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RESEARCH ARTICLE

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Behavior of Spanish durum wheat genotypes against *Zymoseptoria tritici*: resistance and susceptibility

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Abstract

Aim of study: 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.

Area of study: The experimental study was conducted in growth chambers at the IFAPA facilities in Córdoba (Spain).

Material and methods: The percentage of necrotic leaf area, the disease severity, and the pycnidium 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.

Main results: 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.

Research highlights: Provided these resources of resistance in Spanish durum wheat genotypes, future breeding programs could be developed, incorporating both agronomic traits and resistance to STB.

Additional key words: Septoria leaf blotch; plant breeding; foliar disease; germplasm

Abbreviations used: DS (disease severity); EU (European Union); IFAPA (Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica); ITS (internal transcribed spacer); NLA (necrotic leaf area); PDA (Potato-Dextrose-Agar); RAEA (Red Andaluza de Experimentación Agraria); RH (relative humidity); STB (Septoria tritici blotch)

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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 (http://www.fao.org/faostat/es/#home). In the European Union (EU) only, wheat covers approximately 26 million hectares with a production of 155 million tons (https://ec.europa.eu/eurostat/). 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 (https://www.wheatinitiative.org/) 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 colonized 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 & Mcdonald, 2005), northern France, Germany, and the United Kingdom, where wheat yield losses can reach 50% in susceptible cultivars (Fones & 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 & 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 & 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 characterization 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 & Solís, 2003), Extremadura, and Catalonia, becoming an important threat, especially for durum wheat cultivars located in Southern Spain (Royo & 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 durum wheat material are available for farmers. Hence, the main objective of this paper was to identify new sources of resistance to *Z. tritici* for being included in current wheat breeding programs. In particular, we analyzed the incidence of STB in 22 durum wheat breeding lines and

26 commercial cultivars from Spain, under controlled conditions in growth chambers.

Material and methods

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.*, 2019), and 22 were breeding lines, belonging to the wheat breeding program being developed at IFAPA (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 (Table S1 [suppl]). 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, characterized 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 (Fig. 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).

Pathogen isolation and molecular characterization

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 & 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 aluminum foil and placed on a shaker at 210 rpm and 18 °C in the dark.

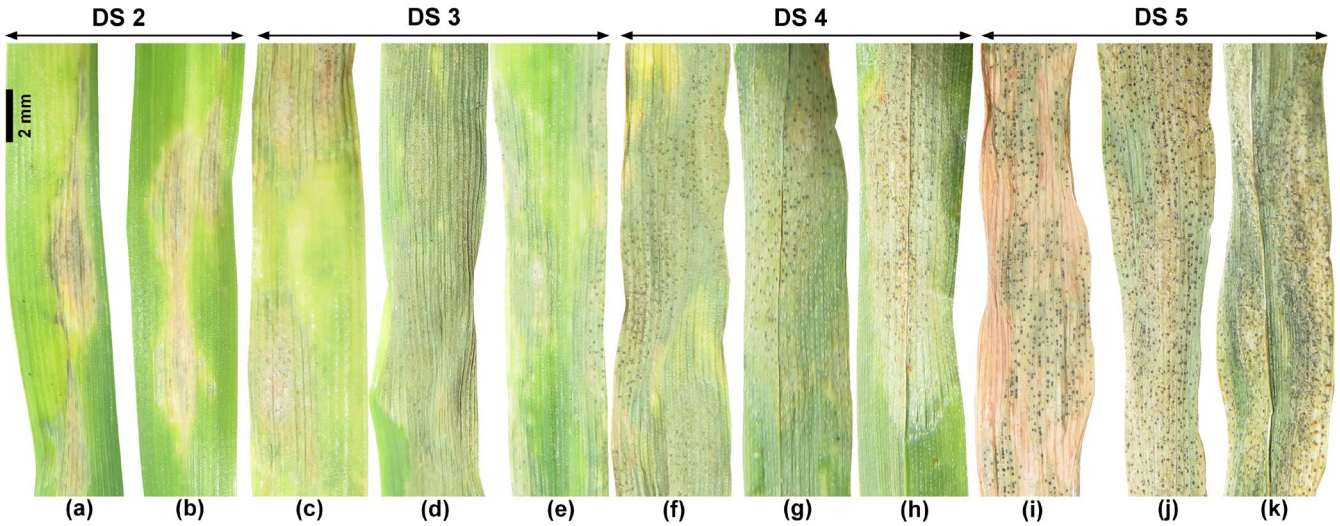

Figure 1. Example of leaves infected with Septoria tritici blotch (STB), showing diverse disease severity (DS) scores. Leaves from breeding lines, commercial cultivars, and control-checks with different DS scores: DS 2, (a) 'RGT Rumbadur' and (b) 'RGT Voilur'; DS 3, (c) 'LG Origen', (d) 'BL 39', and (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.

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 FavorPrep™ 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 at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ITS sequences were deposited at the NCBI database under the SUB9540116 accession number.

Inoculation assays

Our inoculation procedure was developed as described by Stewart & 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.

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 pycnidium 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 (Fig. S1 [suppl]).

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 color 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 analyzed and in two independent experiments.

Data analyses

The experimental design was developed as random blocks. The percentage of NLA covered with pycnidia

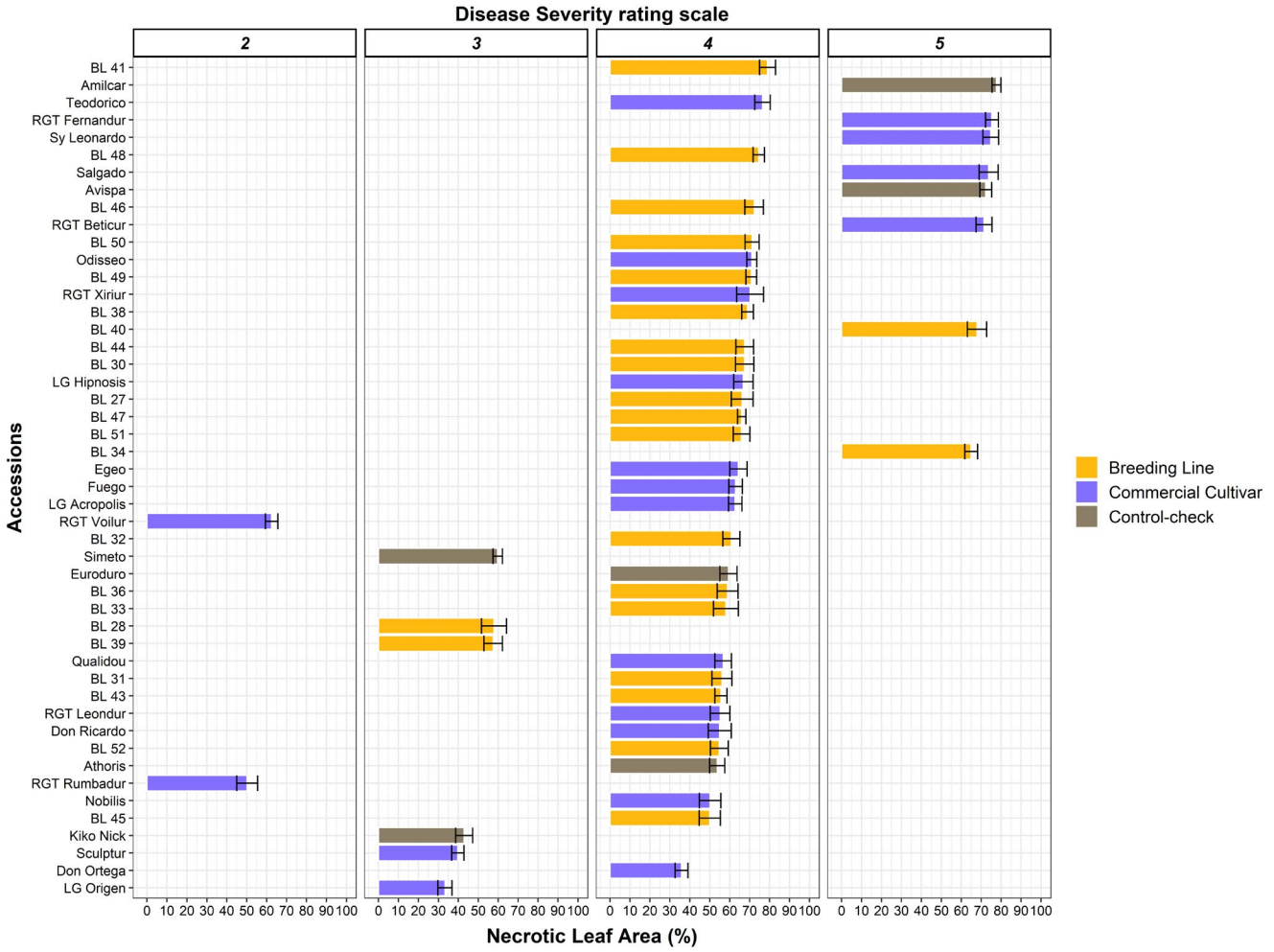

Figure 2. Septoria tritici blotch (STB) infection in durum wheat accessions. Mean percentage of necrotic leaf area (NLA), presented in columns, and disease severity (DS) rating scale, presented as numbers at the top of the figure for breeding lines, commercial cultivars, and control-checks. Accessions 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.

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 = x$^{1/2}$. ANOVA and Tukey's honestly significant difference (HSD) test were performed on these data. Pycnidium size was analyzed 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).

Results

STB infection studies

The development and the aggressiveness of STB in 48 durum wheat accession is shown in Fig. 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% (Fig. 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 (Fig. 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 (Fig. 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 organized to analyze their distribution according to DS and group (Fig. 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 (Fig. 3).

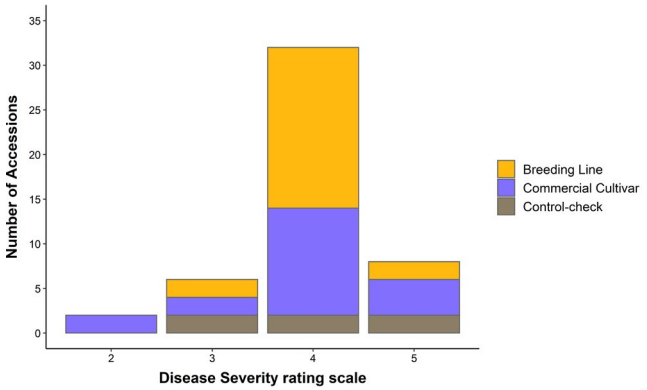

Figure 3. Classification of accessions according their disease severity (DS) (McCartney *et al.*, 2002), characterized as breeding lines, commercial cultivars and control-checks.

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 pycnidium development. An example of leaves showing DS scores of 2-5 amongst breeding lines, commercial cultivars, and control-check cultivars is shown in Fig. 1. Accessions with a DS score of 2 expressed some chlorotic and necrotic lesions without pycnidial development, being considered as resistant (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 1i-k).

Images were then analyzed 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 1). Images were cropped into NLA sections of 25 mm^2 and then analyzed. '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 analyzed, 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 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 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 analyzed,

Table 1. Pycnidium parameters in NLA lesions of 25 mm² in accessions with different DS scores [1].

Accessions	DS	NLA covered with pycnidia (%)	Pycnidium count (P)	Pycnidia/NLA (P/mm²)	Pycnidium size (mm² × 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, $p<0.05$).

respectively, which translates to 6.92, 7.92, and 8.40 pycnidia per mm² of NLA (Table 1). All accessions analyzed 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 mm² of NLA were statistically different among accessions classified with a DS of 3, 4, and 5 (Table 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 1 also shows the parameters analyzed 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 mm² 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 mm² 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.

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 colonized 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 & Mundt, 2016), explaining the current incidence of STB in Spanish wheat-cropping areas, especially in Andalusia (Royo & 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 colonization of leaf tissue by the fungus, but also presented a significant reduction in pycnidium 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 pycnidium 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 (Fig. 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 & 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 analyzed with specific image software (Fig. 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 pycnidium 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 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 & 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 & McDonald (2014), whereby scanned images of infected leaves are analyzed 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 defense 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.

References

Banke S, Mcdonald BA, 2005. Migration patterns among global populations of the pathogenic fungus *Mycosphaerella graminicola*. Mol Ecol 14: 1881-1896. https://doi.org/10.1111/j.1365-294X.2005.02536.x

Benbelkacem A, Djenadi C, Meamiche H, 2016. Mitigation of the global threat of Septoria Leaf Blotch of cereals in Algeria. Int J Res Stud Agric Sci 2: 28-35. https://doi.org/10.20431/2455-6224.0202005

Berraies S, Ammar K, Gharbi MS, Yahyaoui A, Rezgui S, 2014. Quantitative inheritance of resistance to Septoria Tritici Blotch in durum wheat in Tunisia. Chil J Agric Res 74: 35-40. https://doi.org/10.4067/S0718-58392014000100006

Brading PA, Verstappen ECP, Kema GHJ, Brown JKM, 2002. A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the Septoria Tritici Blotch pathogen. Phytopathology 92: 439-445. https://doi.org/10.1094/PHYTO.2002.92.4.439

Brown JKM, Chartrain L, Lasserre-Zuber P, Saintenac C, 2015. Genetics of resistance to *Zymoseptoria tritici* and applications to wheat breeding. Fungal Genet Biol 79: 33-41. https://doi.org/10.1016/j.fgb.2015.04.017

Castilla A, Perea F, Sillero JC, Basallote E, Soriano MT, Canseco E, 2019. Resultados de ensayos de nuevas variedades de trigo duro en Andalucía Campaña 2018/2019. Junta de Andalucía. Cons Agric Ganad Pesc Des Sost, IFAPA, 41 p. http://www.servifapa.es [15 Jun 2020].

Cátedra M, Solís I, 2003. Effect of a fungicide treatment on yield and quality parameters of new varieties of durum wheat (*Triticum turgidum* L. ssp. *durum*) and bread wheat (*Triticum aestivum* L.) in western Andalusia. Span J Agric Res 1: 19-26. https://doi.org/10.5424/sjar/2003013-31

Chartrain L, Brading PA, Makepeace JC, Brown JKM, 2004. Sources of resistance to Septoria Tritici Blotch and implications for wheat breeding. Plant Pathol 53: 454-460. https://doi.org/10.1111/j.1365-3059.2004.01052.x

Chedli RBH, M'barek SB, Yahyaoui A, Kehel Z, Rezgui S, 2018. Occurrence of Septoria Tritici Blotch (*Zymoseptoria tritici*) disease on durum wheat, triticale, and bread wheat in northern Tunisia. Chil J Agric Res 78: 559-568.

Ferjaoui S, M'Barek SB, Bahri B, Slimane RB, Hamza S, 2015. Identification of resistance sources to Septoria Tritici blotch in old Tunisian durum wheat germplasm applied for the analysis of the *Zymoseptoria tritici*-durum wheat interaction. J Plant Pathol 97: 471-481.

Fones H, Gurr S, 2015. The impact of Septoria Tritici Blotch disease on wheat: An EU perspective. Fungal Genet Biol 79: 3-7. https://doi.org/10.1016/j.fgb.2015.04.004

Gerard GS, Börner A, Lohwasser U, Simón MR, 2017. Genome-wide association mapping of genetic factors controlling Septoria Tritici Blotch resistance and their associations with plant height and heading date in wheat. Euphytica 213 (27): 1-16. https://doi.org/10.1007/s10681-016-1820-1

Ghaneie A, Mehrabi R, Safaie N, Abrinbana M, Saidi A, Aghaee M, 2012. Genetic variation for resistance to Septoria Tritici Blotch in Iranian tetraploid wheat landraces. Eur J Plant Pathol 132: 191-202. https://doi.org/10.1007/s10658-011-9862-7

Hosseinnezhad A, Khodarahmi M, Rezaee S, Mehrabi R, Roohparvar R, 2014. Effectiveness determination of wheat genotypes and Stb resistance genes against Iranian *Mycosphaerella graminicola* isolates. Arch Phytopath Plant Prot 47: 2051-2069. https://doi.org/10.1080/03235408.2013.868696

Karisto P, Hund A, Yu K, Anderegg J, Walter A, Mascher F, *et al.*, 2018. Ranking quantitative resistance to Septoria Tritici Blotch in elite wheat cultivars using automated image analysis. Phytopathology 108: 568-581. https://doi.org/10.1094/PHYTO-04-17-0163-R

Kema GHJ, Verstappen ECP, Waalwijk C, 2000. Avirulence in the wheat Septoria Tritici Leaf Blotch fungus *Mycosphaerella graminicola* is controlled by a single locus. Mol Plant-Microbe Interact 13: 1375-1379. https://doi.org/10.1094/MPMI.2000.13.12.1375

Kidane YG, Hailemariam BN, Mengistu DK, Fadda C, Pè ME, Dell'Acqua M, 2017. Genome-wide association study of Septoria Tritici Blotch resistance in Ethiopian durum wheat landraces. Front Plant Sci 8: 1-12. https://doi.org/10.3389/fpls.2017.01586

Linde CC, Zhan J, McDonald BA, 2002. Population structure of *Mycosphaerella graminicola*: From lesions to continents. Phytopathology 92: 946-955. https://doi.org/10.1094/PHYTO.2002.92.9.946

McCartney CA, Brûlé-Babel AL, Lamari L, 2002. Inheritance of race-specific resistance to *Mycosphaerella graminicola* in wheat. Phytopathology 92: 138-144. https://doi.org/10.1094/PHYTO.2002.92.2.138

McDonald BA, Mundt CC, 2016. How knowledge of pathogen population biology informs management of Septoria Tritici Blotch. Phytopathology 106: 948-955. https://doi.org/10.1094/PHYTO-03-16-0131-RVW

Niks RE, Qi X, Marcel TC, 2015. Quantitative resistance to biotrophic filamentous plant pathogens: concepts, misconceptions, and mechanisms. Annu Rev Phytopathol 53: 445-470. https://doi.org/10.1146/annurev-phyto-080614-115928

Oerke EC, 2006. Crop losses to pests. J Agric Sci 144: 31-43. https://doi.org/10.1017/S0021859605005708

Quaedvlieg W, Kema GHJ, Groenewald JZ, Verkley GJM, Seifbarghi S, Razavi M, *et al.*, 2011. Zymoseptoria gen. nov.: A new genus to accommodate Septoria-like species occurring on graminicolous hosts. Persoonia Mol Phylogeny Evol Fungi 26: 57-69. https://doi.org/10.3767/003158511X571841

R Core Team, 2020. R: A language and environment for statistical computing. R Found. Stat. Comput. Vienna, Austria. https://www.R-project.org/ [15 Jun 2020].

Royo C, Briceño-Félix GA, 2011. Wheat breeding in Spain. In: The world wheat book: A history of wheat breeding; Bonjean AP, Angus WJ, van Ginkel M (eds.). pp: 121-154. Lavoisier Publ, Paris.

Royo C, Soriano JM, Alvaro F, 2017. Wheat: A crop in the bottom of the mediterranean diet pyramid. In: Mediterranean identities - Environment, society, culture; Fuerst-Bjeliš B (ed.). pp: 381-399. InTech, Rijeka (Croatia). https://doi.org/10.5772/intechopen.69184

Schneider C, Rasband W, Eliceiri K, 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671-675. https://doi.org/10.1038/nmeth.2089

Somai-Jemmali L, Randoux B, Siah A, Magnin-Robert M, Halama P, Reignault P, Hamada W, 2017a. Similar infection process and induced defense patterns during compatible interactions between *Zymoseptoria tritici* and both bread and durum wheat species. Eur J Plant Pathol 147: 787-801. https://doi.org/10.1007/s10658-016-1043-2

Somai-Jemmali L, Siah A, Harbaoui K, Fergaoui S, Randoux B, Magnin-Robert M, *et al.*, 2017b. Correlation of fungal penetration, CWDE activities and defense-related genes with resistance of durum wheat cultivars to *Zymoseptoria tritici*. Physiol Mol Plant Pathol 100: 117-125. https://doi.org/10.1016/j.pmpp.2017.08.003

Soriano JM, Malosetti M, Roselló M, Sorrells ME, Royo C, 2017. Dissecting the old Mediterranean durum wheat genetic architecture for phenology, biomass and yield formation by association mapping and QTL meta-analysis. PLoS One 12: 1-19. https://doi.org/10.1371/journal.pone.0178290

Stewart EL, McDonald BA, 2014. Measuring quantitative virulence in the wheat pathogen *Zymoseptoria tritici* using high-throughput automated image analysis. Phytopathology 104: 985-992. https://doi.org/10.1094/PHYTO-11-13-0328-R

Stewart EL, Hagerty CH, Mikaberidze A, Mundt CC, Zhong Z, McDonald BA, 2016. An improved method for measuring quantitative resistance to the wheat pathogen *Zymoseptoria tritici* using high-throughput automated image analysis. Phytopathology 106: 782-788. https://doi.org/10.1094/PHYTO-01-16-0018-R

Suffert F, Sache I, Lannou C, 2011. Early stages of Septoria Tritici Blotch epidemics of winter wheat: Build-up, overseasoning, and release of primary inoculum. Plant Pathol 60: 166-177. https://doi.org/10.1111/j.1365-3059.2010.02369.x

Suffert F, Sache I, Lannou C, 2013. Assessment of quantitative traits of aggressiveness in *Mycosphaerella graminicola* on adult wheat plants. Plant Pathol 62: 1330-1341. https://doi.org/10.1111/ppa.12050

Torriani SFF, Melichar JPE, Mills C, Pain N, Sierotzki H, Courbot M, 2015. *Zymoseptoria tritici*: A major threat to wheat production, integrated approaches to control. Fungal Genet Biol 79: 8-12. https://doi.org/10.1016/j.fgb.2015.04.010

Ünal G, Kayim M, Ay T, Yones AM, 2017. Evaluation of disease intensity and molecular identification of *Zymoseptoria tritici* causing Septoria Leaf Blotch on wheat in the eastern mediterranean region of Turkey. Turk J Agric For 41: 405-413. https://doi.org/10.3906/tar-1703-74

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: A guide to methods and applications; Innis MA *et al.* (eds). pp: 315-322. Academic Press, San Diego, CA, USA. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Zadoks JC, Chang TT, Konzak CF, 1974. A decimal code for the growth stage of cereals. Weed Res 14: 415-421. https://doi.org/10.1111/j.1365-3180.1974.tb01084.x