UNIVERSIDAD DE CÓRDOBA

Programa de doctorado en
Biociencias y Ciencias Agroalimentarias

Tesis Doctoral

Efecto de la modificación de parámetros ambientales sobre la resistencia y susceptibilidad de líneas de trigo a varios hongos fitopatógenos

Doctoral Thesis

Wheat resistance and susceptibility against different fungal phytopathogens under changing weather conditions

Autor

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Directores

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Junio 2023

TITULO: *Wheat resistance and susceptibility against different fungal phytopathogens under changing weather conditions*

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TESIS DOCTORAL

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DOCTORAL THESIS

Wheat resistance and susceptibility against different fungal phytopathogens under changing weather conditions

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TÍTULO DE LA TESIS: Efecto de la modificación de parámetros ambientales sobre la resistencia y susceptibilidad de líneas de trigo a varios hongos fitopatógenos

DOCTORANDO: Juan Rafael Porras Pérez

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS
(se hará mención a la evolución y desarrollo de la tesis, así como a los trabajos y publicaciones derivados de la misma)

Los Dres. Josefina C. Sillero Sánchez de Puerta y Alejandro Pérez de Luque, Investigadores Titulares del Área de Mejora Vegetal y Biotecnología del Instituto de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA), como directores de la Tesis Doctoral titulada "***Efecto de la modificación de parámetros ambientales sobre la resistencia y susceptibilidad de líneas de trigo a varios hongos fitopatógenos***", y realizada por el alumno Juan Rafael Porras Pérez, adscrito del Programa de Doctorado "*Biociencias y Ciencias Agroalimentarias*"

INFORMAN

Que la investigación se ha realizado en el Centro IFAPA "Alameda del Obispo", dentro del Área de Mejora Vegetal y Biotecnología, y ha abordado aspectos relacionados con la identificación y caracterización en trigo de fuentes de resistencia genética frente a diferentes enfermedades fúngicas foliares. Además, se ha estudiado cómo se ven afectadas las interacciones del cultivo de trigo con sus respectivos patógenos en condiciones previstas de cambio climático. Esta investigación ha sido completada con éxito como lo demuestran las aportaciones realizadas en forma de publicaciones científicas y de divulgación, que se detallan en el presente informe. A lo largo de su doctorado ha presentado los resultados obtenidos en diversos foros científicos y técnicos, nacionales e internacionales, trabajando tanto en estudios relacionados directamente con su tesis doctoral, como en otros estudios ejecutados por colaboradores del grupo de investigación de IFAPA al que pertenece. Dos de los capítulos de este trabajo se han plasmado en publicaciones en revistas indexadas en el JCR y otros dos capítulos han sido enviados a otras dos revistas y se encuentran ahora mismo en revisión. Todo ello avala la calidad del trabajo realizado. Por último, señalar que el doctorando ha demostrado una extraordinaria capacidad de trabajo, mostrando en todo momento gran iniciativa propia, entusiasmo e interés por el aprendizaje de todas las técnicas que se han manejado para realizar los trabajos.

Todos los objetivos planteados en la memoria del plan de investigación se han cubierto muy satisfactoriamente por lo que se **informa favorablemente** para la exposición y defensa de la Tesis Doctoral.

Publicaciones en revistas ISI elaboradas durante el periodo de tesis doctoral y directamente relacionadas con el contenido de la tesis:

Porras, R., Pérez-de-Luque, A., Sillero, J. C., Miguel-Rojas, C. (2021). Behavior of Spanish durum wheat genotypes against *Zymoseptoria tritici*: resistance and susceptibility. *Spanish Journal of Agricultural Research*, *19*(3), e1002.

Índice de impacto (2021): 1.233, posición 37/59 (Q3) en el área Agriculture, Multidisciplinary.

Porras, R., Miguel-Rojas, C., Pérez-de-Luque, A., Sillero, J. C. (2022). Macro- and Microscopic Characterization of Components of Resistance against *Puccinia striiformis* f. sp. *tritici* in a Collection of Spanish Bread Wheat Cultivars. *Agronomy*, *12*(5), 1239.

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Publicaciones en revistas ISI elaboradas durante el periodo de doctorado relacionadas con el contenido de la tesis aunque no se incluyan en este documento:

Lorite, I. J., Castilla, A., Cabezas, J. M., Alza, J., Santos, C., **Porras, R.**, Gabaldón-Leal, C., Muñoz-Marchal, E., Sillero, J. C. (2023). Analyzing the impact of extreme heat events and drought on wheat yield and protein concentration, and adaptation strategies using long-term cultivar trials under semi-arid conditions. *Agricultural and Forest Meteorology*, *329*, 109279.

Índice de impacto (2021): 6.424, posición 7/90 (Q1) en el área Agronomy.

Cabrera, A., **Porras, R.**, Palomino, C., Sillero, J. C. (2023). Introgression of Seedling Plant Resistance to Leaf Rust from *Agropyron cristatum* into Wheat by Induced Homoeologous Recombination. *Agronomy*, *13*(2), 334.

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Comunicaciones orales en congresos realizadas durante el periodo de tesis doctoral directamente relacionadas con el contenido de la tesis:

Porras, R.; Miguel-Rojas, C.; Pérez-de-Luque, A.; Sillero, J. C. 2020. Identification of sources of resistance to *Zymoseptoria tritici* in Spanish durum wheat cultivars. III Simposio Español de Fisiología y Mejora de Cereales (SEFIMEC). University of Pamplona, November 2020.

Porras, R.; Miguel-Rojas, C.; Lorite, I. J.; Pérez-de-Luque, A.; Sillero, J. C. 2023. Behaviour of Durum Wheat against Leaf Rust under Climate Change Conditions of increasing Temperature and [CO_2]. V Symposium on Cereal Physiology and Breeding. University of Lleida, May 2023.

Otras comunicaciones orales en congresos realizadas durante el periodo de tesis doctoral:

Sillero, J. C., **Porras, R.**, Palomino, C., Cabrera, A. 2022. Introgresión en trigo de resistencia genética a roya de la hoja procedente de *Agropyron cristatum* L. mediante inducción de recombinación homeóloga. XI Seminario de Citogenética de la Sociedad Española de Genética. Jaén, junio 2022.

Participaciones en forma de póster en congresos realizadas durante el periodo de tesis doctoral directamente relacionadas con el contenido de la tesis:

Porras, R.; Miguel-Rojas, C.; Pérez-de-Luque, A.; Sillero, J. C. 2018. Wheat adaptation to climate change through genetic breeding programs in Spain. I Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias. University of Almeria, December 2018.

Porras, R.; Miguel-Rojas, C.; Pérez-de-Luque, A.; Sillero, J. C. 2019. Evaluation of a collection of durum wheat accessions against two isolates of *Puccinia triticina*. II Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias. University of Almeria, October 2019.

Porras, R.; Miguel-Rojas, C.; Pérez-de-Luque, A.; Sillero, J. C. 2019. Preliminary evaluation of a collection of durum wheat accessions against two isolates of *Puccinia triticina*. II Simposio Español de Fisiología y Mejora de Cereales (SEFIMEC). University of Cordoba, March 2019.

Porras, R.; Miguel-Rojas, C.; Pérez-de-Luque, A.; Sillero, J. C. 2021. Characterization of Spanish bread wheat genotypes against *Puccinia striiformis*: resistance and susceptibility. IV Symposium on Physiology and Breeding of Cereals. University of Pamplona, December 2021.

Publicaciones en revistas de divulgación realizadas durante el periodo de tesis doctoral directamente relacionadas con el contenido de la tesis:

Porras, Rafael; Pérez-de-Luque, Alejandro; Sillero, Josefina C. and Miguel-Rojas, Cristina. 2023. Situación actual de las royas del trigo en España. Vida Rural, en revisión.

Participaciones en eventos de divulgación científica durante el periodo de tesis doctoral directamente relacionadas con el contenido de la tesis:

Participación como voluntario en el evento “Paseo por la Ciencia”, celebrado en Córdoba el 6 de abril de 2019.

Participación como voluntario en el evento “Noche Europea de los Investigadores”, celebrado en Córdoba el 27 de septiembre de 2019.

Participación como voluntario en el evento “Café con Ciencia”, celebrado en Córdoba el 12 de noviembre de 2019.

Participación como responsable de mesa de experimentos en el evento “Ciencia y Música bajo las Estrellas”, celebrado en Córdoba el 1 de octubre de 2021.

Participación como voluntario en el evento “Noche Europea de los Investigadores”, celebrado en Córdoba el 30 de septiembre de 2022.

Participación como responsable de taller de Microscopía y Microorganismos en el evento “XVII Paseo por la Ciencia”, celebrado en Córdoba el 15 de abril de 2023.

Por todo ello, se autoriza la exposición y defensa de la Tesis Doctoral.

Córdoba, 21 de junio de 2023

Firma de los directores

Fdo.: Josefina C. Sillero Sánchez de Puerta Fdo: Alejandro Pérez de Luque

TÍTULO DE LA TESIS: Efecto de la modificación de parámetros ambientales sobre la resistencia y susceptibilidad de líneas de trigo a varios hongos fitopatógenos

DOCTORANDO: Juan Rafael Porras Pérez

INFORME RAZONADO DE LA TUTORA DE LA TESIS
(se hará mención a la evolución y desarrollo de la tesis, así como a los trabajos y publicaciones derivados de la misma)

La Dra. Adoración Cabrera Caballero, Catedrática de la Universidad de Córdoba, informa que el ingeniero agrónomo D. Juan Rafael Porras Pérez ha realizado bajo mi supervisión la Tesis Doctoral titulada "Efecto de la modificación de parámetros ambientales sobre la resistencia y susceptibilidad de líneas de trigo a varios hongos fitopatógenos".

El doctorando ha cubierto satisfactoriamente los objetivos de su tesis y ha alcanzado las competencias esperables en un grado de doctor. Además, su producción científica es muy adecuada, por lo que ratifico el informe de sus directores y la evalúo favorablemente.

Por todo ello, se autoriza la presentación y defensa de la Tesis Doctoral.

Córdoba, 21 de junio de 2023

Firma de la tutora

Fdo.: Adoración Cabrera Caballero

"La inspiración existe,

pero tiene que encontrarte trabajando"

Pablo Picasso

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Quisiera dedicar estas líneas para dar las gracias a todas aquellas personas que me han ayudado durante este camino, el cual ha culminado en esta tesis doctoral.

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A: Appressorium
APR: Adult-plant resistance
ASR: All-stage/seedling resistance
CO_2: Carbon dioxide
CS: Colony size
CST: Colonised stomata but not yet transformed into pycnidia
DP: Spores leading to a direct penetration
dpi: days post inoculation
DS: Disease severity
EA–: Early-aborted colonies without necrosis
EA+: Early-aborted colonies associated with necrosis
EC: Epidermal cell
EST–: Established colonies without necrosis
EST+: Established colonies associated with necrosis
GENVCE: Grupo Para la Evaluación de Nuevas Variedades de Cultivos Extensivos en España
GRRC: Global Rust Reference Center
GT: Germinative tube
GTS: Germinative tubes reaching stoma
GU: Germinated urediniospore
H: Haustorium
HMC: Haustorial mother cell
HR: Hypersensitive response
HSD: Honestly Significant Difference
HTAP: High-temperature adult-plant resistance
IF: Infection frequency
IFAPA: Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica
IH: Infection hyphae
IT: Infection type
ITS: Internal transcribed spacer
JA: Jasmonic acid
L: Length
LGT: Lost germinative tubes
LP0: number of hours from inoculation until the first appearance of spores in the uredinia breaking the leaf epidermis
LP50: number of hours from inoculation to the appearance of 50% of the total uredinia breaking leaf epidermis

LSD: Least Significant Difference
MAPA: Ministerio de Agricultura, Pesca y Alimentación
MC: Mesophyll cell
NCS: Non-colonised stomata
NLA: Necrotic leaf area
NP: Spores without penetration
P: Colonised stomata transformed into pycnidia
PCR: Polymerase chain reaction
PDA: Potato-Dextrose-Agar
PDB: Potato-Dextrose-Broth
PLACL: Percentage of leaf area covered by lesions
PR: Partial resistance
PS: Pustule size
Pyc/leaf: Frequency of pycnidia per unit of leaf area
Pyc/lesion: Frequency of pycnidia per unit of lesion area
QTL: Quantitative trait loci
R: Resistance
RAEA: Red Andaluza de Experimentación Agraria
RCP: Representative Concentration Pathway
RH: Relative humidity
RHy: Runner hypha
ROS: Reactive Oxygen species
SE: Standard error
SP: Spores leading to a stomatal penetration
SSV: Substomatal vesicle
ST: Stoma
STB: Septoria tritici blotch
U: Uredinium
W: Width

RESUMEN

El cultivo del trigo es considerado uno de los más esenciales para la alimentación mundial, aportando una fracción nutricional y calórica básica para la humanidad, siendo además el que mayor superficie agrícola ocupa. La producción de trigo, así como de otros cultivos agrícolas en todo el mundo, está seriamente comprometida debido a los incrementos de temperatura y descenso de las precipitaciones derivadas del cambio climático. Concretamente, según las predicciones, estos factores climáticos serán más severos en zonas del sur de Europa, como son los países de la Cuenca Mediterránea. En España, la mayor parte del trigo se cultiva en secano, lo que implicaría una reducción sustancial de la producción en dichas condiciones previstas de incrementos de temperatura y reducción de las precipitaciones. Además, estos cambios ambientales afectarían no solo al cultivo del trigo (morfología, fisiología y producción) *per se*, sino también al desarrollo de los organismos que les causan enfermedades (patógenos) y a las interacciones entre ambos.

Entre los factores ambientales que más afectan al desarrollo de las enfermedades, la temperatura, la concentración de CO_2 y el déficit hídrico (sequía) son los que sufrirán mayores alteraciones en las condiciones previstas de cambio climático. De hecho, existen evidencias de que las enfermedades pueden desarrollar mayores síntomas en condiciones de elevada temperatura o [CO_2], provocando una rápida evolución de nuevos patotipos, o que la resistencia de las variedades se vea superada en menor tiempo. Aparte de esto, el hecho de una posible distribución geográfica de razas de patógenos mejor adaptadas a ambientes más cálidos, supone un gran riesgo para las variedades más susceptibles cultivadas en zonas donde ocurren dichas condiciones climáticas. Asimismo, es conocido que algunos genes de resistencia en trigo pierden su eficacia frente a patógenos específicos con temperaturas elevadas, lo que supone también un riesgo potencial para el cultivo del trigo. Por último, se debe tener en cuenta que tanto el trigo como los patógenos pueden aclimatarse a las condiciones cambiantes de clima, situación que podría derivar en un aumento o disminución de la incidencia de las enfermedades dependiendo de múltiples factores como el ciclo de vida del patógeno (biotrofo, necrotrofo, hemibiotrofo), el genotipo de la planta o el grado de intensidad y duración de los factores ambientales. Además de todos estos condicionantes, existe una falta de estudios de campo para evaluar de forma realista los efectos de diferentes factores abióticos actuando de manera simultánea sobre el desarrollo de enfermedades, lo que dificulta aún más el conocimiento de los efectos concretos del cambio climático en las relaciones planta-patógeno. Por todo ello, y teniendo en cuenta que los hongos fitopatógenos ya causan actualmente importantes pérdidas de producción, existe la necesidad de conocer pormenorizadamente cómo se verían afectadas las interacciones del cultivo del trigo con sus respectivos patógenos en las condiciones previstas de cambio climático.

Para el estudio de dichas interacciones, en esta investigación se han llevado a cabo diversas metodologías de aislamiento, inoculación y análisis de tres enfermedades fúngicas de trigo (septoria, roya de la hoja y roya amarilla), tanto a nivel macro como microscópico. Asimismo, el crecimiento de las plantas de trigo, tanto antes como después de ser inoculadas con los

diferentes patógenos, se realizó en cámaras climáticas e invernaderos de clima controlado, los cuales permitieron aplicar diferentes condiciones de temperatura y concentración de CO_2.

En primer lugar, se ha conseguido el aislamiento, inoculación y posterior desarrollo de la enfermedad de esos tres hongos fitopatógenos de trigo en condiciones óptimas de desarrollo para cada uno de ellos de acuerdo a métodos de varios autores, con algunas modificaciones (Soleiman *et al*., 2014; Sørensen *et al*., 2014; Stewart and McDonald, 2014; Sørensen *et al*., 2017). En segundo lugar, estos métodos permitieron el correcto desarrollo de las tres enfermedades en los genotipos de trigo objetivo de evaluación en esta investigación, tanto variedades cultivadas como líneas de mejora genética. A su vez, la evaluación visual de los síntomas de las tres enfermedades permitió la clasificación de dichos genotipos según su resistencia o susceptibilidad a cada una de ellas (McNeal *et al*., 1971; McCartney *et al*., 2002; Suffert *et al*., 2013; Sørensen *et al*., 2014).

Una vez clasificados, se seleccionaron varios genotipos con diversos grados de respuesta frente a las enfermedades para la evaluación de componentes de resistencia macroscópicos (incluyendo análisis de imagen) (Sørensen *et al*., 2014; Stewart and McDonald, 2014; Karisto *et al*., 2018) y microscópicos (Moldenhauer *et al*., 2008; Bozkurt *et al*., 2010; Soleiman *et al*., 2014; Somai-Jemmali *et al*., 2017) bajo condiciones ambientales óptimas para el crecimiento fúngico. De esta manera se consiguió caracterizar los genotipos de una forma más exacta en su respuesta frente a enfermedades, lo que podría mejorar los procesos de mejora genética vegetal a través del fenotipado, o identificar con antelación la aparición de nuevos patotipos más virulentos de estos patógenos.

Por último, gracias a la realización de dichas evaluaciones macro y microscópicas de componentes de resistencia, se valoró pormenorizadamente el efecto de las condiciones ambientales de incremento de temperatura y [CO_2] en las interacciones planta-patógeno en genotipos con distintos patrones de resistencia y susceptibilidad frente a septoria y roya de la hoja. Así, se comprobó que las condiciones de incremento de temperatura y [CO_2], si bien no modificaron la expresión general de resistencia o susceptibilidad de los genotipos estudiados frente a dichas enfermedades, sí influyeron en cierta medida tanto en la capacidad infectiva de los patógenos (positiva o negativamente) como en la respuesta de los genotipos a nivel fisiológico. De hecho, el uso de métodos de evaluación macro y microscópicos fue esencial para la determinación de dichos efectos, debido a que las interacciones planta-patógeno variaron en función del patógeno, del genotipo y de las condiciones ambientales de estudio.

En resumen, esta Tesis Doctoral establece unos resultados novedosos al evaluar, por primera vez, mediante métodos macro y microscópicos, los efectos derivados del incremento de temperatura y [CO_2] actuando simultáneamente en las interacciones del trigo con sus patógenos más importantes, con el objetivo de emular como serán dichas interacciones en un futuro contexto de cambio climático.

SUMMARY

Wheat is considered the one of most important cultivated crops in the world, providing an essential source of calories, plant-derived proteins and nutrients to mankind. Its production, as those of other crops worldwide, is seriously threatened due to increase in temperature and decrease in rainfall patterns derived from climate change. Particularly, these weather changes would be, according to some predictions, more severe in South European areas such as countries of the Mediterranean Basin. In Spain, where most of the cultivated wheat area is rainfed, these predicted weather conditions of increasing temperatures and reduced rainfall would imply a substantial reduction in wheat production. Additionally, these environmental changes would affect not only wheat cultivation (morphology, physiology and production) *per se*, but also pathogens development and their interaction with wheat.

Amongst abiotic factors that strongly influence pathogens development, temperature, [CO_2] and water deficit would suffer great variations due to climate change. In fact, it is known that fungal diseases could cause higher disease incidence at elevated temperatures or [CO_2], which would favour the evolution of new races and faster resistance breakdown. In this sense, there is evidence that some resistant genes lose their efficacy at elevated temperatures and that some pathogen races, which are more adapted to warmer climates, cause severe epidemics after a geographic distribution to other more suitable zones. Finally, it must be taken into account that wheat and its pathogens can adapt themselves to changing weather conditions, which would increase or decrease disease incidence depending on multiple factors such as lifestyle of pathogens (biotroph, necrotroph, hemibiotroph), plant genotype, or timing and intensity of abiotic factors. In addition to these facts, there is a lack of realistic field studies assessing the effects of simultaneous abiotic factors on disease development, which hampers even more the knowledge about climate change effects on wheat-pathogen interactions. Therefore, and considering that fungal pathogens already cause important losses of wheat production, there is an urgent need to know in detail how wheat-pathogen interactions will be affected under climate change conditions.

In order to study these interactions, this research has been conducted through diverse methodologies of isolation, inoculation and analysis, both macroscopic and microscopic, of three fungal diseases of wheat (septoria tritici blotch, leaf (brown) rust and yellow (stripe) rust). Additionally, climate chambers and a greenhouse with full environmental control of weather conditions such as temperature and [CO_2] were used in the growth and development of wheat plants and their diseases.

Firstly, three fungal pathogens of wheat were isolated, inoculated and developed disease symptoms in wheat plants in their respective optimum conditions, according to diverse authors, with modifications (Soleiman *et al*., 2014; Sørensen *et al*., 2014; Stewart and McDonald, 2014; Sørensen *et al*., 2017). Secondly, the three fungal diseases were conducted in bread and durum wheat genotypes of study in this research, ranging from Spanish commercial cultivars to breeding lines. At the same time, visual evaluation of disease symptoms led to the classification

of wheat genotypes according to their resistance or susceptibility traits (McNeal *et al*., 1971; McCartney *et al*., 2002; Suffert *et al*., 2013; Sørensen *et al*., 2014).

Once wheat genotypes were classified, some of them with different response to each disease were selected for macroscopic (using image analysis) (Sørensen *et al*., 2014; Stewart and McDonald, 2014; Karisto *et al*., 2018) and microscopic (Moldenhauer *et al*., 2008; Bozkurt *et al*., 2010; Soleiman *et al*., 2014; Somai-Jemmali *et al*., 2017) evaluations of components of resistance of wheat-pathogen interactions under optimum conditions of fungal development. As a result, wheat genotypes were precisely characterised in their response to diverse diseases, which would improve breeding research through phenotyping or identify new virulent pathogen pathotypes in advance.

Finally, thanks to these macroscopic and microscopic evaluations of components of resistance, it was possible to precisely assess the effects derived from expected far future weather conditions of increasing temperature and [CO_2] on interactions of selected wheat genotypes against both septoria tritici blotch and leaf rust diseases. Thus, although it was observed that general responses of resistance and susceptibility of currently used selected wheat genotypes did not change after a continuous exposure for both, plants and diseases, to elevated temperature and [CO_2], these weather conditions certainly influenced both fungal infection capability (positively or negatively) and plant physiological responses to some extent. In fact, due to wheat-pathogen interactions varied regarding the pathogen, genotype and weather conditions studied, macro and microscopic evaluations were essential to determine these effects.

In summary, this Ph.D. Thesis obtains novel results assessing, for the first time and using macro and microscopic methods, the effects derived from the exposure to simultaneously increasing temperature and [CO_2] on the interactions of wheat against their most important pathogens, which try to resemble, as well as possible, how wheat-pathogen interactions would be in the future context of climate change.

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1. General Introduction

1.1. Wheat importance and cultivation under global climate change conditions of increasing temperature and CO_2 concentration

Wheat is one of the leading food crops, providing an essential source of calories (20%), plant-derived proteins (>25%), amino acids, minerals, vitamins (especially vitamin B), phytochemicals and fibre components in human diets (Shewry and Hey, 2015). This crop is unrivalled in its range of cultivation and diversity, being widely adapted to diverse growth conditions, especially in temperate climates. Thus, wheat production accounted for 770 million tons on 221 million hectares in 2021 worldwide, being mainly produced in China, India, Russia and the United States of America. In the European Union, main wheat producers were France (36 million tons), Germany (22 million tons), Turkey (18 million tons), Poland (12 million tons), and Spain (almost 9 million tons on 2 million hectares) (FAOSTAT, 2023; Figure 1.1).

Wheat is divided into two main species used for food production: bread (common) wheat (*Triticum aestivum* L.), which is used mainly for baking, and durum wheat (*Triticum turgidum* L. subsp. *durum*), which is used for pasta and traditional Mediterranean dishes. From them, bread wheat is the largest cultivated wheat species worldwide, covering 94% of the total cultivated wheat area, while durum wheat is cultivated over 17 million hectares with a global production of 38 million tons, being the European Union the largest producer (8 million tonnes in 2021), highlighting Italy, Turkey, France and Spain (1 million tons on 0.26 million hectares) (EUROSTAT, 2023). Despite its reduced production in comparison with bread wheat, durum wheat species are considered a staple crop in countries of the Mediterranean Basin due to its traditional presence in diet habits (flour, pasta and semolina). In fact, these countries are the largest importers and consumers of durum wheat products, accounting about 60% of the global durum wheat cropping area and 75% of the global durum wheat production (Royo *et al*., 2017).

Although wheat occupies a key position in global cereal production, the predicted expansion of the global population up to 9.7 billion people by 2050 (World Bank, 2023) would imply an increase of the global demand of wheat consumption by about 60% in 2050. Indeed, despite several policies regarding food security have been implemented, accomplishing this objective of production is a demanding task because current wheat yields are limited by diverse abiotic and biotic constraints. Abiotic stresses, such as high temperatures or drought, affect wheat cultivation depending on the region and environmental conditions, causing physiological and biochemical alterations that ultimately reduce wheat yields, even more than biotic stresses (Chaudhry and Sidhu, 2022). However, these environmental conditions, which characterise different wheat-growing regions, have been suffering variations due to global climate change, defined by shifts in weather patterns and an increase in frequency and magnitude of extreme events such as heat waves, droughts, heavy precipitation, or flooding.

Climate change is caused by both natural and human activities that have increased the emission of carbon dioxide (CO_2) and other gases to the atmosphere and whose concentration is expected to rise further up to 730-1000 ppm by 2100 (Pachauri *et al*., 2014). Indeed, this

continuous increase in carbon dioxide concentration ([CO_2]) is predicted to increase global temperature up to 3.7°C due to the greenhouse effect phenomenon (Pachauri *et al*., 2014), being this increase higher for Europe (4.5 to 5.5°C) depending on the CO_2 emission scenario (Jacob *et al*., 2014). Thus, climate change needs to be urgently considered since its derived abiotic factors, such as high temperature or elevated [CO_2], are predicted to affect plants cultivation, productivity and interactions with pest, pathogens and weeds (Chaudhry and Sidhu, 2022).

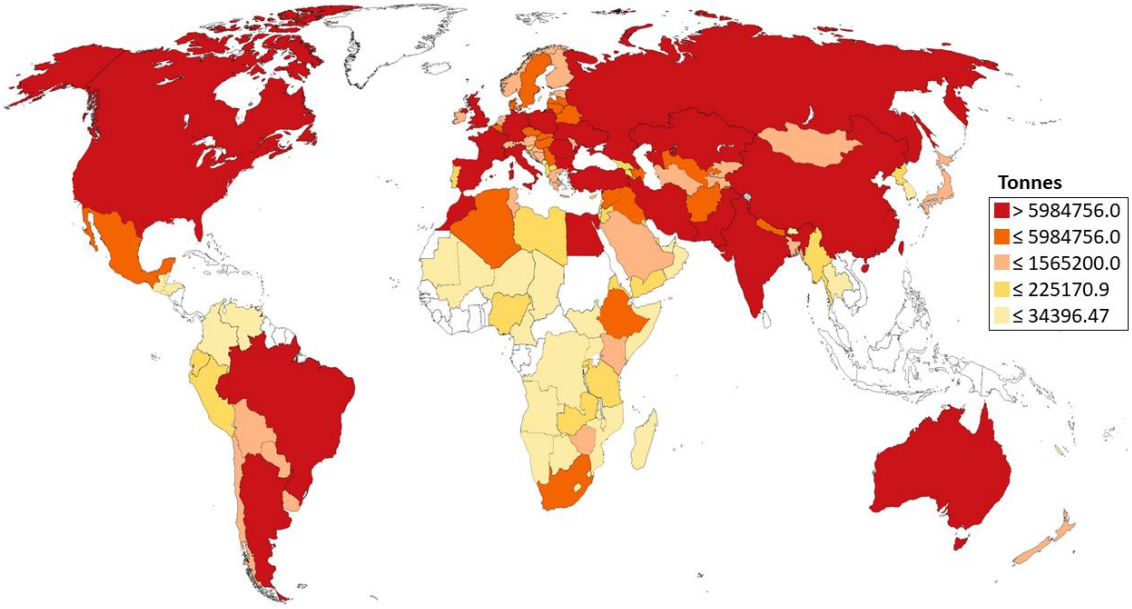

Figure 1.1. Wheat production worldwide in 2021, adapted from FAOSTAT.

High temperature is one of the primary threats to wheat derived from climate change, usually characterised by accelerated plant development and leaf senescence, resulting in a reduced photosynthetic area and plant biomass (Akter and Islam, 2017). It also damages photosynthetic machinery and reduces water-use efficiency, increasing reactive oxygen species (ROS) production and accumulation of other stress metabolites (Akter and Islam, 2017). In later development stages, elevated temperatures may also cause reproductive failure during flowering stage or adversely affect grain formation and filling and yield loss (Akter and Islam, 2017). In addition to yield loss, high temperature also affects grain quality traits basically by limiting grain assimilation supply, remobilization of nutrients and starch biosynthesis and deposition, among other effects (Wang and Liu, 2021). Considering all these effects, some crop models predict a 6% decrease in wheat production, equivalent to a possible reduction of 42 Mt/°C (Asseng *et al*., 2015).

Additionally, the effect of elevated [CO_2] on wheat has been largely studied. In general, high [CO_2] decreases stomatal conductance, greatly improving water-use efficiency, and stimulates photosynthesis, biomass and yield in C_3 species such as wheat (Broberg *et al*., 2019). Despite this increase in yield, some studies supported that elevated [CO_2] reduces grain quality, mainly due to a reduction in amino acids concentration and protein content, leading to a reduced nutritional value of wheat (Loladze, 2014). However, other studies argued that the exposure to

elevated [CO_2] over prolonged periods (days to weeks) produce a reduction in the stimulation of photosynthesis in C_3 plants, known as photosynthetic acclimation, which reduces potential growth and yield. Therefore, the responses of wheat derived from elevated [CO_2] vary greatly depending on the experiment conditions, such as [CO_2] level (ranging from 600 ppm to 800 ppm) and exposure period, growth stage (vegetative growth, anthesis, grain filling or maturity) or additional environmental stress factors (high temperature, drought, or low nitrogen supply), which complicate predictions of how wheat production will be affected by future rising of [CO_2].

1.2. Wheat main diseases nowadays: importance, infection process and resistance

In addition to abiotic factors, wheat cultivation constantly faces also biotic stresses, which include attacks by various pest and pathogens such as fungi, bacteria, oomycetes, viruses or nematodes, leading in a great obstacle for wheat production worldwide (Savary *et al*., 2019). Amongst these biotic stresses, plant diseases cause more than 21% of wheat losses on average, being fungal pathogens such as wheat rusts, blotch diseases, wheat scab, wheat blast or powdery mildew among others, considered the most detrimental (Singh *et al*., 2016; Figure 1.2).

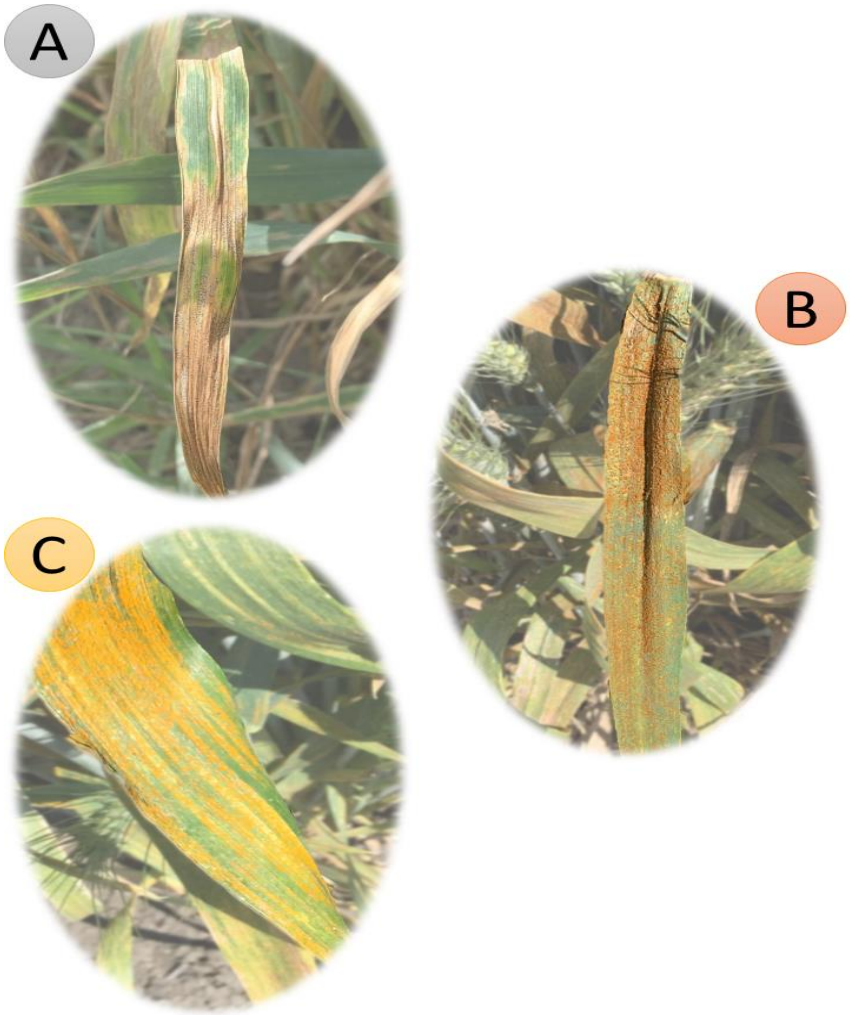

Figure 1.2. Main fungal diseases of wheat: (**A**) Septoria tritici blotch; (**B**) Leaf (brown) rust; (**C**) Yellow (stripe) rust.

In particular, cereal rusts are catalogued as the main biotic constraint in wheat, existing three of them that attack this crop, namely leaf (brown), yellow (stripe) and stem (black) rusts, which are responsible for remarkable yield losses globally. Cereal rust fungi are obligate biotrophs due to their method to gain nutrients from living host cells and heteroecious parasites, which require two taxonomically unrelated hosts to complete their life cycle. In fact, cereal rusts have five distinct stages of teliospores, basidiospores and urediniospores on cereal hosts, and pycnidiospores and aeciospores on the alternate hosts, being then macrocyclic fungi (Kolmer, 2013; Figure 1.3). On the other hand, Septoria tritici blotch (STB) disease, caused by a hemibiotrophic pathogen which develops a biotrophic phase followed by a necrotrophic one, is one of the most common wheat diseases in our climatic conditions due to its adaptation ability and fungicide resistance (Torriani *et al*., 2015; McDonald and Mundt, 2016). Moreover, due to this pathogen usually develops many cycles of sexual (pseudothecia) and asexual (pycnidia) reproduction during the growing season and can survive in wheat debris, epidemics could develop rapidly, specially thanks to dispersal of secondary inoculum (asexual pycnidiospores) mainly by rain-splash (Ponomarenko *et al*., 2011; Figure 1.4). Unfortunately, although stem rust (caused by the fungus *Puccinia graminis* f. sp. *tritici*) has been reported to cause unexpected damages in some wheat-growing areas in Europe, as in the case of Spain (Pérez-Méndez *et al*., 2022) during the last three growing seasons, the present Ph.D. Thesis has not included research with this wheat disease.

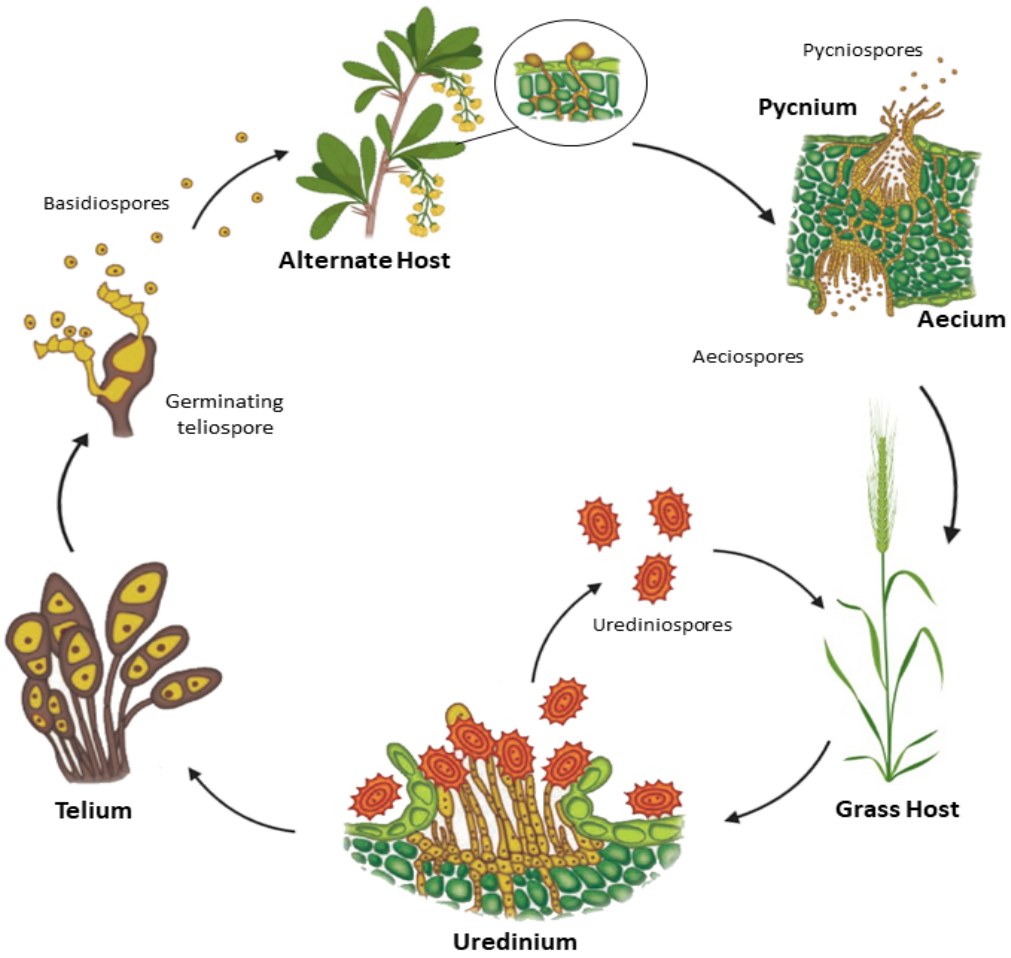

Figure 1.3. Life cycle of a heteroecious, macrocyclic cereal rust, adapted from Kolmer (2013).

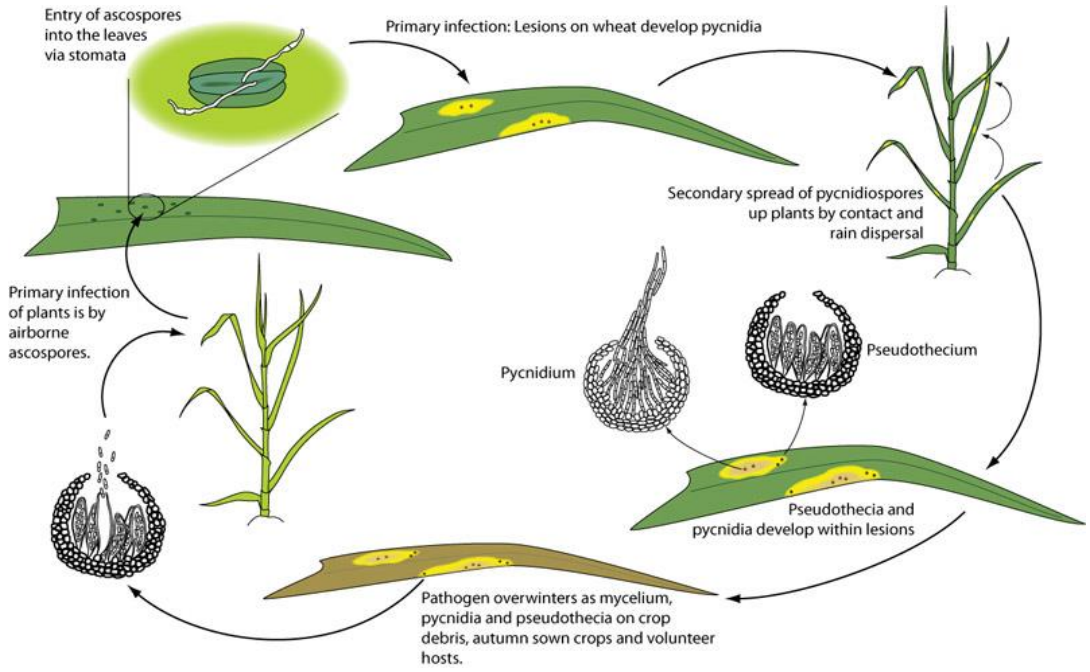

Figure 1.4. Disease cycle of Septoria tritici blotch (STB). Source: Ponomarenko *et al*. (2011).

1.2.1. Leaf (brown) rust

Leaf (brown) rust, caused by the fungus *Puccinia triticina* Erikss., is frequently present in almost all wheat growing areas, being one of the most common and widespread wheat rust (Huerta-Espino *et al*., 2011). In fact, this wide geographic distribution and its high frequency of occurrence make leaf rust responsible for more crop damage worldwide than other rusts. Therefore, leaf rust occurs in locations such as United States, Canada, Mexico (being one of the most important wheat diseases), South America (Argentina, Brazil, Chile), China, India, Russia, North (Morocco, Egypt, Tunisia) and South Africa or Australia (Chai *et al*., 2020; Figure 1.5). However, despite *P. triticina* does not generally cause extremely severe damage, in some cases the early onset of infection can result in yield losses higher than 50% (Huerta-Espino *et al*., 2011). Leaf rust causes the reduction of the photosynthetic activity of infected leaves, which usually result in yield losses through decreasing both number of kernels and kernel weights (Bolton *et al*., 2008) which, according to predictions of Chai *et al*. (2020), would account for about 8.6 million metric tons annually worldwide.

Leaf rust infection process on wheat (Figure 1.6) begins via urediniospore germ tube, which grows in the leaf surface and forms an appressorium (A) over a stomatal aperture. Then, it develops a substomatal vesicle (SSV) from which an infection hypha (IH) grows towards mesophyll cells (MC), forming a haustorial mother cell (HMC) that adheres to the host cell wall (Bolton *et al*., 2008). HMC develops a specialised hypha within the host cell that acts as a feeding structure for the fungus, called haustorium (H). Finally, new infecting hyphae continue growing, resulting in a branching network of fungal mycelium. This growing mycelium rise to form the uredinia (U; known also as pustules) at 7-10 days post infection, which produce at last new orange-red urediniospores released through the uredinia epidermis that develop infection in other leaves or plants by airborne dispersion (Bolton *et al*., 2008).

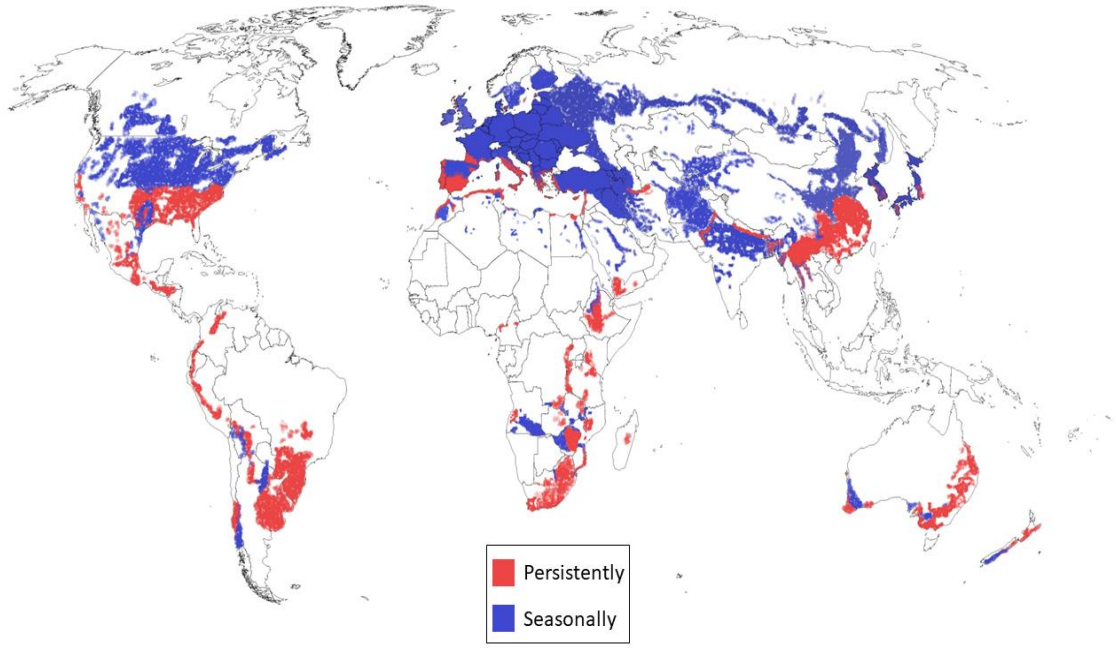

Figure 1.5. Modelled global climate suitability for *Puccinia triticina*, adapted from Chai *et al*. (2020).

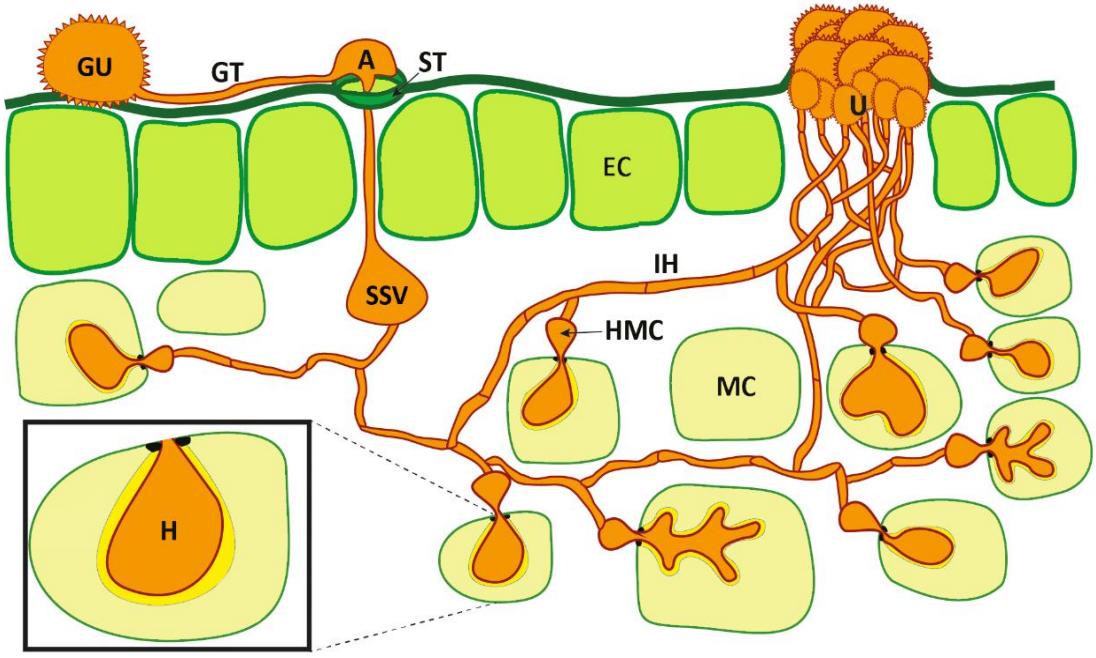

Figure 1.6. Representation of the asexual infection cycle of *Puccinia* spp. on wheat: germinated urediniospore (GU); germinative tube (GT); appressorium (A); stoma (ST); substomatal vesicle (SSV); infection hyphae (IH); haustorial mother cell (HMC); haustorium (H); uredinium (U); epidermal cell (EC); mesophyll cell (MC). Source: Garnica *et al*. (2014).

Currently, the most efficient, sustainable, and nonchemical approach to face leaf rust (as well as other diseases), is through genetic breeding, which allows plant breeders to develop new wheat cultivars that incorporate new resistance traits. Thus, numerous genes of resistance to *P. triticina* have been isolated, identified and named like *Lr* genes (McIntosh *et al*., 2020), being most of them effective in both seedling and adult plant stage, expressing hypersensitive response (HR) (Kolmer, 2013). In addition to HR, which acts as a gene-for-gene interaction with the pathogen, partial resistance (PR) is considered a polygenic, race non-specific, and of durable nature type of resistance (Martínez-Moreno *et al*., 2022), characterised by a slower development of the disease and uredinia of variable size surrounded by varying amounts of chlorosis. Unfortunately, the highly variable nature of *P. triticina* leads to the occurrence of new virulent strains of the pathogen, which eventually overcome the effectiveness of resistance genes (Huerta-Espino *et al*., 2011; Chai *et al*., 2020).

1.2.2. Yellow (stripe) rust

Yellow (stripe) rust, caused by the fungus *Puccinia striiformis* Westend f. sp. *tritici* stands as another serious wheat rust disease worldwide. This disease has undergone important global expansion in the last 50 years, affecting approximately 88% of the world's wheat production, causing losses of 5.5 million tons per year, being frequently presented in the United States, China, India, Australia, United Kingdom or Mexico (Chen, 2020). Contrary to leaf rust, stripe rust is considered a low-temperature disease because most favourable conditions for large-scale epidemics are cool humid climates (Chen, 2020), being ideal disease development conditions between 12-15°C. Despite these optimum conditions, in the past two decades new races adapted to warmer climates have emerged (Figure 1.7), spreading into new territories and producing epidemics in warmer areas where the disease was previously infrequent or absent (Chen *et al*., 2014). This situation, coupled with its long-distance dissemination capability, its high rates of mutation from avirulence to virulence, and the existence of recombinant and highly diverse populations, have made yellow rust a major threat for wheat cultivation.

The infection process of *P. striiformis* is similar to leaf rust (Figure 1.6) but with some differences. Specifically, germ tubes from urediniospores penetrate the stomata without differentiating appressoria, and then, the SSVs form three primary IH that develop HMCs at the point of contact with host mesophyll or epidermal cells (Bozkurt *et al*., 2010). These HMCs penetrate the host cell walls and form haustoria by invaginating the plasma membrane. Unlike leaf rust, the secondary infection occurs via development of intercellular runner hyphae throughout the leaf, which develops an extensive hyphal network. Finally, sporulation (characteristic yellow-orange uredinia appearing in long, narrow stripes on leaves) starts approximately from 12 to 14 days under favourable conditions (Chen *et al*., 2014), leading to desiccation of leaves and reduction of plant growth. Moreover, infection during the grain-filling stage could severely reduce kernel number, size and weight, leading to losses of yield and grain quality (Chen, 2020).

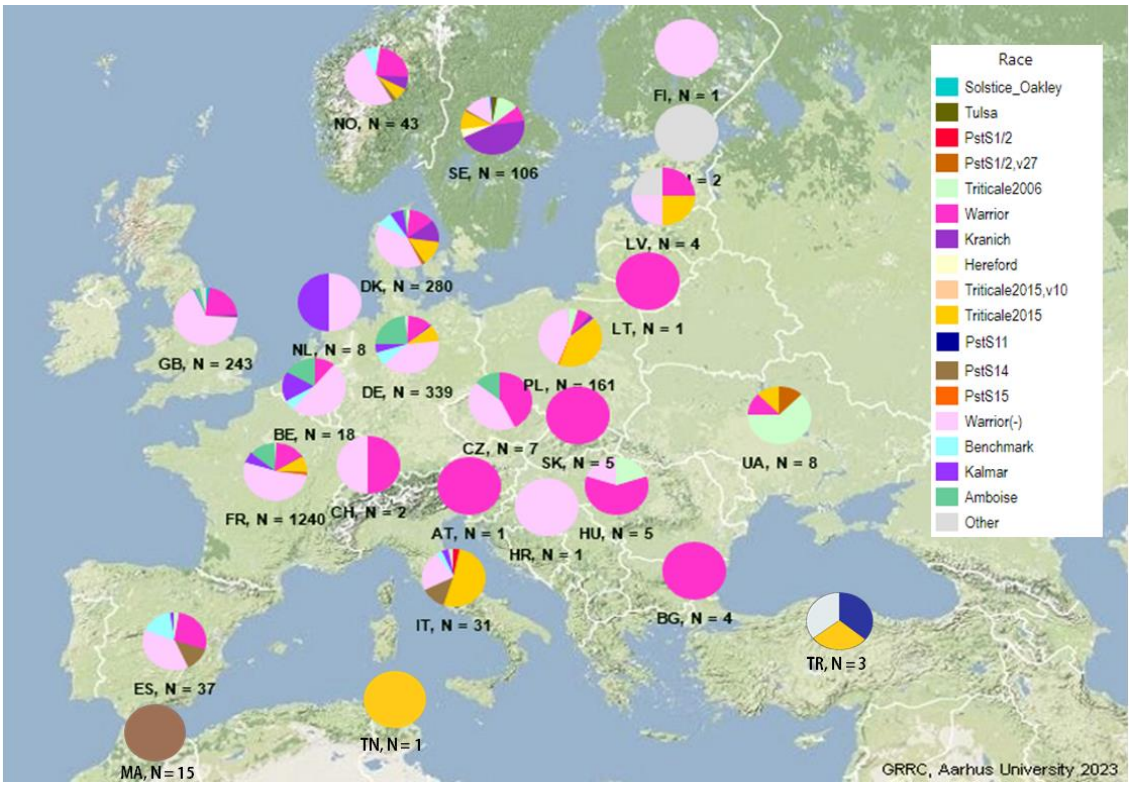

Figure 1.7. Frequency map of *Puccinia striiformis* f. sp. *tritici* races in countries of Europe and the Mediterranean Basin from 2014 to 2022. Number of samples is indicated as "N". Source: Global Rust Reference Center (GRRC, 2023).

Similar to leaf rust, resistance breeding strategies are the most appropriate method to prevent epidemics in the short and long-term future, and to avoid the huge cost of chemical control of this disease (at least 1 billion USD annually; Chen, 2020). In fact, more than 80 yellow rust resistance genes (*Yr*), have been identified and catalogued through different wheat species, most of them derived from common wheat (McIntosh *et al*., 2020). Thus, two major types of resistance are commonly used to control *P. striiformis* in wheat, all-stage/seedling resistance (ASR) and adult plant resistance (APR). The first type is expressed through all growth stages of the host, being characterised by a strong to moderate immune response (HR) that restrict pathogen development, sporulation and infection. However, this kind of resistance is often race-specific and conferred by single resistance genes with major effects, which can be overcome by new virulent races. Oppositely, plants with APR are susceptible in seedling stage, developing varying levels of resistance in post-seedling stages. APR may be race-specific or non-race-specific, and is considered more durable than seedling resistance due to its polygenic nature, often showing partial resistance (slow-rusting) patterns (Chen *et al*., 2014). Lastly, the expression of some APR genes may depend on temperature, named high-temperature adult-plant (HTAP) resistance, which is only effective at warm temperatures in the adult developmental stages (Chen, 2013). In summary, the most powerful strategy to combat wheat yellow rust is the combination of classical qualitative (ASR) and nonclassical quantitative (APR) resistance genes.

1.2.3. Septoria tritici blotch

In addition to wheat rusts, Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici* (Desm.) (Quaedvlieg *et al*., 2011), previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt.), has also emerged as a significant threat to wheat production, mainly due to its rapid evolution and adaptation to diverse agricultural conditions (McDonald and Mundt, 2016). Therefore, this disease is well-developed in temperate climates with cool, wet weather, such as wheat growing areas of North America, northern France, Germany, and the United Kingdom, but also extends to hot dry climates such as areas of the Mediterranean Basin, North Africa, or Iran. Moreover, its adaptability implies resistance to multiple fungicides, which results in wheat yield losses up to 50% in susceptible cultivars, requiring intensive chemical control measures that could reach, only in the European Union, an annual cost of ~1 billion euros (70% annual cereal fungicide usage) (Torriani *et al*., 2015). In addition to the resistance to fungicides, the lifestyle of the pathogen, which develops a long asymptomatic phase prior to the proliferation of disease symptoms, could be another factor that hampers the right application of chemical strategies to control this disease.

In this sense, *Z. tritici* is considered a hemibiotrophic pathogen, which develops a symptomless phase considered as biotrophic, followed by a necrotrophic phase (Figure 1.8). Thus, the infection process starts with the primary source of inoculum (ascospores or pycnidiospores, depending on environmental conditions) developing hyphae that enter host tissue mainly via stomata. Once entered into substomatal cavity (after 12-24 hours), fungal hyphae colonise the intercellular space surrounding the mesophyll cells in a long symptomless phase (biotrophic) that typically extends for 10-15 days after infection depending on wheat genotype-fungal isolate combination. The transition to the necrotrophic phase is marked by the formation of pycnidia (asexual fruiting bodies) in the colonised substomatal cavities, stage in which the infection causes chlorotic lesions, usually followed by later necrotic blotches on wheat, mainly due to extensive host programmed cell death (Steinberg, 2015). Furthermore, the fungus releases cell-wall degrading enzymes, as well as proteases, leading to an increase of nutrient content from the dying host tissue in the apoplastic space that allows rapid fungal growth and proliferation (Somai-Jemmali *et al*., 2017b). Finally, mature pycnidia produce multi-cellular pycnidiospores that are released by water splash and able to spread the infection to other leaves and plants.

Similar to rusts, wheat resistance to *Z. tritici*, which has increased over the last two decades, can be either qualitative (isolate-specific), which depends on major genes with a large effect according to a gene-for-gene interaction, or quantitative (isolate-nonspecific), which develops a partially resistant phenotype controlled by several or many genes with moderate to small effects (Brown *et al*., 2015). Thus, considering the variation of resistance to STB in the field, quantitative resistance plays an important role in wheat breeding due to its effectiveness against several pathotypes of the pathogen and its durability (Brown *et al*., 2015). The effectiveness of this type of resistance could be gradually eroded, but at a slower rate and to a lesser extent than the rapid evolution of *Z. tritici* virulence in the cases of gene-for-gene interactions. Both types of resistance have been extensively studied in bread wheat, for which at least 22 major genes (*Stb* genes) conferring qualitative resistance, together with 167 quantitative trait loci (QTLs), have been identified and mapped to date (Louriki *et al*., 2021). However, although recent studies have

found effective sources of resistance against *Z. tritici* in durum wheat landraces of Tunisia, the durum wheat-*Z. tritici* interaction has been, in general, poorly investigated.

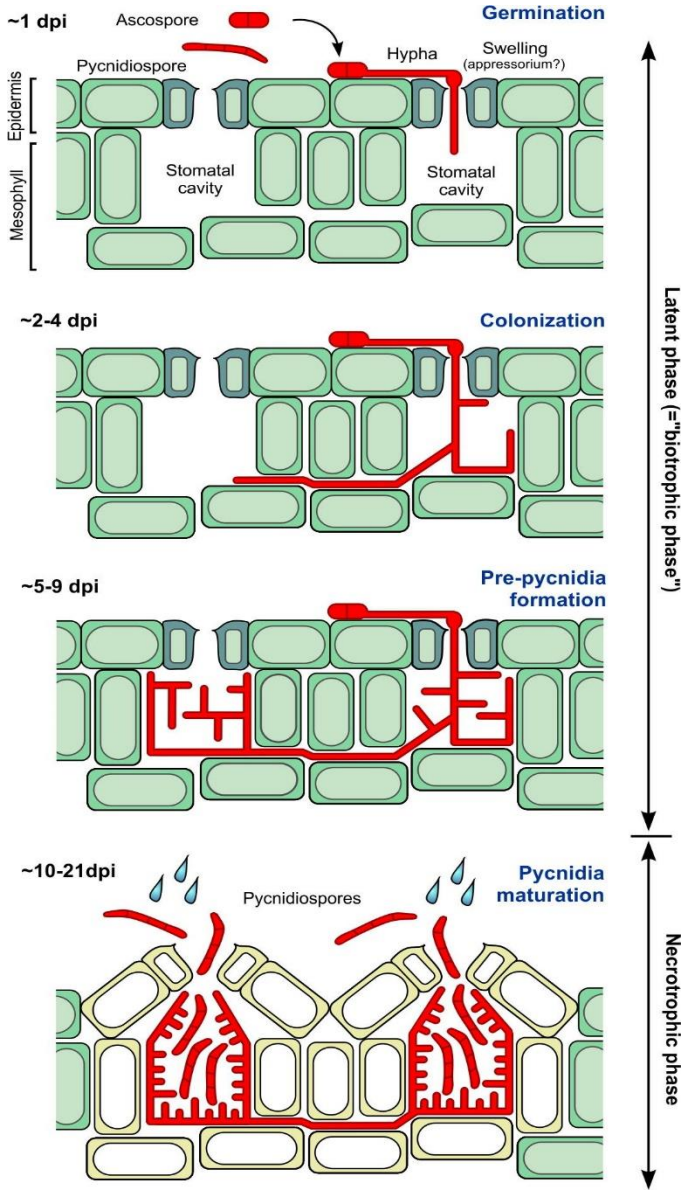

Figure 1.8. Infection stages of the fungal pathogen *Zymoseptoria tritici* on wheat. Source: Steinberg (2015).

1.3. Wheat-pathogen interactions in the context of climate change

Interactions between wheat and its pathogens will be probably influenced, positively or negatively, by future climate change alterations in temperature, [CO_2] and water regimes, which would modify plant development and resistance pathways, on one side, and pathogen virulence mechanisms and life cycle, on the other side (Velásquez *et al*., 2018). Despite this feasible influence, not many realistic field studies about the effects of simultaneous abiotic and biotic stresses in wheat have been conducted to date (Suzuki *et al*., 2014; Ramegowda and Senthil-Kumar, 2015). In addition, these studies often show dissimilar results about the effects of

expected abiotic factors on different wheat-pathogen interactions, which make disease predictions under future climate change a challenging task. In fact, these variable results could be due to plant responses to simultaneous stresses controlled by different, and sometimes opposing, signalling pathways between abiotic and biotic stress, leading plants to develop a specific and unique response for each situation. Lastly, in the case of diverse abiotic and/or biotic stresses occurring at different time intervals, the concept of plant acclimation (or cross tolerance/susceptibility) must be considered because prolonged exposure to an abiotic stress could reduce or enhance a subsequent pathogen attack (Bostock *et al*., 2014; Ramegowda and Senthil-Kumar, 2015). Therefore, there is an urgent need for study empirically how combined environmental factors expected in future climate change would influence plant immunity, pathogen virulence and disease development in wheat-pathogen interactions, which is essential to develop adaptation and mitigation strategies to face the most relevant wheat diseases, together with disease management options (Juroszek and von Tiedemann, 2011).

In order to identify changes in wheat-pathogen interactions under future climate change conditions, the identification of macro and microscopic components of resistance postulates as an essential process. In general, wheat fungal diseases are commonly evaluated through visual (subjective) assessment, determining usual parameters such as percentage of leaf covered with disease symptoms or indexes derived from plant-pathogen interactions. However, in recent years some studies developed assessment for plant diseases through image analysis, evaluating precisely macroscopic symptoms of multiple diseases (Stewart and McDonald, 2014; Karisto *et al*., 2018; Gallego-Sánchez *et al*., 2020). These image-based analyses allow the exploration of more dimensions of disease phenotypes, such as quantification of fungal reproduction structures, distinction among genotypes with different levels of disease severity, or improved reproducibility and sensitivity in disease quantification. Additionally, microscopic evaluation of plant-pathogen interactions is commonly developed through histopathological methods, identifying morphological and molecular changes in plants and pathogens at the cell and tissue levels (Soleiman *et al*., 2014; Somai-Jemmali *et al*., 2017a; 2017b; Sørensen *et al*., 2017). Thus, some microscopic components of resistance can be identified, such as cell wall strengthening through cellular lignification and callose deposition, generation of ROS, or the presence and characteristics of host defence responses in the form of HR, amongst others.

In summary, identifying both macro and microscopic components of resistance can be considered a fundamental step to thoroughly assess the effects of expected abiotic factors derived from climate change, such as elevated temperature and [CO_2], in diverse wheat-pathogen interactions.

1.4. Objectives

The main objective of this Ph.D. Thesis is to evaluate the behaviour of different wheat-pathogen interactions under conditions of increasing temperature and [CO_2] derived from predicted climate change, focusing on current wheat germplasm collections from Spain. In order to do this, macroscopic and microscopic assessments were carried out in three of the most important wheat fungal pathogens: *Zymoseptoria tritici, Puccinia triticina* and *Puccinia striiformis* f. sp. *tritici*.

This general objective is subdivided by four specific objectives:

- ➢ To identify and select resistant and susceptible accessions against the pathogen *Zymoseptoria tritici* in a collection of durum wheat germplasm under controlled conditions (Chapter 2).
- ➢ To evaluate the wheat-pathogen interactions of selected durum wheat accessions against *Zymoseptoria tritici* under conditions of increasing temperature and [CO_2] in greenhouse experiments (Chapter 3).
- ➢ To identify and select resistant and susceptible accessions against the pathogen *Puccinia triticina* in a collection of durum wheat germplasm under controlled conditions, and then to evaluate the wheat-pathogen interactions of these selected accessions under conditions of increasing temperature and [CO_2] in greenhouse experiments (Chapter 4).
- ➢ To identify and select resistant and susceptible accessions against the pathogen *Puccinia striiformis* f. sp. *tritici* in a collection of bread wheat germplasm under controlled conditions (Chapter 5).

1.5. Thesis Outline

Chapter 1 includes the introduction to the issue of this investigation, followed by the description of the research objectives in this Ph.D. Thesis. Chapters 2 to 5 include diverse wheat-pathogen interactions and their macroscopic and microscopic evaluations, as well as assessment of the effects of increasing temperature and [CO_2] weather conditions on these interactions. Chapters 2 and 5 have been published in international scientific journals, whereas contents of Chapters 3 and 4 have been submitted for publication to international journals and are under revision. The last Chapter 6 summarises the main conclusions of this doctoral Thesis.

Chapter 2 evaluates the behaviour of a durum wheat germplasm collection against the fungal pathogen *Zymoseptoria tritici*, first classifying resistant and susceptible genotypes and then using image analysis to assess quantitatively their disease patterns. Moreover, some genotypes were also classified quantitatively according to their disease patterns using image analysis. These evaluations conclude that current Spanish durum wheat material is, overall, susceptible to STB disease.

Chapter 3 continues the research of Chapter 2 and evaluates the effects of increasing temperatures and [CO_2] on the interactions of durum wheat against the pathogen *Zymoseptoria tritici* through macro- and microscopic evaluation methods. Thanks to these evaluations it was possible to find out that, under these weather conditions, durum wheat genotypes expressed and maintained resistance traits to the *Z. tritici* infection. In addition, the exposure to increasing temperature severely affected infection establishment and development, whereas elevated [CO_2] slightly improved fungal infection.

Chapter 4 evaluates the behaviour of a durum wheat germplasm collection against the fungal pathogen *Puccinia triticina*, to classify and select resistant and susceptible genotypes. Then, this Chapter focuses on the effects of increasing temperature and [CO_2] on interactions of durum wheat against the pathogen *P. triticina* using both macroscopic and microscopic

evaluation methods. Thus, although durum wheat genotypes exposed to these changing weather conditions maintained their general resistant and susceptible traits against leaf rust infection, elevated temperature induced host physiological responses that restrict fungal development to some extent, whereas the combination with elevated [CO_2] could both slightly improve these host responses or favours fungal development. Additionally, the exposure to increasing temperature severely reduced infection establishment of *P. triticina*.

Chapter 5 assesses the responses of a bread wheat germplasm collection against the fungal pathogen *Puccinia striiformis* f. sp. *tritici*, classifying these genotypes, and using macroscopic and microscopic evaluations to quantitatively analyse some of them. These results conclude on one side, that Spanish bread wheat germplasm shows resistance to current *P. striiformis* isolates, and on the other side, that detailed evaluations would be essential to detect the appearance of new virulent races more adapted to warmer climates.

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Chapter 2. Behaviour of Spanish durum wheat genotypes against *Zymoseptoria tritici*: resistance and susceptibility

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Abstract

Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici*, is one of the most important wheat diseases worldwide, affecting both bread and durum wheat. The lack of knowledge about the interaction of durum wheat with *Z. tritici*, together with limited resources of resistant durum wheat material, have both led to a rising threat for durum wheat cultivation, particularly in the Mediterranean Basin. In Spain, STB has increased its incidence in the last few years, leading to higher costs of fungicide applications to control the disease. Therefore, identification of new sources of resistance through wheat breeding stands out as an efficient method of facing STB. In order to identify sources of resistance to *Z. tritici*, the percentage of necrotic leaf area, the disease severity and the pycnidia development through image analysis were evaluated from 48 durum wheat Spanish accessions (breeding lines and commercial cultivars) in growth chambers against an isolate of *Z. tritici* from Córdoba. Amongst them, two breeding lines and six commercial cultivars showed resistant responses by limiting STB development through the leaf or its reproduction ability, while the other 40 accessions presented a susceptible response. Provided these resources of resistance in Spanish durum wheat genotypes, future breeding programs could be developed, incorporating both agronomic traits and resistance to STB.

Key words: Septoria tritici blotch; plant breeding; foliar disease; germplasm

2.1. Introduction

Wheat is the most widespread crop, growing on about 214 million hectares globally, leading the production of cereals with 734 million tons, and providing a large proportion of human diets (FAOSTAT, 2021). In the European Union (EU) only, wheat covers approximately 26 million hectares with a production of 155 million tons (EUROSTAT, 2021). Wheat cultivation is divided into two main wheat species: bread (common) wheat (*Triticum aestivum* L.), which is used mainly for baking, covers 94% of the total cultivated wheat area, while durum wheat (*Triticum turgidum* L. subsp. *durum*), which is used for pasta and traditional Mediterranean dishes (Royo *et al.*, 2017), covers approximately 13 million hectares. Global demand of wheat consumption is expected to increase by up to 60% by 2050 (The Wheat Initiative, 2021) due to the growth of the human population. Increases in wheat yield or changes in agricultural practices are not enough to achieve this aim; better protection against pest and pathogens is also necessary, as they could cause wheat losses of up to 10% and 20% of total wheat production in the future, respectively (Oerke, 2006).

One of the most important constraints which could prevent the increase in global wheat production is the Septoria tritici blotch (STB) disease, caused by the fungus *Zymoseptoria tritici* (Desm.) (Quaedvlieg *et al.*, 2011), previously known as *Septoria tritici* (teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt.). *Z. tritici* can be considered as a hemibiotrophic fungus because it colonizes the intercellular space surrounding the mesophyll cells without visible symptoms, which typically occurs for 10-15 days after infection (biotrophic phase). This biotrophic phase is followed by a necrotrophic phase, in which the infection causes chlorotic lesions and later necrotic blotches on wheat, bearing fruiting bodies called pycnidia in the colonised substomatal cavities (Somai-Jemmali *et al.*, 2017b). This disease affects both common and durum wheat, and its development is improved in temperate climates with cool, wet weather, such as in North America (Linde *et al.*, 2002; Banke and McDonald, 2005), northern France, Germany, and the United Kingdom, where wheat yield losses can reach 50% in susceptible cultivars (Fones and Gurr, 2015). However, distribution of *Z. tritici* not only covers temperate climates where wheat cropping plays an important role, but also extends to hot dry climates such as the Mediterranean Basin, North Africa, or Iran, where durum wheat cultivation stands out because of its importance in the Mediterranean diet (Hosseinnezhad *et al.*, 2014; Benbelkacem *et al.*, 2016; Ünal *et al.*, 2017; Chedli *et al.*, 2018). This global spread of *Z. tritici* is due to its rapid evolution and adaptation to diverse agricultural conditions (McDonald and Mundt, 2016). This ability of adaptation also implies a resistance to multiple fungicides, which causes an annual cost of ~1 billion euros (70% annual cereal fungicide usage) in the EU only, thereby representing one of the most important foliar diseases of wheat (Torriani *et al.*, 2015).

Given this ever-growing magnitude of STB in wheat cultivation, breeding programs for wheat resistance are postulated as an effective, economical, and environmentally sustainable approach to face *Z. tritici* using new resistant wheat lines. Wheat resistance to STB, which has increased over the last two decades, can be either qualitative (isolate-specific), which depends on major genes with a large effect according to a gene-for-gene interaction (Kema *et al.*, 2000; Brading *et al.*, 2002), or quantitative (isolate-nonspecific), which develops a partial phenotype controlled by several or many genes with moderate to small effects (Brown *et al.*, 2015). Quantitative resistance plays an important role in wheat breeding due to its effectiveness

against all genotypes of the pathogen and its durability (Brown *et al.*, 2015; Niks *et al.*, 2015). In fact, many studies evaluated the resistance of wheat cultivars against *Z. tritici* through quantitative scoring (Chartrain *et al.*, 2004; Suffert *et al.*, 2013; Gerard *et al.*, 2017). This scoring method, which is based on a subjective visual evaluation, can be supported or even improved by using current methods of image analysis (Stewart and McDonald, 2014; Stewart *et al.*, 2016). Both types of resistance have been extensively studied in bread wheat, for which 21 major genes (*Stb* genes) conferring qualitative resistance, together with 167 quantitative trait loci (QTLs), have been identified and mapped to date (Brown *et al.*, 2015). However, the interaction between *Z. tritici* and durum wheat has been poorly investigated (Somai-Jemmali *et al.*, 2017a), resulting in an absence of any *Stb* genes identified in durum wheat.

This lack of characterisation of resistance genes implies difficulties in finding durable sources of resistance to STB in durum wheat, leading to a great threat for durum wheat production in many cropping areas where its importance exceeds that of bread wheat, such as the Mediterranean Basin, which is the largest durum-wheat-producing area in the world, with about 60% of the global durum wheat cropping area and 75% of the global durum wheat production (Royo *et al.*, 2017). Moreover, this region is the most significant durum import market and the largest consumer of durum wheat products (Soriano *et al.*, 2017). Considering this economic relevance, STB becomes a major constraint for durum wheat production in the Mediterranean Basin and Eastern and Central Africa (Berraies *et al.*, 2014; Ferjaoui *et al.*, 2015; Kidane *et al.*, 2017). In the case of Spain, situated in the Mediterranean Basin, STB has led to high levels of disease severity in some of its wheat-growing regions such as Andalusia (Cátedra and Solís, 2003), Extremadura, and Catalonia, becoming an important threat, especially for durum wheat cultivars located in Southern Spain (Royo and Briceño-Félix, 2011). In summary, the severity and spread of *Z. tritici*, together with the absence of resistance material and virulence knowledge of this disease, emphasize the necessity to investigate new genetic sources of resistance among Spanish wheat accessions, which may also incorporate valuable agronomic traits.

Although *Z. tritici* is increasing its prevalence in Spain in the last few years, limited resources of resistance of durum wheat material are available for farmers. Hence, the main objective of this research was to identify new sources of resistance to *Z. tritici* for being included in current wheat breeding programs. In particular, we analysed the incidence of STB in 22 durum wheat breeding lines and 26 commercial cultivars from Spain, under controlled conditions in growth chambers.

2.2. Material and Methods

2.2.1. Plant material

In our study, we evaluated 48 durum wheat (*T. turgidum* spp. *durum*) accessions against an isolate of the hemibiotrophic fungus *Z. tritici.* Amongst them, 26 accessions were commercial cultivars, selected from the Red Andaluza de Experimentación Agraria (RAEA) (Castilla *et al.*, 2020), and 22 were breeding lines, belonging to the wheat breeding program being developed at IFAPA (Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica), Spain. From the RAEA commercial cultivars studied, six were considered

as control- checks due to their role as historical checks in Spanish durum wheat experiments. The other 22 were recently registered varieties (Supplementary Table S2.1). After a first assessment of disease susceptibility, the historical check 'Amilcar' was selected as the reference susceptible control accession.

Nine wheat accessions with diverse disease severity (DS) rating scale were randomly selected for the image analysis assays. The DS rating scale goes from 0 to 5 (McCartney *et al.*, 2002), where 0 = immune with no visible symptoms, 1 = highly resistant with hypersensitive flecking, 2 = resistant with small chlorotic or necrotic lesions and no pycnidial development, 3 = moderately resistant, characterised by coalescence of chlorotic and necrotic lesions with slight pycnidial development, 4 = susceptible with moderate pycnidial development and coalesced necrotic lesions, and 5 = very susceptible with large, abundant pycnidia and extensively coalesced necrotic lesions (Figure 2.1). These accessions were: 'LG Origen', 'BL 39', 'Sculptur' (DS 3), 'Athoris', 'BL 36', 'BL 33' (DS 4), 'Avispa', 'BL 34' and 'Amilcar' (DS 5).

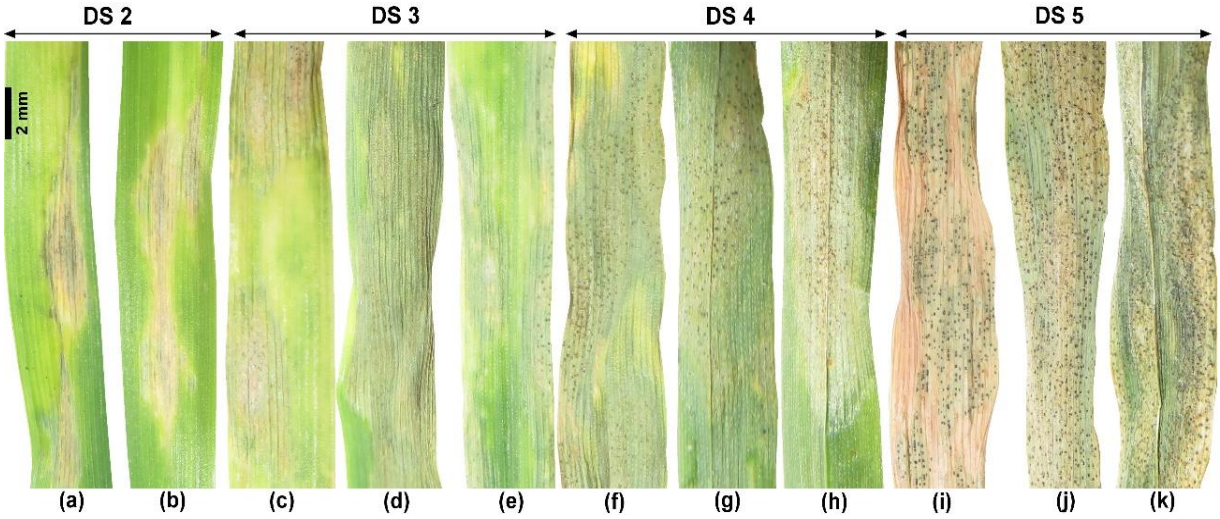

Figure 2.1. Example of leaves infected with Septoria tritici blotch (STB) showing diverse disease severity (DS) scores. Leaves from breeding lines, commercial cultivars, and control-checks with different DS scores: DS 2, (**a**) 'RGT Rumbadur' and (**b**) 'RGT Voilur'; DS 3, (**c**) 'LG Origen', (**d**) 'BL 39', and (**e**) 'Sculptur'; DS 4, (**f**) 'Athoris', (**g**) 'BL 36', and (**h**) 'BL 33'; DS 5, (**i**) 'Avispa', (**j**) 'BL 34', and (**k**) 'Amilcar'. Disease severity is presented according to McCartney *et al*. (2002), see Material and Methods for rating scale.

2.2.2. Pathogen isolation and molecular characterisation

Zymoseptoria tritici was isolated from a naturally infected field of Santaella, Córdoba (Spain). Infected leaves were cut into pieces and placed horizontally in a square culture dish (120 mm × 120 mm) containing wet filter paper to maintain high humidity for 24 or 48 h. Mature pycnidia were observed on the leaf surface, as well as the conidia, in a gelatinous matrix of cirri. We used a sterile needle and a dissecting microscope to collect cirri from the pycnidia and transfer them onto potato dextrose-agar (PDA; Difco Laboratories Inc.) plates, supplemented with streptomycin (50 mg/L) and chloramphenicol (250 mg/L). PDA plates were incubated for 48 h at 20°C in the dark. The colonies grew in the form of yeast-like spores. Once the colony began to darken, the spores were ready to harvest. These colonies were subsequently

transferred with a sterile needle to PDA plates every 48 h to finally isolate the fungus *Z. tritici* avoiding cross-contamination from other microorganisms. Stocks of *Z. tritici* were obtained according to the procedure developed by Stewart and McDonald (2014) for fungal-isolate retrieval, with minor modifications. Colonies were collected using a sterile scalpel, cutting them into small pieces of PDA, and mixing them in a 250 mL conical flask containing 50 mL of yeast-malt-sucrose liquid medium (4 g of yeast extract, 4 g of malt extract, 4 g of sucrose, and 1 L of H_2O). Flasks were then sealed with cotton stoppers and aluminium foil and placed on a shaker at 210 rpm and 18°C in the dark. After 8 to 10 days of growth, 25 mL of spore concentrate was collected in a 50 mL Falcon tube and centrifuged at 3500 rpm (Eppendorf Centrifuge 5810R) at room temperature for 15 min to precipitate the spores. After removing the supernatant, the spore pellet was thoroughly mixed with distilled water. Fungal stocks were stored as microconidial suspensions at -80°C with 30% glycerol until needed.

Molecular identification of *Z. tritici* isolate was based on the amplification and sequencing of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). Mycelium for DNA extraction was grown on PDB (Scharlab, S.L. Spain). Genomic DNA was extracted using the Plant DNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. A 290 bp fragment of the ITS region was amplified using the pair primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS2 (5'GCT GCG TTC TTC ATC GAT GC 3') (White *et al.*, 1990). PCR products were purified using the FavorPrepTM purification kit (FAVORGEN, China). DNA was Sanger sequenced at SCAI facility at University of Córdoba (Spain) with the forward and reserve primers as in PCR. Sequences were subjected to BLAST analysis (BLAST, 2021) at NCBI (National Center for Biotechnology Information, United States). The ITS sequences were deposited at the NCBI database under the SUB9540116 accession number.

2.2.3. Inoculation assays

Our inoculation procedure was developed as described by Stewart and McDonald (2014), with minor modifications. Seeds of the 48 durum wheat accessions mentioned above (commercial cultivars and breeding lines) were sown in 8 × 7 × 7 cm pots containing a mix (1:1, v/v) of commercial compost (Suliflor SF1 substrat; Suliflor Lithuania) and sand. Pots were placed in trays and incubated in a growth chamber at 21°C and 70% relative humidity (RH), with 16 h of light. At the same time, fungal spores were retrieved from the spore suspension at -80°C, before adding 150 µL of the spore suspension to a 250 mL conical flask containing 100 mL of yeast-malt-sucrose medium. The flask was then placed on a shaker as described above to obtain fresh spores as inoculum. After 16 days, seedlings were inoculated when the second and third-leaves emerged (growth stage Z13; Zadoks *et al.*, 1974) in a spore suspension prepared with distilled water, to which Tween-20 was added (0.1%). Spore concentration was measured using a hemocytometer, and conidia were adjusted to 10^7 spores mL^{-1}. Four seedlings of each accession were inoculated with 30 mL of spore solution using a hand sprayer until the solution ran off the leaves. A total of 192 (8 leaves used per accession) plants were inoculated in each biological replicate. Once leaves were totally dry, plants were sealed in clear plastic bags to provide 100% RH, maintained for 48 h in a growth chamber at 22/18°C day/night with a 16 h photoperiod. After 48 h, the plastic bags were removed, and plants were kept in the same conditions with a humidity level of 75-80% using humidifiers to promote infection. The

susceptible control check 'Amilcar' was inoculated following the same treatment, under the same conditions and with the same replications as the other accessions. This experiment was performed three times with similar results.

2.2.4. Disease assessment

At 20 days post inoculation, the second and third-leaves of each plant were evaluated. The infection process was quantitatively scored as the percentage of necrotic leaf area (NLA), including both sporulating (Chartrain *et al.*, 2004) and nonsporulating areas (Suffert *et al.*, 2013; Gerard *et al.*, 2017). The percentage of NLA also included chlorotic areas of accessions which did not develop necrosis. In addition, seedling reactions were qualitatively scored using the DS rating scale from 0 to 5 (McCartney *et al.*, 2002). Reaction types 0-3 were considered resistant, while reaction types 4 and 5 were considered susceptible. Although reaction type 3 includes pycnidia development, it is considered resistant because the growth and sporulation of the fungus is quite restricted, and the chlorotic reaction is similar to the chlorotic blotches of reaction type 2. The same analysis was performed with adult plants (2 months) in a growth chamber to confirm the absence of variation in resistance with respect to the seedlings (Supplementary Figure S2.1).

Once all plants were scored, 2-cm sections of the middles of leaves with STB symptoms were cut and photographed using a digital camera (Canon Powershot SX710 HS) and a ruler as guidance for measurements. Leaves from three accessions with a DS of 2, 3, 4, and 5 were randomly selected in order to support the DS rating scale through image analysis using the imaging software NIS Elements (vers. 4.50; Nikon Instruments Inc.). First, images were cropped into NLA sections of 25 mm^2 bearing pycnidia. Then, the software permitted a colour threshold analysis that distinguished pycnidia amongst the STB lesions. Subsequently, the number and area of pycnidia were measured using established size and circularity parameters. In order to double-check the images, manual selection of pycnidia was carried out in cases where the pycnidia were not selected according to the established parameters. Lastly, the total pycnidial area (mm^2), number of pycnidia, and pycnidia size (mm^2) were calculated. For pycnidium assessment, three cuts per accession were analysed and in two independent experiments.

2.2.5. Data analyses

The experimental design was developed as random blocks. The percentage of NLA covered with pycnidia was transformed according to the formula $y = \log(x)$, whereas pycnidium count and the number of pycnidia per mm^2 of NLA were transformed according to the formula $y = \sqrt{(x)}$. ANOVA and Tukey's honestly significant difference (HSD) test were performed on these data. Pycnidium size was analysed using the Kruskal-Wallis test. Data analyses and figures were carried out using R software (R Core Team, 2020), NIS-Elements, and ImageJ (Schneider *et al.*, 2012).

2.3. Results

2.3.1. STB infection studies

The development and the aggressiveness of STB in 48 durum wheat accession is shown in Figure 2.2. The accessions expressed, on average, 62.2% NLA, with most accessions falling in the range of 55-75%, indicating the great development capability of *Z. tritici* amongst them. Accession 'BL 41', which showed the highest NLA value (79%), belonged to the group of selected lines from our current breeding program, while line 'BL 45' showed the lowest NLA value (50%). Breeding lines presented, on average, 64% NLA. Commercial cultivars also expressed a high percentage of NLA, with an average value of 60.5% in this group. Most commercial cultivars had an NLA ranging from 55% to 75%, except for 'LG Origen' (33.3%), 'Don Ortega' (35.8%), 'Sculptur' (39.8%), 'Nobilis' (50.2%), and 'RGT Rumbadur' (50.2%). Cultivar 'Teodorico' presented the highest NLA value amongst commercial cultivars (76.4%), followed by 'RGT Fernandur' and 'Sy Leonardo', with 75.4% and 74.8% NLA, respectively. Lastly, the control-check cultivars (selected from commercial cultivars) showed mean levels of NLA ranging from 42.9% ('Kiko Nick') to 77.7% ('Amilcar'). This group of accessions presented similar NLA values to the breeding lines and the commercial cultivars, with an average NLA value of 61% (Figure 2.2).

Disease severity scores showed differences among the accessions studied. Most of the breeding lines (18 accessions out of 22) presented a DS of 4 (susceptible), which denotes significant fungus reproduction capability in the form of pycnidia in the necrotic lesions. In addition, two breeding lines, 'BL 34' and 'BL 40', expressed a DS of 5 (very susceptible) with high reproduction of the fungus and extensively coalesced lesions (Figure 2.2). In contrast, breeding lines 'BL 28' and 'BL 39' presented a DS score of 3 (moderately resistant), indicating that the development of *Z. tritici* produced lesions in the form of necrosis but to lesser extent and with limited presence of pycnidia. Commercial cultivars exhibited more variability in DS scores, showing accessions ranging from 2 to 5 (Figure 2.2). This group presented more lines with a DS of 5 (very susceptible) than the breeding lines; however, in contrast, the included accessions that exhibited resistance (DS of 2), such as 'RGT Rumbadur' and 'RGT Voilur', only showed necrotic or chlorotic lesions without the presence of pycnidia. Commercial cultivars also included two accessions with moderate resistance (DS of 3): 'LG Origen' and 'Sculptur'. Regarding control-check cultivars, each DS score (except for a DS of 2) featured two accessions; 'Amilcar' and 'Avispa' showed a DS of 5, 'Athoris' and 'Euroduro' showed a DS of 4, and 'Kiko Nick' and 'Simeto' showed a DS of 3. It should be noted that none of the accessions studied presented a DS of 0 (immune with no visible symptoms) or 1 (highly resistant with hypersensitive flecking). Lastly, all studied accessions were organised to analyse their distribution according to DS and group (Figure 2.3). In total, 40 out of 48 accessions (83.3%) could be considered susceptible to STB as they showed a DS of 4 or 5, while only 8 out of 48 accessions (16.6%) exhibited resistance with a DS of 2 or 3 (Figure 2.3).

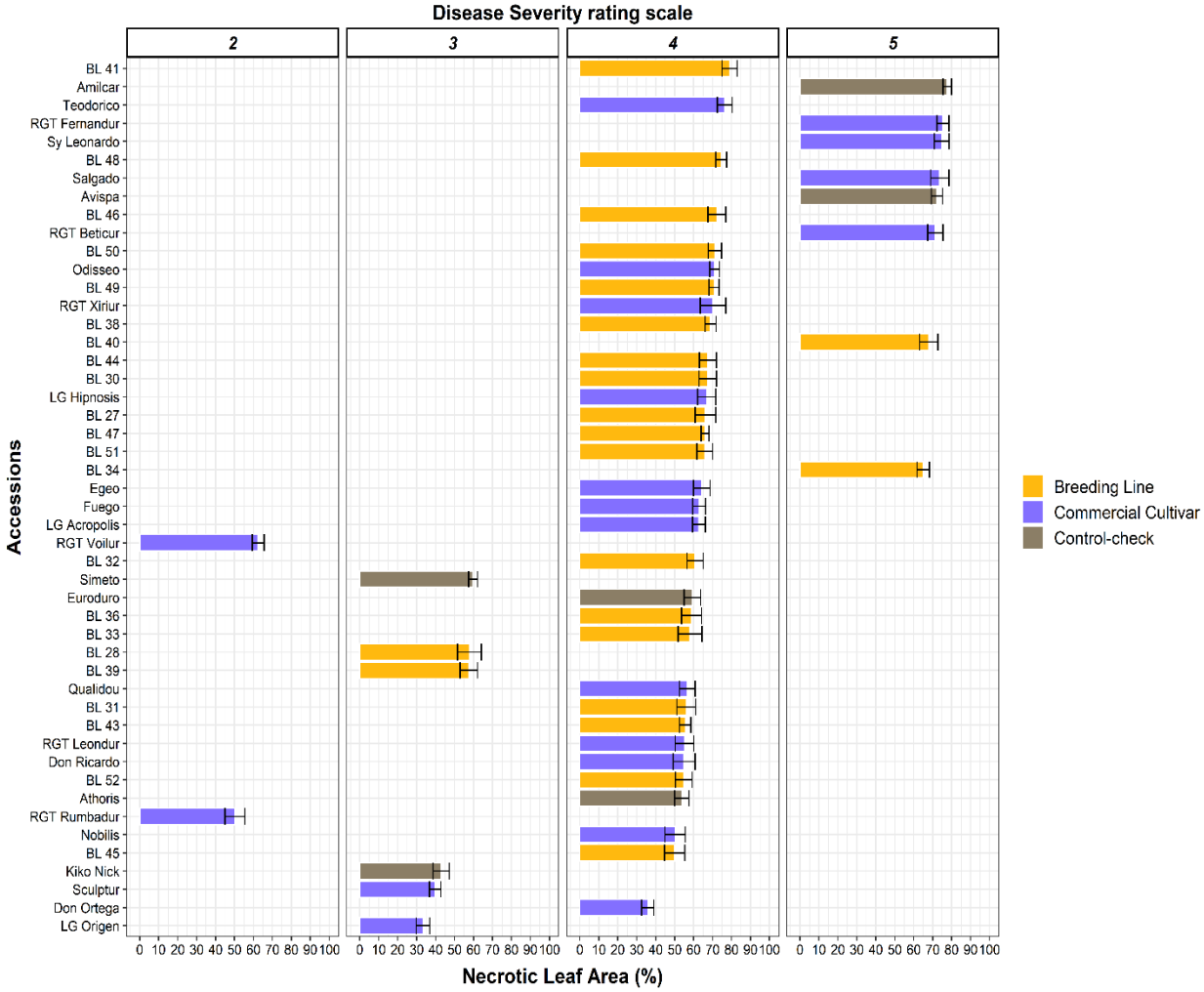

Figure 2.2. Septoria tritici blotch (STB) infection in durum wheat accessions. Mean percentage of necrotic leaf area (NLA), presented in columns, and disease severity (DS) rating scale, presented as numbers at the top of the figure for breeding lines, commercial cultivars, and control-checks. Accessions were arranged according to their mean percentage of NLA and classified by DS in panels. DS is presented according to McCartney *et al.* (2002), see Material and Methods for rating scale. Error bars represent the SE calculated from three independent experiments with eight replicates each.

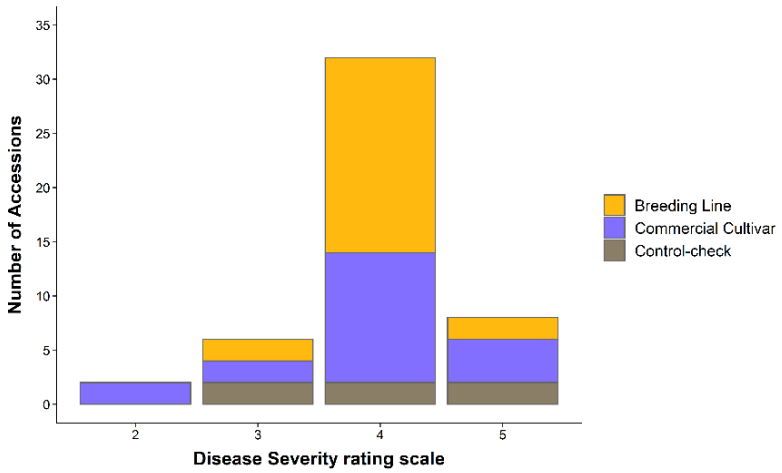

Figure 2.3. Classification of accessions according to their disease severity (DS) (McCartney *et al.*, 2002), characterised as breeding lines, commercial cultivars and control-checks.

2.3.2. Evaluation of STB symptoms through image analysis

Leaves from 9 accessions, which presented diverse DS symptoms ranging from 3 to 5, were selected for image analysis with the objective of better understanding pycnidial development through wheat genotypes. The peak of NLA was reached a couple of days before the peak of pycnidia development. An example of leaves showing DS scores of 2-5 amongst breeding lines, commercial cultivars, and control-check cultivars is shown in Figure 2.1. Accessions with a DS score of 2 expressed some chlorotic and necrotic lesions without pycnidial development, being considered as resistant (Figure 2.1a,b). However, in addition to accessions with a DS of 3 showing similar lesions to accessions with a DS of 2, they developed a few pycnidia (Figure 2.1c-e). The accessions with a DS of 4 presented moderate necrotic and coalescent lesions, while the presence of pycnidia was increased compared to accessions with a DS of 3 (Figure 2.1f-h). Lastly, accessions presenting a DS score of 5 developed a high density of necrotic lesions toward the leaf, in addition to a higher number of pycnidia compared to accessions with DS scores of 3 and 4 (Figure 2.1i-k).

Images were then analysed using image analysis software (NIS-Elements; Nikon Instruments Inc.) in order to obtain numeric parameters, to precisely describe the quantitative differences in STB symptoms amongst the accessions studied (Table 2.1). Images were cropped into NLA sections of 25 mm^2 and then analysed. 'LG Origen', 'BL 39', and 'Sculptur' were classified with a DS of 3 and, through image analysis, they showed mean NLA values of 0.92%, 0.83%, and 1.12% covered with pycnidia, respectively. In the same NLA sections analysed, these accessions presented, on average, pycnidium counts of 65 ('LG Origen'), 85 ('BL 39'), and 90 ('Sculptur'), implying mean values of 2.59, 3.40, and 3.59 pycnidia per mm^2 of NLA, respectively (Table 2.1). Pycnidial parameters of accessions with a DS of 3 presented lower values in comparison with accessions with a DS of 4 or 5. 'Avispa', 'BL 34', and 'Amilcar' were classified with a DS of 5 and showed mean values of 11.93, 14.25, and 16.71 pycnidia per mm^2 of NLA, with total counts, on average, of 298, 356, and 418, respectively. These accessions also expressed higher mean values of NLA covered with pycnidia compared to accessions with a DS of 3, whereby 'Avispa', 'BL 34', and 'Amilcar' presented values of 5.70%, 4.83%, and 5.34%, respectively (Table 2.1). 'Athoris', 'BL 36', and 'BL 33' were classified with a DS of 4 and presented moderate pycnidial development, with numeric values located between those of accessions with a DS of 3 and a DS of 5. These accessions presented, on average, 2.04% ('Athoris'), 2.71% ('BL 36'), and 2.50% ('BL 33') NLA covered with pycnidia. 'Athoris', 'BL 36', and 'BL 33' presented, on average, pycnidium counts of 173, 198, and 210 in the NLA sections analysed, respectively, which translates to 6.92, 7.92, and 8.40 pycnidia per mm^2 of NLA (Table 2.1). All accessions analysed expressed statistically similar values of pycnidium size.

However, the remaining parameters showed statistical differences among the accessions. Mean pycnidium count and, therefore, mean number of pycnidia per mm^2 of NLA were statistically different among accessions classified with a DS of 3, 4, and 5 (Table 2.1). Moreover, some accessions classified in the same DS group showed differences, such as 'Avispa' and 'Amilcar'. Similarly, the percentage of NLA covered with pycnidia was statistically different in accessions with a DS of 3, 4, and 5. Only 'BL 36' (DS of 4) and 'BL 34' (DS of 5) were not statistically different. Table 2.1 also shows the parameters analysed in terms of their mean values grouped by DS score, showing statistical differences for accessions with a DS of 3, 4, and 5 for all

parameters evaluated except for pycnidium size. Pycnidium count increased, on average, from 80 in DS 3, to 194 in DS 4 and 357 in DS 5, thereby implying a similar increase in the number of pycnidia per mm^2 of NLA, with values of 3.19 (DS of 3), 7.75 (DS of 4), and 14.30 (DS of 5). These increases in pycnidium count and the number of pycnidia per mm^2 of NLA were correlated with a similar increase in the NLA covered with pycnidia, with average values of 0.96% in accessions with a DS of 3, 2.41% in accessions with a DS of 4, and 5.29% in accessions with a DS of 5.

Table 2.1. Pycnidium parameters in NLA lesions of 25 mm^2 in accessions with different DS scores [1].

Accessions	DS	NLA covered with pycnidia (%)	Pycnidium count (Pyc)	Pycnidia/NLA (Pyc/mm^2)	Pycnidium size ($mm^2 \times 10^{-3}$)
LG Origen	3	0.92 ± 0.23 a	65 ± 16.44 a	2.59 ± 0.66 a	3.59 ± 0.42 a
BL 39	3	0.83 ± 0.19 a	85 ± 35.37 a	3.40 ± 1.41 a	2.57 ± 0.53 a
Sculptur	3	1.12 ± 0.45 a	90 ± 31.75 a	3.59 ± 1.27 a	3.09 ± 0.35 a
	Mean	0.96 ± 0.30 a	80 ± 27.66 a	3.19 ± 1.11 a	3.08 ± 0.58 a
Athoris	4	2.04 ± 0.37 b	173 ± 15.72 b	6.92 ± 0.63 b	2.93 ± 0.26 a
BL 36	4	2.71 ± 0.50 bc	198 ± 4.58 b	7.92 ± 0.18 b	3.43 ± 0.67 a
BL 33	4	2.50 ± 0.57 b	210 ± 45.43 b	8.40 ± 1.82 b	3.00 ± 0.64 a
	Mean	2.41 ± 0.52 b	194 ± 29.16 b	7.75 ± 1.17 b	3.12 ± 0.53 a
Avispa	5	5.70 ± 1.03 d	298 ± 30.07 c	11.93 ± 1.20 c	4.83 ± 1.13 a
BL 34	5	4.83 ± 1.26 cd	356 ± 114.15 cd	14.25 ± 4.57 cd	3.45 ± 0.47 a
Amilcar	5	5.34 ± 1.43 d	418 ± 43.73 d	16.71 ± 1.75 d	3.18 ± 0.67 a
	Mean	5.29 ± 1.14 c	357 ± 81.44 c	14.30 ± 3.26 c	3.82 ± 1.04 a

DS = disease severity; NLA = necrotic leaf area. [1] Values are means ± standard deviation for three leaves evaluated through image analysis in three accessions classified per DS score. Data with the same letter within a column are not significantly different (HSD, $p<0.05$).

2.4. Discussion

In our study, the incidence of STB in the durum wheat accessions was, in general, severe. Most of the durum wheat accessions studied presented 50% or higher mean values of NLA, which suggests an elevated development of *Z. tritici* in the leaf tissue amongst groups with diverse genetic origin (breeding lines and commercial cultivars). These results of high STB progression through diverse durum wheat accessions are in accordance with results in other studies (Ghaneie *et al.*, 2012; Chedli *et al.*, 2018). In addition to the high disease incidence in most of the commercial cultivars (and control-checks) studied (60% NLA or higher), it could be noted that they showed a DS score corresponding to them being susceptible (DS of 4) or very susceptible (DS of 5). These disease parameters imply that STB would produce not only severe infections through colonised leaf tissue, leading to yield losses, but also great persistence and reproduction capability in the form of pycnidia, increasing the sexual recombination of the fungus and its possibilities of overcoming wheat resistance (McDonald and Mundt, 2016), explaining the current incidence of STB in Spanish wheat cropping areas, especially in Andalusia (Royo and Briceño-Félix, 2011). Breeding lines presented the same disease patterns as

commercial cultivars and control-checks in terms of NLA and DS score; however, in comparison, they almost all (18 of 22 lines) presented a DS of 4 (susceptible). This DS score in breeding lines, together with the high levels of NLA mentioned, highlights the lack of resistant resources in current Spanish wheat-breeding programs to face *Z. tritici*; therefore, there is a necessity to research and introduce resistant wheat genetic material against this pathogen in tailored programs, as seen in other Mediterranean areas (Ghaneie *et al.*, 2012; Ferjaoui *et al.*, 2015; Kidane *et al.*, 2017).

Although *Z. tritici* presented, in general, high disease incidence in our studied accessions, we found some promising sources of resistance amongst them, such as in some control-check cultivars. The occurrence of these resistance patterns in our selected control-check cultivars is explained by the fact that they were chosen because of their role as historical checks, underscoring the shortage of studies related to the resistance and susceptibility of Spanish durum wheat against STB from which we could have selected control-check accessions with a certain degree of resistance. There were some commercial and control-check cultivars which expressed reduced mean values of NLA in comparison with the remaining accessions studied, such as 'LG Origen' (33.3%), 'Don Ortega' (35.8%), 'Sculptur' (39.8%), and 'Kiko Nick' (42.9%). These cultivars (except for 'Don Ortega') also exhibited a DS of 3, suggesting that they not only restricted the colonisation of leaf tissue by the fungus, but also presented a significant reduction in pycnidia development, similar to breeding lines 'BL 28' and 'BL 39' and the control-check cultivar 'Simeto', which, despite their higher mean values of NLA, also expressed a DS of 3, being promising resources of resistance (McCartney *et al.*, 2002; Chartrain *et al.*, 2004). Finally, two commercial cultivars, 'RGT Rumbadur' and 'RGT Voilur', exhibited a DS score of 2, the lowest DS score in our study, which denotes an absence of pycnidia in their developed lesions. Although these cultivars presented 50-60% mean values of NLA, their type of resistance prevents subsequent infections via an inhibition of pycnidia development and, thus, pycnidiospores, which spread the infection via rain splash to other plants. Moreover, this type of resistance could prevent the sexual reproduction of *Z. tritici* by inhibiting pseudothecium formation, which represents the main source of primary inoculum via ascospores dispersed by wind, being an essential resource to control STB in the field (Suffert *et al.*, 2011). Thus, commercial cultivars evaluated which presented resistance patterns could develop an advantage in fields with high STB incidence. Both, commercial cultivars and breeding lines can provide a valuable source of resistance for breeding programs.

The combination of both evaluation methods used in our study, *i.e.*, qualitative (DS; McCartney *et al.*, 2002) and quantitative (NLA and presence or absence of pycnidia; Suffert *et al.*, 2013), permitted the classification of our studied accessions on the basis of their resistance patterns (Figure 2.2). However, quantitative traits among different accessions or even among diverse *Z. tritici* isolates may be difficult to visually evaluate in a growth chamber or in the field (Karisto *et al.*, 2018). In order to precisely assess these quantitative differences, image analysis emerged as a powerful tool in some recent studies (Stewart and McDonald, 2014; Stewart *et al.*, 2016; Karisto *et al.*, 2018). In our work, we used a camera to obtain images of infected leaves, which were later analysed with specific image software (Figure 2.1). Due to this analysis being more thorough in comparison with visual scoring, it enabled the accurate evaluation of various quantitative parameters of STB disease, thereby allowing the classification of accessions on the

basis of their DS score. Our results showed statistical differences in quantitative parameters for accessions classified with different DS scores and, as we expected, accessions with a DS of 3 represented the lowest values, considering their partial resistance with slight pycnidia development. Thus, accessions classified with a DS of 3 showed a lower percentage of NLA covered with pycnidia, as well as a lower pycnidium count and a lower number of pycnidia per mm^2 of NLA, than accessions with a DS of 4, which then showed lower values than accessions with a DS of 5 (Table 2.1). This increase in the values of quantitative parameters from accessions with a DS of 3 to those with a DS of 4 and 5 mainly corresponded to an increase in pycnidium count, considering that the pycnidium size among accessions did not present statistical differences. However, our results also showed statistical differences for the quantitative parameters studied amongst accessions classified with the same DS score, which highlights the value of image analysis as tool not only in the detection of quantitative differences amongst accessions classified with the same DS score, but also in the possible classification of accessions on the basis of only qualitative traits (Stewart and McDonald, 2014; Stewart *et al.*, 2016; Karisto *et al.*, 2018). In future studies, our evaluation of STB symptoms is expected to follow the method developed by Stewart and McDonald (2014), whereby scanned images of infected leaves are analysed through a macro process, leading to an automatic analysis of quantitative STB disease parameters in several leaves. In addition, considering the lack of knowledge about the *Z. tritici*-durum wheat interaction (Somai-Jemmali *et al.*, 2017a), studies of the fungal infection process and its associated plant defence mechanisms in our resistant accessions represent the next step to a better understanding of durum wheat resistance to *Z. tritici*.

The present study evaluated the incidence of the fungus *Z. tritici*, which is the causal agent of one of the most important diseases in wheat, in both commercial cultivars and breeding lines currently available in Spain. The limited sources of resistance found in our 48 studied accessions, evaluated visually and through image analysis, highlights the importance of the incorporation of these sources of resistance against STB into future durum wheat breeding programs. In addition, due to the scarcity of studies related to the resistance and susceptibility of durum wheat against STB, our work represents a novel contribution to the selection of sources of resistance amongst cultivars which currently have valuable agronomic traits in Spain, leading to advantages when facing STB.

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Chapter 3. Characterisation of durum wheat resistance against Septoria tritici blotch under climate change conditions of increasing temperature and [CO_2]

3.1. Introduction

Wheat is considered one of the most important food crops to human populations as it is consumed worldwide and provides substantial amounts of components which are essential or beneficial for health (Shewry and Hey, 2015). Due to its key position in global cereal production and its elevated range of cultivation and diversity, this essential crop is constantly threatened by diverse biotic stresses during the growing season, including attacks by pest and pathogens, such as fungi, bacteria, oomycetes, viruses, nematodes and herbivores, leading in a great constraint for wheat production worldwide (Strange and Scott, 2005; Oerke, 2006; Savary *et al*., 2019). Amongst these biotic stresses, plant diseases cause more than 21% of wheat losses on average (Savary *et al*., 2019), being fungal pathogens such as wheat rusts, blotch diseases, wheat scab, wheat blast or powdery mildew among others, considered the most detrimental (Singh *et al*., 2016; Figueroa *et al*., 2018).

One of the most devastating wheat fungal diseases is the Septoria tritici blotch (STB) disease, caused by the fungus *Zymoseptoria tritici* (Desm.) (Quaedvlieg *et al*., 2011). This major disease is present in all wheat-growing areas of the world and causes important yield losses up to 50% under conducive weather conditions in both common and durum wheat (McDonald and Mundt, 2016). *Z. tritici* normally develops well in temperate climates with cool, wet weather where bread cultivation is significant, such as in North America (Estep *et al*., 2015), northern France, Germany, or the United Kingdom (Fones and Gurr, 2015). However, this pathogen also extends to hot dry climates such as wheat-growing areas of the Mediterranean Basin or North Africa, where durum wheat importance exceeds that of bread wheat due to its common use in the traditional Mediterranean diet (Royo and Briceño-Félix, 2011; Ben M'Barek *et al*., 2022). This adaptation and speciation of *Z. tritici* to various agro-ecosystems is thanks to its high genome plasticity and diversity, which maintain pathogenic fitness even when it loses accessory chromosomes (Wittenberg *et al*., 2009; Goodwin *et al*., 2011), and its active sexual reproduction, which accelerates its evolution (McDonald and Mundt, 2016). This situation has hampered the implementation of an efficient strategy to control STB disease, limiting the efficacy of the chemical control. In fact, STB is probably the most economically important wheat disease in Europe, with an estimated ~€1 billion per year in fungicide expenditure directed toward its control (Torriani *et al*., 2015).

Additionally to fungicides application, protection against STB disease has been traditionally achieved through the use of resistant wheat cultivars. Thus, breeding programs emerge as an effective, environmentally sustainable and cost-reducing measure to control this disease in comparison to fungicide control. In STB, two types of resistances have been described: qualitative (race-specific) resistance is controlled by major genes with a large effect according to a gene-for-gene interaction (Brading *et al*., 2002; McCartney *et al*., 2002), whereas

quantitative (non race-specific) resistance, which develops a partially resistant phenotype, is conferred by a large number of quantitative trait loci (QTL) with moderate to small effects (Brown *et al*., 2015). Both types of resistance have been thoroughly studied in bread wheat, for which 22 major genes (*Stb* genes) conferring qualitative resistance, together with 167 QTLs, have been identified and mapped to date (Louriki *et al*., 2021). In fact, quantitative resistance plays an important role in wheat breeding against *Z. tritici*, being durable and effective against several pathotypes of this pathogen (Brown *et al*., 2015). This type of resistance has been usually evaluated through visual (subjective) quantitative scoring of *Z. tritici* lesions bearing fungal reproductive structures (Kema *et al*., 1996a). However, due to fungal lesions being absent from reproductive structures in some cases, quantitative resistance has also been evaluated measuring lesions and reproductive structures separately (Suffert *et al*., 2013; Gerard *et al*., 2017; Porras *et al*., 2021). Fortunately, recent automated image analysis methods have postulated as an essential tool to precisely evaluate not only the amount of damage of a *Z. tritici* isolate to the host, but also its epidemic potential, obtaining an overall measurement of pathogen virulence (Stewart *et al*., 2016; Karisto *et al*., 2018). Additionally, microscopic studies which determine fungal infection process and subsequent plant defence patterns, together with biochemical and molecular analyses, which examined fungal enzymes or host defence-related genes, remarkably increase knowledge about the wheat-*Z. tritici* interactions (Somai-Jemmali *et al*., 2017a; 2017b).

However, these interactions between wheat and *Z. tritici* would be affected by alterations in environmental conditions derived from global climate change, which is mainly characterised by increasing temperature, [CO_2] and drought (Chaudhry and Sidhu, 2022). In fact, alterations in temperature, [CO_2] and water regimes are supposed to modify plant development and resistance pathways, on one side, and pathogen virulence mechanisms and life cycle, on the other side (Velásquez *et al*., 2018), lastly influencing in the whole wheat-pathogen interactions (Pérez-Méndez *et al*., 2022). In order to assess this influence, disease risk simulation studies, where crop disease models have been linked to climate projections, have been commonly developed for diverse global locations, showing diverse outputs for STB incidence across European wheat-growing areas (Juroszek and von Tiedemann, 2015; Miedaner and Juroszek, 2021). Moreover, although researchers recognise that extreme weather events, which are characteristic of climate change, will have large impacts on disease severity and yield loss, its projections are still at the beginning (Newbery *et al*., 2016).

Disease models are based on the results of experimental investigations which, thanks to the use of diverse facilities (free-air CO_2 enrichment systems, phytotrons or greenhouses), assess the effect of one or several simultaneous abiotic factors, such as high temperature and elevated [CO_2], on plant-pathogen interactions (Newbery *et al*., 2016). However, at the moment there are few realistic field studies that assess the effects resulting from combining simultaneous increasing temperature and [CO_2] with biotic stresses in plants (Suzuki *et al*., 2014) and, in the case of wheat, they often obtained varied results regarding different wheat-pathogen interactions (Melloy *et al*., 2014; Matić *et al*., 2018; Hay *et al*., 2021). These variations are in consequence of plants expressing a tailored physiological and molecular response when facing multiple stresses, in which occurs antagonistic signalling pathways between abiotic and biotic factors (Suzuki *et al*., 2014; Atkinson *et al*., 2015) such as the role of some phytohormones

(Castroverde and Dina, 2021; Kim *et al*., 2022). However, although some studies have evaluated the effect of increasing temperature (Hess and Shaner, 1987; Wainshilbaum and Lipps, 1991; Magboul *et al*., 1992; Chungu *et al*., 2001) and [CO_2] (Váry *et al*., 2015) in wheat-*Z. tritici* interactions, none of them has assessed the effect of both abiotic factors acting simultaneously. Therefore, considering this lack and the variability in experimental assessments, coupled with the uncertainty of disease risk simulation studies, it is likely that predicting wheat-*Z. tritici* interactions under climate change conditions would be a complex task.

Besides this situation, it is necessary considering that the interaction of durum wheat against *Z. tritici* has been poorly investigated (Somai-Jemmali *et al*., 2017a), leading to the absence of any *Stb* gene identified in durum wheat species. This lack of resistant resources against STB disease in durum wheat species implies a great risk for general wheat production, especially in wheat-growing regions of the Mediterranean Basin, such as Spain (Royo and Briceño-Félix, 2011) or Tunisia (Ben M'Barek *et al*., 2022), which altogether stand out for being the largest durum-wheat-producing areas in the world, with about 60% and 75% of the global durum wheat cropping area and production, respectively (Royo *et al*., 2017). Additionally, these regions are considered hotspots of climate change, where temperature warming, extreme events and changes in precipitation regimes are likely to occur (Diffenbaugh and Giorgi, 2012; Trnka *et al*., 2014). In this context, durum wheat cultivation would be severely affected by both abiotic factors derived from climate change and *Z. tritici* pathogen, which is characterised by evolving and adapting rapidly to changing environments (McDonald and Mundt, 2016). Therefore, there is an urgent necessity to precisely know how future conditions of climate change would affect durum wheat-*Z. tritici* interactions.

The aim of this study was to characterise the response of durum wheat accessions with diverse resistance traits against STB disease under increasing temperature and [CO_2] weather conditions, which simulated the far future period of 2070-2099 for the wheat growing region of Córdoba, Spain.

3.2. Materials and Methods

3.2.1. Plant material

In this study, 3 durum wheat accessions (*T. turgidum* spp. *durum*) selected from the germplasm collection used in Chapter 2, 'Sy Leonardo', 'LG Origen' and 'RGT Rumbadur', were evaluated against a local isolate of *Zymoseptoria tritici* under baseline (control) and climate change conditions. These accessions were commercial Spanish cultivars registered in the Spanish MAPA (Ministerio de Agricultura, Pesca y Alimentación) catalogue. Additionally, they were classified in Chapter 2 according to their reactions against *Z. tritici* infection using the DS (disease severity) rating scale from 0 to 5 (McCartney *et al*., 2002), being 'RGT Rumbadur', classified with a DS value of 2, and 'LG Origen', with a DS value of 3, considered as resistant accessions and 'Sy Leonardo', with a DS value of 5, as susceptible.

3.2.2. Pathogen isolation

The fungus *Zymoseptoria tritici* was isolated from naturally infected wheat leaves collected in Santaella, Córdoba (Spain), according to procedures of Stewart and McDonald (2014) and the isolate was molecularly identified with the accession number SUB9540116 at the NCBI website (NCBI, 2021). The fungus isolation, purification, multiplication and conservation procedures were performed as were previously described in Chapter 2. Fungal stocks were stored as microconidial suspensions at -80°C with 30% glycerol until needed for the inoculation of durum wheat plants.

3.2.3. Greenhouse conditioning and design of climate environments

Plants of the three selected durum wheat accessions were grown in greenhouses with full environmental control of temperature and [CO_2]. To establish these weather and [CO_2] conditions, the greenhouses were equipped with air conditioning and dehumidification systems, and CO_2 supply circuits, all controlled by temperature, humidity, and CO_2 sensors, with a fully automated CO_2 injection process to maintain the CO_2 target levels (Sysclima, version 9.4, INTA CROP TECHNOLOGY S.L., Murcia, Spain). The established weather conditions were designed to resemble a standard spring day, which is the expected growth period of *Z. tritici* in the wheat growing area of Córdoba.

Since average temperatures may not always be an accurate predictor of the potential for an infection (Velásquez *et al*., 2018), in our study we carried out a variation of temperature throughout the day, reaching an established maximum and minimum value. Thus, for the baseline, the maximum and minimum temperature values were obtained from the nearest meteorological station, located in Córdoba and belonging to the Spanish State Meteorological Agency, with average values of 24°C and 10°C, respectively. Likewise, the value of [CO_2] was set at around 420-450 ppm, the level currently observed outdoors. Moreover, in order to define the weather conditions for the far future period (2070-2099), the Representative Concentration Pathway RCP8.5 and an ensemble of five climate models (GFDL-CM3, GISS-E2-R, HadGEM2-ES, MIROC5 and MPI-ESM-MR) were taken into account, resulting in an average maximum and minimum temperatures around 30°C and 15°C, respectively, and an average [CO_2] around 620-650 ppm.

Having established the above weather and [CO_2] conditions, five sets of plants from three durum wheat accessions were exposed to three different environments, each in separate greenhouses, to assess *Z. tritici* infection. Under baseline conditions (environment B), plants were exposed to a maximum temperature of 24°C and [CO_2] around 420-450 ppm. For the far future scenario, two possible environments were established: under increasing temperature (environment 1), plants were exposed to a maximum temperature of 30°C and [CO_2] around 420-450 ppm; and under increasing temperature and [CO_2] (environment 2), plants were exposed to a maximum temperature of 30°C and elevated [CO_2] around 620-650 ppm.

One set of plants was grown, incubated and maintained for evaluation under baseline weather conditions (set SB), and four sets of plants were grown under far future weather

conditions: two sets at elevated temperature and two at both elevated temperature and [CO_2]. As high temperatures could critically affect the penetration success of *Z. tritici* to the host tissue, two of the four sets of plants under far future conditions were inoculated and incubated under baseline weather conditions, before returning to their respective far future conditions (sets S1 and S2, respectively). The other two sets of plants were grown, inoculated, incubated and maintained for evaluation under their corresponding far future weather conditions (sets S1G and S2G, respectively).

3.2.4. Inoculation assays

Seeds of the three selected durum wheat accessions were sown in 8 × 7 × 7 cm pots containing a mix (1:1, v/v) of commercial compost and sand. Pots were placed in trays and incubated in a growth chamber at 21°C with a 14-hour photoperiod to germinate the plants for 6 days, and then, seedlings were transferred to different greenhouses with diverse weather conditions described above (environments B, 1 and 2) for 15 days until the third leaf was completely unfolded. Meanwhile, fresh spores of *Z. tritici* were obtained from the spore suspension stored at -80°C according to procedure described by Stewart and McDonald (2014), with minor modifications. Thus, a spore suspension was prepared with distilled water and Tween-20 (0.1%) and adjusted to 10^7 spores mL^{-1}. Then, a total of 180 plants (12 per accession and plant set) were inoculated with 7.5 mL per plant of the prepared spore solution of *Z. tritici,* using a hand-sprayer until the solution ran off the leaves. Once leaves were totally dry, plants were sealed in clear plastic bags to provide 100% RH for the disease incubation for 48 h. Three sets of plants (SB, S1 and S2) were inoculated and incubated on environment B, while two sets of plants were inoculated and incubated on their corresponding environments 1 or 2 (S1G and S2G, respectively). Finally, plastic bags were removed, and plants were kept in their respective environments for 21 days for subsequent macroscopic and microscopic evaluations. Macroscopic and microscopic experiments were performed three times each.

3.2.5. Assessment of macroscopic components of resistance

In order to precisely assess macroscopic disease symptoms of STB in wheat plants and those possible changes in the wheat-*Z. tritici* interactions derived from increasing temperature and [CO_2], image-based analysis was developed in this study. Several A4 pages which contained a list of sample names according to each accession and plant were printed as templates as described by Stewart *et al*. (2016). Each template contains fixed reference points used to set the image scale and boxes within which to mount the leaves. Each box contains the sample names in text as well as encoded as a QR code. Then, the third leaf of 6 plants per accession, plant set and replication were cut at 21 days post inoculation (dpi), mounted on these A4 templates and stored at 4°C for 2 to 3 days with absorbent paper placed between each sheet of leaves and pressed with approximately 5 kg. Once leaves were flattened, templates were digitally scanned at a resolution of 1200 dots per inch using a flatbed scanner (Canon CanoScan LiDE 400, Tokyo, Japan) and saved as “jpeg” images.

Once leaves were scanned, images were analysed with the software ImageJ (Wayne Rasband, NIH, MD, USA; Schneider *et al*., 2012) using the macro instructions first described by

Stewart *et al*. (2016) and later modified by Karisto *et al*. (2018). The maximum length of leaf area scanned in each box was 17 cm. For each leaf, the following parameters were automatically recorded from the scanned image: total leaf area, necrotic and chlorotic leaf area, number of pycnidia and their positions on the leaf. Despite obtaining a high efficiency in the identification of pycnidia presented in *Z. tritici* lesions, some of these pycnidia were not counted by the software, being then manually counted to increase the accuracy of the measurements. Thus, we calculated the percentage of leaf area covered by lesions (PLACL), the frequency of pycnidia per unit of lesion area (Pyc/lesion) and the frequency of pycnidia per unit of leaf area (Pyc/leaf).

3.2.6. Assessment of microscopic components of resistance

Central leaf segments (~ 6 cm) of inoculated third-leaves were cut in 3 leaves per accession, plant set and replication at 4 and 21 dpi. Samples were processed as described in Shetty *et al*. (2003) and then examined using a Nikon microscope (Nikon, Tokyo, Japan). Samples were cleared on filter paper saturated with a mixture of absolute ethanol/glacial acetic acid (3:1, v/v) for 24-48 h. After clearing, the leaves were transferred to filter paper saturated with lactoglycerol (lactic acid/glycerol/water, 1:1:1, v/v) where they were stored until examination. For localization of fungal structures, leaves were stained with 0.1% Evans blue in lactoglycerol. In samples collected at 4 dpi, 400 *Z. tritici* spores were observed and classified as spores leading to a stomatal penetration (SP), spores leading to a direct penetration (DP) and spores without penetration (NP) (Somai-Jemmali *et al*., 2017a; 2017b). Only germinated spores were counted. In samples collected at 21 dpi, 450 fungal stages were observed and classified as non-colonised stomata (NCS), colonised stomata but not yet transformed into pycnidia (CST) and colonised stomata transformed into pycnidia (P) (Somai-Jemmali *et al*., 2017a; 2017b). Both spores and fungal stages of development were photographed using a Nikon DS-Fi1 camera (Nikon, Tokyo, Japan).

3.2.7. Statistical analysis

The experimental design was developed as randomised blocks. Macroscopic and microscopic parameters whose data did not achieve normality and homogeneity requirements amongst different environments for each accession were transformed for statistical analysis with ANOVA test, and back transformed for presentation. However, parameters whose data could not achieve those requirements using transformations were analysed through nonparametric Kruskal-Wallis test. Thus, data from the macroscopic parameter PLACL were transformed according to the formula $y = \sqrt{(x)}$ in our three studied accessions and analysed using ANOVA and LSD (Least Significant Difference) tests. However, data from the other two macroscopic parameters, Pyc/lesion and Pyc/leaf, were analysed using Kruskal-Wallis test.

In terms of microscopic parameters, data from percentages of different spores and fungal stages of development were analysed using ANOVA and Duncan tests for the three selected accessions. Data from microscopic spore development stages of SP and DP in accessions 'Sy Leonardo' and 'RGT Rumbadur', coupled with data of SP in accession 'LG Origen', were transformed according to the formula $y = \log(x)$. In addition, data from NP in accessions 'Sy Leonardo' and 'RGT Rumbadur' were transformed according to the formula $y = \arcsin(\sqrt{(x/100)})$.

Finally, data from fungal development NCS in 'Sy Leonardo' were transformed according to the formula y = log(x), whereas data from P in 'LG Origen' were transformed according to the formula y = arcsin(√(x/100)). Data processing, statistical analyses and figure design were carried out using R (R Core Team, 2023) and ImageJ (Schneider *et al*., 2012) softwares.

3.3. Results

3.3.1. Macroscopic components of resistance to *Z. tritici* infection under climate change conditions

The automated analysis of *Z. tritici*-infected leaves carried out in this study led to precisely assessing the differences in macroscopic disease symptoms in durum wheat accessions exposed to diverse weather conditions (Figure 3.1). Thus, we evaluated three components of STB infection such as percentage of leaf area covered by lesions (PLACL), the frequency of pycnidia per unit of lesion area (Pyc/lesion) and the frequency of pycnidia per unit of leaf area (Pyc/leaf) (Table 3.1).

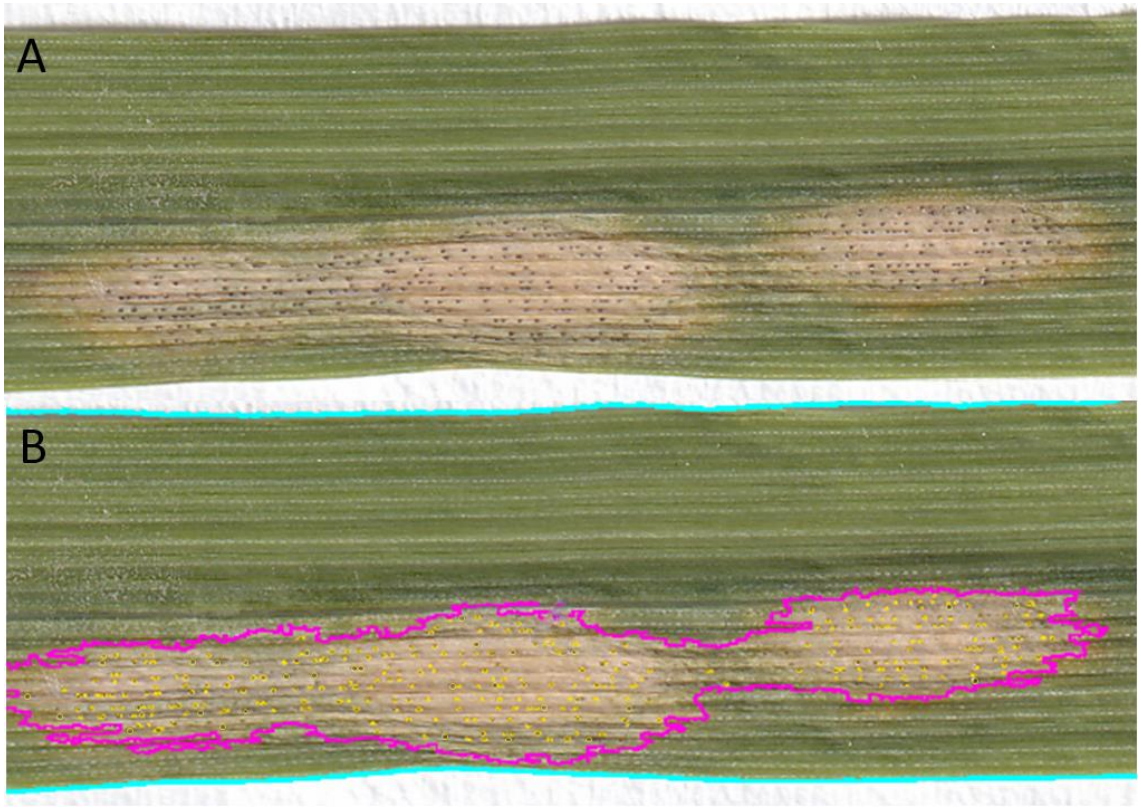

Figure 3.1. Output example of Septoria tritici blotch (STB) image analysis developed on selected durum wheat accessions. (**A**) Original leaf; (**B**) Analysed leaf in which leaf area, lesion area and pycnidia were selected in blue, purple and yellow colours, respectively.

The susceptible accession 'Sy Leonardo' showed the greatest differences in macroscopic parameters amongst weather conditions, expressing SB plants higher values in comparison to the other plant sets for the three studied parameters. Thus, 'Sy Leonardo' showed statistically

lower PLACL values in S1 and S2 plants (21.71% and 23.29%, respectively) in comparison to SB plants (37.48%). In addition, S1G and S2G plants, which were grown, inoculated and incubated under environment 1 and 2, respectively, expressed a relevant reduction of the lesion area caused by *Z. tritici*, showing PLACL values of 5.12% and 9.24%. This same pattern of reduced values across plant sets occurred also for Pyc/lesion and Pyc/leaf parameters. Thus, for Pyc/lesion, S1 and S2 plants expressed reduced values (226.0 and 266.08, respectively) followed by S1G and S2G (105.05 and 139.21, respectively) compared with SB plants (601.27). Similarly, for Pyc/leaf parameter, values ranged from 222.77 in SB plants to 61.81 and 47.01 in S2 and S1 plants, respectively, being the values in S2G (11.92) and S1G (4.43) plants severely reduced.

Table 3.1. Macroscopic image analysis of *Z. tritici* infection in three selected durum wheat accessions under baseline and climate change environments [1].

Accession	Environmental Set	PLACL (%)	Pyc/lesion (Pyc/cm^2)	Pyc/leaf (Pyc/cm^2)
Sy Leonardo	SB	37.48 (6.11 ± 0.09) a	601.27 ± 33.09 a	222.77 ± 10.81 a
	S1	21.71 (4.61 ± 0.17) b	226.00 ± 27.66 b	47.01 ± 5.81 b
	S2	23.29 (4.79 ± 0.14) b	266.08 ± 33.85 b	61.81 ± 8.67 b
	S1G	5.12 (2.21 ± 0.12) d	105.05 ± 18.32 c	4.43 ± 0.63 c
	S2G	9.24 (2.97 ± 0.16) c	139.21 ± 18.75 c	11.92 ± 1.83 c
LG Origen	SB	9.09 (2.94 ± 0.16) a	70.83 ± 23.85 a	4.63 ± 0.91 a
	S1	1.61 (1.17 ± 0.12) b	82.05 ± 32.36 ab	1.43 ± 0.52 b
	S2	2.19 (1.36 ± 0.14) b	47.91 ± 20.67 ab	1.71 ± 0.87 b
	S1G	0.73 (0.56 ± 0.16) c	2.00 ± 1.70 b	0.05 ± 0.37 b
	S2G	1.75 (1.26 ± 0.10) b	44.82 ± 24.46 b	0.83 ± 0.49 b
RGT Rumbadur	SB	8.88 (2.95 ± 0.10) a	12.59 ± 2.11 ab	1.18 ± 0.21 a
	S1	5.17 (2.22 ± 0.12) b	12.41 ± 1.65 a	0.60 ± 0.08 a
	S2	8.67 (2.88 ± 0.15) a	7.60 ± 1.06 ab	0.66 ± 0.12 a
	S1G	1.76 (1.25 ± 0.10) c	10.44 ± 2.56 ab	0.22 ± 0.06 b
	S2G	2.42 (1.51 ± 0.09) c	5.49 ± 1.38 b	0.13 ± 0.03 b

[1] Values are mean ± standard error for six leaves evaluated for each accession and environmental set in three different experiments. Transformed data ± standard error are shown in parenthesis. Data with the same letter within an accession and column are not statistically different (LSD and Kruskal-Wallis tests, $p < 0.05$). PLACL, percentage of leaf area covered by lesions; Pyc/lesion, frequency of pycnidia per unit of lesion area; Pyc/leaf, frequency of pycnidia per unit of leaf area.

The moderately resistant accession 'LG Origen' showed statistically lower PLACL values in all plant sets exposed to future weather conditions in comparison with SB plants (9.09%). In this accession, the reduction across plant sets was more pronounced than in 'Sy Leonardo', showing values ranging from 2.19% for S2 plants to 0.73% in S1G plants. For Pyc/lesion, S1 and S2 plants expressed non-significantly different values (82.05 and 47.91, respectively) in comparison to SB plants (70.83), whereas S1G and S2G plants did show reduced values (2.00 and 44.82, respectively). In contrast, all plant sets exposed to future weather conditions showed relevantly lower values for Pyc/leaf in comparison to SB plants (4.63), being these values non-statistically significant amongst them.

Despite resistant accession 'RGT Rumbadur' showed similar PLACL values in SB plants in comparison to 'LG Origen', the values observed for the other plant sets were not as greatly

reduced as for 'LG Origen'. In fact, 'RGT Rumbadur' showed statistically similar values in S2 plants (8.67%) than in SB plants (8.88%), followed by significantly reduced values of the other sets of plants. Moreover, this accession developed Pyc/lesion values ranging from 12.59 in SB plants to 5.49 in S2G plants. Lastly, 'RGT Rumbadur' developed the lowest Pyc/leaf values for SB plants (1.18) of the three studied accessions, showing similar values for S1 and S2 plants (0.60 and 0.66), whereas S1G and S2G plants expressed statistically lower values (0.22 and 0.13).

3.3.2. Microscopic components of resistance to *Z. tritici* infection under climate change conditions

Different stages of spores (SP; DP; NP) and fungal development (NCS; CST; P) were identified during the microscopic evaluation of *Z. tritici* infection at 4 and 21 dpi, respectively (Figure 3.2), and then analysed as percentages (Figure 3.3, 3.4 and Supplementary Table S3.1).

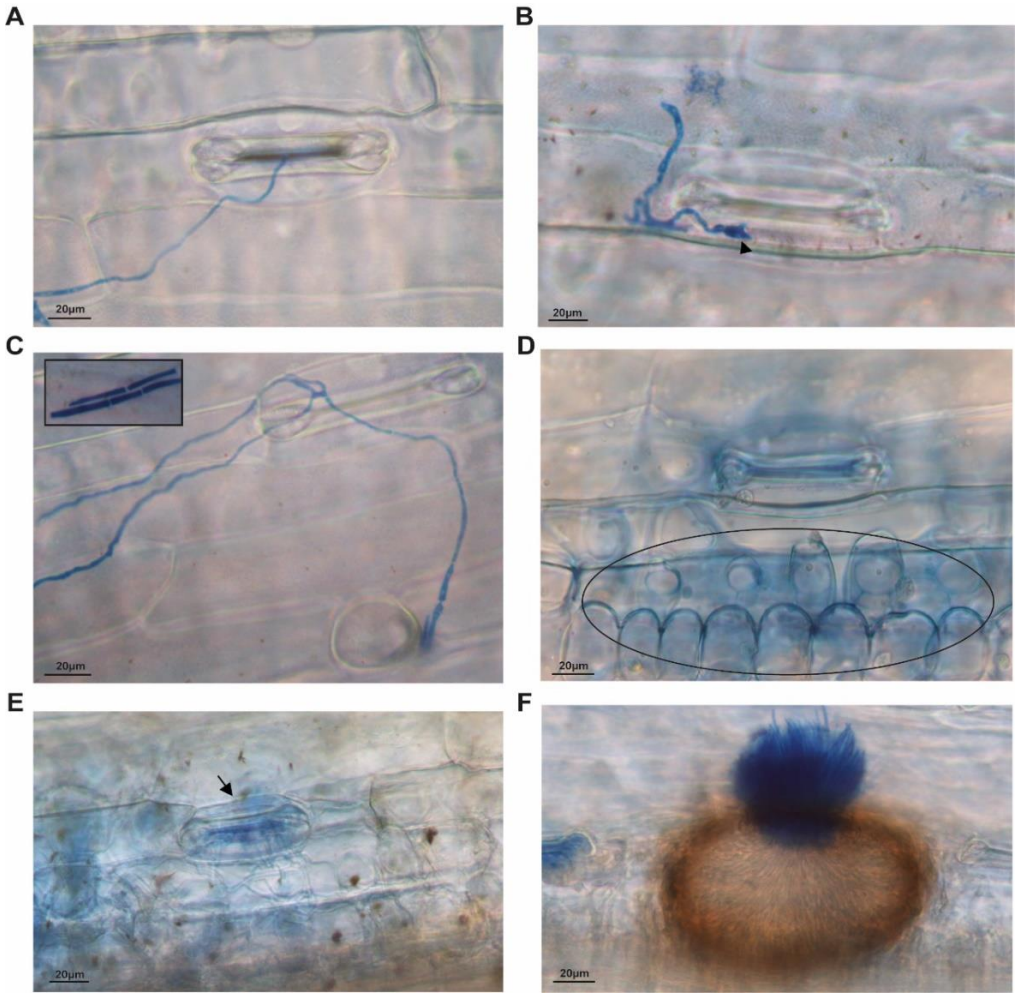

Figure 3.2. Microscopic observation of spore germination, growth and penetration attempt of *Z. tritici* at 4 dpi, and mesophyll colonisation and pycnidium structure at 21 dpi was classified as: (**A**) spores leading to a stomatal penetration (SP); (**B**) spores leading to a direct penetration (DP). Appressorium-like structure formed by an infectious germ tube between stomatal guard cells and epidermal cells over stomata (arrowhead); (**C**) spores without penetration (NP) with non-germinated spore in small square; (**D**) non-colonised stomata (NCS). Longitudinal intercellular growth of infection hyphae growing around mesophyll cells (selected area); (**E**) colonised stomata but not yet transformed into pycnidia (CST). Abundant hyphal growth within the substomatal cavity (arrow); (**F**) colonised stomata transformed into pycnidia (P).

In our microscopic results, the three selected accessions showed similar distribution patterns in the percentages of SP, DP and NP stages observed at 4 dpi (Figure 3.3). Accession 'Sy Leonardo' expressed statistically higher values in SB plants for SP (16.85%) and DP (25.57%) stages in comparison to the rest of plant sets for both stages (Figure 3.3A). Thus, SP values ranged from 8.48% in S1 plants to 5.14% in S2G plants, whereas DP values ranged from 11.48% in S1 plants to 5.26% in S1G plants, being this last set and S2G plant set also statistically different from S1 and S2 plants. By contrast, SB plants presented a reduced value of the NP stage in comparison to the other sets, showing a percentage far from S1 and S2 plants (80.04% and 81.65%, respectively) and even more distant from S1G and S2G plants (88.52% and 89.00%, respectively).

In 'LG Origen' accession, only SB plants differed significantly compared to the other sets for SP and DP stages, showing elevated percentages of 23.98% and 21.34%, respectively (Figure 3.3B). Plant sets exposed to future weather conditions did not have statistical differences amongst them for both stages, ranging their values from 5.13% in S2G plants to 7.02% in S2 plants for SP stage, and from 6.22% in S2G to 10.77% in S1 for DP stage. For the NP stage, following the same trend as in 'Sy Leonardo', SB plants showed the lowest value (54.68%) while the other plant sets presented higher percentages statistically different. Thus, S1 and S2 plants expressed similar values (82.57% and 82.29%, respectively) showing these two sets statistically different values than S1G and S2G plants (87.06% and 88.65%, respectively).

The accession 'RGT Rumbadur' showed a similar pattern for SP stage as in the other two accessions, being SB value (15.75%) statistically different from the rest, ranging from 3.83% in S2G plants to 6.62% in S2 plants (Figure 3.3C). For the DP stage, plants exposed to environment B expressed also the greatest value (25.80%), showing relevant differences with the other plant sets, which ranged from 8.27% in S2G plants to 12.15% in S2 plants, being only these two sets statistically different between them. Lastly, plant sets exposed to future weather conditions showed the highest NP values, being all non-statistically different amongst them except S2 (81.23%) and S2G plants (87.90%), whereas SB plants expressed a significantly reduced NP value (58.45%).

Concerning percentages of fungal stages of development obtained at 21 dpi (Figure 3.4), susceptible accession 'Sy Leonardo' expressed relevantly higher NCS percentages in plant sets exposed to future weather conditions in comparison to SB plants (16.59%), showing simultaneously non-statistical differences amongst them, except for S1G (33.19%) and S2G (23.01%) plants (Figure 3.4A). For the CST stage, SB plants also showed the lowest value (24.37%), followed by S2 (27.78%), S1G (34.66%), S1 (35.93%) and S2G (42.71%) sets. However, for P stage, SB plants developed the statistically highest value (59.04%) in comparison with the plants exposed to future weather conditions, which showed values ranging from 43.85% in S2 plants to 32.15% in S1G plants, being these two sets relevantly different between them.

In 'LG Origen' accession, S1G plant set showed an outstanding value in the NCS stage (97.56%), being significantly higher than S2G (66.52%), S1 (51.45%), S2 (50.37%) and SB (37.90%) plant sets (Figure 3.4B). By contrast, for the CST stage, the S1G plant set expressed the significantly lowest value (2.44%), whereas the other plant sets showed values ranging from 13.70% in S1G plants to 35.98% in SB plants, being also these last two sets statistically different.

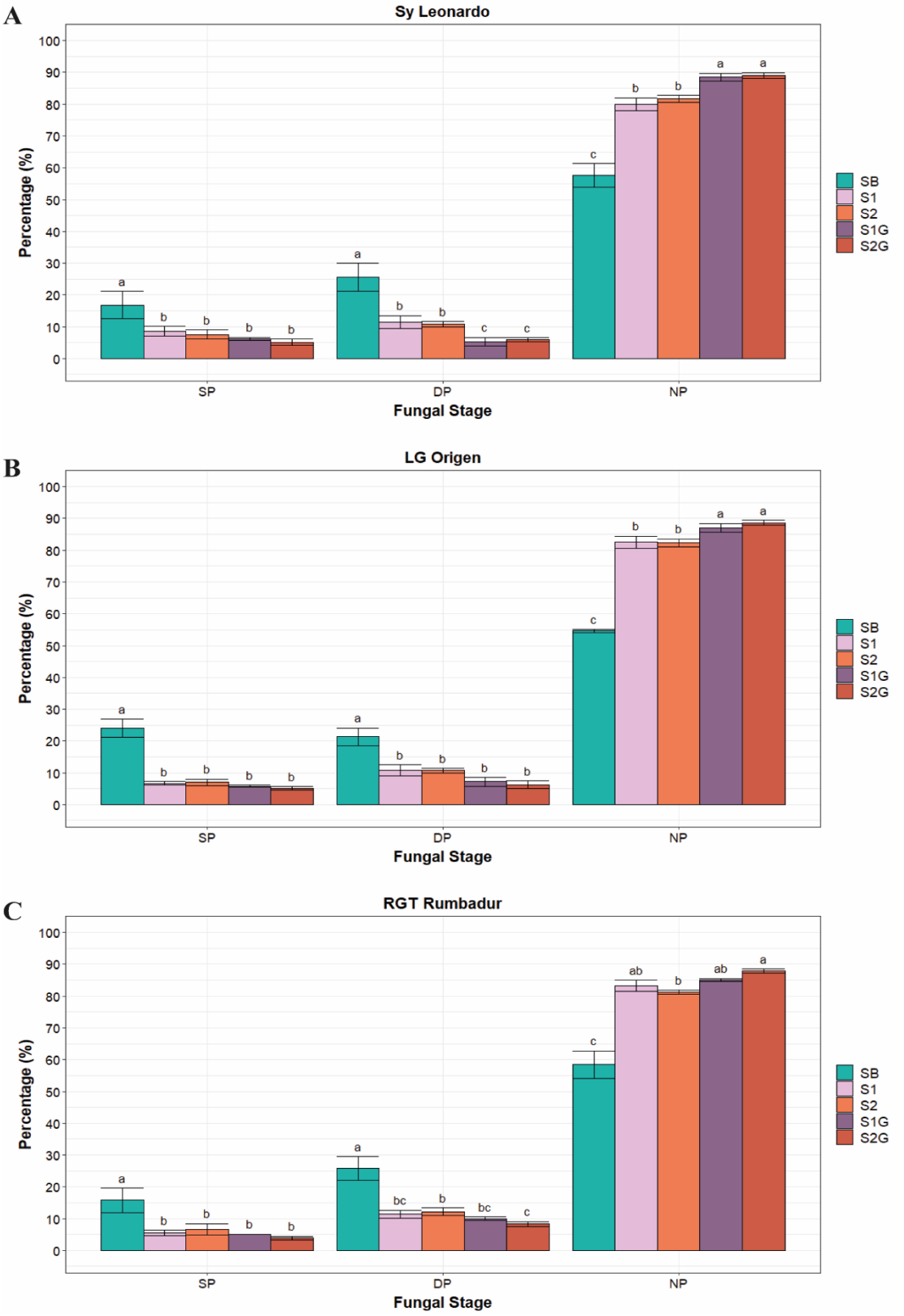

Figure 3.3. Microscopic stages of spores of *Z. tritici* presented as mean percentages in three selected durum wheat accessions (**A**) 'Sy Leonardo', (**B**) 'LG Origen' and (**C**) 'RGT Rumbadur' under baseline (SB) and climate change environments (S1, S2, S1G, and S2G). Error bars represent the standard error calculated from three independent experiments. Data with the same letter within a fungal stage and accession are not significantly different (Duncan test, $p < 0.05$). SP, spores leading to a stomatal penetration; DP, spores leading to a direct penetration; NP, spores without penetration.

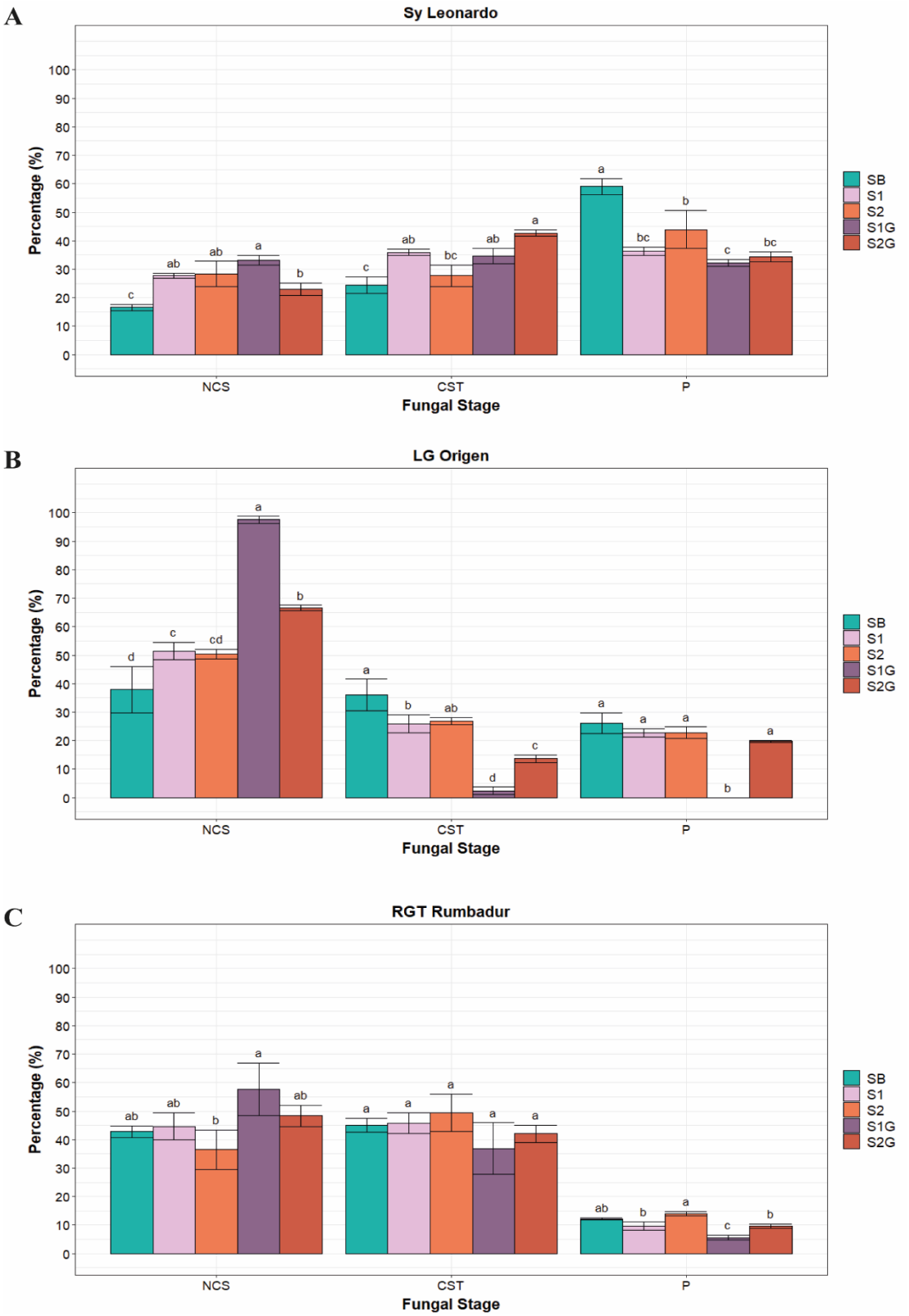

Figure 3.4. Microscopic stages of fungal development of *Z. tritici* presented as mean percentages in three selected durum wheat accessions (**A**) 'Sy Leonardo', (**B**) 'LG Origen' and (**C**) 'RGT Rumbadur' under baseline (SB) and climate change environments (S1, S2, S1G, and S2G). Error bars represent the standard error calculated from three independent experiments. Data with the same letter within a fungal stage and accession are not significantly different (Duncan test, $p < 0.05$). NCS, non-colonised stomata; CST, colonised stomata but not yet transformed into pycnidia; P, colonised stomata transformed into pycnidia.

Then, considering the P stage, S1G plants also developed the statistically lowest score, expressing a null value (0.00%), whereas the other plant sets showed similar values, ranging from 19.78% in S2G plants to 26.12% in SB plants.

'RGT Rumbadur' accession developed the slightest differences amongst plant sets for the three fungal stages studied (Figure 3.4C), expressing NCS values ranging from 36.45% in S2 plants to 57.60% in S1G plants, being only these two sets statistically different between them. For the CST stage, all sets were non-statistically different amongst them, with values ranging from 36.83% in S1G plants to 49.48% in S2 plants. Lastly, 'RGT Rumbadur' accession, considered as resistant, developed reduced P values in all plant sets, outstanding SB and S2 plant sets with 12.15% and 14.07% values respectively, followed by S1 (9.62%) and S2G plants (9.61%), both statistically different compared to S2 plants. The lowest P value was observed in S1G plants (5.57%), being different to the other plant sets.

3.4. Discussion

Zymoseptoria tritici has become a serious threat for wheat cultivation worldwide thanks to its speciation to diverse agro-ecosystems (McDonald and Mundt, 2016), which would imply an uncertain scenario for the wheat-*Z. tritici* interactions in the context of future climate change, especially for durum wheat species mainly cultivated in regions considered as hotspots of climate change (Diffenbaugh and Giorgi, 2012; Trnka *et al*., 2014). Although some experimental studies have assessed the effects of increasing temperature (Hess and Shaner, 1987; Wainshilbaum and Lipps, 1991; Magboul *et al*., 1992) and [CO_2] (Váry *et al*., 2015) in the wheat-*Z. tritici* pathosystem, there is scarce knowledge about how the combination of these two abiotic factors, or the diurnal fluctuating temperature as well as the occurrence of heat events would influence STB disease (Velásquez *et al*., 2018). Therefore, this study conducted the infection of Spanish durum wheat commercial cultivars against *Z. tritici* under diverse weather conditions of increasing (and fluctuating) temperature and [CO_2] to elucidate, through macroscopic and microscopic evaluations, the feasible durum wheat-*Z. tritici* interactions. Additionally, in order to assess how the even more frequent occurrence of heat waves caused by climate change would affect STB disease, expected weather conditions were conducted during both disease establishment and development (S1G and S2G plant sets).

3.4.1. Fungal penetration success at elevated temperature and [CO_2]

In order to assess the capability of *Z. tritici* to penetrate the host plants and cause infection, microscopic observations were developed at 4 dpi, similar to previous studies carried out in durum wheat cultivars (Somai-Jemmali *et al*., 2017a; 2017b). S1 and S2 plants of the three accessions, which were inoculated and incubated under baseline weather conditions, similarly to SB plants, expressed significantly higher NP values compared to SB plants. These values could be explained considering that once the spores germinate, *Z. tritici* hyphae are capable of growing epiphytically on the leaf surface for several days (up to 10) (Fones *et al*., 2017). Therefore, S1 and S2 plants, which were returned after 48 h of disease incubation to environment 1 and 2, respectively, were then exposed to maximum temperatures of 30°C until observation at 4 dpi. This period of exposure could hamper both stomatal (SP) and direct (DP) penetration of

germinated spores, according to the fact that temperature seems to have a strong effect on *Z. tritici* spore viability and survival (Suffert *et al*., 2011; Chaloner *et al*., 2019; Francisco *et al*., 2019). Evidently, S1G and S2G plants, which were inoculated and incubated under future weather conditions, developed even higher NP percentages for the three accessions, showing an increase in failing to penetrate into the host up to 34% compared to SB plants. Moreover, it seems that DP event was more restricted in S1G and S2G plants, especially in susceptible accession 'Sy Leonardo', possibly due to elevated temperature reducing fungal capability of forming appressorium-like structures (Shetty *et al*., 2003; Francisco *et al*., 2019). Considering all these results, we may suggest on one side, that increasing temperature up to 30°C would severely affect the ability of *Z. tritici* to establish the infection in durum wheat plants and, on the other side, that the presence of elevated [CO_2] did not modify these fungal patterns. In addition, since the three accessions, which developed diverse response to infection, expressed similar patterns of fungal penetration, it seems that temperature affected *Z. tritici* behaviour (Wainshilbaum and Lipps, 1991; Suffert *et al*., 2011) more than wheat plants physiology or interactions between both at this stage of the infection process (Eastburn *et al*., 2011; Elad and Pertot, 2014).

3.4.2. Final disease development at elevated temperature and [CO_2]

In order to measure the damage caused by *Z. tritici*, quantification of final chlorotic and necrotic lesions (PLACL) has been usually considered in image-based analyses (Stewart *et al*., 2014; 2016; Karisto *et al*., 2018; Yates *et al*., 2019). In this study, PLACL values observed in plants exposed to future weather conditions in the three studied accessions can be mainly explained regarding the reduced spore penetration in microscopic results obtained at 4 dpi. Thus, considering that temperature seems to have a strong effect on *Z. tritici* spore viability and survival (Suffert *et al*., 2011; Chaloner *et al*., 2019; Francisco *et al*., 2019), it is very likely that spores could develop more successful infection sites in S1 and S2 plants rather than in S1G and S2G plants, even after observations made at 4 dpi (Fones *et al*., 2017). This fact can explain the reduced PLACL values observed in S1G and S2G plants in comparison to S1 and S2 plants for the three accessions. In addition, several studies consider optimum temperature for disease development under greenhouse conditions ranging from 17 to 25°C and relative humidity should be ≥ 85% (Hess and Shaner, 1987; Wainshilbaum and Lipps, 1991; Magboul *et al*., 1992; Ghaffary *et al*., 2018). Therefore, once disease was established, it is feasible that post inoculation maximum daily temperature in environments 1 and 2, coupled with a suboptimal relative humidity for disease development, contributed to reducing the subsequent disease progression in plants exposed to these environments until evaluation at 21 dpi. Contrary to this study, Wainshilbaum and Lipps (1991) found that wheat plants incubated and evaluated at 29°C did not express almost any *Z. tritici* symptoms for final disease evaluation. This is possibly because the temperature range of that study was constantly 29°C during both the incubation process and disease development, whereas in our study the temperature changes throughout the day, highlighting the importance of resembling natural field conditions as well as possible to assess plant-pathogen interactions (Velásquez *et al*., 2018).

Particularly, in 'LG Origen' accession, considered moderately resistant, the reduction of PLACL values in S1 and S2 plants was greater in proportion compared to 'Sy Leonardo' and 'RGT

Rumbadur'. Considering that microscopic parameters observed at 4 dpi and weather conditions were similar to the three accessions, it could be suggested a remarkable post penetration reduction of the disease in this accession. In this sense, elevated temperature has been found to modulate plant resistance against pathogens, increasing or decreasing it in terms of both basal and race-specific resistance (Cheng *et al*., 2013). Additionally, increasing temperature is known to upregulate jasmonic acid (JA) synthesis (Kim *et al*., 2022), which activates immunity against necrotrophic pathogens (Castroverde and Dina, 2021). Considering this and *Z. tritici* being categorised as a latent necrotrophic pathogen according to recent studies (Sánchez-Vallet *et al*., 2015; Orton *et al*., 2017), it could be possible that partial resistance of 'LG Origen' could be favoured at elevated temperatures through JA-induced defence responses.

In addition to these results, it seems that the presence of elevated [CO_2] in environment 2 led plants to express higher PLACL values in comparison to plants exposed to environment 1. Indeed, some studies supported that exposure to elevated [CO_2] increased disease severity in wheat plants against necrotrophic pathogens such as *Z. tritici* or *Fusarium pseudograminearum*, even in resistant cultivars (Melloy *et al*., 2014; Váry *et al*., 2015). This enhanced expression of STB disease could originate from alterations in host physiology produced at high levels of [CO_2] (Manning and von Tiedemann, 1995; Eastburn *et al*., 2011). In fact, it is known that elevated [CO_2] enhanced photosynthetic efficiency of plants, especially in C_3 plants as wheat (Chaudhry and Sidhu, 2022), which increase carbohydrate supply, finally resulting in elevated starch and sugar levels in leaf tissue (Eastburn *et al*., 2011; Loladze, 2014). Considering this situation and the study of Yang *et al*. (2013), which showed that there was enhanced sugar production in leaves during the symptomatic stage of STB disease, it is possible that *Z. tritici* mobilises leaf sugars for nutritional gain and, therefore, could be favoured by the increased levels of sugar presented in leaf tissue at elevated [CO_2]. Additionally, there are studies which indicate that exposure to elevated [CO_2] suppress biosynthesis of stress-induced JA (Bazinet *et al*., 2022), which is the main phytohormone responsible for plant defence response against necrotrophic pathogens, as mentioned above.

Despite these facts, plants exposed to elevated [CO_2] (and temperature) in this study did not express higher *Z. tritici* disease symptoms than plants exposed to baseline conditions, but rather than plants exposed to elevated temperature. Therefore, a feasible explanation is that simultaneous exposure to both elevated temperature and [CO_2] forced S2 and S2G plants to develop a unique response during the subsequent *Z. tritici* infection which, according to previous studies, could be regulated by antagonistic plant physiological mechanisms and signalling pathways as well as varied regarding plant genotypes, pathogen biology, and/or timing and intensity of simultaneous abiotic factors (Suzuki *et al*., 2014; Atkinson *et al*., 2015). Indeed, differences in PLACL values were not equal amongst accessions, indicating that diverse environmental conditions could differently influence wheat resistance against *Z. tritici* (Orton *et al*., 2017; Yates *et al*., 2019). Thus, it seems that relevant differences in susceptible and moderately resistant accessions arise between S2G and S1G plants, highlighting the beneficial effect of elevated [CO_2] for the fungus when its development was hampered by elevated temperature during the incubation process and host defence responses were not complete. By contrast, in resistant accession 'RGT Rumbadur', despite exposure to elevated temperature hampered disease development (observed in S1 plants), it seems that simultaneous exposure to

elevated [CO_2] counteracted this reduction, showing S2 plants similar PLACL values than SB plants. Therefore, it is possible that predicted increase in [CO_2] would compromise STB resistant cultivars (Volk *et al*., 2010; Váry *et al*., 2015) even if they would be exposed to simultaneous elevated temperature during disease progression.

3.4.3. Pycnidia development at elevated temperature and [CO_2]

Lesions caused by *Z. tritici* finally develop asexual reproduction structures, called pycnidia (Kema *et al*., 1996b), which contain asexual pycnidiospores that disperse the disease to other leaves and plants (Suffert *et al*., 2011). Therefore, the restriction of pycnidia development can be considered a key component of resistance against *Z. tritici* (Kema *et al*., 1996a; Eyal, 1999; Suffert *et al*., 2013). However, some studies have shown that resistance which reduces pathogen damage to host tissue (indicated by PLACL) can be independent of resistance that minimises pathogen reproduction (indicated by Pyc/lesion; Karisto *et al*., 2018; Yates *et al*., 2019). Additionally, there is little knowledge about how environmental factors would affect formation and functionality of these fruiting bodies (Eyal, 1999).

In microscopic results, plants of the susceptible accession 'Sy Leonardo' developed, under future weather conditions, relevantly higher non-colonised stomata values (NCS), on one side, together with a significantly reduced proportion of colonised stomata which finally transformed into pycnidia (P), on the other side. These values were confirmed macroscopically with a great reduction of Pyc/lesion values in S1 and S2 plants, being this reduction even more severe in S1G and S2G plants. Therefore, it could be suggested that the elevated maximum temperature of 30°C affects not only the progression of the disease, but also the ability of the fungus to develop pycnidia (Hess and Shaner, 1987; Wainshilbaum and Lipps, 1991; Magboul *et al*., 1992). However, the reduction of Pyc/lesion values in S1G and S2G plants cannot be explained through microscopic P data values. In this sense, a feasible explanation is that a relevant number of microscopic colonised stomata classified as pycnidia (P stage) were arrested or immature due to elevated temperature (Shearer and Wilcoxson, 1978), which considerably reduces its detection by macroscopic image analysis. This is contrary to the commonly observed pycnidia evolution, which normally leads to pycnidia becoming smaller as the density increases on necrotic lesions (Eyal *et al*., 1987). Therefore, it is likely that exposure to elevated temperature during the incubation process, as well as during subsequent disease progression, may lead to reduced pycnidia number and maturity in S1G and S2G plants in susceptible accession. Considering these results altogether, it seems that both *Z. tritici* reproduction and dispersal capability would be severely affected in plants exposed to future weather conditions through reduced pycnidia number on one side, and decreased pycnidia size on the other, which would both compromise pycnidiospores production rate and size for subsequent disease cycles (Gough, 1978; Suffert *et al*., 2011, 2013; Stewart *et al*., 2018; Yates *et al*., 2019). Lastly, although it seems that elevated [CO_2] could slightly improve the formation of pycnidia in S2 and S2G plants in comparison to S1 and S1G plants, respectively, these values were irrelevant, suggesting that elevated [CO_2] mainly improved fungal colonisation to a certain extent (Yang *et al*., 2013; Váry *et al*., 2015; Bazinet *et al*., 2022) rather than pycnidia development.

Then, although moderately resistant accession 'LG Origen' showed reduced Pyc/lesion values in comparison to 'Sy Leonardo', these values were slightly different amongst SB plants and plants exposed to future weather conditions. Concretely, only S1G and S2G plants expressed significantly reduced values in comparison to SB plants, confirmed microscopically in higher NCS values and lower CST and P values, especially in S1G plants. In fact, the supposed reinforced resistance expression of 'LG Origen' under elevated temperature (Cheng *et al*., 2013; Kim *et al*., 2022), coupled with the unfavourable inoculation conditions for a subsequent disease development of S1G plants (Hess and Shaner, 1987; Wainshilbaum and Lipps, 1991; Magboul *et al*., 1992) could be observed in its significantly higher NCS value. Additionally, although S2 and S2G plants expressed similar microscopic P values in comparison to SB plants, they also showed reduced Pyc/lesion values. This indicated that, as for 'Sy Leonardo' accession, elevated [CO_2] mainly improved the fungal colonisation of the leaves instead of developing mature pycnidia.

'RGT Rumbadur' accession showed very reduced pycnidia development amongst diverse weather conditions, which is considered a resistance trait against *Z. tritici* (Kema *et al*., 1996a; Eyal, 1999; Suffert *et al*., 2013; Karisto *et al*., 2018) and, obviously, standing out compared to the other two accessions. Focusing on microscopic analysis, NCS and CST values were not statistically different between SB plants and the other plant sets. Indeed, it could be observed that, overall, CST values were higher in the resistant accession in comparison to the other two evaluated accessions, which means that mycelium was generally restricted to the substomatal cavity, and this is supposed to be a specific characteristic of some resistant cultivars (Kema *et al*., 1996b). In addition, only S1G plants varied statistically in microscopic P values in comparison to SB plants, whereas these differences did not occur in the Pyc/lesion parameter. This lack of relevant differences in both microscopic and macroscopic parameters suggest that future weather conditions carried in this study would barely change pycnidia development in the resistant accession. In this sense, although differences were insignificant, a possible explanation for the lower Pyc/lesion values of S2 and S2G plants compared to S1 and S1G plants could be that elevated [CO_2] increased STB colonisation in 'RGT Rumbadur' plants to the detriment of mature pycnidia development, as mentioned above for 'Sy Leonardo' and 'LG Origen' accessions.

Finally, the Pyc/leaf parameter was obtained in order to assess how future weather conditions would affect pycnidia development regarding the whole plant and to identify differences compared to pycnidia development restricted to STB lesions. In this sense, differences observed in Pyc/leaf values amongst plants exposed to diverse weather conditions were almost similar to those obtained in Pyc/lesion values for the three accessions, especially for 'Sy Leonardo'. In fact, the susceptible accession showed statistically similar reduction patterns in plants exposed to future weather conditions than for Pyc/lesion parameter, mainly as a combination of reduced PLACL values, pycnidia number and, probably, pycnidia size. Then, the severe reduction of PLACL values in 'LG Origen' accession led to reduced Pyc/leaf values for plant sets exposed to future weather conditions, showing irrelevant differences amongst them. Finally, as 'RGT Rumbadur' developed such a low number of pycnidia, it was the accession with fewer changes in Pyc/leaf values through plant sets, mainly due to reduced PLACL values. Additionally, although the effect of elevated [CO_2] in the increase of STB colonisation to the detriment of mature pycnidia development could be observed in S2 and S2G plants, their

Pyc/leaf values did not show relevant differences in comparison to S1 and S1G plants. These results suggest that pycnidia development at the leaf level of plants exposed to changing conditions of increasing temperature and [CO_2] would vary mainly regarding the lesion surface in which pycnidia could develop, which is mainly affected by temperature, and that variations due to elevated [CO_2] would be negligible at this level. Therefore, to detect possible differences under changing environmental conditions, moderately resistant and resistant accessions would require microscopic and specific analysis of lesions caused by *Z. tritici* to a greater extent than susceptible accessions.

In conclusion, the most important fact in our study is that exposure to elevated maximum temperature alone or in combination with elevated [CO_2] not only did not suppress the general defence response in our studied accessions 'LG Origen' (moderately resistant) and 'RGT Rumbadur' (resistant), but also the disease severity was reduced in these accessions as well as in 'Sy Leonardo' accession (susceptible). This indicates that increasing temperature could mainly affect *Z. tritici* virulence rather than plant physiology, especially in the processes of disease establishment and pycnidia formation and maturation, which would severely hamper subsequent infection cycles of STB. Concretely, this obstacle was even worse for *Z. tritici* in the case of plants exposed to climate change conditions during the whole disease process. By contrast, despite the adverse effect of elevated temperature, simultaneous exposure to elevated [CO_2] could induce physiological and molecular alterations in the host plant that eventually benefit *Z. tritici* disease development in terms of leaf tissue colonisation, especially in the resistant accession, which would threaten resistant cultivars under predicted [CO_2] increase. Finally, due to varied results regarding diverse accessions and abiotic stresses observed in this study, it should be noted that for assessment of climate change effects on wheat-*Z. tritici* interactions, it is essential performing experiments in weather conditions that are as realistic as possible and using both, macro and microscopic methods of disease analysis.

3.5. References

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Chapter 4. Characterisation of durum wheat resistance against leaf rust under climate change conditions of increasing temperature and [CO_2]

4.1. Introduction

Wheat is considered one of the most important crops in the world, accounting for 771 million tons of production on 221 million hectares in 2021 and represents an essential source of calories and protein in the human diets (FAOSTAT, 2023). Therefore, the predicted expansion of the global population up to 9.6 billion people by 2050 (World Bank, 2023) would imply an increase of the global demand of wheat consumption. However, accomplishing this objective of production is a challenging task because current wheat yields are limited by diverse abiotic and biotic constraints.

Abiotic stresses, such as drought or high temperatures, affect wheat cultivation depending on the region and environmental conditions, causing physiological and biochemical alterations that ultimately reduce wheat yields, even more than biotic stresses (Abhinandan *et al*., 2018; Hossain *et al*., 2021). Indeed, environmental conditions have suffered variations due to global climate change (Pachauri *et al*., 2014), which could be considered an important constraint for plant growth and development through abiotic stresses such as high temperature and drought (Chaudhry and Sidhu, 2022), reducing wheat yield by 20-30% due to the impact of extreme events (Hossain *et al*., 2021). Concretely, the increase in carbon dioxide concentration [CO_2] is expected to increase global temperature by up to 3.7°C (Pachauri *et al*., 2014), and, according to projections for Europe, this increase could be between 4.5 and 5.5°C, depending on the CO_2 emission scenario (Jacob *et al*., 2014). In addition, wheat is constantly exposed to biotic stresses, which are a major constraint to wheat production worldwide (Strange and Scott, 2005; Oerke, 2006; Savary *et al*., 2019). Amongst them, plant diseases cause more than 21% of wheat losses on average (Savary *et al*., 2019), with fungal pathogens such as wheat rusts considered to be the most detrimental (Sharma, 2012; Singh *et al*., 2016; Figueroa *et al*., 2018).

Leaf (brown) rust, caused by the biotrophic fungus *Puccinia triticina* Erikss., is one of the most common and harmful rust of wheat (Huerta-Espino *et al*., 2011; Chai *et al*., 2020). Leaf rust infections reduce the photosynthetic activity of infected leaves, which usually results in yield losses (~8.6 million tons annually (Chai *et al*., 2020)) through decreasing number of kernels and lower kernel weights (Bolton *et al*., 2008). Currently, the most efficient, sustainable, and nonchemical approach to face leaf rust is through genetic breeding (Pérez-Méndez *et al*., 2022). In fact, several genes of resistance to *P. triticina* have been isolated, identified and named as *Lr* genes (~80) (McIntosh *et al*., 2020). Some genes are race-specific, acting in a gene-for-gene manner with the pathogen, and are associated with a qualitative hypersensitive response (HR), while others are non-race specific and develop a partial resistance response (PR), showing durable incomplete resistance, characterised by a slower development of the disease (Martínez-Moreno *et al*., 2022). Thus, to discover potential sources of resistance, the identification of both macro and microscopic components of resistance is considered essential. In fact, although leaf

rust evaluation has been generally achieved through visual assessment (Kolmer, 1996; Soleiman *et al*., 2014b; Martínez-Moreno *et al*., 2022), image analysis has emerged as a precise tool for quantitatively evaluating wheat rusts (Gallego-Sánchez *et al*., 2020; Porras *et al*., 2022; Cabrera *et al*., 2023). Additionally, histopathological methods allow the identification of microscopic components of resistance to wheat rusts such as generation of reactive oxygen species (ROS), or the presence and development of HR amongst others (Wang *et al*., 2013; Wesp-Guterres *et al*., 2013; Soleiman *et al*., 2014b). Thus, this detailed level of analysis regarding interactions of wheat against its rust pathogens would be necessary not only in finding new sources of resistance, but also in assessing how abiotic factors, which are predicted to be affected by climate change, would influence in wheat-leaf rust interactions.

Indeed, wheat-pathogen interactions are likely to be influenced by future climate change alterations in temperature, [CO_2] and water regimes (Pérez-Méndez *et al*., 2022), which would modify plant development and resistance pathways, on one side, and pathogen virulence mechanisms and life cycle, on the other side (Velásquez *et al*., 2018). Thus, disease risk simulation studies postulate as an essential tool to predict these climate impacts on wheat-pathogen interaction worldwide (Juroszek and von Tiedemann, 2013; 2015; Miedaner and Juroszek, 2021), but they involve a certain degree of uncertainty (Gouache *et al*., 2013). This, combined with a lack of realistic field studies about the effects resulting from the combination of simultaneous abiotic and biotic stresses in wheat (Suzuki *et al*., 2014; Ramegowda and Senthil-Kumar, 2015), make disease predictions under future climate change a challenging task. In fact, these studies are far from finding a similar response about the effects of expected abiotic factors, such as high temperature and elevated [CO_2], on wheat-pathogen interactions (von Tiedemann and Firsching, 2000; Bencze *et al*., 2013; Melloy *et al*., 2014; Mikkelsen *et al*., 2015; Váry *et al*., 2015; Matić *et al*., 2018; Hay *et al*., 2021), possibly due to diverse plant genotypes, pathogen lifestyles, and/or timing and intensity of simultaneous abiotic factors (Suzuki *et al*., 2014). In this sense, plants have a tailored physiological and molecular response to multiple stresses, which cannot be elucidated from studies of individual stresses, mainly due to the occurrence of antagonistic signalling pathways between abiotic and biotic stresses (Suzuki *et al*., 2014; Atkinson *et al*., 2015), such as the role of some phytohormones in this kind of responses (Bostock *et al*., 2014). Moreover, the concept of plant acclimation (or cross tolerance/susceptibility) needs to be considered since prolonged exposure to an abiotic stress could reduce or enhance a subsequent pathogen attack (Bostock *et al*., 2014; Ramegowda and Senthil-Kumar, 2015). It is therefore necessary to study empirically how the combined environmental factors expected in the future climate change scenarios would affect plant immunity, pathogen virulence, and disease development in wheat-pathogen interactions.

This is particularly true for several European wheat-growing areas with Mediterranean-like climatic characteristics, such as some Spanish regions, where not only an increase in leaf rust disease is predicted by 2050 (Miedaner and Juroszek, 2021), but also a major constraint on wheat production due to abiotic factors. Furthermore, as these regions are considered hotspots of climate change, temperature warming, extreme events and changes in precipitation regimes are likely to occur (Diffenbaugh and Giorgi, 2012; Trnka *et al*., 2014). Therefore, this situation would particularly affect the cultivation of durum wheat, which is considered a staple crop in Mediterranean countries (Xynias *et al*., 2020; Ceglar *et al*., 2021). In some Spanish growing areas,

the appearance of new virulent races of leaf rust (Soleiman *et al*., 2016) coupled with the threat of climate change (Velásquez *et al*., 2018; Pérez-Méndez *et al*., 2022; Lorite *et al*., 2023) could severely hamper future durum wheat production.

The aim of this study was to identify macro and microscopic components of resistance against leaf rust in durum wheat accessions with different levels of resistance, using greenhouse experiments that simulated the predicted conditions of increased temperature and [CO_2] in the far future period of 2070-2099 for the wheat growing region of Córdoba, Spain.

4.2. Materials and Methods

4.2.1. Plant material

In our study we evaluated 45 durum wheat (*T. turgidum* spp. *durum*) accessions against a local isolate of leaf rust (*P. triticina*). As in Chapter 2, 22 out of 45 evaluated accessions were breeding lines which belong to the wheat breeding program developed at IFAPA (Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica) Spain, and the other 23 accessions were commercial Spanish cultivars, registered in the Spanish MAPA (Ministerio de Agricultura, Pesca y Alimentación) catalogue (Supplementary Table S4.1), either recently registered or widely cultivated by Spanish farmers.

4.2.2. Pathogen isolation

P. triticina isolate used in this study was collected in a naturally infected field at Santaella (Córdoba, Spain) in 2018. Spores from the infected leaves were inoculated onto uninfected plants of susceptible cultivar 'Qualidou' to purify the inoculum. Plants were placed in a humidity chamber at 21°C to provide 100% relative humidity (RH) and kept for 24 h. Then, plants were transferred to a growth chamber at 21°C day/night with 70% RH and 14 h photoperiod for 9 days. When individual pustules appeared, a single-pustule isolate was obtained and multiplied in 14-day-old 'Qualidou' plants in order to increase the amount of spores for further inocula. Plants were inoculated with spores mixed with pure talc (1:20 v/v) using a manual airbrush spray and incubated as described above. Then, spores of leaf rust were collected using a vacuum bomb and stored at -80°C until inoculation experiments. Finally, this leaf rust isolate showed virulence/avirulence on the following Lr genes: *Lr1*, *Lr2c*, *Lr3*, *Lr3bg*, *Lr3ka*, *Lr10*, *Lr11*, *Lr12*, *Lr14a*, *Lr14b*, *Lr18*, *Lr20*, *Lr22a*, *Lr23*, *Lr30*, *Lr33*, *Lr34*, *Lr35*, *Lr37*, *Lr45*, *LrB* / *Lr2a*, *Lr2b*, *Lr9*, *Lr13*, *Lr15*, *Lr16*, *Lr17*, *Lr19*, *Lr21*, *Lr24*, *Lr25*, *Lr26*, *Lr28*, *Lr32*, *Lr36*, *LrW*.

4.2.3. Screening of the durum wheat germplasm collection

Seeds of 45 durum wheat accessions were sown in 8 x 7 x 7 cm pots containing a mix (1:1 v/v) of commercial compost (Suliflor SF1 substrate; Suliflor, Radviliškis, Lithuania) and sand. Pots were then placed in trays and incubated in a growth chamber at 21°C day/night with a 14-h photoperiod for germination. After 12 days, when the second leaf was completely unfolded, four seedlings of each accession were inoculated with leaf rust spores mixed with pure talc (1:20 v/v) using a manual airbrush spray, and incubated in a humidity chamber as described above. A

total of 180 plants were uniformly inoculated with 80 mg of leaf rust spores. The experiment was performed three times. The same analysis was performed with adult plants (two months) to confirm the absence of significant variations in resistance or susceptibility traits compared to seedlings (Supplementary Figure S4.1). For disease assessment, the second leaf of each plant (fifth leaf for adult plants) was evaluated 9 days post inoculation (dpi). The infection process was recorded as the percentage of each leaf with disease symptoms (pustules, chlorosis and necrosis), referred to as disease severity (DS). In addition, seedling reactions were registered using a disease scoring scale (0-9) for infection type (IT; McNeal *et al*., 1971), where 0 = no visible disease symptoms (immune), 1 = minor chlorotic and necrotic flecks, 2 = chlorotic and necrotic flecks without sporulation, 3-4 = chlorotic and necrotic areas with limited sporulation, 5-6 = chlorotic and necrotic areas with moderate sporulation, 7 = abundant sporulation with moderate chlorosis, 8-9 = abundant and dense sporulation without notable chlorosis and necrosis. Infection types 0-6 were considered resistant, while types 7-9 were considered susceptible.

After this preliminary disease screening, three durum wheat accessions with different responses to infection, ranging from resistant to susceptible, were selected for assessing macro and microscopic components of resistance to leaf rust under baseline (control) and climate change conditions. These accessions were: 'BL 28', 'BL 38' and 'Qualidou'.

4.2.4. Greenhouse conditioning and design of climate environments

Plants of the three selected durum wheat accessions were grown in greenhouses with full environmental control of temperature and [CO_2] as described in Chapter 3, thanks to air conditioning systems and CO_2 supply circuits controlled by sensors. The established weather conditions were also designed as it was defined in Chapter 3 with the objective to resemble a standard spring day, which is the expected growth period of *P. triticina* in the wheat growing area of Córdoba. Therefore, this study carried out a variation of temperature throughout the day, reaching an established maximum and minimum value. As in experiments of Chapter 3, baseline conditions were established with maximum and minimum temperature values of 24°C and 10°C, respectively, whereas weather conditions for the far future period (2070-2099) resulted in maximum and minimum temperatures around 30°C and 15°C. Additionally, [CO_2] levels were set at 420-450 ppm for baseline conditions (from levels currently observed outdoors) and 620-650 ppm for far future weather conditions.

Having established the above temperature and [CO_2] conditions, plants from three durum wheat accessions were exposed to three different environments, each in separate greenhouses, to assess *P. triticina* infection, similar to experiments of Chapter 3. Under baseline conditions (environment B), plants were exposed to a maximum temperature of 24°C and [CO_2] around 420-450 ppm. For the far future scenario, two possible environments were established: under increasing temperature (environment 1), plants were exposed to a maximum temperature of 30°C and [CO_2] around 420-450 ppm; and under increasing temperature and [CO_2] (environment 2), plants were exposed to a maximum temperature of 30°C and elevated [CO_2] around 620-650 ppm.

Thus, five sets of plants were identically exposed to these environments as described in Chapter 3. One set of plants was grown, incubated and maintained for disease evaluation under baseline weather conditions (set SB), and four sets of plants were grown under far future weather conditions: two sets at elevated temperature and two at both elevated temperature and [CO_2]. As high temperatures could critically affect the penetration success of *P. triticina* to the host tissue, two of the four sets of plants exposed to future weather conditions were inoculated and incubated under baseline weather conditions, before returning to their respective far future conditions (sets S1 and S2, respectively). The other two sets of plants were grown, inoculated, incubated and maintained for disease evaluation under their corresponding far future weather conditions (sets S1G and S2G, respectively).

4.2.5. Inoculation assays for evaluation of components of resistance

Seeds of the three selected durum wheat accessions were sown in 30 x 20 x 7 cm trays containing the mix of commercial compost and sand (1:1, v/v) described above. Trays were first incubated at 21°C with a 14-h photoperiod in a growth chamber to germinate the plants for 6 days, and then, seedlings were transferred to different greenhouse with diverse weather conditions described above (environments B, 1 and 2) for 15 days until the third leaf was completely unfolded. Then, following Sørensen *et al*. (2017) with minor modifications, third-leaves were fixed horizontally (adaxial surface up) on a foam board with metal clips. A total of 27 leaves (9 per accession) were fixed in each one of five trays (one per plant set) for subsequent macro and microscopic evaluations, being evaluated a total of 135 leaves per replication. Each tray was inoculated with 4 mg of leaf rust spores (with a spore deposition of 280-300 spores per cm^2) mixed with pure talc (1:20 v/v) using a settling tower which led to a uniform inoculation of the leaves. Then, trays were covered with black plastic bags to maintain a 100% RH and darkness for the leaf rust inoculation for 24 h. Three sets of plants (SB, S1 and S2) were incubated on environment B, while two sets of plants were incubated on their corresponding environments 1 or 2 (S1G and S2G, respectively). Finally, plastic bags were removed, and plants were kept in their respective environments for 9 days. Macroscopic and microscopic experiments were performed three times each.

4.2.6. Assessment of macroscopic components of resistance

Leaf segments of 2 cm long were marked at 5 dpi, before the appearance of rust pustules, in third-leaves of four plants per accession, plant set and replication. Then, the number of visible pustules breaking the leaf epidermis in the marked segments were recorded at different time intervals until the number no longer increased. A portable camera (IPEVO DO-CAM, Sunnyvale, CA, USA) equipped with a hand-lens was used to count the rust pustules, taking photos of leaf segments through different time intervals. Thus, latency period (LP50) was calculated as the number of hours from the day of inoculation to the appearance of 50% of the total pustules breaking leaf epidermis in the marked segments. In addition, five leaves per accession, plant set and replication were detached, placed on black sheets of cardboard, and digitally scanned (Canon CanoScan LiDE 400, Tokyo, Japan) at 1200 ppi of resolution after 9 dpi, similar to Cabrera *et al*. (2023). The image analysis software Fiji (Wayne Rasband, NIH, MD, USA; Schindelin *et al*., 2012) was used for analysing 4 cm^2 of individual scanned leaves. The parameters analysed, based

on Porras *et al*. (2022) for *Puccinia striiformis* with modifications, were Infection Frequency (IF, number of pustules per cm^2 of leaf), Mean Pustule Size (cm^2), Total Pustule Area relative to leaf area (%), Total Disease Area (pustule area plus chlorosis and necrosis areas) relative to leaf area (%) and Pustule Development Rate (proportion of pustule area relative to Total Disease Area (%)). Areas of pustules and disease symptoms were determined by the colour thresholding option using the default method with the HSB colour space setting.

4.2.7. Assessment of microscopic components of resistance

Central leaf segments (~ 6 cm) of third-leaves placed on cork pedestals mentioned above were cut at 5 dpi in four leaves per accession, plant set and replication. Samples were processed as described in Soleiman *et al*. (2014b) with minor modifications. Briefly, leaves were fixed and cleared through boiling for 5 min in lactophenol/ethanol (1:2) and stored overnight at room temperature. Samples were then transferred into a saturated chloral hydrate solution (5:2 wt/v) for 24 h. Then, they were washed once with 50% ethanol for 30 min, twice with 0.5M NaOH for 15 min each, rinsed three times in distilled water (10 min each), and soaked in 0.1 M Tris buffer (pH 8.5) for 30 min. Leaf segments were stained with Uvitex 2B fluorescent dye (Polysciences, Bergstrasse, Germany) using a 0.1% working solution, followed by rinsing four times with distilled water. Samples were immersed in a solution of 25% glycerol for 30 min and stored until observed. Leaf segments were examined using Nikon epifluorescence equipment (Nikon, Tokyo, Japan), with a V-2 A filter (excitation filter 380-420 nm, barrier filter 430 nm).

Fungal colonies were classified based on Soleiman *et al*. (2014b) as early-aborted (EA), when spores developed a substomatal vesicle (SSV), a primary infection hypha and no more than 6 haustorial mother cells (HMC), and established (EST), when spores developed a SSV and a primary infection hypha with more than 6 HMC. In both development stages, the presence (+) or absence (−) of plant cell death autofluorescence (necrosis) was considered to establish early-aborted colonies associated to necrosis (EA+) or not (EA–), and established colonies associated to necrosis (EST+) or not (EST–). A total of 150 spores in four leaves per accession, plant set and replication were evaluated and classified according to the mentioned fungal stages of development. Only spores which formed an infection site were counted. Fungal stages of development were photographed using a Nikon DS-Fi1 camera (Nikon, Tokyo, Japan). In addition, 40 established infection units in four leaves per accession, plant set and replication were measured in their length (L) and width (W) using a micrometer. In the resistant accession 'BL 38', only 10 to 20 established infection units per plant set and replication were measured due to the reduced occurrence of this fungal stage. Colony size (CS) was calculated as the geometric mean of L and W, $CS = \sqrt{(1/4 \times \pi \times L \times W)}$ (Rubiales and Niks, 1995).

4.2.8. Statistical analysis

The experimental design was developed as randomised blocks. Macroscopic and microscopic parameters whose data did not achieve normality and homogeneity requirements amongst different environments for each accession were transformed for statistical analysis with ANOVA test, and back transformed for presentation. However, parameters whose data could not achieve those requirements using transformations were analysed through

nonparametric Kruskal-Wallis test. Thus, data from macroscopic parameter LP50 in accession 'Qualidou' was analysed using ANOVA and Dunnett T3 test, while the rest of macroscopic parameters in this accession were analysed using ANOVA and LSD (Least Significant Difference) tests. Data from IF, Total Pustule Area and Total Disease Area in accession 'Qualidou' were transformed according to the formula $y = \sqrt{(x)}$. Macroscopic data from Total Disease Area were also transformed according to the formula $y = \log(x)$ and $y = \sqrt{(x)}$ in 'BL 28' and 'BL 38' accessions, respectively, and analysed using ANOVA and LSD test. Finally, macroscopic parameters IF, Mean Pustule Size, Total Pustule Area and Pustule Development Rate in accession 'BL 28' were analysed using Kruskal-Wallis test. In terms of microscopic parameters, data from percentages of different fungal stages and CS parameters were analysed using ANOVA and Duncan tests for the three selected accessions. Data from microscopic fungal stages EA– and EA+ in accession 'Qualidou', and EST+ in accession 'BL 38' were transformed according to the formula $y = \sqrt{(x)}$. Data processing, statistical analyses and figure design were carried out using R software (R Core Team, 2023) and Fiji (Schindelin *et al*., 2012).

4.3. Results

4.3.1. Response of durum wheat germplasm to leaf rust infection

The 45 durum wheat accessions were evaluated against *P. triticina* disease and classified according to their percentage of DS and IT (Figure 4.1). There was a high proportion of accessions (33 in total) presenting a susceptible response (IT 7-9) under optimum conditions of fungal infection, possibly due to the recent emergence of new virulent races in recent years not only in Spain (Soleiman *et al*., 2016), but also in other Mediterranean countries such as France (Goyeau *et al*., 2012). Amongst them, 19 accessions were breeding lines and 14 were commercial cultivars. Seventeen accessions showed an IT value of 7, developing abundant sporulation but with the appearance of few chlorosis surrounding the pustules, while the rest of accessions showed IT values of 8 and 9, developing abundant and dense sporulation without chlorosis. Almost all breeding lines (19 out of 22) expressed IT 7-9 values, a quite concerning fact for the current Spanish breeding programs of durum wheat, which presented barely any sources of resistance against leaf rust (Martínez-Moreno, *et al*., 2021) in comparison with bread wheat (Soleiman *et al*., 2014a; 2014b).

The remaining 12 accessions showed diverse value of resistant response to *P. triticina* infection, highlighting accessions with an incompatible response in the form of minor chlorotic flecks (IT 1) such as 'BL 38' (breeding line), 'Fuego' and 'Teodorico' (commercial cultivars). Other two commercial cultivars ('LG Acropolis' and 'Amilcar') and the breeding line 'BL 28' expressed a high resistant response, showing chlorosis and necrosis surrounding limited sporulation (IT 3-4), whereas other commercial cultivars and the breeding line 'BL 45' exhibited a moderately resistant response in the form of chlorosis and necrosis surrounding moderate sporulation (IT 5-6).

Three suitable accessions were selected for the climate change experiments which present resistant and susceptible reactions against leaf rust. Thus, breeding line 'BL 38' (IT 1) was chosen as highly resistant, breeding line 'BL 28' (IT 4) as moderately resistant, and commercial cultivar 'Qualidou' (IT 9) as susceptible.

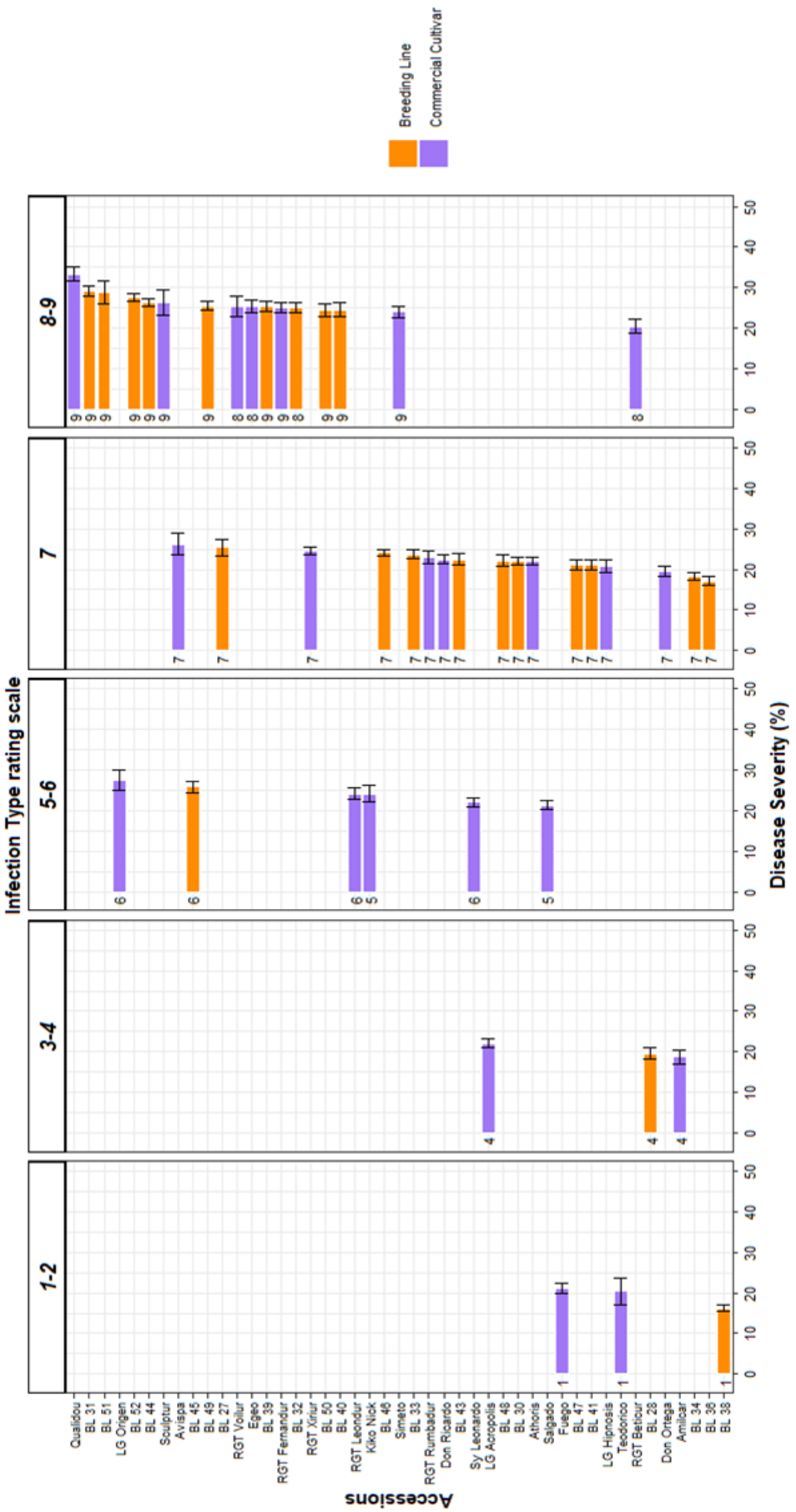

Figure 4.1. *P. triticina* infection in durum wheat breeding lines and commercial cultivars. Mean percentage of disease severity (DS), presented in columns, and infection type (IT) rating scale, presented as numbers at the top of the figure. Accessions were arranged according to their mean percentage of DS and classified according to IT in panels. The IT scale is presented according to McNeal *et al.* (1971), where 0 = no visible disease symptoms (immune), 1 = minor chlorotic and necrotic flecks, 2 = chlorotic and necrotic flecks without sporulation, 3-4 = chlorotic and necrotic areas with limited sporulation, 5-6 = chlorotic and necrotic areas with moderate sporulation, 7 = abundant sporulation with moderate chlorosis, 8-9 = abundant and dense sporulation without notable chlorosis and necrosis. Infection types 0-6 were considered resistant, while types 7-9 were considered susceptible. Error bars represent the standard error calculated from three independent experiments with four replicates each.

4.3.2. Macroscopic components of resistance to *P. triticina* infection under climate change conditions

Selected accessions were macroscopically evaluated to characterise components of resistance to leaf rust infection under diverse weather conditions. Thus, we evaluated diverse components of *P. triticina* infection through image analysis, such as IF, Mean Pustule Size, Total Pustule Area, Total Disease Area and Pustule Development Rate (Table 4.1 and Figure 4.2).

The susceptible accession 'Qualidou' showed the greatest differences in macroscopic parameters amongst weather conditions. This accession showed the lowest IF value for the S1G (18.98 pustules/cm^2) set, compared to the SB set (76.27 pustules/cm^2). In contrast, it developed the highest IF value for the S1 set (102.29 pustules/cm^2). For IF parameter, all data were statistically different amongst them. For the Mean Pustule Size parameter, this accession developed significantly lower values for all sets considering far future weather conditions than SB set (1.36 x 10^{-3} cm^2). It was observed closer values amongst S1, S1G and S2 sets (around 0.97 x 10^{-3} cm^2) except for the set S2G (0.71 x 10^{-3} cm^2) which was statistically different. Referred to the Total Pustule Area parameter, accession 'Qualidou' expressed similar values for SB and S1 sets (10.28% and 10.12%, respectively), which were relevantly higher than values in S2 set (8.63%) and, especially, in S1G and S2G sets (1.88% and 2.09%, respectively). In addition, chlorotic and necrotic areas were identified through image analysis, and together with pustule area, were recorded as Total Disease Area (Table 4.1). Therefore, the highest values in 'Qualidou' accession were expressed in sets S1 and S2 (28.26% and 27.31% values, respectively), being significantly lower in the SB set (20.54%), followed by S2G (12.35%) and S1G (7.83%) sets. Lastly, it was determined which fraction of the disease area was considered as pustule area, being this parameter named as Pustule Development Rate. This value was significantly higher for the SB set (49.77%) in comparison to the other sets. The 'Qualidou' accession developed similar values for S1 and S2 sets (35.80% and 31.51%, respectively), whereas S1G and S2G sets showed even more reduced values (23.47% and 17.12%, respectively), both statistically different between them and with the other sets.

'BL 28' accession scored reduced values for all macroscopic parameters studied (Table 4.1). The IF parameter showed the highest value for the S1 set (3.44 pustules/cm^2), while the lowest one for the S2G set (0.18 pustules/cm^2). Regarding Mean Pustule Size parameter, we found very similar values amongst sets, ranging from 0.25 x 10^{-3} cm^2 to 0.31 x 10^{-3} cm^2. Similarly to IF parameter, Total Pustule Area parameter presented from the highest value for S1 set (0.10%) to the lowest one in S2G set (0.00%). Interestingly, the Total Disease Area values were relevantly higher for all sets in 'BL 28' accession compared with Total Pustule Area values, in concordance with its supposed moderately resistant behaviour. S1G and S2G sets showed slightly lower values, statistically different between them and with the other sets. Finally, accession 'BL 28' showed reduced Pustule Development Rate values statistically non-significant.

Leaves from accession 'BL 38' did not develop pustules in none of the sets of this study (Table 4.1). Due to this absence of pustules, IF, Mean Pustule Size, Total Pustule Area and Pustule Development Rate parameters were not collected (accounted with dash). Thus, only the Total Disease Area parameter was measured in this accession. The highest values were displayed for

Table 4.1. Macroscopic image analysis of *P. triticina* infection in three selected durum wheat accessions under baseline and climate change environments [1].

Accession	Environmental Set	IF (Pustules/cm^2)	Mean Pustule Size (cm^2 x 10^{-3})	Total Pustule Area (%)	Total Disease Area (%)	Pustule Development Rate (%)
Qualidou	SB	76.27 (8.67 ± 0.29) c	1.36 ± 0.038 a	10.28 (3.18 ± 0.10) a	20.54 (4.52 ± 0.10) b	49.77 ± 2.08 a
	S1	102.29 (10.09 ± 0.19) a	0.99 ± 0.028 b	10.12 (3.17 ± 0.07) a	28.26 (5.31 ± 0.06) a	35. 80 ± 1.35 b
	S2	89.51 (9.42 ± 0.24) b	0.96 ± 0.026 b	8.63 (2.92 ± 0.08) b	27.31 (5.21 ± 0.10) a	31.51 ± 1.18 b
	S1G	18.98 (4.30 ± 0.19) e	0.97 ± 0.050 b	1.88 (1.34 ± 0.08) c	7.83 (2.77 ± 0.10) d	23.47 ± 1.69 c
	S2G	28.44 (5.28 ± 0.20) d	0.71 ± 0.041 c	2.09 (1.41 ± 0.08) c	12.35 (3.49 ± 0.10) c	17.12 ± 1.70 d
BL 28	SB	1.24 ± 0.28 a	0.25 ± 0.019 a	0.03 ± 0.01 a	6.49 (0.78 ± 0.05) a	0.48 ± 0.10 a
	S1	3.44 ± 1.00 a	0.28 ± 0.019 a	0.10 ± 0.03 a	6.79 (0.80 ± 0.04) a	1.29 ± 0.39 a
	S2	1.00 ± 0.37 ab	0.26 ± 0.015 a	0.03 ± 0.01 ab	5.56 (0.71 ± 0.05) a	0.37 ± 0.14 a
	S1G	0.39 ± 0.13 ab	0.31 ± 0.032 a	0.01 ± 0.00 ab	1.04 (-0.05 ± 0.07) c	1.06 ± 0.38 a
	S2G	0.18 ± 0.08 b	0.25 ± 0.025 a	0.00 ± 0.00 b	2.05 (0.28 ± 0.04) b	0.21 ± 0.09 a
BL 38	SB	-	-	-	1.44 (1.18 ± 0.05) b	-
	S1	-	-	-	2.10 (1.43 ± 0.04) a	-
	S2	-	-	-	2.00 (1.40 ± 0.04) a	-
	S1G	-	-	-	0.47 (0.68 ± 0.03) d	-
	S2G	-	-	-	0.78 (0.87 ± 0.04) c	-

[1] Values are mean ± standard error for five leaves evaluated for each accession and environmental set in three different experiments. Transformed data ± standard error are shown in parenthesis. Data with the same letter within an accession and column are not statistically different (LSD and Kruskal-Wallis tests, $p < 0.05$). Dash (-) means no data were measured since there was no pustule development.

S1 and S2 sets (2.10% and 2.00%, respectively), while the lowest ones under S2G and S1G sets (0.78% and 0.47%, respectively).

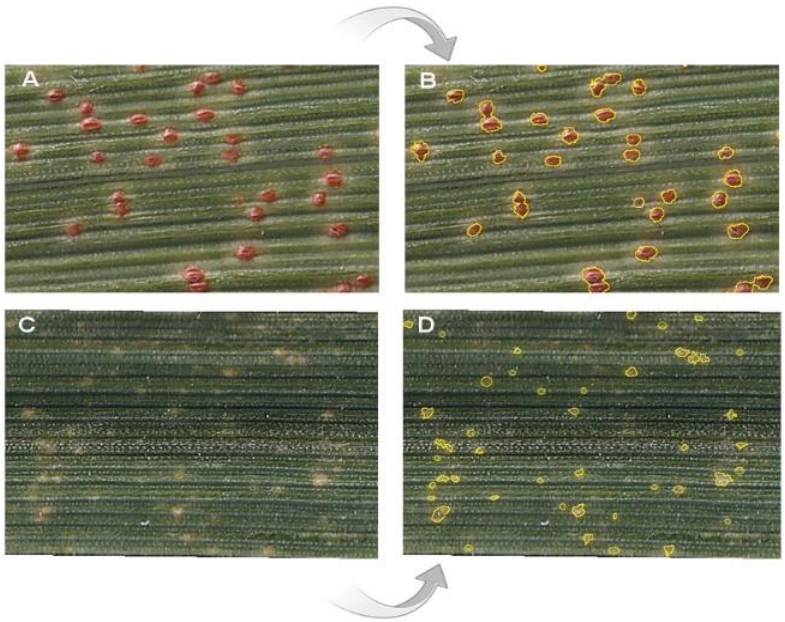

Figure 4.2. Output example of *P. triticina* image analysis developed on selected durum wheat accessions. (**A**) Original leaf of accession 'Qualidou'; (**B**) Analysed leaf of accession 'Qualidou'; (**C**) Original leaf of accession 'BL 38'; (**D**) Analysed leaf of accession 'BL 38'. Pustules and chlorotic and necrotic lesions caused by *P. triticina* were selected in yellow colour.

In addition, we also evaluated the latency period (LP50, Table 4.2). The susceptible accession 'Qualidou' showed a LP50 value of 172.65 h under environment B (SB set), while plants belonging to S1 and S2 sets expressed shortened LP50 values, with 171.66 and 167.83 h, respectively. However, S1G and S2G plants, which were inoculated and incubated in greenhouses, showed significantly longer LP50 values than the other three sets (185.67 and 186.21 h, respectively). Unfortunately, accession 'BL 28' developed such a scarce quantity of pustules with small size in all weather conditions, that an acceptable measurement of LP50 data was not feasible. In this sense, *P. triticina* did not develop pustules either in 'BL 38' accession, making data collection for LP50 values impossible to carry out, similarly to some parameters of Table 4.1.

Table 4.2. Latency Period (LP50) in durum wheat accession 'Qualidou' under baseline and climate change environments [1].

Accession	Environmental Set	LP50 (h)
Qualidou	SB	172.65 ± 1.06 b
	S1	171.66 ± 1.39 bc
	S2	167.83 ± 0.75 c
	S1G	185.67 ± 3.28 a
	S2G	186.21 ± 1.61 a

[1] Values are mean ± standard error for four leaves evaluated for each environmental set in three different experiments. Data with the same letter within a column are not statistically different (Dunnett T3 test, $p < 0.05$).

4.3.3. Microscopic components of resistance to *P. triticina* infection under climate change conditions

Different stages of fungal development were identified during microscopic evaluation of the *P. triticina* infection (EA–; EA+; EST–; EST+) (Figure 4.3) and then analysed as percentages (Figure 4.4 and Supplementary Table S4.2). The Susceptible accession 'Qualidou' expressed the greatest differences amongst weather conditions (Figure 4.4A). Thus, it could be observed that established colonies were the main fungal stage, and that presence (+) or absence (–) of necrotic cells in these colonies varied regarding sets. The percentage of EST– observed under environment B (SB, 66.38%) was significantly higher than those recorded for sets under far future weather conditions. In contrast, this accession showed the lowest EST+ percentage (15.36%) for the SB set, being this value statistically different compared with the higher ones exhibited under far future weather conditions. Despite 'Qualidou' accession expressed a susceptible response against leaf rust, it also developed early-aborted colonies with (EA+) and without (EA–) the presence of necrosis, both non-significantly different amongst sets.

'BL 28' accession also showed non-significant differences amongst fungal stages EA+ and EA– (Figure 4.4B), although the presence of necrotic cells led to higher percentages of EA+ fungal stage for all sets. Fungal stage EST– presented the lowest values of the 'BL 28' accession, with an average score of 3.07% amongst sets. Lastly, EST+ (established colonies with necrosis) was the most abundant stage, showing the greatest differences between sets too. Thus, the highest EST+ percentage occurred for the SB set (67.20%), being the lowest percentage observed for S1G (46.66%).

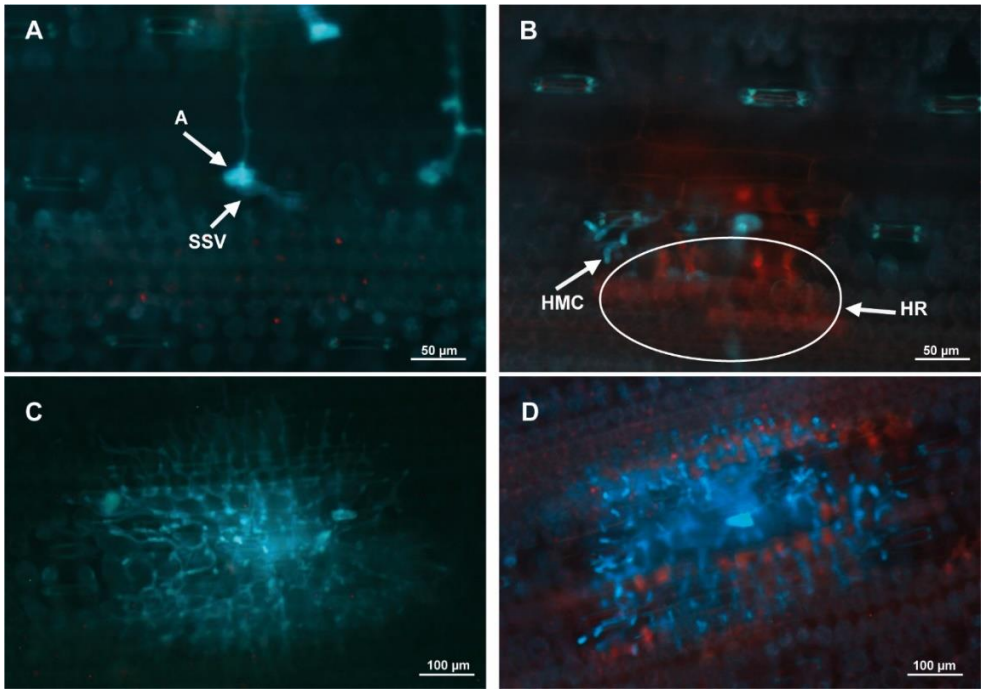

Figure 4.3. Microscopically observed fungal stages of *P. triticina* and plant cellular responses were classified as: (**A**) early-aborted colony without necrosis (EA–); (**B**) early-aborted colony associated with necrosis (EA+); (**C**) established colony without necrosis (EST–); (**D**) established colony associated with necrosis (EST+). A, appressorium; SSV, substomatal vesicle; HMC, haustorial mother cell; HR, hypersensitive response.

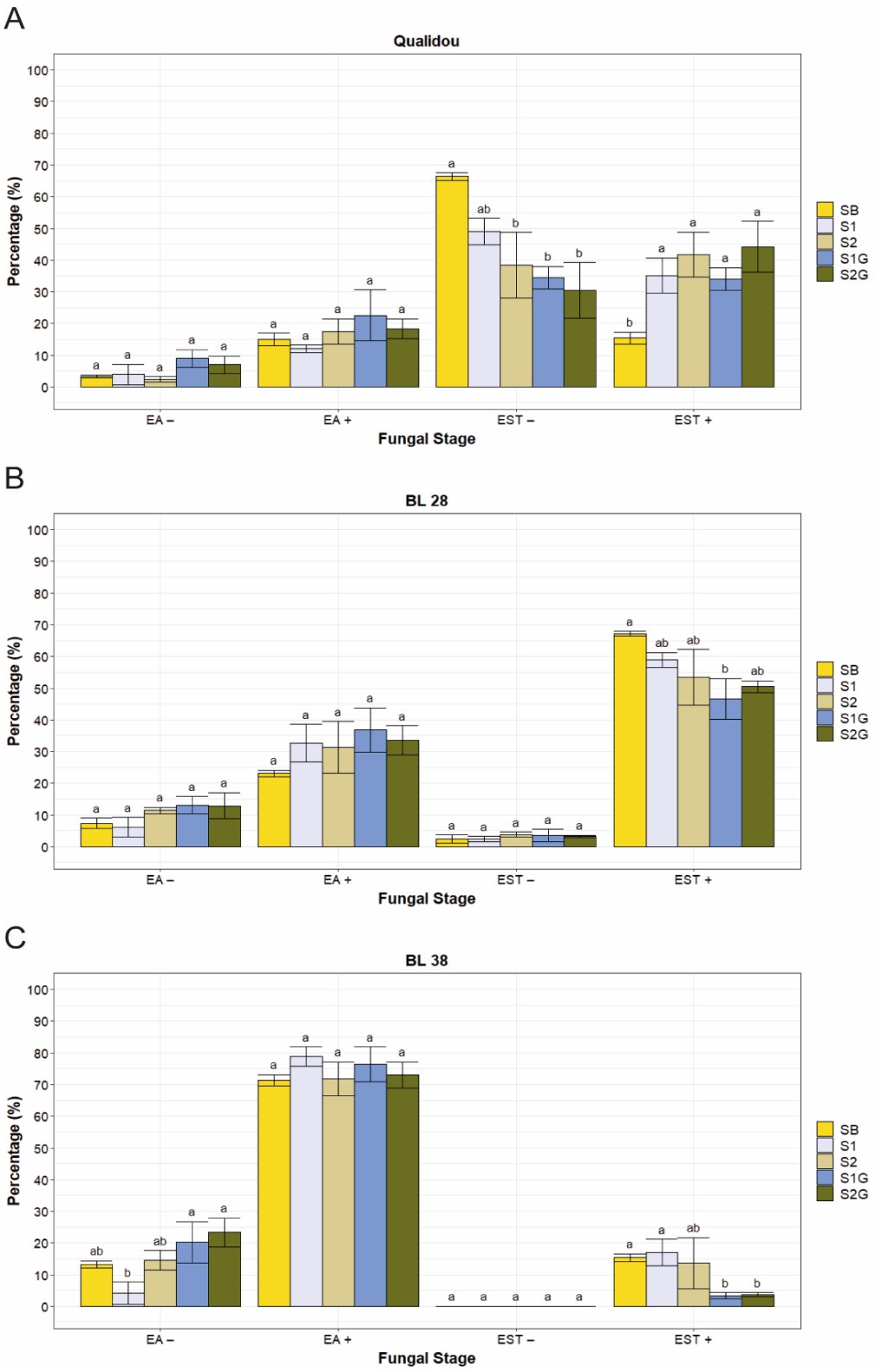

Figure 4.4. Microscopic fungal stages of *P. triticina* presented as mean percentages in three selected durum wheat accessions (**A**) 'Qualidou', (**B**) 'BL 28' and (**C**) 'BL 38' under baseline (SB) and climate change environments (S1, S2, S1G and S2G). Error bars represent the standard error calculated from three independent experiments. Data with the same letter within a fungal stage and accession are not significantly different (Duncan test, $p < 0.05$). EA–, early-aborted colonies without necrosis; EA+, early-aborted colonies associated with necrosis; EST–, established colonies without necrosis; EST+, established colonies associated with necrosis.

The analysis of the resistant accession 'BL 38' (Figure 4.4C) revealed the EA+ fungal stage as the most prominent one. However, non-statistically significant values amongst sets were shown. Oppositely, EST– fungal stage was absent. Significant differences amongst sets were just observed in the fungal stages EA– and EST+. 'BL 38' accession scored 4.20% in the S1 set for the EA– fungal stage, a value statistically different from values in S1G and S2G sets. Finally, the significantly lowest percentages for S1G and S2G sets were presented for the fungal stage EST+.

The colony size (CS) of established colonies (EST– and EST+) were also measured to study the effect of far future weather conditions in the microscopic colony development of leaf rust (Table 4.3). The susceptible accession 'Qualidou' developed a compatible reaction to *P. triticina* and exhibited diverse CS values amongst sets. In fact, plants exposed to environment B showed the highest CS value (SB set, 0.411 mm^2), which was statistically different from CS values of the other sets. In addition, sets S1 and S2 showed statistically higher values than the ones in S1G and S2G sets. In contrast, 'BL 28' and 'BL 38' accessions showed moderately and highly resistant reactions to *P. triticina*, respectively, and did not generally develop statistically different values in CS amongst sets (Table 4.3).

Table 4.3. Colony Size (CS) of *P. triticina* established colonies (EST– and EST+) in three durum wheat selected accessions under baseline and climate change environments [1].

Accession	Environmental Set	Colony Size (mm^2)
Qualidou	SB	0.411 ± 0.012 a
	S1	0.351 ± 0.014 b
	S2	0.328 ± 0.018 b
	S1G	0.243 ± 0.022 c
	S2G	0.241 ± 0.018 c
BL 28	SB	0.191 ± 0.010 a
	S1	0.174 ± 0.005 ab
	S2	0.167 ± 0.023 ab
	S1G	0.151 ± 0.009 ab
	S2G	0.145 ± 0.009 b
BL 38	SB	0.112 ± 0.001 a
	S1	0.127 ± 0.001 a
	S2	0.122 ± 0.011 a
	S1G	0.113 ± 0.008 a
	S2G	0.112 ± 0.002 a

[1] Values are mean ± standard error for four leaves evaluated for each accession and environmental set in three different experiments. Data with the same letter within an accession and column are not statistically different (Duncan test, $p < 0.05$).

4.4. Discussion

Durum wheat cultivation is currently threatened by abiotic and biotic stresses. In fact, the increased risk of wheat leaf rust, coupled with the effects of increased temperature and [CO_2] due to climate change, would lead to an uncertain scenario for durum wheat cultivation in the short and long term, especially in hotspots of climate change such as Mediterranean countries (Velásquez *et al*., 2018; Pérez-Méndez *et al*., 2022; Lorite *et al*., 2023). Although recent studies have evaluated the effects of elevated temperatures (Kolmer, 1996; Singh and Huerta-Espino, 2003) and elevated [CO_2] (von Tiedemann and Firsching, 2000; Bencze *et al*., 2013) in wheat-leaf rust interaction, not many have conducted diurnal fluctuating temperature cycles or even the combination of two abiotic factors. Considering this lack of research in plant-pathogen interactions (Atkinson *et al*., 2015; Velásquez *et al*., 2018), the present study is, to the best of our knowledge, the first to include the effects of the expected diurnal variation of maximum temperature and continuous elevated [CO_2] in the durum wheat-leaf rust pathosystem.

4.4.1. Disease development at elevated temperature (set S1)

Temperature is one of the most important factors influencing the life cycle of pathogens and their interactions with plants (Cheng *et al*., 2013; Pandey *et al*., 2017). Our environment 1 was used to test the individual effect of increased maximum temperature in durum wheat-leaf rust interaction. To ensure the infection, plants of this assay were inoculated and incubated during 24 h under baseline weather conditions (plant set S1). In susceptible accession 'Qualidou', S1 plants expressed elevated IF values compared to plants which were grown, inoculated and incubated in environment B (SB plants). This suggests that elevated temperatures during plant growth resulted in physiological changes that favoured leaf rust penetration success and subsequent infection sites formation in 'Qualidou' accession. Thus, higher temperatures increase the evapotranspiration rate (Swelam, *et al*., 2011) and then, if the water status of the plant is correct, the stomatal aperture is induced, which favours fungal invasion of the host, as stomata are the main gateway for leaf rust to infect wheat (Bolton *et al*., 2008). This increased penetration success could be the reason for the slightly higher macroscopic values obtained in plants of 'BL 28' and 'BL 38' accessions at elevated temperatures.

Once established, fungal development was accelerated in 'Qualidou' accession according to its shortened LP50 value compared to SB plants, a common effect of elevated temperatures in this pathogen (Wójtowicz *et al*., 2017). This accelerated life cycle would increase the risk of higher sporulation, and subsequently the number of disease cycles, favouring the adaptation and appearance of new pathotypes (Chakraborty *et al*., 2011; Chakraborty, 2013; Velásquez *et al*., 2018). However, Mean Pustule Size value in 'Qualidou' accession was relevantly lower in S1 than in SB plants, confirmed microscopically by the lower CS value of established colonies and the elevated proportion of EST+ observed at 5 dpi. This resulted in increased chlorotic and necrotic areas (Total Disease Area value), which reduced photosynthesis and assimilates, leading leaf senescence and, subsequently, reducing yield potential in accession 'Qualidou'. Therefore, considering the lower Pustule Development Rate value in comparison with SB plants, we might suggest this higher Total Disease Area value was due to host responses which restricted fungal development. Thus, considering also that temperature had little effect on pustule size (Singh

and Huerta-Espino, 2003), the higher proportion of established colonies surrounded by necrotic cells (EST+) indicated the occurrence of some kind of host responses that restricted fungal development under elevated temperatures. Conversely, 'BL 28' (moderately resistant) and 'BL 38' (resistant) accessions did not show relevant differences for microscopic parameters compared to SB plants.

In this sense, prolonged exposure to abiotic stresses could lead to a priming or weakening of basal defence in plants prior to pathogen infection (Bostock *et al*., 2014; Ramegowda and Senthil-Kumar, 2015; Pandey *et al*., 2017). In fact, some durum wheat cultivars could prevent or minimise the detrimental effects of higher temperatures through basal or acquired (after acclimation) thermotolerance (Rampino *et al*., 2009), changing their transcriptome, proteome, metabolome or lipidome, and taking advantage against pathogens. Thus, prolonged elevated temperatures could reduce photosynthesis, generate ROS, and trigger programmed cell death (Chaudhry and Sidhu, 2022). However, our observations detected this programmed cell death surrounding leaf rust colonies, indicating that supposed ROS generation could act as a signalling molecule to mediate temperature stress responses (Chaudhry and Sidhu, 2022), such as induction of pathogen-associated defence genes (Mikkelsen *et al*., 2015; Petrov *et al*., 2015). Lastly, phytohormone crosstalk affected by elevated temperatures (Atkinson *et al*., 2015; Abhinandan *et al*., 2018;), together with the generation of ROS, could be a possible reason for elevated temperature-mediated cross-tolerance for subsequent leaf rust infections in 'Qualidou' response (Ben Rejeb *et al*., 2014).

4.4.2. Disease development at elevated temperature and [CO_2] (set S2)

One of the most studied abiotic factors in plant diseases is elevated [CO_2], but its effects vary from increasing (Bencze *et al*., 2013; Melloy *et al*., 2014; Váry *et al*., 2015) to decreasing (Hibberd *et al*., 1996; von Tiedemann and Firsching, 2000; Matić *et al*., 2018;) the incidence of different wheat diseases. In addition, few studies consider the effects of both elevated [CO_2] and temperature together (Melloy *et al*., 2014; Mikkelsen *et al*., 2015; Matić *et al*., 2018; Hay *et al*., 2021), which would most closely resemble the effects of expected field climatic conditions in wheat-leaf rust interactions (Velásquez *et al*., 2018). For that reason, plants of selected accessions were exposed to both elevated temperature and [CO_2] (environment 2, 620-650 ppm) to assess their effects in the durum wheat-leaf rust interactions. To ensure infection, plants of this assay were inoculated and incubated during 24 h under baseline weather conditions (plant set S2).

Exposure of plants to elevated [CO_2] for prolonged periods (days to weeks) reduces stomatal conductance and evapotranspiration, thereby increasing canopy temperature (Kimball and Bernacchi, 2006). This situation forced S2 plants to develop a unique response during a subsequent leaf rust infection, which may reduce fungal penetration success through a partial stomatal closure (Manning and von Tiedemann, 1995; Eastburn *et al*., 2011), confirmed by reduced IF values in 'Qualidou' and 'BL 28' accessions in comparison with S1 plants. Despite this reduction, the IF parameter remained higher in the 'Qualidou' accession (susceptible) compared to SB plants (Bencze *et al*., 2013). In addition, elevated [CO_2] stimulates photosynthesis, especially in C_3 plants as wheat (Chaudhry and Sidhu, 2022), increasing the production of sugar,

starch and other carbohydrates (Eastburn *et al*., 2011; Loladze, 2014). On one hand, this additional carbohydrate accumulation in the leaf tissue could facilitate nutrient acquisition by the fungus (Manning and von Tiedemann, 1995; Eastburn *et al*., 2011; Velásquez *et al*., 2018), as it was observed in the 'Qualidou' accession with a reduction of the LP50 value under environment 2, in agreement with faster growth of other biotrophic fungus under elevated [CO_2] conditions (Hibberd *et al*., 1996). On the other hand, this carbohydrate production might act as an elicitor of defence responses such as enhancement of ROS network and phytohormonal control (Eastburn *et al*., 2011; Matić *et al*., 2018), as it was detected in 'Qualidou' plants lower values of CS and EST–, coupled with higher values of EST+, and reduced values of Mean Pustule Size, Total Pustule Area, and Pustule Development Rate compared to SB plants.

Therefore, considering both elevated abiotic factors (temperature and [CO_2]) induce changes in primary metabolism of plants through photosynthetic efficiency (Hay *et al*., 2021), and elevated [CO_2] may not increase wheat rusts incidence (von Tiedemann and Firsching, 2000; Chakraborty *et al*., 2011), our results suggest that temperature was the main abiotic factor modulating the response of selected accessions against leaf rust infection. This may be due to plants prioritising their response to the most hampering abiotic stress (Atkinson *et al*., 2015). Thus, it is feasible that elevated temperatures and [CO_2] (environment 2), enhance those host physiological and molecular defence responses that are only expressed under elevated temperatures in the environment 1 (Eastburn *et al*., 2011; Cheng *et al*., 2013; Matić *et al*., 2018). In fact, the susceptible accession 'Qualidou' was the only one presenting some parameters with statistical differences in S2 plants compared to S1 plants. A lower Total Pustule Area value was shown, probably due to a lower IF value and, more likely, due to the aforementioned enhanced host defence responses that limited fungal development and subsequent pustule formation, and sporulation potential (Chakraborty *et al*., 2011; Chakraborty, 2013; Velásquez *et al*., 2018). Interestingly, this lower value did not significantly reduce the Total Disease Area, supporting the fact that S2 'Qualidou' plants expressed some enhanced defence responses against leaf rust disease, although this would ultimately reduce host photosynthetic area and yield (Chai *et al*., 2020). Lastly, considering that the effects of elevated [CO_2] on disease incidence could be related to the lifestyle of the pathogens (biotroph vs necrotrophy) (Velásquez *et al*., 2018), our results are in agreement with other studies developed on the biotrophic fungus powdery mildew (Komáromi *et al*., 2013; Matić *et al*., 2018).

4.4.3. Inoculation, incubation and disease development at elevated temperature and [CO_2] (sets S1G and S2G)

Leaf rust infection generally starts at night to ensure the correct disease establishment (Singh *et al*., 2002). However, the even more frequent occurrence of heat waves coupled with changes in rainfall patterns caused by climate change (Diffenbaugh and Giorgi, 2012; Trnka *et al*., 2014) would affect this crucial process to some extent, particularly in Mediterranean growing areas. For this reason, plants in sets S1G and S2G were grown, inoculated and incubated in environments 1 and 2, respectively, to assess the effect of abiotic factors on leaf rust disease establishment.

Interestingly, the three selected accessions showed a significant reduction in leaf rust symptoms in S1G and S2G plants compared to SB plants, especially for the macroscopic parameters IF, Total Pustule Area, Total Disease Area, and Pustule Development Rate. In fact, based on empirical studies (Wiese, 1979; Kramer and Eversmeyer, 1992; de Vallavieille-Pope *et al*., 1995), the increased temperature reached during the inoculation process could affect the germination and penetration success of leaf rust spores, thus reducing disease symptoms. Therefore, it can be assumed that increments of maximum temperatures (30°C) during the inoculation process could reduce spore germination by up to 50% (Kramer and Eversmeyer, 1992). This reduction could lead a delay in the disease establishment, explaining the relevantly longer LP50 values in S1G and S2G plants of the 'Qualidou' accession, as opposed to those in S1 and S2 plants. In addition, no significant differences of EA– and EA+ values were observed in comparison with SB plants in any accession, indicating a reduction of infection sites in S1G and S2G plants prior to the establishment of host-pathogen infection. Furthermore, considering that elevated temperatures (30-35°C) did not negatively affect the fungus once it had entered the host (Singh *et al*., 2002), our results suggest that the inoculation process for S1G and S2G plants could also slightly weaken the subsequent disease progression. This could be observed in the reduced proportion of EST– in the 'Qualidou' accession and EST+ in 'BL 28' and 'BL 38' accessions, together with lower CS values in the three selected accessions, all values compared to SB plants. However, these data were statistically relevant mainly in 'Qualidou' accession and only for EST+ in 'BL 38' accession.

Finally, S2G plants showed relevant differences in some macroscopic parameters compared to S1G plants, especially higher values of Total Disease Area for the three accessions, affecting more host photosynthetic area and reducing yield (Chai *et al*., 2020). However, non-statistically significant differences in Total Pustule Area in 'Qualidou' (susceptible) and 'BL 28' (moderately resistant) accessions were shown in S1G and S2G plants, suggesting that elevated [CO_2] enhanced even more the host responses against leaf rust infection in S2G plants. This is particularly evident in the 'Qualidou' accession, which also showed relevantly lower Mean Pustule Size and Pustule Development Rate values in comparison to S1G.

In conclusion, the most important fact in our study is that elevated maximum temperatures alone or in combination with elevated [CO_2] did not suppress the general defence response in our studied accessions 'BL 28' (moderately resistant) and 'BL 38' (resistant), nor did it cause the loss of susceptibility in 'Qualidou' plants during *P. triticina* infection. This suggests that the genetic resistance background of these accessions was not temperature-sensitive (Kaul and Shaner, 1989; Statler and Christianson, 1993; Chakraborty *et al*., 2011;) or the timing and intensity of abiotic stresses were not sufficient to affect it (Kane *et al*., 2013; Suzuki *et al*., 2014; Velásquez *et al*., 2018). Therefore, variations in macro and microscopic components of resistance in plants exposed to environments 1 and 2 were due to abiotic factors affecting durum wheat-leaf rust interactions mainly through modifications in the host and/or pathogen biology and physiology (Chakraborty *et al*., 2011). In contrast, leaf rust disease was greatly reduced when plants were inoculated and incubated under environments 1 and 2 (S1G and S2G plants), mimicking possible future heat events and disturbed rainfall patterns, suggesting that climate change would affect key stages of *P. triticina* and thus subsequent disease incidence in Mediterranean regions.

4.5. References

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Chapter 5. Macro- and microscopic characterisation of components of resistance against *Puccinia striiformis* f. sp. *tritici* in a collection of Spanish bread wheat cultivars

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Abstract

Yellow (stripe) rust, caused by the biotrophic fungus *Puccinia striiformis* f. sp. *tritici*, stands as the most serious wheat disease worldwide, affecting approximately 88% of world wheat production. Even though yellow rust generally develops in cool humid weather conditions, the expansion of new races adapted to warmer climates threatens zones where severe *P. striiformis* epidemics were infrequent, such as Andalusian wheat cropping areas. In order to characterise yellow rust resistance mechanisms in Spanish germplasm, our study evaluated 19 Spanish bread wheat cultivars against *P. striiformis* under controlled conditions for percentage of disease severity (DS) and infection type (IT). From this visual evaluation, 74% of evaluated cultivars showed resistant responses against *P. striiformis* infection with only five cultivars considered susceptible. Subsequently, macroscopic and microscopic components of resistance were identified through image analysis and histological studies, respectively, in six selected cultivars. Macroscopic parameters such as total pustule area and total affected area (%), together with microscopic parameters such as early-aborted and established microcolonies regarding plant cell death responses (%), and microcolony length (μm), were identified as capable of differentiating cultivars quantitatively. Thus, these parameters could be used as a basis for screening resistant responses in future breeding programs, complementary to physiology, genetic and biochemical studies of plant-*P. striiformis* interaction. Finally, our study seems to be the first macroscopic and microscopic characterisation of *P. striiformis* infection in a collection of Spanish bread wheat cultivars in controlled conditions.

Key words: yellow (stripe) rust; plant breeding; plant resistance; foliar disease; *Triticum aestivum*

5.1. Introduction

Wheat is considered the most widespread crop in the world, accounting for 760 million tons of production on 219 million hectares globally in 2020 and being of great importance in human diets (FAOSTAT, 2022). This crop cultivation is divided into bread (common) wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. subsp. *durum*), which are both expected to increase in consumption by up to 60% by 2050 due to human population growth (The Wheat Initiative, 2022). However, this requirement for wheat production could be hard to achieve due to diverse abiotic and biotic stresses, especially fungal diseases, which cause more than 21% of wheat yield losses on average (Savary *et al*., 2019). In particular, cereal rusts are considered among the most damaging diseases, causing important yield losses globally (Ellis *et al*., 2014). One of them, the wheat yellow (stripe) rust, caused by the biotrophic fungus *Puccinia striiformis* Westend f. sp. *tritici*, stands as the most serious wheat disease worldwide (Chen, 2020). It was present in more than 60 countries in 2000 to 2009 with diverse cropping systems, growing seasons and germplasm characteristics (Wellings, 2011). This disease has undergone important global expansion in the last 50 years, and it is believed to affect approximately 88% of the world's wheat production, causing losses of 5.5 million tons per year (Beddow *et al*., 2015).

P. striiformis is a biotrophic, macrocyclic and heteroecious fungus that depends on a living primary host (wheat or grasses) and an alternate host (*Berberis* or *Mahonia* spp.) for growth and reproduction (Chen *et al*., 2014). The infection process on cereals begins via an urediniospore germ tube, which penetrates the stoma without differentiating an appressorium (unlike other rust fungi), and then differentiates into a substomatal vesicle (SSV) within the stomatal cavity (Moldenhauer *et al*., 2006). This vesicle forms three primary infection hyphae that induce the development of haustorial mother cells (HMC) at the point of contact with host mesophyll or epidermal cells, then penetrating the host cell walls and forming haustoria by invaginating the plasma membrane (Hovmøller *et al*., 2011). The secondary infection occurs via development of intercellular runner hyphae (RHy) throughout the leaf, thus developing an extensive hyphal network, producing further haustoria (Bozkurt *et al*., 2010). Finally, at the same time as the secondary infection develops, a pustule bed is established, from which an uredinium develops. The plant responses commonly appear from six to eight days after infection in the form of chlorosis, whereas sporulation (characteristic yellow-orange uredinia appearing in long, narrow stripes on leaves) starts approximately from 12 to 14 days under favourable conditions (Chen *et al*., 2014), leading to desiccation of leaves.

The most favourable conditions for large-scale yellow rust epidemics are temperate regions with cool humid climates (Chen, 2020); therefore, the disease is considered a low-temperature disease. Thus, *P. striiformis* urediniospores germinate rapidly in optimum conditions of dew surfaces and a temperature range between 7 and 12°C, with ideal disease development conditions (from infection to sporulation) between 12-15°C, which taken together, are temperature ranges approximately 10°C lower on average than those for other wheat rusts (Singh *et al*., 2002). Despite these optimum conditions for yellow rust development, in the past two decades new races adapted to warmer climates have emerged, spreading into new territories and producing epidemics in warmer areas where the disease was previously infrequent or absent (Chen *et al*., 2014). This situation, together with its long-distance dissemination capability, its high rates of mutation from avirulence to virulence, and the

existence of recombinant and highly diverse populations (Ali *et al*., 2017), is leading to a control cost for the disease of more than 1 billion USD annually (Chen, 2020). For that reason, the control of this disease through resistance breeding strategies stands as an efficient, economical and environmentally friendly method to prevent epidemics in the short and long-term future.

The genetic control of wheat yellow rust was achieved thanks to the steady work of plant breeders and pathologists on the identification of yellow rust (*Yr*) resistance (*R*) genes over the last 100 years (GrainGenes, 2022). In general, *R* genes in plants encode nucleotide-binding site leucine-rich repeat proteins that recognise specific pathogen effectors (avirulence proteins) (Jamil *et al*., 2020). Usually, the *P. striiformis*-wheat interaction follows a classical gene-for-gene model. Recognition between *R* genes and avirulence (*Avr*) genes may lead to effector-triggered immunity, resulting in a resistant phenotype (incompatible interaction). Additionally, when effectors do not recognise *Avr* genes, a susceptible (compatible interaction) phenotype may evolve (Jones and Dangl, 2006). Two major types of resistance, all-stage resistance (ASR) and adult-plant resistance (APR), have been characterised and used in breeding programs for resistant wheat cultivars against *P. striiformis*. ASR is effective through all growth stages, being characterised by a strong to moderate immune response that restricts pathogen development, sporulation and infection. This kind of resistance is qualitatively inherited and easily incorporated into adapted cultivars. However, since it counters only one or a few *P. striiformis* races, new virulent races rapidly overcome it and varieties become susceptible after a few years (Schwessinger, 2017; Wang and Chen, 2017; Jamil *et al*., 2020). On the other hand, APR is non-race specific, working against more than one race of the pathogen, a durable type of resistance due to its polygenic nature (Saleem *et al*., 2015). Specifically, APR genes are described as slow-rusting genes, leading to delayed infection and spore germination (Guo *et al*., 2008). Currently, the most powerful strategy to combat wheat yellow rust is the combination of classical qualitative *R* genes and nonclassical quantitative *R* genes.

The *P. striiformis* clonal population present in Europe has largely been replaced since 2011 by diverse new lineages known as Warrior, Kranich and Warrior(–), causing increased epidemics on multiple wheat varieties according to various studies (Hubbard *et al*., 2015; Hovmøller *et al*., 2016; Ali *et al*., 2017; Bueno-Sancho *et al*., 2017). Currently, according to the Global Rust Reference Center (GRRC, 2022), PstS10 is the most prevalent genetic group on bread wheat, dominated by a single race, but the continuous appearance of new races by 2020 resulted in quantitative shifts in rust susceptibility in some local wheat varieties, overcoming long-standing resistance genes. In Europe, Spain stands among the main bread wheat cultivation producers, with 7.3 million tons of yield in 1.6 million hectares (EUROSTAT, 2022). Thus, yellow rust disease, along with its new races, presents a serious threat to Spanish bread wheat cropping areas. At first, yellow rust epidemics generally occurred locally or regionally, and only occasionally became severe (GENVCE, 2022), possibly due to the use of varieties with genes resistant to the *P. striiformis* races present in that moment, together with the absence of optimum conditions for yellow rust development. However, the appearance of new races adapted to warmer climates and with higher aggressiveness (Milus *et al*., 2009) is leading a new perspective on managing this disease in Spain. In fact, yellow rust was first found in 2008 and 2009 in Northern Spain (Martínez-Moreno and Solís, 2019), but quickly spread to the Southern and Eastern regions, where severe epidemics of *P. striiformis* occurred in areas of Castilla y Leon, Aragon and

Andalusia by 2013 and 2014 (Almacellas and Álvaro, 2015). In 2018, a new yellow rust race named PstS14, which is virulent across various resistance genes, was detected in Spain (Martínez-Moreno and Solís, 2019). To date, the GRRC has catalogued the Spanish isolates in the Warrior(–) (with genetic group PstS10) and PstS14 races.

In this context of possible yellow rust epidemic threats in Spanish wheat cropping areas, it appears necessary to conduct a detailed study of the infection process of *P. striiformis* in Spanish bread wheat varieties under controlled conditions, thus obtaining a complete assessment. Hence, different evaluation methods should be considered, both macroscopic and microscopic, to identify components of resistance that might help to find diverse sources of resistance to *P. striiformis* and determine the most effective, economical and environmentally friendly method to control yellow rust (Singh *et al*., 2016). The most common method to evaluate this disease is through visual assessment, a subjective evaluation which generally measures parameters such as disease severity (DS, percentage of infected leaf area) or infection type (IT, index of plant-pathogen interactions determined by the proportion of host response versus fungus sporulation) (Chen, 2020). However, in recent years some studies developed assessments for plant diseases through diverse image analysis systems, leading to important progress (Mutka and Bart, 2015; Bock *et al*., 2020; Porras *et al*., 2021). These image-based analysis systems allowed for the exploration of more dimensions of disease phenotypes, such as quantification of fungal reproduction structures (Stewart and McDonald, 2014), distinction among genotypes with different levels of disease severity (Xie *et al*., 2012), or improved reproducibility and sensitivity in disease quantification (Bock *et al*., 2008). Moreover, the development of diverse software for image analysis permitted a high degree of automation in crop disease assessments (Lamari, 2002; Schindelin *et al*., 2012) and, therefore, the possibility of developing specific applications adapted to different crop diseases (Pethybridge and Nelson, 2015; Gallego-Sánchez *et al*., 2020). Concretely, some studies developed methods for the macroscopic evaluation of *P. striiformis* through image analysis, which led to the identification of resistant responses among different cultivars or even the aggressiveness of diverse *P. striiformis* races, evaluating parameters such as IT, latency period, infection frequency (IF), pustule size, disease area, etc. (Sørensen *et al*., 2014; Hovmøller *et al*., 2017; Sørensen *et al*., 2017b). In addition to this, microscopic evaluation of plant-pathogen interaction is commonly studied through histopathological methods, investigating morphological changes in plants and pathogens at the cell and tissue levels using light, electron or fluorescence microscopy (Zhao *et al*., 2019). These methods provide valuable information regarding the pathogen infection process, such as the tracking of its development through the host tissue, the identification and quantification of fungal infection structures, and the recognition of host defence responses in the form of hypersensitive response (HR) in the host cells or the deposition of lignin and callose compounds (Moldenhauer *et al*., 2006; 2008; Bozkurt *et al*., 2010; Soleiman *et al*., 2014; Sørensen *et al*., 2017a), as well as the screening of resistance genes based on wheat phenotype (Jagger *et al*., 2011).

In summary, taking into consideration that macroscopic and microscopic methods of yellow rust evaluation are valuable tools for finding sources of resistance to *P. striiformis*, together with the incoming threat of new races adapted to warmer climates in Spain, it is appropriate to conduct a specific assessment of yellow rust infection in Spanish bread wheat cultivars. For that

reason, the main objective of this study was to evaluate a group of Spanish bread wheat cultivars against a local isolate of *P. striiformis* under controlled climatic conditions through both macroscopic and microscopic methods, in order to determine the sources and mechanisms of resistance available among the studied accessions.

5.2. Materials and Methods

5.2.1. Plant material

In our study we evaluated 19 bread wheat (*Triticum aestivum* L.) accessions against a local isolate of yellow rust (*Puccinia striiformis* f. sp. *tritici*) collected from Santaella (Córdoba, Spain) in 2020 with a virulence/avirulence spectrum as follows: *Yr1, Yr2, Yr3, Yr6, Yr7, Yr9, Yr17, Yr18, Yr25, Yr27, Yr32, Sp, AvS / Yr4, Yr5, Yr8, Yr10, Yr15, Yr24, Yr26, Yr33, YrAmb*. All accessions studied were commercial Spanish cultivars, registered in the Spanish MAPA (Ministerio de Agricultura, Pesca y Alimentación) catalogue (Supplementary Table S5.1), either recently registered or widely cultivated by Spanish farmers. After preliminary disease screening, six wheat accessions with diverse and representative infection types (ITs) were selected for microscopic and macroscopic assays of components of resistance. These accessions were: 'Rota', 'Galera', 'Artur Nick', 'Ecodesal', 'Califa Sur' and 'Nogal'.

5.2.2. Pathogen isolation

P. striiformis f. sp. *tritici* was isolated from a naturally infected field of Santaella, Córdoba (Spain). Spores from naturally infected leaves were placed onto uninfected plants of susceptible cultivar 'Califa Sur' in order to purify the inoculum. Plants were then misted with distilled water without run-off before being sealed in dark plastic bags to provide 100% relative humidity (RH), and kept for 24 h in a cool chamber at 8°C. After 24 h, plastic bags were removed and plants were transferred to an incubator at 17/13°C day/night, with 70% RH and a 16 h photoperiod, for 15 days. When individual pustules appeared, a single-pustule isolate was obtained by inoculation of new uninfected 'Califa Sur' plants. In order to increase the amount of spores for further inocula, 14-day-old 'Califa Sur' plants were inoculated with spores mixed with talc (1:20 *v/v*) using a manual airbrush spray, and incubated as described above. Spores of yellow rust were collected from infected leaves using a vacuum bomb, placed in a desiccator at 4°C for 4 or 5 days, and then stored at -80°C until inoculation experiments.

5.2.3. Screening of bread wheat germplasm

5.2.3.1. Inoculation assays for evaluation of disease severity (DS) and infection type (IT)

Seeds of 19 bread wheat accessions were sown in 8 x 7 x 7 cm pots containing a mix (1:1, *v/v*) of commercial compost (Suliflor SF1 substrate; Suliflor, Radviliškis, Lithuania) and sand. Pots were placed in trays and incubated in a growth chamber at 21°C with a 14 h photoperiod for germination. After seven days, seedlings were transferred to an incubator at 17/13°C day/night, 70% RH, and 16 h of light to acclimate the plants during five days until the second leaf was

halfway unfolded. Then, six seedlings of each accession were inoculated with yellow rust spores mixed with talc (1:20 wt/wt) using a manual airbrush spray, as described in Section 5.2.2. A total of 114 plants were uniformly inoculated with 20 mg of yellow rust spores. The experiment was performed three times. The same analysis was performed twice with adult plants (two months) to confirm the absence of variation in resistance or susceptibility compared to seedlings (Supplementary Figure S5.1).

5.2.3.2. Disease assessment via DS and IT

The second leaf of each plant was evaluated 15 days post inoculation (dpi) for seedling plants, and the fifth leaf for adult plants. The infection process was quantitatively scored as the percentage of each leaf with disease symptoms (pustules, chlorosis and necrosis), referred to as disease severity (DS). In addition, seedling reactions were qualitatively scored using a disease scoring scale (0-9) for infection type (IT) (McNeal, *et al*., 1971), where 0 = no visible disease symptoms (immune), 1 = minor chlorotic and necrotic flecks, 2 = chlorotic and necrotic flecks without sporulation, 3-4 = chlorotic and necrotic areas with limited sporulation, 5-6 = chlorotic and necrotic areas with moderate sporulation, 7 = abundant sporulation with moderate chlorosis, 8-9 = abundant and dense sporulation without notable chlorosis and necrosis. Infection types 0-6 were considered resistant, while types 7-9 were considered susceptible.

5.2.4. Characterisation of the responses to yellow rust

5.2.4.1. Inoculation assays for evaluation of macroscopic and microscopic components of resistance

Seeds of the six selected bread wheat accessions were sown in 30 x 20 x 7 cm trays containing the mix of commercial compost and sand (1:1, v/v) as in Chapter 4. Trays were incubated at 21°C with a 14 h photoperiod to germinate and grow the plants. After 16 days, seedings were transferred to an incubator at 17/13°C day/night, 70% RH, and 16 h light to acclimate the plants for five days until the third leaf was completely unfolded. Following Sørensen *et al*. (2017b) with minor modifications, the third-leaves of four plants per accession were fixed horizontally (adaxial surface up) on a foam board with metal clips. A total of 24 leaves were fixed per tray for subsequent macro or microscopic evaluations. Each tray was inoculated with 4 mg of yellow rust spores mixed with talc (1:20 wt/wt) using a settling tower to inoculate the leaves uniformly. The inoculation assay was continued as described in the pathogen isolation section. Microscopic experiments were performed three times while macroscopic experiments were performed two times.

In order to study the progression of the disease *in planta* through the leaves of the six selected accessions, an additional tray with fixed leaves was inoculated as described above. Before inoculation, leaves were fully covered with a plastic lid except for a 1 cm length section in the middle of the leaves, where spores settled. After inoculation, the plastic lid was removed, and plants were kept in the same condition as previously described for 15-19 days. This experiment was performed two times.

5.2.4.2. Assessment of macroscopic components of resistance

The procedure followed was similar to that in Sørensen *et al*. (2014) with minor modifications. Third-leaves of each plant, fixed on cork pedestals, were detached 15 dpi, placed on sheets of black cardboard (A4), and digitally scanned (Canon CanoScan LiDE 400, Tokyo, Japan) at 1200 ppi resolution. The image analysis software Fiji (Schindelin, *et al*., 2012) was used for analysing the following parameters: infection frequency (IF, number of pustules per cm^2 of leaf), mean pustule size (cm^2), total pustule area relative to leaf area (%), total affected area (chlorosis and necrosis) relative to leaf area (%), and proportion of pustule area relative to total disease area (pustule area plus affected area). Image analysis was conducted on 6 cm^2 of individual leaves, four leaves per accession. Areas of pustules and disease symptoms were determined by the colour thresholding option using the default method with the HSB colour space setting.

In addition, the third-leaves covered before inoculation except for a 1 cm length area were studied at different time intervals to determine latency period (LP0) and final lesion length of *P. striiformis* (Sørensen *et al*., 2014). Evaluation of LP0 started eight days after inoculation with the examination of inoculation sites with a hand lens at 24 h intervals for 19 days. LP0 was defined as the time interval (hours) from inoculation until the first appearance of spores in the uredinia breaking the leaf epidermis. Evaluation of lesion growth started at the same time as LP0 for individual leaves and was carried out by marking the expanding edge of the lesion using a waterproof felt-tip pen (Staedtler, Nuremberg, Germany). This marking was repeated at 4-day time intervals for three consecutive measurements. Once final markings were carried out, leaves were detached and digitally scanned as previously mentioned. Total length of each lesion was evaluated using Fiji software (Schindelin, *et al*., 2012).

5.2.4.3. Assessment of microscopic components of resistance

The central leaf segments (approximately 6 cm) of third-leaves placed on foam pedestals were cut at seven dpi in four leaves per accession and replication to evaluate the fungal development stages in different accessions. Samples were processed as described in Chapter 4 and then examined using a using Nikon epifluorescence equipment (Nikon, Tokyo, Japan), with a V-2 A filter (excitation filter 380-420 nm, barrier filter 430 nm).

Fungal development and associated plant responses were classified into the following developmental stages based on Soleiman *et al*. (2014), with modifications adapted to *P. striiformis* according to Bozkurt *et al*. (2010): (i) spores developing a germinative tube without ending in a stoma were considered lost germinative tubes (LGT); (ii) spores developing a germinative tube ending in a stoma without exhibiting a substomatal vesicle (SSV) were considered germinative tubes reaching stoma (GTS); (iii) spores developing a SSV and a runner hypha (RHy), with no more than six haustorial mother cells (HMC), were considered early-aborted microcolonies without necrosis (EA–); (iv) spores developing a SSV and RHy, exhibiting notable plant cell death autofluorescence (hypersensitive response, HR) with no more than six HMC, were considered early-aborted microcolonies exhibiting necrosis (EA+); (v) spores developing a SSV and RHy with more than six HMC without necrosis were considered established

microcolonies without necrosis (EST–); (vi) spores developing an established microcolony exhibiting notable HR were considered established microcolonies with necrosis (EST+). A total of 300 spores in four leaves per accession and replication were evaluated and classified according to their stage of development. Only germinated spores were counted. These fungal developmental stages were photographed using a Nikon DS-Fi1 camera (Nikon, Tokyo, Japan). In addition, 40 established microcolonies in four leaves per accession and replication were measured to their maximum colony length, parallel to the length of the leaves, to analyse the size of endophytically growing infection structures according to Moldenhauer *et al*. (2008).

5.2.5. Statistical analysis

The experimental design was developed as randomised blocks. In macroscopic experiments, data from the total affected area were transformed using the formula $y = \sqrt{(x)}$ and then analysed using ANOVA and LSD (Least Significant Difference) tests, as were the other studied macroscopic parameters, except for the LP0, which was analysed using the Kruskal-Wallis test. These analyses included cultivars (considering treatment) and replications (four plants) for each macroscopic parameter examined in two experiments (considered as different blocks). Similarly, for microscopy experiments, percentages of GTS and EA+ were transformed according to the formula $y = \sqrt{(x)}$, and back-transformed for presentation. Percentages of EST– and EST+ for colony length analysis were transformed according to the formula $y = \arcsin(\sqrt{(x/100)})$. Microscopic analyses also included cultivars (considering treatment) and replicates (300 spores and 40 established microcolonies in four leaves) from three experiments (considered as different blocks). All microscopic data were analysed using ANOVA and LSD tests. Data processing, statistical analyses and figure design were carried out using R software (R Core Team, 2022) and Fiji (Schindelin, *et al*., 2012).

5.3. Results and Discussion

5.3.1. Resistance and susceptibility responses to *P. striiformis* infection amongst bread wheat accessions

Our study classified 19 Spanish bread wheat accessions as either resistant or susceptible to yellow rust infection according to their percentage of DS and disease scoring scale of IT (Figure 5.1). Among them, 5 out of 19 (26%) accessions were considered susceptible, developing abundant sporulation with moderate chlorosis (IT 7) to abundant and dense sporulation without notable chlorosis or necrosis (IT 8-9). However, two of them, 'Nogal' (IT 9) and 'Califa Sur' (IT 8), expressed the most severe symptoms. The susceptible response observed in 'Nogal' is consistent with field data collected by the GENVCE (Grupo Para la Evaluación de Nuevas Variedades de Cultivos Extensivos en España) network in 2019 and 2020. The other 14 evaluated accessions (74%) showed either partially resistant or resistant responses. Specifically, seven genotypes were considered partially resistant, exhibiting chlorotic and necrotic areas with limited to moderate development of pustules (IT 3-6), whereas seven accessions were considered highly resistant (IT 0-2) with no development of pustules, highlighting their role as sources of resistance in future breeding programs. In fact, it is significant that 14 out 19 accessions evaluated against *P. striiformis* showed various degrees of resistant responses under

optimum conditions of infection, indicating that yellow rust resistance genes present in the genetic pool of Spanish bread wheat cultivars possibly have not yet been overcome (Martínez-Moreno and Solís, 2019). These results contrast with those recently reported in Spanish landraces (Martínez-Moreno *et al*., 2022), where only 7% of the screened accessions, inoculated with a yellow rust isolate with a similar virulence/avirulence spectrum to the one used in this study, displayed resistant IT.

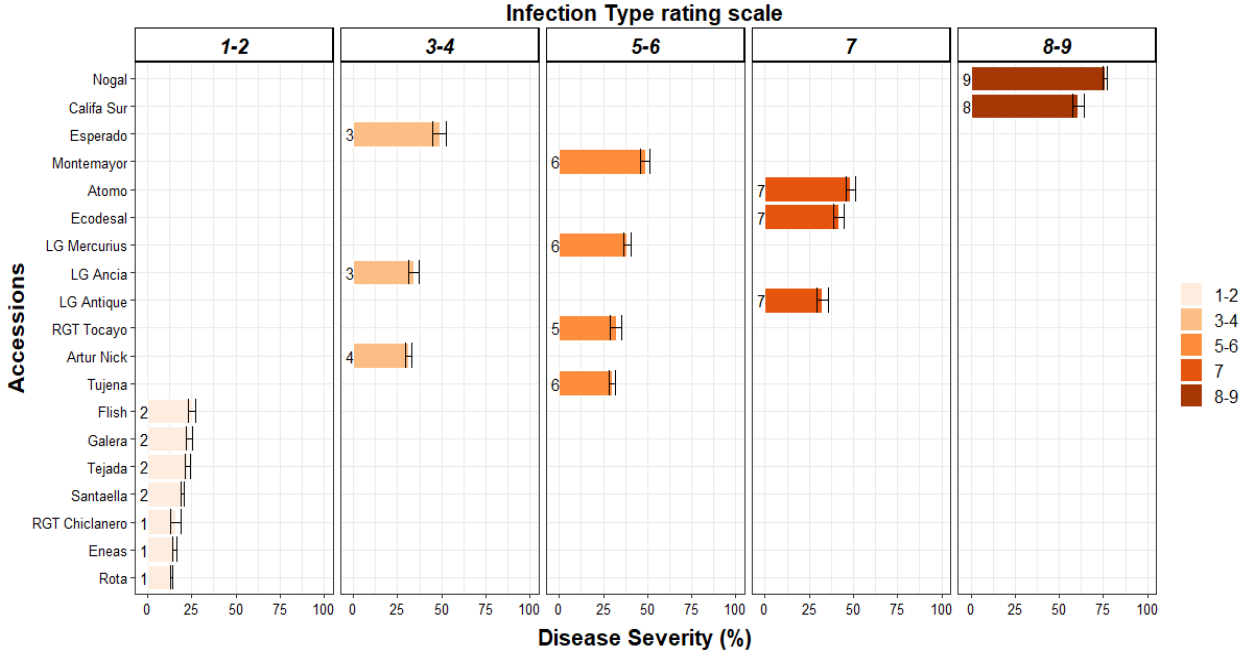

Figure 5.1. *P. striiformis* infection in bread wheat accessions. Mean percentage of disease severity (DS), presented in columns, and infection type (IT) rating scale, presented as numbers at the top of the figure. Accessions were arranged according to their mean percentage of DS and classified according to IT in panels. The IT scale is presented according to McNeal *et al*. (1971), where 0 = no visible disease symptoms (immune), 1 = minor chlorotic and necrotic flecks, 2 = chlorotic and necrotic flecks without sporulation, 3-4 = chlorotic and necrotic areas with limited sporulation, 5-6 = chlorotic and necrotic areas with moderate sporulation, 7 = abundant sporulation with moderate chlorosis, 8-9 = abundant and dense sporulation without notable chlorosis and necrosis. Error bars represent the standard error calculated from three independent experiments with six replicates each.

The cultivar 'Nogal' showed the highest mean DS value among the studied accessions (76%), followed by 'Califa Sur' (61%), both classified as susceptible with IT 9 and 8, respectively. It is worth highlighting that both accessions, presenting the highest DS values, also scored the highest IT, suggesting elevated colonisation of the fungus through leaves together with increased sporulation. 'Atomo', 'Ecodesal' and 'LG Antique' expressed on average DS values of 48, 42 and 32%, respectively, and were also classified as susceptible accessions displaying IT 7. Regarding accessions which presented moderately resistant responses to *P. striiformis* infection due to moderate sporulation, 'Montemayor' (IT 6) developed, on average, a DS value of 49%, followed by 'LG Mercurius' (DS 39%, IT 6), 'RGT Tocayo' (DS 32%, IT 5) and 'Tujena' (DS 30%, IT 6). In addition, 'Esperado' (IT 3), 'LG Ancia' (IT 4) and 'Artur Nick' (IT 4) accessions were also considered moderately resistant, but with limited sporulation of *P. striiformis*, developing DS values of 49, 34 and 31%, respectively. Remarkably, 'Esperado' accession, despite having the third highest DS value, showed a quite low IT value (3). Finally, among the accessions considered

resistant, 'Flish' presented the highest mean value of DS (25%), followed by 'Galera' (24%), 'Tejada' (23%), and 'Santaella' (20%), all categorised as IT 2. Only three accessions exhibited IT 1 values, 'RGT Chiclanero', 'Eneas' and 'Rota', developing, on average, DS values of 16%, 15% and 14%, respectively. Taking into account all the results shown here, it can be stated that our visual evaluation permitted us to classify of yellow rust infection through diverse parameters: (i) the fungal colonisation of leaves (DS), which ultimately causes desiccation of leaves and plant stunting (Chen, 2005), and (ii) the IT scale for classification of resistant responses in the form of chlorotic and necrotic areas, and the sporulation capability of the fungus, leading to infection of other leaves, plants or cropping areas (Ali *et al*., 2017; Chen, 2020). Although visual evaluation of DS or IT is a subjective and laborious assessment that can often be inaccurate (El Jarroudi *et al*., 2015), it is still considered a useful and quick first approach to determine disease extent and plant-pathogen interaction on leaves and plants for breeders, farmers and technicians.

Six accessions with interesting responses to yellow rust, from highly resistant to susceptible behavior, were selected to macro- and microscopically characterise resistance to this pathogen. The accessions were 'Rota', 'Galera', 'Artur Nick', 'Ecodesal', 'Califa Sur' and 'Nogal'.

5.3.2. Macroscopic components of resistance to *P. striiformis* infection

These six accessions were macroscopically evaluated to quantitatively identify components of resistance to yellow rust infection. Figure 5.2 shows their IT scores. Then, diverse components of *P. striiformis* infection were evaluated through image analysis, such as IF (pustules/cm^2), mean pustule size, total pustule area, total affected area, and proportion of pustule area relative to total disease area (Table 5.1).

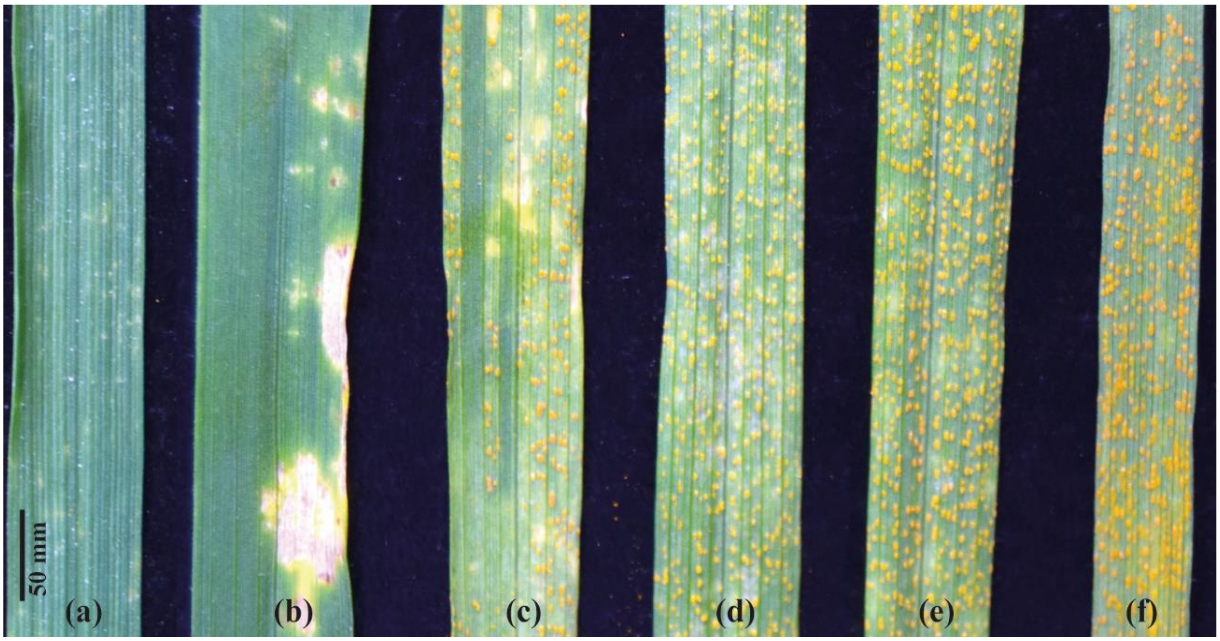

Figure 5.2. Examples of leaves infected with *P. striiformis* showing diverse infection type (IT) scores. Leaves from accessions (**a**) 'Rota' (IT 1), (**b**) 'Galera' (IT 2), (**c**) 'Artur Nick' (IT 4), (**d**) 'Ecodesal' (IT 7), (**e**) 'Califa Sur' (IT 8) and (**f**) 'Nogal' (IT 9).

Table 5.1. Macroscopic image analysis of *P. striiformis* infection in six selected bread wheat accessions [1].

Accession	IF (Pustules/cm^2)	Mean Pustule Size (cm^2 × 10^{-3})	Total Pustule Area (%)	Total Affected Area (%)	Pustule Area/ Disease Area (%)
Nogal (IT 9) [2]	230.85 ± 9.50 a	0.49 ± 0.03 a	11.14 ± 0.57 a	1.64 (1.10 ± 0.25) c	88.37 ± 4.10 a
Califa Sur (IT 8)	205.27 ± 8.22 a	0.48 ± 0.01 a	9.91 ± 0.49 a	5.98 (2.39 ± 0.20) b	63.21 ± 4.58 b
Ecodesal (IT 7)	153.44 ± 9.27 b	0.46 ± 0.01 a	7.00 ± 0.52 b	2.92 (1.60 ± 0.23) c	73.40 ± 5.34 b
Artur Nick (IT 4)	42.27 ± 8.93 c	0.39 ± 0.03 b	1.70 ± 0.45 c	24.02 (4.79 ± 0.39) a	7.08 ± 1.71 c
Galera (IT 2)	-	-	-	8.22 (2.75 ± 0.31) b	-
Rota (IT 1)	-	-	-	1.51 (1.17 ± 0.14) c	-

[1] Values are mean ± standard error for four leaves evaluated in two independent experiments. Transformed data ± standard error are shown in parenthesis. Data with the same letter within a column are not significantly different (LSD, $p < 0.05$). Dash (-) means no data were measured since there was no pustule development.[2] Infection type (IT) data was scored by visual analysis. IF, infection frequency.

The susceptible cultivars 'Nogal' (IT 9) and 'Califa Sur' (IT 8) showed a fully compatible reaction to the yellow rust and displayed the highest mean IF values with 230 and 205 pustules/cm^2, respectively. Cultivar 'Ecodesal' (IT 7) showed an intermediate reaction with 153 pustules/cm^2, while partially resistant accession 'Artur Nick' (IT 4) displayed the lowest IF with 42 pustules/cm^2. In the particular case of the 'Ecodesal' accession, it developed sporulation ranging from moderate in some cases to abundant in others, without exhibiting notable chlorosis or necrosis, making its IT classification with the McNeal scale more difficult (McNeal *et al*., 1971) through initial visual assessment. 'Ecodesal' was finally scored as IT 7, focusing on the sporulation capability of *P. striiformis* in this cultivar according to macroscopic evaluation through image analysis, which permitted an objective classification in comparison with visual assessment (Bock *et al*., 2010). This measurement subsequently led us to quantitatively separate accessions based on IF, which was more accurate than visual assessment of DS to estimate rust infection (Xie *et al*., 2012; Gallego-Sánchez *et al*., 2020), and also helped to more precisely define the initial IT scores obtained by visual assessment. Additionally, since 'Galera' (IT 2) and 'Rota' (IT 1) accessions were found to be highly resistant to *P. striiformis*, pustules were not developed and no measurements of IF or mean pustule size could be taken.

Moreover, average values of mean pustule size exhibited statistical differences between the susceptible accessions, 'Nogal' (0.49 x 10^{-3} cm^2), 'Califa Sur' (0.48) and 'Ecodesal' (0.46), and the less susceptible 'Artur Nick', which developed the smallest pustules (0.39 x 10^{-3} cm^2), possibly due to its partially resistant phenotype (IT 4). It was also analysed the total pustule area as a proportion of leaf area, finding similar differences as in the IF parameter. 'Nogal' expressed the highest mean value with 11%, followed by 'Califa Sur' (9.9%). A lower value was observed in 'Ecodesal' (7%), and the lowest value was recorded in 'Artur Nick' (1.7%). 'Galera' and 'Rota' accessions did not show any data since pustules were not developed. At this point, it is important to highlight that the count of pustules in *P. striiformis* infection presented some difficulties regarding the identification of single pustules due to pustule coalescence, and manual curation of the data was needed, especially in very susceptible accessions. For that reason, the measurement of total pustule area (as a percentage of the leaf area analysed), which did not indicate the number of pustules, required a less complex process of analysis in comparison with IF, and yielded a similar classification of accessions, making it a preferable parameter to take into consideration for quantifying *P. striiformis* sporulation in image analysis applications (Gallego-Sánchez *et al*., 2020).

Image analysis also permitted measurement of the total affected area (chlorosis and necrosis) without sporulation of *P. striiformis*. This parameter could be used to identify partially resistant accessions such as 'Artur Nick' (IT 4), which expressed the highest mean value (24%) and was statistically different from the rest of the accessions categorised as susceptible or resistant. This quantitative identification of chlorosis and necrosis also distinguished accessions with different resistant responses, such as 'Galera' (8.2%) with IT 2 and 'Rota' (1.51%) with IT 1, as well as accessions with different susceptible responses such as 'Nogal' (1.64%) and 'Ecodesal' (2.92%), which expressed smaller affected areas compared to 'Califa Sur' (5.9%). However, statistical analysis in this parameter among accessions did not differentiate, for example, 'Nogal' or 'Califa Sur' (susceptible phenotypes, IT 9 and 8, respectively) from 'Rota' and 'Galera' (resistant phenotypes, IT 1 and 2, respectively), and it was necessary to take into consideration the proportion of pustule area relative to total disease area. This parameter helped us to understand what quantity of yellow rust infection produced sporulation. Therefore, this measurement led to statistical differences in sporulation development between the susceptible accessions 'Nogal' and 'Califa Sur' (88.37% and 63.21%, respectively), compared to the resistant ones 'Rota' and 'Galera' (no data reported since pustules were not developed).

Furthermore, the progression of yellow rust infection produced by this local isolate was evaluated in four of the six selected accessions where the pustules appeared well developed and sporulation could be observed. The macroscopic parameters latency period (LP0) and final lesion length were measured (Table 5.2). LP0 expressed differences between 'Nogal' (IT 9) and 'Califa Sur' (IT 8) (264 h on average) and 'Ecodesal' (IT 7) (303.75 h), all of which were considered susceptible accessions. Surprisingly, 'Ecodesal' developed a statistically longer LP0 than 'Nogal' and 'Califa Sur', which could explain its macroscopic classification as IT 7, with moderate sporulation in some cases and abundant in others. Finally, the longest LP0 was recorded in 'Artur Nick' (306 h on average). The longer LP0 developed by the 'Artur Nick' accession, together with a lower mean pustule size value than found in susceptible accessions, suggest a partial resistance behavior (Singh and Huerta-Espino, 2003; Soleiman *et al*., 2014). Regarding final lesion length, the shorter the LP0, the longer the final lesion length observed and vice versa. Thus, 'Califa Sur' and 'Nogal' developed the largest mean values of final lesion length, 63.69 and 60.58 mm respectively, showing statistical differences from 'Ecodesal' (43 mm) and 'Arthur Nick' (44 mm). These results revealed that the progression of yellow rust infection was more intense in 'Nogal' and 'Califa Sur' due to shorter LP0 values in comparison with 'Ecodesal' and 'Artur Nick', which ultimately developed bigger final lesions. The resistant accessions, 'Rota' and 'Galera', did not produce pustules and no measurements were available.

Table 5.2. Macroscopic components of disease progression of *P. striiformis* infection in four selected bread wheat accessions [1].

Accession	LP0 (h)	Final Lesion Length (mm)
Nogal	264 ± 0.00 a	60.58 ± 3.05 a
Califa Sur	264 ± 0.00 a	63.63 ± 2.47 a
Ecodesal	303.75 ± 8.56 b	42.89 ± 4.59 b
Artur Nick	306 ± 10.88 b	44.18 ± 6.34 b

[1] Values are mean ± standard error for four leaves evaluated in two independent experiments. Data with the same letter within a column are not significantly different (Kruskal-Wallis and LSD tests, $p < 0.05$). LP0, latency period.

5.3.3. Microscopic components of resistance to *P. striiformis* infection

In addition to the macroscopic analyses, microscopic evaluation was carried out in order to compare micro- and macroscopic phenotypes, similarly to other studies developed for yellow rust (Bozkurt *et al.*, 2010; Jagger *et al*., 2011), leaf rust (Wang *et al*., 2013) and stem rust (Wang *et al.*, 2015). The accessions used to evaluate macroscopic components were also examined for microscopic components of resistance to *P. striiformis* infection. Different fungal stages were observed (LGT, GTS, EA+, EST− and EST+) (Figure 5.3), and then analysed as percentages (Figure 5.4).

First of all, measurements of spores which developed lost germinative tubes (LGT) presented similar mean values in the 'Nogal' (34%), 'Galera' (35%), 'Rota' (36%) and 'Califa Sur' (39%) accessions, whereas 'Artur Nick' and 'Ecodesal' had values of 48 and 58%, respectively (Figure 5.4). Additionally, mean values of spores which developed germinative tubes reaching stoma (GTS) were higher in the 'Rota' (17%), 'Artur Nick' (15%) and 'Ecodesal' (12%) accessions. The rest of the accessions showed lower mean values with 'Califa Sur' expressing 8%, 'Galera' 7% and 'Nogal' 6%. Thus, both of these pre-infection parameters (LGT and GTS as a set) together expressed mean percentage values from 40% in 'Nogal' to 70% in 'Ecodesal', in concordance with studies developed by de Vallavieille-Pope *et al*. (1995; 2002), which found a reduction in *P. striiformis* penetration efficiency in comparison with other wheat rusts, such as *Puccinia triticina*. Accessions 'Ecodesal' and 'Artur Nick' presented elevated values of LGT plus GTS, which could explain their lower IF relative to the susceptible accessions as a consequence of a higher proportion of spores that did not form infection sites (70% and 63%, respectively). These low penetration values in 'Ecodesal' and 'Artur Nick' could be explained by a multifactorial component, where in addition to the reduced *P. striiformis* penetration efficiency, diverse circumstances related to the leaf surface played key roles. Leaf surface is the first physical and chemical barrier against fungal infection (Walters, 2006), and elements such as density, wax and morphology of leaf trichomes and stomata (Rubiales and Niks, 1996; Imboden *et al*., 2018; Gupt *et al*., 2021), along with the expression of antifungal compounds in trichomes (Calo *et al*., 2006), are actively involved in halting fungal infection. In this context, 'Rota' expressed the highest mean value of GTS, although without statistical differences with 'Ecodesal' and 'Artur Nick', suggesting the presence of an obstacle mentioned above to the *P. striiformis* penetration process, which needs to be determined via further research.

Furthermore, spores which formed infection sites through SSV development were counted and classified, showing clear differences among accessions. Spores that formed early-aborted microcolonies absent plant cell death response (EA–) were highly infrequent in all studied accessions, and were not analysed in this study. Early-aborted microcolonies showing plant cell death autofluorescence (EA+) presented higher mean values in accessions considered resistant, such as 'Rota' (26%, IT 1) and 'Galera' (16%, IT 2). These resistant accessions developed incompatible interactions with *P. striiformis* to some extent, similarly to other incompatible host-*P. striiformis* interactions shown in other studies (Moldenhauer *et al*., 2006; 2008; Bozkurt *et al*., 2010; Sørensen *et al*., 2017a; Saleem *et al.*, 2019). In fact, the EA+ mean value developed by 'Rota' suggests early recognition of the pathogen, triggering an intensive HR, which did not permit fungal growth, in comparison with 'Galera' (also resistant). This condition correlates with a different macroscopic phenotype with IT 1 in 'Rota' and IT 2 in 'Galera', similar to the results

obtained by Saleem *et al*. (2019). However, the evidence for early pathogen recognition needs further analysis at different time points previous to seven dpi to elucidate the evolution of host responses to yellow rust infection (Sørensen *et al*., 2017a; Saleem *et al*., 2019). By contrast, partially resistant and susceptible accessions exhibited lower EA+ values, indicating that spores that caused infection sites finally developed, in general, established microcolonies. In particular, 'Califa Sur' (IT 8) showed a remarkably higher mean value of EA+ (8%) in comparison with 'Nogal' (0.5%; IT 9), which was also in concordance with macroscopic results (total affected area) and reinforced the link between macroscopic phenotype and histopathological parameters. The partially resistant accession 'Artur Nick' (IT 4) and the moderately susceptible 'Ecodesal' (IT 7) also displayed low EA+ percentages (5 and 3%, respectively).

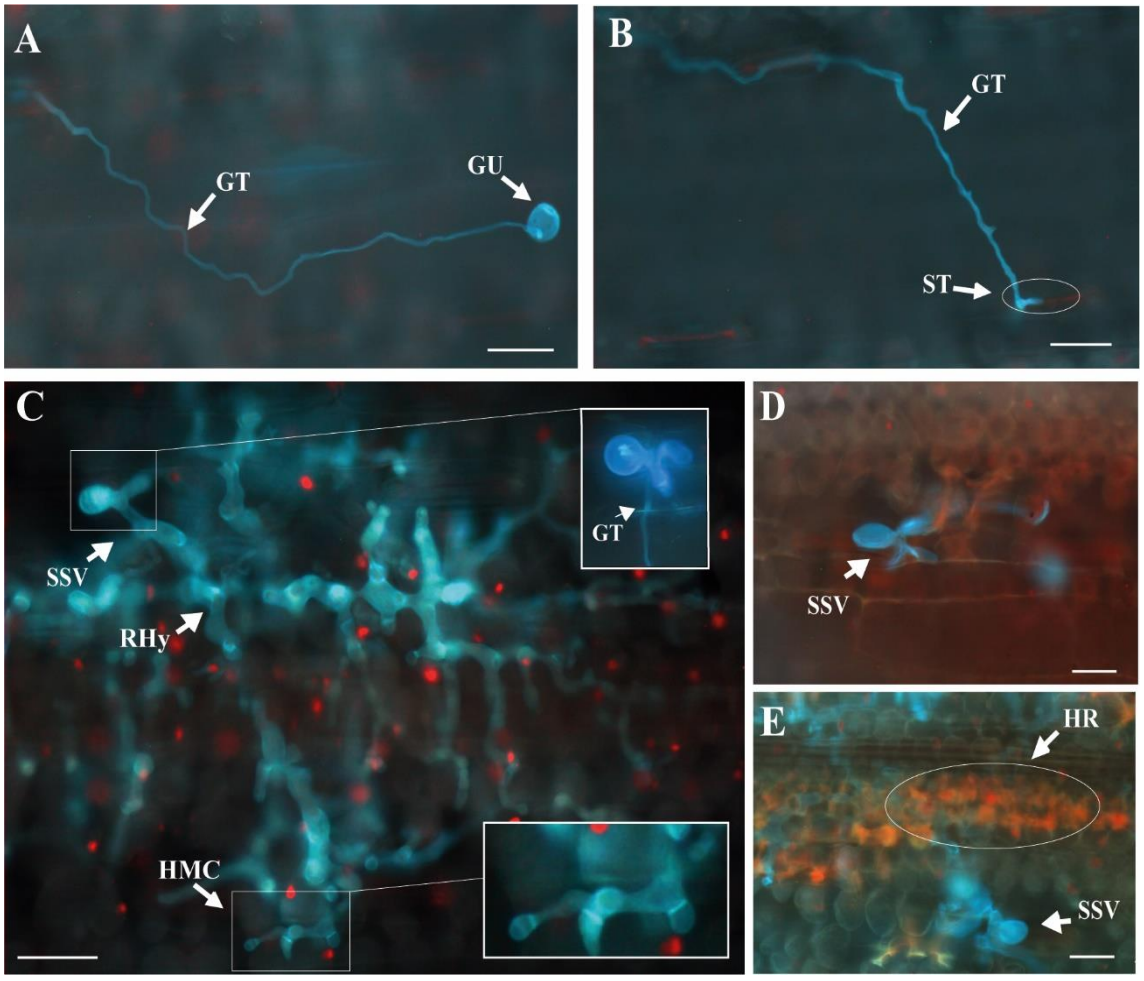

Figure 5.3. Microscopic fungal stages of *P. striiformis* and plant cellular responses were classified as: (**A**) lost germinative tube (LGT), showing the germinated urediniospore (GU) and the germinative tube (GT); (**B**) germinative tube reaching stoma (GTS), showing a germinative tube ending in a stoma (ST) without establishing a substomatal vesicle (SSV); (**C**) established microcolony without necrosis (EST–), with a detailed SSV, developing runner hyphae (RHy) and haustorial mother cells (HMC); (**D**) early-aborted colony associated with hypersensitive response (EA+); (**E**) established microcolony associated with necrosis (EST+), also developing SSV with RHy and HMC associated with hypersensitive response. HR, hypersensitive response. Scale, 50 µm.

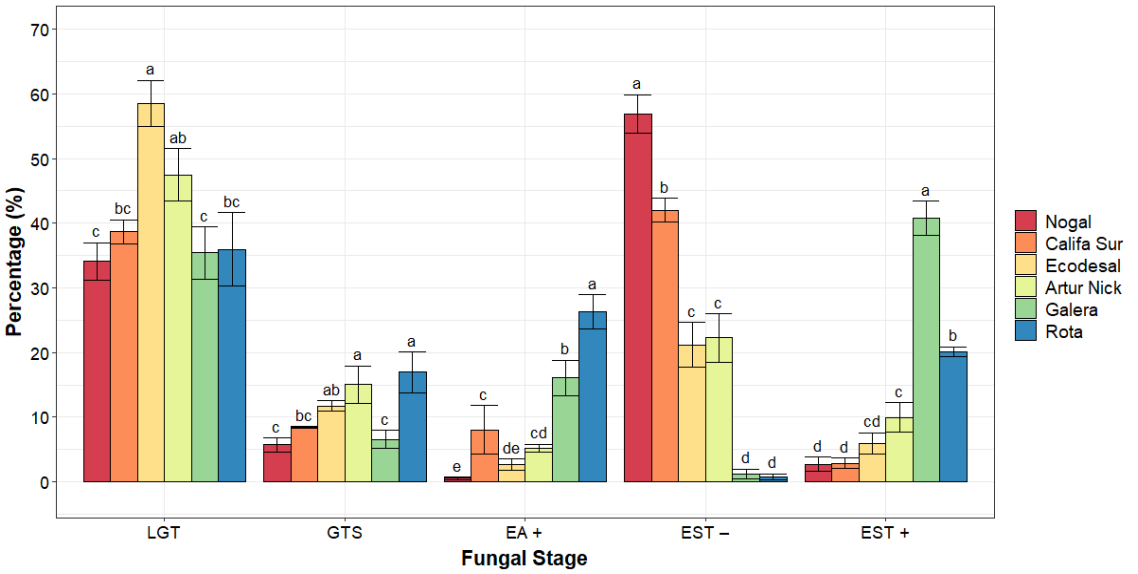

Figure 5.4. Microscopic fungal stages of *P. striiformis* in six selected bread wheat accessions presented as mean percentages. Error bars represent the standard error calculated from three independent experiments. Data with the same letter within a fungal stage are not significantly different (LSD test, $p < 0.05$). LGT, lost germinated tubes; GTS, germinate tubes reaching stoma; EA+, early-aborted microcolonies with necrosis; EST−, established microcolonies without necrosis; EST+, established microcolonies with necrosis.

Regarding mean values of spores which formed established microcolonies without plant cell death (EST–), susceptible accessions 'Nogal' (57%) and 'Califa Sur' (42%) showed statistical differences corresponding with their macroscopic differences obtained through visual IT (9 and 8, respectively) and total affected area (1.6% and 6%, respectively). IF and total pustule area did not show differences. 'Ecodesal', also classified as susceptible, developed significantly lower mean values of EST– (21%) compared to 'Nogal' and 'Califa Sur', possibly due to the higher LGT and GTS values observed in this accession. However, this difference in EST– values relative to 'Nogal' and 'Califa Sur' was in the form of macroscopic results related to sporulation parameters (IF and total pustule area). Similarly, 'Artur Nick', considered partially resistant, did not express differences in EST– values (22%) compared to 'Ecodesal', despite the clear macroscopic differences in IF, total pustule area and total affected area. Thus, this microscopic parameter (EST–) could be considered helpful for classifying susceptible genotypes which presented differences at the microscopic level but were not clearly correlated in macroscopic measurements, and likewise for the microscopic parameter of established microcolonies associated with plant cell death responses used for incompatible interactions (Bozkurt *et al*., 2010; Jagger *et al*., 2011). As we expected, 'Galera' and 'Rota' exhibited the lowest values of EST– (1% in both accessions), manifesting macroscopic responses without development of pustules.

Finally, spores developing established microcolonies associated with HR (EST+) presented mean values that differentiated accessions statistically. 'Galera' presented the highest mean value with 41%, with EST+ being the principal microscopic component of its resistance. This value in 'Galera' is possibly due to an initial delay in the activation of HR against infection, which permitted fungal development until later stages, when HR then appeared in the majority of established microcolonies. This delayed activation of the HR response and the subsequent fungal

growth could suggest an indirect recognition of the pathogen by the host, characterised by a macroscopic phenotype of IT 2, similar to that described by Saleem *et al*. (2019) in a wheat near-isogenic line with *Yr*6 resistance gene at 8 dpi. However, this supposed delay in the expression of HR needs further analysis at previous time points, as in the cases of early pathogen recognition in accessions with relevant values of EA+, such as 'Rota' (Sørensen *et al*., 2017a; Saleem *et al*., 2019). In particular, 'Rota' also presented a remarkable value of EST+ (20%), which could suggest that the initial expression of HR by the host, shown in the EA+ values, stopped fungal development prior the RHy formation in some cases, whereas the fungus was capable of developing RHy and at least six HMCs before being enclosed by HR in other cases.

The accessions that eventually developed pustules in their macroscopic IT also expressed lower values of EST+ in comparison with 'Rota' and 'Galera', leading in the end to lower HR at a microscopic level. Thus, 'Artur Nick', as a partially resistant accession, expressed a mean value of 10% of EST+, statistically different from susceptible accessions 'Ecodesal' (6%), 'Califa Sur' (3%) and 'Nogal' (2.5%), correlating with its macroscopic results. Although 'Ecodesal' was considered susceptible, it showed a different EST+ value in comparison with 'Nogal' and 'Califa Sur', possibly due to a slight expression of HR. Because of that, this accession could be considered moderately susceptible. Values of EST+ were not statistically different between susceptible accessions. In contrast, they could be used to differentiate susceptible from partially resistant accessions with incompatible interactions (IT 3-6) such as 'Artur Nick' and, most relevantly, EST+ could classify accessions considered resistant (IT 1-2) with statistical differences in the presence of HR in their established microcolonies (Bozkurt *et al*., 2010; Jagger *et al*., 2011).

Once we completed the identification and quantification of the main fungal stages developed by *P. striiformis* in our studied accessions, we performed measurements of colony length in their established microcolonies in order to quantify the extension of the fungus through leaf tissue, taking into account the presence of HR (Figure 5.5), similar to previous studies (Moldenhauer *et al*., 2006; 2008).

Data presented in Table 5.3 show analysis of the colony length of established *P. striiformis* microcolonies. 'Nogal' and 'Califa Sur', both susceptible, expressed the highest mean values of colony length (868 and 822 µm respectively) and the highest EST– values amongst our studied accessions (96 and 94%, respectively), in accord with their macroscopic IT scale values (9 and 8, respectively) and their sporulation parameters. Their reduced proportions of established microcolonies that developed HR (EST+, 4 and 6%, respectively) indicated that fungus could grow extensively in these accessions. These results together, both high colony length values and diminished number of microcolonies with HR, could serve as factors for differentiating susceptible accessions from others which developed reduced infection severity, such as 'Ecodesal' (IT 7) with a shorter colony length (646 µm) and a higher percentage of established microcolonies with HR (23%). However, 'Ecodesal' did not show relevant macroscopic affected areas with chlorosis or necrosis, suggesting that these HR were weakly presented, allowing the extension of fungal development. This fact, together with the statistical similarity of 'Ecodesal' (IT 7) to 'Artur Nick' (IT 4) in the parameters of colony length (538 µm) and proportion of established microcolonies associated with HR (31%), could indicate a quantitative increase (current or subsequent) in HR development in 'Artur Nick' compared with 'Ecodesal', which corresponded with their final developed macroscopic phenotype. Thus, since in our study the

evaluation of HR was conducted qualitatively, we could not establish the degree of expression of these responses. For that reason, a quantitative characterisation of these parameters in future studies could shed some light on the differences between accessions that developed different degrees of incompatible interactions with yellow rust, similar to the studies developed by Saleem *et al*. (2019), where the percentage of microcolonies covered with autofluorescence responses in genotypes with diverse *Yr* genes was measured.

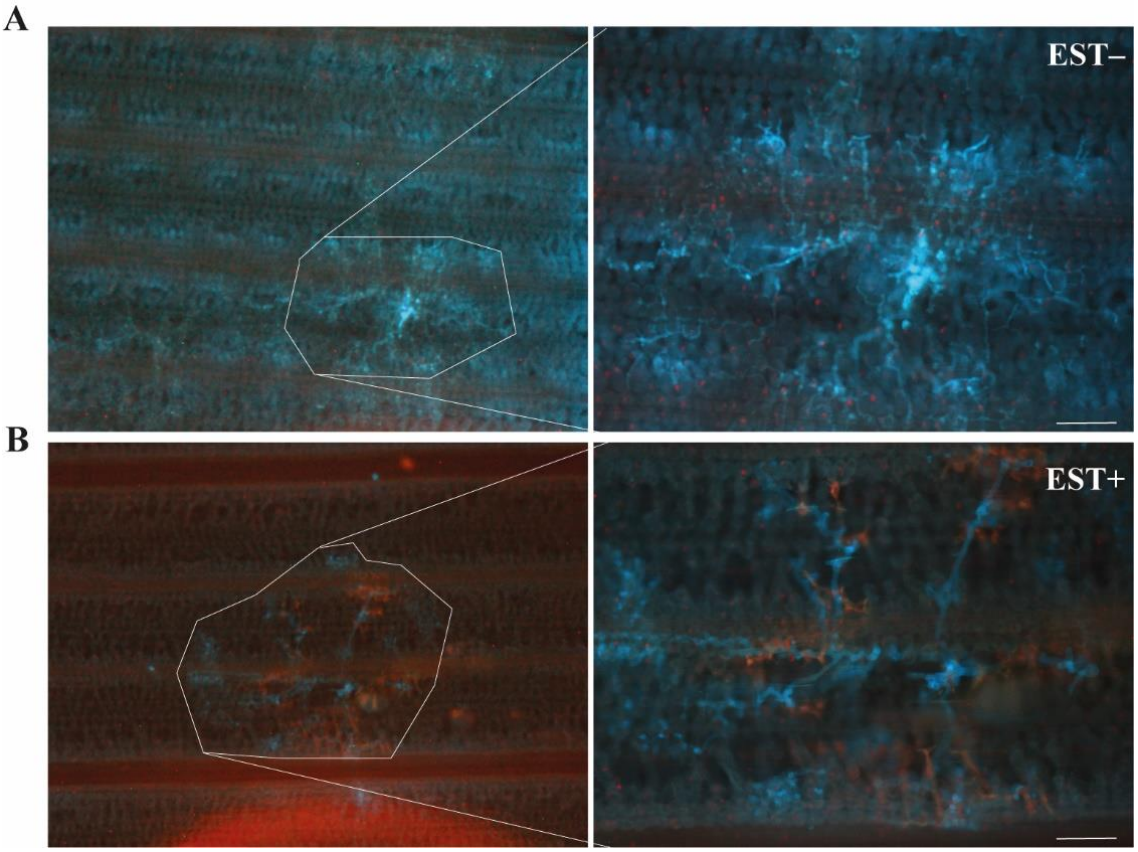

Figure 5.5. (**A**) Examples of established *P. striiformis* microcolonies without necrosis (EST–, upper panel) and (**B**) with necrosis (EST+, lower panel). Left panels show 4 × images with delimited microcolonies magnified at 10 × in the right panels. Scale, 100 µm.

Table 5.3. Analysis of the colony length of established *P. striiformis* microcolonies and their distribution relative to total observed established microcolonies in six selected bread wheat accessions [1].

Accession	Colony Length (µm)	EST– (%)	EST+ (%)
Nogal	867.92 ± 34.31 a	95.61 (0.20 ± 0.04) a	4.39 (1.37 ± 0.04) c
Califa Sur	822.26 ± 18.85 ab	93.73 (0.25 ± 0.03) a	6.27 (1.32 ± 0.03) c
Ecodesal	646.61 ± 58.45 cd	77.07 (0.49 ± 0.10) b	22.93 (1.08 ± 0.10) b
Artur Nick	537.86 ± 38.51 d	68.42 (0.59 ± 0.09) b	31.58 (0.98 ± 0.09) b
Galera	739.33 ± 44.93 bc	2.91 (1.43 ± 0.07) c	97.09 (0.14 ± 0.07) a
Rota	368.99 ± 19.33 e	3.68 (1.42 ± 0.08) c	96.32 (0.15 ± 0.08) a

[1] Values are mean ± standard error for four leaves evaluated in three independent experiments. Transformed data ± standard error are shown in parentheses. Data with the same letter within a column are not significantly different (LSD, $p < 0.05$). EST–, established microcolonies without necrosis; EST+, established microcolonies with necrosis.

On the other hand, the hypersensitive accession ´Rota´ (IT 1), which developed macroscopically small chlorotic and necrotic flecks, exhibited the lowest mean colony length value (369 µm), statistically different from the other accessions, and a remarkably elevated percentage of EST+ microcolonies (96%). These results suggest an early inhibition of fungal growth by the host during the infection process associated with HR, similar to previous histological studies developed by Kang *et al*. (2003), where the low IT scores expressed by *P. striiformis* resistant wheat cultivars were related to the inhibition of fungal growth. Finally, although 'Galera' (IT 2) showed colony length values statistically similar to susceptible accessions (739 µm), it developed the highest percentage of EST+ microcolonies (97%), thus showing differences not only from susceptible accessions, but also from the resistant accession 'Rota'. These results suggest that 'Galera' displayed delayed HR, thus leading to higher fungal development compared to the faster defence pattern of the 'Rota' accession. In addition, future studies focusing on the variation in colony size and HR as quantitative values (percentage) at different time points during the infection process could more precisely characterise the microscopic phenotypes of accessions resistant to yellow rust, such as 'Galera'. In this context, other studied parameters extending the knowledge of the *P. striiformis*-wheat interaction at the microscopic level would be the accounting of HMC per infection unit (Zhang *et al*., 2012) or the deposition of compounds such as lignin or callose (Moldenhauer *et al*., 2006; 2008). Thus, the study of diverse microscopic components of resistance to *P. striiformis* provide relevant information that correlates with the macroscopic expression of the disease, leading to an improvement in the understanding of plant-pathogen interaction for future breeding purposes, complementing the predominant genetic, biochemical and physiological studies (Sørensen *et al*., 2017a).

In summary, this study could be considered as the first macroscopic and microscopic characterisation of *P. striiformis* infection in a collection of Spanish bread wheat cultivars under controlled conditions. Using visual evaluation to analyse parameters such as DS and IT, the cultivars were classified in a range from susceptible to highly resistant depending on their disease expression patterns. Then, several macro- and microscopic parameters of resistance were defined, resulting in measurements that differentiated our studied cultivars quantitatively. Thus, such differences could be considered of particular relevance to find and characterise resistant responses against yellow rust. Moreover, this could help us gain a better knowledge of plant-pathogen interactions, especially considering the quick adaptation of *P. striiformis* to warmer climates. Despite the progress made in this work, further research should focus on the study of early *P. striiformis* recognition by the resistant accessions, prior to 7 dpi, together with physiological study of the leaf surfaces of accessions with low *P. striiformis* penetration values in order to elucidate the evolution of host responses to and morphological changes in yellow rust infection. The continuous evolution of *P. striiformis* allows it to spread into diverse territories and cultivars in Spain, requiring more detailed evaluation of cultivars' responses to control them and adapt new breeding cultivars through genetic, biochemical and physiological studies. In conclusion, this study represents an important new step in the research of plant-pathogen interactions for future breeding purposes regarding the new *P. striiformis* threat in the Spanish cultivars, reinforcing the link between macroscopic phenotypes and histopathological parameters.

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Chapter 6. General Conclusions

The research developed in this Ph.D. Thesis include, on one side, the precise characterisation of the responses of Spanish wheat genotypes against its main fungal pathogens (*Zymoseptoria tritici*, *Puccinia triticina* and *Puccinia striiformis* f. sp. *tritici*) and, on the other side, the subsequent assessment of the effects derived from increasing temperature and [CO_2] on the interactions of selected wheat genotypes against the pathogens *Z. tritici* and *P. triticina*, being both studies accomplished thanks to the use of macroscopic and microscopic evaluations. Thus, the conclusions obtained from this research are the following:

- Wheat genotypes can be precisely classified regarding their response against the pathogens *Z. tritici*, *P. triticina* and *P. striiformis* f. sp. *tritici* using macroscopic and microscopic evaluations, improving breeding research through phenotyping, identifying the occurrence of new virulent pathotypes in advance or assessing the effects derived from environmental alterations in wheat-pathogen interactions.

- Image analysis emerge as a powerful tool for a quick and semi-automated screening of a high number of plants regarding their response against different fungal pathogens.

- Current durum wheat Spanish genotypes presented, in general, susceptible responses against local isolates of *Z. tritici* and *P. triticina* pathogens under optimum conditions for fungal development, whereas current bread wheat Spanish genotypes developed, in general, resistant responses against *P. striiformis* f. sp. *tritici.*

- The exposure of wheat-*Z. tritici* pathosystem to elevated temperature (set S1) reduced disease incidence of *Z. tritici,* mainly affecting pathogen virulence rather than plant physiology, especially at the stages of host penetration and pycnidia formation and maturation (reducing pycnidiospores number and size), which would severely hamper subsequent infection cycles of *Z. tritici*.

- The simultaneous exposure of wheat-*Z. tritici* pathosystem to elevated temperature and [CO_2] (set S2) increased *Z. tritici* leaf tissue colonisation in comparison to weather conditions of elevated temperature (set S1), especially in the resistant accession, but this improvement did not occur in comparison to baseline conditions (set SB). This suggests that temperature was the main abiotic factor modulating the response of this pathosystem, in which elevated [CO_2] slightly favoured fungal development.

- The exposure of wheat-*P. triticina* pathosystem to elevated temperature (set S1) improved host defence responses (hypersensitive response-HR) against *P. triticina* to some extent, especially in the susceptible accession, in which it was observed not only a reduced available host photosynthetic area, but also a reduced pustule development ability of the fungus.

- The exposure of wheat-*P. triticina* pathosystem to both elevated temperature and [CO_2] (set S2) slightly speeded up not only the development rate of *P. triticina* via shorter latency period (LP50), but also host defence responses (HR), especially in the susceptible accession. Concretely, these host defence responses were observed through slightly different values of macro- and microscopic components of resistance in comparison to plants exposed to elevated temperature (set S1), indicating that temperature was the main abiotic factor modulating the response of selected accessions against *P. triticina* infection.

- *Z. tritici* and *P. triticina* were both severely affected by increasing temperature occurring at the beginning of the infection process (spore germination and penetration into host; sets S1G and S2G), which greatly reduced subsequent disease development at different stages.

APPENDIX: Supplementary Materials

A.I. Supplementary Tables

Table S2.1. Commercial cultivars and breeding lines used in Chapter 2 and their source of origin.

Breeding lines		Commercial cultivars	
Accession	**Breeding program origin**	**Accession**	**Company**
BL 27	IFAPA [1]	Amilcar	Semillas Guadalsem S.L.
BL 28	CIMMYT [2]	Athoris	Limagrain Ibérica S.A.
BL 30	CIMMYT	Avispa	Limagrain Ibérica S.A.
BL 31	IFAPA	Don Ortega	Agrovegetal S.A.
BL 32	IFAPA	Don Ricardo	Agrovegetal S.A.
BL 33	IFAPA	Egeo	MAS Seeds
BL 34	CIMMYT	Euroduro	Eurosemillas S.A.
BL 36	CIMMYT	Fuego	Semillas Guadalsem S.L.
BL 38	CIMMYT	Kiko Nick	Limagrain Ibérica S.A.
BL 39	CIMMYT	LG Acropolis	Limagrain Ibérica S.A.
BL 40	CIMMYT	LG Hipnosis	Limagrain Ibérica S.A.
BL 41	CIMMYT	LG Origen	Limagrain Ibérica S.A.
BL 43	CIMMYT	Nobilis	Limagrain Ibérica S.A.
BL 44	IFAPA	Odisseo	Syngenta S.A.
BL 45	IFAPA	Qualidou	Florimond Desprez Ibérica S.A.
BL 46	CIMMYT	RGT Beticur	RAGT Ibérica S.L.U.
BL 47	IFAPA	RGT Fernandur	RAGT Ibérica S.L.U.
BL 48	IFAPA	RGT Leondur	RAGT Ibérica S.L.U.
BL 49	CIMMYT	RGT Rumbadur	RAGT Ibérica S.L.U.
BL 50	CIMMYT	RGT Voilur	RAGT Ibérica S.L.U.
BL 51	CIMMYT	RGT Xiriur	RAGT Ibérica S.L.U.
BL 52	IFAPA	Salgado	AGRUSA
		Sculptur	RAGT Ibérica S.L.U.
		Simeto	PROSEME S.R.L.
		Sy Leonardo	Syngenta S.A.
		Teodorico	MAS Seeds

[1] IFAPA: Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica. Centro Alameda del Obispo, Córdoba (Spain).

[2] CIMMYT: Centro Internacional de Mejoramiento de Maíz y Trigo, El Batán, Texcoco, México.

Table S3.1. Microscopic stages of spores and fungal development of the *Z. tritici* infection in three selected durum wheat accessions under baseline and climate change environments [1].

Accession	Environmental Set	4 dpi			21 dpi		
		SP (%)	DP (%)	NP (%)	NCS (%)	CST (%)	P (%)
Sy Leonardo	SB	16.85 ± 4.26 a	25.57 ± 4.42 a	57.58 ± 3.80 c	16.59 ± 1.09 c	24.37 ± 2.87 c	59.04 ± 2.82 a
	S1	8.48 ± 1.56 b	11.48 ± 1.99 b	80.04 ± 1.97 b	27.70 ± 0.82 ab	35.93 ± 1.12 ab	36.37 ± 1.44 bc
	S2	7.58 ± 1.37 b	10.77 ± 0.82 b	81.65 ± 1.06 b	28.37 ± 4.42 ab	27.78 ± 3.74 bc	43.85 ± 6.71 b
	S1G	6.22 ± 0.42 b	5.26 ± 1.34 c	88.52 ± 1.20 a	33.19 ± 1.74 a	34.66 ± 2.63 ab	32.15 ± 1.30 c
	S2G	5.14 ± 0.94 b	5.86 ± 0.65 c	89.00 ± 0.92 a	23.01 ± 2.12 b	42.71 ± 1.11 a	34.28 ± 1.69 bc
LG Origen	SB	23.98 ± 2.86) a	21.34 ± 2.80 a	54.68 ± 0.50 c	37.90 ± 8.05 d	35.98 ± 5.55 a	26.12 ± 3.68 a
	S1	6.66 ± 0.55) b	10.77 ± 1.73 b	82.57 ± 1.86 b	51.45 ± 3.12 c	25.85 ± 3.17 b	22.70 ± 1.53 a
	S2	7.02 ± 1.00) b	10.69 ± 0.74 b	82.29 ± 1.24 b	50.37 ± 1.80 cd	26.82 ± 1.19 ab	22.81 ± 1.96 a
	S1G	5.78 ± 0.29) b	7.16 ± 1.36 b	87.06 ± 1.37 a	97.56 ± 1.24 a	2.44 ± 1.24 d	0.00 ± 0.00 b
	S2G	5.13 ± 0.49) b	6.22 ± 1.25 b	88.65 ± 0.78 a	66.52 ± 0.98 b	13.70 ± 1.32 c	19.78 ± 0.34 a
RGT Rumbadur	SB	15.75 ± 3.82) a	25.80 ± 3.73 a	58.45 ± 4.35 c	42.74 ± 2.12 ab	45.11 ± 2.40 a	12.15 ± 0.30 ab
	S1	5.41 ± 0.87) b	11.35 ± 1.25 bc	83.24 ± 1.78 ab	44.64 ± 4.71 ab	45.74 ± 3.66 a	9.62 ± 1.39 b
	S2	6.62 ± 1.84) b	12.15 ± 1.22 b	81.23 ± 0.66 b	36.45 ± 6.86 b	49.48 ± 6.51 a	14.07 ± 0.75 a
	S1G	5.00 ± 0.06) b	10.00 ± 0.51 bc	85.00 ± 0.52 ab	57.60 ± 9.18 a	36.83 ± 9.06 a	5.57 ± 0.86 c
	S2G	3.83 ± 0.62) b	8.27 ± 0.75 c	87.90 ± 0.66 a	48.37 ± 3.77 ab	42.02 ± 3.04 a	9.61 ± 0.73 b

[1] Values are mean ± standard error for three leaves evaluated for each accession, time point and environmental set in three different experiments. Data with the same letter within an accession and column are not statistically different (Duncan test, $p < 0.05$). SP, spores leading to a stomatal penetration; DP, spores leading to a direct penetration; NP, spores without penetration; NCS, non-colonised stomata; CST, colonised stomata but not yet transformed into pycnidia; P, colonised stomata transformed into pycnidia.

Table S4.1. Commercial cultivars and breeding lines used in Chapter 4 and their source of origin.

Breeding lines		Commercial cultivars	
Accession	**Breeding program origin**	**Accession**	**Company**
BL 27	IFAPA [1]	Amilcar	Semillas Guadalsem S.L.
BL 28	CIMMYT [2]	Athoris	Limagrain Ibérica S.A.
BL 30	CIMMYT	Avispa	Limagrain Ibérica S.A.
BL 31	IFAPA	Don Ortega	Agrovegetal S.A.
BL 32	IFAPA	Don Ricardo	Agrovegetal S.A.
BL 33	IFAPA	Egeo	MAS Seeds
BL 34	CIMMYT	Fuego	Semillas Guadalsem S.L.
BL 36	CIMMYT	Kiko Nick	Limagrain Ibérica S.A.
BL 38	CIMMYT	LG Acropolis	Limagrain Ibérica S.A.
BL 39	CIMMYT	LG Hipnosis	Limagrain Ibérica S.A.
BL 40	CIMMYT	LG Origen	Limagrain Ibérica S.A.
BL 41	CIMMYT	Qualidou	Florimond Desprez Ibérica S.A.
BL 43	CIMMYT	RGT Beticur	RAGT Ibérica S.L.U.
BL 44	IFAPA	RGT Fernandur	RAGT Ibérica S.L.U.
BL 45	IFAPA	RGT Leondur	RAGT Ibérica S.L.U.
BL 46	CIMMYT	RGT Rumbadur	RAGT Ibérica S.L.U.
BL 47	IFAPA	RGT Voilur	RAGT Ibérica S.L.U.
BL 48	IFAPA	RGT Xiriur	RAGT Ibérica S.L.U.
BL 49	CIMMYT	Salgado	AGRUSA
BL 50	CIMMYT	Sculptur	RAGT Ibérica S.L.U.
BL 51	CIMMYT	Simeto	PROSEME S.R.L.
BL 52	IFAPA	Sy Leonardo	Syngenta S.A.
		Teodorico	MAS Seeds

[1] IFAPA: Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica. Centro Alameda del Obispo, Córdoba (Spain)

[2] CIMMYT: Centro Internacional de Mejoramiento de Maíz y Trigo, El Batán, Texcoco, México.

Table S4.2. Microscopic fungal stages of *P. triticina* infection in three selected durum wheat accessions under baseline and climate change environments [1].

Accession	Environmental Set	EA– (%)	EA+ (%)	EST– (%)	EST+ (%)
Qualidou	SB	3.27 ± 0.52 a	14.99 ± 1.93 a	66.38 ± 1.16 a	15.36 ± 1.86 b
	S1	3.85 ± 3.28 a	12.02 ± 1.20 a	49.04 ± 4.17 ab	35.09 ± 5.63 a
	S2	2.35 ± 0.88 a	17.45 ± 4.05 a	38.40 ± 10.39 b	41.80 ± 7.08 a
	S1G	8.95 ± 2.82 a	22.55 ± 8.08 a	34.47 ± 3.56 b	34.03 ± 3.55 a
	S2G	6.95 ± 2.83 a	18.34 ± 3.15 a	30.48 ± 8.81 b	44.23 ± 8.07 a
BL 28	SB	7.32 ± 1.59 a	23.06 ± 1.03 a	2.42 ± 1.27 a	67.20 ± 0.78 a
	S1	6.13 ± 3.09 a	32.60 ± 5.93 a	2.40 ± 0.87 a	58.88 ± 2.38 ab
	S2	11.40 ± 0.97 a	31.35 ± 8.15 a	3.80 ± 0.70 a	53.45 ± 8.76 ab
	S1G	13.10 ± 2.67 a	36.75 ± 7.00 a	3.49 ± 1.91 a	46.66 ± 6.40 b
	S2G	12.86 ± 4.12 a	33.45 ± 4.63 a	3.22 ± 0.32 a	50.46 ± 1.96 ab
BL 38	SB	13.29 ± 1.11 ab	71.33 ± 1.81 a	0.00 ± 0.00 a	15.38 ± 1.24 a
	S1	4.20 ± 3.50 b	78.81 ± 3.00 a	0.00 ± 0.00 a	16.99 ± 4.14 a
	S2	14.56 ± 3.01 ab	71.85 ± 5.31 a	0.00 ± 0.00 a	13.59 ± 8.08 ab
	S1G	20.26 ± 6.54 a	76.37 ± 5.53 a	0.00 ± 0.00 a	3.37 ± 1.02 b
	S2G	23.31 ± 4.55 a	73.01 ± 4.11 a	0.00 ± 0.00 a	3.68 ± 0.66 b

[1] Values are mean ± standard error for four leaves evaluated for each environmental set in three different experiments. Data with the same letter within an accession and column are not statistically different (Duncan test, $p < 0.05$). EA–, early-aborted colonies without necrosis; EA+, early-aborted colonies associated with necrosis; EST–, established colonies without necrosis; EST+, established colonies associated with necrosis.

Table S5.1. Commercial cultivars used in Chapter 5 and their source of origin [1].

Reference number	Accession	Breeder	Keeper
19990240	Artur Nick	Limagrain Europe	Limagrain Iberica, S.A.
20090316	Atomo	Limagrain Iberica, S.A.	Limagrain Iberica, S.A.
19970151	Califa Sur	Limagrain Europe	Limagrain Iberica, S.A.
20180178	Ecodesal	INIA/IFAPA/IRTA/ITACYL	IRTA (G. Cataluña)
20090257	Eneas	Different keepers (INIA, IRTA, IFAPA, ITACyL)	Different keepers
20170158	Esperado	INIA-OTRI (INIA, IRTA, IFAPA, ITACyL)	IRTA (G. Cataluña)
20160252	Flish	Eurosemillas	Different keepers
19970152	Galera	Limagrain Europe	Limagrain Iberica, S.A.
20170175	LG Ancia	Limagrain Europe	Limagrain Iberica, S.A.
20160210	LG Antique	Limagrain Europe	Limagrain Europe
20170174	LG Mercurius	Limagrain Europe	Limagrain Iberica, S.A.
20170171	Montemayor	Agrovegetal, S.A.	Agrovegetal, S.A.
20040245	Nogal	Florimond desprez veuve et fils	Florimond desprez veuve et fils
20170185	RGT Chiclanero	RAGT 2n S.A.S	RAGT 2n S.A.S
20130239	RGT Tocayo	RAGT 2n S.A.S	RAGT 2n S.A.S
20180212	Rota	Agrovegetal, S.A.	Agrovegetal, S.A.
20170170	Santaella	Agrovegetal, S.A.	Agrovegetal, S.A.
20060153	Tejada	Agrovegetal, S.A.	Agrovegetal, S.A.
20140229	Tujena	Agrovegetal, S.A.	Agrovegetal, S.A.

[1] Variety details at the Spanish MAPA (Ministerio de Agricultura, Pesca y Alimentación) catalogue web: https://www.mapa.gob.es/app/regVar/BusRegVar.aspx?id=es.

A.II. Supplementary Figures

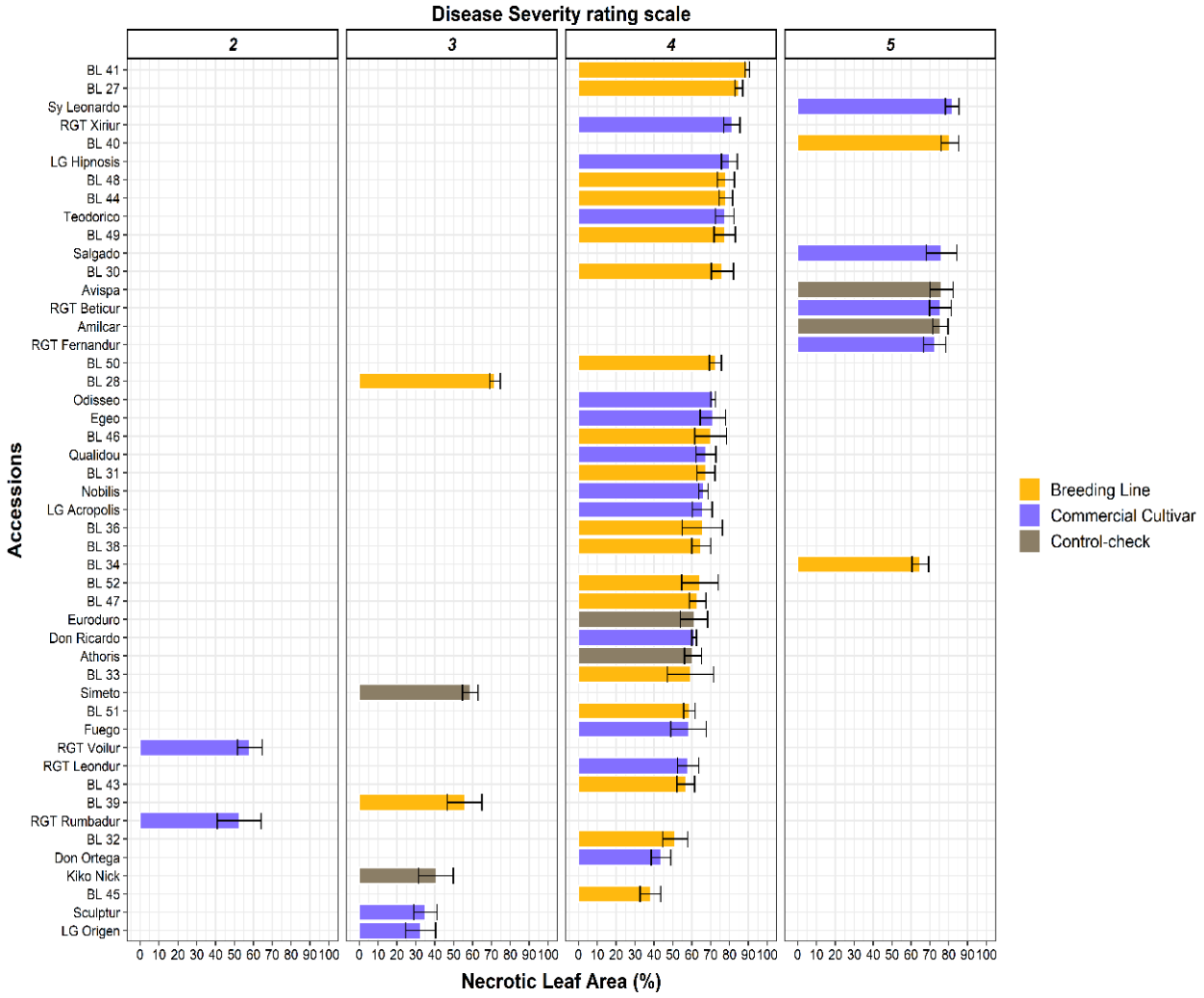

Figure S2.1. Septoria tritici blotch (STB) infection in adult plants of durum wheat accessions. Mean percentage of necrotic leaf area (NLA), presented in columns, and disease severity (DS) rating scale, presented as numbers at the top of the figure for breeding lines, commercial cultivars, and control-checks. Accessions were arranged according to their mean percentage of NLA and classified by DS in panels. DS is presented according to McCartney *et al.* (2002), see Material and Methods for rating scale. Error bars represent the SE calculated from three independent experiments with eight replicates each.

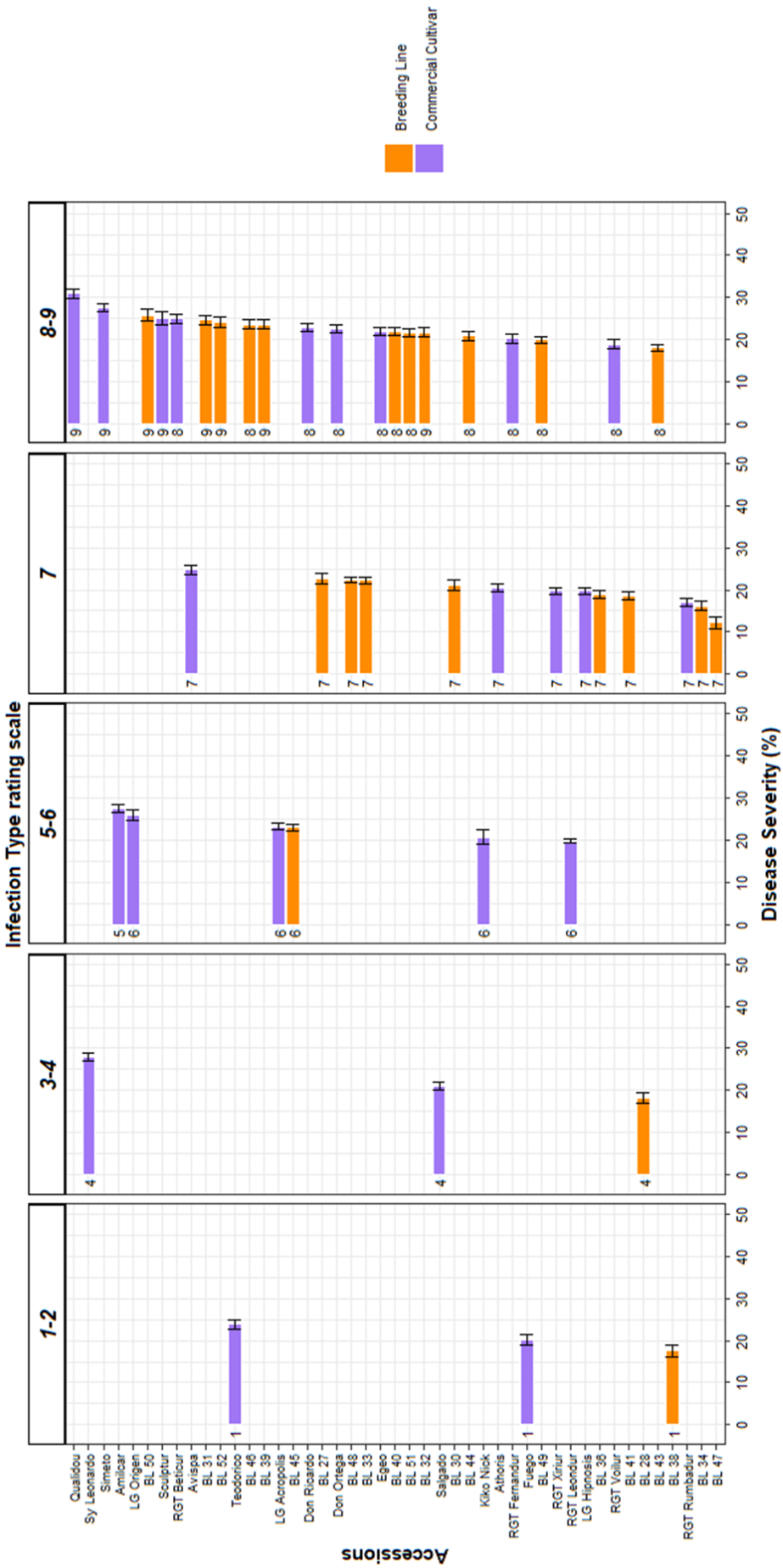

Figure S4.1. *P. triticina* infection in adult plants of durum wheat breeding lines and commercial cultivars. Mean percentage of disease severity (DS), presented in columns, and infection type (IT) rating scale, presented as numbers at the top of the figure. Accessions were arranged according to their mean percentage of DS and classified according to IT in panels. The IT scale is presented according to McNeal *et al.* (1971), where 0 = no visible disease symptoms (immune), 1 = minor chlorotic and necrotic flecks, 2 = chlorotic and necrotic flecks without sporulation, 3-4 = chlorotic and necrotic areas with limited sporulation, 5-6 = chlorotic and necrotic areas with moderate sporulation, 7 = abundant sporulation with moderate chlorosis, 8-9 = abundant and dense sporulation without notable chlorosis and necrosis. Infection types 0-6 were considered resistant, while types 7-9 were considered susceptible. Error bars represent the standard error calculated from three independent experiments with four replicates each.

Figure S5.1. *P. striiformis* infection in adult plants of bread wheat accessions. Mean percentage of disease severity (DS), presented in columns, and infection type (IT) rating scale, presented as numbers at the top of the figure. Accessions were arranged according to their mean percentage of DS and classified according to IT in panels. The IT scale is presented according to McNeal *et al*. (1971), where 0 = no visible disease symptoms (immune), 1 = minor chlorotic and necrotic flecks, 2 = chlorotic and necrotic flecks without sporulation, 3-4 = chlorotic and necrotic areas with limited sporulation, 5-6 = chlorotic and necrotic areas with moderate sporulation, 7 = abundant sporulation with moderate chlorosis, 8-9 = abundant and dense sporulation without notable chlorosis and necrosis. Error bars represent the standard error calculated from three independent experiments with six replicates each.