Antagonistic yeasts and thermotherapy as seed treatments to control Fusarium fujikuroi on rice

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ABSTRACT

Bakanae disease, caused by *Fusarium fujikuroi,* is the most important seedborne disease of rice. Biological control and physical treatments can be effective tools to control seedborne diseases. Sixty-two isolates of yeasts and yeast-like fungi were obtained from different rice seeds. Four yeast isolates were selected in dual culture assays for mycelial growth inhibition, and in seed tests for reduction of infection rate. The isolates R23 and R26 were identified as *Metschnikowia pulcherrima*, the isolate R9 as *Pichia guilliermondii*, and the isolate SB1 as *Sporidiobolus pararoseus*. Rice seeds treated with *P. guilliermondii* R9, *M. pulcherrima* R23 and R26 significantly reduced the infection rate of *F. fujikuroi,* compared to some commercial biofungicides. The four selected yeasts reduced the bakanae disease severity in rice plants grown in greenhouse trials. Antagonist seed dressing resulted in reduction of the disease index from 93.0% in the untreated control to 20.0% in *P. guilliermondii* R9 treated seeds, and to 28.5% in *M. pulcherrima* R23 treated seeds. Selected antagonists were also used in combination with thermotherapy, which contributed to increase their efficacy. Thus, *P. guilliermondii* R9 and *M. pulcherrima* R23 combined with thermotherapy at 60 °C for 10 min decreased the bakanae disease index below 5%, and improved the seed germination rate compared to the single treatments, showing a seed priming effect. This is the first report about the use of antagonistic yeasts for seed dressing of rice to control *F. fujikuroi* on rice seeds, and biological treatment may be improved through combination with thermotherapy.

Keywords: *Fusarium fujikuroi, Metschnikowia pulcherrima, Oryza sativa, Pichia guilliermondii, Sporidiobolus pararoseus,* thermotherapy

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important crops worldwide. Its consumption has been steadily increasing and rice is the staple food for about half of the world's population (White, 1994). Overall, rice covers a surface of 163.4 x 10^6 ha, with a production of 718.3 x 10^6 tonnes (FAOSTAT, 2011). *Fusarium fujikuroi* Nirenberg [teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura], the causal agent of bakanae disease, is of high economic importance on rice (Carter et al., 2008). The impact of bakanae disease is significant in Asian countries, and the importance of *F. fujikuroi* has recently grown also in Europe (Spadaro et al., 2012). Typical bakanae disease symptoms include yellowing, stunting, stem elongation, and root and crown rot (Hsieh et al., 1977; Webster and Gunnell, 1992). Crop losses caused by the disease may reach up to 40% in epidemic cases (Ou, 1987). The fungus overwinters in infected seeds or crop residues (Ou, 1985; Dodan et al., 1994; Biswas and Das, 2003). The disease, originating from infected seeds, could be prevented by using healthy or treated seeds. Chemical seed dressing is the most common strategy adopted to control rice seedborne pathogens (Thobunluepop, 2008), though it might favour the occurrence of fungicide-resistant strains and it could be harmful to beneficial organisms (Munkvold, 2009). Moreover, it is sometimes hard to find chemical compounds which selectively control the fungal pathogen, without damaging the seed. Fungicide toxicity has been associated with the production of phytotoxic compounds reducing seed ageing and viability (Han, 2000; Thobunluepop, 2008). Chlorophyll and N content, such as nodulation, might also be affected in plants originating from fungicide-treated seeds (Aamil et al., 2004).

Biological control, based on the use antagonistic microorganisms, can be an effective and sustainable strategy to control plant diseases (Adams, 1990). Biocontrol has been used against different *Fusarium* spp. (Gilardi et al., 2005; Franco et al., 2011; Matarese et al., 2012). Microbial antagonists use different mechanisms to control plant pathogens, including nutritional competition, parasitism, secretion of antibiotics, or induction of localized and systemic resistance in host plants (Spadaro and Gullino, 2005; Chincholkar and Mukerji, 2007). Seed dressing with microorganisms can protect against seedborne pathogens (Amein et al., 2008; Tinivella et al., 2009; Madiha et al., 2012). Bacterial antagonists have been used to control fungal seedborne diseases of cereals, such as *Drechslera teres, Microdochium nivale* and *Tilletia caries* (Hökeberg et al., 1997). There are also few reports of bacterial application for the control of *F. fujikuroi* on rice (Kazempour and Elahinia, 2007; Li et al., 2006; Zhang et al., 2010a). However, to the best of our knowledge, there are no reports on the use of yeasts for rice seed dressing against *F. fujikuroi*, though previous studies

demonstrated that yeasts, isolated from different rice organs, can be used to control rice sheath blight (Shahjahan et al., 2001).

Physical methods, such as hot water, dry heat and hot steam, represent another valuable alternative strategy to control fungal diseases. Physical methods have been applied to control several seedborne fungal pathogens on cereals (Forsberg et al., 2003; Gilbert et al., 2005), and vegetables (Nega et al., 2003; Schmitt et al., 2009; Koch et al., 2010). There is also one report about hot water dip (HWD) and hot-humid air use to control *F. fujikuroi* on rice seeds, where bakanae disease was reduced by around 90%, both in laboratory tests and field trials (Titone et al., 2004).

The aims of this work were the selection and identification of yeasts with potential antagonistic activity against *F. fujikuroi* on rice, and the evaluation of their biocontrol efficacy both *in vitro* and *in vivo*. Furthermore, the selected biocontrol agents and thermotherapy were compared, alone or in combination, for their efficacy against bakanae disease.

2. Materials and methods

2.1. Seeds and microorganisms

Rice seeds cv Galileo naturally infected with *F. fujikuroi* (over 95%, data not shown), previously tested on blotter paper, were used throughout this study.

For dual culture test, the strain CSV1 of *F. fujikuroi*, previously identified through TEF sequencing (Accession no. KC121067) and selected for its high level of virulence on rice, was used (Matić et al., 2013).

Epiphytic yeasts were isolated from rice seeds. Seeds were placed in flasks containing sterile Ringer solution and kept on a rotary shaker at 150 rpm for 30 min. Serial dilutions of these suspensions were transferred into Petri dishes containing potato dextrose agar (PDA, Merck, Darmstadt, Germany) with 50 mg l^{-1} streptomycin sulphate (Merck). After 48 h incubation at room temperature (25° C), single colonies of yeasts and yeast-like fungi were selected, and 62 isolates were streaked onto NYDA (8 g nutrient broth, 5 g yeast extract, 10 g glucose, and 20 g agar per l of distilled water).

2.2. Screening in dual culture and on seeds in vitro

Inhibition of mycelial growth of *F. fujikuroi* by isolated yeasts was tested according to Zhang et al. (2010b) with minor modifications. For the dual culture assay (*F. fujikuroi* + yeast), a loop of 2-day-old yeast cell suspension grown on NYDA (25 °C) was streaked on a 90 mm diameter Petri Dish with PDA at 30 mm from the border. A 8 mm mycelium disk of *F. fujikuroi*, grown on

Komada (1975) at 25 °C for 5 days was put at 26 mm from the opposite border and at 26 mm from the yeast streak. For single culture assay, a mycelium disk of *F. fujikuroi* was put on PDA plate as a control. Three replicates were used both for dual and single culture assays. Plates were incubated at 25 °C for 10 days for daily observations of the mycelial growth of *F. fujikuroi*. Inhibition of *F. fujikuroi* growth was measured as percentage inhibition of radial growth (PIRG) following the formula (Fokkema, 1976): R1-R2/R1 x 100, where R1 corresponds to the radial growth of the fungus towards opposite site, and R2 corresponds to the radial growth of the fungus towards the antagonist.

The biocontrol efficacy of the yeast strains was also tested on seeds naturally infected by *F. fujikuroi* in Petri dishes. Rice seeds were immersed in 1% NaOCl for 5 min, washed under tap water, and air-dried. Cell suspensions for seed treatment were prepared by growing the selected yeast strains in YEMS (Spadaro et al., 2010) at 25 °C for 48 h on a rotary shaker (200 rpm). Cells were collected by centrifugation at 5000 g for 10 min, re-suspended in Ringer solution, quantified with a Bürker chamber to reach a standard concentration of 10^8 cells ml^{-1}. The seeds were then immersed in the antagonist suspension on a rotary shaker at 90 rpm for 15 min, air dried, and placed in Petri dishes with Komada medium. Twenty-five seeds were placed per Petri dish and three dishes were prepared per treatment. The dishes were kept at 25 °C for 5 and 10 days to evaluate visually and under light microscope the infection rate by *F. fujikuroi*. The biocontrol efficacy test on seeds was carried out twice.

2.3. Molecular and morphological identification

Four yeasts were selected for their antagonistic activity in dual culture assays, and they were identified by sequencing the internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2), according to White et al. (1990). Total yeast DNA was extracted with the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The ITS regions were amplified by using universal primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'). Twenty ng yeast DNA were used for the PCR reaction with a mixture (40 µl) containing 10X PCR buffer (Qiagen, Düsseldorf, Germany), 0.5 µM each primer, 0.5 mM dNTPs (Qiagen), and 2 U Taq DNA polymerase (Qiagen). The PCR program was: 5 min denaturation at 94 °C; 35 cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min; and 7 min final extension at 72 °C. PCR products were purified with QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, and sequenced in both directions at the BMR Genomics Centre (Padova, Italy). ITS sequences were blasted and deposited in GenBank (www.ncbi.nlm.nih.gov; Table 1). Morphological

identification was performed through microscope observations of the yeast cells and morphological examination of the colonies.

2.4. Antagonistic activity on seeds

Four selected yeasts were evaluated for their antagonistic efficacy *in vitro* on a larger number of seeds. Yeast cell suspensions were prepared as indicated above. Seeds were disinfected, treated with the antagonist cell suspension at 10^8 cells $ml^{-1}$for 15 min, air dried and placed in Petri dishes on filter paper (for seed germination tests) and Komada medium (for biocontrol efficacy tests). The controls (Table 2) were three commercial biofungicides (Mycostop Mix, Verdera, Espoo, Finland, 10^8 cfu g^{-1} *Streptomyces griseoviridis* strain K61, 8 g kg^{-1} seed; Serenade, Intrachem Bio Italia, Cesena, Italy, 5.13×10^{10} cfu g^{-1} *Bacillus subtilis* strain QST 713, 10 g kg^{-1} seed; and Ekoseed Pro, Nufarm Ltd, Victoria, Australia, mycorrhizas 30%, rhizobacteria 9.9×10^8 cfu g^{-1}, *Trichoderma* spp. 5×10^7 cfu g^{-1}, 2 g kg^{-1} seed), and a chemical fungicide (prochloraz, Octave, Bayer CropScience GmbH, Wolfenbüttel, Germany, 50% a.i., 0.25 g a.i. kg^{-1} seed).

The seed germination tests were performed in Petri dishes on 2 layers filter paper. After moistening the paper with 6 ml sterile water, 25 seeds were placed in dishes. The dishes were incubated in 12/12 h (day/night) regime at room temperature (25 °C). The percentage of germinated seeds was counted 5 and 10 days after treatment.

To perform the phytopathological analysis, 25 seeds were placed on Komada medium in Petri dishes at 25 °C. The *F. fujikuroi* infection rate was assessed 5 and 10 days after treatment. Seeds were inspected visually and by microscope for the presence of mycelium of *F. fujikuroi*. The germination test and the phytopathological analysis were performed twice. Nine replicates were used in each experiment.

2.5. Antagonistic activity on plants

Rice seeds were dipped in the cell suspension of each antagonist (10^8 cells ml^{-1}), shaken (90 rpm) for 15 min, and air-dried. Seeds were sown in plastic pots (50 seeds per pot) containing sterile soil (60% peat and 40% sand). The pots were placed following a randomised block design in a greenhouse (24-26° C day: 16-18° C night). Germination rate was evaluated and disease symptoms were scored twice, 14 and 28 days after sowing, using a 0-4 scale: 0 – asymptomatic plants; 1 - plants with chlorotic leaves and delayed growth; 2 - plants with thin and elongated internodes; 3 - plants with crown necrosis and severe bakanae symptoms; 4 – dead plants due to bakanae disease (Amatulli et al., 2010; Fig. 1). Three replicates were used in each trial. The biocontrol efficacy on rice plants was tested twice.

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216 *2.6. Influence of thermotherapy on seeds and on plants*

217 Thermotherapy by hot water dipping (HWD) was performed by seed immersion in a water bath
218 heated at 60, 65, 70 and 75 °C for 5, 10, 15 and 20 min. HWD was followed by air-drying for 2 h.
219 Seed germination tests were performed in Petri dishes with 2 layers of filter paper on 25 seeds per
220 dish. The percentage of germinated seeds was counted 5 and 10 days after treatment. To test the
221 infection rate, 25 seeds were placed on Komada medium in Petri dishes for 5 and 10 days. The
222 germination test and the phytopathological analysis were performed twice. Nine replicates were
223 used in each experiment.

224 In a second trial, seeds were sown in plastic pots (three replicates) by adopting a randomised block
225 design and grown in greenhouse under the same conditions described above. Germination rate and
226 disease symptoms were scored twice, 14 and 28 days after sowing. The efficacy on rice plants was
227 tested twice.

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229 *2.7. Combination of antagonists and thermotherapy in controlled trials*

230 Rice seeds were treated by a combination of the most efficient thermotherapy methods with each
231 antagonist as follows. The seeds were placed in a water bath at 60 °C for 5 or 10 min, and air-dried
232 for 2 h. Then, the seeds were dipped in flasks with the yeast cell suspensions (10^8 cells ml^{-1}) on a
233 rotary shaker (90 rpm) for 15 min. The seeds, after air drying, were sown in plastic pots by adopting
234 a randomised block design, and the plants were grown in greenhouse under same conditions as
235 described above. The treatments included single (antagonists and thermotherapy) and combined
236 (antagonist + thermotherapy) applications. Prochloraz (Octave) was used as a chemical standard.
237 Three replicates were used in each trial. Germination rate and disease symptoms were scored twice,
238 14 and 28 days after sowing. The efficacy on rice plants was tested twice.

239

240 *2.8. Statistical analysis*

241 Disease index scores were used to calculate the McKinney index (Mi; McKinney, 1923)
242 expressing the weighted average of the disease severity as actual percentage in terms of maximum
243 disease severity. The index was calculated by using the following formula: $Mi=[\Sigma df/T_nD]x100$,
244 where d is the degree of disease severity, f its frequency, Tn the total number of the plants examined
245 (healthy and diseased), and D the highest degree of disease intensity occurring on the empirical
246 scale. Data of germination and disease index were arcsin square root transformed before statistical
247 analysis. Statistical significance was judged at the level of P-value <0.05. When the analysis of

variance was statistically significant, Duncan's Multiple Range Test was used to separate the means. Data were analysed using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA).

Limpel's formula described by Richer (1987) was used to evaluate synergistic interactions between antagonist and thermotherapy treatments. The formula used was $Ee = X + Y - (XY/100)$, where *E*e corresponds to the expected effect from additive responses of two treatments, and X and Y present the percentages of disease reduction obtained when each agent was applied alone. Thus, synergism existed if the combination of the two agents gave a value of disease reduction superior than *Ee*.

3. Results

3.1. Antagonist screening

Sixty-two isolates of yeasts and yeast-like fungi, obtained from rice seeds, were evaluated against *F. fujikuroi* for mycelium growth inhibition in dual culture assay and for reduction of infection rate on naturally contaminated seeds (data not shown). Percentage inhibition of *F. fujikuroi* radial growth was reported for the four isolates selected for their biocontrol potential: R9, R23, R26, and SB1 (Fig. 2a). The highest PIRG value (around 50%) was obtained at different days of incubation by isolates R23, R26 and SB1, whereas the maximum PIRG was lower for isolate R9 (29% after 10 days incubation; Fig. 2b). R26 isolate provided a more consistent inhibition of *F. fujikuroi* growth (between the 2nd and 8th day of incubation), whereas R23 and SB1 were more active at the beginning (1st or 2nd day) and the end of incubation period (from 8th to 10th day).

3.2. Molecular and morphological identification

Morphological (colony) and microscope (cell shape and size) observations of the four selected antagonists were performed (Table 1). The four selected isolates were also identified by sequencing the ribosomal regions ITS1-5.8S-ITS2 with universal primers ITS-1 and ITS-4 (Table 1). BLASTN analysis showed that isolates R23 and R26 had the highest similarity (98% for both isolates) to *Metschnikowia pulcherrima* (Accession no. AY235809), whereas isolate R9 showed the highest similarity (99%) to *Pichia guilliermondii* (Accession no. DQ663478). The sequence of isolate SB1 was identical (100%) to *Sporidiobolus pararoseus* (Accession no. JQ425362). The sequencing results confirmed morphological and microspore observations.

3.3. Efficacy of antagonistic yeasts on seeds

The four selected yeasts were evaluated for their antagonistic potential on rice seeds naturally infected by *F. fujikuroi* incubated *in vitro* on Komada medium (Table 2). Rice seeds treated with *P. guilliermondii* R9, *M. pulcherrima* R23 and R26 significantly reduced the infection rate of *F. fujikuroi* (14.3%, 16.7%, and 18.0%, respectively) compared to the commercial biofungicides used in this study (ranging from 28.3% to 35.0%) (Table 3). *S. pararoseus* SB1 was less effective in the control of *F. fujikuroi* and it provided results similar to the biofungicide Serenade (*B. subtilis*).

When antagonistic yeasts were applied, rice seeds germination increased compared both to control seeds and seeds treated with biofungicides. Treatment with R9 caused the highest germination rate (99.5%) compared to the other treatments tested (Table 3).

3.4. Efficacy of antagonistic yeasts on plants

Selected yeasts were able to significantly reduce the severity of bakanae disease in rice plants also *in vivo* under greenhouse conditions (Table 4). R9 treatment reduced the McKinney disease index (D.I.) to 20.4%, and R23 to 28.5% compared to 93.0% in the untreated control. *P. guilliermondii* R9 and *M. pulcherrima* R23 were statistically more efficient than the commercial biofungicides Serenade (*B. subtilis*) and Mycostop Mix (*S. griseoviridis*). Although *M. pulcherrima* R26 significantly reduced *F. fujikuroi* D.I. (52.5%), it was less effective compared to R9 and R23 and the treated plants showed yellowing.

Germination of rice seeds increased when the seeds were treated with the four antagonists compared to the untreated seeds and to the seeds treated with the biofungicides, similarly to the results of *in vitro* studies. The highest germination rate was obtained with *P. guilliermondii* R9 (99.2%) (Table 4).

3.5. Influence of thermotherapy on seeds and on plants

Thermotherapy applied with HWD was used to control *F. fujikuroi* infection in rice seeds *in vitro* and *in vivo*. The highest reduction of *F. fujikuroi* infection was obtained with HWD at 60 °C for 5 and 10 min *in vitro*, without affecting the germination rate (above 95 %) (Supplementary Fig. 1).

In greenhouse experiments, the most efficient treatments in reducing bakanae and increasing the germination rate confirmed to be the treatments of rice seeds at 60 °C for 5 and 10 min (Fig. 3). Heat treatments at 65 °C for different times were also efficient in reducing the D.I., but they were accompanied by a lower germination rate compared to the treatments at 60 °C. HWD at 70 °C (15 and 20 min) and 75 °C (5, 10, 15 and 20 min) completely inhibited the seed germination.

3.6. Combination of antagonists and thermotherapy in controlled trials

In greenhouse trials, the efficacy of the combination of antagonists and thermotherapy was evaluated against bakanae disease 14 and 28 days after sowing. Antagonists and thermotherapy alone were used as single treatments. The best results were obtained when antagonists were applied together with HWD, in particular *P. guilliermondii* R9 with HWD at 60°C for 10 min and *M. pulcherrima* R23 with the same thermal treatment (Fig. 4). When the combination of antagonists and thermotherapy was applied, the D.I. was reduced below 5.0% (with the exception of SB1), followed by thermotherapy alone (D.I.: 15.3-19.7%), and R9 and R23 antagonists alone (D.I.: 21.0 and 27.8%, respectively). The Limpel's formula permitted to find a synergism between the antagonists and thermotherapy in reducing bakanae disease. Thus, *M. pulcherrima* R26 acted synergistically with both thermal treatments (60 °C for 5 min and 10 min) in reducing bakanae disease (Supplemental table 1). *M. pulcherrima* R23 and *S. pararoseus* SB1 showed synergism with thermotherapy at 60 °C for 5 min. On the opposite, *P. guilliermondii* R9 did not show synergism with the thermal treatments in reducing bakanae incidence. The germination rate was increased by the combination of treatments compared to the single treatments and to the untreated control (Table 5).

4. Discussion

Four new yeast biocontrol agents effective against *F. fujikuroi*, the causal agent of bakanae disease, were isolated from rice, identified and evaluated for their efficacy both *in vitro* and *in vivo*. This study represents the first report of use of *M. pulcherrima*, *P. guilliermondii* and *S. pararoseus* against the seedborne pathogen *F. fujikuroi*. Previously, other yeast biocontrol agents, such as *Pichia anomala*, have been used on cereals with a different objective, i.e. to prevent mold spoilage and enhance the preservation of moist grain in storage systems (Petersson and Schnürer, 1995).

P. guilliermondii, *M. pulcherrima* and *S. pararoseus* have been commonly isolated from the fruit carposphere (Droby et al., 1993; Janisiewicz et al., 2010), but there are very limited reports about their isolation from cereal seeds (Petersson and Schnürer, 1998). *P. guilliermondii* was reported to be an effective biocontrol agent against *Aspergillus flavus*, fungi of stored grains (Paster et al., 1993; Druvefors and Schnürer, 2005), and mould diseases (Guetsky et al., 2002; Lahlali et al., 2011; Zhang et al., 2011a), with some activity also in the biodegradation of mycotoxins (Patharajan et al., 2011). Tenacious attachment, along with secretion of cell wall degrading enzymes, such as β-(1–3)-glucanase, play a role in the biocontrol activity of *P. guilliermondii* (Wisniewski et al., 1991; Zhang et al., 2011b). On the opposite, the main mode of action of the biocontrol agent *P. anomala* in the grain system, was the antifungal action of metabolites, such as ethyl acetate and ethanol (Druvefors et al., 2005).

M. pulcherrima might be used as an effective biocontrol agent against postharvest diseases of fruit and some human pathogens as well (Schena et al., 2000; Janisiewicz et al., 2001; Grebenisan et al., 2006; Spadaro et al., 2008). *Metschnikowia* spp. antagonise the pathogens through competition for limited nutrients, such as iron (Saravanakumar et al., 2008) or production hydrolases, such as chitinases, involved in the fungal cell-wall degradation (Saravanakumar et al., 2009).

S. pararoseus has been reported to efficiently suppress postharvest decay diseases on fruits of kinnow, nectarine and strawberry (Sharma et al., 2008; Janisiewicz et al., 2010; Huang et al., 2012). *S. pararoseus* mainly acts against pathogens by competing for scarce nutrients and by production of volatile organic compounds (VOCs) (Sharma et al., 2008; Huang et al., 2012).

The present study demonstrated that *P. guilliermondii, M. pulcherrima,* and *S. pararoseus* may play a role in the suppression of *F. fujikuroi* infection on rice. Both dual culture and seed assays showed that the selected antagonists inhibited the mycelium growth of *F. fujikuroi.* However, among the four tested yeast strains, the best results in the control of *F. fujikuroi* were obtained with *P. guilliermondii* R9. *P. guilliermondii* R9 was more efficient in *in vivo* studies compared to the dual culture assay, which might be explained by its possible involvement in inducing rice defence mechanisms that should be further investigated. The other antagonists effective in reducing the infection rate were *M. pulcherrima* R23 and R26. *M. pulcherrima* R26 was less efficient than R23, though they belong to the same species. Although *S. pararoseus* SB1 reduced *F. fujikuroi* infection rate and disease severity, both in *in vitro* and *in vivo* assays, its effectiveness was lower compared to the other three antagonists.

Understanding the mechanism of action is crucial to develop optimal commercial formulations and application procedures in order to maximize the efficacy of biocontrol agents (Spadaro and Gullino, 2005). The modes of action through which the selected antagonists supressed *F. fujikuroi* will be investigated in future, with particular interest in the hydrolases of *M. pulcherrima* and *P. guilliermondii*, and the VOCs produced by *S. pararoseus*, which previously showed to be involved in their antagonistic activity against other fungal pathogens.

It should be evidenced, that prochloraz, used as a chemical standard in *in vivo* assays, permitted a slightly higher efficacy compared to the selected antagonists, but the use of the biocontrol agents could be considered a feasible eco-friendly alternative choice against the seedborne *F. fujikuroi.*

In vitro studies permitted a rapid screening for biocontrol efficiency of the four selected antagonists. None of the antagonists negatively affected germination, indicating the lack of production of secondary phytotoxic metabolites. Moreover, it seems that treatment with antagonists had a positive effect on seed germination, since the germination percentage was higher compared to

the control treatment and to the treatment with commercial biofungicides, indicating a priming effect of the biocontrol agents.

Also HWD treatment was investigated for the control of bakanae disease, and it was compared and combined with biocontrol agents. The most efficient HWD treatments *in vivo* were at 60 °C for 5 min and 10 min. Both treatments showed an effectiveness similar to R9 in *F. fujikuroi* control, and they kept a high germination rate.

When the thermal treatments were combined with the antagonists *P. guilliermondii* R9 and *M. pulcherrima* R23 in greenhouse conditions, both the control of *F. fujikuroi* and the germination rate increased. Furthermore, synergistic interactions between antagonist and thermal treatment in reduction of bakanae disease were found in four out of eight combined treatments, which additionally confirmed an advantage in using antagonists and thermotherapy together. Similar results were obtained by the combination of antagonists and thermotherapy in the control of postharvest diseases of fruit (Karabulut et al., 2002; Spadaro et al., 2004; Zhang et al., 2010c), while there is limited information on combined use of yeast and heat treatments on seeds against fungal pathogens.

In this study, the biocontrol agents and thermotherapy were investigated on the susceptible cultivar of rice 'Galileo'. Further investigations to assess the effectiveness of biocontrol agents and HWD on a wider number of cultivars with various degree of resistance should be performed. Bakanae disease depends on many factors, including the rice cultivar, the time of sowing and flowering, and the rice cropping system (Ghazanfar et al., 2013). Some rice cultivars are resistant to bakanae disease, while other cultivars, such as 'Dorella' and 'Galileo', are susceptible to *F. fujikuroi* (Matić et al., 2013).

In conclusion, to control bakanae disease on rice, the combination of antagonists and thermotherapy should be considered a feasible and effective strategy to be further investigated and to be implemented in commercial large-scale use.

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592 **TABLES**

593

594 **Table 1.**

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Isolate	Colony	Cell shape and size	ITS sequence (Accession no.)	Species
R23	Cream	Ovoid (3.13 x 6.38-2.27 x 4.28 µm)	KC456055	*Metschnikowia pulcherrima*
R26	Cream	Ovoid (3.13 x 6.38-2.27 x 4.28 µm)	KC456056	*Metschnikowia pulcherrima*
R9	White to cream	Ovoid (3.00 x 6.96-1.48 x 4.81 µm)	KC456057	*Pichia guilliermondii*
SB1	Red-orange	Elongated (4.03 x 7.72-2.28 x 3.63 µm)	KC456058	*Sporidiobolus pararoseus*

598 **Table 2.**
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Species and/or product name	Strain	Producer/Institution	Concentration/quantity applied per 5 g of seed
Pichia guilliermondii	R9	Agroinnova	10^8 cells ml^{-1}
Metschnikowia pulcherrima	R23	Agroinnova	10^8 cells ml^{-1}
Metschnikowia pulcherrima	R26	Agroinnova	10^8 cells ml^{-1}
Sporidiobolus pararoseus	SB1	Agroinnova	10^8 cells ml^{-1}
Streptomyces griseoviridis (Mycostop mix)	K61	Verdera	40 mg
Bacillus subtilis (Serenade)	QST 713	Intrachem Bio Italia	50 mg
Glomus caledonium GM 24 *Glomus coronatum* GU 53 *Glomus intraradices* GB 67 *Bacillus subtilis* BA 41 *Streptomyces* spp. SB 15 *Trichoderma harzianum* TH 02 *Pseudomonas proradix* 10 (Ekoseed Pro)	Mix	Nufarm	10 mg

Table 3.

Isolate/commercial product	Germination (%)*	Infection (%)*
M. pulcherrima R23	95.8 abc	16.7 b
M. pulcherrima R26	92.3 abc	18.0 b
P. guilliermondii R9	99.5 c	14.3 b
S. pararoseus SB1	97.6 abc	30.7 cd
B. subtilis QST 713 (Serenade**)	87.5 abc	31.7 cd
S. griseoviridis K61 (Mycostop mix**)	85.8 ab	35.0 d
Microorganism mixture (Ekoseed Pro**)	85.0 a	28.3 c
Prochloraz (Octave***)	98.3 bc	1.3 a
Untreated seed (control)	87.5 abc	97.7 e

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$), ** biofungicides. *** fungicide

Table 4.

Isolate/commercial product	Germination (%)*	Disease index (0-100)*
M. pulcherrima R23	94.2 ab	28.5 b
M. pulcherrima R26	90.0 ab	52.5 bc
P. guilliermondii R9	99.2 b	20.4 b
S. pararoseus SB1	96.7 ab	66.1 cd
B. subtilis QST 713 (Serenade**)	85.8 a	65.3 cd
S. griseoviridis K61 (Mycostop mix**)	84.2 a	71.2 cd
Microorganism mixture (Ekoseed Pro**)	84.2 a	54.8 bc
Prochloraz (Octave***)	95.8 ab	4.3 a
Untreated control	85.0 a	93.0 d

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$), ** biofungicides, *** fungicide

Table 5.

Treatment	Germination (%)*	
M. pulcherrima R23	93.3	bcd
M. pulcherrima R26	91.7	ab
P. guilliermondii R9	99.0	de
S. pararoseus SB1	94.2	bcde
60 °C 5 min	91.7	ab
60 °C 10 min	92.5	bc
M. pulcherrima R23 + 60 °C 5 min	94.2	bcde
M. pulcherrima R26 + 60 °C 5 min	92.5	bc
P. guilliermondii R9 + 60 °C 5 min	98.3	cde
S. pararoseus SB1 + 60 °C 5 min	97.5	bcde
M. pulcherrima R23 + 60 °C 10 min	95.8	bcde
M. pulcherrima R26 + 60 °C 10 min	95.0	bcde
P. guilliermondii R9 + 60 °C 10 min	100.0	e
S. pararoseus SB1 + 60 °C 10 min	98.3	cde
Prochloraz (Octave**)	96.7	bcde
Untreated control	87.0	a

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$), ** fungicide

Supplemental table 1.

Treatment	Disease reduction (%)	*E*e value	Activity
Prochloraz (Octave)	80.5	-	-
P. guilliermondii R9+60 °Cx10'	79.1	86.1	Additive
M. pulcherrima R23+60 °Cx10'	78.5	83.8	Additive
P. guilliermondii R9+60 °Cx5'	78.3	84.3	Additive
M. pulcherrima R26+60 °Cx10'	78.0	76.4	Synergistic
M. pulcherrima R23+60 °Cx5'	77.8	81.7	Synergistic
M. pulcherrima R26+60 °Cx5'	75.5	73.3	Synergistic
S. pararoseus SB1+60 °Cx10'	69.0	70.5	Additive
S. pararoseus SB1+60 °Cx5'	68.8	66.7	Synergistic
60 °Cx10'	65.5	-	-
60 °Cx5'	61.0	-	-
P. guilliermondii R9	59.8	-	-
M. pulcherrima R23	53.0	-	-
M. pulcherrima R26	31.5	-	-
S. pararoseus SB1	14.5	-	-
Untreated control	0	-	-

Figure captions

Fig. 1. Symptomatology induced on 21-day old rice plants (cultivar Galileo) by artificially infection of *Fusarium fujikuroi*. Symptoms of the bakanae disease were evaluated by the following scale: symptomless plants (0), plants with chlorotic leaves and delayed growth (1), plants with thin and elongated internodes (2), plants with crown necrosis (3), and dead plants (4).

Fig. 2. Inhibition of the growth of *F. fujikuroi*: (a) on PDA plates by *M. pulcherrima* R23, *M. pulcherrima* R26, *P. guilliermondii* R9, and *S. pararoseus* SB1, and (b) expressed through the radial mycelial growth. *F. fujikuroi* alone was used as a control in Fig. 2a. Fungus and yeasts were grown at 25 ºC for 10 days.

Fig. 3. Efficacy of thermotherapy on germination of rice seeds naturally infected with *F. fujikuroi* and bakanae disease index on rice plants grown *in vivo* under greenhouse conditions. Disease index and germination were measured 28 days after sowing. Error bars show standard deviations for triplicate assays. Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$).

Fig. 4. Influence of thermotherapy (60 °C for 5 min and 10 min), antagonists (*M. pulcherrima* R23 and R26, *P. guilliermondii* R9, and *S. pararoseus* SB1) and the combination of thermotherapy and antagonists on disease index on rice plants grown *in vivo* (in greenhouse). Prochloraz (Octave) was used as a chemical standard. Disease index was measured 28 days after sowing. Error bars show standard deviations for triplicate assays. Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$).

Supplementary Fig. 1. Effect of thermotherapy on germination (%) and on infection rate (%) of rice seeds naturally infected with *F. fujikuroi*. Germination and infection rates were measured 10 days after treatment. Error bars show standard deviations for triplicate assays. Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$).

Table captions

Table 1. Morphological and molecular characterization of the four selected antagonistic yeasts.

Table 2. Antagonistic yeasts and commercial bio-fungicides tested.

Table 3. Influence of antagonists on rice seed germination (%) and *F. fujikuroi* infection rate (%) *in vitro.* Germination was measured 10 days after treatment.

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$), ** biofungicides. *** fungicide

Table 4. Influence of antagonists on rice germination and bakanae disease index *in vivo.* Germination (%) and disease index (McKinney, 0-100) were measured 28 days after sowing.

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$), ** biofungicides, *** fungicide

Table 5. Influence of combination of antagonists and thermotherapy on rice germination *in vivo.* Germination (%) was measured 28 days after sowing.

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$), ** fungicide

Supplemental table 1. Interactions between antagonist and thermotherapy using Limpel's formula. Synergistic activity existed when the combination of the two agents gave a value of disease reduction superior than *Ee*.