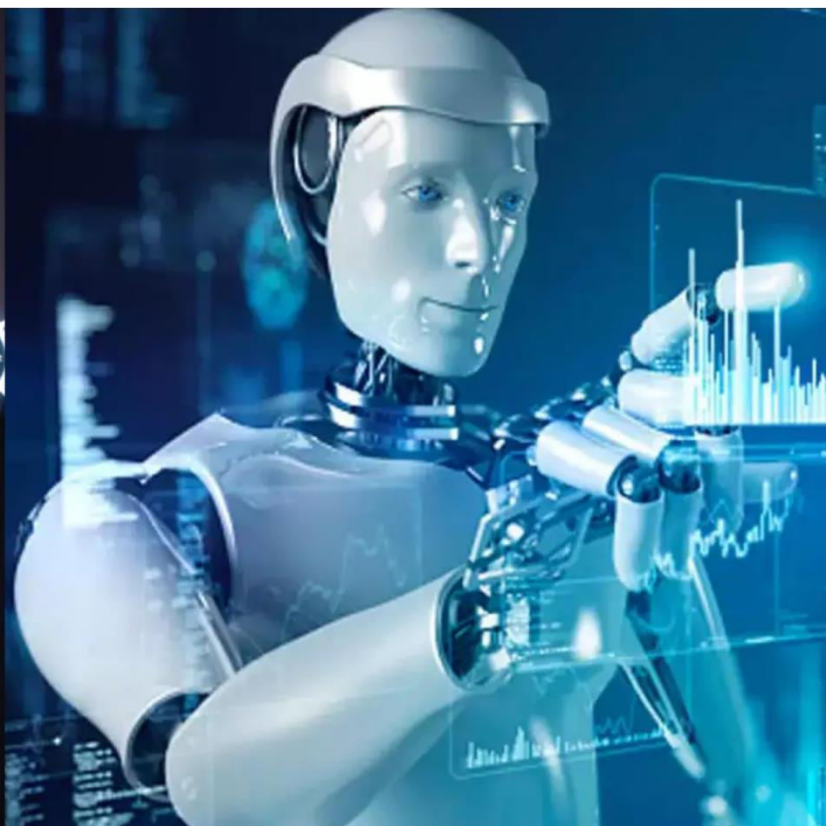




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Metabolomic Alterations in *Raphanus sativus* under Industrial Effluent Stress: A Biochemical Perspective

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ABSTRACT: Waste water from certain industries (such as the textile industry, the pharmaceutical and chemical industry) can cause a high degree of phytotoxic stress if it is used in irrigation. The biochemical and metabolomic changes of *Raphanus sativus* L. were assessed in this study. It was fed to a series of graded concentrations of actual industrial effluents from the Patancheru Industrial Development Area, Telangana, India (r). The effluent had a very high organic load (BOD5 1,240 mg/L; COD 3,580 mg/L) plus high levels of Pb (4.8 mg/L), Cd (2.1 mg/L) and Cr (6.3 mg/L) and a strong acidity (pH 3.2). The plant used for this experiment was radish grown under controlled greenhouse conditions with five replicates per treatment for 21 days exposed to 0, 25, 50, 75 and 100% (v/v) effluent. At 100% effluent, the germination rate was 21.3% in comparison to 96.7% in the control, and shoot length was reduced from 14.2 to 3.1 cm and root length was reduced from 9.8 to 1.4 cm. The amount of total chlorophyll decreased from 4.26 to 0.93 mg/g FW. Oxidative stress increased sharply, as shown by H₂O₂ (4.2 to 38.7 μ mol/g FW), MDA (12.4 to 68.3 nmol/g FW) and electrolyte leakage (8.4 to 62.8%). The antioxidant enzymes exhibited a biphasic response, first increasing up to 75% effluent and then decreasing at 100% effluent, which showed defence saturation. The levels of Proline and the soluble sugars significantly rose, indicating metabolic reprogramming and osmoprotection. The treatment effects were significant ($p < .05$) by one-way ANOVA with Tukey HSD, with a germination EC₅₀ of 68.4% effluent. The results show that there is a definite trend towards biochemical adaptation to oxidative injury with increasing concentration of industrial effluents.

KEYWORDS: *Raphanus sativus*; industrial effluent; phytotoxicity; oxidative stress; antioxidant enzymes; metabolomics; heavy metals; proline.

I. INTRODUCTION

With the rapid industrialisation, the volume of surface water discharge of chemically complex effluents has been heightened, especially in peri-urban areas where water scarcity has been a driver for the reuse of effluents within the agricultural environment. Acidic or alkaline residues, high dissolved solids content, persistent organics, dyes and heavy metals are typical contaminants found in effluents from textile dyeing, pharmaceutical synthesis, electroplating, and chemical manufacturing. Such mixtures when introduced into agricultural soil modify the nutrient balance, water relations, membrane stability and plant metabolism, some of which are not predictable through single contaminant studies (Singh & Agarwal, 2022; Khan et al., 2022; Huang & Jiang, 2022).

Raphanus sativus L. *R. sativus* (Brassicaceae) is a short-duration root crop that is popular in South Asia and has been used in phytotoxicity testing due to its quick germination and root response which has been found to be sensitive to pollutants (Soni et al., 2023; Santos et al., 2023). Heavy metals like Cd, Pb and Cr can interfere with the transport of nutrients, chlorophyll biosynthesis and photosynthetic electron flow, and increase the production of reactive oxygen species (ROS). Too much superoxide and H₂O₂ lead to lipid peroxidation, protein oxidation and membrane leakage. Plants overcome it by their antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and by the presence of glutathione, proline, phenolics and flavonoids (Mittler et al., 2022; Singh et al., 2023).

Metabolomics, on the other hand, provides a systems level view in that it identifies changes in primary and secondary metabolite pools that occur in response to environmental stress. Amino acid, sugars, phenylpropanoids and metal stress related metabolites have been reported to change in Brassicaceae under metal stress, but changes under real mixed industrial effluents is less documented (Kumar et al., 2022; Mehrotra et al., 2022; Rai & Lee, 2022). The present study thus evaluated the actual effluent of Patancheru industries along with evaluating germination, growth, photosynthetic, oxidative stress, antioxidant and metabolomic parameters in *R. sativus* using physicochemical analysis and metabolomics.

1.1 Objectives

The study had five aims: (i) to characterise the physicochemical and heavy metal content of the effluent as per IS 2490; (ii) to quantify the concentration dependent effects on germination, growth and biomass; (iii) to assess the responses of photosynthetic pigment and gas exchange; (iv) to measure the accumulation of ROS, MDA and electrolyte leakage; and (v) to measure the enzymatic and non-enzymatic antioxidants responses; (vi) to identify major metabolomic changes with increasing levels of effluent stress.

II. MATERIALS AND METHODS

2.1 Effluent sampling and characterisation

The sample of Industrial effluents was collected from Common Effluent Treatment Plant inlet of Patancheru Industrial Development Area in Sangareddy District during October-November 2024. Grab samples were taken in duplicate or triplicate, in acid-washed 5L polyethylene containers, according to APHA methods, and were stored at 4°C and were analysed within 48 hours. Treatments were made at 0, 25, 50, 75 and 100% (v/v) distilled water based. The parameters such as pH, EC, BOD₅, COD and TDS were measured using calibrated meters and appropriate standard APHA methods. Atomic absorption spectrophotometry with certified reference standards was used to quantify the elements Pb, Cd, Cr, Ni, Zn and Cu. The obtained results were compared with the tolerance limit of IS 2490 (APHA, 2017; BIS, 2022).

2.2 The growth of plants is measured by their experiment.

Agricultural cultivars of *R. sativus* L. are certified. Seeds of Pusa Chetki were surface sterilised and subjected to five different treatments of effluent along with 5 replications (25 experimental units). Germination was observed for 10 days under controlled light and temperature and the pot-grown plants were kept for 21 days in sterilised river sand and irrigated once every two days with the corresponding treatment solution. Shoot and root length, fresh mass and dry mass and leaf area were measured at harvest. The temperature in the greenhouse was kept around 24-28°C and humidity about 60-70%.

2.3 Photosynthetic, oxidative and antioxidant assays.

Chlorophyll a, chlorophyll b and carotenoids were extracted in 80 acetone and measured according to absorbance at 663, 645 and 470 nm (Lichtenthaler, 1987). Net photosynthesis and stomatal conductance were determined for fully expanded leaves. The amount of hydrogen peroxide was determined using the method described by titanium sulphate, MDA was determined by the assay method of Thiobarbituric acid reactive substances (Heath & Packer, 1968; Bradford, 1976), and the amount of electrolyte leakage by the conductivity method before and after autoclaving. The activities of SOD, CAT, POD, APX and GR were determined by well-established spectrophotometric methods (Giannopolitis & Ries, 1977; Chance & Maehly, 1955; Nakano & Asada, 1981; Foyer & Halliwell, 1976; Ellman, 1959).

2.4 Metabolomic indicators and statistical analysis of the results.

The quantities of Proline, total soluble sugars (TSS), total phenolics (TP) and flavonoids (TF) were determined using the acid-ninhydrin method, anthrone reaction, folin-ciocalteu and AlCl₃ methods respectively. Major phenolic compounds were targeted by HPLC profiling. Data were presented as mean $\pm$ SE (n = 5), and subjected to one-way ANOVA followed by Tukey HSD (p < .05) post hoc testing, while Pearson correlations, principal component analysis (PCA) and four-parameter logistic dose-response modelling for EC₅₀ estimation were used to supplement the analysis.

III. RESULTS

3.1 Effluent quality

The total effluent that was collected exhibited high level of physicochemical non compliance. Its pH was 3.2 ± 0.14 , BOD5 was $1,240 \pm 38.5$ mg/L and COD was $3,580 \pm 97.2$ mg/L. TDS reached $4,620 \pm 112.4$ mg/L and electrical conductivity 7.8 ± 0.34 mS/cm. Pb, Cd and Cr concentrations were 4.8, 2.1 and 6.3 mg/L, corresponding to 48-, 210- and 126-fold exceedances of the stated permissible limits. The combined acidity and organic loading, salinity and polymetallic stress therefore occurred rather than a single toxicant exposure.

Table.1. Physicochemical characteristics of Patancheru industrial effluent

Parameter	Observed	IS 2490 limit	Status
pH	3.2 ± 0.14	6.5-8.5	Non-compliant
BOD5 (mg/L)	$1,240 \pm 38.5$	30	Non-compliant
COD (mg/L)	$3,580 \pm 97.2$	250	Non-compliant
TDS (mg/L)	$4,620 \pm 112.4$	500	Non-compliant
Pb (mg/L)	4.8 ± 0.32	0.1	Non-compliant
Cd (mg/L)	2.1 ± 0.18	0.01	Non-compliant
Cr (mg/L)	6.3 ± 0.44	0.05	Non-compliant
EC (mS/cm)	7.8 ± 0.34	2.5	Non-compliant

Note. Values are mean $\pm$ SD (n = 3).

3.2 Growth and photosynthetic responses

The higher the concentration of effluent, the more the germination and vegetative growth decreased. Germination fell from $96.7 \pm 1.2\%$ in the control to $21.3 \pm 3.8\%$ at 100% effluent. The estimated EC50 for germination inhibition was 68.4% effluent ($R^2 = .994$). Root length decreased by 85.7% while shoot length decreased by 78.2% indicating that the roots were more sensitive. There was also a drop of over 80% in both fresh and dry weights at full-strength effluent. The decline in total chlorophyll was from 4.26 to 0.93 mg/g FW whereas net photosynthesis dropped from 18.4 to 2.9 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ and stomatal conductance from 0.38 to 0.05 $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$. These changes suggest multi-layered effects of pigments stability, stomatal function and photosynthesis metabolism.

Table.2. Selected growth, photosynthetic and oxidative-stress responses

Parameter	0%	25%	50%	75%	100%
Germination (%)	96.7	88.3	72.4	48.6	21.3
Shoot length (cm)	14.2	12.1	9.4	6.2	3.1
Root length (cm)	9.8	7.9	5.3	3.1	1.4
Total chlorophyll (mg/g FW)	4.26	3.72	2.91	1.83	0.93
Net photosynthesis	18.4	15.8	11.3	6.7	2.9
MDA (nmol/g FW)	12.4	18.7	31.2	49.8	68.3
H2O2 ($\mu\text{mol/g FW}$)	4.2	7.8	14.6	24.3	38.7

Parameter	0%	25%	50%	75%	100%
Electrolyte leakage (%)	8.4	16.2	28.7	44.1	62.8
Proline ($\mu\text{mol/g}$ FW)	14.3	26.8	48.4	74.6	98.2

Note. Values shown are treatment means; the original experiment reported significant treatment effects relative to control at $p < .05$ or better for the indicated parameters.

3.3 Oxidative stress and antioxidant defence

The concentration of the oxidative damage increased markedly with the concentration. The production of H_2O_2 increased by about 9.2-fold while MDA and electrolyte leakage increased from 8.4 to 62.8%, thus confirming the injury to the membranes by ROS. The protein content decreased from 24.8 to 5.4 mg/g FW. There was a strong correlation between H_2O_2 and MDA ($r = .987$, $p < .001$), which corroborated the relationship between the formation of ROS and the extent of lipid peroxidation. However, proline and soluble sugars have increased gradually and reached 98.2 $\mu\text{mol/g}$ FW and 82.1 mg/g FW, respectively, at 100% effluent. The response of proline to effluent concentration was almost linear, which suggests a significant metabolic switch towards osmoprotection and stress response.

All 5 antioxidant enzymes showed a biphasic response. The activity was higher at lower effluent levels and decreased at 100%. The SOD activity reached the highest level at 75%, followed by 42.1 U/mg protein, and then decreased at 24.7. The CAT activity peaked at 75% at 28.4, and then dropped to 24.7. The same pattern could be seen in POD, APX and GR with glutathione following the enzymatic response. The reduction at full strength effluent suggests that antioxidant induction was unable to keep up with the ROS production at the highest toxicant concentration (Farid et al., 2022).

Table.3. Antioxidant enzyme response under graded effluent stress

Parameter	0%	25%	50%	75%	100%
SOD (U/mg protein)	14.2	22.8	34.6	42.1	38.4
CAT (μmol $\text{H}_2\text{O}_2/\text{min/mg}$)	8.4	14.6	22.8	28.4	24.7
POD (U/mg protein)	6.2	11.4	19.3	26.8	22.1
APX (nmol/min/mg)	4.8	8.9	14.2	18.7	15.3
GR (nmol NADPH/min/mg)	3.1	5.8	9.4	12.6	10.1
GSH ($\mu\text{mol/g}$ FW)	2.8	4.6	7.8	10.4	8.2

Note. All enzyme variables were reported as highly significant across treatments ($p < .001$).

3.4 Metabolomic reprogramming

Stress reprogramming was observed in both primary and secondary metabolites. Proline was found to be 6.9-fold higher and soluble sugars 4.4-fold higher in comparison to control and proceeded to full strength. Proline and soluble sugars were 6.9- and 4.4-fold higher, respectively, than control at full strength. The HPLC profiling revealed the presence of chlorogenic, caffeic, ferulic acid, p-coumaric acid, sinapic acid, kaempferol and quercetin. The highest amount of total phenolics and flavonoids were observed in the intermediate stress groups, with sinapic acid having the highest recorded fold change (3.8-fold at 50% effluent). The pattern indicates that at sub-lethal stress levels, defence

pathways involving the phenylpropanoids and Brassicaceae are triggered, but at high doses of toxicant, defence pathways become exhausted (Mehrotra et al., 2022; Brunetti et al., 2023). PCA revealed biochemical signatures that supported treatment, PC1 accounted for 74.3% of the variance, being dominated by variables related to oxidative damage/osmoprotectants versus growth and chlorophyll, and PC2 accounted for 14.8% of the variance, dominated by antioxidant enzyme activity.

IV. DISCUSSION

Overall, the data show an adaptive defence to severe phytotoxicity transition as a function of concentration in *R. sativus*. The effluent itself was a source of several stressors: low pH levels can affect the ability of the seeds to imbibe water and perform enzyme activity; high dissolved solids are an osmotic stress and ionic stress; and Cd, Pb and Cr are responsible for impairing nutrient uptake, redox balance and cellular metabolism. This is a result of the combined mechanisms that led to the progressive increase in biological response with increasing effluent concentration (Kumar & Sharma, 2022; Rhoads & Bhatt, 2022).

Root elongation was more sensitive than shoot elongation, which was indicative of direct contact with the root and, as the roots contact and bind and accumulate metals in cell-wall and apoplastic sites, this was more sensitive than the shoot elongation. This steep drop in germination at high concentrations also suggests some inhibition of early metabolic activation. The EC₅₀ (68.4%) shows the concentration at which germination is significantly inhibited in the experiment, but biochemical changes were detected at 25% effluent.

This loss of chlorophyll and gas exchange offers a significant mechanistic connection between the pollutant exposure and biomass lost. The toxicity of Pb, Cd, and ROS can disrupt chlorophyll biosynthesis, which relies on Mg, and/or cause oxidative damage to the photosynthetic membranes (Chen et al., 2022; Hussain et al., 2023). Concurrently, a decrease in stomatal conductance limits CO₂ uptake. A simultaneous reduction of pigments, photosynthetic and growth rates are thus indicative of both stomatal and non-stomatal limitations.

The most consistent indicator of injury was the amount of ROS generated. As H₂O₂ increased, the MDA levels increased and electrolyte leakage increased, which shows that the membranes of lipids were damaged by oxidation and ionic homeostasis was lost. This is confirmed by the correlation between H₂O₂ and MDA. The first line of defense was effective: SOD transforms superoxide to H₂O₂, CAT, APX and POD remove peroxide, and GR helps to keep the glutathione-dependent redox cycle (Mittler et al., 2022). All measured enzyme activities, however, had decreased at 100% effluent indicating either saturation or impairment of antioxidant capacity. This is a biphasic profile with biochemical tipping point at about 75% effluent and partial failure at full strength.

The enzyme response was followed by metabolic reprogramming. Soluble sugars may play a role in osmoprotection and/or energy redistribution, accumulation of proline may involve roles in osmotic adjustment and protein stabilisation and/or ROS buffering. There is a correlation between higher phenolics and flavonoids with higher stress and higher antioxidant/metal chelating capacity in the intermediate stress. The reduction in these secondary metabolites in later stages suggest that this is an extra cost to the plant due to high exposure to effluents. In addition to the PCA separation of treatments, these results highlight that the metabolomic changes are not individual responses but rather part of an integrated stress syndrome which includes growth, photosynthesis, redox metabolism, and secondary defence.

The results challenge the notion that dilution is sufficient to ensure the safety of industrial wastewater for vegetable irrigation from an environmental point of view. Several growth and biochemical characteristics were affected even at 25% effluent and undiluted effluent was found to be far exceeding the stated standards. As a result, rapid plant biomarkers like proline, MDA, electrolyte leakage, and antioxidant enzyme activities, may be used in conjunction with traditional water quality measurements to assess effluent phytotoxicity in agricultural areas where effluent is used for irrigation (Garcia-Sanchez et al., 2022; Prasad et al., 2022).

The environmental implications and research are presented in Section 4.1.

The Patancheru results are not just important for a specific crop assay, but also demonstrate a convergence of the conventional physicochemical monitoring and the plant biochemical endpoints to the same risk signal. The large values of organic load and metal concentrations were correlated with consistent variations in germination, chlorophyll, ROS, membrane integrity, antioxidant activity and accumulation of osmoprotectants. This multi-level agreement reinforces

the importance of using the available tools for assessing effluent-irrigated soils, such as integrated biomarker approaches, and the benefits of using metabolomics indicators alongside more traditional water-quality testing (Pandey & Kumar, 2022; Singh et al., 2023).

The experiment also demonstrates that consideration of the toxicity of complex effluents by one metal alone is incomplete. The presence of multiple metals, salts and organic contaminants can result in synergistic or antagonistic effects to the metabolism, which are different from the individual effects. A coordinated regulation of redox and metal-detoxification and secondary-metabolism pathways under polymetallic stress was also observed in Brassicaceae integrated transcriptome-metabolome studies (Ozturk et al., 2023; Thakur et al., 2023). Future studies should, therefore, relate controlled bioassays to soil Chemistry, rhizosphere microbiology, metal uptake by plant parts and metal exposure in food chains to achieve field-level risk assessment. These extensions are especially beneficial for peri-industrial vegetable cultivation, where the plants might be irrigated at low levels for an extended period with repeated cropping cycle (Chauhan et al., 2022; Verma & Sharma, 2024).

V. CONCLUSION

The effects of Patancheru industrial effluent collected from the effluent corridor were severe and dose-dependent and induced biochemical and metabolomic stress in *R. sativus*. The effluent was highly acidic, high BOD, COD, TDS and EC and contained the concentration of different pollutants such as Pb, Cd and Cr which is higher than the permissible limit. As the concentration of effluent increased, the germination was slowly reduced and the growth of roots and shoots, and the biomass, chlorophyll, and photosynthetic performance were depressed. Meanwhile, the levels of H₂O₂, MDA and electrolyte leakage were significantly elevated, indicating oxidative damage to the membranes. As major osmoprotective response, proline and soluble sugars continued to increase in a dose dependent manner, whereas antioxidant enzymes and glutathione showed a biphasic response with maximum induction at 75% effluent and a decrease at 100%. Further, there was evidence of activation of secondary-metabolite defence, as seen in phenolic and flavonoid responses, at intermediate stress. A PCA separated the groups of treatments according to oxidative damage, growth inhibition and antioxidant activity. The study overall indicates that a definite biochemical shift from compensation to toxicity occurs, and suggests the possibility of using *R. sativus* as an effective bioindicator of complex industrial effluent stress. In the future, untargeted LC-MS/MS, transcriptomics, soil microbiome analysis and tracking metals in plant-soil-food chain could be combined to further support ecological risk assessment.

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