ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

A Fungal Consortium Mitigating Arsenic Uptake In Rice Under Cultivation In Arsenic- Contaminated Soil

Manyata Sharma[1,2], Saloni Rishi[2], Pankaj Kumar Srivastava[2], Deepak Chand Sharma[1*]

[1] Dr. Shakuntala Misra National Rehabilitation University, Lucknow, India

[2] Environmental Technology Division, CSIR-National Botanical Research Institute, Lucknow, India

***Corresponding Author:** Deepak Chand Sharma

Email: dcsharma@dsmnru.ac.in, dcsharma@gmail.com

Abstract

A total of fifteen novel arsenic tolerant soil fungal strains were isolated from the paddy fields of West Bengal, India and were checked and approved for producing a marked decline in the amount of arsenic present in our previous study. In our present study, four leading strains out of those fifteen showing excellent arsenic removal potential were selected for developing a talc-based soil fungal consortia formulation with extended shelf life and creating a bio-inoculant arsenic remediation package for their soil applications and field trials. Initially, plant growth promoting potentials and compatibility characterization was performed to see the stimulating and inhibitory effects respectively. For establishing talc as the most suitable carrier in the field, a pot experiment was set up using a rice variety sarjoo-52 treated with carrier-based inoculum of the strains. Talc was established as the best carrier augmenting the desired fungal formulation. The shelf-life study was conducted and these formulations were found viable for a period of 180 days. The log mean value these of these formulations increased with further addition of enrichment additives. Out of the total additives checked for growth promotion abilities in broth, sucrose and $CaCO_3$ at a concentration of 0.5% gave the best results and improvised the shelf life of the formulation. These fungi serve a dual purpose in amelioration of arsenic uptake by the rice plants along with their excellent roles in plant growth promotion. The carrier-based approach will further increase the durability of the formulated product and nullifying/reducing arsenic toxicity will help in gaining a better yield. The purpose of the work is to produce a preparation of a microbial formulation containing live fungi for the removal of arsenic, and plant growth promotion in crops grown in arsenic- contaminated fields. The study focussed on producing a fungal consortium formulation comprising of four novel strains namely *Westerdykella aurantiaca* (FNBR-FA 03), *Trichoderma longibrachiatum* (FNBR-FA 06), *Lasiodiplodia* sp. (FNBR-FA 13), *Rhizopus delemer* (FNBR-FA 19). The preparation is having synergistic effects on arsenic amelioration. This fungal formulation has enhanced efficiency and is a stable delivery system, which is cost-effective and commercially viable.

Keywords: Arsenic, Fungi, Consortium, PGPR, Bioremediation

INTRODUCTION

Arsenic (As) is an extremely potent toxic metalloid and is classified as non-threshold class I human carcinogen by International Agency for Research on Cancer. Arsenic ranks highest in the

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

Priority List of Hazardous Substances by Agency for Toxic Substances and Disease Registry's (ATSDR, 2020). Arsenic is geogenically present in the environment and is therefore considered a global issue (Raju, 2022). Humans are exposed to arsenic through consumption of agricultural crops and vegetables irrigated by arsenic contaminated ground water (Bhatti et al., 2013). The arsenic levels in food crops are often found above permissible limit by WHO (1.0 mg kg^{-1}) (WHO, 2001). Arsenic in rice (Oryza sativa L) is considered as a major source of arsenic exposure in humans (Mondal et al., 2020; Signes-Pastor et al., 2021). Higher levels of inorganic arsenic have been reported in rice (raw and cooked) and baby foods prepared from rice in many developed countries (US HoR, 2021). The toxic effect of arsenic is majorly due to its superabundant inorganic species- arsenate (V) and arsenite (III) amongst which arsenite being the most toxic. These species are more predominant in soils than the organic species [monomethyl arsonic acid (MMA) and dimethyl arsenic acid (DMA)] (Zhao et al., 2010). The toxicity levels of arsenic species are as follows: arsine > organic trivalent species > inorganic arsenite > inorganic arsenate > organic species (DMA and MMA). In plants, arsenic directly connects with the cellular compartment and warps the physiological processes required for proper cell functioning. Arsenic phytotoxicity often results in stunted growth and yield loss in plants (Li et al., 2006). Arsenic levels in rice may be additionally elevated if rice is grown on arsenic-contaminated soils, probably increasing arsenic exposure, particularly in Bangladesh and the population of Indo-Gangetic Plains. The average soil arsenic contamination around the world is found in ranges from 10 to 2470 mg kg^{-1} (Dahlawi et al., 2018). Bangladesh and a few areas of India (West Bengal) depend on As-contaminated groundwater for the irrigation of major crops like rice which is a dietary staple for almost half of the population of the world as compared to other cereals because of waterlogged growth conditions and transport of As(III) through

extremely efficient silicon transport pathway (Zhao et al., 2010). Various physical and chemical strategies like coagulation, membrane separation, adsorption or precipitation, ion-exchange and stabilization techniques are used for arsenic decontamination. These methods often result in generation of toxic by-products, have high operational cost and are inefficient under natural conditions. Besides these numerous mitigation strategies, the development of low arsenic rice cultivars through breeding and transgenics and nutrient supplementation like silicon and phosphate have additionally been used to reduce arsenic uptake by rice (Zhao et al., 2010). Removing arsenic from polluted soils and groundwater with the assistance of microorganisms is an economical approach for future developments due to its environmental congeniality and low capital investments that offer a sustainable option to address the matter while not compromising the projected demand for rice. Biological treatment of arsenic is a pragmatic approach of remediation over conventional methods. Microorganisms can bioaccumulate, bio-adsorb, bio-transform (methylation and oxidation) and bioremediate arsenic. Microorganisms are capable of biotransforming As(III) to As(V) with the help of enzymes arsenic oxidase (Andreoni et al., 2012). They have some developed biochemical mechanisms where in arsenic oxyanions, either is an electron acceptor As(V) for anaerobic respiration or as donor As(III) for supporting chemoautotrophic fixation of CO_2 into cell carbon. Both viable and non-viable biomass of fungi have been reported to take away radioactive elements, heavy metals, and metal cyanide

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

complexes. Fungi have an advantage over bacteria for being used in the bioremediation of impure soils because of more surface area to volume ratio, more biomass, longer lifecycle in soil and extended hyphal networks (Mohd et al., 2017). Moreover, fungi which are tolerant to metals are capable of surviving very adverse conditions along with the native bacteria. The wide metabolic competency of fungi results in their broad applications for bioremediation of various contaminants. The amino, hydroxyl, carboxyl, phosphate and sulphate, groups, which bind metal(loid) ions are present on the polysaccharides and proteins of cell walls of fungi. Fungi like, *Fusarium, Trichoderma* and *Penicillium* are found to methylate inorganic arsenic to organic species like MMA, DMA and TMAO or TMA (Srivastava et al, 2011). However, the contribution of fungi in reducing stress imposed by arsenic on rice plants is a less explored area because of an extremely variant soil and aquifer chemical composition. However, fungi are capable of tolerating metal(loid) in the soil therefore bio-augmenting those promising native soil fungi can be a successful approach for treating arsenic-contaminated soils and development of arsenic mycoremediation technology. The combination of two or more technologies will work in synergy for good results. This makes microorganisms like fungi pivotal players in restricting metals entry into the food chain by exploiting many bioremediation strategies. Gentry et al., (2004) recommended numerous new methods such as bio-augmentation with cells embedded in several carriers like alginate, resulting in an inflated persistence and activity of microorganisms following application into the soils. Analysis of their physiological and metabolic responses to metal stress is also required for consortium formulation applicable in bioremediation methods like bio-augmentation (Haferburg and Kothe 2010). Fan et al. (2014) worked on the method of application of a bio-augmentation technique to a clay loam soil which was contaminants with total petroleum hydrocarbons using yeast strain isolated from contaminated soil. An important multistep procedure is concerned with developing a formulation of inoculant which has one or several special microorganism strains in an exceedingly appropriate carrier, providing a secure atmosphere to guard against the usually harsh conditions throughout storage and providing extended sustainability once introduced into the target soils. Pandey et al. (2009) gave a method of co-bio-augmentation as a novel technique that comprised of a consortium of various microbial strains with distinct metabolic potentials. As compared to organic contaminants, metal(loid)s is not easily removed by microbial or chemical treatments and exist for a longer time. The bioavailability of metal(loid)s thus plays an important role in remediating contaminants from target soils. The rectification of soils polluted with heavy metal(loid)s through manipulation of their bioavailability employing a variety of soil enrichments and soil microorganisms is often done. Immobilizing amendments such as causative agents and materials, like fungal biomass, reduces the bio-availability and quality of metal(loid)s. Immobilization agents are often used for diminishing the transfer of metal(loid)s into the food chain through plant uptake. The arsenic immobilization in contaminated soils can be achieved using fungal bio-sorbent inoculums to soils as the bio-augmentation approach. Bio-augmentation is the most commonly adopted remediation technology. But up to now a large number of studies showed that introduced microorganisms often suffer sustainability issues and do not survive in the environment thus do not serve the purpose of an improved remediation process Thus, the objective of this study was to develop a consortia based bio-formulation in the best suitable carrier for efficient field applications using

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

culturable soil fungi isolated from the agricultural soils of lower Indo-Gangetic Plains (IGP) West Bengal, India having arsenic tolerance and removal efficiencies (Srivastava et al. 2011) and to find out a promising novel product having an extended shelf-life and a higher arsenic mycoremediation potential. For in-situ bioremediation of heavy metal(loid)s contaminated soils, biostimulation and bioaugmentation are emerging as the two very efficient strategies in the present scenario. Soil microbes are involved in different detoxification mechanisms for remediating arsenic contamination e.g., biosorption, bioaccumulation, biomethylation, biovolatilization, valence transformation, etc. (Srivastava et al., 2011). Bioremediation is giving environmental-friendly, efficient, and economical technology for the removal/ reduction of arsenic from polluted soil and water compared to physio-chemical treatments. All bioremediation strategies depend on fundamental inherent metabolic pathways of the microbes for reducing the toxicant effects of the waste products by conversion to lesser toxicant products; mineralization of the pollutants thoroughly; and inactivation of the pollutant by reducing its mobility. Understanding the mechanism concerned in segregation and hyperaccumulation is extremely vital for developing new methods of bioremediation. This will yield great stimulating insights regarding the mechanisms concerned with the regulation of microbial responses to heavy metals, that successively improve our information of metabolic regulation underneath metal stress and also the use of fungi for bioremediation. While bacteria have made sensible leads to bioremediation in the past, the employment of fungi to cleanse soils has recently been established. Fungi are ubiquitous and dominant organisms in many soils with metabolic diversity which makes them economical agents of bioremediation. Therefore, the application of plant growth-promoting fungi having tolerance to As can be an economical strategy to cut back As load from edible crop plants growing in As contaminated areas. The aim of the study was to isolate As tolerant fungal strains from contaminated soils and investigate their role in the amelioration of arsenic uptake by the rice plants. This was a low-cost, highly efficient, and eco-friendly mitigation strategy for the removal of arsenic pollution. In this study, the current paddy cultivation practice at high arsenic multi-locations in West Bengal was coupled with proposed bioremediation technology using arsenic remediator indigenous soil fungal strains, which reduced the arsenic toxicity/uptake into arsenic sensitive/moderately tolerant paddy crop varieties. This resulted in bioremediation-based safe farming practice for remediation of arsenic contamination to paddy crops through demonstration of a reduction in the bioavailable soil arsenic content and low/no grain arsenic in the paddy grains. For the viable formulation to be successful, it was vital that the agents (fungi) survive and remain active for a period sufficient for them to become established and produce anticipated effects. The formulation should help as a buffer to an organism from adverse conditions (Hasan and Ayres, 1990). It is also important that the fungal consortium is formulated in such a way that it can be easily used and applied by farmers. Thus, a critical step in the carrier-based inoculum is the selection of the appropriate carrier that can effectively deliver the adequate inoculum to the soil. Traditionally peat has been used as an inoculant carrier, while others include oils, inorganic clays (talc and vermiculite), and cellulose derivatives like hydroxyl ethyl and carboxyl methyl cellulose (Digat, 1989), polymers like alginate beads are also used. Crop residues like saw dust etc. have also been used as a carrier (Singh, et al., 2007; Arora et al., 2008*).* In this

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

study, talc was chosen as the suitable carrier material based on the earlier studies (Singh and Nautiyal, 2012).

Materials and Methods

Chemicals and Equipment's

The chemicals used in the study are of analytical and molecular grade procured from Hi-media, India. The stock solutions for As (III) and As (V) was prepared by dissolving sodium arsenite and sodium arsenate salts (Hi-media) in autoclaved Milli-Q and were syringe filtered (0.2 µm) in laminar chambers. The stock solution of standards was stored in ambered coloured bottles at 4°C.

Collection of soil samples

Arsenic contaminated paddy fields (9-15 mg kg^{-1}) from West Bengal, India were selected for the study. Rhizospheric soil samples (n=3) were collected from the paddy plants in sterile collection bags in random manner. The soil samples were bought to laboratory in thermo boxes (4°C) for isolation of fungal strains.

Isolation of arsenic tolerant fungal strains

For the isolation of arsenic tolerant fungal strains, 0.1 g of rhizospheric soil samples were serially diluted in autoclaved saline solution (0.8%). A 100 µl aliquots from each dilution was spread on Rose Bengal agar plates (RBA) in triplicate. The plates were incubated at 28±2°C for 3 days. Morphologically distinct fungal colonies were selected and sub-cultured on RBA plates until pure colonies were obtained. A total of fifteen fungal cultures were obtained which were further screened to four fungi as reported in our previous studies (Srivastava et al., 2012). The selected fungi are *Westerdykella aurantiaca* (FNBR-FA03), *Trichoderma longibrachiatum* (FNBR-FA06), *Lasiodiplodia* sp. (FNBR-FA13), *Rhizopus delemer* (FNBR-FA19) reported for arsenic tolerance (up to 10000 µg ml^{-1}) (Srivastava et al., 2012). The obtained pure fungal cultures were maintained on potato dextrose agar (PDA) plates and stored in PDA slants at 4°C.

Characterization of plant growth promotion traits of fungal strains

The fungal isolates were characterized for plant growth promoting traits such as phosphate solubilization, indole acetic acid (IAA), siderophore production and ACC-deaminase activity.

Phosphate solubilization assay

Phosphate solubilization assay was performed following Fiske and Subbarow method (1925). The fungal strains were grown on agar plates prepared with NBRI-P medium comprising of the following: $MgSO_4.7H_2O$, 0.25 g L^{-1}; tricalcium phosphate (TCP), 5.0 g L^{-1}; $MgCl_2.6H_2O$, 5.0 g L^{-1}; glucose, 10 g L^{-1}; KCl, 0.2 g L^{-1}; $(NH_4)_2SO_4$, 0.1 g L^{-1}. TCP was used as a sole source phosphate. The plates were inoculated with pure fungal agar plugs and incubated at 28°C for 7 days. After 3rd, 5th, and 7th days of incubation, measurements for the solubilization haloes around the colonies and diameters of the colonies were recorded. To obtain the reading for cleared zone,

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

total diameter of halo zone was subtracted from the diameter of the culture zone. For quantitative estimation of phosphate solubilization by fungal strains, liquid NBRI-P medium was used (Mehta and Nautiyal, 2001). Active growing fungal culture of each fungal strain was inoculated in potato dextrose broth and incubated at 28±2°C in an incubator shaker at 120 rpm for 15 days. Phosphate solubilization by each fungal strain was estimated by fetching 1 mL aliquot from each culture flasks on the 3rd, 5th, 7th, 10th and 15th day respectively. The samples were centrifuged at 10,000 rpm for 10 min and the supernatant of each culture was analyzed for phosphate concentration. The phosphate solubilization activity was measured using spectrophotometer at O.D 660 nm and expressed as equivalent phosphate (µg mL^{1}).

Siderophore production assay

For detection of the siderophore production, Universal Chrome Azurol S (CAS) agar medium was prepared (Schwyn and Neiland, 1987). Potato dextrose agar medium was supplemented with 1.21 mg mL^{-1} CAS, 0.273 mg mL^{-1} $FeCl_3$ and 1.81 mg mL^{-1} CTAB as solution A, B and C respectively which were autoclaved separately and added aseptically in laminar chambers. Discs (5 mm diameter) of actively growing cultures of fungal strains were placed on the CAS plates and kept in incubator at 30°C for 5 days. The blue colour of the medium was decolourized to orange coloured halo zone surrounding the fungal disc indicating the production of siderophore. The observations for the halo zone were recorded on 1st day, 3rd day, and 5th day of incubation. For quantification of siderophore, 3 days old fungal cultures were centrifuged 10,000 rpm for 15 min and the cell free supernatant was subjected to detection and estimation of siderophore. Quantitative estimation was done by CAS shuttle assay, 0.5 ml of culture supernatant was mixed with 0.5 ml of CAS reagent. Absorbance was measured at 630 nm against a reference consisting of 0.5 ml of un-inoculated broth and 0.5 ml CAS reagent. Siderophore produced by strains was measured in percent siderophore unit (psu).

The content of siderophore in the aliquot was calculated by using the formula: -

% Siderophore units (psu)= [(Ar-As)/Ar*100]

Indole acetic acid (IAA) assay

The estimation of auxin concentration was determined using Van Urk Salkowski reagent following Salkowaski's method (Ehmann, 1977). The fungal strains inoculated in potato dextrose media supplemented with the following: SDS, 0.06 g L^{-1}; tryptophan, 1.02 g L^{-1} and glycerol 1000 µl L^{-1}. The fungal cultures were incubated at 28±2°C in an incubator shaker at 120 rpm for 21 days. Periodically, an aliquot of 1.5 mL was withdrawn from each culture flask on 7th, 14th and 21st day. The fungal cultures were centrifuged at 10,000 rpm for 10 min and cell free supernatant was harvested. From the supernatant, 1 mL aliquot was fetched and mixed with 0.05 ml orthophosphoric acid and 2 mL of Salkowaski's reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid solution). The reaction mixture was incubated at room temperature for 30 min in dark conditions. The optical density (O.D) was recorded at 530 nm and the auxin produced was expressed as equivalent IAA (µg mL^{-1}).

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

ACC deaminase enzyme

For ACC-deaminase activity in fungal strains, Dworkin and Foster minimal salt (DF) media was prepared using the following: $MgSO_4.7H_2O$, 0.2 g L^{-1}; KH_2PO_4, 4.0 g L^{-1}; Na_2HPO_4, 6.0 g L^{-1}; glucose, 2.0 g L^{-1}; gluconic acid, 2.0 g L^{-1} and citric acid, 2.0 g L^{-1}. With trace elements: $FeSO_4.7H_2O$, 1 mg L^{-1}; $MnSO_4.H_2O$, 11.19 mg L^{-1}; $ZnSO_4.7H_2O$, 124.6 mg L^{-1}; H_3BO_3, 10 mg L^{-1}; $CuSO_4.5H_2O$, 78.22 mg L^{-1}; MoO_3, 10 mg L^{-1}; pH 7.2 (Dworkin and Foster 1958). One set was prepared by DF Media Salts with trace elements and 2.0 g L^{-1} $(NH_4)_2SO_4$ as a nitrogen source. While in another set the nitrogen source was replaced by a 0.5 M solution of ACC. The fungal culture disk was inoculated on the DF media plates and incubated for 1 day at 25±2°C. The plates were observed for ACC deaminase activity. For quantification of ACC deaminase activity, Penrose and Glick, 2003 method was used. The fungal cultures were harvested and washed twice with DF media. The fungal cultures were centrifuged at 10,000 rpm at 4˚C for 4 min and washed using 1 ml 0.1M Tris HCl (pH 7.6). The pellets were stored at -20˚C for 30 min. The pellets were again washed using 1 ml 0.1 M Tris HCl (pH 7.6) and resuspended in 600 µl of 0.1 M Tris HCl (pH 8.5). To this reaction mixture 30 µl toluene was added and vortexed. The sample was then divided into three sets. One set was used for protein estimation following the Bradford method and the rest of the two for ACC assay. To another set, 20 µl of 0.5 M ACC was added and other as blank, vortexed and incubated at 30˚C for 15 min, vortexed and centrifuged at 10,000 rpm at 4˚C for 5 min. Aliquot (200 µl) was taken in fresh glass tubes, added 400 µl 0.56 M HCl,150 µl DNPH, vortexed, incubated for 30 min, and added 1mL of 2N NaOH. Absorbance was recorded at 540nm.

Arsenic biomethylation and oxidation assay

Arsenic tolerant in fungal strains were characterized for their arsenic biomethylation and oxidation properties. The qualitative estimation of arsenic biomethylation was done following silver nitrate assay for which 3 days old fungal culture broths were centrifuged at 8000 rpm for 5 min and cell free supernatant was collected. For the assay, 20 µl of the culture supernatant was pipetted in 96-well plate with 80 µl of 0.2 M Tris-HCL buffer (pH 7.4) and As(III) with final concentrations of between 0.2-0.6 mM was added. The microtiter plates were incubated for 3 days at 28±2°C in dark conditions. The cultures were then supplemented with 100 µl of 0.1 and 0.2 M $AgNO_3$ to each well. The culture supernatant in microwell plate were observed for colour change to brown indicating a positive result for arsenic methylation.

For qualitative estimation of arsenite and arsenate reduction and oxidation, fungal strains were inoculated in potato dextrose broth along with 800 µM $NaAsO_2$ and $NaAsO_4$ (Salmassi et al., 2002). The broth was incubated at 28±2°C in incubator shaker at 120 rpm for 5 days. The culture supernatant was centrifuged at 5,000 rpm for 5 min. The supernatant was harvested and 1 mL of culture supernatant with 30 µL of 0.01 m L^{-1} $KMnO_4$ was added to microcentrifuge tubes and vortexed. The tubes were observed for colour change from yellow to pink or vice versa. The presence of yellow colour indicates the reduction of As(V) to As(III). Moreover, the screening for the activity of arsenite oxidase using $NaAsO_2$ as a substrate was done. The results were indicated positive for oxidation of As(III) to As(V), if pink colour was observed.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

Fungal compatibility assay

A compatibility assay was performed to see the inhibitory effects of the all the strains over each other if any, by inoculating the strains formulated in talc on potato dextrose media plates (PDA) supplemented with an antibiotic solution of penicillin and streptomycin (25 mg mL^{-1}). The co-culturing was done on PDA plates under laboratory conditions at 28± 2°C for an incubation period of 7 d.

Fungal culture growth conditions

For the optimization of fungal growth conditions, fungal cultures were grown in potato dextrose broth (PDB) and incubated at 28±2ºC. Optimum growth of *Rhizopus* and *Trichoderma* is obtained in 5-7 days, and of *Lasiodiplodia* and *Westerdykella* in 10-12 days under stationary conditions.

Preparation of fungal consortia formulation

For the preparation of the fungal consortium formulation, the fungal strains were grown in PDB at 28±2°C under aeriated condition in incubator shaker at 120 rpm till the spore count of each culture reached 10^{-8} spore mL^{-1}. Before the preparation of formulation, talc was autoclaved thrice to decrease the chances of contamination. The fungal cultures biomass was mixed in equal amounts with talc in sterilized tray in laminar chambers to avoid any chances of contamination. The prepared talc formulations were added to sterilized plastic bottles and stored at 10±2°C/dispatched. The co-culturing of the strains is discouraged owing to the different lifecycle patterns or time taken for optimum growth and not because of any incompatibility.

Selection of the carrier material

The talc-based formulations were prepared and evaluated to screen their arsenic removal efficacy in field trials. All four strains were firstly mass cultured in the modified PDB with selected amendments at 28±2ºC under static conditions. *Rhizopus* and *Trichoderma* are fast-growing fungal strains therefore were inoculated 5-7 days before the harvesting date, while *Lasiodiplodia* and *Westerdykella* were inoculated 8-10 days before the harvesting date. The fungal biomass was harvested by straining through autoclaved muslin cloth under sterile conditions till the excess moisture was removed. The equal proportion of biomass of all the four fungal cultures is blended, homogenized and added to the sterilized carrier material in a 1:5 ratio w/w (approximately 200 g biomass in 1000 g carrier) mixing thoroughly in sterilized trays maintaining moisture of 8-12%. The formulation is allowed to cure at 28±2ºC for 2 days and then stored at 10±2ºC until its further use.

Shelf-life study and the role of enrichment additives

The shelf-life studies of the fungal strains were carried out individually as well as in consortium for 12 months at 28±2ºC. Initially, the amendments were screened at high temperatures (40±2°C) in their respective broth supplemented along with the additives. Erlenmeyer flasks of potato-dextrose broth (PDB) were autoclaved for 20 min at 121°C thereafter added with antibiotic solution, and then inoculated individually with 1 mL spore suspension (10^{8} spore mL^{-1}) prepared from pure

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

fungal strains. Talc formulations were prepared as explained above. The shelf life was determined by enumerating the fungal population by the plate-count method. Dilutions were prepared by taking 0.1 g formulation in 1 mL of 0.8% sterile saline (NaCl), vortexed and further diluted to get the final dilution of 10^{-6}. From these dilutions, 100 µl was spread on sterile PDA plates. These plates were incubated at 28±2°C and observed regularly for the presence of microbial colonies. This was done for a period of one year and values were recorded.

The Population (CFU ml^{-1}) was counted and converted as (CFU g^{-1} of the carrier) as follows.

CFU ml^{-1} of carrier = No of colonies/amount plated * dilution factor

For extended shelf life and enhanced field efficiency of the bio-formulation, different enrichment additives at various concentrations were used. A total of nine different additives amendments used in the study. The additives used were boric acid, potassium chloride, sucrose, calcium sulphate, calcium chloride, sodium chloride, sodium carbonate, calcium carbonate and potassium sulphate. These additives were screened and the best two were chosen based on the dry biomass weight (g) of fungi and were used in augmentation of fungal formulation. For this assay, amendments were supplemented in potato dextrose broth media in Erlenmeyer flasks and inoculated individually with 1 mL spore suspension (10^8 spore mL^{-1}) made from individual pure broth cultures of the four fungal strains. Fungi were kept at 40±2°C at 120 rpm in an incubator shaker for the first 7 days and fungal biomass was filtered (40 mm mesh cloth) and washed thoroughly thrice with de-ionized water, and then dried to a consistent value at 45-50°C. Fresh and dry biomass for each treatment was recorded. The formulations of each fungal strain and their consortium as treatments were applied to the respective experimental pots placed in the net house. The sampling of the treatment, as well as control plants, was done and changes in various vegetative, biochemical and microbiological parameters were recorded. Talc-based consortium formulation as well as all the four individual fungal strains were evaluated for the changes in shelf life under storage conditions. Fungi in the formulations were enumerated for viable cells as CFU by the plate-count method (Singh et al., 2014; Rai et al., 2016). For extended shelf-life enhanced field efficiency of bio-formulation, different enrichment additives at various concentrations were screened and the best two were chosen. All four strains were eventually compared. The effect of an improved formulation amended with additives and augmented with a suitable carrier on the shelf life was noted.

Application of the product: Individual and combined effects of each fungal strain on plants in arsenic mitigation and crop growth.

The four fungal arsenic remediating strains were tested under net-house and field conditions for their plant growth-promoting abilities in arsenic-stressed soils. Observations were also recorded for any pathogenic symptoms on the test crop-rice. Under net house conditions, the experiment was conducted in 6 replicates using earthen pots. The soil was spiked with 10 mg kg^{-1} arsenic using arsenate salt and was allowed to acclimatize for 1 week. The seed treatment with the fungal inoculums was given to rice seeds by seed dip method and were allowed to germinate. Two-week-old seedlings of paddy were shifted to the pots after the root dip treatment with fungal inoculums.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

The inoculum strength was adjusted to 10^8 spore mL^{-1} for the seed-soak and the root-dip treatments. The pots were watered with arsenic spiked tap water (100 µg L^{-1}) as and when required.

The treatments included two sets of the following treatments (i) Control; (ii) Fungal Strain 1; (iii) Fungal Strain 2; (iv) Fungal Strain 3; (v) Fungal Strain 4; (vi) Fungal consortium

Spore suspension of fungal strains was prepared by harvesting spores and mycelia from 5-10 days old culture plates of individual strains. The spore suspension was maintained at a spore count of $1x10^8$ spores mL^{-1} by enumerating using a haemocytometer. For the preparation of the consortium, all the four fungal cultures were mixed in equal proportion and were used as inoculum. The prepared fungal inoculum and the consortium were used for the seedling treatment. For seedling treatment, roots of the 15-day-old nursery were dipped for 30 min in 100 ml inoculum before transplantation (3 seedling/hill/; 3 hills/pot) in arsenic spiked soil (AsIII, 20 ppm). The pots were watered with arsenic spiked tap water (100 µg/l) as and when required. A control set was irrigated with arsenic-free water. All the following parameters of rice plants were recorded at the time of harvesting.

Effect of consortia formulation on vegetative parameters of paddy plants

The vegetative parameters in paddy crop plants were collected from the experimental pots and analysed for vegetative growth attributes (viz. shoot length, root length, dry biomass, numbers of tillers per plant, numbers of spikelets per ear).

Estimation of arsenic in plant tissue samples

The total arsenic estimation in the tissues of the rice plant (grains, root, tiller) was done using Inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500). For the estimation of arsenic in rice plant, the plant biomass was oven dried at 105°C for 3 days. The samples were finely crushed to fine powder using motor pestle and weight (0.25 g) in triplicates. The samples were digested using nitric acid (Hi media, India) and hydrogen peroxide (Merck, India) in a microwave-assisted digestion unit (MARS 6, CEM Corporation, USA) (Srivastava et al., 2011). Digested samples were diluted using Milli-Q and filtered using 0.2 µm syringe filter and analysis through ICP-MS. Quality assurance and quality control of ICP-MS analysis were undertaken using NIST CRM number 1568b (rice flour).

Physio-chemical analysis of soil samples

Soil samples were collected and analyzed for evaluating microbial count in each treatment. Total CFU was determined by enumerating the fungal population by the plate-count method. Dilutions were prepared by taking 0.1 g formulation in 1 mL of 0.8% sterile saline (NaCl), shaken well by vortexing and further diluted to get the final dilution of 10^{-6}. From these dilutions, 100 µl was spread on sterile RBA plates. These plates were incubated at 28±2°C and observed regularly for the presence of microbial colonies. Physicochemical characterization (pH, water holding capacity, soil texture electrical conductivity, bulk density, total organic carbon, microbial biomass carbon, and N, P, K, Na, Ca) and biochemical characterization (dehydrogenase activity, cellulase activity, β-

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

glucosidase activity, phosphatase activity and protease activity) was performed according to the standard methods (Black, 1965; Dick, 2011). Total organic carbon (TOC) (Jackson, 1962) and microbial biomass carbon (MBC) were done by the chloroform-fumigation-extraction method (Vance *et al*., 1987). Soil samples extracted in 2% (w/v) Na-bicarbonate solution was used to determine the available phosphorus (P). Available potassium (K) was determined using a flame photometer (Systronix-128) after extracting samples in the 2% (w/v) ammonium acetate solution. Selected soil enzymatic activities were determined in each freshly grounded and sieved (<2 mm) rhizosphere soil sample using four replicates. Dehydrogenase activity (DHA) was assayed following the method of Pepper et al. (22) by the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) and expressed in µg triphenyl formazan (g soil)$^{-1}$h^{-1}. Alkaline phosphatase activity and β-glucosidase activity were measured using the substrate analogue para-nitrophenyl-β-D-glucopyranoside (ρ-NPG) based on determining the quantity of released p-nitrophenol after incubation of each gram of soil with ρ-NPG solution for 1 h at 37°C.

Ergosterol estimation in rhizospheric soil samples

For ergosterol estimation in rhizospheric soil samples, 12.5 g of moist soil samples was added in 30 mL methanol (5°C) and sonicated on ice for 4 min. The sample was centrifuged at 6000 rpm for 15 min at 4°C. The sample was saponified using 5 g potassium hydroxide and were then kept on water bath for reflux at 80°C for 1 hr and then cooled at 5°C. For ergosterol extraction, 30 mL hexane was used followed by re-extraction in methanol. The samples were concentrated using rotor evaporator and re-dissolved in HPLC grade methanol. For HPLC, methanol was used as a solvent, with run time of 12 min and injection volume of 20 µl (Shimadzu, Japan).

Multi-location Trials during Boro- and Aman- seasons at arsenic-contaminated sites of West Bengal

Another aspect of this study was conducted in two rice seasons, Boro and Aman in the fields of West Bengal, India. For field trails, the fungal formulations were prepared: (1) separately growing the selected fungal strains and allowing them to sporulate under conditions favoring the production of maximum viable spores. (2) Mixing the sporulated biomass with sterilized carrier material, viz., talc. This preparation is first re-suspended and then applied as a seed coating. The spores germinate and colonize roots to reduce arsenic in the target soil-plant rhizoplane and also promote plant growth.

On-farm/ in-situ trials using different approaches of consortium inoculum treatment viz., seed dip, root soak and soil priming were conducted. Micro-plot field experiments and soil culture preparation with arsenic sensitive/ moderately tolerant paddy varieties using inoculants consortium formulation (as root- and seed- soak treatments and soil application) to demonstrate low/no grain arsenic and reduction in bioavailable soil arsenic fraction at highly arsenic- contaminated sites in West Bengal during paddy cultivation season was done. The field trials using soil fungal consortium formulation have been conducted at three different villages (Gotera, Ghetugachhi and Pipli) during Boro (summer) and Aman (rainfed) – paddy growing seasons. In the Boro- and Aman-Rice trials, three treatments were given viz., the control (without any treatment) (T1), microbial

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

formulation application to paddy crop through seed-soak only (T2), and microbial formulation application to paddy crop through both seed-soak and root-dip method (T3). Four locally-grown rice varieties were chosen for the trial viz., IR-36, Triguna, Khitiz and Lalat, which were found to be widely grown arsenic accumulating varieties in earlier studies. The mature rice plants were harvested. The rice plant parts (root, tiller/shoot and grains) and soil samples for total arsenic content were digested using a microwave-assisted digestion system. The total arsenic estimation in these plant parts and soil samples were estimated using ICP-MS.

Results and Discussions

Identification and characterization of plant growth promoting traits of the fungal strains

A total of four fungal strains selected for the study were *Westerdykella aurantiaca* (FNBR-FA03), *Trichoderma longibrachiatum* (FNBR-FA06), *Lasiodiplodia* sp. (FNBR-FA13), *Rhizopus delemer* (FNBR-FA19) based on their arsenic tolerance (up to 10000 µg mL^{-1}) as reported earlier by Srivastava *et al.*, 2012. All the four fungal strains showed plant growth promoting traits and arsenic detoxification properties as shown in Table 1.

The phosphate solubilization property might help in competing with arsenic uptake by P uptake. IAA regulates plant growth and developmental. ACC deaminase activity helps in ameliorating arsenic stress during seed germination and enhances seedling germination. The siderophore can chelate to arsenic in the rhizosphere region therefore may results in reducing the bioavailable form of arsenic. *Westerdykella aurantiaca* (strain FNBR-FA03) was isolated from arsenic contaminated agricultural soils collected from birnagar, West Bengal, India. It was reported for its arsenite methyltransferase activity that can volatilize arsenic from the rhizosphere soil (Verma et al., 2016). *W. aurantiaca* was found to possess phosphate solubilization activity (12.54 µg mL^{1}), indole acetic acid activity (57.16 µg mL^{-1}), ACC deaminases activity (111.04 nMAKB µg of $protein^{-1}$) and siderophore production (78.65%). The siderophore production in *W. aurantiaca* was found to increase by 1.01% in the presence of arsenic stress (79.45%). *Trichoderma longibrachiatum* (strain FNBR-FA06) was isolated from arsenic contaminated agricultural soils collected from Barasat, West Bengal, India. *Trichoderma longibrachiatum* possessed arsenite oxidase activity and displayed phosphate solubilization activity (16.01 µg ml^{-1}), indole acetic acid activity (4.03 µg ml^{-1}), ACC deaminases activity (2722 nMAKB µg of $protein^{-1}$) and siderophore production (16.47%). The siderophore production in *T. longibrachiatum* was found to increase by 34.7% in the presence of arsenic stress (22.19%). *Lasiodiplodia* sp. (strain FNBR-FA 13) was isolated from arsenic contaminated agricultural soils collected from Sonarpur, West Bengal, India. It is reported as a bioaccumulator of arsenic and helps in sequestering arsenic in its mycelia to reduce the bioavailability of arsenic to the plants (Jaiswal et al., 2019). *Lasiodiplodia* sp. was found to have phosphate solubilization activity (14.77 µg mL^{-1}), indole acetic acid activity (14.93

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

µg mL^{-1}), ACC deaminases activity (1604.92 nMAKB µg of $protein^{-1}$) and siderophore production (12.73%). The siderophore production in *W. aurantiaca* was found to increase by 53.18 % in the presence of arsenic stress (19.5%). *Rhizopus delemer* (strain FNBR-FA 19) was isolated from arsenic contaminated agricultural soils collected from Birnagar, West Bengal, India. *Rhizopus delemer* possessed arsenite oxidase activity and displayed phosphate solubilization activity (123.21 µg mL^{-1}), indole acetic acid activity (21.46 µg mL^{-1}), ACC deaminases activity (1361.26 nMAKB µg of $protein^{-1}$) and siderophore production (15.78%). The siderophore production in *Rhizopus delemer* was found to decrease by -16.9 % in the presence of arsenic stress (13.12%).

Table 1: Different traits of individual microbial strain w.r.t. arsenic detoxification and plant growth promotion traits

Microbial Strain	Isolation site	Phosphate solubilisation activity (µg/ml)	Indole Acetic Acid production (µg/ml)	ACC Deaminase activity (nMAKB/ µg of protein)	Siderophore production % (+As/-As)	Arsenic tolerance/ remediation mechanism
Westerdykella aurantiaca FNBR_FA 3	Arsenic contaminated fields of West Bengal (WB)	12.54±1.18	57.16±0.69	111.04±0.81	79.45/ 78.65	Methylation (Qualitative)
Trichoderma longibrachiatum FNBR_FA 6	Arsenic contaminated fields of WB	16.01±1.31	4.03±0.50	2722.0±0.51	22.19/ 16.47	Oxidation (Qualitative)
Lasiodiplodia sp. FNBR-FA 13	Arsenic contaminated fields WB	14.77±0.79	14.93±4.30	1604.92±0.14	19.50/ 12.73	Bioaccumulation (Jaiswal et al., 2018)
Rhizopus delemer FNBR_FA 19	Arsenic contaminated fields of WB	123.21± 1.57	21.46±1.85	1361.26±0.67	13.12/ 15.78	Oxidation (Qualitative)

Compatibility assay for arsenic tolerant fungal strains used in the formulation

The compatibility of selected fungal strains was checked by the direct confrontation method inoculating the agar discs containing actively growing fungal cultures to the peripheral edges of the culture plates. In our study, all the selected fungal strains, *Westerdykella aurantiaca* FNBR_FA03, *Trichoderma longibrachiatum* FNBR_FA06, *Lasiodiplodia* sp. FNBR_FA13 and *Rhizopus delemer* FNBR_FA19 in all the possible combinations were found to be compatible with each other (Figure 1).

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

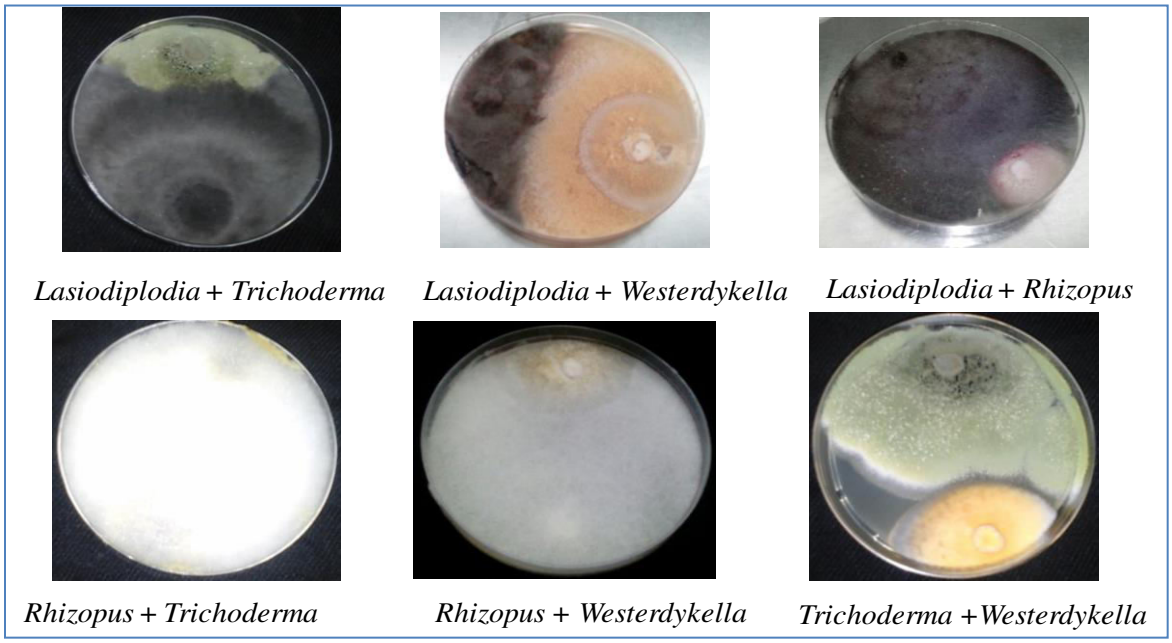

Figure 1. Compatibility among fungal strains in the fungal component of the formulation

Screening and selection of amendments for fungal components

The fungal media broths (PDB) were supplemented with nine different amendments along with control. The amendments were added at the rate of 0.5% and the screening of amendments was carried out in at 40±2°C temperature. Different amendments were selected based on the dry weight biomass (g) after 30 days of incubation (Table 2). Increase or decreases in the dry biomass weight (g) of all the fungal strains were observed. Amongst all the amendments, sucrose (0.5%) and calcium carbonate (0.5%) were found to produce best results. Sucrose being a chemotactic substance for microbial colonization helps framing the rhizosphere microbiota (Tian et al., 2022). Out of all the amendments, sucrose was found to increase the dry weight biomass of all the fungal strains. Sucrose increased the biomass of *Westerdykella aurantiaca* FNBR_FA03 by 23.6 %, *Trichoderma longibrachiatum* FNBR_FA06 by 20.8 %, *Lasiodiplodia* sp. FNBR_FA13 by 21.4 % and *Rhizopus delemer* FNBR_FA19 by 232.6 %. Second amendment, calcium carbonate was found to increase the biomass dry weight (g) of all the fungal strains except *Trichoderma longibrachiatum* FNBR_FA06 and *Lasiodiplodia* sp. FNBR_FA13.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

Table 2. Screening of Amendments for Fungal Strains

Fungal Biomass (dry weight biomass (g) after 30 days at 40±2°C					
Sl. No.	**Name of Amendments**	***Trichoderma longibrachiatum***	***Westerdykella aurantiaca***	***Lasiodiplodia* sp.**	***Rhizopus delmer***
1	Control	0.5924±0.012	0.2961±0.012	0.5805±0.099	0.1572±0.011
2	Boric acid	0.3553±0.024	0.2388±0.014	0.3409±0.064	0.0029±0.001
3	Potassium Chloride	0.3268±0.016	0.2663±0.013	0.3191±0.043	0.1801±0.091
4	Sucrose	0.7156±0.033	0.3661±0.018	0.7050±0.016	0.5228±0.084
5	Calcium Sulphate	0.2844±0.011	0.2066±0.017	0.2044±0.017	0.1599±0.073
6	Calcium Chloride	0.3301±0.012	0.2296±0.011	0.3105±0.019	0.1434±0.041
7	Sodium Chloride	0.3806±0.016	0.2213±0.011	0.2766±0.011	0.1417±0.028
8	Sodium Carbonate	0.3613±0.034	0.3130±0.012	0.3018±0.011	0.1489±0.034
9	Calcium Carbonate	0.4061±0.045	0.3207±0.033	0.3959±0.045	0.5728±0.042
10	Potassium Sulphate	0.3884±0.020	0.2401±0.098	0.3858±0.072	0.1341±0.011

Calcium carbonate increased the biomass of *Westerdykella aurantiaca* FNBR_FA03 by 8.3% and *Rhizopus delemer* FNBR_FA19 by 264.37 % and decreased the biomass dry weight of *Trichoderma longibrachiatum* FNBR_FA06 by 31.44 % and *Lasiodiplodia* sp. FNBR_FA13 by 31.08 % when compared with control.

Shelf life of the talc-based formulation with the selected amendments

The shelf-life studies of the fungal strains were observed as CFU Log_{10} per g^{-1} of talc formulation (Table 3).

After the screening of the amendments at high temperatures (40±2°C), shelf life of the fungal strains was observed for a period of 180 days at 28±2°C and 10±2°C. The results showed higher CFU Log_{10} g^{-1} values till 180 days at 28±2°C in comparison to 10±2°C. At 28±2°C, the CFU count gradually decreased from day 30 in all the fungal strains except in *Westerdykella aurantiaca* FNBR_FA03 in which the CFU value increased by day 30 from 8.09 to 8.28 CFU Log_{10} g^{-1}. The CFU Log_{10} g^{-1} values in *Westerdykella aurantiaca* FNBR_FA03 ranged between 8.09 at day 0 to 7.35 at day 180 at 28±2°C. Whereas at 10±2°C, the CFU values ranged between 7.41 at day 0 to 6.29 at day 180. The CFU Log_{10} g^{-1}

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

Table 3. Shelf life of Fungal Strains as CFU Log_{10} per g of the talc formulation (Fungal Component)

Fungal Strains	Time Period (in days)						
	0	30	60	90	120	150	180
Room Temperature (28±2°C)							
FNBR-FA 03	8.09	8.28	7.78	7.61	7.53	7.41	7.35
FNBR-FA 06	8.05	7.93	7.74	7.53	7.29	7.17	7.01
FNBR-FA 13	8.33	7.22	6.99	6.77	6.58	6.43	6.2
FNBR-FA 19	8.57	8.18	7.73	6.43	6.36	6.21	6.09
Storage Temperature (10±2°C)							
FNBR-FA 03	7.41	7.32	7.17	7.03	6.91	6.73	6.29
FNBR-FA 06	7.35	7.19	7.08	6.93	6.87	6.69	6.13
FNBR-FA 13	6.79	6.66	6.43	6.27	5.91	5.37	5.21
FNBR-FA 19	7.54	7.48	7.31	6.99	6.47	5.98	5.73

values in *Trichoderma longibrachiatum* FNBR_FA06 ranged between 8.05 at day 0 and 7.01 at day 180 at 28±2°C. Whereas at 10±2°C, the CFU values were 7.35 at day 0 and 6.13 at day 180. The CFU Log_{10} g^{-1} values in *Lasiodiplodia* sp. FNBR_FA13 were 8.33 at day 0 and 6.2 at day 180 at 28±2°C. Whereas at 10±2°C, the CFU values were 6.79 at day 0 and 5.21 at day 180. The CFU Log_{10} g^{-1} values in *Rhizopus delemer* FNBR_FA19 was 8.57 at day 0 and 6.09 at day 180 at 28±2°C. Whereas at 10±2°C, the CFU values were 7.54 at day 0 and 5.73 at day 180.

Effects of the fungal consortia on the growth of rice plants under the arsenic stressed condition in a net-house

Effect of arsenic stress on the vegetative characteristics of rice plants (root length, shoot length, number of tillers, number of panicles, panicle length, number of spikelet and dry weight) is shown in table 4.

The results from the study shows (A) change in the vegetative characteristics of rice plants when treated individually with all four fungal strains and their consortium in both arsenic treated and untreated plants and (B) Effect of arsenic stress on rice plant in combination with treatment with all four fungal strains and their consortium. The result shows a significant ($p<0.05$) increase in the vegetative characteristics of rice plants when enriched with fungal consortium in both the arsenic untreated and treated rice plants in comparison with treatment with individual fungal strains.

Table 4: Effects of microbial inoculum on the growth of rice plants

Vegetative characteristics of rice plants							
	Root	Shoot	Number	Number of	Panicle	Number of	Dry

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

	Length(cm)	Length(cm)	of Tillers	Panicles	length	Spikelet	Weight(g)
With Arsenic							
FNBR_FA 3	46.79	23.01	16.60	88.57	143.37	240.65	166.98
FNBR_FA 6	91.35	35.27	30.14	117.14	196.98	130.76	319.80
FNBR_FA 13	61.03	15.31	12.38	188.57	283.13	295.60	355.66
FNBR_FA 19	71.21	40.19	56.06	200.00	257.22	389.01	376.41
Consortia talc	127.87	89.28	116.21	337.14	450.00	537.36	675.47
Without Arsenic							
FNBR_FA 3	50.04	11.22	25.48	67.50	15.00	267.00	68.42
FNBR_FA 6	97.09	24.71	39.78	67.50	20.69	233.00	131.58
FNBR_FA 13	73.61	17.96	18.28	67.50	32.24	267.00	215.79
FNBR_FA 19	64.78	40.44	50.54	232.50	32.24	333.00	291.05
Consortia talc	155.96	89.75	53.76	475.00	83.97	760.00	396.32

In the arsenic untreated rice plant, the addition of fungal consortium increased the root length (60.6-211.6 %), shoot length (121.9-699.9 %), number of tillers (6.4-110.9 %), number of panicles (104.3-603.7 %), panicle length (160.4-459.8 %), number of spikelet (128.2-226.2 %) and dry weight (36.2-492.2 %) in comparison with treatment with individual fungal strains. In arsenic treated rice plant, the addition of fungal consortium increased the root length (39.9-173.3 %), shoot length (122.1-483.1 %), number of tillers (107.3-838.6 %), number of panicles (68.57-280.6 %), panicle length (58.9-213.8 %), number of spikelet (38-310.6 %) and dry weight (76.6-293.7 %) in comparison with treatment with individual fungal strains.

Effect of arsenic treatment along with fungal strain treatment (individual and consortium) on rice plant was also examined in net-house studies. The arsenic stressed rice plant treated with *Westerdykella aurantiaca* FNBR_FA03 displayed increase in shoot length by 105.4 %, number of panicles by 31.2 %, panicle length by 855.8 % and dry weight by 142%. Whereas decrease in root length (-6.5 %), number of tillers (-34.8 %) and number of spikelet (-9.86 %) was observed when compared to arsenic untreated rice plant. In arsenic stressed rice plant treated with *Trichoderma longibrachiatum* FNBR_FA06 displayed increase in shoot length (42.7 %), number of panicles (73.5 %), panicle length (852.1 %) and dry weight (143.1 %). Whereas decrease in root length (-5.9 %), number of tillers (-24.2 %) and number of spikelet (-43.8 %) was observed in comparison to arsenic untreated rice plant. Arsenic stressed rice plant treated with *Lasiodiplodia* sp. FNBR_FA13

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

displayed increase in number of panicles (179.4 %), panicle length (778.2 %), number of spikelet (10.7 %) and dry weight (64.8 %). Whereas decrease in root length (-17.1 %), shoot length (-14.7 %), number of tillers (-32.3 %) was observed when compared with arsenic untreated rice plant. In arsenic stressed rice plant treated with *Rhizopus delemer* FNBR_FA19 displayed increase in root length (9.9 %), number of tillers (10.9 %), panicle length (697.8 %), number of spikelet (16.8 %) and dry weight (29.3 %). Whereas decrease in shoot length (-0.6 %) and number of panicles (-13.9 %) when compared with arsenic untreated plant. In arsenic treated rice plant along with consortium of all four fungal strains there was increase in number of tillers (116.1 %), panicle length (435.9 %) and dry weight (70.43 %). Whereas decrease in root length (-18 %), shoot length (-7.5 %), number of panicles (-29 %) and number of spikelet (-29.29 %) when compared with arsenic untreated rice plant.

Effects of microbial inoculum on arsenic and other multi-elements (µg kg^{-1}) in rice plant

The role of talc based fungal cultures and consortium formulations was studied in rice plants tissues (grain, root and tiller) grown on arsenic untreated and treated soil (Table 5). The effect on the uptake of arsenic, iron, selenium, zinc and manganese by the rice plant was assessed in this study.

In rice plant, there was reduction in the accumulation of arsenic observed in all the fungal and consortium inoculated rice plant

Table 5: Effects of microbial inoculum on arsenic and other microelements (µg/kg) of rice plants

(A). Rice Grains					
	Arsenic	**Iron**	**Selenium**	**Zinc**	**Manganese**
With Arsenic					
Control	1.59^{b}±0.13	7.57^{ab}±0.65	116.7^{b}±9.30	7.36^{a}±0.32	0.09^{b}±0.00
FNBR_FA 3	1.26^{b}±0.09	20.95^{bc}±2.15	493.6^{bc}±22.45	28.99^{bc}±1.24	0.16^{bc}±0.01
FNBR_FA 6	0.70^{bc}±0.06	24.85^{bc}±2.30	376.6^{bc}±12.67	15.03^{b}±0.82	0.12^{b}±0.01
FNBR_FA 13	0.24^{b}±0.02	16.08^{b}±1.30	251.9^{bc}±19.21	19.45^{b}±1.12	0.12^{b}±0.01
FNBR_FA 19	0.30^{b}±0.02	24.12^{bc}±1.34	287.2^{bc}±15.67	55.48^{c}±4.11	0.18^{bc}±0.01
Consortia talc	0.077^{bc}±0.03	16.37^{b}±1.31	211.5^{bc}±9.43	18.89^{b}±0.81	0.13^{b}±0.01
Without Arsenic					
Control	ND	14.57^{b}±1.23	286.7^{bc}±13.24	17.36^{b}±1.13	0.39^{c}±0.03
FNBR_FA 3	ND	26.00^{bc}±2.43	348.9^{bc}±23.69	18.43^{b}±0.99	0.02^{a}±0.00
FNBR_FA 6	ND	17.48^{b}±1.24	278.7^{bc}±21.23	21.91^{b}±1.57	0.07^{ab}±0.00
FNBR_FA 13	ND	15.22^{b}±1.30	195.2^{ab}±8.32	35.01^{bc}±2.57	0.17^{b}±0.01
FNBR_FA 19	ND	19.21^{bc}±1.10	255.5^{bc}±12.52	59.50^{abc}±3.24	0.02^{a}±0.00
Consortia talc	ND	30.24^{bc}±2.38	392.5^{bc}±23.34	25.68^{bc}±2.13	0.25^{bc}±0.01
(B). Rice Roots					
	Arsenic	**Iron**	**Selenium**	**Zinc**	**Manganese**
With Arsenic					
Control	18.85^{bc}±1.11	17.70^{a}±1.05	1303.08^{ab}±76	76.90^{ab}±4.54	0.13^{abc}±0.01
FNBR_FA 3	11.32^{abc}±0.99	51.82^{bc}±4.51	4761.00^{bcd}±414	195.90^{bc}±17.04	0.22^{ab}±0.01
FNBR_FA 6	14.03^{abc}±1.24	47.75^{bc}±4.21	6057.00^{cd}±534	223.26^{bc}±19.69	0.19^{ab}±0.02
FNBR_FA 13	17.36^{bc}±1.44	43.07^{bc}±2.18	3838.20^{bc}±194	170.82^{abc}±8.64	0.22^{ab}±0.01

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

FNBR_FA 19	15.35^{bc}±1.35	46.21^{bc}±4.05	4538.40^{bcd}±397	200.46^{bc}±17.56	0. 25^{ab}±0.02
Consortia talc	3.72^{a}±1.66	70.22^{cd}±6.28	3810.60^{bc}±226	189.18^{bc}±11.24	0. 37^{b}±0.02
Without Arsenic					
Control	ND	27.11^{ab}±0.90	2477.04^{ab}±82	82.07^{a}±6.72	0.18^{ab}±0.02
FNBR_FA 3	ND	35.00^{bc}±2.23	3759.00^{bc}±239	177.96^{bc}±11.35	0.20^{ab}±0.01
FNBR_FA 6	ND	40.72^{c}±3.41	4462.20^{bc}±373	173.16^{bc}±14.48	0.38^{bc}±0.02
FNBR_FA 13	ND	35.13^{bc}±2.70	4522.80^{bc}±347	571.98^{c}±43.96	0.22^{ab}±0.02
FNBR_FA 19	ND	33.07^{bc}±3.36	4408.80^{bc}±316	298.14^{ab}±21.38	0.38^{bc}±0.01
Consortia talc	ND	34.47^{bc}±3.11	4434.00^{bc}±399	193.80^{bc}±17.48	0.41^{bc}±0.01
(C). Rice Tillers					
	Arsenic	**Iron**	**Selenium**	**Zinc**	**Manganese**
With Arsenic					
Control	43.48^{bc}±2.45	30.47^{ab}±2.89	114.88^{a}±8.04	19.69^{a}±1.26	0.20^{a}±0.01
FNBR_FA 3	34.22^{bc}±1.93	35.99^{bc}±2.27	226.78^{b}±9.50	31.95^{ab}±2.04	0.59^{bc}±0.04
FNBR_FA 6	24.26^{bc}±1.37	39.51^{bc}±1.61	153.89^{ab}±10.43	43.67^{bc}±2.79	0.63^{bc}±0.04
FNBR_FA 13	21.48^{ab}±1.21	38.69^{bc}±1.43	241.76^{bc}±10.21	20.66^{a}±1.32	0.47^{ab}±0.03
FNBR_FA 19	25.14^{abc}±1.42	32.71^{ab}±1.67	625.80^{cd}±8.64	64.09^{c}±4.10	0.29^{a}±0.02
Consortia talc	15.14^{a}±0.85	37.93^{bc}±1.01	137.62^{ab}±10.01	25.83^{ab}±1.65	0.53^{bc}±0.03
Without Arsenic					
Control	ND	33.25^{a}±2.21	128.76^{ab}±8.78	15.48^{a}±0.99	0.11^{a}±0.01
FNBR_FA 3	ND	50.71^{ab}±3.37	376.06^{cd}±13.39	96.32^{c}±6.16	0.31^{bc}±0.02
FNBR_FA 6	ND	58.70^{ab}±3.90	553.92^{cde}±15.50	48.09^{bc}±3.08	0.48^{bc}±0.03
FNBR_FA 13	ND	104.60^{c}±6.95	698.56^{de}±27.62	92.25^{c}±5.90	0.26^{ab}±0.02
FNBR_FA 19	ND	118.63^{c}±7.88	749.49^{de}±31.32	34.86^{ab}±2.23	0.34^{bc}±0.02
Consortia talc	ND	51.59^{ab}±3.43	279.29^{bc}±13.62	57.93^{bc}±3.71	0.33^{bc}±0.02

when compared to control. The control rice grain was found to accumulate 1.59 µg kg^{-1} of arsenic. Whereas with the inoculation of individual fungal cultures in the rice plants, the uptake of arsenic decreased significantly ($p < 0.05$) between 0.24-1.26 µg kg^{-1}. In the consortium treated rice plant, 95.2% reduction in arsenic uptake was observed. The arsenic accumulation in consortium treated rice grains was only 0.077 µg kg^{-1}.

The arsenic in the roots of the control rice plant was 18.85 µg kg^{-1}. Whereas with the inoculation of fungal cultures in the rice plants, the uptake of arsenic in roots decreased significantly ($p < 0.05$) between 11.32-17.36 µg kg^{-1}. In consortium treated rice plants root, there was -80.2 % decrease in arsenic accumulation when compared to control. The arsenic in the roots of the consortium treated rice plants decreased to only 3.72 µg kg^{-1}.

In the arsenic treated rice plant, the tillers accumulated 43.48 µg kg^{-1} of arsenic in control samples. The arsenic accumulation in the tillers of rice plant treated with individual fungal strains were found to be 21.48-34.22 µg kg^{-1}. Whereas treatment with consortium talc decreased the arsenic content in tiller by -65.2 %. The arsenic accumulation in consortium treated rice plants tillers was found to be 15.14 µg kg^{-1}. In this study we also found out that the maximum arsenic accumulation was found as rice tillers > rice roots > rice grains. The treatment of rice plants with fungal consortium showed increase in the multielement levels (iron, selenium, zinc and manganese) in the rice grains in both arsenic treated and untreated rice plants when compared with their controls (Table 4).

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

Field trial using a novel talc-based microbial formulation

For the assessment of the efficacy of fungal consortium, field trails were conducted for two years in Boro and Aman season in West Bengal, India. The rice plants were assessed for percentage change in vegetative growth, plant yield, microbial parameters and arsenic accumulation as shown in Table 6. The percentage changes in vegetative growth, plant yield, microbial parameters and arsenic accumulation were observed in T2 treatment (seed soak) and T3 treatments (root soak) rice plants in comparison to T1 (control). The percentage change in the Boro and Aman field trails in T2 treated rice plant showed increase in the root, shoot, panicle and flag leaf length were 22-25 %, 4-5 %, 11-23 %,12-32 % respectively. The number of tillers, panicles and spikelet increased by 13-17 %, 15-20 % and 19-27 % respectively. For rice plant yield, weight of 1000 seeds (filled+unfilled), weight of 1000 filled seeds, weight of 1000 grains and

Table 6. Effects of talc-based microbial formulation on the growth of rice plants during rice-rice cropping seasons

Traits	Parameters	Range of % change Boro-Aman Trials in T2 treatment in comparison with T1 (control)	Range of % change Boro-Aman Trials T3 treatment in comparison with T1 (control)
Vegetative	Root length (cm)	22-25	28-31
	Shoot length (cm)	4-5	3-6
	Panicle Length (cm)	11-23	10-25
	Flag leaf length (cm)	12-32	20-35
	Numbers of tillers	13-17	20-25
	Numbers of panicles	15-20	20-25
	Numbers of spikelets	19-27	30-35
Yield	Weight of 1000 seeds (filled+unfilled) (g)	10-17	15-20
	Weight of 1000 filled seeds (g)	16-35	20-40
	Weight of 1000 grains (g)	18-36	20-45
	Yield per sq. m.	2-3	3-5
Microbial	Rhizosphere fungal cfu Log_{10}	7-15	10-20
	Rhizosphere soil Ergosterol	105-140	120-150

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

	(mg/kg)		
Arsenic	Grain arsenic content (mg/kg)	(-) 24 to (-) 54	(-) 30 to (-) 60
	Tiller arsenic content (mg/kg)	(-) 39 to (-) 64	(-) 45 to (-) 70
	Root arsenic content (mg/kg)	(-) 41 to (-) 58	(-) 50 to (-) 65
	Soil bioavailable arsenic content (mg/kg)	(-) 30 to (-) 41	(-) 40 to (-) 55

yield per sq.m. increased by 10-17 %, 16-35 %, 18-36 %, 2-3 % respectively. The rhizospheric CFU Log_{10} values increased by 7-15 % and rhizospheric ergosterol levels increased by 105-140 %. The arsenic contents in the grains, tillers, roots and soil (bioavailable arsenic) decreased significantly ($p < 0.05$) by (-) 24 - (-) 54 %, (-) 39 – (-) 64 %, (-) 41- (-) 58 % and (-) 30 – (-) 41 % respectively. In the T3 treated rice plant, the percentage changes in the rice plants parameters were observed for Boro and Aman seasons in comparison to T1 (control). The percentage change in the Boro and Aman field trails in T3 treated rice plant showed increase in the root, shoot, panicle and flag leaf length were 28-31 %, 3-6 %, 10-25 %, 20-35 % respectively. The number of tillers, panicles and spikelet increased by 20-25 %, 20-25 % and 30-35 % respectively. For rice plant yield, weight of 1000 seeds (filled+unfilled), weight of 1000 filled seeds, weight of 1000 grains and yield per sq.m. increased by 15-20 %, 20-40 %, 20-45 %, 3-5 % respectively. The rhizospheric CFU Log_{10} values increased by 10-20 % and rhizospheric ergosterol levels increased by 120-150 %. The arsenic contents in the grains, tillers, roots and soil (bioavailable arsenic) decreased significantly ($p < 0.05$) by (-) 30 - (-) 60 %, (-) 45 – (-) 70 %, (-) 50 - (-) 65 % and (-) 40 – (-) 55 % respectively.

CONCLUSION

The work leads to a process of bioaugmentation developed for safe crop cultivation by remediating arsenic contamination/ uptake to crops. The concept can be easily integrated with the present crop cultivation practices in the target regions. Paddy crop, especially arsenic sensitive/ moderately tolerant varieties were used to reveal low/no grain arsenic using proposed bioaugmentation technology based on novel indigenous soil fungal strains at targeted high arsenic sites in the state of West Bengal. Potent arsenic remediating biofertilizer formulation is not available to date for restricting arsenic uptake in rice phytomass and further into the rice grains. The multi-location trials of our developed fungal formulation have shown encouraging results. The reconnaissance was made to arsenic-prone sites in West Bengal. Three villages were recognized where the soil arsenic contents were on the higher side. The selection of paddy fields in these three villages to conduct multilocation trials under the project was done. The consortia formulations on talc were prepared, checked for enhanced efficacy, viability, and compatibility assays were done. The prepared soil fungal formulations were tested for their role in restricting arsenic uptake into rice phytomass in boro- and aman- seasons. Based upon four consecutive paddy cropping seasons, a highly efficient soil fungal arsenic remediating biofertilizer formulation was reported.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

The scope of the developed technology of soil fungal formulation can be increased by applying it to other vegetable crops so that it becomes a wider range of products for restricting arsenic uptake in cultivating crops and promoting crop growth with yield as well.

REFERENCES

A. Thakkar et.al 2014 "Development of microbial consortia as a biocontrol agent for effective management of fungal diseases in Glycine max L." Archives of Phytopathology and Plant Protection 48(6): DOI: 10.1080/0323540.893638

Abedin, M. J., M. S. Cresser, et al. (2002a). "Arsenic accumulation and metabolism in rice (Oryza sativa L.)." Environ Sci Technol 36(5): 962-8

Agency for Toxic Substances and Disease Registry (ATSDR) Toxicological Profile for Metals. Atlanta, GA: Centers for Disease Control; 2002.

An Efficient Method for Qualitative Screening of Phosphate-Solubilizing Bacteria; August 2001 Current Microbiology 43(1):51-6 DOI: 10.1007/s00284001025

and plant. Ecotoxicol. Environ. Saf. 176, 108–118.

Arora, N.K., Naraian, R. and Maheshwari, D.K. (2008) Sawdust as a superior carrier for production of multipurpose bioinoculant using plant growth-promoting rhizobial and pseudomonad strains and their impact on the productivity of Trifolium repense. Curr Sci 95, 91–94

Aspergillus niger. Bioresour. Technol. 70, 95–104.

ATSDR, 2020. Agency for Toxic Substances and Disease Registry justification of appropriation estimates for Appropriations Committees fiscal year 2021. Agency for Toxic Substances and

Disease Registry (ATSDR), Department of Health and Human Services, United States.

B.M. Sandikar and R.S. Awasthi,2010."Preparation and Shelf-life Study of Pseudomonas and Bacillus Bioformulations Against Phytopathogenic Pythium and Fusarium species International Journal of Plant Protection, Vol. 2 No. 2 : 251-254

Bhatti, S.M., Anderson, C.W.N., Stewart, R.B., Robinson, B.H., 2013. Risk assessment of vegetables irrigated with arsenic-contaminated water. Environ. Sci. Process. Impacts 15 (10), 1866–1875.

challenges in the use of mineral nutrition for minimizing arsenic toxicity and accumulation

Dahlawi, S., Naeem, A., Iqbal, M., Farooq, M.A., Bibi, S., Rengel, Z., 2018. Opportunities and

Digat, B. (1989) Strategies for seed bacterization. Acta Hort 253, 121–130

Fan, M.-Y., R.-J. Xie, et al. (2014). "Bioremediation of petroleum-contaminated soil by a combined system of biostimulational-bioaugmentation with yeast." Environmental technology 35(4): 391-399.

Fungal mediated biotransformation reduces toxicity of arsenic to soil dwellingmicroorganism

Geneva, p. 224

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

Hasan, S. and Ayres, P.G. (1990) The control of weeds through fungi: principles and prospects. New Phytol 115, 201–222

in rice: a critical review. Chemosphere 194, 171–188.

Li, W.X., Chen, T.B., Huang, Z.C., Lei, M., Liao, X.Y., 2006. Effect of arsenic on chloroplast ultrastructure and calcium distribution in arsenic hyperaccumulator Pteris vittata L. Chemosphere 62 (5), 803–809.

Littera, P., M. Urík, et al. "Removal of arsenic from aqueous environments by native and chemically modified biomass of Aspergillus niger and Neosartorya fischeri." Environmental technology 32(11): 1215-1222.

Mathivanan,N. R. Prabavathy,V. R. Vijayanandraj,2005" Application of Talc Formulations of Pseudomonas fluorescens Migula and Trichoderma viride Pers. ex S.F. Gray Decrease the Sheath Blight Disease and Enhance the Plant Growth and Yield in Rice. DOI: 10.1111/j.1439-0434.01042

Meenu Gupta et.al 2014 "Shelf life study of formulations of fungal and bacterial antagonists as bioinoculants." Agric. Sci. Digest., 34 (4) : 281 - 284, doi: 10.5958/0976- 0547.01020.9

Mohd, S., Kushwaha, A.S., Shukla, J, Mandrah, K., Shankar, J., Arjari, N., Saxena, P.N., Khare,

Mondal, D., Periche, R., Tineo, B., Bermejo, L.A., Rahman, M.M., Siddique, A.B., Rahman, M.A., Solis, J.L., Cruz, G.J.F., 2020. Arsenic in Peruvian rice cultivated in the major rice growing region of Tumbes River basin. Chemosphere 241, 1–8.

P., Narayan, R., Dixit, S., Siddiqui, M.H., Tuteja, N., Das, M., Roy, S.K., Kumar, M., 2019.

Pindi PK, et.al(2012) "Liquid Microbial Consortium- A Potential Tool for Sustainable Soil Health." J Biofertil Biopestici 3:124. doi:10.4172/2155-6202.1000124

Raju, N.J., 2022. Arsenic in the geo-environment: a review of sources, geochemical processes, toxicity and removal technologies. Environ. Res. 203, 111782

Signes-Pastor, A.J., Guti´errez-Gonzalez, ´ E., García-Villarino, M., Rodríguez-Cabrera, F. D., Lopez-Moreno, ´ J.J., Varea-Jim´enez, E., Pastor-Barriuso, R., Pollan, ´ M., NavasAcien, A., P´erez-Gomez, ´ B., Karagas, M.R., 2021. Toenails as a biomarker of exposure to arsenic: a review. Environ. Res 195, 110286.

Singh and Nautiyal, 2012: Process for preparing a talc-based Trichoderma formulation. Patent Application Number 1986/DEL/2011

Singh, A., Srivastava, S. and Singh, H.B. (2007). Effect of substrates on growth and shelf life of Trichoderma harzianum and its use in biocontrol of diseases. Biores Technol 98, 470–473.

Smith, A.H., Lopipero, P.A., Bates, M.N., and Steinmaus, C.M. (2002a). Arsenic Epidemiology and Drinking Water Standards

Srivastava, P. K., A. Vaish, et al. (2011). "Biological removal of arsenic pollution by soil fungi." Science of the Total Environment 409(12): 2430-2442.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11,S Iss 3, Dec 2022

Tian, Y., Liu, Y., Yue, L., Uwaremwe, C., Zhao, X., Zhou, Q., Wang, Y., & Wang, R. (2022). Bacterial Inoculant and Sucrose Amendments Improve the Growth of *Rheum palmatum* L. by Reprograming Its Metabolite Composition and Altering Its Soil Microbial Community. *International Journal of Molecular Sciences*, *23*(3). https://doi.org/10.3390/ijms23031694.

US HoR, 2021. Baby foods are tainted with dangerous levels of arsenic, lead, cadmium, and mercury. Staff Report, Subcommittee on Economic and Consumer Policy, Committee on Oversight and Reform, U.S. House of Representatives, United States Congress, Washington D.C

V. Andreoni, R. Zanchi, L. Cavalca, A. Corsini, C. Romagnoli, E. Canzi; Arsenite oxidation in ancylobacter dichloromethanicus As3-1b strain: detection of genes involved in arsenite oxidation and CO_2 fixation. Curr. Microbiol., 65 (2012), pp. 212-218

WHO, 2001. Arsenic and arsenic compounds. Environ. Health Criteria. World Health Organization,

Zhao FJ, McGrath SP, Meharg AA (2010) Arsenic as a food chain contaminant: Mechanisms of plant uptake and metabolism and mitigation strategies. Annual Review of Plant Biology 61: 535–559.