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Salinity is a prevailing factor for amelioration of wheat blast by biocontrol agents

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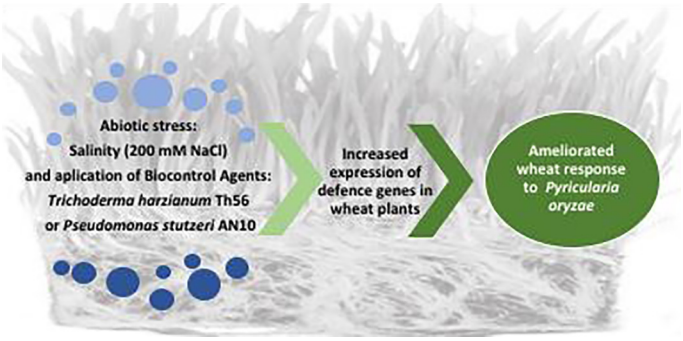
GRAPHICAL ABSTRACT



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ABSTRACT

Plants exposed to combined abiotic and biotic stress conditions may show enhanced pathogen resistance. Biocontrol agents (BCAs) can further contribute to ameliorate plant responses to these complex situations. In recent years, wheat production has been challenged by the expansion of salt-affected soils as well as by severe outbreaks of emerging diseases such as wheat blast. Here, the role of two BCAs, *Pseudomonas stutzeri* AN10 and *Trichoderma harzianum* Th56, was examined in salt-stressed seedlings infected with two *Pyricularia oryzae* isolates of contrasting aggressiveness. BCAs did not enhance plant tolerance to high salt stress. However, BCAs improved performance of salt-stressed wheat plants infected with the less aggressive *Pyricularia* isolate. The lower infection in salt-stressed BCAs-treated plants could be due to a salt-induced priming state required to trigger an early expression of plant defence genes.

1. Introduction

Pyricularia oryzae (Magnaporthe *oryzae* Couch) is a species complex of hemi-biotrophic, pathogenic fungi with a high degree of host specificity which causes blast disease in many *Gramineae* family species, including staple cereal crops such as rice and wheat (Couch et al., 2005; Tosa et al., 2006). Wheat grain infection by *P. oryzae* decreases yield and quality, causing important losses in susceptible cultivars (Kohli et al., 2011). Wheat blast is severely threatening wheat production in several South American countries (Kohli et al., 2011). In Argentina,

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Pyricularia oryzae isolates were recently recorded on wheat, barley, and other herbaceous plants (Perelló et al., 2015), and the severity of wheat blast and associated effects on grain quality and 1000 grain weight were documented (Perelló et al., 2017; Martínez et al., 2018). In 2016, an outbreak in Bangladesh caused losses of up to 90% in infected wheat fields (Callaway, 2016). This alarming event further reinforced the need to control this devastating disease, launching initiatives such as the Open Wheat Blast website (www.wheatblast.net).

Control of *Pyricularia* disease is challenging, as the fungus presents a high genetic diversity and it can recombine by parasexual reproduction to rapidly develop new races, thus hampering the development of resistant wheat cultivars (Monsur and Kusaba, 2018). Moreover, the use of fungicides has not been effective in susceptible wheat varieties so far (Kohli et al., 2011; Maciel, 2011). Under these circumstances, the use of BCAs is recommended as an alternative approach to enhance plant health and to avoid the environmental risks associated to the use of chemical pesticides (Woan-Fei Law et al., 2017).

Under field conditions, crops are challenged by both biotic and abiotic stress conditions. This constitutes a key issue, as plant responses to combined stresses are different from those to individual stress factors (Atkinson and Urwin, 2012). In arid and semi-arid regions all around the world, soil salinization is one of the most limiting factors hampering wheat productivity under both irrigated and rain-fed conditions (Colmer et al., 2006). Mujeeb-Kazi and Díaz de León (2002) reported that about 8–10% of wheat cultivating land was affected by salinization in India, Pakistan, Iran, Egypt, Libya and Mexico.

Both positive and detrimental effects of soil salinity on the plant's response to pathogen stress have been reported. DiLeo et al. (2010) found that a brief salinity stress made tomato and chrysanthemum plants more susceptible to *Phytophthora* spp. infection. These authors reported a relationship between an increase in root ABA and the predisposed state. Moreover, disease enhancement was also triggered by exogenous ABA concentrations. In contrast, different salts, in a concentration-dependent manner, and exogenous ABA, enhanced the response of barley to *Blumeria graminis* (Wiese et al., 2004).

Among the BCAs, *Trichoderma* and *Pseudomonas* have been reported to be effective against diseases caused by pathogens as well as ameliorating the plant's response to salinity. A recent study (Ali and Nadarajah, 2014) reported that *Trichoderma* inhibited blast disease in rice. *Trichoderma* can directly inhibit pathogens by producing toxic compounds or by competing with these microbes for space and resources. Moreover, *Trichoderma* may indirectly hamper pathogen development in target plants by triggering induced systemic resistance (ISR) (Benítez et al., 2004). ISR results from the complex cross talk amongst different stress hormone-triggered signalling pathways (Mathys et al., 2012; Niu et al., 2011). Moreover, colonization of *Arabidopsis* and cucumber roots by *Trichoderma* improved seed germination under saline conditions through mechanisms that involved the reduction of ethylene (ET) levels and the promotion of the antioxidant capacity (Brotman et al., 2013). The rhizobacteria genus *Pseudomonas* also owns direct mechanisms for pathogen control such as effective antimicrobial secondary compounds i.e. antibiotics as phloroglucinols, pyrrolnitrin and phenazines (Chin-A-Woeng et al, 2003) and indirect mechanisms triggering ISR (Van Loon, 2007). Recently, Spence et al. (2014) reported that a *Pseudomonas chlororaphis* isolate protected rice against blast disease by triggering a mechanism dependent on jasmonic acid (JA) and ET signalling, which decreased the severity of the infection. In addition, *Pseudomonas* spp. also ameliorated plant responses to salt. Different mechanisms have been reported on the effect of *Pseudomonas* inoculation in salt-stressed plants, which include, among others, increased K^+ and Ca^{2+} uptake, higher concentrations of compatible solutes, enhanced 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and reduced lipid peroxidation and superoxide dismutase (SOD) activity (reviewed by Shrivastava and Kumar, 2015).

Stress tolerance has been mostly focused on plant responses to individual stresses; thus, the understanding of a plant's ability to adapt to combined stresses is limited. The relationship amongst pathogen, antagonists and biotic stress and their relative role in the response of crops, such as wheat, are less studied and still deficient. Mohamed and Haggag (2006) and Bheemaraya et al. (2013) pointed out that growth and physiological state of *Trichoderma* sp. is affected by both biotic and abiotic stress factors in the natural environment. Recent results (Guo et al. 2018) provided theoretical support and a practical reference for the development of *Trichoderma* strains to alleviate saline or alkaline stress and to improve the management of fungal diseases in saline or alkaline soils.

On the other hand, *Trichoderma harzianum* Th56 (Perelló et al., 2017) and *Pseudomonas stutzeri* AN10 (García-Valdés et al., 1988) are microorganisms of interest in our laboratories for its potential role in different processes, amongst them, the possible beneficial effects on plant growth and development, and on plant responses to stress conditions.

Based on this background, the aim of this work was to explore the possible ameliorating action of two BCAs, *Trichoderma harzianum* and *Pseudomonas stutzeri*, on the response of salt-stressed wheat plants to *Pyricularia oryzae* isolates that differ in virulence. The relative expression of selected stress-response related genes was used as a marker of the sensitivity/resistance response of the wheat plants.

2. Materials and methods

2.1. Blast pathogens

Two local Argentinian isolates from field-grown wheat samples showing blast symptoms, named UNLP2 and UNLP10 were used in this study. These strains were previously characterized by morphological and epidemiological analysis (Perelló et al. 2015). They were selected, amongst others, based on their contrasting aggressiveness profile in wheat according previous results of pathogenicity tests performed with different Argentinian isolates inoculated on seedlings and adults (spike) wheat plants under greenhouse conditions (Perelló et al. 2017).

2.2. Molecular characterization of Pyricularia isolates: DNA extraction, PCR amplification and sequencing

Conidia were scraped off from a plate and mixed with ethanol. After removing the ethanol, they were homogenized in lysis buffer with 150 mg of sea sand. DNA was extracted using a NucleoSpin® Plant II (Macherey-Nagel, Düren, Germany) kit according to the manufacturer's instructions and quantified at 260 nm.

Molecular characterization of both *Pyricularia* isolates was performed by DNA sequencing of an internal fragment of the 28S *rDNA* gene. The PCR amplification of a 330-bp of the 3′ end of the 28S rDNA gene was performed as previously described (Qi and Yang, 2002). The amplified products were purified with Multiscreen HTS PCR 96-well filter plates (Millipore). Sequencing reactions were carried out using the ABI Prism BigDye Terminator version 3.1 following manufactures instructions, and the sequences were read with an automatic sequence analyser (3130 genetic analyzer; Applied Biosystems). Sequence analysis was performed using Sequence Scanner Software 2 (http://www.appliedbiosystems.com). Sequence comparison with GenBank nucleotide database was performed using the blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Trichoderma, Pseudomonas and Pyricularia cultivation and inoculum preparation

Trichoderma harzianum Th56 was grown on potato dextrose agar 2% (PDA) plates at 25 °C. This *Trichoderma* isolate, Th56, was obtained from wheat grain microflora and maintained at the culture collection of CIDEFI-FCAyFUNLP (Argentina) at 5 °C due its previous good performance against other wheat pathogens (Perelló et al., 2017). Conidia

were scraped off the plates and collected in distilled water and filtrated with gauze. Concentrations of propagules in suspensions were standardized with the aid of the haemocytometer to 1×10^8 conidia ml^{-1} with Ringer solution.

Pseudomonas stutzeri isolate AN10R was a Rifampicin-resistant spontaneous mutant of *Pseudomonas stutzeri* AN10 (García-Valdés et al., 1988) that was obtained after plating strain AN10 on LB agar (10 g peptone, 5 g yeast extract, 5 g sodium chloride, 15 g agar per litre) containing 70 μg ml^{-1} of rifampicin. We decided to use this Rif^R derivative to easily follow its presence during the experiment. Isolate AN10R was routinely grown on LB agar plates at 30 °C. To prepare the inoculum, bacteria were scraped off the plates and its concentration was adjusted to 1×10^8 cfu ml^{-1} with Ringer solution based on their optical density at 600 nm.

Pyricularia inoculum was prepared from isolates UNLP2 or UNLP10 (Perelló et al., 2015) and grown on PDA plates at 25 °C and kept on a photoperiod of 12 h light:12 h dark to induce growth for 10 days. Conidia were suspended in sterile distilled water and their concentration was adjusted to $5x10^4$ spores ml^{-1} with Ringer solution.

2.4. Plant culture and salinity treatments

Two experiments were carried out in a biosafety level 2 (BSL-2) room at the UIB. *Triticum aestivum* cv. Baguette 18 seeds were imbibed in distilled water for three days. Following, three treatments were set up by soaking the germinating seeds in 0.5 L of the following solutions for 30 min.: Ringer's solution (no BCA), 1×10^8 ufc ml^{-1} of *Trichoderma harzianum* Th56 (Tricho) and 1×10^8 ufc ml^{-1} of *Pseudomonas stutzeri* AN10 (Pseudo). Fifteen seeds were planted per pot in perlite: vermiculite 1:1 in 13 × 10 cm pots (12 pots per BCA treatment) and kept at field capacity with modified Hoagland nutrient solution with 1 mM NaCl (Epstein, 1972). Plants were grown in a growth room at 25 °C, 50% RH, 8 h light:16 h dark photoperiod and PAR 300 μM photons $m^{-2}s^{-1}$ provided by Sylvania Gro-Lux® fluorescent tubes.

Three days after planting, two saline treatments were set up: 1 and 200 mM NaCl. The severe salinity treatment was imposed in a step-wise manner, adding daily increments of 50 mM NaCl during four consecutive days to half of the pots per BCA treatment. Saline treatment was imposed by immersing the pots in the salinized solution and let them drain fully by gravity. Once the final salt concentration was reached, plants were watered alternatively every other day with nutrient solution or distilled water.

2.5. Pyricularia inoculation and disease rating

Twenty days after planting, one third of the pots, holding plants at 3rd leaf stage were inoculated by spraying the leaves until run-off with either distilled water or a spore suspension of *P. oryzae* UNLP2 or UNLP10. After inoculation, all pots were individually kept under transparent covers with zip closures for 2 days to keep plants at saturated relative humidity to favour fungal infection. To further promote the initial development of the fungus, plants were kept in the dark for the first 24 h after the inoculation, followed by an 8 h dark:16 h light photoperiod.

The plants were examined daily for lesion development and progression. At 7 days after pathogen inoculation, disease severity was assessed by rating of the percentage of leaf area showing characteristics symptoms of blast disease (Severity (%) = [diseased leaf area/total leaf area] × 100). It was rated in five seedlings at the 3-leaf stage per pot in all pots holding pathogen-inoculated plants and shown as the average between the first and second leaf lesion percentages.

2.6. Plant growth

Leaf biomass was recorded 7 days after pathogen inoculation in seedlings at the 3-leaf stage. Leaf biomass was individually recorded in five representative plants per pot.

2.7. RNA extraction, cDNA synthesis and qPCR

The effect of salinity, inoculation with BCAs and/or *Pyricularia* isolates on the expressions of pathogenesis-related proteins (*PR2*) and (*PRPI*), phenyl ammonium lyase (*PAL*), chitinase 2 (*CHI2*), guaiacol peroxidase 2 (*POX2*) and lypoxigenase (*LOX*) defence genes was examined 24 h after pathogen inoculation. Combined first and second leaves from three plants per pot in each treatment were harvested and directly immersed into liquid nitrogen, homogenized to fine powder and stored at −80 °C until use. Total RNA of each plant (around 100 mg) was extracted using a NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Extracted RNA was quantified and quality controlled with Nanodrop 2000 (Thermo Scientific, DE, USA). The synthesis of cDNA (adjusted to 1 μg) was obtained with iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA). The cDNA (1:5) was used as a template for quantitative PCRs using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA) and reaction was developed on a CFX384 Real-Time System (Bio-Rad, CA, USA). The plates were edited by the software CFX manager version 3.1. The primers used for the expression are detailed in Table 1. The expression of target genes was normalized to the expression level of *Actin* gene. Treatment influence on relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each sample was three-technically replicated on each reaction.

2.8. Experimental design and data analysis

Two 3 × 2 × 3 factorial experiments of identical design were conducted consisting in 2 salinity treatments (S): 1 and 200 mM NaCl; 3 BCAs treatments (B): no BCA, Tricho and Pseudo; and 3 pathogen treatments (P): no pathogen, UNLP2 and UNLP1 arranged in a randomized block design providing two replications (pots) for each treatment combination (S^*B^*P). Combined data from the two experiments are presented in the results. A three-way ANOVA was run to examine the S^*B^*P interactions. At each salt concentration, data from all studied variables were analysed by two-way ANOVA and means from 1 and 200 mM-treated plants for each BCA and pathogen treatment were compared using Fisher's test ($P < 0.05$) using Statistica 7 (StatSoft Inc., Tulsa, OK, USA). In the figures, error bars represent SE, and significant differences amongst groups are marked by different letters.

Table 1
Primer sequences used to quantify the relative expression of defence marker genes in leaves of *T. aestivum*.

Primers	Putative function	Sequence 5′-3′ (Fw/Rv)	References
Ta PR 2	β-1,3-glucanase	ACAGAGATAGGCGACGAGGA CCATCCTCCCGGCCATAAAG	Ha et al. (2016)
Ta Chi2	Chitinase	AGGAAAATCAACAGTGGCGA TTGCTAGATTCTTGATCGAC	Li et al. (2010)
Ta PRP I	*PDF1.2* homology	TCGCAGAGCCACAACTTCAA CGCAAGTGCTTCTGCAAGAG	Ha et al. (2016)
Ta LOX	Lipoxygenase	CGACCCGCAGCTGTTGA CCCTTGTGATCGGAGGTGTT	Cruz et al. (2015)
Ta PAL	Phenylalanine ammonia-lyase	CGTCAAGAGCTGTGTGAAGATGG GGTAGTTGGAGCTGCAAGGGTC	Zhang et al. (2011)
Ta POX 2	Peroxidase	CAACGACACCACCGACAACA AAGGTGAACTCGTGATGGAC	Li et al. (2010)
Ta Actin	Reference gene	GCTGTTCCAGCCATCTCATGT GTTTCCTGGAATTGCTGATC	Li et al. (2010)

Table 2
Analysis of variance of the effects of salt (S), BCAs (B) and *P. oryzae* infection (P) on leaf fresh weight (LFW), leaf infection severity (LIS) and the expression of pathogenesis-related protein (*PRPI*), chitinase 2 (*CHI2*), lipoxygenase (*LOX*), phenyl ammonium lyase (*PAL*), guaiacol peroxidase (*POX2*), pathogenesis-related protein (*PR2*). Bold values are significant ($P < 0.05$).

Sources of variation	*F* values							
	LFW	LIS	*PRPI*	*CHI2*	*LOX*	*PAL*	*POX2*	*PR2*
S	**207,7**	**85,7**	**15,0**	**23,9**	**31,8**	1,7	**20,9**	0,05
B	**4,7**	**34,9**	**12,3**	**11,5**	**8,5**	**6,1**	2,7	1,56
P	**229,6**	**798,5**	**50,7**	**33,5**	**31,0**	**69,3**	**12,6**	2,32
S*B	0,8	**3,5**	**10,5**	**12,0**	**13,2**	**10,8**	**5,8**	**3,18**
S*P	**58,0**	**45,9**	**16,7**	**20,6**	**28,9**	**4,9**	**10,9**	**5,69**
B*P	**7,8**	**52,3**	**19,6**	**10,3**	**11,9**	**28,5**	**5,5**	**4,32**
S*B*P	**11,6**	**16,1**	**10,3**	**10,1**	**13,0**	**7,4**	**3,3**	**4,37**

3. Results

3.1. Blast pathogens molecular identification

The DNA sequencing of an internal fragment of the 28S *rDNA* gene showed 100% homology with the corresponding *Pyricularia oryzae* GenBank sequence (AB026819.1). This confirms that the UNLP2 and UNPL10 pathogenic isolates belonged to *Pyricularia oryzae* species.

3.2. Leaf growth response to severe salinity, BCAs and P. oryzae inoculation

A significant three-way interaction was found amongst salt, BCAs and *Pyricularia* isolates on leaf fresh weight and leaf infection severity (Table 2).

Salinity markedly decreased plant growth (see no pathogen treatment in Fig. 1a and b). No BCA no pathogen-treated plants grown at 200 mM NaCl for 20 days had 51% lower leaf fresh weight than controls (Fig. 2a and b). Nonetheless, differences in fresh weight between salt treatments could have partly be due to lower relative leaf water content in 200 mM NaCl-treated plants. For each salt concentration, a significant interaction was found between BCAs and *Pyricularia* isolates on leaf fresh weight (Table S1). Neither *Pseudomonas* nor *Trichoderma* ameliorated the inhibitory effect of 200 mM NaCl on leaf growth (Fig. 2b). In plants not exposed to the pathogen, only seed inoculation with *Pseudomonas* significantly increased leaf fresh weight (38%), while no difference in leaf growth between *Trichoderma* and no BCA plants were found (Fig. 2a).

Both *Pyricularia* isolates showed compatible interactions with wheat cv. Baguette 18. The wheat leaves affected by *Pyricularia* disease presented clear necrotic lesions with or without chlorosis symptoms. Nonetheless, isolate UNLP10 was much more aggressive than UNLP2 (Fig. 1a and b). Two days after inoculation, visual symptoms of blast disease were already important and spreading fast. Within both isolates, the infection started and was much more intense at the leaf apex, where lesions were so frequent that almost contacted one another and, from there, the symptoms advanced to the leaf base (Fig. 1c).

The effect of *Pyricularia* disease on leaf growth decreased in 200 mM NaCl-treated plants, with leaf fresh weight showing a 39% and 65% decline in *Pyricularia* isolates UNLP2 and UNLP10 with respect to 200 mM NaCl no pathogen-inoculated plants (Fig. 2b and c). The BCA treatment had a significant different effect on the plant response to blast disease (Table S1). In 1 mM NaCl UNLP2-inoculated plants, *Trichoderma* reduced leaf growth inhibition and leaf infection severity, while *Pseudomonas* showed no effect (Fig. 2a and c). Remarkably, BCAs neutralized the inhibitory effect of *Pyricularia* UNLP2 in 200 mM NaCl plants and therefore, in the BCAs treatments no significant differences in leaf fresh weight was found between plants that had been inoculated, or not, with the *Pyricularia* UNLP2 isolate (Fig. 2b). In contrast, BCAs did not significantly improve leaf growth in 200 mM NaCl-treated plants inoculated with the more aggressive isolate UNLP10 (Fig. 2b). Nonetheless, *Pseudomonas* and *Trichoderma* reduced leaf infection severity in 200 mM NaCl-treated plants inoculated with UNLP10 (Fig. 2d).

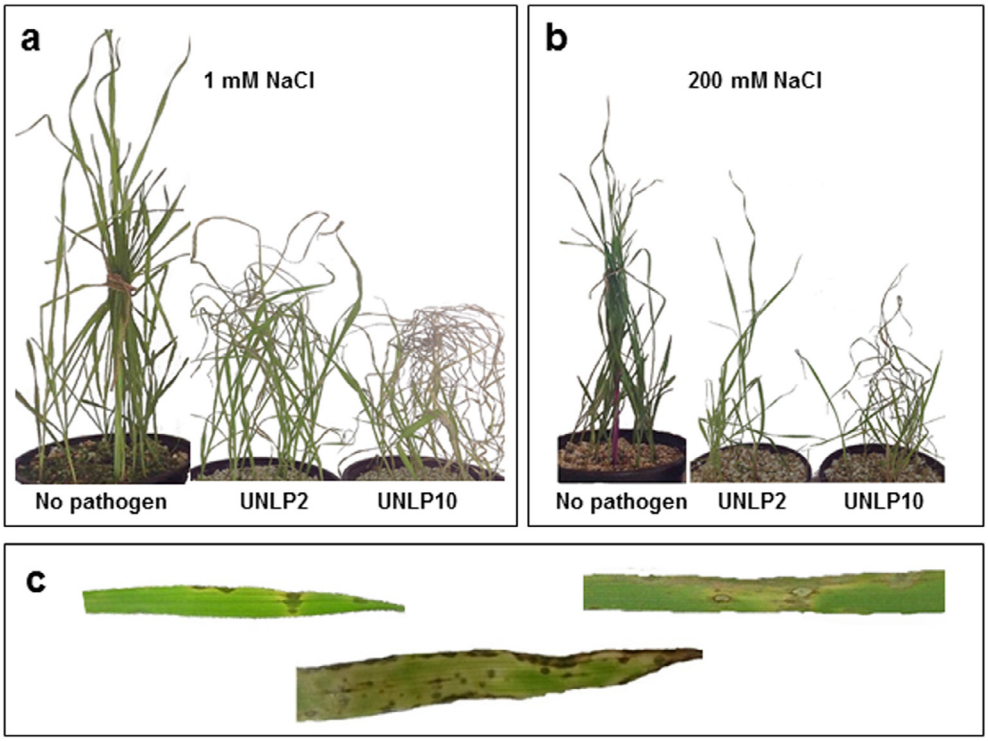

Fig. 1. Wheat plants cv. Baguette18 grown for 27 days at 1 mM (a) or 200 mM (b) aCl, seven days after inoculation with *P. oryzae* isolates UNLP2 (left) or UNLP10 (right). Wheat blast symptoms (c).

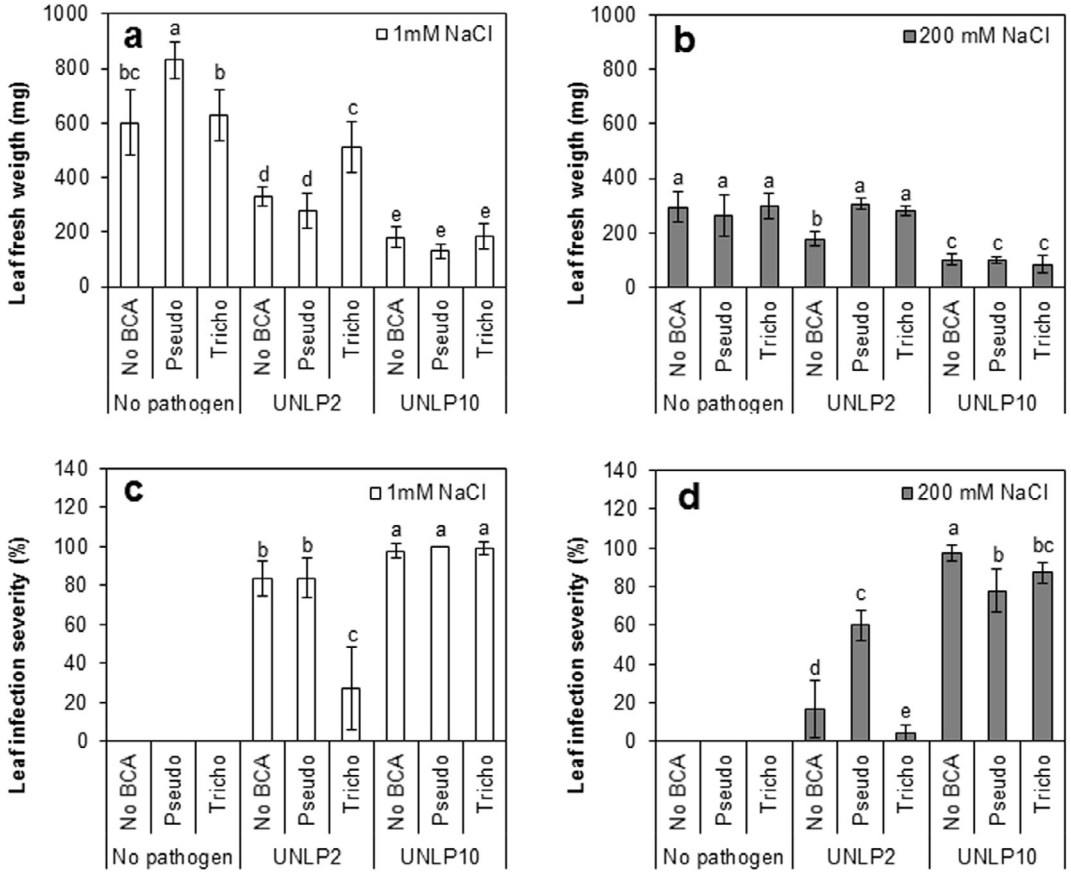

Fig. 2. Leaf fresh weight and leaf infection severity of wheat plants cv. Baguette 18 grown with 1 mM Na Cl (a and c) or 200 mM NaCl (b and d) for 27 days, 7 days after inoculation with two *P. oryzae* isolates, UNLP2 or UNLP10. Before planting, one third of the seeds were inoculated with *P. stutzeri* AN10 or *T. harzianum* Th56. Data are means of four replications from two independent experiments. Each replication consists of five plants. Two-way ANOVA followed by Fisher's LSD test were performed. Error bars represent SE and significant differences are marked by different letters ($p \leq 0.05$).

3.3. Salt, BCAs and P. oryzae effects on the expression of wheat defence genes

The effects of high salt, *P. stutzeri* AN10 and *T. harzianum* Th56 in the relative expression of defence genes of wheat plants was examined 24 h after the inoculation (24 hai) with *P. oryzae* (Figs. 3 and 4). Previous results in compatible interactions showed that rice cells containing *P. oryzae* invasive hyphae plasmolyzed at 27 hai (Kankanala et al., 2007), and consequently, defence pathway activation analysis should be conducted before 27 hai (Bagnaresi et al., 2012).

The expression of most of the defence genes studied in wheat cv. Baguette 18 was significantly affected by salinity, BCAs and *Pyricularia* treatments and for the two-way and three-way interactions (Tables 2).

In the no pathogen treatments, 200 mM NaCl did not triggered the relative expression of defence genes. On the contrary, the expression of *PAL* was decreased by 200 mM NaCl, with no differences amongst BCAs treatments (Figs. 3 and 4).

In plants grown at 1 mM NaCl, a significant interaction between BCAs and *Pyricularia* isolates in the expression of the defence genes was only found for the SA-dependent signalling marker genes PRPI and PAL, while in 200 mM-treated plants, the interaction was significant for all tested genes (Table S1). *P. oryzae* isolate UNLP2 triggered the expression of defence genes almost exclusively in BCAs-inoculated 200 mM NaCl-stressed plants. *CHI2*, *LOX* and, *POX2* gene markers of the JA/ET, JA and ET signalling pathways, respectively, showed an increased expression in 200 mM NaCl -stressed plants inoculated with *Pseudomonas* and even a higher increase in *Trichoderma*-inoculated plants (Fig. 4b, d and f). A similar pattern was found for the SA-dependent gene markers *PR2*, *PRPI* and PAL with some changes. *PR2* did not increase in 200 mM NaCl -stressed plants inoculated with *Pseudomonas* (Fig. 3b) and *PRP1* and *PAL* increased in both salt and BCAs treatments (Fig. 3d and f).

Defence gene expression was little affected in plants inoculated with the most aggressive *P. oryzae* isolate, UNLP10. Nonetheless, a significant increase was found in the expression of *PAL*. The relative expression of this gene highly increased in 1 mM NaCl no BCA-treated plants and in 200 mM NaCl plants inoculated with *Trichoderma* (Fig. 3e and f).

4. Discussion

Under field conditions, crop plants are exposed to multiple stress situations; nonetheless, still few studies in the literature have focused on the plant response to simultaneous biotic and abiotic stress situations (Atkinson and Urwin, 2012). Combined stresses trigger a cross talk among different cell signalling pathways yielding either additive or antagonistic interactions (Asselbergh et al., 2008; Atkinson and Urwin, 2012; Mittler and Blumwald, 2010). Here, we tested the response of 200 mM NaCl -stressed wheat seedlings to the infection of the devastating pathogenic fungus, *P. oryzae*, in the presence or absence of BCAs.

Wheat is a fairly salt tolerant crop, which shows a high variability to saline conditions (Munns et al., 2000). The salt threshold concentration below which wheat does not show a growth reduction was set to 60–80 mM NaCl (Mass and Hoffman, 1977). The commercial cultivar used in this study, cv. Baguette 18, showed a 50% decreased in seedling growth at 200 mM NaCl. The reason for using this high salt concentration was the need to grow plants under a high relative humidity to favour fungal spread (An et al., 2001). The same salt concentration in a dryer atmosphere may have caused a substantially stronger plant

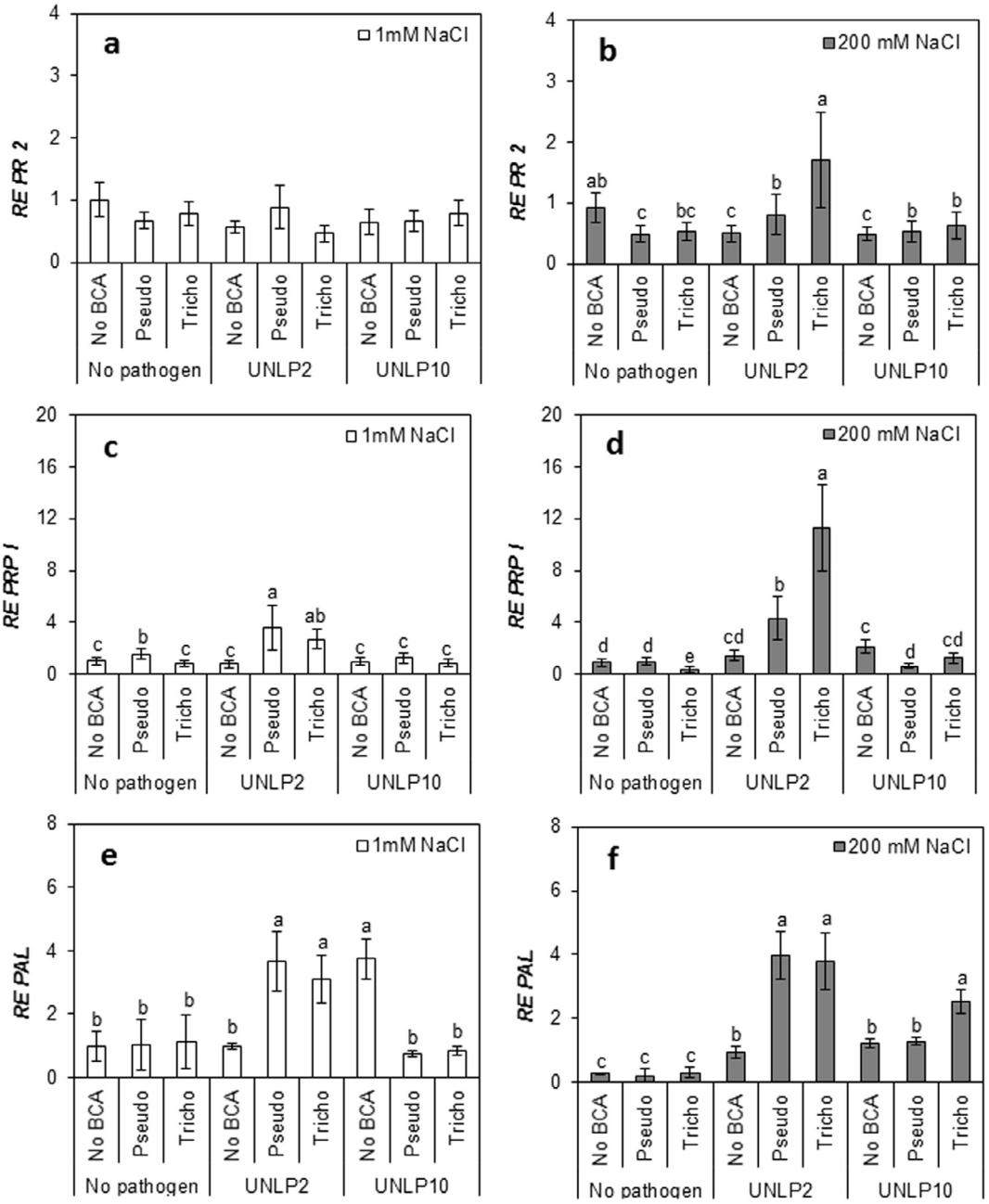

Fig. 3. Relative expression of defence marker genes *PR2*, *PRPI* and *PAL* in leaves of wheat plants cv. Baguette 18 grown with 1 mM Na Cl (a, c and e) or 200 mM NaCl (b, d and f) for 27 days, 7 days after inoculation with two *P. oryzae* isolates, UNLP2 or UNLP10. Before planting, one third of the seeds were inoculated with *P. stutzeri* AN10 or *T. harzianum* Th56. *Actin* gene from *T. aestivum* was used as a reference gene and no pathogen no BCA group was set as a calibrator (RE = 1). Data are means of three replications from two independent experiments. Each replication consists of five plants. Two-way ANOVA followed by Fisher's LSD test were performed. Error bars represent SE and significant differences are marked by different letters ($p \leq 0.05$).

growth inhibitory effect.

Many *Pseudomonas* are qualified as plant growth-promoting rhizobacteria, as they have found to enhance plant growth and health (Glick, 1995; Haas and Défago, 2005). In this line, *Pseudomonas stutzeri* AN10 greatly promoted leaf growth in non-salinized plants. This growth promoting activity in wheat seedlings could be related to the capacity of *P. stutzeri* AN10 for indole-3-acetic acid production, which has already been identified in different strains of this species (Desnoues et al., 2003; Pedraza et al., 2004).

Trichoderma species are fungi widely used as biofertilizer and BCAs due to their positive effect on plant development and productivity (Harman, 2006). On wheat plants, the effectiveness of *Trichoderma* spp. against seedborne and foliar fungal pathogens was previously demonstrated under greenhouse and field conditions (Larrán et al., 2016; Perelló and Mónaco, 2007; Perelló and Dal Bello, 2011). However, in our experimental conditions, *T. harzianum* Th56 did not affect plant growth in 1 mM NaCl plants. Moreover, neither *P. stutzeri* AN10 nor *T. harzianum* Th56 ameliorated the plant's response to 200 mM NaCl. Mastouri et al. (2010) reported that *T. harzianum* alleviated salt stress in tomato germinating seeds and seedlings, while *Trichoderma asperelloides* was found to enhance *Arabidopsis* tolerance to salt stress by reducing the ethylene levels and activating the antioxidant defences (Brotman et al., 2013). However, the *Trichoderma*'s beneficial effects reported by these authors were found at much lower salt treatments than the one used here. This high salinity could also have negatively affected the PGPR activity of *Pseudomonas*, as the salt concentration

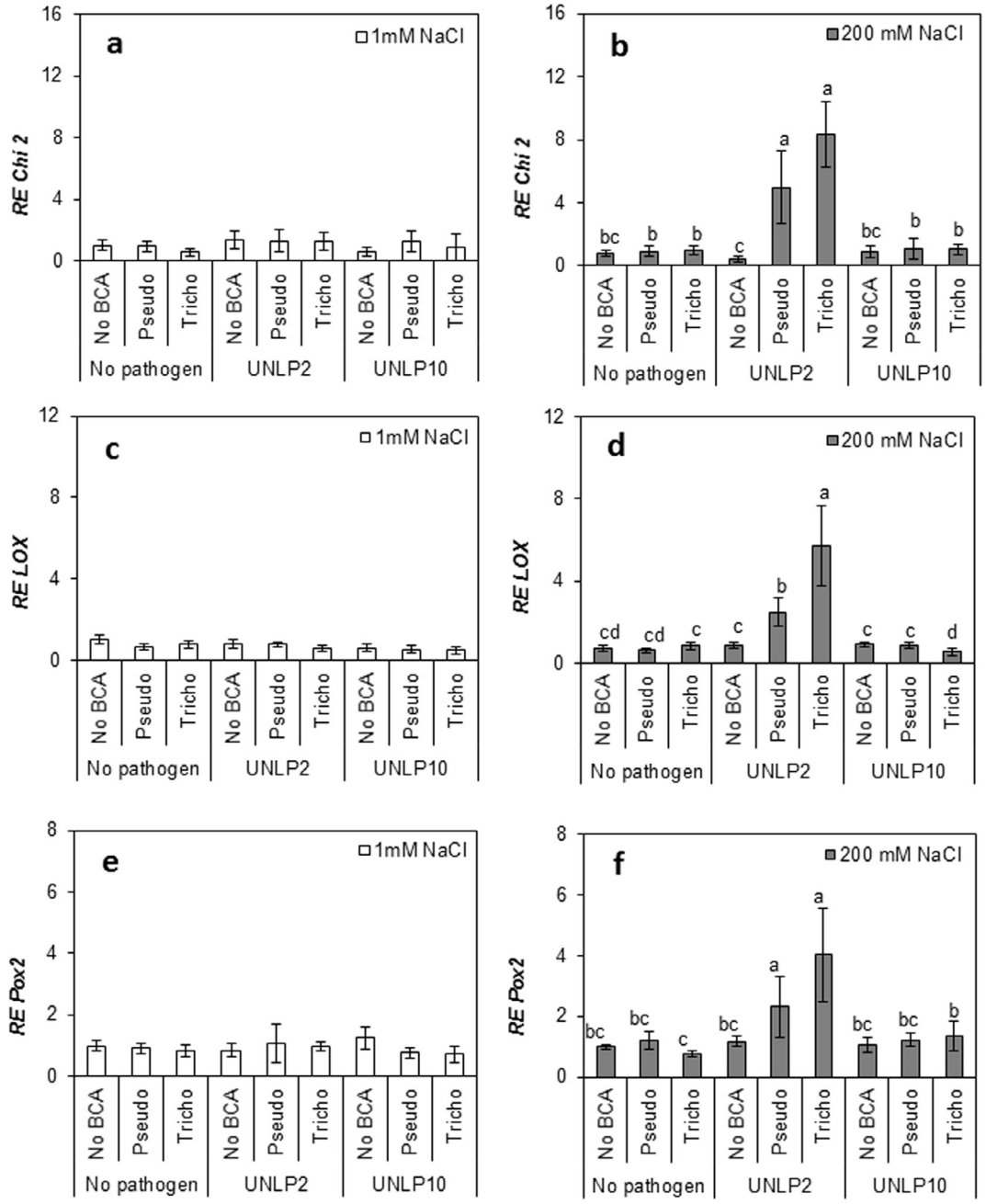

Fig. 4. Relative expression of defence marker genes *CHI2*, *LOX* and *POX2* in leaves of wheat plants cv. Baguette 18 grown with 1 mM Na Cl (a, c and e) or 200 mM NaCl (b, d and f) for 27 days, 7 days after inoculation with two *P. oryzae* isolates, UNLP2 or UNLP10. Before planting, one third of the seeds were inoculated with *P. stutzeri* AN10 or *T. harzianum* Th56. *Actin* gene from *T. aestivum* was used as a reference gene and no pathogen no BCA group was set as a calibrator (RE = 1). Data are means of three replications from two independent experiments. Each replication consists of five plants. Two-way ANOVA followed by Fisher's LSD test were performed. Error bars represent SE and significant differences are marked by different letters ($p \leq 0.05$).

used in this study, 200 mM, is well above the range of optimal NaCl concentrations that were reported to produce an optimal IAA concentration in different *Pseudomonads* (Deshwal and Kumar, 2013).

Salinity increases ABA concentration in plant tissues by suppressing the SA defence pathway and therefore, making salt-stressed plants more susceptible to pathogens (Thaler and Bostock, 2004). Moreover, exogenous application of ABA enhanced rice susceptibility to *Magnaporthe grisea* (Koga et al., 2004). In addition, salinity can seep out plant energy resources and increase its susceptibility to pathogens (Mittler and Blumwald, 2010). Notably, our results showed that at high salinity, *Pyricularia*-induced leaf growth inhibition was lower than in plants exposed to 1 mM NaCl plants. In wheat, both salinity and infection with *Blumeria graminis* have been reported to activate the transcription of the ethylene-responsive factor *ERF1*. Moreover, the overexpression of *TaERF1* triggered the expression of *PR2* gene in non-stressed plants and improved the tolerance to pathogen and salt stress in transgenic plants (Xu et al., 2007). In contrast, our results showed no increased expression of *PR2* gene in wheat plants subjected to either salt stress or to *Pyricularia* infection.

Trichoderma and *Pseudomonas* are BCAs with confirmed high capacity to ameliorate the plant response to pathogens (Howell, 2003; Santoyo et al., 2012). Here, in 1 mM NaCl-treated plants, only *Trichoderma* ameliorated the leaf response to *Pyricularia* disease caused by the isolate UNLP2. This could be due to a direct interaction of *Trichoderma*

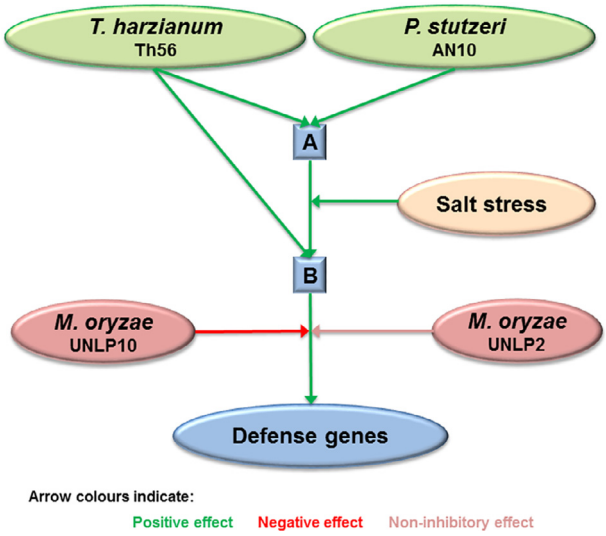

Fig. 5. Illustration of possible mechanisms involved in the contrasting response of wheat plants cv. Baguette 18 to the inoculation with the *P. oryzae* isolates, UNLP2 and UNLP10. The decreased severity and ameliorated leaf growth response of wheat plants to *P. oryzae* infection found in salt-stressed *P. stutzeri*-inoculated plants and in salt- and non-salt treated *T. harzianum*-inoculated plants suggests the sequential participation of at least two signalling components, A and B. Nonetheless, the effect of the B component is highly dependent on the *Pyricularia* isolates.

with this less virulent *Pyricularia* isolate (Punja and Utkhede, 2003), while no beneficial *Trichoderma* effect was found in plants infected with the isolate UNLP10. However, only in plants inoculated with the isolate UNLP2, *Trichoderma* triggered the plant biotic defence response (Conrath, 2011).

The different response of *Pyricularia* isolates UNLP2 and UNLP10 to the BCAs is consistent with the presence of diverse resistance capacities within pathogen populations. In fact, none of the two pathogen isolates had the same resistance phenotype, illustrating variation in the potential for effective antagonist suppression within the Argentinian pathogen population. This variation represents a significant challenge for consistent biological control of wheat blast. Information about the extent of diversity in resistance to antagonist inhibition within fields or across the wheat agricultural regions will be crucial to identifying optimal antagonist combinations that may be both most likely to suppress local *Pyricularia* populations and least likely to select for substantial short-term resistance to suppression.

Although some studies have considered that the ISR is independent of SA and associated to ET and JA (van Loon et al., 1998; Pieterse et al., 2009), our results at 24 hai showed an increase in the expression of the marker genes of the SA-dependent signalling pathway, *PRP1* and especially of the gene that codifies for *PAL*. These results agree with the ISR induced by *Trichoderma hamatum* in *Botrytis cinerea*-infected *Arabidopsis* plants, which showed enhanced expression of *PR2* and *PAL* (Mathys et al., 2012). Our results support a role for the SA pathway in the *Trichoderma*-induced ISR in wheat seedling inoculated with *P. oryzae*. Notably, in these non-salinized plants, no *Trichoderma*-enhanced effect was found in the expression of the marker genes of the JA, ET or JA/ET dependent signalling pathways.

In salt-stressed seedlings, the ameliorating effect of both BCAs on the leaf growth response to *Pyricularia* isolate UNLP2 was related with SA, but also with JA and ET. Both *Pseudomonas* and *Trichoderma*, in addition to an increase expression of the SA-dependent marker genes, showed higher expression of the JA/ET, JA and ET marker genes *CHI2*, *LOX* and *POX2*, respectively. This agrees with previous studies reporting that BCAs-induced ISR was simultaneously triggered by SA, JA and ET signalling pathways (Niu et al., 2011).

PAL was the only defence gene whose relative expression was increased in plants inoculated with the most virulent *Pyricularia* isolates, UNLP10. Although enhanced transcripts of PAL were found in 1 mM and in 200 mM NaCl- treated plants inoculated with UNLP10, these plants showed no amelioration of leaf growth.

In addition to other virulence mechanisms, *Pyricularia* UNLP10 could have evolved a more efficient system than isolate UNLP2 for an effector-mediated manipulation of the plant's hormone homeostasis; for example, targeting plant hormones that are primarily involved in plant defence, thus causing the specific increased susceptibility of wheat plants to this isolate. This effector-activated susceptibility conforms to the interactions between host plants and hemibiotrophic bacteria and fungi such as *P. oryzae* (reviewed by Kazan et al., 2014). The exchange of molecular signals between plants and pathogens builds highly complex networks with interactions that are nowadays still poorly understood. In infected rice plants with *P. oryzae*, a total of 851 genes encoding predicted effector proteins have been identified (Liu et al., 2013).

To summarize, a scheme of possible mechanisms underlying the complex interacting signalling network triggered by high salinity, BCAs and *P. oryzae* infection is shown in Fig. 5. BCAs were prevailing priming factors (state A), however, they only induced plant defence responses to subsequent *P. oryzae* attack in salt-stressed plants, which indicates the need to assess another priming state (B) to overcome the *P. oryzae* effector-induced susceptibility. Even though, the final expression of marker genes for the biotic defence pathways was dependent on the *Pyricularia* isolate. This is supported by the fact that priming of wheat seedlings by both BCAs and salinity was not able to overcome the effector-mediated restrain on defence hormone signalling exerted by the most virulent *Pyricularia* isolate, UNLP10.

These differences in effective disease suppression by BCAs reflect the complex array of factors likely to influence the success of antagonists in suppressing pathogens, including, variation in *Pyricularia* populations and salinity conditions, especially in their response to antagonists. Therefore, in order to better understand the impact of stress combinations on wheat plants when BCAs are co-occurring modulating the blast disease expression, further experiments are imperative to assess its effects under field conditions. Breeders and field pathologists can use results from such studies to better analyse the performance of the resistant/tolerant wheat genotypes under field conditions, particularly in climatic change scenarios where concurrence of stresses is prevalent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biocontrol.2018.07.003.

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