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Targeted isolation of biocontrol agents from plants through phytopathogen co-culture and pathogen enrichment

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Abstract

In a long-term symbiotic relationship between plants and pathogens, plants have evolved to harbor beneficially endophytic microbiomes, thereby conferring them the ability to resist infection by pathogens. This prompted us to establish a phytopathogen-based co-culture platform for the targeted isolation of potential biocontrol agents from plants via specific pathogen enrichment. In this study, we investigated three different phytopathogenic systems, including kiwifruit, turfgrass, and rice, and their infectious bacterial and/or fungal pathogens. By using the developed mono- or co-enrichment platform, we efficiently isolated three antimicrobial agents, including *Bacillus safensis* ZK-1 against *Pseudomonas syringae* pv. *actinidiae* that causes kiwifruit canker, *Pseudomonas alcaligenes* ZK-2 against *Clarireedia paspali* that causes dollar spot disease in turfgrass, and *Bacillus velezensis* ZK-3 against rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* and rice blast fungus *Magnaporthe oryzae*. We believe that the phytopathogenic co-culture and pathogen enrichment platform developed here is versatile and effective for the isolation of potential biocontrol agents with specific or broad-spectrum antimicrobial activities from plants of interest in a targeted and large-scale manner.

Keywords: Phytopathogen, Co-culture, Pathogen enrichment, Biocontrol, Phytosystem

Background

Of the five major crops (rice, wheat, maize, potato and soybean) that feed more than 7 billion people each year, about 17–30% of yield losses are due to various diseases or pests (Godfray et al. 2010). The scale of this crop loss greatly undermines global food security and underscores the needs to develop more effective methods to increase the efficiency of plant disease control (Fisher et al. 2018; Avery et al. 2019). The utilization of disease-resistant plant cultivars and crop rotation are the main non-chemical and preventive methods for plant disease management to date (Barzman et al. 2015; Collinge et al. 2019). Indeed, chemical pesticides with preventive and therapeutic effects have long been used as the primary tool against crop-destroying pathogens (Collinge et al. 2019; Cullen et al. 2019). However, their costs, residual toxicity to humans and animals, and pathogen resistance make them unsatisfactory (Chambers et al. 2014; Fisher et al. 2018; Wang et al. 2021). Biological control involving natural biocontrol agents (BCA) as well as their antimicrobial metabolites has received increasing attention and is emerging as an important tool for integrated pest management (IPM). This is because BCA-based pesticides are inexpensive

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to produce and have low toxicity compared to those chemically produced agents (Ortiz and Sansinenea, 2021). However, one of the primary factors hindering large-scale implementation of biocontrol agents is the lack of efficient and commercialized BCA (Raymaekers et al. 2020). Therefore, identification of new BCAs is a key step in the development of commercial biocontrol products that requires efficient and robust screening methods (Raymaekers et al. 2020).

Over the past three decades, many BCAs have been screened and/or identified by various means. In the early stage, environmental samples from agricultural or non-agricultural soil and water, diseased or heathy plant tissues, plant rhizosphere and endophytes were selected for cultivation on liquid or agar media (Foldes et al. 2000; Berget al. 2005; Huang et al. 2013; Raymaekers et al. 2020). The isolated and purified BCAs were further selected for in vitro antagonistic assays, in which a well-known method called dual-culture assay was devised to co-culture BCA candidates of interest with target pathogens on agar plates (Pliego et al. 2011; Sales et al. 2016). The inhibition of growth zones of pathogens were then calculated to assess the respective antagonistic capacity of BCA candidates against pathogens (Ramesh and Phadke 2012). Using this approach, a large number of live BCAs have been identified, including BCAs from non-pathogenic viruses, bacteria and fungi, represented by the biocontrol *Bacillus*, *Pseudomonas* and *Trichoderma* species (Kamilova 2009; Lugtenberg and Kamilova 2009; Fira et al. 2018). These live BCAs can secrete abundant diffusible antimicrobial factors to the surrounding environment to inhibit the growth of pathogens, including lyases and various volatile or non-volatile antibiotic metabolites (Cao et al. 2018; Schulz et al. 2002; Stinson et al. 2003; Tokpah et al. 2016).

BCAs identified by typical co-culture assays can be further screened and applied to plants in the field or greenhouse to evaluate their efficacy in protecting plant from target pathogen infection under more natural conditions (Comby et al. 2017). For instance, Abraham et al. (2010) tested 60 yeast and 92 *Bacillus* isolates against the post-harvest pathogen *Penicillium digitatum* on oranges for their biocontrol potential, and found that 10 yeasts and 10 bacterial isolates can reduce infection surface area by more than 50%. This plant-based in vivo assay is also suitable for successful screening BCAs against pathogens that are difficult to grow in vitro, such as biographs that requires live host cells for proliferation (Zhang et al. 2017; Raymaekers et al. 2020). The authors screened 239 bacterial grapevine endophytes using grape leaf discs to determine their antagonism against the biotrophic pathogen *Plasmopara viticola* that causes downy mildew, and successful selected two isolates that further demonstrated their utility in protecting grapevines from infection with this disease.

Moreover, an effective "marker-based" screening strategy for BCA was designed recently (Tokpah et al. 2016; Raymaekers et al. 2020). In terms of pathogens, the initial goal of screening was to use bacterial virulence factors (i.e. lyases) that can interfere with or be blocked by BCAs as probes (Kapat et al. 1998). For example, Schoonbeek et al. (2007) screened and identified four BCA bacteria that can use calcium oxalate (the calcium oxalate salt) as the sole carbon source on the plate. Oxalic acid is a key factor required for full pathogenicity of two fungal pathogens (*Sclerotinia sclerotiorum* and *Botrytis cinerea*). Further plant-based bioassays confirmed that these four isolates indeed exhibited remarkable biocontrol capacity in protecting *Arabidopsis thaliana*, cucumber, tomato and grapevine from *S. sclerotiorum* and *B. cinerea* (Schoonbeek et al. 2007). In another earlier study, Hoster et al. (2005) reported that after enriching soil samples in the medium with chitin (the major component of fungal cell walls) as the sole carbon, the authors isolated a new *Streptomyces* strain with antifungal activity. In addition to screening potential BCAs with the ability to directly target pathogenicity factors, "marker-based" method is also useful for screening BCAs that are highly competitive with pathogens. One such representative was documented in an earlier study (Kamilova et al. 2005), in which the authors used plant root colonization as a marker. In this case, the authors inoculated a crude mixture of rhizosphere bacteria from two different rhizosphere samples onto sterile seedlings of two plants (cucumber and tomato) to enrich for bacteria with enhanced competitive root tip colonization. Using this method, the authors finally identified four such isolates that protect tomato from fungal infections that cause crown and root rot. In plants, the "marker-based" strategy is also suitable for screening BCAs that can induce plant immune responses to prevent pathogen infection. For example, by using reactive oxygen species (ROS) as probes to mimic plant's innate immune response to pathogen infection, Zahid et al. (2017) successfully identified 16 ROS-inducing compounds that elicited induced systemic resistance (ISR) in the model plant (*A. thaliana*) against bacterial infection (*Pseudomonas syringae* pv. *tomato* DC3000).

However, as far as we know, almost all previously-characterized BCA screening methods do not involve the enrichment of live pathogens. The long history of host–pathogen symbiosis prompted us to hypothesize that a particular plant host may have some or specific BCA potential to protect itself from one or more pathogens. This idea is supported by a recent report showing that *Sphingomonas melonis*, a bacterial member of the

rice seed endophyte, accumulates in disease-resistant rice seeds and spreads across generations to confer resistance to disease-susceptible phenotypes by producing anthranilic acid (Matsumoto et al. 2021). To test the hypothesis proposed by Matsumoto et al. (2021), we selected three different phypopathogen co-culture systems and developed their respective pathogen mono- and co-enrichment platforms. Using the developed techniques, we can effectively isolate potential BCAs from respective host plants. These isolated BCAs are either against the same pathogen used in enrichment assays or have broad-spectrum antimicrobial activity. The platform developed in this study for targeted isolation of BCA is easy to operate, cost-effective, and appears to be widely applicable to multiple host–pathogen phytosystems.

Results

Isolation of antibacterial *Bacillus safensis* ZK-1 from kiwifruit by mono-enrichment of *Pseudomonas syringae* pv. *actinidiae*

To test the hypothesis that plants harboring endophytic BCAs resist pathogen infection during symbiotic relationship with pathogens, we first developed a bacterial pathogen mono-enrichment (BPME) approach using *Pseudomonas syringae* pv. *actinidiae* (*Psa*) strain C48, which causes kiwifruit bacterial canker, to isolate naturally-occurring *Psa*-inhibiting bacteria from kiwifruit. As shown in Fig. 1, after finishing the surface sterilization, the mature kiwifruits were fully digested by a high-speed blender, and then the supernatant (juice) was collected. To promote the enrichment of kiwifruit-derived bacteria that inhibit *Psa* growth, we added a cell suspension of *Psa* strain C48 (OD_{600}, 1.0) to the prepared kiwifruit supernatant and cultured for 2 days (Fig. 1 left panel, step "a" in S3#). The resultant mixture was spread on LB plates, and bacterial colonies grown under the test conditions were selected for plate-based antibacterial testing against C48. Screening among 20 randomly selected colonies allowed us to identify ZK-1 as an antagonist of C48, as evidenced by the observation of strong growth inhibition zones around the original inoculation site of ZK-1 on C48-imbedded LB plates (Fig. 2a). None of the other 19 isolates showed any antibacterial activity under similar test conditions (Additional file 1: Figure S1). Similar growth inhibitory effect was observed when *P. syringae* pv. *tomato* DC3000, a *Psa* phylogenetically-related species, was applied (Fig. 2a). Under similar test conditions, strain ZK-1 also inhibited the growth of non-pathogenic *E. coli* Top10, although the zone of inhibition appeared to be smaller than that of C48 or DC3000 (Fig. 2a). These results indicate that the antibacterial factor(s) produced by isolated ZK-1 can diffuse into the media under the testing conditions. Thus, addition of cell-free supernatant of ZK-1 inhibited the growth of C48 or *LacZ*-labelled *E. coli* Top10 in LB liquid broth compared to the control (fresh LB broth) (Fig. 2a). Interestingly, on PDA plates, strain ZK-1 failed to inhibit the growth of five selected filamentous fungal pathogens, while known antifungal controls (*Lysobacter enzymogenes* OH11 and *B. subtilis* NCD-2) could accomplish this task (Fig. 2b). These results indicate that the bacterial *Psa*-dependent BPME approach is effective in facilitating the targeted isolation of the antibacterial agent ZK-1 from kiwifruit, providing experimental evidence that the developed BPME can be used for the targeted isolation of host-derived biocontrol agents. Meanwhile, as a control method, the traditional BCA isolation method without pathogen enrichment was also carried out accordingly. We isolated a total of 55 BCA strains based on traditional approach including 22, 13 and 20 strains from kiwifruit, forage seeds and rice seeds, respectively (Additional file 1: Figure S2). We randomly selected 32 isolates from the three aforementioned fruit or seed materials and performed a plate-based antifungal/antibacterial assay. Unfortunately, we failed to find any effective biocontrol isolates (Additional file 1: Figure S2).

A BlastN search using the 16S rDNA gene of ZK-1 as the query sequence revealed that ZK-1 is a member of genus *Bacillus*. Further phylogenetic tree analysis based on a commonly-applied taxonomical marker gene, termed the housekeeping *gryB* of ZK-1 and other reported *Bacillus* species, led to the identification of ZK-1 as *Bacillus safensis* (Additional file 1: Figure S3). For a comprehensive phylogenetic comparison, the genome sequence of strain ZK-1 was uploaded to TYGS (Type Strain Genome Server, https://tygs.dsmz.de). Cut-off thresholds for dDDH (Digital DNA–DNA Hybridization) and ANI (Average Nucleotide Identity) were higher than 70% and 95%, respectively, for prokaryotic species delineation (Meier-Kolthoff et al. 2019) (Additional file 1: Figure S4). Therefore, the results of genome derivation based on the dDDH and ANI values of strain ZK-1 and closely related strains confirmed that strain ZK-1 was close to *B. safensis* FO-36b. In addition, the phylogenomic tree reconstructed on TYGS provided further evidence for the taxonomic position of this strain within *B. safensis* species (Fig. 2c). Notably, there are few studies on this antibacterial agent ZK-1, and most of the available strains of this species show antifungal activity (Singh et al. 2013; Prakash and Arora 2021). Thus, strain ZK-1 represents an unidentified antibacterial agent of *B. safensis*, supporting the

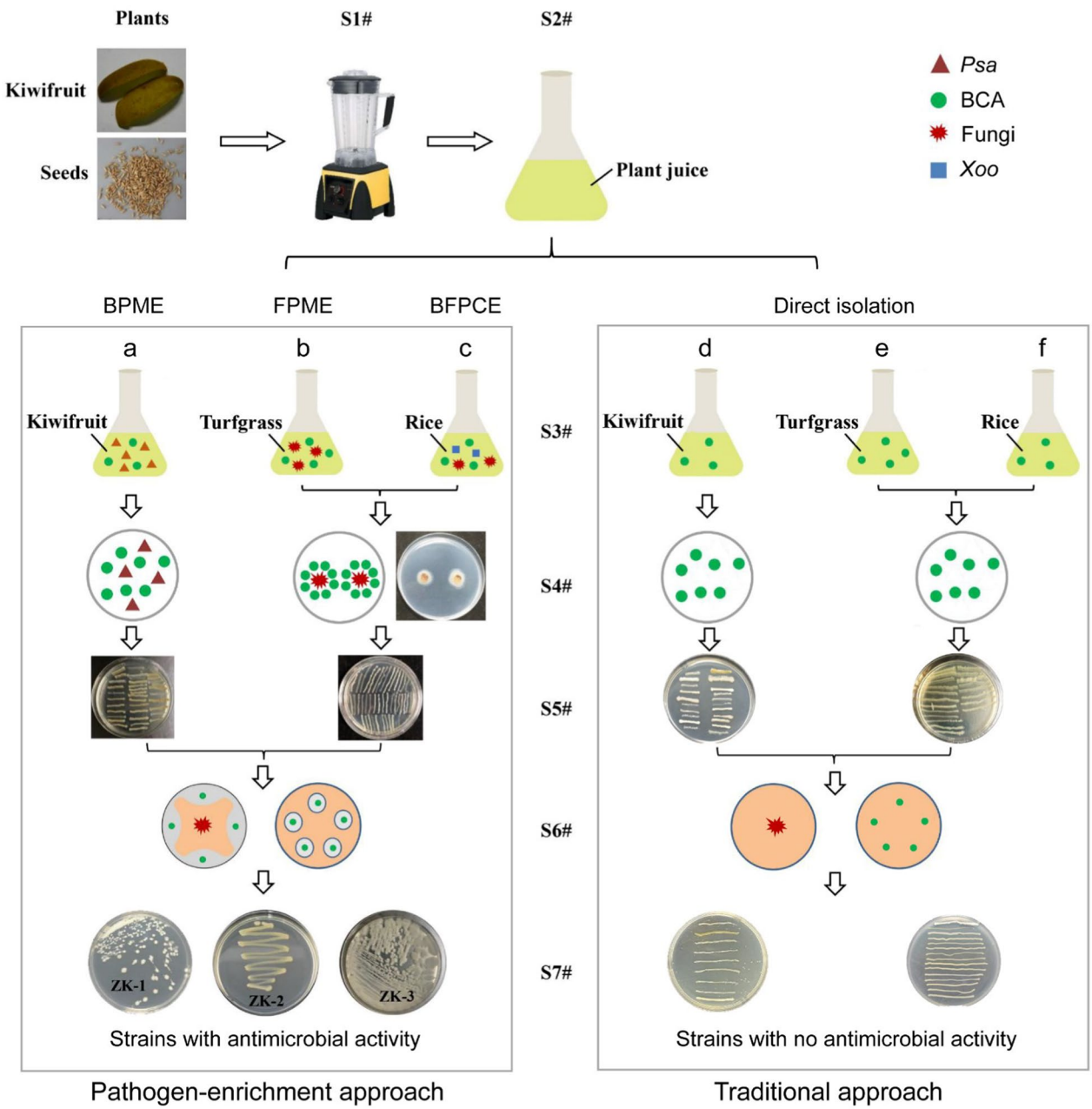

Fig. 1 Schematic diagram illustrating the targeted isolation of potential biocontrol agents from a plant-pathogen co-culture system by enriching for specific pathogens. The plant samples of interest were selected for surface sterilization with 75% ethanol, and then a high-speed blender (S1#) was used to crush the sample with sterilized water to produce a sample liquid mixture called plant juice (S2#). Left panel: Targeted isolation based on pathogen-enrichment approach. The corresponding pathogen for kiwifruit (**a**), turfgrass (**b**) or rice (**c**) in the following steps was added into the corresponding plant juice prior to co-inoculation (S3#). In this process, potential host-derived biocontrol agents (BCAs) were expected to be enriched by "eating" fed bacterial pathogens or by attaching and preying on fungal mycelia (S4#). This pathogen enrichment process can be directed to isolate a range of host-derived bacteria (S5#). These bacteria were randomly selected and tested for their antibacterial and/or antifungal activity by a typical method called the dual-culture method (S6#), resulting in the isolation of 3 potential bacterial biocontrol agents, named antibacterial ZK-1 derived from kiwifruit, antifungal ZK-2 derived from turfgrass seeds, and antibacterial and antifungal ZK-3 derived from rice seeds (S7#). Right panel: Direct isolation based on traditional approach. The plant juice of kiwifruit (**d**), turfgrass (**e**) or rice (**f**) without any pathogens (S3#). The following steps (S4#–S7#) were similar to the left panel

(See figure on next page.)

Fig. 2 Targeted isolation of antibacterial *Bacillus safensis* ZK-1 from kiwifruit by mono-enrichment of the pathogen *Pseudomonas syringae* pv. *actinidiae* (*Psa*). **a** Antibacterial activity of live cells (A–C) or cell-free supernatant (D, E) of ZK-1 against three selected pathogenic or non-pathogenic bacteria. A, B and C, LB plates pre-inoculated with *Psa* C48, *P. syringae* pv. *tomato* DC3000 and *E. coli* Top10, respectively; D, LB liquid culture of *Psa* C48 treated with ZK1 supernatant; E, LB liquid culture of the LacZ-labelled *E. coli* Top10 treated with ZK1 supernatant. "-", without supernatant, "+", with supernatant and X-gal. **b** Antifungal test of ZK-1 against five selected fungal pathogens. 1# and 2#, positive controls with known antifungal activity (1#, *Lysobacter enzymogenes* OH11; 2#, *Bacillus subtilis* NCD-2); 3#, ZK-1; 4#, *E. coli* Top10 (negative control); F, G, H, I and J, PDA plates pre-inoculated with *Diaporthe actinidiae*, *Botrytis cinerea*, *Corynespora cassiicola*, *Alternaria alternata* and *Neofusicoccum parvum*, respectively. **c** Phylogenetic tree constructed based on the genome sequences of strain ZK-1 in TYGS (https://tygs.dsmz.de/). Trees were inferred from GBDP distances computed from genome sequences using FastME 2.1.6.1 (Lefort et al. 2015). Branch lengths were scaled according to the GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60% from 100 replicates, with an average branch support of 98.5%. The tree was rooted in the middle (Farris et al. 1972)

effectiveness of the developed BPME method in isolating pathogen-enriched and host-derived BCAs.

An antifungal *Pseudomonas alcaligenes* ZK-2 as a fungal pathogen isolated from turfgrass seeds by a mono-enrichment approach

Next, we aimed to develop a fungal pathogen mono-enrichment (FPME) method for the targeted isolation of antifungal agents from plants. For this purpose, we selected a phytosystem comprising turfgrass and its fungal pathogen *Clarireedia paspali* lt486, which causes epidemics in turfgrass plants (Hu et al. 2018). In this experiment (Fig. 1), turfgrass seeds were surface sterilized and completely crushed with a high-speed mixer, and the supernatant was collected by centrifugation after adding sterilized water. Three washed fungal mycelial pellets of strain lt486 were added to the prepared seed-derived supernatant and incubated for 4 days (Fig. 1 left panel, step "b" in S3#). This step aimed to enrich seed-derived bacteria that can colonize fungal hyphae. Afterwards, the fungal pellets were picked and rinsed 3 times with sterilized water to remove those bacteria that failed to colonize the fungal mycelium tightly. After this step, the obtained fungal pellets were directly inoculated on PDA plates without antibiotics, enabling the growth of fungal colonizing bacteria. Next, these colonizing bacteria were further isolated, and each purified colony was used for antifungal testing against *C. paspali* lt486. After screening ten bacterial strains, we found that ZK-2 is an antifungal agent. On PDA plates, it inhibited the growth of fungus lt486 by secreting a diffusible antifungal factor, such as the positive controls of *L. enzymogenes* OH11 and *B. subtilis* NCD-2 (Fig. 3a). Interestingly, strain ZK-2 seems to exhibit a narrower antifungal spectrum. Among the three selected fungal pathogens, only the growth of *M. oryzae* that causes rice blast was inhibited by ZK-2 (Fig. 3a). The narrow-spectrum antifungal ability of ZK-2 indicated that it may produce unique and unkown antifungal toxins. When testing the potential antibacterial ability of ZK-2 against three phytopathogens available in the laboratory—*P. syringae* pv. *tomato* DC3000, *Acidovorax citrulli* xjl12 and *Xanthomonas campestris* pv. *campestris* 8004, we found that ZK-2 failed to inhibit their growth and no visible antibacterial zone was observed on LB plates (Fig. 3b). These results revealed that FPME, a method based on plant-pathogen co-culture and fungal enrichment, is also practical for the targeted isolation of unique antifungal agents from host plants. Finally, a BlastN search using the 16S rDNA sequence of ZK-2 as a query revealed that ZK-2 is a member of the genus *Pseudomonas*. A *gryB*-based phylogenetic tree confirmed this finding and identified ZK-2 as *Pseudomonas alcaligenes* (Additional file 1: Figure S5). To more accurately determine the phylogenetic position of strain ZK-2, multilocus sequence analysis (MLSA) based on the sequences of the 16S rDNA, *gyrB*, *rpoB* and *rpoD* was performed as previously described (Lalucat et al. 2020). The sequences of the 16S rDNA (1406 bp), *gyrB* (1040 bp), *rpoB* (1140) and *rpoD* (582 bp) genes were retrieved from GenBank deposits or whole genome sequences. Species closely related to ZK-2 based on 16S rDNA similarity were aligned with ZK-2. *Cellvibrio japonicas* DSM 16015 ^{T} was selected as the outgroup. The sequence data of the four indicated genes were concatenated and subjected to phylogenetic analysis using MEGA 6.0, and the resulting 4170 bp were used to reconstruct a phylogenetic trees based on neighbor-joining and maximum-likelihood methods. Bootstrap analysis was then performed using 1000 replications. Strain ZK-2 was found to locate within the monophyletic cluster of *P. sagittaria* JCM 18195 ^{T} and is closely related to *P. alcaligenes* NBRC 14159 ^{T}. The bootstrap value was higher than 80% (Fig. 3c), indicating that ZK-2 belongs to *P. alcaligenes*.

A broad-spectrum antimicrobial *Bacillus velezensis* ZK-3 was isolated from rice seeds via co-enrichment of bacterial and fungal pathogens

The above results demonstrated that the developed BPME or FPME method can effectively use the host–pathogen interaction system for the targeted isolation of

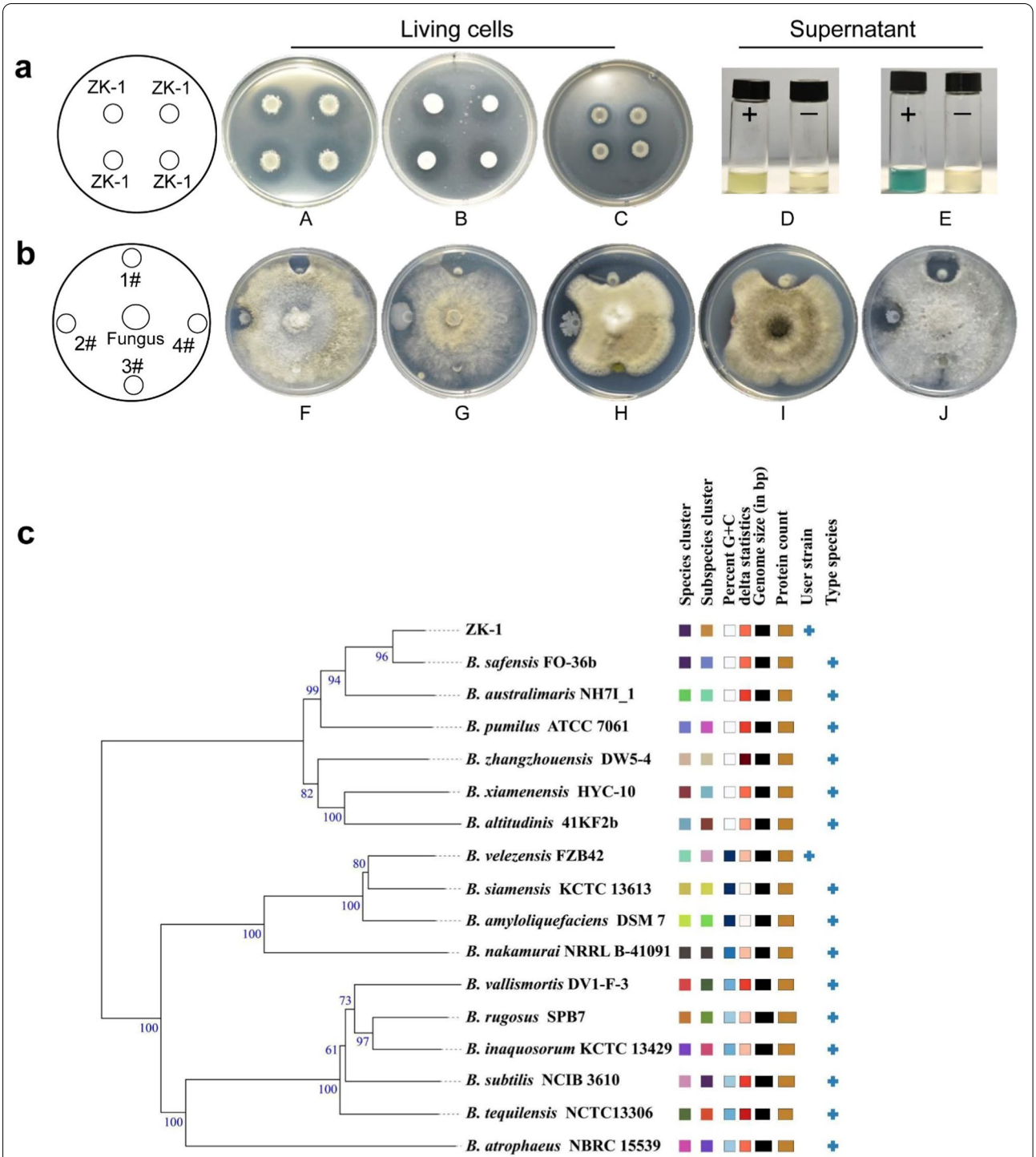

Fig. 2 (See legend on previous page.)

antibacterial or antifungal agents of interest. In the next studies, we aimed to combine BPME and FPME to create a bacterial and fungal pathogen co-enrichment (BFPCE) method with the goal of targeted isolation of bacterial agents that can express both antibacterial and antifungal activities. To achieve this, we selected a rice-pathogen phytosystem comprising the rice blast fungal pathogen *M. oryzae* Guy11 and the rice bacterial blight pathogen

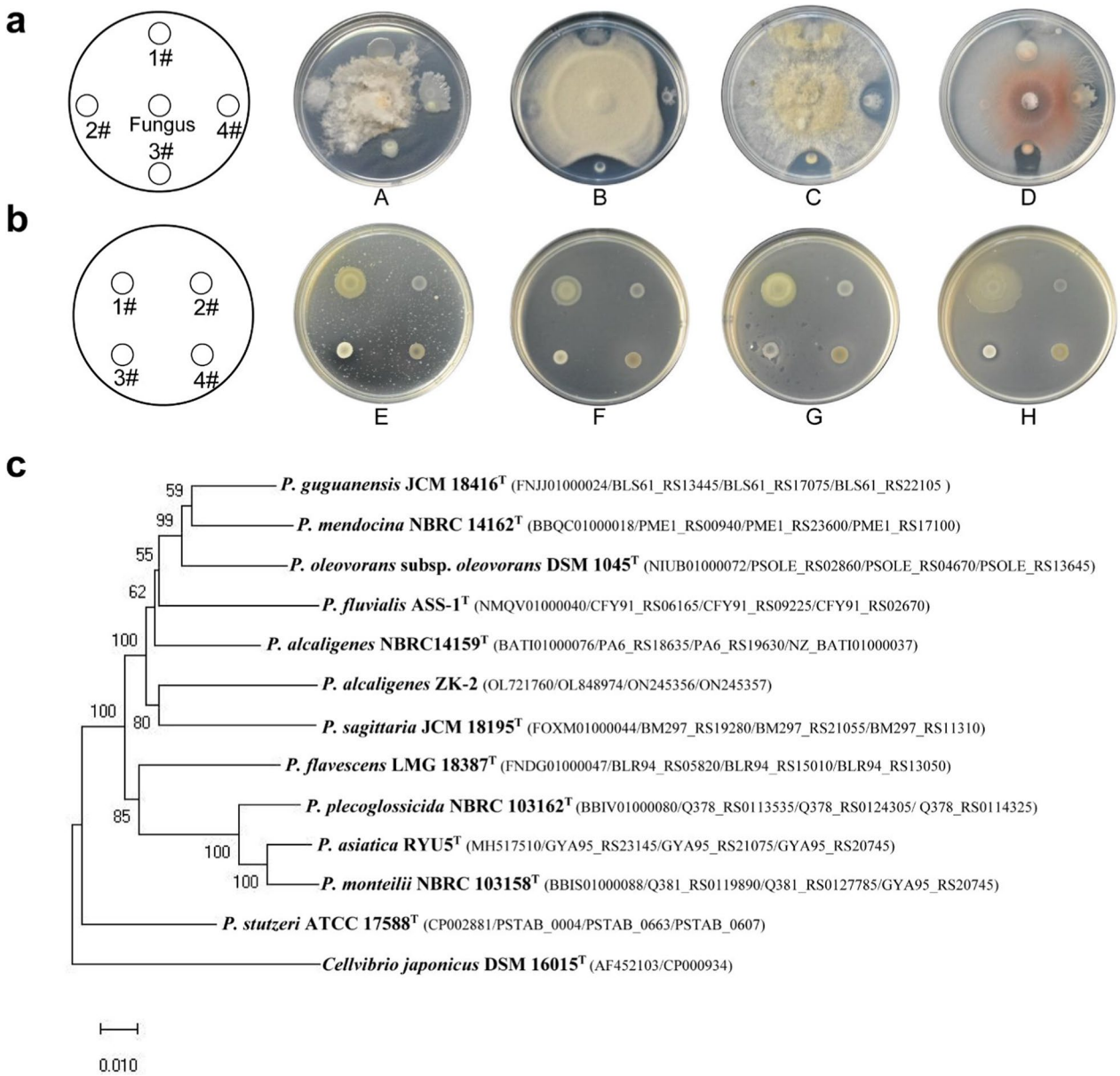

Fig. 3 Isolation of antifungal *Pseudomonas alcaligenes* ZK-2 from turfgrass seeds by mono-enrichment of the pathogenic fungus *Clarireedia paspali*. **a** Antifungal test of ZK-2 against four selected fungal pathogens. 1#, ZK-2; 2#, *E. coli* Top10; 3# and 4#, positive controls (3#, *Bacillus subtilis* NCD-2; 4#, *Lysobacter enzymogenes* OH11) exhibit known antifungal activity but no antibacterial activity. A, B, C and D, PDA plates pre-inoculated with *Clarireedia paspali*, *Magnaporthe oryzae*, *Botrytis cinerea* and *Fusarium verticillioides*, respectively. **b** Antibacterial activity of ZK-2 against four selected pathogenic or non-pathogenic bacteria. E, F, G and H, LB plates pre-inoculated with *Psa* C48, *Pseudomonas syringae* pv. *tomato* DC3000, *Acidovorax citrulli* xjl12 and *Xanthomonas campestris* 8004, respectively. **c** Maximum-likelihood phylogenetic tree constructed based on housekeeping genes 16S rDNA, *gyrB*, *rpoD* and *rpoB,* showing the relationship between strain ZK-2 and closely related strains. *Cellvibrio japonicas* DSM 16015 T was used as the outgroup. The sequences of 16S rDNA (1406 bp), *gyrB* (1040 bp), *rpoB* (1142 bp) and *rpoD* (585 bp) genes were retrieved from GenBank deposits or whole genome sequences. GenBank accession numbers are given in parentheses in the following order: 16S rDNA, *gyrB*, *rpoB* and *rpoD* genes). Numbers at nodes are bootstrap values from 1000 repetitions (≥ 50%). Strain ZK-2 is highlighted in bold. Bar, 0.01 substitutions per nucleotide position

X. oryzae pv. *oryzae* PXO99A. In this assay (Fig. 1), as in the case of turfgrass seeds described above, a supernatant derived from rice seeds (RS) was obtained. We first added the cell suspension of PXO99A to the RS-derived supernatant for 2 days, and then added 10 fungal mycelial pellets of Guy11 for co-incubation for an additional

4 days (Fig. 1 left panel, step "c" in S3#). These two steps are designed to enrich RS-derived beneficial bacteria that can use both PXO99A and Guy11 as survival nutrients in aqueous solution. After that, we picked out Guy11 pellets, washed 3 times with sterile water, and inoculated on PDA plates without antibiotics for 3 days. Similarly, the purified bacterial colonies were isolated from RS-derived bacteria attached to the Guy11 fungal mycelium. These purified bacteria were individually selected for antibacterial and antifungal testing. After screening 12 randomly selected isolates, we observed that strain ZK-3 is an ideal antibacterial and antifungal agent. When we inoculated ZK-3 cells onto the surface of LB plates embedded with *X. oryzae* pv. *oryzae* PXO99A, *X. campestris* pv. *campestris* 8004 or *A. citrulli* xjl12, we found that they all produced clear growth inhibition zones, suggesting that ZK-3 may exhibit a broad antibacterial spectrum by secreting multiple antibacterial factors into the medium (Fig. 4a). Further, when inoculating ZK-3 cell-free supernatant onto LB plates carrying PXO99A, 8004 or xjl12, we also observed growth-inhibition zones (Fig. 4b). In a plate-based challenge assays of ZK-3 with fungal pathogens, we observed that ZK-3 can also produce diffusible antifungal toxins into culture media to inhibit the growth of four selected filamentous fungal pathogens—*M. oryzae*, *C. paspali*, *Botrytis cinerea* and *Valsa pyri* (Fig. 4b). These fungal-growth inhibitions were further confirmed by applying cell-free supernatant of ZK-3 (Fig. 4c). Finally, based on sequence analysis of its 16S rDNA and *gyrB*-based phylogenetic tree, we identified strain ZK-3 as *Bacillus velezensis* (Additional file 1: Figure S6). Further genome-derived result based on dDDH and ANI values of strain ZK-3 and closely related strains validated that strain ZK-3 is close to *B. velezensis* FZB42. Moreover, the phylogenomic tree reconstructed on TYGS further confirmed the taxonomic position of the strain ZK-3 within the *B. velezensis* species (Additional file 1: Figure S4 and Fig. 4d). Together, these findings revealed that the developed BFPCE method, involving co-enrichment of bacteria and fungi, is suitable and effective for the isolation of broad-spectrum antimicrobial agents from target host.

Discussion

Although BCAs have been widely recognized as an important tool for more sustainable disease management and as a valuable alternative/complement to classical pesticides, they still represent only a small fraction (less than 10%) of the global crop protection market (Raymaekers et al. 2020). One of the critical steps in the development of new BCA-based commercial products is the identification of suitable candidates through rapid and robust screening methods. Previous screening systems for BCAs were mainly based on direct antagonism (i.e. antibiosis and competition) of BCAs with target pathogen or indirect effects of BCAs on plants by triggering plant immune responses (Köhl et al. 2019). In this study, we have established a new platform to isolate BCAs directly from plants via phytopathogen co-culture and pathogen enrichment. The development of this platform is primarily based on the symbiotic relationship between host plants and pathogens, where a particular host plant is expected to harbor one or more BCAs that are antagonistic to pathogens that can infect the host. Using these developed techniques, we effectively discovered three BCAs (ZK-1, ZK-2 and ZK-3) that exhibited either a narrow antibacterial/antifungal spectrum or a broad antibacterial spectrum against the pathogen originally used for enrichment assays.

Besides serving as a new, stand-alone BCA screening strategy, the developed pathogen enrichment method can also be combined with other classical screening systems to increase the screening efficacy of BCA while saving cost and time. For example, any pathogen of interest can be co-cultured with any plant or environmental sample in sterile water to enrich those microbes that can digest the tested pathogen as a source of nutrients for their survival. This step not only eliminates non-enriched microbes in the sample of interest through nutritional competition, but also allows pathogen-enriched microbes to dominate the population, facilitating their further selection. Pathogen-enriched and host-derived microbes can be used directly for subsequent BCA screening, using traditional co-culture tool to enhance their efficacy. Notably, the original "pathogen" can be changed to "Secreted Pathogenicity Factor, SPF" to enrich the desired SPF-targeted

(See figure on next page.)

Fig. 4 Targeted isolation of antibacterial and antifungal *Bacillus velezensis* ZK-3 from rice seeds by co-enrichment of the pathogen *Xanthomonas oryzae* and the fungal pathogen *Magnaporthe oryzae*. **a** Antibacterial activity of live cells (A–C) or cell-free supernatant (D–F) of ZK-3 against three selected pathogens. A and D, LB plate pre-inoculated with *Xanthomonas oryzae* PXO99; B and E, LB plate pre-inoculated with *Xanthomonas campestris* 8004; C and F, LB plate pre-inoculated with *Acidovorax citrulli* xjl12. **b, c** Antifungal test of the live cells (**b**) or cell-free supernatant (**c**) of ZK-3 against four selected fungal pathogens. G, H, I and J, PDA plates pre-inoculated with *Magnaporthe oryzae*, *Clarireedia paspali*, *Botrytis cinerea* and *Valsa pyri,* respectively. 1#, ZK-3; 2# and 3#, positive controls (2#, *Lysobacter enzymogenes* OH11; 3#, *Bacillus subtilis* NCD-2) with known antifungal activity; 4#, negative control, *E. coli* Top10. **d** Phylogenomic tree based on the genome sequences of strain ZK-1 in TYGS (https://tygs.dsmz.de/). Trees were inferred from GBDP distances computed from genome sequences with FastME 2.1.6.1 (Lefort et al. 2015). Branch lengths are scaled according to the GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60% from 100 replicates, with an average branch support of 98.5%. The tree was rooted at the midpoint (Farris et al. 1972)

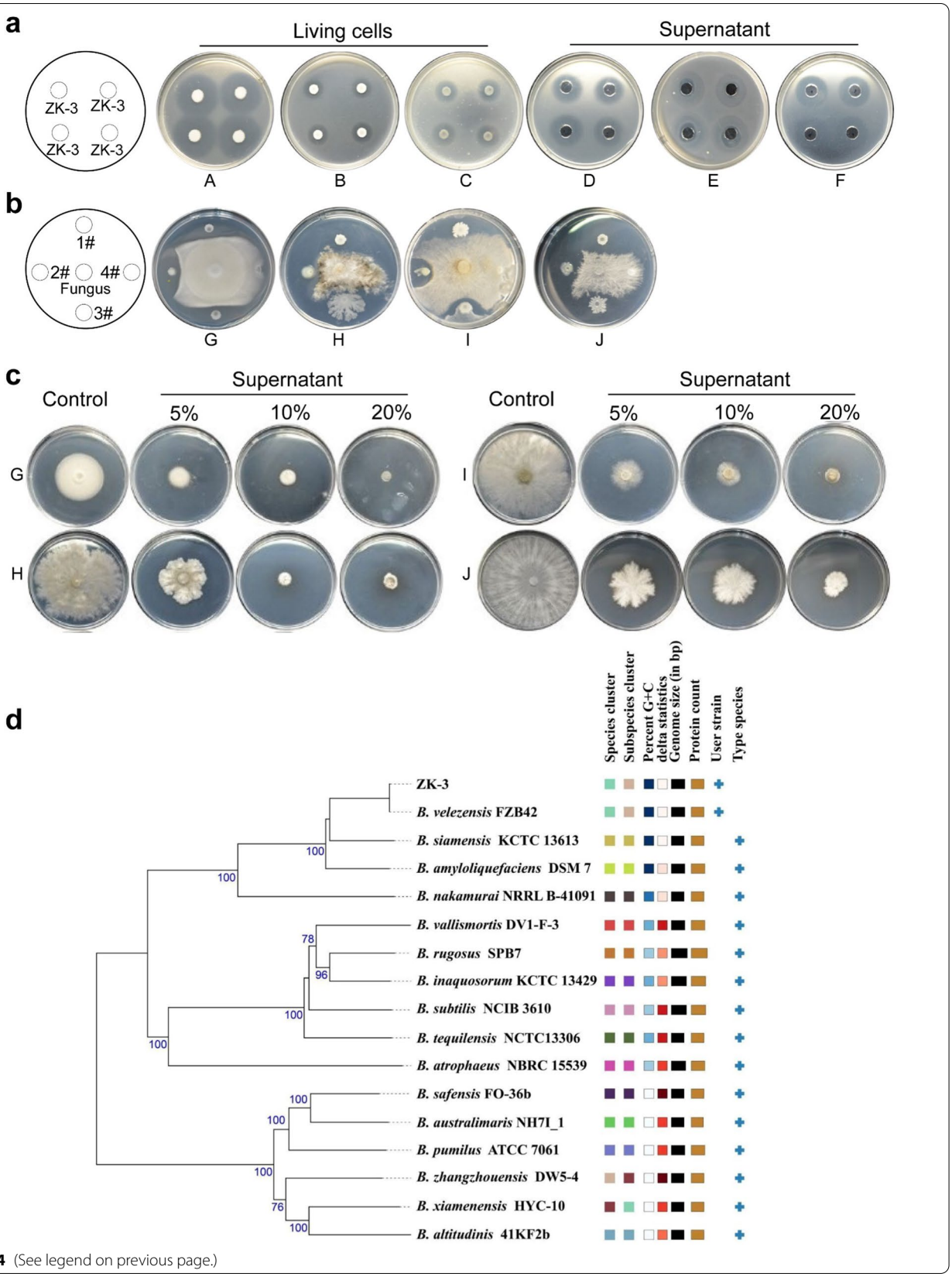

Fig. 4 (See legend on previous page.)

microbes from the sample of interest. These SPF-enriched microbes could expand the screening efficacy of BCA to reduce pathogenic infection of host plants by targeting pathogenic SPF production.

In principle, pathogen-enriched and host-derived BCAs have a similar ecological niche to pathogens, favoring their natural colonization on hosts. Compared with foreign BCAs, this ability is believed to enhance its biocontrol efficacy against ecologically relevant pathogens. Moreover, the discovery of host-derived BCA also provides an alternative route to identify new antimicrobial compounds produced by BCA. For example, the diffusible antibacterial factor produced by the *B. safensis* ZK-1 could inhibit the growth of *Psa*, but was ineffective against several fungal pathogens tested in this study (Fig. 2). To the best of our knowledge, there are few studies on biocontrol agent of *B. safensis,* a species that mainly produces antifungal compounds (Prakash and Arora 2021). Therefore, *B. safensis* ZK-1 is most likely to synthesize unique antibacterial compounds. A similar situation applies to *P. alcaligenes* ZK-2 isolated from turfgrass seeds rich in the fungal pathogen *C. paspali*. This strain secretes an unknown antifungal factor to inhibit the growth of *C. paspali* and *M. oryzae*, but not the other fungal pathogens and bacteria tested in this work (Fig. 3). This suggests that strain ZK-2 might synthesize unique antifungal compounds. Unlike strains ZK-1 and ZK-2, isolated *B. velezensis* ZK-3 likely produces secondary metabolites with both antifungal and antibacterial activities (Fig. 4). Understanding the structural basis of the antimicrobial compounds produced by ZK-1, ZK-2 and ZK-3 will undoubtedly help answer the above questions.

Conclusions

During the long-term symbiotic events between host plants and pathogens, a particular host plant is expected to harbor one or more BCAs against the host-infecting pathogen. To this end, this study developed a simple and inexpensive technique involving mono- or co-enrichment of pathogens to isolate potential BCA from their respective host plants in a targeted manner. Using this developed technology, we could effectively isolate three BCAs with antibacterial and/or antifungal activity from three different host plants. The developed technique is generic and, with modification by changing the host and/or pathogens of interest, allows the targeted isolation of BCA from diverse host–pathogen phytosystems.

Methods

Plant material, microbial strains and growth conditions

Plant materials (kiwifruit, turfgrass and rice seeds) were provided by colleagues or stored in the laboratory. All bacterial and fungal pathogens used in this study are available in the laboratory with their detailed information described in Additional file 1: Table S1. Unless otherwise stated, all bacterial strains were grown in Luria–Bertani (LB) medium at 28 °C, and all fungal strains were grown in potato dextrose agar (PDA) medium at 25 °C.

Targeted isolation of antimicrobial agents based on co-culture of plant and pathogen

Three plant-pathogen co-culture phytosystems were used for targeted isolation of antimicrobial agents from hosts by mono- or co-enrichment of the corresponding pathogens. The first selected phytosystem comprises kiwifruit and *Psa* that severely infects kiwifruit and causes global bacterial canker (Wang et al. 2021). Briefly, five mature kiwifruits were surface-sterilized with 75% alcohol for 2 min, then washed with sterile water. This process was repeated 3 times. After that, the pretreated kiwifruits were cut into small pieces with a sterilized scalpel, transferred to a high-speed blender and supplemented with 50 mL sterilized water, and finally fully digested according to the operation manual. A total of 10 mL of the digested mixture was then transferred to a 100 mL glass Erlenmeyer flask supplemented with 1 mL of cell suspension (OD_{600}, 1.0) of *Psa* strain C48. The resulting kiwifruit-C48 mixture was incubated in a shaker (60 rpm) at 28 °C for 2 days. It is believed that during this process, the kiwifruit-derived bacteria capable "eating" (relying upon) C48 for survival will be enriched. After this step, a mixture of 100 μL was removed and smeared on LB plates. After3 days of incubation, the resulting colonies were used for plate-based antibacterial test against C48 on agar plates.

The second phytosystem includes host plant, turfgrass and the infectious fungal pathogen *Clarireedia paspali* (Hu et al. 2018). In brief, 100 turfgrass seeds were surface-sterilized with 75% alcohol for 2 min, then washed twice with water. After that, all surface-sterilized seeds were transferred to a high-speed blender supplemented with 100 mL of sterilized water, and then fully digested according to the operation manual. A total of 25 mL digested mixture was transferred to a 100 mL glass Erlenmeyer flask supplemented with 10 fungal mycelial plugs of *C. paspali* strain lt486. The resulting seed-lt486 mixture was then incubated in a shaker (60 rpm) at 25 °C for 2 days. We hypothesized that during such co-incubation, bacteria derived from turfgrass seeds capable of "eating" (relying upon) the fungus lt486 for survival would accumulate around the fungal mycelial plugs. The fungal mycelial plugs with seed-derived bacteria were then rinsed twice with sterilized water to remove those bacteria that could not colonize the fungal mycelium. The resulting plugs/bacteria were then inoculated on LB plates. After incubating for 3 days, the bacteria enriched

around the fungal plugs were individually transferred to fresh LB plates by using a transfer loop, and then incubated at 28 °C for another 3 days to obtain purified bacterial colonies. Finally, the purified colonies were randomly selected and tested on agar plates for their antifungal activity against lt486.

The third selected phytosystem consists of the host plant rice and two infectious pathogens, the rice blast fungus *M. oryzae* and the bacterial rice blight pathogen *Xoo* (Wang et al. 2018; Li et al. 2020). In short, 100 rice seeds were surface sterilized with 75% alcohol for 2 min, then washed twice with sterilized water. After that, all seeds were transferred to a high-speed blender supplemented with 100 mL of sterilized water, and then fully digested according to the operation manual. A total of 25 mL of digested mixture was then transferred to a 100 mL glass Erlenmeyer flask supplemented with 1 mL cell suspension (OD_{600}, 1.0) of *Xoo* strain PXO99A. This mixture was then incubated in a shaker (60 rpm) at 28 °C for 2 days to enrich those rice seed-derived bacteria that could "eat" PXO99A, followed by the addition of 10 mycelial plugs of *M. oryzae* strain Guy11 for additional incubation for 3 days at 25 °C in a shaker (60 rpm). This step aimed to further enrich the rice seed-derived bacteria that can use the fungal mycelium as a source of nutrient for growth. Afterwards, the obtained mycelial plugs were rinsed twice with sterilized water, and then inoculated on LB plates for 3 days to enrich the growth of bacteria around the fungal plugs. These bacteria were individually transferred to fresh LB plates using a transfer loop, and incubated at 28 °C for 3 days to obtain purified bacterial colonies, which were then tested on agar plates for their antimicrobial activities against PXO99A and/or Guy11.

Antifungal and antibacterial assays based on live BCA cells

In the fungal inhibition assay, plugs (2 mm in diameter) cut from the edges of a 5-day-old colonies of selected fungal pathogen were transferred from PDA to the centre of a fresh PDA petri dish. Subsequently, 2 µL of bacterial cell suspension (OD_{600}, 1.0) of interest was inoculated on the edge of petri dishes previously inoculated with the fungal pathogen. After 3 days of incubation at 25 °C, the antagonistic activity was revealed by the inhibition zone around the colonies. In the bacterial inhibition assay, 1 mL overnight culture of selected phytopathogenic bacterium was mixed with 25 mL of molten LB agar (LA) medium and poured into a petri dish. Once solidification, 2 µL of cell suspensions (OD_{600}, 1.0) of various plant-derived bacteria isolated in this study were spot-inoculated on the surface of LA culture dishes, each containing the indicator phytopathogen. After 3 days of incubation at 28 °C as previously described (Yang et al. 2020; Shen et al. 2021), zones of inhibition in antifungal and antibacterial assays were recorded using a Nikon camera (D7100, Japan). All experiments were carried out 3 times with 3 replicates for each treatment.

Antifungal and/or antibacterial assay based on cell-free supernatant

The bacterial strains tested in this study were individually cultured in fresh LB broth (25 mL) at 28 °C for 2 days. After centrifugation (6000 rpm) at room temperature, cells were removed and the corresponding supernatant from each strain was collected. These supernatants were further filtered by using 0.22 µM filter to produce cell-free supernatants (10 mL). In the following assays, 1 mL of the cell-free supernatant of the ZK-1 strain was mixed with an equal volume of fresh LB broth with or without the addition of 100 µg/mL X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside), followed by inoculation of 100 µL overnight culture of *Psa* strain C48 or the LacZ-labeled *E. coli* strain Top10 (Shen et al. 2021) for antibacterial assay. For supernatant- based antifungal assay, cell-free supernatants of ZK-3 were collected as described above. Different volumes of the resulting ZK-3 supernatant were added to the PDA agar plates. Various filamentous fungal plugs excised from 3-day-old colonies on PDA plates were inoculated in the center of fresh PDA plates containing 5, 10 or 20% (volume/volume) of ZK-3 cell-free supernatant. Growth of fungal mycelia was detected after 5 days of culturing at 25 °C without shaking. All experiments were carried out 3 times, with 3 replicates for each treatment.

Molecular identification and phylogenetic analysis

Three purified single colonies with bacterial biocontrol potential (named as ZK-1, ZK-2 and ZK-3, respectively) were picked and inoculated individually onto fresh LB liquid broth at 28 °C for 2 days. Cells of each strain were collected by centrifugation (12,000 rpm) at room temperature. Genomic DNA of each strain was extracted by using a commercial kit (DP302, Tiangen, China). For molecular identification, the 16S rDNA gene and the house-keeping gene *gyrB* encoding the DNA gyrase subunit B of each strain were PCR-amplified using the corresponding universal primers (Additional file 1: Table S2). Each PCR product was then purified using Takara MiniBEST DNA fragment purification kit (No. 9761, Shanghai, China). Each purified DNA fragment obtained was sequenced by GENEWIZ Company (Suzhou, China) and compared with the DNA sequences available in the NCBI

database using the BLASTN program. In this study, the 16S rDNA gene was used to classify the strains at the genus level, and the house-keeping gene *gyrB* was used to confirm and generate a phylogenetic tree. In short, *gyrB* DNA sequences from the NCBI database were compared with the ClustalW program in MEGA 7.0 (Kumar et al. 2016). As previously described, the *gyrB*-based phylogenetic tree was generated by using the nearest neighbour data analysis method with 1000 bootstrap replicates,

MLSA analysis

Genome sequence data for strains ZK-1 and ZK-3 was uploaded to the Type Strain Genome Server (TYGS) available at https:// tygs. dsmz. de (Meier-Kolthoff et al. 2019) for genome-wide based taxonomic analysis. DNA-DNA relatedness values were estimated from genome sequences by digital DNA–DNA hybridization (dDDH) using Formula 2 of the Genome-to-Genome Distance Calculator 2.1 (Meier-Kolthoff et al. 2013). Average Nucleotide Identity (ANI) values were calculated using the ANI Calculator (www.ezbiocloud.net/tools/ani) (Yoon et al. 2017a, b). Genome-based phylogeny was inferred from available type strains of *Bacillus* species with full genome sequences using TYGS (Meier-Kolthoff et al. 2019

The 16S rDNA sequence of strain ZK-2 was PCR amplified as described by Weisburg et al. (1991). To reveal phylogenetic relationship, the obtained sequence (1406 bp) was compared with those of other type strains available in the EzBioCloud server (Yoon et al. 2017a, b). Four house-keeping gene (16S rDNA, *gyrB*, *rpoB* and *rpoD*) sequences were directly downloaded from GenBank or retrieved from draft/complete genome sequences. Multiple alignments with sequences from closely related strains were performed using the Clustal W in MEGA version 6.0 software (Tamura et al. 2013). Phylogenetic trees were reconstructed with maximum likelihood (Felsenstein. 1981) in MEGA 6.0 (Tamura et al. 2013), or with FastME (Lefort et al. 2015) based on genome blast distance phylogeny (GBDP) distances calculated from genome sequences. For these, all pairwise genome comparisons were conducted using GBDP and intergenomic distances inferred under algorithm 'trimming' and the distance formula d5 (Meier-Kolthoff et al. 2013).Trees were rooted at the midpoint (Farris et al. 1972). Branch supports were inferred from 100 bootstrap replicates. All strains used for constructing the phylogenetic trees are listed in Additional file 1: Tables S3–S5.

Abbreviations

BCA: Biocontrol agent; *Psa*: *Pseudomonas syringae* pv. *actinidiae*; *Xoo*: *Xanthomonas oryzae* pv. *oryzae*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00124-2.

Additional file1: Figure S1. Isolated bacterial strains from kiwifruit via *Psa* enrichment. **Figure S2**. Traditional method without pathogen enrichment for screening potential BCA strains. **Figure S3**. Phylogenetic analysis and taxonomic position of ZK-1 based on the housekeeping gene *gyrB*. **Figure S4**. ZK-1 and ZK-3 identification based on ANI and DDH. **Figure S5**. Phylogenetic analysis and taxonomic position of ZK-2 based on the housekeeping gene *gyrB*. **Figure S6**. Phylogenetic analysis and taxonomic position of ZK-3 based on the housekeeping gene *gyrB*. **Table S1**. Strains and plasmids used in this study. **Table S2**. Primers used in this study. **Table S3** Strains information for generating phylogenomic tree in Fig. 2c and Fig. 4d. **Table S4**. Strains information for generating phylogenomic tree in Fig. 3c. **Table S5**. GenBank accession numbers of *gyrB* in Additional file 1: Figures S3–S6.

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Author contributions

GQ and CZ conceived the project. GQ and CZ designed experiments. BW, LL and YL carried out experiments. BW, LL and YL analyzed data and prepared figures and tables. GQ and XS wrote the manuscript. GQ, XS and CZ revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The sequence data of this study have been submitted to NCBI GenBank with the following accession numbers: OL721755 (16S rDNA of ZK-1), OL848973 (*gryB* of ZK-1), OL721760 (16S rDNA of ZK-2), OL848974 (*gryB* of ZK-2), ON245356 (*rpoB* of ZK-2), ON245357 (*rpoD* of ZK-2), OL721756 (16S rDNA of ZK-3) and OL848975 (*gyrB* of ZK-3). The genome of ZK-1 and ZK-3 were submitted to NCBI with submission ID CP095759 and CP095760, respectively. All other data needed to evaluate the conclusions in the paper are provided in the paper or supporting information.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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