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Short Notes

Different inoculation methods affect components of Fusarium head blight resistance in wheat

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Summary. Fusarium head blight (FHB) is one of the most important fungal diseases of cereals, and *Fusarium graminearum* is the most damaging FHB pathogen. infection is linked to host anthesis, and symptoms include necrosis, bleaching of heads and shrivelled kernels. No fully effective fungicides are available for FHB control, so utilization of other mitigation measures, such as the use of resistant cultivars, is necessary for FHB management. Resistance to FHB is quantitative and multigenic and five components of resistance (Type I, II, III, IV and V) have been described. The main problem in testing for FHB resistance is reproducibility, so necessary tools for breeding resistant cultivars are reliable inoculation methods and the testing for different FHB-associated characteristics. We screened three Italian wheat genotypes, 'Palesio', 'Claudio' and 'Marco Aurelio', for Type I, Type II, and, in part, for Type V resistances, with both phenotypic (% of disease incidence and severity, thousand kernel weight (TKW) and molecular (quantification of fungal biomass with Real-Time *q*PCR) approaches, using spray and point inoculation protocols. Results underlined that 'Palesio' bread wheat showed Type I tolerance to initial infection, and 'Marco Aurelio' durum wheat showed an important Type II resistance to disease spread when spray-inoculated (27% disease severity). Quantification of fungal biomass showed that differentiation among the three wheat cultivars was best visualized when spray inoculation was used. TKW data showed that % yield loss was greater after point inoculations, except in 'Marco Aurelio', which was not affected by inoculation method. This study has highlighted the complexity of testing for FHB resistance, and demonstrated the necessity to use as many resistance screening protocols as possible.

Keywords. *Fusarium graminearum*, *Triticum aestivum*, *Triticum durum*, spray inoculation, point inoculation.

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INTRODUCTION

Wheat is one the most cultivated crops, followed by rice and maize. Modern wheat cultivars include two species: hexaploid bread wheat, *Triticum aestivum* L. (AABBDD), and tetraploid, durum-type wheat, *T. turgidum* subsp. *durum* (Desfontaines) Husnache (AABB) used for pasta and low-rising bread

(Doebley *et al*., 2006; Dubcovsky and Dvorak, 2007; Charmet, 2011; Feldman and Levy, 2012). Bread wheat accounts for 95% of world wheat production, while durum wheat is the remaining 5%. Wheat accounts for more than 20% of total human food calories. Wheat crops are extensively grown, on 17% of all crop areas, and is the staple food for 40% of the world's population, mainly in Europe, North America and the western and northern parts of Asia (Peng *et al*., 2011).

Fusarium head blight (FHB) is one of the most important fungal diseases of grain crops, including wheat, barley and maize (Goswami and Kistler, 2004; Osborne and Stein, 2007; van der Lee *et al*., 2015). FHB is caused by the *Fusarium graminearum* Species Complex (FGSC), which comprises 16 different species. These produce various mycotoxins, including deoxynivalenol (DON) and zearalenone (ZEA), which are toxic to humans and animals (Desjardins and Proctor, 2007; Foroud and Eudes, 2009;, Walter *et al*., 2010; Darwish *et al*., 2014).

During the past decade numerous FHB epidemics have been reported worldwide, causing significant economic losses (millions to billions of $US) (McMullen *et al*., 2012; Wegulo *et al*., 2015). The spectrum of *Fusarium* spp. causing FHB on wheat varies at the regional level depending on weather conditions, especially during host anthesis. Fungal growth is favoured by high temperatures and humidity, and abundant rain, during the growing season which favour pathogen infection, and can lead to significant yield losses. Given the current global warming associated with increased temperatures, major epidemics of the *Fusarium* diseases are likely (Vaughan *et al*., 2016; Khaledi *et al*., 2017).

Fusarium graminearum Schwabe is the predominant FHB pathogen, but its infection biology is yet to be fully understood. Airborne spores are transported by rain and wind to host floral tissues, where, at anthesis, they proliferate and spread rapidly intracellularly throughout the host spikelets, down into the rachial nodes and ultimately up and down the rachides until FHB symptoms are clear, involving necrosis and bleaching of heads causing shrivelled kernels (Nelson *et al*., 1994; Dweba *et al*., 2017).

In Italy, FHB on wheat has occurred each year since 1995, at varying levels of incidence and severity depending on the year, the region and the wheat genotype involved. (Pancaldi *et al.,* 2010). The disease has been reported mostly in the Northern-Central regions of Italy, and there is evidence indicating that the prevalent FHB species have shifted from *F. culmorum* (W. G. Smith) Saccardo to *F. graminearum* and *F. poae* (Peck) Wollenweber (Shah *et al*., 2005). FHB incidence and severity increase from the South to the North of Italy, and is closely related to the amounts of precipitation during wheat anthesis (Covarelli *et al*., 2015). Regarding mycotoxin production, DON is the most frequently found in Italy, and, as for disease incidence, occurrence of this mycotoxin increases from Southern to Northern Italian regions (Aureli *et al*., 2015). Since durum wheat is grown more widely than bread wheat in Italy, but also is more susceptible to FHB than bead wheat, mycotoxin accumulation in kernels is of particular concern as a food safety issue (Boutigny *et al*., 2008).

Chemical control of FHB using appropriate effective fungicides and correct application methods and timing are feasible for reducing disease severity (Blandino *et al.,* 2012). However, no fully effective FHB fungicide is available (Haidukowski *et al*., 2012), and the application window is very narrow, spanning just a few days around host anthesis (Mesterházy *et al*., 2003). Therefore, while new and eco-sustainable plant protection strategies are being developed (Fortunati *et al.,* 2019), the utilization of resistant genotypes remains important, and is possibly the most effective strategy for FHB control (D'Mello *et al*., 1999).

Resistance to FHB in wheat and other cereals has quantitative and multigenic characteristics (Zhu *et al*., 1999; Gervais *et al*., 2003; Massman *et al*., 2011). It is a non-trivial task for plant breeders to develop FHB-resistant and productive wheat cultivars, since plant breeding requires two essential pre-conditions: availability of genetic resources carrying positive alleles for the trait of interest and reliable testing methods that allow breeders to identify the desired genotypes (Buerstmayr *et al*., 2014; Steiner *et al*., 2017). Two main components of resistance have been described: Type I resistance operates against initial infections and Type II against the spread of symptoms induced by pathogens within their hosts (Schroeder and Christensen, 1963). Furthermore, Type I and Type II resistances vary independently among genotypes (Schroeder and Christensen, 1963). Three other types of FHB resistances have been described, but these are still not well understood. Type III resistance is the host plant's ability to degrade DON (Miller and Arnison, 1986), Type IV is the host's ability to tolerate high DON concentrations (Wang and Miller, 1988), and Type V is resistance to kernel infection, evaluated by analysing grain samples post-harvest for incidence of diseased kernels (Mesterházy, 1995). Gilbert and Tekauz (2000) distinguished between resistance (host ability to prevent infection) and tolerance (host ability to mitigate the infection, with low impacts on yield), and attributed Type IV and V resistances as FHB tolerance.

The most important goal in FHB resistance breeding is that resistant varieties should develop low symptom severity and simultaneously low mycotoxin contamination (Bai *et al*., 2001; Snijders, 2004; Wilde *et al*., 2007). In the second half of the 20th Century, large numbers of varieties, breeding lines and germplasm accessions were evaluated for FHB resistance. Quantitative variation in FHB susceptibility was detected, but no genotype was immune (Miller and Arnison, 1986; Wang and Miller, 1988; Buerstmayr *et al*., 1999). Durum wheat was also more susceptible than bread wheat, where almost no variation in resistance to FHB has been found within historic and current *T. durum,* with most lines being susceptible, even among large germplasm collections of several thousand lines (Otto *et al*., 2002; Stack *et al*., 2002; Ghavami *et al*., 2011; Prat *et al*., 2014).

One of the main problems in testing for FHB resistance is the lack of reproducibility of results (Groth *et al*., 1999; McCallum and Tekauz, 2002; Geddes *et al*., 2008). The chief goal is to measure differences in genetic resistance, taking into account non-genetic factors, which can lead to errors in the results. Under natural conditions, infection pressure is usually not uniform in time and space, while in FHB resistance screenings, infection is achieved by applying uniform inoculum pressure over time (at flowering) and space (in greenhouses) (Campbell and Lipps, 1998). A necessary tool for breeding resistant lines is a reliable inoculation method enabling accurate quantitative disease assessment. Further, since FHB resistance is a complex quantitative trait, a single and simple method for measuring FHB resistance is sometimes insufficient (Buerstmayr *et al*., 2014).

The objectives of the present study were to screen for Type I and Type II resistances in three prominent Italian wheat cultivars, whose FHB responses were unclear, by using phenotyping and molecular tools to assess FHB incidence and severity. Real-Time *q*PCR (FHB Type II resistance) and measurement of thousand kernel weight (TKW) (FHB Type V resistance) were carried out to quantify the fungal biomass in wheat chaff and rachides, and to assess the impacts of *F. graminearum* infection on yield loss.

MATERIALS AND METHODS

Plant material and growth conditions

Italian wheat genotypes 'Palesio' (bread wheat), and 'Marco Aurelio' and 'Claudio' (durum wheat) were grown in a greenhouse, following the protocol developed by Watson *et al.* (2018), with modifications. Seeds were surface sterilized with sodium hypochlorite (0.5% v/v) for 20 min and then rinsed twice for 5 min. in sterile distilled water. Seeds were then germinated in the dark on paper imbibed with sterile distilled water for 15 d at 4°C to break dormancy, followed by 2 d at room temperature. Subsequently, seedlings were transferred to 40 × 20 cm pots (20 plants for each pot), filled with TYPical Brill soil, and were grown at 16–20°C until boot stage, 20–24°C during anthesis, and 24–29°C until maturity. The plants were fertilised to avoid nitrogen deficit, by providing ammonium nitrate at the following proportions and plant stages: 20% at sowing, 40% at tillering and 40% at heading.

Fungal material, inoculum preparation and infection techniques

The highly virulent and DON-producing isolate of *F. graminearum,* wild type 3824 (Mandalà *et al*., 2019), was cultured at 21°C on potato dextrose agar (PDA) and on synthetic nutrient poor agar (SNA) (Urban *et al*., 2002) to obtain macroconidia for inoculum preparation. To prepare inocula, after a minimum of 10 d on SNA, conidia were scraped with a glass rod after pipetting 1 mL of sterile distilled water onto the surface of each Petri dish. The resulting conidium suspension was recovered, and the concentration measured using a Thoma Chamber (0.100 mm depth and 0.0025 mm^2). Inocula were prepared in sterile distilled water supplemented with 0.05% (v/v) of Tween-20. Two inoculum methods and several conidium concentrations were tested: spray inoculation to evaluate Type I FHB resistance, and point inoculation to evaluate Type II resistance, and 500, 1,500 or 2,500 conidia per spike (c/s), to assess dependent disease pressure responses in symptom development. Conidium concentrations were prepared following the protocol of Stein *et al.* (2009). 10 µL of conidium suspension was applied to the central spike floret of each plant for point inoculations (using a laboratory pipette), or 100 µL of conidium suspension was applied to plants (using a manual nasal sprayer) for spray inoculations. Thus, 5×10^4, 15×10^4 and 25×10^4 conidia mL^{-1} concentrations were prepared for point inoculations, while 5×10^3, 15×10^3 and 25×10^3 conidia mL^{-1} were prepared for spray inoculation, in order to inoculate each test plant with 500, 1,500 or 2,500 conidia for each spike using the two both inoculation techniques. The spikes on the main culms (one spike per plant) were inoculated during anthesis (Zadok stage 69: Zadoks *et al*., 1974), at greenhouse temperatures ranging from 20–24°C. Subsequently, the spikes were sprayed with sterile distilled water and covered with clear plastic bags for 48 h to maintain (> 80%) high humidity. Unin-

oculated control plants were treated with sterile distilled water supplemented with 0.05 % (v/v) of Tween-20. Disease incidence (%) was determined for the spray inoculated plants by counting the numbers of bleached spikes at 3, 9, 15 and 21 d post infection (dpi). Disease severity (%) was determined for both spray and point inoculated plants by counting the numbers of bleached spikelets for each inoculated spike from 3 to 21 dpi. All inoculation trials were performed in three replicates, and each replicate contained 20 spikes for each variable (genotype × conidium concentration × inoculation technique).

Fungal biomass quantification

At 21 dpi, the 2,500 c/s (the strongest disease pressure condition) point and spray inoculated spikes were collected and immediately stored in liquid nitrogen, for quantification of *F. graminearum* DNA in the chaff and rachis tissues. Fungal DNA quantification was performed following the protocol of Horevaj *et al.* (2011) and Siou *et al.* (2014). Material to obtain the *F. graminearum* calibration curve (60 mg of fresh mycelium) and the wheat calibration curve (60 mg of uninoculated wheat material) and total inoculated wheat chaff and rachis (60 mg of inoculated wheat material), were finely ground using mortars and pestles plus liquid nitrogen, and were stored at -80°C until DNA extractions. Total wheat and fungal DNA extraction were performed following the protocol for the Invisorb® Spin Plant Mini Kit (Stratec Molecular), and DNA was quantified with a Qubit™ fluorometer 1.01 (Invitrogen) using the Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific). DNA from inoculated samples was diluted to 10 ng μL^{-1}, while fungal and wheat calibration curves were obtained preparing three serial 1:10 dilutions from fresh fungal mycelium and uninoculated wheat material DNAs. Real-Time *q*PCR was performed following the instructions from Rotor Gene Q (Qiagen) and Xpert Fast SYBR (uni) Master Mix (Grisp). Real-Time *q*PCR amplification conditions included: an initial denaturation step of 3 min at 95°C; 35 cycles of 5 sec denaturation at 95°C, 30 sec of annealing at 61°C and 20 sec of elongation at 72°C. A final melt cycle was performed to confirm the amplicons unicity. Real-Time *q*PCR was performed using the primer pair Tri6_10F/Tri6_4R for *F. graminearum* DNA quantification (Horevaj *et al.,* 2011), and Act_77F/Act_312R for wheat DNA quantification (Mandalà *et al.*, 2019). Three biological replicates were analysed for each quantification and from each of these, three technical replicates were obtained and tested. Results are reported as ng of fungal DNA per ng of plant DNA.

Thousand kernel weight (TKW)

At maturity, the 20 spikes from 2,500 c/s (the greatest disease pressure condition) for point and spray inoculated plants, and the uninoculated control plants, were collected and stored at 4°C. the spikes were hand threshed to separate kernels from the chaff. Kernels were then weighed to determine and calculate the TKW. The TKW of inoculated plant kernels was then compared with the TKW of control plant kernels, to estimate the percent yield loss due to inoculations.

Statistical analyses

Data were subjected to analyses of variance (ANOVA). The following data were compared: disease incidence (%) among different conidium inoculation concentrations and genotypes for spray inoculated plants; disease severity (%) among different conidium inoculation concentrations, inoculation methods and genotypes; fungal DNA concentrations (ng of fungal DNA per ng of plant DNA); and yield loss (%) between 2,500 c/s spray- and point-inoculated plants. Two levels of significance ($P < 0.05$ and $P < 0.01$) were computed to assess the significance of the F values. When significant F values were observed, a pairwise analysis was carried out using the Tukey Honestly Significant Difference test (Tukey test) at the 0.95 or 0.99 confidence levels.

RESULTS

Several conidium concentrations were tested to assess disease pressure responses in symptom development. The ANOVA test showed that there were no statistically significant differences in trends of FHB incidence (Type I) or severity (Type II) from the different conidium concentrations in the three cultivars tested. Incidence reached 100% between 15 and 21 dpi in all the three wheat cultivars. For each conidium concentration tested; disease severity also reached 100% between 15 and 21 dpi, when the wheat spikes were point inoculated. From the spray inoculations, FHB severity for 'Marco Aurelio' reached 44% at 21 dpi, while severity on 'Palesio' and 'Claudio' was also 100% at 21 dpi.

Figure 1 presents results obtained from the phenotypic evaluations of symptoms. Figures 1a, 1b and 1c show the genotype comparisons, for assessment of FHB differential responses connected to resistance genotype diversity, while Figures 1d, 1e and 1f show the inoculation method comparisons, for assessment of differences in symptom severity (Type II). Figure 1a shows the

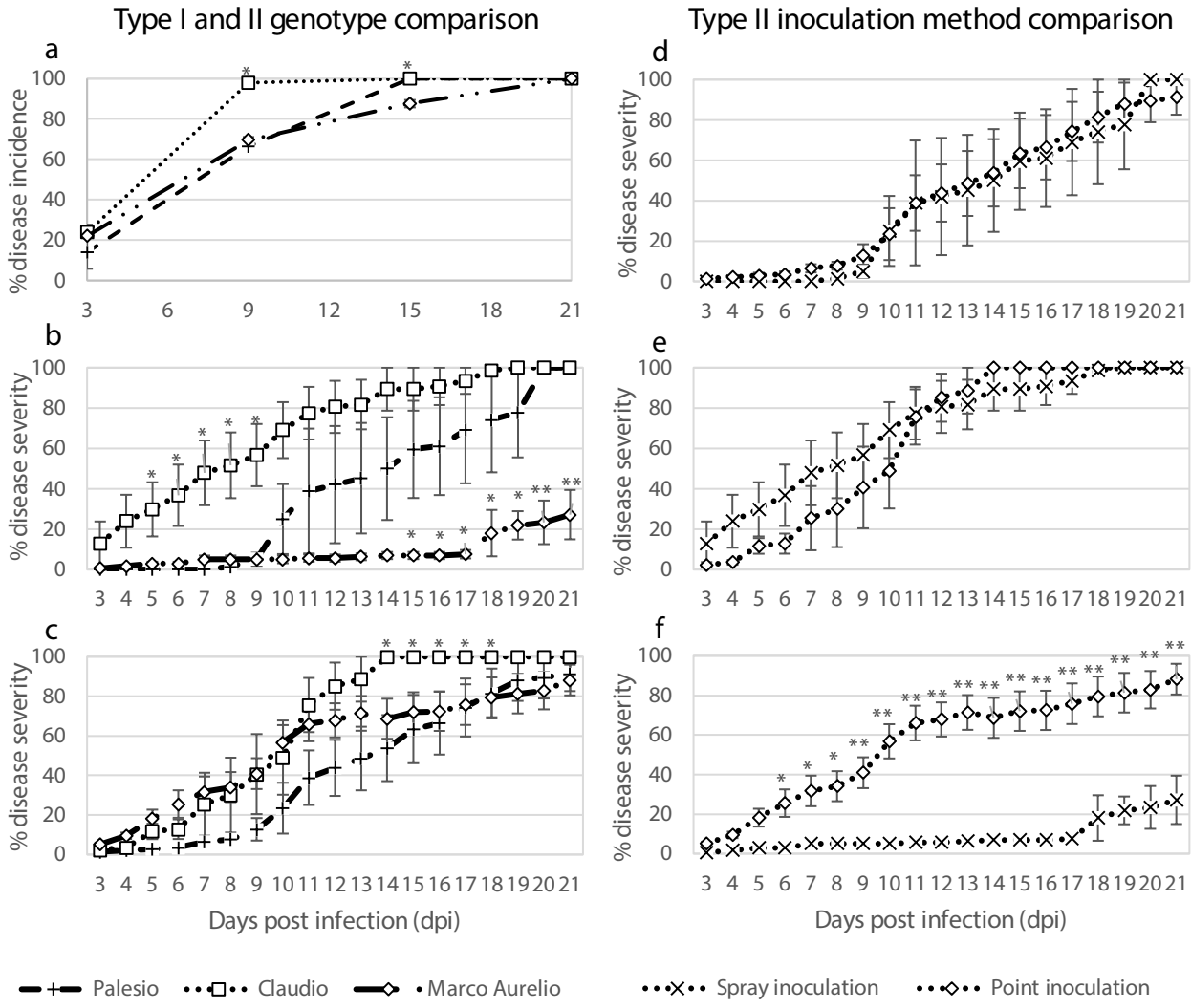

Figure 1. *Fusarium graminearum* symptom development during 21 dpi, following inoculation of three Italian wheat genotypes with 2,500 conidia per spike. a) % disease incidence (Type I); b) % disease severity after spray inoculation (Type II); c) % disease severity after point inoculation (Type II); d), e) and f) % disease severity (Type II) between spray and point inoculation methods for, respectively, 'Palesio, 'Claudio' and 'Marco Aurelio'. Data represent averages and standard errors for three independent replicates with at least 20 plants for each genotype × inoculation combination. Statistical analyses were performed according to a one way analysis of variance (ANOVA) with the Tukey test at a 0.95 confidence level and (*) $P < 0.05$, and at a 0.99 confidence level and (**) $P < 0.01$.

variation in disease incidence at 3, 9, 15 and 21 dpi, for 2,500 c/s, inoculated onto the three Italian wheat cultivars tested. 'Claudio' was the most susceptible reaching 98% of symptomatic spikes at 9 dpi ($P < 0.05$). At 15 dpi, 'Marco Aurelio' showed reduced symptom progression ($P < 0.05$), suggesting Type I tolerance for most of the trial duration.

Figures 1b and 1c indicate the FHB severity trend comparisons between the genotypes at 2,500 c/s. The spray inoculation technique (Figure 1b) gave disease severity at 9 dpi of 5% in 'Palesio' and 'Marco Aurelio', and 57% in 'Claudio'. This indicated the presence of initial Type II resistance in 'Palesio' and 'Marco Aurelio' ($P < 0.05$). From 10 dpi until the end of the trial, the statistically significant differences in symptoms between 'Claudio' and 'Palesio' disappeared, due to the high variability of results obtained with the spray inoculation method. In contrast, symptoms in 'Marco Aurelio' reached a maximum of 27% at 21 dpi ($P < 0.05$ for 15 and 19 dpi and $P < 0.01$ for 20 and 21 dpi), compared to 'Claudio' and 'Palesio'.

Figure 1c shows the same genotype comparisons as previously described, but after point inoculations, to assess putative FHB Type II resistance under more

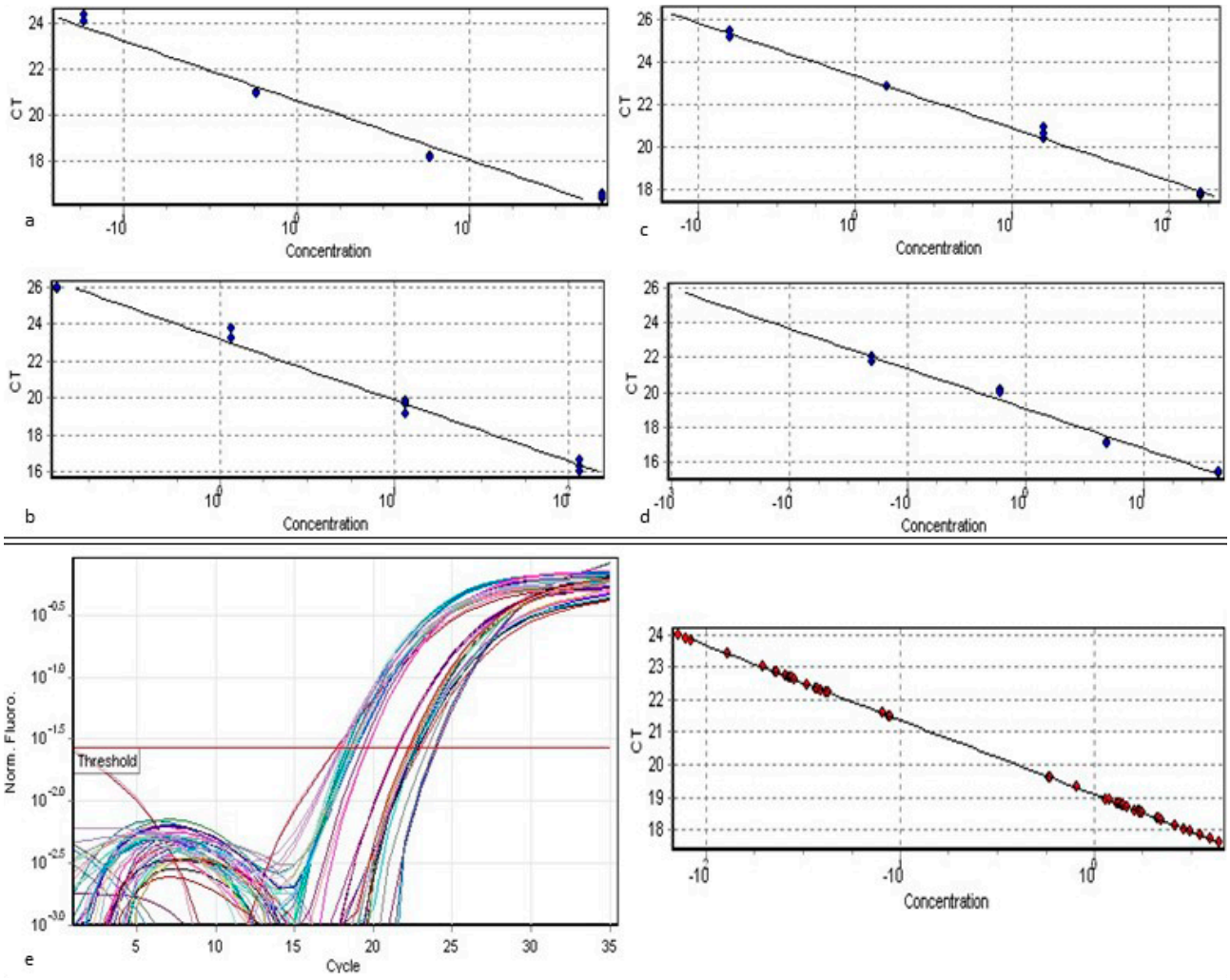

Figure 2. Standard curves resulting from Real-Time *q*PCR quantifications: a), b) and c) show, respectively, *Act* standard curves for wheat genotypes 'Palesio', 'Claudio' and 'Marco Aurelio' pure DNAs. d) shows the *Tri6* standard curve for *F. graminearum* pure DNA. e) shows amplification curves of the *Tri6* gene (left) and interpolations with the standard curve (right).

aggressive disease conditions. Between 3 and 13 dpi, no statistically significant differences were observed among the three wheat cultivars. Subsequently, 'Claudio' again demonstrated high susceptibility, reaching 100% severity at 14 dpi, while significantly less symptom development was observed on 'Palesio' and 'Marco Aurelio' at 14-18 dpi ($P < 0.05$). These results confirm that Type II resistance was present in 'Palesio' and 'Marco Aurelio' under harsh disease conditions. However, at 19–21 dpi, no symptom differences were detected among the three wheat genotypes.

Disease severity differences observed between the spray and point inoculation methods at 2,500 c/s for the three wheat genotypes are shown in Figures 1d, 1e and 1f. Figures 1d and 1e show the severity progression, respectively, in 'Palesio' and 'Claudio'. Under both spray and point inoculation, these two wheat cultivars did not show any FHB resistance or tolerance. In contrast, Figure 1f shows the severity trend in 'Marco Aurelio', where, starting from 6 to 8 dpi, symptom progression was less after spray inoculation than point inoculation ($P < 0.05$). These differences in symptom development were enhanced from 9 to 21 dpi ($P < 0.01$), and at the end of the trial, disease severity reached 27% after spray inoculation, and 88 % after point inoculation.

Additional estimations of FHB Type II tolerance or resistance were made using fungal DNA quantification after spray and point inoculations with 2,500 c/s. Figure 2 shows the Real-Time *q*PCR curves: Figures 2a, 2b, 2c and 2d show standard curves for DNA quantification

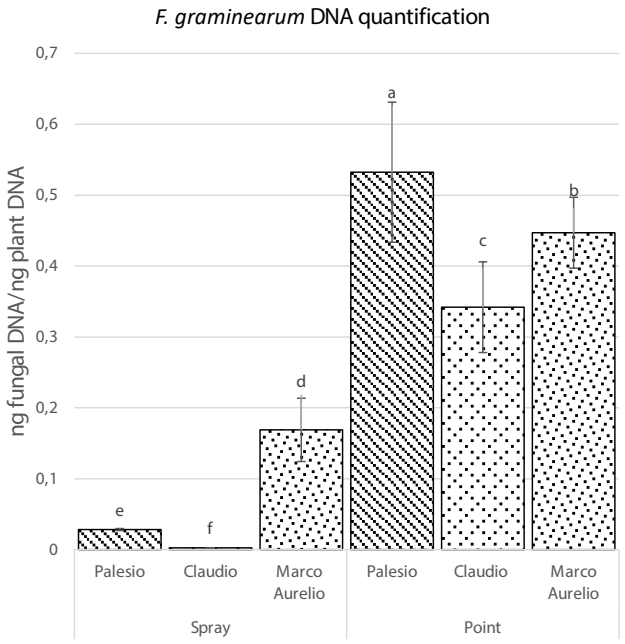

Figure 3. *Fusarium graminearum* biomass quantification from wheat chaff and rachides sampled from spikes that were either spray or point inoculated with 2,500 conidia per spike. Data represents averages and standard errors for three independent technical replicates derived from three independent biological replicates for each genotype × inoculation method. Statistical analyses were performed according to two way analysis of variance (ANOVA), with Tukey test at 0.95 confidence level ($P < 0.05$).

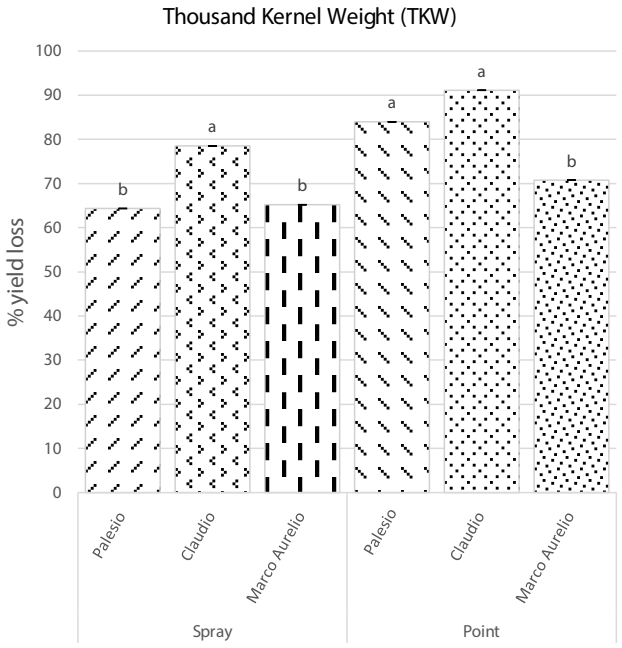

Figure 4. Yield losses (%) based on TKWs for inoculated and control plants after spray or point inoculations with *F. graminearum* at 2,500 conidia per spike. Data represent averages and standard errors for three independent replicates performed with at least 20 plants for each genotype × inoculation combination. Statistical analyses were performed according to two way analysis of variance (ANOVA) with Tukey test at 0.95 confidence level ($P < 0.05$).

of the *Actin* gene ('Palesio', 'Claudio', 'Marco Aurelio'), and the *Tri6* gene (*F. graminearum*). Figure 2e shows the amplifications results. Final results (Figure 3) showed that spray inoculation resulted in a less accumulation of fungal DNA in the wheat chaff and rachis than from point inoculation. 'Palesio' (0.028 ± 0.00146 ng of fungal DNA ng^{-1} per plant DNA and 'Claudio' (0.0025 ± 0.00012 ng) had less pathogen DNA than 'Marco Aurelio', (0.168 ± 0.0446 ng). After point inoculations, greater fungal DNA concentrations were detected, reflecting the aggressiveness of this inoculation method. 'Claudio' again had reduced pathogen accumulation (0.342 ± 0.064 ng of fungal DNA/ng per plant DNA) compared to 'Marco Aurelio' (0.447 ± 0.05 ng) and 'Palesio' (0.532 ± 0.099 ng).

TKW was measured for kernels derived from 2,500 c/s inoculated and uninoculated plants, to determine the pathogen impacts on potential wheat yields for the wheat genotypes. Figure 4 shows results reported as % yield losses. Point inoculations resulted in greater losses compared to spray inoculations ($P < 0.05$), since losses from point inoculations reached 84% in 'Palesio', 91% in 'Claudio' and 71% in 'Marco Aurelio'. These differences were not significant with 'Marco Aurelio', where spray and point inoculation had similar effects. 'Claudio' was more susceptible after spray (79% yield loss) or point (91%) inoculation, while 'Marco Aurelio' was the least affected cultivar, under both spray (65% yield loss) and point inoculation (71%). However, all the yield losses were very high for all of the inoculation and cultivar treatments.

DISCUSSION

FHB is normally a sporadic disease of wheat and other cereals, because infection and colonisation by *Fusarium* spp. are largely dependent on the prevailing weather conditions, which also determine disease severity (Xu, 2003; Burlakoti *et al.*, 2010). The risks of infection are associated with warm and humid conditions (Xu and Nicholson, 2009). As a result, FHB incidence and severity usually vary from year to year (Sutton, 1982), and from region to region (Jelinek *et al.,* 1989).

To develop resistant cereal germplasm, artificial inoculation is essential to ensure disease development, to optimise host genotypic differentiation, and to reduce

the influence of host morphological characters that can contribute to disease avoidance (Mesterházy, 1995; Vaughan *et al.,* 2016).

Evaluating FHB resistance using natural infections is often not possible as disease incidence and severity vary over time and space due to changes in environmental conditions such as temperature and precipitation that are difficult to control (Mesterházy *et al.*, 2003; Kriss *et al.*, 2012).

Obtaining consistent differentiation of FHB resistance levels relies on the use of comparative inoculation methods and different screening tests because of FHB's multigenic nature and complexity (Parry *et al.*, 1995; Browne, 2009). In the present study, Type I and Type II FHB responses were evaluated in three Italian wheat cultivars, to identify the presence of genotypic resistance/tolerance, and to assess two screening protocols, by testing different aspects and components of FHB resistance.

Different conidium concentrations in inocula were assessed to evaluate disease incidence and severity responses connected to different disease pressure. FHB incidence increased in response to conidium concentration, but the differences observed were not statistically significant. Differences in disease severity were more pronounced after spray inoculations, but again, these differences were not statistically significant. These observations could be due to increases in disease development with increasing conidium concentration until a maximum was reached where additional inoculum does not increase the level of disease (Stein *et al.,* 2009). We also observed that results obtained from spray inoculations were characterized by high variability, compared to point inoculations. Kiecana and Mielniczuk (2013) explained that, despite spray inoculation resembling natural routes to infection by FHB pathogens, disease assessment could be arduous due to heterogeneity of conidium spatial location on wheat heads. When a pathogen is spray-inoculated, inoculum can also partially germinate, resulting in reduced symptom development (Parry *et al.*, 1995; Al Masri *et al.*, 2017). This could be due to close relationships between pathogen assessment and plant phenological stage. First establishment of FHB is related to host floret anthesis, which is not uniform within each spike: anthesis begins in the central floret, and then occurs in the upper and lower flowers (Dweba *et al.*, 2017; Kheiri *et al.*, 2019). Point inoculations, on the other hand, is reported to be more environmentally stable and results from this method are more reproducible, since the inoculum is applied directly into the central florets at anthesis. This ensures that equal amounts of inoculum are delivered to individual plants and reduces the chance of disease escape, which has been observed after spray inoculations (Engle *et al.*, 2007; Geddes *et al.*, 2008; Mesterházy *et al.*, 2015). Despite this advantage, point inoculation does not represent the most natural source of *F. graminearum* inoculum and is more labour intensive and time-consuming to carry out. However, point inoculation likely mimics the fungal inoculum transferred onto cereal florets by tiny insects such as aphids and thrips that are often found in wheat crops (Usele *et al.*, 2013; Imathiu *et al.*, 2014; Sørensen *et al.*, 2016).

The genotype comparisons showed that 'Claudio' durum wheat was the most Type I and Type II susceptible, 'Palesio' bread wheat had initial Type I and II resistance after spray inoculation, and Type II tolerance after point inoculation. 'Marco Aurelio' durum wheat showed Type I tolerance and Type II resistance after spray inoculation, and Type II tolerance after point inoculation. These results are similar to those of Miedaner *et al.* (2003). They tested the covariation between spray and point inoculations, and compared host heritability of reactions to pathogens for the two methods. Point and spray inoculations resulted in similar mean disease severities among host genotypes, while the most important source of variance was observed between inoculation methods, reflecting the different disease severities achieved with the two methods.

We observed differential FHB responses within the diverse wheat genotypes analysed. To our knowledge, there are no published studies on the FHB reactions of 'Palesio' and 'Marco Aurelio' wheat cultivars, so our results cannot be compared to others. Amoriello *et al.* (2018) screened a number of durum wheat cultivars, including 'Claudio', for resistance to DON contamination, and 'Claudio' was one of the most contaminated. The ability to degrade DON has been described as Type III FHB resistance, and tolerance of high DON concentrations as Type IV resistance'.(Mesterházy, 1995; 2002; Gunupuru *et al.*, 2017). We cannot associate the susceptibility we observed as resulting from high DON accumulation, since we did not analyse mycotoxin content as an FHB resistance factor.

Regarding the Type II tolerance observed in 'Palesio', it is known that bread wheat is naturally more resistant than durum wheat, because of its hexaploid nature (Buerstmayr *et al.*, 2014; Haile *et al.*, 2019). Nevertheless, the most interesting FHB Type II responses were observed in 'Marco Aurelio' durum wheat. Sources of resistance are limited in durum wheat (Stack *et al.*, 2002) and they reside mainly in other cultivated tetraploid wheat subspecies, such as ancient cereal crops (Oliver *et al.,* 2008). Some of these old cereal crops are *Triticum*

turgidum subspecies, such as *T. turgidum* subsp. *turgidum*, which is native to Mediterranean countries, as is 'Marco Aurelio'. It can therefore be assumed that the Italian wheat genotype 'Marco Aurelio' possess some FHB resistance characteristics that also occur in the ancient cereals.

Results from fungal biomass quantification of the wheat chaff and rachides between the two inoculation techniques reflect the results obtained from the phenotypic evaluations of disease severity. When spray inoculated, wheat cultivars gave better contrast of pathogen spread, than when they were point inoculated. Kumar *et al.* (2015) assessed a Real-Time *q*PCR technique to detect and quantify *F. graminearum* biomass in rachides of barley and wheat resistant and susceptible cultivars, using the *Tri6* gene for fungal quantification and the *Actin* gene for the normalization, as done here. They observed that disease severity could not discriminate resistance before 9 dpi, while spikelet resistance was discriminated in all the wheat and barley genotypes tested, based on *q*PCR quantification of the fungal biomass. We therefore conclude that the wheat cultivars used in our study possess levels of tolerance to pathogen colonisation when spray inoculated, but not when point inoculated. These results agree with those of Brennan *et al.* (2005), who showed that visual disease assessment clearly reflected yield losses, but that no significant relationship was present between symptom severity and fungal DNA content in grain.

The phenotypic FHB scoring after spray inoculation at 2,500 c/s (disease incidence and severity) revealed that 'Claudio' durum wheat reached 100% of diseased spikes and spikelets sooner than the other cultivars, and was also likely best limit pathogen spread. The rapid bleaching of spikes but low amounts of fungal DNA could be explained in several ways. During pathogen infection and FHB development, plant vessels become blocked, preventing water and nutrient supplies as a defence mechanism, and causing sudden spike wilting (Kang and Buchenauer, 2000; Kheiri *et al.*, 2019). head bleaching due to natural absence of water and nutrients could be confused with FHB symptoms (Zwart *et al.*, 2008). Regarding the low amount of fungal biomass present in the chaff and rachides, it is known that DON is an important virulence factor that facilitates infection spread (Bai *et al.*, 2002; Jansen *et al.*, 2005). Ilgen *et al.* (2009) observed that the *Tri5* gene, which controls the trichodeine synthase involved in DON synthesis, is highly induced in the transit zone of host rachis nodes, where the rachilla and rachides divide. It could be possible that wheat genotypes possessing Type III or Type IV resistances (ability to degrade DON and to tolerate high DON levels) will give contrasting pathogen spread into the host vessels. TKW evaluation revealed that 'Marco Aurelio' was the least damaged wheat genotype, after spray and point inoculations. Resistance evaluation concentrates on visual head disease symptoms, since most QTL analyses have evaluated this trait. The TKWs were generally neglected in early wheat breeding programmes for resistance to FHB. Resistance to kernel infection arises from the premise that those genotypes should have a resistance type that does not affect levels, but their TKW values differ significantly. Therefore, it is not by chance that yield stability was developed as a major trait in plant breeding. However, FHB resistance, like yield, is governed by many QTLs, and infection severity has a strong impact on yield. It is therefore necessary to consider TKW in FHB resistance screening protocols (Canci *et al.*, 2004; Mesterházy *et al,.* 2015).

Current wheat breeding programmes for FHB resistance focus more on Type II than Type I resistance (Buerstmayr *et al.*, 2003; Burlakoti *et al.*, 2010; Xiao *et al.*, 2016). Type II resistance has also been reported to be more genetically and environmentally stable than Type I resistance, and provide a more reliable indication of cultivar resistance (Bai and Shaner, 2004). It is desirable, when possible, to replicate FHB resistance testing within and across environments (years and/or locations), in order to obtain meaningful results and to assess reproducibility of the data obtained (Buerstmayr *et al.*, 2014). Different FHB screening methods do not provide answer to the same question. FHB traits differ, and visual symptoms, fungal biomass and TKWs do not closely follow the similar patterns (Mesterházy *et al.,* 2015). No genotype in the present study was completely resistant to FHB. Thus, most genotypes probably quantitatively combine different levels of FHB resistances. This supports the assumption that FHB resistance types are probably governed by different loci and measure different resistance reactions, as has been suggested by Schroeder and Christensen (1963). For routine screening of FHB resistance in large breeding populations, a fast, cheap, and reliable inoculation method is desirable. Spray inoculation is advantageous over point inoculation. It is based on whole-plot inoculation (Martin *et al.*, 2017), which has similarity to natural disease situations and requires less time and labour for inoculation and disease assessments. Spray inoculation can also be useful to establish rapid and low cost assays to evaluate FHB resistance using wheat seedlings (Soresi *et al.*, 2015). However, spray inoculation is environmentally influenced, resulting in high variability and less replicable results. Point inoculation mimics infection through insects, but it is genetically and environmentally stable (Imathiu *et*

al., 2014). From our experience, we suggest, when possible, that both inoculation methods are used, and that as many FHB characteristics as possible are evaluated, since we observed differences in responses within inoculation techniques and the parameters assessed. In addition, screening different FHB parameters allowed us to recognize the FHB responses in 'Marco Aurelio', one of the most cultivated durum wheat cultivars in Italy.

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