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Melatonin and plant growth-promoting rhizobacteria alleviate the cadmium and arsenic stresses and increase the growth of *Spinacia oleracea* L.

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Abstract: Melatonin (*N*-acetyl-5-methoxytryptamine) is a recently discovered natural product that helps the plant to cope with environmental stresses. In the same way, plant growth-promoting rhizobacteria colonise plant roots and enhance plant stress tolerance. To study the impact of exogenous melatonin and *Bacillus licheniformis* on the growth of *Spinacia oleracea* L. seedlings were treated with 100 μmol exogenous melatonin and *B. licheniformis* under cadmium (Cd) and arsenic (As) stresses by a pot experiment. Different plant growth parameters, antioxidant enzymes, and lipid peroxidation were studied. The results showed that melatonin application and *B. licheniformis* inoculation alleviated As and Cd toxicity by significantly reducing the negative impacts of stresses and increasing the fresh and dry weight as well as preventing the damage to the chlorophyll content of *S. oleracea* L. Moreover, supplementation of melatonin, and *B. licheniformis*, enhanced activities of antioxidant enzymes superoxide dismutase, peroxidase, catalase, thus acting as a line of defense against As and Cd stresses. Similarly, lipid peroxidation was also inhibited by exogenous melatonin and *B. licheniformis* inoculation. Exogenous application of melatonin and inoculating roots of *S. oleracea* L. with *B. licheniformis* found to ameliorate the harmful effects of As and Cd contamination.

Keywords: spinach; toxic elements; phytomelatonin; stressful environment; plant hormone; contaminated soil

Plants being sessile are more vulnerable to environmental stresses like heat, cold, contaminants, and drought stress. To cope with these stresses, recently, a new molecule has been discovered in plants named phytomelatonin that helps plant in to survive in a stressful environment (Fan et al. 2018). Melatonin (*N*-acetyl-5-methoxytryptamine) is a low molecular weight tryptophan-derived natural product, produced in all living organisms (Back et al. 2016, Choi et al. 2017). It is involved in seed germination, biomass production, increased photosynthesis, senescence delay, delayed flowering, fruit ripening, and biotic and abiotic stress responses (Lee et al. 2017, Shi et al. 2017, Wang et al. 2017). Melatonin enhances the tolerance of plants to abiotic and biotic stresses and also reactive oxygen species (ROS) scavenger and strong antioxidant (Li et al. 2012, Liu et al. 2016). Melatonin helps plants under oxidative stress through redox enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione reductase (GR) (Arnao and Hernández-Ruiz 2014, Arnao and Hernandez 2018a). Melatonin acts as a significant inducer of gene expression associated to plant hormones like auxin, abscisic acid, cytokinin (Arnao and Hernandez 2018b). These plant hormones also have a protective mechanism against heavy metal toxicity in plants, such as stomatal closure directed by abscisic acid (ABA), eventually, reduce

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the toxic metals uptake and its translocation from root to shoot (Bücker-Neto et al. 2017).

Plant growth-promoting rhizobacteria (PGPR) are soil bacteria inhabiting rhizospheres of the plant which improve the growth of a plant through different processes such as phosphorus solubilisation, nitrogen fixation, secretion of antibiotics, synthesis of the lytic enzymes, induction of systemic resistance, siderophores production (Zhou et al. 2016). Moreover, they enhance plant growth by the increasing rate of germination, root growth, chlorophyll content, protein content, nitrogen content, biomass, yield, tolerance to abiotic and biotic stresses, delaying leaf senescence (Dobbelaere et al. 2003). Additionally, they also possess different metal resistance processes like biosorption, exclusion, precipitation, active removal, or accumulation in external and intracellular spaces (Ullah et al. 2015). Cadmium (Cd) is a heavy metal that is considered as one of the key pollutants because of its toxicity (Byeon et al. 2015). Similarly, arsenic contamination is a global challenge due to its high toxicity and carcinogenicity (Shahid et al. 2018). The application of melatonin and PGPR in agriculture enhances plant growth under abiotic and biotic stress without disturbing the natural soil ecosystem. They both are non-toxic substances that increase and stimulate processes involved in plant growth and development (Asif et al. 2019).

The above literature showed that melatonin and PGPR could be a sustainable solution for plant growth. Therefore, we developed the following hypothesis in the form of questions for this study; (*i*) how the *Spinacia oleracea* L. respond under high As and Cd concentration as well as how the melatonin and PGPR could help and/or harm plant while reducing these stresses? Previous studies showed that melatonin and PGPR tolerate heavy metals, but no one has utilised the eco-friendly PGPR and non-toxic substance (melatonin) simultaneously under high As and Cd concentration. In this study, we determined the phenotypic characteristics, the response of antioxidant enzymes and lipid peroxidation of *S. oleracea* L. in response to melatonin and PGPR under high cadmium and arsenic concentrations.

MATERIAL AND METHODS

Experimental setup. Pots having 6 kg soil (pH 6.5; electrical conductivity 220 µs/cm; organic matter 1.13%; 18.5 g C/kg; 1.1 g N/kg; S 0.01%) was applied with 1.5 L of different concentration (25, 75 and 125 ppm) of cadmium ($CdCl_2$) and arsenic (($Na_2HAsO_4 \cdot 7\ H_2O$) separately. Disinfected spinach seeds (Green Top W.) were sown in properly labeled pots (control and treated) after one week of the above treatments. Treatments consisted of 5 replicates with 10 seeds/pot, and all pots were placed in complete randomised design under natural radiation with 11/13 h dark/light cycle under a temperature of 23 ± 4 °C and relative humidity of 60 ± 5%. In the first part of the experiment, melatonin treatment of 100 µmol was given to 14 days old seedling for 7 days. Melatonin (*N*-acetyl-5 methoxytryptamine) used in this study was purchased from Sigma-Aldrich (St. Louis, USA). *Bacillus licheniformis* identified in our lab previously was used in this study. In the second part, plants were then inoculated with 50 mL of the solution of *B. licheniformis* (4.6×10^6 cells/mL) pre-grown in tryptic soy broth for 72 h at 35 °C. The same quantity (0.5 L) of distilled water was used to irrigate the control (C) and all treated (T) plants every three days. After 4 weeks of treatments, plants were harvested for analysis, and some plants were pulverised in liquid nitrogen and stored at –80 °C for antioxidant analysis.

Measurement of morphological characteristics. Different morphological parameters like the number of plants per pot, number of leaves per plant, plant height, root length, leaf blade length were measured in control and all treatments. Fresh biomass (FW) was measured directly after harvesting. Dry biomass (DW) was measured by drying of samples in a hot air oven at 65 °C for 48 h. Total chlorophyll was extracted with 80% acetone. Absorbance measured at 663 nm and 645 nm was used to determine total chlorophyll content.

Antioxidant enzyme analysis and lipid peroxidation. Fresh plant leaves (0.2 g) were ground in 50 mmol phosphate buffer using pestle and mortar. The ground mixture was then centrifuged at 11 000 *g* for 20 min at 4 °C and collect the supernatant and used it for SOD, CAT, POD, and malondialdehyde content (MDA) analysis. SOD was measured by using the protocol of Kakkar et al. (1984). CAT was measured by a protocol of Brennan and Frenkel (1977). POD was determined by using pyrogallol by following the protocol of Reddy et al. (1995).

Lipid peroxidation was measured by MDA produced, which was extracted by using the method as described by Heath and Packer (1968).

Statistical analysis. Experiments were conducted in a complete randomised design. Data were expressed as means of three replicates. Statistical analysis was

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performed by one-way ANOVA, and Tukey's test ($P < 0.05$) was performed to evaluate the treatment effect. Graphs were prepared by using GraphPad Prism8 software (San Diego, USA).

RESULTS

Morphological characteristics. Melatonin application without any metal treatment (T) increased 14% seedling growth as compared to control. When melatonin was applied to cadmium treated plants, a greater increase of 13% at 25 ppm was observed, and a 17% increase at 75 ppm arsenic stressed plants. When the roots of *S. oleracea* were inoculated with *B. licheniformis*, there was no significant difference observed in the percentage of seedling growth under both Cd as well as As stress (Figure 1A). Melatonin treated non-stressed plants T has a 10% increase in the number of leaves as compared to control. Melatonin treated plants at 25 ppm As has a 19% increase in no. of leaves. *B. licheniformis* inoculated plants under non-stress conditions have a 19% increase in no. of leaves than control (Figure 1B). Melatonin application enhances plant height significantly in non-stressed plants with a 42% increase in the height of T as compared to control. However, there is a significant decrease in plant height with increasing Cd and As concentration. While plants inoculated with *B. licheniformis* under non-stressed conditions has a 39% increase in height than control. Under 75 ppm Cd, plants have a greater height than other treatments while with increasing As concentration, plant height decreases in *B. licheniformis* inoculated plants (Figure 2A). There exists a strong negative correlation between the concentration of As and Cd in the presence of melatonin or *B. licheniformis* treatment and plant height Mel + Cd (–0.97), As + Mel (–0.86), Cd + *B. licheniformis* (–0.71) and As + *B. licheniformis* (–0.99). Exogenous melatonin resulted in a 42% increase in root length under non-stress conditions T, and under Cd stress, the highest increase in root length was found at 25 ppm, which is 3.5% increase as compared to control. *B. licheniformis* inoculated plants T has a 59% increase in root length. *B. licheniformis* inoculated plants under Cd stress has decreased root length while there are 45, 38, and 28% increase in root length at 25, 75, and 125 ppm As stress, respectively (Figure 2B).

Melatonin treatment significantly increased 75% fresh weight of the plant in non-stressed plants T. Melatonin treatment under 75 ppm Cd has the highest 47% increase in FW as compared to control and under 25 ppm As there was 48% increase in fresh weight. The same was observed in plants inoculated with *B. licheniformis* under non-stress conditions T with a significant increase of 47% FW as compared to control. In *B. licheniformis* inoculated plants, a 28% increase in FW under 75 ppm Cd while under As treatment, plants with 25 ppm As has a 38% increase in FW (Figure 2C). Melatonin treated plants T has 33% increased dry weight in non-stress conditions than control while 34% increased under Cd concentration of 75 ppm and 33% increased under 25 ppm As. While *B. licheniformis* inoculated plants without stress T has a significant 59% increased dry weight in *B. licheniformis* inoculated plants, 75 ppm Cd has the highest DW while 25 ppm As has highest dry weight as compared to other treatments (Figure 2D). There was a negative correlation between the concentration of As and Cd in the presence of melatonin or

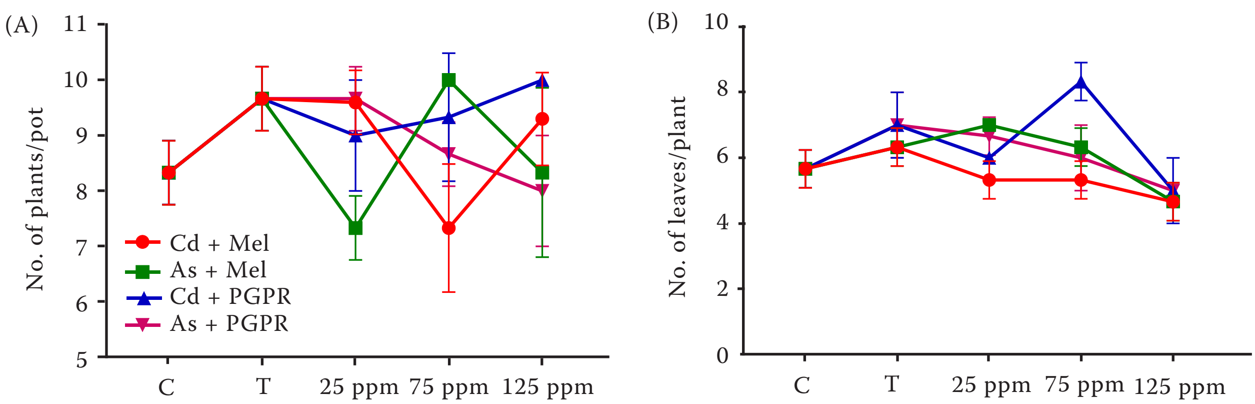

Figure 1. The effect of melatonin (Mel) and *Bacillus licheniformis* under cadmium (Cd) and arsenic (As) stress on (A) no. of plants/pot and (B) no. of leaves per plant. C – control with no As or Cd, no melatonin, no *B. licheniformis*; T – melatonin or *B. licheniformis* but no As or Cd; PGPR – plant growth-promoting rhizobacteria

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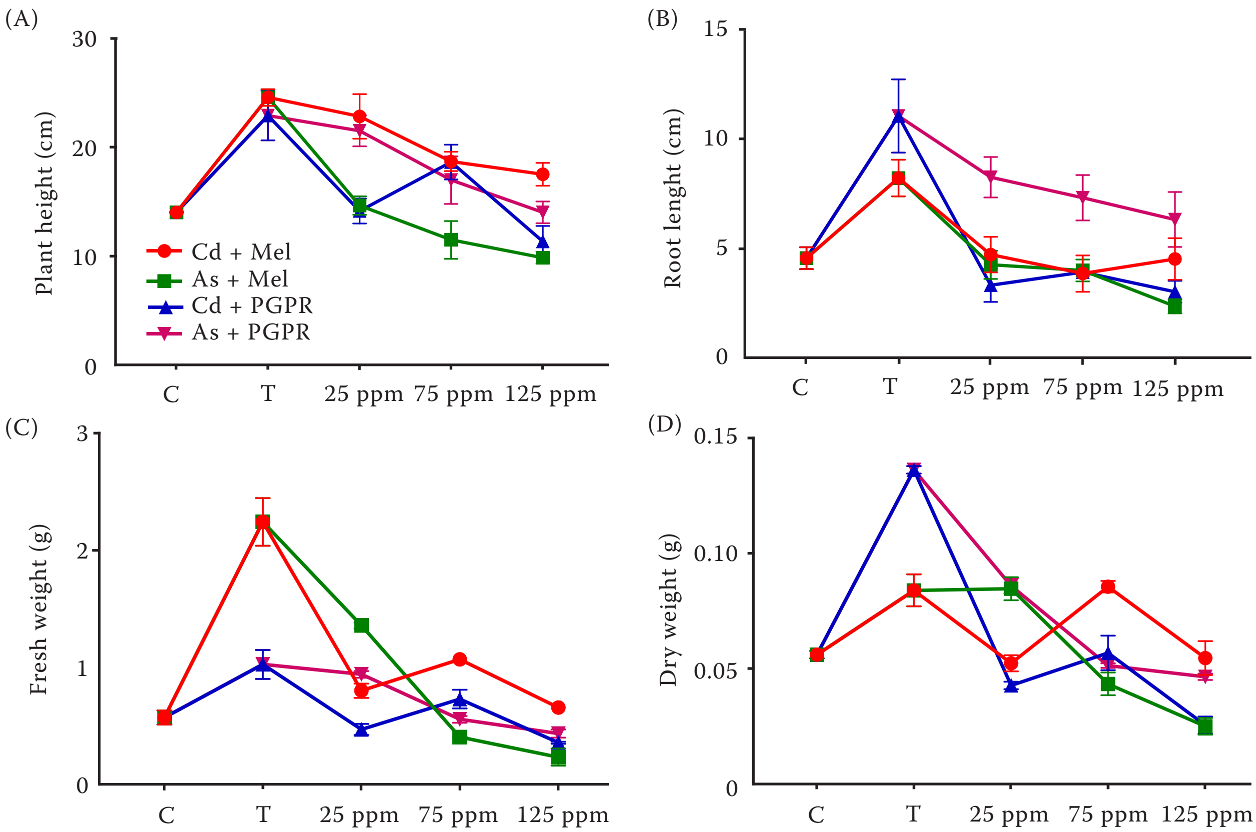

Figure 2. The effect of melatonin (Mel) and *Bacillus licheniformis* under cadmium (Cd) stress and arsenic (As) stress on (A) plant height of *Spinacia oleracea* L.; (B) root length; (C) fresh weight of *Spinacia oleracea* L. and (D) dry weight. C – control with no As or Cd, no melatonin, no *B. licheniformis*; T – melatonin or *B. licheniformis* but no As or Cd; PGPR – plant growth-promoting rhizobacteria

B. licheniformis treatment and fresh weight Mel + Cd (–0.71), As + Mel (–0.93), Cd + *B. licheniformis* (–0.69) and As + *B. licheniformis* (–0.97) and dry weight Mel + Cd (–0.33), As + Mel (–0.97), Cd + *B. licheniformis* (–0.75) and As + *B. licheniformis* (–0.89).

Total chlorophyll content. *B. licheniformis* inoculated plants under non-stress conditions have the highest chlorophyll content that is 33.6 mg/g than melatonin treated plants. In the presence of 25 ppm Cd, plants have chlorophyll content 12.2 mg/g. Similarly, under 25 ppm As has the highest chlorophyll content that is 17.3 mg/g. In *B. licheniformis* inoculated plants, under 25 ppm Cd has the highest chlorophyll content that is 15.6 mg/g while 25 ppm As has significantly high 29 mg/g chlorophyll content (Figure 3). There exists a strong negative correlation between the concentration of As and Cd in the presence of melatonin or *B. licheniformis* treatment and chlorophyll content in plants with Mel + Cd (–0.80), As + Mel (–0.90), Cd + *B. licheniformis* (–0.69) and As + *B. licheniformis* (–0.96).

Antioxidant enzymes measurements. Melatonin and PGPR enhance antioxidant activity under Cd and As stress. Amounts of SOD was analysed in control and all treatments. All three Cd treated plants with exogenous melatonin have a significantly high SOD amount as compared to control with the highest value recorded at 25 ppm Cd that is 116.9 units/g FW. Similarly, plants under As stress has the highest SOD content that was treated with melatonin, with the highest value at 75 ppm As that is 143.6 units/g FW. *B. licheniformis* inoculated plants under Cd stress has produced high SOD content at 125 ppm that is 87.7 units/g FW as compared to control having a value of 23.57 units/g FW. Similarly, *B. licheniformis* inoculated plants under As stress has the highest SOD content at 25 ppm and 75 ppm that is 37.7 units/g FW (Figure 4A). When melatonin is applied to plants under Cd stress, 75 ppm Cd has a significantly high CAT amount of 44.1 units/g FW as compared to control, which has 8.6 units/g FW. Similarly, in

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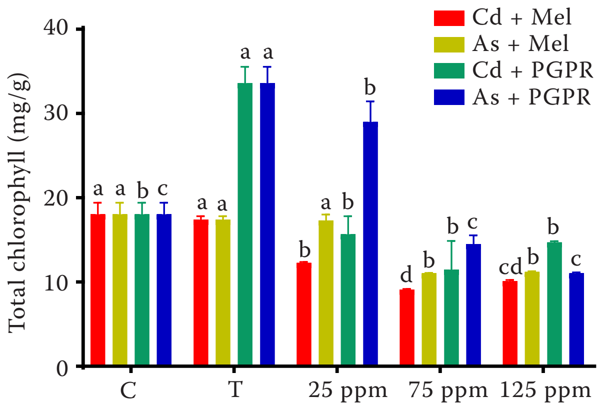

Figure 3. The effect of melatonin (Mel) and *Bacillus licheniformis* under cadmium (Cd) and arsenic (As) stress on total chlorophyll of *Spinacia oleracea* L. C – control with no As or Cd, no melatonin, no *B. licheniformis*; T – melatonin or *B. licheniformis* but no As or Cd. Vertical bars represent ± standard error; PGPR – plant growth-promoting rhizobacteria

As treated plants, CAT activity increases with As stress with the highest value of CAT at 125 ppm As, i.e., 256.6 units/g FW. In *B. licheniformis* inoculated plants under Cd stress, we observed high CAT values for 125 ppm Cd that is 101.8 units/g FW. However, *B. licheniformis* inoculated plants under As stress, the highest amount of CAT was produced at 125 ppm As which is 143.2 units/g FW (Figure 4B). Melatonin treated plants under Cd stress has the highest POD at 125 ppm Cd that is 698.5 units/g FW. Under As stress, 125 ppm As treatment has significantly high POD, that is 494.6 units/g FW. The same trend was observed for *B. licheniformis* inoculated plants under Cd stress with significantly high value at 125 ppm Cd that is 577.3 units/g FW while 125 ppm As has 490.3 units/g FW (Figure 4C). MDA is an indicator of lipid peroxidation. In Cd treated plants, high MDA content was observed at 75 ppm Cd, i.e., 36.12 µmol/g. But under As stress in the presence of melato-

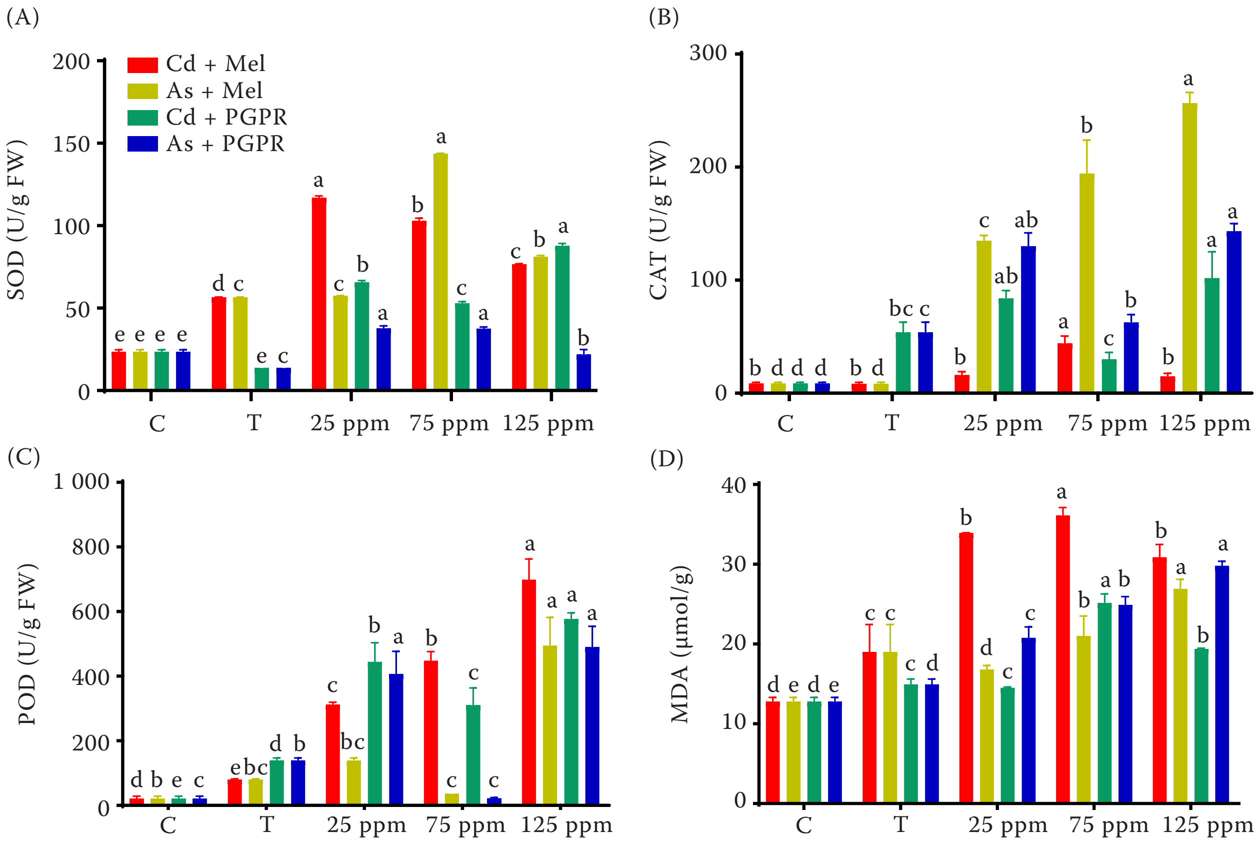

Figure 4. The effect of melatonin (Mel) and *Bacillus licheniformis* under cadmium (Cd) stress and arsenic (As) stress on (A) superoxide dismutase (SOD) activity in *Spinacia oleracea* L.; (B) catalase (CAT) activity; (C) peroxidase (POD) activity in *Spinacia oleracea* L. and (D) malondialdehyde content (MDA) level. C – control with no As or Cd, no melatonin, no *B. licheniformis*; T – melatonin or *B. licheniformis* but no As or Cd. Vertical bars represent ± standard error; PGPR – plant growth-promoting rhizobacteria; FW – fresh weight

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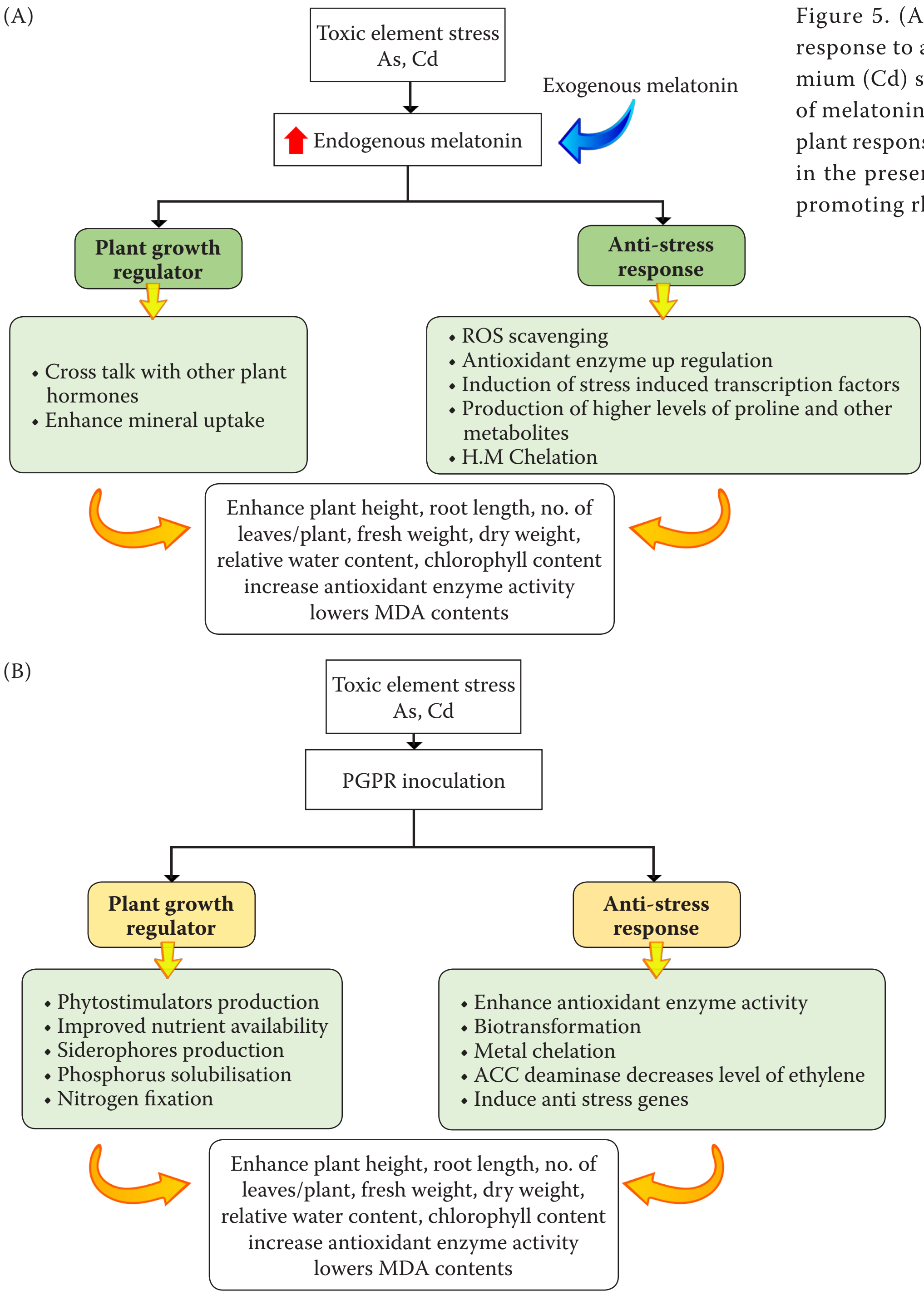

Figure 5. (A) Overview of plant response to arsenic (As) and cadmium (Cd) stress in the presence of melatonin, and (B) overview of plant response to As and Cd stress in the presence of plant growth-promoting rhizobacteria (PGPR)

nin have the highest MDA content at 125 ppm As that is 26.9 µmol/g. Under Cd stress, when *B. licheniformis* inoculated into roots of *S. oleracea* significantly high MDA content was observed at 75 ppm Cd, i.e., 25.1 µmol/g. However, in the presence of As stress, *B. licheniformis* inoculation has the highest MDA content at 125 ppm As which is 29.8 µmol/g (Figure 4C).

DISCUSSION

The research on the impact of exogenous melatonin on various crops and vegetables has been widely performed, but the current study opens a new area of research by studying the role of melatonin and PGPR both on As and Cd accumulation in *S. oleracea*. It was observed that melatonin and *B. licheniformis* both were

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involved in enhancement of some growth parameters significantly as compared to control. They also help the plant to cope with Cd and As stresses by increasing activity of antioxidants SOD, CAT, and POD.

Melatonin is synthesised from tryptophan like indole-3-acetic acid (IAA), so that is why it also plays an important role in plant growth and development. It may also crosstalk with other phytohormones leading to enhanced plant growth. It also enhances the mineral element uptake of plants (Liu et al. 2016). In this experiment, exogenous melatonin enhanced the germination percentage, no. of leaves/plant, plant height, root length of *S. oleracea* compared to control. Similarly, the strong negative correlations were identified between different As and Cd concentrations and different growth parameters like plant height, root length, fresh weight, weight, and chlorophyll content. This showed that both melatonin and *B. licheniformis* enhance growth parameters more efficiently at low metal concentrations. Previous studies also showed that melatonin is involved in promoting a percentage of seed germination, seedling growth, morphology, and nutrient element content in cucumber and wheat plants under different stresses (Zhang et al. 2013, Qiao et al. 2019). It was observed in our study that there is more increase in fresh weight in melatonin treated plants as compared to *B. licheniformis* but *vice versa* for dry weight under non-stress conditions. This is because melatonin preserves high water content in plants. The above literature and our study confirmed that melatonin has not a specific role in the growth of a specific plant, but it increases the growth of almost all plants (Figure 5A). In our study, chlorophyll content was enhanced under different Cd and As treatments as compared to control. This is because melatonin preserves chlorophyll by downregulation of the expression of chlorophyllase (CLH1), pheide, and oxygenase (PAO) enzymes that play a role in the degradation of chlorophyll (Zhang et al. 2015).

Numerous soil-inhabiting bacteria, including PGPR (Figure 5B), are able to tolerate heavy metal stresses and play significant roles in mobilisation and immobilisation of toxic heavy metals. In the current study, it was concluded that *B. licheniformis* enhance the plant height, root length, fresh weight, dry weight under As and Cd stress. Hahm et al. (2017) also found a significant increase in plant height, fresh weight, dry weight, and chlorophyll contents in abiotic stressed pepper plants inoculated with *Brevibacterium iodinum*, *Rickettsia massiliae*, and *Microbacterium oleivorans.* PGPR stimulates plant growth and development by different processes, such as by increasing nutrient uptake, siderophores production, nitrogen fixation, phosphorus solubilisation, producing phytohormones, and producing secondary metabolites that work as insecticides (Asif et al. 2019). Results of current studies show that there is a significant increase in root length, plant height, fresh weight, dry weight, and chlorophyll content in plants applied with melatonin or *B. licheniformis* without any metal treatment while metals treated plants with melatonin application or *B. licheniformis* application also enhanced physiological parameters and chlorophyll content significantly as compared to control.

As and Cd stresses induce plants to synthesise a large amount of ROS that inhibit plant growth (Cui et al. 2012, Khan et al. 2015). The plant produces ROS under stressful conditions, and excessive ROS generation leads to oxidative stress. Oxidative stress is a main damaging factor when plants are exposed to a wide range of environmental stresses. Melatonin and its metabolites act as free radical scavengers and directly scavenge ROS and enhance mitochondrial oxidative phosphorylation efficiency. It also enhances the activities of antioxidant enzymes like SOD, POD, CAT, and GR. Melatonin also upregulates the anti-stress related genes (Zhang et al. 2013, Asif et al. 2019). PGPRs produce various plant hormones that enhance plant growth, cell division, and help plants in tolerating different abiotic and biotic stresses. When plants come across environmental stress, ethylene production enhanced, which eventually reduces plant growth. PGPR having 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase reduces the level of ethylene, consequently increasing plant growth under stressful conditions (Asif et al. 2019). In the present study, exogenous melatonin application and PGPR inoculation to spinach plants resulted in increased antioxidant activities and a decrease in MDA content under As and Cd stress. The results of this study and previous studies confirmed the role of melatonin in plant defense. It was concluded that both melatonin and *B. licheniformis* helps the plant to cope with As and Cd stress by increasing the activity of antioxidant.

In conclusion, this is the first study on the application of melatonin and *B. licheniformis* inoculation into roots of *S. oleracea* L., and it showed that plant growth increased after the application of melatonin and *B. licheniformis* under non-stress as well as high-stress conditions without showing any visible toxic effects on plants. Both enhance the chlorophyll contents and

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increase activities of antioxidants SOD, POD and CAT under As and Cd stresses. However, MDA contents are decreased under As and Cd stresses. The results of this study showed that the *S. oleracea* could be grown on As and Cd contaminated soils, but melatonin and PGPR application will be necessary that will improve the growth of the plant and alleviate both stresses.

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