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Cultivar mixtures for STB control



## Improved control of *Septoria tritici* blotch in durum wheat using cultivar mixtures

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## **Abstract**

Mixtures of cultivars with contrasting levels of disease resistance are capable of suppressing infectious diseases in wheat, as demonstrated in numerous field experiments. Most studies focused on airborne pathogens in bread wheat, while splash-dispersed pathogens have received less attention, and no studies have been conducted in durum wheat. We conducted a field experiment in Tunisia, a major durum wheat producer in the Mediterranean region, to evaluate the performance of cultivar mixtures in controlling the polycyclic, splash-dispersed disease *Septoria tritici* blotch (STB) in durum wheat. To measure STB severity, we used a novel, high-throughput method based on digital analysis of images captured from 1284 infected leaves collected from 42 experimental plots. This method allowed us to quantify pathogen reproduction on wheat leaves and to acquire a large dataset that exceeds previous studies with respect to accuracy and statistical power. Our analyses show that introducing only 25% of a disease-resistant cultivar into a pure stand of a susceptible cultivar provides a substantial reduction in disease -- down to the level of resistant pure stands -- and a corresponding mitigation of yield loss. However, adding a second resistant cultivar to the mixture did not further improve disease control, contrary to predictions of epidemiological theory. Susceptible cultivars are often agronomically superior to resistant cultivars. Hence, if mixtures with only a moderate proportion of the resistant cultivar provide similar degree of disease control as resistant pure stands, as our analysis indicates, such mixtures are more likely to be accepted by growers.

## Introduction

Reduction of biodiversity due to human activities has been linked with increased outbreaks of pests and infectious diseases in wildlife and humans (King & Lively 2012; Ostfeld & Keesing 2012; Civitello et al., 2015). In agroecosystems, a deliberate introduction of genetic diversity into crop plant populations is proposed as a promising way to suppress plant disease epidemics (Wolfe, 1985; Finckh et al., 2000; Mundt, 2002) and enhance the overall crop function (Newton et al., 2009). A possible way to diversify crop plants is to grow two or more genetically distinct cultivars of the same crop concurrently within the same field. This can be achieved by mixing seeds of different cultivars before sowing, thereby creating a physical cultivar mixture.

The idea behind cultivar mixtures is that genetic, physiological, structural, and phenological diversity among the components of the mixture (i.e., among different cultivars that comprise the mixture) may drive beneficial interactions not only between cultivars but also between cultivars and environments (Kiær et al., 2009; Newton et al., 2009; Borg et al., 2018). As a result, cultivar mixtures have proven to improve the resilience to biotic and abiotic stresses in crops and boost yield and its stability compared to pure stands, especially under low pesticide cropping systems (Kiær et al., 2009; Smithson and Lenné, 1996; Borg et al., 2018). Mixtures can also enhance product quality, if the components of the mixture are chosen appropriately (Finckh et al., 2000; Mundt, 2002). For these reasons, cultivation of cultivar mixtures has gained interest in several countries (Borg et al., 2018; de Vallavieille-Pope et al., 2006; Finckh & Wolfe, 1997; Wolfe et al., 2008).

Cultivar mixtures suppress the development of disease epidemics when the mixture components have contrasting levels of resistance to the targeted disease (Wolfe, 1985; Finckh & Wolfe, 2006; Gigot et al., 2013). Consequently, most studies investigated mixtures of disease-susceptible and disease-resistant cultivars (cf. Garrett & Mundt (1999) and Mikaberidze et al. (2015) for discussion on mixtures that contain two or more resistant cultivars). The most important mechanisms of disease reduction in cultivar mixtures are the dilution (or density) effect, the barrier effect, induced resistance (Wolfe, 1985; Finckh et al., 2000), and competition among pathogen strains (Garrett & Mundt 1999). From an evolutionary perspective, appropriately designed mixtures are expected to hamper adaptation of the pathogen and increase the durability of the resistance genes deployed (Finckh et al., 2000; Mundt, 2002).

Many studies presented convincing empirical evidence that cultivar mixtures provide effective control of airborne cereal diseases, particularly rusts and mildews, as reviewed by Wolfe (1985), Finckh et al. (2000), and Mundt (2002). See also a meta-analysis of 11 publications on stripe (yellow) rust of wheat

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(Huang et al., 2012). Effect of cultivar mixtures on splash-dispersed cereal diseases is less studied and the disease reduction by mixtures appears less consistent and is on average lower in magnitude compared to airborne diseases (Jeger et al., 1981b; Mundt et al., 1994; Newton et al., 1997; Garrett & Mundt, 1999).

Here, we investigated *Septoria tritici* blotch (STB) disease, which is predominantly splash-dispersed. STB is one of the major threats to wheat production worldwide. It is caused by the fungal pathogen *Zymoseptoria tritici*. The infection cycle of the fungus starts when asexual pycnidiospores or airborne sexual ascospores land on a susceptible wheat leaf. The asymptomatic phase lasts for about 8-14 days. Karisto et al. (2019) observed an extreme asynchronicity in the duration of the asymptomatic phase: lesions appeared continuously during a period of up to 3 weeks, even when a single pathogen strain was used to infect a single host variety. The switch to necrotrophy leads to a collapse and death of the host mesophyll cells usually between 12 and 18 days after penetration (Kema et al., 1996; Duncan & Howard, 2000; Steinberg, 2015). Within necrotic lesions, the fungus begins to reproduce asexually and later sexually (Eyal et al. 1987; Eyal 1999; Ponomarenko et al., 2011). Airborne ascospores typically constitute the primary inoculum released from wheat debris (Suffert & Sache, 2011; Morais et al., 2016). Pycnidiospores are splash-dispersed and are transmitted more locally infecting nearby plants. They are the main secondary inoculum and drive the disease progress during the growing season (Kema et al. 1996; Hunter et al. 1999; Eyal 1999; McDonald & Linde 2002; Ponomarenko et al. 2011). Under conducive conditions, this polycyclic pathogen can complete up to six asexual infection cycles during one growing season. Because of *Z. tritici*'s mixed reproductive system, large population sizes and long-distance dispersal, its populations are extremely diverse (Linde et al., 2002; Hartmann et al., 2018).

Tunisia is a key durum wheat producer in the Mediterranean region but is also the largest per capita wheat consumer in the world (FAO, 2017). Wheat yields in Tunisia have steadily increased over the last century (Gharbi & El Felah, 2013; El Felah & Gharbi, 2014), but remain largely untapped despite their potential value (Latiri et al., 2010). The production is highly affected by drought and diseases and to compensate the deficit, the country has to import 25% of durum wheat regularly in order to cover the local consumption (FAO, 2017; Gharbi & El Felah, 2013). The main growing areas of durum wheat are located in the Northern part of the country under rain-fed conditions of a subhumid climate (Ammar et al., 2011), favourable for fungal diseases. Among these, STB poses an especially serious threat to Tunisian wheat production. Control of STB in Tunisia relies largely on fungicides and resistant cultivars. The use of chemical compounds has been adopted by Tunisian durum wheat growers at a slower pace as compared to bread wheat growers in Europe. With the first occurrences of strobilurin (QoI)

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resistance in Tunisia reported in 2012 (Boukef et al., 2012), current fungicide treatments in Tunisia rely mainly on DMIs (demethylation inhibitors) or mixtures of DMIs with strobilurins (Taher et al., 2014). At the same time, chemical control is costly and poses substantial risks to the environment and human health. Both factors give the farmers an incentive to reduce or avoid the use of fungicides.

The vast majority of commercial cultivars in Tunisia are highly susceptible to STB and new, STB-resistant cultivars are released at a very slow rate (Ammar et al., 2011). The variety Karim, released in 1980, covers more than 60% of the durum wheat acreage (Ammar et al., 2011; Ben Hamouda et al., 2016; Rezgui et al., 2008; Ben Mohamed et al., 2000). Even though highly susceptible to STB, favorable agricultural properties combined with its relatively low gluten and low yellow flour content (Ammar et al., 2011) made it the farmer's favorite. In the last decade, a few STB-resistant cultivars were released in Tunisia, including 'Salim' that was registered in 2010. At the time of release, Salim showed resistance to STB (Gharbi & El Felah, 2013), but it is gradually becoming more susceptible (Bel Hadj Chedli et al., 2018). Among recently imported cultivars released in Tunisia, 'Monastir' is resistant to STB (SOSEM, 2018; Bel Hadj Chedli et al., 2018).

Currently, the two methods to control STB, fungicides and a few resistant cultivars, available to Tunisian farmers are not providing satisfactory levels of disease control. The country experiences serious recurrent epidemics of STB with yield losses reaching up to 40% (Berraies et al., 2014). Large part of the reason is that many farmers simply do not use either of the two control methods and neglect crop rotation practices. In addition, farmers often cannot afford fungicides. Those farmers who do use either of the two control methods exert a strong directional selection on pathogen populations. As a result, both fungicides and resistant cultivars are likely to rapidly lose efficacy against *Z. tritici* that has a high evolutionary potential (McDonald & Linde, 2002). Thus, there is an urgent need to reconsider the way STB is controlled in Tunisia and devise management strategies that are not only efficient and sustainable, but also likely to be accepted by growers. Cultivar mixtures may prove to be such a strategy that is particularly well-suited to Tunisian conditions.

Despite the importance of durum wheat, the bulk of research on host resistance to STB has been conducted in bread wheat (*Triticum aestivum*) (Kollers et al., 2013; Brown et al., 2015; Karisto et al., 2018; Yates et al., 2018). At present, our understanding of genetics and molecular basis of STB-resistance in durum wheat is limited. However, this situation may improve with the recent studies on Ethiopian durum wheat landraces (Kidane et al., 2017), Tunisian landraces (Aouini, 2018), and the publication of the fully assembled durum wheat genome (Maccaferri et al., 2019).

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Similarly, cultivar mixtures for controlling STB have been studied in bread wheat but not in durum wheat (Mundt et al., 1995; Mille & Jouan, 1997; Cowger & Mundt, 2002; Mille et al., 2006; Gigot et al., 2013; Vidal et al., 2017). In most cases, STB severity in cultivar mixtures is moderately reduced compared to the expectation based on the severity in the pure stands, although Cowger & Mundt (2002) did not observe a consistent pattern. In particular, Gigot et al. (2013) found that a susceptible cultivar was consistently protected in a mixture under low to moderate STB levels. Subsequent greenhouse experimentation (Vidal et al., 2017) and a modelling study (Vidal et al., 2018) have demonstrated the importance of canopy structure for the efficacy of a mixture.

The previous studies have not systematically investigated the effect of the proportion of different cultivars in the mixture (mixing proportion) on its efficacy against STB. This investigation is important both fundamentally and practically. Fundamentally, it provides a more robust and reliable measure of mixture efficacy across the whole range of mixing ratios, which may help to distinguish between different mechanisms of disease reduction in mixtures. From a practical perspective, growers may accept more easily mixtures that contain a small proportion of the resistant cultivar but still provide satisfactory disease control.

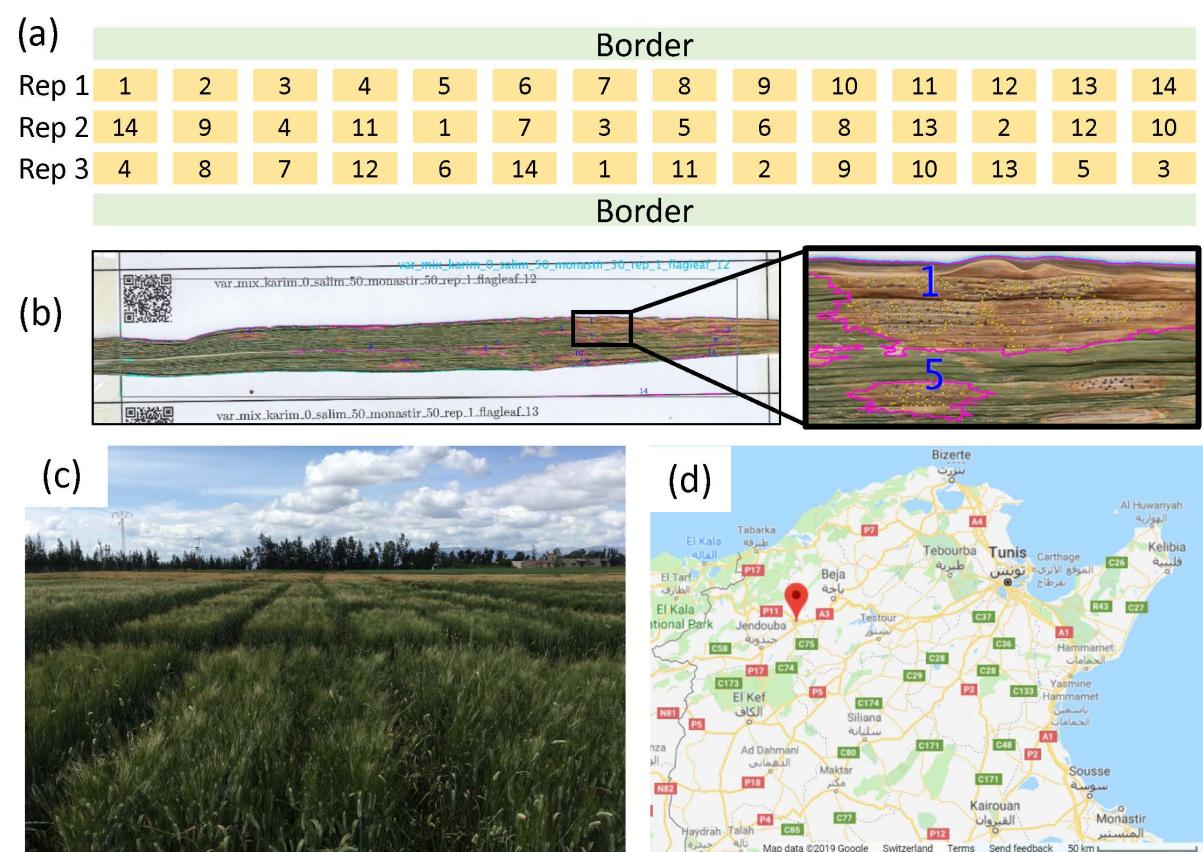
The number of different cultivars, or components, that comprise a mixture is another important parameter that affects the efficacy of disease control. Mille & Jouan (1997) and Mille et al. (2006) considered mixtures with more than two components to control STB, but they only used equal proportions of cultivars in mixtures. For this reason, the proportion of the susceptible component varied between two-way, three-way and four-way mixtures. This precluded consistent testing of whether more than one resistant component in a mixture further improves disease control, as predicted by epidemiological modelling (Mikaberidze et al., 2015).

The objectives of this study were to fill the gaps in current knowledge identified above. We investigate how the proportion of the cultivars in the mixture influences the efficacy of the mixture in controlling STB. Additionally, we determine whether a mixture that contains two resistant components improves the control of STB compared to a mixture with only one resistant component.

## Materials and methods

### Study area and experimental design

Field experiment was conducted during 2017-2018 wheat growing season at the CRP Wheat Septoria Precision Phenotyping Platform -- experimental station of Kodia, which is located in the semi-arid region (36°32'51.89"N, 9°0'40.73"E, governorate of Jendouba, Tunisia, ure 1d). The average annual rainfall in this area varies from 400 to 500 mm and the temperature usually ranges between 9.8°C (average minimum temperature) and 33°C (average maximum temperature). Daily temperature in the governorate of Jendouba and daily precipitation at the experimental station are shown in Figure S1. This region is considered to be a natural hot spot for STB disease.



**Figure 1.** (a) Experimental field layout. Orange: experimental plots (1.5m x 3.5m), numbers correspond to different treatments (Table 1), each of them replicated three times. Green: border plots planted with cultivars Nasr and INRAT100. (b) Example of the automated leaf image analysis. Data is assigned to leaf label based on the QR-code. Cyan lines represent the leaf boundary, purple lines show lesion boundaries, and yellow circles represent detected pycnidia. Small lesions are not analysed. (c) A photograph of the experimental plots. (d) Location of the experimental site. The map from Google.

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Three commercial durum wheat cultivars (Karim, Salim and Monastir) were chosen based on their contrasting scores of resistance to STB, similar earliness and plant height. The susceptible cultivar Karim was originally selected from an introduced F4 bulk from CIMMYT. Cultivar Salim was selected from a cross made in Tunisia in 1993. Cultivar Monastir was imported from France and released in 2012 (SOSEM, 2018). In addition to the three cultivars and their mixtures, we included pure stands of INRAT100, a promising variety that has been registered in 2017 but is not yet released. There were 14 treatments in total: four pure stands, seven two-way mixtures and three three-way mixtures (Table 1). The mixtures with Karim contained always 25%, 50% or 75% of Karim, the rest being resistant cultivars.

### Preparation of plant and fungal material

Seed of different cultivars were mixed in the drill according to the proportions of seed numbers corresponding to each treatment. Seed numbers were adjusted by weighing the seeds and using thousand-kernel-weight (TKW) of each cultivar. The seeds were sown on December 27, 2017, on plots of 5.25 m<sup>2</sup> (3.5 m long, six rows, 25 cm row width) with a precision seeder at a density of 400 seeds m<sup>-2</sup>, which is comparable to common practices in the region. Subsequently, mean density of spikes was found to be 381 spikes m<sup>-2</sup>, and it exhibited no differences between treatments (Kruskal-Wallis p=0.57). The experimental layout was a randomised complete block design with three replicates. Spatial arrangement of treatments and replicates is shown in Figure 1a.

Standard agronomic practices were used to ensure adequate crop development. Herbicide treatment with a mixture of Traxos (1.2 L ha<sup>-1</sup>, Syngenta) and Zoom (180g ha<sup>-1</sup>, Syngenta) was applied on January 25, 2018, at tillering stage corresponding to GS13 [according to Zadoks scale (Zadoks et al., 1974)]. Manual and mechanical weeding was performed on March 19, 2018, (corresponding to stem elongation). Nitrogen fertilizer ammonium nitrate was applied three times at rates 120 kg ha<sup>-1</sup>, 150 kg ha<sup>-1</sup> and 150 kg ha<sup>-1</sup>, on February 1, February 25 and April 1, 2018, respectively. No fungicides or insecticides were applied.

The inoculum of *Z. tritici* was produced in the wheat Septoria platform laboratory according to Ferjaoui et al. (2015) with slight modifications. Six isolates were obtained from infected leaves of durum wheat collected in the same region, and used to prepare the inoculum. The isolates were grown for 6 to 8 days on potato dextrose agar. Inoculum was prepared in 250 mL yeast-glucose liquid medium (30 g glucose, 10 g yeast in 1 L of water). The flasks were inoculated with fresh pieces of *Z. tritici* colonies from agar plates and incubated in a rotary shaker at 100 rpm, at 15°C for 5-7 days. The inoculum concentration was adjusted to 10<sup>6</sup> spores mL<sup>-1</sup> and was the resulting spore suspension was supplemented with 0.1 % of Tween 20 (Merck, UK) prior to inoculation in the field. Approximately 700

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mL of the spore suspension was applied per plot using a sprayer (Efco AT800, Italy). Wheat plants were inoculated three times, on February 27, March 9, and March 20, 2018, corresponding to tillering stage (from GS13 to GS26).

### Disease assessment

Disease levels were assessed two times, at  $t_1$ , on 22 April (GS 61) on flag-1 leaves (the leaf below the flag leaf) and at  $t_2$ , on 9 May (GS 75) on flag leaves. From each plot, 24 leaves were collected at random, without considering their infection status. The collector bias was minimized by a stringent collection protocol: first a spike was chosen at random without looking at leaves and only after that the leaf below this spike was collected. During the collection, the leaves were placed in paper envelopes and kept on ice in a thermo-insulated box. After collection, the leaves were taken to the lab and kept at 4-5°C for one to three days before inspection. The leaves were then inspected visually for the presence of pycnidia as a sign of STB infection. The absence of pycnidia was interpreted as absence of STB, even if necrotic lesions were visible. In this way, STB incidence was estimated as the proportion of infected leaves in each plot. After visual examination, the infected leaves were mounted on paper sheets and scanned, as described by Karisto et al. (2018).

Scanned leaf images were analysed using ImageJ software (Schindelin et al., 2015) with the help of the automated image analysis macro originally developed by Stewart and McDonald (2014) and further improved by Stewart et al. (2016) and Karisto et al. (2018). The program quantified necrotic leaf area and counted pycnidia on each infected leaf as measures of conditional severity of STB, i.e. the severity only on infected leaves. From this raw data, three quantities were computed: (i) the percentage of leaf area covered by lesions (PLACL), (ii) the density of pycnidia per  $\text{cm}^2$  of lesion area ( $\rho_{\text{lesion}}$ ), and (iii) the density of pycnidia per  $\text{cm}^2$  of total leaf area ( $\rho_{\text{leaf}}$ ). The three quantities characterise different aspects of conditional severity of STB. PLACL measures the damage induced by the pathogen to the host,  $\rho_{\text{lesion}}$  measures the degree of pathogen reproduction within the host, and  $\rho_{\text{leaf}}$  is the product of PLACL and  $\rho_{\text{lesion}}$  that incorporates both host damage and pathogen reproduction (Karisto et al., 2018). To estimate the full, unconditional severity, each of the three measures of conditional severity were multiplied by disease incidence of the corresponding plot.

At both time points, 200 leaf images were selected to test the accuracy of the automated image analysis. They were first inspected qualitatively for lesion and pycnidia detection errors (i.e. substantial underestimation or overestimation). After excluding erroneous leaves, the accuracy of automated counting of pycnidia was evaluated by counting pycnidia manually on 20 leaves at each time point and comparing the manual counts to the estimates from the image analysis. The leaves for both qualitative

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and quantitative evaluation were selected with a stratified random sampling based on 10 classes for pycnidia counts (limits of the classes were: 0, 60, 130, 210, 300, 400, 550, 700, 900, 1500, 10000) to ensure a satisfactory coverage of the entire distribution of pycnidia counts. To quantify the accuracy of automated pycnidia counts with respect to manual counts, we calculated the concordance correlation coefficient (Lin 1989), Pearson's and Spearman's correlation coefficients, and the average error of automated pycnidia counts .

## **Yield assessment**

In all plots, the grain yield ( $\text{kg ha}^{-1}$ ) and the TKW (g) were measured. When the plants in all plots had reached physiological maturity (Zadoks et al., 1974), the entire plot area was harvested. Grain yield was calculated by determining the total grain weight in the harvested area ( $\text{kg ha}^{-1}$ ). TKW was estimated by weighing 500 kernels.

## **Statistical analysis**

Treatments were compared to each other based on the three measures of the full STB severity described above: PLACL,  $\rho_{\text{lesion}}$ , and  $\rho_{\text{leaf}}$ . From now on, we will use these three measures of severity to refer to full severity, unless specified otherwise. The comparisons were performed in three groups: two-way mixtures of Karim and Salim (treatments 1–5); two-way mixtures of Karim and Monastir (treatments 5–9); and three-way mixture treatments (5, 10–13, Table 1).

The data was analysed using Python programming language (version 3.5.2, <https://www.python.org>), using the open-source packages scipy (version 1.0.1; Jones et al., 2001), numpy (version 1.14.3) and matplotlib (version 2.2.3). The source code developed for the analysis is available as a supplement (Text S1). Presence of global differences between medians of treatments was tested by Kruskal-Wallis test implemented as `scipy.stats.kruskal` function. Post-hoc comparisons were conducted with multiple Dunn's test with the Bonferroni correction using the library `scikit_posthocs` (version 0.3.8). Separate comparisons were conducted for each of the two time points. Due to the limited number of replicates, strong conclusions cannot be drawn from individual treatments and time points. Hence, after performing the comparisons within each of the three groups separately, all data except for treatment 14 were pooled at each time point based on the proportion of the susceptible cultivar Karim in the mixture. The analysis of the pooled data was conducted in a similar fashion as the analysis within the individual treatment groups described above. The aim of this analysis was to identify general patterns of how disease levels depend on the proportion of the resistant cultivar in the mixture.

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Finally, we tested for mixture effects with the following procedure. Expected levels of disease and yield were calculated for each mixture based on the average of the two pure stands (100% susceptible and 0% susceptible), weighted according to their proportions in the mixture (linear expectation). Note that in three-way mixtures the resistant cultivars were always in equal proportions and thus 0% susceptible corresponds to treatment 10 (Salim 50%, Monastir 50%, see Table 1). The deviations from the linear expectation were calculated for each plot. Then the treatments were pooled based on the percentage of the susceptible cultivar as above and Wilcoxon signed rank test (scipy.stats.wilcoxon) was used to determine if those deviations were symmetric around zero. Comparison of treatments with respect to yield was based on TKW and grain yield.

## Results

### Overview of the dataset

*Septoria tritici* blotch severity was assessed at two time points on all 42 plots. In total, 2021 leaves were inspected for the presence of the disease. Total number of infected leaves included in the image analysis for measuring severity was 1284 (646 leaves at  $t_1$  for flag-1; 638 leaves at  $t_2$  for flag). Based on the qualitative check, 10 leaves from  $t_1$  collection and 26 leaves from  $t_2$  collection were excluded from the dataset. The most common reasons for exclusion were overestimation of lesion area due to insect damage and overestimation of pycnidia counts due to dirt on leaves. Overall, the pycnidia counts were consistent with the manual counting (concordance correlation coefficient = 0.94;  $r_{\text{spearman}} = 0.90$ ,  $p = 5 \times 10^{-15}$ ; Fig. S2). The final data set based on 1248 leaf images is available from the Dryad Digital Repository (xxx link to be inserted after the acceptance of the manuscript).

We observed considerable disease levels on both flag-1 leaves at  $t_1$  and flag leaves at  $t_2$ . Mean incidence over all plot measurements was 64% (range from 8.3% to 100%). Average percentage of leaf area covered by lesions (PLACL) over two timepoints based on plot measurements was 16% (range from 0.25% to 62%), average pycnidia density within lesions ( $p_{\text{lesion}}$ ) was 75 pycnidia  $\text{cm}^{-2}$  (range from 6 to 213 pycnidia  $\text{cm}^{-2}$ ) and average pycnidia count per leaf was 326 (range from 6.2 to 1497). Disease levels were higher in the pure stands of the susceptible cultivar (Karim) than in the pure stands of the two resistant cultivars (Salim and Monastir) or mixtures. Better performance of the resistant cultivars and mixtures compared to Karim was consistent between the two time points (compare left and right columns of Fig. S3 for pure stands and Fig. 3 for mixtures).

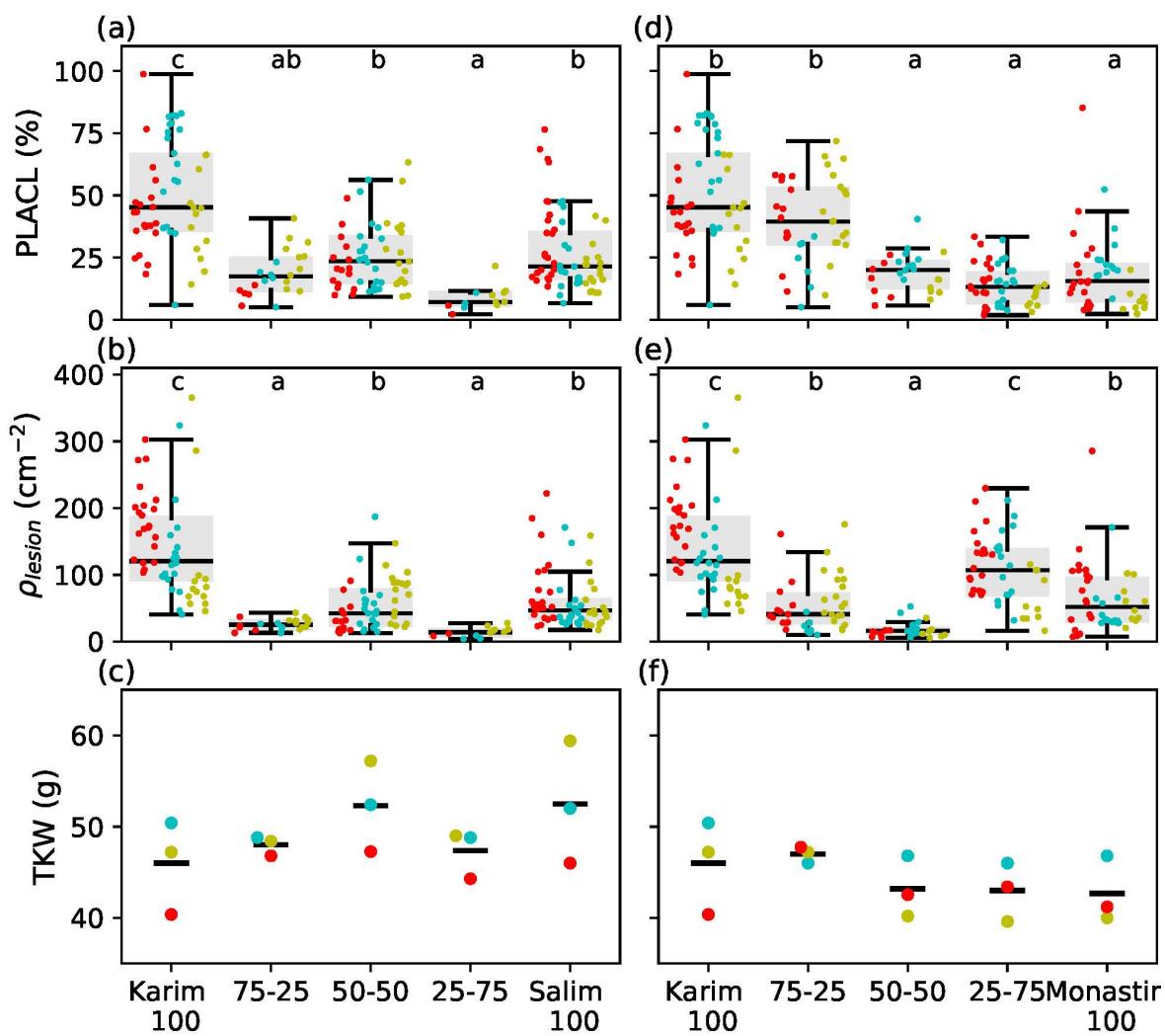
### Performance of the mixtures

Analysis of disease levels in durum wheat mixtures revealed a general pattern where the pure stand of the susceptible cultivar Karim had the highest disease levels and treatments including a resistant

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cultivar had significantly lower disease levels. In most cases, introducing only 25% of a resistant component to the susceptible pure stand was enough to suppress the disease to levels not significantly different from the resistant pure stand. These trends were generally true for all three measures of severity, both two-way and three-way mixtures and the two time points with a few exceptions (see supplementary Fig. S4 for  $t_1$  and Fig. S5 for  $t_2$ ). Results for the two-way mixtures at  $t_2$  are shown for PLACL (Fig. 2a, d) and  $\rho_{lesion}$  (Fig. 2b, e) to illustrate the general tendencies.



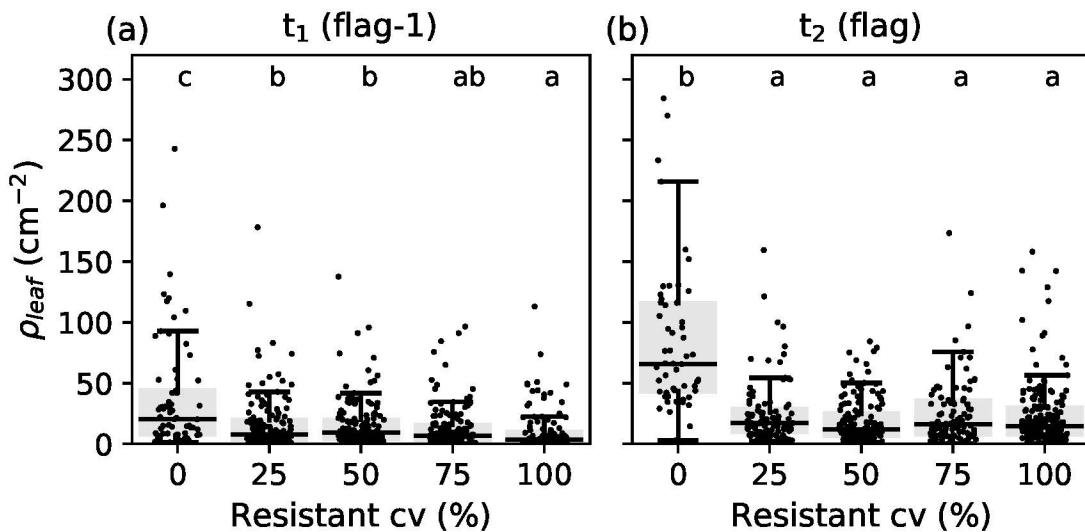
**Figure 2.** STB severity at  $t_2$  (flag leaves) and yield in Karim-Salim two-way mixtures (panels a-c) and Karim-Monastir two-way mixtures (d-f). STB severity measured as PLACL (a, d) and  $\rho_{lesion}$  (b, e). Yield measured in TKW (c, f). Red, cyan and yellow dots represent first, second and third replicate plots, respectively. The box plots show median (horizontal black line) and quartiles (grey box) of the data, whiskers extend outside the box up to a maximum of 1.5 times the interquartile range or to an extreme of the distribution if that is closer. Letters above the box plots are shown to indicate statistical significance of pairwise differences ( $p < 0.05$ ) if the global test indicated presence of differences ( $p < 0.05$ ). Box plots and letters in the following plots follow the same structure and logic. Black bars on yield data (c, f) show means of three replicates.

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For yield measures, there was neither a clear increase or decrease when the proportion of the susceptible cultivar Karim was decreased from 100% to zero, because the susceptible cultivar Karim has intermediate yield in terms of both TKW and grain yield compared to Salim and Monastir. Salim (resistant) had higher TKW and lower grain yield than Karim, while Monastir (resistant) had lower TKW but higher grain yield than Karim (Fig. 2c, f and Fig. S5j, k, m, n show the yield measurements).

To consolidate our observations of disease severity patterns, we pooled the treatments based on the proportion of the susceptible cultivar. The susceptible cultivar alone had higher severity than other treatments and the other treatments were not significantly different from each other at  $t_2$  (Fig. 3;  $t_1$ : global Kruskal-Wallis test  $W=61.2$ ,  $p=1.7 \times 10^{-12}$ ;  $t_2$ :  $W=98.1$ ,  $p=2.5 \times 10^{-20}$ , pairwise comparisons:  $p < 10^{-10}$  when comparing susceptible Karim to others, and  $p > 0.26$  for other comparisons). Comparisons with pooled data for all variables are shown in Figure S6.



**Figure 3.** Disease severity patterns measured in terms of  $\rho_{leaf}$  versus the proportion of resistant component. Data from different treatments is pooled based on the proportion of the resistant component in the mixture. Panel (a) shows  $t_1$  data, panel (b) shows  $t_2$  data.

### Mixture effects

If each component of a mixture performed independently of other components, then the overall disease severity in a mixed stand would be given by the sum of disease severities in corresponding pure stands weighted by the mixing proportions. However, mixture effects may improve the overall performance compared to the sum of individual components. To determine the magnitude of mixture effects, we compared disease severity and yield observed in mixed stands to linear expectations based

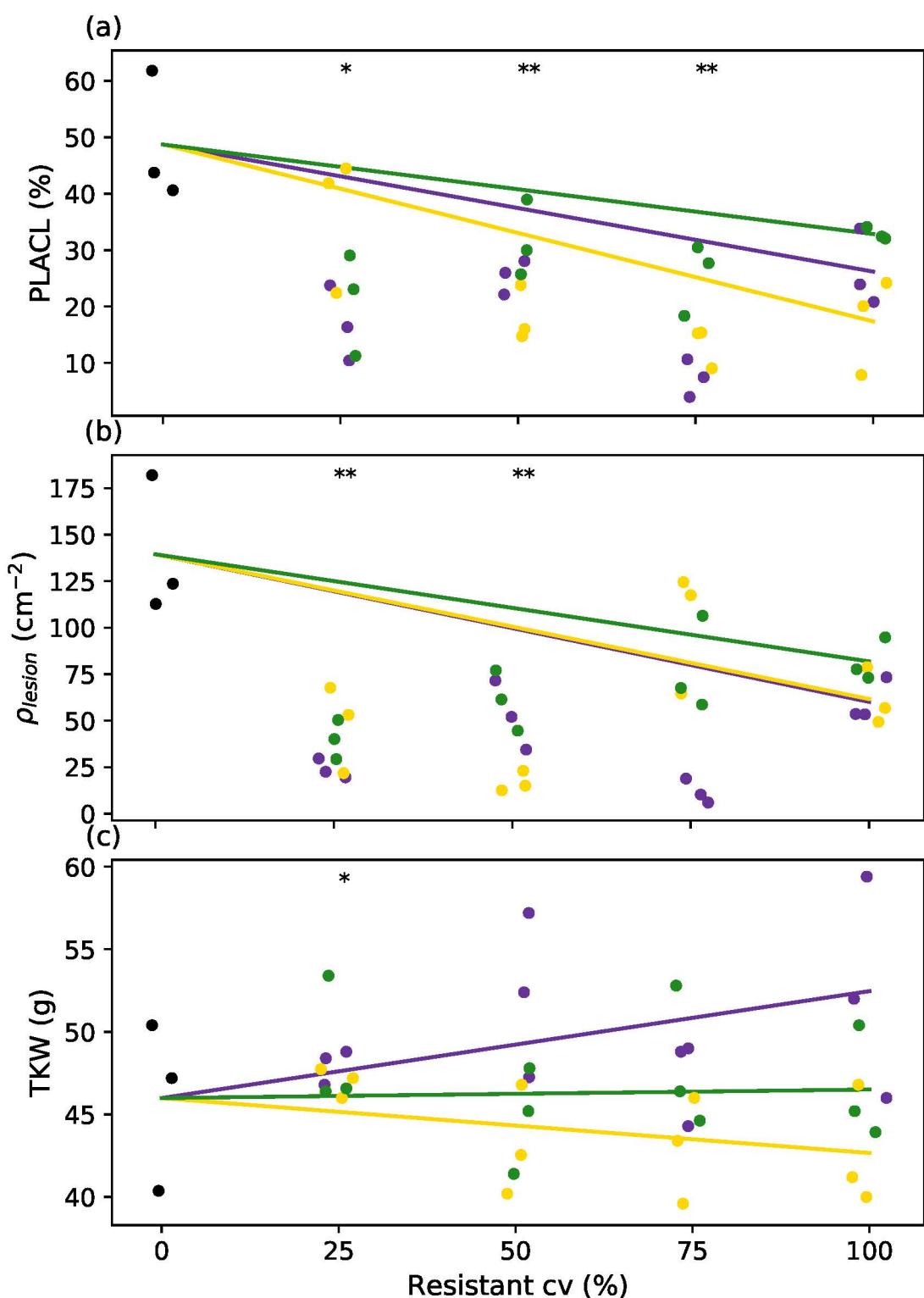
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on measurements in pure stands. Comparing mixture treatments to linear expectations of each treatment group (Karim-Salim; Karim-Monastir; three-way) showed that adding 25% of resistance to susceptible Karim resulted in significant beneficial mixture effects for yield and disease levels ( $t_2$ : Fig. 4; PLACL  $p=0.021$ , mean effect = -18%-points;  $\rho_{lesion}$   $p=0.0077$ , mean effect = -84 pycnidia  $cm^{-2}$ ; TKW  $p=0.028$ , mean effect = +1.6g). Additionally, the host damage levels (PLACL) were lower than expected also at 50% and 75% of resistance ( $p=0.0077$ , for both, mean effects -12%-point and -16%-point, respectively), and the pathogen reproduction ( $\rho_{lesion}$ ) was lower than expected at 50% resistance ( $p=0.0077$ , mean effect = -60 pycnidia  $cm^{-2}$ ). See Figure S7 for a full picture of mixture effects on STB severity and yield at both time points.

#### **Effect of the number of resistant components in the mixture**

To determine whether adding a second resistant cultivar to a two-way mixture of a resistant and a susceptible cultivars leads to a further reduction of disease, we compared the STB severity and yield in two- and three-way mixtures at a constant proportion of the susceptible cultivar Karim (collection  $t_1$ : Fig. S8; collection  $t_2$ : Fig. S9; yield data: Fig. S10). For example, at 75% of Karim, we compared the three-way mixture with 75%/12.5%/12.5% proportions of Karim/Salim/Monastir with each of the corresponding two-way mixtures 75%/25% Karim/Salim and 75%/25% Karim/Monastir. In some cases, three-way mixtures had a slightly higher STB severity than both corresponding two-way mixtures; in other cases, three-way mixtures had an intermediate severity with respect to corresponding two-way mixtures. Interestingly, in none of the cases, a three-way mixture exhibited a significant reduction in STB severity with respect to both corresponding two-way mixtures.



**Figure 4.** Non-linear mixture effects on STB severity at  $t_2$  (panels (a) and (b)) and on yield (c). Each point represents one plot and lines show linear expectations for each group of treatments. Purple, yellow and green dots and lines represent Karim-Salim, Karim-Monastir and three-way mixtures, respectively. Black dots represent data for the susceptible cultivar Karim. Significant deviations from the linear expectation are indicated with asterisks: \* for  $p < 0.05$  and \*\* for  $p < 0.01$ .

## Discussion

Our analyses show that introducing only 25 % of a disease-resistant cultivar into a pure stand of a susceptible cultivar provides a substantial reduction in disease levels -- down to the level of resistant pure stands -- and a corresponding mitigation of the yield loss. To measure the severity of *Septoria tritici* blotch in the field, we used a novel, high-throughput method based on digital analysis of leaf images. This method allowed us to acquire a large dataset that exceeds previous studies with respect to accuracy and statistical power.

The observed pattern matches qualitatively with the findings of Jeger *et al.* (1981b), who investigated the effect of cultivar mixtures on the epidemic development of *Septoria nodorum* blotch (SNB) caused by the necrotrophic fungal pathogen *Parastagonospora nodorum* (Oliver *et al.*, 2012; Quaedvlieg *et al.*, 2013). Jeger *et al.* (1981b) mixed an SNB-resistant with an SNB-susceptible cultivar at different proportions and reported that the 25%/75% resistant/susceptible mixture "reduced disease levels effectively to that found in the resistant pure stand". This similarity in outcomes for *P. nodorum* and *Z. tritici* suggests that there may be a general underlying mechanism. Since the two pathogens are largely similar in terms of their epidemiology, infection biology and population genetic structure, further studies on cultivar mixtures affecting these and other similar pathogens are required to establish whether this pattern holds more generally and to determine which characteristics of the pathogen are responsible for this effect.

Short-range splash dispersal that dominates the spread of both *P. nodorum* and *Z. tritici* may be one such characteristic. The pattern described above may be favouring the barrier effect rather than the dilution effect as the dominant mechanism of disease reduction in mixtures in our experiment. This is because the dilution effect is expected to cause a gradual decrease in the level of disease when the proportion of resistant plants is increased, as predicted for example by the discrete-time population model of Jeger *et al.*, (1981a). In contrast, the barrier effect may result in an abrupt, threshold-like drop in the level of disease at a certain critical proportion of the resistant cultivar in the mixture. At this critical proportion, the connectivity between susceptible plants is disrupted and their population is subdivided into isolated patches. Similar phenomena have been studied extensively in ecology in the context of habitat loss and fragmentation by adapting the conceptual framework of percolation theory (Bascompte & Sole, 1996; Swift & Hannon, 2010), but have not received attention in studies of cultivar mixtures. This mechanism is expected to be of importance in pathogens with sufficiently short range dispersal (for example, splash dispersal in *Z. tritici* and *P. nodorum* on wheat and *Rhynchosporium secalis* on barley, or dispersal of many soil-borne pathogens), but be less prominent in air-borne pathogens such as those causing rust and mildew diseases. Theoretical prediction of the critical

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proportion of resistant plants that causes fragmentation of susceptible plant population requires quantitative characterisation of pathogen's dispersal under field conditions, but such measurements in *Z. tritici* and *P. nodorum* are still lacking.

Adding a second resistant cultivar to a two-way mixture (which makes it a three-way mixture) did not provide a significant additional reduction of disease compared to two-way mixtures of resistant and susceptible cultivars, contrary to the theoretical prediction (Mikaberidze et al., 2015). A possible explanation for this discrepancy could be that either (i) the pathogen population did not possess a sufficient degree of specialisation with respect to the two resistant cultivars and therefore the pathogen population have not been partitioned enough between the two resistant cultivars to result in a measurable reduction of disease. Alternatively, (ii) the resistance of the two cultivars has a largely overlapping genetic basis. Both of these scenarios violate the assumptions of the model (Mikaberidze et al., 2015).

Although we did not observe a significant reduction of disease when a second resistant component was added to two-way mixtures, this can have an important effect on the adaptation of pathogen population to host resistances. If the resistance in the two resistant cultivars is conferred by different genes, then a three-way mixture is expected to impose less selection on the pathogen population compared to a two-way mixture with the same proportion of the susceptible component, in this way extending the durability of host resistances.

One limitation of the present study is that the measurements of yield in the absence of disease were not available. For this reason, we could not evaluate the effect of disease on yield and in this way disentangle the effects of cultivar mixtures on yield through reduction in disease and through other means, for example due to an increase to abiotic stress tolerance provided by a mixture. Future field experiments may be able to overcome this limitation by incorporating a fungicide-treated control treatment.

To conclude, our study (together with the study of Jeger et al. (1981b)) contributes to establishing of a practically useful rule of thumb, according to which adding 25% of resistant plants to the susceptible pure stand provides substantial protection from disease, not different from the resistant pure stand. Such mixtures may have an important advantage with respect to planting resistant pure stands: they are more likely to be used by growers if the susceptible cultivar is agronomically superior compared to the resistant cultivar and is generally more accepted by growers. Future studies will need to consider even smaller proportions of the resistant component in mixtures to determine the threshold more accurately and provide a more solid scientific basis for the choice of mixing proportions to control STB.

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## Supporting information

**Supplementary File S1.** Additional figures that present all variables, time points, and treatments extending the figures presented in the main text. Effect of number of mixture components, test of data quality, and weather conditions are also presented.

**Supplementary Text S1.** Python source code for the data handling, statistical analysis, and figure production.

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