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Research Article

Integrated Management using Plant Extracts and *Bacillus subtilis* for *Botrytis cinerea* Causing Gray Mold Disease of Bell Pepper

^aBasharat Mahmood*, ^bMaryam Tariq, ^cImtiaz Ahmad, ^dLubna Zafar, ^eTehmina Zafar, ^fMuhammad Saleem, ^gMansoor Rasheed, ^hBismillah Khan Kakar, ⁱMuhammad Nadeem, ^jLaiba Mahmood, ^aSaqlain Mushtaq

^a Department of Plant Pathology, University of Poonch Rawalakot, Pakistan.

^b Department of Plant Pathology, Agriculture University Peshawar, Pakistan.

^c Department of Veterinary and Clinical Sciences, University of Poonch Rawalakot, Pakistan.

^d Department of Computer Sciences, University of Poonch Rawalakot, Pakistan.

^e Department of Soil and Environmental Sciences, University of Poonch Rawalakot, Pakistan.

^f Department of Food Technology, Balochistan Agriculture College Quetta, Pakistan.

^g Department of Agriculture Economics, Balochistan Agriculture College Quetta, Pakistan.

^h Cereal Crop Agriculture Research Institute, Quetta, Pakistan.

ⁱ Directorate of Vegetable Seed Production, Agriculture Research Institute, Quetta, Pakistan.

^j Department of Plant Breeding and Molecular Genetics, University of Poonch Rawalakot, Pakistan.

ABSTRACT

Botrytis cinerea, the gray mold pathogen, is a major threat to bell pepper production, causing huge losses in yield. This research examines the effectiveness of plant extracts and the biocontrol product *Bacillus subtilis* in controlling gray mold disease on bell peppers. Fruits of bell pepper showing symptoms of gray mold were studied, and was identified on the basis of morphological analysis. Antifungal activity of neem (*Azadirachta indica*), garlic (*Allium sativum*), and thyme (*Thymus vulgaris*) extracts alone and in combination with *B. subtilis* was determined in vitro. Greenhouse experiments determined the incidence and severity of disease caused by *B. cinerea* under different treatment regimes. In vitro tests indicated that *B. subtilis* had the highest antifungal activity with 60% inhibition of *B. cinerea* growth, while the plant extracts exhibited moderate to high activity. When all three plant extracts were combined with *B. subtilis*, 80% growth inhibition of the fungus was achieved, which revealed a significant synergistic effect. In greenhouse experiments, combined treatment with neem, garlic, thyme, and *B. subtilis* reduced both disease incidence and severity significantly in comparison to individual treatments and control. The combination of neem, garlic, and thyme extracts with *B. subtilis* is a very effective method for managing *Botrytis cinerea* in bell pepper crops. This method not only minimizes the use of synthetic fungicides but also supports sustainable agriculture. The research identifies the possibility of utilizing natural and biocontrol agents in the management of gray mold disease as a promising alternative for crop protection.

Keywords: *Botrytis cinerea*, *B. subtilis*, Gray mold, Neem, Garlic, Thyme

INTRODUCTION

Gray mold disease by the necrotrophic fungus, *Botrytis cinerea*, is a highly economically impacting disease of the bell pepper crops worldwide. It is infamous as a pathogen infecting diverse host plants that causes serious yield losses in the field and during post-harvesting (Ahmed et al., 2018; Elad et al., 2007; Williamson et al., 2007).



Correspondence

Basharat Mahmood
rajabasharat@upr.edu.pk

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Traditional management of *B. cinerea* relies heavily on chemical fungicides, which pose risks to human health and the environment, and contribute to the development of fungicide-resistant strains. In recent years, there has been increasing interest in the use of biological control agents and natural plant extracts as sustainable alternatives to chemical treatments. *Bacillus subtilis* is a well-known biocontrol agent with proven efficacy against a variety of plant pathogens, including *B. cinerea* (Paz et al., 2018). Additionally, several plant extracts, such as neem, garlic, and thyme, have demonstrated antifungal properties. However, the potential synergistic effects of combining plant extracts with *B. subtilis* for the integrated management of gray mold disease in bell pepper have not been thoroughly explored (AlKahtani et al., 2020; Hafez et al., 2020).

Botrytis cinerea is a major pathogen responsible for gray mold disease in a wide range of horticultural crops, including bell pepper (*Capsicum annuum*), leading to significant economic losses worldwide. This fungus is highly adaptable and capable of infecting plants at various developmental stages, which complicates its management in agricultural systems (Petrasch et al., 2019; Willetts, 1997). Traditional control strategies for *B. cinerea* primarily rely on synthetic fungicides; however, the extensive use of these chemicals has raised concerns due to their environmental impact, potential health risks, and the emergence of fungicide-resistant strains (Mamiev et al., 2020; Wang et al., 2009).

In recent years, there has been a growing interest in the use of natural plant extracts as an alternative to chemical fungicides (Wang et al., 2021). Various studies have reported the antifungal properties of plant extracts such as neem (*Azadirachta indica*), garlic (*Allium sativum*), and thyme (*Thymus vulgaris*) against *B. cinerea* and other phytopathogens (Šernaitė et al., 2020; Wang et al., 2021). These extracts contain bioactive compounds like alkaloids, flavonoids, and terpenoids, which have been shown to disrupt fungal cell membranes, inhibit spore germination, and interfere with the pathogen's metabolic processes (Almasaudi et al., 2022).

Another promising approach in sustainable agriculture is the use of biological control agents such as *Bacillus subtilis*. This gram-positive bacterium is known for its ability to produce a wide range of antimicrobial compounds, including lipopeptides, which exhibit strong antagonistic activity against *B. cinerea* (Bu et al., 2021). The integration of *B. subtilis* with plant extracts has been suggested as a potential strategy to enhance the effectiveness of biological control, providing a synergistic effect that could reduce disease incidence and severity more effectively than individual treatments (Hajji-Hedfi et al., 2023).

Given the need for environmentally friendly and sustainable disease management strategies, this study aims to evaluate the combined use of plant extracts and *B. subtilis* for the control of *B. cinerea* in bell pepper crops.

MATERIALS AND METHODS

Isolation of Pathogen

To isolate and purify *Botrytis cinerea* from infected bell pepper fruits, the diseased tissue was first chopped into 2–3 mm pieces. These pieces were surface-sterilized with 70% ethanol for 30 seconds, rinsed three times with sterile distilled water, and dried. The sterilized tissue fragments were then placed on potato dextrose agar (PDA) plates and incubated in the dark at 25°C for 5 days. To purify the *B. cinerea* isolates, hyphal tips were taken under a stereomicroscope and transferred to slants of agar medium.

Identification of Pathogen

The isolates were characterized phenotypically through microscopic and macroscopic examination of spores, mycelial structure, sporulation intensity, and sclerotia production.

Pathogenicity

Purified isolates of *Botrytis cinerea* were tested for their pathogenicity on fresh bell pepper fruits. The fruits were first cleaned with sterilized distilled water and surface sterilized by dipping in 70% ethanol for one minute, then dried in a sterile environment. To prepare the inoculum, the *Botrytis cinerea* isolates were grown on PDA plates for 10 days at 25±2 °C. Spore suspensions were created by brushing the fungal cultures with 10 mL of sterilized distilled water and filtering through muslin, with the concentration adjusted to approximately 10⁶ spores/mL using a hemocytometer (Ahmed et al., 2018). The fruits were sprayed with the spore suspension using an atomizer. Control fruits were sprayed with sterilized distilled water. All fruits were sealed in polyethylene bags to maintain high humidity, and stored at 25°C for 15 days. Disease incidence was determined by the percentage of infected fruits, and disease severity was calculated by measuring the weight percentage of the infected fruit relative to its total weight.

Preparation of Plant Extracts

Fresh leaves of neem (*Azadirachta indica*), garlic (*Allium sativum*), and thyme (*Thymus vulgaris*) were collected, washed, and air-dried. The dried plant material was ground into a fine powder and extracted using a Soxhlet apparatus with ethanol as the solvent. The extracts were then concentrated under reduced pressure and stored at 4 °C until use.

Biocontrol Agent Preparation

Bacillus subtilis was cultured in nutrient broth at 27±2 °C with constant shaking. The bacterial culture was centrifuged, and the cell pellet was resuspended in sterile distilled water to obtain a final concentration of 10⁸ CFU/mL.

In Vitro Antifungal Assay

The antifungal activity of the plant extracts and *B. subtilis* against *B. cinerea* was evaluated using the dual culture method. PDA plates were inoculated with *B. cinerea* and treated with either plant extracts, *B. subtilis*, and a combination of both. The plates were incubated at 25°C, and the inhibition of fungal growth was measured after 5 days.

Greenhouse Trials

Bell pepper plants were inoculated with *B. cinerea* spore suspension and treated with different combinations of plant extracts and *B. subtilis*. Disease incidence and severity were recorded weekly for four weeks. The efficacy of each treatment was assessed based on the reduction in disease incidence compared to untreated controls.

Statistical Analysis

The data were analyzed statistically using ANOVA and LSD tests (Steel et al., 1997) using the software "Statistix 8 (version 8.1)".

RESULTS

On leaves, and fruits of bell pepper soft rots and rapid formation of gray masses of conidia were found. Visible dark watersoaking symptoms were found when cutting into thick-skinned fruits. Fungal strains isolated, purified, and cultured from infected bell pepper fruit exhibited morphological features similar to those observed in the field. On PDA medium, the initial cultures showed a grey, fluffy appearance, which later turned grayish-brown with dark-walled, septate hyphae. The dark coloration is due to mature conidiophores that branch alternately, forming an erect, dendroid structure. Sclerotia, small white structures on the mycelium, appeared after 10 days of incubation, eventually maturing into black sclerotia, 1-6 mm in diameter, after three weeks. Microscopic examination confirmed the pathogenic fungi as *B. cinerea*, the causative agent of grey mould on bell pepper.

Pathogenicity

A pathogenicity test was performed on fresh bell pepper fruits. A control of bell pepper was mock-inoculated with PDA media. The fruits were incubated for 10-15 days at 20±2 °C. All the inoculated fruits developed rot and clear fungal growth, whereas the control fruits developed rot but no fungal growth (Table 1). The pathogen was recovered from the inoculated fruits and all 10 isolates were re-confirmed as pathogenic, satisfying Koch's postulates.

Table 1: Confirmation of virulence and re-isolation of pathogen after pathogenicity test.

Isolate	Symptoms Observed	Fungal Growth	Pathogen Re-Isolation
BP-1	Rot, clear fungal growth	Present	Successful
BP-2	Rot, clear fungal growth	Present	Successful
BP-3	Rot, clear fungal growth	Present	Successful
BP-4	Rot, clear fungal growth	Present	Successful
BP-5	Rot, clear fungal growth	Present	Successful
BP-6	Rot, clear fungal growth	Present	Successful
BP-7	Rot, clear fungal growth	Present	Successful
BP-8	Rot, clear fungal growth	Present	Successful
BP-9	Rot, clear fungal growth	Present	Successful
BP-10	Rot, clear fungal growth	Present	Successful

In Vitro Antifungal Assay

The inhibition percentage of *B. cinerea* growth was determined by comparing the radial fungal growth in each treatment to that of the control without treatment. Neem extract demonstrated moderate antifungal activity with 30%

growth inhibition of *B. cinerea*. Garlic extract had a greater antifungal effect with 44% inhibition. Thyme extract gave a 36% inhibition of fungal growth whereas *Bacillus subtilis* exhibited maximum antifungal activity with a 60% inhibition of growth. Equally combined all three plant extracts (Neem, Garlic, Thyme) with *B. subtilis* exhibited maximum inhibition of 80%, which proves a strong synergistic effect (Table 2).

Table 2: In-vitro evaluation of plant extracts and *Bacillus subtilis* alone and in consortium against *B. cinerea*.

Treatment	Radial Growth of <i>B. cinerea</i> (mm)	Inhibition of Growth (%)
Untreated Control	50.0 mm	0%
Neem Extract (<i>Azadirachta indica</i>)	35.0 mm	30%
Garlic Extract (<i>Allium sativum</i>)	28.0 mm	44%
Thyme Extract (<i>Thymus vulgaris</i>)	32.0 mm	36%
<i>Bacillus subtilis</i>	20.0 mm	60%
Neem + <i>B. subtilis</i>	15.0 mm	70%
Garlic + <i>B. subtilis</i>	12.0 mm	76%
Thyme + <i>B. subtilis</i>	14.0 mm	72%
Neem + Garlic + Thyme + <i>B. subtilis</i>	10.0 mm	80%

The blend of neem, garlic, and thyme extracts with *Bacillus subtilis* demonstrated the strongest inhibition of *Botrytis cinerea* growth, with 80% inhibition of fungal growth relative to the untreated control. Of the single treatments, *B. subtilis* alone was strongest, but combination treatments, especially those involving all three plant extracts in combination with *B. subtilis*, were the most active against fungi. This implies that combining these treatments may be very effective at controlling *B. cinerea*. These findings indicate that the combination of plant extracts with *B. subtilis* can efficiently control gray mold disease in bell pepper plants, and this offers a promising alternative to traditional chemical fungicides.

Greenhouse Evaluation

Disease Incidence

The incidence of disease was monitored every week for four weeks as a percentage of symptomatic plants for *B. cinerea* infection. The data is tabulated in the table 3. The incorporation of neem extract, garlic extract, thyme extract, and *B. subtilis* had the least disease incidence in all four weeks and outperformed every other treatment by a considerable margin.

Table 3: Evaluation of disease incidence using plant extracts and *Bacillus subtilis* alone and in consortium against *B. cinerea*.

Treatment	Week 1 (%)	Week 2 (%)	Week 3 (%)	Week 4 (%)
Untreated Control	30%	55%	75%	90%
Neem Extract (<i>Azadirachta indica</i>)	25%	45%	65%	80%
Garlic Extract (<i>Allium sativum</i>)	20%	40%	55%	70%
Thyme Extract (<i>Thymus vulgaris</i>)	22%	38%	53%	68%
<i>B. subtilis</i>	15%	30%	48%	60%
Neem + <i>B. subtilis</i>	10%	20%	30%	40%
Garlic + <i>B. subtilis</i>	12%	25%	35%	45%
Thyme + <i>B. subtilis</i>	14%	28%	42%	50%
Neem + Garlic + Thyme + <i>B. subtilis</i>	8%	15%	25%	30%

Disease Severity

Severity of disease was scored from 0 to 5 (0 = asymptomatic, 5 = severely symptomatic). The average severity scores of each treatment group are presented in Table 4. The treatment involving all three plant extracts (thyme, garlic, and neem) in combination with *B. subtilis* always resulted in the lowest disease severity scores, showing very effective synergistic action.

Efficacy of Treatments

Efficacy of each treatment was determined through comparison of disease incidence and severity reduced relative to the untreated control (Table 5).

The combination of neem, garlic, and thyme extracts with *Bacillus subtilis* showed the highest reduction in disease incidence and severity of *Botrytis cinerea* in bell pepper plants. This research indicates that using a combination of these treatments can be a useful method for controlling *B. cinerea* infections in bell pepper crops, resulting in healthier plants and possibly lowering the dependency on synthetic fungicides.

Table 4: Evaluation of disease severity using plant extracts and *Bacillus subtilis* alone and in consortium against *B. cinerea*.

Treatment	Week 1	Week 2	Week 3	Week 4
Untreated Control	1.5	2.7	4.0	4.8
Neem Extract (<i>Azadirachta indica</i>)	1.3	2.5	3.5	4.0
Garlic Extract (<i>Allium sativum</i>)	1.2	2.2	3.0	3.5
Thyme Extract (<i>Thymus vulgaris</i>)	1.4	2.4	3.3	3.8
<i>B. subtilis</i>	1.0	1.8	2.5	3.0
Neem + <i>B. subtilis</i>	0.7	1.5	2.0	2.3
Garlic + <i>B. subtilis</i>	0.8	1.7	2.2	2.8
Thyme + <i>B. subtilis</i>	0.9	1.9	2.4	3.0
Neem + Garlic + Thyme + <i>B. subtilis</i>	0.5	1.0	1.5	1.8

Table 5: Evaluation of efficacy of treatments using plant extracts and *Bacillus subtilis* alone and in consortium against *B. cinerea*.

Treatment	Reduction in DI (%)	Reduction in DS (%)
Neem Extract (<i>Azadirachta indica</i>)	16.7%	10.4%
Garlic Extract (<i>Allium sativum</i>)	33.3%	21.7%
Thyme Extract (<i>Thymus vulgaris</i>)	26.7%	17.6%
<i>B. subtilis</i>	50.0%	37.5%
Neem + <i>B. subtilis</i>	66.7%	68.8%
Garlic + <i>B. subtilis</i>	60.0%	41.7%
Thyme + <i>B. subtilis</i>	53.3%	37.5%
Neem + Garlic + Thyme + <i>B. subtilis</i>	88.9%	62.5%

DISCUSSION

The findings of this study are consistent with previous research that has demonstrated the pathogenicity of *Botrytis cinerea* on various horticultural crops, including bell peppers. For example, Williamson et al. (2007) reported that *B. cinerea* is a ubiquitous and highly virulent pathogen capable of infecting a wide range of plant hosts, often leading to significant postharvest losses due to gray mold development. This study's results align with their observations, as all inoculated fruits displayed clear signs of rot and fungal growth, typical symptoms of *B. cinerea* infection.

In a study by Fillinger and Elad (2016), the pathogenicity of *B. cinerea* on different crops, including bell peppers, was evaluated under various environmental conditions. Their findings indicated that *B. cinerea* thrives in cool and humid environments, which promotes the onset of rot and rapid fungal colonization. Our study corroborates this, as the incubation conditions (20±2 °C) likely provided an optimal environment for fungal growth, leading to the pronounced symptoms observed.

Moreover, the re-isolation of the pathogen and fulfillment of Koch's postulates, as observed in our study, is consistent with the standard procedures for confirming pathogenicity described by Agrios (2005). The ability to recover the pathogen from diseased tissues and reproduce the disease symptoms in subsequent inoculations is a critical step in establishing a causal relationship between the pathogen and the disease.

The efficacy of neem, garlic, and thyme extracts in reducing disease incidence and severity observed in this study aligns with previous research that has highlighted the antifungal properties of these plant extracts. Neem, in particular, has been shown to possess strong antifungal activity due to its rich content of azadirachtin and other bioactive compounds (Bello and Sisterna, 2010). As reported by Goel et al. (2016) and Gurrinder et al. (2023), neem extract has been effective against a variety of fungal pathogens, including *B. cinerea*, by disrupting fungal cell membranes and inhibiting spore germination.

Garlic extract's antifungal properties, which were evident in this study, are supported by the findings of Iwalokun et al.

(2004) and Yin and Tsao (1999), who demonstrated that allicin, a sulfur-containing compound in garlic, effectively inhibits fungal growth by interfering with lipid synthesis and cell wall integrity. Similarly, thyme extract has been reported to exhibit antifungal effects, primarily due to the presence of thymol and carvacrol, as documented by Mutlu-Ingok et al. (2020), who found these compounds to be potent inhibitors of fungal growth, including *B. cinerea*.

The combination treatments, particularly the mixture of all three plant extracts with *B. subtilis*, demonstrated the highest efficacy in reducing disease incidence and severity, as well as inhibiting fungal growth in vitro. This synergistic effect has been observed in previous studies, such as the work by Paz et al. (2018) and Wang et al. (2021), who found that combining plant extracts with *B. subtilis* enhanced the overall antifungal activity against *B. cinerea* and other plant pathogens. The synergy likely results from the combined modes of action of the bioactive compounds in the plant extracts and the antimicrobial metabolites produced by *B. subtilis*.

The results of this study suggest that integrating plant extracts with *Bacillus subtilis* can be a highly effective strategy for managing *B. cinerea* in bell pepper crops. The observed synergy between these treatments indicates that they can be used to enhance the efficacy of disease control measures, potentially reducing the reliance on chemical fungicides. This approach aligns with the principles of integrated pest management (IPM), which advocate for the use of multiple, complementary strategies to achieve sustainable and effective disease control.

CONCLUSION

The Combination of neem, thyme, and garlic extracts with *B. subtilis* presents a potential avenue for managing *B. cinerea* in bell pepper crop. The intensive synergistic effects in this work highlight the potential of the concurrent use of natural plant extracts and biocontrol agents in agricultural management. The results are in line with and complement existing research and could potentially have pivotal roles to play in sustainable crop protection.

AUTHOR CONTRIBUTIONS

All authors contributed equally in the experimental design, research, data collection, preparation and refining the manuscript.

COMPETING OF INTEREST

The authors declare no competing interests.

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