has also been shown to improve growth and stress tolerance, resulting in higher crop yields [61–65].

3.2. Physiological Factors Influencing BER and TB Incidence

During agricultural production, an appropriate nutrient management is fundamental for the control of BER and TB. It has been observed that when some nutrients, such as K, P, and Mg, are applied above a certain concentration in the nutrient solution (80, 400, and 500 mg L^{-1}), they could decrease Ca^{2+} uptake and increase BER incidence [66]. Indeed, reducing K^{+} supply in combination with the use of fertilizers such as $Ca(NO_3)_2$ has been shown to reduce the incidence of BER during soilless tomato production. This occurs directly to the antagonistic effect between the cations in the growing medium, so that by reducing the K^{+} concentration, the absorption and mobility of Ca^{2+} can increase [67,68]. It has also been demonstrated that the use of organic fertilizers reduced the incidence of BER [69,70]. The authors found that organic fertilizers not only acted as nutrient sources and increased crop yield, but reduced the effect of BER, probably because they improved

Ca^{2+} absorption and translocation. Ronga and co-authors [70] also suggested that since one of the organic fertilizers they used had milled rice bran with high levels of abscisic acid (ABA), it was possible that the surplus of ABA increased fruit Ca^{2+} uptake directly, as previously reported in tomato fruits [50].

Using pericarp discs from tomato fruits [71], it was shown that exogenously applied Ca^{2+} inhibited BER symptom development in a concentration-dependent manner, but increased symptom severity in tomato fruits when Ca^{2+} was applied to whole plants in the irrigation solution [71]. Unexpectedly, increasing the Ca^{2+} levels of tomato fruits through the expression of the vacuolar H^+/Ca^{2+} antiporter, cation exchanger 1 (CAX1), from *Arabidopsis thaliana*, dramatically increased the occurrence of BER. These latter results suggest that altered Ca^{2+} homeostasis between cytosolic, apoplastic, and vacuolar Ca^{2+} pools might disrupt calcium signaling and lead to localized cell death and enhanced BER incidence [72]. In romaine lettuce cultivars grown in greenhouse conditions, foliar applications of Ca^{2+} resulted in a significant decrease in TB symptoms, which correlated with increased Ca^{2+} concentration in their young leaves as compared with non-treated controls [73]. Several authors have suggested that pectin methylesterases (PME) might be involved in Ca^{2+} transport in tomato plants [74]. They found that silencing PME reduced the concentration of Ca^{2+} bound to the cell wall and improved fruit tolerance to BER [74]. The overexpression of PME was shown to result in Ca^{2+} translocation into cell membranes and, consequently, to Ca^{2+} deficiency in most plant organs, thus enhancing BER incidence in the fruits. Other studies have suggested that the increase in PME synthesis and PME activity overlapped with the critical period for BER development [20]. Taken together, these results indicate that tightly regulated Ca^{2+} homeostasis during periods of rapid growth is required to minimize BER and TB incidence in tomato and lettuce, respectively.

Two stages are involved in fruit growth: cell division influenced by auxin signaling, and cell expansion which is synergistically regulated by auxins and gibberellins (GAs). Fruit ripening occurs when auxin and GA levels decrease with a continuous increase in ABA and ethylene [75]. Phytohormones also regulate a plethora of plant responses to cope with abiotic stress factors [76–78]. Some of these hormones, such as ABA or GAs, have a direct influence on BER [18]. However, a mild level of stress, resulting from one or more interacting environmental factors, does not always result in a certain degree of BER [18]. Rather, it appears that rapid fruit growth promotes a high predisposition to BER and subsequent critical stress is required to trigger cell death [26].

Nevertheless, while it is possible that certain stress conditions may produce hormonal imbalances, it may be likely that hormones involved in cell expansion and fruit development have indirect effects on the incidence of BER. The highest concentration of auxins and GAs in the fruit occurs before cell expansion [79]. The application of auxins and/or GAs is known to increase cell division, rapid fruit growth and BER incidence [80,81]. Therefore, the acceleration of fruit growth and the inability of the plant to supply sufficient Ca^{2+} to the fast-growing fruit could explain the effects of auxins and GAs on BER incidence in most cases.

Although several studies have suggested possible processes by which ABA and GAs regulate BER development in fruit tissue, many of the molecular components involved remain unknown [82]. GAs and ABA can control the expression of genes and gene networks leading to independent and/or antagonistic responses that influence fruit susceptibility to BER [83]. In tomato plants treated with GAs, the expression of genes involved in Ca^{2+} transport and consequently, the concentration of water-soluble Ca^{2+}, was reduced and the incidence of BER concomitantly increased [84]. In turn, the addition of an inhibitor of GA biosynthesis reduced BER in fruits due to increased membrane resistance, thereby decreasing the entry of reactive oxygen (ROS) and other toxic compounds into the fruit [74].

ABA is the main hormone involved in plant stress response. Wang et al. observed that ABA levels negatively correlated with Ca^{2+}, suggesting that ABA plays a regulatory role in response to TB in *Brassica rapa* L. ssp. *pekinensis* [85]. Evidence has been provided indicating an antagonistic interaction between GAs and ABA in the coordination of cation

exchange activity (e.g., CAX1) in the tonoplast and thus in the incidence of BER [46,82]. The tomato *procera* (*pro*) mutant, which shows a constitutive GA response, showed a higher BER incidence due to a combined lower Ca^{2+} translocation to the fruit and a reduced delivery of water and nutrients to the fruit, as a result of competition between vegetative organs and fruits for the available Ca^{2+} [86].

Ethylene has also been proposed to be involved in the induction of BER [18]. Ethylene, in addition to its effect on fruit ripening, is known to be involved in the initiation of wound and pathogen responses via Ca^{2+} signals [87]. Early ethylene production, premature ripening, necrosis, and cell death in the apical region of the fruit, have also been found to be symptoms directly related to BER [20,26,88]. However, it is also possible that ethylene and other stress factors that increase ROS production may influence BER, subsequent Ca^{2+} concentration increase, and rapid cell expansion [88]. In persimmon fruits, salinity stress increased ethylene production, which resulted in necrotic lesions in the calyx resembling BER, but the link with endogenous Ca^{2+} levels has not yet been established [89].

3.3. Genetic Factors Influencing BER and TB Incidence

Crop yields are strongly affected by abiotic stress caused by drought, salinity, and high temperatures. Plants respond to these stressors through various biochemical and physiological adaptations, some of which are the result of changes in gene expression [90]. In addition, many studies have emphasized that susceptibility to BER and TB is highly genotype-dependent [19,91]. In tomato, for example, pear tomatoes are more susceptible to BER than round tomatoes, and BER is never observed in cherry tomatoes [19]. In addition, a strong variation in the incidence of TB between different lettuce cultivars has been reported [92,93] that has been used for the development of TB resistant varieties through targeted breeding [92,94,95].

The use of genomic tools has allowed the identification of quantitative trait loci (QTL) for TB incidence in various recombinant inbred line (RIL) populations of lettuce and the subsequent development of linked molecular markers [96]. A major QTL accounts for up to 70% of the phenotypic variance for TB incidence in lettuce. By comparing lines with contrasting haplotypes, the genetic region was narrowed down to a genomic region containing 12 genes, two of which encoded proteins with sequence similarity to Ca^{2+} transporters. These studies will allow the development of molecular markers to introgress the major resistance alleles found into new cultivars of TB-sensitive iceberg genotypes [96,97]. However, more research is needed to identify the underlying candidate genes for these QTL and to assess the effect of their introgression in other lettuce cultivars. Conversely, only a few studies have been conducted on the incidence of TB in hydroponically grown lettuce [98,99].

Ca^{2+} deficiency in maize causes leaf tip rot, which is similar to TB in lettuce. Two maize lines, B73 and Mo17, differed in their Ca^{2+} deficiency symptoms. In a recent study by Wang and coauthors [100], it was suggested that ammonium reduced the seedling's ability to absorb Ca^{2+}, which ultimately caused the observed Ca^{2+} deficiency phenotype in the leaf tip. To identify a QTL associated with Ca^{2+} deficiency in maize leaves, the authors used a RIL mapping population of 276 lines derived from a cross between B73 and Mo17 maize genotypes. Five QTL associated with a variation in the Ca^{2+} deficiency trait were identified, and some candidate genes were selected for further studies [100].

The slow growth rate and the high concentration of Ca^{2+} observed in the fruits of the IL8–3 line, which contain a small chromosome segment of the wild relative *Solanum pennellii* in the tomato cultivar M82, could be related to the low incidence of BER observed in the IL8–3 line [101]. The results of this study suggest that the main factors contributing to the difference in BER incidence between M82 and IL8–3 were fruit growth rate and Ca^{2+} availability (but also other elements, including K^+ and B^+) during the early stages of fruit enlargement [102].

In a recent systematic review published by Kuronuma and Watanabe [84], the authors discussed the latest studies aimed at the identification of genes associated with BER and TB

by QTL and transcriptomic analysis. Despite these recent advances, the causative genes for Ca^{2+} deficiency disorders in most crops are not yet known and await further investigation.

4. Solutions to Alleviate Ca-Related Disorders in Soilless Production Systems

In the present section, we will briefly introduce farmers to the tools available to minimize some physiological disorders, such as BER and TB, the incidence of which is likely to increase in the coming decades due to climate change. In intensive production systems, new strategies must be applied to mitigate these Ca-related disorders, in order to synergize crop and environmental factors to achieve efficient production with higher yields [15].

The use of smart management practices could help mitigate these Ca-related disorders but could also be useful in lessening the impact of climate change on crop productivity through better nutrient management [15,103,104]. Continuous monitoring of soilless production systems using low-cost sensors, as well as data-integration management approaches, will be key for establishing criteria and aiding decision-making during crop production [105]. It is now possible to automate a hydroponic growing system using cheap sensors that monitor and control environmental parameters such as light intensity, relative humidity, as well as pH, electrical conductivity, and temperature of the nutrient solution [106–108]. Hasan et al. [109] used drones to detect diseases in tomatoes by analyzing foliar images, which allowed them to adjust the treatment to the most affected regions of the crop. These technologies are based on the need to apply artificial intelligence techniques, such as machine learning, that requires training the initial model with a large amount of data and then using the information gathered from the crops to make predictions [109]. Indeed, the use of sensors that measure physiological processes such as photosynthesis, transpiration, and leaf stomatal conductance, has made it possible to detect and quantify the impact of drought stress in tomato plants [104]. In a recent study, the continuous monitoring of tomatoes grown in an NFT soilless system was performed by combining Netatmo sensors for greenhouse microclimate data collection, with daily fertilizer usage data [110]. Based on these data and on crop yield, the authors concluded that a cost-effective and simplified smart agriculture system allows farmers to apply accurate crop production planning and decision making of cultivation activities, such as maintaining a well-balanced microclimate environment [110]. These tools allow us to remotely or automatically adjust the different abiotic factors that, as mentioned above, can trigger the appearance of BER or TB in crops. It has also been observed that regulation of the size of air bubbles in the hydroponic could increase crop yield [111]. In this sense, it has been shown that the production of microbubbles through specific injectors would facilitate the arrival of oxygen to the finest roots, which is necessary for the effective absorption of essential nutrients and plant growth [111].

New strategies have recently been studied to reduce soil contamination due to the excessive use of agrochemicals. It has been proposed that mitigating excess of plant nutrients by using nanoparticles could lead to more precise nutrition and reduced fertilization in both conventional and hydroponic cropping systems [106]. Nanoparticles have been used as slow-release fertilizers [112,113] or for the elaboration of specific biopesticides [114,115]. In this sense, nanoparticles may provide nutrients in a more soluble and available form to plants [116], and some studies have also found that the use of carbon nanotubes as a soil amendment can double tomato yields and increase agricultural production under certain conditions. Strategies such as the use of nanoparticles for fertilization could help deliver nutrients very precisely, especially at different physiological stages, and thus avoid the effects caused by nutrient imbalances in certain phases of the plant growth cycle, which are more sensitive to the appearance of BER and TB. However, it is not yet clear how the soil ecosystem may be affected by such practices, and therefore, a thorough investigation of the impact and assessment of toxicity at all levels of the ecosystem is required [117].

Plant growth-promoting rhizobacteria (PGPRs) have been used in hydroponic growing systems as biofertilizers and/or biocontrol agents with variable success [106,109,118].

Tomato plants treated with potassium-releasing PGPRs showed a greater reduction in BER levels than untreated plants, which ultimately increased yield in terms of fruit size and weight [119]. In another study, tomato plants treated with *Pseudomonas* sp. LSW25R showed a 61% reduction in BER incidence in a hydroponic system, possibly due to increased Ca^{2+} uptake in their roots [120]. In addition, the exogenous application of ABA to tomato crops has been shown to reduce BER incidence at different Ca^{2+} concentrations in the nutrient solution [23,46]. Additionally, the foliar application of Ca^{2+} in lettuce was found to significantly reduce TB incidence [73] Taken together, the implementation of these strategies could enhance crop production and reduce the excess use of fertilizers [121].

From a genetics point of view, identifying genotypes with a high resilience to nutritional disorders, especially Ca^{2+}, and introgressing the causative genes through breeding, may alleviate physiological disorders such as BER and TB [84]. Targeted breeding combined with the application of precision tools in soilless cultivation will provide us with higher yields, especially in terms of fruit quality in the case of tomato [94], as well as of leaf and head quality in the case of lettuce [92,93].

5. Conclusions

The present review summarizes the factors and mechanisms that trigger TB and BER, and this knowledge can be used for the development of new strategies that could help us mitigate these Ca-related physiological disorders. On the one hand, this evidence can be used to develop new cultivars that are highly tolerant to the factors that cause BER and TB. On the other hand, we propose that soilless cultivation offers many advantages over conventional cultivation, as it allows for the detailed monitoring of physiological processes and nutritional balance of plants using remote sensors. The proposed multidisciplinary strategy to reduce BER and TB levels will bring us higher yields and better quality of the final product.

Author Contributions: Conceptualization, V.B., J.R.A.-M. and J.M.P.-P.; writing—original draft preparation, V.B. and J.R.A.-M.; writing—review and editing, J.M.P.-P.; supervision, J.R.A.-M. and J.M.P.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: We are grateful to Jorge Benítez Vega (Bayer CropScience) for his support and Mario Fon for his help with English editing.

Conflicts of Interest: The authors declare no conflict of interest.

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Genotype-Dependent Tipburn Severity during Lettuce Hydroponic Culture Is Associated with Altered Nutrient Leaf Content

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Abstract: Cultivated lettuce (*Lactuca sativa* L.) is one of the most important leafy vegetables in the world, and most of the production is concentrated in the Mediterranean Basin. Hydroponics has been successfully utilized for lettuce cultivation, which could contribute to the diversification of production methods and the reduction of water consumption and excessive fertilization. We devised a low-cost procedure for closed hydroponic cultivation and easy phenotyping of root and shoot attributes of lettuce. We studied 12 lettuce genotypes of the crisphead and oak-leaf subtypes, which differed on their tipburn resistance, for three growing seasons (Fall, Winter, and Spring). We found interesting genotype × environment (G × E) interactions for some of the studied traits during early growth. By analyzing tipburn incidence and leaf nutrient content, we were able to identify a number of nutrient traits that were highly correlated with cultivar- and genotype-dependent tipburn. Our experimental setup will allow evaluating different lettuce genotypes in defined nutrient solutions to select for tipburn-tolerant and highly productive genotypes that are suitable for hydroponics.

Keywords: *Lactuca sativa* L.; crisphead; oak-leaf; root system architecture; tipburn; nutritional imbalance

Citation: Birlanga, V.; Acosta-Motos, J.R.; Pérez-Pérez, J.M. Genotype-Dependent Tipburn Severity during Lettuce Hydroponic Culture Is Associated with Altered Nutrient Leaf Content. *Agronomy* **2021**, *11*, 616. https://doi.org/10.3390/agronomy11040616

Academic Editor: Dimitrios Savvas

Received: 4 February 2021
Accepted: 22 March 2021
Published: 24 March 2021

1. Introduction

Cultivated lettuce (*Lactuca sativa* L.; Asteraceae), which is usually consumed fresh, is one of the most important leafy vegetables in the world. Commercial lettuce varieties are classified based on head and leaf characteristics, and some of the most common horticultural types are romaine, iceberg (also named as crisphead; CHD), oak-leaf (i.e., green oak; GOAK, and red oak; ROAK), and butterhead. Breeding new lettuce cultivars involves manual pollination of genetically stable (i.e., pure) parent lines with agronomic traits of interest, followed by selection based on plant phenotyping and genotyping [1,2]. The availability of detailed genetic maps of cultivated lettuce [3–7] has allowed significant progress for mapping agronomically-important traits and promoted the development of marker-assisted selection (MAS) and candidate gene identification in these species [8]. Several studies have shown that most breeding target traits, such as disease resistance [9], postharvest discoloration [10], thermotolerance in seed germination [7], or water and nitrate capture [6], are complex traits and thus controlled by quantitative trait loci (QTL).

Spain is the third-largest producer of lettuce and chicory in the world after China and the USA, with a total of c.a. 1.1 million tons, with an area of 35,360 hectares dedicated for their cultivation [11]. Most of the production is focused on the southeastern Mediterranean region, with a temperate climate that allows lettuce cultivation throughout the year, making Spain the world's largest lettuce exporting country. However, water scarcity and soil availability are limiting factors for plant cultivation, and inadequate irrigation and fertilization management has increased the environmental impact of agricultural exploitation in this

region [12,13]. Therefore, to contribute to sustainable lettuce production, there is a strong requirement for the development of new forms of farming to increase the crop's resource use efficiency and the reduction of production costs. Floating systems or closed hydroponic methods have been successfully engaged for lettuce cultivation [14–16]. This technique allows for the precise control of water and mineral nutrition, saves soil and labor costs, and provides shorter harvest cycles, high product quality, and good consumer acceptance [16].

Tipburn is defined as the localized necrosis found on the distal margins of rapidly expanding leaves. It is a serious problem in controlled lettuce production, as it reduces the quality and shelf life of fresh lettuce, hence resulting in severe economic losses [17–19]. Tipburn is influenced by many environmental factors, such as light intensity, air temperature, and soil conditions, and is considered a calcium deficiency-related physiological disorder, which is usually associated with rapidly growing tissues [17,18,20,21]. In addition, a strong variation for tipburn incidence among different lettuce cultivars has been reported [19,22], allowing for the development of tipburn-resistant varieties [19,23,24]. The use of genomic tools has enabled the identification of QTL for tipburn incidence in several recombinant inbred line (RIL) populations and the development of linked molecular markers [25,26]. However, further research is needed to identify the underlying candidate genes for these QTL and the effect of their introgression into other lettuce cultivars. Also, only a few studies on tipburn incidence have been carried out in lettuce grown in hydroponics [27,28].

Within the framework of a company-based breeding program for lettuce, we devised a low-cost procedure for closed hydroponic cultivation and easy phenotyping of root and shoot attributes during early growth in three growing seasons (Fall, Winter, and Spring). A representative sample of lettuce varieties from different cultivars (CHD, GOAK and ROAK) were selected based on contrasting agronomically relevant traits such as tipburn tolerance. Our results allowed us to define genotype × environment (G × E) interactions for some of the studied traits, and to establish a strong correlation between leaf nutrient content and tipburn incidence, which may help to reduce leaf damage through adequate fertilization management.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

We selected 12 lettuce genotypes from the breeding program at Monsanto Agriculture Spain S.L.U. (Murcia, Spain) which differed on their tipburn resistance as visually scored at the company's experimental station (37°41′47.6″ N 1°01′55.2″ W, Murcia, Spain; Table 1). We included four genotypes of the *Lactuca sativa* var. *capitata* L., hereafter referred as crisphead (CHD) or iceberg cultivar; and eight genotypes from *Lactuca sativa* var. *crispa* L., which differed in their leaf color, and which were assigned either to the green oak (GOAK) or to the red oak (ROAK) subtypes (Table 1). Seeds from the cultivars used in this work are available upon request to V.B.

Seedlings were sown in 198-well trays filled with moistened 80% perlite and 20% substrate (FloraGard) and were incubated in darkness for 3 days at 10 ± 2 °C and 75% relative humidity. Germinated trays were transferred to the nursery chamber set at 20 ± 2 °C, 65% relative humidity, and under natural photoperiod (Table S1) until the seedlings had 2–3 true leaves (10 mm; Figure 1a). For each cultivar and experiment, eight randomly-selected seedlings were then transferred to 3 L sealed and opaque pots filled with nutrient solution [29] (Table S2); with an eventual air pump (5 × 2.5 L) for hydroponic growth [30] in a multi-tunnel greenhouse at the company's experimental station and under environmental conditions (0 days after planting, dap; Figure 1b). As previous results indicated that lettuce growth was strongly affected by N application [17], we adjusted the nutrient solution for optimal N supply. To avoid contamination, the nutrient solution was renewed every two weeks.

Table 1. Some details of the lettuce genotypes used in this study.

Cultivar	Genotype	Description	Tipburn Phenotype [18]
CHD	C1	Collected in Summer, crispy leaves	Light
CHD	C3	Collected in late Fall, dark green leaves and ovate leaves, big size head	Severe
CHD	C7	Collected in Winter, dark green leaves and ovate leaves, big size head	Medium
CHD	C8	Collected in late Fall, medium-size head	Medium
GOAK	G1	Collected in Fall, indoor production Voluminous and compact lettuce, strong against bolting	Light
GOAK	G3	Collected in Winter and Spring, indoor and outdoor production. Round shape, dense filling, high weight, strong against bolting	Severe
GOAK	G5	Collected in Spring and Fall, upright and compact leaves, slow bolting	Light
GOAK	G6	Collected in Spring and Summer, indoor production, dark green color, strong against bolting	Medium
ROAK	R2	Collected in Fall and Winter, slight red, small and bit cylindrical heads	Severe
ROAK	R3	Collected in Fall and Spring, dark green color, medium volume	Medium
ROAK	R4	Collected in Fall, good vigor and volume	Light
ROAK	R5	Collected in Fall and Spring, good vigor and volume, strong against bolting	Light

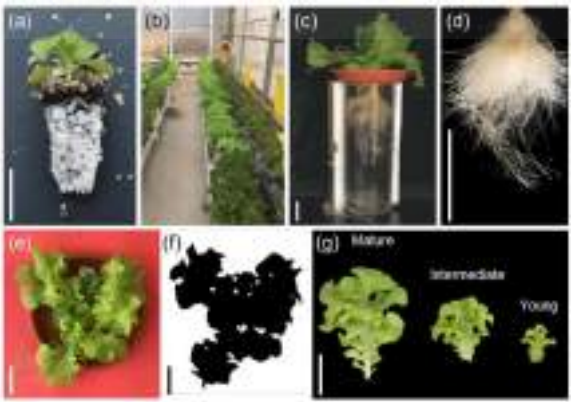

Figure 1. Experimental design for studying growth, tipburn phenotypes, and nutrient concentrations in different lettuce genotypes. (**a**) A representative image of a young seedling from the nursery chamber. (**b**) General view of the hydroponic system used for lettuce cultivation at the experimental station. (**c**) Glass cylinder vase used for image acquisition. (**d**,**e**) A representative image of the roots (**d**) and the shoot (**e**) of a plant grown in hydroponics for 21 days. (**f**) Image segmentation files obtained with Image J software. (**g**) A representative image of leaves collected for nutrient concentration analysis. Scale bars: 50 mm.

2.2. Image Analysis

Five randomly chosen plants were periodically taken for image analyses during the hydroponic culture at 0, 7, 14, 21, 28, 35, and 45 dap. To minimize light variation, a photography box was used with illumination from below. Plants were transferred to a glass cylinder vase (12 × 28 cm) filled with nutrient solution (Figure 1c) and their root and shoot system were respectively imaged (Figure 1d,e) with a still smartphone camera (iPhone 6s, 12 MP f2.2) and saved as an RGB color image in jpeg format (1200 × 2800 pixels). Root area (RA) was measured using the GiA Roots software [31] as described elsewhere [32]. For the shoot area (SA) measurement, the background of the image was removed using Adobe Photoshop CS3, and images were batch-processed using Image J [33] (Figure 1f). Raw measurements were exported to Excel spreadsheets for data analysis.

2.3. Tipburn Evaluation

From 14 dap onwards, tipburn severity (TS) was assessed weekly in individual plants by scoring the presence of necrotic symptoms on the edges of leaves on a scale from 1 to 9, where 1 was no tipburn and 9 was severe tipburn (Figure S1). To obtain these scores, five plants were evaluated per cultivar and season. In addition, tipburn incidence (TBI) was calculated to verify agreement with TS as previously described in [18] with the following formula:

$$\text{TBI} = \frac{(\text{n plants severe tipburn} \times 5 + \text{n plants medium tipburn} \times 3 + \text{n plants light tipburn})}{\text{n plants} \times 5} \times 100$$

2.4. Growth Parameters and Nutrient Content Analysis

At the end of the experiment (45 dap), each plant was separated into shoots and roots to measure their fresh weight (FW). Dry weight (DW) was measured in samples that were oven-dried at 80 °C for 72 h. Root and leaf water content (WC) were determined as: $\frac{(FW-DW)}{FW} \times 100$. Stem length and leaf number were also documented.

For nutrient concentration analysis, we randomly selected three 21 dap plants from the Spring season, and mature (M), intermediate (I), and juvenile (J) leaves were collected from each plant and imaged for SA determination (see Section 2.2; Figure 1g). FW, DW, and WC were measured as described above.

The measurement of different macronutrients: potassium (K), calcium (Ca), phosphorus (P), sulfur (S), magnesium (Mg), sodium (Na), and micronutrients: iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), was carried out in a digestion extract containing 100 mg of tissue powder and 50 mL of a mix of HNO_3:$HClO_4$ (2:1 v/v) using an inductively coupled plasma optical emission spectrometer (ICP-OES IRIS INTREPID II XDL, Thermo Fisher Scientific Inc., Loughborough, UK) at the Ionomic Services of the CEBAS-CSIC (Murcia, Spain) [34].

2.5. Statistical Analysis

The descriptive statistics (mean, standard error of the mean (SEM), etc.) calculated for samples and different tests described below were performed by using the StatGraphics Centurion XV software (StatPoint Technologies, Inc., Warrenton, VA, USA). The Kolmogorov–Smirnov [35] and Shapiro tests were performed to check the normality of the data by analyzing the goodness-of-fit between the distribution of the data and a given theoretical normal distribution. In addition, to check the homogeneity of the variance, the Bartlett and Levene tests were applied. The data with a normal distribution were analyzed by a one-way ANOVA followed by Fisher's LSD (least significant differences) multiple range Test [36] to separate the treatment means, thereby detecting significant differences (p-value < 0.01). Non-parametric tests were used when necessary. In that case, the median was used instead of the mean, and the data were subjected to the Kruskal–Wallis test (p-value < 0.01). Heatmaps were processed using the pheatmap package in R [37]. Neighbor-joining distance matrixes between genotypes (rows) and between samples (columns) were automatically

calculated from average values to build the dendrograms and the heatmap representation. Graphs were drawn with GraphPad Prism 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Quantitative Analysis of Root and Shoot Area during Hydroponic Growth

We followed the growth of the studied lettuce cultivars grown on hydroponic culture by periodically imaging the root and the shoot system between 0 and 35 dap (see Section 2). Estimated root and shoot areas (RA and SA) in the studied CHD genotypes exponentially increased between 0 and 35 dap, following a season-dependent pattern (p-value = 0.002; Figure 2a,b and Table S3). The highest growth rate of the RA occurred during Spring for C3 (Figure S2). Instead, C1 and C7 showed the highest SA growth rate during Fall (Figure S2). In all seasons, C7 and C8 usually showed the lowest RA values, while C3 exhibited the highest RA values at 35 dap (RA_{35}; Figure 2a,c and Figure S3a). In agreement with what was found for RA_{35}, the C8 genotype showed the smallest SA values at 35 dap (SA_{35}) in every season (Figure 2b and Figure S3b), while the SA_{35} values for C7 were much higher in Spring than those in Winter or Fall (Figure 2b,d), despite the RA_{35} in C7 lagging behind in every season (Figure 2a,c). In contrast, the shoot growth and root growth rates of C1 were similar in every season and normally higher than in the other CHD genotypes studied (Figure S2).

Regarding the GOAK genotypes, the estimated RA exponentially increased between 0 and 35 dap. The highest RA growth was observed in Spring (p-value = 0.000), followed by Fall, while in Winter, a slower growth was observed (Figure 3a,c and Figure S4a and Table S3). RA_{35} was similar in all the genotypes in Fall (p-value = 0.624), and Spring (p-value = 0.321), and also slightly significantly different (p-value = 0.046) in Winter (Figure 3a and Figure S4a). RA growth values were quite similar in all GOAK genotypes, with the extreme values shown by G5 in Winter and G3 in Spring (Figure S2a and Table S3). The highest growth rates of the SA were observed during the Fall (Figure S2b and Table S4), and the SA_{35} values significantly differed between GOAK genotypes in every season (Figure 3b and Figure S4b). Overall, G5 showed significantly higher SA_{35} values than the G1 and G6 genotypes, but in Spring, only the SA_{35} values of G5 were significantly higher than the other GOAK genotypes (Figure 3b,d and Figure S4b).

In the ROAK cultivars, we did not find significant differences in the RA_{35} values between ROAK genotypes in Winter (p-value = 0.999) or Spring (p-value = 0.645; Figure 4a and Figure S5a and Table S3). Consistent with the differences observed for RA_{35} values in Fall (Figure 4a and Figure S5a), the lowest growth rate of the RA occurred for the R4 and R5 genotypes in this season (Figure S2a and Table S3).

Conversely, the growth rate of SA was much lower during Spring for all the ROAK genotypes (Figure S2b and Table S3), which also showed similar RA_{35} values in this season (Figure 4b and Figure S5b). The R3 and R4 genotypes showed contrasting RA_{35} and SA_{35} values in Fall (Figure 4a,b), while the R5 genotype showed the smallest RA_{35} and SA_{35} values in this season (Figure 4a,b and Figure S5a,b).

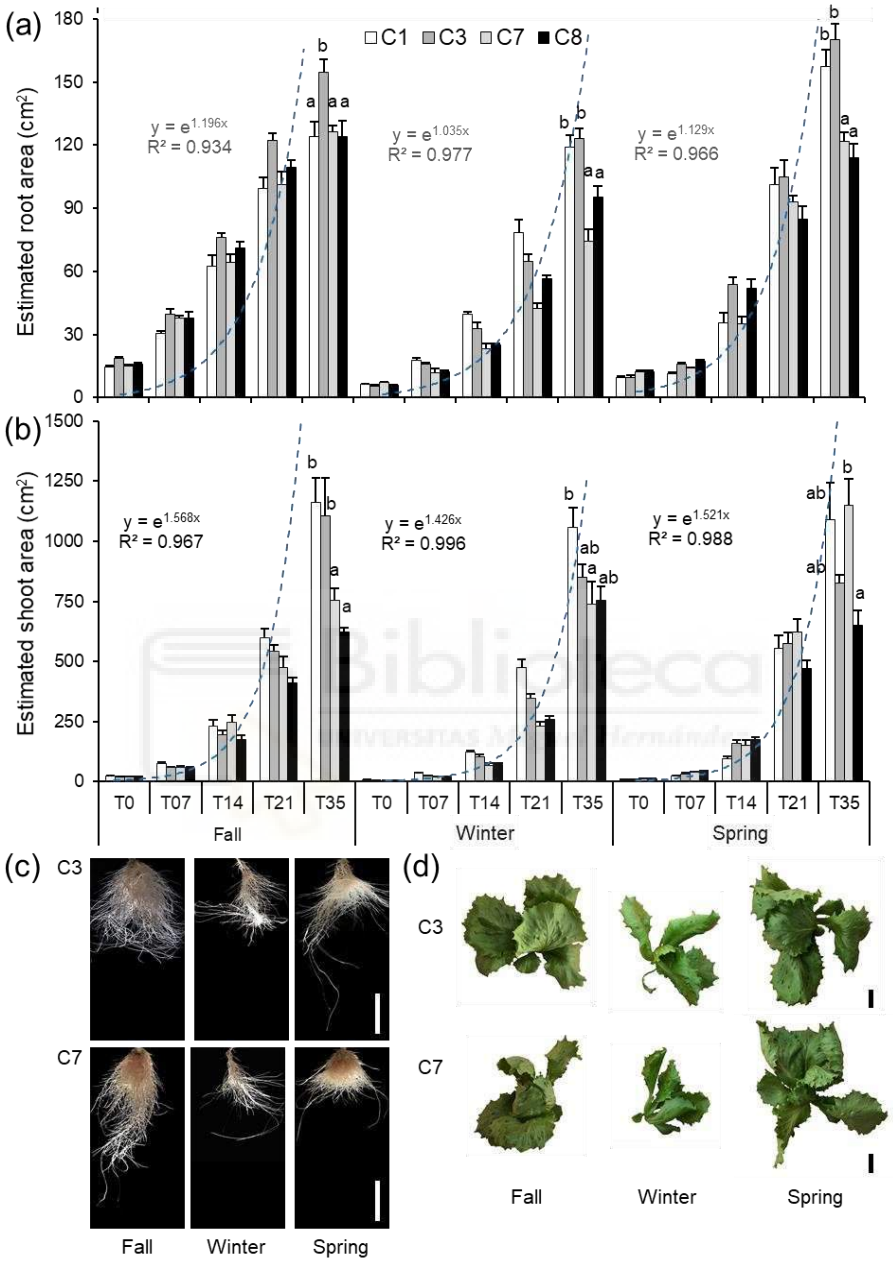

Figure 2. Quantitative analysis of root and shoot area in the studied CHD genotypes during hydroponic growth. (**a**) Average root area (cm^2) and (**b**) average shoot area (cm^2) values in the studied lines (C1, C3, C7, and C8) between 0 (T0) and 35 (T35) days after planting (dap). Theoretical exponential growth curves are depicted in blue. Different letters indicate significant differences at 35 dap (LSD; *p*-value < 0.01). (**c**,**d**) Representative images of the root (**c**) and shoot (**d**) system of C3 and C7 genotypes at 14 and 21 dap, respectively. Scale bars: 50 mm.

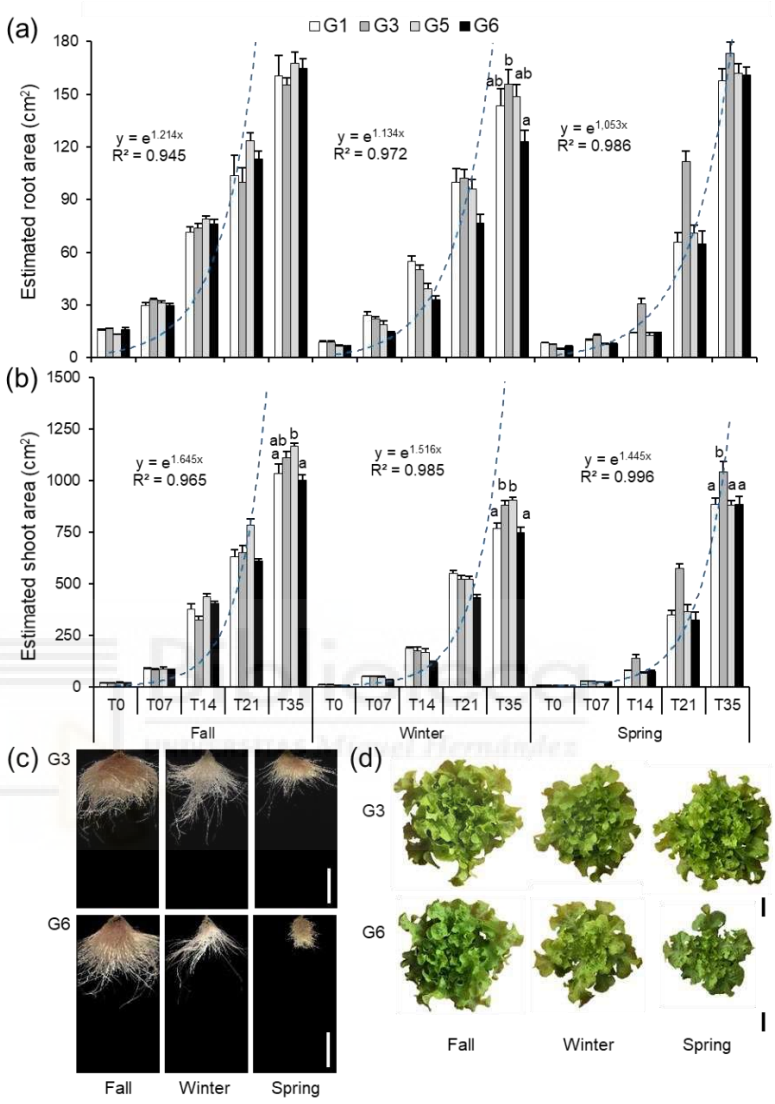

Figure 3. Quantitative analysis of root and shoot area in the studied GOAK genotypes during hydroponic growth. (**a**) Average root area (cm^2) and (**b**) average shoot area (cm^2) values in the studied lines (G1, G3, G5, and G6) between 0 (T0) and 35 (T35) dap. Theoretical exponential growth curves are depicted in blue. Different letters indicate significant differences at 35 dap (LSD; p-value < 0.01). (**c**,**d**) Representative images of the root (**c**) and shoot (**d**) system of G3 and G6 genotypes at 14 and 21 dap, respectively. Scale bars: 50 mm.

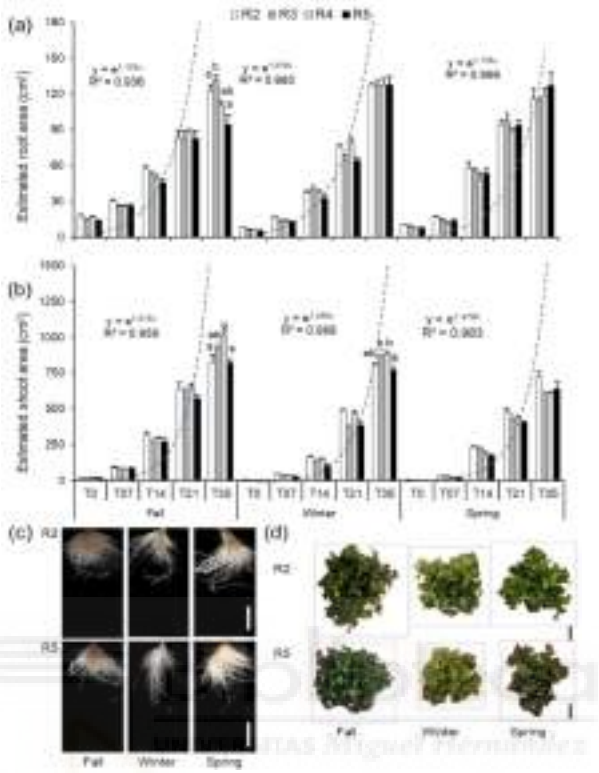

Figure 4. Quantitative analysis of root and shoot area in the studied ROAK genotypes during hydroponic growth. (**a**) Average root area (cm^2) and (**b**) average shoot area (cm^2) values in the studied lines (R2, R3, R4 and R5) between 0 (T0) and 35 (T35) dap. Theoretical exponential growth curves are depicted in blue. Different letters indicate significant differences between genotypes at 35 dap (LSD; *p*-value < 0.01). (**c**,**d**) Representative images of the root (**c**) and shoot (**d**) system of R2 and R5 genotypes at 14 and 21 dap, respectively. Scale bars: 50 mm.

3.2. Variations in Root and Shoot Weights in the Studied Lettuce Cultivars

We measured several growth-related traits of the root and the shoot system at 45 dap (see Materials and Methods; Table S4 and Figure S6a). Root FW and shoot FW were found to be dependent on the cultivar type (*p*-value = 0.000) and the growing season (*p*-value = 0.002). The GOAK genotypes had significantly heavier root systems (FW = 33.50 ± 0.94 g; DW = 1.39 ± 0.05 g; *n* = 57) than those from ROAK (FW = 24.20 ± 0.58 g; DW = 1.19 ± 0.05 g; *n* = 59) or CHD (FW = 24.60 ± 0.85 g; DW = 1.26 ± 0.06 g; *n* = 60), being the largest in Spring for GOAK and CHD (Figure S6b). As for the CHD genotypes, C1 had significantly (*p*-value < 0.01) heavier root systems (FW = 28.60 ± 1.80 g; DW = 1.63 ± 0.26 g; *n* = 15) than C8 (FW = 21.10 ± 1.02 g; DW = 0.97 ± 0.07 g; *n* = 15; Figure 5a). Among the eight *L. sativa* var. *crispa* genotypes studied, G3 showed the heaviest root system (FW = 41.20 ± 2.03 g; DW = 1.56 ± 0.05 g; *n* = 14), while the R5 root system was the lightest one (FW = 21.00 ± 1.07 g; DW = 0.96 ± 0.08 g; *n* = 14; Figure 5a). Despite the FW and DW values being highly correlated overall (Figure S6b), the root DW values were significantly (*p*-value = 0.000) higher in Winter (1.56 ± 0.06 g; *n* = 59), with the lowest values found in Fall (1.00 ± 0.34 g; *n* = 60), but these were not strongly dependent (*p*-value = 0.014) on the type of cultivar (CHD, GOAK or ROAK).

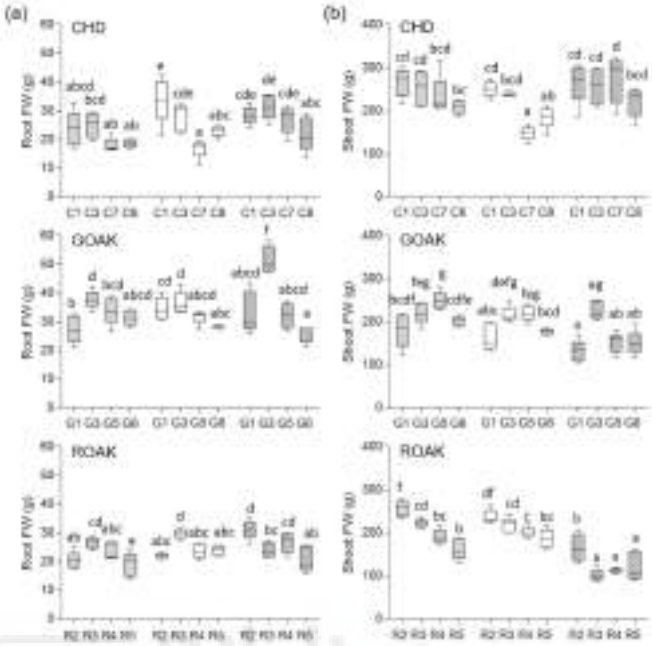

Figure 5. Fresh weight of the studied genotypes at the end of the experiment. (**a**) Root FW and (**b**) shoot FW (Fall, grey-filled bars; Winter, white-filled bars; Spring, lined-filled bars). Different letters indicate significant (p-value < 0.01) differences between CHD, GOAK and ROAK samples.

Despite their small root systems, shoot weights were significantly (p-value = 0.000) higher in the CHD genotypes (FW = 233.30 ± 6.18 g; DW = 8.84 ± 0.30 g; n = 59) than those in GOAK (FW = 190.90 ± 5.36 g; DW = 7.81 ± 0.25 g; n = 60) or ROAK (FW = 182.10 ± 6.72 g; DW = 7.71 ± 0.19 g; n = 59), even though the CHD shoots had significantly (p-value = 0.000) less leaves (15.90 ± 0.50; n = 20) than GOAK shoots (29.80 ± 0.64; n = 60) or ROAK (28.40 ± 0.42; n = 60) shoots (Table S4). Remarkably, a statistically significant (p-value = 0.000) G × E interaction affected shoot FW in GOAK and ROAK genotypes. The shoot FW values from G3 and R2 were much higher than other GOAK or ROAK genotypes only in Spring (Figure 5b). The shoot DW values were also significantly (p-value = 0.000) higher in Winter (9.27 ± 0.26 g; n = 59), but surprisingly, the lowest shoot DW values were found in Spring (7.00 ± 0.21 g; n = 59). The root-to-shoot ratio (R:S ratio) steadily increased from Fall to Spring in the CHD and ROAK cultivars, while non-significant differences were found for the R:S ratio of GOAK in Winter and Spring (Figure S6c).

Root water content (RWC) varied from 94.50 ± 0.36% in C1 to 96.10 ± 0.20% in G3, with a clear effect of the growing season, with lower RWC values in Winter and higher RWC values in Fall (Table S4). Also, shoot water content (SWC) was significantly (p-value = 0.001) lower in ROAK cultivars (95.50 ± 0.15%; n = 57), with the highest values found in C8 (96.50 ± 0.21%; n = 15). Intriguingly, water content (both in the shoot and in the root) was negatively and significantly (p-value = 0.000) correlated with root DW and most considerably in Winter (Figure S6d).

3.3. Tipburn Severity during Hydroponic Growth

Tipburn was scored weekly on cultivars grown in hydroponic culture by means of a visual scaling rate (Figure S1), and it was found that the symptoms steadily increased from 3–4 weeks after planting onwards (Table S5). We found a significant G × E interaction

for tipburn severity in the CHD genotypes (p-value = 0.000), with a higher contribution of the Fall and Spring seasons on the scores at 45 dap (Figure 6a and Table S5). While C3 showed higher scores in every season (6.3 ± 0.5; n = 18), others only showed tipburn symptoms during Spring (C8) or Fall (C7 and C1). Additionally, tipburn phenotypes were highly variable within individual plants in C1, C3, and C8 (Table S5), as estimated by their variance values at 45 dap (7.22; n = 54) compared with those of C7 (2.24; n = 18). The tipburn severity in the ROAK genotypes (measured at 45 dap) was not dependent on the growing season (p-value = 0.296), but a significant dependency on the genotype was observed (p-value = 0.000), with R2 leaves showing similar tipburn symptoms and much higher scores (8.0 ± 0.9; n = 18) than those of the other ROAK genotypes studied (2.1 ± 1.5; n = 54). On the other hand, tipburn symptoms of the GOAK leaves were dependent on the growing season and the genotype (p-value = 0.041), with higher scores in Spring for G3 and G6 (5.0 ± 2.4; n = 16) as compared with the other GOAK genotypes studied (1.8 ± 1.2; n = 56).

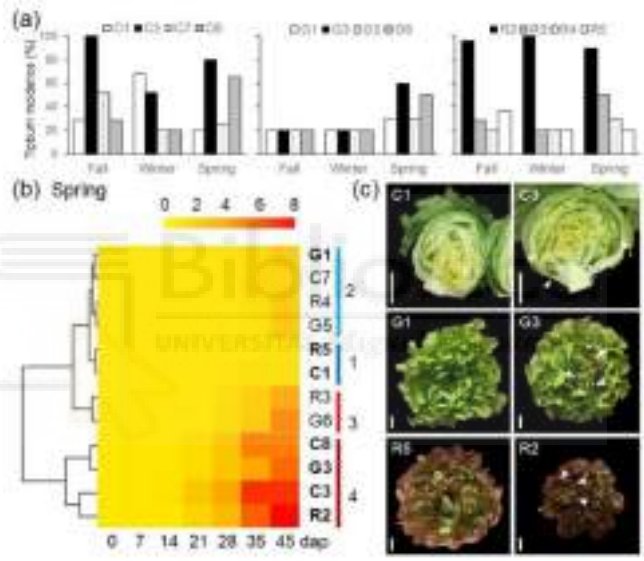

Figure 6. Tip-burn assessment in the studied genotypes. (**a**) Tipburn incidence of the studied genotypes at 45 days after planting (dap) (**b**) Heatmap of tipburn scoring values during the Spring season. Colored bars indicate the severity of the tipburn phenotypes in the studied genotypes, from highly tolerant (0, yellow) to highly sensitive (8, red). Genotypes were grouped into four groups (tolerant: 1,2; sensitive: 3,4). (**c**) Representative images of rosettes of genotypes with extreme tipburn phenotypes (tolerant, left panels; sensitive: right panels) at 45 dap. White arrowheads point to regions where tipburn lesions are present. Scale bars: 50 mm.

Next, we classified the studied genotypes based on their tipburn severity symptoms during the Spring season into four groups: (i) highly tolerant (R5, C1), (ii) intermediate (C7, R4, G1, G5), (iii) sensitive (R3, G6), and (iv) highly sensitive (C8, G3, R2, C3) (Figure 6b). Some highly sensitive genotypes, such as R2 or C3, obtained higher scores in all the seasons, while others, such as C8, G3, or G6 only showed tipburn symptoms during the Spring season (Table S5). Representative pictures of some of the most tolerant and most sensitive genotypes for tipburn symptoms are shown in Figure 6c.

3.4. Leaf Nutrient Variation in the Spring Season in the Studied Lettuce Cultivars

We determined nutrient concentration in mature, intermediate, and juvenile leaves of the studied genotypes in the Spring season at the end of the experiment (Table S6). For

the studied nutrients, most of the variation was found associated with leaf type (*p*-value between 0.0000 (Ca, P, Mg, Na, Fe, Mn, and Cu) and 0.0078 (K)) or cultivar type (p-value between 0.0000 (K, P, S, Mg, Na, Fe, Mn, and Cu) and 0.0029 (Zn)) (Tables 2 and 3). In the CHD cultivar, we found the lowest nutrient levels, while in the GOAK cultivars, their nutrient levels were significantly higher. Nutrient concentrations in the ROAK cultivar were intermediate and similar to GOAK, except for P, where the highest values were observed (Table 2). In regard to the studied macronutrients (K, Ca, P, S, Mg, and Na), all cultivars showed higher P levels in the juvenile leaves, while the Ca, Mg, and Na levels were higher in the mature leaves, albeit not significant for Mg or Na in CHD (Table 2). K levels were significantly higher in mature leaves in the GOAK and ROAK cultivars than in intermediate or juvenile leaves; S showed similar behavior in the CHD and ROAK cultivars with higher concentrations in the juvenile leaves than the GOAK cultivar but without significant differences (Table 2). For the studied micronutrients (Fe, Mn, Zn, and Cu), we found significantly higher concentrations of Mn and Cu in mature leaves of all the cultivars. On the other hand, Fe was significantly higher in mature leaves in GOAK and ROAK, and Zn showed a contrasting behavior in these two cultivars, with significantly higher levels in juvenile leaves in ROAK (Table 2). We next analyzed the mature-to-juvenile (M/J) ratio and found higher values of Ca, Na, and Mn in mature leaves irrespectively of the cultivar. Interestingly, the CHD cultivar showed low ($\leq$1) M/J ratios of K, S, Mg, Fe, Zn, and Cu as compared to those in the GOAK and ROAK cultivars (Tables 2 and 3).

Considering the nutrient concentration of all leaves, we found significant differences between the studied genotypes (Table 4). Overall, the CHD genotypes contained a lower nutrient concentration than the ROAK and GOAK genotypes (Table 4). G3 and R4 had the highest nutrient concentration, which almost doubled those found in C1 and C7 (Table 4). We did not find a clear association between total nutrient concentration and tipburn scores (Figure 6a,b).

After analyzing each nutrient individually, we found that all three cultivars showed similar trends for macronutrient (K > Ca > P > S > Mg > Na) and micronutrient (Fe > Mn > Zn > Cu) concentrations (see percentage in italics in Tables 5 and 6). However, some genotypes displayed substantial differences in the amounts of specific nutrients compared with those in other genotypes of the same cultivar. As regards the CHD genotypes, C1 and C7 had higher concentrations or percentages for all the nutrients analyzed except for Mn, Zn, and Cu, where C1 showed the highest percentages. Comparing the most differentiated genotypes (C1 and C7 vs. C3 and C8), we observed significant differences in K (*p*-value = 0.0003), Ca (*p*-value = 0.0047), S (*p*-value = 0.0026), Mg (*p*-value = 0.0070), Na (*p*-value = 0.0024), and Mn (*p*-value = 0.0025). We classified the CHD genotypes based on their statistically significant nutrient levels, as follows: C1 > C7 > C8 = C3 (Tables 5 and 6). In relation to the GOAK genotypes, G3 contained higher levels of most nutrients compared to those found in G5, G1, and G6 (Tables 5 and 6). Conversely, G6 showed lower levels of some macronutrients (K, P, S, Mg, and Na), being the most malnourished GOAK genotype but without showing significant differences with respect to G1 to G5, which were nutritionally more balanced (Table 5). We found a significantly (*p*-value = 0.0004) higher nutrient concentration in G3 as compared to those in G5, G1, and G6. We ordered the GOAK genotypes as regards to their statistically significant nutrient levels, as follows: G3 > G1 > G5 > G6 Table 6). We did not find significant differences between the ROAK genotypes in regards to total macronutrient and micronutrient content (*p*-value = 0.0650). However, we observed significant differences between R4 and the other ROAK genotypes for P (*p*-value = 0.0021) and S (*p*-value = 0.0040), which resulted in sorting the ROAK genotypes based on significant P and S nutrient concentrations from R4 > R5 > R2 > R3 (Tables 5 and 6). Tipburn incidence and tipburn scores (Figure 6a,b) associated with the R2 and R3 genotypes containing lower P and S content (Table 5).

Table 2. Analysis at the cultivar level (CHD, GOAK, and ROAK) and by leaf type of the macronutrients studied in this trial (ppm). Percentages to the proportion of each nutrient in each type of leaf. Data are means of 12 replicates with different letters for each column indicating significant differences (p-value < 0.01) as determined by LSD multiple comparisons test in different types of leaves analyzed.

	CHD						GOAK						ROAK					
Leaf Type	**K**	**Ca**	**P**	**S**	**Mg**	**Na**	**K**	**Ca**	**P**	**S**	**Mg**	**Na**	**K**	**Ca**	**P**	**S**	**Mg**	**Na**
Mature	1349.00 a 67.52%	340.68 b 17.05%	92.26 a 4.62%	101.45 b 5.08%	65.91 a 3.30%	38.02 b 1.90%	3290.63 b 66.49%	794.50 b 16.05%	228.85 a 4.62%	309.97 b 6.26%	185.36 b 3.75%	109.80 b 2.22%	3204.74 b 69.85%	608.37 b 13.26%	272.64 a 5.94%	179.97 ab 3.92%	184.19 b 4.01%	115.74 c 2.52%
Intermediate	1400.84 a 72.72%	201.41 a 10.46%	137.12 b 7.12%	92.88 a 4.82%	56.78 a 2.95%	30.11 ab 1.56%	2228.40 a 72.06%	316.88 a 10.25%	208.25 a 6.73%	177.64 a 5.74%	105.75 a 3.42%	41.41 a 1.34%	2328.67 a 73.58%	243.09 a 7.68%	283.54 b 8.96%	139.78 a 4.42%	95.44 a 3.02%	64.21 b 2.03%
Juvenile	1657.43 a 73.72%	155.36 a 6.91%	218.00 c 9.70%	121.04 b 5.38%	64.44 a 2.87%	22.90 a 1.02%	2415.40 ab 72.69%	227.05 a 6.83%	326.71 b 9.83%	199.86 a 6.01%	111.22 a 3.35%	28.52 a 0.86%	2424.82 a 72.62%	141.51 a 4.24%	440.74 b 13.20%	192.42 b 5.76%	104.57 a 3.13%	23.21 a 0.69%
p-value	0.1711	0.0001	0.0000	0.1196	0.4857	0.0211	0.0609	0.0001	0.0159	0.0164	0.0133	0.0001	0.0373	0.0000	0.0012	0.0332	0.0000	0.0000
M/J ratio	0.81	2.19	0.42	0.84	1.02	1.66	1.36	3.50	0.70	1.55	1.67	3.85	1.32	4.30	0.62	1.02	1.76	4.99

Table 3. Analysis at the cultivar level (CHD, GOAK, and ROAK) and by leaf type of the micronutrients studied in this trial (ppm). Percentages to the proportion of each nutrient in each type of leaf. Data are means of 12 replicates with different letters for each column indicating significant differences (p-value < 0.01) as determined by LSD multiple comparisons test in different types of leaves analyzed.

	CHD				GOAK				ROAK			
Leaf Type	**Fe**	**Mn**	**Zn**	**Cu**	**Fe**	**Mn**	**Zn**	**Cu**	**Fe**	**Mn**	**Zn**	**Cu**
Mature	4.92 a 0.25%	3.90 b 0.20%	1.67 ab 0.08%	0.23 b 0.01%	16.72 b 0.34%	8.89 b 0.18%	3.35 b 0.07%	0.61 b 0.01%	13.57 b 0.30%	6.75 b 0.15%	1.54 a 0.03%	0.37 b 0.01%
Intermediate	4.12 a 0.21%	1.99 a 0.10%	1.17 a 0.06%	0.13 a 0.01%	8.37 a 0.27%	3.61 a 0.12%	1.67 a 0.05%	0.28 a 0.01%	6.01 a 0.19%	2.45 a 0.08%	1.28 a 0.04%	0.12 a 0.00%
Juvenile	5.23 a 0.23%	1.85 a 0.08%	1.93 b 0.09%	0.21 b 0.01%	8.58 a 0.26%	3.00 a 0.12%	2.35 ab 0.09%	0.30 a 0.01%	6.92 a 0.21%	2.04 a 0.06%	2.50 b 0.07%	0.19 a 0.01%
p-value	0.4398	0.0015	0.0505	0.0029	0.0096	0.0001	0.0431	0.0066	0.0002	0.0000	0.0006	0.0000
M/J ratio	0.94	2.11	0.87	1.09	1.95	2.96	1.43	2.03	1.96	3.31	0.62	1.95

Table 4. Global analysis using all nutrients (ppm) at the cultivar and genotype level. Data are means ± SEM of 9 replicates with different letters for each column (genotypes) or rows (cultivars) indicating significant differences (p-value < 0.01) as determined by LSD multiple comparisons test.

C1	7670.09 ± 634.81 b	G1	10,310.58 ± 338.97 a	R2	10,446.25 ± 804.42 a
C3	5222.15 ± 182.30 a	G3	15,708.86 ± 1099.30 b	R3	10,015.28 ± 1445.66 a
C7	6833.17 ± 294.16 b	G5	10,011.790 ± 990.28 a	R4	13,405.84 ± 1389.01 a
C8	4966.47 ± 392.13 a	G6	9424.43 ± 1482.28 a	R5	9924.48 ± 661.77 a
p-value	0.0004		0.0004		0.0650
Average	6172.97 ± 381.09 a		11,363.91 ± 886.52 b		11,091.40 ± 691.06 b

Table 5. Individual analysis of each macronutrient studied in this trial (ppm) at the genotype level for each cultivar. Percentages refer to the proportion of each nutrient in each type of genotype. Data are means of 9 replicates with different letters for each column indicating significant differences (p-value < 0.01) as determined by LSD multiple comparisons test in the four genotypes analyzed per cultivar (p-value).

CHD	K	Ca	P	S	Mg	Na	GOAK	K	Ca	P	S	Mg	Na	ROAK	K	Ca	P	S	Mg	Na
C1	5493.20 b 71.62%	918.54 b 11.98%	468.06 b 6.10%	390.30 b 5.09%	237.80 b 3.10%	128.51 c 1.68%	G1	7171.16 a 69.55%	1310.47 a 13.05%	655.84 b 6.53%	614.46 a 6.12%	339.65 a 3.38%	170.41 b 1.70%	R2	7649.10 a 73.22%	917.81 ab 8.79%	726.56 a 6.96%	559.32 b 5.35%	396.68 a 3.80%	145.85 a 1.40%
C3	3806.33 a 72.89%	542.29 a 10.38%	361.88 a 6.93%	268.90 a 5.15%	151.76 a 2.91%	69.21 ab 1.33%	G3	10721.92 b 68.25%	2184.11 b 13.90%	886.81 b 5.65%	944.94 b 6.01%	598.40 b 3.81%	287.79 b 1.83%	R3	7129.90 a 71.19%	990.55 ab 9.89%	883.95 b 8.83%	400.07 a 3.99%	377.61 a 3.77%	190.05 ab 1.90%
C7	4849.21 b 70.97%	755.94 b 11.06%	549.96 b 8.05%	355.06 b 5.20%	193.96 b 2.84%	99.52 bc 1.46%	G5	7094.33 a 70.86%	902.42 a 9.01%	858.71 b 8.58%	628.49 a 6.28%	339.99 a 3.40%	140.02 a 1.40%	R4	9485.96 a 70.76%	1253.07b 9.35%	1267.85 c 9.46%	622.57 b 4.64%	441.41 a 3.29%	289.24 b 2.16%
C8	3480.33 a 70.08%	573.01 a 11.54%	409.62 a 8.25%	247.21 a 4.98%	165.00 a 3.32%	66.90 a 1.35%	G6	6750.30 a 71.63%	956.47 a 10.15%	653.87 b 6.94%	561.99 a 5.96%	331.25 a 3.51%	120.72 a 1.28%	R5	7188.79 a 72.43%	784.60 a 7.91%	981.54 b 9.89%	445.11 a 4.48%	323.07 a 3.26%	164.03 a 1.65%
Total	4407.27	697.45	447.38	315.37	187.13	91.03	Total	7934.43	1338.42	763.81	687.47	402.32	179.73	Total	7958.22	992.97	996.92	512.18	384.20	203.16
p-value	0.0003	0.0047	0.0251	0.0026	0.007	0.0024	p-value	0.0012	0.0002	0.2007	0.0009	0.0001	0.0002	p-value	0.1335	0.0879	0.0021	0.004	0.1973	0.0152

Table 6. Individual analysis of each macronutrient studied in this trial (ppm) at the genotype level for each cultivar. Percentages refer to the proportion of each nutrient in each type of genotype. Data are means of 9 replicates with different letters for each column indicating significant differences (p-value < 0.01) as determined by LSD multiple comparisons test in the four genotypes analyzed per cultivar (p-value).

CHD	Fe	Mn	Zn	Cu	GOAK	Fe	Mn	Zn	Cu	ROAK	Fe	Mn	Zn	Cu
C1	15.43 a 0.20%	11.34 b 0.15%	6.15 b 0.08%	0.75 b 0.01%	G1	28.40 a 0.28%	13.17 a 0.13%	6.09 a 0.06%	0.92 a 0.01%	R2	31.74 a 0.30%	14.83 b 0.14%	3.83 a 0.04%	0.53 a 0.01%
C3	11.73 a 0.22%	5.54 a 0.10%	4.01 a 0.08%	0.50 a 0.01%	G3	49.52 b 0.32%	23.19 b 0.15%	10.37 b 0.07%	1.59 b 0.01%	R3	25.85 a 0.26%	11.85 ab 0.12%	4.81 a 0.05%	0.64 a 0.01%
C7	17.34 a 0.25%	7.34 a 0.11%	4.32 a 0.06%	0.52 a 0.01%	G5	27.97 a 0.28%	12.07 a 0.12%	6.67 a 0.07%	1.12 ab 0.01%	R4	27.05 a 0.20%	11.47 ab 0.09%	6.44 a 0.05%	0.79 a 0.01%
C8	12.57 a 0.25%	6.72 a 0.14%	4.59 a 0.09%	0.50 a 0.01%	G6	28.80 a 0.31%	13.61 a 0.14%	6.31 a 0.07%	1.12 ab 0.01%	R5	22.87 a 0.23%	8.24 a 0.08%	5.53 a 0.06%	0.69 a 0.01%
Total	14.27	7.74	4.77	0.57	Total	33.67	15.51	7.36	1.19	Total	26.50	11.25	5.32	0.68
p-value	0.051	0.0025	0.0235	0.0147	p-value	0.0016	0.0003	0.0026	0.0214	p-value	0.3675	0.1888	0.1744	0.2709

We wondered whether differences in nutrient levels between mature and juvenile leaves could account for the observed differences in tipburn scores and tipburn incidence in the GOAK and ROAK genotypes (Figure 6a,b). We found that G1 and R5, with the lowest tipburn scores, showed a mild decrease in K, Ca, Mg, Fe, and Mn concentrations between mature and juvenile leaves (Figure 7). The genotypes with the highest tipburn scores, G3 and R2, showed higher differences in K, Ca, Mg, Fe, and Mn concentrations between mature and juvenile leaves (Figure 7). A similar trend was found for S and Cu, although the R2 genotype showed higher S and Cu levels in juvenile leaves than the other three genotypes (Figure 7). Na concentration was also higher in mature leaves, and their levels were similarly reduced in juvenile leaves in the four genotypes (Figure 7). On the other hand, P and Zn showed the highest levels in juvenile leaves except for the G3 genotype (Figure 7).

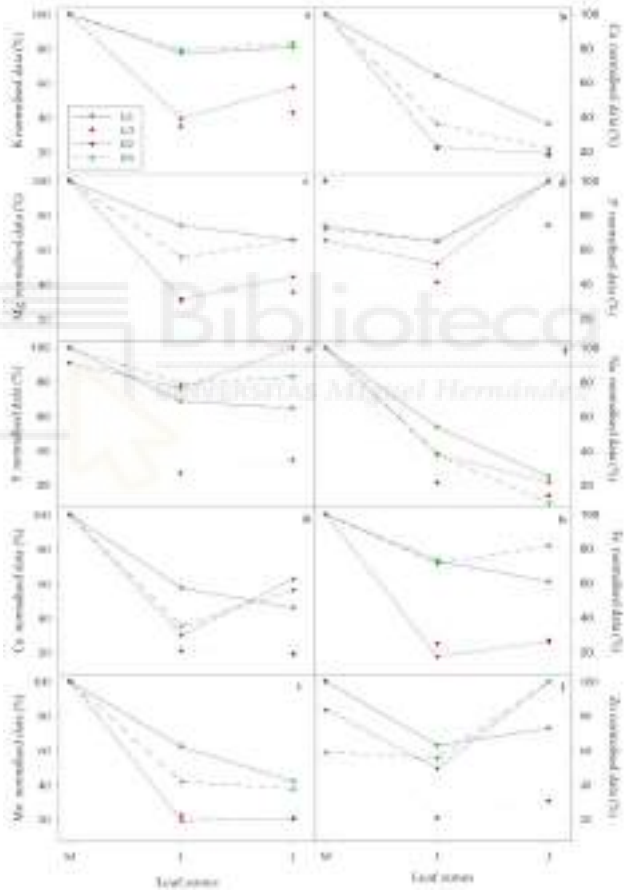

Figure 7. Nutrient analysis of the studied macronutrients in different leaves (M, mature; I, intermediate; J, juvenile). Only the most divergent genotypes for tipburn scoring and tipburn incidence for GOAK (G1 and G3) and ROAK (R2 and R5) cultivars are shown. The nutrients analyzed were (**a**) Potassium (K), (**b**) Calcium (Ca), (**c**) Magnesium (Mg), (**d**) Phosphorus (P), (**e**) Sulphur (S), (**f**) Sodium (Na), (**g**) Copper (Cu), (**h**) Iron (Fe), (**i**) Manganese (Mn) and (**j**) Zinc (Zn). Data are normalized as regards the leaf with the highest amount for a given nutrient.

4. Discussion

There is an increased demand for fresh, locally grown, and safe vegetables among the EU consumers [38]. However, intensive agricultural exploitation might lead to water shortage and soil salinization, among other environmental damages [39]. The greenhouse production of vegetables in closed hydroponic systems is a resource-efficient technique for the production of high-quality and high-yield crops [40]. Here, we devised a low-cost hydroponic system (i.e., floating rafts) for lettuce cultivation, which was used to evaluate the early growth and quality parameters of 12 genotypes from different lettuce cultivars (CHD, GOAK, and ROAK) in three growing seasons (Fall, Winter, and Spring). These genotypes were selected based on agronomically-relevant traits.

Research on the role of root system architecture (RSA) traits that could enhance nutrient and water use efficiency has not received broad attention in lettuce breeding programs until quite recently [6]. We found striking differences among the studied lettuce cultivars in regard to their root system (Figure 2a,c, Figure 3a,c, and Figure 4a,c). The CHD genotypes showed deeper roots as compared to those from GOAK and ROAK. As it is known that deeper roots are crucial for improving drought resistance in plants [41,42], CHD cultivars may be more drought tolerant than GOAK and ROAK genotypes, although this hypothesis could not be directly tested in our hydroponics system. On the other hand, GOAK and ROAK root systems were heavier and more superficial than those in the CHD cultivar. Indeed, GOAK and ROAK are oak-leaf cultivars located on the same genetic clade [43], which are mainly differentiated by their leaf anthocyanin content [1]. The differences in the RSA of the CHD and GOAK/ROAK cultivars may thus account for the genotype-dependent behavior of cultivated lettuce in saline soils [44] or in response to water and nutrient deficiency [6]. Our experimental setup will allow evaluating growth responses under different soil stresses through the adjustment of the nutrient solution and/or the experimental conditions (i.e., temperature, aeration, etc.). In addition, the contrasting RSA phenotypes of the G3 and R5 genotypes (Figures 3c and 4c), with a three-fold difference in their root fresh-weight during the Spring season (Figure 5), may be used for the identification of the genetic determinants involved in RSA variation in the oak-leaf lettuce clade through the implementation of QTL mapping. We estimated the shoot growth rates of lettuce in hydroponic culture through dedicated image analysis (Figure S2). Overall, shoot growth was much lower during Winter than in Spring or Fall, which is in agreement with previous studies where higher temperatures and high irradiance were found to be key factors, which affected growth product quality in these species [16,22]. However, we found an interesting G × E interaction for the estimated SA in some of the studied genotypes. On the one hand, the ROAK genotypes showed lower SA values in Spring than in Winter (Figure 4b). On the other hand, SA values in C7 were highly affected by the growing season, as higher growth occurred during Spring for this genotype (Figure 2b). However, the SA and RA values estimated from images were inaccurate descriptors of yield, as confirmed by the low correlations found between FW and DW values of the shoot system and the root system at the end of the experiment (Figure S6). We found that FW was highly correlated with DW (both for root or shoot) for all the studied cultivars and during the different growing seasons (Figure S6), and that their WC variation ranged from 92% to 98% (Table S4). Interestingly, we found that WC (either in roots or shoots) was negatively correlated with root DW but not with shoot DW, which suggest that thinner roots may be more efficient in water uptake in lettuce plants grown in hydroponics, as compared to those plants grown in soil where root diameter may be directly related to the ability to penetrate the drying soil [41]. In addition, the R:S ratio allowed us to identify genotypes with contrasting yield genotypes, such as C7 and R3 (Table S4). While C7 had the lowest R:S values (and hence higher yield) in Fall, R3 showed the highest R:S ratio (thus lower yield) during Spring, indicating a G × E interaction for this trait, as well.

In Spain and Italy, two of the fifth largest lettuce and chicory producers in the world [11], greenhouse lettuce production is often limited by the extent of tipburn and premature bolting. Tipburn is a physiological disorder characterized by necrotic lesions at

the margins of the developing leaves, resulting from a localized Ca deficiency [45]. Tipburn development in lettuce depends on environmental factors that promote growth [46]. Ca translocation from the roots to the shoots occurs through the xylem due to transpiration, and Ca cannot be mobilized from older leaves to younger ones [47]. Some of the climatic factors that characterize the Mediterranean region, specifically high temperature, high radiation, and long photoperiod, lead to the rapid shoot growth of lettuce, which cannot match Ca translocation from the roots. The lettuce genotypes studied in this work were selected based on their contrasting tipburn incidence when grown in soil. A previous study using a small number of lettuce cultivars grown in hydroponics showed that tipburn was not observed in the late Winter season, whereas it was severe during Spring [48]. We found that tipburn incidence was higher during Spring but lower in Winter for most of the studied cultivars grown in hydroponics (Figure 6a). And we also observed that the CHD cultivars showed a higher variation for tipburn incidence as compared to the studied oak-leaf types (GOAK and ROAK). These results were consistent with the greater genetic variability for tipburn responses found in the CHD cultivar as a result of earlier breeding efforts for tipburn tolerance in this cultivar [19,22,24,25]. In a recent study [28], early bolting and tipburn behavior were studied on 18 genotypes from different lettuce cultivars grown in hydroponics at high temperature and extensive differences were also observed among them. Hence, the combined effect of high growth rates and high temperatures during Spring may lead to the reduced nutrient supply to the developing leaves, resulting in the observed enhancement of the tipburn severity during Spring. Only two of the studied genotypes, C3 and R2 showed severe tipburn symptoms in every season (Figure 6a,c). Another two genotypes, C8 and G3 showed intermediate-to-severe tipburn symptoms only during Spring (Figure 6a,c). On the other hand, C1, G1, and R5 showed tipburn tolerance when grown in hydroponics (Figure 6a,c). These results perfectly matched the tipburn severity symptoms found in the studied genotypes when grown in soil (V.B., unpublished), which validates our experimental setup for the fast and high-throughput evaluation of tipburn responses in lettuce germplasm collections grown in hydroponics.

To investigate the nutritional causes of tipburn incidence during Spring in the studied genotypes, we measured the levels of several macro and micronutrients in leaves of different ages at the end of the experiment (45 dap; Table S6). Ca and Na levels showed the highest M/J ratio, irrespectively of the cultivar type (Table 2), which is consistent with the low Ca mobilization from mature tissues [45] and the higher Na accumulation in older leaves [49]. On the other hand, P displayed the lowest M/J values within the studied macronutrients (Table 2), with lower P levels in the CHD than in the GOAK/ROAK genotypes. These latter results could be explained by the differences in RSA between the studied cultivars, as root responses to low phosphate favor the exploration of the shallower part of the soil, where phosphate tends to be more abundant [50]. We noticed that the CHD genotypes contained a lower nutrient concentration than the GOAK and ROAK ones (Table 4). Nutritional differences between lettuce cultivars have been described previously [51]. The tipburn incidence and tipburn scores of the CHD genotypes perfectly matched their total nutrient content, hence, the genotypes with the lowest nutrient levels (C3 and C8) showed severe tipburn symptoms (Figure 6a,b and Tables 5 and 6). Our results suggest that tipburn in the studied CHD genotypes may be related to some nutrient imbalance, as has been proposed earlier in lettuce [52]. The high growth rates observed during Spring for C3 and C8, combined with their contrasting R:S ratios, may result in decreased Ca concentrations in leaves and thus increased tipburn, as has been previously reported in other lettuce genotypes [21,45]. Because of the restricted Ca transport within the head-enclosed leaves of the CHD genotypes, Ca levels are much lower in the leaf margins, where tipburn symptoms arise early; high K levels in this region might also contribute to enhanced tipburn in CHD genotypes, as suggested previously [45]. Overall, the studied ROAK and GOAK genotypes were less sensitive to tipburn, which was consistent with previous results which suggested a narrow genetic variation for this trait in oak-leaf type cultivars [19]. R2 and G3, which displayed severe tipburn during the Spring season, were

characterized by a strong decrease in K levels between mature and juvenile leaves, as compared with the tipburn tolerant G1 and R5 genotypes (Figure 7). To assess whether tipburn in the studied lettuce genotypes is caused by an altered Ca/K homeostasis, we plan to evaluate tipburn susceptibility using an in vitro evaluation system [24], with some modifications.

We also found striking differences in regard to the studied micronutrients (Fe, Mn, Zn, and Cu) depending on cultivar type and genotypes (Tables 3 and 6). Alterations in micronutrient homeostasis (such as Fe and Mn) have commonly been associated with the appearance of shoot tip necrosis during pistachio in vitro culture [53], which very much resembles the tipburn symptoms found in the ROAK and GOAK genotypes. Fe and Mn levels in leaves, as well as their M/J ratios, were much higher in the GOAK and ROAK genotypes than in the CHD ones (Table 3). Consistent with previous results on K levels, Fe and Mn levels strongly decreased in the R2 and G3 genotypes from mature to juvenile leaves (Figure 7), suggesting that a nutritional unbalance of some micronutrients (Fe and Mn) could explain tipburn in oak-leaf susceptible genotypes. Further experiments with additional ROAK and GOAK genotypes will allow us to confirm this hypothesis.

5. Conclusions

We devised a multi-factorial approach for the study of several growth and quality traits of lettuce (*Lactuca sativa* L.) using a low-cost and high-throughput scalable hydroponic system. By analyzing tipburn incidence and leaf nutrient content, we were able to identify a number of nutrient traits that were highly correlated with cultivar- and genotype-dependent tipburn, suggesting that tipburn is a complex trait in this species. Indeed, the genetic dissection of tipburn resistance in lettuce has recently gained from a detailed study using seven RIL populations in multiple environments and years that allowed the identification of two major QTL affecting this trait [26]. The forthcoming availability of linked molecular markers will allow the evaluation of our germplasm collection.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/4/616/s1, Figure S1: A representation of the scale used for tipburn severity assessment; Figure S2: The growth rate of the studied cultivars between 0 and 35 dap; Figure S3: Representative images of the studied CHD genotypes; Figure S4: Representative images of the studied GOAK genotypes; Figure S5: Representative images of the studied ROAK genotypes; Figure S6: Growth quantification of the studied genotypes at 45 dap; Table S1: Details of the experimental design used; Table S2: Nutrient solution composition; Table S3:, Raw data of root and shoot area; Table S4: raw data of root and shoot weights; Table S5, Raw data of tipburn phenotype assessment, Table S6: Raw data of the nutrient analysis.

Author Contributions: Conceptualization, V.B., J.R.A.-M., and J.M.P.-P.; data curation, V.B. and J.R.A.-M.; funding acquisition, J.M.P.-P.; investigation, V.B.; methodology, V.B. and J.R.A.-M.; supervision, J.M.P.-P.; validation, V.B., J.R.A.-M., and J.M.P.-P.; writing—original draft, J.R.A.-M. and J.M.P.-P.; writing—review and editing, V.B., J.R.A.-M., and J.M.P.-P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available online at www.mdpi.com/xxx/s1.

Acknowledgments: We are grateful to María José Ñíguez Gómez (Universidad Miguel Hernández) for her expert technical assistance, Jorge Benítez Vega (Bayer CropScience) for his support in the experimental designs and genotype information, José Antonio Hernández for his help with nutrient analyses at the CEBAS-CSIC Ionomics laboratory and Mario Fon for his help with English editing.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure S1

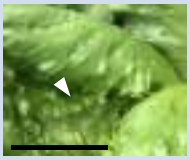
Scale rate	1	2-3	4-5	6-7	8-9
Example					
Description	No tipburn	Light tipburn	Moderate tipburn	Severe tipburn	Highly severe tipburn

Figure S1. A representation of the scale used for tipburn severity assessment. White arrow points to an area where tipburn lesions are present. Scale bars: 50 mm.

Figure S2

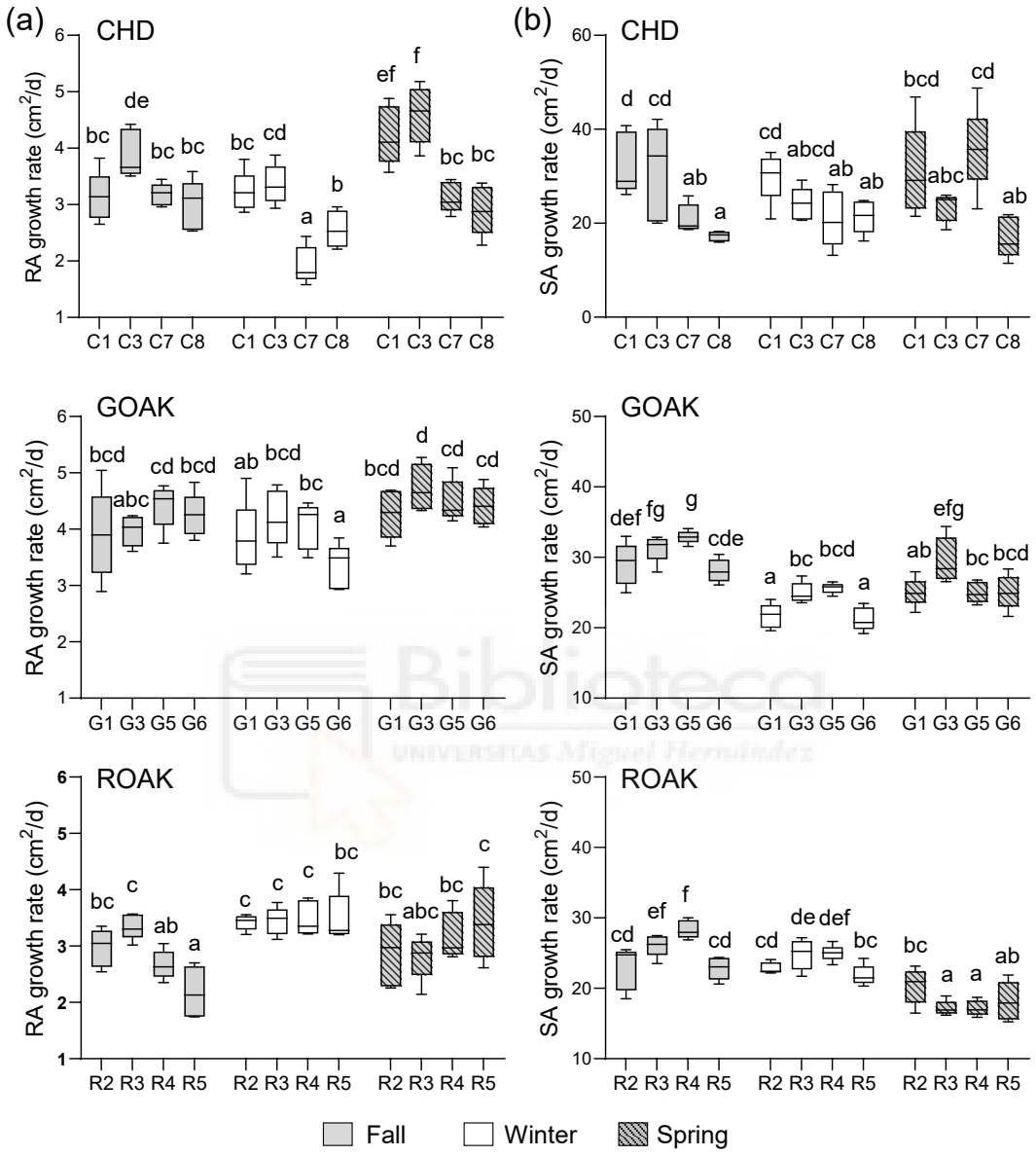


Figure S2. Growth rate of the studied cultivars between 0 and 35 dap. (**a**) Root area growth rate and (**b**) shoot area (SA) growth rate. Letters indicate significant (p-value<0.01) differences between CHD, GOAK and ROAK.

Figure S3

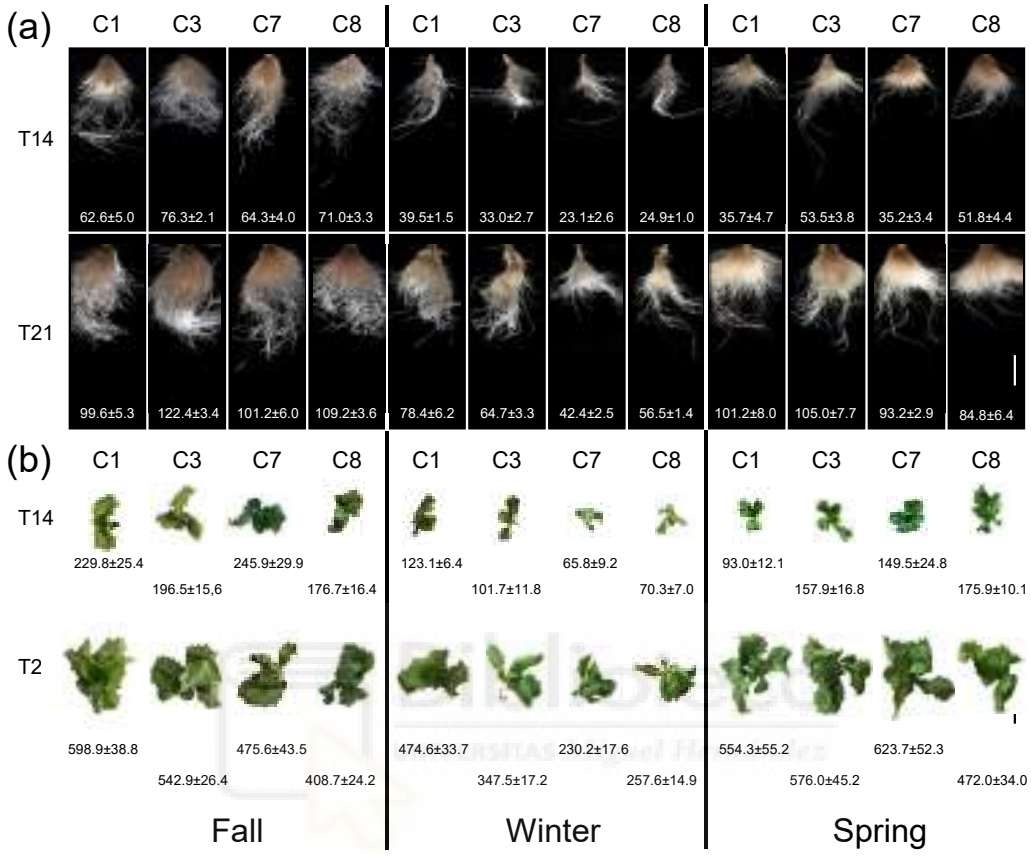


Figure S3. Representative images of the root (**a**) and the shoot (**b**) system of the studied CHD cultivars at 14 (T14) and 21 (T21) days after planting. respectively. Scale bar: 50 mm.

Figure S4

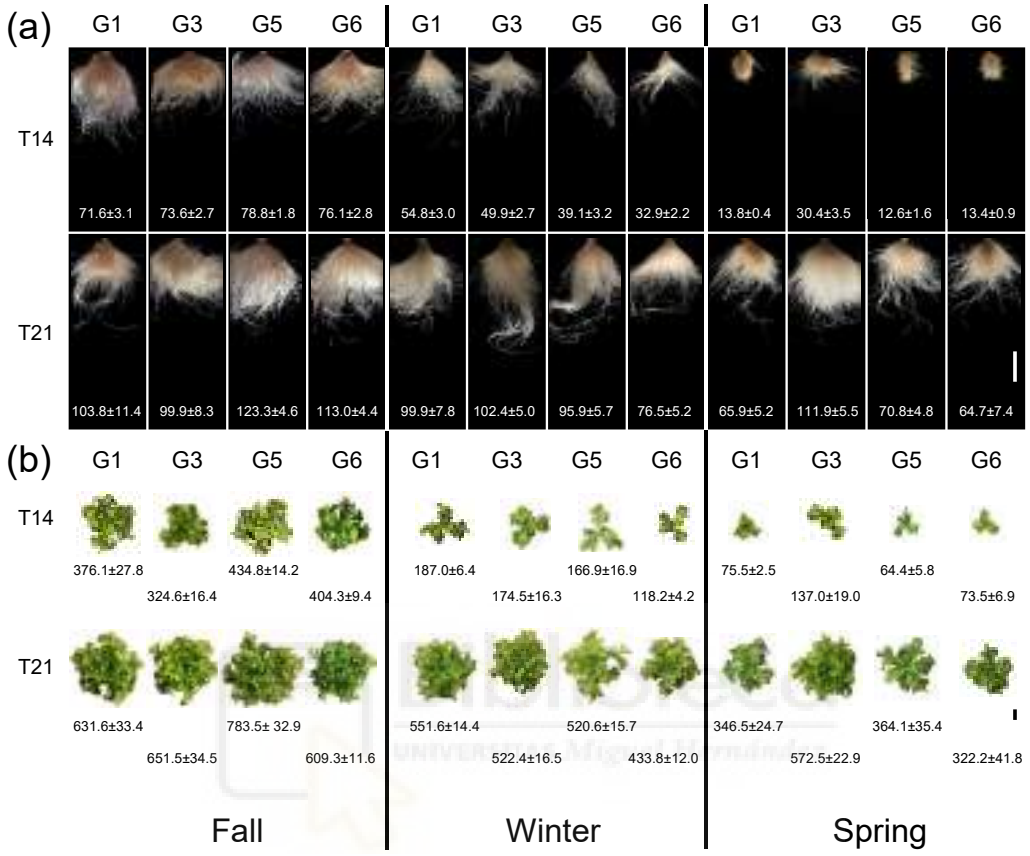


Figure S4. Representative images of the studied GOAK genotypes. (**a,b**) Details of the root (**a**) and the shoot (**b**) system of the studied GOAK cultivars at 14 and 21 dap. respectively. Scale bars: 50 mm.

Figure S5

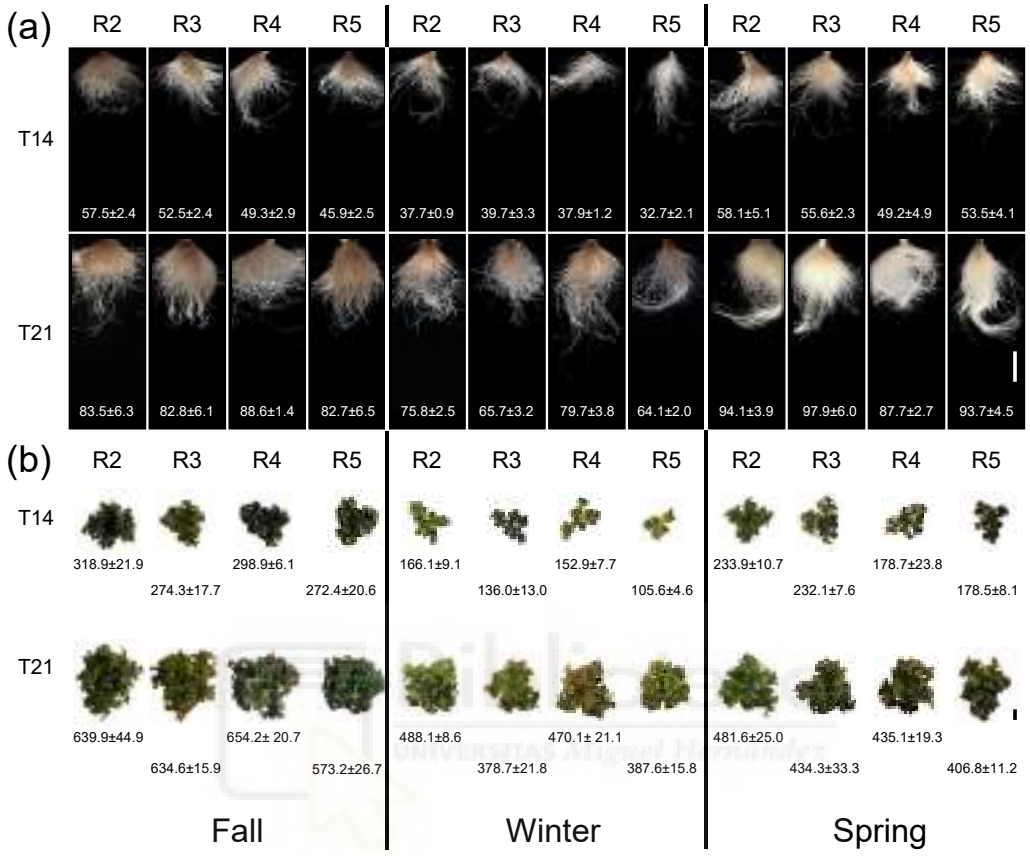


Figure S5. Representative images of the studied ROAK genotypes. (**a,b**) Details of the root (**a**) and the shoot (**b**) system of the studied ROAK cultivars at 14 (T14) and 21 (T21) dap. Scale bars: 50 mm.

Figure S6

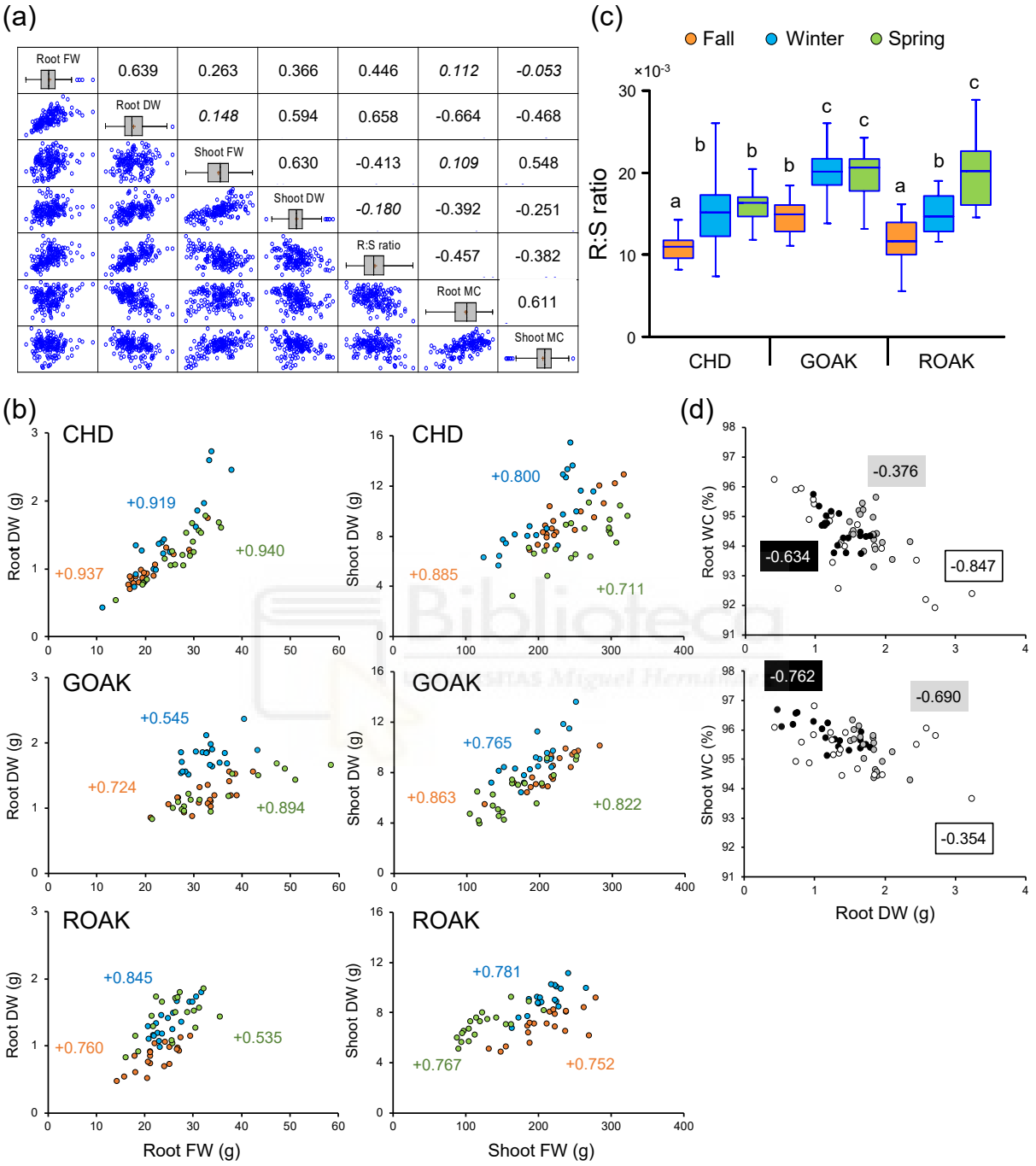


Figure S5. Growth quantification of the studied cultivars at 45 dap. (**a**) Scatter plots of measured traits as defined in Materials and Methods. Numbers in the upper diagonal matrix indicate Pearson's correlation between pairs of traits. (**b**) Relationship between root FW and root DW (left) and root FW and shoot FW (right). Numbers indicate Person's correlation in the studied seasons (Fall, orange; Winter, blue; Spring, green). (**c**) Box-plot of R:S ratio. Letters indicate significant (p-value<0.01) differences between samples. (**d**) Relationship between root DW and root WC (up) and shoot WC (down). Numbers indicate Person's correlation in the studied cultivars (CHD, white; GOAK, grey; ROAK, black).

Table S1. Details of the experimental design used.

Experiment	Season	Nursery chamber	Hydroponic growth	Photopheriod (light/dark)	Air temperature (°C)[1]
I	Fall	21/09/2018-18/10/2018	18/10/2018–02/12/2018	11 h / 13 h	19.7± 3.5
II	Winter	02/11/2018-03/12/2018	03/12/2018–20/01/2019	10 h / 14 h	15.5±1.5
III	Spring	18/02/19-20/03/2019	20/03/2019–04/05/2019	13 h / 11 h	20.2±2.5

[1] average temperature per day.

Table S2. Nutrient solution composition (mg/L)[1].

NO_3	NH_4	P	K	Ca	Mg	Na	SO_4	Cl	Fe	B	Cu	Zn	Mn	Mo
223.7	28.0	61.9	388.3	180.4	24.3	0.3	79.7	0.0	2.2	0.3	0.1	0.3	0.3	0.1

[1] pH 6.0 and 2.37 dS/m of electrical conductivity.

7. AGRADECIMIENTOS

En primer lugar, agradecer a mi director de tesis José Manuel Pérez y mi codirector José Ramón Acosta Motos, por confiar en mí y por darme la oportunidad de trabajar juntos y crear proyectos comunes. Por acercarme cada vez más a la ciencia y enseñarme a que el sacrificio y el trabajo duro son los motores del desarrollo personal y profesional.

A José Ramón Acosta Motos por haber accedido a supervisar mi trabajo y colaborar, gracias por tus sabios consejos y espero seguir enriqueciéndome de las cosas que me quedan por aprender de ti.

A Jorge Benítez Vega por animarme a esta aventura, por la confianza incondicional y ver siempre el lado positivo de las cosas, sus consejos y su afán de ensañar. Por mover cielo y tierra para cumplir los objetivos y estar a mi lado siempre, no sé qué hubiera hecho sin ti.

A Aurora Alaguero, María Salud Justamante, Sergio Ibañez y Victoria Giménez Moya, por compartir trabajo, ensayos y duros momentos, risas e instantes inolvidables.

A Victoria Giménez Moya, por su ayuda ilimitada, por su amistad y trabajo en equipo, siempre dispuesta a todo, ¡eres la mejor!

A mi hermana y a mis padres, por animarme a descansar, apoyarme en los momentos duros y a continuar este largo camino. Por su paciencia, consejos y acompañarme incansablemente a todos los lugares donde proponga.

A la empresa Bayer, por acceder a colaborar con la universidad UMH y desarrollar el proyecto de Doctorado Industrial, a todas las personas que me han apoyado en estos momentos, compañeros de trabajo y amigos. Gracias.