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Characterization of *Stemphylium vesicarium* isolates from pear orchards in Emilia Romagna (Italy) and assessment of potential microbial biocontrol agents

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Abstract: Brown spot of pear caused by *Stemphylium vesicarium* is one of the most important fungal diseases in Europe, as it may cause more than 90 % yield losses. A set of ten *S. vesicarium* isolates from diseased pear fruits cv. Abbé Fétel, collected in Emilia-Romagna orchards, was molecularly identified, showing high genetic similarity. At pathogenic level, *S. vesicarium* isolates showed two main degrees of virulence (i. e., "low" and "high"). The most virulent *S. vesicarium* isolate was then used for assessing the biocontrol of six bacterial isolates, by means of *in vitro* tests and detached pear fruit assays. The two most promising bacteria, belonging to the genera *Bacillus* and *Pseudomonas,* highlighted their potential as putative microbial biocontrol agents for implementing a sustainable control of *S. vesicarium*.

Key words: brown spot of pear, bacterial antagonists, biological control

Introduction

Stemphylium vesicarium (Wallroth) (Simmons, 1969), the causal agent of brown spot of pear (BSP), is responsible for important economic losses in the main producing areas of Italy, such as in the Emilia Romagna region, on the highly susceptible pear cultivar Abbé Fétel (Bugiani, 2022). Climatic changes, such as increased average temperatures and an altered rainfalls pattern, can foster *S. vesicarium* inoculum build-up and disease severity, therefore leading to an increased economic impact of this fungal disease (Moragrega et al., 2018). BSP integrated management includes sanitation practices (e. g., leaf litter removal), aimed to decrease the inoculum potential, and multiple fungicides sprays during the cropping season. However, the disease pressure and the development of resistance traits to chemical fungicides by *S. vesicarium*, call attention to the need for sustainable complementary or alternative strategies in the management of BSP (Soriato et al., 2024). Our study aimed to characterize *S. vesicarium* isolates from Emilia Romagna orchards, at a molecular and pathogenic level, and to search for carposphere epiphytes as possible microbial biocontrol agents (mBCAs).

Materials and methods

***Stemphylium vesicarium* isolation and identification**

Stemphylium spp. were isolated from diseased pear fruits, cv. Abbé Fétel, collected in summer 2023 and 2024 in four Emilia Romagna orchards. The isolates were morphologically characterized and molecularly identified by means of the partial amplification and sequencing of the internal transcribed spacer (*ITS*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and elongation factor (*EF*) regions (Cortiello et al., 2023). All sequences were subjected to BLASTn search (https://blast.ncbi.nlm.nih.gov/), followed by phylogenetic analyses through multiple-sequence alignment using the MEGA11 software.

***Stemphylium vesicarium* strains bioassay on detached fruits**

The BSP severity was assessed for each *S. vesicarium* isolate on detached fruits, according to a bioassay described by Kohl et al. (2009), with modifications. Three biological replicates were used, each consisting of two fruits.

Dual and double culture assays

The antimicrobial activity of a set of four bacterial isolates from the pear carposphere was tested *in vitro* against *S. vesicarium* Sv 2021, both in dual culture and double plate assays for direct antagonism and emission of volatile organic compounds (VOCs), respectively (Xhemali et al., 2023).

Bioassay on detached fruits

In a second bioassay on detached fruits, the efficacy of bacterial isolates to control BSP severity was assessed. Experimental conditions and procedures were similar to the first bioassay. Briefly, 40 µl droplets of bacterial suspension (1×10^8 CFU/ml) were spotted four times on the fruit surface; sterile distilled water was used as a control. After 24 hours, 40 µl of *S. vesicarium* Sv 2021 conidial suspension were spotted on each mBCAs pre-treated point on fruits.

Statistical analysis

The collected data were subjected to ANOVA and Tuckey's tests at $p \leq 0.05$, using SPSS 15.0 for Windows® (SPSS Inc., Chicago, IL).

Results and discussion

Identification and pathogenic characterization of Stemphylium vesicarium isolates

A total of 10 fungal isolates were collected from symptomatic pear fruits. For all isolates, (*i*) morphological characteristics of both colonies and spores were consistent with *S. vesicarium* (Simmons, 1969); (*ii*) BLAST analysis for ITS, GPDH and EF gene sequences showed 99-100 % identity with *S. vesicarium* ATCC 18521. Phylogenetic analysis revealed a high genetic similarity among the *S. vesicarium* strains, with nine isolates that clustered together with the ex-type strain *S. vesicarium* ATCC 18521^{EX-T}. The strain Sv 2261 clustered separately in a second clade, despite having a very low genetic distance (Figure 1 A). All ten *S. vesicarium* strains were able to reproduce the typical symptoms of the BSP on detached pear fruits, with 100 % incidence. The pathogenic characterization tests showed the presence of two *S. vesicarium* groups with "high" and "low" virulence (Figure 1 B). Three strains displayed a significantly lower virulence ($p < 0.05$) in comparison to others. The Sv 2021, belonging to a

"high" virulence group and showing the higher disease score (13.84 ± 2.12), was selected for further experiments with mBCAs.

The genetic similarity and the presence of different degrees of virulence observed among our *S. vesicarium* isolates from pear were consistent with previous studies (Temperini et al., 2022).

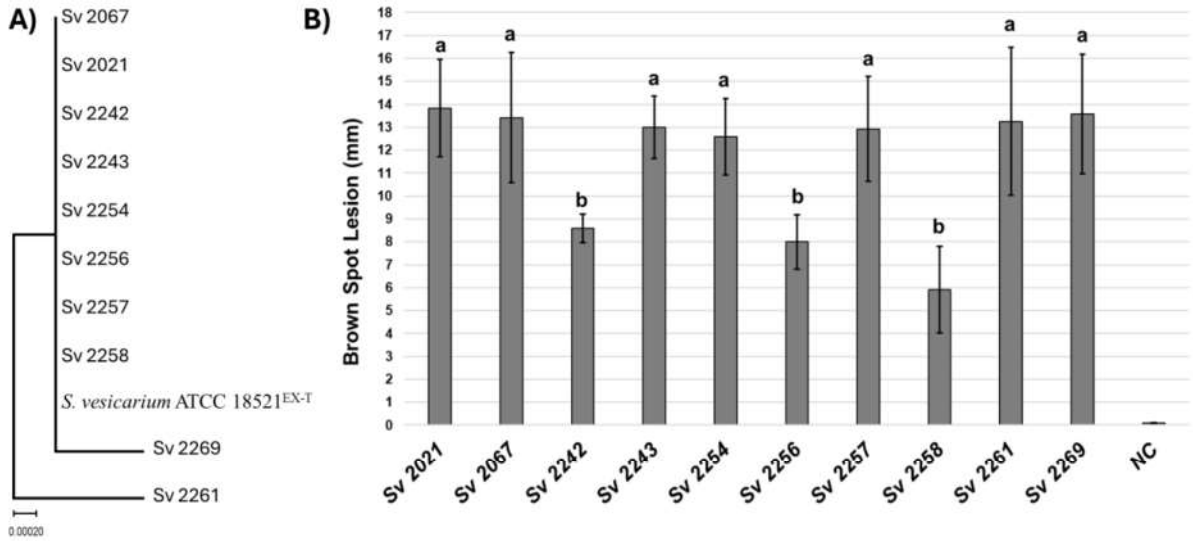

Figure 1. A) Phylogenetic tree based on combined ITS, GAPDH and EF sequences of the 10 *Stemphylium* isolates with the ex-type strain *S. vesicarium* ATCC 18521^{EX-T}. B) Evaluation of BSP disease severity on detached pear fruits infected with *S. vesicarium* isolates. The different letters indicate statistically significant differences among isolates according to the one-way ANOVA test and Tukey's HSD test ($p < 0.05$).

Biocontrol potential of bacterial isolates

The selected bacterial isolates were identified as members of the genera *Bacillus* (DLS188, DLS321 and DLS323) and *Pseudomonas* (DLS329) (Table 1). Regarding their direct antagonistic ability, dual plate assay showed that *Bacillus* sp. DLS321 was the most effective in reducing *S. vesicarium* growth (74.29 %). Meanwhile, *Pseudomonas* sp. DLS329 was the most performing in both double plate assay (VOCs inhibiting mycelial growth), with a reduction rate up to 29.89 % and bioassay on detached fruits (80.37 %).

Intriguingly, for these bacterial isolates the *in vitro* and pear fruit bioassay results were not always closely correlated. Therefore, further studies are needed to investigate the mechanisms by which these mBCAs might exert their adverse effects against *S vesicarium* (Kohl et al., 2020). Overall, these findings highlight the potential of strains *Bacillus* sp. DLS323 and *Pseudomonas* sp. DLS329 as promising biocontrol agents. Future experiments will focus on *in planta* validation to confirm their effectiveness in controlling BSP.

Table 1. Identity and effect of 4 bacterial isolates on *S. vesicarium* Sv 2021 mycelia growth inhibition (MGI) *in vitro* (dual and double plate assays) and BSP disease severity reduction on detached pear fruits. The different letters indicate statistically significant differences among treatments according to the one-way ANOVA test and Tukey's HSD test ($p < 0.05$).

Strain	Identity (BLASTn 16S rRNA)	Dual plate MGI (%)	Double plate MGI (%)	BSP severity reduction on detached fruits (%)
DLS188	*Bacillus* sp.	72.41[a]	16.67[c]	26.30[c]
DLS321	*Bacillus* sp.	74.29[a]	24.46[b]	11.48[c]
DLS329	*Pseudomonas* sp.	21.00[b]	29.89[a]	80.37[a]
DLS323	*Bacillus* sp.	70.11[a]	22.96[b]	33.33[b]

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