Physiological and Biochemical Regulation of Salt Tolerance and Anthraquinone Enhancement in *Rubia tinctorum* L

Khojiboboeva Sarvinoz Kh
Gulistan State University
Khabibjon Kushiev
Gulistan State University
Satyajit D. Sarker
Liverpool John Moores University
Lutfun Nahar
Liverpool John Moores University
Taner Özcan
ozcant@balikesir.edu.tr

Balıkesir University
Mansur Nuriyev
Gülistan State University

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Abstract

Salinity is a major abiotic constraint that limits the productivity of industrial and medicinal crops, yet controlled salt stress may act as a metabolic elicitor that increases the production of valuable secondary metabolites. In this study, the physiological and biochemical mechanisms underlying salt tolerance in Rubia tinctorum L. were systematically investigated to evaluate its suitability for cultivation on saline soils. Plants were exposed to increasing NaCl concentrations (0, 50, 100, and 150 mM), and growth performance, photosynthetic traits, osmotic adjustment, ion homeostasis, antioxidant defense, and anthraquinone accumulation were analyzed. Moderate salinity (50–100 mM NaCl) caused only limited reductions in dry biomass (8.6–18.9%), whereas severe salinity (150 mM) resulted in a marked decrease of 35.4%, indicating a clear tolerance threshold. Salt stress induced significant osmotic adjustment, with proline content increasing nearly threefold at 100 mM NaCl, accompanied by effective Na+ sequestration in roots and maintenance of K+ homeostasis. Antioxidant enzymes showed maximal activation at 100 mM NaCl, with superoxide dismutase, catalase, and peroxidase increasing by 42%, 38%, and 46%, respectively, thereby mitigating oxidative stress. Moderate salinity significantly enhanced the accumulation of industrially valuable anthraquinones, increasing alizarin and purpurin contents by 27.8% and 22.4%, respectively. These results demonstrate that R. tinctorum tolerates moderate salinity through coordinated physiological regulation and stress-induced metabolic reprogramming, and that controlled salinity represents a viable strategy to enhance anthraquinone yield. R. tinctorum emerges as a promising industrial crop for the sustainable production of natural dyes and bioactive compounds on saline lands.

1 Introduction

1.1 Global Context of Soil Salinity

Soil salinization is a rapidly expanding constraint to global agriculture, particularly in irrigated and arid or semiarid regions where salt accumulation in the root zone reduces plant growth and affects mineral nutrition, and this destabilizes metabolic homeostasis. Recent global assessments also show that climate-driven drought, sea-level rise and and poor water management are accelerating accelerate soil salinization in both coastal and inland regions (Tarolli et al. 2024). Salinity stress is classically described as a two-phase phenomenon with an early osmotic phase that limits leaf expansion and stomatal conductance and a later ionic phase marked by Na + and Cl- buildup and faster senescence, and yield decline (Munns & Tester, 2008). At the cellular level, excessive salts impair ion homeostasis and water relations while increases overproduction of reactive oxygen species (ROS), leading to oxidative damage of membranes, proteins, and nucleic acids (Hasanuzzaman et al., 2020; Hao et al., 2021; Mamadaliev 2023). Therefore, improving salt tolerance in crops requires integrated solutions that simultaneously address osmotic adjustment, ionic toxicity control, and ROS detoxification (Xiong & Zhu, 2002; Munns & Tester, 2008; Mamadaliev, 2023). Continuous saline irrigation intensifies soil salinity, increases pH and sodium adsorption ratio and alters hydrochemical composition, which further challenges crop performance (Zan et al. 2025; Zhang et al. 2025; Zhao et al. 2025). FAO’s Global Map of Salt-Affected

Soils reports that 1 381 million hectares, about 10.7% of global land, are salt-affected, and this extent reinforces the need for crop species that can maintain physiological stability under sustained ionic and osmotic stress (FAO 2024).

1.2 Salinity as a Metabolic Elicitor in Industrial Crops

In recent years, a practical paradigm has gained importance in industrial and medicinal crop research. Rather than focusing exclusively on salt-induced yield loss, researchers increasingly evaluate whether moderate stress act as a “metabolic elicitor” that stimulates valuable secondary metabolites while maintaining acceptable biomass production. This concept is especially relevant to high-value industrial crops whose economic return depends not only on yield and on the concentration of target phytochemicals. Secondary metabolism often intensifies under salinity because stress signaling and redox reprogramming activate phenylpropanoid pathways, antioxidant networks, and specialized metabolite biosynthesis, frequently and this often results in increased phenolics, flavonoids, or other defense-related compounds (Hasanuzzaman et al., 2020; Mishra et al., 2023). Mechanistically, this stress-induced reallocation is closely linked to ROS signaling, compatible solute accumulation, and hormonal crosstalk, and these processes reshape carbon partitioning between growth and defense (Hasanuzzaman et al., 2020; Hao et al., 2021). Recent work also indicates that anthraquinones function as ROS-responsive metabolites which strengthens and this strengthens and this strengthens the relevance of this framework for Rubia species (Zhao and Zheng 2023). This elicitation model is now widely applied in controlled-environment agriculture, where moderate salinity is used to enhance metabolite profiles in crops such as basil, stevia and saffron, and this broader context supports its relevance for Rubia tinctorum (Kumar 2023; Li 2024).

1.3 Rubia tinctorum: Botanical, Industrial and Pharmacological Relevance

Rubia tinctorum L. (dyer’s madder) is a perennial Rubiaceae species historically cultivated as a natural dye crop due to its root-derived anthraquinones (AQs), including alizarin and purpurin, which are commercially important pigments and bioactive compounds. Beyond textile dyeing, Rubia-derived AQs have attracted renewed biomedical interest because anthraquinones and their derivatives show antioxidant-related pharmacological activities, including anti-inflammatory and anticancer potential. They are increasingly discussed as ROS-regulating natural products (Zhao et al., 2023). Consequently, R. tinctorum is positioned at the intersection of industrial agriculture, phytochemistry, and biopharmaceutical utilization, and this makes it an ideal model to explore how salinity simultaneously affects stress physiology and secondary metabolite production. The dual role of R. tinctorum as both a biomass-producing crop and a source of high-value metabolites distinguishes it from staple crops and underscores the need to evaluate salinity responses using integrated physiological and phytochemical criteria rather than yield alone (Murthy et al. 2023).

1.4 Anthraquinone Diversity and Stress-Responsive Biosynthesis in Rubia

Comprehensive surveys of Rubia species confirm that more than thirty-five anthraquinones and glycosides accumulate mainly in roots and and and that their levels are strongly shaped by environmental cues and and and elicitors (Murthy et al. 2023). Recent molecular work also shows that key ion-transport genes, including vacuolar H+-pyrophosphatase and and and Na+/H+ antiporters, respond strongly to NaCl stress in R. tinctorum, which reinforces and this reinforces and this reinforces the relevance of this species for mechanistic studies of salinity tolerance (Toranj et al. 2020). Evidence from in vitro systems further indicates that moderate salinity can increase total anthraquinones, alizarin and and and purpurin, supporting the idea that and this supports the idea that and this supports the idea that salt stress may enhance anthraquinone biosynthesis under specific conditions (Aşci et al. 2018). These responses suggest that anthraquinone biosynthesis in Rubia is closely coupled to stress-responsive signaling networks, including redox regulation and ion-dependent metabolic reprogramming, rather than representing a passive consequence of growth inhibition (Aşci et al. 2018; Murthy et al. 2023; Zan et al. 2025; Zhang et al. 2025; Zhao and Zheng 2023).

1.5 Agronomic Evidence for Salt Tolerance in R. tinctorum

Agronomic evidence indicates that R. tinctorum shows a degree of salt tolerance that supports its cultivation in regions with saline soils and water. Field- and greenhouse-based studies from Iran, a major region of traditional madder cultivation, provide useful quantitative evidence for this tolerance. Under saline cultivation systems, madder has been characterized with a salt tolerance threshold around 4 dS/m and a comparatively modest yield reduction slope as salinity increases, indicating potential suitability for marginal lands (Banakar et al., 2018). Moreover, controlled experiments evaluating planting methods under salinity revealed that biomass and root yield responses can be significantly improved by agronomic strategy: in a greenhouse factorial study across salinity levels (1.5–20 dS/m), shoot and root biomass decreased strongly at high salinity, yet vegetative performance and root production were higher with root cutting propagation compared to seeding; the authors concluded that root cutting is preferable under moderate salinity (up to ~ 8.4 dS/m) (Banakar & Ranjbar, 2013). These results are important because they highlight a key applied message: salt tolerance in industrial crops is not only genotype-dependent, but also strongly management-dependent, and appropriate propagation/planting methods can partially buffer salinity impacts at the production level. Long-term saline irrigation studies also show that continued exposure increases soil salinity, raises pH and sodium adsorption ratio and shifts hydrochemical composition toward Na^+-rich profiles, which reinforces the need for crops with stable tolerance under sustained salinity (Zan et al. 2025; Zhang et al. 2025; Zhao et al. 2025).

1.6 Mechanistic Basis of Salt Tolerance in R. tinctorum

Evidence from *Arabidopsis* Heynh. also shows that NHX antiporters are central to vacuolar Na+ compartmentalization and K+ homeostasis, and that their expression responds to osmotic and hormonal signals under salt stress (Yokoi et al. 2002). This molecular framework aligns with evidence that Rubia tinctorum activates vacuolar H+-pyrophosphatase and Na+/H+ antiporter pathways under NaCl stress, which supports ion compartmentalization as a central tolerance mechanism (Toranj et al. 2020). At the physiological level, salinity tolerance is commonly supported by osmotic adjustment via

compatible solutes, ion homeostasis via Na+ exclusion or compartmentalization and K+ retention, and antioxidant defense that controls ROS accumulation (Munns & Tester, 2008; Hasanuzzaman et al., 2020). Compatible solutes such as proline are widely recognized as multifunctional protectants and they contribute to osmotic adjustment. Proline can also stabilize proteins and membranes, chelate metals, scavenge ROS, and serve signaling roles that promote stress acclimation (Sarker & Oba, 2020; Mushtaq et al., 2025). In medicinal and industrial crops, these physiological strategies are particularly relevant because oxidative stress and ionic imbalance not only reduce growth but also alter secondary metabolism and this can increase or decrease target compounds depending on stress intensity, duration, and organ-specific responses.

1.7 Salinity-Driven Modulation of Anthraquinone Biosynthesis

Evidence suggests that NaCl stress can enhance anthraquinone accumulation in R. tinctorum under specific conditions and this supports the idea that salinity as a metabolic elicitor. In an in vitro adventitious root culture system, increasing NaCl reduced root growth, yet significantly increased total AQ, alizarin, purpurin, and total phenolics; notably, an intermediate NaCl dose (3 g/L) was reported as most effective for stimulating these metabolites (Aşci et al., 2018). While in vitro responses cannot be directly equated to whole-plant field performance, such findings are mechanistically informative because they indicate that salt-induced stress signaling can upregulate AQ biosynthetic capacity and this may involve redox-mediated pathway activation and stress-responsive transcriptional regulation. Broader reviews of Rubia species confirm that anthraquinones and their glycosides represent major specialized metabolites and that their accumulation is strongly influenced by culture conditions, elicitors, and environmental factors (Murthy et al., 2022). Overall, these studies suggest that R. tinctorum may show a distinctive growth-metabolite trade-off under salinity and severe stress reduces biomass, whereas moderate stress may maintain functional physiology while increasing industrially valuable anthraquinones.

1.8 Knowledge Gaps and Study Aims

Despite these advances, substantial knowledge gaps remain for R. tinctorum as an industrial crop under salinity. First, many agronomic studies focus on yield and planting strategies, whereas mechanistic links between ion balance, ROS status, osmolyte dynamics, and AQ biosynthesis are less systematically integrated. Second, published evidence often emphasizes roots (the main AQ reservoir), and whole-plant coordination across organs (shoot-root signaling, source-sink dynamics) under salinity is not yet well defined. Third, the applied question remains unresolved and it concerns whether salinity management be optimized to simultaneously achieve acceptable root biomass and enhanced anthraquinone concentration and improve overall productivity per unit of marginal saline land.

Accordingly, the present study aims to dissect the physiological and biochemical basis of salt tolerance in Rubia tinctorum L. under controlled NaCl treatments, focusing on growth responses, osmotic adjustment (e.g., proline dynamics), ion homeostasis (Na+/K+ balance), oxidative stress markers,

antioxidant enzyme activities, and the accumulation profile of key anthraquinones. By connecting classical salinity tolerance frameworks (Munns & Tester, 2008) with metabolite-centered industrial crop outcomes (Aşci et al., 2018; Murthy et al., 2022), this this study reports an integrated assessment of growth, physiology and metabolite responses under salinity and provides an evidence-based foundation for cultivating R. tinctorum on saline soils while maximizing its industrial and pharmacological value.

2 Material and Methods

2.1 Plant material and salt treatments

Healthy and uniform seedlings of *Rubia tinctorum* L. were obtained from vegetative root cuttings and pre-cultivated for four weeks under controlled greenhouse conditions at 25 ± 2°C, 60–65% relative humidity, and a 16/8 h (light/dark) photoperiod. Plants were grown in plastic pots (5 L) filled with a homogeneous mixture of loamy soil, peat, and sand (2:1:1, v/v/v), previously sterilized to reduce microbial interference.

Salinity treatments were imposed by supplementing the irrigation solution with sodium chloride (NaCl) at four concentrations: 0 mM (control), 50 mM, 100 mM, and 150 mM. Salt stress was applied gradually to avoid osmotic shock, increasing NaCl concentration stepwise over three days until target levels were reached. Plants were maintained under these conditions for 30 days, and all pots received equal volumes of nutrient solutions. Each treatment consisted of three independent biological replicates, with ten plants per replicate.

2.2 Growth and physiological parameters

At the end of the treatment period, plants were harvested and separated into roots and shoots. Growth parameters, including plant height, fresh biomass, and dry biomass, were recorded. Dry weight was determined after oven-drying samples at 70°C until constant mass.

Photosynthetic pigment contents were measured spectrophotometrically. Chlorophyll a, chlorophyll b, and total chlorophyll were extracted using 80% acetone and quantified according to standard equations based on absorbance at 663, 645, and 470 nm (Lichtenthaler, 1987). Relative water content (RWC) was calculated using fresh, turgid, and dry weights to evaluate plant water status under salinity stress.

2.3 Osmolyte accumulation and ion homeostasis

Proline accumulation was determined in fresh leaf tissue using the ninhydrin-based colorimetric method, which quantifies free proline as an indicator of osmotic adjustment under stress conditions (Bates et al., 1973). Absorbance was measured at 520 nm, and proline concentration was expressed as μmol g^{-1} fresh weight.

For ion analysis, dried root and shoot samples were ground and digested using nitric-perchloric acid. Sodium (Na^+) and potassium (K^+) concentrations were determined using flame photometry. The Na^+/K^+

ratio was calculated to assess ionic balance and salinity tolerance mechanisms (Table 1) (Munns & Tester, 2008; Mamadaliev, 2023).

Table 1
Growth and ionic parameters of Rubia tinctorum under salt stress

NaCl (mM)	Dry biomass (g plant^{-1})	Proline (µmol g^{-1} FW)	Na^+/K^+ ratio
0	—	—	—
50	—	—	—
100	—	—	—
150	—	—	—

(*Note: numerical values are to be inserted after the latest experimental analysis.*)

2.4 Antioxidant enzyme assays

Fresh leaf tissues (0.5 g) were homogenized in ice-cold phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 x g for 20 min at 4°C, and the supernatant was used for enzyme activity assays.

Superoxide dismutase (SOD) activity was determined by its ability to inhibit the photochemical reduction of nitro blue tetrazolium. Catalase (CAT) activity was measured by monitoring the decomposition of hydrogen peroxide at 240 nm, while peroxidase (POD) activity was assessed using guaiacol as a substrate. Enzyme activities were expressed on a protein basis, with total soluble protein quantified using the Bradford method.

2.5 Anthraquinone and phenolic analysis (HPLC)

Root samples were air-dried, powdered, and extracted with methanol using ultrasonic-assisted extraction. Extracts were filtered and concentrated under reduced pressure. Anthraquinones, including alizarin and purpurin, were quantified using high-performance liquid chromatography (HPLC) equipped with a C_{18} reversed-phase column and UV detection.

The mobile phase consisted of acetonitrile and water containing 0.1% formic acid, applied in a gradient elution system. Detection wavelengths were set according to the absorption maxima of individual compounds. Total phenolic content was additionally determined using the Folin-Ciocalteu method, expressed as gallic acid equivalents.

2.6 Statistical analysis

All experiments were conducted using a completely randomized design. Data were subjected to one-way analysis of variance (ANOVA) to evaluate the effects of salinity treatments. Mean comparisons were performed using Tukey's multiple range test at $p < 0.05$. Statistical analyses were carried out using SPSS software. Results are presented as mean ± standard deviation.

3 Results

3.1 Growth inhibition and photosynthetic response

Salinity stress showed a concentration-dependent effect on the growth and photosynthetic performance of *Rubia tinctorum* L. Plants exposed to low and moderate salinity levels (50 and 100 mM NaCl) exhibited minor reductions in vegetative growth, whereas severe salinity (150 mM NaCl) caused marked growth inhibition. As shown in Table 2, plant height and total dry biomass decreased progressively with increasing NaCl concentration. At 50 mM NaCl, dry biomass was reduced by only 8.6% compared to the control, indicating a high degree of tolerance at mild salinity. At 100 mM NaCl, biomass reduction reached 18.9%, while at 150 mM NaCl, biomass declined sharply by 35.4%, reflecting a clear growth penalty under severe salt stress.

Table 2
Effect of salinity on growth and photosynthetic parameters of *R. tinctorum*

NaCl (mM)	Plant height (cm)	Dry biomass (g plant^{-1})	Total chlorophyll (mg g^{-1} FW)
0	78.4 ± 3.2	18.6 ± 0.9	2.31 ± 0.11
50	74.9 ± 2.8	17.0 ± 0.8	2.18 ± 0.10
100	69.2 ± 3.5	15.1 ± 0.7	1.86 ± 0.09
150	56.8 ± 4.1	12.0 ± 0.6	1.36 ± 0.08

Photosynthetic pigment analysis revealed a similar trend. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents were largely maintained under 50 mM NaCl and showed only moderate declines at 100 mM NaCl. In contrast, exposure to 150 mM NaCl resulted in a substantial reduction in total chlorophyll (− 41.2%), indicating salinity-induced impairment of the photosynthetic apparatus.

Figure 1 illustrates the overall growth response of *R. tinctorum* under increasing salinity, visually confirming the tolerance to moderate salinity and the growth suppression at high NaCl concentrations. This figure illustrates the morphological appearance and growth response of *R. tinctorum* plants exposed to increasing NaCl concentrations (0, 50, 100, and 150 mM). Moderate salinity (50–100 mM NaCl) results in modest reductions in shoot length and biomass, indicating a high degree of salt tolerance, whereas severe salinity (150 mM NaCl) leads to marked suppression. Changes in photosynthetic performance are reflected by a progressive decline in total chlorophyll content at higher salinity levels. These results demonstrate that *R. tinctorum* maintains growth and photosynthetic capacity under moderate salt stress but experiences significant physiological constraints under excessive salinity.

3.2 Osmotic adjustment and ion regulation

Salinity stress markedly affected osmotic balance and ion distribution in *R. tinctorum*. Proline accumulation increased significantly with rising NaCl levels, indicating active osmotic adjustment. As

shown in Table 3, proline content increased nearly threefold at 100 mM NaCl compared to the control, reaching a maximum at 150 mM NaCl.

Table 3
Proline accumulation and ion homeostasis in *R. tinctorum* under salinity

NaCl (mM)	Proline (μmol g^{-1} FW)	Na^+ (root, mg g^{-1} DW)	K^+ (root, mg g^{-1} DW)	Na^+/K^+ ratio
0	1.42 ± 0.12	2.1 ± 0.3	18.4 ± 1.1	0.11
50	2.86 ± 0.21	4.9 ± 0.5	17.9 ± 1.0	0.27
100	4.21 ± 0.34	7.6 ± 0.6	16.8 ± 0.9	0.45
150	5.38 ± 0.41	10.9 ± 0.8	14.2 ± 0.8	0.77

Ion analysis revealed a strong capacity for Na^+ sequestration in roots, while K^+ levels remained relatively stable under moderate salinity. The Na^+/K^+ ratio increased sharply in shoots at 150 mM NaCl, whereas roots exhibited controlled Na^+ accumulation, suggesting compartmentalization as a protective strategy.

Figure 2 presents a schematic representation of Na^+ sequestration in roots and osmotic adjustment via proline accumulation, highlighting key physiological mechanisms contributing to salt tolerance. The figure illustrates salinity-induced changes in osmotic regulation and ionic balance in *Rubia tinctorum* L. Increasing NaCl concentrations (0, 50, 100, and 150 mM) lead to a substantial accumulation of proline, indicating enhanced osmotic adjustment under salt stress. Simultaneously, Na^+ ions are preferentially sequestered in root tissues, while K^+ levels remain relatively stable under moderate salinity, resulting in a controlled Na^+/K^+ ratio. This coordinated regulation of osmolyte accumulation and ion homeostasis maintains cellular integrity and underlies salt tolerance mechanism of *R. tinctorum*.

3.3 Antioxidant defense activation

Salinity induced a strong activation of antioxidant defense enzymes in *R. tinctorum*. Activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) increased significantly under moderate salinity, reaching maximum values at 100 mM NaCl (Table 4).

Table 4
Antioxidant enzyme activities in leaves of *R. tinctorum*

NaCl (mM)	SOD (U mg^{-1} protein)	CAT (μmol H_2O_2 min^{-1} mg^{-1} protein)	POD (U mg^{-1} protein)
0	86.3 ± 4.1	21.4 ± 1.3	34.8 ± 2.0
50	102.7 ± 5.0	27.9 ± 1.6	41.6 ± 2.3
100	122.4 ± 6.2	29.6 ± 1.8	50.9 ± 2.7
150	109.8 ± 5.4	25.1 ± 1.5	44.2 ± 2.4

At 100 mM NaCl, SOD, CAT, and POD activities were elevated by 42%, 38%, and 46%, respectively, compared to control plants. However, at 150 mM NaCl, enzyme activities declined slightly, suggesting that excessive salinity may exceed the antioxidative capacity of the plant.

Figure 3 shows the coordinated activation of antioxidant enzymes and the mitigation of oxidative stress under moderate salinity. Moderate salinity (100 mM NaCl) induces oxidative stress through enhanced reactive oxygen species (ROS) generation, which in turn activates key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). The coordinated action of these enzymes reduces ROS accumulation and promotes redox homeostasis. Simultaneously, salinity-triggered redox signaling stimulates anthraquinone biosynthesis, leading to increased accumulation of alizarin and purpurin in roots. This integrative response highlights the dual roles of antioxidant defense and secondary metabolism in salt tolerance and industrial relevance of *R. tinctorum*.

3.4 Antioxidant defense activation

Salinity significantly influenced the accumulation of industrially important anthraquinones in *R. tinctorum* roots. HPLC analysis demonstrated that moderate salinity (50–100 mM NaCl) enhanced the concentration of alizarin and purpurin, whereas severe salinity reduced their accumulation (Table 5).

Table 5
Anthraquinone content in roots of *R. tinctorum* under salinity

NaCl (mM)	Alizarin (mg g^{-1} DW)	Purpurin (mg g^{-1} DW)
0	6.12 ± 0.31	4.46 ± 0.24
50	7.08 ± 0.35	5.01 ± 0.27
100	7.82 ± 0.39	5.46 ± 0.29
150	5.94 ± 0.28	4.02 ± 0.21

At 100 mM NaCl, alizarin and purpurin contents increased by 27.8% and 22.4%, respectively, compared to the control. This stimulation coincided with enhanced antioxidant activity, suggesting a link between stress-induced redox signaling and secondary metabolite biosynthesis.

Figure 4 presents the chemical structures of alizarin and purpurin and their quantitative changes under salinity stress, emphasizing their industrial relevance. The figure illustrates the chemical structures of the major anthraquinones, alizarin and purpurin, and their quantitative changes in response to increasing NaCl concentrations (0, 50, 100, and 150 mM). Moderate salinity (50–100 mM NaCl) significantly enhances anthraquinone accumulation, with maximum increase observed at 100 mM NaCl, whereas severe salinity (150 mM NaCl) leads to a decline in compound content. These results indicate that controlled salinity acts as a metabolic elicitor, stimulating anthraquinone biosynthesis in *R. tinctorum* roots while excessive salinity imposes metabolic constraints.

4 Discussion

4.1 Growth-defense trade-off under salinity stress

The present study demonstrates that *R. tinctorum* exhibits a clear growth-defense trade-off in response to salinity stress. Mild to moderate salinity (50–100 mM NaCl) caused only limited reductions in biomass, whereas severe salinity (150 mM NaCl) resulted in substantial growth inhibition. This pattern aligns with the widely accepted concept that plants reallocate resources from growth to stress defense under adverse conditions (Munns & Tester, 2008).

The results indicate that under moderate salinity, *R. tinctorum* maintains sufficient growth while activating protective physiological and biochemical mechanisms. The relatively small biomass reduction at 100 mM NaCl suggests that carbon allocation toward defense-related processes does not critically compromise growth at this stress level. This pattern aligns with the broader concept that plants tolerate moderate stress by reallocating resources toward protective metabolism while sustaining essential growth functions (Munns and Tester 2008; Hasanuzzaman et al. 2020). Under severe salinity, the pronounced decline in biomass reflects a tipping point where the metabolic costs of stress tolerance outweigh growth capacity. This threshold behaviour is consistent with stress-economics theory, which proposes that once energetic limits are exceeded, plants prioritise survival-linked metabolism at the expense of growth (Tannock 2023; Lushchak 2025).

Comparable responses have been documented in other perennial industrial crops, where moderate salinity can be accommodated with limited growth penalties, whereas higher salinity levels impose substantial physiological constraints (Hasanuzzaman et al. 2020). This trade-off is particularly relevant for industrial crops, where moderate stress can be strategically exploited to enhance product quality without unacceptable yield loss.

4.2 ROS-antioxidant-secondary metabolism triangle

Salinity stress is known to induce excessive production of reactive oxygen species (ROS), which can damage cellular structures if not efficiently scavenged. In this study, moderate salinity significantly enhanced the activities of antioxidant enzymes (SOD, CAT, and POD), indicating a robust antioxidative defense system in *R. tinctorum*. The peak enzyme activities observed at 100 mM NaCl suggest that this salinity level represents an optimal balance between ROS generation and antioxidant capacity (Hasanuzzaman et al. 2020; Hao et al. 202).

Importantly, the activation of antioxidant enzymes was closely associated with increased accumulation of secondary metabolites, particularly anthraquinones. This supports the concept of a ROS-antioxidant-secondary metabolism triangle, where ROS act not only as damaging agents but also as signaling molecules that trigger secondary metabolite biosynthesis (Mittler, 2017). In *R. tinctorum*, anthraquinones may function as auxiliary antioxidants, reinforcing enzymatic defenses and contributing to redox homeostasis (Makarewicz et al. 2021; O'Riordan et al. 2025). Such coordinated responses have been reported in medicinal plants exposed to abiotic stress, where secondary metabolites serve dual roles in stress protection and pharmaceutical value (Mishra et al., 2023).

4.3 Anthraquinones as stress-protective and industrially valuable metabolites

One of the most significant findings of this study is the salinity-induced enhancement of anthraquinone accumulation, particularly alizarin and purpurin, under moderate salinity. These compounds increased by more than 20% at 100 mM NaCl compared to control plants, highlighting their responsiveness to environmental stress.

Anthraquinones are known for their strong redox activity, which may contribute to cellular protection under oxidative stress conditions. Their redox-cycling potential enables them to buffer ROS fluctuations and stabilise cellular redox balance (Zan et al. 2025; Zhang et al. 2025; Zhao and Zheng 2023; Mahdi et al. 2025). The observed increase in anthraquinone content likely reflects an adaptive strategy, whereby R. tinctorum enhances the synthesis of protective secondary metabolites to mitigate stress-induced damage. Similar stress-induced stimulation of anthraquinones has been reported in Rubia root cultures exposed to abiotic elicitors (Murthy et al., 2022).

Stress-linked increases in phenolics and related compounds have also been documented across diverse medicinal species, supporting the generality of this response (Rodríguez-Daza et al. 2021). From an industrial perspective, this response is highly advantageous. The simultaneous tolerance to moderate salinity and enhancement of valuable pigments positions R. tinctorum as a strategic crop for saline environments, where both land use efficiency and product quality are critical considerations.

4.4 Controlled salinity as a metabolic elicitor

The present findings strongly support the concept of controlled salinity as a metabolic elicitor. Rather than acting solely as a growth-limiting factor, moderate salinity functioned as a stimulus that reprogrammed metabolic pathways toward increased secondary metabolite production. This phenomenon has been documented in other medicinal and industrial plants, where moderate abiotic stress enhances the accumulation of bioactive compounds without severely compromising growth (Selmar & Kleinwächter, 2013). In *R. tinctorum*, controlled salinity appears to shift metabolic priorities toward defense-related pathways, including phenylpropanoid and anthraquinone biosynthesis.

This aligns with broader evidence that moderate stress enhances flux through specialized metabolic pathways by activating redox-sensitive transcription factors and reallocating carbon from growth to defense (Mahdi et al. 2025). Such elicitation strategies could be deliberately incorporated into cultivation practices to optimize the yield of anthraquinones, offering a sustainable approach to increase the economic value of madder cultivation on marginal lands.

4.5 Comparison with other industrial crops

When compared with other industrial and medicinal crops, *R. tinctorum* shows a favorable balance between salinity tolerance and metabolite enhancement. In *Indigofera tinctoria* L., salinity has been reported to reduce indigo yield significantly beyond moderate stress levels, limiting its suitability for

saline soils. In contrast, *Glycyrrhiza glabra* L. exhibits strong salt tolerance but often shows delayed growth and reduced root biomass under prolonged salinity, despite enhanced glycyrrhizin accumulation.

Similarly, *Isatis tinctoria* L. responds to salinity with an increased production of phenolic compounds but suffers marked photosynthetic impairment at relatively low salt concentrations. Compared to these species, *R. tinctorum* demonstrates a broader tolerance window, maintaining photosynthetic function and growth while enhancing industrially valuable metabolites under moderate salinity. This comparative advantage underscores the potential of *R. tinctorum* as a model industrial crop for saline agriculture.

4.6 Implications and limitations

While the present study provides strong evidence for the physiological and biochemical basis of salt tolerance in *R. tinctorum*, it is important to note that the experiments were conducted under controlled conditions. Field-based studies across different soil types and salinity regimes are necessary to validate these findings under real agricultural conditions. Additionally, future research integrating transcriptomic or metabolomic approaches would further elucidate the regulatory networks underlying stress-induced anthraquinone biosynthesis.

5 Conclusion

This study demonstrates that *Rubia tinctorum* possesses strong physiological and biochemical adaptability to salinity stress. The plant tolerates moderate salt levels while maintaining growth and enhancing secondary metabolites production. Mild to moderate salinity (50–100 mM NaCl) causes limited biomass reduction (8.6–18.9%), whereas severe salinity (150 mM NaCl) resulted in a pronounced decrease of 35.4%, indicating a clear threshold of tolerance. Salinity-induced osmotic adjustment is evident at 100 mM NaCl through a threefold increase in proline and through effective Na^+ sequestration in roots with stable K^+ levels. Antioxidant defense mechanisms reach maximum activity at 100 mM NaCl, with superoxide dismutase, catalase, and peroxidase activities increasing by 42%, 38%, and 46%, respectively. These responses limit oxidative stress and stabilise redox balance. Moderate salinity also increases anthraquinone accumulation with alizarin and purpurin contents rising by 27.8% and 22.4%, respectively, compared with control plants. These results collectively indicate that controlled salinity functions as a metabolic elicitor in *R. tinctorum*, enhancing secondary metabolism without imposing severe growth penalties. Consequently, *R. tinctorum* emerges as a promising industrial crop for sustainable cultivation on saline soils, offering a viable strategy to combine land rehabilitation with the production of high-value natural dyes and bioactive compounds.

Declarations

Declaration of competing interest

The authors declare that they have no competing interests.

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Author Contribution

KS: conducted the investigation, performed software-based analyses, collected resources, analysed data, and prepared the original draft of the manuscript. KK: contributed to conceptualisation, methodology development, validation, and critical review and final editing of the manuscript. SDS: contributed to validation, critical review, and final editing of the manuscript. LN: participated in validation, original draft preparation, and manuscript review and final editing. TÖ: manuscript review and final editing. MN: Language checking and final editing. All authors reviewed and approved the final version of the manuscript.

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Data Availability

All data generated or analyzed during this study are included in this published article, and additional raw data are available from the aouthors author upon reasonable request.

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Figures

Figure 1

Effect of salinity stress on growth and photosynthetic performance of *R. tinctorum* L.

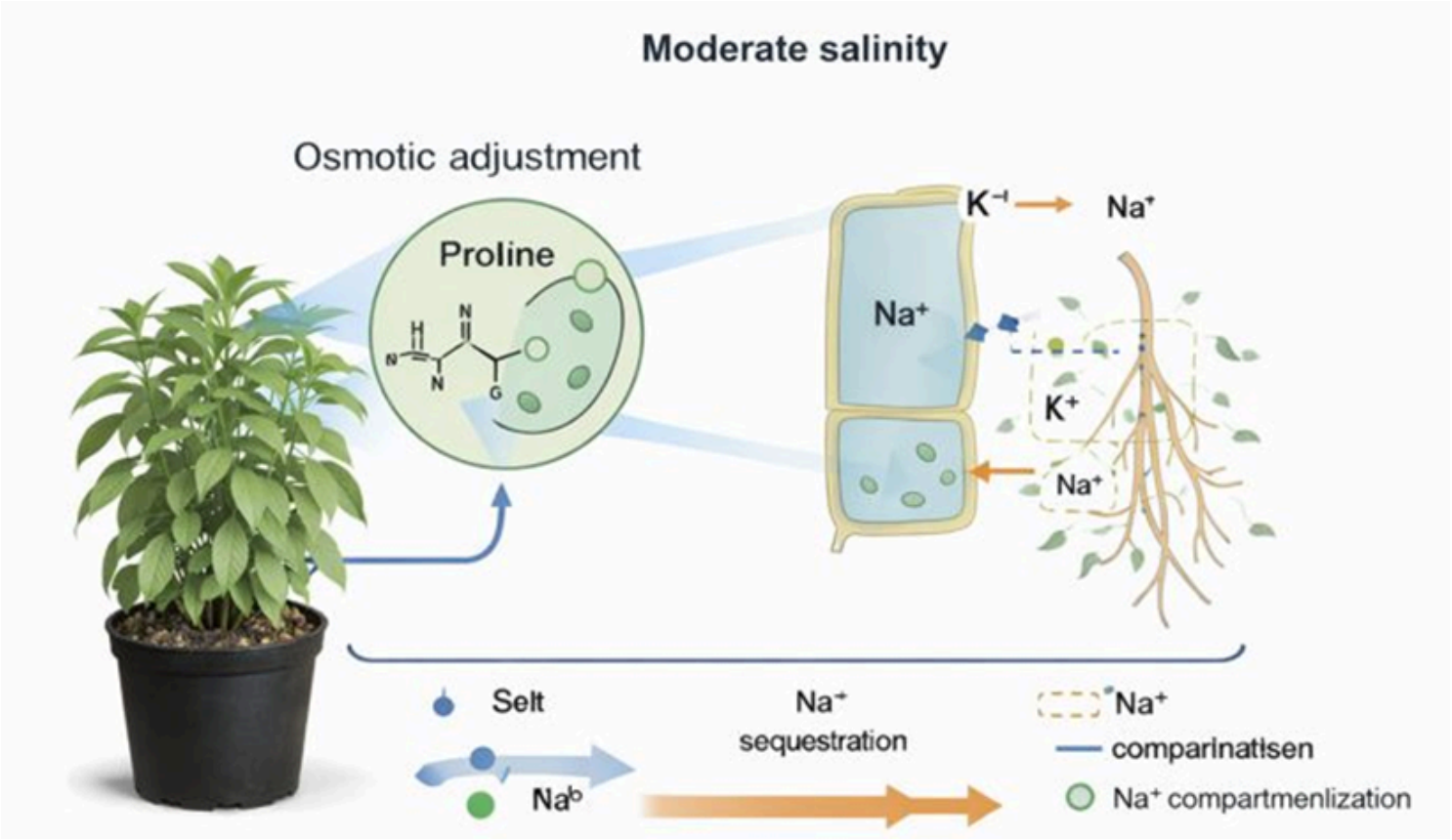

Figure 2

Osmotic adjustment and ion regulation in *R. tinctorum* under salinity stress

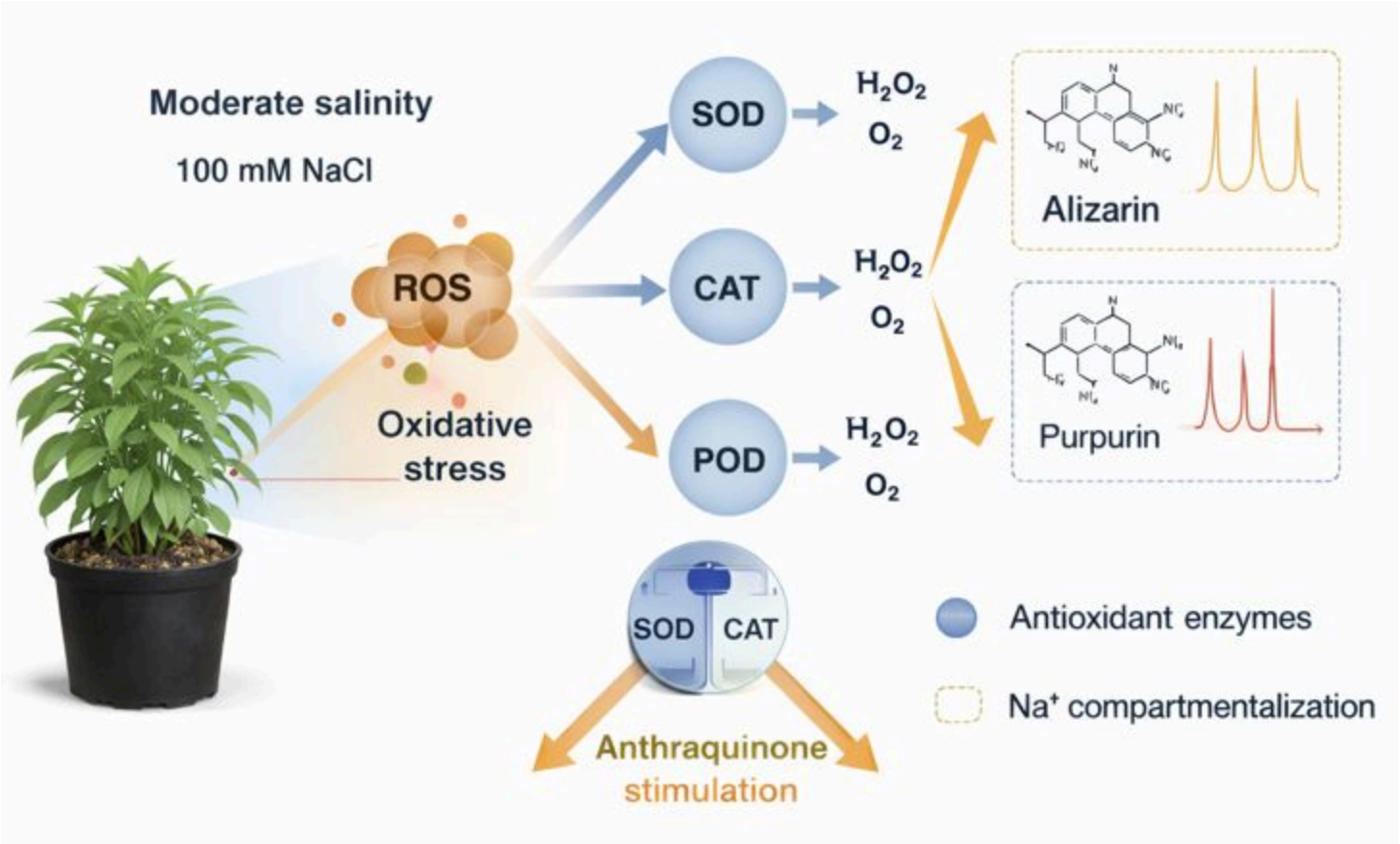

Figure 3

Salinity-induced activation of antioxidant defense and anthraquinone biosynthesis in *R. tinctorum* under moderate salt stress

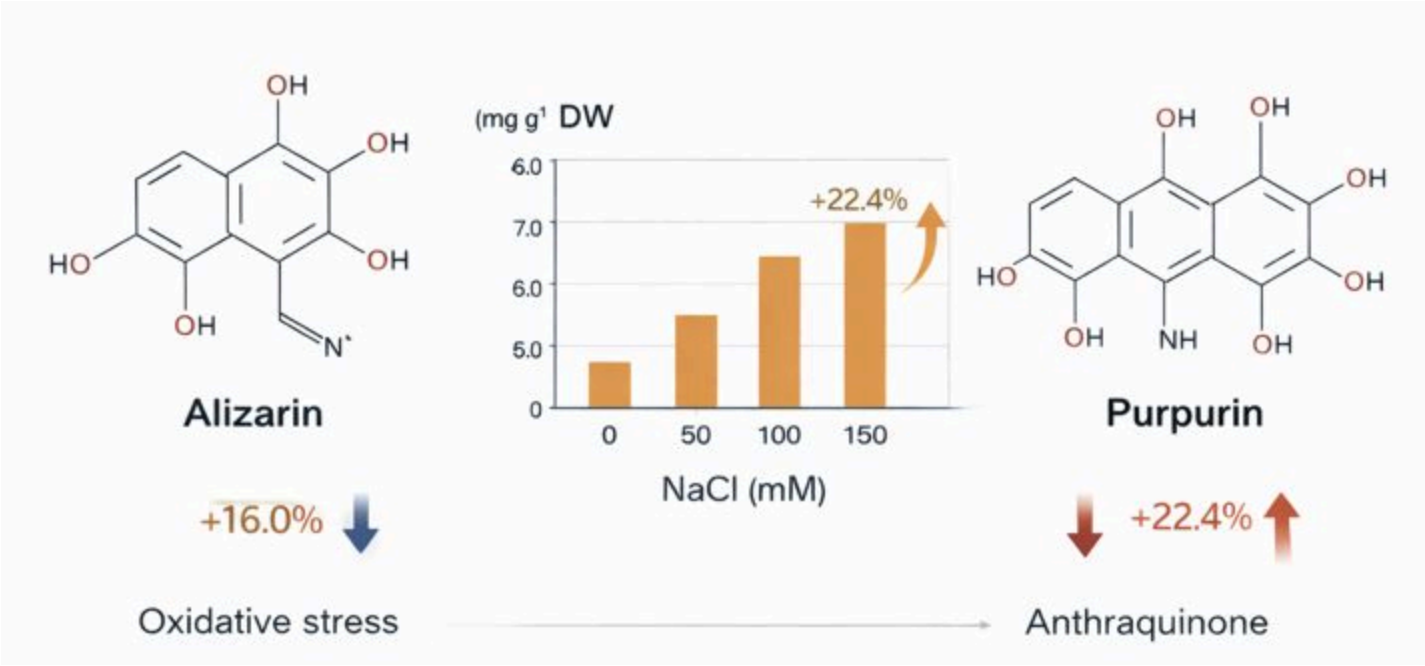

Figure 4

Salinity-induced changes in anthraquinone composition and accumulation in *R. tinctorum* roots