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Blast resistance R genes pyramiding in temperate japonica rice

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Abstract:	A major issue in rice production is the control of Pyricularia oryzae , the causal agent of rice blast. Genotypes with R resistant leucine-rich repeat (LRR) genes control specific races of the parasite. However, the resistance is overcome by the pathogen, over a sufficiently long time. In temperate areas, current cultivated varieties have a largely insufficient field resistance. This prompted us to undertake the pyramiding of the R resistance genes Pib , Piz , Pik , Pita 2, and Piz-t in temperate japonica materials. Two lines were produced, SJKK and SJKT-2 , that have each four pyramided genes. They are fully resistant to rice blast when tested in the field and in greenhouse. However, the tropical origin of the R donor genotypes added complexity to the pyramiding exercise. The results point to a lack of fitness costs in pyramided lines.
Response to Reviewers:	LETTER TO THE EDITOR Dear Dr. Jiankang Wang with this letter we submit a revised version of our manuscript entitled " Blast resistance R gene pyramiding in temperate japonica rice", authored by Gabriele Orasen, Raffaella Greco, Enrico Puja, Carlo Pozzi and Maria Rosaria Stile, to be considered for publication in Euphytica. Taking into account the criticisms from the reviewers, we have extensively reshaped the whole manuscript to improve readability. The Appendix was removed, and the relevant information concerning the breeding actions leading to the production of the two pyramided lines SJKK and SJKT-2 were inserted in the main text (new added Table 4). At the same time, all information considered as not relevant to this objective, have been removed not to create confusion. All sections have been modified; in particular "Material and Methods" and "Results" have been extensively reorganised in order to present the results in a more logical way. A new paragraph has been created in the Results section describing the pyramiding program. Results have been

described more in details and discussed in the Discussion section. Moreover, the manuscript has been revised by an English professional editor to improve language.
Please find below the responses to your comments:
1)The text has been modified (see response A to Reviewer 2).
2)Table 2 has been modified, now the primer sequences are in capital format.
3)Table A3 has been merged to Tables A1 and A2 to create a new Table 4 in the main text (see response F to Reviewer 2). The field has been removed, as it was considered not relevant.
We hope that the paper, in its present revised form, will satisfy the reviewers criticism and fulfil the standards requirements for publication in Euphytica.
With kind regards,
Prof. Carlo Pozzi

RESPONSE TO REVIEWER 1
1.In our work, we claim that the two lines with multiple resistances can be the source of R genes for temperate rice material. However, we partially agree with the comment of the Reviewer and the sentence of line 36 " North-West China produces 20% of total harvested rice (IRRI 2014)" has been removed as it was considered not relevant.
2.One of the major mechanisms of fungal evolution is recombination between races. Multiple-resistance genotypes reduce the probability that two fungal races can meet in the same plant. The text has been modified as follows: "The presence of a set of different R resistance genes in the same plant blocks the infection from multiple pathogen races, thus avoiding fungal evolution by preventing recombination between different fungal races. This allows a broad and more durable tolerance to the disease".
3.As suggested by the Reviewer, the text has been modified as follows: "Our aim was to produce, through pyramiding of R genes, blast resistant rice varieties adapted to the European pedoclimatic conditions, specifically cold tolerant and with reduced photosensitivity".
4.The text has been modified as follows: “The conclusion is that, in crosses where japonica lines are donor or R genes, the selection of genotypes adapted to temperate condition is difficult, when the target is a high degree of blast resistance.”
5.The text has been modified as follows: “obtaining materials with several R genes, competitive in the Italian environment”.
6.Table 1 has been modified as suggested and a new column was added.
7.The Reviewer is right. The text has been extensively modified (see the new "Material and Methods" and "Results" sections). We believe that the new text is now clear.
8.See response to point 7.
9.See response to point 7.
10.The Reviewer is right. The two lines with four homozygous R genes produced, SJKK and SJKT-2, are now cited in the text specifically. The gene Pita2 has now the same symbol across the manuscript.
11.The text has been modified (see the second paragraph of the new "Results" section).
12.It is specified in the text that five R genes were used, with the target to select lines with four different genes in the same plant. We believe that the new text is now clear.
13.Working with R genes, it is well known that a single gene controls one pathogen race. On the contrary, a single race of the fungus may be infective in more than one gene, in genotypes with multiple R genes. The text has not been modified.
14.The text has been modified to specify the conditions used.
15.The text has been corrected as suggested by the Reviewer.
16.The text has been corrected as suggested by the Reviewer.

RESPONSE TO REVIEWER 2

a)The text has been modified as follows: “Statistical evaluation ….. was performed by two-way ANOVA using the function “aov” in the R STATS package (R Development Core Team 2010).”
b)The blast disease symptoms were similar in the greenhouse and in the field, and the scale used for the scoring was the same. The following text has been added to the

	Results section: “The data obtained from natural blast infection in the field were consistent with those obtained with artificial inoculation”. c)Pyramided lines have not been texted for yield response because this will require multiple locations and years; moreover, these lines were created only to be used as multiple R gene donors. d)The heterozygous status of R genes in pyramided lines was almost always equivalent in level of infection to the homozygous situation. e)As suggested, the manuscript has been extensively modified and corrected by a professional English editor to improve readability. f)To improve understandability, tables A1, A2, A3 have been merged into Table 4, included in the main text.

Blast resistance *R* genes pyramiding in temperate japonica rice

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Major Keywords: Rice Blast - *LRR* genes

RUNNING TITLE: *R* genes pyramiding in rice

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We thank Prof. Francesco Salamini for the critical evaluation of the manuscript and for discussion of the results. We also acknowledge Dr. Mario Lo Pinto, who maintained the contacts between Agroalimentare Sud and Bertone Sementi S.p.A. The latter company, in addition to carrying out the crossing activities, provided the greenhouse and field facilities, and supported the project financially.

The two pyramided lines produced in this work (*SJKK* and *SJKT-2*) are protected by a patent certificate for industrial invention (Italian Ministry of Economic Development N. 102015000076118, May 5th, 2018).

ABSTRACT

A major issue in rice production is the control of *Pyricularia oryzae*, the causal agent of rice blast. Genotypes with *R* resistant leucine-rich repeat (LRR) genes control specific races of the parasite. However, the resistance is overcome by the pathogen, over a sufficiently long time. In temperate areas, current cultivated varieties have a largely insufficient field resistance. This prompted us to undertake the pyramiding of the *R* resistance genes *Pib*, *Piz*, *Pik*, *Pita*2, and *Piz-t* in temperate japonica materials. Two lines were produced, *SJKK* and *SJKT-2*, that have each four pyramided genes. They are fully resistant to rice blast when tested in the field and in greenhouse. However, the tropical origin of the *R* donor genotypes added complexity to the pyramiding exercise. The results point to a lack of fitness costs in pyramided lines.

Keywords: Rice Blast - LRR genes - *R* gene pyramiding - temperate germplasm - greenhouse and field trials.

INTRODUCTION

Due to its nutritional profile and competitive price, rice represents the major source of proteins and calories for half of the world population (Biselli et al. 2014; Parengam et al. 2010; Zhang et al. 2014). Rice production is mainly concentrated in sub-tropical regions, but the crop is grown also in temperate climates: Italy produces more than 50% of the 4.2 million tons of European rice (Conaf 2015), about 0.32 % of the total world rice production (FAO, 2016). Temperate japonica varieties are also grown in the USA, in other European countries, and in Japan.

One of the major agronomic challenges to rice production is represented by the pathogen *Pyricularia oryzae*, the causal agent of the disease commonly known as rice blast. The disease causes significant yield losses all around the world. Symptoms of rice blast are evident on leaves, leaf sheaths, culms and panicles, leading to plant weakness and a high level of sterility. In the absence of genetic factors conferring resistance to specific *Pyricularia oryzae* strains, yield losses up to 80% are reported (Faivre-Rampant et al. 2010). In Italy, rice blast control is based on fungicide treatments, which are expensive both in economic and environmental terms. Moreover, control agencies are imposing a progressive reduction in their use, thus triggering the need for genetic-based solutions.

Resistance to blast is conferred by dominant genes belonging to the leucine-rich repeat (LRR) class, called *R* genes. The presence in the plant of a major *R* gene prevents infection by a race of *P. oryzae* harboring the corresponding avirulence (*Avr*) gene (Flor 1971). However, *R*-mediated plant resistance is usually lost with time, because the pathogen can evolve new races by gene recombination or by mutating avirulence genes (Farman 2007; Roumen et al. 1997; Starnes et al. 2012). The presence of a set of different *R* resistance genes in the same plant blocks the infection from multiple pathogen races, thus avoiding fungal evolution by preventing recombination between different fungal races. This allows a broad and more durable tolerance to the disease. This approach was successfully followed in the pyramiding of four *R* resistance genes in a rice variety which, however, is not adapted to European climate conditions (Cho et al. 2007). Our aim was to produce, through pyramiding of *R* genes, blast resistant rice varieties adapted to the European pedoclimatic conditions, specifically cold tolerant and with reduced photosensitivity (Okumoto et al. 1996; Ichitani et al. 1997). This task was not easy due to the risk of introducing, along with the *R* genes, negative traits contributed by the *R* gene donors. Examples of rice cultivars with durable resistance to the rice blast disease are rare (Ballini et al. 2008). Moreover, besides *R* genes, 350 QTLs have been reported to be responsible for rice blast control (reviewed in Ballini et al. 2008). This suggests that effective pyramided major *R* genes must coexist with an appropriate QTL context. The conclusion is that in crosses where japonica lines are donors of *R* genes, the selection of genotypes adapted to temperate conditions is difficult, when the target is a high degree of blast resistance. The work described here was supported by the breeding company Bertone Sementi S.p.A. and had two targets: 1) producing lines with several pyramided *R* genes to evaluate their reaction to major

pathogen strains present in the Italian, European and Mediterranean cultivation areas; 2) obtaining materials with multiple *R* genes competitive in the Italian rice environments. Target 1) is the subject of this work. Target 2) is currently ongoing.

MATERIALS AND METHODS

Plant material

The five rice varieties, donors of *R* genes to be pyramided, were Saber, Kusabue, Katy, Jefferson and Toride. Their choice was based on the effectiveness of the *R* alleles which they carry against the main races of *Pyricularia oryzae* present in Italy and Europe (Faivre-Rampant et al. 2010; Roumen et al. 1997; Tacconi et al. 2010). The resistance donors are listed in Table 1, and their resistance genes are specified. The introgression of the five *R* genes into the progeny plants throughout the pyramiding program was monitored by Marker Assisted Selection (MAS, described later). Plants were grown at Bertone Sementi, Alessandria, in greenhouses and growth chambers. Crosses were made using the vacuum technique (Lupotto et al. 2008). Plants with unsuitable characteristics, such as a too long life-cycle or excessive tendency to lodging, were gradually discarded.

The adaptation of the *R* pyramided lines to the Italian pedoclimatic conditions was assessed with two experiments carried out in greenhouses and fields in 2017 and 2018. For local adaptation evaluation, the date of panicle emergence and the number of days from sowing to flowering (Fogliatto et al. 2012) were recorded, along with other agronomic traits, such as plant height and canopy structure.

Marker Assisted Selection (MAS)

Genomic DNA was prepared from leaves of young plantlets using the NucleoSpin PlantII kit (Macherey-Nagel), according to the manufacturer's instructions. Plants were screened for the presence of five major blast resistance genes (*Pib*, *Piz*, *Pik*, *Piz-t*, *Pita2*) using the PCR molecular markers described in Table 2. PCR amplifications were carried out as indicated in Tacconi et al. (2010). The resulting fragments were size-fractionated on 1.5% agarose gels and presence/absence visually scored.

Pathogenicity tests

Greenhouse. Artificial blast infection was based on inoculation with the *Pyricularia oryzae* strains IT2, IT3 and IT10, inactive in presence of *Pi1*, *Pita*2, *Pib*, *Pik-p*, *Pita*, *Piz*, *Piz-t*, and *Pi33* (Faivre-Rampant et al. 2010; Tacconi et al. 2010). These strains were selected based on their pathogenicity, as described by Roumen et al. (1997) and Faivre-Rampant et al. (2010). Fungal strains were grown in 20 ml Petri dishes with a suitable growing medium prepared with 20 g per L of rice seed flour, 2.5 g per L of yeast extract, 1.5% agar (Merck) and 500,000

units of Penicillin G (SIGMA) added after autoclaving. Cultures were grown in a growth chamber with a 12h photoperiod and a constant temperature of 25°C for 7–9 days prior to inoculation (Tacconi et al. 2010; Faivre-Rampant et al. 2010). Seeds of resistant and susceptible lines were germinated in a greenhouse in pots, under controlled conditions (12h photoperiod, 27°C day/22°C night temperature). Nitrogen fertilization (8.6 g of N equivalent in 1 l of water) was applied to soil two days before inoculation. A conidial suspension (50,000 conidia ml^{-1}) was sprayed two weeks after sowing, at stage 1.3 of the BBCH scale (Lancashire et al. 1991). After 7 days, presence/absence of necrosis and lesions on rice leaves were recorded, according to the infection scale of Roumen et al. (1997) (Figure 1).

Field. Field resistance to natural blast infection of selected genotypes was assessed at Bianzè, Vercelli, during summer 2015. The trial was based on 50 square meter plots in triplicate, with tolerant and susceptible varieties organized in three independent randomizations (Huyhn and Feldt 1970). The susceptible variety Maratelli was used as natural spreader of the infection. The trial was sown on silty-sand soil, with low clay content, and 150 U of 23-0-30 (N, P, K) fertilizer was applied. Leaf blast symptoms were visually scored using the same infection scale as above (Roumen et al. 1997).

Statistical Analysis

Statistical evaluation of the response of rice genotypes to greenhouse infection with the blast strains IT2, IT3 and IT10 was performed by two-way ANOVA using the function “aov” in the R STATS package (R Development Core Team 2010). The same was done for field collected infection data.

RESULTS

State of Italian genotypes with respect to R resistance genes

At first, we investigated the presence of five major *R* resistance genes, *Pib*, *Piz*, *Pik*, *Piz-t*, *Pita2*, in five Italian varieties. These varieties represented the range of rice genotypes cultivated in the country (Table 3). Four of the five lines tested carried only one *R* gene, either *Pib* or *Piz*. The results demonstrate that, in temperate rice materials, *R* alleles are largely absent, at least when the five genetic loci screened are considered. This finding prompted us to undertake the enrichment of local cultivated germplasm with dominant resistance genes active against *Pyricularia*.

Pyramiding of multiple R resistance genes in Italian genotypes

131 The foreign *R* genes donor varieties Jefferson, Katy, Kusabue, Saber and Toride, entered the pyramiding
132 program targeted to the creation of temperate rice genotypes carrying at least four resistance genes. Marker
133 assisted selection was used at each step of the breeding program to monitor the introgression of the various
134 *R* genes.
135 The work started with the pairwise crossing of the foreign *R* donor genotypes. The resulting hybrids were
136 then crossed to five lines adapted to the pedoclimatic conditions of the European cultivated area (Table 3).
137 Selected F1 progeny plants accumulating *R* genes in a temperate x exotic background were further
138 intercrossed. Their F1 progenies were tested for the presence of *R* genes and in 2010 plants homozygous or
139 heterozygous for more than one *R* gene were obtained. Those lines were self-pollinated and further
140 intercrossed during 2011, allowing the selection of 162 plants with *Pib*, *Piz* and *Pik* in different double or
141 triple combinations (Table 4). Starting from 2012, those multiple *R* lines were self-pollinated to obtain
142 homozygous *R* alleles. Further crosses to the exotic donors Toride and Katy were also made to introgress the
143 genes *Piz-t* and *Pita2*, which were found to be difficult to transfer. At the end of 2013, three lines with four
144 *R* genes were isolated (Table 4) that, upon selfing, originated two multiple resistance lines each containing
145 four *R* genes in a homozygous state, *SJKK* and *SJKT-2*. The *SJKK* line is a semi-early long grain B rice,
146 homozygous for the *R* genes introduced from Saber (*Pib*), Jefferson (*Piz*), Kusabue (*Pik*) and Katy (*Pita*2). The
147 *SJKT-2* line is an early long grain A rice, homozygous for the *R* genes deriving from Saber (*Pib*), Jefferson (*Piz*),
148 Kusabue (*Pik*) and Toride (*Piz-t*).
149 In addition, 17 lines homozygous for *Pib, Piz*, *Pik* and four lines containing the Toride and Katy alleles (*Piz-t*,
150 *Pita2*) were obtained as sources of resistant genes for further crosses with varieties of interest.
151
152 *Resistance to rice blast of lines adapted to temperate climates and carrying multiple R genes*

153 The two multiple resistance genotypes resulting from the pyramiding project *SJKK* and *SJKT-2* were evaluated
154 for their response to artificial and natural blast infection. The two lines were infected in the greenhouse with
155 conidial suspensions of three *P. oryzae* strains, along with the susceptible rice cultivars Maratelli, Carnaroli
156 and Vialone Nano. As expected, the latter three varieties were highly susceptible to the disease, with average
157 infection scores between 5 and 6 (Table 5). Maratelli showed large leaf lesions without dark margins, while
158 the other two varieties were characterized by large brown-bordered leaf lesions. On the contrary, the two
159 multiple *R* lines had an average infection score between 0 and 2, showing an almost complete absence of
160 lesions. The *SJKK* line appeared slightly more resistant compared to *SJKT-2*. The data obtained from natural
161 blast infection in the field were consistent with those obtained with artificial inoculation (Table 5). These
162 results reflect the well-known constitutive presence at a high level of infection of the rice blast disease in
163 northern Italian fields. Because of this, we assumed that the resistance response to blast infection of the
164 pyramided lines *SJKK* and *SJKT-2* depended only on their *R* genotype, therefore the trial was considered
165 conclusive and not repeated further. The statistical analysis of the data reported in Table 5 supports a large

166 difference among the genotypes tested (P value <0.001). The treatments with *P. oryzae* strains did not
167 generate significant effects (P value = 0.216). The Genotype x Treatment interaction had a P value of 0.048,
168 significant at P <0.05.
169
170 To assess whether the pyramided lines *SJKK* and *SJKT-2* were adapted to the temperate climate, they were
171 phenotyped for several agronomic traits (panicle emergence, total plant height, canopy structure) and relying
172 on an index represented by the duration of the first part of the life cycle (days from sowing to flowering).
173 This index was selected because panicle emergence, considered to occur when at least 50% of the plants
174 have extruded 1/3 of the panicles (Volante et al. 2017), shows a sufficient degree of genetic variability when
175 assessed in a large collection of Italian varieties (Fogliatto et al. 2012). The two resistant lines *SJKK* and *SJKT-*
176 *2* flowered respectively 106 and 93 days after sowing (average of greenhouse and field evaluations). These
177 values are shared by several Italian commercial varieties like Baldo, Carnaroli, Carnise, Gigante Vercelli,
178 Jefferson, Katy, Kusabue, Maratelli, Roma, Ronaldo, Thaibonnet, Ulisse and Vialone Nano. The above-cited
179 varieties flower between 91 and 104 days after sowing (Ben Hassen et al. 2018). Seven of them belong to the
180 temperate japonica group, based on the study conducted on 391 rice accessions by Biscarini et al. (2016). It
181 is concluded that the two *R* pyramided lines share with temperate japonica materials the length of the
182 growing cycle.
183

184 # DISCUSSION

185

186 In the first two years of the project, the use of MAS allowed the selection of plants with multiple *R* genes.
187 These genotypes were self-pollinated and intercrossed: their progenies were analyzed with molecular
188 markers until lines were found with four different *R* genes in a homozygous state. Due to the use, in the
189 original crosses, of genotypes adapted to temperate pedoclimatic conditions, segregating resistant
190 phenotypes with traits typical for rice grown in Italy were selected.
191 A major challenge for plant breeders is to accumulate in new cultivars as many resistances as possible to
192 pests and diseases (Stam et al. 2014; Wiesner-Hanks and Nelson 2016). However, in a given plant species,
193 the genes participating in the resistance mechanisms can be so numerous as to become a challenge for a
194 successful breeding program. The rapid development of technologies, such as Clustered Regularly
195 Interspaced Short Palindromic Repeats(CRISPR/Cas), particularly in its multiplexing version, significantly
196 contributes to the scope of this approach (Andolfo et al. 2016; Arora and Narula 2017; Fonfara et al. 2016;
197 Mishra et al. 2018; Zaidi et al. 2018).
198 Currently, under monocultural management (Andow 1983), the incidence of pathogen mutation and genome
199 recombination opposes a long-lasting resistance to major diseases. In the case of rice blast, it is well known

that the fungus causing the disease has overcome major *R* resistance genes introduced, over time, in the breeding materials (Miah et al. 2013). The problem is the discrepancy between the long time required to move *R* genes into rice varieties, and the speed at which the fungus can overcome the gene being transferred. Strategies devised to durably control plant diseases can be based on multiline varieties (Abe 2004), each different for a single monogenic resistance. The aim is to exploit a wide array of resistance genes to influence the epidemic development. The concept has been applied, among other crops, in oat (Mundt 2002) and wheat (Gill 1984). Similarly, mixtures of disease-susceptible and resistant varieties with a large genetic heterogeneity, have been proposed as an ecological approach to disease control (Browning and Frey 1969; Castro 2001; Wolfe 1985). Successful cases are those of rice (Zhu et al. 2000; Zhu et al. 2005), wheat (Cox et al. 2004), and barley (Mundt et al. 1994).

Conceptually, genotype heterogeneity can be reached by pyramiding, in a single genotype, major resistance genes active against multiple races of the same pathogen. At the root of this approach is the consideration that, the same as for *R* genes and alleles, several avirulence loci of the pathogen can mutate independently. Thus, the probability that a pathogen will overcome an entire set of *R* genes is the product of the mutation frequencies of those pathogen avirulence genes, each matching a plant *R* resistance gene (Vera Cruz et al. 2000). It is also clear that the presence of a group of resistance genes in the same plant can prevent the infection from multiple races, thus avoiding fungal evolution due to recombination among different races. The result of a pyramiding program should be the achievement of a more durable resistance.

Our approach for the effective management of rice blast was focused on the combination of a broad spectrum of *R* genes (Kiyosawa 1982; Wang et al. 2014). Such a program is justified by the frequently claimed absence of rice varieties both adapted to the European climates and characterized by a field resistance to rice blast (Ali et al. 2016; Srivastava et al. 2017). Our work demonstrates that Italian temperate material is largely defective in terms of resistance alleles at five *R* loci, while the pyramided lines *SJKK* and *SJKT-2* are fully resistant. The complication in achieving this goal, is that the *R* donor genotypes were in large part of tropical origin and, as such, poorly adapted to the Italian environments (Cho et al. 2007). Recent work indicates that pyramiding *R* genes into a single line may have fitness costs (Deng et al. 2017), such as photoperiod sensitivity and lower yield. In our case, the multiple *R* lines were characterized by traits common to local adapted materials.

When tested in the field, successful cases of pyramiding are rare (Mundt 2018), and not all resistance genes are equally able to provide durability when combined to other resistance genes. The best examples are found for stem rust resistance in wheat, where varieties resistant to the *Ug99* rust race have been developed that are based on 4-5 minor genes (Ellis et al. 2014; Mago et al. 2011; Singh et al. 2015; Singh et al. 2011). Other pyramiding experiments concern tropical rice lines resistant to rice blight (Chukwu et al. 2019; Singh et al. 2001). The pyramided lines described in this work showed almost complete resistance to blast both under artificial infection and natural infection in the field.

235 The selection of the genes to be included in a pyramiding effort must be carefully planned, and the use of
236 molecular markers is essential (Burdon et al. 2014; Joshi and Nayak 2010; Pink 2002). In rice, lines hosting
237 key genomic regions associated with rice blast resistance have been developed through conventional and
238 marker assisted breeding (Chen et al. 2008; Miah et al. 2013; Peng and Khushg 2015). Molecular markers
239 have been developed for many rice blast resistance genes, including those used in our program (Ashkani et
240 al. 2011; Fjellstrom et al. 2004; Hayasaka et al. 1996; Hayashi et al. 2006; Jia et al. 2002; Nakamura et al.
241 1997; Wang et al. 1999; Zhou et al. 2006). *Pi1*, *Piz-5* and *Pita*2 have been pyramided into cultivars with broad
242 spectrum resistance (Hittalmani et al. 2000).
243 Our work provides evidence, for the first time, of the possibility of pyramiding several *R* genes into rice
244 genetic backgrounds adapted to European growing conditions. A second target of the program was to move
245 at least four *R* genes to germplasm competitive with superior varieties currently cultivated. The breeding
246 work is still in progress.
247

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TABLES

TABLE1. *R* resistance genes used in pyramiding and the rice donor lines carrying them.

Gene	Cultivar	Species	Type	References
Pib	Saber	*O.sativa*	tropical japonica	(Hayashi et al. 2006; Shakiba et al. 2017)
Pik	Kusabue	*O.sativa*	temperate japonica	(Fukuhara 1999; Hayashi et al. 2006)
Pita2	Katy	*O.sativa*	temperate japonica	(Fjellstrom et al. 2004; Jia et al. 2003)
Piz	Jefferson	*O.sativa*	tropical japonica	(Fjellstrom et al. 2004; Volante et al. 2017; Das et al. 2012)
Piz-t	Toride	*O.sativa*	japonica	(Das et al. 2012; Thakur et al. 2012)

TABLE 2. Molecular markers used to monitor the presence of the five *R* genes during MAS.

Marker	Gene	Chr	Forward primer	Reverse primer	References
*Pib*5	*Pib*	2L	CTACTGCTCTCGCTCCGAATTCC	CAGAATTTTGTCAGGAACCTGCC	(Tacconi et al. 2010)
K2167	*Pik*	11L	CGTGCTGTCGCCTGAATCTG	CACGAACAAGAGTGTGTCGG	(Hayashi et al. 2006)
*Pita*3	*Pita2*	12L	AGTCGTGCGATGCGAGGACAGAAAC	GCATTCTCCAACCCTTTTGCATGCAT	(Tacconi et al. 2010)
Z4794	*Piz*	6S	TGAATGTGAGAGGTTGACTGTGG	CACGCCACCCTTCAATGGAGACT	(Hayashi et al. 2006)
ZT56591	*Piz-t*	6S	TTGCTGAGCCATTGTTAAACA	ATCTCTTCATATATATGAAGGCCAC	(Hayashi et al. 2006)

TABLE 3. Temperate Italian genotypes recipient of *R* resistance genes and their genetic state at the five *R* loci used in pyramiding.

Variety/Breeding line	Code	Profile	*R* gene				
			Pib	*Pik*	*Pita2*	*Piz*	*Piz-t*
Line 0	AL	long grain	-	-	-	+	-
Line 1	LI	indica	+	-	-	-	-
Line 2	LL	long grain	-	-	-	-	-
Line 3	AR	aromatic	+	-	-	-	-
ISTA F6	IS	long grain	-	-	-	+	-

460 **TABLE 4.** Number of *R* genes pyramided at various stages of the breeding program.

Year	Temperate background	*R* gene	N° of pyramided *R* alleles	N° of plants	Code
2011	AL, LI, AR	*Pik, Piz*	2	133	
		Pib, Piz	2	6	
		Pib, Pik	2	8	
		Pib, Pik, Piz	3	15	
2013	AL, LI, AR	*Piz-t, Pita2*	2	4	
		Pib, Pik, Piz	3	17	
		Pib, Pik, Piz, Pita2	4	1	
		Pib, Pik, Piz, Piz-t	4	2	
2014	AL, LI	*Pib, Pik, Piz, Pita2*	4	1	*SJKK*
		Pib, Pik, Piz, Piz-t	4	1	*SJKT-2*

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465 **TABLE 5.** Infection scores for blast artificial and natural infection on leaves of the
466 two pyramided lines *SJKK* and *SJKT-2* and the susceptible rice varieties Carnaroli,
467 Maratelli and Vialone Nano.
468

Line / Variety	Greenhouse				Field
	IT2	IT3	IT10	Mean	
SJKK	0	0	0	0	0
SJKT-2	0	2	0	0.7	1
Carnaroli	5	5	5	5	5
Maratelli	6	6	6	6	6
Vialone Nano	5	5	5	5	5

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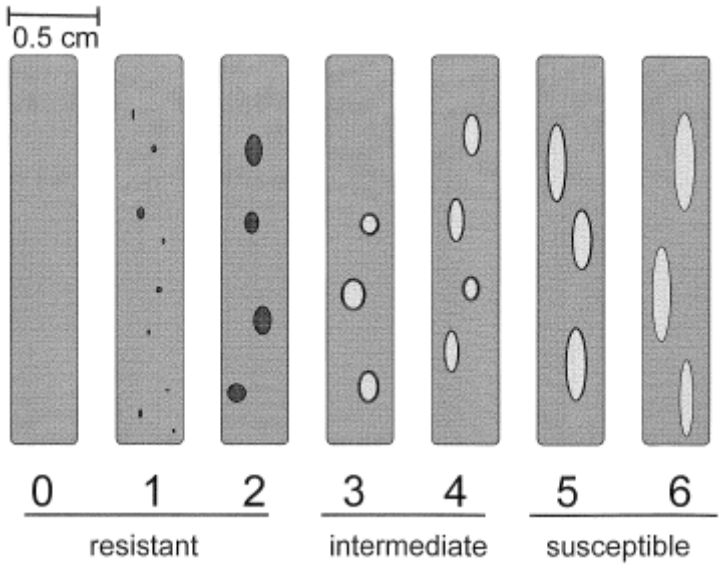
FIGURES

FIGURE 1. Scoring of symptoms induced by the blast pathogen *Pyricularia oryzae* on rice leaves. 0-2: dark, resistant type lesions without sporulation. 3-5: sporulating lesions with grey centre and dark margin. 6: susceptible, sporulating lesions without dark margin (Roumen et al. 1997).



CONFLICT OF INTEREST

The work was sponsored by a private company. GO works for the sponsoring company, while the other Authors declare no conflict of interest.

CONTRIBUTION OF AUTHORS

GO, RG and EP performed the experiments. GO, RG and CP wrote the manuscript with a review and input from the rest of the team. MRS and EP conceived the project. MRS directed the project.