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CADMIUM-RESISTANT FUNGI IDENTIFIED BY INTERNAL TRANSCRIBED SPACER GENE SEQUENCING ON *VICIA FABA* GROWTH IN CADMIUM-CONTAMINATED SOILS

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Abstract

Cadmium (Cd^{+2}) is a heavy metal that has harmful effects on plants. This study aims to isolate and assess the effect of inoculation of contaminated soil with selected fungi on the responses of *Vicia faba* plants to Cd stress. Twelve isolates of fungi isolated from Cd-contaminated soil were screened for their resistance to Cd^{+2} using the minimum inhibitory concentration (MIC) approach. Four strains having high MIC values (up to 640 µg/ml) were subjected to molecular identification based on internal transcribed spacer (ITS) gene sequencing. The selected fungal strains were *Aspergillus flavus*, *Penicillium rubens*, *Talaromyces stipitatus*, and *Trichoderma lixii*. Our findings demonstrated that the morphological traits (shoot and root length, shoot and root fresh and dry weight, leaf area) of *Vicia faba* were significantly reduced under stress of Cd^{+2} element. Similar trend was observed in antioxidant enzymes [Catalase (CAT), Peroxidase (POD) and Polyphenol oxidase (PPO)] and chlorophyll a and b content when compared to un-stressed plants. Cd^{+2} caused chromosomal aberrations and a substantial boost in proline level. Nevertheless, inoculation with fungal strains mitigates the adverse effect of Cd on plant growth at the morphological, physiological and genetic levels compared to non-inoculated plants. Eventually, the inoculation of fungus into Cd-contaminated soil significantly enhanced plant growth and minimized the negative side effect of Cd.

Key words: Internal transcribed spacer, Gene sequencing; Heavy metals, *Vicia faba*, Cadmium-resistant fungi, Sustainability.

Introduction

Heavy metals pose a serious threat to the environment. Besides, they also represent a substantial danger to soil and water quality and plant and animal nutrition (Genchi *et al.*, 2020; Eldamaty *et al.*, 2021). Localized mining, industry, and agriculture are the leading causes of elevated toxic metals in soils) Rahimzadeh *et al.*, 2017 (Overexposure to some metals may adversely affect on plant's physiology, metabolism, growth, and senescence (Stone, 2019; Peng & Shahidi, 2021). Due to its potential toxicity to flora and wildlife at low quantities, cadmium (Cd) is of particular concern (Peng & Shahidi, 2021). Plant roots rapidly absorb Cd, and it alters the structural and functional characteristics of plants, inhibits seed germination (Nouairi *et al.*, 2019) and root elongation (Ali *et al.*, 2014), influences the physiological mechanisms that affect plant metabolism including respiration, photosynthesis, water transport, and gas exchange (Tang *et al.*, 2018), chlorophyll synthesis (Jia *et al.*, 2015), and interferes with the antioxidant defense system by generating more reactive oxygen species (ROS) (Gallego & Benavides, 2019). Additionally, under Cd-stress, cytogenetic abnormalities are more common and the mitotic index is shown to be lower (Vladimirovich *et al.*, 2021).

Alternative processing techniques for eliminating the heavy metal ions from polluted areas including bioremediation. When naturally occurring organisms (plants, bacteria, and fungi) break down environmental contaminants into less hazardous forms, this process is called bioremediation (Siddiquee *et al.*, 2015). In addition to providing nutrients to plant hosts, endophytic fungi also offer practical techniques for reducing heavy metals toxicity through multiple ways, such as sequestration, biomineralization, chelation, and biotransformation (Ali *et al.*, 2019). Due to their increased biomass and enhanced production of secondary metabolites when exposed to heavy metals, endophytic fungi are compatible with bioremediation techniques (Khan *et al.*, 2016). Furthermore, the area and good binding characteristics of fungi allow them to take heavy metals through their cell walls, such as *Fusarium*, *Penicillium*, *Aspergillus*, *Phanerochaete*, *Verticillium*, and others from *Zygomycota* and *Basidiomycota* (Iram *et al.*, 2012). One of the most important legume crops, *Vicia faba* L., is utilized to feed people and animals. Since 1982, *Vicia faba* has been a model test material often used to detect and diagnose contaminant genotoxicity (Chen *et al.*, 2020). Because Cd can build up in plants and enter human body through the chain of food, causing poisoning and jeopardizing human health, it is crucial to develop a successful technique for cleaning up Cd-contaminated soils. Although several fungi have been identified as effective in heavy metal uptake, there is a lack of comprehensive data comparing the efficiency of different species in bioremediation. Understanding which specific fungi are most effective in different environments or levels of contamination is crucial at the level of morphological, physiological and cytogenetically parameters. The present study was designed to: isolate Cd-resistant fungi from contaminated soil with identifying via ITS gene sequencing. Assess co-inoculation with *Vicia faba* for bioremediation

Materials and Methods

Isolation of fungi: Three distinct areas with polluted soils (Talkha, Qalyubia Governorate (31.05700, 31.38083); Nawag, Gharbia Governorate (30.86818, 31.00423); and

Quesna, Menoufia Governorate (30.56281, 31.15929) in Egypt) were considered sources of the soil samples. Ninety milliliters of sterilized distilled water was used to suspend 10 grams of well-mixed samples. Plates containing PDA media were cultured from serial dilutions and incubated for 7 days at 30°C.

Minimum inhibitory concentration (MIC): Applying the hole approach, the minimal concentration of the metal that could prevent fungal growth was estimated. PDA medium plates were infected with a suspension of fungal spores (100 µl). A circular hole was made and the heavy metal solution (40 mg/L to 640 mg/L) was poured through these holes. Plates were incubated at 30°C for five days, and growth measurements were recorded daily (Akbar *et al.*, 2022).

Molecular identification fungi

DNA extraction and PCR reaction: The total DNA was extracted according to Abed (Abed, 2013). A 30 ng of extracted genomic DNA was used as a template, along with 50 µl of 1X reaction buffer, 1.5 mM MgCl2, 1U Taq DNA polymerase (Promega), 2.5 mM dNTPs, and 30 pmol of the ITS gene's forward (F) and reverse (R) primers (F: 5′TCCGTAGGTGAACCTGCGG3′ and R: 5′TCCTCCG CTTATTGATATGC3′, coded (ITS-1) and (ITS-4) . PCR amplification conditions, identification and purification was performed as mentioned by Sharaf-Eldin *et al*., (Sharaf-Eldin *et al.*, 2023).

ITS sequencing analysis: Following the manufacturer's instructions of Big Dye™ Terminator Cycle Sequencing Kits, the resulting PCR products were sequenced in an automated sequencer (ABI PRISM 3730XL sequencer, Microgen Company, Korea). The generated sequences were aligned with data available in the GenBank using the online BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Using the neighbor-joining method and data matrix, the phylogenetic tree was built by MEGA X software (Mega, 2018).

Pots experiment: *V. faba* seeds (Sakha 1) were purchased from the Sakha Agricultural Research Station (SARS), Food Legumes Research Section, Kafr El-Sheikh, Egypt. Three replicates for each treatment were achieved; each replicate contained 10 seeds and was planted in plastic pots filled with peat moss. Before planting, the soil was contaminated with Cd at the concentration of 15 mg / 120 g peat moss (El-Mahdy *et al.*, 2021) and inoculated with the selected tolerant fungi strains (10 ml of 5×10^6 spore). The experiment design included 6 groups. The first group was considered a negative control without any treatment, whereas the second group was a positive control (cd). The third group was inoculated with *Aspergillus flavus* and cd (AfCd), and the fourth group was infected by *Penicillium rubens* with cd (PrCd).

Furthermore, the fifth group included *Talaromyces stipitatus* coupled with cd (TaCd), whereas the last group had *Trichoderma lixii* mixed with cd (TriCd). After two weeks of soil inoculation with fungi, *V. faba* (10 seeds) was planted in each replicate and inoculated with 10 ml of the same spore suspension (5×10^6). After 3 weeks, the plants were harvested and stored at - 80 ºC for further determination.

Morphological parameters: The morphological parameters, including shoot and root length (cm), shoot fresh and dry weight (g), root fresh and dry weight (g) and leaf area (cm^2) were evaluated.

Physiological parameters: Proline content was determined spectrophotometrically at 520 nm, according to Bates *et al*., (Bates *et al.*, 1973). Chlorophyll levels were also estimated using the spectrophotometeric (Jenway 6305 UV/Visible) method, according to HK (HK, 1985).

Antioxidants enzymes: Catalase activity (CAT) assay was carried out according to Aebi (Aebi, 1984). Similarly, peroxidase activity (POD) was estimated at 420 nm according to the described method by Chance & Maehly (1955). In contrast, polyphenol oxidase activity (PPO) was assessed at 420 nm and 25°C, according to Duckworth & Coleman method (Duckworth & Coleman, 1970).

Cytological analysis: The root tips were cut and fixed for twenty-four hours in acetic acid and ethanol (1:3) (Omar *et al.*, 2023). Following the pretreatment, 70% ethanol was administered to the samples and root tips were stained using 2% of the aceto-carmine dye, as described previously by Zedan & Omar (2019).

Data analysis: Data were presented as means ±standard deviation (SD). The Duncan multiple range test was implemented to examine the significance of differences between means after the One-Way Analysis of Variance (ANOVA) using the software SPSS for Windows (version 20). At a *p*-value of 0.05, the results were significantly different.

Nucleotide sequences: The generated sequences of ITS-1 were deposited into the GenBank under the following accession numbers: OR807414 - OR807415 - OR807416 - OR807417.

Results

Fungi isolation and determination of minimum inhibitory concentration (MIC): The total count of isolated fungi in soil samples was 5.3×10^3, 2.3×10^3 and 5.33×10^4 in Talkha, Quesna and Nawag locations, respectively. The isolated fungi were taken and identified based on the morphology and color of colonies. The results of MIC for twelve-isolated fungi at concentrations ranging from 40 to 640 mg/L from Cd are presented in Table 1. The isolates a, c, f, g, i and j gave inhibition zones reached 8.5, 8.5, 1.7, 1.25, 1.65, and 1.55 cm, respectively. On the other hand, b, d, e, h, k and l isolates exhibited zero inhibition ,reflecting high resistance to Cd metal. For more selection, the six selected fungi were examined for more tests, including resisting other heavy metals such as Pb, as shown in Table 2.

Table 1. Minimum inhibitory concentrations for fungi isolates in media containing Cd.

S. No.	Fungal isolates	Cd con. (640 mg/L)	No.	Fungal isolates	Cd con. (640 mg/L)
1.	Isolate a	8.5	7	Isolate g	1.25
2.	Isolate b	0	8	Isolate h	0
3.	Isolate c	8.5	9	Isolate i	1.65
4.	Isolate d	0	10	Isolate j	1.55
5.	Isolate e	0	11	Isolate k	0
6.	isolate f	1.7	12	Isolate l	0

Table 2. The inhibition zone for fungi isolates in media contains Pb or Cd heavy metals.

S. No.	Fungi isolates	Pb at 640 mg/L	Pb at 1280 mg/L	Cd at 640 mg/L
1.	Isolate a	8.5		8.5
2.	Isolate b	8.5		0
3.	isolate c	3.45		8.5
4.	Isolate d	8.5		0
5.	Isolate e	0	0	0
6.	Isolate f	0	8.5	1.7
7.	Isolate g	0	5.5	1.25
8.	Isolate h	0	0	0
9.	Isolate i	0	0	1.65
10.	Isolate j	8.5		1.55
11.	Isolate k	0	2.85	0
12.	Isolate l	0	3.5	0

The letter "a" to "l" refers to the fungi isolates, the black rows refer to the strain that isn't undertaken for more tests because it is not more resistant to low doses of Pb, and the rows with blue is the selected strains for more studies

Table 3. Similarity percentage of each fungal strain and the accession numbers.

Isolate code	Scientific name	Accession no.	Similarity %
E	*Aspergillus flavus*	MF681598	99.81%
I	*Talaromyces stipitatus*	MH857208	100%
H	*Penicillium rubens*	MT558923	100%
K	*Trichoderma lixii*	MT446172	95.29%

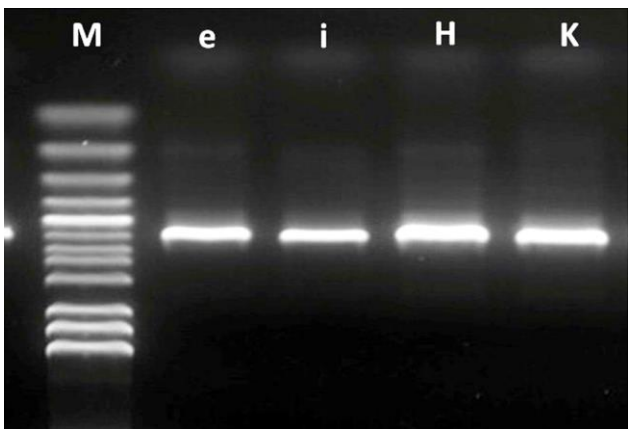

Fig. 1. ITS PCR amplification of fungal isolate. A band of ~700 bp was obtained against the 100 pb marker.

Molecular identification of fungal isolates based on ITS gene sequencing: Using the ITS1 and ITS4 primers, the fungal ITS region was amplified from the genomic DNA of four isolates, selected based on their morphological and Cd-resistance features. PCR yielded a product with ~700 bp from each isolate (Fig. 1). After that PCR product was purified, Big Dye™ Terminator version 3.1 was used to sequence the DNA on an ABI Prism 3730XL platform. A sequence search was applied using the BLAST standard nucleotide-nucleotide basic local alignment search tool to identify the fungal strain from ITS sequencing. To validate the initial identification of the tested sequence, the homology should be more than 95% with the referenced culture. The outcomes from alignment exhibited similarity, recorded at 99.81%, with the reference strain *Aspergillus flavus* for fungal isolate (e) (Table 3, Fig 2).

The fungal isolate coded (i) had 100% similarity to the reference strain *Talaromyces stipitatus*. Additionally, *Penicillium rubens* explored a similarity percent of 100% with the fungal isolate H, and the isolate (k) was identified as *Trichoderma lixii* with a similarity percent of 95.29%.

Growth parameters: The growth parameters (shoot and root length, shoot and root fresh and dry weight, leaf area) of faba bean plants were significantly reduced by Cd stress relatively to control plants (73.77%, 53.88%, 75.06%, 43.74% , 58.60%, 72.91% and 65.34% in comparison with the control respectively). Applying *Aspergillus flavus* (Af), *Penicillium rubens* (Pr), *Talaromyces stipitatus* (Tas) and *Trichoderma lixii* (Tri) treatments revealed marked improvement in the growth parameter when compared to cd-stressed plants (Fig. 3).

PrCd and TasCd treatments had the highest values in shoot length compared to the other treatments and Cd treatment (129.63% and 128.89%, respectively). In contrast, TasCd treatment recorded the best value in fresh and dry weights of shoot and root (165.48%, 171.38%, 269.04 and 181.32% respectively). As for root length, AfCd and TasCd

treatments displayed the uppermost value (183.16 and 183.98%, respectively). Finally, the leaf area increased under PrCd and TasCd treatments, and the maximum value was recorded in comparison to other fungal strains and Cd treatment (166.27% and 168.50%, respectively).

Physiological parameters

Proline content and antioxidant enzyme activity: The proline concentration and antioxidant enzyme activity (catalase, peroxidase and polyphenol oxidase) were evaluated to assess the metal-induced oxidative stress (Fig. 4). Faba bean plants treated with Cd markedly increased proline content (256.07%) and decreased the activity of catalase (CAT, 69.43%), peroxidase (POX, 72.95%) and polyphenol oxidase (PPO, 71.15%) compared to non-stressed plants (control). On the other hand, faba bean plants exposed to fungi induced a marked boost in CAT, POX and PPO activity and minimized proline content except for the *Trichoderma lixii* (TriCd) strain that produced the maximum amount of proline content compared to stressed plants (114.84%) and control (294.07%). The fungal strain *Trichoderma lixii* (TriCd) strain revealed the best value in CAT activity (263.78%), whereas *Talaromyces stipitatus* (TasCd) strain displayed the highest value in POX activity (579.36%). Otherwise, PPO activity reached the highest significant increment under *Aspergillus flavus* (AfCd) treatment (285.77%) compared to stressed plants.

Chrolophyll: Cd toxicity caused a reduction in chlorophyll "a" (77.50%) and chlorophyll "b" (95.39 %) of stressed faba bean plants when compared to non-treated plants (Fig. 5). On the contrary, the inoculated plants with the isolated fungal strains enhanced the leaf chlorophyll content compared to positive control (non-stressed plants). The highest content of Chll a and b was assigned to the treatment Cd + *Talaromyces stipitatus* (TasCd, 144.70, 165.42% respectively) followed by *Trichoderma lixii* (TriCd) in the case of chll a measurement and AfCd implementation in chll b evaluation.

Cytological studies: In the present study, the mitotic phases of plant cells under both Cd-stress and inoculated with fungal strains were calculated. The best percentage of prophase (73.45%) was observed in Cd coupled with *Aspergillus flavus* (AfCd) treatment. In contrast, the uppermost percentage of metaphase (25.29%), anaphase (23.92%) and telophase (15.61%) were detected in Cd + *Talaromyces stipitatus* (TasCd), Cd and Cd + *Trichoderma lixii* (TriCd) treatments, respectively (Table 4). The mitotic index and chromosomal aberrations percentage under cd stress are given in Table 5. Cells under Cd stress showed the least mitotic index (4.99%) and the highest abnormality index (46.46%). Cd induced different types of chromosomal aberration such as C-mitosis, pole-to-pole metaphase, bridges, disrupted, fragment, laggard, star anaphase and chromosome stickiness in the cells (Fig. 6). Present findings demonstrated that inoculation with fungal strains significantly alleviated the toxicity impact of Cd at the level of mitotic index and chromosomal abnormalities in root tip cells. Implemented Cd + *Aspergillus flavus* (AfCd) exhibited the top percentage (12.77%) of mitotic index, followed by control and Cd+*Penicillium rubens* (PrCd), respectively. Similarly, the lowest value (16.63%) of chromosomal abnormalities were found in Cd+ *Talaromyces stipitatus* (TasCd) treatment, followed by plants treated with Cd +*Aspergillus flavus* (AfCd).

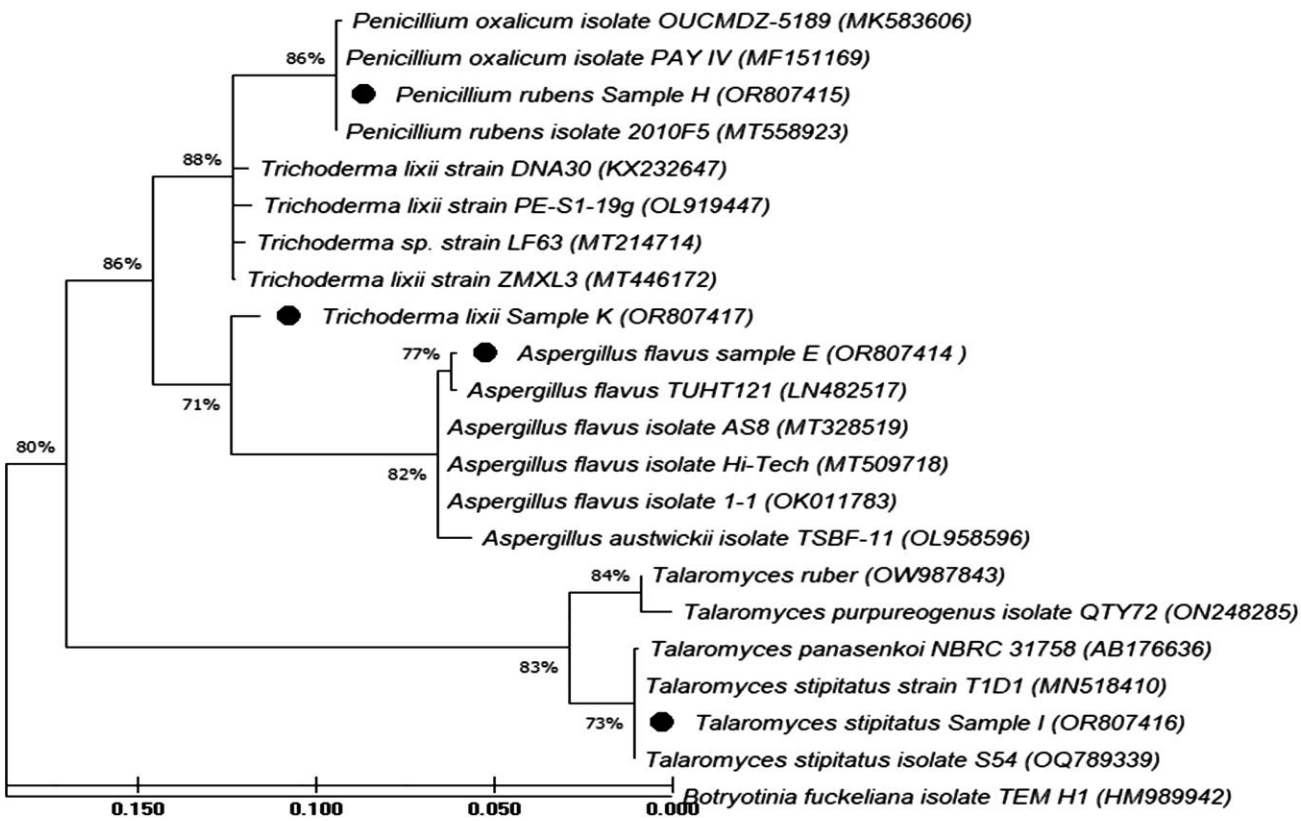

Fig. 2. The molecular phylogenetic tree of four isolated fungal strains (Sample H, K, E and I) using ITS-1 gene sequences.

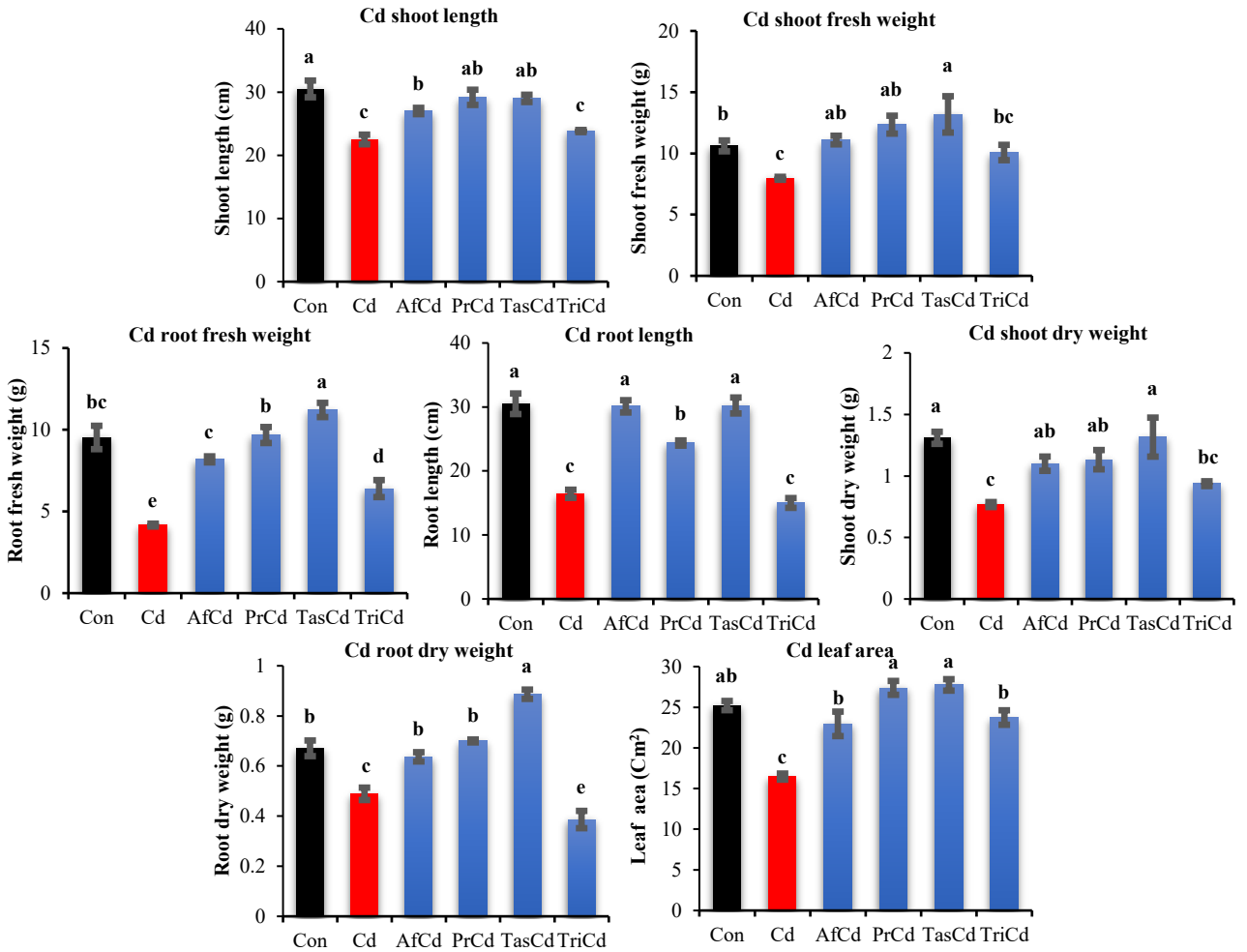

Fig. 3. Changes in the growth parameters of *Vicia faba* under normal and Cd treatment without or with fungi inoculum. Con, Control; Cd, Cadmium; AfCd, *Aspergillus flavus* with Cd; PrCd, *Penicillium rubens* with Cd; TasCd, *Talaromyces stipitatus* with Cd; TriCd, *Trichoderma lixii* with Cd. Values with different letters were considered significant.

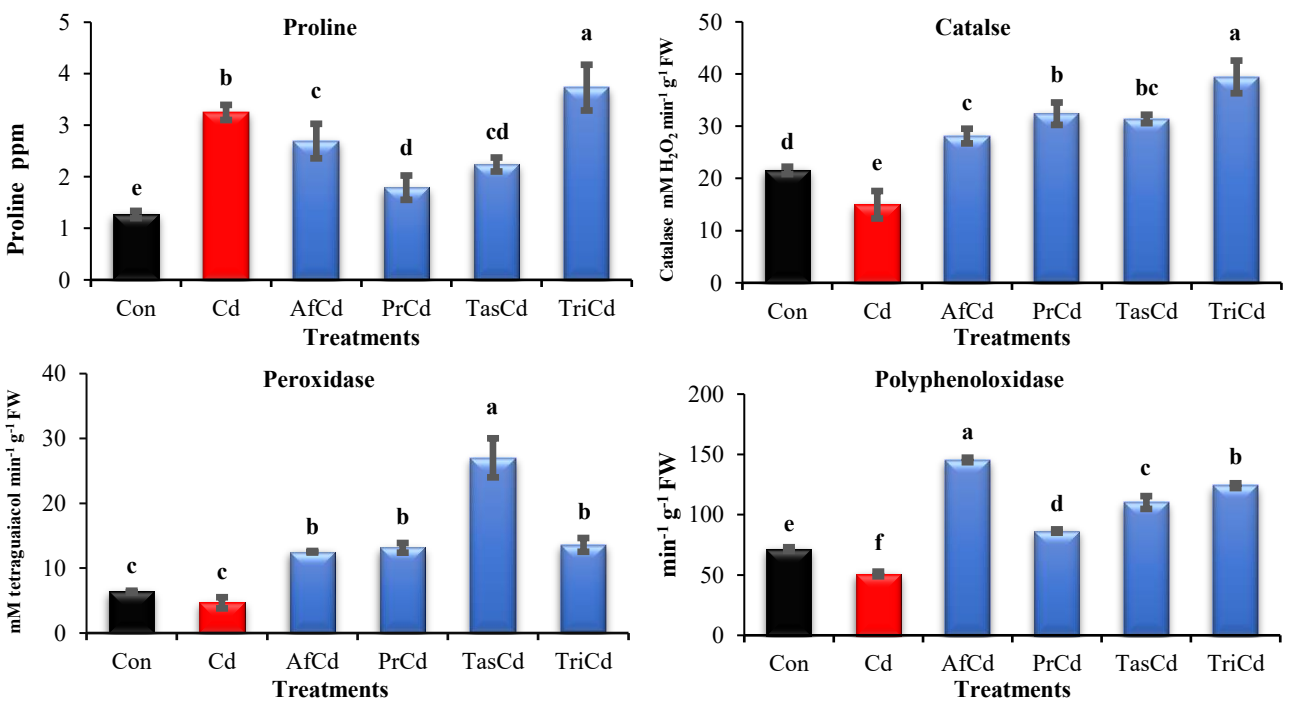

Fig. 4. Changes in proline concentration, catalase, peroxidase and polyphenol oxidase activities in seedlings of *Vicia faba* under control and cd treatment without or with fungi inoculum. Con, control; Cd, cadmium; AfCd, *Aspergillus flavus* with Cd; PrCd, *Penicillium rubens* with Cd; TasCd, *Talaromyces stipitatus* with Cd; TriCd, *Trichoderma lixii* with Cd. Values with different letters were considered significant.

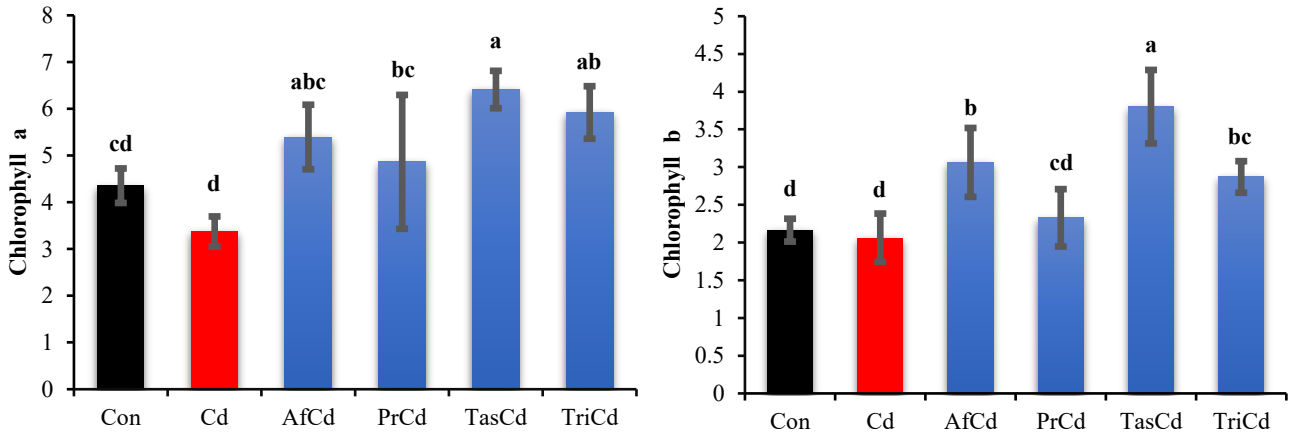

Fig. 5. Ameliorating effect of fungi inoculum on chlorophyll a and b content of *Vicia faba* under Cd stress.. Con, control; Cd, cadmium; AfCd, Aspergillus flavus with Cd; PrCd, Penicillium rubens with Cd; TasCd, Talaromyces stipitatus with Cd; TriCd, Trichoderma lixii with Cd. There is no statistical difference at $p<0.05$ (Duncan Multiple Range Test) when bars with the same letters are followed.

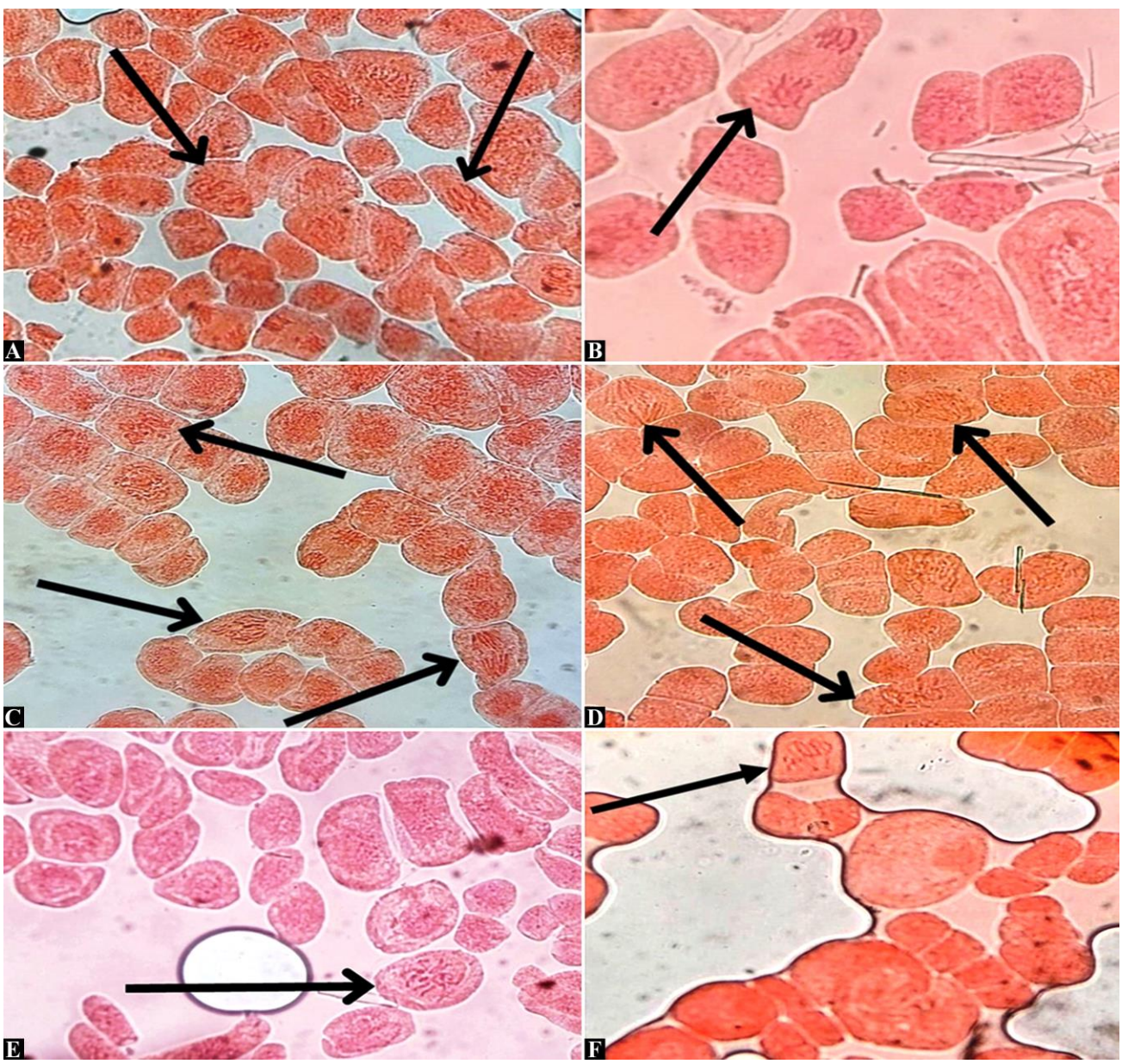

Fig. 6. Types of abnormalities observed in *V. faba* root tips cells under Cd stress: A, C-metaphase and disrupted metaphase; B, Fragment in telophase; C, Stickiness, fragment in metaphase, disrupted and bridge in anaphase; D, bridge, laggard chromosome and disrupted in anaphase; E, Star anaphase; F, Pole-to-pole metaphase.

Table 4. Cytological indicators of *Vicia faba*-root tips treated with Cd and inoculated with heavy metal-tolerant fungal strains.

Treatments	Examined cells	Dividing cells	Abnormal cells	Mitotic phase (%)			
				Prophase	Metaphase	Anaphase	Telophase
Control	3252	310	-	70	14.83	11.29	3.37
Cd	3250	163	94	46.62	25.15	23.92	4.29
AfCd	3037	388	77	73.45	9.02	12.37	5.15
PrCd	3071	280	85	65.71	13.92	11.07	9.28
TasCd	3083	170	28	61.17	25.29	13.52	0
TriCd	3126	269	80	59.10	14.49	10.78	15.61

Cd, cadmium; AfCd, *Aspergillus flavus* with Cd; PrCd, *Penicillium rubens* with Cd; TasCd, *Talaromyces stipitatus* with Cd; TriCd, *Trichoderma lixii* with Cd

Table 5. Mitotic index, percentage and types of abnormalities of *V. faba* under Cd stress and inoculated plants with fungi in the presence of Cd.

Treatments	Mitotic aberration (%)								Mitotic index	Abnormalities
	S	D	F	B	C-M	PP	ST	L	(%)	(%)
Control	-	-	-	-	-	-	-	-	9.60 ± 1.96^{b}	00.00 ± 0.00^{d}
Cd	26.59	30.85	7.44	7.44	17.02	3.19	3.19	4.25	4.99 ± 0.38^{c}	46.46 ± 6.09^{a}
AfCd	24.67	23.37	7.79	12.98	14.28	1.29	2.59	12.98	12.77 ± 3.06^{a}	20.73 ± 3.91^{c}
PrCd	44.70	14.11	8.23	4.70	21.17	-	1.17	5.88	9.11 ± 1.75^{b}	30.02 ± 4.05^{b}
TasCd	7.14	17.85	-	28.57	46.42	-	-	-	5.47 ± 0.60^{c}	16.63 ± 5.99^{c}
TriCd	43.75	17.5	6.25	11.25	17.5	-	1.25	2.5	8.59 ± 0.56^{b}	29.59 ± 2.36^{b}
Sig									0.00	0.00

Cd, cadmium; AfCd, *Aspergillus flavus* with Cd; PrCd, *Penicillium rubens* with Cd; TasCd, *Talaromyces stipitatus* with Cd; TriCd, *Trichoderma lixii* with Cd. S: Stickiness, D: Disrupted, F, Fragment, B: bridge, C-M: C-metaphase, PP: Pole-to-pole metaphase, ST: Star anaphase, L: Laggard. Values with different letters are considered significant.

Discussion

Fungi are a better choice for bioremediation because they can handle toxic heavy metals, grow very quickly, and have great metal-binding qualities (Dhankhar & Hooda, 2011; Fu & Wang, 2011). The minimum inhibitory concentration (MIC) can measure an organism's tolerance to heavy metals. In the current study, four isolates from fungi were selected for identification and further studies based on the high MIC that reached 640 µg/ml. Sequence analysis of E, I, H, and K strains revealed > 99.81%, 100%, 100% and 95.29% similarity with *Aspergillus flavus* (MF681598), *Talaromyces stipitatus* (MH857208), *Penicillium rubens* (MT558923) *and Trichoderma lixii* (MT446172), respectively.

One of heavy metal that contributes to minimizing plant productivity is Cd, which is non-essential and hazardous to the environment (El-Beltagi & Mohamed, 2013). In the current investigation, faba bean plants treated with Cd substantially reduced growth parameters compared to control (non-treated plants). These results align with those found by El-Mahdy *et al.*, (2021), who observed a significant reduction in the morphological characteristics in bean plants after treatment with Cd and Pb. Furthermore, our results meet the same findings of decreasing the growth parameters under Cd implementation obtained by Dutta *et al*., (2018) and Khanna *et al*., (2019). This could be explained by a drop in water potential, nutrient content, and a blockage in the proton pumps, which further hinders cell elongation division and lowers the dry weight of plants (Sarathambal *et al.*, 2017). On the contrary, adding fungi to the soil and plants actively helped absorb heavy metals and reduced their toxic concentration (El-Mahdy *et al.*, 2021). By lowering their translocation from the roots to the top portion of the tomato plants, *Aspergillus flavus* improved the growth characteristics of tomato seedlings under Cd and Cr stress (Aziz *et al.*, 2021). *Penicillium* sp. has been demonstrated to increase plant growth by enhancing nutrient uptake (Christie *et al.*, 2004). These results align with previous research demonstrating that beneficial fungi enhanced plant development in disturbed and polluted environments (Prasad *et al.*, 2011). Additionally, *Triticum aestivum* development and related plant parameters were greatly improved by sewage sludge treatments and soil incubation with *Talaromyces pinophilus* (El-Shahir *et al.*, 2021). This happened because *Talaromyces pinophilus* treatment maximized the amount of minerals and improved microbial activation, resulting in excessed nutrient availability, absorption, and root distribution (Abo-Baker & El-Tayeh, 2017),(Kabesh *et al.*, 2009). *Trichoderma sp.* can directly stimulate plant growth and development by raising the rate of germination, dry weight, flowering, and vigor, all of which can indirectly boost the plant's resilience to biotic and abiotic stress (Shoresh & Harman, 2010; Stewart & Hill, 2014).

In the present study, there was an increase in proline with Cd treatment, while *Aspergillus, Penicillium and Talaromyces* inoculation recovered the natural content of proline. Similarly, Aziz *et al*., (2021) and El-Mahdy *et al*., (2021) found that *Aspergillus* and *Penicillium* were doing the same action. On the other hand, *Trichoderma lixii* significantly improved the proline content in faba bean plants under Cd conditions.

The results also agreed with El-Mahdy *et al*. (2021) who disclosed that Cd caused a significant decrease in

CAT and POX whereas an elevated level of SOD was detected in faba bean-stressed plants. Likewise, it was noticed that *Helianthus annuus* seedlings and the maize plants exposed to heavy metal stress and Cd stress respectively. The enzyme activity was decreased (SOD, POD, and CAT) (Wang *et al.*, 2016; Devi *et al.*, 2017). This could be explained by attaching non-essential heavy metals to the enzyme's active site, excessive ROS production, enzyme breakdown, or enzyme inactivation (Filek *et al.*, 2008). Conversely, plants inoculated with endophytic fungi showed a large rise in CAT, POX, and PPO compared to non-inoculated plants. Similar results were reported by El-Mahdy *et al*., (2021) who found that the activity of POX and CAT was boosted in faba bean inoculated with *Aspergillus niger and Penicillium chrysosporium* under heavy metal stress. Remarkably, the increased activities of CAT, SOD and POD were recorded in *Triticum aestivum* plants incubated with *Talaromyces pinophilus*, following the sewage sludge amendment at a greater level (El-Shahir *et al.*, 2021). The activities of SOD, CAT, and POD were significantly increased in wheat seedlings with the application of the strain of *Trichoderma longibrachiatum* under salt stress (Zhang *et al.*, 2016) that are very necessary to dismutate H_2O_2 to H_2O and molecular oxygen in the cells (El-Shahir *et al.*, 2021).

According to the current study, the decline in chloroplast and photosynthetic apparatus content brought on by Cd stress may be caused by protein complex instability, the breakdown of the photosynthetic electron transport chain, the inability of chloroplasts, or the enzymes involved in chloroplast production. By interacting with sulphydryl-requiring enzymes in the system, Cd suppresses heme biosynthesis and chlorophyll synthesis (Pandey *et al.*, 2007). The obtained findings coincide with those of (Yang *et al.*, 2015; Rasouli-Sadaghiani *et al.*, 2019; Sofy *et al.*, 2020; Saadaoui *et al.*, 2022). The inoculation with fungi strains enhanced the chlorophyll content in faba bean plants under Cd stress. *Talaromyces pinophilus* and *Trichoderma longibrachiatum* promoted the photosynthetic pigments, as observed by (El-Shahir *et al.*, 2021). The inoculation might prevent production of ROS and building up in plant tissue (Zhang *et al.*, 2016), besides increasing plants' uptake of nutrients by solubilizing phosphate and releasing essential chemicals (Elhindi *et al.*, 2017). Fungal isolates confer cadmium (Cd) tolerance to *Vicia faba* through several mechanisms: Sequestration, fungi sequester Cd within their cells using compounds like metallothioneins, reducing its availability to plants (Sácký *et al.*, 2022) Biomineralization: Fungi can convert soluble Cd into insoluble forms, immobilizing it and decreasing toxicity (Wang *et al.*, 2023).

The cytotoxic or genotoxic effects were more severe due to the increased concentration of Cd ions (Lyu *et al.*, 2020) thus, enhanced the cytogenetic abnormalities' output (Vladimirovich *et al.*, 2021). Chromosomal abnormalities and the reduction of the mitotic index were among the negative effects of Cd on mitosis. Vladimirovich et al. (Vladimirovich *et al.*, 2021) observed that cytogenetic anomaly frequencies were elevated and the mitotic index was reduced under Cd^{2+} stress. Some Researchers attributed this disruption to proteins that regulate the proper organization of chromosomes (Liu *et al.*, 2003; Souguir *et al.*, 2008; Saxena *et al.*, 2010). Fungal strain inoculation produced a higher mitotic index and decreased the chromosomal aberrations. Fungi are known for their ability to degrade environmental pollutants, such as heavy metals and aromatic compounds (Zhang *et al.*, 2020). By breaking down these harmful substances, fungi can reduce the genotoxic effects that lead to chromosomal aberrations(Pereira *et al.*, 2014). Also, fungi produce a variety of secondary metabolites, many of which have antioxidant properties. These metabolites can neutralize free radicals and reactive oxygen species (ROS) that are known to cause DNA damage and chromosomal aberrations (Pinar & Rodríguez-Couto, 2024).

To minimize the rate of aberration and enhance mitosis index, the seeds were simultaneously inoculated with *Bacillus* sp. (AS03) and *Rhizobium* sp. (AS05) (Dhali *et al.*, 2022). Plants with endophytic microbes may withstand a variety of environmental challenges (Smith *et al.*, 2008; Santoyo *et al.*, 2016; Dhali *et al.*, 2021). Moreover, *Aspergillus flavus*, which is associated with plants, expressed the SlGSH1 and SlPCS1 genes, which helped them establish resistance against Cd toxicity. Both genes contributed to metal chelation and reduced the toxicity of Cd (Aziz *et al.*, 2021). In light of that , a cytological study of *Coriandrumsativum* showed that the highest cell division frequency is observed under *Pseudomonas putida* treatment, which increased the cell division in *Coriander* root tip cells due to having a high source of auxins production (Jha *et al.*, 2018).

Conclusions

We isolated a patch of tolerant fungi in the contaminated soil with Cd. Six tolerant strains could reduce the side effects of Cd. These fungi were isolated and selected based on the molecular sequences. Cd negatively affected the morphological, physiological, and genetic properties of faba bean. The Cd-tolerant fungi, especially *Talaromyces stipitatus* supported faba bean plants to recover their morphological and physiological traits and reduced the chromosomal aberration rate induced by cadmium stress. Incorporating these fungal strains into agricultural practices could improve crop resilience and productivity in Cd-contaminated areas. Assessing the ecological implications of introducing these fungal strains into the environment, ensuring that they do not disrupt local ecosystems.

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