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Article

Tramesan elicits Durum Wheat Defence against the Septoria Disease Complex

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Abstract: The Septoria Leaf Blotch Complex (SLBC), caused by the two ascomycetes *Zymoseptoria tritici* and *Parastagonospora nodorum*, can reduce global yearly yield of wheat by up to 50%. In the last decade in Italy, SLBC incidence has increased; notably, durum wheat has proven to be more susceptible than common wheat. Field fungicide treatment can efficiently control these pathogens, but it leads to the emergence of resistant strains and adversely affects human and animal health, and the environment. Our previous studies indicated that active compounds produced by *Trametes versicolor* can restrict the growth of mycotoxigenic fungi and the biosynthesis of their secondary metabolites (e.g. mycotoxins). Specifically, we identified Tramesan: a 23 KDa α-heteropolysaccharide secreted by *T. versicolor* that acts as a pro-antioxidant molecule in animal cells, fungi, and plants.

Foliar-spraying of Tramesan (3.3 μM) in SLBC-susceptible varieties of durum significantly diminished symptoms of Stagonospora Nodorum Blotch (SNB) and Septoria Tritici Blotch (STB) by 75% and 65%, respectively. Tests were conducted under controlled conditions as well as in field. We show that Tramesan elicits wheat defence against SNB and STB augmenting the synthesis of defence-related hormones, notably JA and SA, that in turn switch on the expression of markers of defence (*PR1, PR4 inter alia*). In field experiments, yield of durum wheat plants treated with Tramesan was similar to that of untreated ones. The results suggest the use of Tramesan for protecting durum wheat against SLBC.

Keywords: biostimulant; plant defence; mushrooms; antioxidant; septoriosis; wheat

1. Introduction

Wheat is the main source of plant proteins in human diet. Plant diseases such as rusts, head blight, powdery mildew, and leaf blotch negatively affect wheat yield. Among these diseases, leaf blotch is caused by fungal pathogens, including *Parastagonospora nodorum* and *Zymoseptoria tritici* [1,2] and can significantly alter the taste, smell, and texture of the grains [3]. Septoria Leaf Blotch Complex (SLBC), one of the main diseases of wheat, may cause up to 50% of crop loss in years of severe epidemics [4].

Parastagonospora nodorum is a necrotrophic pathogen of both common and durum wheat. It causes necrotic leaf spots and thus hampers photosynthesis. To infect the plant, *P. nodorum* produces several necrotrophic effectors: Tox1, Tox3, and ToxA are small secreted proteins that alter or suppress the host immune response [5]. These effectors interact in a reverse manner with respect to the "gene-for-gene" model, with corresponding sensitivity loci in wheat [6,7]. *Zymoseptoria tritici* is a hemibiotroph—parasitic in living tissue and persistent in dead tissue—that commonly undergoes

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sexual reproduction; local populations are extremely variable and can rapidly adapt to fungicides [8]. By causing necrosis in the leaves, *Z. tritici* reduces the wheat's grain-filling capacity and, as a consequence, yield.

Most recently, the molecular basis of the interaction of wheat (specifically common wheat) with SLBC are in the spotlight [9,10]. Some problems in the control of this disease have been elucidated through these studies: emersion of resistance to fungicide; the basis of the switch in the lifestyle of *Z. tritici;* the introduction of susceptibility traits by 50s and 80s breeding programs [11,12].

P. nodorum and *Z. tritici* infections account for approximately 70% of annual cereal fungicide (>€400 million) usage in the European Union (EU) [3]. To counter them, chemical fungicides such as strobilurins, triazoles, and imidazoles are widely used; however, resistant fungal strains have been emerging [13]. The European regulation 128/2009 governing the use of pesticides (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do? uri=OJ:L:2009:309:0071:0086:EN:PDF) recently eliminated several fungicides from the market to limit the environmental and health-related issues caused by chemical pesticides. At present, the employment of non-synthetic (natural) compounds in agriculture is increasing worldwide [14]: volatile molecules [(2,6-dichloroisonicotinic acid (INA), benzothiadiazole (BTH) commercialized as BION®], mineral nutrient and non-protein amino acids such as DL-3-amino-n-butanoic acid (BABA), and cellular or molecular elicitors perceived as PAMPS, MAMPS or DAMPS [15]. Regarding the latter elicitors, they provide a systemic and broad range, albeit partial, resistance to several diseases. This resistance is presumed to be based on the induction of the innate immune system of the host. These responses are typically associated with systemic acquired resistance, induced systemic resistance (ISR), and mycorrhiza-induced resistance. These different types of resistance help the plant to contain the attacker and are characterized, for instance, by the direct induction of antimicrobial proteins [16]. Nevertheless, plant capacity to challenge insults such as pathogen attack can be enhanced without a direct induction of defenses, for instance by conditioning plants "for boosted responses against pathogens" (cited from reference 17). This targeted induction of host defence is called "defence priming." Since adaptive plasticity prevents a defence-response in the absence of a challenge [17], defence priming allows the immune system to respond to stimuli with little energy expenditure. Defence priming responses are challenge-specific [18] and by enhancing plants' resistance, they enable a more effective response to subsequent attacks [19]. Plants can be "primed" with select elicitors to respond to the onset of a future attack in a faster and stronger way compared to non-primed plants [20].

In this study, we explore the possibility that purified Tramesan, a 23KDa α-heteropolysaccaride secreted by the lignin degrading basidiomycete *Trametes versicolor* [21] could act as an elicitor of durum wheat' defences. We tested Tramesan in controlled conditions as well as in the field to verify its efficacy in eliciting durum wheat in reacting more promptly against SLBC. We posit that Tramesan significantly reduces SBLC severity, by eliciting the innate defence of durum wheat.

2. Materials and Methods

2.1. Preparation of Fungal Inoculum

The fungal pathogen *Parastagonospora nodorum* strain 15465 was isolated at Council for Agricultural Research and Economics – Research Centre for Engineering and Agro-Food processing, CREA-IT, and submitted to the Culture Collection Agri-Food Important Toxigenic Fungi-Item, Institute of Science of Food Production (ISPA), National Research Council (CNR), Bari, Italy (http://server.ispa.cnr.it/ITEM/Collection/). The fungus, stored at -20°C, was revitalized by transferring it into fresh Potato Dextrose Agar plates (41g/L PDA, Formedium) and incubated at 20 °C with a photoperiod of 12 hours for 10 days. After the growth, conidia were transferred with sterile needles to Petri dishes containing PDA and placed in a growth cabinet under the same temperature and light conditions. After the incubation period, sterile distilled water was added to each plate and the spores were gently scraped with special glass rods. The obtained spore suspension was filtered with a sterile gauze and diluted to obtain a concentration of 10^6 spores/mL [22]. Tween 20 surfactant was added to the suspension (0.1% v/v) to increase the adhesion to leaves.

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Zymoseptoria tritici strain 18258, was isolated at CREA-IT and stored at -20 °C. The pathogen was revitalized transferring it in fresh PDA medium (Potato Dextrose Agar 39g/L HiMedia) and incubated at 18 °C with a photoperiod of 12 hours for 10 days. After the incubation period, sterile distilled water was added to each plate and the spores were scraped gently with special glass rods. The spores were transferred in yeast- sucrose liquid medium (yeast extract 10 g/L; sucrose 10 g/L) and left in shaking for 7 days at 18 °C with permanent light. The spores were collected by centrifugation at 5000 rpm for 5 minutes at 15 °C, washed twice with sterile distilled water and resuspended with an $MgSO_4$ solution containing Tween 20 surfactant (0.1% v/v). The concentration was adjusted to 10^7 spores/mL.

2.2. Tramesan Preparation

Trametes versicolor strain C (https://www.cabi.org/services/microbial-services/culture-collection-microorganism-supply/) is preserved on Potato Dextrose Agar (PDA, Difco) at 4 °C and renewed every 60 days within the frame of the collection of the Botanical Garden of Rome. *T. versicolor* was grown for 7 days on PDA in Petri dishes, at 25 °C. After 7 days, 3 plugs of 1 cm diameter with biomass (mycelium + medium) uniformly grown were collected and added, under sterile conditions, to 100 mL of Potato Dextrose Broth (PDB) under shaking at 150 rpm for 14 days at 25 °C. After 14 days of growth, the entire mass (liquid medium + mycelium) was homogenized using a Waring blender (speed 5 for 3 pulses of 2 min each), and added at 5% v/v to PDB liquid medium and grown for 14 days. Tramesan was purified as described by [21] and 2.3 g of lyophilized Tramesan was dissolved in 1 L of distilled sterile water. This solution was used to spray wheat plants in greenhouse. Plants were treated with Tramesan at 3.3 µM according to [21]. Tramesan was formulated with 0.1% v/v of Tween 20 as surfactant to increase its adhesion to leaves.

2.3. Trials in Phytotron

In a phytotron, kernels of two Italian commercial varieties (Svevo and Duilio; Syngenta Italia and SIS società italiana sementi, respectively) of durum wheat (*T. turgidum* subsp. *durum* (Desf. Husn.) moderately susceptible to the SLBC were superficially disinfected with a sodium hypochlorite solution (0.1% v/v) by shaking for 10 minutes and washed three times with sterile distilled water. The disinfected seeds were placed in Petri dishes containing water/agarose (2% w/v) and incubated for 24 hours at 20 °C, 24 hours at 4 °C, and 48 hours at 20 °C for their germination. The kernels were transferred in two times autoclaved (20 min at 121 °C) soil mixture (20 L of soil/5 L of perlite), in pots of 0.5 L. In the chamber, temperature, humidity, and light were regulated to have the following conditions: temperature of 20 °C, humidity of 80%, and light exposure for 18 h with 150 µmol of photon $m^{-2}s^{-1}$ from 6 am to 10 pm. The pots were positioned on a rotary floor, so that different plants were subject to the same conditions. The plants (n=48; 24 var. Svevo, 24 var. Duilio for each experimental condition) were irrigated three times a week. Four different experimental conditions were used: 1) plants non-inoculated and non-treated with Tramesan (Mock); 2) plants treated with Tramesan (T); 3) plants inoculated with *P. nodorum* or *Z. tritici* (Inf); 4) plants treated and inoculated with *P. nodorum* or *Z. tritici* (T + Inf). Tramesan (100 mL per 64 pots; 3.3 µM) was sprayed on seedlings of the 2 varieties at the 2-3 leaf stage (second leaf fully expanded), 48 hours prior to pathogen inoculation. The inoculation of wheat plants with foliar pathogens was carried out by spraying conidial suspensions (*P. nodorum*: 10^6 spores/mL; *Z. tritici*: 10^7 spores/mL) containing Tween 20 surfactant (0.1% v/v) as previously described [22]. The inoculated plants were covered with black plastic sheets for 24 h and with transparent plastic sheets in the subsequent 24 h, providing a humid saturated atmosphere necessary for spore germination and penetration into the leaves. Trials were conducted on a 9-day observation period. To assess SNB or STB symptoms, a severity index (numerical rating from 1 to 5) was adopted following Liu's scale [23], where 0 represents absence of symptoms and 5 indicates large coalescent lesions. Results from this scale were then transformed into percentage of lesioned area (as reported by Liu) using the arc sen $\sqrt{(x)}$ formula. To study the early effect of Tramesan on infected wheat, the seedling leaves were collected for subsequent analysis from -48 h to 9 days after infection (dai).

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2.4. Calibration Curve of P. nodorum and Z. tritici and Quantification of Fungal DNA by Real-time PCR

Total DNA was extracted from spores of *P. nodorum* and of *Z. tritici.* The spores (10^8) were collected by pipetting 50µl of Triton-X100 (Sigma-Aldrich, US) in Petri dishes (9 cm) completely covered with conidiating mycelia. Then, 500µL of CTAB buffer (1M Tris at pH 8, 5M NaCl, 0.5M EDTA, 2% w/v CTAB) and glass beads (425–600 µm; Sigma-Aldrich, US) were added to the spores/Triton-X100 mix in 2.0 mL tubes and DNA was extracted as previously described (Iori et al., 2015). Real time PCR was performed in a LineGene K PCR detection system (Bioer, PRC) with the following cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min to amplify β-tubulin gene (for_TGGGTACGCTTTTGATCTCC; rev_AACGAGGTGGTTCAGGTCAC) of *Parastagonospora nodorum* (acc. n. KF252679). Cycling of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 62 °C for 30 s and 72 °C for 1 min to amplify GAPDH gene (for_TCCGTCGTTGACTTGACCTG; rev_TCTGAACTCAACGGTCGCTT) of *Zymoseptoria tritici* (acc. n. XM_003855615). Standard calibration was performed plotting the real-time PCR (RT-PCR) signals obtained for *S. nodorum* and *Z. tritici* genomic DNA extracted from fungal strains in the concentration range of 5 pg to 50 ng. The equation describing the increase of DNA concentration was calculated ($y = 3\times10^8e - 0.6396x$, $R^2=0.9967$ for *P. nodorum*; $y = 2\times10^6e - 0.756x$, $R^2 = 0.9864$ for *Z. tritici*) and used afterwards as a reference standard for the extrapolation of quantitative information for DNA targets of unknown concentrations. The efficiency of the PCR reaction (102%) was obtained from calibration curves slope ($E = 10^{-1/slope} - 1$). For quantifying the fungal amount within the plant tissues, total DNA was extracted from the wheat leaves (n=2 leaves/plants; n=196 plants) at different times after pathogen inoculation (0-9 days after inoculation; dai) according to the Färber method with minor modifications [24]. RT-PCR (Bioer, PRC) as indicated above used total DNA extracted from wheat leaves for PCR quantification of the amount of fungal DNA within plant tissues. RT-PCR amplification reactions were carried out in three technical replicates, and the results were expressed as ng of target DNA/µg of total DNA.

2.5. Analysis of Expression of Plant Defence-related Genes

For plant gene expression analysis, aliquots of 25 mg of lyophilized wheat leaves were ground in liquid nitrogen and treated for RNA extraction. RNA extraction was performed with the TRI REAGENT method (Sigma-Aldrich, USA) according to manufacturer's instructions; cDNA was obtained using first strand cDNA synthesis SUPER SCRIPT II RT-PCR kit (Invitrogen, USA). RT-PCR was performed as described by [24]. To identify target genes and primer (Supplementary Table 1) homologs in wheat, we used the NCBI, DFCI Wheat Gene Index, an International Wheat Genome Sequencing Consortium chromosome survey sequence repository at URGI INRA databases, and specific references (for the homologues of *cerk1* see [25]; for the homologue of *mpk3* see [26]. The β-tubulin gene of *T. turgidum* subsp. *durum* (acc. N. AJ971820.1; for 5'- GCTGCTGTATTGCAGTTGGC-3'; rev 5'- AAGGAATCCCTGCAGACCAG-3') was employed as a housekeeping reference to normalize the expression of the target genes in the wheat leaves. Previous trials showed that Tramesan was effective in limiting the growth of pathogens and in reducing the symptoms on leaves. Ruling out a direct antimycotic effect of Tramesan (M. Reverberi, unpublished results) on *P. nodorum* and *Z. tritici,* we carried out experimental tests to evaluate whether this exo-polysaccharide was able to induce defence responses in wheat plants. In this regard we analysed the expression of some marker genes of defence response in the durum wheat variety Svevo: *PR1, PR4, PR9, CHIT2, MCA2, NADPH-ox, PAL, CERK1* and *MPK3.* Indeed, this variety resulted the more susceptible to both pathogens (Table 1; Fig. 1).

Table 1. Disease severity at the second leaf stage according to Liu's scale (0-5) in the durum wheat varieties (Svevo and Duilio) inoculated with *P. nodorum* or *Z. tritici.* **Mock**: without Tramesan, non-inoculated. **T**: treated with Tramesan; **Tr+Inf**: treated with Tramesan, inoculated with the pathogen; **Inf**: inoculated with the pathogen. Fisher test on n=48 × 2 biological repetitions.

	Durum wheat			
	P. nodorum		***Z. tritici***	
	Svevo	Duilio	Svevo	Duilio

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mock	0^{a}	0^{a}	0^{a}	0^{a}
T	0^{a}	0_{a}	0^{a}	0^{a}
Tr+Inf	1^{ab}	1^{ab}	0.7^{ab}	0.7^{ab}
Inf	4^{b}	2^{b}	2^{b}	1.7^{b}
F test (P value)	*2.5×10^{-5}*	*0.031*	*0.0015*	*0.025*

The relative expression of wheat genes was calculated following the $2^{-\Delta\Delta Ct}$ method using untreated samples as calibrators. RT-PCR was prepared in 20 µL reaction mixture containing SYBR green JumpStart Taq Ready Mix 1X (Sigma-Aldrich, USA), 3 mM $MgCl_2$ and 0.5 mM β-tubulin. RT-PCR was performed in a LineGene K PCR detection system (Bioer, PRC). *PR1, PR4, PR9, CHIT2, MCA2, NADPH-ox, PAL, CERK1*, and *MPK3* expressions were evaluated at inoculation time (t_0) and at 24 hours after pathogen inoculation (hai) on Svevo artificially infected with: 1) *P. nodorum* not treated (Inf Pn) or treated (Tr+Inf Pn) with 3.3 µM of Tramesan or 2) *Z. tritici* not treated (Inf Zt) or treated (Tr+Inf Zt) with 3.3 µM of Tramesan.

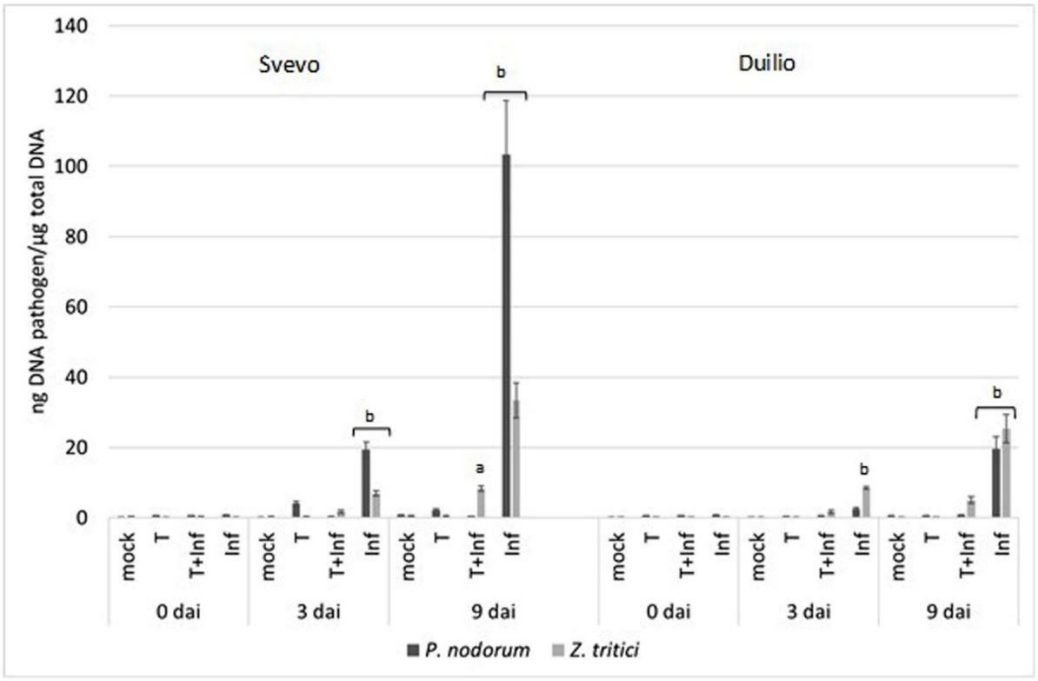

Figure 1. *P. nodorum* and *Z. tritici* growth assessed by qPCR. At 0, 3, and 9 days after inoculation (dai) in durum wheat (vars. Svevo and Duilio). **Mock**: without Tramesan, non-inoculated. **T**: treated with Tramesan; **T+Inf**: treated with Tramesan, inoculated with the pathogen; **Inf**: inoculated with the pathogen. Values represent the mean ± SE as described in the methods section. Small capital letter in the chart represent the significantly different groups ($p < 0.05$; Fisher test).

2.6. Plant Hormones Analysis

Salicylic acid (SA) and jasmonic acid (JA) were extracted from wheat leaves in all the experimental conditions described above. The quantification was done by the addition of the internal standard 1-Naphthaleneacetic acid (NAA MW 186.21 g mol^{-1}), at 5 µM final concentration. An amount of 30 mg of lyophilized durum wheat leaves was ground with liquid nitrogen, mortar, and pestle. Hormones were extracted with 750 µL of MeOH:H_2O:HOAc (90:9:1, v/v/v), mixed and centrifuged for 1 min at 10,000 rpm. The supernatant was collected, and the extraction was repeated. Pooled supernatants were dried under nitrogen gas flux. The dried samples were resuspended in 200 µL of 0.05% HOAc in H_2O-Acetonitrile (85:15, v/v). The analysis of SA and JA was performed with the LC-MS/MS Agilent 6420. The acquisition was in MRM negative ion mode $[M-H]^-$. Chromatographic separation was performed with a Zorbax ECLIPSE XDB-C18 rapid resolution HT 4.6 × 50 mm 1.8 µm p.s. column (Agilent Technologies) at room temperature, and the injected volume was 10 µL. The mobile phases consisted of A: H_2O containing 0.05% HOAc, and B: Acetonitrile. The elution gradient was as follows: 0-3 min 15% B, 3-5 min 100% B, 5-6 min 100% B, 6-7 min 15% B, 7-8

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min 15% B. The gradient was followed by 5 min for re-equilibration. The flow-rate was constant at 0.6 mL·min^{-1}. The injector needle was washed with the mobile phase in the wash at the end of each HPLC run. Nitrogen was used as the nebulizing and desolvation gas. The temperature and flow of the drying gas were 350 °C and 10 mL·min^{-1}, respectively, with a nebulization pressure of 20 psi. The capillary and cone voltage was 4000 V. The main transition and qualifier ions are 137.2→92.9, 137.2→64.8 for SA (CE 20, FV 135), 209.2→59.1, 209.2→41.3 for JA (CE 28 FV 135) and 245→180.8 for the internal standard NAA (CE 16 and FV 100). To quantify hormone levels, a STD curve was generated for both compounds in the 0.01 μM to 10 μM range. Trend curves are linear within this range and equation correlating relative (area) abundance and molarity are $y = 3432.2x$, $R^2 = 0.9973$ for SA and $y = 541.22x$, $R^2 = 0.9813$ for JA, respectively.

2.7. In-field Artificial Infection Trials

Field trials in the growing season 2014-2015 were performed in Montelibretti (CREA-IT) and in Rome using durum wheat variety Svevo and the same strains of *P. nodorum* and *Z. tritici* used for artificial inoculations (see "Phytotron trials"). In the crop season 2014-2015, we have 12 plots in two different fields located in Rome (lat. 41.969277; long. 12.464256; m.a.s.l. 20) and in Montelibretti (RM) (lat. 42.129287; long. 12.63969; m.a.s.l. 25) at *CREA–Research Centre for Engineering and Agro-Food processing*, CREA-IT. Svevo wheat variety was sown (viable seeds; 450/m2) in plots of 1.5 m × 2 m (3 m^2) using a randomized block design with three replicates. Different experimental conditions were set up (Table 2).

Table 2. Different experimental conditions used on durum wheat cultivars to study the effect of Tramesan on SNB and STB in Montelibretti (Rome) and in Rome (Italy) field trials performed by the CREA-IT in the 2014-15 growing season.

Treatment	Application time (GS)	Active ingredient	Dose (mL/m^2)
Untreated control (non-inoculated and non-treated)	--	Water	40
T	47	Tramesan	40
inoculated with *P. nodorum*	49	water	40
T and inoculated with *P. nodorum*	47; 49	Tramesan	40
Untreated control (non-inoculated and non-treated)	37	Water	40
T	37	Tramesan	40
inoculated with *Z. tritici*	39	water	40
T and inoculated with *Z. tritici*	37; GS 39	Tramesan	40

Each plot in the two fields contained n = 250 plants (plants were fewer than seed probably for the considerable presence of birds at the time of sowing). The trials were performed in three plots *per* thesis (mock, T, Inf, T+Inf). Inoculation for the artificial contamination in the field plots was prepared as indicated in "Preparation of fungal inoculum." As in Table 2, the plants were treated with Tramesan by spraying at booting growth stage GS 47 for SNB trials, and at stem elongation growth stage GS 37 for STB trial. The inoculation of wheat plants with foliar pathogens was carried out by spraying conidial suspensions (*P. nodorum*: 10^6 spores/mL at growth stage GS 49; *Z. tritici*: 10^7 spores/mL at growth stage GS39), containing Tween 20 surfactant (0.1% v/v) as previously described [22]. The inoculated plots were covered with transparent plastic sheets for 48 h, providing a humid, saturated atmosphere necessary for spore germination and penetration into the leaves. Over that plastic sheet, a plastic shade was used to protect the plants from direct sunlight. Infections were assessed by visual observations of symptoms as STB and SNB severity on flag leaf at growth stage GS 83, according to Iori *et al*., 2015. In the field, diseases were assessed every 7-10 days until ripening stages. All field trials were harvested at maturity.

2.8. Statistics

Data were analysed by ANOVA and, in case of statistical significance, Fisher's multiple comparison tests between means were performed (XLSTAT, Addinsoft).

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3. Results

3.1. Phytotron Trials show that Tramesan reduces the Pathogen Load and SLBC Disease Severity

The SNB and STB severity on the leaves of two commercial varieties of durum wheat, Svevo and Duilio, was calculated based on Liu's scale, a 0-to-5 qualitative lesion-type rating scale that assesses symptoms on leaves at 7 dai (Tab. 1 and Fig. 1). Results showed that plants treated with Tramesan had fewer symptoms compared to the untreated ones, and that Tramesan reduces disease severity by *P. nodorum* (up to 75%) more than that by *Z. tritici* (up to 65%), even if these differences are not statistically significant (p=0.06). Svevo was more susceptible to the disease (mainly *P. nodorum*) than Duilio. By quantifying the fungal DNA within leaf tissues at 0, 3, and 9 dai (Fig. 1), we found that the pathogen load dramatically decreased under Tramesan treatment by up to 99% for *P. nodorum*, and up to 75% for *Z. tritici*.

3.2. Tramesan affects the Expression of Defence Genes and Hormones in Durum Wheat Leaves

Leaves were harvested at 48 hours after treatment. RT-PCR showed a significant difference in gene expression in plants treated with Tramesan; notably, gene expression enhancement occurred at 48 hours after treatment compared to untreated plants. This significant difference of expression between Tramesan-treated and untreated plants was found in almost all the defence genes analysed (Fig. 2).

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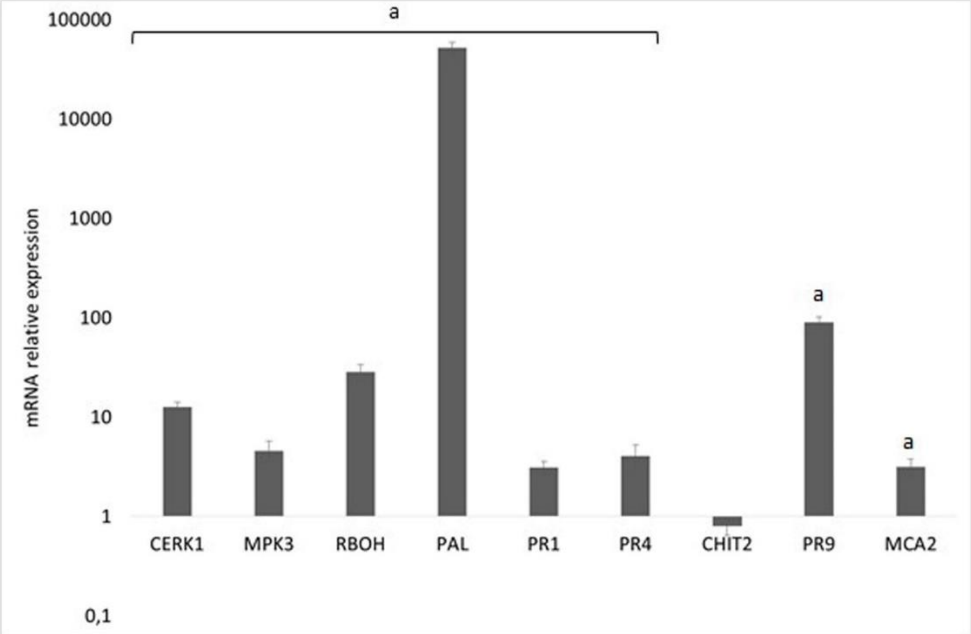

Figure 2. Expression profile of transcripts of genes *PR1*, *PR3*, *PR4*, *PR9*, *MCA2*, *NADPHox*, *PAL*, *CERK1*, and *MPK3* in durum wheat var. Svevo at 48 h after treatment with Tramesan (3.3 μM). Expression is relative to values in untreated plants (control) and values represent the mean ± SE as described in the methods section. Small capital letter in the chart represent the significantly different groups ($p < 0.05$; Fisher test).

The expression of these genes could be indicative of a defence priming *status* of the plant, as elsewhere suggested [20]. The measurement of the content of defence-related hormones such as SA and JA provided straightforward results (Fig. 3; Supplementary Table 2). Notably, JA was induced at an approximately 3-fold higher concentration at 48 hours after Tramesan application on plants. SA was down-modulated both in the control (mock) and treated (T) plants, the latter registering a lower decrease in SA compared to the mock.

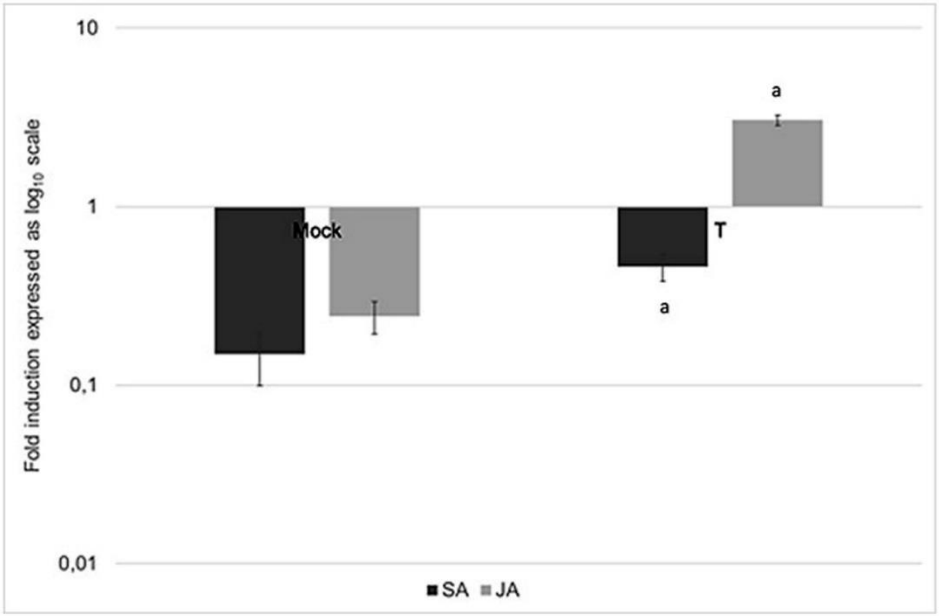

Figure 3. Fold induction (reported in $\log_{10}$ scale) of salicylic acid (SA) and jasmonic (JA) acid in durum wheat var. Svevo, after 48 h treatment (T) or untreated (mock) with Tramesan (3.3 μM). Values represent the mean ± SE as described in the methods section. Small capital letter in the chart represent the significantly different groups ($p < 0.05$; Fisher test).

3.3. Tramesan elicits the Plant Defence

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Recognition and signal transduction-related *cerk1* and *mpk3* expressions were triggered by fungal infection and were even more enhanced in plants treated with Tramesan (Fig. 4A). Fungal infections depressed both SA and JA production at 24 hai (Fold Change (FC) in infected *versus* non-infected samples: 0.15 for SA and 0.4 for JA). Pre-infection treatment with Tramesan (Tr+Inf) had little effect on SA (FC of 0.25 for Tr+Inf *versus* non-infected control) but triggered JA biosynthesis (FC of 6.2 for Tr+Inf *versus* non-infected control) (Supplementary Table 2).

Potential indicators of plant response to damage, phenylalanine ammonia lyase *PAL* that is the main hub for the phenylpropanoid pathway and metacaspase-encoding *MCA2* that is active in the programmed cell death induced by biotic stress, were expressed at high levels even in the absence of the pathogen; while *PAL* did not change its expression profile after fungal infection, *MCA2* was significantly upregulated after fungal inoculation. *P. nodorum* infection down-regulated the expression of the respiratory burst oxidase (*RBOH*); the product of this gene represents an indicator of stress response in plants. Similarly, *Z. tritici* infection pushed down *RBOH* expression; only in this latter case did Tramesan aid its stimulation (Fig. 4B). This could be explained by the set of genes under the putative control of SA and JA, even when different outcomes were observed between the two pathogens. In fact, under *P. nodorum* infection, the expression of SA-dependent pathway *PR1* and *PR9* decreased in both treatments (Inf Pn and TR+Inf Pn), whereas the JA-modulated *PR4* and *Chit2* showed an opposite trend: unaffected or slightly down-regulated in Inf Pn and strongly up-regulated in Tr+Inf Pn (Fig. 4 C-D). On the contrary, Tramesan, in the presence of *Z. tritici*, up-regulated SA-dependent genes (e.g. *PR1*), whereas JA-controlled genes (e.g. *PR4*) were almost unaffected (Fig. 4G).

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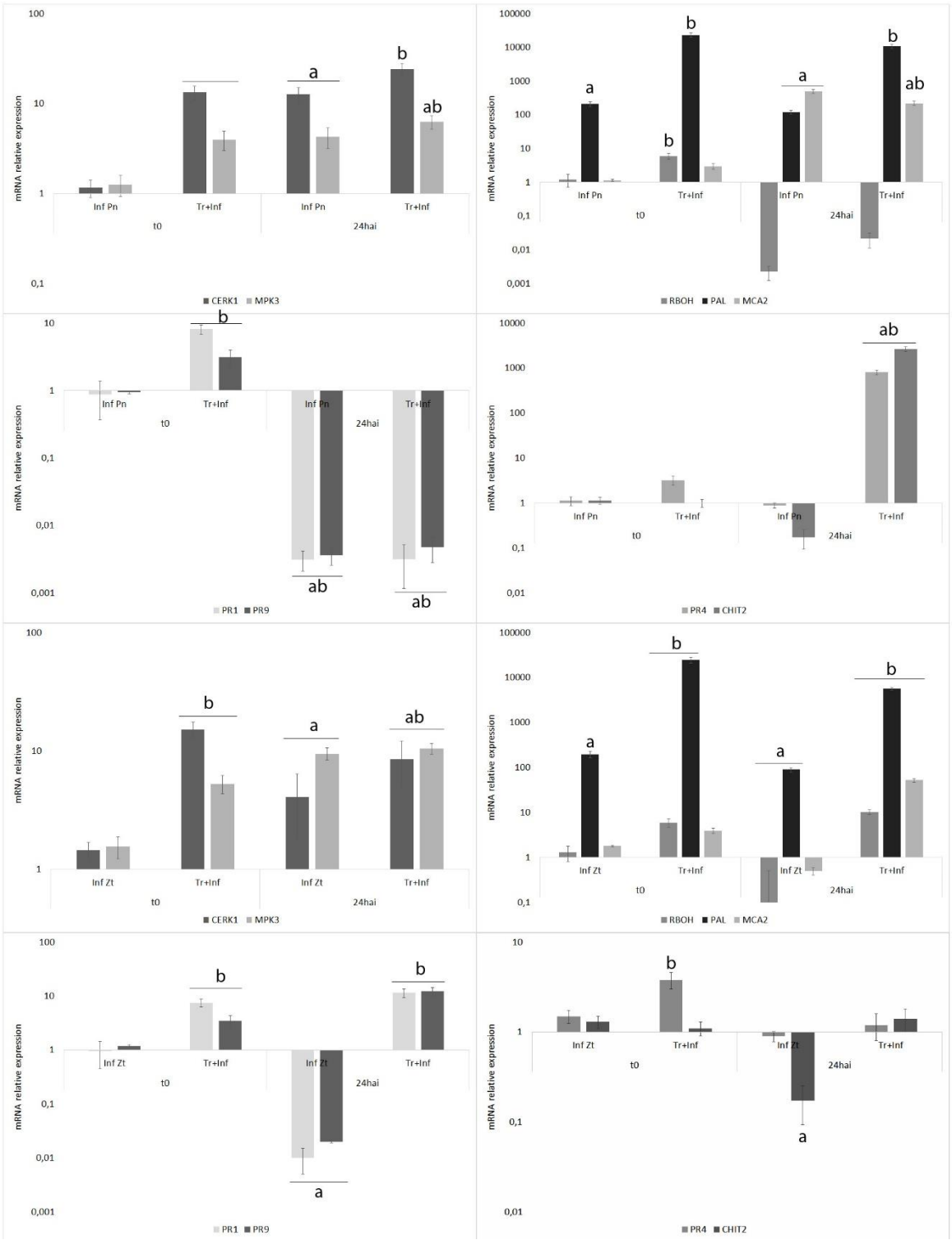

Figure 4 A-H. Expression profile of transcripts at 0 and 24 hours after infection (hai) of genes *PR1*, *PR3*, *PR4*, *PR9*, *MCA2*, *RBOH*, *PAL*, *CERK1*, and *MPK3* in durum wheat var. Svevo infected with *P. nodorum* (Inf Pn) or *Z. tritici* (Inf Zt) and infected with pathogens and pre-treated (-48 hai) with Tramesan (Tr+Inf). Expression is relative to values in untreated plants (CT-; without Tramesan, non-inoculated) and values represent the mean ± SE as described in the methods section. Small capital letter in the charts represent the significantly different groups ($p<0.05$; Fisher test).

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3.4. Field Trials confirm that Tramesan reduced the SLBC Disease Severity

The plants treated with Tramesan showed a lower severity of disease (Table 3), even if results were less intense than those under controlled growth conditions (symptoms decreased by up to 25% for *P. nodorum* and up to 30% for *Z. tritici*). Furthermore, we analysed the yield of the harvested grain, proving that the Tramesan treatment (5.29 ± 0.05 t/ha) did not affect it compared to the untreated plots (5.28 ± 0.08 t/ha).

Table 3. SNB and **STB** severity on flag leaf (%) in durum wheat (var. Svevo) at growth stage GS83 grown in Montelibretti (Rome) and in Rome fields in the 2014-2015 growing season. **Mock**: without Tramesan, non-inoculated. **T**: treated with Tramesan; **T+INF**: treated with Tramesan, inoculated with the pathogen (SNB: *P. nodorum;* STB: *Z. tritici*); **INF**: inoculated with the pathogen. Values represent the mean ± SE of three technical repetitions (n=3).

	Svevo	
	Severity on flag leaf (%) SNB	**Severity on flag leaf (%) STB**
Mock	3.2^{ab}	1.2^{a}
T	0.8^{a}	0.5^{a}
Tr + Inf	28.5^{b}	32.4^{b}
Inf	36.5^{c}	47.5^{c}
F test (P value)	4.65×10^{-4}	1.73×10^{-5}

Means followed by different letters are significantly different (the level of significance P is shown in the table).

4. Discussion

Septoria Disease Complex is a major issue for European wheat farmers. Excessive cost of fungicides and the pathogens' emerging fungicide resistance are consistent threats to the wheat yield. Even though several loci for resistance to SLBC were identified so far, a deficit in availability of resistant lines still remains [12]. One of the major problem to develop stable resistant lines could rely on the complexity of the SLBC disease. The two principal cause of this disease, *Z. tritici* and *P. nodorum*, play differently their role as pathogen of wheat. Namely, the main differences in the pathogenic process emerge in the initial stage of infection: the hemibiotrophic *Z. tritici* skips the plant recognition system by camouflaging its PAMP (e.g. chitin with Mg3LysM); the necrotrophic *P. nodorum* does not possess such "weapons" but in sensitive genotype, induce straight plant cell death (e.g. ToxA *versus* Tsn1 *sensitivity* gene) [10]. Nonetheless, twenty major *Stb* genes providing a qualitative resistance to SLBC have been identified so far [11]. Therefore, it is reasonable to suppose that novel SLBC-resistant lines could be on the market within few years; nowadays the problem of SLBC is becoming more and more a constraint for wheat production worldwide even because the emergence of *Z. tritici* and *P. nodorum* resistant to QI or SDHI is increasing [12].

We show that Tramesan—a basidiomycete-derived exo-polysaccharide—could represent a natural, user-friendly, low-cost alternative to protect crops. Specifically, our results indicate that Tramesan supports wheat plants in controlling SLBC causal agents: *P. nodorum* and *Z. tritici.* However, Tramesan's mode of action is still an outstanding question.

We report that Tramesan is effective at augmenting the synthesis of defence-related hormones, notably JA, and of some markers of plant defences (*PR1* and *PR4 inter alia*). This effect occurs within 48 hours after Tramesan is applied, albeit not all the markers tested react linearly: the mitogen-activated protein MPK3 and the LysM receptor kinase CERK1 display high basal levels [27,28] even if induced by Tramesan and fungal infection. In contrast, *PAL*, *MCA2,* and *RBOH* are evidently controlled by additional inputs other than *stress* [ABA(-TAS14) and auxin(-PIN2) [29,30]. The set of genes putatively regulated by SA, such as *PR1* and *PR9,* or by JA, such as *PR3* and *PR4* [31,32], showed a straight correlation with the hormone levels. In the case of SA-controlled genes, NPR1 is the most likely mediator of the stimulus [33–36].

This set of data did not allow to settle the "nature" of our elicitation process: Tramesan induce *per se* a high basal activation of plant defenses. In this perspective, Tramesan can be envisioned as a MAMP that plant recognize as non-self biomolecule for inducing its defenses. The sought for

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compounds, mainly microbe-derived, able to induce efficiently plant defenses is gaining momentum under the light of elucidating the induced systemic resistance and defense priming patterns [16,19]. Our results did not allow to assess if Tramesan could induce an IR or prime plant defenses and further study are needed. Some hints could suggest than is more than a "simple" MAMP: the onset of these defences appears modulated in time, triggered by subsequent infection and effectively reduces the pathogen growth and, consequently, the symptoms of disease. Is it costly? Probably not; yield data from field experiments suggest that Tramesan did not affect durum wheat production. We hypothesize that Tramesan is recognized by specific receptors that, in turn, activate pathways leading to an antioxidant response in the host. Tramesan acts in several organisms (mammals, fungi, and plants) as an activator of antioxidant defenses[21]; when employed against mycotoxigenic fungi, it switches off aflatoxin synthesis—a process closely related to the antioxidant system of the cell—in *Aspergillus* section Flavi [37]. Therefore, along with inducing plant defenses, Tramesan can lower the reactive oxygen species (ROS) that circulate at the plant-pathogen interface, thus creating a less favorable environment for the necrotrophic growth of plant pathogens such as *P. nodorum*. Several receptors in several organisms are known to act as ligands of fungal glucans and, in turn, trigger various responses ranging from innate immunity to cell death or symbiosis [38–40]. Most recently, Gubaeva and colleagues hypothesise the "slipped sandwich" model for explaining the ability of AtCERK1 in bound and transduce oligochitosan (the most effective with degree of polymerization up to 7- DP7) to induce defence reactions in *A. thaliana* [41]. Similar results, i.e. the identification of a receptor kinase proteins putatively able to bound oligo-chitosan was found in common wheat [42].

In this context, we postulate that Tramesan acts as a ligand for still unknown inter-kingdom conserved receptors, and this recognition would elicit host defences to limit pathogen development and disease. The ability that Tramesan demonstrated in modulating cell antioxidation in other systems and the well-established relation between plant defence onset and antioxidants modulation [42] suggest that the triggering of defence-related hormones and genes occur at the very early steps following pathogen recognition.

Further studies in the field, under natural infection conditions, should allow us to better assess the priming and memory effect of Tramesan in host plants actually does occur and its real effectiveness in controlling SLBC.

Supplementary Materials: The following are available online at xxx, Table S1: List of primers used within this study. Table S2: Average values (biological duplicate-technical triplicate) of relative abundance (normalised on NAA as ISTD) of salicylic and jasmonic acids in leaves of durum wheat var. Svevo, at different times (0-9 dai) pre- and after the inoculation with the pathogens P. nodorum (Inf Pn) or Z. tritici (Inf Zt) in wheat plants treated (Tr + Inf Pn and Tr + Inf Zt) or not with Tramesan 3.3 μM).

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