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Research paper

Responses of broad bean to water polluted with three solid raw dyes

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Abstract
Textile dye wastes are significant sources of pollution on a global scale. Numerous plants may survive and degrade various forms of poisons in contaminated settings. To survey broad bean (*Vicia faba* L.) tolerance to three types of dye (Acid Yellow, Acid Red, Direct Blue) at five concentrations (0, 30, 50, 70, and 90 mg/L) during three growing stages (seedling, pre-flowering, flowering), a greenhouse experiment was conducted at the University of Tabriz in 2020. The dye type did not affect the number of pods, fresh roots weight, leaf area, root length, proline content, and superoxide dismutase (SOD) enzyme activity. The control treatment had the highest pods (3.22 numbers per plant), the maximum leaf area (13380 mm^2), and the heaviest root fresh weight (9.40 g per plant). The number of pods per plant decreased by 42.05, 40.01, and 19.30 percent in the Direct Blue, Acid Red, and Acid Yellow, respectively, compared to the control. Increasing the dye concentration decreased the pod number, leaf area, and root fresh weight. SOD activity and proline content increased at the dye concentration of 90 mg/L. Tolerance to maximum dye concentration by broad bean plants and increasing SOD activity and proline content showed that this plant could survive this stressful condition. These findings allow us to propose broad bean as an efficient phytoremediation species.

Keywords: broad bean; dye concentration; dye type; proline; superoxide dismutase

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Introduction

The modern chemical industry began to form in the nineteenth century (Meyer-Thurow 1982). William Henry Perkin created the first synthetic dye, Mauveine, which was used daily and documented the appearance of these xenobiotics. Since then, hundreds of synthetic dyes have been developed and widely used. Synthetic dyes have long been recognized as significant pollutant in wastewater and the environment (Saleh *et al.* 2018). Following several decades of the industrial revolution, a severe threat of contamination and degeneration of vital water resources occurred. The presence of fabric dyes in water fields reduces sunlight dispersion, slowing the photosynthesis of algae and other aquatic herbage. They degrade the water quality due to the low dissolved oxygen concentrations, which have many harmful effects on marine ecosystems (Saratale *et al*. 2011).

In some places of the world, industrial or urban effluent is commonly used in agriculture (Sharma *et al.* 2007). At least twenty million hectares of land in fifty countries are irrigated with untreated or partially treated effluent (Hussain *et al.* 2001).

This wastewater contains a variety of micronutrients required for plant growth. However, numerous studies have been conducted to determine whether treated effluent may be appropriately utilized for crops and vegetable production. However, untreated wastewater has had a detrimental effect on qualitative and quantitative indicators. In certain dyeing operations, both chemicals and water are employed, whereas, in others, just water is used to wash and clean the fabric following chemical treatment. According to Haque (2008), around 50% of effluents are polluted and require treatment, while the remainder can be released immediately or subjected to extremely gentle treatment. Thus, there is a range of possible uses for such effluents in crop production. Additionally, dyeing is a continuous process, so, it will not impair the continuous flow of irrigation water.

Narrow-leaved cattail (*Typha angustifolia*) showed the ability to utilize Reactive Red Forty One at 100–300 mg L^{-1} and achieved a decolorization rate of approximately 60%. (Nilratnisakorn *et al.* 2007). Even lesser duckweed was presented as a substitute for Methylene Blue dye, with repositioning as the primary method (Reema *et al.* 2011). Marine plants such as *Eichhornia crassipes* can remove Direct Dark Blue 6B, Black HY, and Congo Red from the environment (Anjana and Thanga 2011). According to Bahojb *et al.* (2019), broad bean (*Vicia faba* L.) can decolorize Direct Blue at 30 mg/L concentration by increasing catalase (CAT) and peroxidase activity.

Hydroponic systems are another alternative for specialists to grow crops for experimental purposes. Plants cultivated in soilless systems have an advantage because most root systems are accessible for cleanup. Hydroponic systems are more efficient than tissue culture trials and produce more comparable results to those (Aires 2017; Verdoliva *et al.* 2021).

Plants' transformation capacities are critical since they can ultimately contaminate dyes, turn them into non-toxic compounds, and release them into the ecosystem. When confronted with xenobiotics' abiotic stresses, plants have promoted numerous systems (Page and Schwitzguébel 2009). Dye degradation and decolorization are time-consuming and costly processes; thus, developing a practical approach is critical. This experiment sought to determine the effect of dye types and concentrations on the morphological and physiological characteristics of the broad bean.

Materials and Methods

This experiment was performed as factorial based on a randomized complete block design with three replications at a greenhouse of the University of Tabriz during the 2020 growing season. The factors were dye types at three levels (Acid Yellow 17, Acid Red 27, Direct Blue 71) and dye concentrations at five levels (0, 30, 50, 70, and 90 mg/L) (Bahojb-Almasi *et al.* 2019). Three solid natural dye types (Acid Yellow 17, Acid Red 27, Direct Blue 71) were purchased from the Kimia Corporation (Tabriz, Iran) and used as model pollutants. The chemical structure of Acid Red 27 (molecular weight = 604; pKa = 6.5), Acid Yellow 17 (molecular weight = 551; pKa = 5.5), and Direct Blue 71 (molecular weight = 1029.9; pKa = 5.5), are shown in Figure 1. Broad bean seeds were

planted in 3 cm-deep pots (15 cm diameter) and then transferred to a greenhouse with natural light and photoperiod, with an average temperature of 26-28 °C during the day and 15-18 °C during the night, and average humidity of 35-40%. Twenty days after sowing, homogenous seedlings were taken carefully from the pots, washed from the adhering sand with the tap distilled water, and finally blotted gently with tissue paper. One seedling was transferred to 250-ml, wide-mouth bottles containing 200 ml of hydroponic solution (1/10 strength Hoagland solution with electrical conductivity= 1.3 ds m-1 and pH= 6.5-7). Hoagland solution contained 0.88 x 10-3 K2S04; 2 x 10-3 Ca(NO3)2; 0.25 x 10-3 KH2PO4 0.1 x 10-3 KCl; 1 x 10-5 H3BO3; 4 x 10-5 FeEDTA; 1 x 10-6 MnSO4; 1 x 10-6 ZnSO4; 1 x l-7 CuSO4; 1 x 10-8 (NH4)6MoO24. Chakmak and Marschner (1992) nourished the plants in the bottles during their growth and development stages. We chose three distinct stages of growth (seedling, pre-flowering, flowering) for adding different dyes in different concentrations in bottles. To avoid oxygen hypoxia, oxygen was injected by the pipes into bottles.

The number of pods per plant, leaf area, root length, and root fresh weight were measured at the broad bean ripening stage.

The proline content of broad bean leaves was measured according to Bates *et al.* (1973) three days after adding dye concentrations in bottles at each growth stage (Liu *et al.* 2009). The leaf sample (0.05 g) was homogenized in 5 ml of 3% aqueous sulphosalicylic acid, then centrifuged at 1500 g for 10 min. After that, 2 ml of supernatant was added to 2 ml glacial acetic acid and 2 ml acidic ninhydrin. This mixture was boiled in a Bain-marie (at 100 °C for 60 min), cooled at room temperature, and the absorbance of the upper phase was read at 520 nm using toluene as the blank. The proline content of leaves was determined using a standard curve and expressed as mg g^{-1} of fresh tissue weight.

SOD activity was measured by Beyer and Fridovich (1987), three days after adding dye concentrations in bottles at each growth stage by photochemical staining with nitrotetrazolium blue chloride (NBT). The reaction mixture contained 0.5 mL clear supernatant, 2 ml of 0.15 mM ethylene diamine-tetra-acetic acid (EDTA), 20 mM methionine, and 0.12 mM NBT. The test tubes were then placed under a 20-W fluorescent lamp for 15 min, and an identical unilluminated assay mixture served as blank. At the end of the reaction, the absorbance at 560 nm was determined.

Analysis of variance (ANOVA) was performed to test for the significance of the effects of the studied factors. However, before ANOVA, its assumptions were verified. The Duncan Multiple Range Test was used to test the differences between individual means. The data were analyzed using MSTAT-C software. The figures were drawn by Excel 2010.

Results

The dye type significantly changed the number of pods in broad bean plants transferred to dye-polluted water at the seedling and pre-flowering stages (Table 1-3). The dye concentration significantly changed leaf area, root fresh weight,

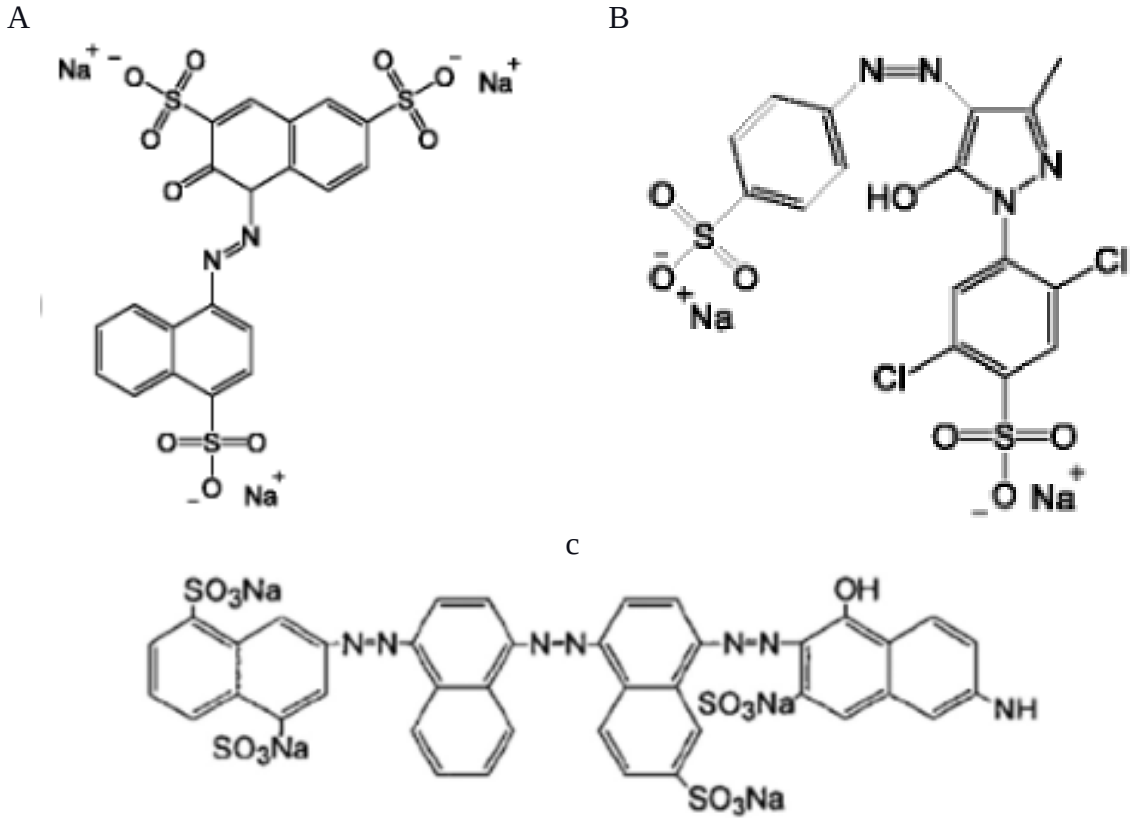

Figure 1. Chemical structure of (a) Acid Red 27, (b) Acid Yellow 17, and (c) Direct Blue 71.

Table 1. Analysis of variance for all characteristics of broad bean (*Vicia faba* L.) plants polluted by different dye concentrations at the seedling stage.

SOV	df	Number of pods	Leaf area	Root fresh weight	Root length	Proline	Superoxide dismutase
Replication	2	1.49^{ns}	296993.36^{ns}	0.09^{ns}	0.07^{ns}	0.002^{ns}	0.001^{ns}
Dye type (A)	2	3.29^{**}	30211.82^{ns}	0.16^{ns}	0.47^{ns}	0.001^{ns}	0.002^{ns}
Dye concentration (B)	4	0.20^{ns}	21165827.14^{**}	15.98^{**}	185.64^{**}	1.54^{**}	0.49^{**}
A × B	8	0.48^{ns}	54577.54^{ns}	0.17^{ns}	0.58^{ns}	0.003*	0.002^{ns}
Error	28	0.41	44900.86	0.23	0.97	0.001	0.001
CV (%)		12.39	2.87	7	2.41	2.34	2.65

ns: Not significant; * and **: Significant at 5% and 1% probability levels, respectively.

Table 2. Analysis of variance for all characteristics of broad bean (*Vicia faba* L.) plants polluted by different dye concentrations at the pre-flowering stage.

SOV	df	Number of pods	Leaf area	Root fresh weight	Root length	Proline	Superoxide dismutase
Replication	2	0.47^{ns}	7704125.09^{ns}	0.08^{ns}	4.29^{ns}	0.007^{ns}	0.023^{ns}
Dye type (A)	2	2.47^{*}	636362.42^{ns}	0.04^{ns}	3.89^{ns}	0.114^{ns}	0.023^{ns}
Dye concentration (B)	4	5.30^{*}	8064857.47^{**}	2.72^{**}	88.8^{**}	1.51^{**}	0.304^{**}
A × B	8	0.22^{ns}	324172.62^{ns}	0.01^{ns}	1.58^{ns}	0.011^{ns}	0.001^{ns}
Error	28	0.21	399461.83	0.02	2.26	0.007	0.002
CV (%)		15.2	5.83	1.57	3.82	3.26	3.162.96

ns: Not significant; * and **: Significant at 5% and 1% probability levels, respectively.

Table 3. Analysis of variance for all characteristics of broad bean (*Vicia faba* L.) plants polluted by different dye concentrations at the flowering stage.

SOV	df	Number of pods	Leaf area	Root fresh weight	Root length	Proline	Superoxide dismutase
Replication	2	0.56^{ns}	8058114.20^{ns}	0.05^{ns}	28.96^{ns}	0.11^{ns}	0.03^{ns}
Dye type (A)	2	0.09^{ns}	699657.27^{ns}	0.03^{ns}	6.16^{ns}	0.07^{ns}	0.03^{ns}
Dye concentration (B)	4	2.42^{**}	8212609.53^{**}	3.07^{**}	115.98^{**}	1.17^{**}	0.29^{**}
A × B	8	0.34^{ns}	348843.27^{ns}	0.01^{ns}	0.54^{ns}	0.005^{ns}	0.001^{ns}
Error	28	0.29	394270.02	0.02	0.39	0.01	0.002
CV (%)		15.23	5.73	1.76	1.89	4.18	2.96

ns: Not significant; * and **: Significant at 5% and 1% probability levels, respectively.

root length, proline, and SOD in broad bean plants transferred to dye-polluted water at all three growth stages (Tables 1-3). The dye type × dye concentration interaction was only significant for the proline content at the seedling stage (Tables 1 and 2).

Number of pods and leaf area

After control, the maximum pod count was achieved in the Acid Yellow dye (Figure 2). The number of pods per plant decreased by 42.05, 40.01, and 19.30 percent in the Direct Blue, Acid Red, and Acid Yellow, respectively, compared to the control (Figure 2). Increasing the dye concentration at the pre-flowering and flowering stages, reduced the number of pods (Figure 3). Increasing the dye concentration to 90 mg/L, the pod number declined to 58.72 and 68.96% after pollution at the pre-flowering and flowering stages, respectively, compared to the control (Figure 3). The dye concentration considerably reduced the leaf area at all three growth phases, with the greatest dye concentration reducing the leaf area by about 75% compared to the control (Table 4).

Weight and length of fresh roots

Increased dye concentration resulted in a decrease in the fresh weight of broad bean roots at all three growth phases (Table 4). The minimum root fresh weight at the seedling stage was obtained at 70 and 90 mg/L of dye concentration (Table 4). The increase in the dye concentration decreased root length compared to the control (Table 5), but dye type did not affect root length at any of the three stages. The pre-flowering stage exhibited the most significant root length (Table 5).

Proline and superoxide dismutase

The dye type had no significant effect on the SOD concentration in any of the three growth phases. In contrast, dye concentration significantly affected this characteristic (Table 5). The highest SOD content was detected at a dye concentration of 90 mg/L, whereas the lowest SOD content was detected at a dye dosage of 0 mg/L or the control (Table 5). The results indicated that dye concentration substantially affected the proline content in all three growth phases. Proline content was enhanced in all three developmental stages by increasing the dye concentration. The relationship between Red 90 mg/L dye concentration and the seedling proline content was determined to be the highest (Figure 4). The highest proline content was observed during the pre-flowering and flowering stages when the dye concentration was 90 mg/L. (Figure 5).

Table 4. The leaf area and root fresh weight of broad bean (*Vicia faba* L.) plants were polluted by dye concentrations at the seedling, pre-flowering, and flowering stages.

	Leaf area (mm^2)			Root fresh weight (g)		
Dye concentration	Seedling	Pre-flowering	Flowering	Seedling	Pre-flowering	Flowering
0 $mg.L^{-1}$	13380± 366.25a	11890±403.65a	12000±401.58a	8.759±0.15a	9.273±0.07a	9.402±0.08a
30 $mg.L^{-1}$	12260±445.11b	11660±303.82a	11790±322.54a	7.596±0.28b	8.931±0.08b	9.027±0.1b
50 $mg.L^{-1}$	11260±459.68c	10840±301.98ab	11030±296.53ab	6.667±0.16bc	8.512±0.05c	8.663±0.05c
70 $mg.L^{-1}$	10370±437.23d	9997±366.63b	10120±360.54b	5.611±0.27c	8.186±0.05d	8.273±0.06d
90 $mg.L^{-1}$	9479±280.37e	9787±202.78b	9883±209.48b	5.694±0.12c	7.911±0.04d	7.993±0.04e

Different letters in each column indicate significant differences among means according to Duncan Multiple Range Test at $p \leq 0.05$.

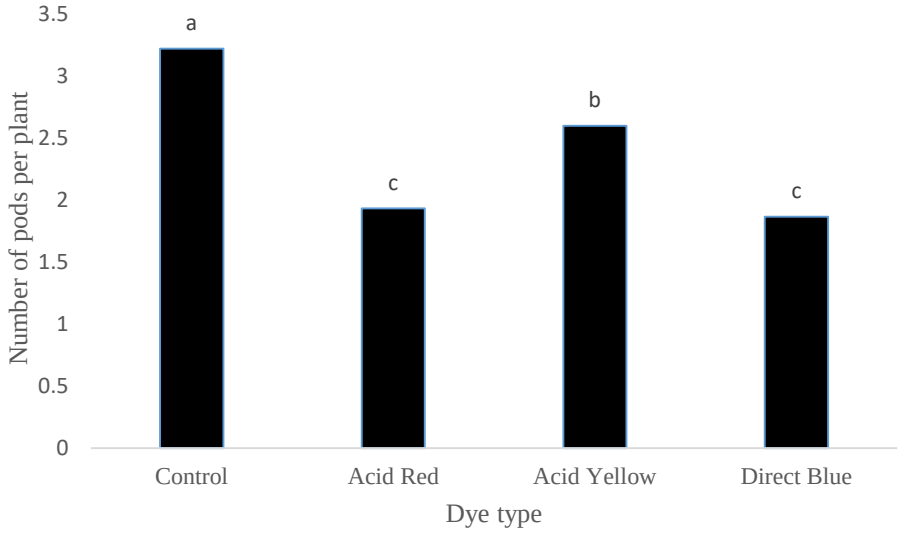

Figure 2. The effect of dye type on the pod number of broad bean (*Vicia faba* L.) plants polluted by different dye concentrations at the pre-flowering stage. Different letters indicate significant differences among means according to Duncan Multiple Range Test at $p \leq 0.05$.

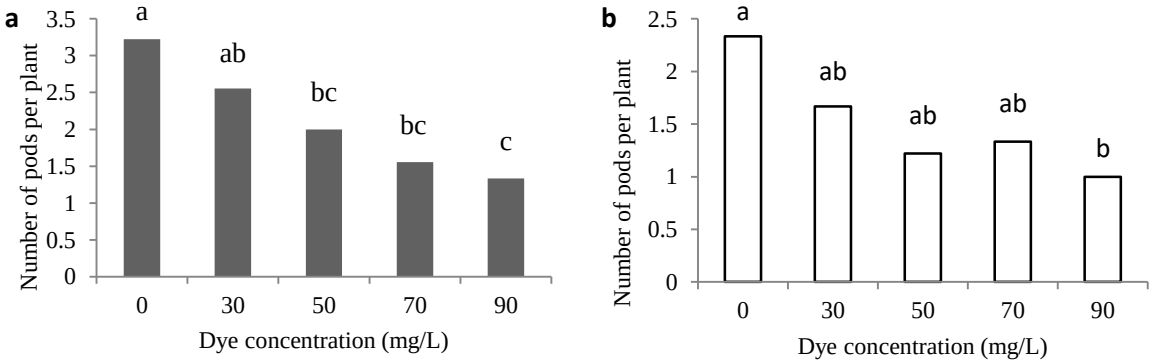

Figure 3. The effect of dye concentration on the number of pods of broad bean (*Vicia faba* L.) plants polluted by dye concentrations at pre-flowering (a) and flowering (b) stages. Different letters indicate significant differences among means according to Duncan Multiple Range Test at $p \leq 0.05$.

Table 5. The effect of dye concentration on superoxide dismutase (SOD) and root length of broad bean (*Vicia faba* L.) polluted by dye concentrations at seedling, pre-flowering, and flowering stages.

	SOD (EU g^{-1} FW)			Root length (cm)		
Dye concentration	Seedling	Pre-flowering	Flowering	Seedling	Pre-flowering	Flowering
0 $mg.L^{-1}$	1.117±0.04e	1.158±0.01d	1.123±0.01c	61±1.07a	67.67±0.66a	69.56±0.7a
30 $mg.L^{-1}$	1.351±0.08d	1.234±0.02d	1.211±0.02c	57.44±0.86b	59.67±0.52b	60.56±0.57b
50 $mg.L^{-1}$	1.449±0.04c	1.382±0.02c	1.352±0.01b	55.44±0.78b	55.33±0.35c	56.11±0.59c
70 $mg.L^{-1}$	1.56±0.08b	1.493±0.02b	1.464±0.02a	50.67±1.54c	50.78±0.49d	52.11±0.47d
90 $mg.L^{-1}$	1.742±0.04a	1.608±0.02a	1.559±0.02a	46.33±1.39d	48.67±0.46e	49.44±0.5e

Different letters in each column indicate significant differences among means according to Duncan Multiple Range Test at $p \leq 0.05$.

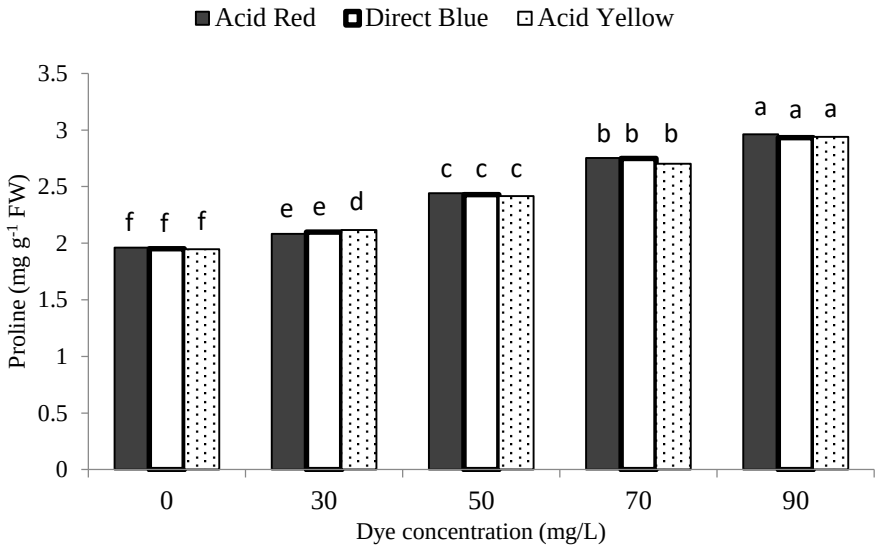

Figure 4. The influence of dye type and concentration on the proline content of the broad bean (*Vicia faba* L.) plants polluted by the dye concentrations at the seedling stage. Different letters indicate significant differences among means according to Duncan Multiple Range Test at $p \leq 0.05$.

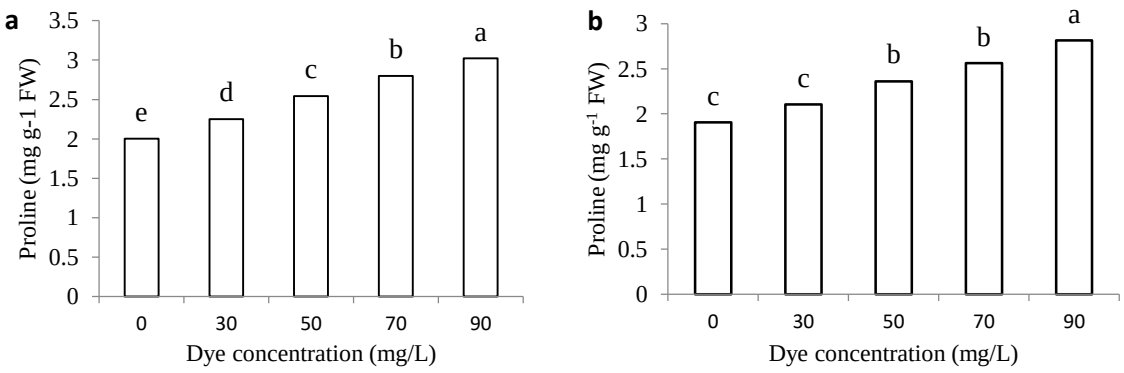

Figure 5. The effect of dye concentration on the proline content of broad bean (*Vicia faba* L.) plants polluted by dye concentrations at pre-flowering (a) and flowering (b) stages. Different letters indicate significant differences among means according to Duncan Multiple Range Test at $p \leq 0.05$.

Discussion

Increasing dye concentration can be toxic for the plant, so high dye concentration has negatively influenced the number of pods and other characteristics. Wastewater changes the pH and EC of the water, potentially shifting the rhizosphere into a saline environment and slowing plant growth (Raziuddin *et al.* 2011). According to our findings, the treated Acid Yellow resulted in the maximum number of pods per plant after dye pollution at the seedling stage (Figure 2). These results agreed with Safi-naz and Shaaban (2015), who reported that the application of treated wastewater enhanced the yield and yield components of sunflowers. The absorption of certain elements found in dyes or wastewaters may increase yield and yield components. However, according to Balseiro-Romero *et al.* (2016), increasing oil pollution inhibited the shoot and root growth of yellow lupin (*Lupinus luteus*).

Antioxidant enzymes reduce reactive oxygen species (ROS) (Kurutas 2016) and protect cells against their toxicity (Dazy *et al.* 2012). Increased free radicals such as superoxide indicate that the plant is experiencing environmental stress (Hassannejad and Porheidar-Ghafarbi 2017). Plants activates SOD, CAT, and glutathione S-transferase in response to stressful conditions to reduce ROS produced by xenobiotics such as textile dyes (Varhsney *et al.* 2012). According to Bahojb-Almasi *et al.* (2019), broad bean plants can alleviate oxidative stress by increasing peroxidase and CAT activities in response to different textile dyes. The rise in the SOD activity identified in this study has also been detected in other plants, including lettuce (*Lactuca sativa*) (Gusman *et al.* 2013) and Eichhornia (*Eichhornia rassipes*) (Andrade *et al.* 2016).

Accumulation of proline is necessary to maintain the bloated state of plant cells (Hassannejad and Porheidar-Ghafarbi 2017). When plants are exposed to environmental stress, proline acts as a signaling molecule to restore cell viability (Liang *et al.* 2013). The soluble sugar accumulation in the plant leaves has the same function as proline in osmotic regulation, leading to the osmotic adjustment and increased tolerance to environmental stresses (Liu *et al.* 2011; Abdoli and Gassemi-Golezani 2021).

Any reduction in the plant water uptake leads to a reduction in the leaf area and size and consequently decreases the plant's dry matter. It is believed that increasing the dye concentration imposes stress to plants and decreases the leaf area. Azmat *et al.* (2009) reported a reduction in the leaf area by Pb poisoning. However, according to Jolly *et al.* (2008), wheat plants irrigated with the treated wastewater grow 2.5-5% higher, and their leaf diameter, root dry weight, seed number, and seed weight increased compared to the control.

The increase in dye concentration decreased the root fresh weight and length in the broad bean plants. However, some studies indicated that some elements in the wastewater result in root elongation. The hairy roots of French marigold can decolorize Reactive Red 198 (Patil *et al.* 2009). Patil *et al.* (2009) found that hairy roots of tobacco, Surattense nightshade, and Indian nightshade decolorized 95, 96, and 86 percent, respectively, in 20 mg L-1 Reactive Red 198 after 12-30 days.

In areas of low dye concentration, the amount of dye is insufficient to interfere with the essential biological functions of broad bean plants. The plant activates tolerance mechanisms and attempts to decolorize the area. When the concentration reaches 70 mg/L and 90 mg/L, the dye concentration becomes hazardous and endangers the plant. As a result, the ability of plants to absorb

dye and degrade is reduced. According to Khataee *et al.* (2012), duckweed (*Lemna minor*) dramatically decolorized Acid Blue 92. Other research in the hydroponic medium has demonstrated that the dyes Acid Blue 92, Basic Red 46, and Reactive Blue 19 were successfully eliminated from water velvet (*Azolla pinnata*) (Khataee *et al.* 2013), watercress (*Nasturtium officinale*) (Torbati *et al.* 2014), and lesser bulrush (*Typha angustifolia* L.)(Mahmood *et al.* 2014).

Conclusions

Increasing dye concentration decreased leaf area, root fresh weight, and pod number. However, it affected root fresh weight and length and proline content after plant pollution at the seedling, pre-flowering, and flowering stages. The results showed that the dye type significantly affected the pod number only after plants transferred at the pre-flowering stage, indicating that Acid Yellow increased pod number compared to other dye types. We concluded that while dye concentration imposes stress on the broad bean plants, the plants can survive by increasing the proline content and SOD activity. These findings suggested that the broad bean has tolerance to dye stress due to the ability to increase antioxidant activities.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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پاسخ‌های باقلا به آب آلوده به سه رنگ خام جامد

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چکیده

پسماندهای رنگ نساجی منابع مهم آلودگی زیست محیطی در مقیاس جهانی هستند. گیاهان متعددی ممکن است تحت این شرایط زنده مانده و شکل های مختلفی از سموم را در محیط‌های آلوده تجزیه کنند. به منظور بررسی تحمّل باقلا (Vicia faba L.)به سه نوع رنگ مختلف (اسیدی زرد، اسیدی قرمز، آبی مستقیم) در پنج دز مختلف (۰، ۳۰، ۵۰، ۷۰ و ۹۰ میلی‌گرم بر لیتر) در طول سه مرحله رشدی (گیاهچه‌ای، قبل از گل‌دهی، گل‌دهی)، آزمایشی گلخانه‌ای در دانشگاه تبریز در سال ۱۳۹۸ انجام شد. نوع رنگ اثر معنی‌داری روی تعداد نیام، وزن تر ریشه، سطح برگ، طول ریشه، محتوی پرولین و میزان فعالیت آنزیم سوپراکسیددسموتاز نداشت. تیمار شاهد بیشترین تعداد نیام (۳,۲۲ عدد در بوته)، بیشترین سطح برگ (۱۳۳۸۰ میلی‌مترمربع) و سنگین‌ترین وزن تر ریشه (۴۰,۹ گرم در بوته) را داشت. تعداد نیام در هر گیاه به ترتیب ۴۲,۰۵، ۴۰,۰۱ و ۱۹,۳۰ درصد در آبی دایرکت، قرمز اسیدی و زرد اسیدی نسبت به شاهد کاهش یافت. افزایش غلظت رنگ باعث کاهش تعداد نیام، سطح برگ، و وزن تر ریشه شد. در غلظت ۹۰ میلی‌گرم در لیتر، فعالیت سوپراکسیددسموتاز و محتوی پرولین افزایش یافت. تحمّل گیاهان باقلا به حداکثر غلظت رنگ و افزایش فعالیت سوپراکسیددسموتاز و محتوی پرولین نشان می‌دهد که این گیاه می‌تواند در این شرایط تنش‌زا سالم بماند. بر اساس این یافته‌ها می‌توان باقلا را به عنوان یک گونه برخوردار از توانایی گیاه -پالایی معرفی کرد.

واژه‌های کلیدی: باقلا؛ پرولین؛ سوپراکسیددسموتاز؛ غلظت رنگ؛ نوع رنگ