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A consortium of alga (*Chlorella vulgaris*) and bacterium (*Pseudomonas putida*) for amelioration of arsenic toxicity in rice: A promising and feasible approach

Surabhi Awasthi[a,b], Reshu Chauhan[a], Sanjay Dwivedi[a], Suchi Srivastava[a], Sudhakar Srivastava[b,*], Rudra Deo Tripathi[a,*]

[a] CSIR – National Botanical Research Institute, Council of Scientific and Industrial Research, Rana Pratap Marg, Lucknow, 226 001, India
[b] Institute of Environment and Sustainable Development, Banaras Hindu University, Varanasi, 221005, India

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ABSTRACT

In the present study, arsenic (As) toxicity amelioration potential of a consortium of plant growth promoting rhizobacterium (*Pseudomonas putida*) and alga (*Chlorella vulgaris*) was evaluated during arsenate (AsV) exposure to rice (*Oryza sativa*) plants for 15 d. The consortium mediated amelioration of As toxicity was evident through improved growth of rice plants (root and shoot length and biomass) and reduced oxidative stress [as level of superoxide radicals ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2) and membrane damage]. The positive responses were attributable to a significant decline in As accumulation in root (94 mg kg^{-1} dw) and shoot (51 mg kg^{-1} dw) in consortium (*P. putida* + *C. vulgaris*) inoculated seedlings as compared to As alone exposed plants (156 and 98 mg kg^{-1} dw, respectively). There were also significant changes in the level of various nutrient elements (Mn, Fe, Co, Zn, Mo and Cu), thiols and in the activities of antioxidant and thiol metabolism enzymes in the consortium inoculated seedlings that allowed the plants to tolerate As stress effectively and achieve better growth. The study demonstrated that consortium of *P. putida* and *C. vulgaris* may alleviate As stress and improve growth of rice seedlings along with reduction in As levels.

1. Introduction

Arsenic (As) is a highly toxic metalloid, which is ubiquitously present in soil and water. Serious contamination of As in South and Southeast Asian countries affects millions of people (Awasthi et al., 2017). In the As affected regions, people have to use As contaminated groundwater for drinking and irrigation purposes. This is a serious health issue for people living in contaminated areas and it generates route for As entry into people from other areas also through food chain (Zhao et al., 2010). Rice in comparison to other cereal crops has been recognized as a catastrophe as it accumulates As in significantly higher amounts than any other cereal crop (Awasthi et al., 2017). This also affects grain quality and yield of rice plants (Dwivedi et al., 2012). Thus, it is critically important to develop effective, feasible and environmentally amicable approaches to alleviate the toxic effects of As on plant growth and to achieve low As accumulation in rice grains.

Microbes present in rhizosphere are known to improve plant growth through effects on bioavailability of essential nutrient elements and involving secretion of specific chemicals/hormones like indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase and siderophores (Idris et al., 2007). Hence, these are referred to as plant growth promoting rhizobacteria (PGPR). Further, these PGPR may also mitigate toxic effects of heavy metals (Lampis et al., 2015). Microbes that are able to grow in As contaminated environment are equipped with genetic architecture, metabolic pathways and enzymatic systems that enable them either to tolerate As toxicity via transformation of toxic inorganic forms into less toxic organic ones or to use As as a part of metabolism (Verma et al., 2016). *Pseudomonas putida* is one of the important rhizosphere microorganisms, which is known to impart beneficial effects on plant growth and development (Ahemad and Kibret, 2014). These bacteria are able to convert insoluble P into available form for plants and are also known to modulate metal(loid) toxicity to plants and promote growth of plants (Khan et al., 2009).

Algae have high potential in accumulating metals within their cells (Monteiro et al., 2012). The presence of different types of binding groups on the algal cell surface i.e. hydroxyl, phosphoryl, carboxyl, sulphuryl, amine, imidazole, sulphate, phosphate, carbohydrate, etc. helps in the biosorption of metals (Richmond and Hu, 2013). *C. vulgaris*

* Corresponding authors.
E-mail addresses: sudhakar.srivastava@gmail.com, sudhakar.iesd@bhu.ac.in (S. Srivastava), tripathird@gmail.com (R.D. Tripathi).

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is a common single-cell green microalgae that tolerates a number of heavy metals and metalloids (Wang et al., 2015) including As through exclusion, binding to cysteine rich peptides/proteins, methylation, and volatilization (Munoz et al., 2016).

Rice grown in As contaminated soil harbors unique As-resistant micro-flora in the rhizosphere. The identification and utilization of suitable micro-flora from As contaminated regions may be advantageous as the micro-flora might have plant growth promoting properties and might reduce As toxicity to plants via biotransformation and bioaccumulation of As by itself and also by alteration of As bioavailability to plants (Kumar and Oommen, 2012; Lakshmanan et al., 2015). There are reports of micro-flora utilization for plant growth promotion, enhanced productivity and for improved metal(loid) resistance (Wani and Khan, 2010). However, the use of a consortium of bacteria and algae has seldom been attempted for the mitigation of As stress in rice. Though, reports about potential role of algal and bacterium consortium against the stress of Cu, Cd, Ni and Zn are available (Munoz and Guieysse, 2006; Loutseti et al., 2009). The synergistic effects of an appropriate consortium might deliver greater benefits *viz.*, bacteria mediated growth improvement of algae (Mouget et al., 1995). The objectives of the present study were (i) to evaluate the effect of the consortium of *P. putida* MTCC5279 and *C. vulgaris* on rice growth during As stress, and (ii) to explore the possible mechanisms of As stress amelioration by *P. putida* + *C. vulgaris* consortium.

2. Material and methods

2.1. Growth of the PGPR and algae biomass

In the present study, strains of PGPR, (*P. putida* MTCC 5279) and microalga (*C. vulgaris*), were used for inoculation in rice plants. For the identification and isolation of As tolerant algal strain, the mixed culture of algae was collected from the soil of As contaminated regions of West Bengal, India and isolated in laboratory conditions. The mixture was spread on algal culture agar plates by following serial dilution. After 7 d, the unialgal cultures were isolated. At this stage microscopic examination was made to identify the unialgal strain i.e. *C. vulgaris* (Desikachary, 1959). *C. vulgaris* was grown and maintained in the BG11 medium containing macronutrients *viz.,* $NaNO_3$ (1.5 g L^{-1}), K_2HPO_4 (0.04 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.075 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.036 g L^{-1}), Na_2CO_3 (0.02 g L^{-1}), Citric acid (0.006 g L^{-1}), EDTA (Na_2 salt, 0.001 g L^{-1}) and micronutrients *viz.,* H_3BO_3 (2.86m g L^{-1}), $MnCl_2{\cdot}4H_2O$ (1.81m g L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.22m g L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.39m g L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.079m g L^{-1}), Co $(NO_3)_2{\cdot}6H_2O$ (0.049m g L^{-1}) in conical flask at 24 ± 2 °C under light intensity of 60 mmol m^{-2} S^{-1} in culture room under controlled condition. To maintain aeration, culture was shaken daily 2–3 times and sub cultured weekly into fresh BG-11 medium (Rippka et al., 1979). The bacterium, *P. putida*, was identified in a previous work at NBRI, Lucknow (Srivastava et al., 2012). *P. putida* was grown in minimal growth medium (M9)/Nutrient Broth (NB, Hi-media) at 28 °C in a rotary shaker. The cultured cells of bacterium and alga were centrifuged and pelleted down. The pellet of both was dissolved in 10 ml of Hewitt medium separately and one ml of each dissolved pellet was used for the inoculation. At this time the growth of algae and bacteria was represented by viable cell count (1% of the log transform value; Fig. 1, value at 0 d). Arsenic stress tolerance of *P. putida* and *C. vulgaris* was determined by growing the culture in their respective medium under different As concentrations followed by their viable cell count determination (CFU/ml) on agar supplemented respective medium plates at different time intervals. The combined growth of *P. putida* and *C. vulgaris* was also tested by incubating the combination of these cultures in Hewitt nutrient medium in Erlenmeyer flasks at 25 ± 2 °C with a light intensity of 60 mmol m^{-2} S^{-1}. Viable cells (CFU/ml) were counted at various time intervals of 0, 7 and 15 d by serial dilution plating on agar supplemented medium plates in triplicate (Gupta et al., 2006) (data not shown).

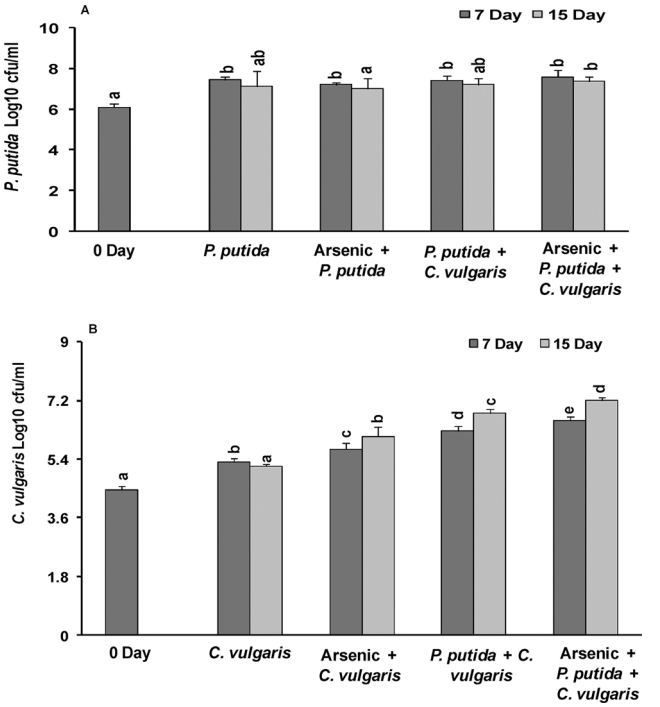

Fig. 1. (A,B) Number of viable cells (CFU/ml) (A) *Pseudomonas putida,* (B) *Chlorella vulgaris* at time intervals of 0, 7 and 15 d by serial dilution plating on agar supplemented NB medium plates in triplicate.

2.2. Plant material and experimental conditions

Oryza sativa L. var. Triguna was used in the present study. The rice seeds of the variety were collected from Rice Research Station Chinsurah, West Bengal. Seeds were disinfected with 0.1% $HgCl_2$ solution for 30 s followed by washing with milli-Q water for 5–6 times. After thorough washing, seeds were soaked in milli-Q water for 24 h. The germinated seeds were transferred to petridishes and kept in culture room at 26 °C for 3–4 d in dark for their germination. Germinated seedlings of uniform length were transferred to trays having fixed PVC cups (4 cm diameter and 5 cm height, ten plants per cup) and grown hydroponically in Hewitt nutrient medium (Liu et al., 2004) for 7 d for acclimatization. The pH (5.5) of the nutrient medium was maintained with the help of 0.1 M KOH or HCl. After 7 d of acclimatization, plants were exposed to different treatments (50 µMAsV), *P. putida* and *C. vulgaris* and co-inoculation of *P. putida* and *C. vulgaris* for 15 d. The experiment was carried out in controlled culture conditions with 14-h, 28 °C day and 20 °C night temperature and with 70% relative humidity. At an interval of 3 d, nutrient medium was changed. All experiments were performed three times. After completion of the experiment at 15 d, the root and shoot length of the plants were measured by a metric scale. Then, plants were harvested, washed with milli-Q, blotted gently, frozen in liquid nitrogen and stored at −80 °C for further analysis of various physiological and biochemical parameters. The analyses of As level, growth, photosynthetic pigment and protein analyses were performed with *P. putida* and *C. vulgaris* alone and consortium. However, other parameters were performed only with consortium of *P. putida* + *C. vulgaris.*

2.3. Photosynthetic pigments

Fresh leaves (200 mg) of control and treated rice plants were used for extraction of photosynthetic pigments in 80% chilled acetone in dark. After centrifugation at 10,000*g* for 10 min at 4 °C, absorbance of