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CONTENTS

In vitro phosphate solubilizing and phyto stimulating potential of Rhizospheric *Trichoderma* from Hilly areas of Kumaun Region

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ABSTRACT: *Trichoderma* spp. is widely recognized as promising bioagent for sustainable agriculture due to their multi- dimensional roles in enhancing plant growth, improving soil nutrient availability, and suppressing a wide range of soil-borne phytopathogens. Their ability to solubilize phosphate, produce phytohormones, and secrete hydrolytic enzymes contribute to improved plant health and productivity. Considering the plant growth promoting mechanisms of *Trichoderma*, eight strains were isolated from rice and guava rhizosphere thriving in Almora and Udham Singh Nagar districts of Uttarakhand. The isolates were assessed *in vitro* for their phosphate solubilizing potential using TCP (10g/L) amended Mineral Salt medium. During qualitative analysis by plate assay, only 3 isolates showing vigorous growth on TCP amended MSM medium were selected and quantified for phosphate solubilization. The concentration of phosphate mobilized from TCP in culture filtrates was maximum in the culture filtrate of the hilly isolate RAF5 showing 417.40 µg/mL and RAF6 showing 377.03µg/mL of phosphate after 72h and 96h of incubation respectively. These two potential strains (RAF5 and RAF6) were further investigated for their phosphatase enzyme production, heavy metal tolerance, phytohormone production, siderophore production and hydrolytic enzyme production. The cultures exhibited tolerance towards Cadmium and Lead up to 50ppm concentration. The study reveals a green strategy, using a potential bioagent *Trichoderma* equipped with both biocontrol and plant growth promoting activities as an alternative to chemical fertilizer, for enhancing plant growth and soil health management in areas contaminated with heavy metals.

Keywords: Heavy metal, phosphate solubilization, phyto stimulation, soil health

Phosphorus being the second most important macronutrient is crucial for important physiological and metabolic functions such as nucleic acid and cell membrane synthesis in plants (Singh and Satyanarayana, 2011). It also remains a limiting factor for crop yield and plant development. Phosphorus is abundantly available in soil but only a small fraction (0.1%) of this is available to plants due to its chelation with divalent cations such as Calcium, Zinc, Aluminium, and Iron resulting into formation of bound forms that are unavailable to the plants (Silva *et al.,* 2023; de Oliveira *et al.,* 2025). The limited availability of plant-accessible phosphorus in soils remains a widespread and persistent challenge across the globe. For decades, chemical phosphate fertilizers have been employed to address this deficiency. However, their continued use has raised serious harmful effects on native soil microflora, as well as potential risks to both human and animal health. Additionally, agricultural runoff often leads to the leaching of these fertilizers into nearby water bodies, contributing to the contamination of both soil and aquatic ecosystems (de Azeredo Morgado *et al.,* 2023). In addition to their impact on soil biology, chemical fertilizers often contain traces of heavy metals such as chromium, arsenic, zinc, and mercury. Over time, these toxic elements can accumulate in the soil and leach into water sources, posing significant environmental hazards and serious health risks such as neurotoxicity and genotoxicity in humans (Wojciechowska *et al.,* 2008; Zwolak *et al.,* 2019). Certain soil microorganisms, including *Trichoderma*, *Aspergillus, Penicillium, Bacillus*, *Pseudomonas*, and *Rhizobium* can convert insoluble forms of phosphorus—such as tricalcium phosphate, zinc phosphate, and phytate—into plant-available forms like orthophosphates ($H_3PO_4^-$ and $H_2PO_4^-$) (Tomar *et al.,* 2017). These microbes facilitate phosphate solubilization through various biochemical mechanisms, including the production of phosphatase and phytase enzymes, secretion of

organic acids, and release of siderophores (Tiwari *et al.,* 2024). *Trichoderma,* a genus of soil dwelling fungi belonging to the phylum Ascomycota, is widely recognized for its dual role in plant growth promotion and biocontrol.

About 300 spp. of *Trichoderma* have been taxonomically classified from different habitats, ecosystems and as endophytes in plants (Paul and Rakshit, 2021). These fungi contribute to nutrient availability by producing phosphatase enzymes that facilitate phosphate solubilization. Additionally, they are known to synthesize plant hormones such as IAA and Gibberellic acid, which support various aspects of plant growth and development. Trichoderma species also produce ACC deaminase, an enzyme that helps alleviate stress in plants by lowering ethylene levels, and they exhibit a degree of tolerance to heavy metals, enhancing their capacity to support plant health under abiotic stress conditions in the rhizosphere (Waghunde *et al.,* 2016; Tian *et al.,* 2021).

The inoculation of *Trichoderma* bioformulation as biofertilizers offers an ecofriendly and sustainable alternative to the use of chemical formulations. *Trichoderma* sp. is also capable of coexisting with other beneficial microbes, such as arbuscular mycorrhizal fungi and certain bacteria, in the plant root zone. Numerous studies have highlighted. effectiveness of *Trichoderma* sp. in controlling plant pathogens like *Fusarium* and *Rhizoctonia* by competing with them for space and nutrients (Tariq *et al.,* 2021; Prismantoro *et al.,* 2024; Mishu *et al.,* 2025).

This suggests that *Trichoderma* can play multiple roles in the soil ecosystem—supporting plant health and suppressing harmful pathogens—without significantly disrupting the surrounding microbial community.

Despite extensive research on *Trichoderma*'s biocontrol potential, its ability to augment plant growth and improve nutrient uptake remains relatively underexplored. In this context, the present study focuses on the assessment of in- *vitro* plant growth promoting activities and phosphate solubilization of rhizospheric *Trichoderma* isolates. Additionally, the research also explores the in vitro heavy metal tolerance potential of *Trichoderma*, indicating its potential future applications in being used as biofertilizer in heavy metal contaminated soils.

MATERIALS AND METHODS

Sampling of soil

The fungal cultures were isolated from the paddy (*Oryza sativa*) and guava plant rhizosphere from Almora (29.8150 º N, 79.2902 º E) and Udham Singh Nagar (28.9610° N, 79.5154° E) district of Uttarakhand, India respectively.

Isolation and assessment of cultures for phosphate solubilization

The soil was serially diluted and pour plating was performed on Trichoderma specific medium (TSM). The distinguish colonies were selected and transferred onto PDA to obtain the pure cultures. The isolates were then qualitatively screened for their inorganic fixed phosphate solubilizing activity using Mineral Salt Medium (MSM) amended with Tri Calcium Phosphate (TCP, 10g/L). A fungal disc (4mm) was placed on the centre of the plate and incubated at 30°C for three days (Kapri *et al.,* 2010). PDA plates inoculated with cultures were taken as control. The cultures showing positive results in plate assay were further quantified using 100 ml of sterilized MSM medium amended with Tri calcium Phosphate (10g/L each) in conical flasks. The flasks were inoculated with 4 fungal discs (4mm) in triplicate. The inoculated flasks were incubated at 37 °C up to 120 h. The samples were withdrawn at every 24 h, centrifuged (Remi M121) at (5,000 rpm) for 10 min at 4 °C. Uninoculated broth was taken as control. The supernatant was used for quantification assays as described by Fiske & Subbarow, Y. (1925).

Enzymatic exploration of Phosphate solubilization by selected cultures

Phosphatase enzyme production

Based on P-solubilization assays, two fungal isolates

exhibiting highest P solubilizing efficiency were selected for phosphatase enzyme production. The assay was carried out in MSM broth medium amended with TCP as bound phosphate form. The samples were withdrawn at every 24 h, followed by centrifugation at 5,000 rpm for 10 min at 4 °C. Uninoculated broth was taken as control. The resulting supernatant was further used to estimate phosphatase enzyme production as described by Kapri *et al.,* 2010**.** The absorbance was recorded at 405nm using U. V. Visible spectrophotometer (Biomate 3S by Thermo scientific). Enzyme activity was expressed in enzyme units (U mL^{-1}), where one unit corresponds to the amount of enzyme catalyzing the formation of 1 µM of product (here p-NP) /minute/mL under experimental conditions.

pH change

The supernatant as obtained earlier during phosphatase enzyme production was used to check the pH drop in the medium using pH meter.

Other plant growth promoting traits exhibited by selected fungal isolates

Direct mechanisms

Ammonia production

The fungal isolates were inoculated in 10 mL peptone water and incubated at 30 ° C for 5 days.1 ml of Nessler's reagent (Himedia) was added to each tube. A colour change ranging from pale yellow to dark brown indicates the presence of ammonia (Dye *et al.,* 1962).

Siderophore production

Qualitative and Quantitative assessment of siderophore production was performed using Schwyn and Neilands (1987) with slight modifications.60.5 mg of CAS dye (Himedia) was dissolved in 50 mL of distilled water and mixed with 10 ml of ferric chloride solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). The solution was stirred and added to 72.9 mg HDTMA dissolved in 40 ml distilled water. The pH of the solution was adjusted to 6.8 using NaOH and HCl, after which the resulting blue dye solution was autoclaved. For plate assay 10mL of dye was mixed with 100 mL malt extract medium to evaluate the siderophore producing ability. One fungal disc (4mm diameter) was inoculated on the centre of CAS agar medium and incubated at 30°C. Uninoculated plate containing CAS dye medium was taken as control. The fungal growth diameters and orange coloured halo zones surrounding the culture growth were measured after 2, 4 and 6 days in triplicates. Siderophore producing index (SPI) was determined by the formula: (coloured zone + colony)/colony diameters (Bilal *et al.,* 2021).

Siderophore quantification was performed by inoculating 3 fungal discs (4mm diameter) in 100mL malt extract medium. Sample was extracted at every 24-hour interval, centrifuged at 10,000 rpm for 15 minutes.500µL supernatant was mixed with 500µL of CAS reagent and absorbance was taken at 630nm using U. V. Visible Spectrophotometer (Thermo scientific Biomate 3S). Uninoculated malt extract medium was taken as control. Percent siderophore units were calculated using the following formula:
% Siderophore Units = (Absorbance of the reference "Absorbance of s ample)×100

IAA production

PDB (Potato Dextrose Broth) supplemented with 1% tryptophan was inoculated with fungal discs (4 mm) and kept in a shaking incubator at 100 rpm and 28°C. Samples were collected every 24 hours, centrifuged, and the supernatant was analysed. For quantification, 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent, and absorbance was measured at 536 nm using U. V. Visible spectrophotometer (Thermo scientific Biomate 3S) (Abdenaceur *et al.,* 2022). Uninoculated PDB supplemented with 1% tryptophan was taken as control. The appearance of a pink colouration indicated a positive result for the assay.

Indirect mechanism

Heavy metal tolerance of the selected isolates

The tolerance of fungal cultures to heavy metals

(cadmium and lead) was assessed using plate assay by measuring radial growth. A fungal disc was placed at the centre of petri plates containing MSM medium supplemented with three concentrations (50 100 and 150 ppm) of Cadmium nitrate and Lead nitrate. The plates were then incubated at 30°C for seven days to observe growth. Uninoculated plates were taken as control. (Zhang *et al.,* 2020).

HCN production

Hydrogen cyanide (HCN) production of fungi was evaluated using Bakker and Schipper's (1987) method. Fungal isolates were streaked onto PDA supplemented with glycine (4.4 g/L). A sterile filter paper soaked in picric acid solution (2.5 g picric acid, 12.5 g Na, CO*f* in 1000 mL distilled water) was affixed to the inner lid of each Petri plate. The plates were sealed with Parafilm and incubated at 30°C for 48 hours. Colour change in the filter paper from yellow to light brown (weak, +), brown (moderate, ++), or reddish-brown (strong, +++) indicated positive HCN production.

Hydrolytic enzyme production
PROTEASE ACTIVITY

Skim milk agar medium was utilized to assess protease activity of selected fungal cultures. Fungal disc (5mm diameter) was kept on the centre of the plate containing skim milk agar medium and incubated at 30°C for 72h hours. Protease activity was confirmed by the appearance of clear halo zones around fungal colonies, reflecting the microbial ability to hydrolyse casein present in the medium. (Berg *et al.,* 2002).

CHITINASE ACTIVITY

The chitinase plate assay was conducted following the protocol described by Roberts and Selitrennikoff (1988).

The preparation of colloidal chitin was performed by adding 5.0 g of chitin to 60 ml of concentrated HCl under continuous magnetic stirring. The resulting mixture was stored overnight in refrigerated conditions to allow acid hydrolysis. The slurry was slowly introduced into 2000 mL of pre-chilled 95% ethanol and maintained at 26°C overnight. The precipitated material was subsequently centrifuged at 3000 rpm for 20 minutes at 4°C. The pellet was then washed multiple times with sterile distilled water to remove residual impurities. The purified colloidal chitin was then stored at 4°C until further use.

To prepare the medium for chitinase activity detection, 4.5 g of the previously prepared colloidal chitin was suspended in 1 L of minimal salts medium (MSM). The pH was adjusted to 4.7, and the medium was sterilized by autoclaving at 121°C for 15 minutes.

Sterile agar plates containing the chitinase detection medium were inoculated with 5 mm diameter plugs of actively growing fungal isolates. The plates were incubated at 28°C for three days. Uninoculated plates were taken as control. Chitinase activity was indicated by the formation of a purple-hued zone surrounding the fungal colonies. The intensity and diameter of the purple zone were recorded as a criterion to evaluate chitinase production after three days of incubation.

Microscopy and Molecular Identification of the selected cultures

The selected fungal cultures were grown on PDA and incubated at 28º C for two days. Plate morphology of the fungus was observed and microscopy of isolates was performed using lactophenol cotton method as described by Leck (1999). A drop of lactophenol cotton blue was placed on a glass slide and a small tuft of fungi was transferred using a sterilized needle. The fungal specimen was gently teased, and a coverslip was carefully placed over it. The slides were observed under the light microscope.
For molecular identification, the fungal DNA was isolated using CTAB method, 5.8s r RNA was amplified and sequencing was performed via Sanger sequencing using capillary electrophoresis (Kumar *et al.,* 2013). BLAST was run using NCBI database,

phylogenetic trees were constructed using Neighbourhood joining method with the help of MEGA 12.0 software.

Statistical Analysis

All the experiments were conducted in triplicate using a completely randomised design. The data obtained was analysed using Analysis of Variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) analysis and mean comparisons were at a significance threshold of $P \leq 0.05$ using SPSS software version 16.0. The alphabets in the graphs and tables denote levels of significance; groups sharing the same letter are not statistically different from each other

RESULTS AND DISCUSSION

Isolation and selection of fungal isolates based on their Phosphate solubilizing potential

Eight distinguish *Trichoderma* colonies (6 from paddy and 2 from guava rhizosphere) were isolated and qualitatively screened for their phosphate solubilizing potential. Out of 8 isolates only 3 isolates i. e., RAF5, RAF6 and RRF3 were able to grow on MSM medium amended with Tri calcium phosphate. No zones were formed around the colonies. The growth diameters of the fungus were recorded in comparison to their growth on their universal medium i. e., PDA. RAF5 showed the maximum growth diameter i. e., 9.0 cm followed by RAF6 (7.5cm) and RRF3 (5.3 cm) in MSM amended with TCP on sixth day of incubation (Table 1). The fungal growth diameter was notably reduced in MSM medium supplemented with TCP in comparison to PDA. This reduction is likely due to the nutrient-rich composition of PDA, which offers more favourable conditions for fungal growth. In contrast, MSM supplemented with TCP imposes phosphate stress, requiring the fungus to activate specific adaptive mechanisms for survival and growth. Previous studies have reported that *Trichoderma* is capable of solubilizing insoluble forms of phosphate in quantitative assays, even in the absence of visible halo zone formation, relying solely on mycelial growth (Fankem *et al.,* 2006).

The concentration of phosphate (µg/mL) in MSM broth supplemented with tricalcium phosphate (TCP) was measured over a 120-hour period, with readings taken at 24-hour intervals (Fig.1). At 24 h, strains RAF5 and RAF6 exhibited significantly elevated phosphate concentrations (194.90 and 231.01 µg/mL respectively), as compared to RRF3 which displayed moderate release (75.46 µg/mL) of phosphate in the medium. A pronounced increased in phosphate concentration was observed at 72 h, in RAF5 i. e., 417.40 µg/mL, whereas the cultures RAF6 and RRF3 exhibited maximum phosphate solubilization (377.03 µg/mL and 365.00 µg/mL respectively) at 96h. By 120 h, phosphate concentrations declined slightly in all strains but remained consistently and significantly higher as compared to control.

Among the tested strains, RAF5 consistently exhibited highest phosphate solubilizing potential, followed by RAF6 and RRF3. These findings align with trends reported by (Rawat and Tewari, 2011; Tiwari *et al.,* 2024). Among all the three isolates, the isolates showing the maximum phosphate solubilizing ability were isolated from the soil of hilly areas. The above results revealed that the fungus residing in the rhizosphere of hilly area plants are more effective phosphate solubilizers than that present in soils of Tarai regions.

Enzymatic mechanism of Phosphate solubilization by selected fungal isolates

Microorganisms secrete phosphatase enzymes that help in the solubilization of bound forms of phosphorus to plant available forms.

Phosphatase activity and pH change

The cultures showing the maximum Phosphate solubilization with time i. e. (RAF-5 and RAF-6 both the hilly area rhizospheric soil) were assessed for their phosphatase enzyme production potential. Phosphatase activity varied with time intervals in both the selected cultures. Alkaline phosphatase

activity showed a marked increase from 24 to 48 hours, reaching peak levels at 72 hours in RAF5 i. e., 10.98 U/mL and at 96h in RAF6 i. e., 12.27 U/mL (Fig.2). The enzyme remained relatively stable through 120 hours in both RAF5 and RAF6. At each time point beyond 24 hours, alkaline phosphatase activity in RAF6 was marginally higher as compared to RAF5.

In contrast, acid phosphatase activity remained significantly lower than alkaline phosphatase across all time intervals. The maximum activity was observed in both the cultures at 72 hours, with RAF5 showing higher activity i. e., 4.7 U/mL as compared to RAF6 i. e., 3.22 U/mL (Fig.2). The pH values, plotted concurrently, remained relatively stable between 6.5 and 7.0, with a minor declining trend over time.

The above results clearly show the dominance of alkaline phosphatase activity over acid phosphatase across the studied time intervals suggesting that the enzyme alkaline phosphatase is solely involved in Phosphate solubilizing mechanisms in both the selected cultures. *Trichoderma* spp. is known to modify their external environment during growth, often shifting the medium toward neutral to slightly alkaline pH due to organic acid metabolism and active nutrient uptake as evident in the above findings. Alkaline phosphatase is optimally active in such conditions, while acid phosphatase activity is suppressed with rising extracellular pH. This pH adjustment might be a possible reason for the predominance of ALP activity in culture filtrates.

Table 1: Mycelial growth (diameter) of the fungal isolates in TCP amended mineral salt medium as compared to PDA (without TCP) during plate assay

S. No.	Cultures	Fungal colony diameter (cm)					
		PDA			Tri calcium Phosphate		
		2d	4d	6d	2d	4d	6d
1.	RAF5	5.0±0.15^{*b}	6.1±0.46^{*a}	9.0±0.68^{*a}	1.2±0.23^{*a}	3.0±0.70^{*b}	9.0±0.28^{*b}
2.	RAF6	4.2±0.38^{*b}	7.2±0.35^{*b}	9.0±0.85^{*a}	1.5±0.07^{*a}	5.0±0.56^{*c}	7.5±0.62^{*b}
3.	RRF3	2.2±0.21^{*a}	4.9±0.59^{*a}	8.0±0.44^{*a}	0.9±0.03^{*a}	1.8±0.08^{*a}	5.3±0.89^{*a}

SE (m)Factor (A)= 0.21 Factor (B)= 0.258 A X B= 0.365 Factor (C)= 0.258 A XC= 0.365 B X C= 0.446 A X B X C = 0.631
C. D. at 5% Factor (A)= 0.605 Factor (B)= 0.502 A X B= 1.048 Factor (C)= 0.406 A X C= 1.021 B X C= 1.040 A X B X C= 1.815

Table 2: Heavy metal tolerance of the selected fungal isolates in Cadmium nitrate and Lead nitrate amended mineral salt medium

S. No.	Cultures	Fungal colony diameter (cm)					
		Cadmium (50ppm)			Lead (50ppm)		
		2d	4d	6d	2d	4d	6d
1.	RAF5	1.00±0.5^{*a}	3.22±0.31^{*b}	9.00±0.70^{*c}	1.55±0.42^{*a}	4.24±0.24^{*b}	9.03±0.15^{*c}
2.	RAF6	1.04±0.6^{*a}	3.07±0.66^{*b}	9.01±0.42^{*c}	1.43±0.11^{*a}	4.44±0.46^{*b}	9.05±0.43^{*c}

SE (m) Factor (A, culture) = 0.197 Factor (B, time) = 0.161 Factor (A X B) = 0.278 Factor (A, culture) = 0.112 Factor (B, time) = 0.137 Factor (A X B) = 0.193
C. D. at 5% Factor (A, culture) = 0.628 Factor (B, time) = 0.511 Factor (A X B) = 0.888 Factor (A, time) = 1.003 Factor (B, culture) = 0.672 Factor (A X B) = 1.419

Table 3: *In vitro* plant growth promoting parameters of the selected fungal isolates

S. No.	Cultures	Ammonia production	HCN production	Protease Activity (Halo zone in cm)	Chitinase activity (Halo zone in cm)	Siderophore Index (2d)
1.	RAF5	++	+	4.53±0.41^{*a}	6.13±0.95^{*a}	2.06±0.82^{*a}
2.	RAF6	-	+	4.87± 1.66^{*a}	6.60±1.01^{*a}	1.10±0.78^{*a}
		SE (m)		0.715	0.463	0.378
		C. D. at 5%		2.523	1.635	1.33

This enzymatic profile aligns with earlier findings that alkaline phosphatases are often more actively involved in phosphate solubilization by *Trichoderma* species as compared to acid phosphatases (Rawat and Tewari, 2011; Tandon *et al.,* 2020). The relatively stable pH throughout the experiment suggests that the fungi maintained microenvironmental buffering despite enzymatic activity. This could be beneficial in agriculture applications for soil pH stability, overall soil health management and nutrient cycling (Rodrýµguez and Fraga, 1999; Jia *et al.,* 2019; Asghar and Kataoka 2021; Tyskiewicz *et al.,* 2022).

Other plant growth promoting activities of selected fungal isolates

Plant growth promoting actions of the microorganism can be direct or indirect. Direct mechanisms include phytohormone production, ammonia production and other related mechanisms that have a direct effect on plant development. Indirect mechanisms include stress tolerance, antibiosis, siderophore and hydrolytic enzyme production which are not directly involved in plant development but help plant in defence against unfavourable conditions, aiding survival and adaptations in plants.

Direct mechanisms

Ammonia and Siderophore production

Ammonia production by microbes is related to the activities like N_2 fixation and ammonification. Out of two selected isolates only one culture i. e., RAF5

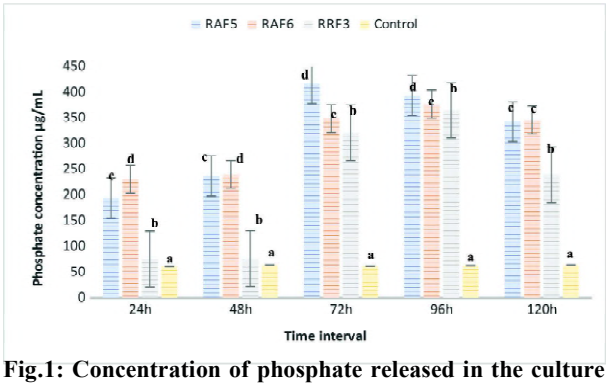

Fig.1: Concentration of phosphate released in the culture filtrates of the fungal isolates growing in Tri calcium phosphate amended MSM medium

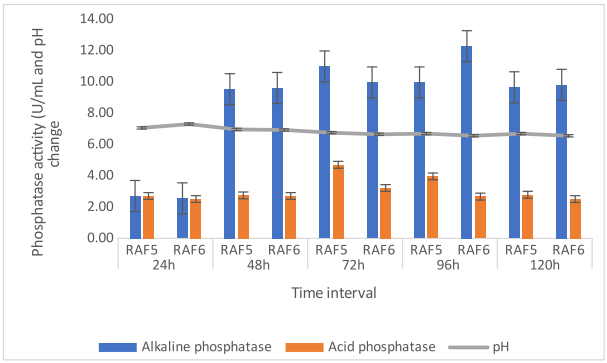

Fig.2: Phosphatase enzyme activity and pH changes during growth of selected fungal isolates in TCP amended broth cultures

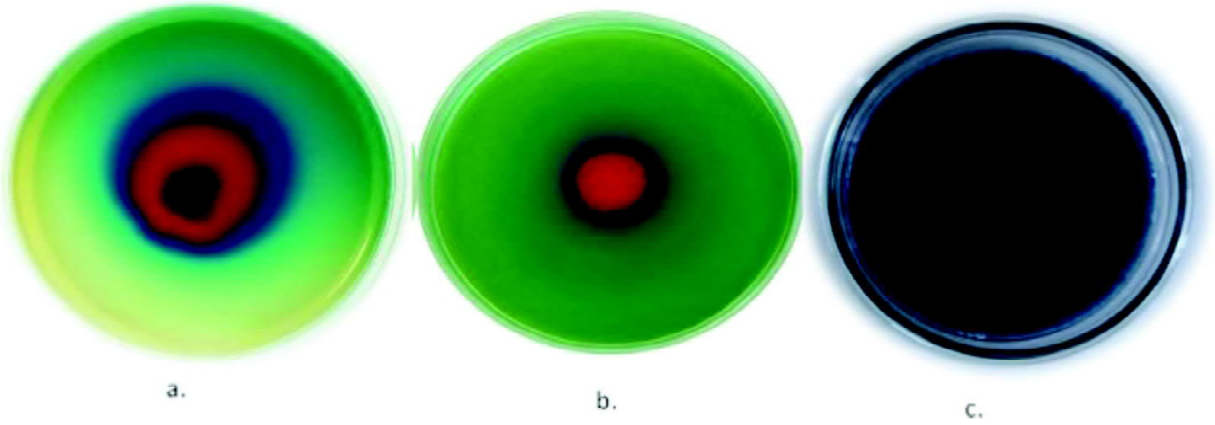

Fig.3: *In vitro* Siderophore production by the fungal isolates RAF5 (a) and RAF6 (b) during plate assay on CAS amended malt extract agar medium (c-control)

showed positive ammonia production.

Siderophores are the ion chelating molecules that chelate iron and zinc and help in the plant survival during deficient conditions (Loper and Buyer, 1999; Saha *et al.,* 2016; Sharma *et al.,* 2023). Both the cultures i. e., RAF5 and RAF6 showed the capability to produce siderophores by making pink halo zones around the colonies on blue coloured CAS dye agar plates (Fig.3) The siderophore index of the isolate RAF5 (2.06) was found to be higher than that of the isolate RAF6 (1.10). The siderophore production during quantification was reported up to 89.88% siderophore units in RAF5 followed by 88.85 % siderophore units in RAF6 after two days of incubation (Fig.4). Comparable levels of siderophore percentage have also been reported by Tiwari *et al*. (2024), supporting the present findings.

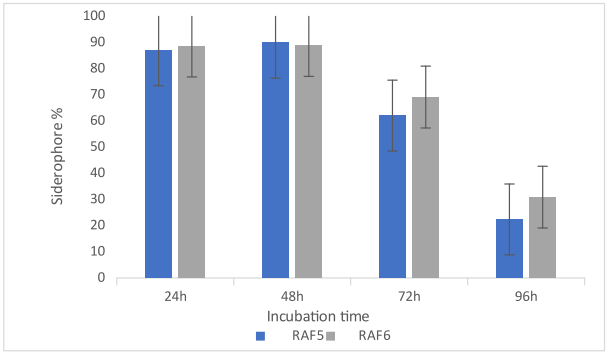

Fig.4: Siderophore production as quantified in the culture filtrates of the selected fungal isolates growing in malt extract medium

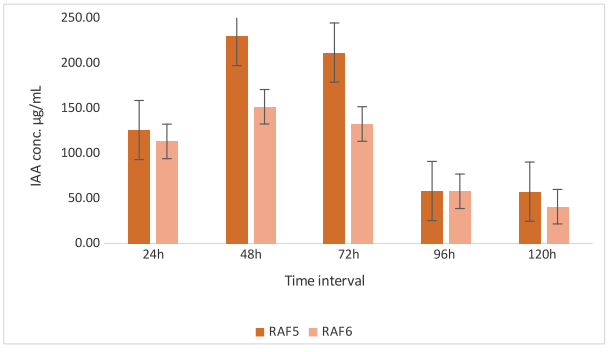

Fig.5: IAA concentrations estimated in the culture filtrates of the selected fungal isolates growing in tryptophan amended medium at different time intervals

IAA Production

IAA production by fungal isolates RAF5 and RAF6 was evaluated at every 24 h time intervals upto 120h. Both isolates demonstrated the highest levels of IAA production at 48 hours, with RAF5 reaching 230.02 µg/mL followed by RAF6, 151.65 µg/mL. (Fig.5) Both the isolates showed a gradual decline in IAA concentrations by 72 hours. RAF5 produced substantially more IAA than RAF6 at nearly all-time intervals, indicating superior auxin-producing potential. This enhanced IAA synthesis may confer greater plant growth-promoting capabilities, especially in rhizosphere interactions where microbial IAA stimulates root elongation and branching.

Indirect mechanisms

Heavy metal tolerance of the selected isolates

Certain heavy metals, including cobalt (Co), copper (Cu), molybdenum (Mo), and manganese (Mn), occur naturally in soil and play essential roles in biological processes when present in trace amounts. However, excessive accumulation of these metals—often due to anthropogenic activities such as overuse of chemical fertilizers—can lead to soil contamination, posing risks to organisms. In contrast,

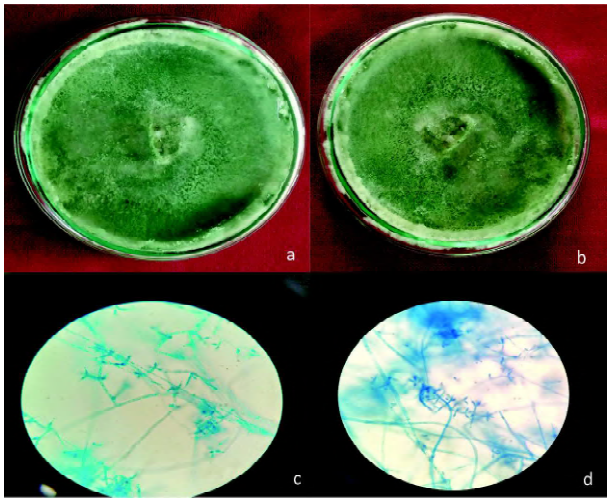

Fig.6: Mycelial growth, sporulation, and microscopic structure of *Trichoderma* isolates RAF5 (a, c) and RAF6 (b, d)

metals like cadmium (Cd) and lead (Pb) lack any known biological functions and are inherently more toxic. Elevated concentrations of these non-essential metals in soil can severely compromise ecosystem health and pose significant threats to both soil integrity and animal well-being (Rodrigues *et al.,* 2017*;* Maldaener *et al.,* 2021). Both the cultures (RAF5 and RAF6) showed mycelial growth in the plates at 50ppm of Cadmium and Lead salts indicating their tolerance towards these metals. No mycelial growth was observed in the increased metal concentrations i. e., 100 and 150 ppm. Both cultures exhibited greater sensitivity to cadmium (Cd) compared to lead (Pb), as evidenced by their reduced growth diameters after 2 and 4 days of incubation on cadmium-containing plates relative to those supplemented with lead salts (Table 2). Heavy metal tolerance of *Trichoderma* strains upto 40 ppm concentration has also been reported by other workers (Nongmaithem *et al.,* 2016). Several studies (de Freitas Lima *et al.,* 2011; Mohsenzadeh and Shahrokhi, 2014; Maldaner *et al.,* 2021) have demonstrated that elevated concentrations of heavy metals exert detrimental effects on hyphal growth and spore production in *Trichoderma* species. This might be the possible reason for the absence of detectable growth at higher heavy metal concentrations observed in the present study.

HCN and Hydrolytic Enzyme production

The ability of *Trichoderma* to act as a biocontrol agent is associated with its potential to produce HCN and hydrolytic enzymes such as chitinase and protease. Both strains, RAF5 and RAF6, were evaluated for their ability to produce these compounds. RAF5 tested positive for HCN production, as well as chitinase and protease activity. After 72 h of incubation, the halo zone diameters of isolate RAF5 were measured as 4.53 cm for protease and 6.13 cm for chitinase. On the other hand, RAF6 only exhibited positive results for protease (4.87cm) and chitinase (6.60cm) activity, with no detectable HCN production (Table3). These findings are

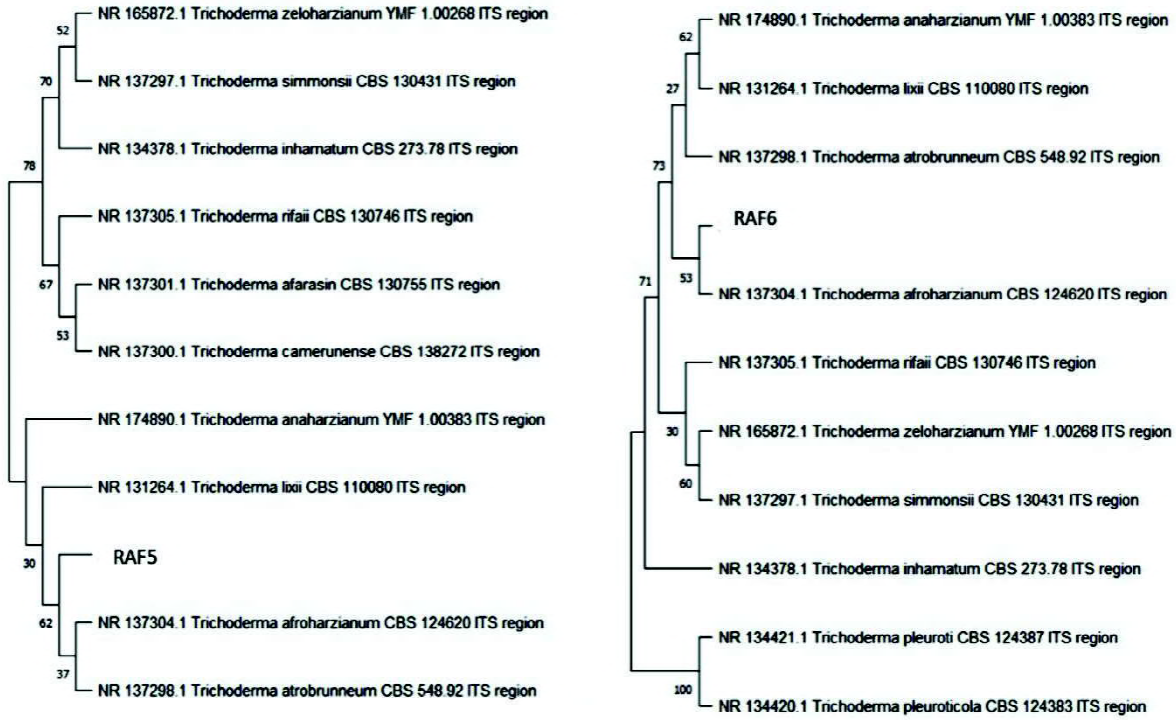

Fig.7: Phylogenetic trees showing the molecular identification of the selected fungal isolates. RAF5 (a) and RAF6 (b)

consistent with those reported by Kamala and Devi (2020).

Morphological, Microscopic and Molecular Characterization of the selected fungal isolates

After three days of incubation, both fungal isolates (RAF5 and RAF6) developed dark green spores on PDA plates. Light microscopy of lactophenol-stained slides revealed key morphological features, including septate hyphae, ampulliform phialides, and densely arranged subglobose conidia (Fig.6). According to Chaverri *et al*. (2015), isolates displaying rapid growth and conidia ranging in color from green to yellow, along with ampulliform phialides and subglobose conidia, are likely members of the genus *Trichoderma*. However, these morphological traits alone are insufficient for species-level identification. To accurately determine the species, the internal transcribed spacer (ITS) region of the fungal DNA was sequenced. Molecular analysis confirmed that both isolates were distinct strains of *Trichoderma afroharzianum*. (Fig.7). Numerous studies have highlighted the potential of *Trichoderma harzianum* and its related species in promoting plant growth through mechanisms such as phosphate solubilization, phytohormone synthesis, and antagonism against various plant pathogens. These beneficial traits make them promising candidates for the development of bioformulations that can be applied in agricultural fields to enhance plant development and improve crop productivity (Altomare *et al.,* 1999; Sharma *et al.,* 2012).

CONCLUSION

The research underscores phosphate-solubilizing potential of *Trichoderma* isolated from the hilly and Tarai regions of Uttarakhand. Among them, the hilly area isolates demonstrated significantly higher solubilization efficiency of phosphate in TCP-amended MSM medium. This activity is primarily attributed to the enzymatic action of alkaline phosphatase. Additionally, both the isolates exhibited siderophore production, phytohormone synthesis, and the ability to produce hydrolytic enzymes. Notably, the strains also showed tolerance to heavy metals such as cadmium and lead at concentrations up to 50 ppm, indicating their suitability for application in contaminated soils. These multifaceted traits suggest that the isolates can enhance nutrient availability, suppress phytopathogens, and contribute to soil remediation. Their ability to withstand abiotic stress factors further supports their robustness under field conditions. Overall, both *Trichoderma* strains evaluated in this study show immense potential as biofertilizers for promoting environmentally sustainable and climate-resilient agriculture.

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REFERENCES

Abdenaceur, R., Farida, B. T., Mourad, D., Rima, H., Zahia, O. and Fatma, S. H. (2022). Effective biofertilizer *Trichoderma* spp. isolates with enzymatic activity and metabolites enhancing plant growth. *International Microbiology*, 25 (4):817–829.

Altomare, C., Norvell, W. A., Bjorkman, T. H., Harman, G. E. (1999). Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus Trichoderma harzianum Rifai 1295-22. *Applied and Environmental Microbiology*, 65 (7): 2926–2933.

Asghar, W., and Kataoka, R. (2021). Effect of co-application of Trichoderma spp. with organic composts on plant growth enhancement, soil enzymes and fungal community in soil. *Archives of Microbiology*, 203 (7): 4281–4291.

Bakker, A. W. and Schipper, B. (1987). Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth

stimulation. *Soil Biology and Biochemistry*, 19: 451–457.

Berg, G., Krechel, M., Ditz, R., Sikora, A. and Hallmann, J. U. (2002). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiology Ecology*, 51 (2): 215–229.

Bilal, S., Hazafa, A., Ashraf, I., Alamri, S., Siddiqui, M. H., Ramzan, A. and Naeem, M. (2021). Comparative effect of inoculation of phosphorus-solubilizing bacteria and phosphorus as sustainable fertilizer on yield and quality of mung bean (*Vigna radiata* L.). *Plants*, 10 (10): 2079.

Chaverri, P., Branco-Rocha, F., Jaklitsch, W., Gazis, R., Degenkolb, T. and Samuels, G. J. (2015). Systematics of the Trichoderma harzianum species complex and the re-identification of commercial biocontrol strains. *Mycologia*, 107 (3): 558–590.

de Azeredo Morgado, M. G., Passos, C. J. S., Garnier, J., De Lima, L. A., de Alcântara Mendes, R., Samson-Brais, É. and Lucotte, M. (2023). Large-scale agriculture and environmental pollution of ground and surface water and sediment by pesticides in the Brazilian Amazon: The case of the Santarém region. *Water, Air and Soil Pollution*, 234 (3):150.

de Freitas Lima, A., Ferreira de Moura, G., Barbosa de Lima, M. A., Mendes de Souza, P., Alves da Silva, C. A., de Campos Takaki, G. M., and do Nascimento, A. E. (2011). Role of the morphology and polyphosphate in Trichoderma harzianum related to cadmium removal. *Molecules*, 16 (3): 2486–2500.

de Oliveira, H. P., de Melo, R. O., Cavalcante, V. S., Monteiro, T. S. A., de Freitas, L. G., Lambers, H., and Valadares, S. V. (2025)). Phosphate fertilizers coated with phosphate-solubilising Trichoderma harzianum increase phosphorus uptake and growth of Zea mays. *Plant and Soil*, 508 (1): 613-624.

Dye, D. W. (1962). The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. *New Zealand Journal of Science*, 5: 393–416.

Fankem, H., Nwaga, D., Deubel, A., Dieng, L., Merbach, W. and Etoa, F. X. (2006). Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. *African Journal of Biotechnology*, 5 (24) :2450-2460.

Fiske, C. H. and Subbarow, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem*, *66* (2):375-400.

Jia, L., Zhang, Q., Song, W., and Chen, J. (2019). Soil application of *Trichoderma asperellum* GDFS1009 granules promotes growth and resistance to *Fusarium graminearu*m in maize. *Journal of Integrative Agriculture*, 18 (3): 599–606.

Kamala, T. H., and Devi, S. I. (2020). Biocontrol properties of indigenous Trichoderma isolates from North-east India against *Fusarium oxysporum* and *Rhizoctonia solani*. *African Journal of Biotechnology*, 11 (34): 8491–8499.

Kumar, S., Rai, S., Maurya, D. K., Kashyap, P. L., Srivastava, A. K. and Anandaraj, M. (2013). Cross-species transferability of microsatellite markers from *Fusarium oxysporum* for the assessment of genetic diversity in *Fusarium udum*. *Phytoparasitica*, 41: 615–622.

Leck, A. (1999). Preparation of lactophenol cotton blue slide mounts. *Community Eye Health*, 12 (30): 24.

Loper, J. E. and Buyer, J. S. (1999). Siderophores in microbial interactions on plant surfaces. *Molecular Plant-Microbe Interactions*, 4: 5–13.

Maldaner, J., Steffen, G. P. K., Missio, E. L., Saldanha, C. W., de Morais, R. M. and Nicoloso, F. T. (2021). Tolerance of Trichoderma isolates to increasing concentrations of heavy metals. *International Journal of Environmental Studies*, 78 (2): 185–197.

Maldaner, J., Steffen, G. P. K., Missio, E. L., Saldanha, C. W., de Morais, R. M., and Nicoloso, F. T. (2021). Tolerance of Tricho-

derma isolates to increasing concentrations of heavy metals. *International Journal of Environmental Studies*, 78 (2): 185–197.

Mishu, N. J., Hasan, M. R., Islam, S. M. N., Nayeema, J., and Hossain, M. M. (2025). Additive effects of Trichoderma isolates for enhancing growth, suppressing southern blight and modulating plant defense enzymes in tomato. *PLoS One*, 20 (7): e0329368.

Mohsenzadeh, F., and Shahrokhi, F. (2014). Biological removing of cadmium from contaminated media by fungal biomass of Trichoderma species. *Journal of Environmental Health Science and Engineering*, 12 (1): 102.

Nongmaithem, N., Roy, A., and Bhattacharya, P. M. (2016). Screening of Trichoderma isolates for their potential of biosorption of nickel and cadmium. *Brazilian Journal of Microbiology*, 47 (2): 305–313.

Paul, S. and Rakshit, A. (2021). Effect of seed biopriming with *Trichoderma viride* strain BHU-2953 for enhancing soil phosphorus solubilization and uptake in soybean (*Glycine max*). *JSSPN*, 21 (2): 1041–1052.

Prismantoro, D., Akbari, S. I., Permadi, N., Dey, U., Anhar, A., Miranti, M. and Doni, F. (2024). The multifaceted roles of *Trichoderma* in managing rice diseases for enhanced productivity and sustainability. *Journal of Agriculture and Food Research*, 18: 101324.

Rawat, R. and Tewari, L. (2011). Effect of abiotic stress on phosphate solubilization by biocontrol fungus Trichoderma sp. *Current Microbiology*, 62: 1521–1526.

Roberts, W. K. and Selitrennikoff, C. P. (1988). Plant and bacterial chitinases differ in antifungal activity. *Journal of General Microbiology*, 134: 169–176.

Rodrigues, T. F., de Pontes, A. S., de Jesus, A. P., da Silva Marques, H. M., de Jeusus Silva, C. C., Barletta, R. V., et al.2017. A ação dos metais pesados provenientes de rejeitos de mineração sobre a saúde e o meio ambiente. Semioses, 11 (2): 82–87.

Rodrýμguez, H. and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, 17 (4–5): 319–339.

Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S. and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. *Environmental Science and Pollution Research*, 23 (5): 3984–3999.

Schwyn, B., and Neilands, J. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry*, 160 (1): 47–56.

Sharma, B., Tiwari, S., Kumar, R., Kumar, M., and Tewari, L. (2023). Eco-friendly detoxification of hazardous Congo red dye using novel fungal strain Trametes flavida WTFP2: deduced enzymatic biomineralization process through combinatorial in silico and in vitro studies. *Journal of Hazardous Materials*, 455: 131503.

Sharma, P., Patel, A. N., Saini, M. K. and Deep, S. (2012). Field demonstration of *Trichoderma harzianum* as a plant growth promoter in wheat (*Triticum aestivum* L). *Journal of Agricultural Science*, 4 (8): 65.

Silva, L. I. D., Pereira, M. C., Carvalho, A. M. X. D., Buttrós, V. H., Pasqual, M. and Dória, J. (2023). Phosphorus-solubilizing microorganisms: A key to sustainable agriculture. *Agriculture*, 13 (2): 462.

Singh, B. and Satyanarayana, T. (2011). Microbial phytases in phosphorus acquisition and plant growth promotion. *Physiology and Molecular Biology of Plants*, 17: 93–103.

Tandon, A., Fatima, T., Shukla, D., Tripathi, P., Srivastava, S. and Singh, P. C. (2020). Phosphate solubilization by *Trichoderma koningiopsis* (NBRI-PR5) under abiotic stress conditions. *Journal of King Saud University – Science*, 32 (1): 791–798.

Tariq Javeed, M., Farooq, T., Al-Hazmi, A. S., Hussain, M. D., Rehman, A. U. (2021). Role of Trichoderma as a biocontrol agent (BCA) of phytoparasitic nematodes and plant growth inducer. *Journal of Invertebrate Pathology,* 183: 107626.

Tian, J., Ge, F., Zhang, D., Deng, S., Liu, X. (2021). Roles of phosphate solubilizing microorganisms from managing soil phosphorus deficiency to mediating biogeochemical P cycle. *Biology*, 10 (2): 158.

Tiwari, S., Sharma, B., Bisht, S., Pant, D., Kumar, S., and Tewari, L. (2024). An eco-friendly approach harnessing Trichoderma lixii ORT2 for reducing chemical phosphatic fertilizer dependency and groundwater phosphorus management through integrated in silico, *in vitro* and omic studies. *Groundwater for Sustainable Development*, 26: 101278.

Tomer, S., Suyal, D. C. and Goel, R. (2017). Biofertilizers: a timely approach for sustainable agriculture. In *Plant-microbe interaction: an approach to sustainable agriculture,* Singapore: Springer, Singapore Pp.375–395.

Waghunde, R. R., Shelake, R. M., Sabalpara, A. N. (2016). *Trichoderma*: A significant fungus for agriculture and environment. *African Journal of Agricultural Research*, 11 (22):1952–1965.

Tycekiewicz, R., Nowak, A., Ozimek, E. and Jaroszuk-Œcise³, J. (2022). Trichoderma: The current status of its application in agriculture for the biocontrol of fungal phytopathogens and stimulation of plant growth. *International Journal of Molecular Sciences*, 23 (4): 2329.

Wojciechowska-Mazurek, M., Starska, K., Bruliñska-Ostrowska, E., Plewa, M., Biernat, U. and Kar³owski, K. (2008). Monitoring of contamination of foodstuffs with elements noxious to human health. Part I. Wheat cereal products, vegetable products, confectionery and products for infants and children (2004 year). *RocznikPZH*, 59: 251–266.

Zhang, D., Yin, C., Abbas, N., Mao, Z. and Zhang, Y. (2020). Multiple heavy metal tolerance and removal by an earthworm gut fungus *Trichoderma brevicompactum* QYCD-6. *Scientific Reports*, 10 (1): 6940.

Zwolak, A., Sarzyñska, M., Szpyrka, E. and Stawarczyk, K. (2019). Sources of soil pollution by heavy metals and their accumulation in vegetables: A review. *Water, Air, and Soil Pollution*, 230: 1–9.

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