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RESEARCH ARTICLE
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Biological Control of Wilt Disease Caused by *F. oxysporum* Schlecht. in Bok Choy (*Brassica campestris* var. *chinensis* L.) using a Combination of Antagonistic Agents

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ABSTRACT

Fusarium wilt is a significant disease caused by Fusarium oxysporum Schlecht, leading to severe crop damage in bok choy (*Brassica campestris var. chinensis* L.). Biological control, which uses biotic entities to reduce pathogen inoculum density, has emerged as a potentially effective method for managing plant diseases. This study aims to evaluate the effectiveness of combinations of antagonistic agents in controlling *Fusarium* wilt in bok choy. The fungal inoculum of *F. oxysporum* was sourced from bok choy, which showed typical symptoms of *Fusarium* wilt. The isolated pathogen was characterized based on its morphological and physiological properties, purified, and stored on PDA media for further testing. The antagonistic isolates were preserved on PDA slants for Trichoderma *harzianum* TR-01 and Gliocladium virens GR-01 and King's B medium for Pseudomonas fluorescence PR-01. The in vitro antagonistic assay was conducted using the dual culture method, while the antagonistic agent's field tests were applied one week before planting, alongside the manure application. The combination of antagonistic agents in the H treatment significantly reduced the incidence of damping-off both before (7.00%) and after appearing at the ground level (7.16%) compared to the control treatment (A) ($P<0.01$). This treatment also extended the disease incubation period to 5 days, showing an increase of 32.86% compared to controls, who showed only 3 days of incubation ($P<0.01$). The pathogen population in treatment H was recorded to be 39.25% lower than in treatment A. In addition, there was an increase in the number of healthy plants by 14%, from 80 plants (A) to 94 plants (H) ($P<0.01$). In vitro dual culture assays revealed inhibition rates of 45.56% for *T. harzianum*, 41.11% for *G. virens*, and 32.22% for *P. fluorescens* ($P<0.05$). Field trials showed that the combined application of these agents significantly reduced disease incidence and improved plant health and yield. The antagonist potential assay of three antagonistic isolates against *Fusarium oxysporum* demonstrated that the combined application of *Gliocladium virens* GR-01, *Trichoderma harzianum* TR-01, and *Pseudomonas fluorescens* PR-01 was most effective in suppressing the pathogen. This integrated biocontrol treatment significantly reduced pre- and post-emergence damping-off, lowered pathogen populations, increased the number of healthy plants, and enhanced plant growth and yield. Overall, this approach offers a sustainable and effective alternative to conventional synthetic fungicides for managing *F. oxysporum*–induced diseases.

Keywords: *Fusarium oxysporum; Trichoderma harzianum*; *Gliocladium virens*; *Pseudomonas; Fluorescence*; bok choy; Prevention strategies, Biological control agents

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INTRODUCTION

Bok choy (*Brassica campestris var. chinensis*) is a leafy vegetable of the *Brassicaceae* family, also known as flowering Chinese cabbage. It has a higher economic value compared to related crops such as cabbage, cauliflower, and broccoli (Guangguang et al., 2023; Tanni et al., 2023). It is widely cultivated throughout Asia, including Indonesia, due to its rich nutritional value and high market demand (Lehar et al., 2024). As public awareness of nutrition grows, consumers are increasingly aware of the importance of including protein-rich vegetables like bok choy in their diets (Wu et al., 2022). Despite its popularity, bok choy production faces serious challenges due to soil-borne diseases, especially *Fusarium* wilt caused by Schlecht's *Fusarium* oxysporum. This pathogen is known for its ability to invade plant vascular tissues, causing yellowing, wilting, and eventually the death of the host. *F.oxysporum* can survive in soil and plant debris for many years through the formation of resistant chlamydospores, making chemical control measures less effective and harmful to the environment (Guo et al., 2023; Elango et al., 2024).

As an alternative, biological control with a sustainable approach to managing soil-borne pathogens is to use antagonistic agents, namely microorganisms that can inhibit the growth or activity of pathogens (Arif et al., 2023; Elango et al., 2024). Several microbial agents have been identified for their antagonistic activity against *F.oxysporum*. *Trichoderma harzianum* is known for its ability to parasitize pathogenic fungi through the production of lytic enzymes such as chitinase and β-glukanase (Chan et al., 2023; Ismaiel et al., 2024). Meanwhile, *Pseudomonas fluorescens* improves plant health and inhibits pathogens through siderophore production, nutrient competition, and the secretion of antimicrobial compounds such as 2,4-diacetylphloroglucinol (Gade and Koche, 2022; Sagala et al., 2023).

The effectiveness of biological control against *Fusarium* wilt disease in bok choy plants is achieved by combining several antagonistic agents, which is a more effective strategy than using one type of agent alone. Several reports of the results of the study stated that *Trichoderma harzianum (T. harzianum)* is an effective biological control agent against *Fusarium* infection in tomato plants, especially by improving the plant's natural defense mechanisms (Rashid et al., 2021). It was further stated by Pattikawa et al., (2020) that *Trichoderma harzianum (T. harzianum)* isolate has antagonistic properties to *Fusarium oxysporum f. sp. Cubense*, the fungus responsible for wilting disease in the kapok banana plant. The use of antagonistic fungi as biological control agents presents a sustainable approach to managing *Fusarium oxysporum f. sp. ciceris*, combining effective pathogen inhibition with environmental safety. Among the fungi tested, *G. virens* showed the strongest antagonist, while *Paecilomyces lilacinus* was the least effective.

Combining these agents can improve the efficacy of biocontrol through synergistic mechanisms. A multistrain biocontrol approach can expand the range of pathogen suppression and minimize the risk of resistance development (Price-Christenson and Yannarel, 2023). In an effort to improve the effectiveness of biological control against *Fusarium* wilt disease in bok choy plants, combining multiple antagonistic agents may be a more effective strategy than using just one type of agent. Therefore, combining these agents is expected to produce synergistic effects that improve control mechanisms and provide more effective protection for plants. This study aims to evaluate the effectiveness of the combined application of *T. harzianum, G. virens*, and *P. fluorescens* in managing *Fusarium* wilt in bok choy both in vitro and field conditions.

MATERIALS & METHODS

Research Sites

The research was conducted in the experimental garden and laboratory of Plant Pests and Diseases of the UPT-Protection of Food Crops and Horticulture, Riau Province. The research took place from October 2023 to January 2024. The field experiment was conducted on ultisol soil with a silty clay loam texture, which is commonly found in the study area. Initial soil analysis revealed moderately acidic conditions with a pH ranging from 5.4 to 5.8. The soil was classified as moderately fertile, containing 1.8–2.2% organic matter, 0.12–0.18% total nitrogen, 15–20ppm available phosphorus, and 80–110ppm exchangeable potassium. These values indicate sufficient baseline fertility to support crop growth while allowing for measurable impacts of treatment. During the experimental period (October 2023 to January 2024), environmental conditions were typical of the region, with average daily temperatures ranging from 26–30°C, relative humidity between 75–85%, and light to moderate rainfall. Such conditions are favorable for both pathogen development and biological control activity, making them suitable for field evaluation of antagonistic agents.

Sources of Pathogen and Antagonist Isolates

The pathogen *F.oxysporum* was isolated from bok choy plants exhibiting characteristic wilt symptoms. Infected samples were aseptically collected and processed using the moist chamber method. Following standard surface sterilization protocols (Guo et al., 2023), plant tissues (1×1cm) were surface-sterilized in 10% sodium hypochlorite solution for 2minutes, rinsed twice with sterile distilled water, and placed in Petri dishes containing moistened Whatman filter paper. The dishes were incubated at room temperature until fungal growth appeared.

The growing fungi were taken with an inoculating loop to be planted on Potato Dextrose Agar (PDA) media containing 50ppm streptomycin antibiotics. The fungi were incubated at room temperature in an incubator for 2-3days. After that, the isolates obtained were characterized based on their morphological and physiological properties, as described by (Manoj et al., 2016; Summerell et al., 2003). The fungi that grew and showed the characteristics of *F.oxysporum* were purified and stored in PDA media for use in subsequent tests. Morphological observation of *F.oxysporum* fungus was conducted using a multimedia microscope. Isolates that grew and were pink under 40-watt neon light (black light) were selected as the wilt type of *F. oxysporum*.

The antagonist isolates *P. fluorescence* PR-01, *T. harzianum* TR-01 and *G. virens* GR-01 are collections from the Plant Pest and Disease Laboratory (LHPT), UPT-Protection of Food Crops and Horticulture Riau. These isolates were stored in a refrigerator on PDA slant agar media for *T. harzianum* TR-01 and *G. virens* GR-01 while *P. fluorescence* PR-01 on King'B media. These isolates had been previously preserved and routinely utilized for biological control research. Prior to application, the isolates were reactivated and propagated under sterile conditions. At the source laboratory, identification of the antagonists was performed based solely on morphological and microscopic characteristics; no molecular identification was conducted. Therefore, to confirm the identity of the fungal isolates (*T. harzianum* TR-01 and *G. virens* GR-01), molecular identification was carried out prior to their use in this study. For field application, all biocontrol agents were prepared at a concentration of 1×10^8 CFU/mL for *P. fluorescens* PR-01 and 1×10^8spores/mL for both *T. harzianum* TR-01 and *G. virens* GR-01. Each microbial suspension was applied in 200mL volumes per 2×2m plot, mixed with manure and administered one week before transplanting.

Molecular Identification

DNA Extraction

Genomic DNA from *F. oxysporum*, *T. harzianum* TR-01 and *G. virens* GR-01 was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005), following the manufacturer's standard protocol. Approximately 50mg of fungal biomass was lysed using Bashing Bead™ buffer and bead-beating to break down the fungal cell walls. After centrifugation, the resulting supernatant was mixed with a DNA binding buffer and transferred to a Zymo-Spin™ II column to facilitate DNA binding (Lee et al., 2022). To remove contaminants, the DNA-bound column was washed twice with DNA wash buffer. Finally, the DNA was eluted with DNA elution buffer and centrifuged to obtain purified DNA. The extracted DNA was stored at –20°C for further use in PCR analysis or sequencing (Mijnendonckx et al., 2024).

DNA Amplification and Electrophoresis

The fungal isolates were identified by amplifying the internal transcribed spacer (ITS) region using PCR with 2X MyTaq HS Red Mix (Bioline, BIO-25048). The universal primers ITS-1 (5′–TCCGTAGGTGAACCTGCGG–3′) and ITS-4 (5′– CTCCGCTTATTGATATGC–3′) were used for the amplification of *F.oxysporum, Trichoderma* spp, and *Gliocladium* sp. (Shamkh et al., 2021; Sukapiring et al., 2024). Each 25µL PCR reaction contained 12.5µL of 2X MyTaq HS Red Mix, 1µL of forward primer, 1µL of reverse primer, 1µL of DNA template, and 9.5µL of nuclease-free water. The PCR conditions were as follows: initial denaturation at 95°C for 1 minute (1cycle), followed by 35cycles of denaturation at 95°C for 10seconds, and a final extension at 72°C for 5minutes. PCR products were separated by electrophoresis on a 0.8% agarose gel in 1× TBE buffer at 75volts for approximately 90minutes. The gel was stained with GelRed and visualized using a UV transilluminator. Digital images were captured with a camera. The presence and size of amplified DNA bands were confirmed by comparison with a DNA ladder marker (Priyashantha and Umashankar, 2021).

In vitro Antagonistic Activity

The antagonistic potential of microbial isolates against *F.oxysporum* was evaluated using the dual culture technique. A 0.5×0.5cm fungal plug of each antagonist was placed on PDA (or King's B for *P. fluorescens*) in a 9cm Petri dish, positioned 3cm from a similarly sized plug of *F. oxysporum*. Control dishes contained only the pathogen. All treatments were replicated three times and incubated at 28 ± 2°C for 7days. The percentage inhibition of pathogen growth was calculated using the following formula (Vinayarani and Prakash, 2018; Mirsam et al., 2021):

$$I = \frac{r1-r2}{r1} \times 100\%$$

Where:
I = inhibition percentage
r1 = the radius of pathogen colonies towards the Petri dish
r2 = the radius of colonies towards the antagonist.

Germination Test

Testing the germination power of bok choy seeds was carried out using the blotter test method according to the standard method determined by the International Rules for Seed Testing (ISTA, 2024). This test was carried out in 4 Petri dishes filled with moist filter paper, each containing 25 again seeds. Observations were made every day until the 15th day, and the seeds that germinated usually were counted. To determine the germination power of seeds, it was calculated using the formula:

$$D = \frac{b}{B} \times 100\ \%$$

Where:
D = Percentage of germination power
b = Number of seeds that germinate normally
B = Number of seeds that germinate.

Field Evaluation of Antagonistic Agents

Field trials were conducted under standard agronomic practices, including land preparation, fertilization, antagonist application, planting and maintenance (irrigation, weeding and plant protection). Field trials were conducted in plots measuring 2x2m, with nine treatments and four replications. The treatments were defined as follows: A = *F.oxysporum* (Fo), B = Fo + *T. harzianum* TR-01 (Th), C = Fo + *G. virens* GR-01 (Gl), D = Fo + *P. fluorescens* PR-01 (Pf), E = Fo + Th + Gl, F = Fo + Th + Pf, G = Fo + Gl + Pf, H = Fo + Gl + Th + Pf, and I = synthetic fungicide (Fi). The antagonistic microbial agents were applied in combination with manure one week prior to transplanting.

Observation parameters are 1) Antagonistic potential (disease incidence); 2) Percentage of healthy (surviving) plants; 3) Plant height (cm); 4) Fresh weight per treatment (kg); 5) pre-emergence dumping off (%); 6) post-emergence dumping off (%); 7) Incubation period) (days); incubation period was observed from planting until the first symptoms appeared in days after inoculation (dsi); 8) Pathogen population (cfu/g$\times10^{-1}$).

The incidence of pre-and post-emergence damping-off and survived plants were calculated according to (Mahartha and Suprapta, 2018; Ketta et al., 2021) as the following formula:

$$S = (\frac{A+B}{B}\ x\ 100\%) - (\ 100\% - \mathrm{D})$$

Where:
S = Incidence of pre-emergence damping-off (%)
A = number of planted seed
B = number of emerged seedlings
D = germination rate (%).

$$K = (\frac{n}{N}\ x\ 100\%)$$

Where:
K = incidence of post-emergence damping-off (%)
n = number of infected seedlings
N = number of emerged seedlings.

Pathogen population was quantified as colony-forming units per gram of soil (cfu/g), and results were expressed in units of cfu/g×10^{-1}. The final number of conidia of *F .oxysporum* was calculated by shaking a mixture of 10g of rhizosphere soil with 90ml of water in an Erlenmeyer flask; the mixture was made into a series of dilutions and then counted with a hemocytometer under a microscope with low magnification (10x).

Pre-emergence damping-off (%):

$$S = \frac{(A-B)}{A}\ x\ 100$$

Where A is the number of seeds planted and B is the number of seedlings emerged

This equation is mathematically equivalent to S = 100% − D, where D = (B/A)×100. We chose this form to present the calculation directly from primary observations (number of seeds planted and germinated), thereby avoiding the indirect step of calculating D first.

Post-emergence damping-off (%):

$$K = \frac{\mathrm{n}}{N}\ x\ 100$$

where n is the number of infected seedlings and N is the number of seedlings emerged.

Data Analysis

The test of antagonist isolates on bok choy growth was arranged a completely randomized (CRD) design with nine treatments and four replications. The observed data were analysed statistically with RStudio software followed by Duncan's New Multiple Range Test (DNMRT)) at the 5% level (P<0.05).

RESULTS

Morphological Characteristics of *F.oxysporum*

The isolated *F. oxysporum* showed distinctive macroscopic and microscopic features. Colonies appeared white to cream in color, dense, with cotton-like mycelial growth that radiated in all directions. Microscopically, macroconidia were crescent-shaped and thin-walled with 2–4septa, while microconidia were oval to kidney-shaped and single-celled. Chlamydospores were present, supporting the identification of the isolate as *F. oxysporum* (Table 1 and Fig. 1).

Table 1: Result of macroscopic and microscopic observations of the fungus *F. oxysporum*

Observation	Observation Result
Macroscopic	
Colony colour	White to cream
Colony shape	Round, smooth surface, dense and thick hyphae so that it looks like cotton
Colony growth direction	Spreads in all directions
Microscopic	
Shape and Colour	
Macroconidia	Crescent-shaped, hyaline
Microconidia	Oval or elliptical, hyaline
Number of Cells	
Macroconidia	2-4cells
Microconidia	One cell
Chlamydospores	Present

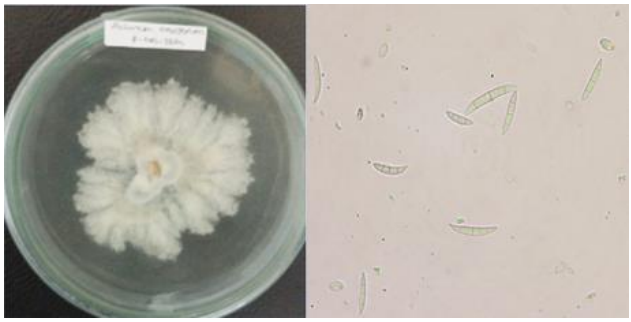

Fig. 1: *F.oxysporum* fungus on 7-day-old PDA media and microscopic observation of fungal conidia.

Molecular Identification

The molecular identification of *F. oxysporum* was conducted using PCR amplification targeting the ribosomal DNA (rDNA) region. The amplification product was visualized through gel electrophoresis and is presented in Fig. 2. A clear and single band was observed in lane 1, corresponding to the *F. oxysporum* isolate, with an estimated amplicon size of approximately 600 base pairs. This band size aligns with the expected fragment length of the ITS region commonly used for fungal identification. No amplification was observed in the No Template Control (NTC), confirming the absence of contamination and the specificity of the reaction. These results support the successful molecular identification of the isolate as *F. oxysporum* and validate the morphological observations. The confirmed identity of the pathogen was used in subsequent antagonistic testing.

Fig. 3 showed the agarose gel electrophoresis of PCR products amplified using ITS1 and ITS4 primers for fungal identification. Lane M (Marker): DNA ladder ranging from 100bp to 3,000bp. NTC (No Template Control): No DNA band observed, indicating the absence of contamination in the PCR reaction. Lane 1 (*T. harzianum* TR-01): A single, sharp DNA band approximately 500bp in size. Lane 2 (*G. virens* GR-01): A single, clear DNA band also around 500bp. Both samples produced a single amplicon of the expected size, indicating successful and specific amplification of the ITS region.

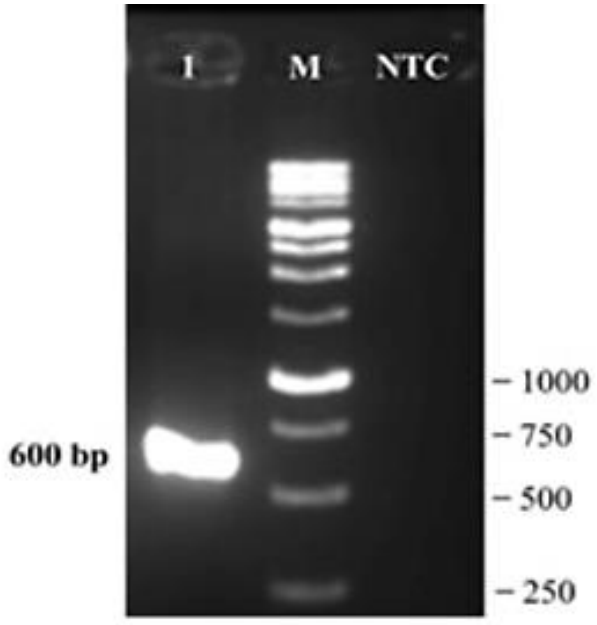

Fig. 2: Agarose gel electrophoresis of ITS PCR products from *Fusarium oxysporum* isolate.Lane M: 100bp DNA ladder; Lane 1: *F. oxysporum* PCR product (~600bp); NTC: No Template Control.

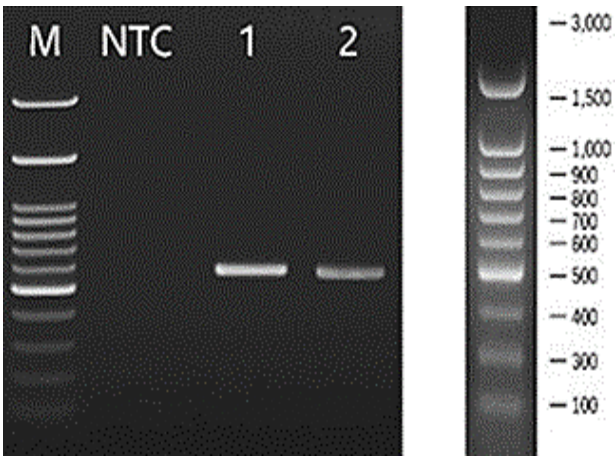

Fig. 3: Agarose gel electrophoresis of ITS PCR products from biocontrol fungal isolates. Lane M: 100bp DNA ladder; Lane 1: *Trichoderma harzianum* TR01 (~500bp); Lane 2: *Gliocladium virens* GR-01 (~500 bp); NTC: No Template Control.

In vitro Antagonistic Activity

The results of the antagonistic potential test of three antagonistic species isolates against the fungus *F. oxysporum* showed that all three isolates could suppress the development of *F. oxysporum* (Fig. 4A). The highest inhibition rate was observed with *T. harzianum* TR-01 (45.56%), followed by *G. virens* GR-01 (41.11%) and *P. fluorescens* PR-01 (32.22%). From the appearance of all treatments on PDA and King's B media, it can be seen that *T. harzianum* TR01 was a mycoparasite against *F. oxysforum* pathogens. At the same time, *G.virens* GR-01 and *P. fluorescens* PR-01 isolates were space competition and nutrition against *F. oxysforum* pathogens (Fig. 4B).

Germination Test

The blotter method revealed that bok choy seeds had a germination rate of 85%, indicating good viability (Fig. 5).

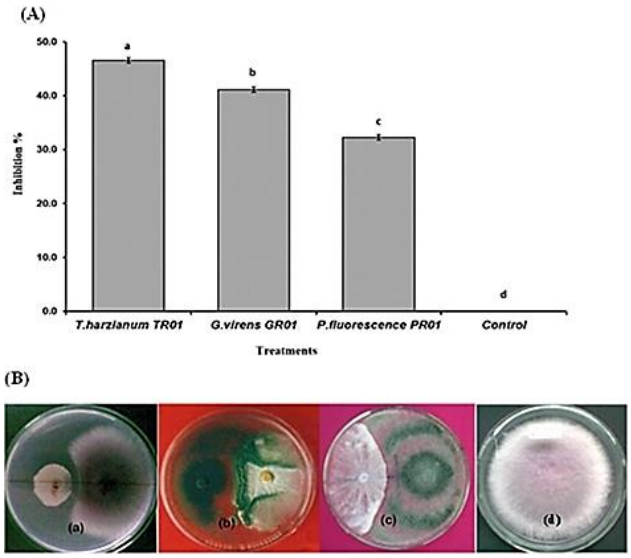

Fig. 4: (A) Percentage of inhibition of the growth of the pathogen *F.oxysforum* against all three isolates of microbial antagonist. (B) Appearance of antagonistic activity of isolates microbial antagonists against pathogens *F.oxysforum.* (a). *Pf + F. oxysforum,* (b). *Tricho + F. oxysforum,* (c). *Glio + F. oxysforum*.(d). *F. oxysforum x* (control).

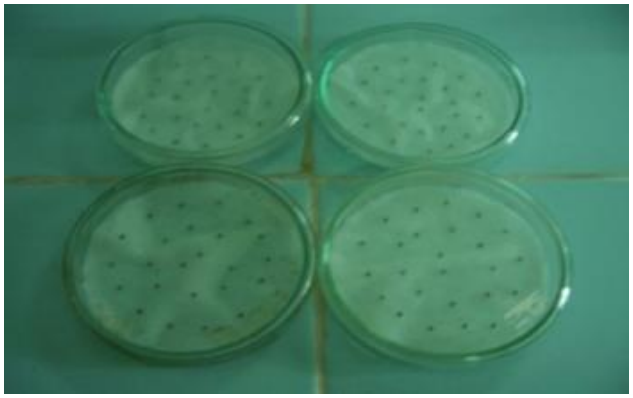

Fig. 5: Testing the germination power of bok choy seeds using the Blotter method in a Petri dish.

Field Testing of Antagonistic Agents

The results in Table 2 show that pre-emergence damping-off, which is the number of plants that die before emerging above the soil surface, had the lowest percentage in treatment H (*F. oxysporum* + *G. virens* + *T. harzianum* + *P. fluorescens*), at 4.75%, followed by treatment F (*F.oxysporum* + *T. harzianum* + *P. fluorescens* Pf) at 5.25%. These results indicate that combining various antagonistic agents (*T. harzianum*, *G. virens*, and *P. fluorescens*) effectively reduces pre-emergence damping-off. For post-emergence damping-off, the number of plants that die after emerging above the soil surface, treatment H again showed the best results with a percentage of 1.31% (Table 2).

The incubation period refers to the time between inoculation and the appearance of disease symptoms. Treatment H also exhibited a more extended incubation period (5.20days), indicating that the combination of antagonistic agents can slow pathogen development. The combination of antagonistic agents has shown significant potential in delaying pathogen progression through various mechanisms (Table 2). The highest pathogen population was observed in treatment A (Fo) with 83.42cfu/g, while the

Table 2: In vivo effect of combinations between several antagonist agents against *F. oxysporum*

Treatment	Mean Pre-emergence damping-off (%)	Mean Post-emergence damping-off (%)	Mean Incubation period (days)	Mean Pathogen population (cfu/g × 10^{-1})	Mean Number of healthy plants (stems)
A	11.75+0.65a	8.50+0.72a	3.50+0.25c	83.42+2.14a	80.00+1.83g
B	6.75+0.50de	2.41+0.33de	5.25+0.21a	52.58+1.37d	91.00+1.41cd
C	7.25+0.48cde	2.43+0.30de	5.00+0.19ab	52.84+1.29d	90.50+1.25d
D	8.00+0.55bc	3.81+0.45cd	4.25+0.22bc	61.58+1.50c	88.50+1.36e
E	5.50+0.40ef	2.65+0.36de	5.25+0.20a	51.42+1.18de	92.00+1.15bc
F	5.25+0.42f	1.85+0.29def	5.00+0.24ab	52.00+1.12de	93.00+1.22abc
G	6.50+0.37de	1.61+0.27ef	5.25+0.18a	52.09+1.10de	92.00+1.08bc
H	4.75+0.35f	1.31+0.22f	5.20+0.17a	50.67+1.05e	94.00+1.10abc
I	8.50+0.53bc	4.92+0.41b	4.25+0.23bc	69.33+1.92b	87.00+1.27f

Values (mean+SD) followed by the same letter in the same column (a,b,c,d,e,f,g) are not significantly different according to DMRT at a 5% error level. The data for plant height and total harvest weight are the retransformed results of log (×+1), √ (×+0.5), and √ (×+0.5), respectively. A = Fo; B = Fo + Th; C = Fo + Gl; D = Fo + Pf; E = Fo + Th + Gl; F = Fo + Th + Pf; G = Fo + Gl + Pf; H = Fo + Gl + Th + Pf; I = Fi. Fo = *F. oxysporum*, Th = *T. harzianum* TR-01, Gl = *G. virens* GR-01, Pf = *P. fluorescens* PR-01, Fi = synthetic fungicide.

Table 3: In vivo effect of combinations between several antagonist agents on yield parameters

Treatment	Plant Height (cm)	Total Harvest Weight (kg)
A	34.75+0.17d	3.85+0.10d
B	44.60+0.13a	5.30+0.08ab
C	44.35+0.13a	5.08+0.05b
D	42.43+0.25b	4.45+0.13cd
E	44.60+0.22a	5.06+0.06b
F	44.45+0.13a	5.25+0.06ab
G	44.78+0.13a	5.30+0.00ab
H	44.48+0.17a	5.68+0.08a
I	36.68+0.13c	4.08+0.10cd

Values (mean+SD) followed by the same letter in the same column (a,b,c,d) are not significantly different according to DMRT at a 5% error level. The data for plant height and total harvest weight are the retransformed results of log (×+1), √ (×+0.5), and √ (×+0.5), respectively. Treatments: A = *F. oxysporum* (Fo); B = Fo + *T. harzianum* TR-01 (Th); C = Fo + *G. virens* GR-01 (Gl); D = Fo + *P. fluorescens* PR-01 (Pf); E = Fo + Th + Gl; F = Fo + Th + Pf; G = Fo + Gl + Pf; H = Fo + Gl + Th + Pf; I = synthetic fungicide (Fi). Fo = *F. oxysporum*, Th = *T. harzianum* TR-01, Gl = *G. virens* GR-01, Pf = *P. fluorescens* PR-01, Fi = synthetic fungicide.

lowest was found in treatment H with 50.67cfu/g (Table 2). The data in Table 3 show that the tallest plants were observed in treatments B, E, F, and G, each with a height of approximately 44.60cm. The highest total harvest weight was observed in treatment H at 5.68kg, followed by treatments B and G, each with around 5.30kg (Table 3).

DISCUSSION

In this study, the application of three biocontrol agents *Trichoderma harzianum* TR-01, *Gliocladium virens* GR-01, and *Pseudomonas fluorescens* PR-01 demonstrated significant effectiveness in suppressing *Fusarium oxysporum* and enhancing bokchoy plant growth. While each agent showed individual efficacy, the combined treatment resulted in the highest disease suppression and plant performance. This synergistic effect likely arises from the complementary mechanisms of action among the antagonists. The combination of *T. harzianum*, *G. virens*, and *P. fluorescens* demonstrated superior disease suppression compared to single-agent treatments. This synergistic effect is likely attributed to the complementary mechanisms of action. While *T. harzianum* and *G. virens* employ mycoparasitism and secretion of cell wall-degrading enzymes (Ghasemi et al., 2020), *P. fluorescens* produces siderophores and diverse antimicrobial metabolites (AMs) that suppress a wide range of pathogens in the rhizosphere. Yang et al. (2025) emphasized the potential of these AMs as biopesticides that not only inhibit pathogenic microbes but also reduce reliance on chemical pesticides. When combined with fungal antagonists, *P. fluorescens* contributes to multi-targeted pathogen suppression and supports sustainable plant disease management.

Similar findings have been reported in tomato and chili, where the co-application of fungal and bacterial biocontrol agents significantly reduced *Fusarium* wilt incidence and enhanced plant growth (Abdelaziz et al., 2022; Chohan et al., 2024). *Trichoderma harzianum*, in particular, has shown superior performance in promoting root and shoot development while effectively suppressing multiple pathogens, including *Fusarium* spp., under greenhouse conditions, even outperforming chemical fungicides (Rashid, 2025; Suwor et al., 2025). In chickpea, *T. asperellum* and two strains of *T. harzianum* reduced *F. oxysporum* growth by up to 42% in vitro and significantly alleviated disease impact in field trials, resulting in improved morphological traits such as shoot length and biomass (Chohan et al., 2024). Collectively, these studies reinforce the potential of integrated microbial consortia as a sustainable strategy for managing *Fusarium* wilt in horticultural crops.

Izquierdo-García and Moreno-Velandia (2024) demonstrated that a microbial consortium of *Trichoderma virens* and *Bacillus velezensis* effectively controlled *Fusarium* wilt in cape gooseberry through complementary mechanisms such as enzyme production, gliotoxin synthesis, and plant tissue colonization. These findings highlight the importance of combining functionally diverse microbes for enhanced biocontrol efficacy. Building on this, our results show that the addition of *G. virens* to a consortium with *T. harzianum* and *P. fluorescens* further improves pathogen suppression, supporting the robustness of a three-agent strategy that can be extended to cruciferous crops like bok choy, thus broadening its applicability in sustainable horticulture. The results of the isolation of *F. oxysporum* showed that it exhibits unique characteristics that highlight its pathogenicity and cultural properties. These traits vary significantly among specific forms, reflecting complex interactions with host plants and environmental factors. Microscopic analysis of *F. oxysporum* revealed distinct morphological features, such as hyphal structures and conidia, which are critical for identification (Li et al., 2015).

The gel electrophoresis results in Fig. 2 showed a clear and distinct DNA band in lane 1, corresponding to the amplified product of *F.oxysporum*. The PCR amplicon in lane

1 is estimated to be approximately 600 base pairs, consistent with the expected size for the *F.oxysporum* ribosomal DNA region when amplified using ITS (Internal Transcribed Spacer) primers such as ITS1/ITS4. This result confirms the successful amplification of the *F.oxysporum* genomic DNA. Molecular identification of *F.oxysporum* using PCR targeting the rDNA-ITS region is a widely accepted method due to its high specificity and sensitivity. According to Guo et al. (2023), amplification of ITS regions allows for reliable differentiation of *Fusarium* species and serves as a primary tool in fungal diagnostics. Moreover, Elango et al. (2024) emphasize that the use of molecular markers, especially in combination with morphological traits, significantly enhances the accuracy of pathogen identification in biological control studies. Additionally, the successful amplification in this study supports the integrity of the DNA extraction protocol and confirms the presence of *F. oxysporum* in the sample used for further antagonistic assays. These results validate the morphological identification performed previously and ensure that subsequent bioassays are conducted with a confirmed pathogen isolate.

The ITS region is one of the most widely used DNA barcodes for fungal identification due to its high variability among species and conserved primer binding sites (Badotti et al., 2017). The PCR amplification of the ITS region using primers ITS1 and ITS4 resulted in clear, single bands around 500 bp for both *G. virens* and *T. harzianum*. These sizes are consistent with previous reports for these genera (Grubišić et al., 2021). The absence of a band in the NTC confirms that there was no contamination in the PCR reaction, ensuring the reliability of the molecular results (Lee et al., 2012). The use of 0.8% agarose gel provided optimal resolution for DNA fragments in the 400–1000bp range. GelRed dye was used for DNA staining due to its high sensitivity and safety compared to traditional ethidium bromide (Huang et al., 2010). The sharp and specific bands observed indicate high-quality DNA and efficient amplification without non-specific products or smearing.

Testing the antagonistic potential of three isolates against the *F .oxysporum* fungus indicated that all isolates were effective in inhibiting the growth of *F. oxysporum*. *T.harzianum* has a strong inhibitory effect on the growth of *F. oxysporum* mycelium due to its ability to compete for space and nutrients. The structure of *T. harzianum*, especially its hyphae and conidia, contributes to its ability to suppress the growth of pathogenic fungi through mechanisms such as mycoparasitism and the production of volatile compounds (Chan et al., 2023). *T. harzianum* can effectively suppress the growth of *F. oxysporum*, significantly reducing mycelial growth and disease severity in tomato plants infected by this pathogen (Kumar et al., 2019; Gade and Koche, 2022). Dual culture experiments revealed that *T.harzianum* inhibited the mycelial growth of *Fusarium* and improved host plant growth parameters, demonstrating its role in enhancing plant health while competing for resources (Sorahinobar et al., 2024). Furthermore, the competitive advantage of *T. harzianum* is reinforced by its ability to induce systemic resistance in plants, boosting their defence mechanisms against *Fusarium* infections (Ismaiel et al., 2024; Pudake et al., 2024). Overall, *T.harzianum* is a potent biocontrol agent, effectively competing with *Fusarium* for critical resources, thus reducing its detrimental effects on plant health. Besides *T.harzianum*, *G.virens*, and *P.fluorescens* also exhibit significant inhibitory effects on *Fusarium* mycelium, primarily through mechanisms of competition for space and nutrients.

P.fluorescens has been shown to suppress the growth of *F.oxysporum* effectively, achieving inhibition rates as high as 83.33% *in vitro*, demonstrating its potential as a biocontrol agent against root pathogens (Al-Aamel and Al-Maliky, 2023). Similarly, *Gliocladium* sp. displays antagonistic activity against various *Fusarium* species, utilizing mechanisms that include competition, lysis, and mycoparasitism, leading to notable growth. The effectiveness of these microorganisms underscores their role in biocontrol strategies, particularly in agricultural systems where *Fusarium* species pose a significant threat to plant health. Overall, the competitive nature of these fungi and bacteria in nutrient acquisition and space occupation supports their ability to effectively inhibit *Fusarium* growth (Podgorska-Kryszczuk et al., 2022).

The results of the bok choy seed germination test indicated a germination rate of 85%. The germination rate of bok choy seeds at 85% indicates a healthy seed viability, which is crucial for successful cultivation. This rate aligns with findings from studies on seed priming techniques that enhance germination performance. The germination percentage of bok choy seeds can be influenced by various priming methods. For instance, hydropriming and bio-nutri-priming have been shown to improve germination rates, with one study reporting an average germination rate of 88.35% under optimal conditions. Priming techniques not only enhance germination rates but also reduce the time to peak germination, indicating improved seed readiness for growth (Phooi et al., 2023).

Our results demonstrated that combining multiple antagonistic agents *P. fluorescens* PR-01, *T. harzianum* TR-01, and *G. virens* GR-01) effectively reduced damping-off on the bok choy plant. This synergistic approach enhances disease resistance and promotes plant growth. Combining *P.fluorescens* with other biocontrol agents like *T. harzianum* further increases its effectiveness, reducing damping-off incidence to as low as 38.33% (Rajendraprasad et al., 2017). *T.harzianum*, combined with other agents, has improved seed germination and reduced disease incidence, thus enhancing overall plant health (Mannai et al., 2020). These agents effectively increase plant height and leaf production in *Amaranthus hybridus*, highlighting their potential in controlling damping-off (Muhammed et al., 2022). In chilli seedlings, these agents reduce post-emergence damping-off by up to 40% (Mannai et al., 2020). The isolates exhibit antagonistic solid activity against *Rhizoctonia solani*, achieving inhibition zones of up to 14.30 mm. Talc-based formulations applied as seed and soil treatments significantly reduce tomato damping-off (Suma et al., 2023).

The incubation period refers to the time between inoculation and the appearance of disease symptoms. The combination treatment of antagonistic agents also showed

a longer incubation period, which proves that this treatment can slow down the development of the pathogen. This approach leverages the synergistic effects of multiple biological control agents, enhancing their effectiveness against plant pathogens. Combining different antagonistic agents can result in more effective disease suppression. For example, using Trichoderma species with bacterial agents has increased overall pathogen inhibition compared to individual applications (Meyer and Roberts, 2002). Antagonistic bacteria such as *P. fluorescens* produce antibiotics that inhibit pathogen growth while competing for resources, thereby limiting pathogen proliferation (Podgorska-Kryszczuk et al., 2022; Sagala et al., 2023). Combining biocontrol agents can achieve up to 100% inhibition of specific pathogens, outperforming single-agent applications (El-Mohamedy et al., 2013). Volatile Organic Compounds (VOCs): Interactions among antagonistic microbes can be mediated by VOCs, which play a crucial role in enhancing pathogen suppression through complex signalling pathways (Rybakova et al., 2022).

The combination of antagonistic agents has significantly reduced pathogen populations, particularly in agricultural environments. The combination of *Saccharomyces cerevisiae* and *Sordaria fimicola* resulted in over 96% inhibition of *Fusarium graminearum*, highlighting the potential of mixed biocontrol agents in disease management (Yesim, 2020). Multiple antagonistic agents can produce additive or synergistic effects, enhancing overall efficacy against pathogens (Drusano, 2017). *P. fluorescens* is well known for its ability to inhibit various plant pathogens through the production of antimicrobial substances such as 2,4-diacetyl phloroglucinol, which improves plant resistance and growth (Sagala et al., 2023). Other bacterial antagonists, such as *Bacillus* and *Trichoderma*, also suppress pathogens by competing for nutrients and producing toxins (Boakye et al., 2022). Our result showed that antagonistic agents not only protect plants from pathogens but may also support plant growth and higher harvest weight. Biological control agents (BCAs) play a dual role in agriculture, protecting crops from pathogens and promoting plant growth. These natural microorganisms, including bacteria and fungi, suppress diseases and enhance plant health and productivity through various mechanisms. BCAs like *Bacillus* and *Pseudomonas* are crucial for managing plant pathogens through mechanisms such as antibiosis, competition, and the production of lytic enzymes. These bacteria inhibit pathogen growth and promote plant health, making them essential for sustainable agriculture. The antagonistic effects mainly result from synthesising diffusible and volatile compounds and lytic enzymes. The antagonistic bacterial strain (B18) could reduce wilt incidence in susceptible chickpea varieties from 90% to 18%, thereby increasing chickpea yield (Elbouazaoui et al., 2022). *Bacillus* and *Pseudomonas* compete for nutrients and space, limiting pathogen access to essential resources (Lee et al., 2023; Zhang et al., 2023). Bacillus strains produce cell wall-degrading enzymes such as chitinases and glucanases, which disrupt pathogen cell integrity (Saini et al., 2024). Combining BCAs with traditional methods can optimize disease control and increase crop yields (Elango et al., 2024).

The higher harvest weight of bok choy indicates that biocontrol agents can also improve crop yields. Biocontrol agents have demonstrated significant potential in boosting crop production by enhancing plant health and resistance to pests and diseases. Integrating these agents into agricultural practices reduces dependence on synthetic chemicals and promotes sustainable farming. Diverse microbial types can minimize inconsistencies in field performance, resulting in more reliable outcomes for food crop production (Pirttilä et al., 2021). Microbial antagonists, such as *P. fluorescens* and *Bacillus* species, effectively suppress plant diseases, thus maintaining both the quality and quantity of harvests (Suprapta, 2012).

Conclusion

Treatments incorporating multiple biocontrol agents—particularly treatment H (*F. oxysporum* + *G. virens* GR-01 + *T. harzianum* TR-01 + *P. fluorescens* PR-01)—were the most effective in suppressing both pre- and post-emergence damping-off, reducing pathogen populations, increasing the number of healthy plants, and enhancing overall plant growth and yield. This integrated biocontrol approach demonstrated superior efficacy compared to the synthetic fungicide (Fi), highlighting its potential as a sustainable and environmentally friendly strategy for managing *F. oxysporum*-induced diseases. The combined application of *Trichoderma harzianum* TR-01, *Gliocladium virens* GR-01, and *Pseudomonas fluorescens* PR-01 significantly suppressed *Fusarium oxysporum* infection in bok choy, with the triple-agent treatment (Gl + Th + Pf) reducing post-emergence damping-off by up to 81.25% and achieving the highest plant survival rate of 91.67%. This treatment also led to the greatest increases in plant height and fresh weight, indicating both disease suppression and growth promotion. These findings support the potential of microbial consortia as effective and sustainable biocontrol strategies in cruciferous crops. Future research should include large-scale field trials to validate the consistency of these results under diverse environmental conditions. Additionally, molecular studies are recommended to explore the plant immune responses induced by this microbial consortium, which could further inform its optimization for broader agricultural applications.

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