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Research Paper

THE EFFICACY OF BACTERIAL AND FUNGAL ANTAGONIST SUSPENSIONS IN CONTROLLING FOLIAR MILDEW DISEASE IN ZUCCHINI PLANTS

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ARTICLE HIGLIGHTS

- Diverse microbial antagonists can be used as alternatives to control foliar disease
- Microbial agents offer zucchini protection against downy mildew causing microbes
- Biocontrol agents effectively control mildew infection in zucchini plants
- Diverse microbial antagonists have potential to control foliar disease in zucchini
- New bio-based strategy supports sustainable crop disease management
- Diverse microbial antagonists are promising for controlling mildew in zucchini

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ABSTRACT

Downy mildew is recognized as a major constraint in zucchini production, caused by obligate fungal-like pathogens that thrive under humid conditions. In this study, the efficacy of selected bacterial (*Lysobacter antibioticus* Bali G, *Pseudomonas corrugata* SAJ6) and fungal (*Trichoderma* sp. Td22) antagonists was evaluated for the management of this foliar disease on zucchini plants as an alternative to chemical fungicides. The efficacy of these bacterial and fungal antagonists against a suspected downy mildew pathogen was assessed on zucchini leaves in a glasshouse. It was found that the antagonists provided 22 - 83% protection ($P < 0.05$) against the pathogen two weeks after application. However, the level of protection declined over time, with 46 - 60% of leaves infected five weeks after pathogen exposure, regardless of treatment. The combination of *Trichoderma* sp. Td22, the most effective agent, with either *Lysobacter antibioticus* Bali G, *Pseudomonas corrugata* SAJ6, or both, was observed to reduce its overall effectiveness. Survival of the biological agents on leaf surfaces was low, although prior research has indicated that survival may not be essential for sustained disease control. Further investigation is required to determine the potential role of these agents in inducing systemic acquired resistance in crops such as grapes and poppies. For commercial application, repeated treatments may be necessary to maintain disease management. Notably, the protection provided by *Trichoderma* sp. Td22 was found to be comparable to that of chemical treatments, representing a promising step toward more sustainable agricultural practices.

Keywords: bacterial antagonists, biocontrol, *Lysobacter antibioticus, Pseudomonas corrugata*, zucchini disease control

INTRODUCTION

Downy mildew is a widespread and economically impactful plant disease that affects various crops, including cucumbers, grapes, cantaloupes, and other cucurbits, across different agricultural regions globally. This disease is caused by obligate fungal-like pathogens, such as *Plasmopara viticola* (Heger *et al.* 2022; Clippinger *et al.* 2024) and *Pseudoperonospora cubensis* (Wallace *et al.* 2020; Sun *et al.* 2022), which predominantly infect green plant tissues, especially the leaves. Typical symptoms include irregular spots on the leaves, which can range from pale green to yellow or brown in color (Newark *et al.* 2019; Purayannur *et al.* 2021). Favorable conditions, including high humidity and moderate temperatures, facilitate rapid disease spread through wind-borne spores or rain splashes, often resulting in large-scale outbreaks.

In severe cases, downy mildew can lead to defoliation, stunted growth, poor fruit quality, and even total crop failure, making it a critical challenge for growers (Purayannur *et al.* 2021; Marone Fassolo *et al.* 2022). As global temperatures and weather patterns shift, the range and frequency of downy mildew outbreaks are projected to increase, emphasizing the need for integrated disease management strategies that combine resistant crop varieties, cultural practices, and sustainable control measures (Singh *et al.* 2023).

Globally, downy mildew significantly impacts food security and agricultural output, particularly in areas where susceptible crops are grown extensively. *Plasmopara viticola* for example, continues to challenge grape production in Europe, North America, and Australia, leading to reduced harvests and increased dependency on fungicides (Koledenkova *et al.* 2022). Similarly, *P. cubensis* is a key limitation for cucurbit farming in Asia, the Americas, and parts of Africa, where recurrent outbreaks cause severe losses to commercial yields (Salcedo *et al.* 2020).

Downy mildew, a destructive disease affecting the foliage of many crops, has traditionally been managed using several strategies. These include crop rotation, which interrupts the pathogen's lifecycle by alternating host availability, and the deployment of resistant plant varieties that utilize genetic traits to fend off infections (Tör *et al.* 2023; Clippinger *et al.* 2024). Efficient irrigation practices, such as drip systems and proper drainage, reduce moisture levels conducive to the disease's growth (Tör *et al.* 2023; Clippinger *et al.* 2024). Chemical fungicides remain a common solution, offering rapid disease suppression during critical periods (Toffolatti *et al.* 2024; Clippinger *et al.* 2024). However, their frequent use has raised concerns over environmental pollution, public health, and the evolution of fungicide-resistant pathogens (Ons *et al.* 2020; Islam *et al.* 2024).

Current research underscores the value of integrated disease management (IDM) approaches that combine various practices to achieve effective and sustainable disease control. Studies by Corkley *et al.* (2022) and Wang *et al.* (2022) demonstrated the advantages of integrating resistant crop varieties with targeted fungicide applications to manage disease outbreaks. This dual approach, leveraging genetic resistance to reduce pathogen pressure and applying fungicides strategically to mitigate severe infections, minimizes reliance on chemicals while sustaining crop yields. Such strategies align with contemporary goals of reducing the environmental impact of agriculture.

In growing concerns about fungicide overuse, biological control options are becoming increasingly popular within IDM frameworks (Ons *et al.* 2020). Compost teas, liquid extracts produced through compost fermentation, represent one promising strategy (Coker & Ozores-Hampton 2021). These solutions are rich in beneficial microorganisms and bioactive compounds that combat pathogens and enhance plant immunity (Ramírez-Gottfried *et al.* 2023). They offer a natural and sustainable alternative or complement to synthetic chemicals, integrating seamlessly into environmentally conscious agricultural practices.

Recent research also highlights the role of specific biocontrol agents, such as *L. antibioticus, P. corrugata*, and *Trichoderma* spp. in managing zucchini diseases such as downy mildew (Ayaz *et al.* 2023). *L. antibioticus* produces antibiotics that suppress *Pseudoperonospora cubensis* (Drenker *et al.* 2023), the pathogen responsible for downy mildew, while *P. corrugata* inhibits spore germination and disease spread through antagonistic interactions (Ramona *et al.* 2020). Meanwhile, *Trichoderma* sp. not only protect plants by colonizing root systems but also enhance systemic resistance, making plants more resilient to infections (Chakraborty *et al.* 2020).

Combining traditional practices with these innovative biological tools provides a balanced way forward (Clippinger *et al.* 2024). This integrated approach reduces harmful inputs while addressing key challenges in plant health management, promoting sustainable and resilient agricultural systems.

The effectiveness of compost teas in managing plant diseases arises primarily from the diversity of their microbial communities, which actively suppress pathogens while supporting plant health (Barghouth *et al.* 2023). Beneficial microbes, such as *Bacillus* and *Pseudomonas* produce antimicrobial compounds that inhibit pathogen growth, while fungi like *Trichoderma* outcompete pathogens for space and nutrients and form protective root barriers (St. Martin *et al.* 2020). Some microorganisms in compost teas also trigger systemic resistance, equipping plants with enhanced defense mechanisms against diverse pathogens (Emannuel Oliveira Vieira *et al.* 2024).

Sarmah *et al.* (2020) emphasized that enriching compost teas with targeted strains, including *Trichoderma* spp. and *Bacillus* spp., boosts their efficacy against a broader spectrum of diseases. This approach addresses common challenges like variability in compost tea effectiveness, which often results from differences in compost quality and brewing conditions. Introducing well-characterized microbial strains ensures consistency and reliability, enhancing the utility of compost teas in agriculture.

Beyond their role in disease management, compost teas promote healthier plants by improving soil quality and nutrient availability (De Corato, 2020; Ramírez-Gottfried *et al.* 2023). Their microorganisms facilitate the release of essential nutrients like nitrogen and phosphorus, boosting plant growth and vitality (Singh *et al.* 2022). By combining disease suppression with nutrient enhancement, compost teas serve as a multifunctional tool that aligns with the goals of sustainable agriculture. Leveraging these biological solutions offers a scalable and eco-friendly way to address modern agricultural challenges.

Based on the above rationale, this research focused on assessing the effectiveness of bacterial suspensions of *Lysobacter antibioticus* Bali G and *Pseudomonas corrugata* SAJ 6 in TSB, as well as a fungal spore suspension of Td_{22} in saline, for controlling downy mildew in zucchini plants. The study aimed to offer alternatives to conventional "compost teas" by using selective microbial antagonists as active agents for disease management in important crops, including, zucchini, grapes, and poppies.

MATERIALS AND METHODS

Bacterial and Fungal Antagonist Isolates

Three antagonistic microorganisms, including *Lysobacter antibioticus* Bali G, *Pseudomonas corrugata* SAJ6, and *Trichoderma* sp. Td_{22} that effective against *Sclerotinia minor* in lettuce plants (Ramona *et al.* 2022), were evaluated in the current study for their potential to manage a foliar mildew disease, downy mildew, in zucchini. The *L. antibioticus* Bali G and *P. corrugata* SAJ6 were obtained from lettuce farms in Bedugul, Bali, Indonesia, whereas the *Trichoderma* sp. Td_{22} was obtained from Dr Dean Metcalf, a senior researcher at Department of Primary Industries, Parks, Water, and Environment (DPIWE) in Tasmania, Australia.

Downy Mildew Isolate

The pathogen analyzed in this research was obtained from diseased grape leaves sourced from the Horticultural Research Centre (HRC) at the University of Tasmania, Australia. The characteristic leaf damage initially suggested identification as downy mildew. As obligate parasites, downy mildew pathogens necessitate the need for living tissue; therefore, the infected leaves were collected just prior to preparing the pathogen suspension in saline solution.

Preparation of Antagonist Suspensions

The bacterial antagonists were grown in a medium containing 0.5% (w/v) trypticase soya broth (OXOID) at 25 °C for 48 hours without agitation, achieving a final concentration of roughly 10^8 cells/mL. The *Trichoderma* sp. Td22 spores were obtained from wood fiber waste (WFW) compost, which had previously supported Td22 cultivation during our previous lettuce or pyrethrum experiments. To extract the spores, the Td22-grown WFW compost was agitated to release most spores in a saline solution at a 1 : 10 (w/v) ratio for around 10 minutes before being utilized. The Trichoderma sp. Td22 spore density obtained was 8.42 ± 0.01 log10 cfu/mL (average of triplicates measurements with an Improved New Bauer Hemacytometer).

Preparation of the Pathogen Suspension

Approximately 10 g of infected grape leaves were placed in 200 mL of sterile saline solution (0.85% NaCl) and shaken thoroughly to release the pathogen from the leaves. The mixture was then sieved with a piece of sterile cloth to remove the leaf debris, before being used in the trials. A density of 2.7×10^7 propagules/mL were obtained following determination with an Improved New Bauer Hemacytometer.

Glasshouse Scale Experiments

Zucchini seeds ('Blackjack' Yates®) were sown in 1.5 L pots containing a steam-sterilized standard potting mix. After 14 days, the seedlings' leaves were sprayed with 2 mL of antagonist suspensions. The study included various combinations of antagonists, such as *L. antibioticus* + *P. corrugata, L. antibioticus* + *Trichoderma* sp. Td_{22}, *P. corrugata* + *Trichoderma* sp. Td_{22}, and a mixture of all three (*L. antibioticus* + *P. corrugata* + *Trichoderma* sp. Td_{22}) in equal proportions (v/v). Three days following the antagonist application, a 2 mL suspension of the pathogen was sprayed onto the leaves. Each treatment consisted of five replicate pots, with each pot holding a single 3 weeks old plant or approximately 10 cm in height. Control groups were either sprayed with only the pathogen or with a saline solution lacking both pathogens and antagonists.

The pots were maintained in a shaded house for eight weeks, with infection levels evaluated at two and five weeks post-pathogen application. To avoid cross-contamination, control pots (A_0B_0) were positioned separately from those exposed to the pathogen. Infection severity on the leaves was assessed using a 0 - 5 scale, as described by Nakasaki *et al.* (1998), where 0 indicated no visible symptoms, 1 represented infection on ≤ 20% of the leaf area, 2 on 21 - 40%, 3 on 41 - 60%, 4 on 61 - 80%, and 5 on 81 - 100%.

Establishment of the Antagonists on the Zucchini Leaves

The experiment was completed six weeks after the pathogen inoculation, with efforts made to recover antagonists from randomly chosen healthy leaves. To assess the colonization of bacterial antagonists, 10 g of leaves from each pot were mixed with 90 mL of saline solution and homogenized for 3 - 5 minutes. Colony-forming units (cfu) were quantified using dilution plating on Trypticase Soya Agar (TSA), followed by incubation at 25 °C for 2 - 5 days. The identities of the bacteria were verified by comparing colony characteristics on TSA with those of the original strains

To evaluate the presence of *Trichoderma* sp. Td_{22}, 20 leaf plugs (~3x3 mm) per treatment were aseptically collected and placed on pectin agar medium (MERCK) containing 60 µg/mL tetracycline. These samples were incubated at 25 °C for 4 - 7 days to allow fungal growth. Emerging fungal colonies were isolated and grown on the same medium to compare their morphology with a *Trichoderma* sp. Td_{22} stock culture. Observations were extended for one week to monitor conidial development for accurate identification.

Data Analysis

The data obtained from this study were analyzed using analysis of variance (ANOVA), which was carried out with the help of Minitab software for Windows. ANOVA enabled the assessment of any significant variations between the treatment groups. To identify specific differences between group means, the least significant difference (LSD) test was employed following the ANOVA procedure. The LSD test is a post-hoc statistical test that compares means to detect significant differences. A significance threshold of $P < 0.05$ was set to determine whether the differences observed were statistically meaningful, ensuring the results were valid and reliable.

RESULTS AND DISCUSSION

The efficacy of the selected antagonistic fungus and bacteria in preventing zucchini leaves from downy mildew infection, as evaluated by the percentage of infected leaves and the disease severity index is presented in Figure 1. The use of antagonists, excluding treatment A_2B_1 (plants treated with *L. antibioticus* and the pathogen), significantly lowered disease incidence compared to the untreated-pathogen control (A_0B_1) two weeks post-infection ($P < 0.05$) (Fig. 1).

The most effective disease suppression was achieved with the fungal antagonist Trichoderma sp. Td_{22} (A_3B_1), providing an 83% reduction in disease (calculated from disease incidence) compared to the control group at two-weeks post-inoculation. However, this effect declined and became statistically insignificant after five weeks. A non-significant synergistic effect ($P > 0.05$) was noted when L. antibioticus and P. corrugata were applied in combination (treatment A_4B_1), resulting in 57% disease protection (calculated from disease incidence; Fig. 1A) relative to the untreated-pathogen control. This was higher (higher protection) than when each bacterial antagonist was applied individually (A_1B_1 or A_2B_1; Fig. 1). When the Trichoderma sp. Td_{22} was combined with the bacterial antagonists, its effectiveness diminished, possibly due to reduced levels of the primary biocidal compounds on leaf surfaces (Poromarto et al. 2021). Additionally, the lack of synergy between the fungal and bacterial antagonists could be from antagonistic interactions, as dual-culture tests showed inhibition zones produced by both bacteria against Trichoderma sp. Td_{22} (Fig. 2). In contrast to our findings, Poveda & Eugui (2022) suggested synergic effect when they were applied in combination in a sustainable agriculture system.

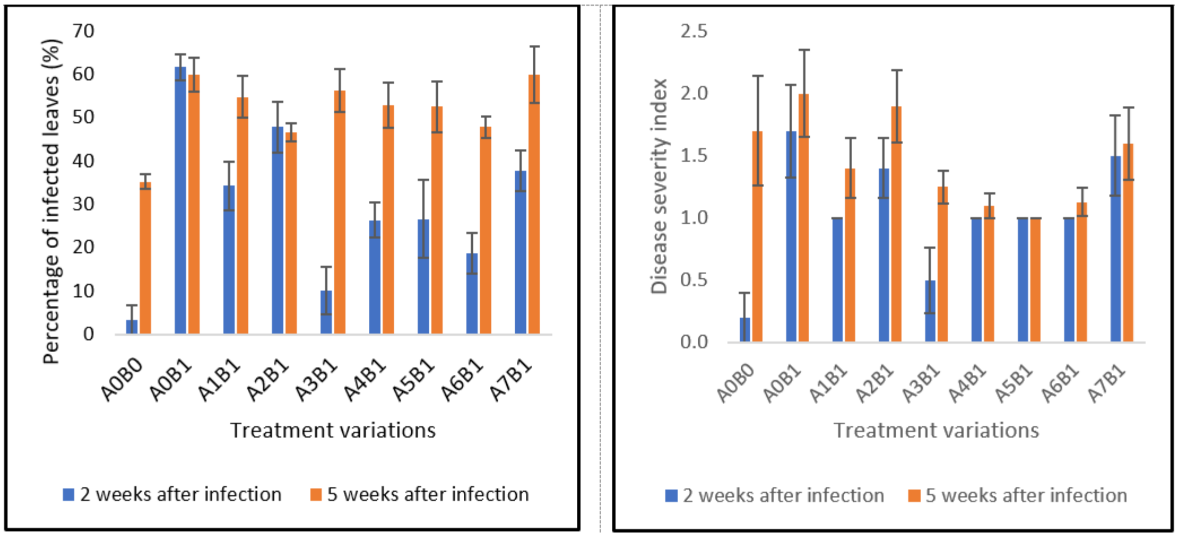

Figure 1 The effectiveness of *Lysobacter antibioticus* Bali G, *Pseudomonas corrugata* SAJ6, and *Trichoderma* sp. Td_{22} to prevent zucchini plants from foliar downy mildew infection in a glasshouse scale experiment

Notes: A = percentage of infected leaves; B = disease severity index; the assessments were conducted at 2 and 5 weeks after infection; treatments applied: A_0B_0 = control group (no antagonist or pathogen applied); A_0B_1 = control treatment (pathogen only applied); A_1B_1 = plants treated with *P. corrugata* and pathogen; A_2B_1 = plants treated with *L. antibioticus* and pathogen; A_3B_1 = plants treated with Td_{22} and pathogen; A_4B_1 = plants treated with a mixture of *L. antibioticus*, *P. corrugata*, and pathogen; A_5B_1 = plants treated with a mixture of *L. antibioticus*, Td_{22}, and pathogen; A_6B_1 = plants treated with a mixture of *P. corrugata*, Td_{22}, and pathogen; A_7B_1 = plants treated with a mixture of all three antagonists and pathogen; Each bar represents the mean of disease ± standard error.

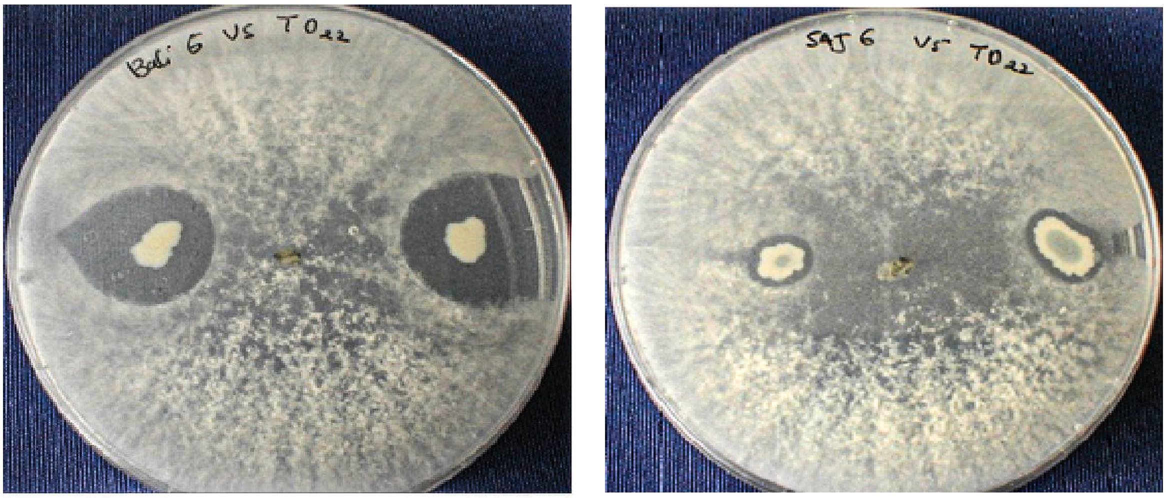

Figure 2 In vitro dual culture assays between *L. antibioticus* Bali G and *Trichoderma* sp. Td_{22} (left) and between *P. corrugata* SAJ 6 and *Trichoderma* sp. Td_{22} (right) on TSA plates after incubation at 25 °C for three days

An interesting phenomenon was observed in this glasshouse trial where some plants in the control group (no antagonist or pathogen applied or A_0B_0) showed disease symptom (Fig. 1). This could be due to cross contamination from the infected leaves nearby (plants in pots treated with pathogen). Water splash during irrigation or the blowing wind could be the main cause of this cross contamination.

The protection shown by the antagonists against downy mildew was no longer significant ($P > 0.05$) after five weeks. At that time, 60% of the leaves in the untreated control group were infected. The survival rate of the applied antagonists on the leaf surfaces was observed to be low. For instance, *Trichoderma* sp. Td_{22} was recovered from only two out of 20 leaf samples taken from treatment A_5B_1 (plants treated with *L. antibioticus*, *Trichoderma* sp. Td_{22}, and the pathogen) five weeks post-application, and it was undetectable in samples from other treatments. Likewise, the bacterial antagonists applied to the leaves were not found (exist) from any treatments after the five-week exposure.

Applying fungal and bacterial antagonists, either individually or in combination, showed potential for managing foliar diseases, such as downy mildew in zucchini plants. Significant reductions in disease incidence ($P < 0.05$) were observed two weeks after the pathogen was introduced (Fig. 1). These findings indicate the possibility of modifying compost to develop specialized "compost teas" or cultivating specific biocontrol agents to manage foliar pathogens.

The application of *Trichoderma* sp. Td_{22} yielded the most successful results in our study, particularly when the strain was cultivated in a mixture of wood fiber waste (WFW) compost and millet seed (80 : 20 w/w), as detailed in our previous research (Ramona *et al.* 2022). The biocontrol agent was stored for approximately 10 months at around 20 °C before being applied in the current study. In efforts to further improve the effectiveness of both fungal and bacterial antagonists, recent research by Brost (2020) suggested that the addition of chelating agents and detergents may enhance the activity of these biocontrol agents. By incorporating such additives, it may be possible to increase the stability, viability, and overall efficacy of microbial antagonists under various application conditions.

Previous studies have pointed out the compatibility challenges between bacterial and fungal antagonists, which can significantly influence the success of biocontrol strategies. El-Sharkawy *et al.* (2021) for example, reported that the combination of *T. harzianum* with *P. fluorescens* reduced the efficacy of the fungus in controlling *Aphanomyces euteiches*, a root rot pathogen in peas. This finding highlights the importance of understanding microbial interactions, as incompatibility can undermine the individual effectiveness of biocontrol agents. On the other hand, research by Amirthalingam *et al.* (2020) and Ntakirutimana *et al.* (2024) showed that using mixed cultures of antagonistic microorganisms often improved disease control. These contrasting outcomes emphasize the need for comprehensive evaluations of compatibility when developing microbial formulations for disease management.

The broad-spectrum potential of *Trichoderma* strains, such as Td_{22}, is particularly significant in this regard. Its ability to combat various fungal pathogens has been consistently demonstrated in several studies, establishing it as a promising biocontrol agent (Ali *et al.* 2021; Kumar *et al.* 2023). In our current study, *Trichoderma* sp. Td_{22} achieved an 83% reduction in zucchini downy mildew during a two-week glasshouse trial, offering a level of protection comparable to chemical fungicides. Such results validate its potential as a sustainable alternative to synthetic treatments. However, achieving similar effectiveness in field conditions remains challenging, as environmental factors often necessitate frequent reapplication to maintain the agent's activity and persistence.

Reapplication intervals for biocontrol agents align closely with those recommended for chemical fungicides. Jones *et al.* (2021) for example, suggested reapplying fungicides every 10 - 14 days to sustain protection against downy mildew. This similarity underscores the practical challenges of deploying biological control agents while highlighting their potential integration into established disease management practices. Optimizing formulations, improving the stability of biocontrol products, and addressing compatibility issues are essential for advancing the reliability and scalability of biological control solutions.

Mildew symptoms observed in the nil-pathogen control group (A_0B_0) after five weeks (Fig. 1) were likely caused by natural infection from spores originating in a nearby vineyard with known disease presence or accidental transfer through human activity. Despite this unintended exposure, the infection levels remained minimal compared to those in inoculated plants, ensuring that the overall conclusions of the study were unaffected. This minimal infection demonstrates the robustness of the experimental setup in isolating key variables under investigation.

The limited persistence of biocontrol agents on leaf surfaces was expected due to several environmental challenges. Factors such as low moisture levels, ultraviolet (UV) radiation from sunlight, and the washing effect of overhead irrigation contributed to the reduced survival of these agents (Devi 2024). These environmental conditions are well-known to limit the effectiveness of biocontrol organisms in outdoor applications. A thorough review by Fedele *et al.* (2020) discussed the impacts of environmental stressors, including humidity, temperature variations, and irrigation practices, on the survival and performance of biocontrol agents in field environments. These findings emphasize the need for improved application techniques and protective formulations to enhance the stability and efficacy of biocontrol agents under field conditions.

CONCLUSION

The biological control agents evaluated in this study, with the exception of *L. antibioticus* Bali G, demonstrated significant effectiveness ($P < 0.05$) in protecting zucchini leaves from downy mildew during the first two weeks of the glasshouse trial. These findings highlighted their potential for managing foliar diseases. It is necessary to maintain protection beyond 14 days by reapplying the biocontrol agents.

The fungal antagonist *Trichoderma* sp. Td_{22} was found to be incompatible with both *L. antibioticus* Bali G and *P. corrugata* SAJ6. In contrast, the combination of *L. antibioticus* Bali G and *P. corrugata* SAJ6 appeared to be compatible but did not significantly enhanced disease control when compared to *P. corrugata* SAJ6 alone. The survival of all three antagonists on zucchini leaf surfaces was notably very low.

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