Study of synergistic effect of combined application of tebuconazole with two biocontrol agents for management of *Fusarium* crown rot in durum wheat

Estudio del efecto sinérgico de la aplicación combinada de tebuconazol con dos agentes de biocontrol para el manejo de la pudrición de la corona por *Fusarium* en trigo duro

Estudo do efeito sinérgico da aplicação combinada de tebuconazol com dois agentes de biocontrole no manejo da podridão-da-colheita por *Fusarium* em trigo duro

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Abstract

The *in vitro* and growth chamber, tests were conducted in order to assess the effects of *Bacillus amyloliquefaciens* B18 and *Bacillus subtilis* S8 strains each alone and in combination with tebuconazole against *Fusarium culmorum* (FC) isolate responsible of *Fusarium* crown rot (FCR) in durum wheat. The *in vitro* growth of B18 and S8 strains was unaffected by 30 µg.mL^{-1} tebuconazole. The *Bacillus* strains (at 10^6 CFU.mL^{-1}) and tebuconazole, each alone, reduced the mycelial growth, this effect was significantly improved when they were combined (inhibition of more than 92 %). In growth chamber experiments, efficacy against FCR was significantly higher when integrating *Bacillus* strains and tebuconazole than by either alone; control efficacy of tebuconazole at 30 µg.mL^{-1} in combination with S8 and B18 strains reached 90.91 and 95.45 %, respectively. The obtained results indicated that combination of tebuconazole with the biocontrol agents B18 and S8 synergistically improved control efficiency of the fungicide against FCR of wheat.

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Resumen

Se realizaron experimentos *in vitro* y en cámara de crecimiento para evaluar los efectos de las cepas *Bacillus amyloliquefaciens* B18 y *Bacillus subtilis* S8, cada una sola y en combinación con tebuconazol, contra el aislado de *Fusarium culmorum* (FC) responsable de la pudrición de la corona por *Fusarium* (FCR) en trigo duro. El crecimiento *in vitro* de las cepas B18 y S8 no se vio afectado por 30 µg.mL^{-1} de tebuconazol. Las cepas de *Bacillus* (10^6 UFC.mL^{-1}) y tebuconazol, cada una sola, redujeron el crecimiento micelial, este efecto mejoró significativamente cuando se combinaron (inhibición de más del 92 %). En experimentos en cámaras de crecimiento, la eficacia contra FCR fue significativamente mayor cuando se integraron cepas de *Bacillus* y tebuconazol que cuando se integraron cualquiera de las dos solas; la eficacia de control de tebuconazol a 30 µg.mL^{-1} en combinación con las cepas S8 y B18 alcanzó el 90,91 y el 95,45 %, respectivamente. Los resultados obtenidos indicaron que la combinación de tebuconazol con los agentes de biocontrol B18 y S8 mejoró sinérgicamente la eficiencia de control del fungicida contra FCR de trigo.

Palabras clave: *Bacillus,* control combinado, fungicida, *Fusarium culmorum,* plántulas de trigo.

Resumo

Os experimentos *in vitro* e em câmara de crescimento foram realizados para avaliar os efeitos das cepas de *Bacillus amyloliquefaciens* B18 e *Bacillus Subtilis* S8 isoladamente e em combinação com tebuconazol contra o isolado de *Fusarium culmorum* (FC) responsável pela podridão da coroa de *Fusarium* (FCR) em trigo duro. O crescimento *in vitro* das cepas B18 e S8 não foi afetado por 30 ug.mL^{-1} de tebuconazol. As cepas de *Bacillus* (10^6 CFU.mL^{-1}) e tebuconazol, cada uma isoladamente, reduziram o crescimento micelial, este efeito foi significativamente melhorado quando foram combinados (inibição de mais de 92 %). Em experimentos de câmara de crescimento, a eficácia contra FCR foi significativamente maior ao integrar cepas de *Bacillus* e tebuconazol do que por qualquer um deles sozinho; a eficácia de controle do tebuconazol a 30 µg.mL^{-1} em combinação com as cepas S8 e B18 atingiu 90,91 e 95,45 %, respectivamente. Os resultados obtidos indicaram que a combinação de tebuconazol com os agentes de biocontrole B18 e S8 melhorou sinergicamente a eficiência de controle do fungicida contra FCR de trigo.

Palabras-chave: *Bacillus,* controle combinado, fungicida*, Fusarium culmorum,* mudas de trigo.

Introduction

Triticum turgidum var. *durum* L., also known as durum wheat, is a crucial crop for the economy crop globally after rice and corn (Food and Agriculture Organization [FAO], 2018). In Algeria, about 4 million tons of wheat were harvested in 2019, an increase of 1.2 million tons compared to 2017 (FAO, 2019). Despite this, Algeria remains one of the important importing countries for this important crop, which is due to the weak yield and the increasing consumer needs in parallel with the increasing demographic growth (Bellout *et al.,* 2020; FAO, 2018).

Every year, most regions that produce wheat suffer large losses due to one of the most economically significant diseases of wheat, the FCR. On average, it caused losses in yield, between 24 and 52 % in durum wheat fields per year (Chekali *et al.,* 2013; Hollaway *et al.,* 2013).

For decades, the primary method of preventing the spread of fungal phytopathogens has been the use of synthetic fungicides (Ceiro-Catasú *et al.,* 2022; Palmieri *et al.,* 2022). Despite this, the control efficacy of azole fungicides has been significantly decreased due to the developed resistance in FCR agents resulting from the excessive use of fungicides (De Chaves *et al.,* 2022; Hellin *et al.,* 2018). For this reason, more effective and environmentally friendly methods for controlling FCR agents must be found. Tebuconazole is a potent multifunctional systemic fungicide that rapidly penetrates all plant parts. It works by preventing the sterol C-14 -demethylation of 2,4-methylenedihydrolanosterol, which is the precursor to ergosterol in fungi. This inhibits the formation of the cell membrane, which results in the pathogen's death (Odds *et al.,* 2003; Shishatskaya *et al.,* 2018). It is proved to be very efficient in decreasing deoxynivalenol (DON) amounts when is using in the control of *Fusarium* Head Blight (Sun *et al.,* 2014). Unfortunately, given the harmful impact of synthetic fungicides, such as the impact on ecosystems and human well-being, it is required to seek to develop alternative control methods. Instead of synthetic fungicides, employing biological control agents (BCAs) to control FCR is viewed as a promising option (Khedher *et al.,* 2020; Lee *et al.,* 2017).

Bacillus amyloliquefaciens and *Bacillus subtilis* are widely recognized for their antifungal activity and they can be promising BCAs against several plant diseases (Khedher *et al.,* 2020; Lee *et al.,* 2017; Zhao *et al.,* 2013; Zhu *et al.,* 2020). BCAs can minimize the frequency and amount of fungicide employed, which reduces the danger of residues and resistance development, but their efficiency is often lower than that of chemical fungicides since it is frequently unstable in field circumstances and may be affected by a variety of factors (Ji *et al.,* 2019; Yu *et al.,* 2017). The combination of *Bacillus*-tebuconazole could lead to a practical method for controlling *F. culmorum* in wheat fields. This study aimed to 1- evaluation of the compatibility of tebuconazole and two antagonistic *Bacillus* strains (B18 and S8) and then 2- evaluation of the efficacy of tebuconazole and *Bacillus* strains (B18 and S8) each alone, and in combination, for the control of *F. culmorum* in *in vitro* experiments (effect on mycelium growth) and finally, in growth chamber experiments (effect on induced FCR on durum wheat seedlings).

Material and methods

Plant material

The wheat seed sample namely: Boutaleb (Harvest season 2020-2021), was kept at room temperature pending use.

Fungicides

Tebuconazole under its commercial formulation (Raxil 60FS®: active ingredient content: 60 g.L^{-1}), was purchased from CASAP company specializes in agricultural products, Algiers - Algeria, which it imports from Bayer CropScience. Stock solutions of tebuconazole (7.5, 15, and 30 µg.mL^{-1}) were made with distilled water and kept at a temperature of 4 °C until use.

Antifungal bacteria

The bacterial strains *Bacillus amyloliquefaciens* (B18) and *Bacillus subtilis* (S8) previously obtained from the wheat rhizosphere from Bordj Bou Arreridj and Setif province respectively, Algeria. Through macro and micro-morphological, biochemical, and

molecular assessment, the bacterial strains were identified (Bencheikh *et al.,* 2022). Bacterial strains were stored in 30 % glycerol at −20 °C. Nutrient agar (NA) medium (Liofilchem)® was used as the standard growth medium for bacteria. They were cultured at 35 °C until colonies appeared.

The bacterial suspension was prepared by the inoculation of the bacteria in the Nutrient Broth (NB) medium for 18 hours. After that, the suspension was diluted in physiological water, until the obtention of a final concentration of 10^6 colony forming units (CFU) per mL.

FCR agent, *Fusarium culmorum*

Using morphological and molecular characterization, the previously obtained *F. culmorum* (FC) isolate, which was acquired from durum wheat seeds, was identified (Bencheikh *et al.,* 2022). The *F. culmorum* isolate was maintained at 25 °C on Potato Sucrose Agar (PSA). According to Bouanaka *et al*. (2021) procedure, the conidial suspension was established, with some modifications, according to the following steps:

1-sterilized distilled water (10 mL) was added to the aerial mycelium of a 15-day-old fungal colony. 2- The colony was carefully scraped in order to retrieve all spores present within the aerial mycelium. 3- The concentration of spores was estimated using the Malassez counting cell (hemocytometer). 4- The appropriate dilutions were made to obtain a final concentration of 10^5 conidia.mL^{-1}.

Tebuconazole and *Bacillus* strain compatibility

After the bacterial suspension was prepared, a tenfold dilution was made in physiological water, then 100 µl from the 10^{-3}, 10^{-4}, and 10^{-5} dilutions were strewn throughout Petri plates containing NA medium (used as control) or NA medium added by 7.5, 15, and 30 µg.mL^{-1} of tebuconazole fungicide. The highest concentration used was taken from the concentrations suggested by the manufacturer, while the other concentrations represent half and a quarter of this concentration. Three plates were used for each test, the plates were incubated at 35 °C for 24 hours and then the colonies were enumerated.

***In vitro* tebuconazole tests for preventing the mycelial development of *F. culmorum* in conjunction with *Bacillus* strains**

Plugs of 6 mm-diameter from *F. culmorum* 7-days-old colony were put on the center of fresh PDA plates containing tebuconazole alone (mixed with the medium), *Bacillus* strain alone (streaked parallelly at a distance of 2.5 cm from either side of the *F. culmorum* plug), or both tebuconazole and *Bacillus* strain. Doses of tebuconazole were 7.5, 15, and 30 µg.mL^{-1}, and the concentration of *Bacillus* strains was 10^6 CFU.mL^{-1}.

PDA plates without tebuconazole and *Bacillus* strains were used as controls. Each treatment received three dishes, which were incubated at 25 °C for 5-7 days (until the mycelium in the control dishes reaches the edge of the dish). The *F. culmorum* colonies' diameters were measured. Using the Vincent (1947) formula, which is given below, the percentages of mycelium growth inhibition by tebuconazole and/or *Bacillus* strains in comparison with the control were estimated.

$$I = \frac{(C - T) \times 100}{C}$$

Where: I= percent inhibition of mycelium growth; C= diameter colony of the control,

T= diameter colony in the presence of tebuconazole and/or *Bacillus* strain.

Growth chamber experiments

Pot experiments were performed in a controlled growth chamber (22 °C ± 3, 12 h/12 h photoperiod of light and dark and 90 % relative humidity, consecutively), in circular 8-cm-diameter pots containing sterilized potting soil (FLORAVA®). In tubes containing NB, at 28 °C for 48 hours, *Bacillus* strain inoculum was prepared. To acquire a final concentration of 10^8 CFU.mL^{-1}, the resulting suspension was diluted with sterile physiological water. Tebuconazole was applied with or without *Bacillus* strains at 7.5, 15, and 30 µg.mL^{-1}.

Three seeds (cv. Boutaleb), surface sterilized (Bencheikh *et al*., 2022) and submerged in the suspension of bacterial strain for 3h, were displayed in each pot. Each seed received one 6 mm diameter plug from a 7-day-old *F. culmorum* culture. The seeds and inocula were carefully buried in sterile soil. The seeds without bacteria and *Fusarium* plugs served as the negative control, whereas the direct contact of a 6 mm-diameter *Fusarium* plug with each seed without bacteria served as the positive control.

Concerning the chemical control, the tebuconazole solution was applied to the surface-sterilized wheat seeds before soaking (with the concentrations cited above) for 15 min. Each seed was in contact with one 6-mm-diameter plug from a culture of *F. culmorum* that was seven days old. On the other hand, seeds soaked in tebuconazole but without *Fusarium* plugs were used as control.

Relating to the combined control, surface-sterilized wheat seeds were submerged in the bacterial suspension supplemented with tebuconazole solution (the same concentrations as the chemical control test) for 3 h. One 6-mm diameter plug from the *Fusarium* 7-days-old culture was put in contact with each surface-sterilized seed.

Three duplicate pots were employed in a factorial totally randomized design, and the plants received two weekly waterings. The seedlings were carefully removed after 21 days, and measurements of the plant height and root weight were taken. For the evaluation of the FCR, a disease scale from 0 to 3 was used (Grey and Mathre, 1984), where:

0 = no discoloration in crown, 1 = 1–25 % of browning in crown, 2 = 25–50 % of browning in crown, 3= more than 50 % of browning in crown. In accordance with the method, the McKinney (1923) index was used to estimate the disease severity (DS):

$$DS = (\sum(a \times b)/n \times N) \times 100$$

Where: a= disease class, b= frequency, n =number of observations, and N = greatest value of the empirical scale adopted (class 3).

The control efficacy of the FCR was calculated using the formula proposed by Ji *et al.* (2019): Disease control efficacy (%) = ((C − T)/C) × 100, where C= disease severity of control; T= disease severity of treatment.

Statistical analysis

The IBM SPSS Statistics V25 program was used to conduct the statistical analysis. With the exception of the growth chamber experiment, all tests employed the one-way ANOVA instead of the two-way ANOVA. The means were compared using the Duncan post hoc test at the 0.05 threshold of risk.

Results and discussion

Compatibility of tebuconazole with *Bacillus* strains

After 24 hours of incubation, the growth of the two tested *Bacillus* strains was not affected by tebuconazole using the NA medium containing 7.5, 15, and 30 µg.mL^{-1} of tebuconazole, and the number of CFU.mL^{-1} was not statistically different compared with the number of CFU.mL^{-1} of the untreated control (table 1).

Table 1. Tebuconazole's impact on *Bacillus* strain growth in NA medium.

Concentration of tebuconazole (µg.mL^{-1})		Number of CFU^{y}.mL^{-1} (x10^{5})	
		S8	B18
Control	0	47.97±2.11a*	50.4±2.3a
F1/4	7.5	50.3±3.4a	48.3±1.4a
F1/2	15	48.47±1.05a	49.7±2.6a
F1	30	49.2±1.6a	51.33±1.7a

[y] CFU: colony-forming unit; * Means in same column followed by the same letter are not significantly different at $p < .05$ as determined by Duncan test.

The attempt to combine antagonistic bacteria with synthetic fungicides depends on their compatibility, which is usually difficult to achieve. In this study, the growth of the two tested *Bacillus* strains (S8 and B18) on the NA medium was not affected by tebuconazole at a concentration of 30 µg.mL^{-1} (table 1). This result indicates the full compatibility of *Bacillus* strains with tebuconazole.

***In vitro* tests of tebuconazole in combination with *Bacillus* strains for preventing *F. culmorum's* mycelial expansion**

Tebuconazole and the two tested *Bacillus* strains substantially ($p>$ .05) reduced the radial mycelial development of *F. culmorum* on PDA dishes and when treated separately (table 2). Combining tebuconazole with *Bacillus* strains significantly increased the rate of inhibition. As an example, the inhibition rate was 65.1 % with B18 strain alone, and 87.84 % with tebuconazole alone at 30 µg.mL^{-1} (F1), but it increased to 93.73 % when the two treatments were combined (table 2). Using Colby's equation (Colby, 1967), each of the anticipated inhibition rates were greater than those obtained when combined with the *Bacillus* strains with the tebuconazole (table 2). These results confirm that the combination of the tested *Bacillus* strains and the tebuconazole gives a synergetic effect.

Table 2. Tebuconazole and other *Bacillus* strains individually and in combination influenced *Fusarium culmorum* growth on PDA plates [x].

Traitement	Diameter (mm)[y]	Inhibition observed (%)	Inhibition expected (%)	Difference
FC	8.5f	-	-	-
S8	3.03e	64.31a	-	-
B18	2.94e	65.10a	-	-
F1	1.03b	87.84d	-	-
F1/2	1.33c	84.31c	-	-
F1/4	1.67d	80.39b	-	-
S8_F1	0.53a	93.73[e]	95.65	+1.93
S8_F1/2	0.57a	93.33[e]	94.41	+1.07
S8_F1/4	0.6a	92.94[e]	93.00	+0.06
B18_F1	0.53a	93.73[e]	97.81	+4.08
B18_F1/2	0.57a	93.33[e]	97.67	+4.34
B18_F1/4	0.6a	92.94[e]	97.54	+4.60

[x] The means in the same column that are separated by the same letter are not considerably different $p < .05$ (Duncan test); [y] Each figure represents the average of three separate trials; [z] Differences, shown by a plus sign, indicate synergistic effects (the percentage decrease seen minus the percentage reduction anticipated).

Growth chamber experiment

In the growth chamber experiments, tebuconazole at 7.5, 15, and 30 µg.mL^{-1} in combination with the *Bacillus* strains (B18 or S8) significantly reduced FCR severity and displayed a noticeable control effect with rates of control efficacies of 59.09, 77.27, and 90.91 % respectively with S8 strain, and of 72.73, 81.82, and 95.45 % respectively with B18 strain (table 3). On the other hand, the results showed that each of the tebuconazole (with all the tested concentrations) and the *Bacillus* strains, can reduce the FCR severity, but with greater effectiveness of the fungicide compared to the *Bacillus* strains, where control efficacies rates were between 27.27 and 81.82 % for the tebuconazole, and between 9.09 and 13.64 % for S8 and B18 strains respectively (table 3).

Through the previous results, it was found that the process of combining tebuconazole with one of the two *Bacillus* strains gave the desired results, which is to provide the best protection against the FCR damages. In addition, the combination (*Bacillus*-tebuconazole) increased significantly both the weight of the fresh roots and plant height in comparison to each treatment separately. On the contrary, no significant differences have been observed between the control efficacy rates of the S8 and the B18 strain each alone or in combination with tebuconazole 15 and 30 µg.mL^{-1}.

Table 3. Effect of tebuconazole and *Bacillus* strains against *Fusarium* crown rot (FCR) in the growth chamber, alone and in combination.

Traitement	Seedlings hight (cm)	Root fresh weight (g)	Disease severity (%)	Control efficacy (%)
Control[y]	11.82±0.44a[z]	0.081±0.002a	81.48g	-
F1/4	16.17±1.21bc	0.095±0.005bc	59.26f	27.27ab
F1/2	17.04±1.42c	0.108±0.01c	44.44e	45.45bc
F1	20.12±0.34d	0.155±0.01ef	14.81abc	81.82de
S8	14.50±1.78b	0.107±0.009c	74.07g	9.09a
S8 + F1/4	16.17±1.21bc	0.095±0.008bc	33.33de	59.09cd
S8 + F1/2	19.37±1.12d	0.145±0.011de	18.52bc	77.27de
S8 + F1	23.61±1.11e	0.197±0.025g	7.41ab	90.91e
B18	12.02±1.6a	0.090±0.012ab	70.37fg	13.64a
B18 + F1/4	17.04±1.42c	0.108±0.09c	22.22cd	72.73de
B18 + F1/2	20.12±0.34d	0.133±0.01d	14.81bc	81.82de
B18 + F1	21.49±1.01d	0.162±0.02f	3.70a	95.45e

[y] Untreated durum wheat seeds with FC1 plug; [z] The values represent the 9 replicates' means and standard errors. At $p < .05$ (Duncan test), values in the same columns that are followed by the same letter are not statistically different.

With the very intensive use of the fungicides, the resistance of *Fusarium* isolates against these latter was highly increased (Yin *et al.,* 2009; Zhang *et al.,* 2013). Therefore, it has become necessary to look for more effective ways to control these pathogens in cereal production. Tebuconazole has been used for controlling FHB in several countries (Sun *et al.,* 2014) and proved its very effectiveness in decreasing the FHB methylenedihydrolanosterol and production of deoxynivalenol (DON). Akgül and Erkiliç (2016), found that wheat seed covered by tebuconazole decreased disease severity (DS) in wheat seedlings' crown compared to the non-treated seeds and they concluded that it was the most effective fungicide with an efficacy rate

of 47.8 %. These findings are in accordance with the obtained *in vitro* and growth chamber results proving the effectiveness of tebuconazole in the limitation of the FCR, where both the *in vitro* growth inhibition rate and control efficacy were more than 80 %. Tebuconazole's mechanism of action was examined, and the findings revealed that it inhibits one of the ergosterol precursors in fungus (DMI's fungicides), the pathogen dies as a result of this activity because it prohibits the establishment of the cell membrane (Odds *et al.,* 2003).

The obtained results proved that the two *Bacillus* strains were effective in reducing the radial growth of the *F. culmorum*, where the inhibition was more than 60 %. Via a number of processes, the species of the genus *Bacillus* can decrease the growth of phytopathogenic fungi and even eradicate them. The most significant of these mechanisms is the release of antifungal compounds like antibiotics, cyanides, and gas products like ammonia (Fira *et al.,* 2018; Lugtenberg *et al.,* 2009; Zhao *et al.,* 2013), and by generating hydrolytic enzymes including cellulase, glucanase, chitinase, and protease that demolish the cell wall (Brzezinska *et al.,* 2020; Yanti *et al.,* 2021), or by enhancing plant development (Huang *et al.,* 2020; Kalam *et al.,* 2020). *B. amyloliquefaciens* and *B. subtilis* have been considered to be promising biocontrol agents with diverse capabilities. Wang *et al.* (2016) reported that the *B. amyloliquefaciens* W19 strain can produce bioorganic fertilizers "BIO6", which could effectively suppress FCR disease in bananas and promote plant growth. On the other hand, *B. subtilis* was able to produce three natural substances called lipopeptides, namely: fengycin, surfactin, and mycosubtilin. These latter have shown an interesting antifungal activity each alone or in combination compared to tebuconazole in the *in vitro* control of two strains of *Venturia inaequalis*, the responsible agent of apple scab (Desmyttere *et al.,* 2019). Previous research revealed that the *B. subtilis* S8 strain and *B. amyloliquefaciens* B18 were potential agents in biocontrol of *F. culmorum* isolates (FC1 and FC2), by producing several hydrolytic enzymes (amylase, pectinase, cellulase, protease, and chitinase) and by producing antifungal metabolites like siderophore and ammonia (Bencheikh *et al*., 2022). Based on all the aforementioned advantages of the antagonistic bacteria and their anti-fungal metabolites, they can be relied upon as ideal alternatives to chemical fungicides (Desmyttere *et al.,* 2019; Ji *et al.,* 2019). While being safe for the environment and effective against FCR, *B. subtilis* and *B. amyloliquefaciens* strains effectiveness is often unstable in the field and may be affected by a variety of factors (Ji *et al.,* 2019; Yu *et al.,* 2017). This finding is confirmed by the obtained results in the growth chamber experiments where low control efficacy rates were obtained when each *Bacillus* strain was used alone. Moreover, the combination of *Bacillus*-tebuconazole revealed a synergistic effect in inhibition of mycelium growth (table 2). Rotolo *et al.* (2018), declared that the integration of synthetic fungicides and biocontrol agents might be a successful plan of action more than the use of each alone. The obtained results demonstrated also that control of *Fusarium* crown rot was significantly improved by combining tebuconazole with *Bacillus* strains S8 or B18.

Conclusion

Through this study, it was proven that *Bacillus* strains (S8 and B18) were completely compatible with tebuconazole and that they can be combined without affecting each other's growth. Furthermore, a synergistic effect was obtained in the laboratory and growth chamber experiments.

The control efficacy of the combination B18-tebuconazole, at half of the concentration suggested by the manufacturer, was too close to that of the combination B18-tebuconazole, at the concentration suggested by the manufacturer, with no significant differences with the combination B18-tebuconazole, at the quarter of the concentration suggested by the manufacturer, or with the combination S8-tebuconazole at half of the concentration suggested by the manufacturer. These findings showed that the amount of fungicide suggested by the manufacturer can be reduced by half if combined with the S8 strain or even by a quarter if combined with the B18 strain.

To determine the optimal application method of these biocontrol agents (B18 and S8 strain) to control FCR in durum wheat production, more research must be carried out on the environmental destiny and behavior of *Bacillus* strains in the field.

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