Enhancement of Nickel Tolerance and Growth in Redgram (*Cajanus cajan*) by *Metapseudomonas otitidis* ASW-04: Physiological and Anatomical Insights.

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Abstract
Nickel (Ni) pollution in agricultural soils, stemming from industrial activities and over-fertilization, presents a considerable risk to crop development and food safety by obstructing physiological and developmental functions in *Cajanus cajan* (redgram). The present investigation sought to assess the capability of the Ni-tolerant PGPR *Metapseudomonas otitidis* ASW-04 in alleviating the detrimental impacts of Ni toxicity on the redgram variety IBT DRG6. Four experimental cohorts were formed: control, Ni-treated (100 ppm), ASW-04 treated, and a combination of Ni + ASW-04 treated. Findings indicated that nickel exposure significantly diminished germination parameters, with the germination index and percentage falling to 27.8±3% and 37%, respectively, while also inhibiting root and shoot development, seed vitality, and biomass production. Conversely, *M. otitidis* ASW-04 inoculation whether administered independently or in conjunction with Ni stress substantially enhanced all evaluated metrics, achieving a germination rate of up to 94%, with notable increases in root and shoot development, as well as superior fresh (2.81±0.5 g) and dry weights (0.072±0.01 g) in the treated seedlings. Anatomical investigations demonstrated that *M. otitidis* ASW-04 treated plants had revitalized vascular tissue diameters in both roots and stems, alongside enhanced stomatal architecture, despite the presence of Ni. The results demonstrated that *M. otitidis* ASW-04 significantly mitigates Ni-induced phytotoxicity and promotes robust growth in redgram via mechanisms that include antioxidative defense, improved nutrient absorption, and Ni sequestration.

Keywords: Redgram, Ni-toxicity, PGPR, M. otitidis ASW-04, Gemination, Stomata

Introduction

Cajanus cajan (Redgram), referred to as pigeon pea, is a multifaceted leguminous plant that holds significant nutritional and economic importance globally and in India. Redgram, being among the most ancient cultivated legume varieties, has served as a fundamental protein source in vegetarian diets, especially in tropical and subtropical areas (Saxena, 2008; Varshney et al., 2010). Its versatility in various agro-climatic environments and suboptimal soils highlights its importance in maintaining food and nutritional security (FAO, 2016). In India, redgram serves as a fundamental dietary element, constituting the foundation of the prevalent cuisine “dal” and is ingested in multiple forms including whole, split, or as flour (Singh et al., 2015). The crop's capacity to deliver vital nutrients at a reasonable price establishes it as a key dietary option for economically disadvantaged communities globally, encompassing Africa, Latin America, and Southeast Asia (FAO, 2016). Worldwide, the

principal cultivators of pigeon pea include India, Myanmar, Malawi, Kenya, and Tanzania, with India representing more than 70% of the total global output (FAOSTAT, 2022). The agricultural area spans approximately 5 million hectares in India, primarily in regions such as Maharashtra, Karnataka, Andhra Pradesh, Madhya Pradesh, and Uttar Pradesh (Das et al., 2022). From a global trade standpoint, redgram serves as an essential export product for India and other significant producers, catering to the nutritional requirements of the South Asian diaspora and various consumers worldwide (FAO, 2016).

In the past few decades, the intensification of industrial operations, inadequate waste management, and overuse of nickel-rich fertilizers have led to heightened nickel deposition in agricultural soils (Seregin and Kozhevnikova, 2006). Nickel (Ni) is acknowledged as a vital micronutrient for flora, engaging in crucial metabolic functions such urease activation and hydrogen metabolism (Marschner, 2012). Yet, its exposure to high Ni levels can impose several physiological, biochemical, and agricultural limitations, ultimately diminishing both growth and yield (Sharma and Agrawal, 2005). At the cellular scale, Ni toxicity can provoke oxidative stress through the reactive oxygen species (ROS), compromising membrane integrity and resulting in cellular injury (Flora et al., 2012). The productivity of redgram declines in elevated Ni environments because of hindered vegetative and reproductive growth. These decreases in yield are frequently ascribed to compromised photosynthetic systems, suppression of critical metabolic enzymes, and limited transport of key nutrients like nitrogen, phosphate, and potassium (Seregin and Kozhevnikova, 2006). In addition to physiological impacts, Ni toxicity in redgram also generates food safety issues, since the excessive buildup of Ni in seeds may provide health hazards to consumers (Kabata-Pendias, 2011). In response to these issues, scientists have investigated various approaches to improve plant Ni tolerance, with the goal of ensuring agricultural yield and protecting food safety in polluted settings.

Genetic methodologies, including selective breeding and molecular genetic modification, have effectively produced cultivars exhibiting natural or artificially induced tolerance to Ni stress (Zhang et al., 2020). In addition to plant-focused approaches, agronomic management techniques include the use of soil amendments such as biochar, zeolites, and organic matter, together with the optimization of nutrient regimes, can alleviate Ni toxicity by modifying metal availability or bolstering plant resilience (Nanda and Abraham, 2022). However, these physical and chemical methods may be financially burdensome or exhibit inconsistent effectiveness in the field, underscoring the necessity for more sustainable and economical alternatives. Consequently, biological therapies have attracted notable interest due to their environmentally sustainable prospects. The application of plant growth-promoting rhizobacteria (PGPR) has surfaced as a viable approach to enhance nickel tolerance in plants (Mishra et al., 2023).

PGPR are advantageous soil microorganisms that inhabit plant roots and promote growth by several methods, such as nutrient solubilization, phytohormone synthesis, and the stimulation of systemic resistance (Goswami et al., 2016). A multitude of research endeavors has evidenced the efficacy of PGPR in augmenting nickel tolerance across diverse plant species. Mishra et al., (2023) found that inoculating wheat with *Pseudomonas aeruginosa* strain S11 increased plant development and decreased Ni uptake by regulating antioxidative enzymes, reducing Ni-induced oxidative stress. Sharma et al., (2023) found that *Bacillus subtilis* and *Rhizobium* spp. produced siderophores and exopolysaccharides that chelate or immobilize Ni in the rhizosphere, increasing chickpea biomass and reducing Ni accumulation. Goswami et al., (2016) found that *Pseudomonas putida* increased mustard plant tolerance to Ni toxicity by increasing phytohormone synthesis and regulation of stress-responsive gene expression. In addition to enhancing Ni-stress resilience, certain PGPR strains have been observed to facilitate critical nutrient absorption and root growth in

conditions of Ni toxicity (Ali et al., 2022). These findings show that various PGPR species induce plant Ni tolerance through robust rhizosphere interactions, increased antioxidant defense, and Ni sequestration. Thus, the present study examined the ability of potent Ni-tolerant bacterial isolate *Metapseudomonas otitidis* ASW-04 to increase redgram's resistance to Ni stress, providing insights into environmentally viable ways to improve crop durability, soil vitality, and yield in polluted environments.

Materials and Methods

Sample collection

Healthy and young redgram cultivar (IBT DRG6) and their seeds were collected from the from the ANGRAU, Guntur, Andhra Pradesh, India. Approximately, 500 gm of seed samples were collected and the collected redgram cultivars were aseptically transferred to the zip bags and transported to the laboratory.

Effect of Ni-tolerant bacterial isolate *M. otitidis* ASW-04 on the redgram germination

Experimental design

The selected redgram cultivar seeds were further examined to find out the possible effects with the *M. otitidis* ASW-04 on the seed germination. The experiment was conducted as four groups. In the first group the redgram cultivars are grown in normal conditions and irrigated with normal water, they are referred as control. In the second group, the seeds of IBT DRG6 cultivar were grown under 100 ppm nickel concentrations. In the third group, the seeds of IBT DRG6 cultivar were grown with only the application of *M. otitidis* ASW-04 with 10^8 cells/mL as bioinoculant. And in the fourth group, the seeds of IBT DRG6 cultivar were grown under 100 ppm of nickel solution and the *M. otitidis* ASW-04 with 10^8 cells/mL as bioinoculant.

Seed germination

Seeds of IBT DRG6 cultivars were washed with tap water and then distilled water. Washed seeds were surface sterilized with 70% ethanol and 2% sodium hypochlorite. Then seeds were kept for germination on moistened filter paper in sterile Petridish. The control seeds were treated with only water. For the second group seeds Ni stress is given by irrigation of 100 ppm $NiSO_4$ solution. The third group seeds were irrigated with normal water and 5 mL of *M. otitidis* ASW-04 suspension with 10^8 cells/mL. As well as, the fourth group seeds were irrigated with 100 ppm $NiSO_4$ solution and 5 mL of *M. otitidis* ASW-04 suspension with 10^8 cells/mL. After initiation of germination seedlings were grown for one week and seed germination potential of *M. otitidis* ASW-04 was established by assessing the germination index, percentage of seed germination, root length, shoot length, seed vigour index, fresh and dry weights of redgram cultivars from four experimental groups. After germination, seedlings were transferred to plastic pots filled with autoclaved vermiculture. The pots were grown at 25ºC under 16 h photoperiod in a green house. The seedlings in all the experimental groups were treated every alternate day with the respective solutions and bacterial inoculum for 3 weeks.

Assessment of seed germination

Fifty soaked seeds of redgram seeds were kept separately for germination on the moist filter paper in a Petridish for each treatment group. Germination was recorded by counted the number of seedlings emerging daily from the day of planting the seeds in the Petridish till the time germination is complete. Thereafter a germination index (G.I.) is measured according to the AOSA, (1983) method.

Growth Performance and Seed vigour index

The growth performance was demonstrated by measuring the root and shoot lengths. Seedlings were uprooted carefully to measure the mean root length and mean shoot height (cm) of plants. The measurement of growth was made on a weekly basis after planting. Shoot height was taken as the distance from the soil surface to the point where the last petioles or leaf left the stem. Seed vigour index is calculated by multiplying germination (%) and seedling length. The seeds showing the higher seed vigour index is considered to be more vigorous (Abdul-Baki and Anderson, 1973).

Determination of fresh and dry weights

For the determination of fresh and dry weights randomly five seedlings were selected from each test group which contain plants showing least growth and height growth. To determine fresh weight, seedlings were carefully removed, rinse with distilled water, blot dry with paper towels, and rapidly weigh each seedling using an analytical balance. Dry weight was determined from each test group by drying entire plant material along with roots, shoots, and leaves in the hot air oven at 60ºC. Then the dried plant was powdered and weight was measured.

Screening of Anatomical changes

The anatomical changes in the root, shoot, and leaf from all the experimental groups was performed by observing under microscope. For anatomical analyses, root, shoot, and the first fully expanded leaves from all the experimental groups collected. The material was fixed in FAA (formaldehyde + acetic acid + ethyl alcohol 70 % 5:5:90 L L^{-1} L^{-1}) (Johansen, 1940) for 72 hours and then kept in ethyl alcohol 70 %. Then the upper and lower epidermis of leaf was peeled and safranine staining mixture was applied (Kraus and Arduim, 1997). For root and stem, cross sections were taken and the sections were stained with a safranine solution at 1%. Glycerin 50 % was used to mount the slides. All slides were examined and photographed using a Lynx light microscope and a Capture 2.4 HDMI camera.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) of 5 subjects in each group. The significance of variation in means across groups was determined using one-way ANOVA. The p-value of <0.05 was considered to be statistically significant. SPSS Windows version 10.0 was used for the descriptive statistical analysis.

Results and Discussion

Germination Index (GI)

Figure 1 present the germination index data for the four treatment groups. These results demonstrated that the application of *M. otitidis* ASW-04 significantly increased the germination index. Conversely, the seedlings grown under 100 ppm Ni stress had a lower germination index. The four treatment groups of IBT DRG6 such as control, 100 ppm Ni-treated, ASW-04 (10^8 cells/mL) treated, and 100 ppm Ni+ ASW-04 (10^8 cells/mL) treated exhibited the germination indices of 72.6±2, 27.8±3, 87.5±3, 66.8±2% respectively. The current study found that the use of *M. otitidis* ASW-04 on the seeds led to a considerable rise in germination index in the seeds grown under Ni-stress.

These outcomes align with the research conducted by Hafeez et al., (2020) who revealed that PGPR inoculation enhanced germination index, germination rate, and seedling vigor in mung bean and wheat varieties subjected to cadmium and lead exposure. Comparable patterns were recorded by Saikia et al., (2022), indicating that PGPR treatment enhanced germination efficacy and stress resilience in rice subjected to environmental heavy

metals. The noted reduction in germination index under 100 ppm Ni treatment (27.8±3) in this study aligns with previous research by Chaturvedi et al., (2021) and Zhu et al., (2019), which indicated that heavy metals may inhibit enzyme function, obstruct water absorption, and provoke oxidative stress during seed germination. In contrast, seeds subjected to ASW-04 (10^8 cells/mL) demonstrated the most elevated GI (87.5±3), but the 100-ppm Ni + ASW-04 group revealed a significantly enhanced GI (66.8±2%) compared to Ni treatment alone, nearing the control measurement (72.6±2). These findings align with earlier research of Mishra et al., (2017) who demonstrated that the plant growth-promoting rhizobacteria (PGPR) improved germination rates under metal stress. The current investigation shown that the utilization of bacterial isolate ASW-04 significantly enhanced the GI of IBT DRG6 redgram seeds, despite exposure to Ni stress.

Germination percentage

The results of germination percentages in the four treatment groups of redgram cultivar were shown in Figure 2. From these results, the greatest seed germination was found in *M. otitidis* ASW-04 treated experimental group, whereas the least germination percentage was observed in the 100-ppm Ni treated group. In the present study, the four treatment groups of IBT DRG6 such as control, 100 ppm Ni-treated, ASW-04 (10^8 cells/mL) treated, and 100 ppm Ni+ ASW-04 (10^8 cells/mL) treated exhibited the germination percentages of 86, 37, 94, 72% respectively. These results demonstrated that the application of *M. otitidis* ASW-04 significantly enhances the percentage of germination in IBT DRG6 cultivar.

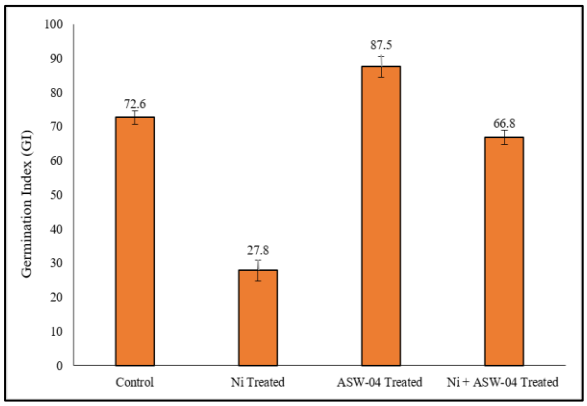

Figure: 1. Germination indices of redgram cultivar (IBT DRG6) under different treatment conditions.

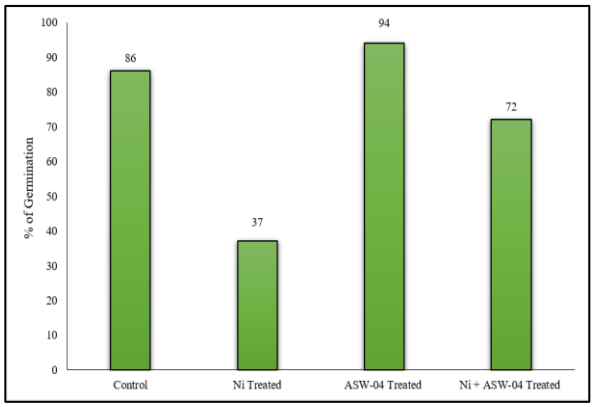

Figure: 2. Germination percentages of redgram cultivar (IBT DRG6) under different treatment conditions.

Table: 1. Germination and growth parameters of IBT DRG6 seedlings under different treatment conditions.

S. No	Parameter	Experimental groups			
		Control	Ni Treated	ASW-04 Treated	Ni + ASW-04 Treated
1	Germination Index (GI)	72.6±2	27.8±3	87.5±3	66.8±2
2	Germination percentage (%)	86	37	94	72
3	Root growth	8.2±1.2	6.3±0.8	9.4±1.1	7.9±0.9
4	Shoot growth	10.3±1.4	5.7±0.6	12.8±1.6	9.3±0.8
5	Seed vigour index	1591	456	2087	1238
6	Fresh weight (gm)	2.35±0.6	1.54±0.8	2.71±0.5	2.11±0.6
7	Dry weight (gm)	0.051±0.01	0.027±0.01	0.072±0.01	0.045±0.01

The findings of seed germination in the present study align with previous research by Khodadadi et al., (2012), who indicated that heavy metals such as Ni adversely affect seed germination by interfering with metabolic processes and cellular structure. Moreover, the utilization of ASW-04 not only alleviated the detrimental impacts of Ni but also elevated germination beyond control benchmarks, corroborating the results of Mishra et al., (2017), who demonstrated that PGPR treatment augmented seed germination and seedling vitality in legumes subjected to heavy metal stress. The observed improvement in germination may be related to the phytohormone synthesis, siderophore secretion, and stress-alleviating mechanisms imparted by the ASW-04 isolate, as previously reported by Glick, (2012) and Ma et al., (2011). Verma et al., (2018) also noted that the application of PGPR strains enhanced the germination rate and growth metrics in chickpeas subjected to cadmium stress. These cumulative results highlight the importance of employing PGPR *M. otitidis* ASW-04, which can significantly improve seed germination of redgram under nickel stress.

Growth Performance

Root growth

In the present study seedlings of redgram cultivar showed significant deviation in root growth from all the experimental groups. The results of root growth for the four treatment groups were shown in Figure 3. From these results, it was observed that the seedlings treated with *M. otitidis* ASW-04 exhibited higher root growth. While, the 100-ppm Ni-treated seedlings shows the lowest root growth. In the present study, the four treatment groups of IBT DRG6 such as control, 100 ppm Ni-treated, ASW-04 (10^8 cells/mL) treated, and 100 ppm Ni+ ASW-04 (10^8 cells/mL) treated exhibited the root growth of 8.2±1.2, 6.3±0.8, 9.4±1.1, and 7.9±0.9 cm respectively.

Shoot growth

The results of shoot growth from the four treatment groups were shown in Figure 4. These results demonstrated that the shoot growth from all the four treatment groups exhibited significant variation. Furthermore, it was observed that the seedlings treated with *M. otitidis* ASW-04 exhibited greater shoot length. While, the 100-ppm Ni-treated seedlings shows the lowest shoot length. In the present study, the four treatment groups of IBT DRG6 such as control, 100 ppm Ni-treated, ASW-04 (10^8 cells/mL) treated, and 100 ppm Ni+ ASW-04 (10^8 cells/mL) treated exhibited the shoot lengths of 10.3±1.4, 5.7±0.6, 12.8±1.6, and 9.3±0.8 cm respectively.

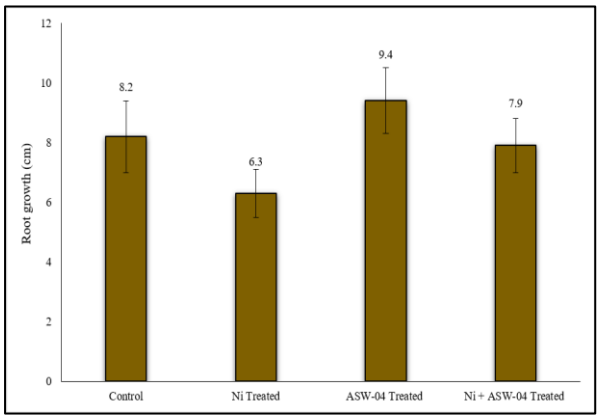

Figure: 3. Root growth of redgram cultivar (IBT DRG6) under different treatment conditions.

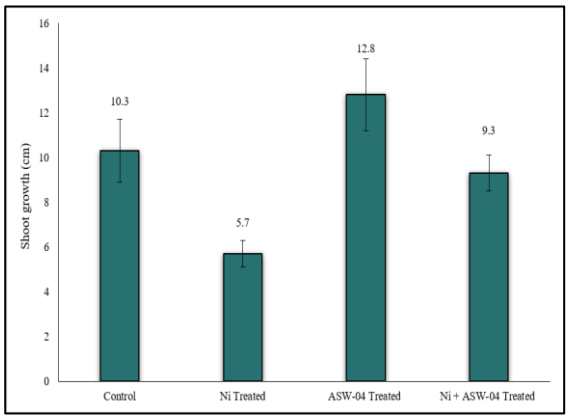

Figure: 4. Shoot growth of redgram cultivar (IBT DRG6) under different treatment conditions.

The current findings related to the minimal growth metrics in Ni-only treated seedlings correspond with earlier studies, including those by Sarma et al., (2020) and Rizvi and Khan, (2021) who indicated that heavy metals impede root and shoot elongation by causing oxidative harm and interfering with nutrient transport. Similarly, Chaturvedi et al., (2021) indicated that heavy metals like Ni impede root elongation and shoot growth by causing an oxidative damage and disrupting cellular division. Hafeez et al., (2020) and Ma et al., (2011) discovered that metal toxicity inhibited the growth of both roots and shoots in mung bean, wheat, and many legumes.

The present research revealed that the application of the *M. otitidis* ASW-04 significantly enhanced both root and shoot development in the IBT DRG6 redgram variety under Ni stress. These findings correspond with earlier studies by Hafeez et al., (2020) and Saikia et al., (2022) who indicated that PGPR-treated plants under cadmium and lead stress exhibited greater root length, shoot height, and biomass. Similarly, Verma et al., (2018) discovered that PGPR inoculation in chickpeas subjected to cadmium stress also enhanced the elongation of primary roots and shoots. Egamberdieva et al., (2019) indicated that the use of rhizobacterial inoculants markedly improved the growth of primary roots and shoots in *Vigna radiata* subjected to multi-metal stress. The research by Dharanivasan et al., (2021) revealed that PGPR extracted from soils susceptible to stress enhanced root structure and shoot growth in redgram under abiotic stress, paralleling our results with *M. otitidis* ASW-04. In a similar vein, Jain et al., (2020) recorded advantages of *Pseudomonas* and *Bacillus* strains for the enhancement of shoot and root development in pigeon pea subjected to lead and arsenic.

Seed vigor index

The results of seed vigor index in the four treatment groups of IBT DRG6 cultivar were shown in Figure 5. From these results, the greatest seed vigor index was found in the seedlings treated with *M. otitidis* ASW-04, whereas least seed vigor index was observed in the 100-ppm nickel treated group. The four treatment groups of IBT DRG6 such as control, 100 ppm Ni-treated, ASW-04 (10^8 cells/mL) treated, and 100 ppm Ni+ ASW-04 (10^8 cells/mL) treated exhibited the seed vigor indices of 1591, 456, 2087, and 1238 respectively. These results demonstrated that the application of *M. otitidis* ASW-04 significantly enhances the seed vigor index in IBT DRG6 cultivar.

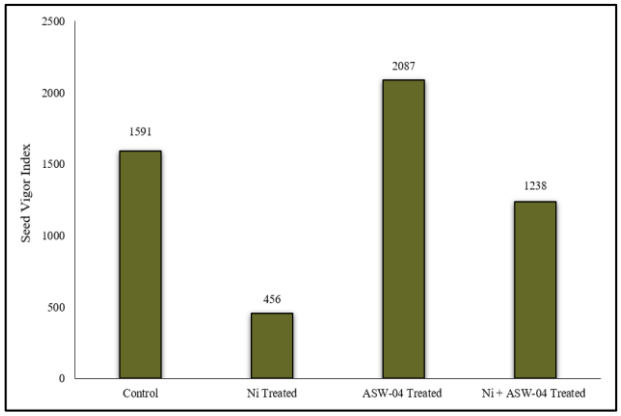

Figure: 5. Seed vigor index of redgram cultivar (IBT DRG6) under different treatment conditions.

The reduction in SVI under Ni stress noted in the present study corresponds with the observations of Rizvi and Khan, (2021) who reported significant decreases in SVI in Phaseolus species exposed to metal toxicity, highlighting the direct correlation between

environmental stresses and reduced seedling vitality. The findings of the current investigation reveal that application of *M. otitidis* ASW-04 significantly improves the Seed Vigor Index (SVI) particularly under nickel stress. These findings demonstrate a correlation with the data of Habib et al., (2023) who noted enhancements in SVI and seedling vigor after PGPR application in mungbean, wheat, and chickpea subjected to heavy metal stress. In a similar vein, Jain et al., (2020) documented the enhanced SVI in pigeon pea subsequent to the application of PGPR in soils tainted with various metals. Rahnama et al., (2023) found that seeds treated with PGPB of *B. cereus* exhibited the greatest vigor and quality indices, measuring 6.55 and 8.45 times superior to the control treatment, respectively.

The findings of Gholami et al., (2009) and Govindappa et al., (2011) indicated an enhancement in the seed vigor index of *Carthamus tinctorius* and *Zea mays* when treated with *Azospirillum* and *Pseudomonas*. Pandey et al., (2018) noted a substantial enhancement in vigor indices (117%, 151.94%) of *Amaranths* due to *Bacillus* strain (BS-58) and documented a rise in seedling vigor indexes by 30% to 60% in barley seeds subjected to PGPR treatments. Pacome et al., (2013) indicated that the co-inoculation of *P. fluorescens* and *P. putida* with maize seeds markedly enhanced the vigor index to 77.54% compared to the control. The rise in these indices may result from enhanced metabolism and the synthesis of auxin and cytokinin, chemicals that promote cell elongation and division by PGPB inoculation (Bharathi et al., 2004). Abdelaziz et al., (2021) discovered that the inoculation of PGPR induces the overexpression of genes associated with stress responses and root architecture formation in maize subjected to Ni stress, paralleling our observations in redgram. These findings collectively underscore the efficacy of PGPR, such *M. otitidis* ASW-04, as sustainable bioinoculants to mitigate heavy metal toxicity and enhance seedling vigor, evidenced by an increased SVI in legumes cultivated under challenging conditions.

Fresh weight

The results of fresh weight from the four treatment groups of IBT DRG6 cultivar were shown in Figure 6. From these results, the greatest fresh weight was found in the seedlings treated with *M. otitidis* ASW-04, whereas least fresh weight was observed in the 100-ppm nickel treated group. The four treatment groups of IBT DRG6 such as control, 100 ppm Ni-treated, ASW-04 (10^8 cells/mL) treated, and 100 ppm Ni+ ASW-04 (10^8 cells/mL) treated exhibited the fresh weights of 2.35±0.6, 1.54±0.8, 2.81±0.5, and 2.11±0.6 gm respectively. These results demonstrated that the application of *M. otitidis* ASW-04 significantly enhances the seedling growth and increases the fresh weight in IBT DRG6 cultivar.

Dry weight

Figure 7 presents the results of dry weight from the four treatment groups of IBT DRG6 cultivar. From these results, the higher dry weight was found in the seedlings treated with *M. otitidis* ASW-04, whereas lower dry weight was observed in the 100-ppm nickel treated group. The four treatment groups of IBT DRG6 such as control, 100 ppm Ni-treated, ASW-04 (10^8 cells/mL) treated, and 100 ppm Ni+ ASW-04 (10^8 cells/mL) treated showed the dry weights of 0.051±0.01, 0.027±0.01, 0.072±0.01, and 0.045±0.01 gm respectively. These results demonstrated that the application of *M. otitidis* ASW-04 significantly enhances the seedling growth and increases the dry weight in IBT DRG6 cultivar.

In the current investigation, seedlings subjected to *M. otitidis* ASW-04 had the greatest fresh and dry weights, whereas seedlings exposed to 100-ppm nickel showed the least biomass accumulation. These findings correspond with the research conducted by Santos et al., (2023) who indicated that growth-promoting rhizobacteria markedly enhanced plant vitality and biomass yield in both standard and stress-affected environments. Consistent with the current results, Bellishree et al., (2014) observed an enhancement in the dry weight

of the shoot (1.13 g) and root (0.08 g) following the treatment of *B. subtilis* Strain BCA-6 in tomatoes. Mandyal et al., (2012) also indicated that bell pepper treated with a *Bacillus* isolate enhanced both plant and root biomass. Garcia-Murillo et al., (2023) asserted that enhancing plant resilience by gene modification or advantageous microorganisms results in significant advancements in biomass production and general plant vitality. Muthuraman et al., (2021) indicated that PGPF and PGPR have markedly enhanced both the fresh and dried biomass of bhendi. The research by Rajamani et al., (2014) indicates that *Sesbania grandiflora* exhibits greater fresh and dry weights when treated with *Bacillus subtilis* and a PGPR mixture. According to Souza et al., (2016) PGPB considerably boosts fresh and dry weights and root volume, potentially attributed to the IAA and cytokinins produced via PGPB that stimulate cell division and elongation. Many studies such as Gholami et al., (2009); Widnyana and Javandira, (2016); Najafi Vafa et al., (2022) have reported that seed priming with PGPB increase plants availability of nutrients and water and consequently increases fresh and dry weight of roots. Chandran et al., (2021) suggested that PGPB improves the availability of nutrients and water to plants by promoting root development and thus the uptake of plant nutrients and water thereby, plant nutrient uptake may improve growth traits and indices.

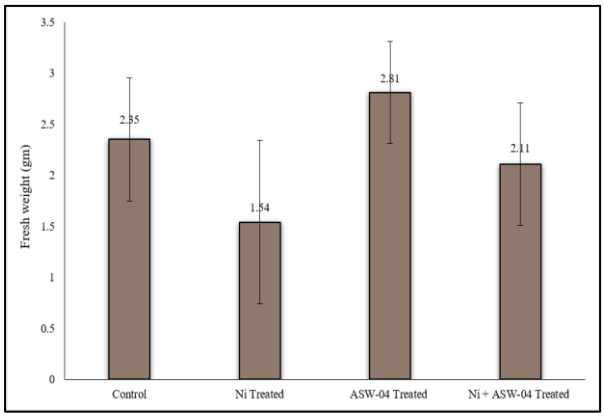

Figure: 6. Fresh weights of redgram cultivar (IBT DRG6) under different treatment conditions.

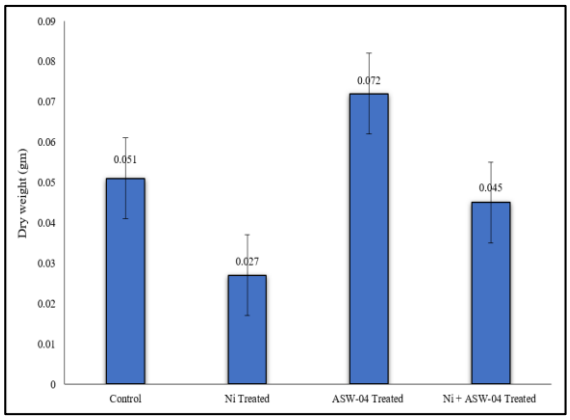

Figure: 7. Dry weights of redgram cultivar (IBT DRG6) under different treatment conditions.

Anatomical changes

Root anatomy

The current study indicates that modifications in root architectural characteristics due to various treatments imply that robust root structures, especially with the administration of *M. otitidis* ASW-04, improve the plant's resilience to nickel toxicity by facilitating superior absorption and effective resource movement. Furthermore, the roots from each treatment group exhibited significant variability in the diameter of vascular tissue.

Under controlled and *M. otitidis* ASW-04 inoculation conditions, the root displayed conventional anatomical features, including a distinct epidermis, cortex, endodermis, pericycle, and centrally positioned vascular structures. The cortical cells had a regular arrangement, with minimum intercellular gaps seen in both cultivars. Exposure to 100 ppm Ni induced significant anatomical changes, characterized by a considerable increase in the thickness of the epidermis and cortex. The vascular tissues, especially the xylem, exhibited greater robustness, hence preserving the overall structural integrity of the root. The intercellular gaps in the cortex were restricted, signifying maintained cellular organization. The ASW-04 treated plants exhibited vascular tissue with a larger diameter, while the Ni-treated plants display a reduced diameter of vascular tissue. The mean diameters of vascular tissue from the roots of the control, Ni-treated, ASW-04-treated, and Ni + ASW-04 treated

groups were measured at 200.86, 208.59, 182.62, and 192.92 µm, respectively. Figure 8 depicts the microscopic images of root anatomy.

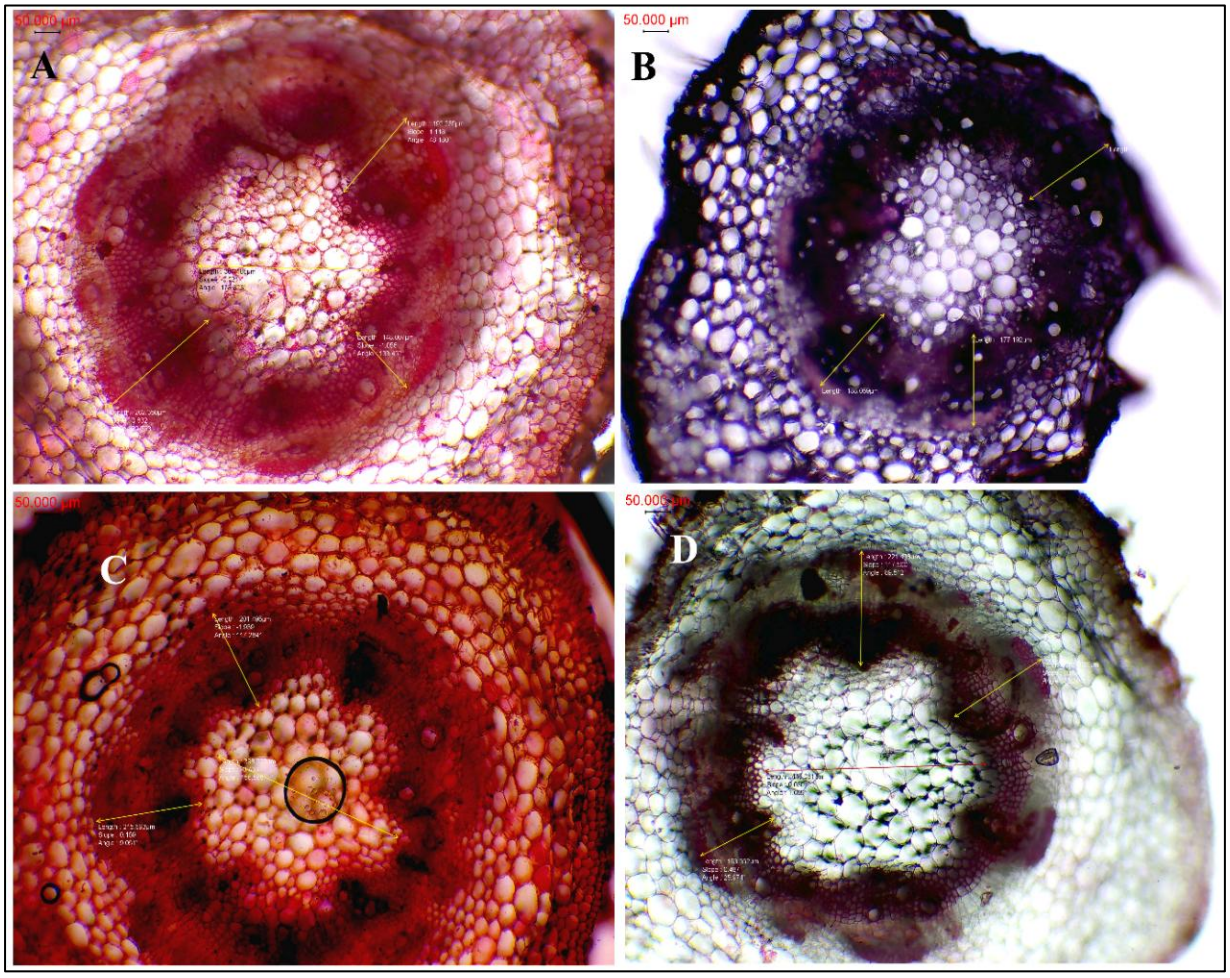

Figure: 8. Representative micrographs of root cross-sections A) control B) Ni-treated C) ASW-04 treated, D) Ni + ASW-04 treated.

Stem anatomy

This study illustrates the alterations in vascular tissue architecture and diameter due to various treatments, emphasizing the influence of nickel toxicity and the beneficial effects of *M. otitidis* ASW-04 on plant vitality. Figure 9 depicts the microscopic images of stem anatomy under different treatment conditions. From these results, it was observed that the control group plants exhibited well-organised vascular bundles and medullary regions with the mean diameter of vascular tissue measuring 125.34 µm. But, under Ni-stress, notable morphological alterations were identified, comprising diminished thickness in the cortex and vascular bundles, expanded intracellular gaps, and a reduction in mean vascular tissue diameter to around 112.69 µm. Conversely, plants subjected to bacterial isolate ASW-04 exhibited superior tissue organisation relative to Ni-treated and control groups, with a significant increase in mean vascular tissue diameter reaching 131.68 µm, alongside greater integrity of the cortex and vascular bundles. Furthermore, the combined administration of Ni+ASW-04 led to a significant recuperation, with stem structure closely mirroring the control and the mean vascular tissue diameter in this group attained 126.27 µm. These findings demonstrated that whereas Ni stress negatively impacts stem anatomy, the application of *M. otitidis* ASW-04 particularly along with Ni, can markedly rehabilitate and improve the vascular tissue architecture in the red gram cultivar.

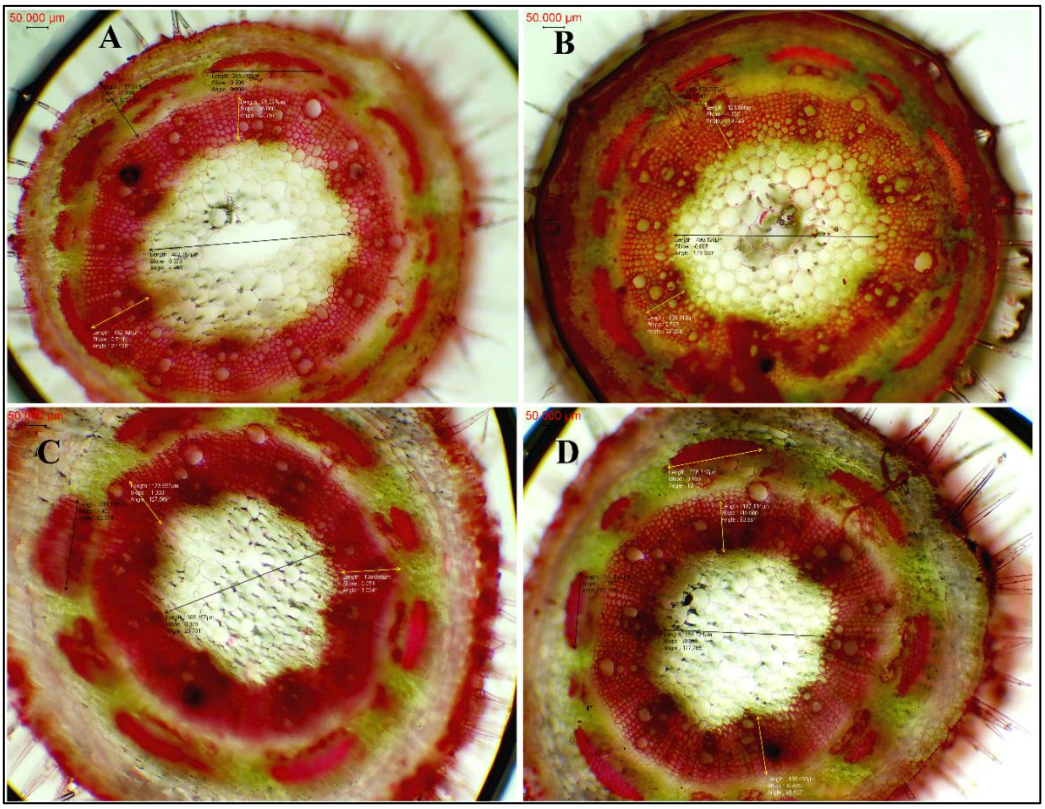

Figure: 9. Representative micrographs of stem cross-sections A) control B) Ni-treated C) ASW-04 treated, D) Ni + ASW-04 treated.

The present study revealed that redgram roots and stem displayed notable structural changes due to nickel stress and showed the beneficial effects of *M. otitidis* ASW-04 treatment. The results align with the findings of Seregin and Kozhevnikova, (2006) and Ahmad et al., (2012) who noted significant thickening of cell walls, especially in the endodermal region, potentially indicating suberization as a defensive mechanism to limit metal absorption. Moreover, Yusuf et al., (2011) found xylem vessels frequently became distorted and decreased in diameter, which may impair water and nutrient transport and eventually affect plant growth. Kumar et al., (2017) reported that the application of PGPR may improve antioxidant enzyme functioning, mitigate oxidative damage, and promote healthier root development. The present results of root anatomy correspond with the findings of Kirkham, (2006) and Choudhary and Sharma, (2013) who stated that improved root architecture not only facilitates stress reduction but also enhances overall plant vitality and yield. As well as the findings of Salisbury and Ross, (1992) and Taiz and Zeiger, (2015) corroborated with the stem anatomy results in the current study, which indicated that the PGPR application facilitates improved stem architecture and effective vascular transport, thereby leading to better resource distribution and adaptive capacity against heavy metal stress.

Leaf Stomata

Microscopic analysis of leaf stomata demonstrated significant morphological variations across all treatment groups. The stomatal measurements from all treatment groups are displayed in Table 2. The results indicated that the length of stomata in control and Ni-treated plants showed almost similar results. However, the abundance and width of the stomata was significantly decreased in Ni-treated plants. In *M. otitidis* ASW-04 treated plants, the abundance and length of stomata was significantly increased in compassion to control group.

The leaves of control plants displayed characteristic stomatal morphology and uniform distribution on both the abaxial and adaxial leaf surfaces, featuring elliptical guard cells and distinctly defined stomatal openings. The average length and width of stomata in control plants were determined to be 101.48 µm and 72.86 µm, respectively. But, the mean length and width of stomata in Ni-treated plants were measured as 98.86 µm and 58.057 µm, respectively. Under nickel stress, notable modifications were detected in stomatal morphology and distribution, including diminished stomatal density, smaller guard cells that were frequently distorted or shriveled, narrower stomatal apertures, and plasmolyzed subsidiary cells in comparison to control plants. These aberrant stomata indicate compromised gas exchange efficiency and stress-induced anatomical alterations.

Redgram cultivar exposed to ASW-04 exhibited enhanced stomatal structures relative to the control group. The ASW-04 treated plant exhibited mean stomatal length and width measurements of 114.61 µm and 70.46 µm, respectively. Moreover, the treatment of ASW-04 in conjunction with nickel stress resulted in significant enhancements in stomatal architecture compared to nickel stress alone. The mean stomatal length and width of the Ni + ASW-04 treated plant were measured at 106.69 µm and 69.87 µm, respectively. Furthermore, guard cells restored a significant portion of their typical size and morphology, exhibiting more regular and open stomatal apertures. Subsidiary cells exhibited enhanced health and turgidity, whereas the incidence of collapsed or aberrant stomata was markedly diminished. The findings indicate that nickel stress negatively impacts the stomatal structure of redgram leaves, resulting in diminished stomatal density, irregular guard cell shape, and compromised stomatal pore opening. Conversely, the administration of *M. otitidis* ASW-04 mitigates these anatomical changes, promoting improved stomatal function even in the presence of Ni stress. Figure 10 depicted the microscopic pictures of stomata from all treatment groups.

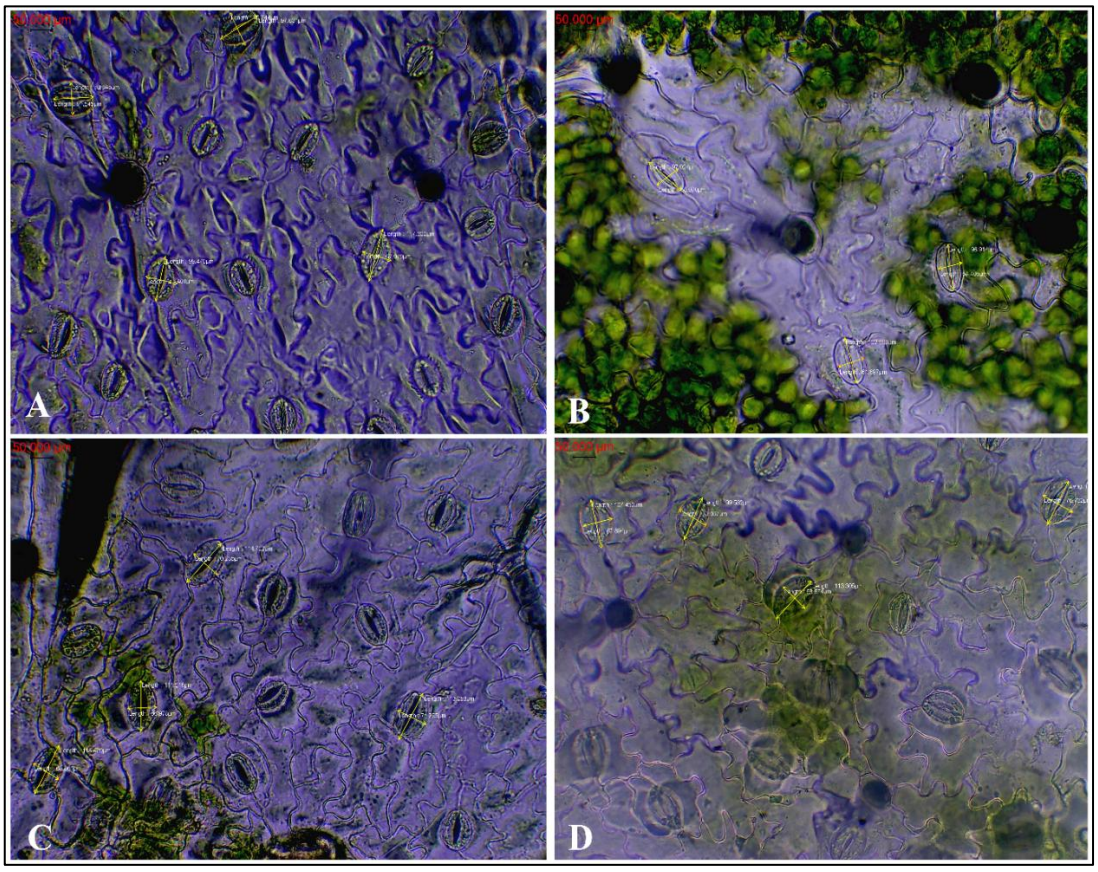

Figure: 10. Representative micrographs of stomatal anatomy A) control B) Ni-treated C) ASW-04 treated, D) Ni + ASW-04 treated.

Table: 2. Stomatal dimensions from all the treatment groups of redgram cultivar.

S. No.	Treatment Group	Stomatal dimensions (µm)	
		Mean Length	Mean Width
1	Control	101.48	72.86
2	Ni treated	98.86	58.05
3	ASW-04 treated	114.61	70.46
4	Ni + ASW-04 treated	106.69	69.87

This study revealed significant variations in the stomatal features of redgram leaves exposed to nickel stress and those inoculated with PGPR under nickel stress. The findings align with earlier research by Sharma and Dubey, (2005) and Ahmad et al., (2012) which indicated that heavy metal stress, hinders stomatal growth and function, resulting in compromised gas exchange and diminished photosynthetic efficiency. Singh et al., (2016) posited that the decrease in stomatal density and modified guard cell morphology under nickel stress may result from metal-induced oxidative stress. Seregin and Kozhevnikova, (2006) asserted that diminished stomatal aperture and the prevalence of aberrant stomata indicate that nickel stress constrains transpiration and carbon dioxide assimilation, thereby impairing plant development and productivity.

The current investigation demonstrated that *M. otitidis* ASW-04 treated plants under nickel stress had enhanced stomatal density, more uniform and turgid guard cells, and a reduced incidence of aberrant stomata in comparison to plants exposed solely to nickel stress. The findings correspond with the studies of Glick, (2012) and Ma et al., (2016) which indicated that PGPR inoculation augments plant tolerance to heavy metal stress by regulating phytohormone levels, enhancing nutrient absorption, and mitigating oxidative damage. Kumar et al., (2017) indicated that PGPR are recognized for enhancing stomatal growth and sustaining cellular homeostasis during stressful circumstances. The beneficial benefits of PGPR may involve mechanisms like the generation of ACC deaminase, siderophores, and exopolysaccharides, which together immobilize metals, thereby safeguarding root tissues and enhancing leaf architecture (Rajkumar et al., 2010).

Conclusion

The current study concludes that Ni-toxicity severely reduces seedling vigor and biomass accumulation, as well as negatively impacts redgram growth, germination, and anatomical integrity. However, administration of Ni-tolerant PGPR *M. otitidis* ASW-04 significantly improves the resistance of redgram under Ni stress. The administration of *M. otitidis* ASW-04 enhanced germination rates, root and shoot development, seed vigor index, and biomass, while simultaneously rehabilitating the vascular and stomatal systems compromised by Ni exposure. The advantageous outcomes are ascribed to the diverse plant growth-enhancing characteristics of *M. otitidis* ASW-04, including phytohormone synthesis, enhanced antioxidative protection, and Ni immobilization in the rhizosphere. The results confirm the efficacy of *M. otitidis* ASW-04 as a sustainable bioinoculant to alleviate Ni stress and enhance crop yield in Ni-contaminated soils.

Conflict of Interest

All the authors declare that they have no conflict of interest

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