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Full Length Research Paper

Marker-assisted pyramiding of *Xa21* and *Xa7* genes conferring resistance to bacterial leaf blight in *indica* cultivar Bacthom7

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Bacterial leaf blight (BLB) of rice is one of the most destructive diseases affecting rice fields. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the causal agent of BLB. Two BLB resistance genes, *Xa21* and *Xa7*, were transferred into the susceptible *indica* cultivar Bacthom7 (BT7) by using marker-assisted selection with markers *pTA248* for *Xa21* and *ID7* for *Xa7*. Improved BT7 lines carrying the two resistance genes were inoculated with three isolates of the *Xoo* from Northern Vietnam and evaluated for agronomic traits. Artificial inoculation of 13 lines with three *Xoo* races identified nine highly resistant lines with wide-spectrum resistance to *Xoo,* including D1, D2, D3, D6, D7, D8, D9, D10 and D12. These lines were similar to recurrent parent BT7 with regard to external appearance, yield performance and grain quality. On the basis of agronomic traits and the level of resistance to BLB, two promising lines, D6 and D9 were further selected. These two lines could efficiently contribute to rice production for food security and food safety in northern Vietnam.

Key words: *Xanthomonas oryzae* pv. *oryzae*, resistance genes, near-isogenic lines, marker-assisted selection (MAS), improved rice lines.

INTRODUCTION

Bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most devastating diseases in rice fields in Asia. The disease has recently become more serious in northern Vietnam because it

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now appears during two crop seasons, especially among hybrid rice varieties. BLB caused yield decreases of up to 60% (Mew et al., 1982). Thousands of hectares of cultivated land are affected by BLB annually in India, with yield losses amounting up to 60% (Sirivastava, 1972). BLB spreads widely during the summer season in coastal areas of northern Vietnam (Plant Protection Department, MARD). Areas affected by BLB increased by 30 to 70% in 2012. Although BLB can occur at any stage and in any organ of rice plants, infection particularly reduces yield at the booting, heading and milk stages. However, practical chemical methods that can be applied to control BLB remain to be established. Therefore, deployment of resistant varieties and integrated pest management are important solutions to controlling BLB. Thus far, a total of 38 BLB-resistant genes have been identified in rice (Khan et al., 2014). Among them, four BLB-resistant genes were mapped on rice chromosome 4 (*Xa1*, *Xa2*, *Xa14* and *Xa25*), one on chromosome 5 (*xa5*), one on chromosome 6 (*Xa7*), one on chromosome 8 (*xa13*) and six on chromosome 11 (*Xa3*, *Xa4*, *Xa10*, *Xa21*, *Xa22* and *Xa23*). The locations of the remaining BLB-resistant genes are still ambiguous.

Near-isogenic lines (NILs) and pyramided lines (PYLs) that are almost identical to parental lines, except target genes, are very useful genetic resources for genetic improvements to rice. The NILs can be used to introduce a target gene into improved rice cultivars without inducing any adverse effects such as sterility or unfavorable linkage drags such as tall plant height (Yara et al., 2010). However, the pathogen can evolve to overcome a resistant cultivar that carries a single resistance gene after large-scale and long-term cultivation. *Xa4* showed resistance to BLB in the Philippines in the 1970s, but this was later overcome (Mew et al., 1992). Recently, *Xa21* caused a reduction in the resistance level of *Xoo* races in the Philippines, India, Korea and China (Lee et al., 1999; Marella et al., 2001; Xu et al., 2012). In contrast, PYLs that carry more than two BLB resistance genes showed more durability and a higher level of resistance to BLB than lines carrying a single resistance gene (Pradhan et al., 2015). PYLs can delay the emergence of virulent *Xoo* races against BLB resistance genes. However, pyramided resistance genes, which show similar reactions to BLB, are difficult to develop through conventional breeding methods. Marker-assisted selection (MAS) has unique advantages to overcome this limitation because MAS relies on DNA polymorphism rather than phenotypic selection (Collard and Mackill, 2008).

Bacthom7 (BT7) is a high-quality cultivar and is widely cultivated in northern Vietnam but is also susceptible to *Xoo*. Among the reported resistance genes, *Xa7* and *Xa21* showed wide-spectrum resistance in Asia (Vera Cruz et al., 2000; Webb et al., 2010). Thus, one of the promising strategies to effectively improve the resistance level of BT7 is pyramiding these two resistance genes. Previously, a BT7-carrying BLB resistance gene *Xa21* (BT7-*Xa21*) was developed and released as a new variety, BT7KBL.

Therefore, the objective of the present study was to improve BLB resistance by pyramiding two resistance genes, *Xa7* and *Xa21* into *indica* cultivar Bacthom7 (BT7). In this study, MAS was applied to improve accuracy as well as efficiency of gene pyramiding.

MATERIALS AND METHODS

BT7-*Xa21*, which carries BLB resistance gene *Xa21* in a genetic background of BT7, was used as recurrent parent. One IR24 NIL, IRBB7 was used as donor parent for *Xa7* and IR24 was used as a susceptible control in BLB resistance evaluation. The materials were planted at Vietnam National University of Agriculture, Hanoi, Vietnam. To produce F_1 plants, BT7-*Xa21* was crossed with IRBB7 (Figure 1). The F_1 plants were backcrossed with BT7-*Xa21*. In the BC_1F_1 generation, MAS was used to select plants with resistance alleles of *Xa21* and *Xa7*. A similar strategy was applied until the BC_4F_2 generation. The BC_4F_2 plants were then self-pollinated to produce a BC_4F_3 generation. Finally, 13 BC_4F_3 lines carrying the two BLB resistance genes *Xa21* and *Xa7* were inoculated with 3 *Xoo* races and evaluated for agronomic traits.

Isolation of Xoo strains and evaluation of BLB resistance level

BLB-infected rice leaf samples were collected in farmers' fields in Tuyen Quang, Nam Dinh, and Thanh Hoa provinces from 2012 to 2014 (Table 1). The isolation, culture and artificial infection was done following Furuya et al. (2012). The infected leaf was cut into 1-cm-long specimens and sterilized with 70% ethanol followed by 1% H_2O_2 solution. Each sample was soaked in 1 ml of distilled water and the solution was streaked on Wakimoto medium. To develop bacterial colonies, the culture was kept on a bench at room temperature for 4 days.

Yellow bacterial colonies were picked and transferred to a new clean Wakimoto medium and further cultured for 2 days. The cultured *Xoo* colonies were diluted to about 10^9 cfu/ml for artificial inoculation. Plant inoculation was carried out by clipping the tip of leaf (about 2 to 3 cm) with scissors that were dipped into the bacterial solution. The lesion lengths (cm) on the inoculated leaves were measured at 18 days after inoculation. The level of resistance was categorized as follows: lesion length <4.0 cm was highly resistant (HR), 4.0 to 8.0 cm was resistant (R), 8.0 to 12.0 cm was moderately resistant (MR), and >12.0 cm was susceptible (S).

DNA isolation and marker-assisted selection

Marker-assisted backcross was conducted to select plants that carried *Xa7* and *Xa21*. At the BC_1F_1 generation, plants homozygous for *Xa21* and heterozygous for *Xa7* were selected. *ID7* marker (forward 5′-ATA TTC ACC AAA TCA TTC CCT C-3′, reverse 5′-ATA CAA CCC TAA ACC CAT CTC A-3′) was applied to select plants that carried *Xa7* (Zhang et al., 2009). *pTA248* markers (forward 5′-AGA CGC GGA AGG GTG GTT CCC GGA-3′, reverse 5′-AGA CGC GGT AAT CGA AAG ATG AAA-3′) linked to *Xa21* were used to select the plants that carried *Xa21* (Williams et al., 1992).

Leaves (1.0 to 2.0 cm long) were harvested at mature or young stages and stored in a deep freezer for long-term storage or a refrigerator for short-term storage until use. Two DNA extraction methods were used: the CTAB method (Varghese et al., 1997) or the TPS method (Monna et al., 2002). The extracted DNA was dissolved into half strength of TE and diluted to 50% with H_2O just before PCR preparation. PCR was conducted in Gene Atlas (Astes,

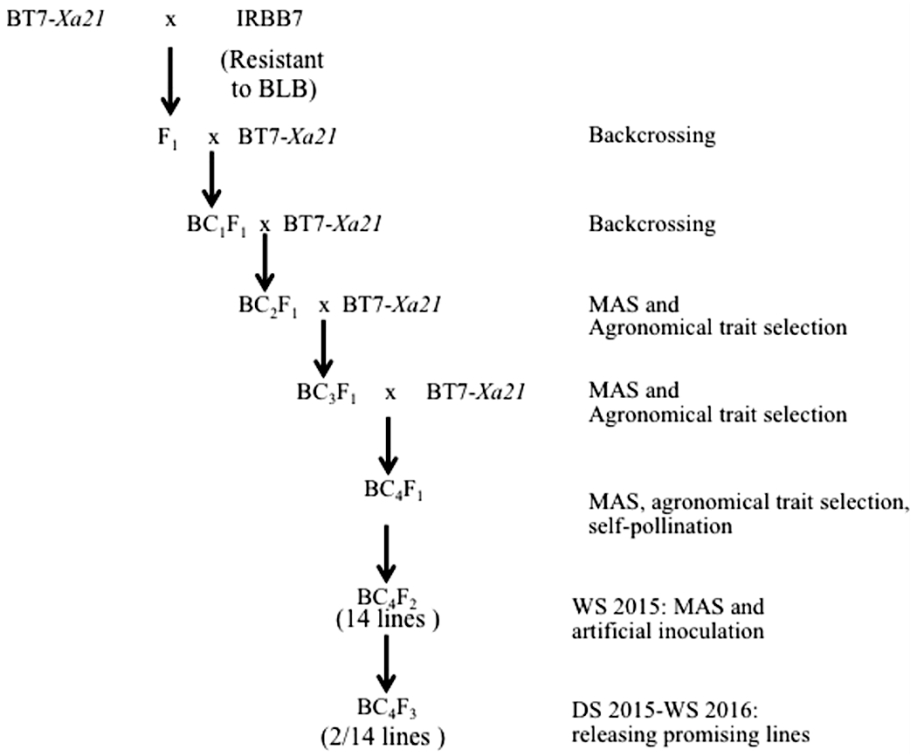

Figure 1. Breeding scheme for marker-assisted backcrossing of *Xa7* in a genetic background of BT7-*Xa21*. The numbers of plants selected for backcrossing or self-pollination and the total plant numbers are indicated in parentheses. MAS, marker-assisted selection, WS, winter-spring season, DS, summer season.

Table 1. *Xoo* races used in this study.

Race No.	Isolate name	Collected on rice varieties	Collection location	Date of collection
Race 3	HUA 012035-3	BC15	Kim Phu, Yen Son, Tuyen Quang	18/09/2012
Race 5	HUA 014042-3	Thai Xuyen	Quang Chau, Quang Xuong, Thanh Hoa	10/10/2014
Race 14	HUA 013031-1	Bac Thom 7	Minh Tan,Vu Ban, Nam Đinh	13/09/2013

Fukuora, Japan). The PCR reaction mixture (10 µl) contained 5 µl of Dream Taq Green PCR Master mix (Thermo Scientific, Waltham, MA, USA), 0.15 µl of primers (0.3 µM each), 2 µl DNA solution and 2.7 µl H_2O. The thermal cycler was programmed as follows: initial denaturation for 2 min at 95°C (*pTA248*) or 5 min at 95°C (*ID7*); followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were separated in 1% agarose gels (*pTA248*) or 2% (*ID7*) by electrophoresis at 100 V for 45 min in TAE buffer. Gels were stained in ethidium bromide solution and then photographed under ultraviolet light.

Evaluation of agronomic traits

Rice lines were evaluated in the field at Vietnam National University of Agriculture, Hanoi, Vietnam during the spring season (January to June) in 2016. Plants were numbered and grown in numerical order in nursery beds that were 5 m in length with row spacing of 20 cm and plants were spaced 20 cm apart. Seven agronomic traits were evaluated in the BC_4F_3 individuals that were homozygous for *Xa7* and *Xa21.* The traits investigated comprised days to heading (DH), plant height (PH), panicle length (PL), number of spikelets per panicle (NSP), number of grains (NG), number of panicles per plant (NPP) and 1000-grain weight (TGW) (Huang et al., 2012; Yara et al., 2010). Aromatic testing was performed according to the method described by Kibria et al. (2008). Briefly, 40 brown rice seeds were placed in a test tube and 5 ml of 1.7% (v/v) KOH was added. The tube was sealed and kept at room temperature for 15 min. Evaluation of aroma was performed by panelists and scored from grades of 1 to 9.

Data analysis

Analysis of variance (ANOVA) was performed to test the differences

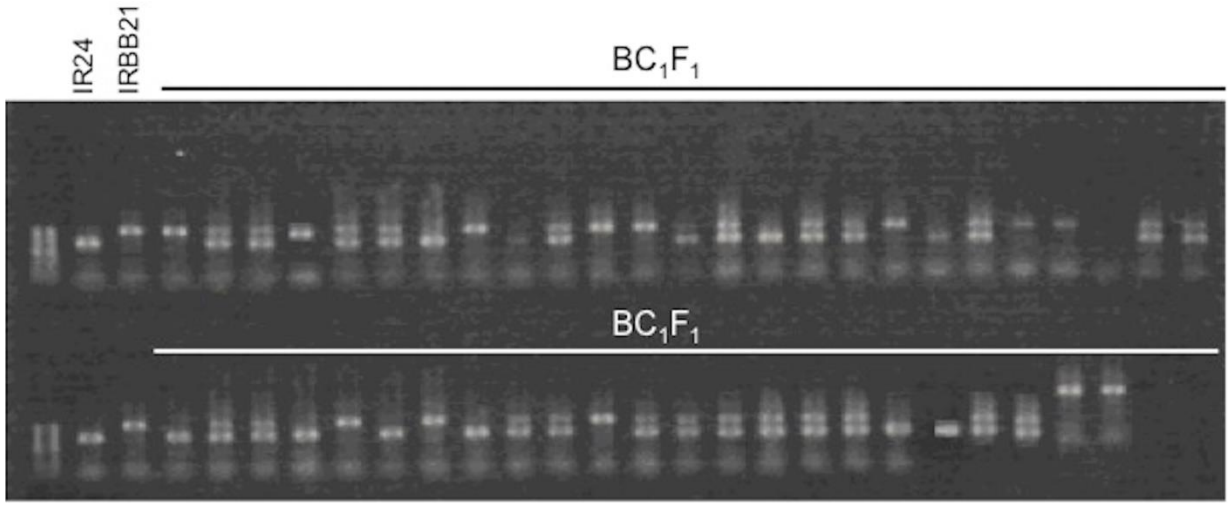

Figure 2. PCR analysis of the parental lines and BC_1F_1 plants. DNA was amplified with *pTA248* that was linked to *Xa21*. IR24 (*xa21*/*xa21*) and IRBB21 (*Xa21*/*Xa21*) were used as controls for PCR amplification.

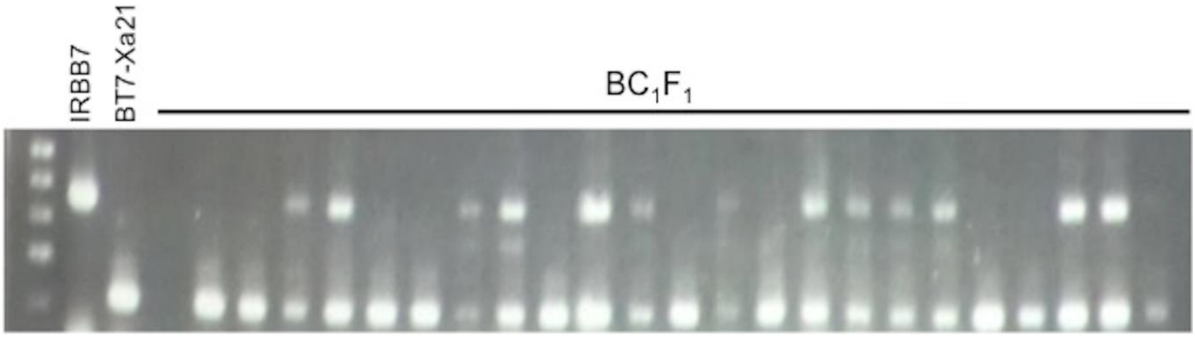

Figure 3. PCR analysis of the parental lines and BC_1F_1 plants. DNA was amplified with *ID7* that was linked to *Xa7*. BT7-*Xa21* (*xa7*/*xa7*) and IRBB7 (*Xa7*/*Xa7*) were used as controls for PCR amplification.

in the response to BLB and agronomic traits among the lines and parents. The values of each line were averaged for 10 individuals in each line.

RESULTS

Marker-assisted selection of *Xa21* and *Xa7*

At the BC_1F_1 generation, a *pTA248* marker was used to select plants that were homozygous for *Xa21* (Figure 2). In total, 21 out of 96 plants were homozygous for *Xa21*. These plants were used for marker-assisted selection of *Xa7* by using an *ID7* marker (Figure 3). Among the 21 plants, 13 were heterozygous at *Xa7*

An evaluation of agronomic traits were performed for 263 BC2F1 plants and 46 plants that were similar to recurrent parents were selected for genotyping. Among the 46 plants, 21 were heterozygous at *Xa7*. These plants were backcrossed to recurrent parents to generate a BC_3F_1 generation. Similarly, among 187 BC_3F_1 plants, 46 were selected for genotyping. Finally, 13 plants that were heterozygous for *Xa7* were backcrossed to recurrent parents to generate a BC_4F_1 generation. The seeds of the BC_4F_1 generation were planted to generate 302 BC_4F_2 lines. Among them, 45 were first selected based on the agronomic traits and were then checked for *Xa7* (Figure 4). Finally, 14 lines were sown separately into 14 BC_4F_3 lines.

Artificial inoculation of BC_4F_3 lines carrying *Xa21* and *Xa7*

Three Xoo races, which were virulent to IR24 were used for inoculation. Nine lines showed high levels of resistance to race 3 (Table 2). Twelve lines were resistant to races 5 and 14, and two lines were moderately resistant to race14. Recurrent parent BT7-*Xa21* was resistant to race 3, moderately resistant to race

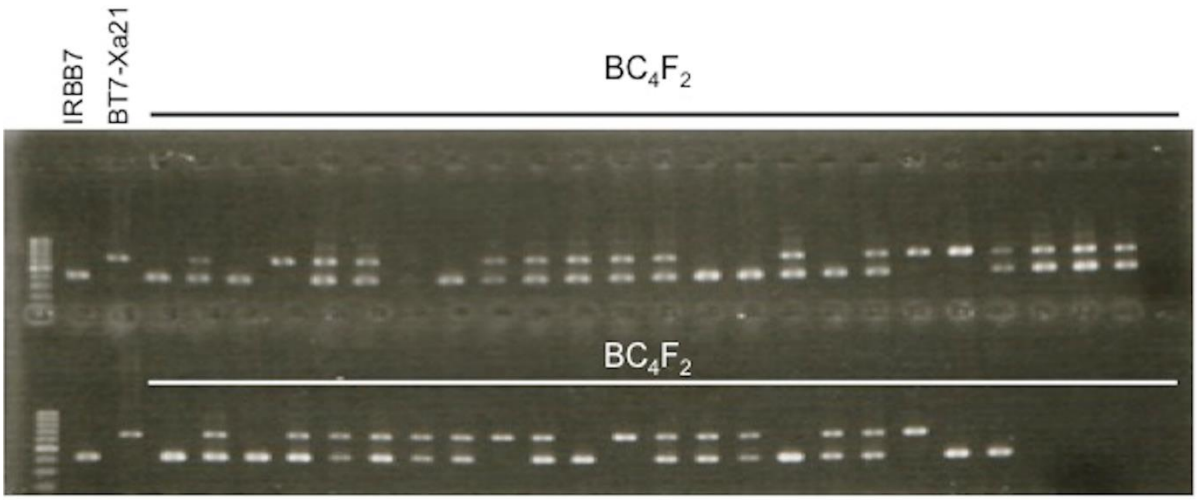

Figure 4. PCR analysis of the parental lines and BC_4F_2 plants. DNA was amplified with *ID7* that was linked to *Xa7*. BT7-*Xa21* (*xa7*/*xa7*) and IRBB7 (*Xa7*/*Xa7*) were used as controls for PCR amplification.

Table 2. Evaluation of BLB resistance of improved lines (BC_4F_3) in winter-spring season in 2015–2016 in Soc Trang province.

Line name	Race 3		Race 5		Race 14	
	LL (cm)	Responds	LL (cm)	Responds	LL (cm)	Responds
D1	2.6	HR	6.3	R	6.7	R
D2	3.1	HR	5.7	R	5.2	R
D3	2.3	HR	5.1	R	6.6	R
D4	2.6	R	6.2	R	10.2	R
D5	5.0	R	5.3	R	8.9	R
D6	1.9	HR	4.6	R	6.4	R
D7	1.7	HR	5.4	R	5.7	R
D8	2.4	HR	6.0	R	6.8	R
D9	2.2	HR	6.8	R	5.7	R
D10	3.5	HR	6.2	R	6.2	R
D11	4.8	R	6.9	R	9.7	R
D12	3.2	HR	7.6	R	8.6	R
D13	4.6	R	10.5	MR	11.3	MR
D14	5.1	R	8.7	MR	10.8	MR
IR24	27.5	S	30.8	S	34.6	S
BT7-*Xa21*	6.2	R	10.2	MR	14.6	S

Where LL: lesion length.

5, and susceptible to race 14. Pyramided lines of BT7 carrying *Xa21* and *Xa7* have acquired novel resistance to race 14 as well as higher resistance to race 3 (e.g., D1, D2, D3, D6, D7, D8, D9, D10 and D12).

Purification and agronomic traits of improved lines at BC_4F_3 generation

Agronomic traits including plant height, tillers per hill, length and width of flag leaf, effective tillers per hill, growth duration, panicle length, number of fruiting seeds per panicle, seed set rate, 1000-grain weight, and yield were evaluated at the BC_4F_3 generation (Tables 3 and 4). All lines examined showed good uniformity with purification scores ranging from 5 to 9 even though uniformity of BT7-*Xa21* was superior to the improved lines. Plant height was classified into a dwarf group, and the difference to recurrent parent ranged from 3.0 (D13) to 10.7 cm (D5). Tillering ability of the lines was similar to the recurrent parent BT7-*Xa21*, and the number of tillers per hill varied from 9.5 (D7) to 10.7 tillers (D10), while

Table 3. Agronomical traits of improved lines (BC_4F_3) in winter-spring season in 2015-2016, Soc Trang province.

Line	Purification (Score)	Plant height	Tillers per hill	Length of flag leaf (cm)	Width of flag leaf (cm)	Effective tillers per hill	Growth duration (days)
D1	9	91.5±4.2	10.3	32.6	1.7	8.5	109±5
D2	5	89.7±2.8	9.6	34.1	1.7	8.2	106±3
D3	5	89.2±3.3	10.6	33.5	1.7	8.7	108±3
D4	9	89.8±2.9	10.1	30.2	1.8	8.4	110±3
D5	9	100.3±3.8	9.7	34.7	1.6	7.9	110±3
D6	5	94.2±2.3	10.4	34.0	1.7	8.3	108±3
D7	9	93.1±2.8	9.5	33.8	1.7	7.8	107±3
D8	5	94.6±2.4	9.9	33.6	1.7	8.1	106±4
D9	5	95.7±2.6	10.3	34.1	1.7	8.2	107±3
D10	9	92.2±3.9	10.7	33.7	1.7	8.6	106±5
D11	9	90.8±3.1	10.4	32.6	1.6	8.2	109±5
D12	9	91.3±4.3	9.8	32.9	1.7	7.6	110±4
D13	5	86.6±2.9	10.3	31.8	1.8	8.4	110±3
D14	9	87.4±3.3	9.6	31.4	1.7	8.1	105±5
BT7	1	89.6±1.3	10.3	33.8	1.7	8.4	107±2

Purification (scores) 1: different plant type <0.25%; 5: different plant type 0.25 to 1%; 9: different plant type >1%. Mean±standard error.

Table 4. Yield and yield components of improved lines in winter-spring season in 2015–2016, Soc Trang province.

Line	Panicle length (cm)	No. of frutfull seeds per panicle	Seed set rate (%)	1000 grain weight (g)	Yield (quintal/ha)
D1	24.2	130.7	88.4	20.2	70.7
D2	25.1	139.1	87.7	20.8	72.0
D3	25.6	134.3	89.2	20.7	68.3
D4	26.6	133.3	86.9	20.3	71.6
D5	26.2	140.4	89.6	20.1	70.2
D6	24.8	136.1	89.7	20.4	72.6
D7	23.6	131.4	90.8	20.6	69.9
D8	25.2	140.1	87.2	20.8	72.5
D9	25.4	142.7	86.9	20.3	73.0
D10	24.7	135.5	88.7	20.4	75.8
D11	24.3	124.5	85.3	20.7	66.6
D12	25.1	139.7	87.4	20.5	65.9
D13	24.6	124.1	82.2	20.3	61.1
D14	25.6	130.0	81.4	20.4	67.7
BT7-*Xa21*	25.5	138.5	88.5	20.0	71.5

that of recurrent parent was 10.3 tillers. Effective tillers per hill varied from 7.6 (D12) to 8.7 (D3). The growth duration of improved lines was similar to the recurrent parent, but uniformity was less than the recurrent parent. Based on these agronomic traits, D2, D3, D6, D8, D9 and D13 were selected as promising lines.

Yield and yield components of improved lines were similar to those of recurrent parent BT7-*Xa21*. Moderate panicle size, high seed set ratios between 81.4 (D14) and 90.8 (D7), small seeds, and number of spikelets per panicle ranging from 124.1 (D13) to 142.7 (D9) were observed. 1000-grain weight varied from 20.1 to 20.8 g. Yield of improved lines varied from 61.1 (quintal/ha) (D13) to 75.8 (quintal/ha) (D10) even though the control was 71.5 (quintal/ha). Finally, D2, D6, D8 and D9 were selected as promising lines for quality evaluation based on response to BLB, phenotypic uniformity, agronomic traits and yield. The D3 and D13 lines were excluded due to some inferior quality traits (data not shown).

Quality evaluation of improved lines at BC_4F_3 generation

BT7-*Xa21* is a high-quality rice variety with slender,

Table 5. Quality traits of the promising lines of BC_4F_3 generation.

Trait	D2	D6	D8	D9	BT7-*Xa21*
Straw color	Brown-yellow	Brown-yellow	Brown-yellow	Brown-yellow	Brown-yellow
Seed length (mm)	6.3	6.2	6.3	6.3	6.2
Length/width	2.8	2.7	2.8	2.8	2.7
Aromatic	3	4	3	4	4

1: Non aromatic, 2: weak aromatic, 3: moderate aromatic, 4: aromatic, 5: strong aromatic.

small, soft, brownish and aromatic grains, and is almost identical to the original cultivar BT7 except for the possession of *Xa21*. Some indicators of high-quality varieties are presented in Table 5. The four selected lines had grain characteristics similar to recurrent parent BT7-*Xa21* including a brown yellow hull, seed length of 6.2 to 6.3 mm, and length/width of 2.7 to 2.8 (Table 5). Two of the promising lines, D6 and D9, had the same level of aroma as produced by recurrent parent BT7-*Xa21*. Based on the level of BLB resistance and agronomic traits, two lines, D6 and D9, were eventually selected as promising lines to be released to farmers' fields.

DISCUSSION

In this study, plants carrying *Xa7* in addition to *Xa21* from BC_1F_1 to BC_4F_2 generations were successfully selected using the MAS technique. Plants carrying two resistance genes show wider resistance than plants carrying single resistance gene. Previously, pyramiding BLB resistance genes, *Xa4* and *xa5,* or *xa5* and *Xa10,* was shown to express higher levels of resistance to BLB than a single gene (Huang et al., 2012). Similarly, the combination of *Xa21* and *Xa7* showed a high level of resistance as well as wide spectrum resistance to BLB (Table 2). Furthermore, the improved lines showed high phenotypic uniformity, semi-dwarf, good tillering, high seed set rate and small seeds like the recurrent parent at the BC_4F_3 generation. This proved that pyramiding two resistance genes *Xa7* and *Xa21* was useful for improving BLB resistance in cultivar BT7. Conventional breeding is laborious, time consuming and difficult to apply when it comes to pyramiding dominant genes with similar reactions to BLB (Collard and Mackill, 2008). he results of this study show that MAS is an effective method to overcome the limitations of phenotypic selection in BLB resistance breeding in rice. Through further improvement of several traits along with additional field trials, the promising D6 and D9 lines will be released as new high quality varieties with improved resistance to BLB.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Superoxide dismutase activity and jasmonic acid during *in vitro-ex vitro* transition of pineapple (*Ananas comosus* (L.) Merr.) micropropagated plantlets

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Recent agriculture is characterized by intensive and cleaning productions, which need seeds with high quality in large quantities bonded by *in vitro* culture labs. Nevertheless, *in vitro ex vitro* transition and during acclimatization losses occur due to the death of plantlets unable to survive this abiotic stress. Reactive oxygen species production during jasmonic acid-induced changes of previous transition was demonstrated. The role of superoxide dismutase in regulation of oxidative metabolism signaling in response to environmental stress is analyzed. Pineapple plantlets treated with jasmonic acid showed higher protein biosynthesis, which can be associated with a better regulated metabolic predisposition to face this phase, when superoxide dismutase activity showed adequate control against this stress in relation to superior water-use efficiency and survival.

Key words: Environmental stress, water-use efficiency, survival.

INTRODUCTION

The presence of reactive oxygen species (ROS) as superoxide radical (O^{-2}) and hydrogen peroxide (H_2O_2) is associated in plants with the normal biochemistry processes as photosynthesis and respiration (Sejima et al., 2014; Huang et al., 2016). The accumulation and high reactivity has a cytotoxic effect by oxidative damage throughout lipid peroxidation and membrane destruction, protein inactivation and DNA mutation (Pospisil and Prasad, 2014). The reduction oxidation cascades (redox) of photosynthetic and respiratory chains of electron transport do not only provide energy for the metabolism, moreover it generates signals about participation in plant regulation of all the biology aspects at gene expression and the translation including chemistry of the enzymes (Kim et al., 2009). Some antioxidative enzymes as superoxide dismutase (SOD) and peroxidase participate in the ROS metabolism in pathogen infection. In plants, ROS are considered the first defense line against

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oxidative stress (Mittler and Blumwald, 2017). The induction or suppression of ROS production in the leaves is related with the antioxidative enzymatic activity diminishing H_2O_2 (by direct decomposition or oxidation) and O^{-2} (by dismutation) levels (El-Khallal, 2007).

The tissue culture changes some morphological characteristics of plantlets such as chemical composition of epicuticular layer (Preece and Sutter, 1991), form and distribution of stomas, tails and leaves structure (Ziv, 1990); also, physiological characteristics as activities of stomas, roots and leaves functionality. These changes raise the adaptation capacity of some plantlets to external conditions and originate not survival in acclimatization phase of significant number of micropropagated plants (Preece and Sutter, 1991) improved in pineapple using temporary immersion (Gonzalez-Olmedo et al., 2005). In this situation, plantlets do not control the excess of epidermical transpiration considered as principal mortality plants factor when they are transferred into the soil conditions (Durkovic and Misalova, 2009). ROS play a very important role in these adaptation processes as ubiquity response messenger in the stress (Apel and Hirt, 2004).

To supply some of these deficits, the use of plant growth regulators is a common practice (Preece and Sutter, 1991). Jasmonic acid (JA) that acts mainly as signal molecule as plant response against many abiotic and biotic stress (Schilmiller and Howe, 2005; Abdala and Cenzano, 2006), could attenuate these effects in pineapple plantlets during *in vitro-ex vitro* transition and SOD activity could be a biological indicator, whose demonstration is the objective of this work.

MATERIALS AND METHODS

The experiment was carried out with pineapple (*Ananas comosus* (L.) Merr.) micropropagated plantlets according to Daquinta and Benegas (1997) during acclimatization phase. Previous at *in vitro-ex vitro* transition, during *in vitro* rooting phase, a group was growing on medium enriched with Biojas® (a JA formulation) at the dose of 1 $mg.L^{-1}$ established because it was the one that achieved the best effects in a previously tested screening. Another group without Biojas® was used as control.

The variables were determined at the beginning of acclimatization (0 day), 14, 28 and 42 days later than the *in vitro-ex vitro* transition. Ten representative plantlets per treatment were used to choose the leaves analyzed.

Soluble proteins extraction, involving enzyme, was carried out using the same procedure. 0.25 g of macerated leaves in liquid nitrogen was aggregated at Tris-HCl buffer 01 M, pH 7.5, with 0.1 $mmol.L^{-1}$ EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 15 mM mercap-toethanol (ME, 1:4) (p:v). Further, 10% polyvinyl polypyrrolidone (PVPP) with respect to fresh weight was added. Homogeneous suspension was centrifuged at 15000 g during 20 min. The supernatant was used as enzymatic extract and to quantify soluble proteins according to Bradford (1976) expressed as mg $Prot.g^{-1}$ fresh weight (FW) referred to Bovine Serum Albumin (BSA) standard curve.

Reaction mixture to determine SOD (EC 1.15.1.1) activity comprised 20 µL of enzymatic extract, 1 mL potassium phosphate (KOH), 50 $mmol.L^{-1}$ buffer, pH 7.6, 0.1 $mmol.L^{-1}$ EDTA, 0.01 $mmol.L^{-1}$ cytochrome C, 0.05 $mmol.L^{-1}$ xanthin, 0.03 unities of xanthin oxidase (EC 1.2.3.22) (SIGMA). Mixture xanthin-xanthin oxidase was used as superoxide radicals source using just as cytochrome C method (550 nm) (extinction molar coefficient 340 = 21.1 $(mmol.L^{-1})^{-1}cm^{-1}$) (Mc Cord and Fridovich, 1969) in spectrophotometer (Pharmacia, LKB). Reaction time was 3 min, enzymatic activity was expressed as µmol of superoxide by $min.g^{-1}$ FW and specific activity was expressed as µmol superoxide by $min.mg^{-1}$ Prot.

Leaf D from the same plantlets from two treatments was used for physiological evaluations realized at the beginning of acclimatization phase and after 14, 28 and 42 days. Photosynthesis (µmol CO_2 $m^{-2}.s.^{-1}$) and transpiration total (mmol H_2O $m^{-2}.s^{-1}$) were measured using CIRAS-2 (Portable System of Photosynthesis, Europe, PP Systems, UK) equipment connected to universal cuvette PLC6 2.5 cm^2. The water-use efficiency (WUE) was estimated at these variables as relationship between photosynthesis and transpiration total.

Survival was estimated as percentage as relationship between the number of alive plantlets in each moment of evaluation and the total number per treatment at the beginning (0 day).

The Statistical Package for Social Sciences (Version 11.5 for Windows, SPSS Inc.) was used to perform statistical significance range test for bi-factorials comparisons or Student's t-test for comparison of two conditions, both at 5% were evaluated using two-way analysis of variance (ANOVA) followed by Tukey's Multiple significance. Normal distribution and homogeneity of variances were evaluated with Kolmogorov-Smirnov and Levene tests, respectively. Some data were mathematically transformed for statistical analyses. Discrete quantitative variables were transformed according to $y' = SQR(y)$ or $y' = SQR(0.5 + y)$. Percentage variables were transformed according to $y' = 2 \arcsin(SQR(y/100))$.

RESULTS AND DISCUSSION

The *in vitro-ex vitro* transition of plants provokes an abiotic stress to them and one of the responses to this situation is related to ROS such as superoxide anion, hydrogen peroxide, etc. At high concentrations, ROS cause abnormalities and in extreme cases may result to cell death of plant tissues (Kim et al., 2009). SOD is the first in plant defense system to transform the superoxide anion into H_2O (Kim et al., 2009).

Figure 1 shows the results of SOD activity determined under effects of 1 $mg.L^{-1}$ and without JA. The results of Figure 1 showed no differences between two groups on the SOD activity in all the evaluated moments. However, in control plantlets, the values of the activity of this enzyme were different at the initial as much as at final evaluation. Plantlets treated with JA increased the enzyme activity of SOD from the first 14 days.

In this period, the same plantlets registered higher soluble protein content than control group (Figure 1 B). Only on the 28 day evaluation, this variable was higher in plantlets not treated with JA. At the end of acclimatization for both groups, this variable decreases to the lowest values of the experiment due to the reduction in the synthesis of these biomolecules, the translocation to other organs or degradation as a consequence of environmental conditions under which the plantlets were grown.

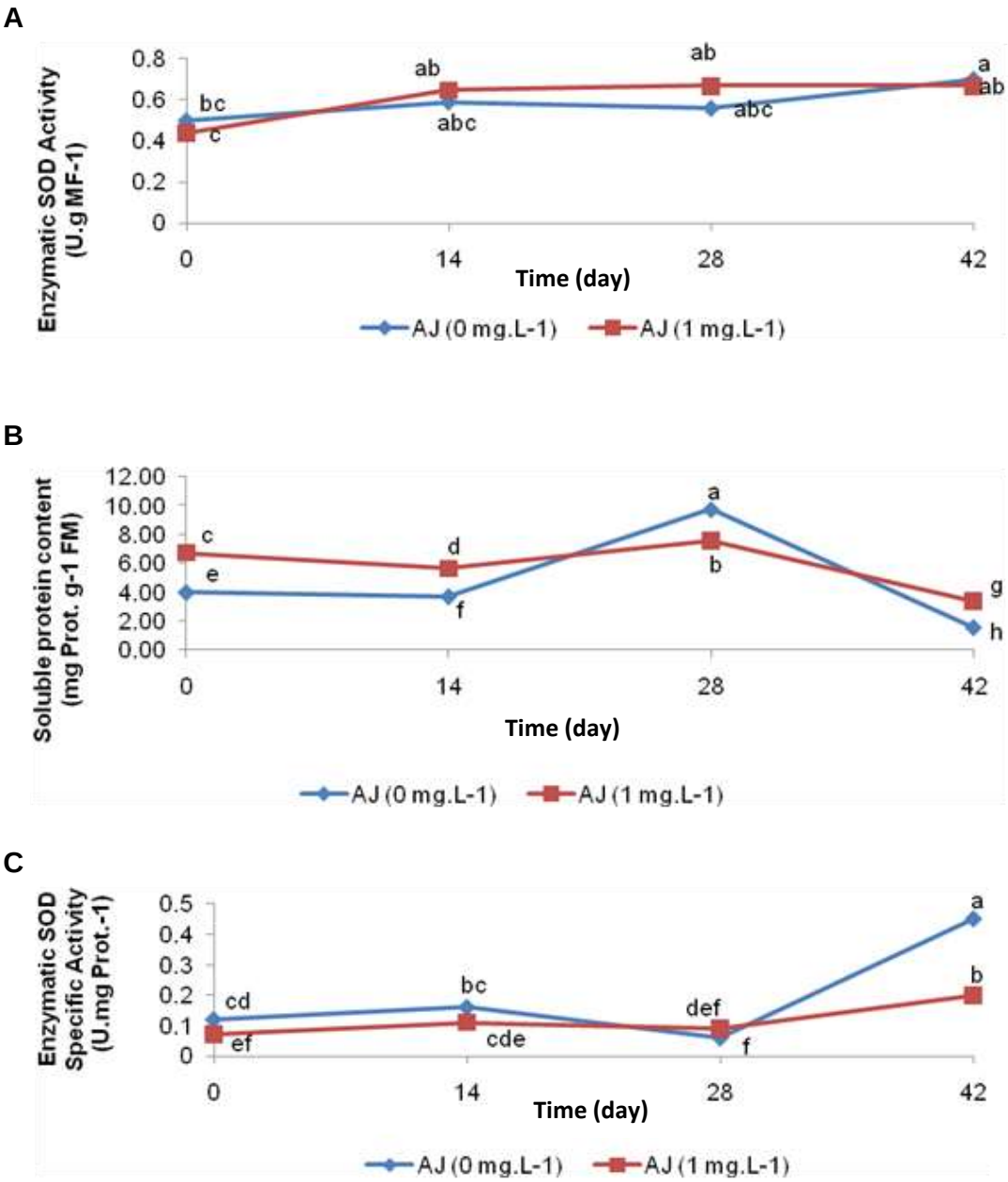

Figure 1. Effect of jasmonic acid on the SOD activity (A) (SE= ±0.02 U.g MF^{-1}), SOD specific activity (C) (SE= ±0.02 U.mg $Prot^{-1}$) and soluble protein content (B) (SE=±0.36 mg $Prot.g^{-1}$ MF), of *Ananas comosus* cv MD-2 plantlets in acclimatization conditions. Means with different letters indicate signification (ANOVA, Tukey test, $p \leq 0.05$). Each datum represents the mean for n=6. One unit (U) corresponds to 1 µmol of superoxide by minute.

As a consequence of the behaviour previously analyzed in Figure 1A and B, the enzymatic SOD specific activity also varied (Figure 1C). Changes registered in this variable are in agreement with the concentration of soluble proteins quantified in the plantlets (Figure 1B) and they deserve a proteomic study of each moment of evaluation. During the transition moment, plantlets treated with JA showed higher protein biosynthesis, which can be associated with a better regulated metabolic predisposition to face this phase (Aragón et al., 2010), which was expressed since the specific activity of SOD lightly increased at the end of evaluation against high increase observed in plantlets without JA in relation to the variable content of soluble protein and therefore with enzymatic specific activity, since the enzymatic activity was the same in both treatments (Figure 1A).

Table 1. Effects of Jasmonic acid (1 mg.L^{-1}) on water use efficiency (mmol CO_2 mol^{-1} H_2O) of MD-2 hybrid pineapple plantlets (*Ananas comosus* (*n* = 40)) in acclimatization conditions.

Treatment	Days after acclimatization			
	0	14	28	42
Without Biojas®	0.61[b]	3.35[a]	6.46[b]	17.29[a]
Biojas®	1.44[a]	3.62[a]	8.37[a]	14.05[a]
SE	0.15	0.12	0.44	0.86

Means within columns followed by the same letters are not significantly different (Student't Test, $p \leq 0.05$).

At the end of acclimatization, the moment where the reduction of metabolic activity and growth rate is observed frequently, among other reasons due to substrate exhaustion, environmental and nutritional factors that resulted to be restrictive. The specific activity of SOD increased for both groups. The increase was higher in the control plantlets because they had a higher enzymatic activity and low protein concentration. This demonstrated the anti-stress effects induced by the JA on pineapple plantlets of this experiment.

Normally, plantlets are stressed during the *in vitro-ex vitro* transition due to changes on environmental conditions such as light and relative humidity (Kozai et al., 2000). As a result, plantlets suffer from abiotic stress that is frequently manifested through dehydration and photo-oxidation (Preece and Sutter, 1991) that provokes changes in the electron transfer chain and thus in redox systems. Light reactions are the most important source of ROS in illuminated mesophyllic cells. Jasmonates induced the degradation of chloroplast proteins, among them ribulosebiphosphate carboxylase/oxygenase subunits (Agrawal et al., 2002). JA through the same mechanisms might have reduced the generation of ROS such as superoxide anion (O^{-2}) that has the capacity to cause oxidative damage to proteins, DNA and lipids.

Low generation of ROS (presumably O^{-2}) in plantlets treated with JA ensures their good growth. Higher ROS production can cause a retarded growth in plants as it was observed in transgenic potato plants with an elevated ROS production by the over expression of chloroplastic Cu/Zn SOD (Kim et al., 2009).

Forty two days after acclimatization, both groups increased the specific activity of SOD with a marked difference among them where control plantlets had higher values. This final moment of acclimatization corresponds to stress factors that provoke metabolic changes as previously analyzed. It is known that early stimulation of antioxidant enzymes during the C3 to CAM change is accompanied by the increase in ROS generation. That is supported by molecular induced analysis during 30 to 40 h treatment with salinity in *M. crystallium* leaves, showing that genes related to stress and antioxidant proteins are among the first to be induced (Kore-eda et al., 2004; Niewiadomska and Borland, 2008).

As we all know, plant will trigger the production of ROS in response to stress. They have a dual effect which is based on their overall cellular amount in plant. If kept in low level, they can function as signaling molecules to transmit information from metabolism to trigger appropriate cellular defense/acclimation response to environmental changes (Mittler, 2017).

Using data not shown on transpiration and photo-synthesis, the water-use efficiency was calculated as shown in Table 1.

JA reduced the transpiration of treated plantlets only at the beginning of acclimatization with significant difference, which can be attributed to stomata conductance. It can be supposed that JA induced stomata closure, as previously has been informed by other authors (Creelman and Mullet, 1997; Evans, 2003). Exogenous MeJA does not appear to antagonize ABA-induced stomatal closure, although the ability of MeJA to regulate stomatal apertures remains controversial (Montillet et al., 2013). Recently, it has been proposed that 12-OPDA (a JA precursor), rather than MeJA, acts in promotion of stomatal closure (Savchenko et al., 2014 but Han et al. (2018) demonstrated the negative regulation of stomatal development.

In this study, the last evaluations of the transpiration recorded similar values in both treatments, which decreased in each evaluation with respect to previous study. At the same time, photosynthesis did not change between the treatments but increased during the experiment, but without significant differences in the last three evaluations. All these joined by the low transpiration rate in plantlets treated with JA to perform their photosynthesis with the higher water-use efficiency (Table 1) especially at the beginning of acclimatization which is the most critical moment of the acclimatization process. WUE was also significantly higher in plantlets treated with Biojas® after 28 days of acclimatization.

JA increased WUE by reducing the transpiration rate without a marked difference on photosynthesis in respect to control plantlets. The low WUE of these plantlets at the beginning of acclimatization is as a result of the incapacity of the plantlets to control excessive water loss through transpiration. In general, the WUE increased during acclimatization in both groups because the plants improved their control on transpiration rate. WUE is the resultant compromise between the maximum o photosynthesis and the minimum transpiration to improve the plant quality (Cernusak et al., 2007), as shown in this

Table 2. Effects of Jasmonic acid (1 mg.L^{-1}) on survival (%) of MD-2 hybrid pineapple plantlets (*Ananas comosus* (*n* = 90)) in acclimatization conditions.

Treatment	Days after acclimatization			
	0	14	28	42
Without Biojas®	100^{a}	98^{ab}	94^{b}	94^{b}
Biojas®	100^{a}	98^{ab}	96^{ab}	96^{ab}

Means followed by the same letters are not significantly different (ANOVA, Tukey Test, p≤ 0.05). Data were transformed according to y´= 2 arcsin (SQR(y/100)).

experiment where plantlets treated with Biojas® comprised intrinsically of the capacity to suffer tolerance to the abiotic stress caused by acclimatization conditions and increased the survival as shown in Table 2.

The higher levels of survival were in line with the efficiency of the methodology used according to Yanes et al. (2000). Nevertheless, plantlets treated with JA reduced the losses by the death of plantlets during 42 days, due to 94% of survival in control which was statistically different while 96% in JA treatment was similar. Another datum that resume the productive value of application BioJas® to save 2% of plantlets during *in vitro-ex vitro* transition in relation to the expression of SOD activity to suffer tolerance to the effects of this abiotic stress, added to the knowledge on the physiology of *ex vitro* pineapple (*A. comosus* var. MD-2) as CAM or C3 regulated by the environmental conditions (Aragón et al., 2012). JA acts mainly as signal molecule as plant response against this abiotic stress and SOD activity could be a biological indicator if studied in line with the performed by Avila et al. (2017) with Ethrel®48 treatment to increase pineapple flowering.

It is known that temperature increased (in this case from 23 to 29°C) during the transition. The influence of temperature on the production of plants can be direct, on the growth of the plant altering its physiology, or indirectly by varying the humidity, the quantities of minerals absorbed by the plant and its transport. Whatever the influence of the thermal increase is in this transit, the results of the application of Biojas® favoured the relationships of the metabolic processes, perhaps as demonstrated by Cejas et al. (2012) in other thermal management required to improve productivity in new climatic scenarios. It would then be a tool to apply in predictive studies (Lobell and Asseng, 2017). Pineapple plantlets treated with JA showed higher protein biosynthesis, which can be associated with a better regulated metabolic predisposition to face this phase, when superoxide dismutase activity showed adequate control against this stress related to superior water-use efficiency and survival.

Thus, based on these results, this study could show the molecular, hormonal, and histological changes that are present right after Biojas® application, providing new insights into how pineapple acclimatization occurs under natural conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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