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Isolation and Screening of Fungal Endophytes for Arsenic-Metal Tolerance Potential and Seedling Growth of Rice (*Oryza sativa* L.) Under Induced Arsenic Stress Condition

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Abstract

Endophytes are one of the symbiotic associations which can be successfully utilized to mitigate negative effects of abiotic stress in agricultural crops**.** Habitat adapted symbiotic fungal endophytes are known to impart heavy metal stress tolerance in host plants. Besides, endophytic fungi secrete secondary metabolites and enhance the plant's ability to tolerate stressful conditions. Therefore, this study was aimed at evaluating fungal endophytes of arsenic stress adapted plants for their arsenic tolerance potential. Present study yielded 83 arsenic tolerant fungal endophytes from the plants grown in arsenic-contaminated areas of Karnataka. These isolates were screened for arsenic tolerance at different arsenic (Sodium arsenite) concentrations (100 ppm to 2000 ppm). Among the isolates, eight endophytes (S1P1R1, S3P1S1, S3P1S3, S4P2L2, S5P1S1, S6P1S3, S8P1L4 and S10P1L1) were found efficiently enduring metal stress up to 2000 ppm with tolerance index >0.5. These isolates were further re-evaluated for arsenic tolerance in biomass assay. Significantly higher biomass was achieved in all eight endophytes at 100 PPM arsenic concentration compared to non arsenic control. However, fungal biomass relatively reduced with subsequent increase in arsenic concentration from 250 ppm to 2000 ppm. Further, these endophytes were evaluated for their ability to alleviate arsenic stress and growth promotion of rice seedlings under *in vitro* arsenic stress condition. Rice seedlings inoculated with fungal endophytes showed significant increase in shoot length under arsenic stress compared to control. Among eight endophytes, the isolate S3P1S1 significantly enhanced shoot and root length of rice. Therefore, data obtained in the present study revealed that the fungal endophyte, isolated from arsenic adapted plant has ability to mitigate arsenic stress and improve the rice growth under arsenic condition.

Keywords : Endophytic fungi, Arsenic, Tolerance, Growth promotion

Heavy metal and metalloid toxicity is a serious environmental and health issue. Among the heavy metals, arsenic (As) is a toxic metalloid steadily increasing in the ground and surface water poses a threat to environmental health. Being a class one human toxin and carcinogen (Khairul *et al*., 2017), arsenic mainly released into the environment by a number of anthropogenic as well as natural activities (Yaghoubian *et al*., 2019). Agriculture land contaminated with arsenic is one of the major problems of developing countries including India, Bangladesh and China; where over-exploitation of groundwater containing arsenic is in practice for cultivation (Ranjan *et al*., 2019). Irrigation practices with groundwater increases surface arsenic and accumulation in crops that is finally consumed by

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animals and humans leading to several health complications such as skin lesions, neurological impairment and cancer (Gao *et al*., 2018). Likewise, arsenic is highly toxic for plant life as it affect the plant water status, generates oxidative stress, alter the hormonal content and inhibits photosynthesis (Farooq *et al*., 2019). Bioconcentration and subsequent biomagnification and high levels of toxicity they impart to biological system indicate the necessity for the remediation of arsenic (Govarthanan *et al.,* 2014).

Plants adapt various metabolic and physiological mechanisms to tolerate or avoid arsenic stress *viz*., upregulation of ascorbic acid-glutathione (AsA-GSH) cycle, antioxidant enzymes and glyoxalase cyclic pathway (Upadhyay *et al*., 2020). Plant associated microbial community, particularly the fungal endophytes play a critical role in assisting host plant to overcome abiotic stresses including heavy metal stress (Kushwaha *et al.,* 2022). Fungal endophytes are known to impart arsenic tolerance in host plant by activating host defense systems such as osmotic adjustment, increasing plant growth by producing growth hormones, scavenging reactive oxygen species (ROS) and producing antioxidant enzymes (Moghaddam *et al.,* 2021). Shukla *et al.* (2023) reported *Piriformospora indica* induce arsenic tolerance in tomato by increasing proline, phytochelatins, thiol compounds and antioxidant activities in host plant. Further, Mohd *et al*. (2017) reported root endophytic fungus, *Piriformospora indica* protects rice plants from arsenic toxicity by not only reducing its availability in the plant but also by restricting arsenic in colonized roots through immobilization of As. Therefore, endophytic fungi mediated bioaccumulation restriction of toxic arsenic might be the plausible solution to prevent it from entering the food chain and protect the plants from arsenic toxicity.

Despite having numerous sound advantages, the investigation has been only restricted to root endophytic fungus, *Piriformospora indica* and arbuscular mycorrhiza fungi. In addition, *m*any studies have reported the benefits of habitat adapted endophytes in plants against abiotic stresses which includes drought, heavy metals and salinity. There fore, fungal endophytes isolated from arsenic stress adapted plants can efficiently impart stress tolerance in crop plants. This study is intended to isolate, screen and evaluate efficient fungal endophytes against arsenic stress in rice.

Material asnd Methods

Collection of Plant Samples from Arsenic Contaminated Regions of Karnataka

Plant samples were collected from previously reported arsenic contaminated regions of Karnataka *viz*., Yadagiri (Chakraborti *et al*., 2013), Raichur (Chakraborti *et al*., 2013), Bellary (Chakraborti *et al*., 2013), Chitradurga (Hebbar *et al*., 2016) and Hassan (Mohan *et al*., 2012). The details of geographical location of the different arsenic contaminated sites, different plant samples collected and endophytes isolated from each plant samples are presented in the Table 1.

Isolation of Fungal Endophytes

The isolation of arsenic resistant fugal endophytes was carried out as per the standard procedure given by Bacon *et al.* (2012). Root and leaf samples were cut in to 1cm bits and surface sterilized using 4 per cent sodium hypochlorite for 45 seconds followed by 70 per cent alcohol for one minute. The sterilized bits were repeatedly washed using sterile water to remove residual chemicals. The surface sterilized root and leaf bits were placed on minimal medium amended with 10 ppm sodium arsenite ($NaAsO_2$) dispensed plates and incubated at 28 °C for 7 days. The 1000 ppm stock solution of arsenic was made in deionized water using $NaAsO_2$. The stock solution of heavy metal was sterilized separately through bacteriological filters and added to sterilized above mentioned media to make the concentration at 10 ppm. The fungal mycelia emerged from the medium were purified and preserved under refrigerator for further study.

Screening of Fungal Endophytes for Arsenic Tolerance

Arsenic metal tolerance of isolated fungal endophytes was determined by following the poisoned

food technique protocol of Rodriguez *et al*. (2008).The fungal mycelial disc of 3 mm size with actively growing hyphae was cut aseptically from each isolate and were inoculated onto Potato Dextrose Agar plates in which 100 µg/ml (ppm) arsenic concentration was achieved by adding $NaAsO_2$. PDA medium agar plates without arsenic were used as controls. The plates were then incubated at 28 °C for seven days. The diameter of growing mycelia was measured daily for seven days.

The Following Observations were Recorded.

Tolerance Index (TI)

Tolerance of fungi was studied by the tolerance index. TI of the isolates was calculated as the diameter of test fungal colony on arsenic incorporated medium divided by the diameter of the colony on medium without arsenic (Oladipo *et al*., 2018).

$$TI = \frac{\text{Mycelial growth in arsenic supplemented plates}}{\text{Mycelial growth in control plates}}$$

Per cent Growth Inhibition

The percent growth inhibition was calculated as per the method given by Vincent, (1927)

$$PI = \frac{C - T}{C} \times 100$$

Where,

PI = Growth inhibition (%)

C = Diameter of colony in control (mm)

T = Diameter of colony in treatment (mm)

Secondary Screening of Fungal Endophytes for Arsenic Tolerance at Different Arsenic Concentration

Preliminary screened arsenic metal-tolerant (100 ppm) endophytic fungal isolates were further screened for arsenic tolerance at different concentrations *viz*., 250, 500, 1000, 1500, 2000 ppm individually on PDA. The fungal isolate on PDA medium without adding any heavy metal is served as control for comparing the growth of fungal isolates on PDA medium containing different concentration of heavy metals. The plates were then incubated at 27 °C for seven days. The diameter of growing mycelia was again measured daily for seven days. Tolerance index and per cent growth inhibition at each arsenic concentration is calculated using formula as mentioned above.

The metal tolerance index reported in the previous study (Oladipo *et al*., 2018), was used to screen the most promising endophytic fungal strains. Efficient strains with tolerance index ≥ 0.5 at 2000 ppm were identified as arsenic tolerant fungal endophytes and further evaluated for their growth promotion under *in vitro* condition.

Fungal arsenic tolerance (Oladipo *et al*., 2018) was rated in the following way:

- 0.00 - 0.29 (very low tolerance)
- 0.30 - 0.49 (low tolerance)
- 0.50 - 0.79 (moderate tolerance)
- 0.80 - 0.99 (high tolerance)
- 1.00 - >1.00 (very high tolerance)

Evaluation of Selected Arsenic Resistant Fungal Endophytes for their Arsenic Tolerance by Biomass Method

Mycelial discs (5mm) of actively growing eight fungal isolates were inoculated in to conical flask containing potato dextrose broth (PDB) amended with sodium arsenite ($NaAsO_2$) at 100, 500, 1000 and 2000 concentrations. The inoculated flasks were placed in a shaker (100 rpm) at 27 °C and incubated up to 7 days. Fungal mycelia were separated by passing the culture broth through pre-weighed Whatman-No.1 filter paper. The filter paper was rinsed in distilled water to remove broth residue. The filter paper with mycelium was dried at 60 °C in hot air oven for 48 hrs to attain constant weight and the final weight of filter paper with mycelium was recorded. The dry weight of the fungal mycelium recorded was the final weight of the filter paper with mycelium minus

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weight of the filter paper. Fungal growth types were assessed as reported by Hutton *et al*. (1996).

Morphological Characters of Selected Arsenic Tolerant Fungal Isolates

The fungal isolates was observed for their colony morphology and the fruiting body and spore characters were studied under microscope using agar slide culture technique (Harris, 1986).

Determination of LC_{50} Value of Arsenic (Sodium Arsenite) for Rice Plant

The method followed for he standardized LC_{50} value was the paper towel method with 4 replications for each treatment. Different concentrations (25ppm, 50 ppm, 75 ppm, 100 ppm 125 ppm and 150 ppm) of arsenic solution was prepared in sterile deionized water (Muddarsu and Manivannan, 2017). Three germination papers were soaked in 400 mL arsenic solution of different concentration and excess solution was removed. For control, sterile distilled water was used. The seeds of rice (*Oryza sativa* L.) variety IR-64 were collected from UAS, GKVK, Bengaluru. The seeds were surface sterilized through 70 per cent ethanol for 30 sec followed by sodium hypochlorite solution (3%) for 3 min and repeatedly washed with sterile water. Ten pre-germinated rice seeds were placed on each germination paper and incubated in room temperature for 14 d. Root, shoot and seedling length was recorded on the 14th day of incubation.

LC_{50} value of arsenic was calculated through probit analysis for the seedling length through statistical software OPSTAT.

Evaluation of Selected Fungal Endophytes for Induction of Arsenic Stress Tolerance in Rice

Rice grains (IR-64) were surface sterilized using sodium hypochlorite (4%) solution followed by 70 per cent ethanol and repeatedly washed with sterile water to remove residual chemicals on the seeds. Then these seeds were pre-germinated in sterile moist blotters. The pre-germinated seeds were treated with fungal spore suspension (10^6 spores/ mL) prepared from 10 days old culture for 3 hours (Zhang *et al*., 2014). The control seeds were treated with sterile distilled water. The pre-germinated seeds were subjected to arsenic stress by placing them on a sterile paper towel soaked in 50 PPM (critical arsenic concentration for crops) sodium arsenite and 75 PPM (LC_{50} value) sodium arsenite solution and incubated at 27 ºC in the growth chamber for 10 days. There were three replications and each replication comprised with twenty seedlings. Root and shoot lengths were recorded after incubation for 10 days.

Statistical Analysis

The data was statistically analysed using WASP: 2.0 (Web Agri Stat Package 2) statistical tool (www.icargoa.res.in/wasp2/index.php) and the means were separated by Duncan Multiple Range Test (DMRT).

Results and Discussion

Binomial approaches using microbe-plant interactions have recently drawn the attention of many research groups, since several works have shown that microorganisms associated with plants can also be relevant for bioremediation strategies, especially those regarding heavy metal pollution. However, there are only a few reports that describe fungal communities associated with hyper accumulator plants and most of them focus only on arbuscular mycorhizae and *Piriformospora indica*. Therefore, current study aimed at exploring the axenically cultivable endophytic fungi present in plants adapted to metal stress and preliminary characterization of their potential roles in *in vitro* arsenic metal tolerance and plant growth promotion.

Isolation of Fungal Endophytes

Habitat adapted symbiosis is an ecological phenomenon where endophytes of plant from different habitats such as geothermal soils, coastal beaches and agricultural fields confer habitat-specific stress tolerance to plants which provide an intergenomic epigenetic mechanism for plant adaptation and survival in high-stress habitats (Rodriguez *et al*., 2008). In some cases, plants are

unable to survive in their native stress habitats without the habitat-adapted fungal endophytes (Rodriguez and Redman, 2008). Therefore in the present investigation, plants grown in arsenic polluted sites were sampled for the isolation of endophytes. Previously reported arsenic contaminated regions of Karnataka *viz*., Kiradalli, Kiradalli tanda, Jainapura and Hegganadoddi of Yadagiri district (Chakraborti *et al*., 2013), Lingasugur and Sindanur of Raichur district (Chakraborti *et al*., 2013), Sandur of Bellary district (Chakraborti *et al*., 2013), Ingaldhal of Chitradurga district (Hebbar *et al*., 2016) and Nuggehalli and Bairapura of Hassan district (Mohan *et al*., 2012) were chosen for the study (Table 1, Plate 1). Interestingly, it was found that the source of arsenic pollution in these regions differed. Rocks

TABLE 1

Details of geographical location of the different arsenic contaminated sites, different plant samples collected and endophytes isolated from each plant samples

Location	Longitude (N)	Latitude (E)	Name of the plant	Isolate code
Kiradalli Tanda, Shorapur (Tq), Yadgiri (Dist.)	16.37'132°	76.36'788°	Paddy (*Oryza sativa* L.)	S1P1R1, S1P1R2, S1P1R3,S1P1R4, S1P1S1, S1P1S2, S1P1S3, S1P1S4
Kiradalli, Shorapur (Tq), Yadgiri (Dist.)	16.35'563°	76.33'832°	Paddy (*Oryza sativa* L.)	S2P1R1, S2P1R2, S2P1S1, S2P1S2, S2P1S3, S2P1S4
Jainapura, Shorapur (Tq), Yadgiri (Dist.)	16.36'813°	76.37'408°	Paddy (*Oryza sativa* L.)	S3P1R1, S3P1R2, S3P1R3, S3P1S1, S3P1S2, S3P1S3
Hegganadoddi, Shorapur (Tq), Yadgiri (Dist.)	16.35'858°	76.38'049°	Paddy (*Oryza sativa* L.)	S4P1R1, S4P1R2, S4P1R3, S4P1S1, S4PIS2, S4P1S3
			Raddish (*Raphanus sativus* L.)	S4P2R1, S4P2R2, S4P2L1, S4P2L2, S4P2L3, S4P2L4
Lingasugur, Lingasugur (Tq), Raichur (Dist.)	16.16'022°	76.52'014°	Paddy (*Oryza sativa* L.)	S5P1R1, S5P1R2, S5P1S1
			Euphorbia hirta L.	S5P2R1, S5P2R2, S5P2R3, S5P2S1, S5P2L1, S5P2L2, S5P2L3, S5P2L4
Sindhanur, Sindhanur (Tq), Raichur (Dist.)	15.78'302°	76,75'600°	Paddy (*Oryza sativa* L.)	S6P1R1, S6P1R2, S6P1S1, S6P1S2, S6P1S3
Sandur, Sandur (Tq), Bellary (Dist.)	15.81'259°	76.54'362°	*Dichrostachys cinerea* (L.)	S7P1S1
			Argentina adenophora	S7P2R1. S7P2R2, S7P2R3, S7P2L1, S7P2L2, S7P2L3
Nuggehalli, Channarayapatna (Tq), Hassan (Dist.)	12.00'395°	76.27'551°	*Solanum nigrum*	S8P1R1, S8P1R2, S8P1R3, S8P1S1, S8P1S2, S8P1L1, S8P1L2, S8P1L3, S8P1L4, S8P1L5
			Maize (*Zea mays* L.)	S8P2R1, S8P2R2, S8P2S1, S8P2S2, S8P2S3, S8P2S4
Ingaldhal, Chitradurga (Tq), Chitradurga (Dist.)	14.10'45°	76.26'678°	*Lantana camera*	S9P1L1, S9P1L2, S9P1L3
Byrapura, Channarayapatna Hassan (Dist.)	12.21'79°	76.89'885°	*Amaranthus*	S10P1R1, S10P1R2, S10P1L1, (Tq), S10P1L2, S10P1L3
			Chenopodium album	S10P2R1, S10P2R2, S10P2R3, S10P2L1

Note : S- Sampling site, P-Plant sample, R-root, S-Shoot/stem, L- Leaf


Kiradalli Tanda, Yadgiri (Dist.)


Kiradalli, Yadgiri (Dist.)


Hegganadoddi, Yadgiri (Dist.)


Lingasugur, Raichur (Dist.)


Sindhanur, Raichur (Dist.)


Byrapura, Hassan (Dist.)


Nuggehalli, Hassan (Dist.)


Sandur, Bellary (Dist.)


Ingaldhal, Chitradurga (Dist.)

Plate 1 : Collection of plant samples from arsenic contaminated sites of Karnataka

(metamorphic rock having laminated, flaky parallel layers of micaceous minerals) in the Raichur, Yadgiri and Bellary districts contain arsenic rich minerals like arseno pyrite (Chakraborti *et al*., 2013). The weathering of rocks and their dissolution, leading to contamination of aquifers. Whereas, mining of the copper and chromium ore in the Chitradurga (Hebbar *et al*., 2016) and Hassan (Mohan *et al*., 2012) respectively have contaminated the aquifers (Rama-mohan *et al*., 2012). Plants adapted in these regions *viz*., *Oryza sativa*, *Raphanus sativus, Euphorbia hirta, Dichrostachys cinerea, Argentina adenophora, Solanum nigrum, Zea mays, Lantana camera, Amaranthus and Chenopodium album* were used in isolation of fungal endophytes.

A total of 83 fungal endophytes were extracted in minimal medium amended with 10 PPM arsenic from the previously mentioned 10 plant species (root, shoot and leaf segments). Among the 83 isolates, 34 were recovered from roots, 26 isolates were isolated from the stem/shoot and 23 from leaf bits. In a study by

Lalancette *et al*. (2019) twelve fungal endophytes were isolated from alder trees that were growing on or near disturbed environments, which later imparted tolerance to Cu, Ni, Zn and As. Hussain *et al*. (2021) isolated nine different endophytic fungal isolates from the ornamental plant, *Chlorophytum comosum* which efficiently endured metal stress up to 1200 µg/mL. These results indicate that even in a toxic environment like heavy metal-polluted soils, fungi show a considerable diversity, probably through interactions with host plants. Further, endophytic fungi known to secrete secondary metabolites and enhance the plant's ability to tolerate stressful conditions. The isolated fungal endophytes were then grown on PDA plates and were grouped on the basis of their morphological traits. The dominant genus in our study was *Fusarium* sp. *Tricoderma* sp. and *Penicillium* sp. Although most of the *Fusarium* species are pathogenic, some can exist as symptom -less endophytes. Plethora of researchers also reported *Fusarium, Penicillium, Aspergillus, Metarhizium, Beauveria and Trichodermaas* the most predominant fungal genera present in the arsenic contaminated soil (Srivastava *et al*., 2021 and Tripathi *et al*., 2020). Similar trend was observed for endophytic fungal diversity in heavy metal contaminated soils (Cd, Cr, Ni, Pb) *i.e.* Fusarium and Aspergillus are dominant endophytic fungal community (Ignatova *et al*., 2021)

Screening of Fungal Endophytes for Arsenic Tolerance at 100 PPM Arsenic Stress

Arsenic and other heavy metals are potentially toxic to fungus, adversely affects the fungal cellular life process, ultimately leading to growth inhibition and death, therefore numerous studies focused on deducing the extent of tolerance of various fungi. Metal tolerance was determined by spiking the media with increasing concentrations of inorganic arsenic chemicals (Oladipo *et al.,* 2018). In *in vitro* assays, sodium arsenite chemical has been used to simulate arsenic stress in culture media. Initially, all the isolated fungal endophytes were screened for arsenic tolerance at 100 PPM sodium arsenite concentration by poison food technique. The isolates grown in arsenic stress condition showed 3 different types (Type-I, Type-II and Type-III) of growth response as defined by Hutton *et al*. (1996) which include: Type I- overall inhibition/ no growth after 7 days; Type II- maximum growth at control and decreased growth in arsenic stress; Type III maximum growth in arsenic induced plates compared to control. Out of 83 isolated fungal endophytes, it was noticed that (Fig. 1), 25 isolates exhibited 100 per cent growth

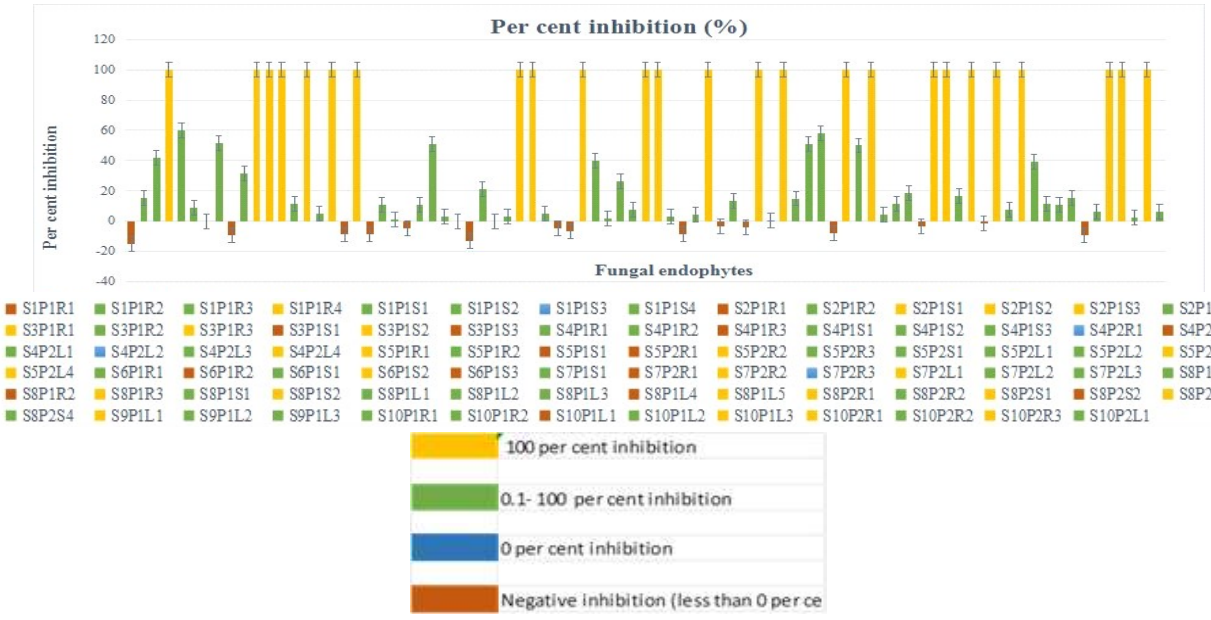

Fig. 1 : Effect of 100 PPM arsenic concentration on per cent growth inhibition of endophytic fungal isolates

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inhibition, suggesting the sensitivity of these isolates to 100 PPM arsenic concentration. Interestingly, Type III of growth was observed in 15 fungal endophytes. They showed increased mycelia growth with supplementation of 100 PPM arsenic compared to control. This observation is in accordance with Lalancette *et al.* (2019). They noted that presence of 50 mg/l arsenic stimulated the growth of a large number of strains, even though arsenic performs no known biological function and recorded median growth rates of the isolates higher than 1 (100%) at 50 mg/l As^{3+} for 12 strains. However, growth rates starts falling at 150 mg/l. Similarly, there are regular reports in the literature suggesting growth-stimulation effects at lower metal concentrations in several fungi. Remaining fungal endophytes showed Type II type of growth *i.e.* reduced growth in the presence of arsenic stress. This is because excessive concentration of accumulated metals causes microbial membrane damage, organelle damage, lipid peroxidation and cell apoptosis contributed by generation of reactive oxygen species (Igiri *et al*., 2018). Similarly, Mohd *et al.* (2017) reported that in the axenic culture, *P. indica* is able to tolerate both sodium arsenate (As V) and sodium arsenite (As III) up to 1 mM. However, the growth of fungus was reduced 30 and 50 per cent in presence of 1.5 mM As V and As III respectively.

Tolerance of fungi to heavy metals was also studied by the determination of tolerance index. Metal tolerance of fungal isolates was rated in the following way: 0.00-0.39 (very low tolerance), 0.40-0.59 (low tolerance), 0.60-0.79 (moderate tolerance), 0.80-0.99 (high tolerance) and 1.00->1.00 (very high tolerance) (Oladipo *et al*., 2018). At 100 PPM sodium arsenite concentration, among the 83 fungal endophytes 35 isolates with 100 per cent inhibition recorded least (0) tolerance index (Fig. 2). The fungal endophytes (15) having Type III growth response recorded tolerance index >1. Highest T.I. was documented in the isolate S1P1R1 (1.15) at 100 ppm arsenic. Likewise, in a study by Ignatova *et al*. (2021), a total of 253 strains of endophytic fungi was isolated and screened for metal tolerance at the initial (100 µg/ml) level of Cd supplemented media. Among them, 23 fungal strains showed tolerance to Cd with TI ranged from 0.24 to 1.12. The endophytic fungi have the ability to lockdown metals by various mechanisms including adsorption by the cells, metal binding to extracellular polysaccharides, sequestration of metals by intracellular phytochelatins and metallothioneins and localization in vacuole, etc., thereby provides tolerance to different toxic heavy metals (Upadhyay *et al*., 2020).

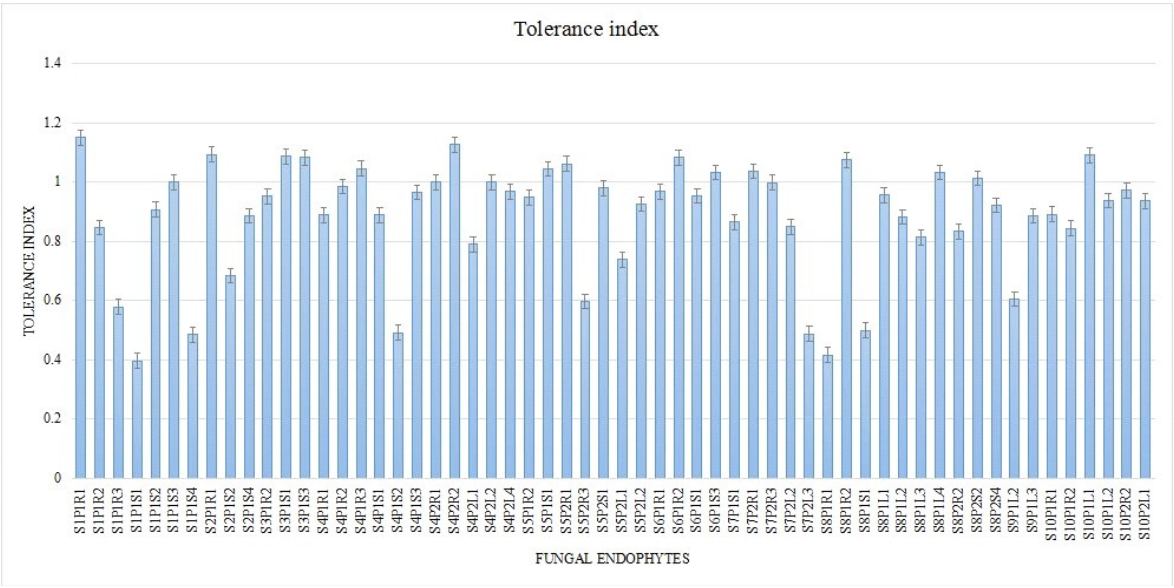

Fig. 2: Tolerance index of isolated fungal endophytes at 100 PPM arsenic stress

Arsenic Tolerance Potential of Screened Endophytic Fungal Isolates at Different Arsenic Stress Condition

Arsenic metal-tolerant (100 ppm) endophytic fungal isolates (58 isolates) were further screened for arsenic tolerance at different concentrations *viz*., 250, 500, 1000, 1500, 2000 ppm individually on PDA (Plate 2). In this study at lower concentration of arsenic (250 PPM), isolates showed resistance and good growth which subsided as the concentrations increased. From the heat map (Fig. 3a, 3b) we can observe increasing the metal concentration beyond the level (250 PPM) resulted in increased per cent growth inhibition in all the 58 isolates indicating the sensitivity of these isolates at higher arsenic concentration. The change in tolerance towards different concentration could be because of the altered endurance approach or resistance mechanisms exhibited by different fungi

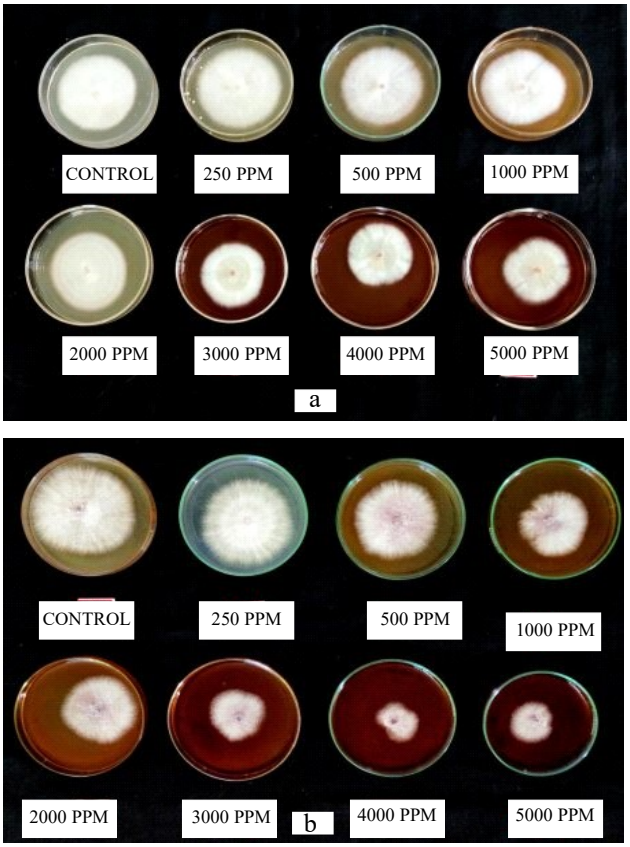

Plate 2 : Arsenic tolerance potential of fungal endophytes a) S1P1S1 b) S3P1S1 at different arsenic concentration (250 PPM, 500 PPM, 1000 PPM and 2000 PPM, 3000 PPM, 4000 PPM and 5000 PPM)

(Zafar *et al*., 2007). To address the effect of higher concentration of metals, Levinskaite (2011) examined the influence of heavy metals such as cadmium, nickel and zinc on the development of the fungi *Penicillium atramentosum* and *P. funiculosum*. He reported all tested stages *i.e.,* spore germination, germ tube growth, radial growth rate and conidiogenesis, were affected by the presence of heavy metals compared to control. Cao *et al.* (2023) demonstrated that endophytic fungal growth diminishes with increase in heavy metal concentration for all metals tested (As, Cu, Zn and Ni). This is because excessive concentration of accumulated metals causes microbial membrane damage, organelle damage, lipid peroxidation and cell apoptosis contributed by generation of reactive oxygen species (Igiri *et al*., 2018).

*Among the 58 isolates, eight isolates (*S1P1R1, S3P1S1, S3P1S3, S4P2L2, S5P1S1, S6P1S3, S8P1L4 and S10P1L1) exhibited remarkably higher tolerance index (more than 0.5) and minimum per cent inhibition (less than 50%) at 2000 PPM arsenic stress. Although tolerance subsides at 2000 ppm *viz*., S1P1R1 (0.69), S3P1S1 (0.56), S3P1S3 (0.63), S4P2L2 (0.88), S5P1S1 (0.55), S6P1S3 (0.65), S8P1L4 (0.63) and S10P1L1 (0.59), the maximum tolerance index was recorded at 250 PPM arsenic in all eight isolates *viz.,* S1P1R1 (1.11), S3P1S1 (1.06), S3P1S3 (1.00), S4P2L2 (1.00), S5P1S1 (1.01), S6P1S3 (1.02), S8P1L4 (0.95) and S10P1L1 (0.98). Our results are in accordance with Tripathi *et al.* (2020), out of 15 isolated fungal strains, only five fungal strains were found resistant to arsenic and survived with tolerance index pattern as 0.956 (sterile mycelial strain) > 0.311 (*Rhizopus* sp.) > 0.306 (*Neocosmospora* sp.) > 0.212 (*Penicillium* sp.) > 0.189 (*Aspergillus* sp.) at 10,000 mg l^{-1} of arsenate. Similarly, Hussain *et al*. (2021) isolated nine different endophytic fungi from the ornamental plant, *Chlorophytum comosum* and observed that among the isolated endophytes, *Aspergillus welwitschiae* (Bk) efficiently endure metal stress *i.e.,* Cr-VI and As-V in the form of $K_2Cr_2O_7$ and Na_3AsO_4 up to 1200 µg/mL and also showed a higher tolerance index. Henceforth, these eight fungal endophytes with

Fig. 3a : Tolerance index

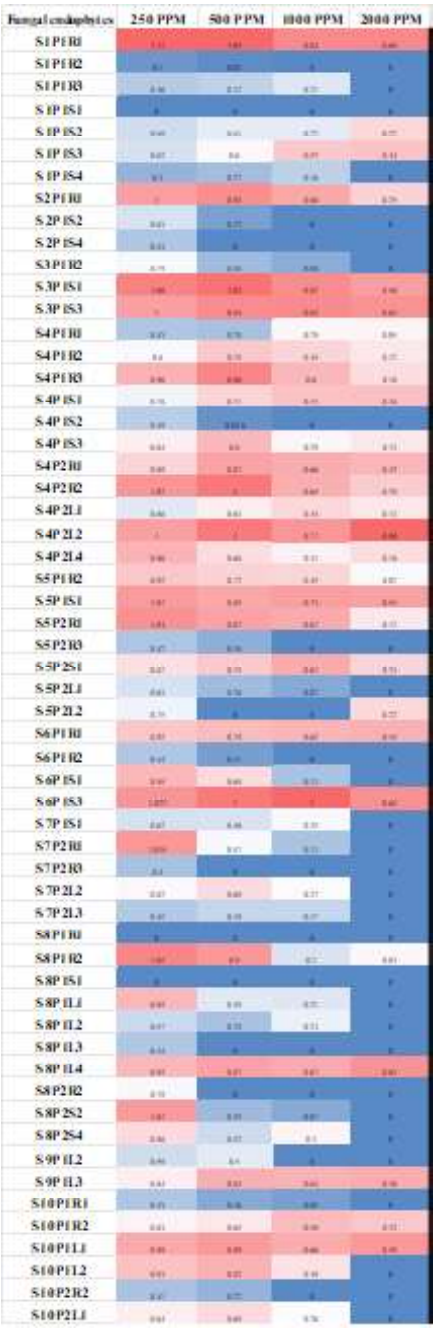

Fig. 3b : Per cent inhibition

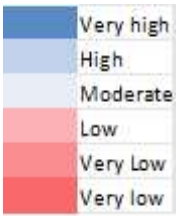

Fig. 3 : Tolerance index and per cent growth inhibition of fungal endophytes at different arsenic concentration (250 PPM, 500 PPM, 1000 PPM and 2000 PPM)

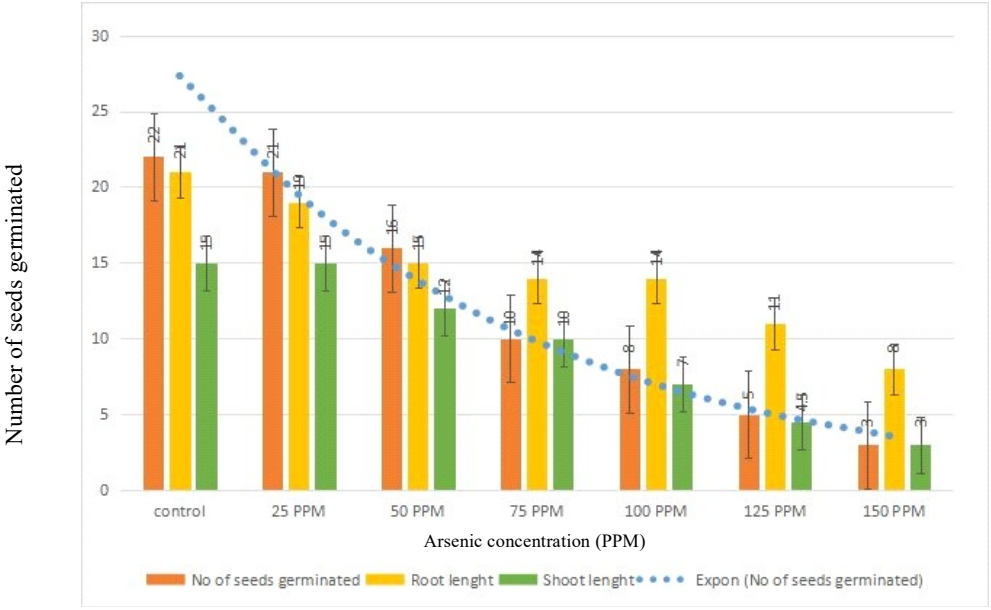

Fig.4: Determination of LC_{50} value of arsenic for rice crop

tolerance index ≥ 0.5 at 2000 ppm (Fig. 4) were identified as arsenic tolerant fungal endophytes by following tolerance index rating of Oladipo *et al*. (2018) and further evaluated for their growth promotion potential under *in vitro* arsenic condition.

Evaluation of Selected Arsenic Tolerant Fungal Endophytes for Arsenic Tolerance by Biomass Method

The selected eight endophytic fungal strains were re-validated for their arsenic tolerance in a biomass method (Plate 3). Similar to poisoned food technique, highest mycelial dry weight in all eight was observed in 100 PPM (Table 2) arsenic stress compared to control, suggesting preferences of these isolates for arsenic for their growth stimulation. Increasing concentration to 500 PPM and subsequently higher levels resulted in decreased mycelial dry weight. Significantly highest fungal biomass (dry weight) was noticed in S6P1S3 irrespective of arsenic concentration *viz*., 239 mg (100 PPM), 207 mg (500 PPM), 143 mg (1000 mg), 98 mg (2000 PPM) and 218 mg (control). Similarly, Cao *et al*. (2023) screened twenty three isolated endophytic fungal strains (fifteen dark septate endophytes and eight non dark septate endophytes) for heavy metal tolerance in minimal medium containing 100 mg/L As(V) and Cd (II) and observed that dry weight of the mycelia decreased with the addition of As(V) and Cd (II).

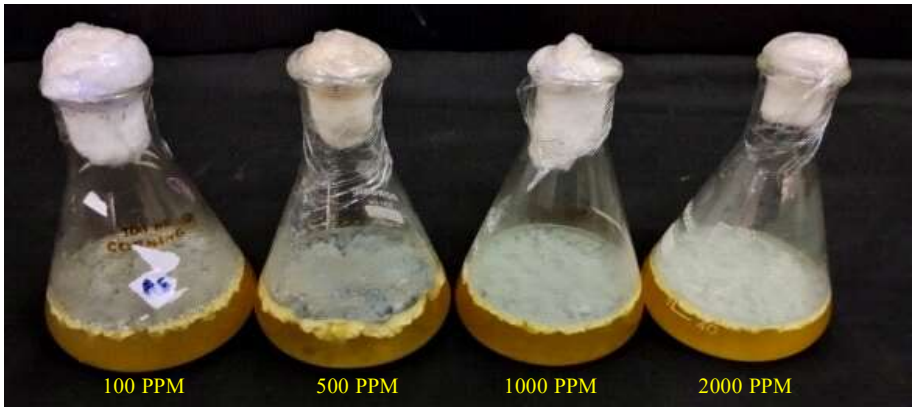

Plate 3 : Biomass of S4P2L2 at different arsenic stress condition

TABLE 2
Evaluation of selected arsenic tolerant fungal endophytes for arsenic tolerance by biomass method

Isolates	Fungal biomass (mg)				
	Control	100 PPM	500 PPM	1000 PPM	2000 PPM
S1P1R1	143 de	169 d	144 c	102 c	81 cd
S3P1S1	120 f	153 ef	111 f	98 c	62 g
S3P1S3	145 de	171 d	112 f	87 de	83 c
S4P2L2	181 b	198 c	185 b	141 a	121 a
S5P1S1	138 e	140 f	135 cd	81 e	76 de
S6P1S3	218 a	239 a	207 a	143 a	98 b
S8P1L4	193 b	213 b	187 b	132 b	86 c
S10P1L1	154 cd	166 de	122 ef	104 c	67 fg

Note: S- Sampling site, P-Plant sample, R-root, S-Shoot/stem, L- Leaf

The eight selected fungal endophytes (S1P1R1, S3P1S1, S3P1S3, S4P2L2, S5P1S1, S6P1S3, S8P1L4 and S10P1L1) when examined for their cultural, microscopic and morphological characteristics (Plate 4. a, b, c, d, e, f, g and h) revealed that four isolates S1P1R1, S3P1S1, S3P1S3 and S10P1L1 belong to the genera of *Fusarium*, S4P2L2 to *Penicillium*, S5P1S1 to *Alternaria* S6P1S3 to *Muco*r and S8P1L4 to *Curvularia* (Watanabe, 2010).

In vitro Evaluation of Selected Fungal Endophytes on Growth of Rice under Induced Arsenic Condition

Fungal endophytes are known to enhance plant growth and biomass under abiotic stress condition (Neekshita *et al*., 2023 and Srikanth *et al.*, 2023). Therefore, eight isolates were further evaluated for their ability to influence on early seedling growth of rice (IR-64) under arsenic stress. To induce arsenic stress, LC_{50} value of arsenic for rice was calculated by probit analysis (Fig. 4). In 75 PPM arsenic stress, number of seeds germinated (10) drastically reduced to 50 per cent compared to seeds germinated in control (22). Further shoot and root length also relatively lower (10 cm and 14 cm respectively) compared to control, 25 ppm and 50 PPM. Therefore 75 PPM identified as LC_{50} value of arsenic for rice. Many researchers suggested that critical arsenic level for plant growth is around 50 PPM. Therefore 50 PPM and 75 PPM arsenic levels were selected for inducing arsenic stress to evaluate efficiency of fungal endophytes in imparting growth promotion under *in vitro.*

The results revealed that seedlings inoculated with fungal endophytes showed significant increase in shoot length compared to un-inoculated seedlings under both 50 PPM and 75 PPM stress (Table 3). Remarkably, fungal endophytes also exhibited influence on the root length of rice seedlings which are not treated with arsenic stress (control). However, both shoot and root length reduced in accordance to arsenic stress. In addition, root hair development was observed in endophyte inoculated seedlings. This indicates that the fungal endophytes might have influenced root architecture by producing plant growth hormones (Verma *et al.,* 2020). Among the eight selected arsenic tolerant fungal endophytes, the isolate S3P1S1 showed highest shoot and root length compared to others under control (24 cm and 19 cm respectively), 50 PPM (20 cm and 18 cm respectively) and 75 PPM arsenic stress (20 cm and 16 cm respectively). It was followed by isolate S4P2L2 which showed significantly higher shoot and root length in 50 PPM and 75 PPM arsenic stress condition. Chen et *al*. (2013) observed inoculation of fungal endophytes improves seed germination and seedling vigor, which is a critical life stage for plant survival and timely seedling establishment, especially in stressful environments. Besides promoting seedling growth, a significant increase in seed germination (69.8%) has been reported with application of endophytic fungi. Further more, endophytic fungi boost plant fitness and other competitive abilities by enhancing growth rate and germination or by increasing the plants' absorption capacity for nutritional elements (Aly *et al*., 2011).

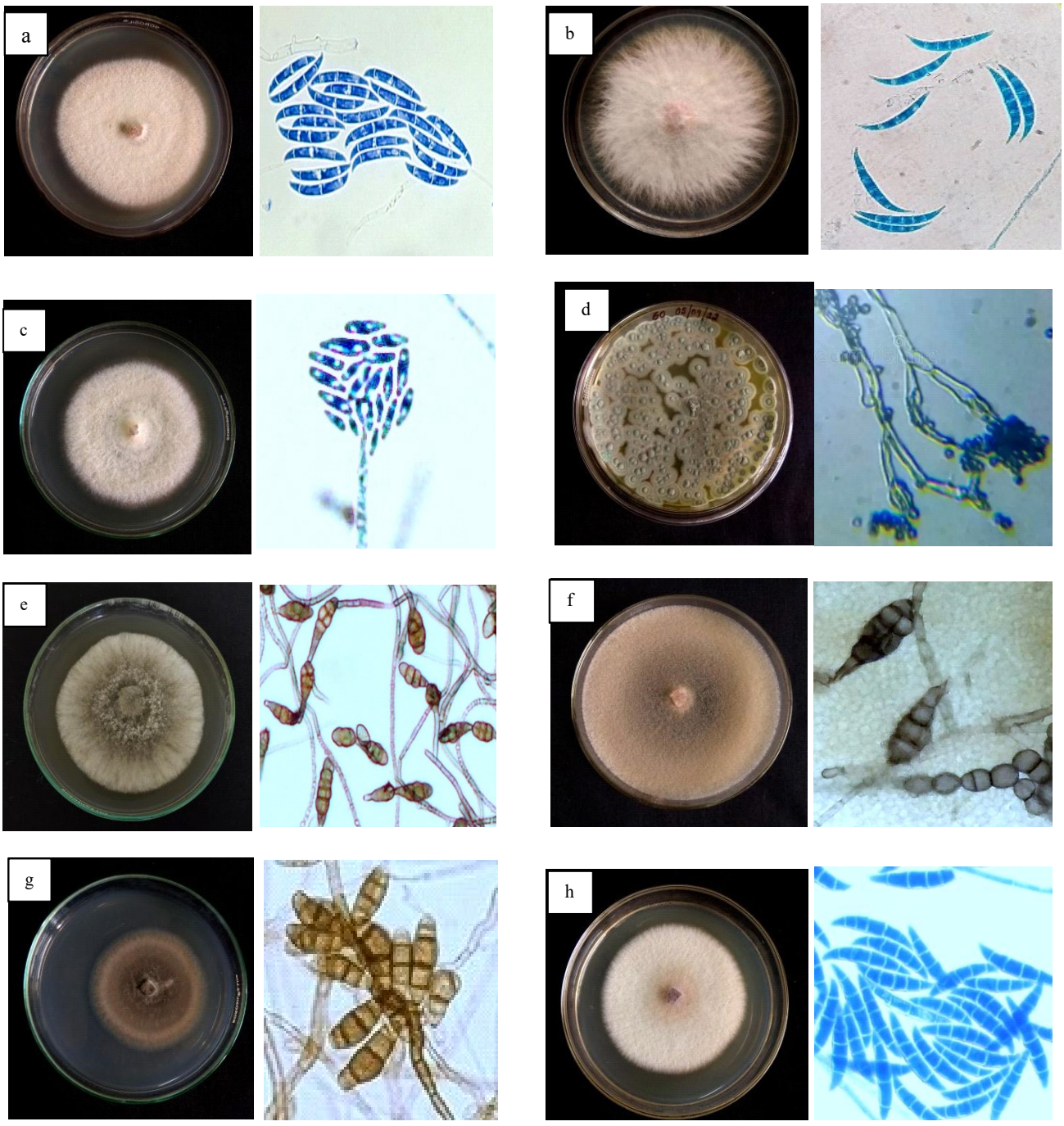

Plate 4 : Microscopic observation of efficient arsenic tolerant fungal endophytes a) S1P1S1 - *Fusarium* b) S3P1S1 - *Fusarium* c) S3P1S3- *Fusarium* d) S4P2L2- *Penicillium* e) S5P1S1 -*Alternaria* f) S6P1S3- *Mucor* g) S8P1L4 - *Curvularia* h) S10P1L1- *Fusarium*

Endophytic fungi and plants have long-standing symbiotic relationships, which clearly explain the importance of endophytes in the host plant stress tolerance during metal stress. Besides, endophytic fungi secrete secondary metabolites and enhance the plant's ability to tolerate stressful conditions. The findings of the current study indicate that fungal endophytes seems to be a promising candidate, which can be used to alleviate the adverse effects of metal stress on the cultivation of rice in metal-polluted areas.

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TABLE 3

Root length and shoot length of rice seedlings as influenced by inoculation of arsenic resistant fungal endophytes at different arsenic concentrations (Paper towel method)

Isolates	Control (0 PPM)		50 PPM		75 PPM	
	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)
Control	19 ef	15 de	15 e	12 f	13 f	11 e
S1P1R1	22 bc	18 ab	19 ab	17 c	18 b	14 b
S3P1S1	24 a	19 a	20 a	18 b	20 a	16 a
S3P1S3	20 de	15 de	17 cd	15 d	14 ef	12 d
S4P2L2	23 ab	17 bc	20 a	19 a	18 b	16 a
S5P1S1	19 ef	14 e	16 de	14 de	15 de	13 c
S6P1S3	18 f	14 e	15 e	12 g	14 ef	12 d
S8P1L4	23 ab	18 ab	18 bc	13 g	17 bc	13 c
S10P1L1	19 ef	16 cd	18 bc	15 d	14 ef	12 d

Note: S- Sampling site, P-Plant sample, R-root, S-Shoot/stem, L- Leaf

Finally, in this report we propose the fungal strains with its endophytic character and plant growth-promotion activity, along with its high metal tolerance ability could be a novel, effective and sustainable bioremediation strategy for arsenic-polluted soils.

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