Effective and durable systemic wheat-induced resistance by a plant-growth-promoting rhizobacteria consortium of Paenibacillus sp. strain B2 and Arthrobacter spp. strain AA against Zymoseptoria tritici and drought stress

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Abstract

For a sustainable environment, plant-growth-promoting rhizobacteria (PGPR) are natural resources considering as one of the most important fungicides' alternatives inducing resistance to plant diseases.

The aim of the present work is to investigate the synergistic promotion effect of a PGPR mixture composed of *Paenibacillus* sp. strain B2 (PB2) and *Arthrobacter* spp. strain AA (AA), referred hereafter as Mix-2, on the wheat growth, resistance to *Zymoseptoria tritici*, the causal agent of Septoria tritici blotch (STB) and drought stress.

The results of the quantitative real-time polymerase chain reaction (qPCR) showed a helpful effect of AA for wheat-root external and internal colonization with PB2. Interestingly, in non-stress conditions, the inoculation of wheat grains with Mix-2 (PB2:AA, 1:1), at sowing, showed an increase in almost all tested cultivars for foliar and root dry biomasses. Under drought stress, contrarily to PB2 in single inoculation, Mix-2 induced a significant tolerance in all tested cultivars for plant dry biomass and root length. On the other hand, both PB2 alone and Mix-2 induced resistance against *Z. tritici* with at least 50% of protection efficiency in all tested cultivars. However, Mix-2-root colonization and -induced resistance were observed at the most mature wheat growth stage. Moreover, Mix-2-induced resistance is characterized by the upregulation of gene markers of the basal defense, defense and cell rescue, reactive oxygen species, jasmonic acid, and phenylpropanoids & phytoalexins pathways. *Pathogenesis-related protein 1* (*PR1*), *chitinase*, *glucanase,* and *flavonoides* are possible gene markers for wheat resistance selection to STB.

To conclude, the endophyte PGPR' consortium of AA and PB2 is a wheat growth promoter and inducer of a durable-systemic resistance to *Z. tritici* and genotype-independent tolerance to drought stress.

Keywords: *Zymoseptoria tritici*, plant-growth-promoting-rhizobacteria (PGPR), *Paenibacillus* sp. strain B2, *Arthrobacter* spp. strain AA, induced systemic resistance, drought stress.

1. Introduction

Plant growth-promoting rhizobacteria (PGPR) is now well recommended as bio-fertilizer and biocontrol products. Also, they are considered as alternatives to pesticides that are discredited by environmental and health concerns. However, almost of PGPR tested in a single inoculation have demonstrated unstable efficiency, depend on pathogen strain and environmental conditions. Indeed, the use of a single strain of PGPR might not be efficient because of soil and environment diversities, agricultural practices, biotic and abiotic stresses [1], host growth stages, and genetic variation among pathogen strains and plant cultivars [2], resulting in low commercialization potential [3]. To avoid these obstacles, one of expected strategy reposes on the combination of several strains that may develop superior biocontrol activities [4], particularly by mixing PGPR with different target pathogens, with different antagonisms, and under different growth conditions [1]. Indeed, the combination of microorganisms can associate different mode of actions and mechanisms able to promote plant growth and reduce diseases (competition, antibiosis, ISR, helpful impact in root colonization) and also may work in addition or synergies [5]. The application of compatible microbial consortium has demonstrated more efficient, stable, reliable activities under variable conditions than individual inoculation [6] and enhanced plant growth and disease suppression [5]. Nandakumar et al. (2001) [7] have demonstrated the additive efficiency of a PGPR-mixture of *Pseudomonas fluorescens* strains against sheath blight in rice. The co-inoculation of *P. fluorescens* Aur 6 and *Chryseobacterium balustinum* Aur 9 has also increased protection efficiency of 50% against blast disease of rice compared to single inoculations under field conditions [8].

Usually, the selection of PGPR mixtures is based on their individual potential on the target plant pathosystems or biological model without unfortunately, taking in consideration the compatibility of those PGPR consortia. Indeed, by competition, PGPR may inhibit each other

as reported by Xu et al. (2010) [9], that disease reduction of *Botrytis cinerea* on strawberry is higher in PGPR single- than in co-inoculation.

Despite the more expensive cost for an industrial product based on a mixture of PGPR than a single strain, the first is more interesting because of its large and stable activity against biotic stress, under natural environment and also in drought conditions, which is an important preoccupation caused by climatic change [10].

Recently, we showed a high efficiency of *Paenibacillus* sp. strain B2 (PB2), a PGPR isolated from sorghum [11], which produces antimicrobial compounds [12] and triggers local and systemic induced resistance (IR) in alfalfa and wheat [2,13,14]. The PB2-IR against Septoria tritici blotch (STB), caused by *Zymoseptoria tritici* is depending on pathogen strain, wheat genotype and growth stage [14]. In single inoculation, PB2 has no effect on plant growth but, in co-inoculation with *Curtobacterium plantarum*, an increase of wheat growth, in one of two tested cultivars, has been observed [2].

The aim of the present investigation was to evaluate (1) the compatibility of a PGPR mixture composed of PB2 and *Arthrobacter* spp. strain AA (AA) referred in this study as Mix-2, (2) Mix-2-growth promotion effect, (3) Mix-2-resistance induced in wheat against biotic (STB) and abiotic (drought) stresses, (4) Mix-2 efficiency, stability and durability over different wheat genotypes, growth stages, and *Z. tritici* strains, and (5) wheat defense reactions as a response to root inoculation with Mix-2.

2. Materials and methods

2.1. Microorganisms and inoculum preparation

Four *Z. tritici* strains were used in this study, i.e. strain IPO323 (provided by Dr F. Suffert, INRAE Versailles-Grignon, France), strain 1193, characterized as a moderate resistant strain to

DMI fungicides with three SNP mutations (M-281-V, A-379-G, I381-V) (Selim, 2017, NCBI GeneBank database accession number KX356102), strains TO256 and ST38 from the authors' laboratory. *Paenibacillus* sp. strain B2 [11] kindly provided by Dr D. van Tuinen, INRAE Dijon, France, and *Arthrobacter* spp. strain AA (AA) by C. Ernenwine, Society SDP, Pinon, France. PGPR inocula were prepared as described in Selim et al. (2005) [12]. Briefly, to carry out the final bacterial inocula, bacterial cells were collected from Luria-Bertani (LB) liquid cultures at optical density $OD_{0.5}$, centrifuged at 2655×g for 10 minutes and at 4°C, washed twice and then suspended in a sterile solution of 10 mM $MgSO_4$ for keeping the bacterial vitality. *Z. tritici* inocula were prepared as described by Selim et al. (2014) [15]. Briefly, sporidia stored at -80°C were transferred to potato dextrose agar medium. After 7 days of incubation at 18°C with a 12-hour photoperiod, mycelia and spores were scraped off the surface and transferred into a liquid yeast-sucrose medium for 5 days at 18°C with permanent light (100 µmol of photon m^{-2} s^{-1}) and shaking (150 rpm). Spores were collected by centrifugation at 2655 g for 10 min at 15°C, washed twice with sterile distilled water and then suspended in 10 mM $MgSO_4$, containing 0.05% Tween 20 as a surfactant. The final concentration was adjusted to 10^6 spores.mL^{-1}. Bacterial cells and fungal spores vitality was checked by spreading 100 mL of inoculum on LB or potato dextrose agar media, respectively.

2.2. Plant material and growth conditions

Fifteen wheat cultivars (Alixan, Trapez, Terroir, Altigo, Rubisco, Expert, Chevron, Hyking, Boregar, Complice, Creek, Cellule, Fructidor, Chevignon and Hyfi with different level of resistance against STB, 4, 4, 5, 5.5, 5.5, 5.5, 5.5, 6, 6, 6, 6, 6.5, 6.5, 7 and 7, respectively (Arvalis Institut du Végétal, 2017), on a scale from 1 (fully susceptible) to 9 (fully resistant)) were used in this study (Table 1). Grains were disinfected according to Samain et al. (2017) [2], with a few modifications, as follows: incubation in a solution of oxytetracycline, streptomycin, penicillin, and ampicillin antibiotics (100 $mg.L^{-1}$ each) overnight to obtain broad-spectrum

activity against bacterial strains, then suspended in 10% calcium hypochlorite solution for 10 minutes and washed three times in autoclaved Milli-Q water after each disinfection step. The sterilized grains were pre-germinated on 0.5% water-agar medium and incubated in darkness at 4°C for 24h, 20°C for 48h, and 4°C for 24h. Germinated seeds were transferred into an inoculum of PB2 or a mixture of PB2 and AA with an equal quantity of each (1:1). Inocula for single- or co-inoculation were adjusted to a final concentration of 10^6 CFU (colony forming units).mL^{-1} of 10 mM $MgSO_4$. One millilitre per grain was used for one hour with light shaking. For the non-inoculated control, grains were immersed in 10 mM $MgSO_4$. After inoculation, grains were transferred into 250-mL pots containing a sterilized soil mixture of silt-loam soil and sand (1:1, v/v). Pots were incubated in a phytotron at 18°C (+/- 2°C), 40% humidity, for a 16-hour photoperiod with 185 µmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes (Philips Master Cool White 80 W//865, Lamotte Beuvron, France). Plants were watered three times a week with 50 mL distilled water per pot.

2.3. Root colonization

The external and internal root colonization by PB2 and AA, composing Mix-2, were quantified at 21 days after sowing (das) (three-leaf growth stage (3-L GS)) on the 4 cultivars Alixan, Altigo, Cellule and Hyfi. External and internal colonization by AA and PB2 were also determined at flag leaf growth stage (FL GS) on Alixan and Cellule, by real time quantitative qPCR using 16S rDNA specific primers (Table 2) for PB2 and AA strains as described in Samain et al. (2019) [14]. Briefly, DNA was extracted from plant roots using a DNeasy 96 Plant kit (Qiagen, USA), according to the manufacturer's protocol. DNA quantity and quality were confirmed on a Nanodrop apparatus (Thermo Fisher Scientific, Waltham, MA, USA). SYBR Green qPCR assays were carried out in a reaction mixture of 25 µL that contained the following: 12.5 µL Universal Quantifast SYBR Green PCR master mix (Qiagen, USA), 0.3 µM of each primer, 50 ng of DNA, and water up to a volume of 25 µL. The conditions of quantitative PCR

were as follows: 5 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. All quantitative PCR was carried out using StepOnePlus (Thermo Fisher Scientific®).

The standard curve, obtained by plotting known amounts of each bacterial DNA against Ct values, was used to determine the PCR's amplification efficiency (Table 2 and Supplementary Figs. 1-3). The resulting regression equations were used to calculate the amounts of PB2 and AA DNA in tested samples.

2.4. Mix-2-growth promotion effect on wheat

The impact of PB2 and Mix-2 on wheat biomass was evaluated 6 weeks after the inoculation with PGPR at sowing by measuring root and foliar dry biomass, after 48h of incubation at 60°C, of the 15 cultivars mentioned above.

2.5. Mix-2-resistance induced in wheat against drought stress

The induction resistance against drought stress in wheat by PB2 and Mix-2 was studied in a phytotron, using Alixan, Altigo, Cellule and Hyfi wheat cultivars, as a response to the inoculation of the pre-germinated grains with PB2 alone or Mix-2 at sowing as mentioned above. Plants were watered with 50 mL/pot twice a week, for 2 weeks, followed by 19 days without watering for the drought stress modalities. To study the plant growth recovery capacity, the drought stressed period was followed with 2 weeks of normal watering with 50 mL/pot twice a week. Foliar and root dry biomass, as well as root length, were measured at the end of the recovery period.

2.6. Mix-2-resistance induced in wheat against Z. tritici

To select the good rational proportion of the strain AA in the Mix-2 with PB2, the four wheat cultivars Alixan, Altigo, Cellule, and Hyfi were inoculated with PB2 in single inoculation (100% PB2) or in co-inoculation with strain AA, as mentioned above, in different ratio (PB2:AA; 100:0%, 75:25%, 50:50%, and 25:75%). Plants were infected at 3-L GS by spraying

a 2 mL of *Z. tritici* strain IPO323 inoculum ($2x10^6$ spores/mL) over the whole plant. Controls were sprayed with a solution of 10 mM $MgSO_4$ containing 0.1% Tween 20 as a surfactant. Seventeen days after infection with *Z. tritici*, leaves were collected and lyophilized to evaluate the protection efficiency as a response to PB2 alone or with strain AA (Mix-2) using qPCR. The DNA extraction and quantification of *Z. tritici* using qPCR was performed as described by Selim et al. (2014) [15]. Briefly, the DNA was extracted from plant leaves using a DNeasy 96 Plant kit (Qiagen, USA), according to the manufacturer's protocol. DNA quantity and quality were confirmed by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). To quantify infection levels of *Z. tritici*, primers and a TaqMan minor groove binder probe (For: GCCTTCCTACCCCACCATGT; Rev: CCTGAATCGCGCATCGTTA; Probe: FAM-TTACGCCAAGACATTC-MGB) were used to target a 63-bp fragment of the *Z. tritici* β-tubulin specific gene (GeneBank accession no. AY547264) [16]. A TaqMan assay was carried out in a 25 µL reaction mixture that contained 12.5 µL of Universal TaqMan PCR Master Mix (Life Technologies SAS, Villebon sur Yvette, France), 0.3 µM of each primer, 0.2 µM of probe, 200 ng of DNA, and water to a volume of 25 µL. The conditions for qPCR determination were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All qPCR experiments were carried out using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific®). qPCR analysis of the *Z. tritici* β-tubulin gene was calibrated from 10^2 to 10^7 copies by serial dilution of the appropriate cloned target sequence, as previously described by Selim et al. (2014) [15].

2.6.3. Stability and durability of Mix-2-resistance induced over wheat genotypes, growth stages and pathogen strains

2.6.3.1. *Mix-2-Z. tritici-wheat genotypes interaction*

Grain sterilization, pre-germination, inoculation with PB2 and Mix-2, and phytotron conditions were carried out, as mentioned above. The 15 wheat cultivars listed above were used and infected at 3-L GS with *Z. tritici* strain IPO323 as mentioned above.

2.6.3.2. *Mix-2-Z. tritici-wheat-growth stages interaction*

Susceptible and resistant non-hybrid wheat cultivars Alixan and Cellule respectively, were used to evaluate the effect of pathogen strains and growth stage on the efficiency of the resistance induced by Mix-2. They were inoculated with one of the four *Z. tritici* strains, IPO323, 1193, TO249, and ST38, at 3-L, tillering (Ti) and FL GS. The inocula of each strain were prepared and applied as mentioned above. Five repetitions were carried out for each condition. Three control modalities were used as non-infected with *Z. tritici* and non-inoculated with Mix-2 (C-), inoculated with Mix-2 without pathogen infection (Mix-2), and non-inoculated with Mix-2 infected with pathogen (MG). Modalities inoculated with Mix-2 and infected with *Z. tritici* (Mix-2/MG) were compared to control modalities. Seventeen days after infection with *Z. tritici*, leaves were collected and lyophilized to evaluate the protection efficiency as a response to Mix-2 using qPCR as mentioned above.

2.7. RNA extraction and relative gene expression quantification by real-time PCR

At the 3-L GS, aerial parts of Alixan and Cellule plants were collected at the time of infection (T0) with *Z. tritici*, at 6, 12, 24, and 48 hours after infection (hai), and at 3, 5, 9, and 11 days after infection (dai) to study the evolution of defense gene expression. Samples were stored directly in liquid nitrogen. RNA extraction and cDNA synthesis were carried out using a RNeasy® Mini Kit and a QuantiTect® Reverse Transcription Kit (Qiagen, USA), respectively, following the manufacturer's protocol. The gene expression of 20 wheat-defense genes was studied using specific primers (Table 2). qPCR conditions were as described by Samain et al. (2017) [2]. Briefly, the Quantifast® SYBR® Green PCR Kit (Qiagen, USA) and the

StepOnePlus Real-Time PCR Systems (Thermo Fisher Scientific®) were used. Amplification conditions consisted of a denaturation cycle (95°C for 5 minutes) and amplification and quantification cycles (95°C for 10 seconds, 60°C for 30 seconds) repeated 40 times. One final step from 60 to 95°C with an increase of 0.2°C s^{-1} was added to obtain a specific denaturation curve for each studied gene (Table 2). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-tubulin (β-TUB) housekeeping genes were used to normalize results and to determine the expression ratio for each cDNA, as described by Ors et al. (2018) [17]. Briefly, expression ratios for each cDNA were calculated for each time point, relative to control at the same time using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001) [18], where ΔΔCt = [Ct Target (Sample) – Ct Reference (Sample)] - [Ct Target (Control) – Ct Reference (Control)] and Ct Reference = geometric mean (Ct GAPDH : Ct β-TUB). Similar amplification efficiencies ranging between 90 and 110% were checked for all the tested primers (Table 2) and expression ratio values of 2 were considered as a minimum to be significantly different from the control.

2.8. Statistical analysis

All experiments were repeated three times and each experiment contained at least five replicates. For all experiments, significant differences were evaluated using the Tukey test at $p \leq 0.05$ with the XLSTAT® statistics program (version 2014, Addinsoft, Paris, France).

3. Results

3.1. Impact of wheat genotypes on root colonization by Mix-2

Wheat root colonization by PB2 and AA were evaluated using qPCR at 21 das on the four cultivars, Alixan, Altigo, Cellule, and Hyfi. The results in Figure 1 show the presence of PB2 and AA in internal and external root of all tested cultivars inoculated with Mix-2. External and

internal root colonization with AA did not show any significant differences between cultivars (Fig. 1), only, PB2 showed a significantly higher external root colonization in Cellule cultivar (614 pg.g^{-1} of root) compared to Altigo and Hyfi cultivars and strain AA (Fig. 1). Contrarily, no significant differences were observed for root internal colonization between PB2 and AA in all cultivars (Fig. 1). Interestingly, the strain AA showed a significant helpful effect explained by an increase for the external root colonization with PB2 in Alixan, Altigo and Cellule with 7.4, 2.5, and 30.4 times, respectively, and for the internal root colonization in Alixan, Cellule, and Hyfi, with 3.2, 3.1, and 2.2 times, respectively, compared to PB2 colonization levels when a single inoculum of PB2 was used (Table 3).

3.2. Effect of wheat growth stage on the root colonization by Mix-2

The impact of wheat growth stage on the root colonization by PB2 and AA, as a response to pre-germinated grains with Mix-2, was investigated at 3-L GS and FL GS growth stages using the most susceptible and resistant cultivars, Alixan and Cellule, respectively.

For the external root colonization with PB2, at 3-L GS, the results showed high root colonization in Alixan and Cellule, with respectively, 256 and 614 pg of PB2 DNA.g^{-1} of root, compared to 32 and 17 pg of AA DNA.g^{-1} of root, without significant differences (Fig. 2). The results at FL GS (Fig. 2) show the presence of the PB2 and AA externally and internally of wheat roots of the two tested cultivars. However, a significant increase of external root colonization with strain AA was observed in Alixan and Cellule reached 721 and 1.2×10^4 pg of DNA.g^{-1} of root, respectively, whereas, the external root colonization with PB2 decreased to 40 and 85 pg of DNA.g-1 of root, respectively (Fig. 2). Internal colonization for AA showed the same evolution, with a significant increase of 15.3 and 74-fold compared to the 3-L GS, for Alixan and Cellule respectively. Likewise, an increase of PB2 internal colonization in Alixan and Cellule with 2- and 8-fold, respectively, compared to the 3-L GS, was observed (Fig. 2). This increase was only significant in Cellule cultivar.

3.3. Effect of Mix-2 on plant growth promotion

The plant growth promotion effect of Mix-2 was evaluated 6 weeks after sowing by measuring dry foliar (DFB) and root biomasses (DRB) in fifteen wheat cultivars (Fig. 3a&b). The results in Figure 3a show an increase in DFB of more than 5% in twelve of the fifteen tested cultivars, with a significant increase in 5 cultivars, compared to the non-inoculated control. For DRB, 9 cultivars showed more than 5% increase and 2 cultivars, Fructidor and Rubisko, had a significant decrease, compared to the non-inoculated control (Fig. 3b).

3.4. Resistance induction by Mix-2 in wheat against drought stress

Tolerance to drought stress induced by PB2 and Mix-2 was evaluated using the four cultivars, Alixan, Altigo, Cellule, and Hyfi, after 19 days of a drought stress without watering followed by 2 weeks of a recovery period with normal watering, compared to the non-inoculated non-stressed controls (Fig. 4a-c). The results showed significant decrease in DFB and DRB in all tested cultivars as response to drought stress and compared to the non-inoculated-non-drought-stress control (Fig. 4a&b). For root length, the results in Fig. 4c show that plants tended to have shorter roots under drought stress conditions.

The DFB results demonstrate no significant differences with the non-inoculated-non-drought-stress control and a significant protective effect by PB2 on 3 of 4 tested cultivars, i.e. Alixan, Altigo and Cellule, with 56%, 65% and 42%, respectively, compared to non-inoculated-drought-stressed control (Fig. 4a). A considerable, non-wheat-genotype dependent and significant protective effect was observed in response to Mix-2 on the four tested cultivars with 57%, 99%, 43%, and 23% in Alixan, Altigo, Cellule, and Hyfi, respectively (Fig. 4a). Furthermore, the results of DRB ((Fig. 4b) showed an increase of 119%, 139%, 41%, and 53% with Mix-2 modalities and of 98%, 88%, 63%, and 0% with PB2 modalities, compared to the non-inoculated-drought-stressed control, in Alixan, Altigo, Cellule and Hyfi, respectively. This

increase in DRB with Mix-2 and PB2 had eliminated the significant reduction effect by drought stress in all tested cultivars except for Cellule with mix2 and Hyfi with PB2. Interestingly, Mix-2 promoted a significant increase, more than 13%, in Alixan cultivar compared to the non-inoculated-non-drought-stress control.

Concerning root length, the PB2 modalities did not show significant differences compared to stressed, non-inoculated plants in the four tested cultivars. However, the significant reduction in root length observed in the water stressed modalities was eliminated in the four tested cultivars in the Mix-2 modalities, with an increase of 93%, 24%, 66%, and 26%, in Alixan, Altigo, Cellule, and Hyfi respectively, compared to the non-inoculated- drought-stress controls (Fig. 4c).

3.5. Mix-2-induced resistance in wheat against Z. tritici

3.5.1 Effect of PB2:AA proportional on the resistance induced by Mix-2

Different PB2:AA rational proportional (100:0%, 75:25%, 50:50%, and 25:75%) in Mix-2, at a final concentration of 10^6 CFU.mL^{-1}, were tested for their protection efficiency against STB (Fig. 5). Plants of Alixan, Altigo, Cellule, and Hyfi wheat cultivars were inoculated with PGPR-inocula at sowing and infected at the 3-L GS with the wild-type strain IPO323 of *Z. tritici*, and disease level was quantified 17 dai using qPCR and expressed in β-tubulin copy number in 100 ng of leaf DNA (BCN_{100ng}), as previously described by Selim et al. (2014) [15]. The level of protection was determined as the percentage of the reduction of BCN_{100ng} in response to root inoculation with the different mixtures of BP2:AA compared to the control infected with *Z. tritici* and non-inoculated with PGPR. The infection levels in controls infected with *Z. tritici* and non-inoculated with PGPR were 10903, 376, 396, and 4444 BCN_{100ng} respectively, in the Alixan, Altigo, Cellule, and Hyfi cultivars. However, these infection levels were strongly reduced in the four tested cultivars with 57-78% in response to the single inoculation with PB2

(Fig. 5), and with 52.3-92.7%, 62.4-83.1%, 0-49%, protection in the PB2:AA mixtures, 75:25%, 50:50%, and 25:75%, respectively. However, the PGPR mixture with the equal proportional ratios of PB2 and AA (50:50% or 1:1) showed a stable high protection efficiency in the four tested cultivars (Fig. 5). Remarkably, the reduction of PB2 proportional in the tested mixtures to 25% decreased strongly the protection efficiency against STB (Fig. 5).

3.5.2. Effect of wheat genotype on the resistance induced by Mix-2

The impact of wheat genotypes on the resistance induced by Mix-2 (PB2:AA, 1:1) against STB was evaluated using 15 wheat cultivars with the same earliness but varied for their resistance to STB. The average of the infection levels in controls infected with *Z. tritici* and non-inoculated with PGPR were 510.7, 164.4, 285.2, 202.0, 253.7, 189.8, 262.8, 213.5, 161.6, 173.8, 164.8, 331.5, 311.5, 259.0, and 192.5 BCN_{100ng}, respectively, in the Alixan, Trapez, Terroir, Altigo, Rubisko, Expert, Chevron, Hyking, Boregar, Complice, Creek, Chevignon, Cellule, Fructidor, and Hyfi cultivars (Fig. 6). The results in Fig. 6 show high protection efficiency (50.5-83.1%) induced by Mix-2, in the all tested cultivars. However, the protective effect induced by Mix-2 was not correlated with the natural resistance level of these cultivars (Fig. 6). The results of the protection level in response to PB2 in a single inoculation did not show significant differences compared to that of Mix-2 in almost all tested wheat cultivars. However, in Alixan, Terroir, Hyking, and Complice, the protection levels induced by PB2 with respectively, 94, 91, 76, and 72%, were significantly higher than that of Mix-2 (Fig. 6).

3.5.3. Effect of wheat-genotype-growth stage–Z. tritici strain interactions on durability of the resistance induced by Mix-2

The most susceptible cultivar (Alixan) and the moderately resistant cultivar (Cellule) were used for this experiment. The resistance induced by Mix-2 (PB2:AA, 1:1) against STB was analyzed against the four *Z. tritici* strains, IPO323, 1193, ST38, and TO256. The durability of the resistance induced was followed at the earlier wheat-growth stages (3-L GS and Ti GS), and

the most mature growth stage (FL GS), corresponding to 38, 59, and 153 das. The disease level was quantified at 17 dai as mentioned above, and we used the 40% protection level as a threshold to indicate the importance of protection against STB in response to root inoculation with Mix-2 [14]. However, the global infection averages of the BCN_{100ng} over the three tested growth stages in the controls without Mix-2 in Alixan were 413, 515, 2950, 2000, and in Cellule 245, 260, 2400, 1200 for the IPO323, TO256, 1193, and ST38 strains, respectively. Remarkably, the BCN_{100ng} was approximately, 50% less in the moderately resistant cultivar Cellule than in the susceptible cultivar Alixan, except for the strain 1193 (Fig. 7 a–d).

The results in Fig. 7a show a non-genotype and non-growth-stage dependent induced resistance against strain IPO323, where the BCN_{100ng} was reduced by Mix-2 more than 53% on the two tested cultivars and at the three tested growth stages. However, no significant differences were observed between the two wheat genotypes.

Similarly, Fig. 7b represents a non-growth-stage dependent induced resistance by Mix-2 against *Z. tritici* strain TO256, in cultivar Alixan, conferring >48% protection over the three tested growth stages. For the cultivar Cellule, Mix-2 conferred 80% and 48% protection against strain TO256 at Ti and FL GS, respectively, but, no protection was observed at 3-L GS (Fig.7b).

For the 1193 strain, a growth-stage-dependent induced resistance was observed in the two tested cultivars in response to root inoculation with Mix-2 (Fig.7c). Indeed, a strong protection efficiency (>64%) was observed in the two tested cultivars, but only at 3-L and FL growth stages. However, with this strain, significant differences between genotypes were observed (Fig. 7c).

Likewise, protection efficiency induced by Mix-2 against the strain ST38 was growth stage but also, genotype dependent, where a strong efficiency was observed at 3-L and FL GS (>51%) in

Alixan, and only at 3-L GS in Cellule (fig.7d). Significant difference between the two cultivars was observed only, at FL GS (fig.7d).

3.6. Gene expression time-course analysis

For more details about the defense mechanisms implicated in wheat genotype-*Z. tritici*-Mix-2 interaction, the expression of the twenty defense related genes were studied in three-week-old plants of the Alixan and Celule as a response to Mix-2 root inoculation and leaves infection with *Z. tritici* IPO323 strain, at T0 (just at the moment of infection), 6, 12, 24, and 48 hai and at 3, 5, 9 and 11 dai. However, genes have shown more significant upregulations in Mix-2/MG modalities than in the MG modalities were labeled with stars in Figs. 8 and 9, and they were used to discriminate the effect of Mix-2 of that of MG strains.

At T0, significant upregulation, 1.9, 4.5, and 2.4-fold of the *PR1*, *LOX*, and *FLAV* genes, respectively, was observed in leaves of the susceptible cultivar Alixan in response to Mix-2 root inoculation and 7.1, 1.7, 2.4, and 5.6-fold of the *PR1*, *CHIT*, *GLU*, and *TLP* genes, respectively, in the cultivar Cellule.

In Alixan, results in Fig. 8 show significant overexpression of genes implicated in the basal defenses (*PR1*, the gene marker of the salicylic acid (SA) pathway, *GLU*, and *TLP*) by an average expression level over studied timing of 5.0, 6.7, and 6.6-fold, respectively, compared to Mix-2-non-inoculated control and 3.0, 3.3, and 2.3 times, respectively, more than that of MG modality; the jasmonic acid (JA) signaling pathway (*LOX*), by 12.7-fold and 11.5 times more than MG; the phytoalexin and phenylpropanoid pathway (*PAL*, *CHS*, and *FLAV*) by 2.3, 2.9, and 23.8-fold, respectively, and 3.8, 7.8, and 4 times than MG; the reactive oxygen species (ROS) pathway (*POX*, *OXO*, *GST*, and *GLP*) by 14.0, 2.1, 3.7, and 3.3-fold, respectively, and 14.5, 3.0, 2.8, and 3.8 times more than MG modality; and defense and cell rescue (*WRKY*) by 10.2-fold, respectively, and 30.9 times more than MG modality. The *PR1* gene was upregulated

over all the tested timings, from 6 hai to 11 dai, whereas *GLU* was upregulated at 6, 12, 48, hai and 11 dai as was *TLP* with 3 and 9 dai in addition. However, almost all studied genes were upregulated earlier between 6 and 24 hai except *LOX*, which upregulated only at 11 dai, *PAL* at 48 hai and 11 dai, *POX* at 48 hai and *OXO* at 9 dai.

In Cellule, the results in Fig. 9 show significant upregulation of genes implicated in basal defenses (*PR1*, *CHIT*, *GLU*, and *TLP*) with average inductions of 9.8, 4.7, 11.8, and 8.5-fold, respectively, compared to the Mix-2-non-inoculated-MG-non-infected control and 4.7, 3.0, 2.2, and 2.7 times more than MG modality; the JA signaling pathway (*LOX* and *AOS*) by 3.1 and 2.7-fold, respectively, and 1.5 and 2.0 times more than MG; the phytoalexin and phenylpropanoid pathway (*PAL*, *CHS*, and *FLAV*) by 2.7, 20, and 20-fold, respectively, and 1.8, 2.9, and 3.8 times more than MG; the ROS pathway (*GPX*) by 27-fold, respectively, and 27.4 times more than MG; and defense and cell rescue (*WCK1* and *rpK*) by 6.4 and 6.5-fold, respectively, and 2.8 and 1.6 times more than MG. Also, almost of these genes were upregulated early, within 6 hai and 3 dai, except for *GPX* which was upregulated at 9 dai.

4. Discussion

Beneficial microorganisms such as PGPR are one of the main explored alternatives to fungicides. Despite their inconsistent efficiency and the limited spectrum activities observed, they still have important consideration in the enhancement of the high, stable and durable non-specific resistance induced [1].

The main objective of our study was to investigate the compatibility of two interesting beneficial bacteria to associate together in a PGPR-mixture, hopping to mutualize their mode of actions. The challenge of this mixture is to be able to (1) establish a stable symbiotic relationship with wheat roots, (2) able to protect wheat against STB, (3) promote plant growth,

and (4) enhance plant tolerance to drought stress. Moreover, this PGPR-mixture has to be stable and durable in interaction with wheat genotype, growth stage, and *Z. tritici'* strains.

The results of the first screening using fifteen wheat cultivars demonstrated the efficiency of Mix-2, which compose of AA and PB2 PGPR strains, to promote wheat growth on almost all cultivars, accompanied with a strong level of protection to STB and drought stress *via* wheat defense mechanisms induction.

Wheat root colonization with PGPR in Mix-2

The results of wheat root colonization with Mix-2 of an equal portion of PB2 and PGPR-strain AA showed that both of them has an ectophytic and endophytic symbiotic relationship with wheat roots. Moreover, wheat roots colonization by these bacteria was durable overall growth stages and less influenced by wheat genotypes, contrarily, to PB2 in single inoculation as previously shown that it was wheat-genotype dependent [2]. Furthermore, compared to wheat root inoculation with a single inoculum of PB2, root colonization by PB2, in presence of AA, increased 2-30 times, demonstrating their compatibility and the helpful effect of AA toward PB2. Interestingly, it has been shown previously that PB2 has also a helping effect on the PGPR *Curtobacterium plantarum*, which indicates more possible advantages when Mix-2 comes into contact with other grain-associated or agricultural soil PGPR [2].

Wheat growth promoting in response to Mix-2

The results showed that Mix-2 promotes wheat growth compared to controls in almost all tested cultivars under non-stress conditions, and in all tested cultivars under drought stress. On the same hand, Mix-2 showed a strong protection efficiency against STB, which reduces subsequently, the high loss of the green area of the infected leaves under field conditions. Taken together, Mix-2 has a high importance for wheat growth especially, under stress conditions and can increase wheat productivity which is well correlated with leaf green area [15]. However,

we previously, showed that PB2 alone has no effect on wheat growth promotion [2], that gives the importance to strain AA in Mix-2 as wheat growth biostimulator. These results indicate the synergistic beneficial impact of combining the PGPR AA with PB2 because of the association of multiples mode of action involved, which results synergistic mechanisms.

Mix-2-resistance induced against drought and STB

Mix-2-resistance induced against drought

The results demonstrated that Mix-2 has the potential to induce tolerance against drought stress on all tested cultivars preventing the loss of plant foliar and root biomass. However, its protection effect was comparable to that obtained by PB2 in single inoculation. But, Mix-2 was less influenced by wheat genotype as observed with PB2 alone. Moreover, Mix-2 eliminated the drought effect on the root length, while, PB2 alone had no any effect. These results are confirming the importance of endophytic PGPR in the triggering of plant tolerance to abiotic stresses, including drought stress, as previously demonstrated [19]. They confirm also, the successful use of Mix-2 in the way to have a synergistic effect on the tolerance induced against drought stress in wheat where, the presence of AA strain slightly increased the protection effect by PB2 for DFB and DRB.

Mix-2 -resistance induced against STB

Mix-2 showed a considerable protective efficiency against STB reducing 50% to 83% of *Z. tritici* DNA in infected leaves in the fifteen tested cultivars. However, the Mix-2-protective effect against STB was not correlated to the rating resistance level of wheat cultivars. It seems that protection against STB as a response to root colonization with Mix-2 is dependent on the presence of PB2 in Mix-2, where, the addition of strain AA to PB2 in Mix-2, did not show significant differences compared to PB2 alone.

Influence of wheat genotype, growth stage, and Z. tritici strain on the durability of the resistance induced by Mix-2

In general, the impact of pathogen strain, wheat-genotype and wheat-growth stage on the efficiency and durability of Mix-2-induced resistance against STB is similar to that previously observed with PB2 in single inoculation [14], with an exception where Mix-2-induced resistance against strains TO256 and ST38, in the cultivar Cellule, greater than that induced by PB2 alone.

However, different kinds of the induced resistance were identified as a response to root colonization with Mix-2 in the two tested cultivars Alixan and Cellule; a non-genotype and non-growth-stage dependent resistance against strains IPO323 and ST38, a non-growth-stage dependent resistance against strain TO256 in Alixan; a genotype dependent resistance against strain TO256 only, at the earlier growth stage 3-L GS; and a growth-stage-dependent resistance against strain 1193.

These results show that Mix-2-induced resistance is durable maintaining its strong efficiency over all wheat-growth stages and especially for the last three leaf layers, which participate directly in the grain filling. The fact that Mix-2 controls some strains in the earlier growth stage and not in later stages, luckily, doesn't influence its importance in the control of *Z. tritici*, as it participates indirectly in the protection by reducing the inoculum of the upper leaf layers, which is the conidia transported from the bottom leaf layers by the impact of rain splash [15].

Defense mechanisms induced in wheat as a response to Mix-2–wheat genotype–Z. tritici interactions

To explain those results, the durability of the overexpression of wheat defense-related genes, especially during the biotrophic infection phase of this pathogen [15], was studied at 3-L GS using the most susceptible and resistant cultivars, Alixan and Cellule, respectively. The strain

IPO323 was chosen in this part of our study, to represent the non-genotype and non-growth-stage induced resistance by Mix-2. Moreover, the earlier growth stage (3-L GS) was chosen for its importance as the source of the inoculum for the upper-leaf layers.

The gene expression results in both cultivars showed the priming induction of the most important plant defense pathways, i.e. basal defenses, JA, SA, phenylpropanoid and phytoalexins and ROS, as a response to wheat root inoculation with Mix-2. The induction of theses defense pathways confirms their importance in the resistance of wheat against STB [2,14].

However, the induction of the genes coding for PR-proteins, *PR1*, *CHIT*, *GLU*, and *TLP*, as a response to Mix-2 in both cultivars, indicates the importance of the lytic degradation activities of chitin and glucan, the main components in the fungal cell wall, that lead to produce monomers known as PAMPs (pathogen-associated-molecular-patterns)-elicitors [20,21]. The combination of wheat basal defenses and other defense pathways induced as a response to Mix-2 and that of PAMPs indicates synergistic defense effects. However, over the studied time course, these defense pathways were identified using the results of gene expression and especially for that significantly upregulated compared to Mix-2/Mg modalities. The upregulation of *LOX* gene in Alixan and *LOX*, *AOS*, and *WCK1* genes in Cellule confirms the activation of the JA pathway; *PAL* and *CHS*, in both cultivars, for the phenylpropanoids and phytoalexins pathway; *POX*, *OXO*, *GST*, and *GLP* in Alixan and only *GPX* in Cellule, for the ROS pathway. Interestingly, the results showed a downregulation of catalase (*CAT*), known by its role in the degradation of the reactive oxygen species to prevent the auto toxicity of plant cells by the ROS accumulation. However, this enzyme is used also as effector by the pathogen to facilitate infection [22]. Furthermore, genes markers of defense and cell rescue are also upregulated as *WRKY* in Alixan and *rpK* in Cellule, codded for a transcription factor and a

related protein kinase, respectively. These genes are known by their implication in signaling, enhancement of plant defense, in stress recognition and in abiotic stress tolerance [23].

Those results are very close to the gene expression induced by a single inoculation with PB2 but some of the overexpressed genes are more upregulated by Mix-2, as *PR1*, *TLP*, and *FLAV* in the two cultivars, *POX* and *GLP* in Alixan and *CHIT*, *GLU*, and *rpK* in Cellule. Furthermore, in the susceptible cultivar Alixan, the upregulation of these genes was more durable in Mix-2 compared to PB2. Confirming those observations, Burkhanova et al. (2017) [24] have demonstrated a similar protection efficiency in wheat against *Stagnospora nodorum* as a response to *Bacillus* spp. in single and in co-inoculation. The resistance induced in their work was associated with a priming upregulation of *peroxidase* and *PR1* genes with more importance for the first in co-inoculation and the same induction level for the second in single and co-inoculation. Taking into consideration our last publications [2,14], we propose the *PR1*, *CHIT*, *GLU*, and *FLAV* as protection gene markers maybe used in the selection for the quantitative disease resistance in wheat.

5. Conclusions

Our previous results showed the importance of *Paenibacillus* sp. strain B2 as a biological control agent and a systemic resistance inducer. Here, we show that its efficiency was improved by co-inoculation with the PGPR *Arthrobacter* spp. strain AA (Mix-2), exhibiting higher, more stable, and more durable protection and reducing the effect of wheat genotype and pathogen strain. Moreover, Mix-2 also promoted plant growth with or in the absence of stress conditions. The results highlight also, the importance of ROS, phenylpropanoids and phytoalexins, SA, and JA pathways combined with basal defenses in the resistance induced in wheat by Mix-2. Finally, we propose the *PR1*, *CHIT*, *GLU*, and *FLAV* as possible gene markers for the selection to the quantitative disease resistance in wheat to STB.

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Author contributions

E.S. carried out the experimental work and wrote the manuscript. S.S. and T.A. revised the manuscript, and managed the experimental design and the PhD research program. S.S., T.A. and C.E. supervised the project.

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Table 1. Wheat cultivars used to study the impact of wheat genotypes on the resistance induced by Mix-2.

Cultivar	Producer	Year	Susceptibility rating*
Alixan	LG	2005	4
Trapez	Unisigma	2009	4
Terroir	Florimond Desprez	2013	5
Altigo	LG	2007	5.5
Rubisko	RAGT	2012	5.5
Expert	Syngenta	2008	5.5
Chevron	Saaten Union	2009	5.5
Complice	Florimond Desprez	2016	6
Creek	Saaten Union	2019	6
Hyking**	Saaten Union	2016	6
Boregar	RAGT	2008	6
Cellule	Florimond Desprez	2012	6.5
Fructidor	Unisigma	2014	6.5
Chevignon	Saaten Union	2017	7
Hyfi**	Saaten Union	2013	7

*The susceptibility rating is on a scale of 1 to 9, where 1 represents 'susceptible' and 9 represents 'resistant' (Arvalis Institut du Végétal, 2017) [25].
****Hybrid cultivars.

Table 2. Oligonucleotide primer sequences of wheat-defense genes and the 16S rDNA for *Paenibacillus* sp. strain B2 and *Arthrobacter* strain AA.

Gene Name	GeneBank accession N°	For/Rev primers (5'-3')**	Tm* (°C)	Amplicon length (bp)	MT* (°C)	PCR Efficiency (%)
Housekeeping genes						
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AF251217	AAGGGCATTTTGGGTTACGTT CCTGTTGTCACCCTGGAAGTC	58.4 58.1	63	79.1	103.98
β-tubulin (B-TUB)	U76897	CTGCCTCCAAGGTTTCCAAGTA GTGTCCATCCCGGAACCA	59.2 58.7	63	81.0	92.70
Cell wall proteins & Basal defense						
Pathogenesis-related protein (PR1)	HQ848391	CATGCACCTTCGTATGCCTAACT TGGCTAATTACGGCATTCCTTT	58.9 59.4	52	79.1	90.25
Chitinase (CHIT)	AB029935	GGGTGGACCTGCTGAACAAT AGAACCATATCGCCGTCTTGA	58.4 58.3	75	84.6	92.35
β-1,3-glucanase (GLU)	DQ090946	TCCTGGGTTCAGAACAATGTCC TTGATGTTGACAGCCGGGTAGT	59.8 60.4	50	78.2	105.35
Thaumatin-like protein (TLP)	CD86039	AGGTAATTTTTTTATTGCCCTGTACTG TTACAGCCGCCGTACTACATGT	58.9 60.3	89	77.7	90.25
JA signaling pathway						
Lipase (LIP)	TaBs117A2	CACAAAATATCGACCCACCAC ACTGGGTATTCGTCTGTCAGC	60 59	149	86.3	100.92
Lipoxygenase (LOX)	U32428	GGGCACCAAGGAGTACAAGGA GCTCGTGATGGTGTGGATGA	59.9 59.1	66	82.2	99.66
Allene oxide synthase (AOS)	AY196004	AGGCCGGAGAGAAGTTCCAC CCGACTTGGTCAGCTCCATC	59.3 59.2	119	88.0	93.65
Phenylpropanoid & Phytoalexin pathway						
Phenylalanine ammonia-lyase (PAL)	AY005474	GTCGATTGAGCGTGAGATCAAC CACGGGAGACGTCGATGAG	58.0 59.0	59	80.5	101.35
Chalcone synthases (CHS)	AY286097	GCGCCTGCGTACTCTTCATC CCTCGGCGGAGCGTTT	60.0 59.0	51	80.8	109.67
Flavonoid 7-O-methyltransferase-like (FLAV)	CA682712	GACAACAAGGAGGCTGTGTATGG GGTGTAATGCAGTTGAATCAAGGA	62.4 59.3	117	80.6	93.43
Reactive oxygen species (ROS)						
Peroxidase (POX)	X85228	TGCTTTGTCCAAGGCTGTGA GACCCGCGTTTTGTTCCA	59.0 59.1	61	79.8	108.68
Oxalate oxidase (OXO)	AJ556991	GCCAGAACCCCGGTATCG GGTGGGTTGGAGCTGAAGAG	60.0 58.0	55	80.8	97.23
Glutathione-s-transferase (GST)	AF397085	CGCTCTGAGCCCCATTCTC GGCTCCCCCAAGCATAGG	59.5 59.0	55	79.1	106.28
Germin-like-protein (GLP)	Y09916	AGGTGAGCTCCTTGTTGGAATC GTTGAACTGGAAGTGCATGAGG	60.3 60.3	121	85.6	91.99
Glutathione peroxidase (GPX)	KM817777	GTTCAGTTTGCCTGCACTCG GTTCCACTTGATGCTGTCGC	58.1 57.9	141	83.3	94.17
Catalase (CAT)	X94352	TTCAAGCAGGCTGGTGAGAG TTTCATGGGTGACACGAGCA	59.8 59.7	106	84.5	103.09
Superoxide dismutase (SOD)	EF392662	TCAGGACCCTCTTGTGACCA CGGCCTCACGTTCTTGTACT	57.6 56.6	100	81.7	102.65
Defense and cell rescue						
Related protein kinase (rpK)	KR611569	TTTTGTTGGGGATCCTGCGT GCTCAGGCTCCTCGTATTGG	61.2 58.5	128	81.5	99.66
WRKY1 transcription factor (WRKY)	EU665424	TGGCGCAAGTATGGTCAGAA CAGCCCTGGTGGGTACATTT	58.9 58.4	77	79.3	101.35
MAP kinase (WCK1)	AF079318	AGTTCGAGATCACGGCCAAGT GAAGGCGTTGGCGATCTTC	59.8 58.8	131	87.6	108.19
16S ribosomal DNA (16S rDNA)						

Paenibacillus sp. strain B2	AJ011687	TCGTAAAGCTCTGTTGCCAGG CTTGAGCAGTTACTCTACAAGACGTTC	59.0 58.0	51	75.45	109.67
Arthrobacter spp. strain AA		GATCTGCGGTGGGTACGG CGGTTCATGTCAAGCCTT	56.5 53.7	380	86.91	104

* Tm, primer's annealing temperature; MT, amplicon's specific melting temperature.

** Primers' reference, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (B-TUB) (Scholtz and Visser, 2013); pathogenesis-related protein (PR1), Chitinase (CHIT), β-1,3-glucanase (GLU), lipoxygenase (LOX), allene oxide synthase (AOS), phenylalanine ammonia-lyase (PAL), chalcone synthases (CHS), peroxidase (POX), oxalate oxidase (OXO) and glutathione-s-transferase (GST) (Ors et al., 2018); thaumatin-like protein (TLP), flavonoid 7-O-methyltransferase-like (FLAV), and germin-like-protein (GLP) (Desmond et al., 2008); lipase (LIP) (Lu et al., 2006); MAP kinase (WCK1) (Sardesai et al., 2005); glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD), related protein kinase (rpK), WRKY1 transcription factor (WRKY), and 16S rDNA of *Paenibacillus* spp. and *Arthrobacter* spp. Primer pairs were designed using the Primer Express® program and tested for secondary structure using the AmplifX® program. All used primers did not show any form of dimerization.

Table 3. *Paenibacillus* sp. strain B2 (PB2) external and internal wheat root colonization increasing ratio as a response to root inoculation with Mix-2 and compared to that in single inoculation with PB2. The values shown are means with SD (n=5).

Wheat cultivar	External root colonization				Enternal root colonization			
	Si	Mix-2		PB2-Mix-2/PB2-Si**	Si	Mix-2		PB2-Mix-2/PB2-Si
	PB2*	PB2	AA		PB2	PB2	AA	
Alixan	129	960	121	7.42 ±5.13	6,5	21	2971	3.19 ±2.47
Altigo	226	565	93	2.50 ±0,63	14,7	12	1289	0.79 ±0.29
Cellule	76	2304	65	30.35 ±10.86	14,4	44	3639	3.08 ±1.38
Hyfi	450	468	130	1.04 ±0.77	20,4	44	146	2.15 ±1.45

* Values are the means of bacterial DNA quantity in pg per 100 ng of root DNA.

** Ratio of wheat root colonization by PB2 when using Mix2 compared to that when using PB2 alone (the helpful effect of the strain AA toward PB2) = Quantity of PB2 DNA in roots inoculated with Mix-2/Quantity of PB2 DNA in roots inoculated with PB2 alone.

Si, the single inoculation of wheat roots with PB2.

Mix-2, the inoculation of wheat roots with a PGPR mixture, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1).

Figures:

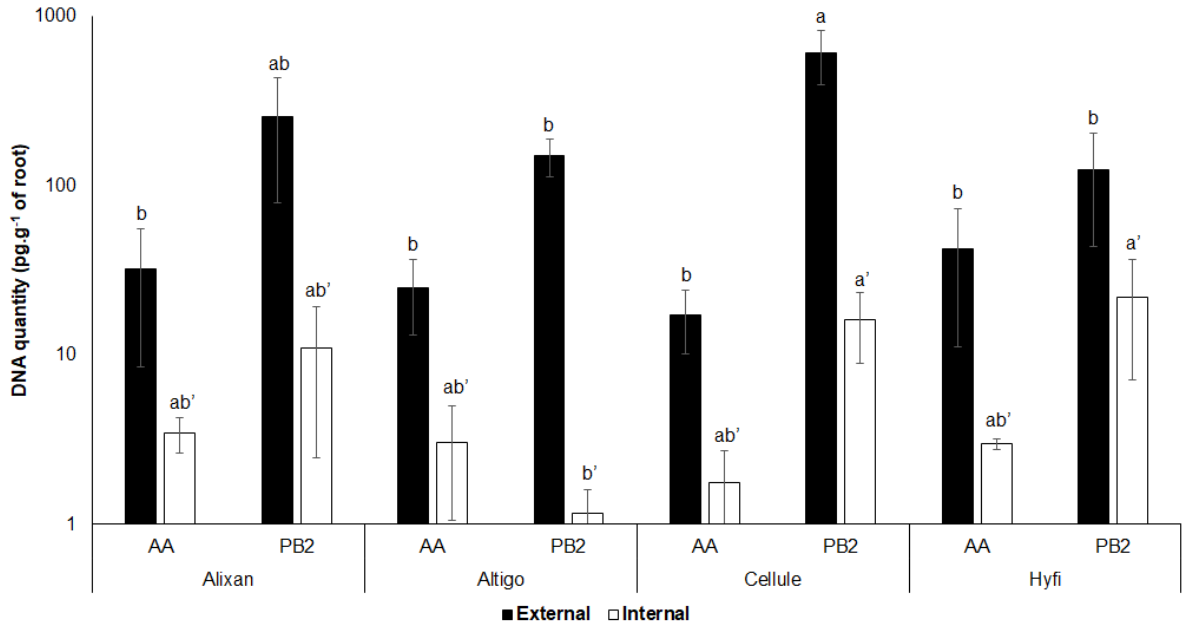


Fig. 1. Root external and internal colonization of Mix-2 composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) in the Alixan, Altigo, Cellule, and Hyfi cultivars. Plants were inoculated with PB2 and Mix-2 by immersing the pre-germinated grains in a suspension of a final concentration of 10^6 CFU.mL^{-1}. The results show the DNA amount per gram of root, determined by qPCR. The values shown are the means of three biological replicates and five technical replicates. Different lower-case letters indicate significant differences between treatments, according to the Tukey test ($p \leq 0.05$).

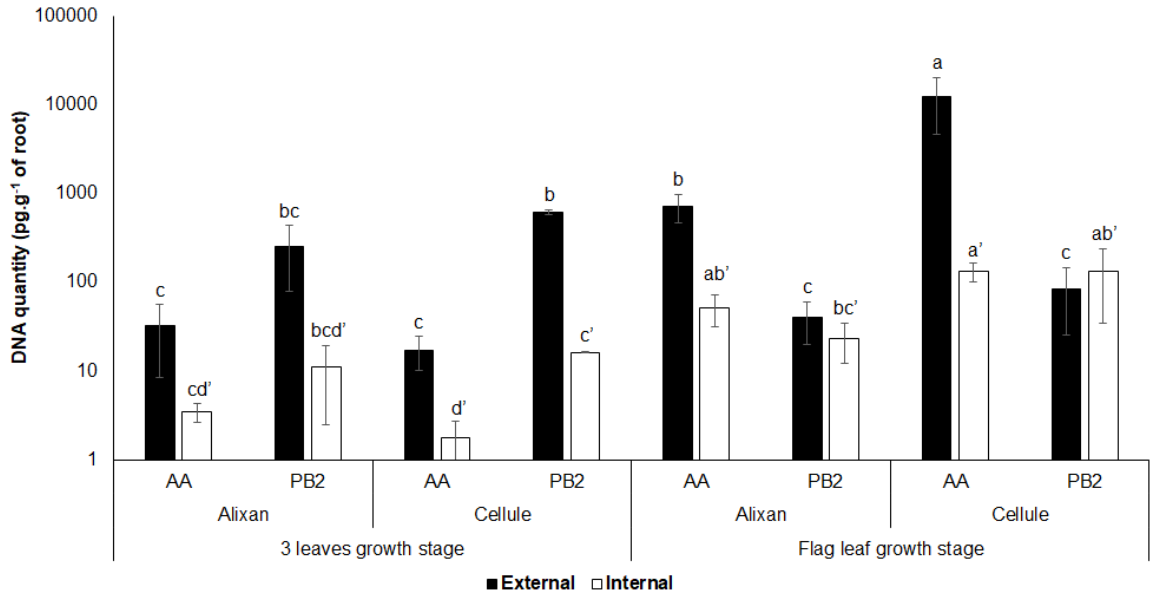


Fig. 2. Evolution of root external and internal colonization of Mix-2, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1), in the Alixan and Cellule wheat cultivars, between the three-leaf (3-L) and flag-leaf (F-L) growth stages. Plants were inoculated with PB2 and Mix-2 by immersing the pre-germinated grains in a suspension of a final concentration of 10^6 CFU.mL^{-1}. Root colonization was determined by qPCR, as the DNA amount of PB2 and AA per gram of root. The values shown are the means of three biological replicates and five technical replicates. Different lower-case letters indicate significant differences between treatments, according to the Tukey test ($p \leq 0.05$).

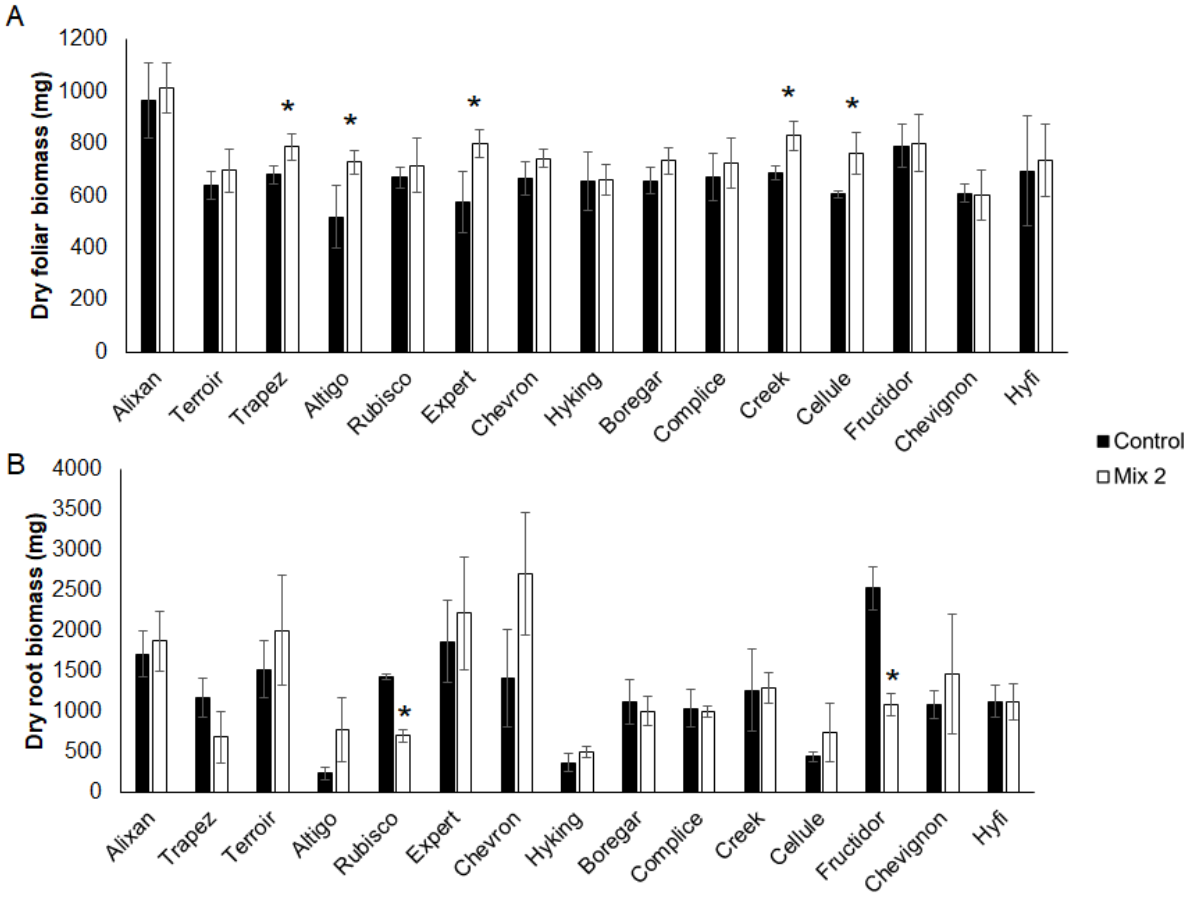


Fig. 3. Impact of wheat root inoculation with Mix-2, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1), on wheat growth. Plants were inoculated with Mix-2 by immersing the pre-germinated grains in a suspension of Mix-2 at a final concentration of 10^6 CFU.mL^{-1}. The foliar (A) and root (B) dry biomass were evaluated 6 weeks after sowing on 15 wheat cultivars. The values shown are the means of three biological replicates and five technical replicates. The values shown are means of three biological replicates and five technical replicates. Different lower-case letters indicate significant differences between treatments, according to the Tukey test ($p \leq 0.05$).

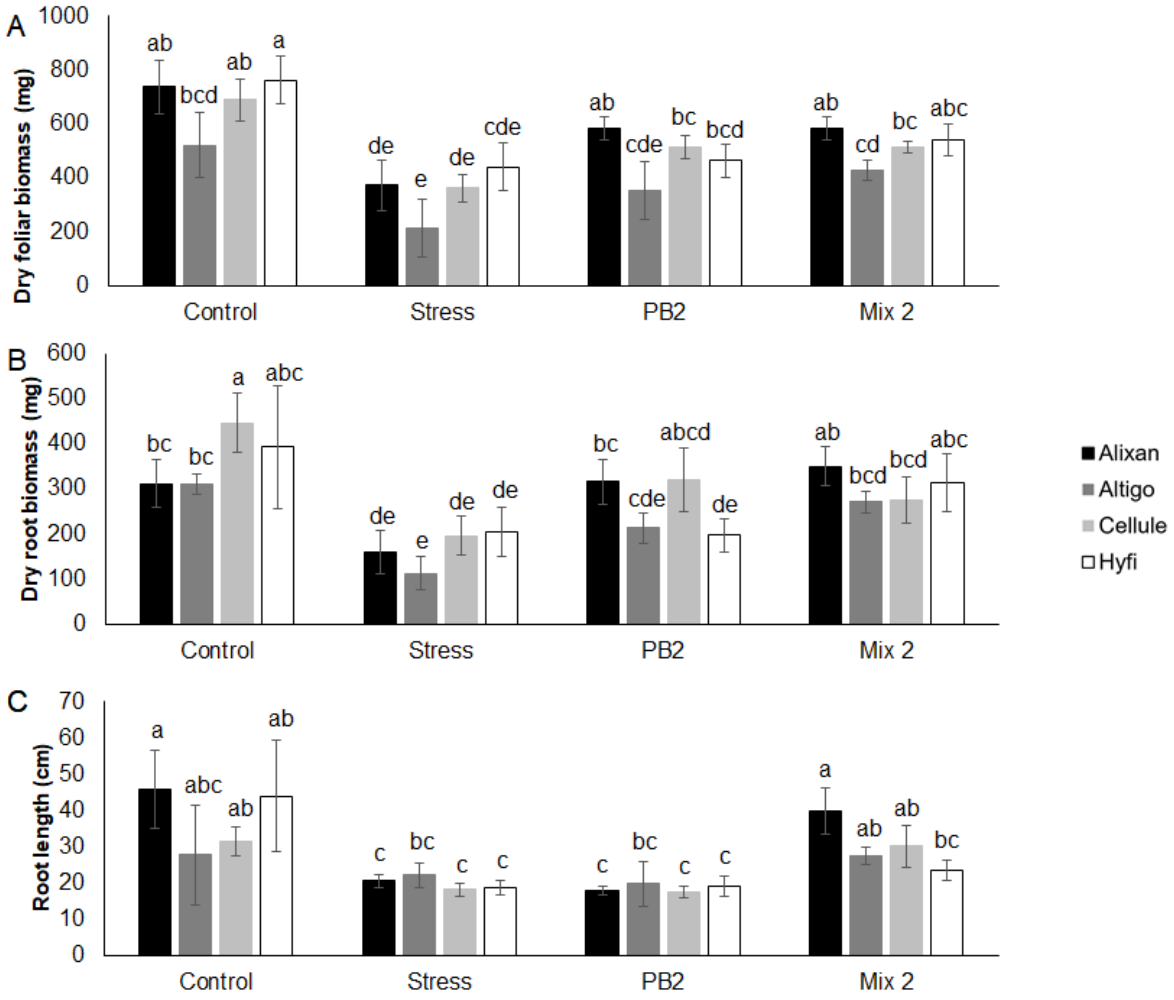


Fig. 4. Impact of Mix-2, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1), on the induced resistance of wheat against drought stress. Plants were inoculated with Mix-2 by immersing the pre-germinated seeds in a suspension of Mix-2 at a final concentration of 10^6 CFU.mL-^{1}. Two weeks after sowing, a drought period of 19 days was applied, followed by a recovery period of 2 weeks. The protection efficiency of Mix-2 against the drought effect on foliar (A) and root (B) biomass, and root length (C) was evaluated. The values shown are the means of three biological replicates and five technical replicates. Different lower-case letters indicate significant differences between treatments, according to the Tukey test ($p \leq 0.05$).

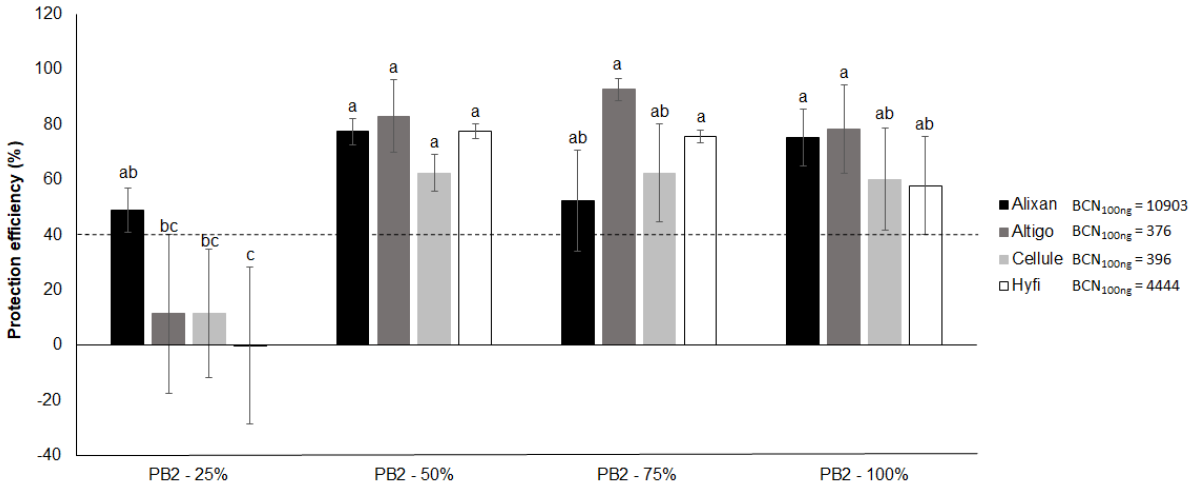


Fig. 5: The effect of rational proportion of *Paenibacillus* sp. strain B2 (PB2) to *Arthrobacter* strain AA (AA) in the mixture PB2:AA (Mix-2) on the protection efficiency against *Z. tritici* strain IPO323, in the Alixan, Altigo, Cellule, and Hyfi cultivars, at the three-leaf growth stage, represented as the reduction percentages of *Z. tritici* β-tubulin copy number in 100 ng of leaf DNA (BCN_{100ng}) at 17 days after infection with *Z. tritici*. Plants were inoculated with Mix-2 by immersing the pre-germinated grains in a suspension of 10^6 CFU.mL^{-1}. The values shown are the means of three biological replicates and five technical replicates. Different lower-case letters indicate significant differences between treatments, according to the Tukey test ($p \leq 0.05$).

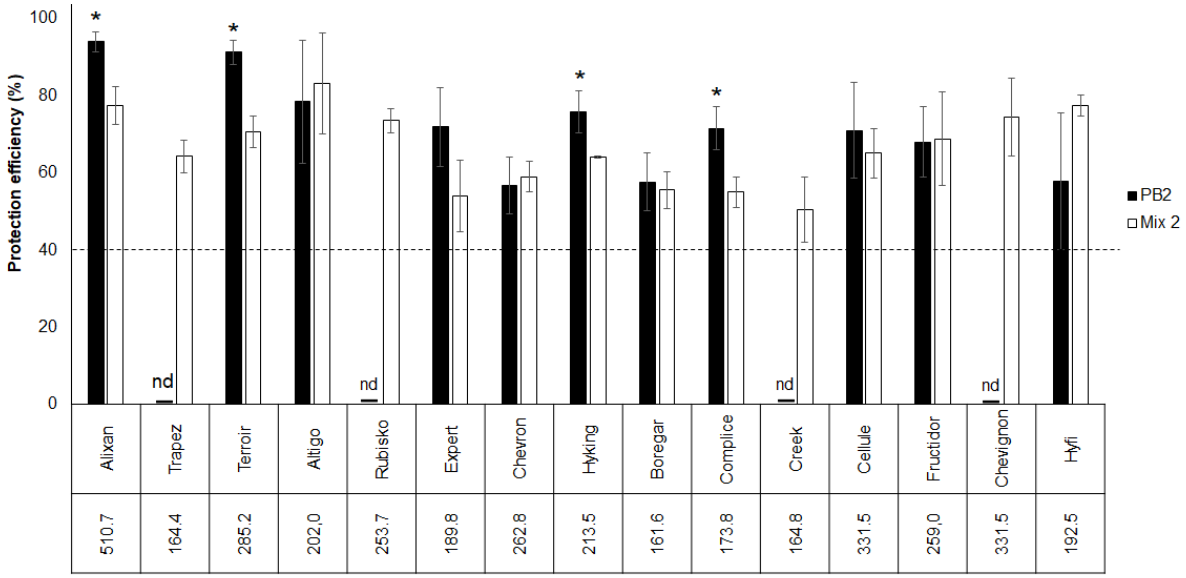


Fig. 6. Protection efficiency induced by Mix-2, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1), against *Z. tritici*, strain IPO323 in fifteen wheat cultivars, at the three-leaf growth stage, represented as the reduction percentages of *Z. tritici* β-tubulin copy number in 100 ng of leaf DNA (BCN_{100ng} DNA) at 17 days after infection with *Z. tritici*. Plants were inoculated with Mix-2 by immersing the pre-germinated seeds in a suspension of 10^6 CFU.mL^{-1}. The values shown are the means of three biological replicates and five technical replicates. Different lower-case letters indicate significant differences between treatments, according to the Tukey test ($p \leq 0.05$).

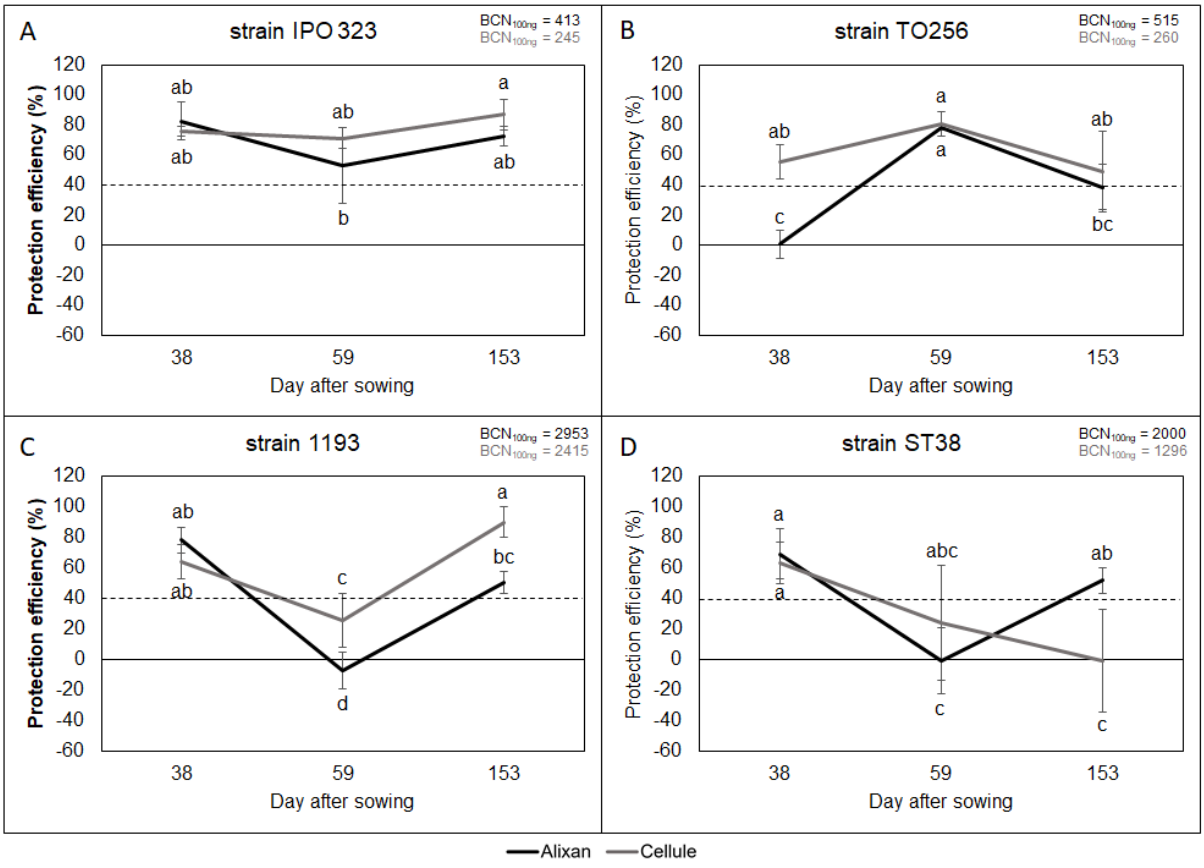


Fig. 7. Protection efficiency induced by Mix-2, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1), against four strains of *Z. tritici*, IPO323 (a), TO256 (b), 1193 (c) and ST38 (d), in the Alixan and Cellule wheat cultivars, at the three-leaf (38 das), tillering (59 dai) and flag-leaf (153 dai) growth stages, represented as the reduction percentages of *Z. tritici* β-tubulin copy number in 100 ng of leaf DNA (BCN_{100ng} DNA) at 17 days after infection with *Z. tritici*. The BCN_{100ng} in controls not inoculated with PB2 or Mix-2 is listed upper the figures. The values shown are the means of three biological replicates and five technical replicates. Different lower-case letters indicate significant differences between treatments, according to the Tukey test ($p \leq 0.05$).

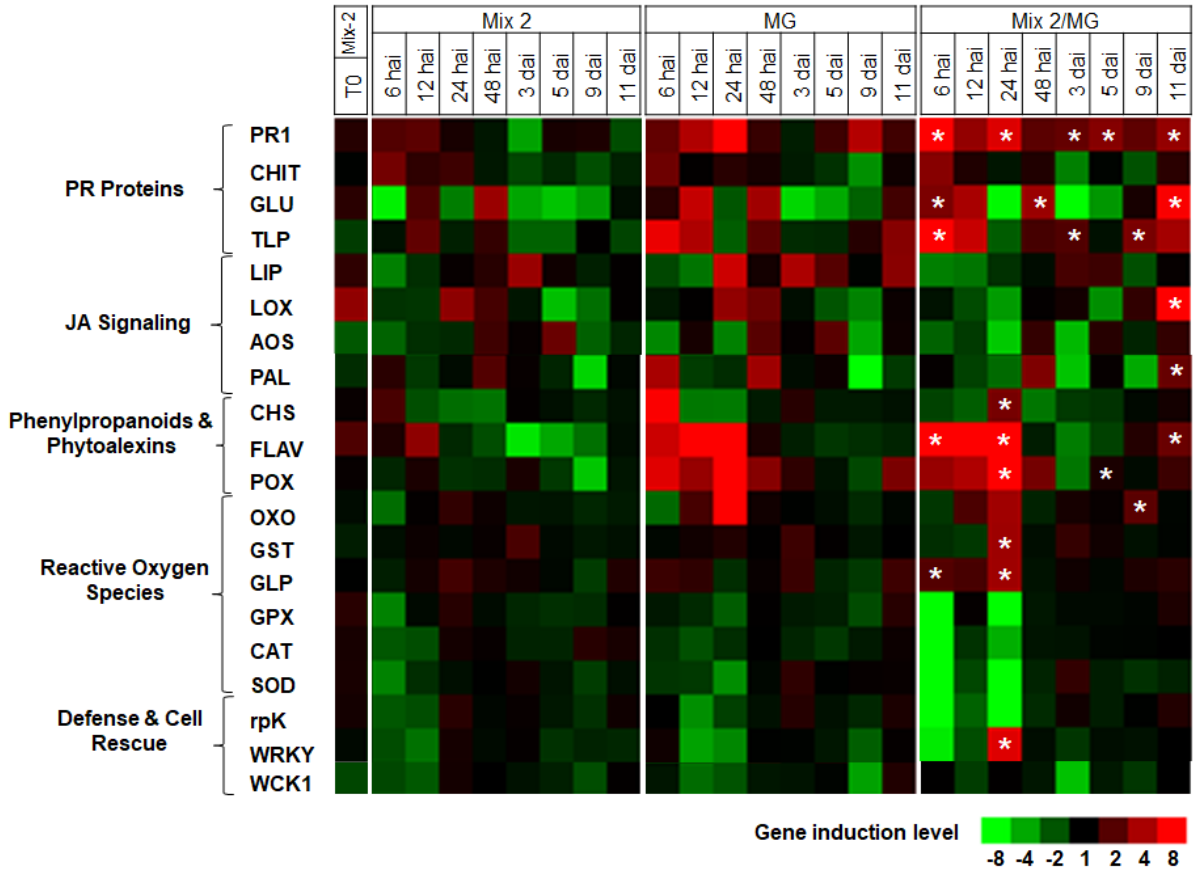


Fig. 8. Time course of the relative expression of wheat defense genes in the susceptible cultivar Alixan at the time of leaf infection (T0) with *Z. tritici* strain IPO323 (MG), 6, 12, 24, and 48 hours after infection (hai), and 3, 5, 9, and 11 days after infection (dai). Plants were inoculated with Mix-2, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1), by immersing the pre-germinated grains in a suspension of 10^6 CFU.mL^{-1}. Gene expression of the following tested modalities, Mix-2-inoculated and MG-non-infected (Mix-2), Mix-2-non-inoculated, and MG-infected (MG), and Mix-2-inoculated and MG-infected (Mix-2/MG), were compared to the Mix-2-non-inoculated and MG-non-infected control modalities. ✯ Stars indicate gene induction ≥2-fold and significant differences between the Mix-2/MG and MG modalities, according to the Tukey test ($p \leq 0.05$). The values shown are the means of three biological replicates and five technical replicates.

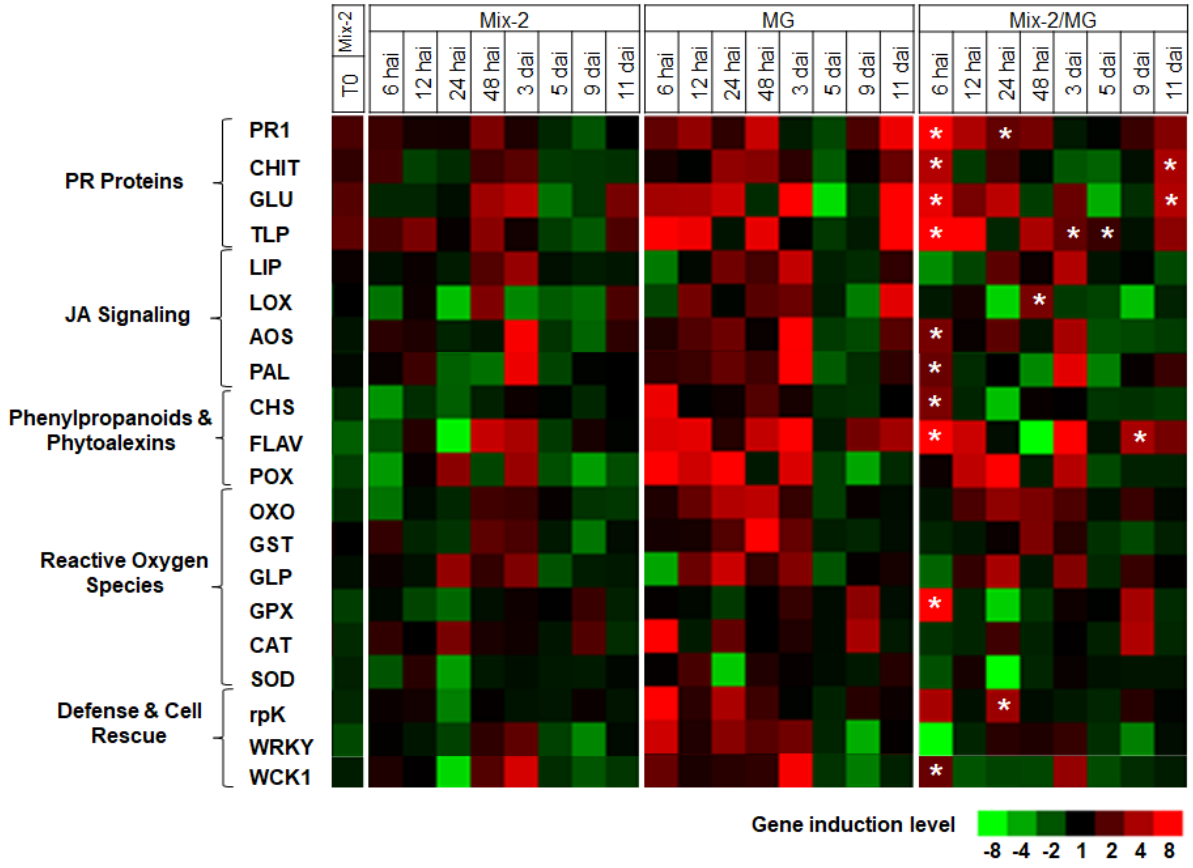
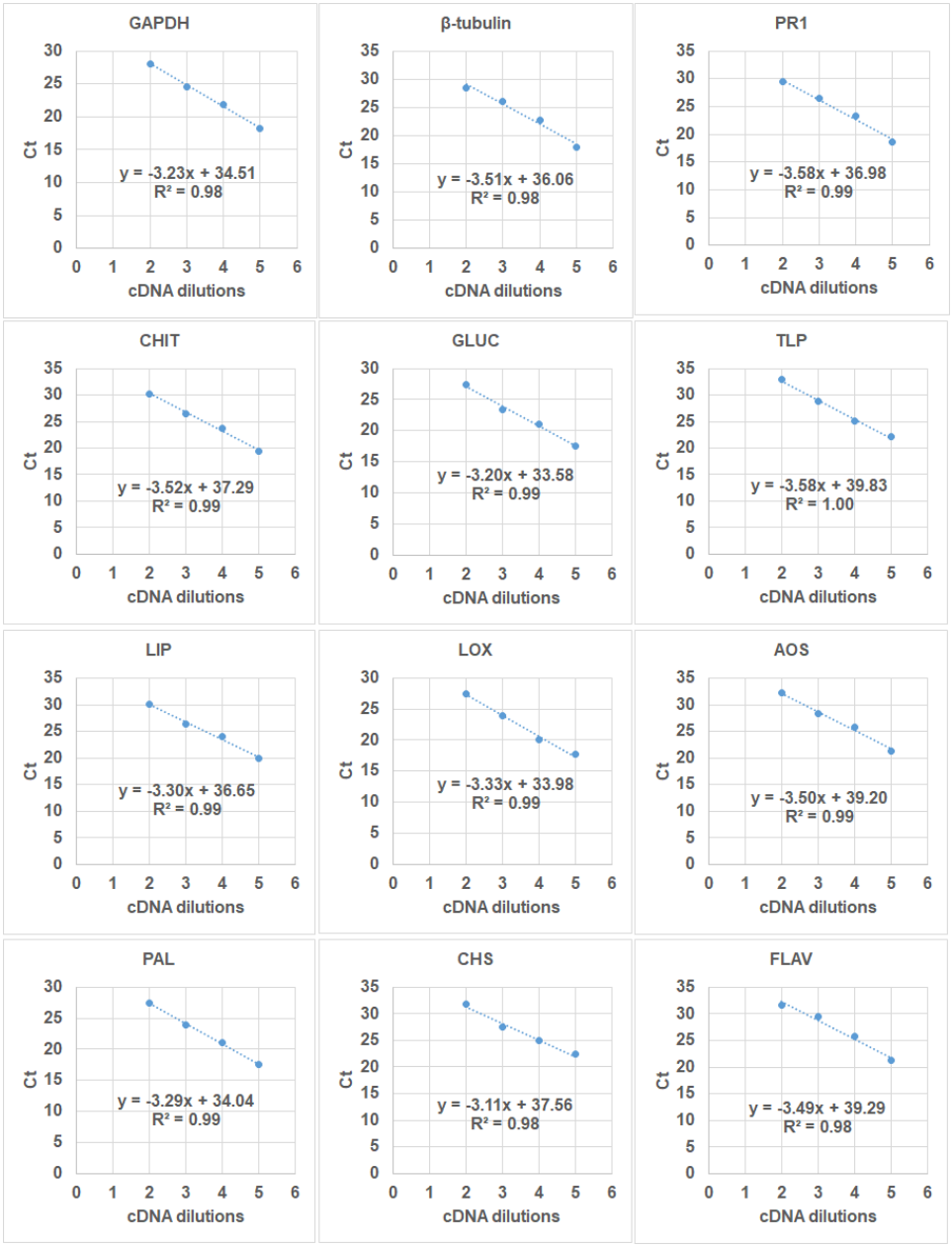
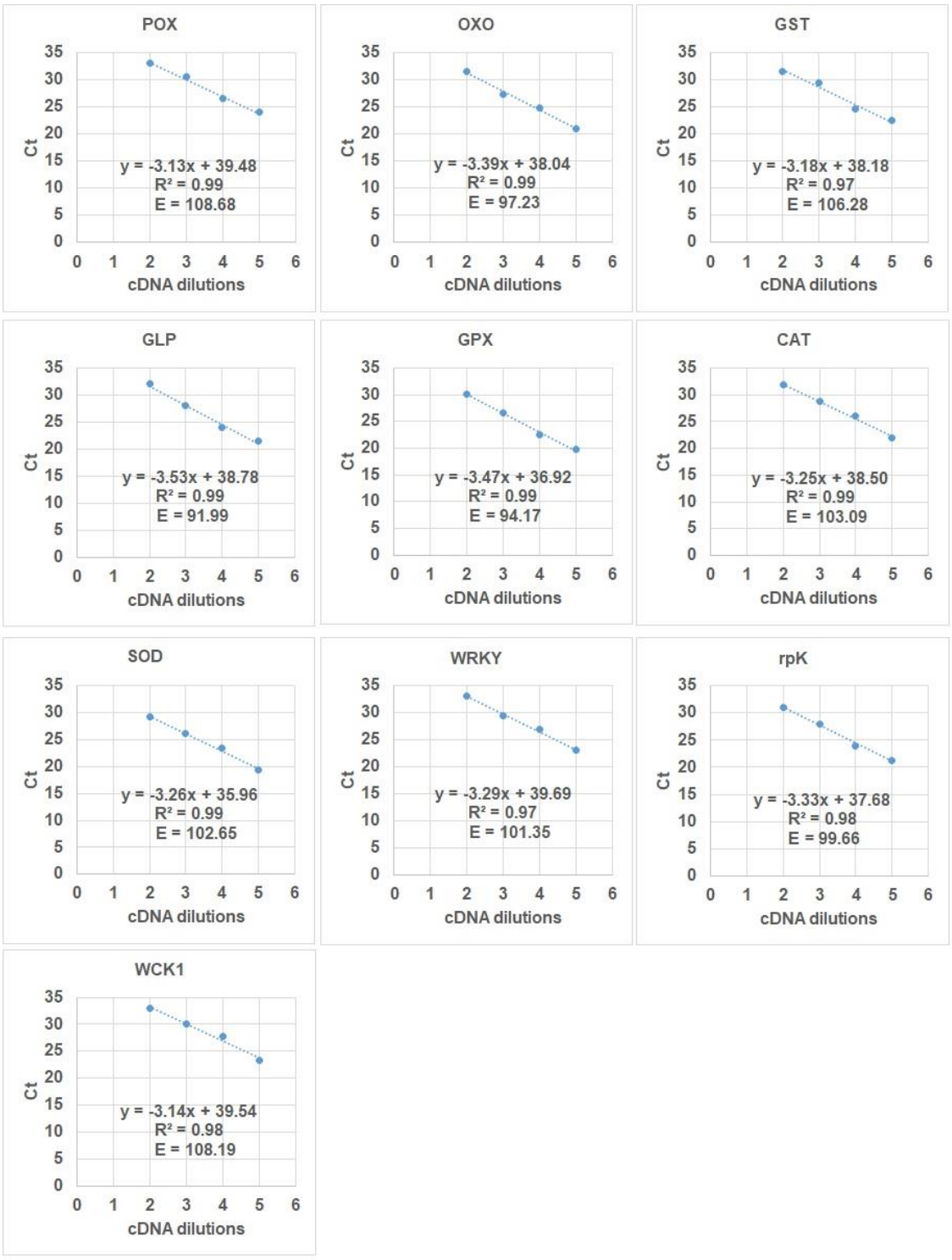


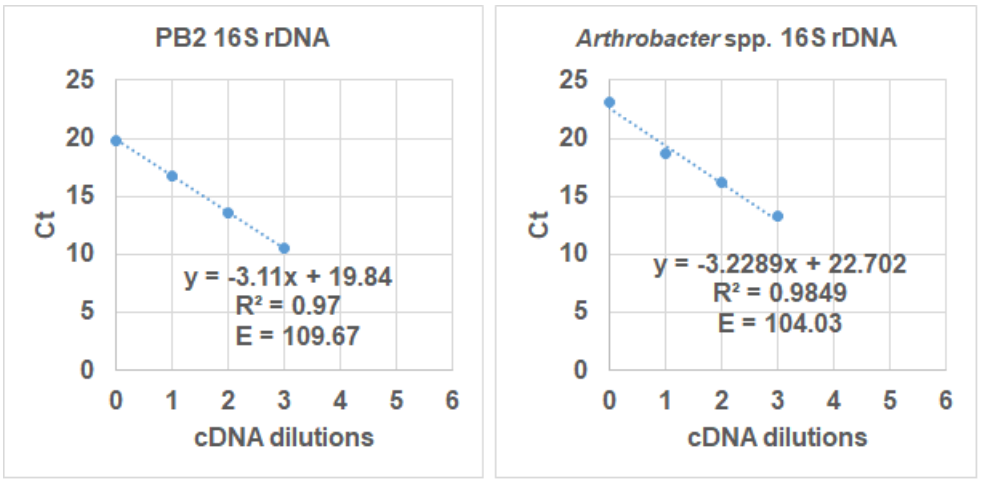
Fig. 9. Time course of the relative expression of wheat defense genes in the moderately resistant cultivar Cellule at the time of leaf infection (T0) with *Z. tritici* strain IPO323 (MG), 6, 12, 24, and 48 hours after infection (hai), and 3, 5, 9, and 11 days after infection (dai). Plants were inoculated with Mix-2, composed of *Arthrobacter* strain AA (AA) and *Paenibacillus* sp. strain B2 (PB2) (1:1), by immersing the pre-germinated grains in a suspension of 10^6 CFU.mL^{-1}. Gene expression of the following tested modalities, Mix-2-inoculated and MG-non-infected (Mix-2), Mix-2-non-inoculated and MG-infected (MG), and Mix-2-inoculated and MG-infected (Mix-2/MG), were compared to the Mix-2-non-inoculated and MG-non-infected control modalities. ☆ Stars indicate gene induction ≥2-fold and significant differences between the Mix-2/MG and MG modalities, according to the Tukey test ($p \leq 0.05$). The values shown are the means of three biological replicates and five technical replicates.



Supplementary Fig. 1. PCR amplification efficiency for each primer pair used in the gene expression study. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin (B-TUB), pathogenesis-related protein (PR1), chitinase (CHIT), β-1,3-glucanase (GLU), thaumatin-like protein (TLP), lipase (LIP), lipoxygenase (LOX), allene oxide synthase (AOS), phenylalanine ammonia-lyase (PAL), chalcone synthases (CHS), and flavonoid 7-O-methyltransferase-like (FLAV).



Supplementary Fig. 2. PCR amplification efficiency for each primer pair used in the gene expression study. Peroxidase (POX), oxalate oxidase (OXO), glutathione-S-transferase (GST), germin-like protein (GLP), glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD), related protein kinase (rpK), WRKY1 transcription factor (WRKY), and MAP kinase (WCK1).



Supplementary Fig. 3. PCR amplification efficiency for each primer pair used in the quantification of 16S rDNA of *Paenibacillus* strain B2 (PB2) and *Arthrobacter* spp. strain AA.