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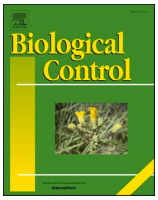

Screening and characterization of bacterial and fungal endophytes as potential biocontrol agents for rice seed dressing against *Fusarium fujikuroi*

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HIGHLIGHTS

- One hundred thirty-five rice endophytes were isolated from rice seeds and shoots.
- Eighteen strains significantly reduced bakanae disease severity *in vivo*.
- Five strains were selected as the most effective and safest BCAs.
- *In vitro* efficacy of selected strains does not correlate with *in vivo* results.
- First report of *Epicoccum catenisporum* and *Methylobacterium oryzae* as BCAs.

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ABSTRACT

Rice bakanae disease, caused by *Fusarium fujikuroi*, nowadays represents the main threat for rice seed producers. With recent European guidelines progressively reducing the use of chemical fungicides, new disease management strategies are urgently needed. The plant endosphere constitutes a source of potential microbial biocontrol agents (BCAs) against fungal pathogens. Endophytic antagonists, with their close association with the host, are more likely to withstand harsh field conditions, the main bottleneck of BCAs selection. For this reason, 135 fungal and bacterial endophytes were isolated from rice seeds and shoots. As previously observed, *in vitro* screenings rarely correlate with biocontrol efficacy *in planta*, thus each isolate was tested *in vivo* under controlled conditions via seed treatment against *F. fujikuroi* to select endophytes that significantly reduced bakanae disease severity. Among the 18 selected isolates, 12 were excluded after a further screening step based on their potential role as plant or human pathogens. The 5 remaining strains, belonging to *Epicoccum layuense*, *Epicoccum catenisporum*, *Microbacterium testaceum* and *Methylobacterium oryzae* species, were tested *in vitro* to assess their capacity to inhibit *F. fujikuroi* through agar diffusible or volatile antimicrobial compounds. The biocontrol efficacy of these endophytes was further confirmed *in vivo*, by observing a significant decrease in disease severity and incidence, as well as an increase in total fresh biomass. This represents the first report of *E. layuense*, *E. catenisporum*, *M. testaceum* and *M. oryzae* strains with biocontrol potential against *F. fujikuroi* as promising biological seed dressing alternatives to chemical fungicides.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important crops as it represents the main staple food for half of the world's population (Prasad et al., 2017). In Europe, Italy is the biggest rice producer, with 218,420 ha of surface dedicated and 1,236,960 t of production (FAOSTAT, 2022). One of the main threats to rice production in Europe is *Fusarium fujikuroi* Nirenberg [teleomorph *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura], causal agent of bakanae disease (Kraehmer et al., 2017). Typical symptoms include chlorosis and abnormal internode elongation due to the overproduction of gibberellic acid, both of fungal and plant origin, during the biotrophic stage (Siciliano et al., 2015; Matić et al., 2016). At later stages, this hemibiotrophic pathogen can cause crown rot or panicle sterility (Piombo et al., 2020; Shakeel et al., 2023). Bakanae is a monocyclic and mainly seedborne disease, resorting to seeds for its inter-field dispersion, whereas sources of secondary inoculum are

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infected or dead plants. *F. fujikuroi* white–pink mycelium profusely colonizes the crown during its necrotrophic phase, especially at the flowering stage, allowing intra-field conidia dispersal through water and wind to infect new hosts and being vertically transmitted to seeds (Amatulli et al., 2010; Matić et al., 2017). Yield losses due to bakanae disease are limited in the EU area, however the European Commission has set restrictive thresholds of plants infected by *F. fujikuroi* in rice seed producers' fields with the Commission Implementing Directive 2012/1/EU, seeking to limit the disease spread (Mongiano et al., 2021; Valente et al., 2017). Although the most reliable solution to avoid bakanae disease onset is chemical seed dressing (Amatulli et al., 2012), the environmental and health risks of pesticide use led the European Parliament to take measures aiming to ban or limit many previously authorized active ingredients with the Regulation (EC) 1007/2009 (Lamichhane et al., 2016). Additionally, the more recent Farm to Fork Strategy, in the framework of the European Green Deal, set two key targets for pesticides, aiming to reduce by 50 % the use and risk of chemical pesticides, as well as to cut by 50 % the use of more hazardous pesticides, referring to the so called "candidates for substitution". In this context, alternative solutions that safely and efficiently reduce the presence and spread of *F. fujikuroi* in the field, are needed especially by seed producers to meet European regulatory limits.

A sustainable bakanae disease management is based on breeding to obtain tolerant rice cultivars (Shakeel et al., 2023), however only a limited number of resistant genotypes are currently available (Valente et al., 2017) and cultivar choice in Italy is highly influenced by the market demand for "risotto" varieties. Additional management solutions explored over time include physical and biological seed treatments, such as thermotherapy (Forsberg et al., 2003), botanical and microbial biopesticides (Mongiano et al., 2021; Shakeel et al., 2023), or a combination of these methods (Matić et al., 2014). Most efforts, however, are focused into the selection of rice-associated microorganisms as potential biocontrol agents (BCAs) against *F. fujikuroi*. Microbial antagonists can rely on different strategies to suppress plant pathogens, namely competition for space and nutrients, antibiosis, hyperparasitism and production of lytic enzymes, host induced resistance, or resident microbiota manipulation (Collinge et al., 2022; Köhl et al., 2019). A number of bacterial (Hossain et al., 2016; Nawaz et al., 2022), yeast (Matić et al., 2014) and fungal (Kato et al., 2012; Ng et al., 2015; Saito et al., 2021) species were able to successfully reduce bakanae disease symptoms *in vivo* through different biocontrol modes of action (MOA), however the screening process prior the selection of candidate antagonistic strains was still *in vitro*-based in most cases, i.e. involving the classic dual culture method. Although *in vitro* assays are cost-effective and high throughput, as they allow to screen large numbers of isolates, it is well established that pathogen inhibition *in vitro* and disease reduction *in vivo* rarely correlate (Hassan et al., 2010; Huang et al., 2020; Rojas et al., 2020). Additionally, faster-growing strains *in vitro*, as well as genera with well-studied antibiosis or competition MOA, e.g. *Bacillus*, *Pseudomonas*, *Trichoderma* and *Clonostachys*, tend to be preferentially selected, thus excluding potential BCA whose biocontrol MOA cannot be detected under such conditions, i.e. induced resistance and microbiota manipulation (Collinge et al., 2019).

Microbes inhabiting inner plant tissues (plant endosphere) without causing disease are becoming increasingly interesting for their plant growth promotion (PGP) features, i.e. nutrient uptake improvement and phytohormone production, in addition to their potential biocontrol activity (Walitang et al., 2019; Chaudhary et al., 2022). Moreover, thanks to their close association to the host, they are theoretically more protected from external abiotic stressors and, thus, have higher chances to withstand harsh field conditions, which is the main bottleneck of BCAs selection (Collinge et al., 2019; Latz et al., 2018). Rice endophytes with a spectrum of biocontrol efficacy against more than one seedborne rice pathogen in addition to *F. fujikuroi* were previously selected (Hossain et al., 2016; Nawaz et al., 2022), however seed treatment was not explored as a delivery system. With this method, the establishment of microbial inoculants, as well as the early elicitation of plant defences against pathogens, were still observed well after seed germination (Koné et al., 2022; Mattei et al., 2022; O'Sullivan et al., 2021).

The aim of this work was to isolate and screen rice endophytes for their biocontrol potential against rice bakanae disease. This study followed an already proposed structured pipeline for untargeted endophytes isolation and *in vivo* mass screening using seed dressing as a delivery method (Latz et al., 2020), but also provides a workflow for both unicellular (bacteria and yeasts) and multicellular (filamentous fungi) microorganisms. The early development of inoculation methods similar to large-scale applications of commercialized strains are essential to reliably select BCAs that can efficiently colonize the host and avoid the disease onset. Strains that significantly reduced disease severity *in vivo* were, then, molecularly identified and characterized *in vitro* to verify their ability to inhibit *F. fujikuroi* colony growth as well as their range of abiotic stress tolerance. Finally, further *in vivo* biocontrol assays were performed to confirm the efficacy of selected strains by evaluating additional variables, such as disease incidence and total fresh biomass.

2. Materials and methods

2.1. Rice endophyte isolation

Seeds of 24 Italian rice experimental and commercial lines, provided by CREA-DC (Vercelli, Italy) and the seed company Sa.Pi.Se. Coop. Agricola (Vercelli, Italy), were used for endophytes isolation (Table 1). Among these, 5 genotypes (Oceano, Unico, S18042, S19048, S19067-1) were also grown in a greenhouse for 4 weeks to sample and isolate from rice aerial tissues. One gram of seed or aerial tissue (pools of leaves and stems) were used. Aerial organs were further cut into smaller pieces (3–5 cm) and collected into 50 ml Falcon tubes for surface sterilization. Seeds were washed with 30 ml of 0.1 % Tween 20 in a Falcon tube, which was then placed on a horizontal shaker at 100 rpm for 1 min; then the suspension was discarded and seeds were submerged into 30 ml of 70 % ethanol in the same tube and placed on a shaker for 2 min, followed by 2 % sodium hypochlorite for 10 min, 0.1 % Tween 20 for 1 min, 70 % ethanol for 2 min and, finally, rinsed 5 times with sterile deionized water (SDW), at least 30 s for each wash. Pools of leaves and stems were surface sterilized with 0.1 % Tween 20 for 30 s, 70 % ethanol for 30 s, 2 % sodium hypochlorite for 1 min, 0.1 % Tween 20 for 30 s, 70 % ethanol for 30 s and rinsed 5 times with SDW. To confirm the absence of epiphytic isolates (Sahu et al., 2021), 100 μl of the final rinsing water for each sample was plated onto Luria-Bertani Agar (LBA, Merck, Germany) and Potato Dextrose Agar (PDA, VWR, Italy) in triplicate and incubated at room temperature (22 ± 2 °C) for 7 d.

For endophytic fungi isolation, surface sterilized plant material (seed or aerial tissue) was further cut into smaller pieces (~3 x 3 mm) with a sterile scalpel and, after drying on sterile filter paper for 1 min, directly plated onto PDA and Malt Extract Agar (MEA, Merck), both supplemented with 0.025 g L^{-1} of streptomycin sulphate, in triplicate. For endophytic bacteria isolation, surface sterilized tissues were ground with sterile mortar and pestle in 9 ml of sterile ¼ strength Ringer solution (Merck). The homogenized suspension was serially diluted and 100 μl of each dilution were spread onto LBA and Reasoner's 2A Agar (R2A, VWR) media in triplicate for bacterial isolation and total microbial count, as described in Walitang et al. (2017). For endophytic yeast isolation, serial dilutions were spread plated on PDA and MEA amended with streptomycin sulphate in triplicate. All plates were incubated at room temperature for up to 14 d and microbial count was performed after 7 d. Single colonies of unique bacterial and yeast morphotypes were streaked, respectively, on LBA and PDA, whereas fungal endophytes emerging from directly plated tissues were subcultured onto PDA. Bacterial and yeast isolates were grown in LB broth and Potato Dextrose Broth (PDB, VWR), respectively, on a shaker (100 rpm) at room temperature for 2 d and stored at −80 °C in 30 % glycerol. Fungal

Table 1
Rice genotypes chosen for endophytes isolation, commercial status and provider, as well as number and type of microbial isolates obtained for each genotype.

Genotype	Commercial status	Provider	Number of isolates obtained		
			Bacteria	Yeasts	Filamentous fungi
45R/22	Breeding line	CREA-DC	2	–	1
Arsenal	Cultivar	CREA-DC	2	–	1
Vialone Nano	Cultivar	CREA-DC	2	1	–
G02220	Breeding line	Sa.Pi.Se. Coop. Agricola	1	–	2
S17023	Breeding line	Sa.Pi.Se. Coop. Agricola	1	–	2
S18042	Breeding line	Sa.Pi.Se. Coop. Agricola	4	2	3
S18050	Breeding line	Sa.Pi.Se. Coop. Agricola	–	–	3
S18052	Breeding line	Sa.Pi.Se. Coop. Agricola	1	–	2
S19026	Breeding line	Sa.Pi.Se. Coop. Agricola	1	–	2
S19048	Breeding line	Sa.Pi.Se. Coop. Agricola	6	2	1
S19067-1	Breeding line	Sa.Pi.Se. Coop. Agricola	7	–	2
S20054	Breeding line	Sa.Pi.Se. Coop. Agricola	1	–	2
Apollo	Cultivar	Sa.Pi.Se. Coop. Agricola	2	–	1
Artemide	Cultivar	Sa.Pi.Se. Coop. Agricola	2	1	–
Balilla	Cultivar	Sa.Pi.Se. Coop. Agricola	2	–	1
Carnise	Cultivar	Sa.Pi.Se. Coop. Agricola	2	–	1
Cerere	Cultivar	Sa.Pi.Se. Coop. Agricola	2	1	–
Ermes	Cultivar	Sa.Pi.Se. Coop. Agricola	2	–	1
Galileo	Cultivar	Sa.Pi.Se. Coop. Agricola	12	2	5
Misaki	Cultivar	Sa.Pi.Se. Coop. Agricola	3	–	–
Oceano	Cultivar	Sa.Pi.Se. Coop. Agricola	7	–	2
Selenio	Cultivar	Sa.Pi.Se. Coop. Agricola	10	8	2
Unico	Cultivar	Sa.Pi.Se. Coop. Agricola	7	–	2
Venere	Cultivar	Sa.Pi.Se. Coop. Agricola	2	1	–

isolates were stored at −80 °C in 40 % glycerol.

2.2. *In vivo* screening for biocontrol efficacy

Mass screening of the endophytes collection, aimed to select potential BCAs, was performed by evaluating disease symptoms reduction *in vivo*. Most fungal isolates were unable to produce a sufficient amount of conidia for mass inoculum production on common sporulation-inducing media such as PDA, MEA, Potato Carrot Agar (PCA) and Oatmeal Agar (OA) (Ritchie, 2001), therefore inoculum preparation and seeds treatment were performed according to Latz et al. (2020), with minor modifications. Endophytic fungi were cultured onto PDA plates at room temperature for 7–14 d, based on their growth rate. Aerial mycelium was collected by flooding the plates with sterile Ringer solution amended with 0.5 % sodium carboxymethyl cellulose (CMC-Na, Merck) as a seed coating agent, followed by scraping the surface with a sterile loop. The suspension was transferred into a Falcon tube and gently mechanically blended, before adjusting the concentration to 10^7 conidia or mycelial fragments ml^{-1} using a Burker chamber. The protocol of Walitang et al. (2017) with few adjustments was followed for bacterial inoculum preparation and seed treatment, and it was adapted for yeast isolates. Bacterial and yeast single colonies were grown in 30 ml of LB and PDB, respectively, into conical flasks and incubated at room temperature on a shaker (100 rpm). After 2 d, culture broth was removed by centrifugation at 6,000 × g for 10 min and cells were resuspended in sterile Ringer solution with 0.5 % CMC-Na. Bacterial inoculum was adjusted to $OD_{600} = 0.5$ (~10^7-10^8 CFU ml^{-1}) using a spectrophotometer, while yeast inoculum was adjusted to 10^7 cells ml^{-1} using a Burker chamber. Rice seeds of bakanae-susceptible cultivar Galileo (Amatulli et al., 2010) were surface sterilized by immersion in 2 % sodium hypochlorite for 30 min on a horizontal shaker, followed by 5 washings with SDW. Seeds were then dipped in the endophyte microbial suspension with shaking (100 rpm) at room temperature for 2 h or overnight, for bacteria/yeast or filamentous fungi, respectively. Treated seeds were dried on sterile filter paper under a laminar hood for 30–60 min.

Highly virulent *F. fujikuroi* strain A5 (TUCC00000714), previously isolated and characterized by Amatulli et al. (2010) and deposited at Turin University Culture Collection, was grown on PDA at room temperature for 7 d. Conidia were harvested using a Ringer solution with 0.5 % CMC-Na by scraping the aerial mycelium, subsequently the suspension was filtered through 2 layers of sterile gauze to remove mycelial fragments and the final concentration was adjusted to 10^5 conidia ml^{-1} using a Burker chamber. Two days after endophyte treatment, rice seeds were inoculated with the pathogen by dipping in the conidial suspension on a horizontal shaker for 30 min, followed by drying on sterile filter paper under a laminar hood for 30–60 min. Three controls were included: I) healthy control, without pathogen inoculation; II) inoculated control, inoculated with *F. fujikuroi*; III) chemical control, dressed with fludioxonil (15 ppm, treatment by immersion with shaking at 100 rpm for 1 h), after pathogen inoculation.

Sowing was performed in a substrate made of 60 % peat and 40 % sand inside plastic trays containing 30 plants split into 3 rows. For each treatment, 30 seeds were divided into 3 biological replicates and sown in 3 different trays with a randomized block design. The trays were placed in a greenhouse under controlled conditions (light regime: 12 h, temperature: 27 ± 5°C day/22 ± 5 °C night) and, after germination, disease severity (DS) was weekly scored using the following scale (Piombo et al., 2020): 0, healthy plant; 1, reduced dimensions, chlorotic leaves; 2, significant dwarfism, significant yellowing, internode elongation; 3, necrosis of the crown; 4, dead or not-germinated plant (**Supplementary** Fig. 1). Germination percentage, as well as total fresh biomass were also recorded for each biological replicate at the end of the assay, when at least half of the plants in the inoculated control were dead (4 weeks post germination). Each experiment was carried out twice.

2.3. Molecular identification

Endophytic isolates that significantly reduced bakanae disease severity *in vivo* were molecularly identified to exclude potential plant pathogens on economically relevant crops. Monohyphal fungal strains were cultured on PDA in Petri dishes for 7–14 d, based on their growth rate at room temperature, and biomass was collected by scraping with a sterile scalpel. Total fungal DNA was extracte using E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, USA) following the manufacturer's instructions, except for samples mechanical disruption, which was performed using a Tissuelyser (Qiagen, Germany) at 25 Hz for 25 min. Universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for the amplification of fungal ITS1, 5.8S and ITS2 regions (White et al., 1990). For species identification, partial amplification of the β-tubulin gene was performed using the Btub2Fd (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Btub4Rd (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) primer pair (Woudenberg et al., 2009). Bacterial endophytes were grown at room temperature in 30 ml of LB on a shaker for 2 d and biomass was retrieved by removing the culture broth through centrifugation at 6,000 × g for 10 min. Total bacterial DNA was extracted using MasterPure™ Complete DNA & RNA Purification Kit (Biosearch Technologies, USA) according to manufacturer's instructions. The partial 16S bacterial marker gene was amplified with FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RD1 (5′-GTTACCTTGTTACGACTT-3′) universal primers (Weisburg et al., 1991).

PCR amplifications were carried out in a total volume of 25 μL containing: 2.5 μL of Buffer 10X, 0.5 (for β-tubulin and 16S) or 1.5 μL (for ITS) of $MgCl_2$, 0.5 μL of dNTPs (10 mM), 0.5 μL of each primer (10 mM), 0.2 μL of Taq DNA polymerase (Qiagen) and 10 ng of template DNA. Thermal cycling programs were set as follows: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 (for ITS and β-tubulin) or 60 s (for 16S) and extension at 72 °C for 45 (for β-tubulin) or 60 s (for ITS and 16S), and a final extension step at 72 °C for 5 min. PCR products were sent for sequencing in both directions at Macrogen Europe (Milan, Italy). The consensus sequences were obtained using the DNA Baser program (Heracle Biosoft S.R.L., Arges, Romania). After cutting the trimmed regions and manual correction, the sequences were previously compared with those deposited in public datasets (National Centre for Biotechnology Information, NCBI) using the BLASTn programme, and afterwards aligned using CLUSTALW through Molecular Evolutionary Genetics Analysis (MEGA6) software. To perform the phylogenetic analysis with the Maximum likelihood (ML) algorithm or Neighbor-Joining (NJ) method, the best-fit nucleotide model was determined using MEGA version 6 and a phylogenetic tree was built using the same program. References sequences (RefSeq) from NCBI were used for phylogeny of 16S region, whereas the phylogeny of *Epicoccum* isolates were performed using the references reported in the last revisions of the genus (Chen et al., 2017; Valenzuela-Lopez et al., 2018; Hou et al., 2020).

2.4. In vitro characterization

Among the molecularly identified promising BCAs, a further selection step was performed based on their growth rate at 37 °C to exclude potential opportunistic human pathogens, according to Latz et al. (2020), with minor adjustments. Each strain was grown on LBA or PDA and incubated at 25 °C and 37 °C to assess the preferred growth temperature. Plates were checked daily for 5 d and strains with faster growth rate at 37 °C were discarded.

2.4.1. F. fujikuroi growth inhibition

Selected endophytic strains were tested *in vitro* for their putative antagonistic mode of action against *F. fujikuroi* using dual culture assay, to test for agar-diffusible antimicrobic compound (Spadaro et al., 2002), and sandwich plate method, to verify the production of volatile antimicrobials (Sipiczki, 2023). In the dual culture assay, a mycelial disk was cut using a sterile cork borer (6 mm diameter) from a 7-day-old *F. fujikuroi* colony and transferred onto a 90 mm PDA plate at 30 mm from the edge. Mycelial disks of 7-day-old endophytic fungi were placed on the opposite side of the plate at 30 mm from both the pathogen and the edge of the plate. Bacterial endophytes were collected with a 10 μl loop from a suspension with OD_{600} adjusted to 0.3 (~10^7 CFU ml^{-1}) and streaked on the opposite side of the plate at 30 mm from the edge.

In the sandwich plate method, *F. fujikuroi* was inoculated in the middle of a 90 mm PDA plate as a mycelial disk. Endophytic strains were inoculated on an independent PDA plate as mycelial disks from 7-day-old fungal colonies or by spread plating 10 μl of bacterial suspension with OD_{600} adjusted to 0.3. The plate containing *F. fujikuroi* was joined with the endophyte plate and sealed with parafilm, allowing the microorganisms to share the same atmosphere.

For both experiments, control plates containing only the pathogen and a PDA disk or 10 μl of LBA were included. For both assays, 5 replicates were used, and plates were incubated at 22 °C for 5 d, before measuring the pathogen colony radius (in cm). *F. fujikuroi* inhibition *in vitro* was calculated, as described in Pellicciaro et al. (2021), as percentage of the pathogen colony size reduction determined by the endophytes compared to the untreated control. The antagonistic activity *in vitro* against *F. fujikuroi* was tested twice for each method.

2.4.2. Temperature and osmotic stress tolerance

Selected promising biocontrol strains were tested for both thermal and osmotic stress tolerance *in vitro*. Tolerance to a range of different temperatures (4, 10, 22, 28 and 37 °C) was evaluated on PDA and LBA plates. Osmotic stress tolerance was tested as described by Jones et al. (2016), by amending fungal and bacterial media, respectively PDA and LBA, with the following KCl concentrations: none (−0.1 MPa), 10.06 g L^{-1} (−1.1 MPa), 17.9 g L^{-1} (−1.9 MPa), 28.44 g L^{-1} (−3.0 MPa) and 36.5 g L^{-1} (−3.9 MPa). Fungal endophytes were inoculated on PDA as a single mycelial disk and, after 5 d incubation, colony diameter was measured (in cm). Bacterial endophytes were inoculated on LBA plates by spotting 10 μl of a microbial suspension with OD_{600} adjusted to 0.3 and its serial dilutions (10^1-10^3) in triplicate on the same plate. After 5 d incubation, scoring was performed by counting the number of dilutions that fully colonized the inoculation spot in at least 2 technical replicates (Fig. 1**a, 1b and 1c**). Incubation temperature was set at 25 °C for all strains and 5 replicates were used for each condition in both assays. Each experiment was performed twice.

2.5. Biocontrol efficacy of selected strains in vivo

Selected endophytic strains were tested *in vivo* to confirm their biocontrol efficacy against *F. fujikuroi*. Seeds treatment with rice endophytes and pathogen inoculation was performed as described above. Bacterial growth curves in LB broth were assessed in order to adjust the inoculum to 10^7 CFU ml^{-1}, corresponding to $OD_{600} = 0.3$ for all strains. Each treatment was composed of 6 biological replicates made of 30 seeds each, for a total of 180 plants. A randomized block design was adopted. In addition, disease incidence was extrapolated from disease severity data as described in Li et al. (2018), thus considering as bakanae infected plants only those displaying symptoms with a disease score of 2 or higher. The efficacy of selected strains was tested twice.

2.6. Statistical analysis

Disease severity data were converted into percentage values as described in Matić et al. (2014), obtaining the weighted average disease severity percentage for each biological replicate based on the frequency of each disease score in the empirical scale. In the screening tests, strains determining a statistically significant disease severity reduction were considered antagonistic only when the effect was significant in both trials. All statistical analyses were performed in R environment version

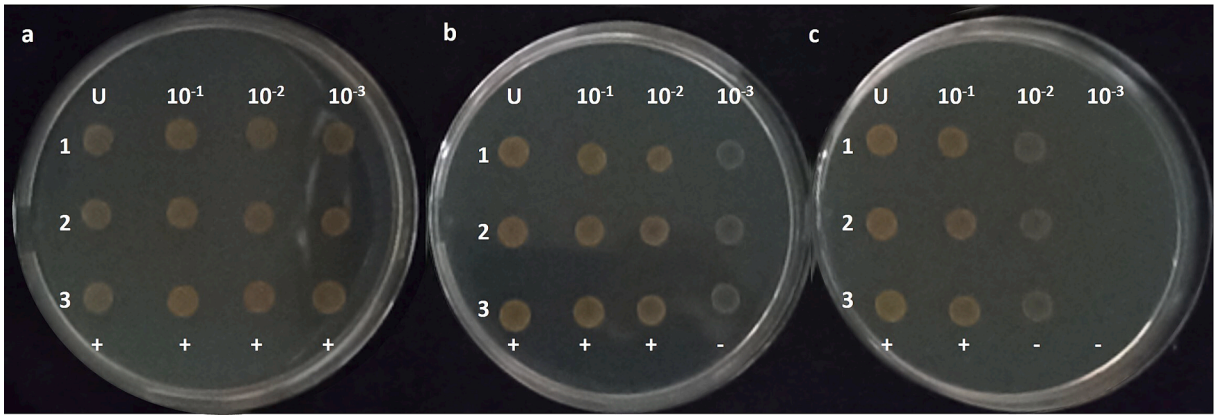

Fig. 1. Visual representation of *in vitro* tolerance to different abiotic stressors on solid medium for unicellular microorganisms. U = undiluted microbial suspension, $10^{-1/-3}$ = serial dilutions of the microbial suspension, 1–3 = technical replicates for each dilution, + = columns displaying at least 2 fully colonized inoculation spots, − = columns displaying less than 2 fully colonized spots after 5 d of incubation. Complete tolerance of the stress condition (a, score: ++++), partial tolerance of the stress condition (b and c, scores of, from left to right, +++ and ++ respectively).

4.3.1 (R Core Team, 2022). Shapiro-Wilk and Levene's tests were performed to check for data normality and homoscedasticity before conducting parametric tests. When the analysis of variance was statistically significant ($P < 0.05$), Tukey's HSD test was used for pairwise comparisons among treatments. Percentage of *F. fujikuroi* growth inhibition *in vitro* data were heteroscedastic, thus Welch's ANOVA was performed, followed by Games-Howell post-hoc test.

3. Results

3.1. Rice endophyte isolation and in vivo screening

One hundred and thirty-five rice endophytes were isolated from rice seeds, leaves, and stems. Total microbial count was performed at least twice for each sample, resulting in microbial concentrations ranging from $\log_{10}$ 5.84 to 8.02 CFU g^{-1} on seed and from $\log_{10}$ 3.42 to 5.63 CFU g^{-1} on aerial organs. In total, 99 endophytes were isolated from seed and 36 from leaves and stems. They included 81 bacteria, 36 filamentous fungi and 18 yeasts (Table 1).

Each isolate was screened in two greenhouse assays, each one consisting of three replicates of 10 plants. Each assay permitted to test 45 isolates (Fig. 2**a, 2b and 2c**). There were no significant differences in germination rate and total fresh biomass in all trials. A statistically significant reduction in disease severity was visible in 18 seed treatments with endophytes ($P \leq 0.05$), 6 from the first screening, 5 from the second one, and 7 from the third *in vivo* screening. Compared to the control (DS ranging from 65.00 % to 66.67 %), treatments allowing the lowest DS were observed with strain RS-GAL41 in the first screening (24.17 %), RL-1948.2 in the second one (26.67 %), and RS-APO3 in the third one (26.67 %). The remaining selected isolates determined a DS ranging from 25 to 30 %. Efficacy of selected BCAs was comparable to chemical seed dressing with fludioxonil ($P \leq 0.05$), that determined a DS ranging from 23.33 to 25.00 % throughout the trials.

3.2. Molecular identification and growth at 37 °C

Eighteen promising BCAs included 7 filamentous fungi and 11 bacteria, which were identified through DNA sequencing. Among the 7 fungal isolates identified, those belonging to species of rice or other crop pathogens were discarded (data not shown), thus only *Epicoccum* spp. RS-GAL41 and RS-1850.3 were maintained for further analyses. None of them was able to grow at 37 °C. The sequences obtained for these strains, RS-GAL41 and RS-1850.3, were blasted in GenBank obtaining 99.40 % homology with *E. sorghinum* (AN MN562463.1) and 100 % homology with *E. layuense* (AN OP715583.1). Whereas through the Maximum likelihood phylogeny using β-tubulin gene the strains were identified as *Epicoccum catenisporum* and *E. layuense* respectively, clustering together with the reference strains with high bootstrap values (99 and 100 % respectively) (**Supplementary** Fig. 2**a**).

Among the 11 bacterial isolates identified, most genera included potential human opportunistic pathogens, leading to the exclusion of all selected bacterial strains with an optimal growth rate at 37 °C, except for three strains. Maximum likelihood phylogeny using 16S sequences led to the identification of strains RL-1948.2 and RS-APO3 as *Microbacterium testaceum* (bootstrap 91 %,) confirming the identification using the BLASTn programme (99.93 and 99.86 % homology, respectively, with reference strain NR_026163.1) (**Supplementary** Fig. 2**b**). The sequence blasted in GenBank for RS-MIS3 did not permit to identify the strain at species level (obtaining 100 % homology with strains of *Methylobacterium* sp., *M. fujisawaense* and *M. oryzae*). Whereas the identification based on Neighbor Joining phylogeny, led to the identification of the strain as *Methylobacterium oryzae* (bootstrap 88 %) (**Supplementary** Fig. 2**c**).

3.3. In vitro antagonism

The 5 selected strains (Table 2) were tested *in vitro* for their antagonistic potential against *F. fujikuroi* (Fig. 3). *E. catenisporum* RS-GAL41 determined a *F. fujikuroi* colony size reduction ($P \leq 0.05$) in the dual culture assay compared to the control, although still displaying a limited efficacy (24.9 % inhibition). *E. layuense* RS-1850.3 and *M. testaceum* RS-APO3 also caused significant *F. fujikuroi* growth inhibition ($P \leq 0.05$, 6.90 % and 8.40 %, respectively). Concerning the sandwich plate assay, *M. testaceum* RL-1948.2 and RS-APO3 were able to significantly reduce the pathogen colony size with putative volatile antimicrobials, allowing a growth inhibition of 10.2 % and 9.0 %, respectively, compared to the control ($P \leq 0.05$). *M. oryzae* RS-MIS3 displayed the lowest antagonistic efficacy with both dual culture and sandwich method (1.80 % and 0.90 %, respectively).

3.4. Tolerance to temperature and osmotic stress

The selected BCAs were also grown at multiple temperatures and salt concentrations to assess their abiotic stress tolerance (Table 3). The optimal growth temperatures were between 22 °C and 28 °C for all strains, except for *M. oryzae* RS-MIS3, growing faster at 22 °C, than at 28 °C. Fungal and *M. testaceum* strains, on the other hand, exhibited a limited ability to grow at 10 °C, whereas only *E. layuense* RS-1850.3 could grow at 4 °C. None of the selected endophytes had the ability to proliferate at 37 °C, except for RL-1948.2 (*M. testaceum*), although with

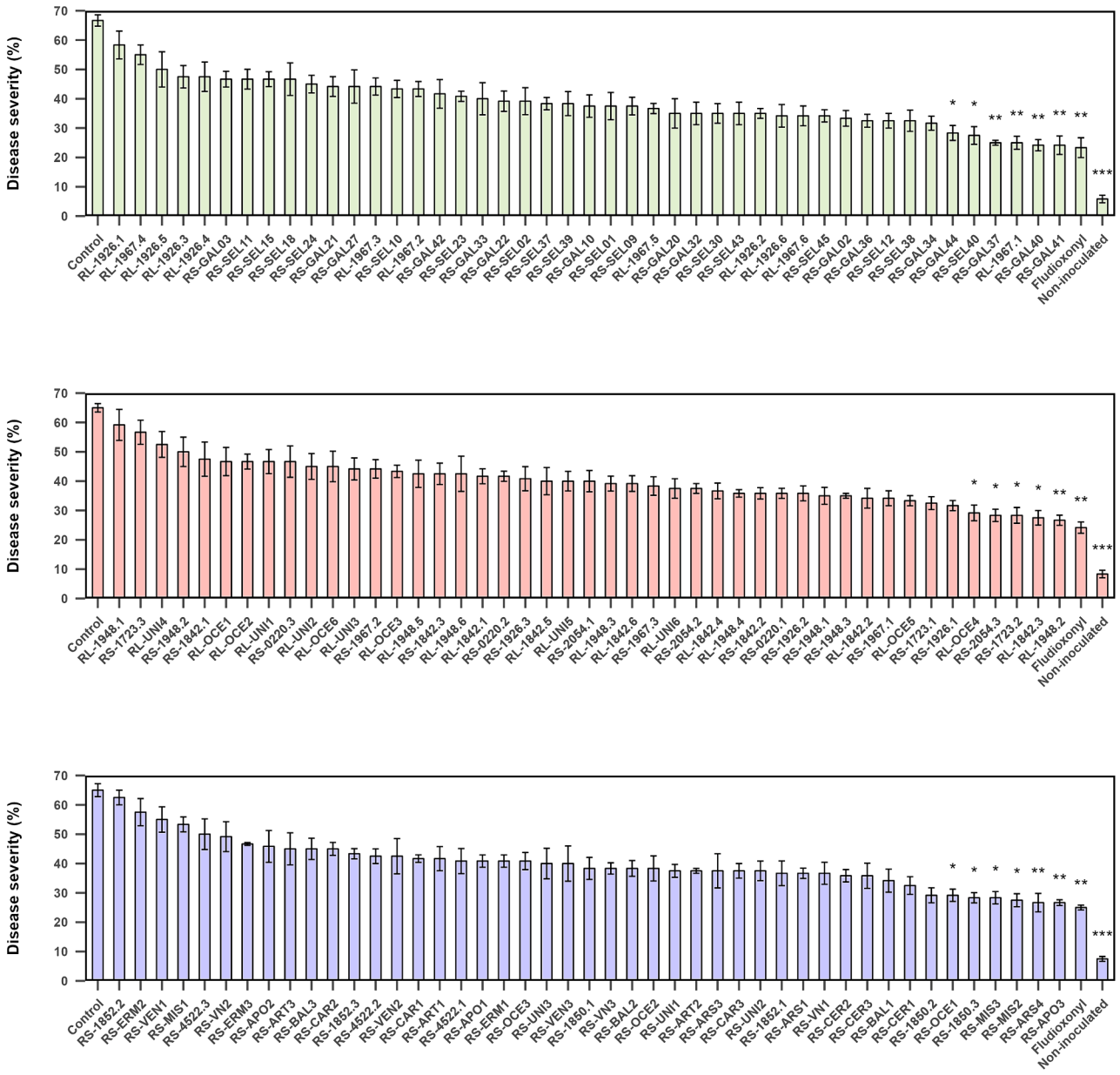

Fig. 2. *In vivo* biocontrol efficacy of rice endophytes against *F. fujikuroi* 4 weeks post germination. First (a), second (b) and third (c) independent screenings with 45 isolates. Each bar represents the average of 2 trials, where for each treatment 3 biological replicates are made of 10 plants each, error bars represent standard errors (SE). One, two and three asterisks indicate significant differences at $P \leq 0.05$, 0.01 and 0.001, respectively, compared to not-treated inoculated control.

Table 2
Selected rice endophytes for their biocontrol efficacy against *F. fujikuroi*.

Strain	Species	Year of isolation	Tissue and cultivar of origin	TUCC code	NCBI accession number
RS-1850.3	*Epicoccum layuense*	2023	Rice seeds cv S18050	TUCC00000696	PP429300
RS-GAL41	*E. catenisporum*	2022	Rice seeds cv Galileo	TUCC00000695	PP429299
RL-1948.2	*Microbacterium testaceum*	2022	Rice shoot cv S19048	TUCC00000699	PP412049
RS-APO3	*Microbacterium testaceum*	2023	Rice seeds cv Apollo	TUCC00000700	PP412050
RS-MIS3	*Methylobacterium oryzae*	2023	Rice seeds cv Misaki	TUCC00000697	PP412048

a lower growth rate compared to 22 and 28 °C. Concerning the simulated osmotic stress tolerance, the strains withstanding the highest KCl concentration in the substrate were the ones belonging to the *Epicoccum* and *Microbacterium* genera, proliferating up to −3.9 MPa. Strain RS-MIS3 (*Methylobacterium oryzae*) could not grow even at the lowest osmotic stress level.

3.5. Biocontrol efficacy and fresh plant biomass in vivo

The 5 selected strains were tested *in vivo* to confirm their biocontrol efficacy against *F. fujikuroi*. Differences in germination rate, ranging from 83.33 to 100.00 %, were not detected. A significant reduction in bakanae disease severity was observed in all treatments ($P \leq 0.05$), with similar values compared to the chemical control (Fig. 4**a**). Additionally,

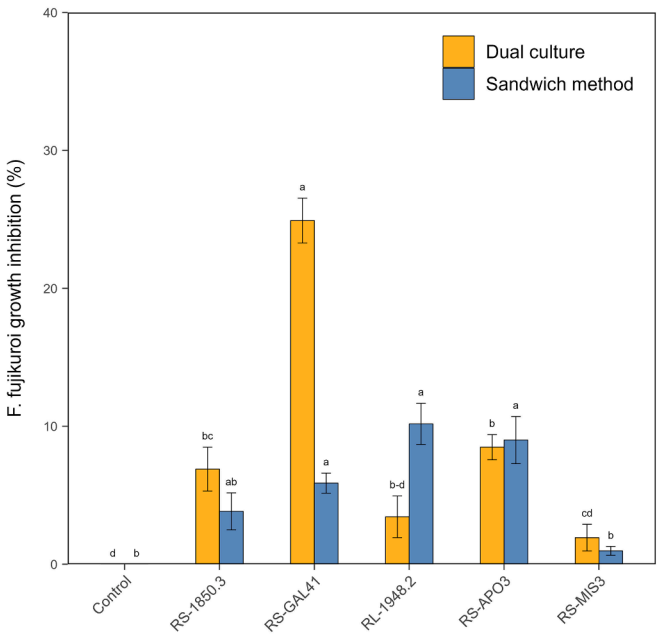

Fig. 3. *In vitro* growth inhibition of *F. fujikuroi* by 5 endophytes, selected based on their biocontrol efficacy *in vivo*, assessed with dual culture (green) and sandwich plate (purple) methods 5 d post inoculation. Each bar represents the average of 2 trials, where each treatment was made of 5 biological replicates. Error bars represent standard errors (SE). Different letters indicate a significant difference ($P < 0.05$) among treatments according to Games-Howell test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

E. layuense RS-1850.3, *E. catenisporum* RS-GAL41 and *M. oryzae* RS-MIS3 determined a significant increase in total fresh biomass ($P \leq 0.05$), comparable to the non-inoculated control (Fig. 4**b**). Treatment with RS-GAL41 led to an average DS of 25.97 %, compared to 60.00 % in the inoculated control, and an average total fresh biomass of 8.35 g compared to 4.10 g in the inoculated control. Despite RS-1850.3 and RS-MIS3 enhanced plant biomass (respectively 8.35 g and 7.48 g), they were less efficient in DS reduction (DS respectively of 35.00 % and 35.28 %). Treatments with *M. testaceum* RL-1948.2 and RS-APO3 did not significantly increase total fresh biomass and determined a lower disease reduction (DS of 33.61 and 31.94 % respectively), compared to the other strains tested.

4. Discussion

An untargeted approach for endophytes isolation was followed obtaining a collection of 135 bacterial, yeast and fungal endophytes. Seeds of 24 Italian genotypes were chosen as the main target for rice endophytes isolation, considering that the inner spermosphere microbiome is known to foster crucial taxa facilitating germination as well as with PGP potential (Shahzad et al., 2018; Verma et al., 2019). Additionally, stems and leaves of 5 of the 24 genotypes were sampled for endophytes isolation after growth in a greenhouse for 4 weeks. Higher amounts of bacterial, rather than fungal or yeast, morphotypes were isolated from both tissues considered. Microbial diversity could be enhanced at the isolation step by increasing the number of media, for instance including nutrient-poor substrates to disadvantage fast growers that may otherwise rapidly grow on the substrate in the plate (Eevers et al., 2015; Latz et al., 2020). Fast growing generalist microbes have been associated to higher adaptability to a wide range of environments, however they also display poor capacity to establish steadily into a specific ecological niche (Berg and Cernava, 2022). Although they may be interesting for their potentially wide spectrum of stress tolerance, a more intimate relationship between a beneficial microbe and its host, as anticipated, should be preferred in this context. In our study, rice seed harboured higher total microbial counts compared to aboveground organs, as previously observed (Hameed et al., 2015; Loaces et al., 2011).

A strategy that allows to more likely pre-select efficient antagonists without resorting to mass *in vitro* screenings was theorized by Collinge et al. (2019), suggesting to perform isolation from ecological niches where the endophyte and the pathogen are interacting, i.e. from healthy plants found in fields with high incidence of the target disease. Following this method, a relatively low number of isolates is sufficient to find potential BCAs, as demonstrated by Latz et al. (2020), who, starting from a collection of 69 fungal endophytes, found that 6 of them (8.7 %) were able to reduce the target symptoms in controlled conditions, and 2 confirmed the results in field conditions. In our screening, among 135 endophytes isolated from rice seeds and shoots, 18 (13.3 %), including 7 fungi and 11 bacteria, were able to significantly reduce bakanae disease in controlled conditions. We chose to collect a large amount of rice endophytes to be screened for different objectives, such as biocontrol potential in this study. We demonstrated that a pipeline based on *in vivo* mass screening before molecular identification and *in vitro* characterization yielded a relatively high percentage of promising BCAs, as previously reported. This workflow, thus, can also be suitable with lower numbers of isolates seeing these chances to find candidate antagonists. Molecular and *in vitro* characterization before *in vivo* screening for biocontrol efficacy should not be excluded as an alternative pipeline, which can be chosen depending on laboratory infrastructure and research aims, e.g. ecological studies. We mainly suggest expanding as much as possible the spectrum of genotypes considered in the isolation approach to enhance the representativeness of the target plant core microbiota, as well as the chances of adaptation on multiple cultivars for large-scale applications.

Molecular identification of the promising BCAs led to the exclusion of 5 strains belonging to *Alternaria*, *Curvularia* and *Gaeumannomyces* genera, as they mainly encompass species causing non-negligible yield losses on economically relevant crops (Hyde et al., 2014; Nagrale et al., 2016; Palma-Guerrero et al., 2021). The 2 remaining strains belonged to the *Epicoccum* genus, which, despite also including a limited number of plant pathogenic species (Taguiam et al., 2021), is mainly known for its wide use in the biological control of plant diseases (Braga et al., 2018). Although among some of these fungal genera opportunistic human pathogenic species were also reported (Krizsán et al., 2015; Sharma and

Table 3
Range of thermal and osmotic stress tolerance of selected endophytes *in vitro*. Fungal growth at different temperatures and osmotic potential were recorded as cm of colony size ± standard deviation (SD). For bacterial endophytes, scoring was performed by counting the number of dilutions that fully colonized from 0 (−) to 4 (++++) inoculation spots, corresponding to undiluted bacterial suspension and its dilutions (10^1-10^3).

Strain	Temperature (°C)					Osmotic potential (−MPa)				
	4	10	22	28	37	0.1	1.1	1.9	3.0	3.9
E. layuense RS-1850.3	1.61 ± 0.07	1.98 ± 0.08	2.95 ± 0.16	2.44 ± 0.08	–	3.32 ± 0.06	3.08 ± 0.11	2.38 ± 0.11	2.17 ± 0.15	2.01 ± 0.11
E. catenisporum RS-GAL41	0.72 ± 0.04	1.49 ± 0.07	6.29 ± 0.08	6.71 ± 0.16	–	6.55 ± 0.11	6.28 ± 0.26	6.23 ± 0.21	5.65 ± 0.12	4.79 ± 0.23
M. testaceum RL-1948.2	–	+	++++	++++	++	++++	++++	++++	++++	+++
M. testaceum RS-APO3	–	+	++++	++++	–	++++	++++	++++	+++	+++
M. oryzae RS-MIS3	–	–	++++	++	–	++++	–	–	–	–

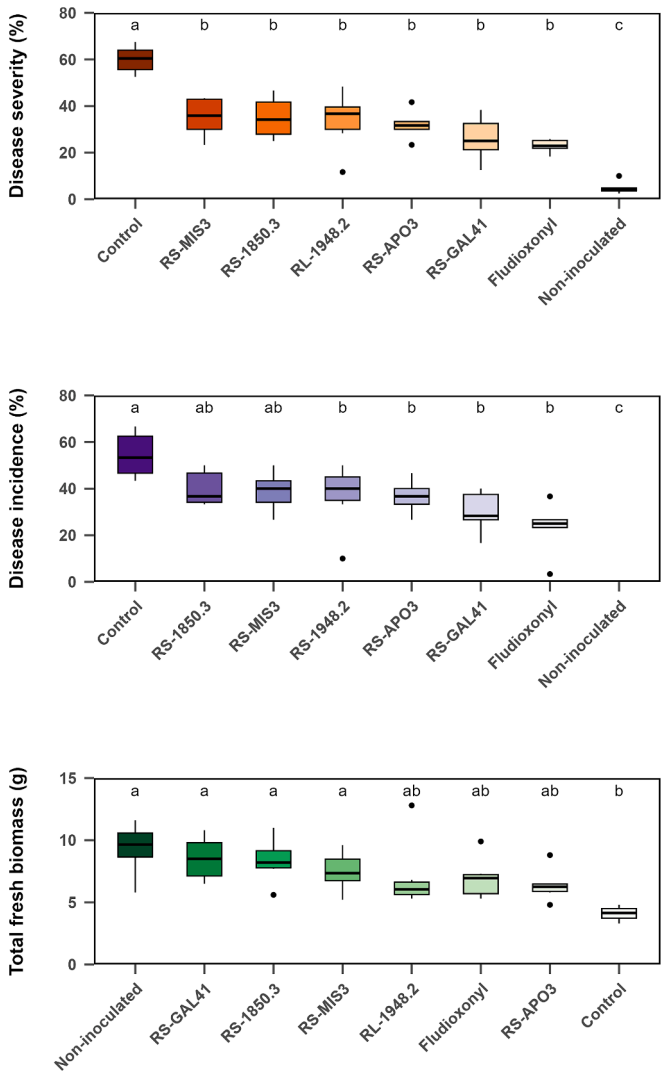

Fig. 4. *In vivo* biocontrol efficacy of 5 selected endophytes against *F. fujikuroi* assessed by evaluating bakanae disease severity (a), incidence (b) and rice plants total fresh biomass (c) 4 weeks post germination. Each boxplot represents the median, first and third quartile, and minimum and maximum of data obtained from 2 trials, where each treatment was composed of 6 biological replicates made of 30 plants each. Different letters indicate significant differences ($P < 0.05$) among treatments according to Tukey's test.

Nonzom, 2021), none of the strains were able to grow at 37 °C on PDA. Among bacterial endophytes, the identified genera were *Burkholderia*, *Curtobacterium*, *Methylobacterium*, *Microbacterium* and *Pseudomonas*. Higher growth rate on LBA at 37 °C was recorded for 7 strains belonging to *Burkholderia*, *Curtobacterium* and *Pseudomonas*, therefore leading to their exclusion from further trials as opportunistic human pathogenic species were previously reported for these genera (Butt and Thomas, 2017; Francis et al., 2011; Moradali et al., 2017). The remaining 3 strains were associated with *Methylobacterium* and *Microbacterium* genera, which include members that have already been investigated as BCAs against plant pathogens (Ardanov et al., 2016; Patel et al., 2022).

The efficacy of *E. catenisporum* RS-GAL41, *E. layuense* RS-1850.3, *M. testaceum* strains RL-1948.2 and RS-APO3, as well as *M. oryzae* RS-MIS3 in bakanae disease reduction *in vivo* was confirmed by increasing the number of biological and technical replicates. The highest biocontrol potential was observed in treatments with RS-GAL41, also leading to a significant increase in total fresh biomass. Conventional chemical treatment, despite determining the highest average disease reduction, did not avoid biomass loss, being the total fresh weight reduced compared to the non-inoculated control and like the inoculated control. Further trials without *F. fujikuroi* inoculation are necessary to assess potential plant growth promoting features of the strains that enhanced biomass production in diseased rice plants. Additionally, upscaling will also require optimizing inoculum stabilization and formulation, which, in our case, could be challenging especially for *Epicoccum* strains, a genus well known for its low sporulation and conidia production (Pascual et al., 1999; Fávaro et al., 2012). However, many suggestions can be found in literature to optimize inoculum quantity (Larena et al., 2004), stability (Larena et al., 2007) and efficacy (Larena et al., 2010) through media and formulation engineering in either solid- or liquid-state fermentation.

Selected rice endophytes were also tested against *F. fujikuroi in vitro* using common assays for mass biocontrol screenings, i.e. dual culture and sandwich plate methods. The strain showing the highest significant *F. fujikuroi* growth inhibition was RS-GAL41, which was more evident with the dual culture method, even though the antagonistic effect was still limited and would have probably led to the exclusion of this strain. Overall, these results highlight the importance of *in vivo* rather than *in vitro*-based screening methods, especially when dealing with promising BCAs that either do not display antagonistic activity *in vitro* or with a biocontrol MOA linked to the host, i.e. induced resistance and/or microbiome manipulation (Köhl et al., 2019; Latz et al., 2018). For this reason, gene expression of defence-related genes and microbiome analyses at different phenological stages are planned to investigate the MOAs of at least a fungal and a bacterial strain with promising biocontrol activity.

Moving from controlled conditions experiments to field trials, abiotic stresses could affect not only the physiology of the plants but also the survival of its microbiota, and in particular of BCAs (Jones et al., 2016; Köhl et al., 2011). For this reason, abiotic stress tolerance was tested for selected endophytes *in vitro* as a further selection factor for the choice of candidate BCAs. All endophytes exhibited optimal growth in mesophilic conditions. *Epicoccum* strains also showed proliferation at 4 °C, a psychrotolerant behaviour previously observed in other members of the genus (Ogórek et al., 2017). Among the selected rice endophytes, the only one with the ability to proliferate at 37 °C, although with a lower growth rate than at mesophilic conditions, was *M. testaceum* RL-1948.2. Despite members of the *Microbacterium* genus have been reported to cause a limited number of opportunistic infections (Amano et al., 2019), there is no record associating this species as pathogenic for humans. Concerning osmotic stress tolerance, which was tested to simulate drought conditions by reducing the water activity of the growth medium, *Epicoccum* and *Microbacterium* strains displayed active proliferation with the widest range of salt concentrations, as previously reported in other members of these genera (De Paula et al., 2022; Walitang et al., 2017). On the other hand, the strain with the lowest adaptability to a range of abiotic stress conditions was *M. oryzae* RS-MIS3, which is in line with the mesophilic and low salt tolerance that characterizes this species (Madhaiyan et al., 2007).

To our knowledge, this is the first report about the efficacy of endophytic *E. catenisporum, E. layuense, M. testaceum* and *M. oryzae* in reducing bakanae disease of rice. Additionally, among the promising BCAs selected in this study, the only species that were already used as BCAs in other pathosystems were *M. testaceum* (Silva et al., 2012) and *E. layuense* (Del Frari et al., 2019). At the genus level, *Epicoccum* species are widely known for their biocontrol potential against various plant pathogens, resorting to both antibiosis and host induced resistance for disease control (Taguiam et al., 2021). A strain of *Epicoccum nigrum* isolated from grape was also employed for rice blast disease control *in vivo* (Koné et al., 2022), where it was observed that, when seed treatment was performed before spray inoculation of both the antagonist and the pathogen, disease symptoms reduction, as well as grain yield, were higher compared to only foliar treatment with the BCA 24 h before the pathogen inoculation. These results highlight the importance of early establishment of beneficial microorganisms in plant tissues to reliably

obtain plant protection and a growth promotion effect. Additionally, seed treatment with microbial inoculants tends to be preferred as a large-scale delivery method compared to foliar or soil application, as it allows to introduce BCAs directly in contact with each individual target plant and does not require any additional action from the farmer (Collinge et al., 2019; Jambhulkar et al., 2016). *Microbacterium testaceum* was previously used for seed priming, in addition to preventive foliar treatment, for rice blast disease reduction through host induced resistance (Patel et al., 2022; Sahu et al., 2021). *Methylobacterium* species, on the other hand, were mainly studied as BCAs against Solanaceae diseases by manipulating the resident microbiota (Ardanov et al., 2016) or modulating phytohormone production (Yim et al., 2013). Further investigation on the specific biocontrol MOA of the rice endophytes selected in this study is required to develop the selected BCAs as potential biofungicides. Next steps will be to assess the efficacy of these strains in field conditions and proceeding with downstream stabilization and formulation.

CRediT authorship contribution statement

Simone Bosco: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Simona Prencipe:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Monica Mezzalama:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Davide Spadaro:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105580.

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