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Effect of coating seeds with *T*richoderma *harzianum* (S. INAT) on the oxidative stress induced by *Fusarium culmorum* in durum wheat

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Abstract - Fusarium crown rot (FCR) is a devastating wheat disease caused by *Fusarium culmorum*. The plants respond to infection by inducing a set of defence related enzymes. In this current study, seed coating with *Trichoderma harzianum*, known as biocontrol agent, was released on durum wheat grains of the cultivar 'Karim' in order to investigate its effects on *F. culmorum* resistance and antioxidant metabolites synthesis, under controlled conditions. Seed coating with a commercial product "Panoramix" was applied as reference, which is based on *T. harzianum* and mycorrhiza. Following Fusarium-inoculation, the results showed that the plants treated by *T. harzianum* induced wheat defence mechanisms by significantly reducing the necrotic symptoms in wheat crown compared to control plants with 50% of rate reduction of severity index at 10dpi (days post infection). The activities of peroxidase, catalase, chitinase as well as phenolic compounds were highly induced since the start of stress in plants treated by *Trichoderma* compared to plants treated by Panoramix. Both treatments significantly decrease 40% the pro-oxidant H_2O_2 content compared to control. These results suggest that *T. harzianum* reduce the H_2O_2 damage by enhancing the antioxidant defence mechanisms in response to the Fusarium challenge. Hence, the seed coating with *T. harzianum* may be considered as a potential strategy to alleviate of the oxidative stress caused by the FCR disease in durum wheat.

Keywords : FCR, *Trichoderma harzianum*, defence response, antioxidant enzymes.

1. Introduction

Fusarium culmorum (wm.g.sm) sacc performs a hemibiotrophic life cycle in wheat seedlings (Petti et al., 2012). *F. culmorum* infects host plants at the initial growing stages, causing rotting of root and crown tissues. In some cases, lesions and browning of the coleoptiles or seedling death may occur. When *F. culmorum* infects wheat plants at later growing stages, brown spots on basal internodes can be observed (Scherm et al., 2013; Balmas et al., 2015, Mohapatra et al., 2017). The pathogen is also the causal agent of Fusarium crown rot (FCR) (Chekali, et al.2013) in wheat field in Tunisia leading to crop loss. Many strategies have been employed to control this disease using the biotic agents to avoid the use of chemicals products. Among the known biocontrol agents, *Trichoderma* spp. is a common and effective fungus used for the management of plant diseases (Benítez et al. 2004). The beneficial potential of *Trichoderma* spp. is gradually gaining attention in the pathogen-biocontrol sector as it is capable to colonise roots and enhance root and shoot development and are known as antagonist to various phytopathogens including *Fusarium* spp (Harman et al., 2004; Oliveira et al., 2018). Their mechanisms include competition for nutrients, mycoparasitism, production of inhibitory volatiles and non-volatile compounds involving the production of hydrolytic enzymes (Benítez et al. 2004) and production of siderophores (Vinale et al. 2013), leading to the induction of plant defense-related enzymes against a wide range of pathogens (Harman, 2011, Harman et al., 2004; Zhang et al., 2016). Seed coating with *Trichoderma spp*. may afford several economic and agronomic advantages through priming the germinating seedling in order to react more rapidly and efficiently to a stress (Umadi et al., 2018), by using beneficial microorganisms and thus improve disease resistance (Lutts et al., 2016), and at the same time tends to outweigh farmers fungicide's expenses on fertilizers and fungicides. Therefore, in the aim of this present work is to investigate the effects of seed coating with *T. harzianum* on *F. culmorum* resistance in durum wheat and to explore the possible induced defense response.

2. Materials and methods

Trichoderma strain

The strain S. INAT of *T. harzianum* (KU710282) used in this assay was isolated and identified in the laboratory of genetics and cereal breeding of INAT. A liquid culture of S. INAT strain was produced by scraping the spores of a culture on a ??? medium in the sterile distilled water and transferring them to a 250 ml Erlenmeyer flask containing 100 ml of PDB (Potato-Dextrose-Broth) medium. The liquid culture was then incubated on a rotary shaker at 110 rpm and 25 ° C. After seven days, conidia suspension was collected by filtration and the concentration was adjusted to 10^6 conidia mL-1.

Cuture of *F. Culmorum* and inoculums preparation

To produce macroconidia of *F. culmorum*, a mixture of barely grains (3:1 by volume) was soaked in water overnight in 250 mL glass bottles. Water was decanted and seeds were autoclaved. Afterwards, seeds were inoculated with F. culmorum mycelium from culture 7 days, and were kept for 2 weeks at 25°C in the dark. Conidia were washed from the kernels and the concentration of the conidial suspension was set to $1x10^5$ mL^{-1}. The plants were inoculated two weeks after sowing by removing the soil around the collar and applying 100ml of the Fc suspension, or water for control plants, both containing 0.02 % (v/v) Tween 20.

Coating seeds and plant inoculation

The durum wheat variety «Karim» was used in this work. It is one of the most used durum wheat cultivars in Tunisia (Rezgui et al. 2000). Seeds were surface disinfected by soaking them in 95% ethanol for 10 s and 5% sodium hypochlorite for 3 min and were rinsed three times in sterile water.

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 prepared conidia suspension of Trichoderma S. INAT. An amount of 400 µl of water was used as a control. Similarly an amount of 400 µl of the product Panoramix was used as reference. The product Panoramix is a biological seed dressing that is marketed by koppert and is a combination of microorganisms and additives which promote plant growth. This product is composed of Mycorrhiza (>10 propagules /ml), Trichoderma spp. (>$1x10^7$ CFU/ml), and Bacillus spp. (2 x 10^7 CFU/ml) which colonize the roots and protect the crop during the entire cultivation season.

The coating mixture was applied progressively to 10 g of wheat seeds in rotation. Coated seeds were sown in pots containing an autoclaved mixture of horticultural compost and sand (1/1, v/v).

Coated seeds were sown in a total of 12 pots (volume of 1 litre) with a density of 4 seeds/pot; (total of 3 pots for each coating treatment). The trials were repeated three times independently, with three replications each. The 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).

Growth parameters

At 7 dpi, roots and shoot lengths, and biomass were measured. Further, total chlorophyll content of each treatment was estimated following the method of Witham et al (1971). Chlorophyll a was measured by reading the absorbance at 663 nm and chlorophyll b was measured by reading the absorbance at 645 nm using UV-vis spectrophotometer. The total chlorophyll (TC) content was calculated using the following formula:

TC (mg. g-1 FW) = (((20.2xA645) +(8.02xA663))/1000xW)) xV

Where **W**: fresh weight, **V:** volume extraction

Disease Scoring

The visible necrosis on the stem base was scored weekly for all treatments (3 plants/repetitions/treatments). For each evaluation, the plants were gently uprooted and the crown disease was scored basing on symptom extension and browning index on a five-class scale (class 0 = healthy stem; class 1 = mild browning on the stem; class 2 = browning on one-half of the stem; class 3 = complete browning of the stem; class 4 = plant death).

Determination of Antioxidants activities

The antioxidant enzymes and the phenolic compounds were performed in the leaf's wheat 3, 7 and 10 days after fusarium-inoculation.

Phenolic compounds quantification

The quantification of the soluble phenolic compounds was determined according to the Folin-Ciocalteu method modified by Singleton et al. (1999). Samples were collected in the same conditions used for the enzymatic analysis. Foliar tissues (200 mg from 3rd leaf), were homogenized in an ice bath with 1.5 ml methanol. The homogenate was centrifuged three times at 10,000×*g* for 5 min; supernatants were collected each time. A 100 µL of the supernatant was added to the reaction mixture containing 50µL of sodium carbonate (20%), 1750 µL of sterile distilled water and 250 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, Germany). The mixture was incubated at 40°C for 30 min and the blue color was read at 760 nm using cathechin (Sigma Aldrich, USA) as a standard. The content of soluble phenolic compounds was expressed in mg-equivalents of cathechin per gram of fresh weight (FW).

Assay of H_2O_2 content

H_2O_2 was extracted and assayed following the method described by Noreen and Ashraf (2009). Fresh shoot tissues (0.1 g) from each sample were homogenised with 2 mL of 0.1% (w/v) cold trichloroacetic acid (TCA), centrifuged at 14,000 g for 15 min and the supernatant was collected. Absorbance of the reaction mixture containing 0.5 mL of the supernatant, 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1 mL of 1 M KI was read at 390 nm. The H_2O_2 content was determined using an extinction coefficient of 0.28 µM−1 cm−1 and expressed as µM g−1 FW.

Peroxidases activity

500 mg of were homogenized in 5 mL of 50 mM K-phosphate buffer (pH 5.5). After centrifugation at 12,000× g for 20 min at 4 °C, the supernatant was collected as the crude enzyme solution. A reaction mixture was prepared by adding 2.9 mL of 50 mM K-phosphate buffer (pH 5.5), 1 mL of H_2O_2 (0.6 M) and 1 mL of 50 Mm guaiacol to 0.1 mL of crude enzyme solution (Egley et al. 1983). Protein content of the crude enzyme solution was determined at 595 nm with bovine serum albumine as the standard using the Bradford assay (Bradford 1976) by mixing 790µL of extraction buffer, 10µL of crude enzyme solution and 200µL of Bradford reagent (Biomatik, Tunisia). Peroxidases activity was determined with guaiacol at 470 nm and expressed in unit mg-1 protein.

Catalase enzyme

Catalase activity was assayed according to the method of Aebi(1974). 100 mg of leaf sample was mixed with 5 mL of sodium phosphate buffer (100 mM, pH 7.0) and ground thoroughly. The homogenate was centrifuged as above. CAT activity was determined by adding 0.2 mL of the enzyme preparation to 3 mL of sodium phosphate buffer containing 0.2 mL H_2O_2 as a substrate. The decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm with a spectrophotometer. One unit was defined as the change in 0.001 absorbance units per minute and the specific activity was expressed as units per gram of fresh weight

Chitinase enzyme

Leaves were frozen in liquid nitrogen and immediately ground to a fine powder with a mortar and pestle. The powder was mixed with 2 mL of buffer (0.05 M NaAc, pH 5.0, 100 lM PMSF) for 1 h (Ride et al., 1990). The reaction mixture was centrifuged at 15,000g for 15 min and the supernatant was collected for enzyme assays. The concentration of the total soluble protein was determined by using the method of Bradford (1976) with bovine serum albumin (BSA) as a standard. The chitinase activity of crude protein was analyzed by using colloid chitin as the substrate. A unit of chitinase activity was defined as the amount of enzyme required for releasing 1 lM N-acetylglucosamine (GlcNac) in 1 h.

Statistical analysis

Analysis of variances was performed by one-way ANOVA test using the SPSS-20 software (SPSS Inc). Means were compared by Duncan's test at the 0.05 level of confidence to show significant differences among treatments.

3. Results

Effect of seed coating on the growth parameters

The treatments affected significantly ($P<0.05$) all the growth parameters (Table1). After Fusarium-infection, Results showed that in control plants, the infection reduces significantly in total biomass and total chlorophyll, and in root and shoot length with more remarkable reduction in root length compared to shoot length (table1). Seed coating with either Trichoderma or Panoramix, induce biomass, total

chlorophyll and root length, compared to both non-infected and infected control (Table1). Seed coating with Panoramix had more remarkable impact on total biomass while seed coating with Trichoderma had more remarkable impact on root and shoot length.

Table 1: Comparative assessment of growth parameters as Biomass (g/seedling), root length (cm), shoot length (cm) and Total Chlorophyll in durum wheat seedling at 7 dpi. Means in the same column followed by letters differ significantly at P < 0.05.

	Biomass (g/seedling)	**Root length (cm)**	**Shoot length (cm)**	**Total Chlorophyll**
Non-infected Control	1,96±0.11bc	8,50±0.48b	9,77±0.24b	39,92±4.37b
Infected control	1,18±0.16c	6,57±0.89c	8,10±0.29c	23,73±0.90b
T. harzianum	3,55±0.49b	12,13±0.53a	13,60±0.65a	76,46±11.82a
Panoramix	2,36±0.43a	9,57±0.32b	9,77±0.44b	66,50±11.82a
ANOVA				
Coating	9,86**	48,48***	48,89***	5259,32**

Sum squares were indicated with Level of significance (LS) (**, significant at P < 0,05; ***significant at P < 0,001)

Effect of seed coating on disease severity

Compared to controls, plants derived from seed coating with *T. harzianum* and Panoramix showed significantly lower disease index at 14 dpi and 21 dpi (figure1).

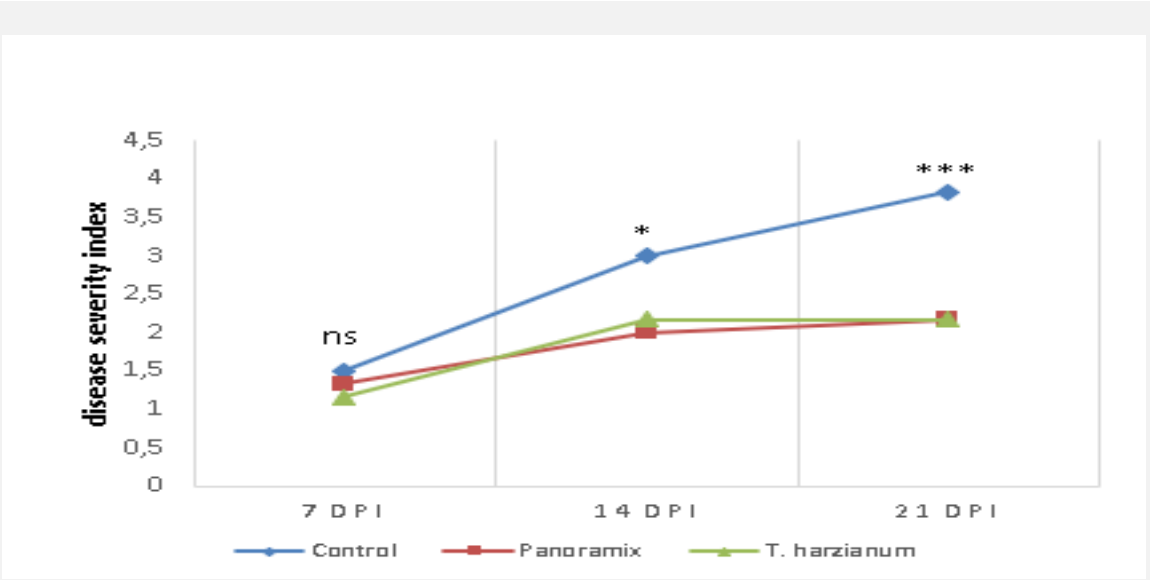

Figure 1: Disease severity in durum wheat stem under *T. harzianum*, Panoramix and control conditions measured at 7, 14, 21 dpi. LS: ns, Not significant; *, P < 0.05; ***, P < 0.001

Effect of the disease and the seed coating on phenolic compound content:

Phenolic compound **(**PC) was significantly affected by the two factors coating (C) and time of analysis (D) and their interaction C x D (Table3). In control plants, the infection with *F. culmorum* decrease slightly PC at 10 dpi. Seed coating with Panoramix induced a higher accumulation of PC at 10 dpi. Differently, seeds coating with *T. harzianum* induced a slight decrease of PC at 3 and 7 dpi associated with the highest recorded accumulation of PC at 10 dpi (Figure 2).

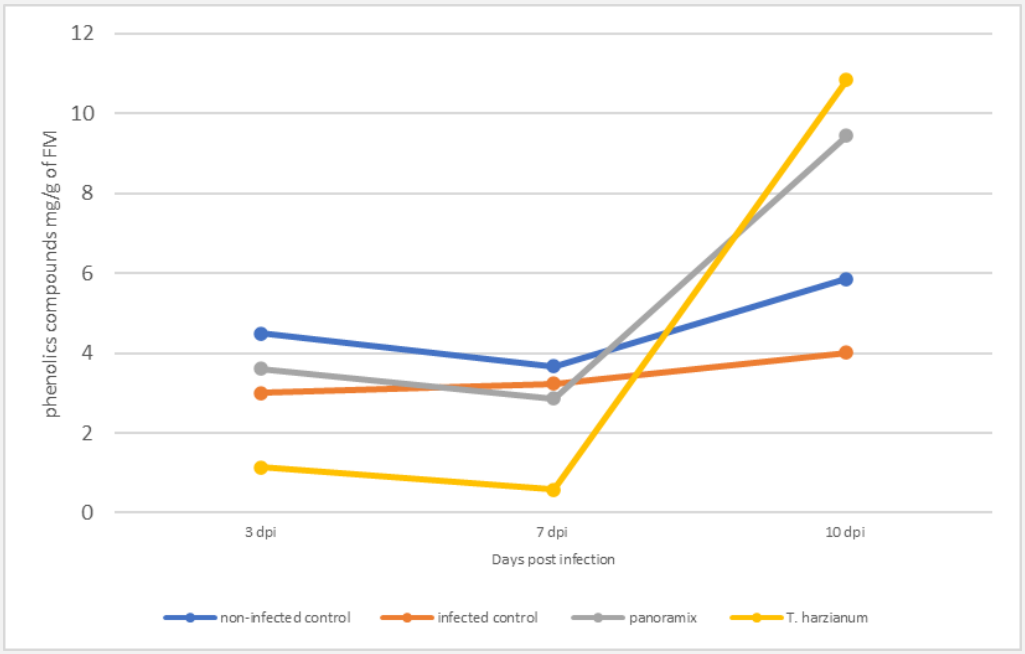

Figure 2: Change in the total phenolic content (PC) in leaves after 7, 14 and 21-days post inoculation by *F. culmorum*

Effect of the disease and the seed coating treatments on H_2O_2 content and enzymatic activity of catalase, chitinase, and peroxidase in wheat plants

The ANOVA analysis showed that H_2O_2content, and the enzymatic activity of catalase, chitinase, and peroxidase were affected significantly by the two factors treatment and time of analysis, and their interaction (TxD) (Table3). In control plants, the inoculation induced: (i) an increase of H_2O_2 content at 7 and 10 dpi (figure3, A); (ii) a slight increase of peroxidase at the beginning of the stress (3 dpi) followed by a decrease at 10 dpi (figure3, B); (iii) a slight increase of catalase activity at 10 dpi (figure3, C); and (iv) a slight increase of chitinases activity at 7 dpi (figure3, D).

Table3: ANOVA for the H_2O_2 content, and enzymatic activity of catalase, chitinase, and peroxidase in inoculated plants with *F. culmorum*

	df	H_2O_2	Catalase activity	Chitinase activity	Peroxidase activity	Phenolic compounds
Treatment (T)	3	1721,22***	23,43***	116,38**	1405,12***	17,06**
dpi(D)	2	25,81 ns	6,55***	134,31***	1593,14***	179,06***
T X D	6	4658,76***	12,16***	155,42**	2041,65***	107,80***

The sum squares values are shown with LS (ns: non-significant, **, P < 0,01; ***, P < 0,001).

Unlike the infected control, plants derived from both of the seed coating treatments induced an early increase in H_2O_2 content at 3 dpi followed by its decrease over time (figure3, A). This was associated with an increase in peroxidase and catalase activities at 7 and 10 dpi (figure3, B, C) lhowever, Trichoderma induced an early increase of peroxidases at 3 dpi (figure3, B), and an increase of chitinases at both 3 and 10 dpi (figure3, C, D); while Panoramix resulted in an early decrease of peroxidases at 3 dpi (figure3, B) with no remarkable change on chitinases (figure3, D).

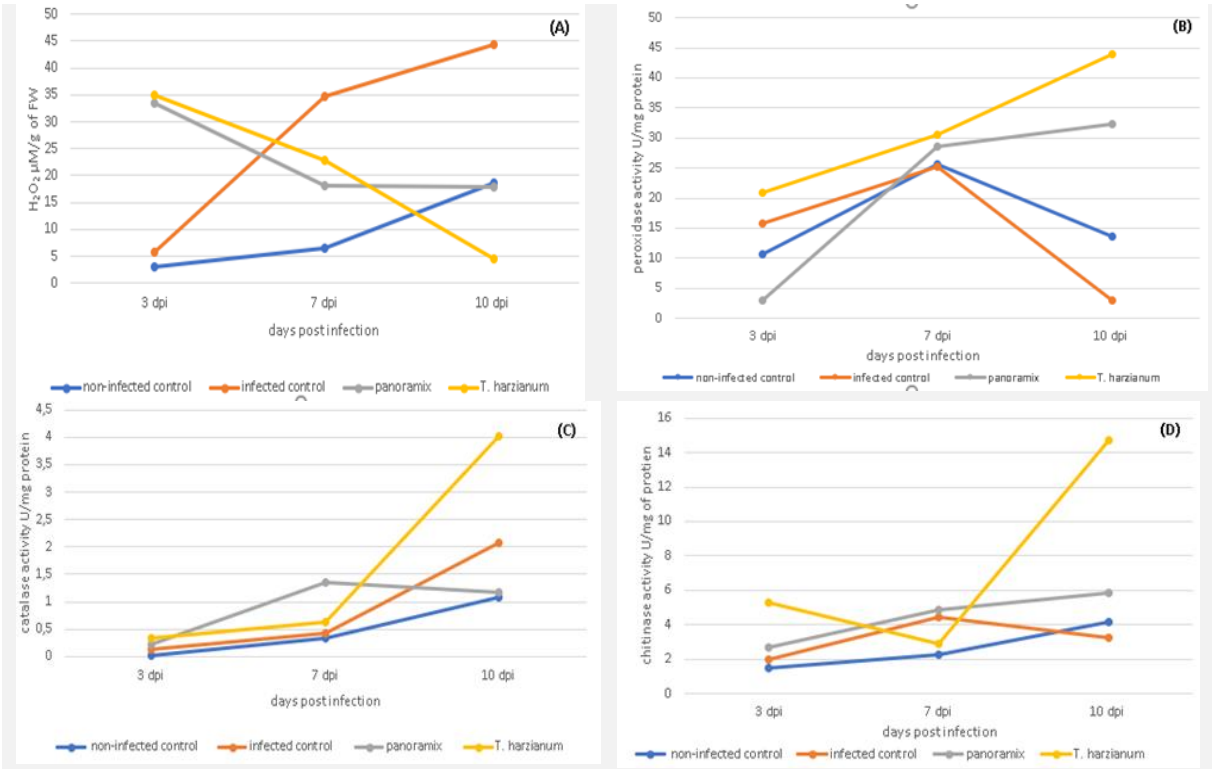

Figure3: Comparative assays of H_2O_2 content, and enzymatic activities of catalases, chitinases, and peroxidases in durum wheat leave tissues following Fusarium-inoculation: (A) H_2O_2; (B) Peroxidases; (C): catalase activity; (D): chitinase activity.

4. Discussion

As already cited, *F. culmorum* performs a hemibiotrophic life cycle in wheat seedling (Petti et al., 2012) and produce lesions on the coleoptile, roots, and subcrown internode and cause browning of the stem bases (Beccari et al, 2011; Chekali et al., 2011). Despite the detrimental impact of the FCR disease in wheat, few studies were focused on the defense response induced by plants following Fusarium-inoculation. The physiological studies of infected plants could help to clarify the pathways involved in the defence signaling. In plants, the resistance to pathogens is often mediated by a complex system of defence responses. Within this network, a variety of antioxidative enzymes, such as, catalase (CAT), and peroxidase (POX) are involved in the detoxification of ROS (Mottalebi et al., 2017). In the present study, the infection by *F. culmorum* resulted mainly by a reduction of root length. This was associated with an increase of peroxidases at 7 dpi, followed by an increase of phenolics compounds at 10 dpi, indeed, peroxidases are oxido-reductive enzymes that participate in the wall-building processes such as oxidation of phenols, suberization, and lignification of host plant cells during the defence reaction against pathogenic agents. Besides, the accumulation of phenolic compounds have been correlated with disease resistance in a number of plant–pathogen interactions (Mohammadi et Kazmi, 2002) . This finding was consistent with the study of (Mohammadi et Kazmi, 2002), who suggested that wheat infected with *F. graminearum* increased activities of peroxidase. Moreover, the production of mycotoxins by *F. culmorum* is believed to play a role in pathogenesis as potent inhibiters of protein synthesis and is postulated to inhibit activation of defence response genes, and can induce complete loss of chloroplast pigments (Wagacha & Muthomi, 2007). This could explain the observed reduction of chlorophyll content and the slight increase of chitinases, phenolic compounds, and catalases.

The potential of *Trichoderma* spp. has already been reported in growth promotion and disease control of plants (Benítez et al. 2004; Chandra Nayaka et al. 2010; Harman, 2011; Vinale et al. 2013). Nevertheless, the potential of seed coating with these beneficial species have been rarely tested. Chandra Nayaka et al. (2010) reported that seed treatment with *Trichoderma* spp. increased the germination rate and vigour index of maize and enhanced growth in the field. The extent to which the seed coating technique with Trichoderma spp and Trichoderma-based products could enhance plant protection and production is yet to be discovered. In this study, seed coating with either *Trichoderma* or Panoramix, increased the seedling biomass, chlorophyll and elongation compared to both non-infected and infected control, and reduced the disease severity. This demonstrates that both treatments have a potential of growth promotion and plant protection against *F. culmorum*. The observed potential of the growth

promotion supports previous studies demonstrating that *Trichoderma* spp. has the ability to colonize plant roots, provide symbiotic relationships with several host plants, and promote plant growth and development (Harman, 2011, Hajieghrari et Mohamed, 2016, Oliveira et al., 2018, Mohapatra et al., 2017). Different mechanisms have been proposed to explain the improvement of plant growth such as the increase of plant nutrient uptake, the ability of mineral solubilization (Altomare et al., 1999), and production of phytohormone and plant growth regulatory material (Vinale et al., 2008; 2013). However, differences were observed concerning the extent of impact between seed coating with *T. harzianum* and with Panoramix; Panoramix had more remarkable impact on total biomass, while *T. harzianum* had more remarkable impact on total chlorophyll and seedling elongation. This difference could be due to the composition of Panoramix which includes not only Trichoderma spp but also Mycorrhiza and *Bacillus* spp. suggesting that the competition between these three different microorganisms is in favour for the biomass yield but not as much for chlorophyll and seedling elongation. Indeed, many species of *Bacillus* have been identified as plant-growth promoting bacteria and/or biocontrol agents (Kan et al., 2018). The most commonly studied is *B. subtilis* which have been known to enhance plant growth by phytohormone production and the acquisition of nutrients such as phosphorous and nitrogen (Kan et al,, 2018). Moreover, Arbuscular mycorrhizal fungi promote host plants growth under stress conditions by mediating a complex of communication events between the plant and the fungus leading to induced photosynthetic rate and enhances the access of roots to a large soil surface area (Begum et al., 2019),
Similar results were obtained when applying *T. harzianum* strain CCTCC-RW0024 (Saravana kumar et al., 2017) and *T. gamsii* T6085 (Sarrocco et al., 2013; Matarese et al., 2012) as potential biocontrol agents against the pathogens *F. graminearum* and *F. culmorum* through inhibiting their growth and reducing the mycotoxin contamination. Other studies reported that two beneficial strains of *Bacillus* protect Tunisian durum wheat against *F. graminearum* (Zalila-Kolsi et al,, 2016). The biocontrol potential of both *Trichoderma* and Panoramix against FCR might be due to the activation of direct mechanisms such as mycoparasitism, antibiosis, competition, and indirect mechanisms by inducing the systemic plant resistance which involve the production of secondary metabolites (Jaber and Ownley, 2018).
Undeniably, the alleviation of FCR disease by *T. harzianum* and Panoramix was associated to a higher induction of antioxidant metabolites and lower accumulation of the pro-oxidant H_2O_2, compared to the control. The production of reactive oxygen species (ROS) is one of the different mechanisms of defence against a wide range of pathogens (Torres, 2010). It is among the earlier cellular responses induced by plant defence response, and at higher rates can cause oxidative damage of cells (Torres, 2010). In this study, both seed coating treatments triggered the accumulation of H_2O_2 at the beginning of the stress. H_2O_2 accumulation could be considered as a temporary signal, it is suggested that both treatments induced seed priming at the germination stage that is reported to make plants react more rapidly and more efficiently to a subsequent stress (Lutts et al., 2016). Indeed, subsequently both treatments maximized defensive properties in host plants depicted by the higher accumulation of peroxidases and phenolic compounds. Peroxidases are one of the enzymes involved in the anti-oxidative defence response, due to her ability to decompose H_2O_2, and play a key role in lignin synthesis (Mohapatra et al.2017). The production of phenolic compounds may relate to PAL activity and is involved in the non-enzymatic resistance mechanism in plants exposed to biotic stressors (Kofalvi et al. 1995; Vinale et al. 2008 ; Mottalbi et al., 2017; Tchameni et al., 2017). Also, these metabolites may be involved in stopping pathogen development or by accelerating the death of cells close to the infection site thus preventing the growth of the pathogen inside the cells (Madadkhah and al. 2012).
Noticeably, it seems that Panoramix and *T. harzianum* triggered different defense responses that could be attributed to the different composition of the products; *T. harzianum* triggered chitinases at 10dpi while Panoramix triggered catalases at 7 dpi. The Chitinases induced by *T. harzianum* has the role to hydrolyse chitin; one of the major cell wall compounds of most of the fungal pathogens, into mono- and oligomers, thus induction of chitinases in plants plays an important role in defense against invading pathogens (Zaho et al., 2008; Kumar et al., 2018). The catalases induced by Panoramix can eliminate the effects of ROS and catalyses the disproportion of highly damaging O_2^- into comparatively less damaging H_2O_2 (Torres, 2010), and its induction could be attributed to the presence of Bacillus spp. and Mychorriza in Panoramix. Other studeis reported that after application of T. harzianum in wheat infected with Mycosphaerella graminicola, they showed increase in the activity of the catalase. The catalse is responsible for the direct dissociation of H_2O_2 in H_2O and O_2, removing this peroxide generated in the peroxisomes by oxidases involved in the oxidation of fatty acids, photorespiration and purine catabolism (Pittner et al., 2019).

Conclusion

In conclusion, our results could contribute to understanding the quantitative plant resistance against hemi biotrophic pathogen. It is conceivable that *Trichoderma* may be capable to induce plant growth following Fusarium-infection and reduce the severity of the disease. Hence, the coating seeds with *T. harzianum* compared the commercial product Panoramix, may be considered as a potential strategy for alleviation of the oxidative stress induced by the infection and may be capable to minimise the excess of ROS through the activation of antioxidant enzymes.

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