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Seed Coating with Thyme Essential Oil or *Paraburkholderia phytofirmans* PsJN Strain: Conferring Septoria Leaf Blotch Resistance and Promotion of Yield and Grain Isotopic Composition in Wheat

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Abstract: Septoria leaf blotch (SLB) is considered one of the most devastating diseases affecting global wheat production. Biostimulant application is among the modern approaches in plant protection to overcome the impact of SLB's fungicide resistance. In this manner, the effect of coating seeds with thyme essential oil or *Paraburkholderia phytofirmans* PsJN strain on SLB severity and yield components (spikes/m^2, straw yield (SY), grain yield (GY) and thousand kernel weight (TKW)) were assessed under field conditions for 3 years. The effect on physiological traits and nitrogen and carbon isotope composition ($\delta^{15}N_{grain}$, $\delta^{13}C_{grain}$) and nitrogen and carbon content (N_{grain}, C_{grain}) of grains was assessed in one year of study. The increasing SLB severity decreased all yield components, increased $\delta^{15}N_{grain}$ and C_{grain} content and slightly decreased $\delta^{13}C_{grain}$ as the resulting effect of *Zymoseptoria tritici* inducing stomatal opening and leaf necrosis. Across the years, both treatments alleviated the SLB adverse impact by reducing SLB severity, increasing spikes/m^2, SY, GY and TKW. Both treatments ameliorated grain quality by increasing C_{grain} content and decreasing $\delta^{13}C_{grain}$ and $\delta^{15}N_{grain}$. The difference between the performance of thyme oil or PsJN strain in terms of intensity and stability is discussed and considered to be linked to the different triggered systemic resistance and the associated amount of costs deriving from resource allocation towards defense processes.

Keywords: Septoria; wheat; *Paraburkholderia phytofirmans*; thyme essential oil; isotope

1. Introduction

Globally, wheat leads all crops in terms of cultivated area and continues to be the most important food grain source for humans [1]. The high consumption of hard (or durum) wheat in some countries is associated with a decrease in wheat production resulting from ongoing climate change causing a rise of drought stress and the emergence of more aggressive pathogens [2], which leads to above-average imports to meet needs for consumption. Septoria leaf blotch (SLB), caused by the hemibiotroph *Zymoseptoria tritici*, constitutes one of the major constraints affecting durum wheat global production resulting in yield losses [3] and shriveled grains, which is undesirable for industries as they result in low flour extraction rates in milling and provide poor quality for feeding livestock [4]. Since the introduction of fungicides in the 1980s, chemical control is currently one of the main approaches used to manage SLB [3,4]. However, fungicide resistance and its associated environmental impact is now a widespread problem [5].

Biostimulants are considered as products modifying biochemical and physiological processes in plants, neutralizing the adverse impact of weather conditions and reducing the occurrence of diseases by stimulating plant growth, strengthening plant defenses and improving nutrition efficiency leading to sustainable crop yield [6]. In this context, this study's interest focused towards assessing the effect of the biostimulants thyme oil and *Paraburkholderia phytofirmans* PsJN strain against SLB severity via the seed coating technique. Our previous experiments revealed that seed coating with both agents induced seed priming associated with increased germination, the emergence of seedlings, shoot and root development, and a decreased root to shoot ratio [7]. Moreover, coating seeds with either thyme oil or *P. phytofirmans* revealed great potential in controlling SLB under controlled conditions [8]. Thyme oil and PsJN strain differed in their mode of action. Thyme oil induced systemic programmed cell death (PCD) with higher frequency of formed papillae, high peroxidases activity and H_2O_2 amount, and low catalase and phenolic compounds, indicating systemic acquired resistance (SAR), and the necrotic area was reduced to 30% with reduced pycnidial density to 1.8%. While PsJN strain encountered hyphae and condensate for biofilm formation, the induced local PCD with less frequency of formed papillae, low peroxidases activity and H_2O_2 amount, and low catalase and phenolic compounds, indicated induced systemic resistance (ISR), and the necrotic area was reduced to 10% with reduced pycnidial density to 9.4%. Despite the potential of biostimulants in achieving disease control under controlled conditions, their performance under field conditions could be less imposing. Hence, the effect of thyme essential oil and PsJN strain under field conditions on SLB severity, yield components and carbon and nitrogen stable isotope composition in durum wheat grains are examined.

2. Materials and Methods

2.1. Plant Material

A Tunisian variety of durum wheat (*Triticum turgidum* L. subsp. *Durum* (Desf) Husn.); 'Karim', known for its sensitivity to SLB, was used.

2.2. Seed Coating Treatment

Just before sowing, the seeds were coated with either thyme essential oil or *Paraburkholderia phytofirmans* PsJN strain. Thyme essential oil was extracted by hydro distillation from dried aerial parts of *Thymbra capitata* (L.) Cav. (chemotype carvacrol, voucher specimen D 1186-3), and harvested during the flowering stage from the plain of Kef (Tunisia, 36°23′ N, 8°79′ E). The obtained essential oil was distributed into 1 mL-amber-glass vials 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. [9]. The concentration of thyme oil was adjusted to 5 ppm before use with adding 0.5% of dimethyl sulfoxide (DMSO) as a solubilizing agent to assure the homogenous application of the essential oil. The bacterial inoculum of *P.phytofirmans* PsJN strain (provided by Pr. Ait Barka, University of Reims, France) was produced by transferring one colony to 20 mL of King's B liquid medium, incubated at 27 °C at 150 rpm for 48 h. The bacteria were collected by centrifugation at 8000 rpm for 5 min and washed and the concentration was adjusted to 10^8 CFU.mL^{-1} before use with phosphate-buffered saline (PBS) (10 mM, pH 6.5). The coating product Agicote Rouge T17 (AEGILOPS Applications, Val de Reuil, France), specific for cereal seeds, containing propane-1,2-diol (5–10%), polyethylene glycol mono(tristyrylphenyl)ether (5–10%), and 1,2-benzisothiasol3(2H)-one (0.0357%), was used [10]. The coating technique consists of preparing the appropriate volume of the coating solution mixture based on the quantity of seeds required for each experimental plot. Each 10 g of wheat seeds required 40 µL of the coating product Agicote Rouge T17 and 400 µL of either thyme oil (5 ppm) or PsJN inoculum (10^8 CFU.mL^{-1}), (400 µL of water was used as a control). Then, the coating mixture was applied progressively to wheat seeds in a continuous rotation, using a portable rotating drum apparatus (SUNCOO, Atlanta, GA, USA) with a speed of 2800 rpm, at an ambient temperature (20 ± 2 °C) until complete adhesion and absorption, to assure the homogeneous distribution of the coating mixture among the seeds. The final concentration of products per seed was 10^{-5} μL of coated thyme oil/seed and 210^4 CFU of coated PsJN strain/seed. Prior to the evaluation of the effect of coating seeds with thyme oil, the effect of the coating product was evaluated in the laboratory. The positive or negative effects of the coating product on seed germination and seedling growth were not detected and its inertness was assured.

2.3. Experimental Design for Field Trials

The experiments were conducted at the experimental station in Oued-Beja (CRGC), located in the sub-humid bioclimatic zone of Tunisia, for three years; 2015–2016 and 2017 under rainfed conditions (Table 1). The soil type of the experimental area is mostly clay loam with pH 7.2 (Table 2). A complete random block design with three replicates was used. The plots size was 1 × 3 m spaced by 1.5 m. Each plot consisted of 6 rows; with a row spacing of 0.15 m. The sowing was carried out in the first week of December at a sowing density of 350 seeds /m^2. The plants were inoculated with 10^7 spores/ml of *Z. tritici* twice. After full emergence of the third leaf and at stem elongation, a CO_2-pressurized knapsack sprayer was used. Nitrogen (ammonium nitrate) was applied at 25 kg N/ha at sowing and at the stem elongation stage.

Table 1. The climatic conditions (temperature, precipitation, humidity) of the three years in the experimental station of Oued Beja.

Climatic Factors	Precipitation (mm)			T Min (°C)			T Max (°C)			Humidity (%)		
year	2015	2016	2017	2015	2016	2017	2015	2016	2017	2015	2016	2017
October	59.2	77.5	32.0	15.03	17.5	14.76	28.92	27.10	28.85	73.5	75.4	76.5
November	39.2	108.8	60.0	10.6	14.06	9.58	24.0	20.14	21.56	72.9	86.6	84.8
December	105.6	21.4	40.8	6.89	11.44	8.04	16.28	17.83	17.16	86.2	90.0	92.8
January	136.2	65	119.2	5.18	5.12	3.46	15.81	17.06	13.31	83.5	88.7	81.1
February	189.0	39.2	96.4	5.20	6.35	4.78	13.69	17.78	17.80	87.1	86.2	76.9
March	77.3	115.6	25.6	7.48	6.52	6.37	17.75	18.63	20.72	83.4	86.1	71.7
April	5.2	23.4	42.4	4.58	6.15	7.52	23.68	24.57	22.71	72.0	78.4	69.4
May	25.0	40.4	23.4	12.9	9.57	11.44	29.07	27.86	29.71	65.9	70.7	56.3
Sum/Average *	636.7	491.3	439.8	8.48	9.58	8.24	21.15	21.37	21.47	78.06	82.76	76.18

* Sums for precipitation; average values for the rest.

Table 2. Soil's physicochemical characteristics of Oued Beja station.

pH	7.2				
Soil type	Vertosol (texture: Clay loam)				
Composition of Soil					
Depth	Clay (%)	Loam (%)	Sand (%)	Mineral N (ppm)	Total N (%)
0–20	67.5	22.5	10	859	0.17
20–40	65	23.7	11.3	934.7	0.16

2.4. Effect of Seed Coating with PsJN Strain and Thyme Oil on Plant Physiology, Disease Control and Yield Components

At anthesis, five leaves within each plot were selected for nondestructive measurements of leaf chlorophyll content, using a portable meter (SPAD 502 plus, Minolta, UK), and stomatal conductance of the flag leaf with a leaf porometer (Decagon, Pullman, Washington, USA). In addition, the following measurements were performed for each plot at the canopy level: The canopy normalized difference vegetation index (NDVI), with a spectroradiometer (GreenSeeker@Trimble, Westminster, Colorado, USA), canopy temperature using an infrared thermometer (Fluke, Everett, Washington, USA). For disease scoring, 15 plants were sampled from each plot, all leaves were taken for assessing the vertical disease progress and estimated for severity according to Eyal et al. [11]. Since the difference in vertical disease progress upon the samples was not observed, the diseases assessment was conducted at the leaf numbered flag leaf-3, the highest leaf showing symptoms. The leaves were scanned, and the images were analyzed using ImageJ software (the National Institute of Mental Health, Bethesda, MD, USA). The extent of the necrotic area was determined, according to Stewart and McDonald [12]. Briefly, the background was removed from each image and the total leaf area and green leaf area in the pixel was calculated using color thresholding in the red-green-blue (RGB) color space as formulated: Septoria severity (%) = (total leaf area-green leaf area)/total leaf area × 100. At harvest, 1 m^2 of each plot was hand harvested, and then straw yield (SY, Mg ha^{-1}), number of spikes/m^2, thousand kernels weight (TKW, g) and grain yield (GY, Mg ha^{-1}) were measured.

2.5. Effect of Seed Coating with PsJN Strain and Thyme Oil on Total Nitrogen and Carbon Content and Stable Carbon and Nitrogen Isotope Composition

The total N and C content and the stable nitrogen isotope signature in the dry matter of the mature grains sampled from each plot of the third field trial (2017) were analyzed at the Scientific Facilities of the University of Barcelona. Approximately 1mg of each sample and reference materials were weighed into tin capsules and measured with an elemental analyzer (Flash1112EA; Thermo Finnigan, Bremen, Germany) coupled with an isotope ratio mass spectrometer (Delta CIRMS, Thermo Finnigan, Bremen, Germany) operating in continuous flow mode in order to determine the total C and N content and the stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopes' ratios. The ratios were expressed in δ notation [13], as δ13C = ($^{13}C/^{12}C$) sample/ ($^{13}C/^{12}C$) standard −1, where sample refers to the plant material and standard to Pee Dee Belemnite (PDB) calcium carbonate, and as $\delta^{15}N$ = ($^{15}N/^{14}N$) sample/ ($^{15}N/^{14}N$) standard −1, where sample refers to plant material and standard refers to N_2 in air.

2.6. Statistical Analysis

The effects of the treatments and years and their interaction on SLB severity and yield components were determined through a two-factor (treatment × year) analysis of variance (ANOVA) with RStudio 1.1.463 (R Foundation for Statistical Computing, Vienna, Austria). The effects of the treatments on physiological traits, yield components and grain stable isotopic compositions were determined through a one-factor ANOVA (treatment). The least significant difference (LSD) test was used to assess the differences between the treatment means. The clustered Pearson correlation matrices were generated in the RStudio environment using the mean values of all traits to study the relationships between all parameters analyzed within each treatment. The data of the non-inoculated control and inoculated control were correlated (Figure 1, IC) assessing for relationship between traits in wheat-*Z. tritici* interaction. The data of the inoculated control and plants treated with PsJN strain were correlated (Figure 1, CB), and the data of the inoculated control and plants treated with thyme oil were correlated (Figure 1, CT) for extracting the potential mode of action of each treatment in conferring disease resistance and yield improvement.

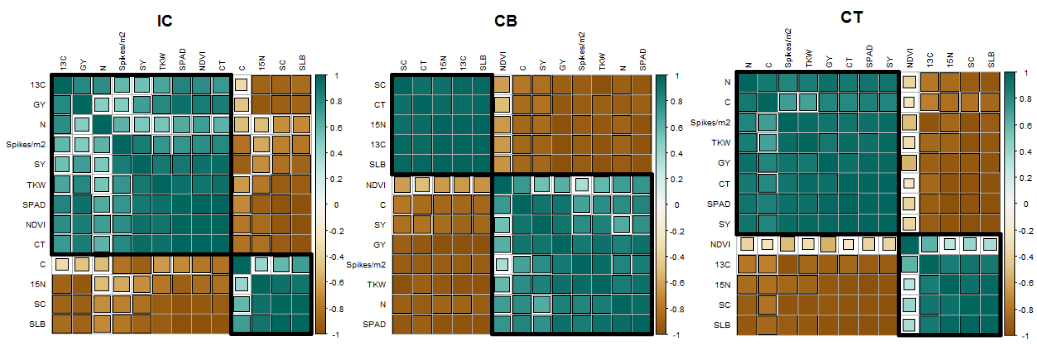

Figure 1. A correlation matrix for physiological traits, yield components and grain stable isotope composition (2017 year of study). Treatments; IC: inoculated control, CB: coated with PsJN strain,

CT: coated with thyme oil. Traits; 13 C: $\delta^{13}C_{grain}$, 15N: $\delta^{15}N_{grain}$, C: C_{grain}, N: N_{grain}, GY: Grain yield, SY: Straw yield, TKW: Thousand kernel weight, CT: Canopy temperature, SLB: SLB severity. The darker, bigger blue squares indicate a stronger positive correlation. The darker, bigger brown squares indicate stronger negative correlation.

3. Results

3.1. Climatic Features and Sources of Variances of 3 Years of Study

The data in Table 1 show that the experimental season 2016 is the season most favoring SLB compared to the other seasons tested. It was characterized by a higher amount of annual precipitations, lower maximal temperatures and high humidity. By contrast, the experimental season 2017, was characterized by drier weather due to a lower amount of precipitation, a higher maximal temperature and lower humidity. In fact, the analysis of variance revealed a highly significant ($p < 0.001$) effect for SLB severity (%), straw yield (SY) and grain yield (GY), and thousand kernel weight (TKW) was also significantly ($p < 0.01$) affected between the years. The effect of treatment (T) and the interaction year x treatment (Y × T) was highly significant ($p < 0.001$) for all four traits (Table 3).

3.2. Effect of SLB Severity on Wheat Yield Components in Control Plants

SLB was spotted in the non-inoculated control. Nevertheless, SLB severity was less compared to the inoculated control (Table 3). Therefore, a comparison between the inoculated control and non-inoculated control revealed that field artificial inoculation of wheat with *Z.tritici* increased SLB severity over naturally occurring levels, facilitating the study of the effect of treatment on wheat yield under infested conditions. Furthermore, SLB severity varied according to the variability in climatic conditions between the years. The highest severity occurred at the driest season 2017. SLB decreased significantly all yield components of the cultivar 'Karim' specifically and compared with the control. The grain yield reduced by 0.2, 0.3, and 0.5 Mg ha^{-1} in 2015, 2016, and 2017 respectively.

3.3. Effect of Seed Coating Treatment on SLB Severity and Yield Components

Both treatments showed a great potential in controlling SLB under field conditions (Table 3). The plants coated with thyme oil reduced SLB severity by 22%, 25.5%, and 53.2% in 2015, 2016, and 2017 respectively compared to the inoculated control. The plants coated with PsJN strain reduced SLB severity by 30%, 24%, and 48.3% in 2015, 2016, and 2017 respectively compared to the inoculated control. In season 2015, when water availability was high, PsJN strain was more efficient than thyme oil in reducing SLB severity. In seasons 2016 and 2017, when water availability decreased, thyme oil was more efficient than PsJN strain in reducing Septoria severity. In fact, a significant treatment by year interaction was observed for SLB.

The treatment with PsJN strain increased all yield components in the 3 seasons, not only with regard to the inoculated control but also compared with the non-inoculate control (Table 3), and the increased intensity varied among the years, most likely due to environmental factors. Contrastingly, thyme oil increased TKW compared to the inoculated control and decreased it compared to the non-inoculated control in all seasons. Furthermore, thyme oil had different effects on GY and SY among the 3 years. In 2015, in which the rainfall was more abundant in the vegetative growth stage (December–February) than the grain filling stage (April), thyme oil increased SY and decreased GY. In 2016, in which rainfall was limited in the vegetative growth stage (December-February) and abundant at the heading and anthesis (March), thyme oil decreased SY and increased GY. In 2017, in which rainfall was abundant in both vegetative growth stage (December–February) and grain filling stage (April), thyme oil increased both SY and GY.

Table 3. Effect of treatments on SLB severity and yield components of durum wheat evaluated in three-year-study.

	SLB Severity (%)				**SY (Mg ha^{-1})**				**GY (Mg ha^{-1})**				**TKW (g)**			
Treatment	NIC	IC	CB	CT	NIC	IC	CB	CT	NIC	IC	CB	CT	NIC	IC	CB	CT
Year																
2015	36.0^{cd}	50.0^{ab}	20.0^{ef}	28.0^{de}	8.7^{c}	6.0^{ef}	13.3^{a}	13.9^{a}	1.8^{ef}	1.6^{fg}	2.0^{de}	1.6^{g}	49.8^{cd}	37.4^{f}	59.1^{a}	48.7^{d}
2016	15.0^{fg}	34.5^{cd}	10.6^{fg}	9.0^{fg}	8.1^{cd}	7.1^{de}	11.4^{b}	6.6^{ef}	2.4^{c}	2.1^{d}	3.0^{a}	2.5^{bc}	51.3^{cd}	48.7^{d}	57.8^{a}	50.2^{cd}
2017	43.5^{bc}	59.1^{a}	10.8^{fg}	5.9^{g}	4.5^{gh}	3.7^{h}	5.7^{fg}	6.1^{ef}	1.7^{fg}	1.2^{h}	2.4^{c}	2.7^{ab}	53.0^{bc}	44.4^{e}	55.6^{ab}	53.2^{bc}
LSD																
2015	16.46	18.85	10.54	11.35	1.47	0.60	0.90	0.98	0.06	0.07	0.02	0.05	6.50	2.46	1.64	2.10
2016	6.66	18.62	2.83	2.10	0.09	0.44	1.21	0.55	0.04	0.02	0.26	0.11	0.20	0.25	3.01	0.19
2017	10.04	25.83	6.80	2.95	0.30	0.20	0.95	0.08	0.12	0.14	0.15	0.31	0.40	2.66	0.66	1.40
ANOVA																
Treatment (T)	45.348 ***				58.35 ***				55.38 ***				43.929 ***			
Year (Y)	16.629 ***				146.48 ***				80.97 ***				5.766 **			
Interaction (T × Y)	4.879 ***				20.60 ***				18.47 ***				6.371 ***			

The F values are shown, and the symbols indicate statistical significance (**, $p < 0.01$; ***, $p < 0.001$), values with different superscript letters are significantly different classes according to the LSD test ($p \leq 0.05$).LSD: least significant difference; SLB: Septoria leaf blotch; SY: Straw yield; GY: Grain yield; TKW: Thousand Kernels weigh; NIC: non-inoculated control; IC: inoculated control; CB: coated with PsJN strain; CT: coated with thyme oil.

3.4. Effect of Seed CoatingTreatment and SLB Severity on Physiological Traits, Yield Components and Grain Isotopic Composition

3.4.1. Effect of SLB in Control Plants

On the control plants inoculated with *Z.tritici*, during vegetative growth, the green leaf area was reduced compared with the other treatments (Figure 2), as shown by the reduction in the canopy vegetation index NDVI, and the decrease in leaf chlorophyll content (SPAD), while stomatal conductance increased and the carbon isotope composition ($\delta^{13}C$) of the grains slightly decreased. At harvest, SLB severity caused a reduction in GY and biomass as well as in the yield components spikes/m^2 and TKW and altered the grain composition by increasing C_{grain} content and $\delta^{15}N_{grain}$ (Table 4). SLB had no effect on N_{grain} content. The behavior of *Z.tritici*, the effect of SLB on the wheat physiological state, and the impact on yield components and grain composition was confirmed by the negative correlation between traits in cluster 1: SPAD, NDVI, spikes/m^2, GY, SY, TKW, canopy temperature, $\delta^{13}C_{grain}$ and the traits in cluster 2: SLB severity, stomatal conductance, C_{grain} content, $\delta^{15}N_{grain}$ (Figure 1, IC).

3.4.2. Effect of Seed Coating with PsJN Strain

Disease resistance was observed and characterized by a higher green leaf area (Figure 2) and SPAD values, and lower SLB severity and stomatal conductance compared to the inoculated control (Table 4). The plant growth promoting effect of coating seeds with PsJN strain was remarkably observed from (i) an increase in SPAD, and NDVI in the vegetative growth phase and increase in SY, GY, TKW at harvest (Table 4), and (ii) the positive correlation among SY, GY, SPAD, spikes/m^2, TKW, NDVI (Figure 1, CB, cluster 1). Concerning grain composition, the coating with PsJN strain and C_{grain} content was positively correlated to SY, GY, SPAD, spikes/m^2, TKW, NDVI (Figure 1, CB, cluster 1), and decreased $\delta^{15}N_{grain}$ and $\delta^{13}C_{grain}$, which is most likely related to a lower canopy temperature, stomatal conductance, and SLB severity compared to the inoculated control (Figure 1, CB, cluster 2). No effect was observed on N_{grain} content.

3.4.3. Effect of Seed Coating with Thyme Oil

Disease resistance was observed and characterized by a higher green leaf area (Figure 2) and SPAD values, lower SLB severity, and a lower stomatal conductance, resulting in a higher canopy temperature compared to the inoculated control. Coating seeds with thyme oil increased GY, SY, spikes/m^2 and TKW compared to the inoculated control (Table 4). Concerning grain composition, thyme oil increased C_{grain} content which was positively correlated to GY, SY, spikes/m^2, TKW, canopy temperature and SPAD. The effect of thyme oil on decreasing $\delta^{13}C_{grain}$ and $\delta^{15}N_{grain}$ content is most likely related to an increase in stomatal conductance mediated by a lower SLB severity (Figure 1, CT, cluster 2) and NDVI was the less correlated trait. No effect was observed on N_{grain} content.

Table 4. Effect of treatments on physiological traits, yield components and grain stable isotope composition in the 2017 year.

2017	SLB Severity (%)	SPAD	NDVI	Canopy Temperature (°C)	Stomatal Conductance (mmol.m^{-2}·s^{-1})	Spikes.m^{-2}	Straw Yield (Mg. ha^{-1})	Grain Yield (Mg. ha^{-1})	Thousand Kernel Weigh (g)	N_{grain} (%, g DW)	Isotopic Composition $\delta^{15}N_{grain}$ (‰)	C_{grain} (%, g DW)	Isotopic Composition $\delta^{13}C_{grain}$ (‰)
NIC	43.56^{b}	49.46^{b}	0.78^{a}	19.70^{a}	157.50^{d}	176.33^{bc}	4.50^{b}	1.75^{b}	53.06^{a}	1.79^{b}	0.77^{b}	35.87^{b}	−24.62^{a}
IC	59.19^{a}	43.20^{c}	0.75^{b}	18.00^{b}	282.36^{a}	154.00^{c}	3.78^{b}	1.21^{c}	44.44^{b}	1.71^{b}	1.34^{a}	40.81^{a}	−24.75^{b}
CB	10.82^{c}	53.20^{a}	0.76^{b}	16.99^{c}	234.53^{b}	232.00^{a}	5.77^{a}	2.48^{a}	55.69^{a}	1.97^{a}	0.05^{c}	44.22^{a}	−25.31^{d}
CT	5.94^{d}	48.63^{b}	0.74^{b}	19.43^{a}	204.80^{c}	193.66^{b}	6.18^{a}	2.77^{a}	53.20^{a}	1.96^{a}	0.62^{b}	42.49^{a}	−24.99^{c}
LSD													
NIC	1.20	0.37	0.05	0.36	12.82	14.57	0.30	0.12	1.40	0.04	0.15	4.81	0.05
IC	0.46	0.36	0.00	0.17	10.26	10.14	0.20	0.14	2.66	0.03	0.14	0.77	0.03
CB	0.58	1.99	0.01	0.15	3.29	24.97	0.95	0.15	0.66	0.07	0.01	1.40	0.02
CT	0.22	0.30	0.01	0.15	4.55	9.81	0.08	0.31	0.40	0.06	0.05	0.27	0.09
ANOVA Treatment	3872.00 ***	46.89 ***	10.03 **	95.03 ***	109.30 ***	12.58 **	14.07 **	37.08 ***	30.10 ***	16.11 ***	69.74 ***	6.039 *	79.45 ***

The F values are shown, and the symbols indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), values with different superscript letters are significantly different classes according to the LSD test ($p \leq 0.05$). LSD: Least significant difference; NIC: non-inoculated control; IC: inoculated control; CB: coated with PsJN strain; CT: coated with thyme oil.

Figure 2. Pearson's correlation matrix of seed coating treatment on SLB symptoms in 3 representative leaves of wheat (2017 year of study). NIC: non-inoculated control, IC: inoculated control, CB: coated with PsJN strain, CT: coated with thyme oil.

4. Discussion

4.1. Effect of Climate on Variability of SLB Severity, Yield Components among the 3 Years

Despite the less favoring conditions for disease development in the dry season 2017, SLB severity was the highest. In fact, one of the fundamental concepts in plant pathology illustrates that plant disease occurrence requires a three-way interaction of a susceptible host, a virulent pathogen and an environment suitable for disease development, which is referred to as the disease triangle [14].The drought and temperature stresses, associated with climatic change as well as anthropogenic air pollutants as is the case of elevated O_3 levels, have the potential to: (i) Accelerate tissue necrosis favoring infection by necrotrophic pathogens, drawing nutrients from dead host tissues; (ii) reduce the major plant defense processes against pathogens due to reduced photosynthate production and the activation of the ABA-responsive signaling pathway [15,16]. SLB significantly decreased straw yield, grain yield and the yield components of the cultivar 'Karim' specifically and compared with the control in the three years of study, which agrees with the sensitive attitude of this cultivar reported [17].

4.2. Effect of SLB on PhysiologicalTraits, Yield Components, and Stable Isotopic Composition

SLB was spotted in the non-inoculated control due to the natural aerial epidemics in the experimental station zone considered as a hot spot for SLB [17]. In season 2017, the green status of plants (SPAD and NDVI) decreased with the increasing SLB severity as expected since symptoms of SLB involve chlorotic and necrotic lesions in leaves, thus reducing the green leaf area. Furthermore, SLB caused a decrease in canopy temperature (CT) and an increase in stomatal conductance (SC). This constitutes a part of *Z.tritici* hemibiotrophic behavior causing early malfunction of stomatal regulation through the stimulation of a stomatal opening leading to an increase in the transpiration rate and energy dissipation, and the subsequent decline of canopy temperature [18]. All these metabolic modifications provoked by SLB are thought to contribute to the decreasing grain yield, straw yield, number of spikes/m^2, and the decreasing grain quality through the modification of TKW, $\delta^{15}N_{grain}$, C_{grain} and $\delta^{13}C_{grain}$.

Carbon content in grains is derived from photosynthetic fixation occurring during grain filling, from diffusion of CO_2 from the air into the leaves (and the non-laminar parts) through stomata and carboxylation by Rubisco, and from earlier-assimilated carbon remobilized from vegetative organs [19]. Through these enzymatic and physical processes, C3 plants discriminate against ^{13}C in favor of ^{12}C leading to lower $\delta^{13}C/\delta^{12}C$ ratio [20]. The values of the $\delta^{13}C/\delta^{12}C$ ratio in C3 plants have been shown to vary depending on the balance between CO_2 diffusive supply (stomatal conductance) and the enzymatic demand for CO_2 (net photosynthetic assimilation), which defines the intercellular versus atmospheric ratio of CO_2 (Ci/Ca) in the photosynthetic organ [19–21]. In this context, multiple mechanisms could be involved in the alteration of carbon metabolism by SLB, decreasing $\delta^{13}C_{grain}$ content and increasing C_{grain} content: (i) The induced stomatal opening by SLB results in an increase of CO_2 supply to carboxylation sites; (ii) during the long latent biotrophic period, and referred as the symptomless growth phase, the pathogen suppresses the plant defense response which consumes the carbon skeleton components resulting in an increase in the carbon reserve [22]; (iii) during the necrotrophic phase, the pathogen releases the early suppressed plant defense resulting in the accumulation of ABA responsible for increasing the carbohydrate content in leaves and for enhancing their remobilization to grains [22,23]; (iv) in the necrotrophic phase, the pathogen causes a decrease in the photosynthetic capacity associated with less chlorophyll resulting in an increase in the Ci/Ca ratio, therefore decreasing the $\delta^{13}C$ [24]. On the other hand, the nitrogen content in grains is derived from direct nitrogen assimilation from roots during grain filling and from remobilization of earlier-assimilated nitrogen from vegetative organs to developing grains [25]. The natural variation of the stable nitrogen isotopes $^{15}N/^{14}N$ assessed through the nitrogen isotope composition ($\delta^{15}N$) is linked to nitrogen sources used by the plant (NH_4^+ uptake will induce ^{15}N enrichment compared to NO_3^-), to the activity of enzymes involved in the assimilation of ammonium (glutamine synthetase, GS) or nitrate (nitrate reductase, NR), to the nature of compounds resulting from nitrogen fractionation. Proteins are generally ^{15}N enriched compared to chlorophyll, lipids, amino sugars and alkaloids [26], and to volatilization, translocation, or nitrogen recycling in the plant [25]. SLB, decreasing $\delta^{15}N_{grain}$ ($^{15}N/^{14}N$) and not influencing total N_{grain} content at the same time, suggests that SLB both increased the isotopic fraction ^{15}N and decreased the isotopic fraction ^{14}N. In this context, multiple mechanisms could be involved in the decrease of the isotopic fraction ^{14}N by SLB: (i) During the long latent biotrophic period, pathogens successfully acquire primary and secondary nitrogen sources available in the living tissues by enzymatic digestion of host cell walls, by invading neighboring cells, or by inducing nutrient leakage from the surrounding tissues [27] resulting in decreased ^{14}N leaf storage in the vegetative growth stage; (ii) at the metabolic level, *Z.tritici* causes a decrease in N assimilation and remobilization via reducing the activity of the enzymes NR, GS and GDH starting from the first phase of infection leading to decreased $^{14}N_{leaf}$ and a resulting decrease in $^{14}N_{grain}$ [28]; (iii) SLB causing chlorotic and necrotic lesions induce N retention in the diseased plant parts, thus decreasing N remobilization to grain resulting in decreased $^{14}N_{grain}$ [29]; (iv) stomatal-opening induced by *Z.tritici* can cause an increase in N compounds volatilization resulting in decreased ^{14}N leaf storage, thus a decrease in later $^{14}N_{grain}$ content [26]. Moreover, the mechanism involved in the increase of the isotopic fraction $^{15}N_{grain}$ tends to be the effect of SLB on increasing grain protein (^{15}N enriched) content as a consequence of the loss of photosynthetic leaf area and, therefore, of carbohydrate availability to the developing grain [26,30].

4.3. Effect of PsJN Strain

Coating seeds with PsJN strain showed a great potential for controlling SLB under field conditions in the three years of study and tends to be the most stable treatment by increasing all yield components (GY, SY, spikes/$m^2$and TKW) despite the different climatic conditions. Disease resistance was associated to the alleviation of the plant damage induced by *Z.tritici* behavior characterized by less stomata openings and enhanced chlorophyll pigmentation observed in the 2017 year of study. This could be referred to the bacterial direct effect in altering the fungal development, and the indirect effect in triggering induced systemic resistance (ISR) within the plant tissues and promoting shoot and root growth [8]. The increase in photosynthesis (SPAD) and yield components is thought to be related to the effect of PsJN strain on: (i) Inducing seed priming resulting in metabolic changes that involve phenolic compound accumulation and growth promotion of root and shoot parts starting from the seedling emergence stage [7]; (ii) decreasing the plant ethylene level by decreasing ACC levels in plants via the bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity resulting in a delay of senescence and prolonged photosynthetic activity of green tissue [31]; (iii) producing the growth regulator indole 3-acetic acid (IAA) that stimulates the development of the root system, thereby increasing nutrient absorption [32].

More specifically, in a way to understand the effect of the interaction PsJN strain-*Z.tritici* on carbon and nitrogen metabolism, the total carbon content (C_{grain}) and fractionation ($\delta^{13}C$, $\delta^{15}N$) were analyzed in the grains. The effect of PsJN strain on decreasing $\delta^{13}C_{grain}$ and increasing C_{grain} content compared with the inoculated and the non-inoculate controls suggests that this effect is mostly related to its potential in improving the plant water status due to the enhanced root development conferring a higher amount of captured water [7,24]. The effect of PsJN strain on decreasing $\delta^{15}N_{grain}$ compared to the inoculated control and simultaneously not influencing total N_{grain} content suggests that PsJN strain both increases the isotopic fraction $^{14}N_{grain}$ and decrease the isotopic fraction $^{15}N_{grain}$ and could be interpreted as: (i) The enhanced N uptake and assimilation during vegetative growth and remobilization during grain filling leading to increased $^{14}N_{grain}$ [25]; (ii) the enhanced photosynthesis and water status leading to nitrogen fractionation into chlorophyll, lipids, amino sugars rather than proteins in the vegetative growth resulting in decreased $\delta^{15}N_{grain}$ [25]; (iii) and/or as the consequence of the alleviation of SLB's adverse effects.

4.4. Effect of Thyme Oil

Coating seeds with thyme oil showed a great potential in controlling SLB under field conditions in the three years of study and seems to be more efficient in controlling SLB compared to PsJN strain according to SLB severity values. The thyme oil effect on yield components tends to be dependent on climatic conditions since the latter had different effects on GY and SY among the 3 years. In 2015, in which rainfall was more abundant in the vegetative growth stage (December–February) than the grain filling stage (April), thyme oil increased SY and decreased GY. In 2016, in which rainfall was limited in the vegetative growth stage (December-February) and abundant at the heading and anthesis (March), thyme oil decreased SY and increased GY. In 2017, in which rainfall was abundant in both vegetative growth stage (December–February) and grain filling stage (April), thyme oil increased both SY and GY. This suggests that thyme oil increases the growth rate of the assimilatory organ dependent on water availability. Thyme oil seems to be ineffective in promoting grain yield when there is an interaction disease x water deficit at the grain filling stage. This is thought to be the side effect of the activation of the systemic acquired resistance SAR [8], which induces the energy allocation towards defense related mechanisms and limits energy availability towards drought-tolerance mechanisms when water deficit occurs at the grain filling stage. According to the 2017 one year of study, disease resistance was branded by the absence of the plant damage induced by *Z.tritici* behavior, resistance was characterized by less stomata opening and the absence of chlorophyll deterioration which is most likely due to thyme oil's direct effect via hampering the fungal development and indirect effect via inducing SAR within plant tissues [8].The thyme oil effect behind enhanced GY, SY, spikes/m^2 and TKW of wheat is thought to be related to both: (i) The elicitor effect inducing seed priming resulting in the metabolic changes that involve peroxidase, phenolic compounds accumulation and the growth promotion of root and shoot parts starting from seedling emergence stage [7]; (ii) the alleviation of SLB's adverse effect. Concerning grain composition, the effect of thyme oil on increasing C_{grain} content and decreasing $\delta^{13}C_{grain}$ suggests that this effect is mostly related to thyme oil's potential in improving the plant water status due to the enhanced root elongation conferring a higher water uptake [7,24]. The thyme oil effect on decreasing $\delta^{15}N_{grain}$ compared to the inoculated control and simultaneously, not influencing the total N_{grain} content suggests that thyme oil both increases the isotopic fraction $^{14}N_{grain}$ and decreases the isotopic fraction $^{15}N_{grain}$ and could be explained by: (i) The enhanced N uptake during vegetative growth as a consequence of the thyme oil priming effect on inducing intracellular acidification of plant cells [7] was found to increase N uptake [33], leading to increased ^{14}N [25]; (ii)the enhanced water status leading to nitrogen fractionation into lipids, amino sugars rather than proteins in the vegetative growth resulting in decreased $\delta^{15}N_{grain}$ [20]; (iii) and/or as the consequence of the alleviation of SLB's adverse effect.

4.5. Comparison between Treatments and Insight to Cost/Gain Balance

The effect of PsJN strain and thyme oil differed among the three years of study. Concerning their effect on crop protection against SLB, in season 2015, when water availability was high, PsJN strain was more efficient than thyme oil in reducing SLB severity. Contrastingly, in seasons 2016 and 2017, when water availability decreased, thyme oil was more efficient than PsJN strain in reducing SLB severity. It is suggested that this difference is most likely due to their different induced type of resistance. Thyme oil triggers systemic acquired resistance causing the systemic stomatal closure [8], thus preserving water content and, by the way, decreasing the drought side effects. However, PsJN strain triggers induced systemic resistance (ISR) causing local stomatal closure only in the presence of a pathogen [8], thus maintaining the normal water dissipation rate. By this way, the energy needed for SLB resistance is expected to decrease due to energy allocation towards drought-tolerance mechanisms when a water deficit occurs, as in the years 2016 and 2017.

The better impact of PsJN strain on yield components and grain composition compared to thyme oil is suggested to be related also to the distinct defence mechanisms and can be explained by the selective cost–benefit scenario of inducible defences [22]. Thyme oil is considered to trigger constitutive defence and PsJN strain is considered to trigger induced defence [8]. The plant defence is a costly business, requiring energy and resources that would otherwise be used for growth and development [22,34]. In this context, the constitutive resistance triggered by thyme oil, where the activation occurs before the onset of the disease, is considered to be a costly advantage causing higher allocation of resources. While, the induced resistance triggered by PsJN strain, where defences are only activated following pathogen attack and only at the site of infection, is considered a less pricey advantage compared to constitutive resistance [22,34].

5. Conclusions

This study revealed that economic losses in durum wheat due to increased SLB severity can result from losses in straw yield, grain yield and grain quality. Coating seeds with either thyme oil or PsJN strain showed potential in counteracting the deleterious effects of SLB and the promotion of straw yield, and grain yield and quality. The data showed that the impact of thyme oil and PsJN differed in terms of intensity and stability. Further, it is considered to be linked to the different growth promoting effects and the different triggered systemic resistance and associated amount of costs deriving from resource allocation towards defense processes. This cost-benefit of induced resistance in the variety 'Karim' of durum wheat gives insight into the worth of studying the effects of PsJN strain or thyme oil in other varieties of wheat in order to seek better interaction which minimizes the costly effect of biostimulants.

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