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PHYTOTOXIC EFFECTS OF SAFFLOWER YELLOW EXPOSURE ON SEED GERMINATION AND EARLY SEEDLING GROWTH OF CANOLA (*BRASSICA NAPUS* L.)

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Abstract

The aim of current investigation was to determine phytotoxic effects of safflower dye (safflower yellow) on seed germination and early seedling growth of canola (*Brassica napus* L.) cv. Rainbow. Safflower yellow was extracted in autoclaved distilled water and was applied at various concentrations (0.312%, 0.625%, 1.25%, 2.5% and 5%) to canola seeds in petri dishes under *axenic* conditions. Phenolics content was detected in dye solutions and was maximum (53 mg gallic acid/g extract) in 5% dye solution. The growth rate of canola was decreased with the increase of safflower dye concentration ($p<0.05$). Maximum inhibition in the activity of enzyme lipase in germinating seeds, germination (%), root and shoot length, root and shoot weight was exhibited by 5% dye solution. In response to the application of safflower dye, canola seedlings accumulated endogenous phenolics. Relative water content and photosynthetic pigments of seedling were reduced to a maximum level by 5% and 2.5% dye solutions. It was inferred that safflower dye exhibited phytotoxic effects at higher concentration on canola. The findings of the current investigation will help in safe disposal of safflower dye found in industrial effluents.

Key words: Key words: Phytotoxicity, Safflower dye, Phenolics content, Canola

Introduction

In the past, dyes were derived by boiling flowers, fruits, leaves and other parts of plants in water and submersing fabrics in a dye bath (Joseph, 1977). The invention of synthetic dyes has reduced use of natural dyes because synthetic dyes are easy to be used, fast in action and are commercially available. However, in several accidents synthetic dyes were recognized toxic and mutagenic (Papita & Siddhartha, 2008). Presently, dyes of plant origin have once again emerged as potential alternatives of synthetic dyes (Shakhatreh, 2013, Jan *et al*., 2011a). However, all of these natural dyes are not ecofriendly. There is possibility of the presence of heavy metals and other toxic materials in natural dyes which can adversely affect the existing flora and fauna. Previous studies have proved that higher concentration of natural dye extracted from rhizomes of turmeric inhibited seed germination and growth of maize (Chauhan *et al*., 2013). Therefore, prior to use, natural dyes need to be tested for toxicity (Samanta & Agarwal, 2009; Jan *et al*., 2011b; Gilani *et al*., 2010). The textile effluents contain a large amount of untreated dye stuffs which is released into neighboring aquatic ecosystems in the form of sludge. The final disposal of this sludge remains a challenge, and its ecotoxicological assessment is important for minimizing its environmental impacts (Chia & Musa, 2014). Safflower is cultivated in more than 60 countries in the world. It has been grown for centuries as a food coloring agent, vegetable oil and for preparation of textile dye (Esendal, 2001). The carthamidin (water soluble yellow pigment) is extracted from petals and is utilized in food products and for other purposes (Machewad *et al*., 2012).

Canola (*Brassica napus* L.), chosen for this study, is an important oilseed crop of Pakistan. The yield and quality of this plant varies with physiological state (Ullah *et al*., 2012). Moreover, this plant is considered as a hyper accumulator of heavy metals and other contaminants (Lassoued *et al*., 2013). Keeping in view the industrial applications of safflower dye and chances of its flow in irrigated water, current investigation was aimed to determine phytotoxic effects of safflower yellow on seed germination and seedling growth of canola.

Materials and Methods

Collection of safflower petals: Safflower (*Carthamus tinctorious* L.) petals were collected from field grown plants in the Department of Plant Sciences, Quaid-i-Azam University Islamabad, air dried for 10d and ground finely in an electric grinder.

Extraction of safflower yellow: The yellow dye in petals was extracted by placing 10g powdered safflower petals in 100 mL autoclaved distilled water and filtered once a day. This process was repeated several times until no yellow dye was left in the filtrate. The 10% stock solution was further diluted to make 5%, 2.5%, 1.25%, 0.625% and 0.312% dye solutions. The various solutions formed were stored in sealed plastic bottles at 4^{o}C for future use in the experiment.

Seed material and growing conditions: Petri dish experiment was carried out in growth chamber of Department of Botany University of Science and Technology Bannu. Seeds of canola (*Brassica napus* L.) cv. Rainbow were obtained from National Agriculture Research Centre Islamabad Pakistan. Seeds of uniform size were selected and surface sterilized with 0.2% solution of mercuric chloride for 2-3 min., with shaking. The seed were subsequently washed thoroughly 3-4 times with autoclaved distilled water and were arranged (20 seeds per petri dish) on filter papers in a laminar flow under sterilized

conditions. The calculated quantity of dye solution was applied to autoclaved filtered papers in Petri dishes. The controlled treatment was made by soaking filter papers in autoclaved distilled water. The petri dishes were covered and placed in a growth chamber at 24°C during 14h light period and 22°C during the 10h dark period. Germinated seeds in all treatments were counted on daily basis for four consecutive days. At 7th day of the experiment, the seedlings were harvested for further analysis.

The following treatments were made:

Treatments	Concentrations used
Control	**Plants supplied with autoclaved distilled water**
T1	Plants supplied with 5% dye solution
T2	Plants supplied with 2.5% dye solution
T3	Plants supplied with 1.25% dye solution
T4	Plants supplied with 0.625% dye solution
T5	Plants supplied with 0.312% dye solution

Total phenolic compounds analysis: Determination of total phenolics content was based on the Folin-Ciocalteau method (Adom & Liu, 2002). Extract (125 µL) were mixed with distilled water (500 µL) and were subsequently added with 125 µL of Folin-Ciocalteau reagent. The mixture was allowed to stand for 6 min. The volume was raised to 3 mL by the addition of 1.25 mL 7% aqueous sodium carbonate and 1 mL distilled water. The absorbance was measured at 760 nm using a spectrophotometer (Hitachi's U-5100 Japan) after reacting for 90 min in dark. Deionized water was used as blank. The measurements were compared to a standard curve prepared by using various concentrations of gallic acid solution. The concentration of total phenolics was expressed as mg gallic acid equivalents / g sample.

Parameters studied: The following parameters were studied.

Seed germination (%): Seed germination (%) was determined according to following formula:

$$\text{Germination \%} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds grown}} \times 100$$

Germination index (GI): It is the estimate of germination rate of seedlings and was calculated as described in association of official seed analysis (Anon., 1983).

GI = No: of seeds germinated at first count +…….No. of seeds germinated at final count / Days of first count +……days of final count

Germination rate index (GRI): GRI for each treatment and replication was calculated as germinate index divided by germinate percentage.

$$\text{GRI} = \frac{\text{Germination index}}{\text{germination percentage}}$$

Determination of lipase activity: Extraction for lipase was carried out according to method of Lin & Huang (1983). The germinating seeds (at 72 h of germination) were homogenized for 10 min in a grinding medium (4 mL/g fresh weight). The grinding medium was composed of 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM MgCl 2,2 mM DTT, and 0.15 M tricine {N-[tris(hydroxymethyl)methyl]- glycine} buffer adjusted to pH 7.5 with KOH. The homogenate was centrifuged for 30 min at 10,000 *g* (Labofuge 400e Heraeus Instruments Germany). After centrifugation, the mixture showed separation into three layers: a fat layer, a supernatant layer, and a pellet. The supernatant layer was taken and used for the assay of lipase activity. The lipase activity was determined as per method of Haas *et al*., (1995). The reaction was initiated by adding 0.58 mL of the supernatant to 5.0 mL of substrate emulsion containing 20 µM $CaCl_2$. The pH (7.5) was maintained by drop wise addition of KOH (0.1 M). The lipase activity was expressed as the release of 1 µg FA/µg protein/min

After harvest, root length and shoot length were measured by using common measuring tape after harvest of the plants (7 days after germination).

Determination of relative water content of seedlings: Relative water content (RWC) of seedlings was estimated by using the method of Gao (2000).

$$\text{RWC (\%)} = \frac{\text{(W-DW)}}{\text{(TW-DW)}} \times 100$$

W represents sample fresh weight; TW represents sample turgid weight; DW represents sample dry weight.

Determination of chlorophyll and carotenoids content of seedlings: The chlorophyll and carotenoids content of leaves were determined by the method of Arnon (1949) as modified by Kirk (1968).

Statistical analyses: Experiment was carried out in complete randomized design (CRD) with three replicates for each treatment. The data were analyzed statistically by analysis of variance technique (one way ANOVA) and treatment means were compared by least significant differences (LSD) test (Steel & Torrie, 1984). Coefficient of correlation was determined using Student Statistix (version 8.1 USA).

Results and Discussion

Phytotoxicity is very old component of agriculture but it is described as allelopathy by Molisch, (1937). A number of phytotoxic substances or allelochemicals have been found in different plant species and in different tissues of plants (Ashrafi *et al*., 2008; Gilani *et al*., 2007). Phenols are the most abundant of such substances affecting seed germination, seedling growth and cell division (Khan *et al*., 2011). Therefore, various safflower yellow dye solutions were screened for their content of total phenolics. Maximum phenolic content (53 mg gallic acid / g extract)was recorded in 5% dye solution followed by 2.5% dye solution (44 mg gallic acid / g extract) and 1.25% dye solution (19 mg gallic acid / g extract) respectively (Fig. 1). Previous studies have shown that greater portion of plant based natural dyes is composed of phenolics (Mirjalili & Karimi, 2013).

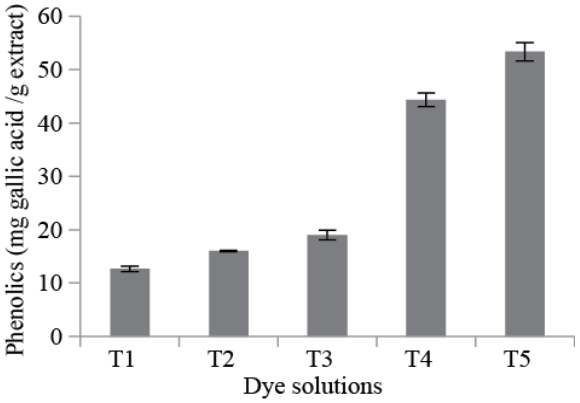

Fig. 1. Total phenolics content of safflower yellow solutions.
Treatments: T1- 0.312% dye solution, T2-0.625% dye solution, T3-1.25% dye solution, T4-2.5% dye solution, T5- 5% dye solution

Phytotoxicity occurs when some substance or a mixture of substances is applied to plants and the target plants suffer some sort of negative effects (Khan *et al*., 2014). Seed germination (%) and activity of enzyme lipase of germinating seeds was significantly inhibited by safflower dye at $p<0.05$. The inhibitory effects of dye on seed germination (%) were higher at 5% concentration. The T5 (5% dye solution) exhibited 19% significant reduction in seed germination (%) as compared with control (Fig. 2). The T4 (2.5% dye solution) showed 14% reduction as compared with control. There were observed non-significant effects of dye on seed germination index and germination rate index. The greater anti germination activity of the dye on canola seeds at higher concentration can be linked with presence of higher quantity of phenolics. The inhibitory effects of phenolic compounds on early development and growth are not surprising because previous authors have described them as effective anti germinating and growth inhibitors (Mizutani, 1999). Baleroni *et al*., (2000) demonstrated that a phenolic compound, p-coumaric acid significantly affected seed germination and growth of canola. The inhibition in seed germination was due to arrest of the lipid mobilization. During current studies reduction in activity of enzyme lipase was evident in response to higher concentration of dye. Allelo chemicals exhibit diverse chemical structures and actions. However, mechanisms involved behind their effects on biochemical and physiological processes of target plants have not been investigated thoroughly (Hussain *et al*., 2010). Lipolytic enzymes are crucial for the biological turnover of lipids during seed germination. The higher activity of lipase is therefore reported during germination of oil seeds (Ejedegba *et al*., 2007; Gadge *et al*., 2011). Therefore, suggested mechanism for the inhibition of seed germination by higher concentration of dye might be correlated with the disruption of enzyme lipase activity involved in hydrolysis of storage lipids necessary for canola seed germination.

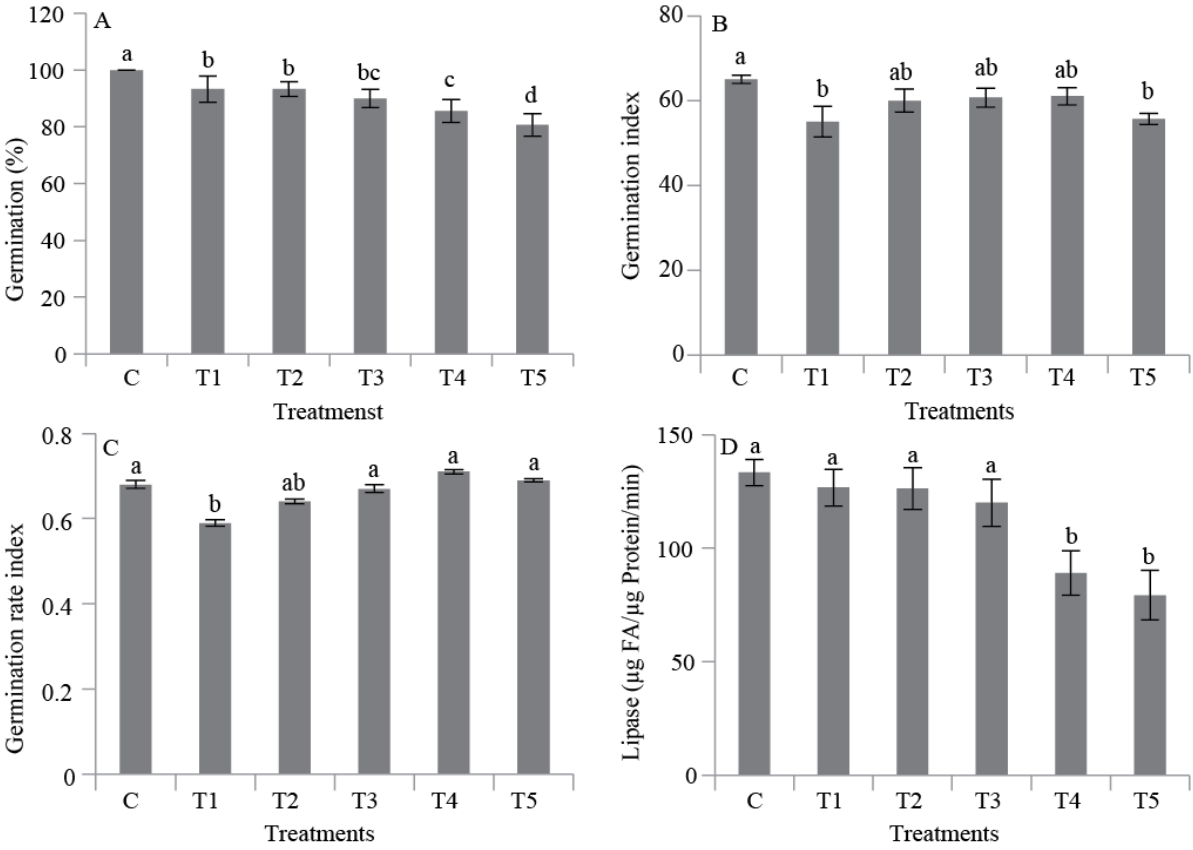

Fig. 2. Effect of Safflower Yellow on (A) seed germination % (B) germination index (C) germination rate index and (D) activity of enzyme lipase.
Treatments: T1- 0.312% dye solution, T2-0.625% dye solution, T3-1.25% dye solution, T4-2.5% dye solution, T5- 5% dye solution

Allelochemicals suppress the mitotic activity of young cells, resulting in inhibition of seedling growth and the effect is dose dependant (Rice, 1984). Results of the present studies showed that higher concentration of dye (T5) was significantly more effective in reducing shoot and root length and seedling fresh and dry weight as compared with control (Fig. 3). These results are in agreement with previous finds of Liu & Lovett (1993) that barley extracts inhibited growth of other plant species.

Effects of safflower yellow on total phenolics content of canola seedlings were stimulatory. However, the seedling relative water content, chlorophyll and carotenoids content was significantly reduced at higher concentration of the safflower yellow as compared with control (Fig. 4). There was observed negative correlation between total phenolics content and leaf relative water content. Phenolic acids cause water stress in plants (Barkosky & Einhellig, 2003) which might be responsible for reduction in leaf relative water content. Safflower yellow was rich source of phenolic compounds and its application enhanced endogenous level of phenolic compounds in canola plants. These results are in agreement with previous studies that cucumber plants supplemented with phenolics exhibited higher content of endogenous phenolics (Muzaffar *et al.,* 2012). Inhibitory effects of dye on chlorophyll content might be because those allelochemicals reduce chlorophyll content in target plants by inhibiting chlorophyll synthesis, stimulating the degradation of chlorophyll or by both the processes (Heidarzade *et al*., 2012).

There was negative and significant correlation (r= -0.884) between total phenolics content of the dye and seed germination (%) (Table 1). Similarly there was negative and significant correlation between total phenolics content and shoot length (r= -0.7862). The root length was also negatively significantly correlated (r=-0.9335) with total phenolics content. There was observed positive and significant correlation (r=0.6014) between shoot length and shoot fresh weight. Seed germination index was positively significantly correlated with germination rate index (r=0.6028). The correlation of seed germination (%) with germination index was also positive and significant (r=0.5741).

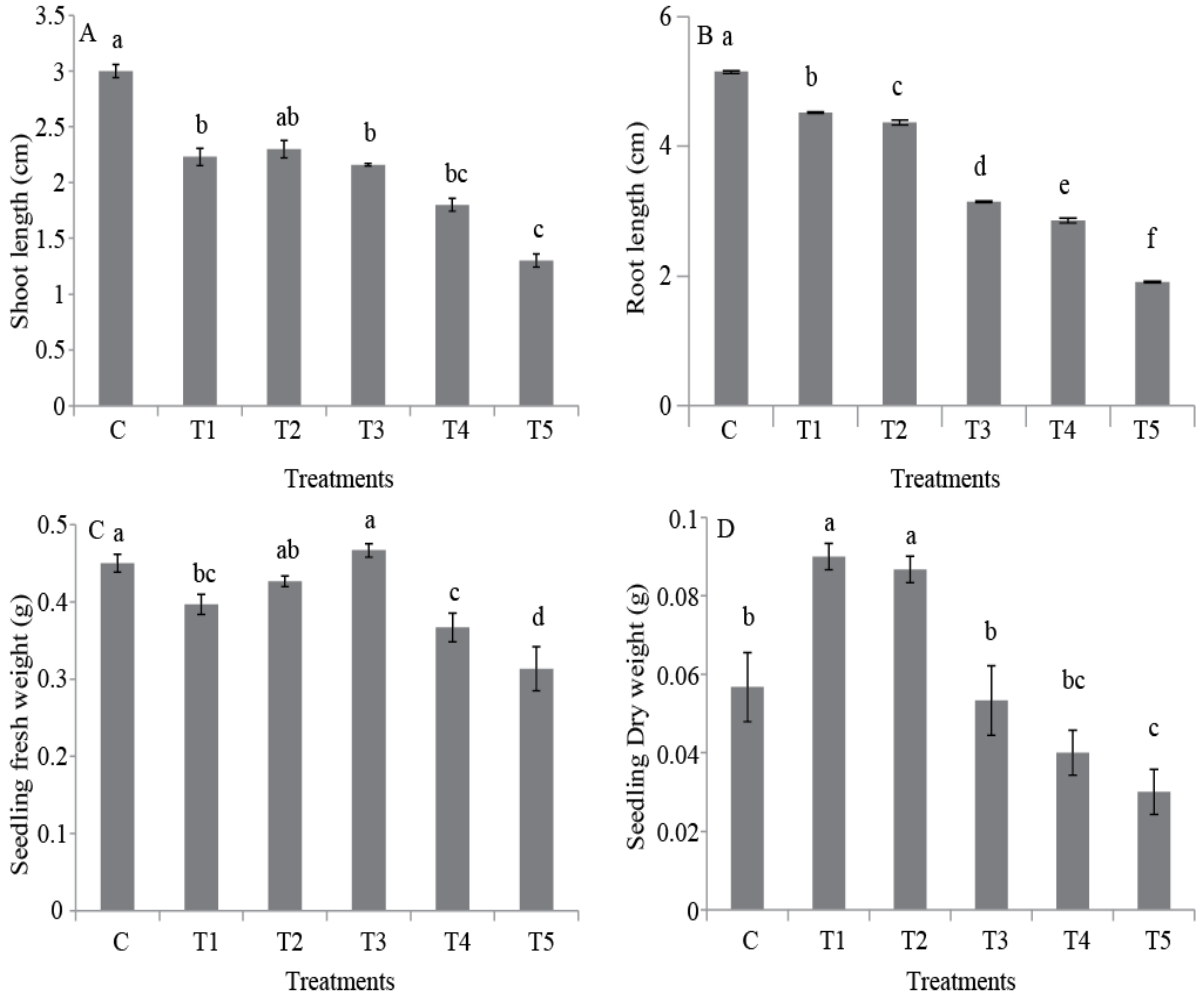

Fig. 3. Effect of Safflower Yellow on (A) shoot length (B) root length (C) seedling fresh weight (D) seedling dry weight.
Treatments: T1- 0.312% dye solution, T2-0.625% dye solution, T3-1.25% dye solution, T4-2.5% dye solution, T5- 5% dye solution

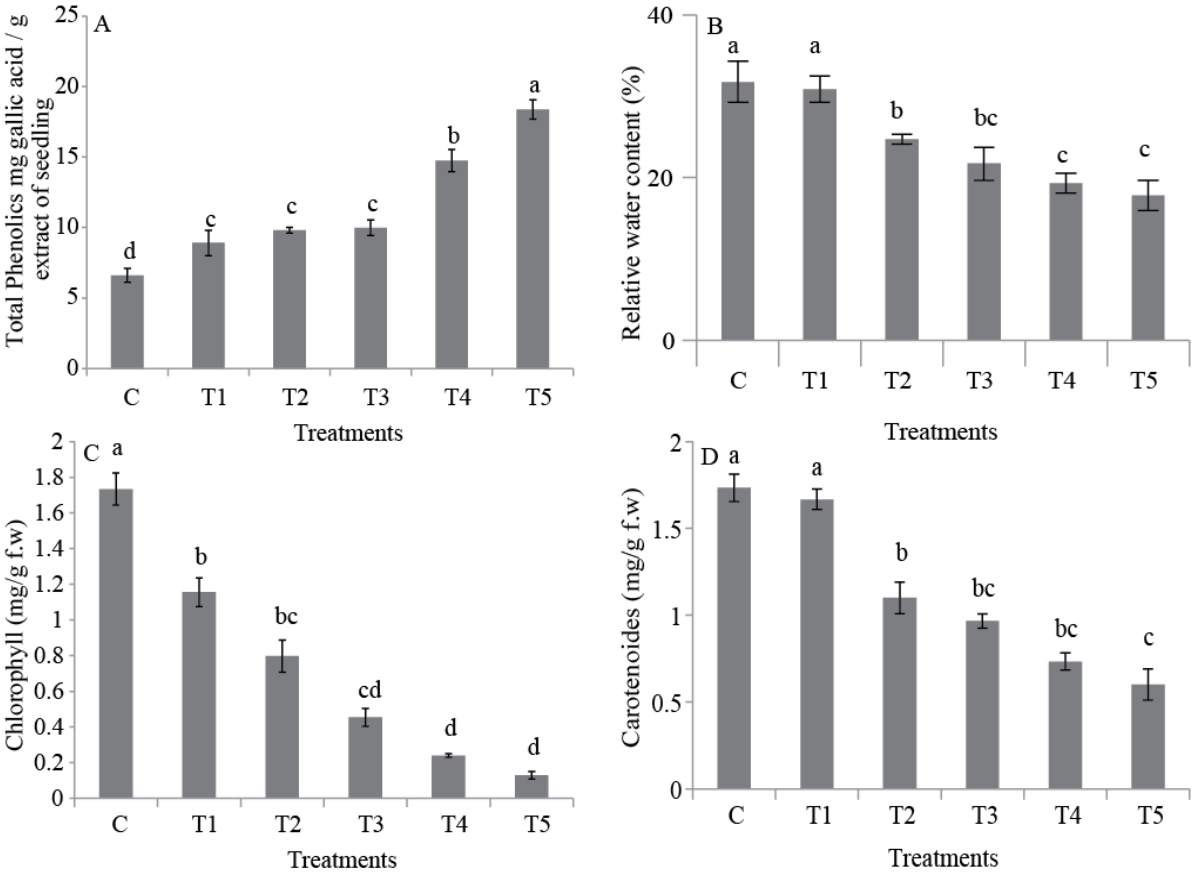

Fig. 4. Effect of Safflower Yellow on (A) total pehnolics content (B) seedling relative water content (C) chlorophyll content (D) Carotenoids content
Treatments: T1- 0.312% dye solution, T2-0.625% dye solution, T3-1.25% dye solution, T4-2.5% dye solution, T5- 5% dye solution

Table 1. Correlations (Pearson).

	DW	FW	GI	GRI	Phenolics	RL	RWC	SG
FW	0.4597							
P-VALUE	0.0549							
GI	-0.0199	0.4042						
	0.9375	0.0962						
GRI	-0.3715	-0.2690	0.6028					
	0.1290	0.2805	0.0081					
Phenolics	-0.6504	-0.7923	-0.3745	0.4310				
	0.0035	0.0001	0.1258	0.0741				
RL	0.6901	0.6258	0.3870	-0.3934	-0.9335			
	0.0015	0.0055	0.1127	0.1063	0.0000			
RWC	-0.4719	0.5542	0.4548	0.0582	-0.2377	0.0415		
	0.0480	0.0170	0.0579	0.8185	0.3422	0.8700		
SG	0.3456	0.7519	0.5741	-0.3067	-0.8840	0.8621	0.4883	
	0.1601	0.0003	0.0127	0.2158	0.0000	0.0000	0.0398	
SL	0.5405	0.6014	0.4557	-0.2658	-0.7862	0.7914	0.1785	0.8141
	0.0206	0.0083	0.0574	0.2864	0.0001	0.0001	0.4785	0.0000

SG: Seed germination (%), GI: Germination index, GRI: Germination rate index, FW: Fresh weight, DW: Dry weight, SL: Shoot length, RL: Root length

Conclusion

Safflower yellow possessed higher content of phenolic compounds. The higher phenolic contents were negatively correlated with seed germination (%), seed germination rate index, shoot and root lengths, shoot and root fresh and dry weights. Reduction was recorded in activity of enzyme lipase of germinating seeds, relative water content and photosynthetic pigments of canola seedlings at higher concentration of dye solution. It was inferred that higher concentration of safflower yellow exhibits phytotoxic effects on canola plants. Further, investigation on other plants particularly weed species will lead to the formulation of some novel natural herbicides.

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