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Biochemical responses of radish (*Raphanus sativus* L.) to irrigation with polluted lake water: A study of photosynthetic pigments and stress metabolites

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Abstract
The biochemical responses of plants to wastewater irrigation reflect the complex interplay between nutrient enrichment and contaminant-induced stress. This study investigated the effects of irrigation with heavy metal-contaminated water from Ramgarh Lake, Gorakhpur, on photosynthetic pigments, stress-related metabolites, and primary metabolic compounds in radish (*Raphanus sativus* L. cv. NRD 45). A two-year pot experiment (2024–2025) compared tap water irrigation (control) with raw wastewater from the lake's outflow channel. Polluted water irrigation significantly enhanced photosynthetic pigment concentrations, with chlorophyll a increasing by 36.75%, chlorophyll b by 45.45%, and total chlorophyll by 38.03%. Carotenoid content rose by 37.07%, indicating improved photosynthetic capacity. Stress-adaptive metabolites showed substantial increases: proline by 102–107%, anthocyanin by 75–83%, total phenolics by 60–66%, and ascorbic acid by 37–39%. Primary metabolites in edible roots also increased, with carbohydrates rising by 24–30% and proteins by approximately 46%. These biochemical changes demonstrate that radish plants mounted a coordinated physiological response to wastewater irrigation, simultaneously benefiting from nutrient enrichment while activating protective mechanisms against heavy metal-induced oxidative stress. The findings highlight the remarkable physiological plasticity of radish under moderate contamination stress and provide insights for managing wastewater-irrigated vegetable production.

Keywords: *Raphanus sativus*, wastewater irrigation, photosynthetic pigments, antioxidant metabolites, proline, heavy metal stress, oxidative stress

Introduction
Plants continuously encounter environmental stresses that disrupt normal physiological processes and reduce growth and productivity. Among these, heavy metal stress is particularly detrimental because it interferes with fundamental biochemical pathways by inducing excessive production of reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide, and hydroxyl radicals (Hasanuzzaman *et al*., 2020; Gill & Tuteja, 2010) [7, 10]. These ROS damage lipids, proteins, nucleic acids, and cellular membranes, ultimately impairing plant metabolism and development. To mitigate oxidative injury, plants have evolved sophisticated antioxidant defence systems comprising both enzymatic and non-enzymatic components (Apel & Hirt, 2004; Sharma *et al*., 2012) [1, 25].

Wastewater irrigation presents a unique scenario where plants are simultaneously exposed to beneficial nutrients and potentially toxic contaminants. The nutrient-rich nature of wastewater—containing nitrogen, phosphorus, potassium, and micronutrients—can stimulate photosynthetic activity, chlorophyll synthesis, and primary metabolism (Marschner, 2012) [17]. However, the presence of heavy metals such as lead, cadmium, and arsenic triggers oxidative stress and activates protective biochemical pathways (Shahid *et al*., 2021) [23]. The net biochemical response depends on the balance between these opposing influences.

Radish (*Raphanus sativus* L.) is an economically important root vegetable cultivated extensively in peri-urban areas where wastewater irrigation is common. Its short growing cycle and direct contact with contaminated soil make it an ideal model for studying plant responses to wastewater-induced stress. While previous research has documented heavy metal accumulation in radish tissues (Rai *et al*., 2019; Zhou *et al*., 2020) [21, 32], limited information exists regarding the biochemical adaptations that enable this crop to maintain growth under moderate contamination stress.

Photosynthetic pigments—chlorophyll a, chlorophyll b, and carotenoids—serve as sensitive indicators of plant physiological status because they directly reflect light-harvesting efficiency and photosynthetic capacity (Taiz *et al*., 2018) [30]. Under heavy metal stress, chlorophyll synthesis is often inhibited due to disruption of chloroplast structure and replacement of magnesium in the chlorophyll molecule (Shomali *et al*., 2024) [26]. However, enhanced nutrient availability through wastewater may counteract these inhibitory effects by providing the nitrogen and magnesium required for pigment biosynthesis.

Stress-related metabolites, including proline, phenolics, anthocyanins, and ascorbic acid, play crucial roles in protecting plants from oxidative damage. Proline functions as an osmoprotectant, free radical scavenger, and metal chelator (Szabados & Savouré, 2010) [29]. Phenolic compounds and anthocyanins neutralize reactive oxygen species and chelate metal ions (Michalak, 2006) [18]. Ascorbic acid detoxifies hydrogen peroxide through the ascorbate-glutathione cycle (Foyer & Noctor, 2011) [6]. The coordinated accumulation of these metabolites reflects the activation of integrated defence mechanisms in response to environmental stress.

This study aimed to: (1) evaluate the effects of irrigation with Ramgarh Lake water on photosynthetic pigment concentrations in radish; (2) quantify changes in stress-related metabolites including proline, phenolics, anthocyanins, and ascorbic acid; (3) assess the impact on primary metabolites (carbohydrates and proteins) in edible roots; and (4) interpret the biochemical responses in the context of wastewater-induced nutrient enrichment and heavy metal stress.

Materials and Methods

1. Study Site and Experimental Design

The study was conducted at Mahatma Gandhi P.G. College, Gorakhpur, Uttar Pradesh, India, over two consecutive years (September 2024 and September 2025). Polluted water was collected from the Gura Nala outflow channel of Ramgarh Lake, which serves as the primary discharge point used by local farmers for irrigation. Control irrigation used tap water.

Radish seeds (*Raphanus sativus* L. cv. NRD 45) were sown in pots (30 cm diameter) containing 5 kg of uniform agricultural soil per pot. A completely randomized design was employed with two treatments: (1) Control—plants irrigated with tap water, and (2) Treated—plants irrigated with raw wastewater from Ramgarh Lake. Each treatment had three replications per year. Plants were harvested at maturity (40 days after sowing) for biochemical analyses.

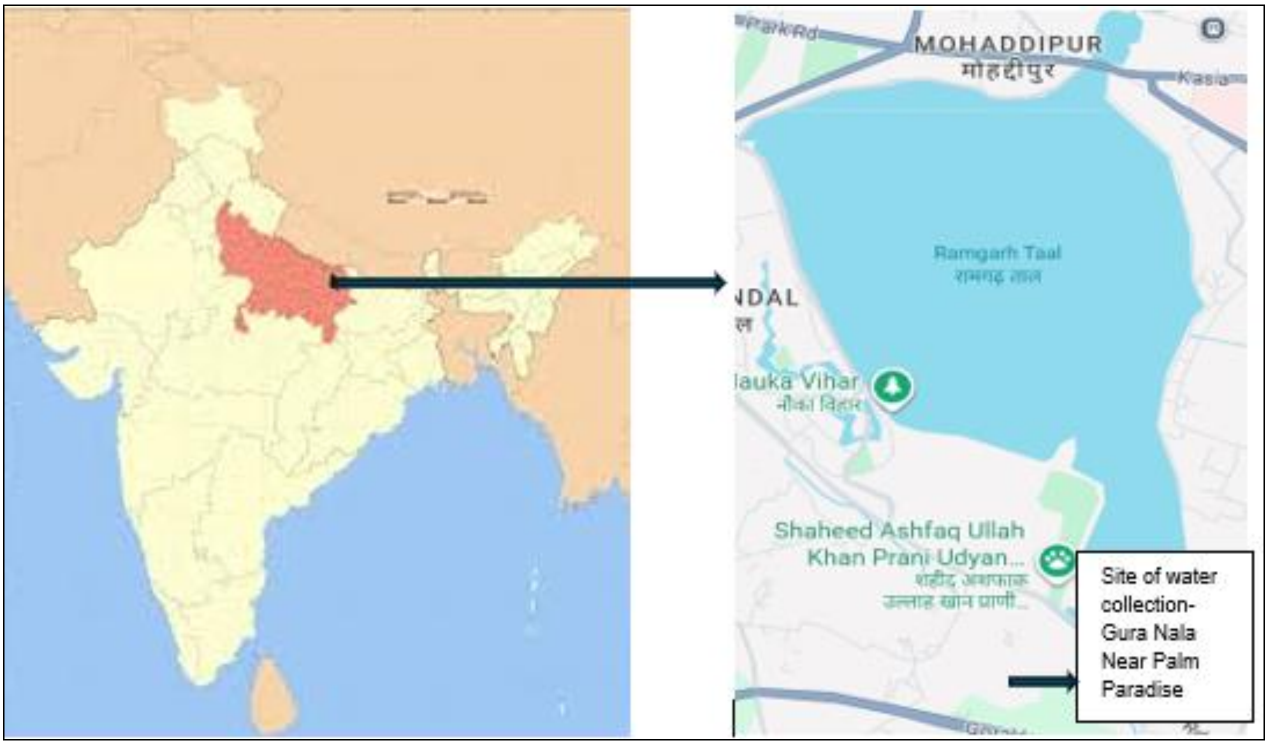

Fig 1: Ramgarh Lake of Gorakhpur showing water collection site for irrigation.

2. Biochemical Analyses

2.1 Photosynthetic Pigments

Fresh leaf samples (100 mg) were homogenized in 80% acetone with a pinch of sodium bicarbonate and centrifuged at 5000 rpm for 10 minutes. The supernatant was collected, and the final volume was made up to 10 ml with 80% acetone. Optical density was recorded at 645 nm and 663 nm on a spectrophotometer. Chlorophyll a, chlorophyll b, and total chlorophyll were calculated according to Arnon (1949) [2]:

- Chlorophyll a (mg/g FW) = $[12.72 \times A_{663} - 2.69 \times A_{645}] \times (V / W \times 1000)$
- Chlorophyll b (mg/g FW) = $[22.90 \times A_{645} - 4.68 \times A_{663}] \times (V / W \times 1000)$
- Total chlorophyll (mg/g FW) = $[8.02 \times A_{663} + 20.29 \times A_{645}] \times (V / W \times 1000)$

Where A = absorbance, V = final volume of extract, W = fresh weight of tissue.

Carotenoid content was determined following Maclachlan and Zalik (1963) [15]. Fresh leaves (100 mg) were extracted in chilled acetone, centrifuged at 3000 rpm for 10 minutes, and the supernatant was kept in dark for 24 hours. Optical density was recorded at 480 nm and 510 nm.

Carotenoids were calculated as

- Carotenoids (mg/g FW) = $[7.6 \times A_{480} - 1.96 \times A_{510}] \times (V / W \times 1000)$

2.2 Anthocyanin Content

Anthocyanin was estimated using methanolic HCl (methanol: distilled water: concentrated HCl in 80:20:1 ratio) following Manchinelli *et al*. (1975) [16]. Fresh leaf tissue (100 mg) was extracted in 80% methanolic HCl and stored at 4°C for 24 hours. After centrifugation at 4000 rpm for 10 minutes, the supernatant was made up to 5 ml with methanolic HCl. Optical density was recorded at 530 nm and 660 nm. Anthocyanin content was calculated as:

- Anthocyanin (mg/g FW) = $A_{530} - 0.25 \times A_{660}$

2.3 Total Phenolics

Total phenolics were estimated using the Folin-Ciocalteu method. Fresh leaf tissue (100 mg) was extracted in 80% methanol and centrifuged. The supernatant was mixed with Folin-Ciocalteu reagent and sodium carbonate solution, incubated for 30 minutes, and absorbance was recorded at 760 nm. Gallic acid was used as standard, and results were expressed as mg gallic acid equivalent (GAE) per gram fresh weight.

2.4 Proline Content

Proline was estimated following Bates *et al*. (1973) [3]. Fresh leaf tissue (100 mg) was homogenized in 3% sulfosalicylic acid and centrifuged. The supernatant was reacted with acid-ninhydrin and glacial acetic acid in a boiling water bath for 1 hour. The reaction mixture was extracted with toluene, and absorbance was recorded at 520 nm. Proline concentration was calculated from a standard curve and expressed as µmol per gram fresh weight.

2.5 Ascorbic Acid

Ascorbic acid was determined by titration against 2,6-dichlorophenol indophenol (DCPIP) following Sadasivam and Manickam (1992) [22]. Fresh tissue (50 mg) was homogenized in 4% oxalic acid and filtered. The extract was titrated with DCPIP solution until a pink colour persisted. Ascorbic acid content was calculated and expressed as mg per 100 g fresh weight.

2.6 Carbohydrate Content

Total carbohydrates were estimated by the anthrone method (Yemm & Willis, 1954) [31]. Dried plant samples (100 mg) were hydrolyzed with 2.5 N HCl in a boiling water bath, neutralized with sodium carbonate, and centrifuged. The supernatant was reacted with anthrone reagent (200 mg anthrone in 100 ml ice-cold 95% H_2SO_4) in a boiling water bath for 8 minutes. Optical density was recorded at 630 nm. Carbohydrate content was calculated from a standard curve and expressed as mg per gram fresh weight.

2.7 Protein Content

Total proteins were estimated following Lowry *et al*. (1951) [14]. Fresh tissue (50 mg) was homogenized in chilled tris-maleate buffer and centrifuged. The supernatant was precipitated with 20% trichloroacetic acid and kept overnight. The pellet was dissolved in 0.1 N NaOH, and the protein solution was reacted with alkaline copper tartarate and Folin-Ciocalteu reagent. Optical density was recorded at 660 nm. Protein content was calculated from a casein standard curve and expressed as mg per gram fresh weight.

3. Statistical Analysis

All data were expressed as mean ± standard deviation (SD) of three replicates. One-way analysis of variance (ANOVA) was performed using SPSS version 20.0. Statistical significance was set at $p<0.05$ and $p<0.01$.

Results

1. Photosynthetic Pigments

Irrigation with polluted water significantly enhanced all photosynthetic pigments in radish leaves compared to the control (Table 1, Figure 2). Chlorophyll a increased by 36.75% during 2024 and 35.95% during 2025, while chlorophyll b showed increases of 45.45% and 46.88% in the respective years. Total chlorophyll rose by 38.03% and 37.19%, respectively. Carotenoid content also increased significantly, with 37.07% and 36.95% enhancements during the two experimental years.

Table 1: Photosynthetic pigment concentrations in radish leaves (mean ± SD, n=3)

Parameter	Season	Control (mg/g FW)	Treated (mg/g FW)	Increase (%)
Chlorophyll a	2024	1.287 ± 0.022	1.760 ± 0.027**	36.75
Chlorophyll a	2025	1.310 ± 0.029	1.781 ± 0.009**	35.95
Chlorophyll b	2024	0.550 ± 0.010	0.800 ± 0.017**	45.45
Chlorophyll b	2025	0.563 ± 0.012	0.827 ± 0.008**	46.88
Total chlorophyll	2024	1.837 ± 0.030	2.535 ± 0.040**	38.03
Total chlorophyll	2025	1.873 ± 0.015	2.569 ± 0.016**	37.19
Carotenoids	2024	0.375 ± 0.007	0.514 ± 0.022**	37.07
Carotenoids	2025	0.387 ± 0.004	0.530 ± 0.010**	36.95

**$p<0.01$ vs control.

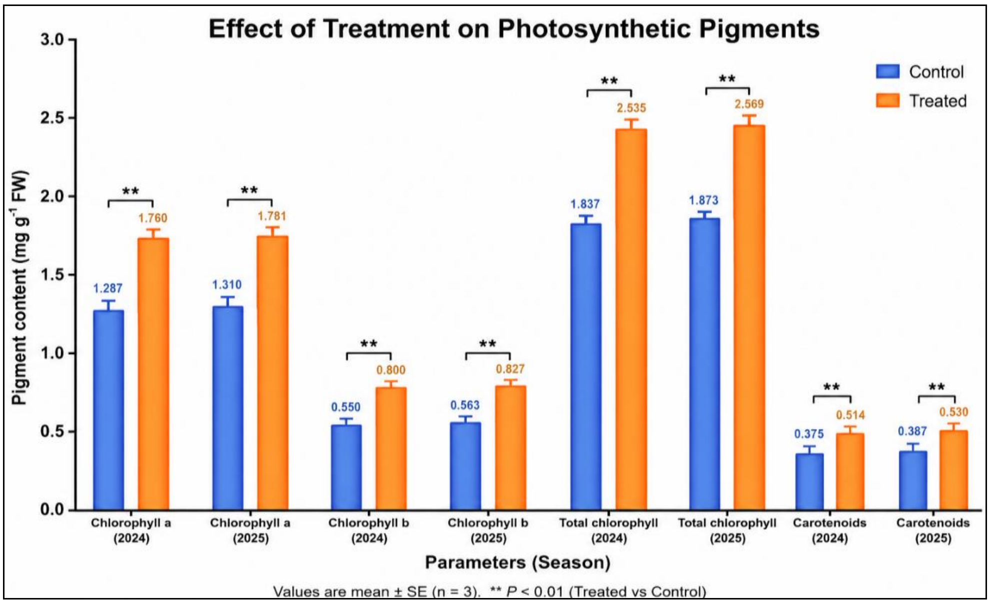

Fig 2. Comparison of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents in control and treated plants during 2024 and 2025. Values are expressed as mean ± SE (mg g^{-1} FW). Treatment significantly increased all photosynthetic pigments compared with the control ($P<0.01$). The greatest enhancement was observed in chlorophyll *b* (45.45–46.88%), while chlorophyll *a*, total chlorophyll, and carotenoids also showed substantial increases (35.95–38.03%).

The magnitude of increase was consistently higher for chlorophyll b than for chlorophyll a, suggesting enhanced development of light-harvesting antenna complexes. Treatment effects were highly significant in both years (ANOVA, $p<0.01$). The consistent response across both experimental seasons confirms the reproducibility of the observed pigment enhancement.

2. Anthocyanin and Total Phenolics

Anthocyanin content increased dramatically under polluted water irrigation (Table 2). During 2024, anthocyanin increased from 0.12 ± 0.00 to 0.21 ± 0.00 mg/g FW, representing a 75.00% increase. During 2025, the increase was from 0.12 ± 0.00 to 0.22 ± 0.01 mg/g FW, corresponding to an 83.33% increase. The treatment effects were highly significant in both years ($p<0.0001$).

Total phenolic content also showed substantial enhancement under polluted water irrigation (Table 2). In 2024, phenolics increased from 1.42 ± 0.01 to 2.27 ± 0.06 mg GAE/g FW, representing a 59.86% increase. In 2025, phenolics increased from 1.43 ± 0.07 to 2.38 ± 0.04 mg GAE/g FW, corresponding to a 66.43% increase. These increases were statistically highly significant ($p<0.0001$).

Table 2: Anthocyanin and total phenolic content in radish leaves (mean ± SD, n=3)

Parameter	Season	Control	Treated	Increase (%)
Anthocyanin (mg/g FW)	2024	0.12 ± 0.00	0.21 ± 0.00**	75.00
Anthocyanin (mg/g FW)	2025	0.12 ± 0.00	0.22 ± 0.01**	83.33
Total phenolics (mg GAE/g FW)	2024	1.42 ± 0.01	2.27 ± 0.06**	59.86
Total phenolics (mg GAE/g FW)	2025	1.43 ± 0.07	2.38 ± 0.04**	66.43

**$p<0.01$ vs control.

The magnitude of increase was greater for anthocyanin than for total phenolics in both years, indicating that the flavonoid pathway was particularly responsive to wastewater-induced stress. The highly significant treatment effects (ANOVA F-values exceeding 500 in most cases) confirm the robustness of these responses.

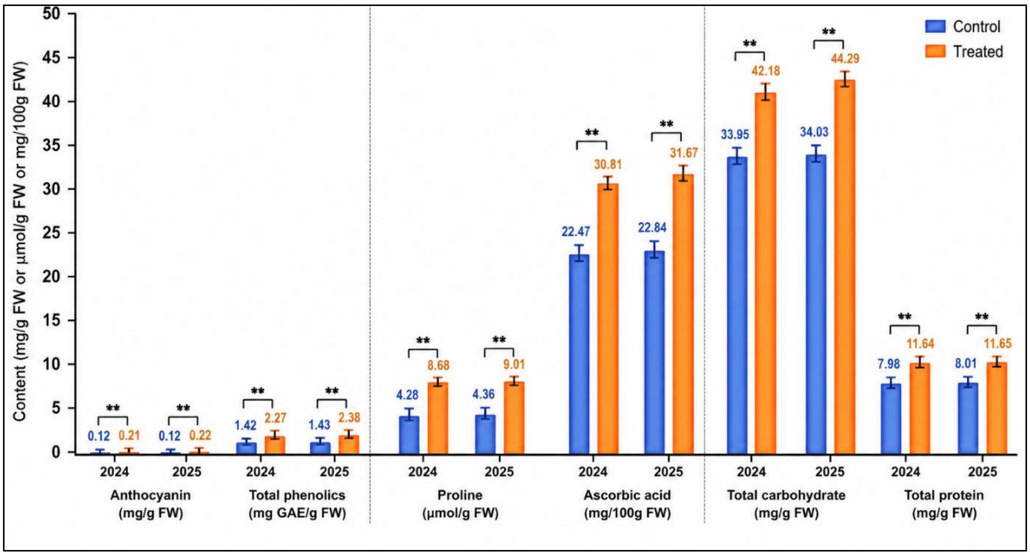

Fig 3: Combined bar chart showing the effects of treatment on anthocyanin, total phenolics, proline, ascorbic acid, total carbohydrate, and total protein contents in radish during the 2024 and 2025 seasons. Data are expressed as mean ± SD (n = 3). Error bars indicate standard deviation, and ** denotes a significant difference from the corresponding control ($P<0.01$).

3. Proline Accumulation

Proline showed the most pronounced increase among all biochemical parameters evaluated (Table 3). During 2024, proline concentration increased from 4.28 ± 0.05 to 8.68 ± 0.09 µmol/g FW, representing a 102.80% increase. During 2025, proline increased from 4.36 ± 0.05 to 9.01 ± 0.03 µmol/g FW, corresponding to a 106.65% increase. These increases were highly significant ($p<0.0001$), with F-values exceeding 5000 in both years.

Table 3: Proline and ascorbic acid content in radish leaves (mean ± SD, n=3).

Parameter	Season	Control	Treated	Increase (%)
Proline (µmol/g FW)	2024	4.28 ± 0.05	8.68 ± 0.09**	102.80
Proline (µmol/g FW)	2025	4.36 ± 0.05	9.01 ± 0.03**	106.65
Ascorbic acid (mg/100g FW)	2024	22.47 ± 0.06	30.81 ± 0.26**	37.12
Ascorbic acid (mg/100g FW)	2025	22.84 ± 0.15	31.67 ± 0.83**	38.66

**$p<0.01$ vs control.

The magnitude of proline accumulation exceeded that of all other stress metabolites, suggesting that osmotic adjustment and free radical scavenging, were primary adaptive responses. The low standard deviations recorded across replicates indicate good reproducibility of the observations.

4. Ascorbic Acid

Ascorbic acid content increased significantly under polluted water irrigation (Table 3). During 2024, ascorbic acid increased from 22.47 ± 0.06 to 30.81 ± 0.26 mg/100g FW, representing a 37.12% increase. During 2025, ascorbic acid increased from 22.84 ± 0.15 to 31.67 ± 0.83 mg/100g FW, corresponding to a 38.66% increase. Both increases were statistically highly significant ($p<0.0001$).

The increase in ascorbic acid content, though substantial, was lower than that observed for proline, phenolics, and anthocyanin. This pattern may reflect the different roles of these metabolites, with ascorbic acid functioning primarily as a hydrogen peroxide detoxifier within the ascorbate-glutathione cycle.

5. Carbohydrate and Protein Content in Edible Roots

Irrigation with polluted water significantly enhanced primary metabolite accumulation in radish storage roots (Table 4). Total carbohydrate content increased by 24.24% during 2024 and 30.15% during 2025. Total protein content showed even greater enhancement, increasing by 45.86% during 2024 and 45.44% during 2025. These differences were highly significant ($p<0.01$).

Table 4: Carbohydrate and protein content in radish edible roots (mean ± SD, n=3)

Parameter	Season	Control (mg/g FW)	Treated (mg/g FW)	Increase (%)
Total carbohydrate	2024	33.95 ± 1.17	42.18 ± 1.52**	24.24
Total carbohydrate	2025	34.03 ± 0.11	44.29 ± 0.58**	30.15
Total protein	2024	7.98 ± 0.23	11.64 ± 0.10**	45.86
Total protein	2025	8.01 ± 0.22	11.65 ± 0.11**	45.44

**$p<0.01$ vs control.

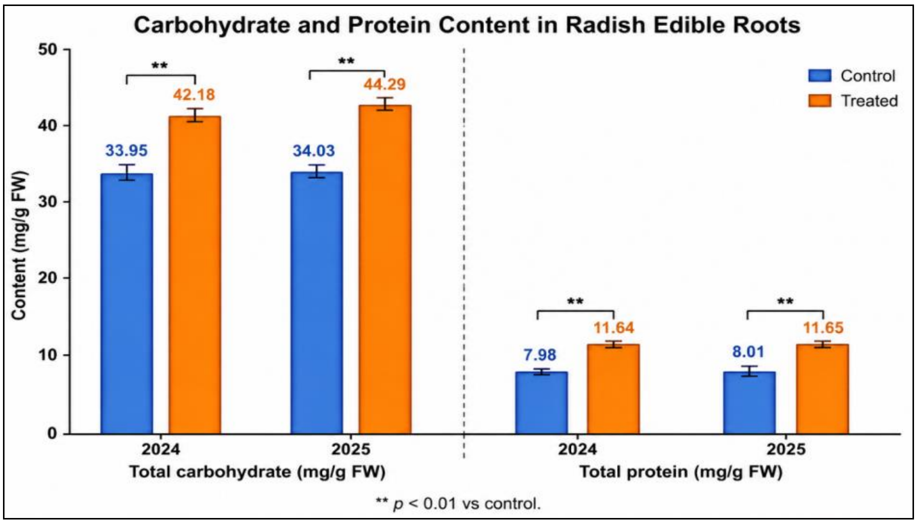

Fig 4: Effect of treatment on total carbohydrate and protein contents in edible roots of radish (*Raphanus sativus* L.) during the 2024 and 2025 seasons. Values are expressed as mean ± SD ($n = 3$). Error bars indicate the standard deviation, and ** denotes a significant difference from the corresponding control ($P<0.01$).

The magnitude of protein enhancement was nearly double that of carbohydrate enhancement, suggesting that nitrogen availability from wastewater strongly stimulated protein biosynthesis. This may reflect increased nitrate assimilation and amino acid synthesis under improved nitrogen nutrition.

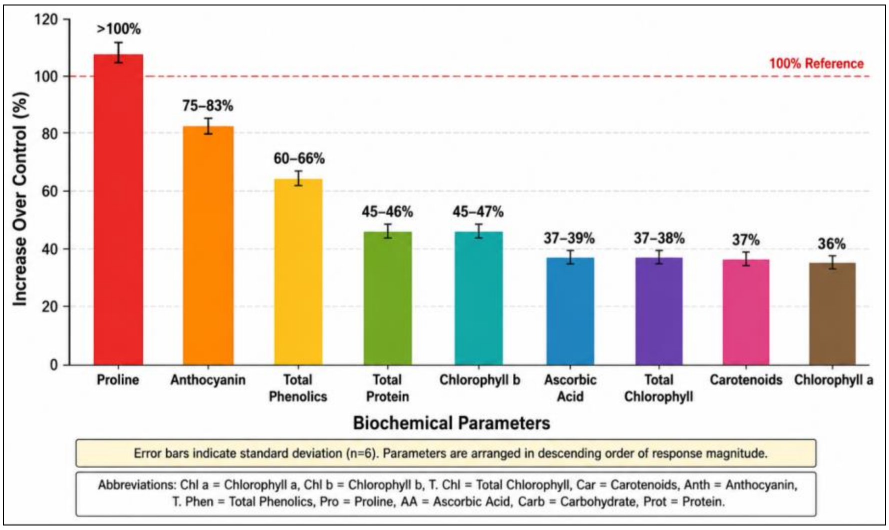

Fig 4: Percentage Increase in Biochemical Parameters of Radish Under Polluted Water Irrigation

6. Summary of Biochemical Responses

Figure 4 presents the relative increases in all biochemical parameters under polluted water irrigation. Proline showed the greatest response (over 100% increase), followed by anthocyanin (75–83%), total phenolics (60–66%), total protein (45–46%), chlorophyll b (45–47%), ascorbic acid (37–39%), total chlorophyll (37–38%), carotenoids (37%), and chlorophyll a (36%). The consistent response patterns across both experimental years confirm the reproducibility of these biochemical changes.

7. Heavy Metal Accumulation in Plant Tissues

Heavy metal concentrations in radish tissues were substantially higher in treated plants than in controls (Table 5). Edible roots accumulated lead (1.58 ppm), cadmium (0.016 ppm), arsenic (0.021 ppm), copper (0.517 ppm), chromium (0.093 ppm), and zinc (0.407 ppm). Lead showed the highest concentration among all metals in both leaves and roots.

Table 5. Heavy metal concentrations in radish tissues (ppm, mean ± SD, n=6; average of two years)

Tissue	Pb	Cd	As	Cu	Cr	Zn
Control leaves	0.127 ± 0.009	0.002 ± 0.001	0.008 ± 0.001	0.113 ± 0.006	0.021 ± 0.001	0.031 ± 0.005
Control root	0.188 ± 0.009	0.003 ± 0.001	0.011 ± 0.001	0.131 ± 0.006	0.018 ± 0.001	0.022 ± 0.001
Treated leaves	1.464 ± 0.038**	0.014 ± 0.001**	0.017 ± 0.001**	0.543 ± 0.018**	0.091 ± 0.005**	0.393 ± 0.011**
Treated root	1.584 ± 0.046**	0.016 ± 0.001**	0.021 ± 0.001**	0.517 ± 0.014**	0.093 ± 0.004**	0.407 ± 0.011**

**p<0.01vs control.

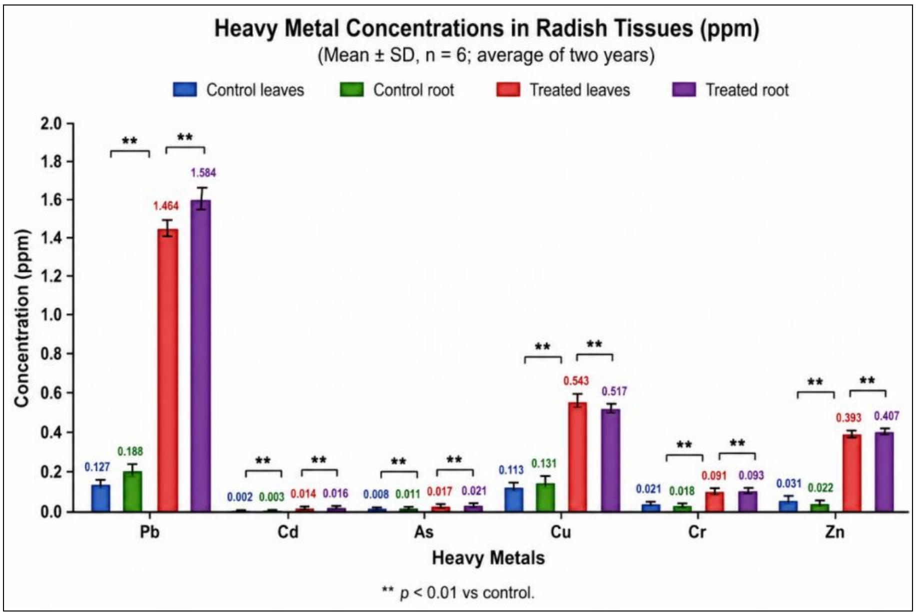

Fig 5: Heavy metal concentrations (Pb, Cd, As, Cu, Cr, and Zn) in radish leaves and edible roots under control and treated conditions. Values are presented as mean ± SD (n = 6; average of two years). Error bars represent the standard deviation, and ** denotes a significant difference from the corresponding control (*P*<0.01).

Discussion

1. Enhanced Photosynthetic Pigments: Nutrient Benefits vs Metal Stress

The significant increases in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids observed in wastewater-irrigated radish indicate that nutrient enrichment enhanced photosynthetic pigment synthesis despite the presence of heavy metals. This finding is consistent with the hypothesis that improved nitrogen and magnesium availability outweighed the inhibitory effects of metal toxicity under the experimental conditions.

Nitrogen is an essential structural component of chlorophyll molecules and constitutes a major proportion of photosynthetic proteins, including Rubisco and thylakoid membrane complexes (Marschner, 2012) [17]. Wastewater from Ramgarh Lake contained appreciable concentrations of nitrate and ammonium, which were readily absorbed and utilized for chlorophyll biosynthesis. Magnesium, the central atom of the chlorophyll molecule, was also supplied through wastewater irrigation, further supporting pigment synthesis.

The relatively greater increase in chlorophyll b (45–47%) compared to chlorophyll a (36%) suggests enhanced development of light-harvesting antenna complexes. Chlorophyll b is primarily associated with light-harvesting complex II (LHC-II), which contains a relatively greater proportion of chlorophyll b than reaction centre complexes (Taiz *et al*., 2018) [30]. Improved nitrogen availability stimulates synthesis of chlorophyll-binding proteins and LHC-II components, thereby increasing light capture efficiency.

Carotenoid enhancement (37%) reflects both improved nutritional status and activation of photoprotective mechanisms. Carotenoids perform dual functions: they expand the range of light absorption and protect chloroplasts against photooxidative damage by quenching triplet chlorophyll molecules and scavenging singlet oxygen (Demmig-Adams & Adams, 2002) [4]. The increased carotenoid content observed in this study may represent an adaptive response to moderate oxidative stress induced by heavy metals.

The absence of chlorophyll degradation under wastewater irrigation contrasts with studies reporting significant pigment reduction under severe heavy metal exposure (Shahid *et al*., 2021; Shomali *et al*., 2024) [23, 26]. This suggests that the concentrations of lead, cadmium, and arsenic in Ramgarh Lake water were insufficient to cause major chloroplast damage or pigment degradation. Instead, nutrient enrichment predominated, resulting in net pigment enhancement.

2. Proline Accumulation: A Robust Stress Marker

The substantial increase in proline content (over 100%) represents the most striking biochemical response to wastewater irrigation. Proline accumulation is one of the most reliable indicators of abiotic stress tolerance in plants and serves multiple protective functions (Szabados & Savouré, 2010; Hayat *et al*., 2012) [11, 29].

Under heavy metal stress, proline accumulation is mediated through activation of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), the rate-limiting enzyme in proline biosynthesis (Kishor *et al*., 2005) [13]. Heavy metal-induced oxidative stress stimulates proline synthesis while simultaneously reducing its degradation, leading to rapid accumulation within plant tissues. The massive proline increase observed in this study (from ~4.3 to ~8.8 μmol/g FW) indicates that radish plants perceived wastewater irrigation as a significant stress signal.

Proline contributes to stress tolerance through multiple mechanisms. As an osmoprotectant, it maintains cellular water balance and stabilizes proteins and biological membranes under osmotic stress (Szabados & Savouré, 2010) [29]. As a free radical scavenger, it directly neutralizes hydroxyl radicals and singlet oxygen, thereby preventing oxidative damage to lipids, proteins, and nucleic acids (Hayat *et al*., 2012) [11]. Furthermore, proline forms complexes with heavy metal ions, reducing their toxicity and limiting their interaction with sensitive metabolic enzymes (Sharma *et al*., 2012) [25].

The magnitude of proline accumulation (102–107% increase) far exceeded that of other stress metabolites, suggesting that osmotic adjustment and free radical scavenging were primary adaptive mechanisms. The consistent response across both years indicates that proline accumulation is a reliable biomarker for assessing heavy metal stress in radish under wastewater irrigation.

3. Phenolic and Anthocyanin Accumulation: Activation of Phenylpropanoid Pathway

The significant increases in total phenolics (60–66%) and anthocyanin (75–83%) indicate activation of the phenylpropanoid pathway in response to wastewater irrigation. This pathway is responsible for biosynthesis of diverse phenolic compounds, including flavonoids, lignin precursors, and phenolic acids, which perform multiple protective functions during environmental stress (Dixon & Paiva, 1995; Michalak, 2006) [5, 18].

Heavy metals stimulate phenylalanine ammonia-lyase (PAL), the key regulatory enzyme of the phenylpropanoid pathway, resulting in increased accumulation of antioxidant phenolics (Sharma *et al*., 2019) [24]. These compounds effectively chelate metal ions, inhibit lipid peroxidation, stabilize plasma membranes, and reduce oxidative injury by scavenging free radicals (Shahid *et al*., 2021) [23]. The pronounced anthocyanin increase (75–83%) suggests that flavonoid biosynthesis was particularly responsive to wastewater-induced stress.

Anthocyanins, water-soluble flavonoid pigments, function as highly effective antioxidants that scavenge reactive oxygen species generated during environmental stress (Gould, 2004). They also absorb excess visible and ultraviolet radiation, thereby protecting chloroplasts from photooxidative damage. The activation of anthocyanin biosynthesis reflects elevated ROS production and the need for enhanced photoprotection under metal stress.

In addition to their antioxidant function, phenolics contribute to cell wall strengthening through lignification, thereby restricting heavy metal movement into sensitive tissues (Mourato *et al*., 2015) [19]. Increased lignin deposition may reduce the translocation of toxic metals from roots to shoots by immobilizing them within the apoplast. Thus, enhanced phenolic accumulation represents an important adaptive strategy that improves both antioxidant protection and heavy metal tolerance.

4. Ascorbic Acid: Hydrogen Peroxide Detoxification

The significant increase in ascorbic acid (37–39%) indicates enhanced antioxidant capacity through the ascorbate-glutathione cycle, which constitutes the principal antioxidant pathway operating within chloroplasts, mitochondria, peroxisomes, and the cytosol (Foyer & Noctor, 2011) [6]. Ascorbic acid detoxifies hydrogen peroxide through the action of ascorbate peroxidase (APX), thereby preventing oxidative damage to membranes and photosynthetic pigments (Noctor & Foyer, 1998) [20].

The increase in ascorbic acid content, though substantial, was lower than that observed for proline, phenolics, and anthocyanin. This pattern may reflect the different roles of these metabolites. While proline and phenolics act as general stress protectants with multiple functions, ascorbic acid functions specifically in hydrogen peroxide detoxification. The 37–39% increase suggests that the ascorbate-glutathione cycle was effectively upregulated, but perhaps less dramatically than other protective pathways.

In addition to its antioxidant activity, ascorbic acid regenerates other antioxidants such as α-tocopherol, maintains chloroplast integrity, supports cell division, and participates in the biosynthesis of plant hormones including gibberellins and ethylene (Smirnoff, 2018) [28]. Increased ascorbic acid therefore contributes not only to stress tolerance but also to continued growth and development under adverse environmental conditions.

5. Primary Metabolite Enhancement: Carbohydrates and Proteins

The significant increases in carbohydrate (24–30%) and protein (~46%) content in radish storage roots indicate that wastewater irrigation enhanced primary metabolism despite simultaneous metal stress. This finding demonstrates that the beneficial effects of nutrient enrichment on carbon and nitrogen assimilation outweighed the inhibitory effects of moderate heavy metal exposure.

The increase in carbohydrate content reflects enhanced photosynthetic activity resulting from improved chlorophyll synthesis and nutrient availability. Greater carbohydrate production from leaves was efficiently translocated to storage roots, where it contributed to root enlargement and dry matter accumulation. The 24–30% increase in carbohydrates is consistent with the improved photosynthetic pigment concentrations observed in leaves.

The substantial protein increase (~46%) indicates enhanced nitrogen assimilation and protein biosynthesis in wastewater-irrigated plants. Nitrogen is the most important nutrient regulating protein metabolism because it forms the basic structural component of amino acids, nucleic acids, and enzymes (Marschner, 2012) [17]. Wastewater irrigation increased the availability of nitrate and ammonium, which were assimilated into amino acids through the GS-GOGAT pathway. Enhanced nitrogen assimilation consequently increased synthesis of structural proteins, photosynthetic enzymes, and metabolic regulators.

The magnitude of protein enhancement (46%) was nearly double that of carbohydrate enhancement (24–30%), suggesting that nitrogen availability strongly stimulated protein biosynthesis. This may reflect the relatively high nitrogen content of the wastewater compared to its carbon or energy content. The increased protein content has important implications for nutritional quality, as proteins are essential dietary components.

6. Integrated Stress Response and Physiological Adaptation

The biochemical changes observed in this study represent a coordinated adaptive response to wastewater irrigation. Nutrient enrichment stimulated chlorophyll synthesis and photosynthetic activity, while heavy metal stress activated protective pathways that minimized oxidative damage. The simultaneous enhancement of photosynthetic pigments, stress metabolites, and primary metabolites reflects the integrated nature of plant stress responses.

The wastewater irrigation simultaneously provides: (1) nutrients (N, Mg) that stimulate chlorophyll synthesis and photosynthesis; (2) heavy metals (Pb, Cd, As) that induce oxidative stress and activate antioxidant defences; and (3) nitrogen that enhances protein synthesis and primary metabolism. The balance between these influences determines the net biochemical response.

The absence of pigment degradation or metabolic inhibition suggests that nutrient enrichment effectively compensated for heavy metal stress under the experimental conditions. Plants therefore maintained—and even enhanced—photosynthetic capacity and primary metabolism while activating protective mechanisms. This remarkable physiological plasticity enabled radish plants to tolerate moderate heavy metal exposure without compromising growth and productivity.

7. Comparison with Previous Studies

The biochemical responses observed in this study are consistent with previous investigations on wastewater irrigation in vegetable crops. Gupta *et al*. (2024) reported increased chlorophyll content in wastewater-irrigated tomatoes, attributing this to improved nitrogen and magnesium nutrition. Singh *et al*. (2023) [27] observed enhanced proline accumulation in vegetables irrigated with municipal wastewater, indicating activation of osmotic adjustment mechanisms.

Hussain *et al*. (2024) [12] documented increased phenolic and anthocyanin content in wastewater-irrigated crops, suggesting activation of phenylpropanoid metabolism under metal stress. The present study extends these findings by quantifying the relative magnitudes of different biochemical responses and demonstrating the coordinated nature of stress adaptation in radish.

Several studies have reported contrasting results under severe heavy metal contamination, where chlorophyll degradation and metabolic inhibition occurred (Shahid *et al*., 2021; Shomali *et al*., 2024) [23, 26]. The absence of such adverse effects in the present study suggests that the concentrations of heavy metals in Ramgarh Lake water were below phytotoxic thresholds, allowing nutrient enrichment to predominate. This highlights the importance of considering contaminant concentrations when interpreting plant responses to wastewater irrigation.

8. Implications for Wastewater Irrigation Management

The biochemical responses observed in this study have important implications for managing wastewater-irrigated vegetable production. The enhanced photosynthetic pigments and primary metabolites indicate that wastewater can effectively substitute for chemical fertilizers, reducing production costs and promoting circular nutrient management. However, the activation of stress metabolites—particularly proline—signals that plants experience significant physiological stress, which may affect long-term productivity and crop quality.

The coordinated enhancement of protective metabolites suggests that radish plants can tolerate moderate heavy metal stress without major yield reductions. However, the cumulative nature of metal accumulation (as documented in the accompanying study) means that protective mechanisms may be overwhelmed over time. Regular monitoring of biochemical markers such as proline could provide early warning of increasing stress levels and guide irrigation management decisions.

The enhanced protein and carbohydrate content in edible roots has implications for nutritional quality. Increased protein content is generally desirable, while increased carbohydrate content may affect taste and processing characteristics. However, the concurrent heavy metal accumulation—particularly lead—presents a food safety concern that outweighs these nutritional benefits.

9. Limitations and Future Research Directions

This study provides valuable insights into the biochemical responses of radish to wastewater irrigation. However, several limitations should be acknowledged. The experiments were conducted in pots under controlled conditions, which may not fully reflect field conditions where additional factors such as climate variability, pest pressure, and soil heterogeneity influence plant responses.

Future research should investigate the long-term biochemical responses to continuous wastewater irrigation, including changes in enzymatic antioxidant activities (superoxide dismutase, catalase, peroxidase). Transcriptomic and metabolomic approaches could elucidate the molecular mechanisms underlying the observed biochemical changes. Field studies across multiple locations and seasons would validate the reproducibility of these responses under diverse environmental conditions.

Additionally, investigations into the interactive effects of heavy metals and nutrients on biochemical pathways would improve understanding of the mechanisms governing stress adaptation. The role of specific heavy metals in triggering antioxidant responses warrants further investigation through controlled exposure experiments.

Conclusion

This study demonstrates that irrigation with polluted water from Ramgarh Lake significantly alters the biochemical profile of radish plants through a coordinated combination of nutrient enhancement and stress adaptation. Photosynthetic pigments—chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids—all increased substantially, indicating improved photosynthetic capacity driven by enhanced nitrogen and magnesium nutrition. Stress-related metabolites—proline, phenolics, anthocyanins, and ascorbic acid—showed marked increases, reflecting activation of protective mechanisms against heavy metal-induced oxidative stress. Primary metabolites in edible roots—carbohydrates and proteins—also increased, indicating enhanced carbon and nitrogen assimilation.

Proline emerged as the most responsive biochemical marker, with over 100% increase under wastewater irrigation. This robust response indicates that radish plants experienced significant osmotic and oxidative stress, despite maintaining enhanced growth and pigment content. The simultaneous enhancement of photosynthetic pigments and stress metabolites reflects the dual nature of wastewater irrigation—providing beneficial nutrients while imposing moderate heavy metal stress.

The coordinated biochemical response enabled radish plants to maintain and enhance physiological performance under moderately contaminated conditions. However, the progressive accumulation of heavy metals in edible tissues (documented in the companion study) represents a food safety concern that cannot be overlooked. The biochemical markers identified in this study may serve as useful indicators for monitoring plant stress under wastewater irrigation and guiding management decisions.

Future research should focus on long-term field studies, enzymatic antioxidant assessments, and molecular investigations to further elucidate the mechanisms governing stress adaptation in wastewater-irrigated vegetable crops.

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