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Triggering Systemic Resistance by seed coating with thyme oil and *Paraburkholderia phytofirmans*PsJN strain against Septoria leaf blotch of durum wheat

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Abstract – Biostimulants inducing plant defences is among the promising approaches to reduce the chemical pesticides use in wheat protection against Septoria leaf blotch caused by the hemibiotrophic *Zymoseptori tritici*. Within this scope, the effect of seed coating with the bacterium *Paraburkholderia phytofirmans* (PsJN strain) or thyme oil on triggering wheat systemic resistance to Septoria leaf blotch under controlled conditions was investigated using histopathological, physiological and biochemical approaches. Both products reducednecrosis, pycnidial density,associated with induction of programmed cell death, papillae accumulation in the biotrophic stage, and lowerperoxidase activity, H_2O_2, catalase and phenolic content in the necrotophic stage. Thyme oilinduced systemic acquired resistancewhile PsJN strain triggered induced systemic resistance to Septoria leaf blotch.

Keywords: Biostimulants; programmed cell death; papillae; peroxidase; catalase; hemibiotrophic

1. Introduction

In Tunisia, durum wheat approximates 49% of the total annual cereal area, with an average yield of 1.6 tons.ha^{-1}between 2000 and 2012 (Nefzaoui et al. 2012). Septoria leaf blotch (SLB) caused by the hemibiotroph *Zymoseptoria tritici*(Desm.) is the primary fungal disease that affects durum wheat throughout causingyield losses estimated amount up to 30% (Berraies et al. 2014) and the use of fungicides is currently one of the main approaches used to manage SLB, however, fungicide resistance is a widespread problem (Jorgensen et al. 2008), thereby requiring new approaches for preventing ingress and colonization of the pathogen in wheat.Seed priming with seed coating technique may afford several economic and agronomic advantages to cultivated plants through priming the germinating seedling in order to react more rapidly and efficiently to a further stress, by using beneficial microorganisms and other biochemical compounds as seed-priming agents, and thus improvedisease resistance (Lutts et al. 2016), and at the same time tends to outweigh farmers fungicide's expenseson fertilizers and fungicides (Porter and Scott 1981). The objective of the present study was thus to examine the effects of seed coating with thyme essential oil and PsJN strain on SLB resistance in wheat plants.*Paraburkholderia phytofirmans* PsJNstrain, an endophytic bacterial stain originally isolated from onion roots, significantly promoted growth of several plants and has been reported as a remarkable biocontrol agent(Esmaeel et al. 2018). Thyme essential oil was reported to have antifungal activities by targeting genes involved in fungal development and virulence of the yeast-like fungus*Z. tritici*, (Ben Jabeur and Hamada 2014; Ben Jabeur et al. 2017), most of which are mediated by thymol or carvacrol, as the major phenolic components of this oil. Our previous studies also reported the effectiveness of thyme essential oil in controlling gray mold and Fusarium wilt, when applied to tomato roots, by inducing systemic acquired resistance in seedlings attributed to peroxidase accumulation (BenJabeur et al. 2015).

2. Material and methods

2.1. Plant material

The Tunisian variety « Karim » of durum wheat, classified as susceptible to SLBbased on field tests (Ferjaoui et al. 2015), was used in this work. Seeds were surface sterilized by soaking seeds in 95% ethanol for 10 s, rinsed three times with sterile water, soaked again in 5% sodium hypochlorite for 3 min, rinsed three times with sterile water, and placed on sterile filter paper to air dry.

2.2. Biotic and abiotic natural products

Thyme essential oil: aerial parts of *Thymus capitatus* (chemotype carvacrol, voucher specimen No. D 1186-3) were harvested from the mountain of Zaghouen. The essential oil was extracted by hydrodistillation and stored at 4 ° C for subsequent use. The chemical composition of the oil was investigated and carvacrol was identified as the major compound according to Ben Jabeur et al. (2015). The concentration of thyme oil was adjusted to 5 ppm before use with adding DMSO as solubilising agent to assure homogenous application of the essential oil.

Paraburkholderia phytofirmans PsJN strain was provided by Pr. Ait Barka, (University of Reims, France). The bacterial inoculum was produced by transferring one colony to 20 ml of King's B liquid medium, in a 50-ml Falcon tube incubated at 27°C at 150 rpm for 48 h. Bacteria were collected by centrifugation at 8000 rpm for 5 min and washed twice with phosphate-buffered saline (PBS) (10 mM, pH 6.5). The pellet was re-suspended in PBS and used as inoculum. The bacterial concentration was estimated by spectrophotometry (600 nm) and adjusted to 10^9CFU mL^{-1} with PBS. The concentration was also checked by serial dilution method and estimation of the number of colonies on King's B agar medium.

2.3. Fungal inoculum preparation

The strain TUN6 of*Z. tritici*,used in this study, is a virulent pathotype of SLB in Tunisia causing susceptibility in several durum wheat genotypes(Medini and Hamza 2008), provided by Pr Sonia Hamza(INAT, Tunisia). Inoculum was produced by scrapping spores and mycelia off the surface of potato dextrose agar (PDA)platesand cultivating them in liquid yeast extract sucrose medium (YES)for 7 days at 18°C on a rotary shaker at 150 rpm. Spores were collected by centrifugation at 5000 g for 5 min, washed twice with sterile distilled water and suspended in sterile distilled water containing 0.1% Tween 20 surfactant. The concentration was adjusted to 10^5 spores.mL^{-1}, for in vitrospore germination assays, and to 10^7 sporesmL^{-1}, for plant inoculation experiment.

2.4. Direct in vitro antifungal potential of thyme oil and PsJN strain

Direct effect of products on mycelia development was evaluated. In Erlenmeyer flasks, 20 mL of YES medium was inoculated with 10^5 spores mL^{-1} of *Z. tritici*. Thyme oil and PsJN strain were added with the concentrations 100 ppm and 10^6CFU mL^{-1}respectively; sterile YES medium was used as control. The assay was performed in triplicate for each sample. The Flasks were incubated at 18°C on a rotary shaker for 5 days and samples were taken for cell viability and for microscopic image analysis.Cell viability (%), was determined with MTT assay according to Tada et al. (1986). MTT is a colorimetric assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and based on the mitochondrial enzyme reduction of tetrazolium dye to detect and determine cell viability.For microscopic image analysis, 3 drops of 5 µL were taken from each samples and inoculated on PDA plates. Plates were incubated for one day at 18°C and then put under microscope for observation and imaging. Microscopic images were taken using Leica LAS EZ software of Leica DM 750 research microscope (Leica Microsystems) and image analysis was performed using the software imageJ (Rasband, W.S., ImageJ; U. S. National Institutes of Health). Mycelial development was determined by measuring the area occupied by the mycelium in control and treated samples according to the following formula; Mycelial development (%) = (mycelium area in pixel/ image area in pixel) x 100.

2.5. In plantastudies of SLB under controlled conditions

2.5.1. Seed coating with thyme oil and PsJN strain

The coating technique consists on preparing the coating solution mixture containing 40 µL of the coating product (Agicote Rouge T17, AEGILOPS Applications, France) and 400 µL of the appropriate dose of thyme oil or PsJN strain (water was used as a control). The coating mixture was applied

progressively to 10 g of wheat seedsin rotation. Seeds were coated with PsJN strain (10^8CFU mL^{-1}) and thyme oil (5 ppm) and were sown in pots under controlled conditions.

2.5.2. Controlled growth conditions, plant inoculation and leaf sampling

Coated seeds were sown in pots containing an autoclaved soil mixture of horticultural compost and sand (1/1, v/v). Pots were incubated in a growth chamber at 22°C (day) and 18°C (night) with 16h photoperiod. Seedlings were watered twice a week with distilled water, and once a week with Hoagland nutritive solution (Hoagland and Arnon 1950). Inoculation occurred following full emergence of the second leaf; 21 days after planting by spraying with *Z. tritici* inoculum (10^7 spores mL^{-1}) and plants were enveloped in a transparent polyethylene bag for 2 days tomaintain a high level of humidity. Disease development was followed by sampling third leaves at 16 days post-inoculation (dpi) for the microscopic observationsand at 21 dpi for biochemical analysis and disease assessment.

2.5.3. Histopathological analysis

Microscopic observations were carried out according to Shetty et al. (2003) with modifications. Three third leaveswere sampled from different pots per each treatment, and were cut and cleared by immersing them overnight in glacial acetic acid and absolute ethanol (1/3, v/v). Then, they were washed three times with distilled water and stored in a solution of lactoglycerol (lactic acid/glycerol/distilled water, 1/1/1, v/v/v) until observation. Coloration was carried out by incubating the leaf sections in 0.1% trypan blue in lactoglycerol for 1h at 50°C. Stained slides were microscopically assessed using Leica microscope. Programmed cell death (PCD) were scored within each leaf section,using the software ImageJ, as PCD (%) = leaf section area- PCD area / leaf section area) X 100, then data of PCD percentage from each section were summarized to get the final PCD percentage per total leaf area. The number of formed papillae per leaf section wasmeasuredusing the "Analyze Particles" function in ImageJ software; the data of formed papillae per leaf section were summarized to get the final formed papillae per total leaf area.

2.5.4. Disease assessment

Third leaves were sampled for disease assessment, scanned and then the extent of both the necrotic area and pycnidial density were measured using the software ImageJ according to Stewart and McDonald (2014). Briefly, the background was removed from each image and total leaf area and green leaf area in pixel was calculated using color thresholding in the RGB (Red Green Blue) color space as formulated: Septoria severity (%) =((total leaf area-green leaf area) /total leaf area) X 100. The number of pycnidia was calculated using the "Analyze Particles" function.

2.5.5. Biochemical analysis

Hydrogen peroxide (H_2O_2) levels were determined as described by Sergiev et al. (1997).Catalase activitywas determined according to Beers and Sizer (1925).Protein concentration was determined according to Bradford (1976).Specific activity of catalase was expressed as $\mu mol.min^{-1}.mg^{-1}P$.Peroxidase activity (guaiacol peroxidase) was determined according to Egley et al. (1983)and expressed as Unit mg^{-1} P,one unit of peroxidase (U) is defined as µmol mL^{-1} oxidized guaiacol per min per mg protein.Total phenolic content was estimated by Folin-Ciocalteu method (Singleton et al. 1999), using Catachol as the standard and expressed as mgg^{-1} FW.

2.5.6. Statistical analysis

The effects of treatments on catalase, peroxidase, hydrogen peroxide, phenolic content, necrotic area, and pycnidial densitywere determined through a one-factor ANOVA (treatment) using the RStudio 1.1.463 (R Foundation for Statistical Computing, Vienna, Austria). The least significant difference (LSD) test was used to assess differences between treatment means.

3. Results

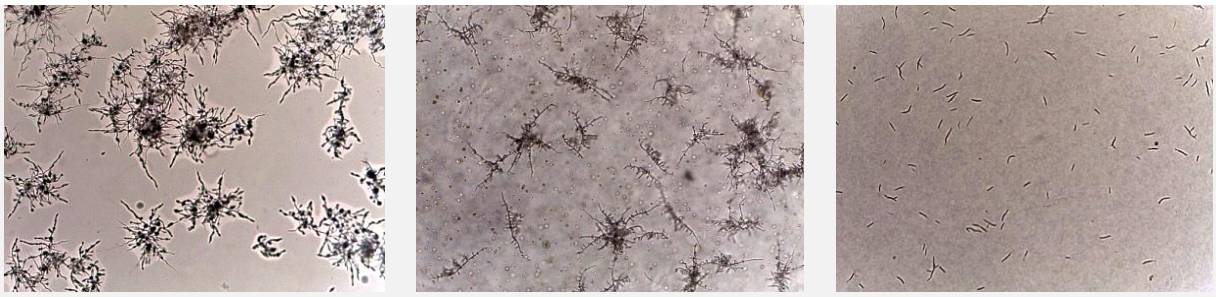
3.1. Direct in vitro antifungal activity of thyme oil and PsJN strain

Analysis of variance (Tab.1) showed that treatments significantly affected cell viability and mycelial development ($P < 0.001$).

Table 1. Effect of thyme oil and PsJN strain on Cell viability and mycelial development of *Z.tritici.* The F values are shown, and the symbols indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Treatment	Control	PsJN strain 10^6 CFU mL^{-1}	Thyme oil (100 ppm)	ANOVA
Cell viability (%)	$86.63^a \pm 4.31$	$75.3^a \pm 3.99$	$48.43^b \pm 8.25$	33.75***
Mycelial development (%)	$14.66^a \pm 1.62$	$7.99^b \pm 1.56$	$1^c \pm 0.2$	82.21***

In control pathogen strain, microscopic captures showed that germ tubes or macropycnidiospores did not grow extensively in length but rather undergoes a process of intense localized microconidiation as production of additional micropycnidiospores formed by lateral budding from hyphae that partly elongates in relatively short filamentous hyphae and accumulate in the center of the dense colony as previously described (Mehrabi et al.2006) (Fig.1. A). In control pathogen strain, mycelial development was around 14% and cell viability was around 86 % (Tab. 1). In the presence of thyme oil, hyphal elongation was hampered and most of germ tubes look degenerated (Fig.1. C), mycelial development was reduced to 1%, and cell viability decreased to around 48 % (Tab. 1). In the presence of PsJN strain, the pathogen showed a different germination pattern; hyphae were straighter and longer, micropycnidiospores formed by lateral budding did not elongate in filamentous hyphae, budding process was rarely observed resulting in a less dense filaments and indicating a higher tendency for filamentous growth rather than microconidiation (Fig.1. B). In contrast, mycelial development was reduced to around 8 % (corresponding to 50% inhibition) while cell viability slightly decreased to around 75 % (corresponding to 8.6 % inhibition).



A B C

Figure1. Comparative analysis of direct effect of thyme oil and PsJN strainon *Z.tritici* growth morphology in vitro. (A) Normal hyphal development of control strain, (B) altered hyphal development in presence of PsJN strain, (C) hampered hyphal development in presence of thyme oil.

3.2. In plantastudies of SLB

3.2.1. Effect of coating seeds with thyme oil and PsJN strain on biotrophic behavior of *Z. tritici*

Analysis of variance (Tab.2) showed that treatments significantly affected PCD percentage ($P < 0.001$). Concerning *Z.tritici* behavior in control plants, microscopical observations showed intercellular growth and hyphal penetration through open stoma (Fig. 2.B) and abundant mycelial growth around epidermal cells and vascular cells (Fig. 2.A, C). Concerning plant defense responsein control plants, there was no trace of Trypan blue staining in cells indicating absence of programmed cell death (PCD) (Fig. 2.A, B, C; Tab.2). In plants treated with PsJN strain, PCD reached 2.33% of the leaf area (Tab.2), and was induced only in challenged sites, as stained in blue (Fig. 2. E, white arrow) associated with systemic closure of intact adjacent stoma (Fig. 2. E, black arrow) was observed. Another phenomenon was observed in *Z.tritici*- PsJN strain -wheat interaction, bacterial cells condensate in clusters in leaf vessel bundle around threatening hyphae (Fig. 2.D), and switched to the biofilm mode of growth when penetration of adapted hyphae occurred (Fig. 2. F).In treated plants with thyme oil, PCD was induced in challenged (Fig. 2. I),and in non challenged (Fig. 2. G, H) leaf sites;characterized by collapse of epidermal and substomatal cells, and reaching an area of 23. 33 % of leaf area (Tab.2).

Table 2. Effect of thyme oil and PsJN strain on PCD percentage and papillae formation. The F values are shown, and the symbols indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Treatment	Control	PsJN strain 10^6 CFU mL^{-1}	Thyme oil (100 ppm)	ANOVA
PCD percentage (%)	0.0^{c}±0.0	2.33^{b}±0.57	23.33^{a}±1.52	557.4***
Papillae number in vessels/leaf	0.33^{b}±0.57	15.33^{a}±1.52	5.33^{b}±0.57	133.8***
Papillae number in epidermis/leaf	2.66^{c}±0.57	1.66^{a}±0.57	2.33^{a}±0.57	9.333 *
Total papillae number/leaf	3.0^{c}±1	17.0^{a}±1	7.66^{b}±1.15	137.2 ***

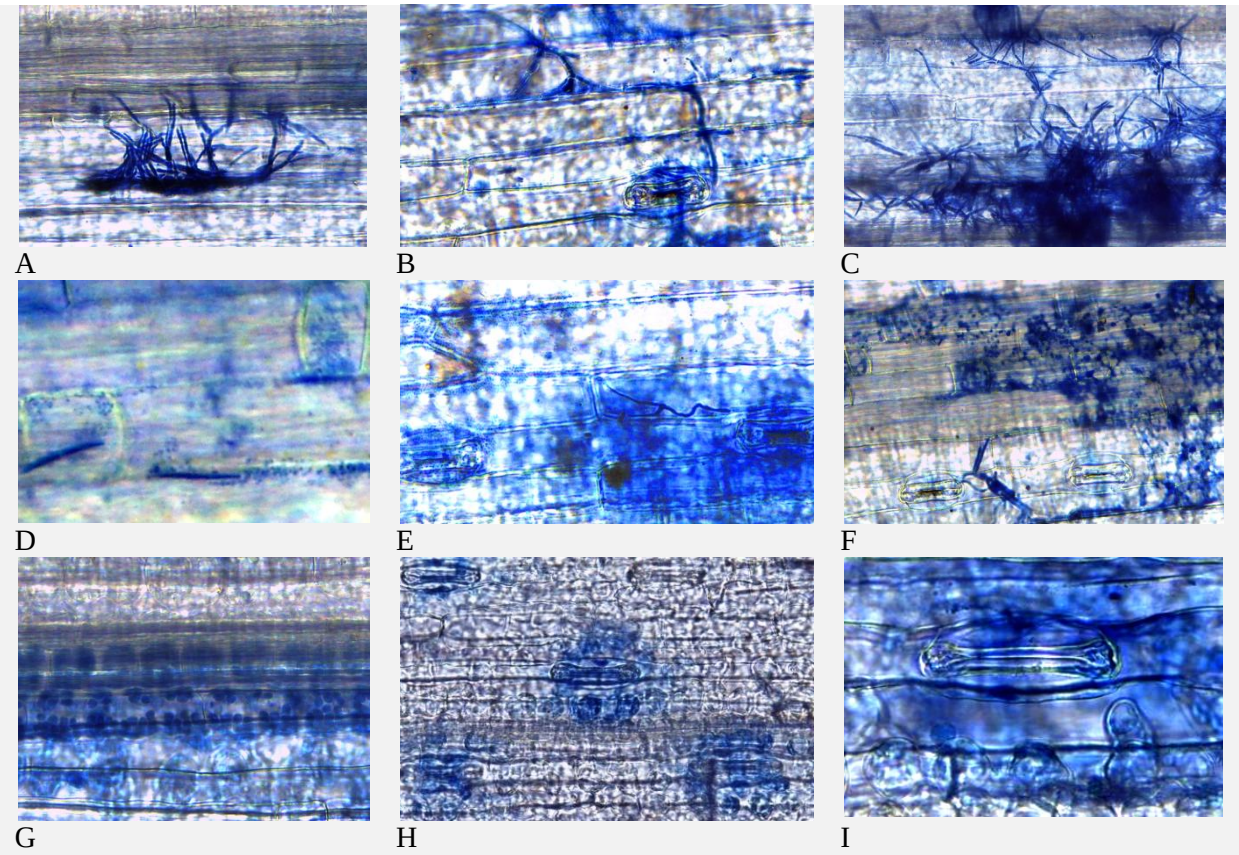


Figure 2. Microscopical symptoms of wheat leaves inoculated with *Z.tritici* sampled at 16 dpi. Panels (A-C): inoculated control plants; panels (D-F): inoculated plants treated with PsJN strain; panels (G-I): inoculated plants treated with thyme oil; (B) intercellular growth and hyphal penetration through open stoma; (A, C) extensive mycelial growth in epidermal cells and around vascular cells; (D) condensation of bacterial cells in clusters around threatening hyphae in vascular cells; (E) Trypan blue staining of induced PCD in challenged leaf site treated with PsJN strain (white arrow) and condensation of bacteria in the adjacent non challenged leaf site (Black arrow); (F) bacterial biofilm formation; (G, H) PCD induced in non challenged leaf tissues treated with thyme oil; (I) PCD induced in challenged leaf sites treated with thyme oil, red arrow refers to hyphae (colored in dark blue).

Papillae accumulation, as another common physical defense mechanism,was observed in plants (Fig. 3), treatment affected significantly papillae number in vessels and total papillae number per leaf ($P < 0.001$) and papillae number in epidermis per leaf ($P < 0.05$).In control plants papillae were rarely observed counting 3 papillae per leaf (Tab.2); 2.66 papillae in epidermal cells (Tab.2; Fig. 3.B),and 0.33 papillae in vessel bundle extensively colonized with pathogen hyphae(Tab.2; Fig. 3.A). Thyme oil induced papillae formation, counting 7.66 papillae per leaf (Tab.2); 5.33 papillae in vessel bundle (Tab.2; Fig. 3. F, I), and 2.33 papillae in epidermal cells (Tab.2; Fig. 3.G, H). PsJN strain highly induced papillae formation, compared to control and thyme oil effect, counting 17 papillae per leaf (Tab.2); 15.33 papillae in vessel bundle (Tab.2; Fig. 3. C, E), and 1.66 papillae in epidermal cells (Tab.2; Fig. 3.D). Multi-vesicular bodies (MVBs) were captured surrounding papillae in plants treated with PsJN strain (Fig. 3.E, red arrows).

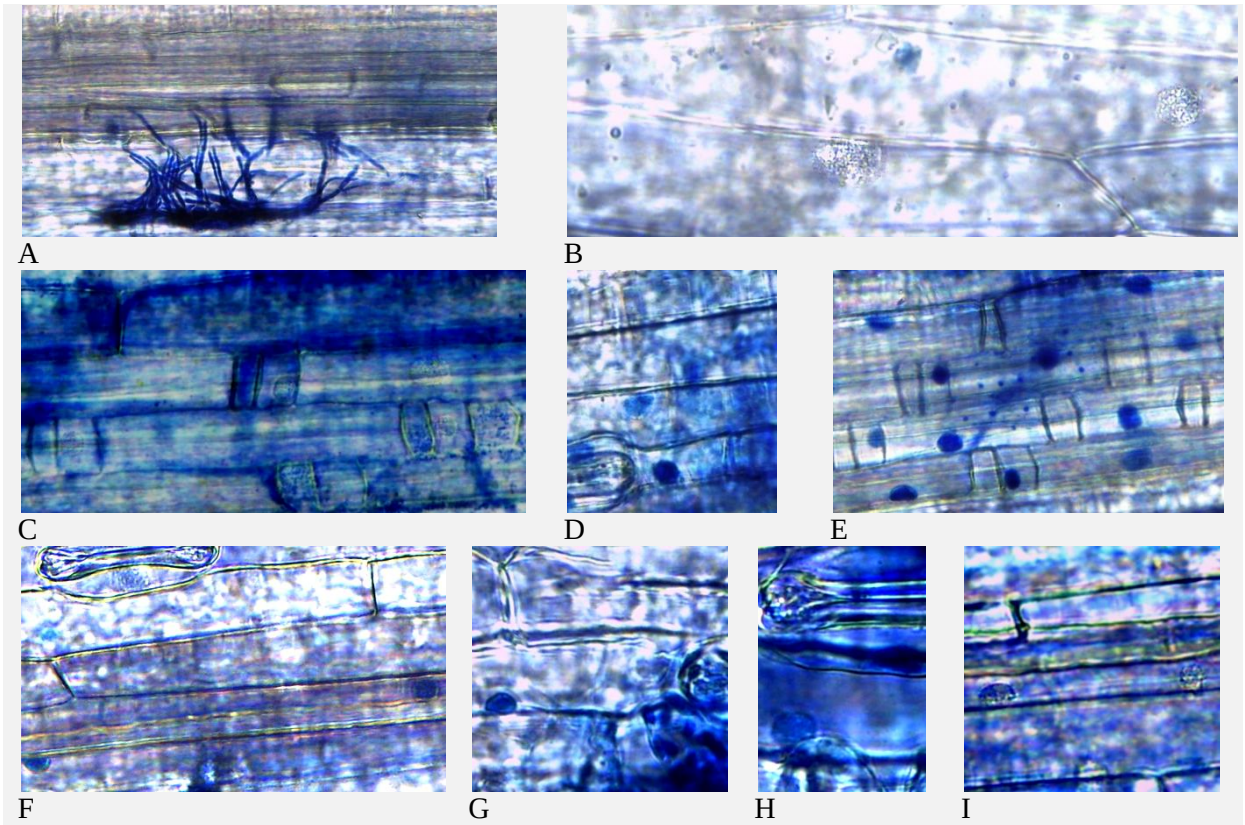


Figure 3. Microscopic, cellular localization of papillae formation in wheat leaves inoculated with *Z.tritici* at 16 dpi. Panels (A-B): inoculated control plants; panels (C-E): inoculated plants treated with PsJN strain; panels (F-I): inoculated plants treated with thyme oil; (A, C, E , F, I): vessel bundle; (B,D,G,H: epidermal substomatal cells; black arrows indicate papillae; r arrows indicates Multi-vesicular bodies (MVBs).

Furthermore, concerning papillae colorization; in treated plants, Trypan blue remain entrapped in papillae in intact cells not undergoing PCD (Fig. 3.D, E, F, G, black arrows), while in cells undergoing PCD, papillae were no longer discernible and color loss is most likely a result of lateral diffusion of the dye in the plasma membrane (Fig.3.C, H, I, black arrows). Contrastingly, in control plants, indiscernible cleared papillae were observed in cells not undergoing PCD (Fig. 3.B, black arrows).

3.2.2. Effect of coating seeds with thyme oil and PsJN strain on the necrotrophic behavior of *Z. tritici*

Effect of coating seeds with thyme oil and PsJN strain on necrotic area and pycnidial density

Analysis of variance (Tab. 3) showed that treatment significantly affected necrotic area and pycnidial density (P<0.001).

Table 3. Results of one-factor ANOVA for necrotic area, and pycnidial density, in inoculated plants with *Z.tritici*. The F values are shown, and the symbols indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Traits	Necrotic area	Pycnidial density
Treatment	527.6***	431.9***

At 21 dpi, macroscopical symptoms became visible on control infected plants as chlorotic and necrotic spots at the tip of the leaves (Fig. 4.C); necrotic leaf area reached 50% (Fig.4.A), with 60% of pycnidial density in necrotic spots (Fig.4.B). Both treatments reduced necrotic area and pycnidial density with relative differences. In treated plants with PsJN strain, necrotic area was reduced to 10% (Fig.4.A), with reduced pycnidial density in necrotic area to 9.4% (Fig.4.B), associated with higher green pigmentation (Fig. 4. D). In treated plants with thyme oil, necrotic area was reduced to 30% (Fig.4.A) with reduced pycnidial density in necrotic area to 1.8% (Fig.4.B), associated withreduced green pigmentation, compared to treated plants with PsJN strain (Fig. 4.E).

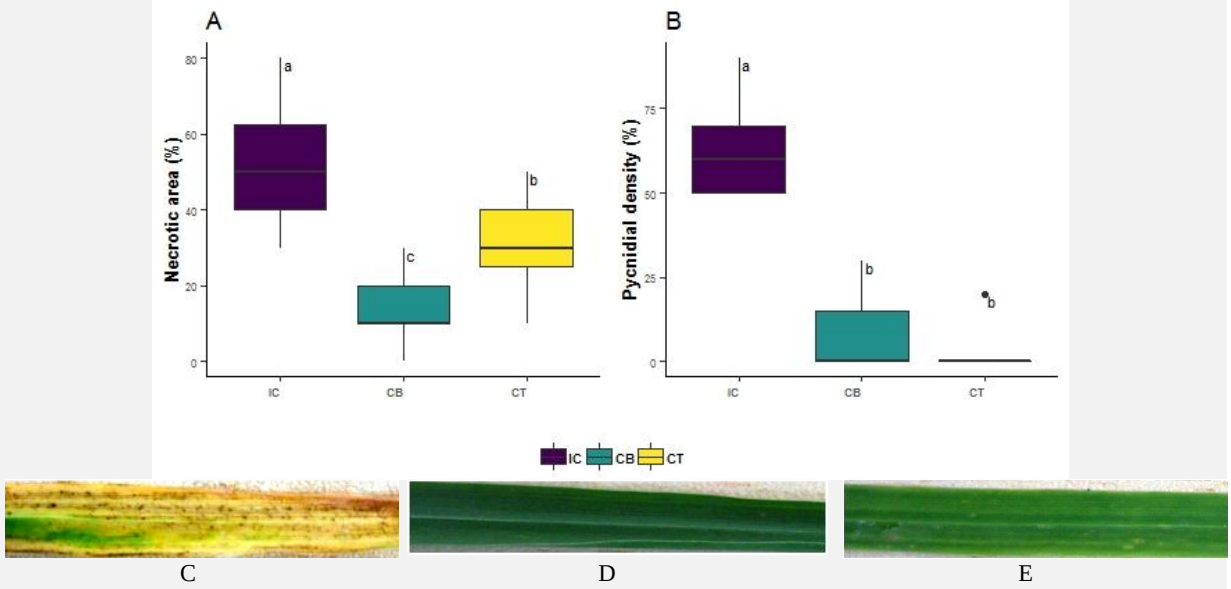


Figure 4. Effect of coating seeds with thyme oil and PsJN strain on necrotic area, and pycnidial density measured with imageJ, in inoculated plants with *Z.tritici* at 21 dpi. IC: inoculated control, CB: coated with PsJN strain, CT: coated with thyme oil, panels (C-E) macroscopical symptoms of inoculated wheat leaves, C: inoculated leaves derived from control coated seeds, D: inoculated leaves derived from seeds coated with PsJN strain, E: inoculated leaves derives from seeds coated with thyme oil.

Effect of coating seeds with thyme oil and PsJN strain on catalase, peroxidase, Hydrogen peroxide, and phenolic compounds

Analysis of variance (Tab. 4) showed that treatment significantly affected catalase, peroxidase, Hydrogen peroxide ($P < 0.001$), and phenolic compounds ($P < 0.01$).

Table 4. Results of one-factor ANOVA for catalase, peroxidase, Hydrogen peroxide, and phenolic compounds, in inoculated plants with *Z.tritici*. The F values are shown, and the symbols indicate statistical significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Traits	H_2O_2	Catalase	Peroxidase	Phenolic content
Treatment	99.9***	45.74***	21.92**	22.25**

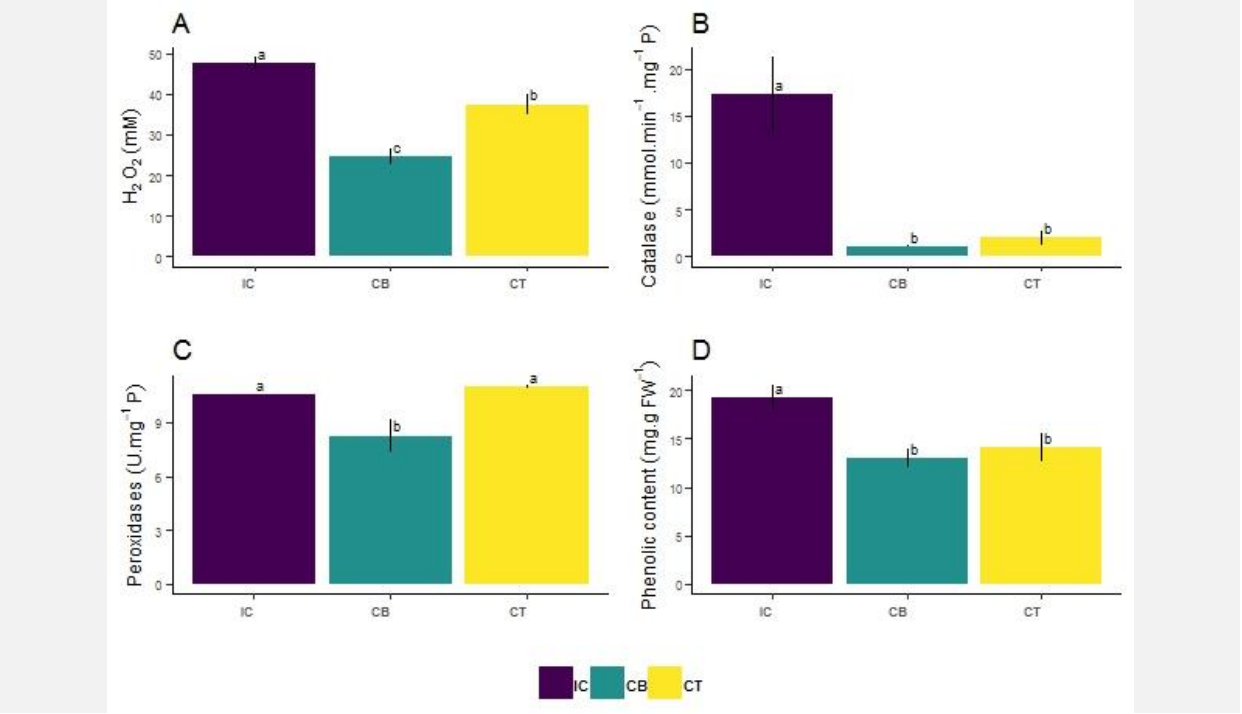


Figure 5. Effect of coating seeds with thyme oil and PsJN strain on catalase, peroxidase, H_2O_2, and phenolic content, in inoculated plants with *Z.tritici* at 21 dpi. IC: inoculated control, CB: coated with PsJN strain, CT: coated with thyme oil.

At 21 dpi, H_2O_2level was elevated, in control infected plants, associated with elevated catalase, peroxidase and phenolic content (Fig. 5). In treated plants, H_2O_2level was lower (Fig. 5.A) associated with lower phenolic content (Fig. 5.D) and muchreduced catalase activity (Fig. 5.B), compared to control. Effect of the treatments in terms ofperoxidase activity (Fig. 5.C) and H_2O_2(Fig. 5.A);peroxidase activity and H_2O_2levelwere reduced in treated plants with PsJN strain compared to treated plants with thyme oil.

4. Discussion

4.1. Direct in vitro antifungal activity

Since bioactive molecules that inhibit the growth of fungal pathogens can be either fungicidal or fungistatic, the effect of thyme oil and PsJN strain on both the viability and development of hyphal cells was assessed The observed direct antifungal effect of thyme oil confirms our previous results revealing its potential to repress genes involved in *Z. tritici* fungal development (Ben Jabeur et al. 2017). The observed effect of PsJN strain on reduction of yeast-like growth could be either due to trophic competition by depriving the pathogen from nutrient liquid culture or due to antibiosis. The contrasting results of mycelial development and cell viability are due to the MTT test characteristics; unable to differentiate strains' cells during bacterial-fungal interactions. Combining this result with microscopic observation, it is concluded that bacterial cell density reduced and altered fungal cell development and that the tetrazolium-salt-MTT test, appropriate for bacterial-fungal interactions, and should be used instead (Patel et al. 2013).

4.2. Effect of coating seeds with thyme oil and PsJN strain on biotrophic behavior of *Z. tritici*

Typical symptoms of the wheat-*Z.tritici* compatible interaction were observed in control infected plants and colonization was successful characterized irregular chlorotic areas on the infected leaves that develop into necrotic lesions covered with dark brown to black pycnidia or pseudothecia of the fungus (Eyal et al. 1987). It has been described thatincompatible responses in the wheat-*Z. tritici* interaction consist of the inability of the fungus to grow within its host characterized by a very little increase in fungal biomass during the later stages of pathogenesis, can still result in necrosis and production of pycnidia but considerably less as compared with that in compatible interactions, and that any restriction or delay in pathogen development can be regarded as a form of resistance (cited in Kema et al. 1996). In this context, we may assume that coating seeds with either PsJN strain or thyme oil resulted in wheat-*Z. tritici* incompatible interaction. Both treatments caused degeneration of mostly all hyphae reflecting altered biosynthesis in the pathogen cell wall. In the case of minor established hyphal penetrations, treatments encountered pathogen progress most likely due to induction of PCD where nutrients or critical host components for replication are considered limiting (Van Aken and Van Breusegem 2015) leading to systemic closure of intact stomata which confirm previous work reporting that PCD is linked to enhanced resistance to several different pathogens and systemic acquired resistance (Walters and Fountaine 2009). Indeed, control infected plants didn't exhibit PCD after fungal infection highlighting the hypervirulence of the strain silencing plant PCD-related defense response (Van Aken and Van Breusegem 2015).Interestingly, thyme oil induced systemic PCD in non challenged leaf indicating its potential to trigger systemic acquired resistance (SAR) (Walters and Fountaine 2009) leading to transient reduced green pigmentation as consequence of the plant sacrificing some of its cells during the PCD response. Bacteria condensate around hyphae and, when penetration of adapted hyphae occurred , PSJN either switched to the biofilm mode of growth or induced local PCD indicating induced systemic resistance (ISR) (Walters and Fountaine 2009) and highlighting the chemotaxis of PsJN straintowards the attractant pathogen. A possible explanation for *Z.tritici*- PsJN straininteraction is that PsJN strainbehaves as an epiphyte by forming clusters of colonies that grew and coalesced around pathogen mycelium to inhibit hyphal development (Miotto-Vilanova et al. 2016). Also, PsJN strainseems to interfere in plant signaling by reducing systemic spread of PCD, probably in order to reduce dissipation of energy since it is an energy-demanding process (Van Aken and Van Breusegem 2015), and to reduce cell damage and the rate of systemic closure of stomata provoked by PCD aiming to maintain photosynthetic efficiency as confirmed by the observedhigher green pigmentation of leaves.

Besides many specialized defense mechanisms, the plant deploy physical defense through the deposition of cell wall appositions, so-called papillae, at sites of interaction with intruding microbial pathogens. The papilla is a complex structure that is formed between the plasma membrane and the

inside of the plant cell wall, commonly composed of phenolics, ROS, cell wall proteins, and callose as one of the most abundant and ubiquitous cell wall polymers (Voigt 2014). Callose deposition and papillae formation was found to be multifaceted defense response, involve coordinated synthetic and oxidative activities (Voigt 2014), depending on growth conditions, hydrogen peroxide (H_2O_2) and ABA production and the challenging pathogen-associated molecular pattern (Luna et al. 2011). In this study, rarely observed papillae in control infected plants indicate the ability of the virulent strain to down regulate papillae formation to shield itself from plant defense as previously observed in Arabidopsis thaliana and tomato in interaction with bacterial PAMP flagellin and *Pseudomonas syringae* respectively and was mainly due to induced ABA accumulation (cited in Luna et al. 2011). The effects of both treatments on inducing papillae accumulation indicates an effective plant's physical defense mechanism and clearly indicates that papillae formation play an active role in stimulants-induced-plant resistance. Furthermore, frequency of formed papillae was higher in plants treated with PsJN strain than those treated with thyme oil suggesting that rate of papillae accumulation is related to the type of elicited systemic resistance of each treatment. Captured Multi-vesicular bodies (MVBs) surrounding papillae in plants treated with PsJN strain confirms their function as reported to be required for transportation and delivery of defense components to the forming papilla in the paramural space between the plasma membrane and the cell wall to be discharged against fungal penetration (Voigt 2014).A detailed understanding of the papillae membrane and trypan blue function would be beneficial for interpretation of papillae differential intensity staining. In fact, trypan blue is a dye that functions on the principle that dying cells have a permeable plasma membrane and thus will not be able to exclude certain dyes while live cells possess intact cell membranes that exclude them (Perry et al. 1997) which make it a good indicator for lack of membrane integrity and disorganization. Our results suggest that dark blue papillae reflecting an intact membrane indicates a papilla that didn't fuse yet to plasma membrane and didn't extrude yet chemicals or enzymes necessary for defense response, whereas cleared papillae indicates papillae's membrane disassembly mostly because diffusion occurred. Cleared disassembled papillae related to cell PCD suggest that their membrane disassembly reflect chemical and/or enzymatic release needed for executing PCD (Van Aken and Van Breusegem 2015). Without doubt, the connection between PCD-based defense and papillae composition remains to be determined.

4.3. Effect of coating seeds with thyme oil and PsJN strain on the necrotrophic behavior of *Z. tritici*

One of the many diverse defence reactions activated in plant tissues in response to pathogen attack is the accumulation of reactive oxygen species (ROS), often termed the oxidative burst. Among ROS species, H_2O_2 has a direct antimicrobial effect and is involved in wall strengthening by facilitating peroxidase reactions, catalyzing molecular cross-links between structural components of cell walls and lignin polymerization, and involved in hypersensitive cell death, phytoalexin production and systemic resistance (Shetty et al. 2007). In this study, the high accumulation of H_2O_2 in control plants at 21 dpi reflected the necrotrophic phase of *Z.tritici* growth, since it was reported that, in a compatible wheat–*Z.tritici* interaction, massive H_2O_2 accumulation, tissue collapse and necrosis occurred during the necrotrophic phase (Shetty et al. 2003).Since high concentration of H_2O_2 has a toxic effect, both of plants and fungi activate detoxification mechanisms including antioxidant enzymes such as peroxidase and catalase and antioxidant compounds like phenolic compounds to reduce H_2O_2 damage effect. Activation of antioxidative enzymes catalase and peroxidase and accumulation of phenolic compounds in sensitive control plants is most likely monitored by the plant trying to survive the harsh oxidative burst initiated by the necrotrophic behavior of the pathogen. In fact, enhanced foliar catalase activity was often associated with susceptibility (Hückelhoven et al. 1998). In treated plants, observed microscopic resistance could also explain the reduction in $H_2O_2$and phenolic compounds amounts and non-induction of H_2O_2-scavenging-catalase; since fungal penetration was already prevented at a very early time point of infection, an activation of subsequent defense mechanisms was not required to induce additional plant responses.Previous studies of plant-PsJN interactions have discussed the ability of PsJN to trigger plant immune response and induce the systemic resistance (ISR) via priming the expression of SA and JA related genes; modulating the level of leaf sugars and accumulating callose deposition after the pathogen challenge (Miotto-Vilanova et al. 2016). Similary, our previous study outlined the potential of thyme essential oil in controlling gray mold and Fusarium wilt and inducing systemic acquired resistance (SAR) in tomato, mostly attributed to peroxidase accumulation

(BenJabeur et al. 2015).Difference in mode of action of both treatments was outlined in H_2O_2-mediated-peroxidase process; peroxidase activity and H_2O_2 amount were higher in plants treated with thyme oil than those in plants treated with PsJN strain. This could be highly associated with their different triggered type of systemic resistance. Our data suggest that thyme oil induced systemic PCD thus triggering higher H_2O_2-scaveging peroxidase activity for detoxification while PsJN strain induced local PCD thus triggering limited H_2O_2-scaveging peroxidase activity.

5. Conclusion

In vitro, thyme oil hampered hyphal elongation while PsJN strainaltered growth pattern by hampering microconidiation. In planta, control plants underwent suppression of (PCD and papillae formation in the biotrophic stage of the fungus, while in the necrotrophic stage accumulation of H_2O_2, activation of peroxidase, catalase and phenolic compounds were observed indicating plant sensitivity, and necrotic leaf area reached 50% with 60% of pycnidial density. In treated plants, pathogen progress was encountered associated with induction of PCD and papillae accumulation. Thyme oil induced systemic PCD with lower frequency of formed papillae, high peroxidase activity and H_2O_2 amount but not as high as control plants, and low catalase and phenolic compounds, indicating SAR, and necroticarea was reduced to 30% with reduced pycnidial density to 1.8%. PsJN strainencountered hyphae and condensate for biofilm formation, induced local PCD with higher frequency of formed papillae, low peroxidase activity and H_2O_2 amount, and low catalase and phenolic compounds, indicating induced ISR, necroticarea was reduced to 10% with reduced pycnidial density to 9.4%. Due to the complexity of interactions among the microbes, bioactive molecules, plant host, and environment, there is the potential that thyme oil and PsJN that confers benefit *in vitro* may have a minimal effect in field; therefore, considerable work will be needed to confirm their effect, as a beneficial agricultural agents, on crop performance of wheat during the whole growing cycle and on yield in hot spot regions for Septoria leaf blotch.

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6. References

Beers RF, Sizer IW (1952) A spectrofluorometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem 195: 133-140.

Ben Jabeur M, Ghabri E, Myriam M, Hamada W (2015) Thyme essential oil as a defense inducer of tomato against gray mold and *Fusarium* wilt.Plant Physiol Biochem94: 35–40.

Ben Jabeur M, Hamada W (2014) Antifungal activity of chemically different essential oils from wild Tunisian *Thymus* spp. Nat Prod Res 7:1-7.

Ben Jabeur M, Somai-Jemmali L, Hamada W (2017) Thyme essential oil as an alternative mechanism: biofungicide-causing sensitivity of *Mycosphaerella graminicola*. J Appl Microbiol 122: 932–939.

Berraies S, Ammar K, Salah Gharbi M, Yahyaoui A, Rezgui S. (2014) Quantitative inheritance of resistance to *Septoria tritici* blotch in durum wheat in Tunisia. Chile J Agri Res 74:35-40.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. Anal Biochem 72: 248–258.

Egley GH, Paul RN, Vaughn KC, Duke SO (1983) Role of peroxidase in the development of water impermeable seed coats in *Sida spinosa* L. Planta 157:224-232.

Esmaeel Q, Miotto L, RondeauM, Leclère V, Clément C, Jacquard C, Sanchez L, A.Barka E (2018) *Paraburkholderia phytofirmans* PsJN-Plants Interaction: From Perception to the Induced Mechanisms. Front Microbiol 9: 1-14.

Eyal Z, Scharen AL, Prescott JM, van Ginkel M (1987) The septoria diseases of wheat: concepts and methods of disease management: CIMMYT. Mexico, DF, 52-70.

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

Hückelhoven R, Kogel KH (1998) Tissue-specific superoxide generation at interaction sites in resistant and susceptible near-isogenic barley lines attacked by the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*).Mol Plant Microbe Interact 11:292-300.

Jørgensen LN, Jahn M, Clark B, Antichi D, Góral T, Schepers H, Lucas P, Gouache D, Hornok L (2008) Report on Best control practices of diseases in winter wheat in 8 countries in the EU. ENDURE Project report DR1,2.

Kema GHJ, Yu D, Rijkenberg FHJ, Shaw MW, Baayen, RP (1996) Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. Phytopathol 86:777-786.

Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J (2011) Callose deposition: a multifaceted plant defense response. Mol Plant Microbe Interact 24: 183-193.

Lutts S, Benincasa P, Wojtyla L, Kubala S, Pace R, Lechowska K, Quinet M, Garnczarska M (2016) Seed priming: New comprehensive approaches for an old empirical technique. In: Araujo S, Balestrazzi A (ed) New challenges in seed biology - Basic and translational research driving seed technology. InTechOpen, Rijeka, Croatia pp 1-46

Medini M, Hamza S (2008) Pathotype and molecular characterization of *Mycosphaerella graminicola* isolates collected from Tunisia, Algeria and Canada. J. Plant Pathol 90: 65-73.

Mehrabi R, Zwiers LH, de Waard MA, Kema GHJ. 2006. MgHog1 regulates dimorphism and pathogenicity in the fungal wheat pathogen *Mycosphaerella graminicola*. Mol Plant Microbe Interact 19: 1262–1269.

Miotto-Vilanova L, Jacquard C, Courteaux B, Wortham L, Michel J, Clément C et al. (2016) *Burkholderia phytofirmans* PsJN confers grapevine resistance against *Botrytis cinerea* via a direct antimicrobial effect combined with a better resource mobilization. Front Plant Sci 7: 1236.

Nefzaoui A, Ketata H, El Mourid M (2012) Agricultural technological and institutional innovations for enhanced adaptation to environmental change in North Africa. In International Perspectives on Global Environmental Change. IntechOpen.

Patel N, Oudemans PV, Hillman BI, Kobayashi DY (2013) Use of the tetrazolium salt MTT to measure cell viability effects of the bacterial antagonist *Lysobacter enzymogenes* on the filamentous fungus *Cryphonectria parasitica*. Antonie Van Leeuwenhoek 103:1271–1280.

Perry SW, Epstein LG, Gelbard HA (1997) Simultaneous in situ detection of apoptosis and necrosis in monolayer cultures by TUNEL and trypan blue staining. Biotechniques 22: 1102-1106.

Porter FE, Scott JM (1981) Plant seed coating.US Patent 4: 251,952.

Sergiev I, Alexieva V, Karanov E (1997) Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. Comt Rend Acad Bulg Sci 51: 121-124.

Shetty NP, Kristensen BK, Newman M-A, Møller K, Gregersen PL, Jørgensen HJL (2003) Association of hydrogen peroxide with restriction of *Septoria tritici* in resistant wheat. Physiol Mol Plant Pathol62:333-346.

Shetty NP, Mehrabi R, Lutken H, Haldrup A, Kema GH, Collinge DB, Jorgensen HJ (2007) Role of hydrogen peroxide during the interaction between the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. New Phytol 174: 637-647.

Singleton VL, Orthofer R, Lamuela-Raventos, RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol 299: 152-178.

Stewart EL, McDonald BA (2014) Measuring quantitative virulence in the wheat pathogen *Zymoseptoria tritici* using high-throughput automated image analysis. Phytopathol 104: 985-992. Tada H, Shiho O, Kuroshima K, Koyama M, Tsukamoto K (1986) An improved colorimetric assay for interleukin 2. J Immunol Methods93: 157-165.

Van Aken O, Van Breusegem F (2015) Licensed to Kill: Mitochondria, Chloroplasts, and Cell Death. Trends Plant Sci 20: 754-766.

Voigt C A (2014) Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. Front. Plant Sci. 5:168.

Walters DR, Fountaine, JM (2009) Practical application of induced resistance to plant diseases: an appraisal of effectiveness under field conditions. *J Agricultural Sci* 147: 523-535.