A Plant Nutrient- and Microbial Protein-Based Resistance Inducer Elicits Wheat Cultivar-Dependent Resistance Against *Zymoseptoria tritici*

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ABSTRACT

The induction of plant defense mechanisms by resistance inducers is an attractive and innovative alternative to reduce the use of fungicides on wheat against *Zymoseptoria tritici*, the responsible agent of Septoria tritici blotch (STB). Under controlled conditions, we investigated the resistance induction in three wheat cultivars with different susceptible levels to STB as a response to a treatment with a sulfur, manganese sulfate, and protein-based resistance inducer (NECTAR Céréales). While no direct antigermination effect of the product was observed in planta, more than 50% reduction of both symptoms and sporulation were recorded on the three tested cultivars. However, an impact of the wheat genotype on resistance induction was highlighted, which affects host penetration, cell colonization, and the production of cell-wall degrading enzymes by the fungus. Moreover, in the most susceptible cultivar Alixan, the product

upregulated *POX2*, *PAL*, *PRI*, and *GLUC* gene expression in both noninoculated and inoculated plants and *CHIT2* in noninoculated plants only. In contrast, defense responses induced in Altigo, the most resistant cultivar, seem to be more specifically mediated by the phenylpropanoid pathway in noninoculated as well as inoculated plants, since *PAL* and *CHS* were most specifically upregulated in this cultivar. In Premio, the moderate resistant cultivar, NECTAR Céréales elicits mainly the octadecanoid pathway, via *LOX* and *AOS* induction in noninoculated plants. We concluded that this complex resistance-inducing product protects wheat against *Z. tritici* by stimulating the cultivar-dependent plant defense mechanisms.

Keywords: defense mechanisms, induced resistance, manganese, wheat, *Zymoseptoria tritici*

Soft winter wheat (*Triticum aestivum* L.) is a major economically important crop in France, and Septoria tritici blotch (STB) is currently one of the most damaging foliar wheat diseases, causing yield losses reaching up to 40% (Eyal 1999; Palmer and Skinner 2002). The infectious process of the hemibiotrophic fungus *Zymoseptoria tritici*, responsible for STB, is characterized by an approximately 2- to 3-week-long symptomless period. Later on, the fungus switches to a necrotrophic phase, associated with an increase of fungal biomass, the formation of pycnidia in colonized substomatal cavities, necrosis (Kema et al. 1996; Ors et al. 2017; Selim et al. 2011, 2014; Siah et al. 2010a), and the production of fungal cell-wall degrading enzymes (CWDE) (Douaiher et al. 2007a, b; Siah et al. 2010a).

At the field level, STB is mainly controlled by the use of fungicides. An alternative to chemical control is breeding for new and, if possible, fully resistant wheat cultivars. The wheat resistance against *Z. tritici* is controlled by (i) gene-for-gene relationships leading to incompatible interactions (Brading et al. 2002) and/or by (ii) nonspecific and partial resistance expressed during compatible

interactions (Chartrain et al. 2004a). While *Z. tritici* is able to overcome specific resistance controlled by 1 of the 18 known *stb* wheat genes (Goodwin 2007; Goodwin and Thompson 2011; Simón et al. 2012), partial resistance, controlled by several quantitative trait loci (QTL), is more durable at the field level (Orton et al. 2011).

Plant resistance is the result of an effective defense against pathogen attack. Defense responses are triggered by the recognition of molecules derived from pathogens. The first level of defense is based on the perception of pathogen-associated molecular pattern (PAMP) or microbe-associated molecular pattern, and is called PAMP-triggered immunity (PTI) (Nürnberger and Scheel 2001; Deller et al. 2011; Henry et al. 2012; Mengiste 2012). To overcome PTI, effectors have emerged in pathogens, that are released within the host during the infection process to suppress PTI (Li et al. 2013), which results in a compatible interaction associated with partial resistance, depending on the degree of effectiveness of triggered defense pathways. In turn, plant receptors can recognize such pathogen effectors, resulting in a second type of immunity referred to as effector triggered immunity (ETI), leading to an incompatible interaction (Jones and Dangl 2006; Deller et al. 2011) and total resistance.

Wheat defenses against *Z. tritici* have been studied during compatible and incompatible interactions, revealing an early induction of peroxidase (POX) activity and H₂O₂ accumulation in wheat cells, associated with wheat resistance to *Z. tritici* (Shetty et al. 2003, 2007). Different defense-related genes, such as genes encoding PR-proteins, were described as early and highly up-regulated during the biotrophic phase of an incompatible interaction, while a slight induction only or a repression were observed during a compatible interaction (Adhikari et al. 2007; Ray et al. 2003; Yang et al. 2013a, b). However, by comparing

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defense-related gene expression in eight combinations of cultivars and isolates leading either to compatible or incompatible interactions, no significant difference was observed in the responses during the symptomless phase of colonization, suggesting that induction of defense gene expression is not specific to incompatible interactions (Orton et al. 2017). Moreover, a comparison between cultivars with different relative levels of susceptibility against *Z. tritici* during compatible interactions highlighted a constitutive expression of defense-associated genes at a higher level in the less susceptible studied cultivars (Ors et al. 2017). Surprisingly, during the symptomless phase of fungal development, many defense genes were induced in the most susceptible cultivar, while no induction or only slight inductions were observed in the less susceptible cultivar (Ors et al. 2017). These results underline the complexity of the mechanisms involved in specific or partial resistances of wheat against *Z. tritici*.

The induction of plant defense mechanisms by resistance inducers is an attractive and innovative alternative to reduce the use of fungicides because induced resistance is a natural and potentially effective, persistent and durable strategy (Samain et al. 2019; Walters et al. 2005, 2008). So far, a number of resistance inducers have been developed, based on either natural or synthetic compounds, that could be used in crop protection at the field (Tayeh et al. 2014; Thakur and Sohal 2013; Vavrina et al. 2004; Walters et al. 2008). Induced resistance could be related to a direct elicitation of defense responses as well as to a sensitization by a priming agent, which results in the expression of earlier and/or stronger basal defense mechanism upon a pathogen attack (Conrath et al. 2006; Walters et al. 2005).

The only resistance inducer currently registered on wheat against STB in France is Vacciplant (Goëmar, France), which contains laminarin (<https://ephy.anses.fr/ppp/iodus-2-cereales/>). NECTAR Céréales is currently commercialized as a complete foliar fertilizer based on sulfur (>25%), manganese sulfate (>2.5%), and cellulase (>0.0015%) (<https://www.agronutrition.com/>). NECTAR Céréales is investigated in this work for its protective efficacy against STB and for its wheat defense activation properties. We compared the resistance induced by this product against a given *Z. tritici* strain in three distinct wheat cultivars characterized by different partial resistance levels in the field. On the one hand, the impact of the treatment on the infection process and the pathogenicity of the fungus were measured by cytological and biochemical approaches during both biotrophic and necrotrophic phases of *Z. tritici*. On the other hand, various defense pathways were investigated at biochemical and molecular levels during the early period following treatment with NECTAR Céréales in both inoculated and non-inoculated plants. The influence of the wheat genotype on the induced resistance level will be discussed.

MATERIALS AND METHODS

Fungal inoculum preparation. *Z. tritici* strain T01193 (GenBank accession number KX356102), isolated from Northern France (Lorgies), is moderately resistant to sterol 14 α -demethylase inhibitor (DMI) fungicides, characterized by three single nucleotide polymorphism mutations (M-281-V, A-379-G, and I-381-V) in the *cyp51* gene encoding for sterol 14 α -demethylase (Selim 2016). Fungal material (in vitro cirrus) of this strain stored at -80°C was activated by transfer to fresh potato dextrose agar (PDA) medium (39 g liter⁻¹; Sigma). After 7 days incubation at 18°C, with a 12-h photoperiod, spore suspensions were prepared by washing the cultures using sterile distilled water. Then, the concentration of spores was adjusted to the desired concentration using a Malassez cell.

Direct antifungal assays. Antifungal activity of NECTAR Céréales (sulfur (>25%), manganese sulfate (>2.5%), and cellulase (>0.0015%) against *Z. tritici* (on both fungal growth and spore germination) was assessed in vitro using PDA medium amended

with different concentrations of the product (1.2, 3.7, 11.1, 33.3, and 100 ml liter⁻¹), using the spotting and spore germination methods described by Siah et al. (2010b). The product was added to the medium at 30°C after autoclaving. The effect on fungal growth was recorded by measuring the colony perpendicular diameters after 10 days incubation at 21°C in the dark, while the effect on spore germination was evaluated after 1 day incubation by determining the percentage of germinated spores calculated from 100 spores under a light microscope (Nikon, Eclipse80i) for each dish (Siah et al. 2010a). Three dishes were used as replicates for each treatment, including controls, within both spotting and spore germination bioassays.

Plant growth and protection tests. Alixan, Premio, and Altigo wheat cultivars were used in this study. These cultivars exhibited various levels of partial resistance against STB under field conditions in 2009 and were noted as 4, 5.5, and 7, respectively, on a scale from 1 (highly susceptible) to 9 (highly resistant). Wheat grains were germinated in Petri dishes and wheat plantlets were grown in the greenhouse as described by Ors et al. (2017). After 3 weeks (when third leaves from the base of plants were fully expanded), plants of each pot were sprayed with 2.5 ml/plant of a solution containing NECTAR Céréales at 8.73 μ l/ml of distilled water. This dose of NECTAR Céréales (previously named FSOV7) was identified by Ors et al. (2012, 2013) as the most effective dose to protect wheat against *Z. tritici*. Control plants were treated with distilled water. Plants were inoculated 48 h after treatment as mentioned in Selim et al. (2014). Briefly, 21-day-old plants were inoculated by spraying a 2-ml *Z. tritici* inoculum (2×10^6 spores/ml) in 10 mM MgSO₄ amended with 0.1% polyoxyethylene-sorbitan monolaurate surfactant (Tween 20, Sigma). Controls were sprayed with 2 ml/plant of the same solution without conidia. Five replications were carried out for each treatment. Plants in pots were covered with transparent polyethylene bags for 3 days and the disease was scored at 21 days after inoculation (dai) by noting the percentage of the third leaf's area covered by lesions (chlorosis and necrosis) and by noting their pycnidial density on a scale from 0 (absence of pycnidia) to 5 (high density of pycnidia) as described by Ors et al. (2017).

Histological analyses. Two-centimeter leaf segments were harvested from the third expanded wheat leaf at different time points (1, 5, and 21 dai) and observed microscopically. Spore germination and leaf penetration by *Z. tritici* was assessed using Fluorescence Brightener 28 (Calcofluor, Sigma) at a wavelength of 365 nm according to Siah et al. (2010a). Mesophyll colonization was investigated using Trypan blue according to Ors et al. (2017). The percentage of germinated spores (GS) was recorded at 1 dai. Total germinated spores with penetrated germ tube (PS) and within PS, penetration through stomata (PSS) were determined at 5 dai. At 21 dai, total colonized substomatal cavities (CS) among 150 randomly chosen cavities were recorded and, within CS, percentages of colonized substomatal cavities bearing pycnidia (CSP) were estimated.

Protein extraction and enzymatic assays. CWDE. Protease, β -1,4-endoglucanase and β -1,4-endoxylanase activities were measured according Ors et al. (2017) and total protein concentrations were determined at 595 nm using the method of Bradford (1976). Bovine serum albumin (Sigma A7906) was used as standard.

Plant enzyme assays. POX activity was measured according to Mitchell et al. (1994) modified by Randoux et al. (2006) and phenylalanine ammonia lyase (PAL) activity according to Randoux et al. (2010) at 0, 12, 24, 48, 54, 60, 72, and 120 h after treatment.

RNA extraction and gene expression quantification by quantitative real-time RT-PCR. The third expanded wheat leaves were sampled at 0, 12, 24, 48, 51, 54, 60, 72, 120, and 168 h after treatment. Total RNA extraction and real time RT-PCR was performed according to Ors et al. (2017) and Livak and Schmittgen (2001) to quantify the expression patterns of nine genes: *PR1*, *CHIT2*, *gluc*, *LOX*, *AOS*, *PAL*, *CHS*, *POX2*, and *OXO*.

The *TUB6* and *GAPDH* genes were used as endogenous reference for each treatment. The primers used in this study were published previously in Ors et al. (2017).

Statistical analysis. All experiments were carried out using at least three technical replications and two biological replicates. Data represent continuous variables and were analyzed using analysis of variance (Tukey's test at $P \leq 0.05$) assuming a normal distribution. Variances were stabilized by logarithmic transformation of data if necessary, as described by Ors et al. (2017). All data were analyzed by Xlstat software (Addinsoft, Paris, France).

RESULTS

Protection efficacy on Alixan, Premio, and Altigo inoculated with *Z. tritici*. In order to evaluate the protection efficacy of NECTAR Céréales against *Z. tritici*, the percentage of leaf area with symptoms and their pycnidial density were assessed on the third fully expanded wheat leaves from the three cultivars at 21 dai (Fig. 1; Supplementary Data File S1). In the nontreated inoculated (nTI) control, the percentage of leaf area with symptoms (Fig. 1A) recorded in Alixan and Premio was not significantly different (41.7 and 42.6%, respectively), but was significantly lower in Altigo (27.7%). In plants treated with NECTAR Céréales, disease severity (10.8, 10.1, and 2.9% in Alixan, Premio, and Altigo, respectively) was significantly lower than in their nTI controls, corresponding to reductions by 74.1, 76.3, and 89.5% on Alixan, Premio, and Altigo, respectively.

In nTI controls, the pycnidial density (Fig. 1B) recorded in Alixan was significantly higher (2.9) than in Premio (1.1) and Altigo (1.1). In plants treated with NECTAR Céréales, a significant reduction of pycnidial density was observed in Alixan, Premio, and Altigo (55.3, 66.3, and 91.3% reductions, respectively).

Figure 2 shows the third wheat leaves harvested at 21 dai from Alixan, Premio, and Altigo nontreated and noninoculated (nTnI), nontreated inoculated with *Z. tritici* (nTI) and treated with NECTAR Céréales before inoculation. In each cultivar, nTI leaves exhibited chlorosis and necrosis related to disease development compared with nTnI leaves. Symptoms were drastically reduced in leaves treated with NECTAR Céréales before inoculation. Furthermore, the NECTAR Céréales-treated noninoculated leaves, especially in Altigo, displayed a lighter green color than the nTnI controls, and chlorosis at the tip (data not shown).

Alteration of the *Z. tritici* infectious process. In order to evaluate the impact of NECTAR Céréales on the development of *Z. tritici* in the three cultivars, five cytological parameters were assessed at different time points after infection (Fig. 3). At 1 dai, more than 60% of fungal spores were germinated on the nTI controls of the three cultivars (Fig. 3A). No significant effect of NECTAR Céréales treatment on spore germination was observed (Fig. 3A). This result found in planta agrees with the in vitro antifungal assays, which showed no significant antifungal effect of NECTAR Céréales on both the growth and spore germination of *Z. tritici* when tested on PDA medium (data not shown).

At 5 dai, the percentage of germinated spores with penetrated germ tube (PS) recorded in Alixan, Premio, and Altigo nTI controls were not significantly different (between 39.3 and 55.9%) (Fig. 3B). Alixan plants treated with NECTAR Céréales had a percentage of PS significantly lower than that of the nTI control (33.3% compared with 53% in nTI) corresponding to a reduction by 37.2% (Fig. 3B). No significant difference was detected for the percentages of penetrations through stomata (PSS) among the three nTI cultivars (between 24.2 and 28.2%) and between NECTAR Céréales-treated plants and their nTI controls (between 15.6 and 26.2%) (Fig. 3C).

Concerning the percentage of total colonized substomatal cavities (CS) recorded at 21dai, no significant difference in Alixan, Premio, and Altigo nTI controls was found (between 72.0 and 79.2%). In Premio and Altigo treated with NECTAR Céréales the

percentage of CS (31.4 and 27.8%, respectively) was significantly lower than in their nTI controls, corresponding to reductions by 56.4 and 61.9%, respectively, but not in Alixan (Fig. 3D). Likewise, in the case of CS bearing pycnidia (CSP), only Premio treated with NECTAR Céréales had a percentage significantly lower (3.8%) than in the nTI control, corresponding to a reduction by 84.1% (Fig. 3E).

CWDE activities. The fungal β -1,4-endoxylanase, β -1,4-endoglucanase, and protease activities were measured in leaves from Alixan, Premio, and Altigo (Tables 1, 2, and 3). At 1, 5, and 11 dai, corresponding to the biotrophic phase, all of these enzyme activities were at low levels and not significantly different from those of nTnI controls.

Later on, the β -1,4-endoxylanase activity levels recorded in Alixan and Premio nTI controls at 17 dai (0.08 and 0.04 units mg^{-1} of total protein) were significantly higher than in their nTnI controls (0.03 and 0.01 units mg^{-1} of total protein, respectively), unlike in Altigo (between 0.02 and 0.03 units mg^{-1} of total protein for both nTI and nTnI) (Table 1). At this time point, enzyme activity, recorded in Alixan and Premio treated with NECTAR Céréales and inoculated, was significantly lower than in their nTI controls, without significant differences from their nTnI controls. At 21 dai, the β -1,4-endoxylanase activity was significantly higher in the three nTI cultivars than in their nTnI controls, and was significantly higher in Alixan than in Premio and Altigo. After treatment, this activity was significantly lower only in Alixan treated with NECTAR Céréales compared with its nTI control.

Similar results were recorded for β -1,4-endoglucanase activity (Table 2) at 17 and 21 dai.

For the protease, at 17 dai, NECTAR Céréales significantly reduced enzyme activity only in the nTI control of the most susceptible cultivar Alixan compared with the nTnI control (Table 3). At 21 dai, this activity reached 2.66 milli-unit mg^{-1} total protein in Alixan nTI plants and was significantly higher than in nTI Premio and Altigo. However, only Premio treated with NECTAR Céréales exhibited an activity significantly reduced (0.07 milli-unit mg^{-1} total protein) compared with its nTI control (Table 3).

Induction of POX and PAL activities. Total POX and PAL activities were measured in Alixan, Premio, and Altigo at 0, 24, 48, and 72 h after treatment with NECTAR Céréales in both infection and noninfection contexts (Figs. 4 and 5). POX activity in plants treated with NECTAR Céréales was significantly higher than in the nTnI control in Alixan (Fig. 4A) at both 48 and 72 h, and in Premio (Fig. 4B) and Altigo (Fig. 4C) at 72 h. In inoculated plants, the POX activity in NECTAR Céréales-treated and inoculated plants was significantly higher in Alixan at 45 and 72 h and in

Fig. 1. Disease severity caused by *Zymoseptoria tritici* strain T01193 on Alixan, Premio, and Altigo wheat cultivars on nontreated inoculated control plants (nTI) and on plants treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) 48 h before inoculation (NECTAR Céréales I). **A**, Symptoms were evaluated by noting the percentage of the third leaf area covered by lesions (chlorosis and necrosis), and **B**, the pycnidial density within symptoms was estimated on a scale from 0 (absence of pycnidia) to 5 (high density of pycnidia) at 21 days after inoculation. Means with the same letter are not significantly different using Tukey's test at $P \leq 0.05$.

Altigo and Premio at 72 h after treatment than in the corresponding nTI plants.

PAL activity recorded on noninoculated plants in Alixan treated with NECTAR Céréales (3.6 nanokatal mg^{-1} of protein) was significantly higher than in nTnI, nTI and in inoculated NECTAR Céréales plants at 72 h after treatment (0.8, 1.5, and 0.7 nanokatal mg^{-1} of protein, respectively) (Fig. 5A). For Premio, NECTAR Céréales-treated plants had a PAL activity (2.1 nanokatal mg^{-1} of protein) significantly higher than the nTnI controls (Fig. 5B). In inoculated plants, the activity recorded in Premio NECTAR Céréales plants (1.3 nanokatal mg^{-1} of protein) was significantly higher than in the nTnI and nTI treatments, but significantly lower than in NECTAR Céréales plants. In Altigo, the PAL activity recorded in NECTAR Céréales plants at 48 and 72 h after treatment (2.1 and 1.1 nanokatal mg^{-1} of protein, respectively) was significantly higher than in the nTnI control (1.3 and 0.9 nanokatal mg^{-1} of protein, respectively) (Fig. 5C). In inoculated plants, the PAL activity recorded at 72 h after treatment in inoculated NECTAR Céréales plants (4.6 nanokatal mg^{-1} of protein) was significantly higher than in NECTAR Céréales plants as well as in nTnI and nTI controls.

Induction of defense-related gene expression in Alixan, Premio, and Altigo in noninoculated plants. We established transcript profiles of a set of nine distinct defense genes associated with four types of wheat defenses: ROS metabolism (*POX2*, *OXO*) (Fig. 6),

octadecanoid (*LOX*, *AOS*) (Fig. 7), phenylpropanoid (*PAL*, *CHS*) (Fig. 8) pathways, and PR protein biosynthesis (*PR1*, *CHIT2*, *GLUC*) (Fig. 9). Gene expression profiles in Alixan, Premio, and Altigo plants were followed at 12, 24, 48, 51, 54, 60, 72, 120, and 168 h after treatment with NECTAR Céréales and were compared with their respective (nTnI) controls (Figs. 6, 7, 8, and 9) for which expression was considered to be at 1. For a meaningful biological approach, significantly differentially expressed transcripts were reported according to a strict selection criterion; namely, a ≥ 2.0 -fold (upregulation) or a < 0.5 -fold (downregulation) change threshold.

Concerning ROS metabolism, the *POX2* gene was induced (≥ 3.2 -fold) in the Alixan cultivar over all the time courses except at 24, 60, and 168 h after treatment; in Altigo, from 3.1- to 17.3-fold at 12, 24, 48, 51, and 60 h after treatment; in Premio, from 4.6- to 12.5-fold at 24, 51, 54, and 60 h after treatment (Fig. 6). The *OXO* gene expression was induced at 60 h (2.7-fold) in Premio, and at 72 h after treatment (4.1- and 6.8-fold, respectively) in Alixan and Altigo.

Concerning the jasmonate pathway, Premio showed an early induction of the *LOX* gene expression level, ranging from 3.6- to 28.8-fold, at 12 to 54 h after treatment (Fig. 7). In Altigo, *LOX* was induced at 24 h after treatment (10.1-fold). Also, *AOS* showed an early significant induction at 5.1- to 77.2-fold, and 5.6- to 11.5-fold in Premio and Altigo, respectively. However, in Alixan, this gene was induced later at 54 and 120 h after treatment with 6.5- and 2.0-fold, respectively (Fig. 7).

Fig. 2. Wheat leaves sampled from Alixan, Premio, and Altigo cultivars in greenhouse conditions at 21 days after inoculation. Plants were either nontreated noninoculated (nTnI), nontreated and inoculated (nTI) with *Zymoseptoria tritici* strain T01193, or treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales® I) 48 h before inoculation.

Fig. 3. Microscopic observation of the infection process of *Zymoseptoria tritici* on Alixan, Premio, and Altigo wheat cultivars treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales I) 48 h before inoculation (NECTAR Céréales I), or nontreated and inoculated (nTI). **A**, Percentage of germinated spores (GS) at 1 day after inoculation (dai); **B**, percentage of total germinated spores with penetrated germ tube (PS) and **C**, penetrating spores through stomata (PSS) at 5 dai; and **D**, percentage of colonized substomatal cavities (CS) and **E**, percentage of pycnidia emerging from colonized substomatal cavities (CSP) at 21 dai. Means with the same letter are not significantly different using Tukey's test at $P \leq 0.05$.

TABLE 1. Time course of β -1,4-endoxylanase activity (units mg^{-1} of total protein) in Alixan, Premio, and Altigo wheat cultivars treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) 48 h before inoculation with *Zymoseptoria tritici* strain T01193^z

Cultivar	Treatment	1 dai	5 dai	11 dai	17 dai	21 dai
Alixan	Nontreated noninoculated	0.01 b	0.01 a	0.01 a	0.03 bc	0.02 d
	Nontreated inoculated	0.02 ab	0.01 a	0.01 a	0.08 a	0.34 a
	Treated inoculated	0.02 ab	0.02 a	0.02 a	0.03 bc	0.10 b
Premio	Nontreated noninoculated	0.02 ab	0.01 a	0.01 a	0.01 d	0.01 d
	Nontreated inoculated	0.01 ab	0.01 a	0.02 a	0.04 ab	0.08 b
	Treated inoculated	0.02 a	0.01 a	0.01 a	0.02 cd	0.07 b
Altigo	Nontreated noninoculated	0.02 ab	0.01 a	0.01 a	0.02 cd	0.02 d
	Nontreated inoculated	0.02 ab	0.01 a	0.01 a	0.03 bc	0.05 bc
	Treated inoculated	0.02 ab	0.01 a	0.02 a	0.03 bc	0.03 cd

^z At each time point (1 to 21 days after inoculation [dai]), activity levels were compared with nontreated noninoculated and nontreated inoculated controls. Means with the same letter at each time point are not significantly different using Tukey's test at $P \leq 0.05$.

TABLE 2. Time course of β -1,4-endoglucanase activity (units mg^{-1} of total protein) in Alixan, Premio, and Altigo wheat cultivars treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) 48 h before inoculation with *Zymoseptoria tritici* strain T01193^z

Cultivar	Treatment	1 dai	5 dai	11 dai	17 dai	21 dai
Alixan	Nontreated noninoculated	0.06 ab	0.08 a	0.08 a	0.10 bc	0.09 ef
	Nontreated inoculated	0.06 ab	0.08 a	0.09 a	0.21 a	0.62 a
	Treated inoculated	0.07 ab	0.09 a	0.1 a	0.12 bc	0.26 b
Premio	Nontreated noninoculated	0.05 b	0.07 a	0.08 a	0.06 d	0.07 f
	Nontreated inoculated	0.06 ab	0.07 a	0.09 a	0.14 ab	0.18 bcd
	Treated inoculated	0.08 a	0.07 a	0.09 a	0.09 cd	0.24 bc
Altigo	Nontreated noninoculated	0.05 b	0.07 a	0.08 a	0.12 bc	0.09 ef
	Nontreated inoculated	0.05 b	0.07 a	0.07 a	0.10 bc	0.15 cd
	Treated inoculated	0.06 ab	0.08 a	0.06 a	0.10 bc	0.12 de

^z At each time point (1 to 21 days after inoculation [dai]), activity levels were compared with nontreated noninoculated and nontreated inoculated controls. Means with the same letter at each time point are not significantly different using Tukey's test at $P \leq 0.05$.

Concerning the salicylic acid (SA) pathway, an early induction of *PAL*, the gene marker of the phenylpropanoid pathway, was recorded in Altigo with a 2.3-fold increase at 12 h after treatment and reaching 35.0-fold at 72 h (Fig. 8). It was induced at 48 to 120 h (ranging from 2.7- to 14.0-fold) in Alixan and at 60 h only in Premio (2.3-fold). Concerning the *CHS* gene, Altigo showed induction at 54 and 72 h (22.1- and 15.0-fold, respectively).

Concerning PR protein biosynthesis, Altigo showed an early induction of *PR1*, the SA pathway marker of systemic acquired resistance, at 24 and 60 h (2.3- and 3.0-fold, respectively) (Fig. 9). In Alixan, *PR1* was induced at 48 and 51 h (6.2- and 18.1-fold, respectively). *CHIT2* gene expression was induced in Altigo at 12 and 72 h (4.6- and 5.9-fold, respectively), whereas in Alixan, it was only induced at 72 h (9.1-fold). No induction was observed in

TABLE 3. Time course of protease activity (milli-units mg⁻¹ of total protein) in Alixan, Premio, and Altigo wheat cultivars treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) 48 h before inoculation with *Zymoseptoria tritici* strain T01193^z

Cultivar	Treatment	1 dai	5 dai	11 dai	17 dai	21 dai
Alixan	Nontreated noninoculated	0.05 ab	0.13 a	0.08 a	0.09 bc	0.04 c
	Nontreated inoculated	0.07 ab	0.08 ab	0.06 ab	0.85 a	2.66 a
	Treated inoculated	0.07 ab	0.09 ab	0.03 ab	0.22 b	1.12 ab
Premio	Nontreated noninoculated	0.10 a	0.13 a	0.03 ab	0.05 c	0.05 c
	Nontreated inoculated	0.05 ab	0.06 ab	0.05 ab	0.07 bc	0.51 b
	Treated inoculated	0.02 b	0.05 ab	0.03 b	0.09 bc	0.07 c
Altigo	Nontreated noninoculated	0.06 ab	0.08 ab	0.06 ab	0.07 bc	0.02 c
	Nontreated inoculated	0.08 a	0.04 b	0.06 ab	0.15 bc	0.72 ab
	Treated inoculated	0.03 ab	0.07 ab	0.05 ab	0.12 bc	0.57 b

^z At each time point (1 to 21 days after inoculation [dai]), activity levels were compared with nontreated noninoculated and nontreated inoculated controls. Means with the same letter at each time point are not significantly different using Tukey's test at $P \leq 0.05$.

Fig. 4. Time course of peroxidase (POX) activity in A, Alixan, B, Premio, and C, Altigo wheat cultivars treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) either noninoculated or inoculated (NECTAR Céréales I) with *Zymoseptoria tritici* strain T01193 compared with nontreated noninoculated (nTnI) and nontreated inoculated (nTI) controls. Inoculation occurred 48 h after treatment (arrow). Means with the same letter are not significantly different for each time point using Tukey's test at $P \leq 0.05$.

Fig. 5. Time course of phenylalanine ammonia lyase (PAL) activity in A, Alixan, B, Premio, and C, Altigo wheat cultivars treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) either noninoculated or inoculated (NECTAR Céréales I) with *Zymoseptoria tritici* strain T01193 compared with nontreated noninoculated (nTnI) and nontreated inoculated (nTI) controls. Inoculation occurred at 48 h after treatment (arrow). Means with the same letter are not significantly different for each time point using Tukey's test at $P \leq 0.05$.

Premio. The *GLUC* gene was induced in Premio at 12 to 54 h with 2.5- to 5.1-fold overexpression. In Alixan, this gene induction was recorded at 24 to 54 h with 2.7-, to 9.8-fold increase and at 60 and 72 h in Altigo with 2.1- and 5.2-fold increase, respectively.

Induction of defense-related gene expression in Alixan, Premio, and Altigo in inoculated plants. The transcription profiles of the same set of gene were also established in plants treated with

NECTAR Céréales and inoculated. Gene expression profiles were followed at 6, 12, and 24 h after inoculation and were compared with nTnI controls (Fig. 10). Only genes that were significantly regulated following infection are displayed.

The *POX2* gene expression level recorded in Alixan NECTAR Céréales-treated and inoculated plants at 6 h after inoculation (68-fold) was significantly higher than in nTI controls (17.8-fold) and

Fig. 6. Relative expressions of *POX2* and *OXO* genes in Alixan, Premio, and Altigo wheat leaves treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) compared with their respective nontreated noninoculated (nTnI) controls at 12, 24, 48, 51, 54, 60, 72, 120, and 168 h after treatment. Means with the same letter are not significantly different for each time point using Tukey's test at $P \leq 0.05$.

Fig. 7. Relative expression of *LOX* and *AOS* genes in Alixan, Premio, and Altigo wheat leaves treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) compared with their respective nontreated noninoculated (nTnI) controls at 12, 24, 48, 51, 54, 60, 72, 120, and 168 h after treatment. Means with the same letter are not significantly different for each time point using Tukey's test at $P \leq 0.05$.

in NECTAR Céréales -treated plants (17.3-fold), whereas no significant increase was recorded in Premio and Altigo.

The *PAL* gene was significantly upregulated in Alixan and Altigo NECTAR Céréales-treated and inoculated plants at 6 h after inoculation (26.7-fold) and at 24 h after inoculation (84.64-fold) relative to the nTI controls (Fig. 10). In Premio, NECTAR Céréales-treated and inoculated plants presented no significant induction of this gene.

The *PR1* gene was significantly upregulated in Alixan NECTAR Céréales-treated and inoculated plants at 12 h after inoculation (7.4-fold) compared with nTI controls (0.4-fold) and NECTAR Céréales-treated plants (1.4-fold). No significant induction of this gene was observed in Premio and Altigo. Concerning the *GLUC* gene, its expression was significantly higher at 6 and 12 h after inoculation in Alixan (7.4- and 2.6-fold, respectively) than in nTI plants (0.9- and 0.5-fold, respectively) and in NECTAR Céréales-treated plants (2.7- and 0.4-fold, respectively). This gene expression level recorded at 12 and 24 h after inoculation in Altigo NECTAR Céréales-treated and inoculated plants (5.9- and 32.5-fold, respectively) was significantly higher than in nTI (2.6- and 0.9-fold, respectively) and in NECTAR Céréales-treated plants (2.1- and 5.2-fold, respectively). In Premio NECTAR Céréales-treated and inoculated plants, the *GLUC* gene expression level was not significantly different from nTI and NECTAR Céréales-treated plants.

DISCUSSION

The present study aimed to evaluate the elicitor potential of a sulfur, manganese sulfate and microbial protein-based commercial product, NECTAR Céréales, against STB, taking into account the wheat genotype response. During the first part of our project, we determined the efficient dose, timing, and number of applications of NECTAR Céréales (previously named FSOV7) to protect wheat against STB (Ors et al. 2012, 2013). Significant reductions >70% of disease symptoms and >50% of pathogen sporulation on the three studied cultivars were obtained when

leaves were pretreated 48 h before inoculation with 30 ml of NECTAR Céréales at the dose of 8.73 µl/ml. While the efficient dose of this product did not show any direct effect on spore germination and mycelium growth, the upregulation of at least one wheat defense gene marker, such as *POX2*, *PAL*, *LOX*, *AOS*, *OZO*, *CHIT2* or *GLUC* genes was recorded in the three studied cultivars after leaf pretreatment. The involvement of these genes in the resistance of wheat to *Z. tritici* was suggested previously in numerous studies (Adhikari et al. 2007; Diani et al. 2009; Ors et al. 2017; Samain et al. 2017; Shetty et al. 2009; Yang et al. 2013b).

Many microbial proteins have been identified as inducers of plant resistance to pathogen attack. The *pebC1* protein from *Botrytis cinerea* reduced gray mold in tomato (Zhang et al. 2010, 2014b), an elicitor and a 75 kDa protein elicitor purified from *Phytophthora palmivora* induced resistance in *Hevea brasiliensis* against this pathogen (Dutsadee and Nunta 2008), the *SsCut* cutinase from *Sclerotinia sclerotiorum* induced defense mechanisms in various plant species (Zhang et al. 2014a) and the *Hrip1* protein from *Alternaria tenuissima* elicited defense mechanisms in tobacco (Kulye et al. 2012). However, minerals and ions are also able to induce defenses in plants against pathogens. Low concentrations of CuSO_4 elicited the production of phytoalexins in grapevine leaves and protected them against *B. cinerea* and *Plasmopara viticola* (Aziz et al. 2006). Moreover, high leaf manganese concentrations induced protection against *Erysiphe necator*, responsible for powdery mildew in grapevine, and the synthesis of proteins related to pathogen resistance (Yao et al. 2012).

Nevertheless, a light chlorosis was observed on wheat leaves treated with NECTAR Céréales. Previously, Fecht-Christoffers et al. (2003) described similar symptoms in cowpea leaves caused by the toxic effect of an excessive manganese supply. The authors reported an increase of *POX* activity and an accumulation of proteins showing homologies to PR-proteins such as glucanase and chitinase. As discussed below, treatment with NECTAR Céréales induces similar accumulations in the wheat cultivars

Fig. 8. Relative expression of *PAL* and *CHS* genes in Alixan, Premio, and Altigo wheat leaves treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) compared with their respective nontreated noninoculated (nTnI) controls at 12, 24, 48, 51, 54, 60, 72, 120, and 168 h after treatment. Means with the same letter are not significantly different for each time point using Tukey's test at $P \leq 0.05$.

Alixan, Premio, and Altigo. It is possible that manganese contained in NECTAR Céréales is at least partially responsible for such induction and possibly involved in the slight toxicity observed during the induced resistance. Abiotic stresses, such as an excessive Mn supply, could induce plant defense mechanisms. Mithöfer et al. (2004) hypothesized that heavy metals as well as biotic stress lead to an accumulation of the reactive oxygen species (ROS) involved in the oxidation of unsaturated fatty acids which initiate the octadecanoid pathway. The authors suggested that the formation of oxylipins (through LOX and AOS activities) could elicit plant secondary metabolism and accumulation of defense compounds as induced by a biotic stress. Taken together, these results suggest that the protection efficacy conferred by NECTAR Céréales on wheat against *Z. tritici* results from the induction of resistance mechanisms that could be attributed to one or several of its components.

Whatever the cultivar, wheat defense responses were activated during the first 72 h after treatment, even if many differences concerning timing and intensity were recorded between cultivars. More specifically, the RT-qPCR analysis showed an important upregulation of *POX2* as a response to NECTAR Céréales pretreatment in the three wheat cultivars, in noninoculated plants. At the biochemical level, our results confirmed the elicitation of POX activity in the three cultivars 72 h after treatment. The *pox* gene

encodes for the peroxidase enzyme which plays an important role in the ROS pathway by catalyzing genes implicated in oxidative radicals. In addition, peroxidases are implicated in lignin production and in plant cell wall reinforcement (Pechanova et al. 2010). This mode of action represents the first physical defense barrier to pathogen attack (Underwood 2012). In addition to lignification, oxidative radicals with other related defense proteins and polysaccharides have restricted strongly cell wall invasion by fungal pathogens including *Verticillium* spp. and *Melampsora* spp. (Pechanova et al. 2010; Floerl et al. 2012). Also, the overexpression of the hydrogen peroxide (H₂O₂)-producing enzyme oxalate oxidase (*OXO*) as a response to NECTAR Céréales pretreatment indicates the implication of H₂O₂ in the induced resistance against STB. Indeed, H₂O₂ has shown direct antagonistic effect against phytopathogenic microorganisms (Peng and Kuc 1992). Previously, a correlation between *OXO* gene expression and resistance to *Sphaerulina musiva* has been reported (Liang et al. 2001). The production of ROS was reported as an earlier response during the biotrophic phase of *Z. tritici* starting at the infection point and expanding to the entire leaf tissue during the necrotrophic phase (Yang et al. 2015). In contrast to necrotrophs, which often enhance ROS production to stimulate programmed cell death and facilitate nutrient uptake (Govrin and Levine 2000), this mode of action could be efficient in stopping the

Fig. 9. Relative expression of *PR1*, *CHIT2*, and *GLUC* genes in Alixan, Premio, and Altigo wheat leaves treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) compared with their respective nontreated noninoculated (nTnl) controls at 12, 24, 48, 51, 54, 60, 72, 120, and 168 h after treatment. Means with the same letter are not significantly different for each time point using Tukey's test at $P \leq 0.05$.

infection process of *Z. tritici*, which reacts as a biotroph during the first 9 days of its contact with wheat plants (Selim et al. 2014).

However, our results showed variations between cultivars at the level of elicitation timing and upregulation intensities of other

tested genes. Such variations were interpreted previously as the impact of the plant genotype (Bruce 2014; Samain et al. 2019; Walters et al. 2005, 2011). In contrast to Alixan and Premio, the most resistant cultivar Altigo treated with NECTAR Céréales

Fig. 10. Relative expression of *POX2*, *PAL*, *PR1*, and *GLUC* genes in Alixan, Premio, and Altigo wheat leaves treated with a plant nutrient- and microbial protein-based resistance inducer (NECTAR Céréales) either noninoculated or inoculated (NECTAR Céréales I) with *Zymoseptoria tritici* strain T01193 compared with nontreated noninoculated (nTnI) and nontreated inoculated (nTI) controls at 6, 12, and 24 h after inoculation. Inoculation occurred at 48 h after treatment. Means with an asterisk are significantly higher than in nontreated noninoculated, nontreated inoculated, and treated noninoculated plants for each cultivar and for each time point using Tukey's test at $P \leq 0.05$.

exhibited an earlier and a significant upregulation of the *PAL* and the *CHS* genes at 12 h in noninoculated plants. The overexpression of *PAL* reached a maximum at 72 h and was associated with a high induction level of *CHS* and with transient but significant inductions of *chit*, *GLUC* and *OXO* genes. The *PAL* and *CHS* genes encode for phenylalanine ammonia-lyase and chalcone synthase enzymes, respectively, that are known as markers of the phytoalexin and phenylpropanoid pathway. Indeed, *PAL* catalyzes phenylalanine as the first step of serial reactions where many other enzymes such as *CHS* are implicated. These reactions result to the biosynthesis of large varieties of compounds with antimicrobial and antioxidant activities, flavonoids, phytoalexins, and lignin (Lamb and Dixon 1997). In addition, the upregulation of *chit* and *GLUC* genes coding for chitinase and glucanase enzymes could be efficient in the control of *Z. tritici* by their known role in the digesting of chitin and β -glucans, respectively, of the fungal cell wall (Samain et al. 2017). Moreover, after inoculation with *Z. tritici*, the same defense pathways are still stimulated in Altigo treated with NECTAR Céréales, since an overexpression of *PAL* and *GLUC* genes was observed during the first 24 h after infection compared with plants treated with NECTAR Céréales only or inoculated with *Z. tritici* only. Hence, the phenylpropanoid pathway and glucanase seem involved in Altigo's defense response induced by NECTAR Céréales against *Z. tritici*.

In the moderately resistant wheat cultivar Premio, any of the tested genes were up-regulated following inoculation after treatment. Defenses mechanisms were mainly induced just after treatment, suggesting an eliciting effect rather than a priming effect of NECTAR Céréales in this cultivar. In the noninoculated plants, *LOX* and *AOS* gene expression were strongly up-regulated at the same times after 12, 24, and 48 h indicating the stimulation of jasmonic acid and ethylene signaling pathways. *LOX* and *AOS* enzymes are implicated in the biosynthesis of the oxylipins (Gottwald et al. 2012) which are involved in pathogen recognition (Wasternack 2007) and in different plant defense mechanisms such as proteinase inhibitor induction, secondary metabolite accumulation and eliciting of other defenses such as PR genes (Blée 2002; Mithöfer et al. 2004; Robert-Seilanianantz et al. 2007). Moreover, some of them, such as thaumatin-like proteins have fungicidal activities inhibiting spore germination and mycelium development (Prost, 2005).

In the most susceptible cultivar Alixan treated with NECTAR Céréales, in noninoculated plants, a significant up-regulation of different genes (such as *POX2*, *OXO*, *PAL*, *PR1*, *CHIT2*, and *GLUC*) as well as an increase of *POX* and *PAL* activities were recorded and sometimes earlier (e.g., *pox* at 12 h) and stronger (e.g., *PR1* 18.1-fold at 51 h) than in Premio and Altigo. Moreover, in inoculated plants, a priming effect of NECTAR Céréales in Alixan was noticed through the induction of an overexpression of *pox*, *PR1*, *PAL*, and *GLUC* at 6 and 12 h after infection. The association of overexpression of *PR1*, *PR2* (β -1,3-glucanase), and *pox*, which have been identified previously as types of PR protein (Van Loon and Van Strien 1999), were observed more in incompatible than in compatible interactions in several cereal pathosystems (Boyd et al. 1994; Desmond et al. 2008) and especially in the wheat–*Z. tritici* pathosystem (Adhikari et al. 2007; Ray et al. 2003; Samain et al. 2019). This suggests that *PR1* proteins may play an important role in the resistance against *Z. tritici* alone or in concert with other defense-related genes or pathways such as phenylpropanoid and ROS pathways. The absence of *PR1* overexpression in the other two cultivars Premio and Altigo as a response to NECTAR Céréales pretreatment contradicts the idea that *PR1* is the wheat protection marker against STB, as previously proposed by Adhikari et al. (2007) and Samain et al. (2019). The percentage of germinated spores with penetrated germ tubes (PS) recorded at 5 days after inoculation was reduced in Alixan treated with NECTAR Céréales compared with nontreated plants, suggesting that in this cultivar, the elicitation of *POX2*, *OXO*, *PR1*, *PAL*, *CHIT2*, and *GLUC* gene

expression as well as the increase of *POX2*, *PR1*, *PAL*, and *GLUC* gene expression in inoculated plants affect the penetration of *Z. tritici*. A high level of gene expression seems to be required, since in this susceptible cultivar, *POX2*, *CHIT2* and *PR1* are already induced after infection and without NECTAR Céréales pretreatment (Ors et al. 2017).

The effect of NECTAR Céréales on the pathogenicity of *Z. tritici* was investigated at the cytological and biochemical levels. Results showed that CWDE activities increased on the three wheat cultivars according to the sporulation level of *Z. tritici* and the highest levels were recorded in the most susceptible cultivar Alixan at 21 days after inoculation. It has been previously reported that CWDE activities are correlated with pycnidial formation (Douaiher et al. 2007a, b; Siah et al. 2010a) and wheat susceptibility (Tian et al. 2009). As a response to pretreatment with NECTAR Céréales, CWDE activities were reduced significantly in Alixan only (except protease activity which was also reduced in Premio). Surprisingly, the pretreatment with NECTAR Céréales affected the colonization of substomatal cavities in Premio and Altigo but not in Alixan, where the reduction of CWDE activities was most important. According to microscopic observations, only direct penetration attempts were reduced in Alixan. At the macroscopic level, leaf areas with symptoms and sporulation level were both reduced in the three cultivars. However, the reduction of CWDE activities cannot be considered as a strict marker of induced resistance as a response to the elicitor pretreatment in wheat against STB since a protection has been obtained without influencing these enzymatic activities.

In conclusion, under controlled conditions, NECTAR Céréales, a manganese, sulfur- and microbial protein-based product, induces a non-genotype-specific wheat resistance conferring more than 70% of protection against *Z. tritici*. Nevertheless, the mechanisms of this induced resistance were genotype dependent and can be linked to an elicitor or both elicitor and priming effects. However, these defense pathways could impact the penetration process and the first days after infection in some cultivars or could interfere later with the fungus spreading in the leaf tissues, leading to symptom and sporulation reduction. It seems that manganese contained in NECTAR Céréales induces an abiotic stress leading to plant defense responses and chlorosis. Such an effect may be reduced at the field level by applying a lower dose or treating more mature plant growth stages.

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