



## Article

# Identification and Characterization of Triple Action Bioagents (TAB) and Their Potency against Fusarium Wilt of Lentil

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**Abstract:** *Fusarium* wilt is a severe disease that plays a significant role in reducing the yield of lentil. Under favorable conditions for disease growth, the disease can cause complete crop failure and can be a crucial limiting issue for lentil cultivation in specific geographical zones. The current work focused on isolating potential bio-agents exhibiting copper oxychloride resistance and evaluating their efficacy in seed treatment for ecologically sustainable management of *Fusarium* wilt of lentil. Seventy biocontrol agent isolates were isolated and tested for resistance by growing them on Potato Dextrose Agar medium (PDA) amended with copper oxychloride at the rate of 2500 ppm. Isolate-H10 and isolate-C9 showed more excellent compatibility with copper oxychloride fungicide with 69 mm and 65 mm radial growths, respectively. The isolates H10 and C9 had the highest inhibitory percentages of 84.30% and 83.94% against *Fusarium oxysporum* f. sp. *lentis*, respectively, and the highest phosphorus solubilization index (PSI). Primers (ITS 1 and ITS 4) identified these putative bioagents as *Trichoderma harzianum* isolate skua-tab-1 and *Penicillium crysogenum* strain Tab2. Sequences were submitted to the NCBI and assigned the accession numbers MK414603 and MK418066. In pot culture, these isolates also demonstrated their superiority in reducing the disease incidence and severity if seeds were treated with H10 and C9 alone or in combination with copper oxychloride fungicide. The two isolated bioagents exhibit three fundamental properties: compatibility with copper oxychloride, antagonistic activity toward the pathogen fall armyworm, and the ability to dissolve phosphorus minerals.

**Keywords:** bio-agents; copper oxychloride; phosphorus solubilization; *Fusarium* wilt; lentil

## 1. Introduction

Lentil (*Lens culinaris* Medik) is among the potential food legume crops in many farming and food systems [1]. Wilt of lentil (*F. oxysporum* f. sp. *lentis*) is a severe disease reducing lentil yield in India and the world [2]. The *Fusarium* wilt causes yield losses up to 50% in India and may cause complete crop failure under favorable conditions for disease

development and can be the primary limiting factor for lentil cultivation in certain crop areas. If *F. oxysporum* f. sp. *lentis* attacks the crop at seedling and pre-pod stages, the plant wilt can be as high as 100 and 67% when it occurs at flowering and pod stage [3]. Lentil wilt is defined by abrupt leaf drooping (more like wilting and damping off), followed by the leaves drying and the seedling's eventual mortality. The root system appears to be robust, although with a lower rate of proliferation and modulation [4]. Disease management can be done through cultural, chemical, biological methods and incorporating resistant varieties in the agricultural system. In the absence of resistance/tolerant varieties, it is difficult to manage the disease caused by soil-borne pathogens because of the complex edaphic environment of physical, chemical, and biological origin [5]. Broad-spectrum fungicides cause environmental pollution and have a detrimental effect on human health.

Moreover, the development of fungicidal resistance in pathogenic fungi is becoming a significant issue, and they alone cannot provide long-term protection and often need repeated applications [6]. Broad-spectrum fungicide sprays offer proper management, but they cause imbalance to the microbial community, harmful effects on the ecosystem and human life due to the presence of carcinogenic chemical residues in the food chain [7]. A beneficial bio-antagonist might be a suitable alternative to reducing the chemical input in the agricultural systems [8]. The use of specific microbes to control diseases biologically may be an alternative or complement to synthetic chemicals [9]. Integrating biological seed protectants with a fungicidal seed treatment that eliminates competitors may enhance the establishment of desired biocontrol agents and provide better control of seed and seedling diseases [10]. Recent studies indicate that copper oxychloride is effective against wilt-causing pathogens in pulses like chickpea [11,12] as well as cotton [13]. Applying potential bioagents with commonly used copper fungicide might be an efficient strategy for managing the lentil wilt [14]. *Trichoderma* sp. effectively inhibits the growth of lentil wilt pathogen [15]. *Trichoderma* sp. successfully hampers the development of lentil wilt pathogen [16]. Owing to the importance of bioagents and copper fungicides in disease control, the objective of this investigation was to isolate, screen, and identify potential bioagents that are resistant to the fungicide copper oxychloride and evaluate their antagonistic potential against lentil fusarium wilt.

## 2. Materials and Methods

### 2.1. Isolation of Pathogen

The isolation of the causal agent was conducted by the tissue sample transfer technique [17]. The symptomatic diseased stems and roots were cut into small bits (2–3 mm) with a sharp, sterilized blade so that each diseased tissue contained a portion of healthy tissue along with it. These bits were subjected to surface sterilization with 1% sodium hypochlorite solution for 30 s, followed by three rinses with distilled sterilized water to remove the remaining traces of sodium hypochlorite. The tissue pieces were blotted, dried, and later transferred aseptically to Potato Dextrose Agar (PDA—potato 200 g; dextrose 20 g; agar 15 g; distilled water 1000 mL) medium in sterilized Petri-plates (9 cm) and incubated at  $25 \pm 1$  °C for seven days. The fungal colonies emanating from bits were examined after seven days of incubation, then transferred on fresh medium in Petri-plates and incubated again at  $25 \pm 1$  °C for periodic observations to record observations like mycelial characteristics color, shape, size, and septation of spores.

### 2.2. Pathogenicity Test

The sick-soil method was used to test the pathogenicity [18]. The sand-maize (3:1) medium was autoclaved for 20 min at 15 psi in a 250 mL flask with 20 mL distilled water, and the media was autoclaved for three consecutive days. To mass multiply the inoculum, each sterilized flask was inoculated with a 7 mm disc of an actively growing 7-day fungal culture and incubated at  $25 \pm 1$  °C for 15 days. The plastic containers were thoroughly washed with sterilized distilled water, dried, and then sprayed with ethanol to prevent saprophytic contamination. Five days before sowing, all plastic pots (15 cm) were filled

with sterilized soil (2 kg pot<sup>-1</sup>) and inoculated with *F. oxysporum* f. sp. *lentis* (10 g kg<sup>-1</sup> soil). Seventy bio-agent isolates were obtained from various locations throughout the Kashmir valley (cultivated, uncultivated, and forest areas). Soil samples from a two and a half square foot (0.23 m<sup>-2</sup>) patch were taken after the land had been pre-treated with 2500 ppm copper oxychloride for 24 h. The samples were kept in polythene bags labeled with the location's name. Serial dilution and plating have been used to isolate the bioagents from these samples. One-pot with sterilized soil in the same quantity was not inoculated and served as a control. In each pot, five seeds of a susceptible lentil variety (Shalimar Masur-1) were sowed (surface sterilized with 1% sodium hypochlorite for two minutes followed by rinsing thrice with sterile distilled water). Irrigation was performed on a regular and adequate basis as needed. Both inoculated and non-inoculated pots were kept in the greenhouse to assess disease incidence and intensity, and three replicates were maintained in each treatment using a completely randomized design (CRD).

### 2.3. Isolation of Copper Oxychloride Resistant Bioagents

Seventy bio-agent isolates were obtained from various locations throughout the Kashmir valley (cultivated, uncultivated, and forest areas). Soil samples from a two-and-a-half square foot (0.23 m<sup>-2</sup>) patch was taken after the land had been pre-treated with 2500 ppm copper oxychloride for 24 h. The samples were kept in polythene bags labeled with the location's name and conveyed to the lab. Serial dilution and plating have been used to isolate the bioagents from these samples. Trichoderma specific medium (TSM—Magnesium sulfate heptahydrate 0.200 g; dipotassium hydrogen phosphate 0.900 g; ammonium nitrate 1.0 g; potassium chloride 0.159 g; dextrose 3 g; rose Bengal 0.150 g; agar 20 g and distilled water 1000 mL) was used as the plating medium for the selective isolation of fungal potential biocontrol agents. Colonies that appeared morphologically distinct were aseptically purified on Potato Dextrose Agar (PDA). Purified isolates were preserved at 4 °C in a refrigerator for further research.

### 2.4. Compatibility of Biocontrol Agents with Copper Oxychloride Fungicide In Vitro

Standard poisoned food technique [14] was adopted to confirm the resistance of isolated biocontrol agents against the copper oxychloride fungicide. The potato dextrose agar (PDA) was amended with the concentration of 2500 ppm copper oxychloride (Blitox 50% WP). 18 mL of amended medium was aseptically poured into the sterilized Petri-plates (9 cm). 18 mL of amended medium was poured onto the sterilized Petri-plates aseptically (9 cm). The plates were inoculated with a 5 mm disc of biocontrol agents that were 7 days old. Each biocontrol isolate and test pathogen was replicated thrice, and the inoculated plates were incubated at 25 ± 1 °C in Bio-Oxygen-Demand (BOD) incubator. After 5 days of inoculation, the biocontrol isolates' radial growth was measured up to 14 days. The experiment was carried out with the help of a CRD design. This method has been shown to be very significant in determining whether a biocontrol fungus is compatible with specific fungicides [19].

### 2.5. In Vitro Screening of Isolated Biocontrol Agents against *F. oxysporum* f. sp. *lentis*

The dual culture approach [20] was used to screen antagonists against *F. oxysporum* f. sp. *lentis*. 20 mL of sterilized melted Potato Dextrose Agar (PDA) medium was poured aseptically into sterilized petri-plates, allowed to solidify. Then, 6 mm discs of the test fungus and biocontrol isolates were cut with a sterilized cork borer, placed on PDA plates at opposite ends of 9 cm petri-plates, and incubated Bio-Oxygen-Demand (BOD) incubator at 25 ± 1 °C for 96 h. For each treatment, three replications were kept. The diameter of the colony of biocontrol isolates and the test fungus were measured following CRD experimental design. Observations of % inhibition of mycelium were recorded using the formula:

$$I = (C - T)/C \times 100$$

where,

I = Percent Inhibition;  
C = Colony diameter (mm) in control;  
T = Colony diameter (mm) in treatment.

### 2.6. Phosphorus Solubilizing Potential of Effective Biocontrol Agents

Ten biocontrol agents (resistant to copper oxychloride fungicide) exhibiting highest antagonistic activity against the pathogen *in vitro* were selected for their phosphorus solubilizing activity. Bioagents were inoculated on sterilized 9 cm petri-plates containing Pikovskaya's Agar (PKA) medium containing the following constituents: glucose 10 g; tri-calcium phosphate (TCP) 5 g; yeast extract 0.5 g; ammonium sulfate 0.5 g; potassium chloride 0.2 g; sodium chloride 0.2 g; magnesium sulfate 0.1 g; ferrous sulfate trace; magnesium sulfate trace; agar 15 g; distilled water 1 l. The ability of bioagents to solubilize tri-calcium phosphate (TCP) on Pikovskaya's agar medium (PKA) was measured quantitatively, determining the solubilization index (SI). The phosphate solubilization index was calculated by measuring the colony diameter (mm) and the halo zone diameter (mm), using the formula as below [21]. Phosphate solubilization index (SI) = (colony diameter (mm) + halo zone diameter (mm))/colony diameter (mm).

### 2.7. Identification of Potential Biocontrol Agents

Two isolates of biocontrol agents (resistant to copper oxychloride fungicide) were chosen from a total of seventy isolates and identified based on cultural and morphological characteristics such as colony characteristics, mycelia growth, color, size, and sporulation of the fungus. Precise molecular identification was conducted by DNA sequencing [22]. Extracted and purified DNA of the sample was quantified by loading 5  $\mu\text{L}$  of DNA of sample mixed with 3  $\mu\text{L}$  of flooding buffer (ready to use) and 10  $\mu\text{L}$  of distilled water into separate wells and on 1% agarose gel. DNA concentration standards were also loaded to estimate the concentration of DNA in each sample. The electrophoresis was carried out at 75 V for 2 h and subjected to a gel documentation system (DNR Bioimaging system, Modi'in-Maccabim-Re'ut, Israel). The concentration of DNA was determined by comparing the intensity of genomic DNA bands with that of known fragments. The isolated DNA template was subjected to PCR amplification using ITS 1 and ITS 4 primers. Under the following thermal conditions: 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 8 min using a peq STAR 2X Thermocycler (PEQLAB, Hampshire, UK). The amplified product was custom sequenced from Genei, Bangalore. The sequence of PCR amplified products obtained from Genei, Bangalore, Karnataka, India, was subjected to NCBI (National Centre of Biotechnology Information) BLAST database, and precise identification of bioagents was established. The sequence was submitted to GenBank and secured.

### 2.8. Mass Multiplication of Potential Bioagents

Pure cultures of potential bioagents were mass multiplied in 250 mL flasks with 150 mL potato dextrose broth for 7 days (PDB). After 7 days, the fungal mats were extracted and mixed for 30 s in 200 mL of distilled water. This stock sample was serially diluted ( $10^{-6}$ ) until it reached a concentration of  $2 \times 10^{-8}$  spores  $\text{mL}^{-1}$ . A fungal biocontrol agent was combined with Kaolinite powder (in a 1:3 ratio) in distilled water, which served as a carrier material. To obtain a homogeneous powder, kaolinite and charcoal were crushed and sieved. Kaolinite: charcoal in the ratio of 4:1 (*w/w*) was autoclaved in a 1000 mL flask for one and half hours at 121.6 °C at 1.5  $\text{kg cm}^{-2}$  for two successive days. The spore suspension (at a rate of  $2 \times 10^6$  spores  $\text{mL}^{-1}$ ) was blended with Kaolinite charcoal powder (1:3 ratio). The resultant powder was stored at 4 °C for further use.

### 2.9. Evaluation of Potential Copper Oxychloride Resistant Bioagents against Fusarium Wilt of Lentil in Pots

Pot experiment trial was conducted in the greenhouse (November–March in consecutive two years 2017–2018 and 2018–2019) at Faculty of Agriculture, SKUAST-K, and Wadura using completely randomized design (CRD). Six treatments, including untreated control, were carried out in this experiment. Three replications were maintained in each treatment. Seed treatments with potential bioagents and copper oxychloride fungicide individually as well as in combination were evaluated in this experiment as per the following treatment details:

(1) TAB-1 + copper oxychloride (lentil seeds were treated with a paste/slurry containing potential bioagent 1 at a rate of  $2 \times 10^8$  spores  $\text{mL}^{-1}$  and Kaolinite charcoal powder (1:3 ratio) at a rate of  $3 \text{ gm kg}^{-1}$  seed + copper oxychloride at a rate of  $2.5 \text{ gm kg}^{-1}$  seed. (2) TAB-2 + copper oxychloride (potential bioagent 2 at a rate of  $2 \times 10^8$  spores  $\text{mL}^{-1}$  and Kaolinite charcoal powder (1:3 ratio) + copper oxychloride at a rate of  $2.5 \text{ gm kg}^{-1}$  seed). (3) TAB-1 only (paste/slurry made of potential bioagent 1 at a rate of  $2 \times 10^8$  spores  $\text{mL}^{-1}$  and Kaolinite charcoal powder (1:3 ratio). (4) TAB-2 (paste/slurry made of potential bioagent 2 at a rate of  $2 \times 10^8$  spores  $\text{mL}^{-1}$  and Kaolinite charcoal powder (1:3 ratio). (5) Copper oxychloride at a rate of  $2.5 \text{ gm kg}^{-1}$  seed only, and (6) control (seeds treated with Kaolinite powder only not having bioagent). Lentil seeds of the susceptible variety Shalimar Masur-1 were used in the study. The seeds were imbibed in water for 24 h before receiving treatment. In the case of seed treatment with copper oxychloride, seeds were coated with copper oxychloride powder at the rate of  $2.5 \text{ gm kg}^{-1}$  seed. Seed treatment with bioagents was conducted by mixing bioagents at the rate of  $3 \text{ gm kg}^{-1}$  seed kaolinite-based bio-formulation. In a treatment combination of copper oxychloride fungicide and bioagents, lentil seeds were first treated with copper oxychloride at the rate of  $2.5 \text{ gm kg}^{-1}$  seed followed by Kaolinite-based bioformulation at the rate of  $3 \text{ gm kg}^{-1}$  of seed. Seeds that were treated with Kaolinite powder only were used as control. Seeds in each treatment were slightly moistened with sterile distilled water so that a thin film of treatment was layered on all seeds. Five seeds from each treatment were sown in pots filled with 2 kg of sterile soil, sand, and FYM in the ratio of 2:1:1 in polyhouse. The soil in each pot was inoculated with *F. oxysporum* f. sp. *lentis* has grown on sand maize medium at the rate of  $10 \text{ gm kg}^{-1}$  soil.

### 2.10. Disease Incidence

Percent of disease incidence was calculated by formula:

$$\text{Disease incidence (\%)} = \frac{\text{number of diseased plants}}{\text{Total number of plants examined}} \times 100$$

### 2.11. Disease Severity

Plants showing wilt symptoms on leaves were categorized using a 1 to 5 scale [23]. Wherein grade value—1 = No symptoms, 2 = yellowing of basal leaves only, 3 = yellowing of 50% foliage, 4 = complete yellowing of foliage, 5 = whole plant or unilateral shoot is wilted.

Percent Disease severity was calculated using the formula:

$$\text{Disease severity index (\%)} = \frac{\sum(n \times v)}{N \times G} \times 100$$

where,

n = number of diseased plants observed;

v = numerical grade value;

N = total number of plants observed;

G = highest grade value.

Observations of incidence and severity were recorded at 45 days.

### 2.12. Statistical Analysis

The data were statistically analyzed using IBM SPSS Statistics Base 22.0, and critical difference value was used to compare the treatment means. Data transformation using arcsine transformation was carried out wherever necessary. Analysis of variance of the data was carried out at a 5% ( $p < 0.05$ ) level of significance.

## 3. Results

### 3.1. Pathogenicity and Morphological Characterization of the Isolated Pathogen

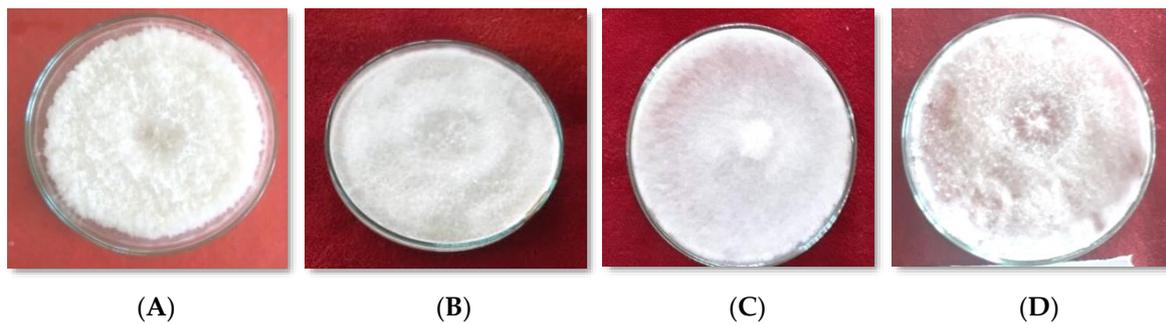
The pathogenicity of the isolated fungus was established by confirming Koch's postulates using the sick soil method (Figure 1A,B). Initiation of typical symptoms of the disease was observed after 16–20 days of inoculation. However, no symptom development was observed in the control. Microscopic observations of the isolated fungus revealed that the mycelium was septate and hyaline with hyphal width of 4.34–5.01  $\mu\text{m}$ . The fungus produced cottony and fluffy growth on PDA initially white and later turned pinkish-white after 25–30 days of incubation (Figure 2A,D). The pathogen produced three types of spores within the culture, i.e., microconidia, macroconidia, and chlamydo spores. Microconidia were hyaline, aseptate, cylindrical to falcate with an ovoid shape. The length  $\times$  breadth of the microconidia ranged from 5.2–(5.6)–6.0  $\times$  2.2–(3.0)  $\times$  3.5  $\mu\text{m}$ . Macroconidia ranging from 27.2–(28.2)–37.5  $\times$  2.6–(3.6)  $\times$  2.6  $\mu\text{m}$  were found to be septate (3–5 septa). They were sickle-shaped with slightly foot-shaped basal cells. Chlamydo spores were produced 12–15 days after incubation in cultures. They were globose, single-celled, aseptate, produced terminally or intercalary with 7.2–10.5  $\times$  8–15.0  $\mu\text{m}$  size (Figure 3A,D).

### 3.2. Isolation of Copper Oxchloride Resistant Biocontrol Agents

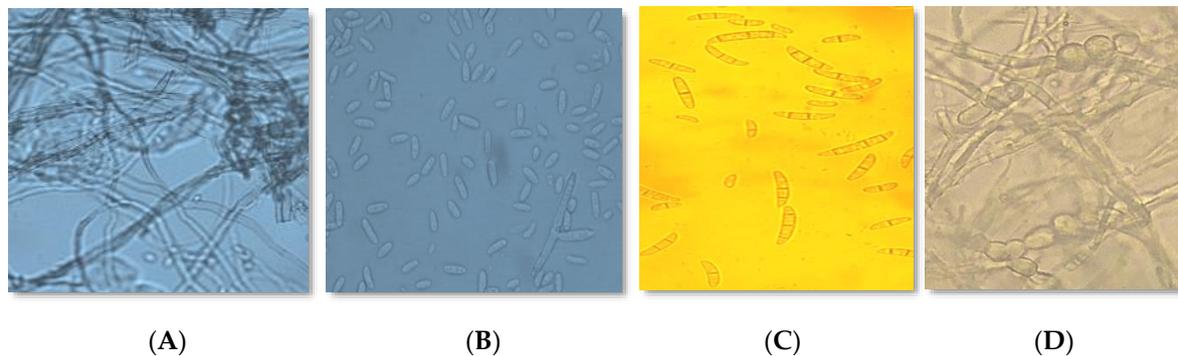
A total of seventy isolates of biocontrol agents were obtained from three locations of Kashmir valley—from forest/cultivated/uncultivated areas of Verinaag (Anantnag district), of natural regions of Wadura and Tarzoo (Baramulla district), and from cultivated/natural areas of Saloor and Safapora (Ganderbal district) (Table 1).



**Figure 1.** Pathogenicity test of *F. oxysporum* f. sp. *lentis* on lentil plant. (A) Uninoculated. (B) Inoculated.



**Figure 2.** Colony characteristics of *F. oxysporum* f. sp. *lentis*; (A) 7-day old colony; (B) 15-day old colony; (C) 25-day old colony; (D) 35-day old colony.



**Figure 3.** Morphological characteristics of *F. oxysporum* f. sp. *lentis* (A) Mycelium; (B) Microconidia; (C) Macroconidia; (D) Chlamydospores ( $\times 400$ ).

**Table 1.** Biocontrol isolates obtained from different regions of Kashmir.

S. No. *	Isolate Code	Place of Collection	S. No.	Isolate Code	Place of Collection	S. No.	Isolate Code	Place of Collection
District Ganderbal			23	L3	Behaama	45	H6	Verinag
1	PH3	Saloor	24	L4	Behaama	46	H7	Verinag
2	PH6	Saloor	25	L5	Behaama	47	H8	Verinag
3	KG-1	Saloor	26	L6	Behaama	49	H10	Verinag
4	KG-3	Saloor	27	L7	Behaama	50	M1	Verinag
5	KG-4	Saloor	28	L8	Behaama	51	M8	Verinag
6	P1	Saloor	29	L9	Behaama	52	M9	Verinag
7	P2	Saloor	District Anantnag			53	W9	Verinag
8	P3	Saloor	30	C1	Verinag	54	W10	Verinag
9	P5	Saloor	31	C2	Verinag	District Baramulla		
10	P6	Saloor	32	C3	Verinag	55	M2	Tarzo
11	P8	Saloor	33	C4	Verinag	56	M3	Tarzo
12	O4	Saloor	34	C5	Verinag	57	M4	Tarzo
13	O2	Saloor	35	C6	Verinag	59	M5	Tarzo
14	O1	Saloor	36	C7	Verinag	60	M6	Tarzo
15	O3	Saloor	37	C8	Verinag	61	M7	Tarzo
16	O6	Saloor	38	C9	Verinag	62	M10	Wadura
17	O5	Saloor	39	C10	Verinag	65	W1	Wadura
18	O10	Saloor	40	C16	Verinag	66	W2	Wadura
19	O5	Saloor	41	Vn-1	Verinag	67	W3	Wadura
20	O9	Saloor	42	Vn-2	Verinag	68	W4	Wadura
21	L1	Saloor	43	H2	Verinag	69	W5	Wadura
22	L2	Saloor	44	H3	Verinag	70	W8	Wadura

\* Serial number.

### 3.3. Compatibility of Biocontrol Agents with Fungicide

Among the seventy bioagents evaluated using the poison food technique, the highest radial growth (mm) was observed in isolate H10 (69 mm) followed by isolate C9 (65 mm) after 5 days of incubation on PDA amended with 2500 ppm copper oxychloride. The most negligible radial growth was observed in isolate M10 (1.0 mm) (Figure 4 and Table 2).

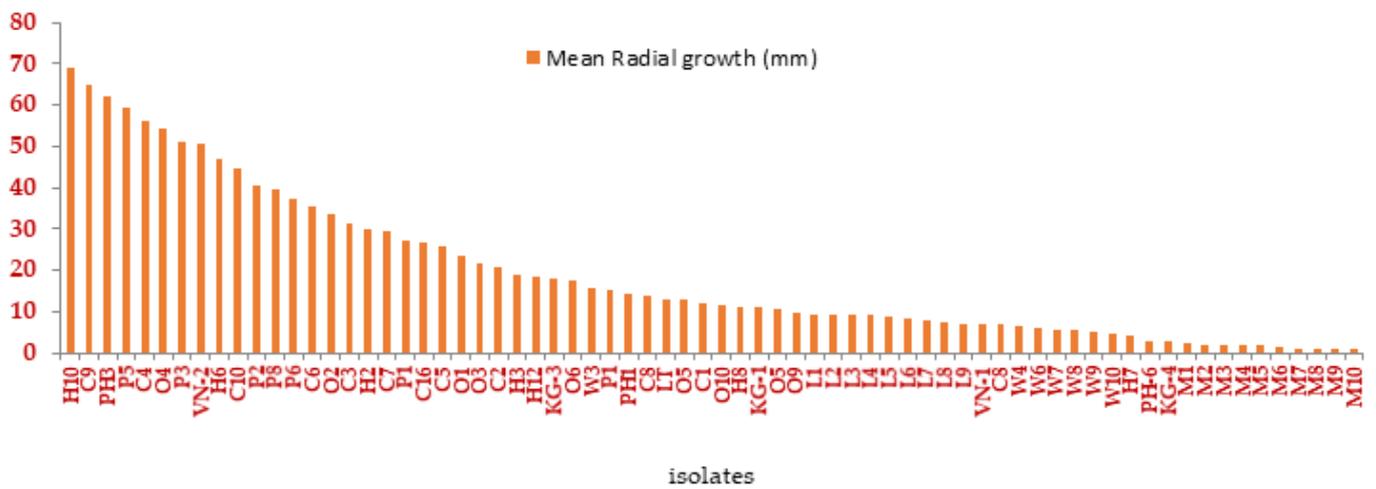
**Table 2.** The radical growth of biocontrol agents on Potato dextrose media (PDA) was amended with 2500 ppm copper oxychloride recorded after 5 days of incubation (Poisoned Food Technique).

Scheme	Bioagents	Mean Radial Growth (mm)	S. No.	Bioagents	Mean Radial Growth (mm)
	H10	69.00 *	38.	KG-1	11.00
	C9	65.00	39.	O5	10.60
	PH3	62.00	40.	O9	9.50
	P5	59.16	41.	L1	9.40
	C4	56.16	42.	L2	9.16
	O4	54.23	43.	L3	9.00
	P3	51.30	44.	L4	9.40
	VN-2	50.73	45.	L5	8.63
	H6	46.93	46.	L6	8.10
	C10	44.50	47.	L7	7.80
	P2	40.33	48.	L8	7.40
	P8	39.56	49.	L9	7.00
	P6	37.50	50.	VN-1	6.70
	C6	35.23	51.	C8	6.70
	O2	33.53	52.	W4	6.40
	C3	31.46	53.	W6	6.00
	H2	29.76	54.	W7	5.56
	C7	29.50	55.	W8	5.40
	P1	27.00	56.	W9	4.90
	C16	26.86	57.	W10	4.50
	C5	25.70	58.	H7	4.00
	O1	23.50	59.	PH-6	3.00
	O3	21.70	60.	KG-4	2.90
	C2	20.73	61.	M1	2.40
	H3	18.93	62.	M2	2.06
	H12	18.26	63.	M3	1.73
	KG-3	18.13	64.	M4	1.66
	O6	17.43	65.	M5	1.63
	W3	15.80	66.	M6	1.40
	P1	15.33	67.	M7	1.16
	PH1	14.50	68.	M8	1.16
	C8	13.96	69.	M9	1.11
	LT	12.86	70.	M10	1.00
	O5	12.70			
	C1	11.96			
	O10	11.43			
	H8	11.13			
				C.D ( $p \leq 0.05$ )	1.3

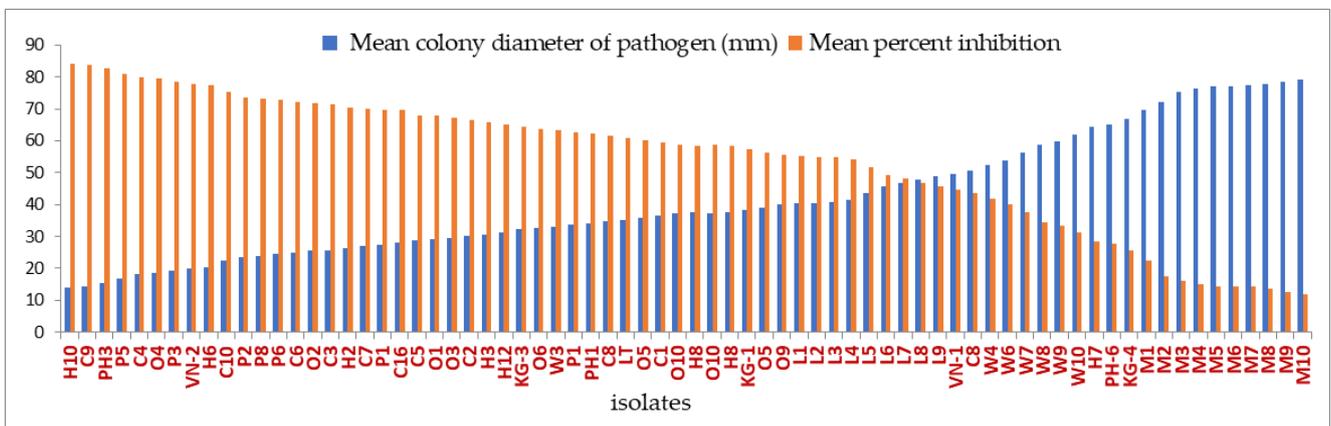
\* Average of three replications.

### 3.4. Dual Culture Assay

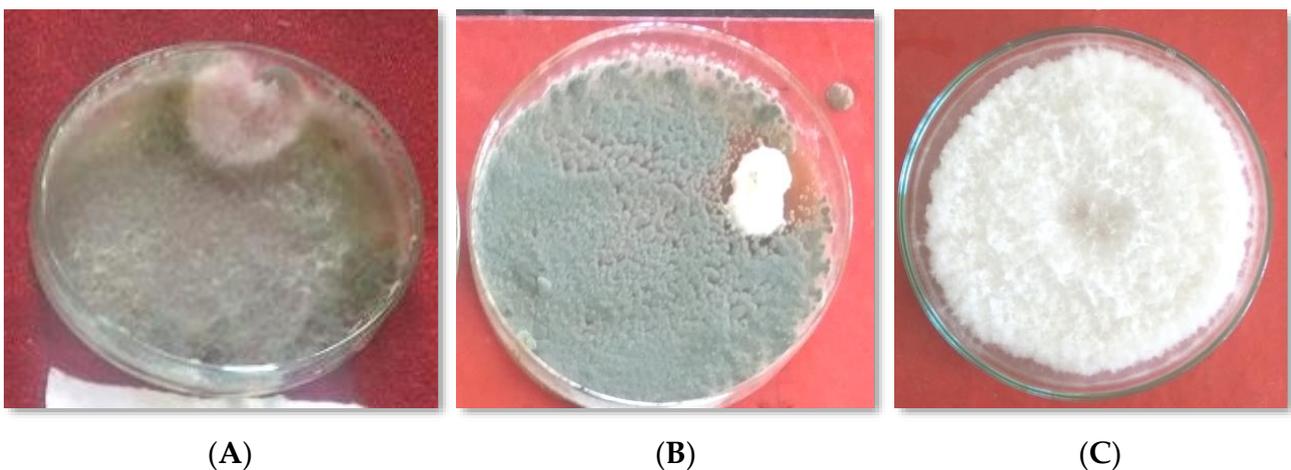
Seventy fungal bioagents were screened in vitro against *F. oxysporum* f. sp. *lentis* for their antagonistic activity via dual culture technique [20] on Potato dextrose agar (PDA). It is evident from the data (Table 3; Figures 5 and 6A–C) that all the bioagents proved antagonistic against *F. oxysporum* f. sp. *lentis*. Minimum colony diameter of pathogen 14.08 mm and 14.41 mm was recorded in dual culture with isolates- H10 (Figure 6A) and C9 (Figure 6B), respectively as compared to control (Figure 6C), where a colony diameter of 85.00 mm was recorded after 7 days. Maximum inhibition percentages of 84.30 and 83.94 of the pathogen were recorded by bioagents- H10 and C9, respectively.



**Figure 4.** Radial growth (y-axis 0 to 80) of biocontrol agents (x-axis-isolate name from H10 to M10) on Potato dextrose media (PDA) amended with 2500 ppm copper oxychloride recorded after 5 days of incubation.



**Figure 5.** In vitro efficacy of bioagents (x-axis isolate name from H10 to M10) against mycelial growth of *F. oxysporum* f. sp. *lentis*.



**Figure 6.** In vitro efficacy of bioagents H10 (A) and C9 (B) against mycelial growth of *F. oxysporum* f. sp. *lentis* (C).

**Table 3.** In vitro efficacy of bioagents against mycelial growth of *Fusarium oxysporum* f. sp. *lentis*.

Bioagents	Mean Colony Diameter of Pathogen (mm)	Mean % Inhibition	Bioagents	Mean Colony Diameter of Pathogen (mm)	Mean % Inhibition
H10	14.08	84.30 * (66.63)	O10	37.20	58.66 (49.96)
C9	14.41	83.94 (66.35)	H8	37.70	58.29 (49.75)
PH3	15.41	82.85 (65.51)	KG-1	38.40	57.33 (49.19)
P5	17.00	81.11 (64.21)	O5	39.16	56.47 (48.70)
C4	18.20	79.75 (63.23)	O9	40.00	55.55 (48.16)
O4	18.50	79.40 (62.98)	L1	40.33	55.18 (47.95)
P3	19.20	78.66 (62.46)	L2	40.53	54.94 (47.82)
VN-2	19.86	77.92 (61.94)	L3	40.83	54.95 (47.82)
H6	20.20	77.55 (61.69)	L4	41.43	54.29 (47.44)
C10	22.30	75.18 (60.09)	L5	43.53	51.62 (45.91)
P2	23.60	73.75 (59.12)	L6	45.66	49.25 (44.55)
P8	24.00	73.33 (58.88)	L7	46.66	48.14 (43.91)
P6	24.50	72.77 (58.16)	L8	47.76	46.92 (43.21)
C6	25.00	72.22 (57.81)	L9	48.80	45.77 (42.55)
O2	25.50	71.66 (57.69)	VN-1	49.73	44.73 (41.96)
C3	25.60	71.48 (57.09)	C8	50.70	43.66 (41.34)
H2	26.50	70.51 (56.76)	W4	52.43	41.73 (40.22)
C7	27.00	70.00 (56.53)	W6	54.00	40.00 (39.21)
P1	27.30	69.62 (55.91)	W7	56.20	37.55 (37.77)
C16	28.20	69.62 (55.57)	W8	58.90	34.55 (35.98)
C5	28.73	68.06 (55.38)	W9	59.80	33.55 (35.38)
O1	29.00	67.77 (55.00)	W10	61.90	31.21 (33.94)
O3	29.56	67.14 (54.53)	H7	64.50	28.33 (32.14)
C2	30.26	66.36 (54.23)	PH-6	65.13	27.62 (31.69)
H3	30.70	65.88 (53.77)	KG-4	66.96	25.58 (30.37)
H12	31.40	65.10 (53.26)	M1	69.83	22.40 (28.23)
KG-3	32.16	64.25 (53.01)	M2	72.13	17.62 (24.80)
O6	32.53	63.84 (52.68)	M3	75.33	16.29 (23.78)
W3	33.03	63.29 (52.22)	M4	76.30	15.22 (22.95)
P1	33.73	62.51 (52.09)	M5	76.93	14.51 (22.38)
PH1	33.93	62.29 (51.59)	M6	77.00	14.44 (22.32)
C8	34.70	61.44 (51.35)	M7	77.46	14.29 (22.20)
LT	35.06	61.03 (50.92)	M8	77.80	13.55 (21.59)
O5	35.73	60.29 (50.42)	M9	78.50	12.77 (20.93)
C1	36.50	59.44 (49.96)	M10	79.26	11.92 (20.18)
O10	37.20	58.66 (49.96)			
H8	37.70	58.29 (49.75)		C.D ( $p \leq 0.05$ )	0.61

\* Average of three replications, values in brackets were arc sine transformed values.

### 3.5. Phosphorus Solubilization Test of Effective Biocontrol Agents

Ten biocontrol agents (H10, C9, PH3, C4, P3, O4, P5, C10, H6, and Vn-2) that proved to be highly antagonistic in dual culture were tested for their solubilizing activity in vitro in a phosphorylation assay. Biocontrol isolates H10 and C9 showed the highest solubilization index (SI) on Pikovsky's medium (PKA), with a phosphate solubilization index of 2.87 and 2.51 for C9 and H10, respectively (Table 4 and Figure 7).

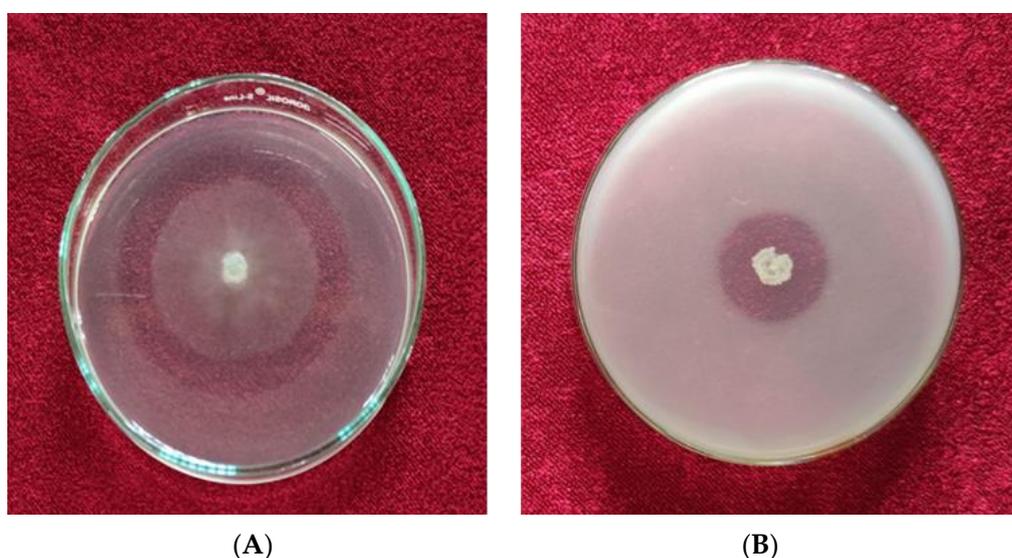
### 3.6. Identification of Efficient Biocontrol Agents

DNA sequencing identified two biocontrol agents effective in the poisoned food technique, dual culture and phosphorus solubilization test. Genie labs sequenced the amplified products acquired after PCR amplification using the ITS-1 and ITS 4 markers. The sequences were identified as *Trichoderma harzianum* isolate skua-tab1 and *Penicillium crysogenum* strain Tab2 after BLAST(632 bp and 576 bp). The sequences of bioagents H10 and C9 have been uploaded onto NCBI as *Trichoderma* sp. Skau-tab-1 and *Penicillium* sp. strain Tab2, respectively, with accession numbers MK414603 and MK418066.

**Table 4.** Phosphorus solubilization potential bioagents.

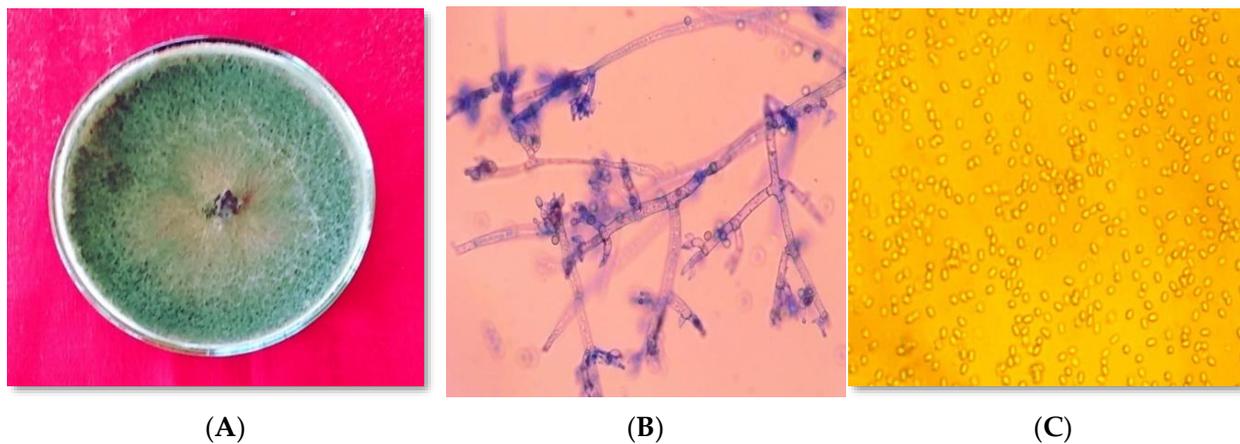
S. No.	Isolates of Bioagents	* Mean Phosphate Solubilization Index (PSI)
1	C9	2.87
2	H10	2.50
3	PH3	2.25
4	C4	2.00
5	P6	1.75
6	O5	1.61
7	P5	1.48
8	C10	1.35
9	H6	1.26
10	Vn-2	1.13
C.D ( $p \leq 0.05$ )		0.22

\* Average of three replicates.

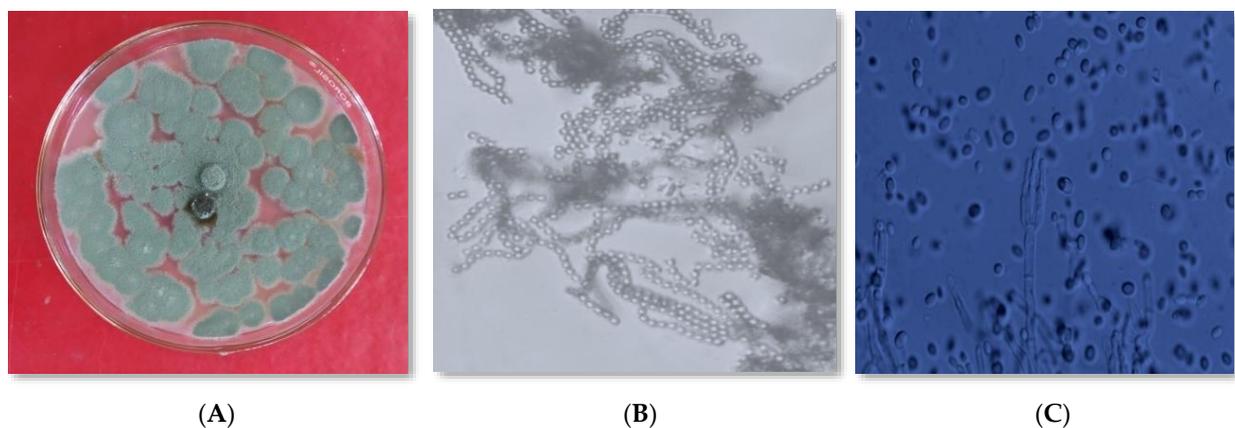
**Figure 7.** Phosphorus solubilization—(A) Holo zone by bioagent H10; (B) Holozone formed by Bioagent C9.

### 3.7. Morphological Characteristics of Biocontrol Agents *Trichoderma harzianum* Isolate Skau-Tab-1 (TAB-1) and *Penicillium crysogenum* sp. Strain Tab-2 (TAB-2)

Using a 5–10 day-old culture, the morphological characteristics of different structures formed by biocontrol agents, such as mycelium, conidia, and phialides, were studied. TAB-1 and TAB-2 pure cultures were examined for colony features. TAB-1 does have a cottony white fungal colony with dark green conidiation near the edges. After 15–20 days of incubation, the fungus formed whitish-green cottony growth on PDA, initially light greenish-white and eventually turned dark green. Microscopic observations of TAB-1 revealed that the hypha was hyaline (Figure 8). Conidia were hyaline, globose to sub-globose and aseptate. The size of the conidia was  $2.8\text{--}(2.6) \times 2.6$  ( $2.4$ )  $\mu\text{m}$ . Phialids were flask-shaped with  $5.0\text{--}(4.5) \times 2.6\text{--}(2.9)$   $\mu\text{m}$ . The fungal colony of bioagent TAB-2 was dull green in color. Microscopic observations revealed that the hyphae were hyaline and branched. The conidia were hyaline, globose, and aseptate (Figure 9). The size of the conidia was  $2.5\text{--}(2.3) \times 5\text{--}(4.6)$   $\mu\text{m}$ . Phialids were flask-shaped with  $7.0\text{--}(6.5) \times 8\text{--}(7.3)$   $\mu\text{m}$ .



**Figure 8.** Morphological characteristics of bioagent TAB-1; (A) 7 days old culture; (B) phialids; (C) conidia ( $\times 400$ ).

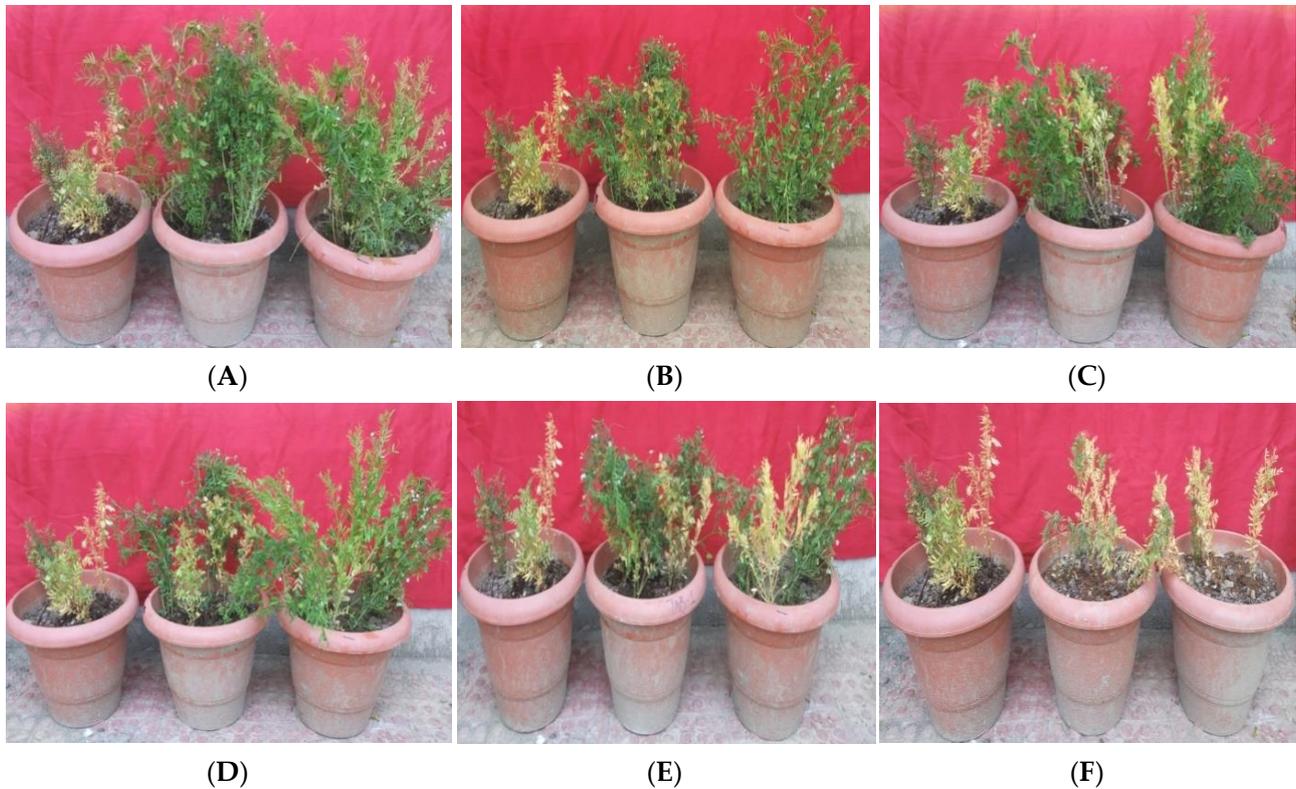


**Figure 9.** Morphological characteristics of bioagent TAB-2; (A) 7 days old culture; (B) phialids; (C) conidia ( $\times 400$ ).

### 3.8. Evaluation of Efficient Copper Oxochloride Resistant Bioagents against Wilt Disease of Lentil in Pots

An experiment was conducted in November 2017–March 2018 to compare the effects of six different seed treatments (copper oxochloride, TAB-1, TAB-2, TAB-1 + copper oxochloride, TAB-2 + copper oxochloride) and one untreated control. The results revealed (Tables 5 and 6) that the treatment combination TAB-1 + Copper oxochloride had the lowest disease incidence and severity (3.24% and 3.3%, respectively), followed by TAB-2 + copper oxochloride with an incidence of 7.72% and severity of 4.74%. Individual treatments of bioagents of TAB-1, TAB-2, and copper oxochloride followed in decreasing sequence, with disease incidences of 10.00%, 13.85%, and 21.32%, respectively, and varying severity of 9.48%, 10.77%, and 14.87%, respectively. In the control treatment, disease incidence was 81.94%, and intensity was 81.67%. In 2018–2019, treatment combination TAB-1 + copper oxochloride had the lowest disease incidence and severity (5.64% and 4.1%, respectively), followed by TAB-2 + copper oxochloride with incidence of 5.62% and severity of 4.14%. Individual treatments of bioagents of TAB-1, TAB-2, and copper oxochloride followed in decreasing sequence, with disease incidences of 12.2%, 17.27%, and 23.12%, respectively, and varying severity of 8.28%, 9.77%, and 13.277%, respectively. In the control treatment, disease incidence was 86.94%, and intensity was 80.21%. Analysis of pooled data of two consecutive years revealed that seed treatment with TAB-1 + copper oxochloride showed the lowest disease incidence (4.44%) and disease intensity (3.7%) followed by TAB-2 + copper oxochloride with disease incidence and intensity of (6.67%) and (4.44%), respectively. These were followed by seed treatment with TAB-1 (disease incidence of 11.11% and disease intensity of 8.88%), TAB-2 (disease incidence of 15.55% and disease intensity of 10.37%),

and copper oxychloride (disease incidence of 22.22% and disease intensity of 14.07%). Control pots showed the highest disease incidence (84.44%) and disease intensity (80.74%) compared to all treatments (Figure 10).



**Figure 10.** Effect of different seed treatments on the *Fusarium* wilt incidence of lentil; (A) TAB-1 + Copper chloride; (B) TAB-2 + copper oxychloride; (C) Copper oxychloride; (D) TAB-1; (E) TAB-2; (F) Control (No treatment).

**Table 5.** Effect of different seed treatments on disease incidence (%) of *Fusarium* wilt disease of lentil.

S. No.	Seed Treatment	2017–2018 % Mean Disease Incidence	2018–2019 % Mean Disease Incidence	Pooled % Mean Disease Incidence
1	TAB-1 + copper oxychloride	3.24 (10.34) *	5.64 (13.76)	4.44 (9.97)
2	TAB-2 + copper oxychloride	7.72 (16.12)	5.62 (13.69)	6.67 (14.96)
3	TAB-1 ( <i>Trichoderma</i> )	10.00 (18.42)	12.2 (20.43)	11.11 (19.25)
4	TAB-2 ( <i>Penicillium</i> )	13.85 (21.84)	17.27 (24.54)	15.55 (23.12)
5	Copper oxychloride	21.32 (27.49)	23.12 (28.72)	22.22 (28.06)
6	Control	81.94 (64.83)	86.94 (68.79)	84.44 (66.83)
	C.D ( $p \leq 0.05$ )	0.835	0.815	3.07

\* Values in brackets were arcsine transformed values.

**Table 6.** Effect of different seed treatments on disease intensity (%) of *Fusarium* wilt lentil disease.

S. No.	Seed Treatment	2017–2018 % Mean Disease Intensity	2018–2019 % Mean Disease Intensity	Pooled % Mean Disease Intensity
1	TAB-1 + copper oxychloride	3.3 (10.42) *	4.1 (11.66)	3.7 (8.13)
2	TAB-2 + copper oxychloride	4.74 (12.56)	4.14 (11.70)	4.44 (11.97)
3	TAB-1 ( <i>Trichoderma</i> )	9.48 (17.92)	8.28 (16.71)	8.88 (17.33)
4	TAB-2 ( <i>Penicillium</i> )	10.77 (19.14)	9.97 (18.40)	10.37 (18.77)
5	Copper oxychloride	14.87 (22.67)	13.27 (21.35)	14.07 (22.00)
6	Control	81.67 (64.62)	80.21 (63.57)	80.74 (59.35)
	C.D ( $p \leq 0.05$ )	0.87	0.97	2.13

\* Values in brackets were arc sine transformed values.

#### 4. Discussion

The present study found that the pathogen associated with wilt in lentils in the Himalayan state of Kashmir was found to be *F. oxysporum* f. sp. *lentis*. A study was conducted to isolate the copper oxychloride resistant bioagents and use them against *Fusarium* wilt of lentils. Bioagents H10 and C9 were found resistant to copper oxychloride at 2500 ppm. This technique proved to be a reliable and effective way to manage the disease on an ecological level. Highly effective antagonistic (copper oxychloride resistant) fungi that were screened by the dual culture test (Figure 5) were further evaluated for phosphorus solubility. It is noteworthy that the top two fungal isolates, H10 and C9, which proved to be highly antagonistic, also had the highest “P” solubilizing activity. Fungal isolates H10 and C9 were selected for further pot culture evaluation to determine if both effectively suppressed the disease alone or combined with the fungicide copper oxychloride. Due to their triple effect, i.e., resistance/compatibility with copper oxychloride fungicide, antagonistic activity, and superior solubilizing activity of “P”, isolates H10 and C9 were renamed TAB-1 and TAB-2 (triple effect biological control). After morpho-cultural characteristics and DNA molecular approaches using ITS-1 and ITS-4, the bioagent TAB-1 (H10) was identified as *Trichoderma harzianum* isolate skua-tab-1 and bioagent TAB-2 (C9) as *Penicillium crysogenum* strain Tab-2. TAB-1 and TAB-2 were found to be more effective in controlling wilt disease in lentils in pot culture trials than untreated control and copper oxychloride fungicide alone. The control variant improved significantly when combined with a fungicide containing copper oxychloride. The current investigation demonstrated that the isolated and described bioagents are essential in integrated disease management and sustainable agriculture, preventing pathogen resistance to synthetic pesticides. Apart from that, they are economically significant due to their excellent field-level applicability compared to any other common antagonistic bioagent and their biofungicidal and bio-fertilizer potentialities (as evidenced by high “P” solubilization indices). As a result, their use will not only minimize losses but will also increase yield through “P” solubilization, as “P” fixation is a common concern in this region’s soils. These identified potential bioagents could very well be helpful in a variety of soil-borne diseases (such as wilts and root rots) in a variety of other crops.

Furthermore, copper fungicide inhibits pathogen growth quickly, and the biocontrol agent establishes itself effectively by the time the copper fungicide’s efficacy has expired. As a result, their combination can give long-term protection against diseases. Copper oxychloride and antagonistic/bio-fertilizer fungi are not only a successful IDM/IPM approach, but they are also an organic management strategy since copper oxychloride is accepted in organic farming systems. The compatibility of bioagents with chemicals and fertilizers has been studied extensively by various researchers.

*Trichoderma harzianum* was found highly compatible with copper oxychloride, phorate, and carbofuran [24]. *Penicillium* spp. EU0013 exhibited resistance against benomyl fungicide. In previous studies, bioagents successfully inhibited the *Fusarium* wilt and showed their ability to solubilize phosphorus by forming a wide zone of clearance on pikoviskya medium (PKA) [21]. *Trichoderma atroviride*, *T. hamatum*, *T. harzianum* and *T. koningii* successfully inhibit the growth of *F. oxysporum* f. sp. *ciceri* and the inhibition mechanisms by these biocontrol agents include competition, mycoparasitism, antibiosis [25]. *Trichoderma harzianum* has ability to induce systemic resistance in plants and is considered a good agricultural biostimulant [26]. *Trichoderma harzianum* and *Trichoderma parareesei* have the ability to promote stress tolerance to salinity and drought in plants [27]. It *Trichoderma parareesei* was previously reported that cumin seed treatment with the combination of carbendazim and *Trichoderma viride* resulted in only 10.6% disease incidence of cumin wilt caused by *F. oxysporum* f. sp. *cumini* [27]. The combination of all three properties, i.e., copper tolerance, antagonism to soil pathogen(s), and highly efficient solubilization of “P”, is a potential novelty explored and offered by this research that could be useful in a wide range of contexts other than the lens wilt disease studied in this case.

## 5. Conclusions

In this work, soil patches were treated with copper oxychloride fungicide at various places throughout the Kashmir valley in an attempt to implement selection pressure on fungi with biocontrol competence. Using *Trichoderma* selective medium, 70 putative biocontrol fungi were identified from the treated soil. The compatibility of all 70 fungal isolates with copper oxychloride was tested once more by growing them on medium with copper oxychloride fungicide. In-vitro testing was conducted for all 70 fungal isolates to assess their antagonistic efficacy against *F. oxysporum* f. sp. *lentis* and invitro phosphorus solubilization. Based on morpho-cultural and ITS region sequencing, two of the top-performing fungal isolates (TAB-1 and TAB-2) were selected and identified as *Trichoderma harzianum* isolate skua-tab1 and *Penicillium crysogenum* strain Tab2, respectively, out of 70 isolates. In the pot culture experiment, both biocontrol agents surpassed the fungicide copper oxychloride alone and combined with the fungicide copper oxychloride. The use of potential biocontrol agents in combination with copper oxychloride is beneficial for better and longer-lasting control and helps in managing pathogen resistance in several different ways, plus copper oxychloride is an environmentally approved chemical. A unique feature of this present study adds value to the microorganisms under investigation as putative solubilizers of “P” making these potential bioagents and biofertilizers simultaneously. Such microorganisms have the potential to be very beneficial against other rots and root rots of various crops, but more thorough testing is needed in the future.

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**Conflicts of Interest:** All authors declare that there is no conflict of interest.

### Abbreviations

IDM: integrated Disease Management; PDA: Potato dextrose agar; TAB: triple action biocontrol agent; p: phosphorus; TSM: *Trichoderma* selective medium; ITS: internal transcribed spacer; PKA: Pikovskaya's agar; F: *Fusarium*.

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