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Sustainable Management of Green Mold Disease of White Button Mushroom Using Botanicals and Biocontrol Agents under Temperate Conditions

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Abstract: Green mold (caused by *Trichoderma harzianum*) is a destructive disease in mushrooms which limits commercial production. The present investigation was carried out to verify the in vitro and in vivo effect of locally available botanicals and bacterial biocontrol agents against this disease. The in vitro evaluation of ethanol extract of botanicals against mycelial growth of *T. harzianum* at 1, 2, and 3% concentrations showed that *Juglans regia* and *Allium sativum* exhibited maximum mycelial growth inhibition of 84.9 and 79.8%, respectively. When the same botanicals were tested against the mycelial growth of *A. bisporus*, it was observed that *J. regia*, *Curcuma longa*, and *Azadirachta mellea* were least inhibitory (4.66–7.4%). From the in vivo evaluation of plant botanicals at 2% concentration, *J. regia* and *C. longa* had the highest average weight (11.8–11.9 g) of a single fruit body and a combined button yield of 11.3–11.9 kg/quintal compost. Among the bacterial bioagents evaluated *in vitro*, *Pseudomonas flourescens*, *Azotobacter* sp., and *Bacillus subtilis* displayed stimulatory effects of varying degrees on the mycelial growth of *A. bisporus* but exhibited antagonistic effects on *T. harzianum*. *B. subtilis*-38, and *P. flourescens*-104. *Azotobacter*-108 caused the highest mycelial growth inhibition of 97.6, 97.4, and 90.3% of *T. harzianum*, respectively. The current study reveals that the integration of botanical and bacterial antagonists in pathogen-infested white button mushroom casing reduces green mold infection with corresponding yield gains.

Keywords: *Agaricus bisporus*; antagonists; biological control agents; botanicals; *Trichoderma harzianum*

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1. Introduction

Mushroom growing is a simple microbiological technique for commercial agro-waste recycling on a wide scale [1]. White button mushrooms (*Agaricus bisporus*) are the most popular mushrooms cultivated globally including India at the higher elevations. It is

produced in great quantities in nations such as the United States, Canada, China, Europe (mainly the western half), Indonesia, Korea, Taiwan, and India. The button mushroom, especially white in color, is the most extensively farmed edible mushroom, accounting for 31.8% of global mushroom output [2].

The yearly mushroom production in India alone is valued to be over 1,20,000 mt, with button mushrooms accounting for 85% of this total [3,4]. The mushroom industry, similar to the fruit and vegetable production industry, is consistently subjected to growing pressure for transformations in the production system. A consumer-driven shift is demanding healthier and safer goods, which includes reductions in the reliance on synthetic fungicides. Mushrooms are cultivated under controlled indoor conditions that facilitate the practical use of integrated disease management programs, including fungicides, botanical and biocontrol substitutes, and agronomical practices to avert outbreaks and the spread of green mold disease [5–7].

Mushroom diseases are the key constraints for rapid mushroom production in the cottage industries in the union territory of Jammu and Kashmir. Among white button mushroom diseases, fungal diseases are of the most serious concern, damaging yield and causing the crop to fail or the quality of the produce to deteriorate [5–8]. Wet bubble, cob web dry bubble, green mold, and competitive molds such as fake truffle, brown plaster mold, olive green mold, yellow molds, ink caps, and others are all responsible for varied degrees of crop losses [9]. Among all these fungal diseases and competitor molds, *Trichoderma harzianum* causing green mold has been reported as a major constraint and common disease that leads to extensive damage to the fungal mycelium and sporocarps [10], with yield losses of 64–67% in cultivated mushrooms [11].

Under severe infestation, green mold prevents the development of button mushrooms by completely colonizing mushroom compost, leading to a severe reduction in yield [12]. This disease is easily recognizable by its green color due to the abundant sporulation of the pathogen, covering large portions of the mushroom farm; in many cases, it covers the complete mushroom bed. At the maturity stage when mushroom buttons develop fully, necrotic brown lesions frequently form [8]. This disease has been present in mushroom production for decades and may cause complete crop losses [13]. The infection spreads so fast and aggressively that even a single contaminated grain in spawn in 45 kg of mushroom compost resulted in a yield loss of 12–46% [14].

In spite of devastating epidemics and consequent yield losses instigated by green mold, insufficient information is available about the management of the disease. In addition, for the management of green mold, only certain pesticides are registered due to their mycelium sensitivity towards several chemicals [15]. Resistance towards pesticides and subsequent high cost also adds problems in button mushroom cultivation [16,17]. In contrast, biological control of mushroom diseases based on the use of natural plant and microbial agents against pathogens is considered safe and should be encouraged [18]. These botanicals and biocontrol agents have unique features, with the potential of being target specific, environmentally friendly, and cost-effective [19].

The aim of the present investigation was to identify locally available and cost-effective botanicals and bacterial biocontrol agents for the in vivo management of green mold in button mushrooms, and to create a treatment strategy that is easy to apply, neutral for white button mushrooms, and with maximum antagonistic potential against green mold under the temperate climatic conditions of the Kashmir Valley.

2. Materials and Methods

2.1. Evaluation of Botanicals

2.1.1. In Vitro Evaluation of Botanicals

Ethanol extracts of ten locally available botanicals were evaluated in vitro for their potential antifungal efficacy (Figure S1 and Table 1) against mycelial growth of *A. bisporus* and *T. harzianum* through poisoned food technique [20]. Each test botanical was tested at three concentrations viz., 1%, 2%, and 3%.

Table 1. List of botanicals evaluated against *A. bisporus* and *T. harzianum.*

Botanical Name	Common Name	Part Used
Juglans regia	Walnut	Hull
Artemesia annua	Sweet wormwood	Foliage
Matricarias spp.	Matricaria	Whole plant
Urtica dioca	Stinging nettle	Whole plant
Mentha longifolia	Horse mint	Whole plant
Lavendula officinalis	Lavender	Whole plant
Curcuma longa	Turmeric	Rhizome
Azadirachta indica	Neem	Leaves
Allium sativum	Garlic	Cloves
Zingiber officinale	Ginger	Rhizome

The collected plant parts were washed in tap water to remove dirt particles, rinsed with sterile distilled water, and then dried under sun shade. Rhizomes of turmeric and walnut hulls were also dried until they became brittle. A fine powder (75 µm) of each botanical was obtained by grinding the shade-dried plant parts with an electric grinder, sieving though a 200 mm sieve, and storing in closed plastic containers for later use.

Twenty grams of each powdered botanical were separately mixed in 200 mL of ethanol (70%) in 250 mL Erlenmeyer glass flasks. All glass flasks were aseptically plugged with sterile cotton, wrapped with aluminum foil, and shaken for 36 h on an electric shaker. The flasks were left undisturbed after shaking for 6 h, allowing the heavily suspended plant parts to settle at the bottom. The extract was transferred to a clean flask by filtering the filtrate through sterilized Whatman No.1 filter paper, and then centrifuged at 5000 rpm (REMI-412LAG) for 15 min. The pellet was discarded, and the ethanol extract of each botanical in supernatant was separately collected. The solvent was allowed to vaporize at 40–50 °C in a rotary evaporator to complete dryness and stored in air-tight bottles [21]. The test concentrations of 1%, 2%, and 3% were achieved by adding appropriate amounts of sterile, distilled water to the standard solution (100%). Three milliliters of each ethanol extract was dispensed in Petri plates, and 25 mL of molten sterile PDA was gently poured over the extract solution. After the solidification of PDA media, 5 mm (diameter) mycelia disks were cut from a fresh 6-day-old culture of *A. bisporus* and *T. harzianum* and were separately inoculated at the center of the Petri plates. Each treatment was replicated thrice, and the experiment was repeated twice under the same conditions. Petri plates without botanical extract served as controls, and percent mycelial growth inhibition was calculated using the formula as below [22]:

$$\text{Per cent growth inhibition} = \frac{C - T}{C} \times 100 \quad (1)$$

where, C = colony diameter in the control plate and T = colony diameter of the inoculated plate.

2.1.2. In Vivo Evaluation of Botanicals

Extracts of *J. regia*, *A. annua*, and *U. dioca,* which were minimum inhibitory to mycelial growth of *A. bisporus* and maximum inhibitory to *T. harizianum*, were evaluated against the green mold pathogen in cultivation trials during spring 2017 and 2018 at the Mushroom Research and Training Centre, SKUAST-K. Mushroom compost was prepared according to the method of Mantel et al. [23], followed by the Long Method, and constituents (wheat straw, 300 kg; rice bran, 50 kg; chicken manure, 150 kg; potash, 2 kg; urea, 5 kg; molasses, 12.5 kg; and gypsum, 15 kg) were used as per Formula SKI-4 of SKUAST-K [24]. Before the green mold pathogen was inoculated, the dried powders of *J. regia, A. sativum*, and *C. longa* were passed across a dual layered muslin cloth and were combined with the casing material at 0.5%, 1%, and 2% to evaluate their efficacy against the disease. The botanicals were mixed with casing material and dispersed over the button mushroom spawn run

compost (10 kg polythene bags). Three replicates of each treatment were carried out, each consisting of an individual polybag. After casing, the polybags were kept in the production room at 23–25 °C for 4 days and then at 15–18 °C for the remaining period before cropping. Controls included treatments with and without botanical admixture, as well as treatments with and without mold pathogen inoculation. Mushrooms were harvested in four flushes. Data on disease severity percentage yield per polybag, total mushroom weight, mushroom yield per quintal compost, and other quality parameters were recorded for a period of 1.5 months.

2.2. Evaluation of Bacterial Biocontrol Agents

2.2.1. In Vitro Evaluation of Potential Bacterial Biocontrol Agents

The axenic cultures of the bacterial isolates of *P. fluorescens*, *B. subtillis*, and *Azotobacter* sp., previously evaluated against the green mold of oyster mushrooms, were procured from the Department of Environmental Sciences, SKUAST-K, Shalimar. These bacterial biocontrol isolates were preserved and multiplied by repeated subculturing on King's B Medium as well as on nutrient agar (NA). The potential antagonistic effect of these isolates was tested via dual culture technique against mycelial growth of *T. harizianum* and *A. bisporus* using the streak plate method [25]. The hot molten sterilized PDA media was poured in Petri plates and allowed to solidify. Bacterial isolates of *P. fluorescens*, *B. subtillis*, and *Azotobacter* sp. were separately streaked in zigzag patterns on the center of the Petri plate. After streaking, 5 mm mycelial discs of both *A. bisporus* (8-day-old culture) and *T. harzianum* (5-day-old culture) were equidistantly placed from both ends of the bacterial streak. The inoculated plates were incubated at 25 ± 2 °C for 3 days. In the control, Petri plates with *A. bisporus* and *T. harzianum* served as checks. Three replicates were maintained for each treatment. Colony diameter of both *T. harizianum* and *A. bisporus* in each treatment was recorded, and the inhibition percentage of the mycelium was calculated as described for the botanicals [22]. From the dual culture assay, the bacterial isolates were grouped into distinct classes [26] with slight modification [27] as below:

1 = Stimulatory; 2 = More stimulatory; 3 = Antagonistic; 4 = Neutral

2.2.2. In Vivo Evaluation

Bacterial antagonists that exhibited maximum mycelial growth inhibition against *T. harzianum* and no/least inhibition of *A. bisporus* were assessed against the green mold pathogen in vivo during cultivation trials of 2017 and 2018 at the Mushroom Research and Training Centre, SKUAST-K. The cell suspension (6×10^6 cfu/mL) of the best bacterial isolate was independently mixed with casing material (0.5%, 1%, and 2% concentration) before inoculating with *T. harzianum*. The inoculated pathogen as well as admixed bacterial antagonist in spawn casing material was spread over spawn run compost filled in 10 kg polythene bags. Each treatment was replicated thrice with each replication involving a single bag. In controls, neither pathogen nor bacterial antagonistic were admixed with compost material. Observations were made for percent disease severity of green mold, and yield in each treatment was estimated for the 1.5 month cropping period. Other quality characters viz., pileus weight and diameter, and stipe weight and diameter were also recorded.

2.3. Statistical Analysis

The experiment was carried out in a completely randomized design (CRD) with 10 treatments for botanicals and 9 treatments for bacterial biocontrol agents with each treatment replicated thrice. The data obtained from in vitro and in vivo studies were subjected to one- and two-way analysis of variance (ANOVA). The critical difference (CD, $p \leq 0.05$) was used to compare treatment means. The number and weight (g) of fruiting bodies per bag per treatment was recorded daily, and mushroom yield data were expressed as kg mushroom per 100 kg compost for up to 1.5 months. Statistical analysis

(Tables S1 and S2) of botanicals and bacterial biocontrol agents from in vitro and in vivo experiments was conducted using R Studio Desktop (version 4.2.1) [28].

3. Results

3.1. In Vitro and In Vivo Efficacy of Botanicals

3.1.1. Effect of Ethanol Extract of Botanicals against *T. harzianum* In Vitro

Ten botanicals viz., *Metricaria* sp, *M. longifolia*, *C. longa*, *J. regia*, *A. annua*, *L. officinalis*, *A. sativum*, *Z. officinale*, *A. mellea*, and *U. dioca* were evaluated for their inhibitory effect against the mycelial growth of *T. harizianum* and *A. bisporus* (Figure S2). Table 2 reveals that all test botanicals inhibited the mycelial growth of *T. harzianum* at each level of concentration. Among the botanicals, *J. regia* and *A. sativum* displayed maximum mycelial growth inhibition percentages of 84.9 and 79.8, respectively, followed by *A. amellea* and *C. longa* with 74.0 and 70.6%, respectively, against the test pathogen, while *Z. officinale* had the lowest growth inhibition (28.5%). Further, the data also show that, on average, lower concentrations showed least mycelial growth inhibition (49.1%), while higher concentrations showed the most growth inhibition (70.6%). At a concentration of 3%, *J. regia* exhibited the highest mycelial growth inhibition of 95.3%, followed by *A. sativum* (90.1%). The next best treatments were *J. regia* and *A. sativum*, both at a concentration of 2%, which inhibited pathogen growth by 81.9–85.8%. At 1%, *Z. officinale* was found the least effective botanical against the pathogen, inhibiting mycelial growth by just 19.3%.

Table 2. In vitro evaluation of botanicals against the green mold pathogen (*Trichoderma harzianum*) at concentrations between 1 and 3%.

Conc. (%) / Botanical	Inhibition of Growth over Control (%)			Mean
	1	2	3	
Juglans regia.	73.8 (59.5)	85.8 (67.8)	95.3 (77.4)	84.9 (68.2) [a]
Artemesia annua	53.7 (47.1)	65.9 (54.3)	76.9 (61.3)	65.5 (54.2) [d]
Mentha longifolia	29.9 (33.1)	38.5 (38.0)	46.9 (43.2)	38.4 (38.1) [h]
Metricaria spp.	41.6 (40.3)	52.6 (45.9)	63.9 (52.8)	52.7 (46.3) [f]
Azadirachtamellea	61.4 (51.5)	74.9 (59.7)	85.7 (67.8)	74.0 (59.6) [c]
Allium sativum	67.3 (55.4)	81.9 (64.6)	90.1 (72.3)	79.8 (64.1) [b]
Urtica dioca	39.9 (39.1)	46.8 (43.1)	58.9 (50.1)	48.5 (44.1) [g]
Curcuma longa	58.9 (50.1)	71.5 (57.8)	81.3 (64.4)	70.6 (57.4) [c]
Lavendula officinalis	45.2 (42.2)	57.9 (49.5)	68.9 (56.0)	57.3 (49.2) [e]
Zingiber officinale	19.3 (26.0)	28.5 (31.8)	37.9 (38.0)	28.6 (31.9) [i]
Mean	49.1 (44.4) [c]	60.4 (51.2) [b]	70.6 (58.3) [a]	
		S.E±	CD. ($p \leq 0.05$)	
Botanical	:	(0.0072)	(0.0204)	
Concentration	:	(0.0040)	(0.0112)	
Botanical × concentration	:	(0.0125)	(0.035)	

Mean of three replicates; values in parentheses are angular transformed; means followed by the same letters are not significantly different at $p = 0.05$.

3.1.2. Effect of Ethanol Extract of Botanicals against Host Mycelium (*A. bisporus*) In Vitro

The ethanol extracts of ten botanicals were also tested for their potential inhibitory effect against *A. bisporus* at the same three concentrations (1%, 2%, and 3%) using poisoned agar technique. The data reveal that all botanicals exhibited substantially less inhibitory effects towards the mycelial growth of *A. bisporus* at each concentration (Table 3). In an overall comparison, *J. regia*, *C. longa,* and *A. mellea* displayed the least amount of mycelial growth inhibition (4.7–7.4%) to *A. bisporus*, followed by *A. annua* and *A. sativum* (12.4–12.9%). *M. longifolia* had the highest degree of inhibition (30.7%). The lower concentrations had a minimum inhibitory effect of 9.0%, while the higher concentrations had a maximum inhibitory effect of 23.3%. There was also a major interaction between the botanicals and the concentrations of their ethanol extracts. *J. regia* at 1 and 2% concentrations, as well as

C. longa at 1% concentration, showed minimum mycelial growth inhibition of 2.7–4.4%, with *A. mellea* at 1% and *C. longa* at 2% concentrations showing growth inhibition of 4.8–5.9%. At a concentration of 3%, *M. longifolia*, *Z. officinale*, and *U. dioca* each showed a maximum growth inhibition of 20.6–30.7%.

Table 3. Efficacy of botanicals against mycelial growth of *Agaricus bisporus* in vitro at concentrations between 1 and 3%.

Botanical \ Conc. (%)	Mycelial Growth Inhibition (%)			Mean
	1	2	3	
Juglans regia.	2.7 (9.4)	4.4 (12.1)	6.9 (15.3)	4.7 (12.3) [a]
Artemesia annua	7.8 (16.2)	11.8 (20.1)	17.7 (24.8)	12.4 (20.4) [c]
Mentha longifolia	17.2 (24.5)	26.9 (31.2)	47.9 (43.8)	30.7 (33.2) [g]
Metricariaspp.	9.7 (18.1)	15.9 (23.5)	26.5 (31.0)	17.8 (24.2) [d]
Azadirachta mellea	4.8 (12.7)	7.91 (16.3)	9.5 (17.9)	7.4 (15.6) [b]
Allium sativum	8.7 (17.2)	12.0 (20.2)	18.1 (25.2)	12.9 (20.9) [c]
Urtica dioca	11.4 (19.7)	19.0 (25.8)	31.6 (34.2)	20.6 (26.6) [e]
Curcuma longa	3.2 (10.3)	5.9 (14.0)	6.9 (15.3)	5.3 (13.2) [a]
Lavendula officinalis	10.1 (18.6)	16.1 (23.7)	27.5 (31.6)	17.9 (24.6) [d]
Zingiber officinale	14.6 (22.4)	21. 6 (27.7)	40.9 (39.7)	25.7 (30.0) [f]
Mean	9.0 (16.9) [c]	14.2 (21.5) [b]	23.3 (27.9) [a]	
		S.E±	CD. ($p \leq 0.05$)	
Botanical	:	(0.0065)	(0.0185)	
Concentration	:	(0.0036)	(0.0101)	
Botanical × concentration	:	(0.0113)	(0.032)	

Mean of three replicates; figures in parentheses are angular transformed; means followed by the same letters are not significantly different at $p = 0.05$.

3.1.3. In Vivo Efficacy of Botanicals

Effect of botanicals on green mold development

When compared to check-I (pathogen infested-untreated), the data (Figure S3 and Table 4) reveal that all botanical treatments significantly decreased the severity of green mold in white button mushrooms during spring 2017 and 2018. From the pooled data, disease reduction of 1.60–3.20% was observed by incorporating *J. regia* or *A. sativum* at a 2% concentration in casing material which resulted in relative disease control of 85.9–93.0%. The next best treatments were *C. longa* at 2.0% and *J. regia* at 1.0% concentrations which showed disease reduction of 5.8 and 6.1% and a relative disease control of 73.1 and 74.3%, respectively. At a 0.5% concentration, *C. longa* and *A. sativum* were the least effective botanical treatments with green mold severity ranging from 10.3 to 13.2% and relative disease control ranging from 41.9 to 54.7%. Maximum diseases severity (24.2%) of green mold was observed in check-I.

Effect of botanicals on yield and yield components

The application of *Curcuma longa*, *A. sativum*, or *J. regia* at 2.0% concentration yielded the least amount of fruiting bodies per kg of mushrooms (85.5–87.4), followed by *Curcuma longa* and *A. sativum* at 1.0% concentration which yielded 89.1 and 91.0 fruit bodies per kg of mushrooms, respectively, which was less than check-I and -II (96.7 and 94.8 fruit bodies kg^{-1} mushroom), respectively. The average fruit body weight of button mushrooms also differed considerably with the use of botanical treatments. *J. regia* and *C. longa* each had the highest average weight of single fruiting bodies (11.8–11.9 g), followed by *J. regia* at 1.0% and *A. sativum* at 2.0% concentration (10.9–10.9 g) compared to 11.4 g in check-II. In the application of *J. regia* and *C. longa*, each had the highest button yield of 11.3–11.9 kg/quintal compost at 2% concentration, compared to check-I and -II where 7.6 and 12.8 kg/quintal compost were obtained, respectively. The next best treatments were *C. longa* and *J. regia* at 1.0% each, and *A. sativum* at 2.0%, with an average yield of 10.3–10.9 kg/quintal compost (Table 5). The least effective botanical, *A. sativum*, yielded just 8.2 kg mushroom/quintal compost at a concentration of 0.5%. Thus, it was observed that the application of botanicals,

as well as reducing green mold infection, also significantly enhances the yield of buttons compared to the check.

Table 4. In vivo efficacy of botanical extracts on severity (%) of green mold of button mushrooms at 0.5 to 2% concentration.

Treatment		Spring 2017	Spring 2018	Pooled	Disease Control (%)
Juglans regia	0.5%	10.7 (3.4)	9.1 (3.2)	9.9 (3.3) ^{f}	56.4
	1.0%	6.3 (2.7)	5.9 (2.6)	6.1 (2.7) ^{d}	73.1
	2.0%	2.1 (1.8)	1.1 (1.5)	1.6 (1.6) ^{b}	93.0
Sub mean		6.4 (2.6)	5.4 (2.4)	5.9 (2.5)	
Allium sativum	0.5%	10.7 (3.4)	9.9 (3.3)	10.3 (3.4)^{f}	54.7
	1.0%	8.3 (3.1)	6.9 (2.8)	7.6 (2.9) ^{e}	66.4
	2.0%	3.9 (2.2)	2.5 (1.9)	3.2 (2.0) ^{c}	85.9
Sub mean		7.6 (2.9)	6.4 (2.7)	7.0 (2.8)	
Curcuma longa	0.5%	13.9 (3.9)	12.5 (3.7)	13.2 (3.8)^{g}	41.9
	1.0%	10.1 (3.3)	9.1 (3.2)	9.6 (3.3) ^{f}	57.6
	2.0%	6.1 (2.7)	5.5 (2.6)	5.8 (2.6) ^{d}	74.3
Sub mean		10.0 (3.3)	9.1 (3.1)	9.6 (3.2)	
Check-I (infested-untreated)		26.9 (5.3)	21.5 (4.7)	24.2 (5.0) ^{g}	-
Check-II (uninfested-untreated)		0.0 (1.0)	0.00 (1.00)	0.00 (1.00) ^{a}	-
C.D ($p \leq 0.05$)					
Treatment combination		0.006	0.007	0.005	
Control v/s rest		0.006	0.006	0.004	
Botanicals		0.019	0.020	0.020	
Concentration		0.019	0.020	0.020	
Botanical × concentration		0.0334	0.035	0.034	

Mean of three replicates; figures in parenthesis are square root transformed values; means followed by the same letters are not significantly different at $p = 0.05$.

Table 5. Impact of botanical extracts on the number and weight of fruiting bodies and button yield under in vivo at concentrations between 0.5 to 2%.

Treatment		No. of Fruit Bodies kg^{-1} Mushroom	Weight of Fruit Bodies (g)	Button Yield kg^{-1} Quintal Compost
Juglans regia	0.5%	93.7 ^{f}	10.9 ^{c}	9.4 ^{e}
	1.0%	91.2 ^{e}	11.5 ^{ab}	10.5 ^{cd}
	2.0%	87.4 ^{bc}	11.9 ^{a}	11.9 ^{b}
Sub mean		90.8	11.4	10.6
Allium sativum	0.5%	93.5 ^{d}	10.1 ^{f}	8.2 ^{g}
	1.0%	91.0 ^{e}	11.0 ^{c}	9.7 ^{e}
	2.0%	86.8 ^{b}	11.4 ^{ab}	10.3 ^{cd}
Sub mean		90.4	10.8	9.4
Curcuma longa	0.5%	94.1 ^{fg}	10.3 ^{e}	8.6 ^{f}
	1.0%	89.1 ^{d}	11.1 ^{c}	10.9 ^{c}
	2.0%	85.5 ^{a}	11.8 ^{a}	11.3 ^{c}
Sub mean		89.6	11.1	10.3
Check I (infested-untreated)		96.7 ^{h}	10.7 ^{d}	7.6 ^{g}
Check II (uninfested-untreated)		94.8 ^{fg}	11.4 ^{ab}	12.8 ^{a}
C.D ($p \leq 0.05$)				
Control v/s rest		0.064	0.035	0.033
Botanicals		0.038	0.021	0.020
Concentration		0.038	0.021	0.020
Botanical × concentration		0.065	0.036	0.035

Mean of three replicates; means followed by the same letters are not significantly different at $p = 0.05$.

Effect of botanicals on quality parameters of sporophores

The application of botanicals in *T. harzianum*-infested casing material had a major impact on sporophore quality parameters viz., the weight and diameter of pileus, and the weight and diameter of stipe (Figure 1 and Table S3).

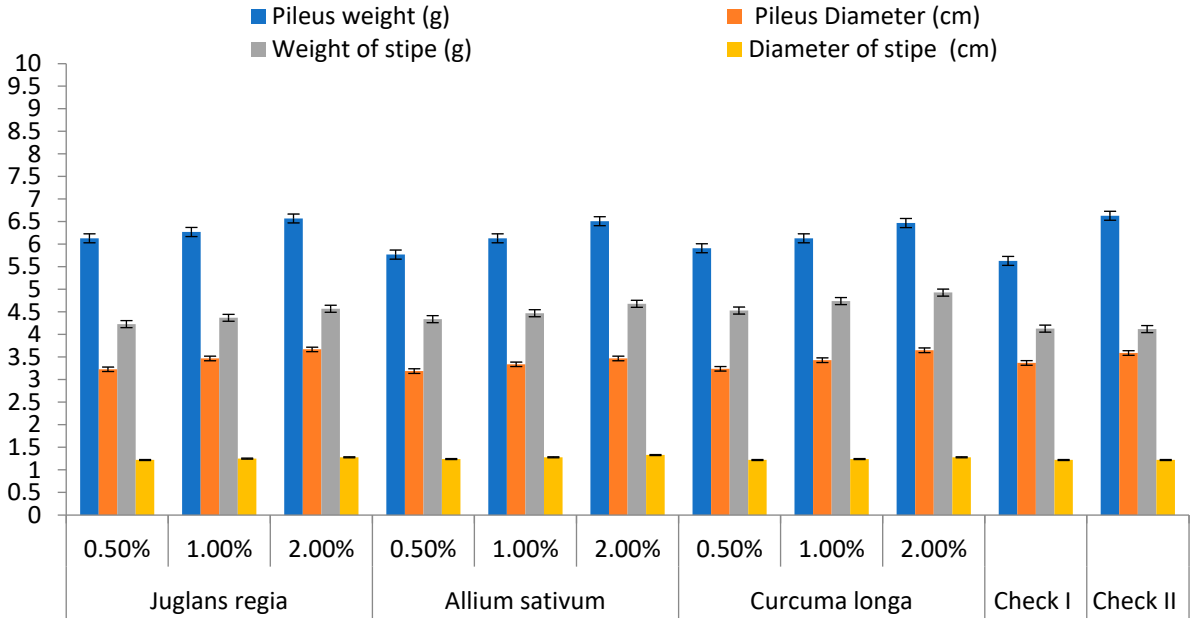

Figure 1. Efficacy of botanical extracts on quality parameters of white button mushrooms (*Agaricus bisporus*) at concentrations between 0.5 to 2%. Values are the mean and standard errors of three replications; Check I = infested-untreated; Check II = uninfested-untreated.

Pileus weight: The pileus weight (5.63 g) in check-I substantially increased to 6.51–6.57 g in the treatments containing 2.0% of *J. regia* or *A. sativum*. The next best botanicals were *C. longa* (2.0%), *J. regia* (1.0%), *A. sativum* (1.0%), and *C. longa* (1.0%), all of which had a pileus weight of 6.13–6.47 g.

Pileus diameter: The pileus diameter of *J. regia* at 2% concentration was the largest among the botanical treatments, measuring 3.67 cm on average, followed by *C. longa* and *A. sativum* at 2.0% concentration, and 3.37 cm in check-I.

Stipe weight: Stipe weight of 4.13 g in check-I significantly increased (4.74–4.93 g) with the application *C. longa* at a concentration of 1–2%. The next best treatments, *A. sativum* and *J. regia*, both at 2.0% concentration, produced stipe weights of 4.57–4.68 g compared to check-II (4.12 g).

Stipe diameter: The stipe diameter of the treatment receiving *A. sativum* at 2.0% concentration was highest (1.33 cm), followed by 1.28 cm in the treatments receiving *A. sativum* at 1% or *J. regia* and *C. longa* each at 2.0% concentration, and 1.22 cm in check-I.

3.2. Efficacy of Bacterial Antagonists against Green Mold

3.2.1. Effect of Bacterial Antagonists against *T. harzianum* and *A. bisporus* In Vitro

The data in Table 6 clearly reveal that none of the tested bacterial isolates of *P. fluorescens*, *B. subtillis*, and *Azotobacter* sp displayed antagonism towards the mycelial growth of *A. bisporus*. Among the bacterial isolates, PS-103, PS-104, Azt-108, BS-34, and BS-38 were more stimulatory towards the mycelial growth of *A. bisporus*. However, *Azotobacter* sp., Azt-106, Azt-117, and *B. subtillis* BS-37 only displayed stimulatory effects on *A. bisporus* growth (Figure S4), and the *P. fluorescens* isolate, PS-105, exhibited neutral effects. In contrast, all the test isolates, except *Azotobacter* sp., Azt-106, and Azt-117 displayed varying degrees of antagonism against *T. harzianum*. Isolate Azt-117 and BS-101 exhibited neutral effects on the growth of *T. harzianum*.

Table 6. In vitro evaluation of bacterial antagonists against mycelial growth of *Agaricus bisporus* and *Trichoderma harzianum.*

Bacterial Isolate	Radial Mycelial Growth (mm) of *A. bisporus*	Percent Growth Stimulation	Radial Mycelial Growth (mm) of *T. harzianum*	Percent Growth Inhibition	Interaction	
					T. harzianum	*A. bisporus*
P. flourescens-103	43.3	28.8	2.3	95.2	A	MS
P. flourescens-104	47.8	38.2	1.3	97.4	A	MS
P. flourescens-105	34.3	13.6	6.7	86.1	A	N
B. subtilis-B-34	44.2	33.9	1.8	96.4	A	MS
B. subtilis-B-37	37.7	31.7	3.2	93.4	A	S
B. subtilis-B-38	58.8	41.3	1.1	97.6	A	MS
Azotobacter-106	32.2	26.7	8.5	82.4	N	S
Azotobacter-108	39.7	35.3	4.7	90.3	A	MS
Azotobacter-117	31.7	23.5	9.6	79.9	N	S
Control	30.0	-	48.0	-		

S = Stimulatory; MS = More stimulatory; N = Neutral; A= Antagonistic with clear inhibition zone.

3.2.2. In Vivo Efficacy of Bacterial Antagonists against Green Mold during Spring 2017 and 2018

Effect on green mold disease development

Figure S3 and Table 7 data reveal that all bacterial antagonists significantly decreased the percentage of green mold disease severity in comparison to pathogen-infested and untreated (check-I). Compared to a green mold disease severity of 15.3% in check-I, disease severity was significantly lowered to 1.2%, yielding relative disease control of 91.9% in the treatment with *B. subtillis* at 2.0% concentration, followed by disease severity of 3.0–3.1% and relative disease control of 79.8–80.3% in the treatment with *P. flourescens* at 2% or *B. subtilis* at 1% concentrations. *B. subtillis* at 0.5% and *P. flourescens* at 1% concentrations were found to be the next best treatments, exhibiting green mold severity of 4.8–6.2%, with relative disease control of 59.2–68.6%. Among the bacterial antagonists, *Azatobacter* sp. at a concentration of 0.5% was found the least effective treatment with high severity of disease (13.2%) and relative disease control of 13.9%.

Table 7. In vivo effect of bacterial antagonists on disease severity (%) of green mold of button mushrooms at concentrations between 0.5 and 2%.

Treatment		Spring 2017	Spring 2018	Pooled	Disease Control (%)
Pseudomonas flourescens-104	0.5%	8.6 (3.1)	7.5 (2.9)	8.0 (3.0) ^{e}	47.3
	1.0%	6.3 (2.7)	6.1 (2.7)	6.2 (2.7) ^{d}	59.2
	2.0%	3.7 (2.2)	2.3 (1.8)	3.0 (2.0) ^{c}	80.3
Sub mean		6.2 (2.7)	5.3 (2.5)	5.8 (2.6)	
Bacillus subtilis-38	0.5%	5.4 (2.5)	4.2 (2.3)	4.8 (2.4) ^{d}	68.6
	1.0%	3.9 (2.2)	2.3 (1.8)	3.1 (2.0) ^{b}	79.8
	2.0%	1.8 (1.7)	0.6 (1.3)	1.2 (1.5) ^{ab}	91.9
Sub mean		3.7 (2.1)	2.4 (1.8)	3.0 (2.0)	
Azotobacter-108	0.5%	13.4 (3.9)	12.9 (3.7)	13.2 (3.8) ^{f}	13.9
	1.0%	10.7 (3.4)	8.5 (3.07)	9.6 (3.2) ^{e}	37.3
	2.0%	7.4 (2.9)	6.3 (2.7)	6.9 (2.8) ^{d}	55.1
Sub mean		10.5 (3.4)	9.2 (3.2)	9.9 (3.3)	
Check-I (infested-untreated)		15.9 (4.1)	14.7 (4.0)	15.3 (4.0) ^{g}	-
Check-II (uninfested-untreated)		0.0 (1.00)	0.0 (1.0)	0.0 (1.0) ^{a}	-
CD ($p \leq 0.05$)					
Treatment combination		0.0070	0.0082	0.0052	
Control v/s rest		0.0066	0.0073	0.0047	
Biocontrol agent		0.0196	0.0197	0.0197	
Concentration		0.0196	0.0197	0.0198	
Biocontrol agent × concentration		0.0340	0.0342	0.0341	

Mean of the three replicates; figures in parentheses are square root transformed values; means followed by the same letters are not significantly different at $p = 0.05$.

Effect on yield and yield components

In vivo application of bacterial antagonist isolates in green mold-infested casing material showed significant and positive effects on yield and yield components viz., the weight and number of mushroom fruiting bodies (Table 8). The results from Table 8 show that the least number (78.5–83.7) of fruiting bodies kg^{-1} mushroom was noted in treatments with *P. fluorescens*-103 at 1% and 2% compared to check-II (93.9). *P. fluorescens*-104 at 0.5% concentration and *B. subtillis*-38 at 2% were the second best treatments with 86.3–86.7 fruit bodies kg^{-1} mushroom in comparison to 94.7 fruit bodies kg^{-1} mushroom obtained in check-I. A mean fruit body weight of 10.3 g obtained in check-I significantly improved (12.3–12.8 g) in *B. subtillis*-38 at 1 and 2%. *P. fluorescens*-104 at 2% and *Azotobacter*-106 at 2% were second best treatments, giving fruit body weight of 11.8–12.1 g. *Pseudomonas fluorescens*-104 at 2% displayed a maximum yield of 11.7 kg^{-1} quintal comparable to that obtained in check-II (12.2 kg^{-1} quintal compost). *Pseudomonas fluorescens*-104 at 1% or *B. subtilis*-38 (2%) was subsequently the best treatment with an average yield of 10.7–10.9 kg^{-1} quintal compost. The application of 0.5–1.0% concentration of *Azotobacter* sp. was found to be the least effective bacterial antagonist, giving a button yield of 6.1–6.7 kg/quintal of compost which is similar to that obtained in check- II (6.2 kg_{-1} quintal compost). The in vivo study of bacterial antagonists revealed that button yields, the mean weight of fruit bodies, and number and weight of fruit bodies considerably increased with the treatments of bacterial antagonists.

Table 8. In vivo effect of bacterial antagonists on yield and yield components of button mushrooms at concentrations between 0.5 and 2%.

Treatment		No. of Fruit Bodies per kg Mushroom	Weight of Fruit Bodies (g)	Button Yield kg^{-1} Quintal Compost
Pseudomonas flourescens-104	0.5%	86.3 ^{c}	10.9 ^{e}	9.7 ^{e}
	1.0%	83.7 ^{b}	11.4 ^{cd}	10.9 ^{c}
	2.0%	78.5 ^{a}	11.8 ^{c}	11.7 ^{b}
Sub mean		82.8	11.4	10.7
Bacillus subtilis-38	0.5%	92.5 ^{e}	11.7 ^{c}	9.3 ^{e}
	1.0%	88.7 ^{d}	12.3 ^{ab}	10.1 ^{d}
	2.0%	86.7 ^{c}	12.8 ^{a}	10.7 ^{c}
Sub mean		89.3	12.3	10.0
Azotobacter-108	0.5%	93.5 ^{f}	11.1 ^{cd}	6.1 ^{g}
	1.0%	92.3 ^{e}	11.7 ^{c}	6.7 ^{g}
	2.0%	88.9 ^{d}	12.1 ^{ab}	7.8 ^{f}
Sub mean		91.6	11.6	6.9
Check-I (infested-untreated)		94.7 ^{g}	10.3 ^{f}	6.2 ^{g}
Check-II (uninfested-untreated)		93.9 ^{f}	10.8 ^{e}	12.2 ^{a}
CD ($p \leq 0.05$)				
Control v/s rest		0.0638	0.0347	0.0343
Biocontrol agent		0.0291	0.0196	0.0201
Concentration		0.0291	0.0196	0.0201
Biocontrol agent × concentration		0.0505	0.0340	0.0349

Mean of three replicates; means followed by the same letters are not significantly different at $p = 0.05$.

Effect of bacterial biocontrol agents on quality parameters of sporophores

The application of bacterial biocontrol isolates in green mold-infested casing material with green mold pathogen considerably affected the quality parameters of sporophore viz., the weight of pileus, diameter of pileus, weight of stipe, and diameter of stipe as shown in Figure 2 and Table S4.

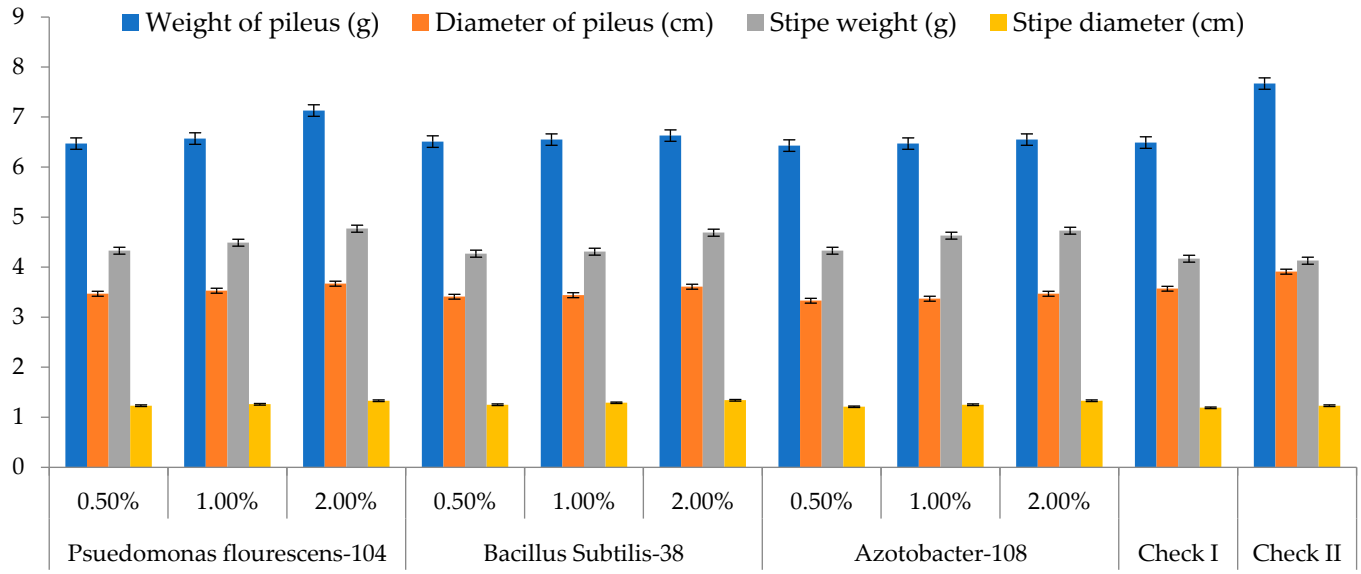

Figure 2. In vivo efficacy of bacterial antagonists on quality parameters of white button mushrooms at concentrations between 0.5 to 2%. Values are the mean and standard errors of three replications; Check-I = infested-untreated; Check-II = uninfested-untreated.

Weight of pileus: Among antagonist bacteria, *P. fluorescens*-104 at 2% concentration showed the maximum weight of pileus (7.13 g), followed by *B.subtilis*-38 at 2.0% and *P. fluorescens* at 1.0% with pileus weight (6.57–6.63 g) compared to check-I where the weight of pileus was 6.49 g.

Diameter of pileus: Among the treatments, *P. flourescens*-104 and *B.subtilis*-38 both at 2% concentration showed the largest pileus diameter (3.61–3.67 cm) that was statistically at par with check-II (3.91 cm). The second best treatment was *Azotobacter*-108 at 2% or *P. flourescens*-103 at 0.5–1.0% concentrations that showed pileus diameters of 3.47–3.53 cm.

Weight of stipe: The stipe weight of 4.17 g obtained in check-I significantly improved from 4.73 to 4.77 g in treatment with *P. fluorescens*-104 or *Azotobacter* sp-108 at 2.0% concentration, followed by *B. subtillis*-38 at 2% and *Azotobacter* sp-108 at 1.0% which showed a stipe weight of 4.63–4.69 g. In check-II, the average stipe weight was 4.13 g.

Diameter of stipe: The diameter of stipe (1.34 cm) was highest in treatment with the application *B. subtilis*-38 at 2.0% compared to check-I (1.19 cm). *Azotobacter*-108 and *P. fluorescens*-104, at 2.0% concentration, were the second best treatments which showed a stipe diameter of 1.33 cm in comparison to the stipe diameter of 1.23 cm obtained in check-II.

4. Discussion

Green mold is widely distributed in mushroom growing countries of the world and generally appears in substrate rich in carbohydrates and deficient in nitrogen [29]. Application of fungicides to reduce the green mold infestation in mushroom cultivation not only increase costs but also leaves unwanted residues. Most fungicides that are still permitted have become unsuccessful in adequately controlling the mold disease of mushrooms because of resistance development [30]. The use of biological management tools such as botanicals and biocontrol agents is necessary for the successful and effective management of green mold disease in the production houses of mushroom [31]. In the present study, botanicals and biological control agents were tested both in vitro as well as in vivo against *T. harzianum* to choose the most effective for disease management. The present study further showed that botanical extracts and bacterial antagonists not only inhibit mycelial growth of the pathogen but also significantly influence the yield and quality parameters of button mushroom. In vitro evaluation of botanicals viz., *Metricaria* sp, *M. longifolia*, *C. longa*,

J. regia, *A. annua*, *L. officinalis*, *A. sativum*, *Z. officinale*, *A. mellea*, and *U. dioca* at 1, 2, and 3% concentrations against *T. harzianum* revealed that *J. regia* showed the highest mycelial growth inhibition of 84.9% and was least inhibitory towards the mycelial growth of *A. bisporus* (4.7–7.4%). Furthermore, under in vivo evaluation, the application of *J. regia* at 2% concentration in casing material resulted in relative disease control of 85.9–93.0%. The antifungal potential of eight botanicals, *A. sativum*, *U. dioca*, *L. esculentum, D. strimonium, Mentha*, and *J. regia* against *T. harzianum* in white button mushrooms was also evaluated by Shah and Nasreen [32], who reported that *J. regia* (52.9% inhibition) displayed maximum suppression, followed by *A. indica* (34.1%) and *A. sativum* (28.4%), whereas *M. longifolia* showed the least inhibition. The antifungal activity of plant extracts against the mold pathogens may be ascribed to antifungal volatile and nonvolatile metabolites such as allicin, ajoene, azadirachtin, nimbidin, nimbinin, nimbolidin, and nimbin, which are responsible for damaging the fungal cell walls [33]

Furthermore, during the in vivo evaluation of botanicals at 2% concentration, *J. regia* and *C. longa* each had the highest average weight (11.8–11.9 g) of a single fruit body and a combined button yield of 11.3–11.9 kg/quintal compost. Several authors reported that some bioactive components of *Azadirachta indica*, *Eucalyptus camaldulensis*, *Cymbopogon marginatus,* and *Citrus lemon* are capable of enhancing the potential yield of mushrooms and slow down the progression of pathogenic microbes in oyster mushroom cultivation [34]. Strong activity has been shown by biochemically active substances such as essential oils from plants, particularly from basil and mint oils, against *T. aggressivum* f. *europaeum* [35]. The study is also in line with the work of Shaiesta et al. [32] and Kumar et al. [36] who reported that *A. indica* was the most effective botanical and showed maximum inhibition against *T. harzianum* without inhibiting the mushroom mycelium. The findings indicate the potential usefulness of *J. regia, A. sativum,* and *C. longa* as amendments or sprays in soil or compost casing to reduce green mold to low levels.

Several bacterial species viz., *Pseudomonas* spp., *Bacillus* spp., and *Actinomycetes* were previously tested for their antifungal activity against *Trichoderma sp*. and showed potential mycelial growth inhibition of *T. harzianum* to a much greater extent and stimulated defense mechanisms through the production of enzymes such as laccases [37]. The use of different concentrations of bacterial antagonists viz., *P. fluorescens*, *Azotobacter* sp., or *B. subtilis* in infested casing material also yielded noticeable control of green mold disease with parallel enhancement in yield and yield components. The present study shows that *Pseudomonas* spp., *Azotobacter* spp., and *B. subtilis* exhibited no confrontational effects on *A. bisporus* in vitro and, simultaneously, exhibited inhibitory effects of varying degrees on the mycelial growth of *T. harzianum,* except for *Azotobacter* spp isolate 117 and 106 that showed neutral effects towards the pathogen. The application of *B. subtillis* at 2.0% concentration significantly displayed disease control of 91.9%, followed by the treatment of *P. flourescens* at 2% and *B. subtilis* at 1% concentrations. Stanojevic et al. [38] reported that bacterial isolates were highly effective against the mycelial growth of *T. aggressivum* f. *europaeum,* indicating their potential use as an effective biocontrol agent for the management of mold diseases. Under in vivo conditions, the application of bacterial antagonist isolates in pathogen-infested casing material showed significant and stimulating effects on mushroom yield and yield components viz., the weight and number of fruit bodies. Aydoğdu et al. [21] also reported that bacterial isolates MSG-15 and MSG-11 caused increases in the yield of white button mushroom of up to 28.9 and 38.7%, respectively, in the treated plots.

5. Conclusions

The ethanol extracts of *J. regia* and *A. sativum* showed significant mycelial inhibition of *T. harzianum* but no/least inhibition of *A. bisporus*. *Juglans regia* at a concentration of 3% was most effective in inhibiting the mold fungus while having the least effect on host mycelium and a positive effect on mushroom yield. Therefore, the present study reveals that a treatment of botanicals/bacterial isolates on compost and soil casing may cause substantial increases in yield by subduing green mold disease.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8090768/s1, Figure S1: Locally available botanicals used against green mold pathogen of button mushroom under in vitro and in vivo condition, Figure S2: In vitro evaluation of ethanol extract of botanical against pathogen (*T. harizianum*) and host (*A. bisporus*), Figure S3. Evaluation of bioassay of bacterial antagonists and botanicals against the green mold disease of white button mushroom, Figure S4: In vitro evaluation of bacterial antagonists against the green mold pathogen (*T. harzianum*) and white button mushroom (*A. bisporus*), Table S1: Mean sum of squares of botanical extracts, TableS2: Mean sum of squares of bacterial antagonists, Table S3. In vivo effect of botanical extracts on quality parameters of white button mushroom (*Agaricus bisporus*) and Table S4. In vivo effect of bacterial antagonists on quality parameters of white button mushroom (*Agaricus bisporus*).

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