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Effect of different concentrations of cadmium metal and cattle manure on morph-physiological attributes of spinach (*Spinacia oleracea* L.) and fenugreek (*Trigonella foenum gracum* L.) varieties

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Abstract

The present study investigates the toxicological effects of cadmium stress on two different varieties of Spinach (*Spinacia oleracea*) and fenugreek (*Trigonella foenum graecum* L). Additionally, it explores the impact of cattle manure application on the morpho-physiological traits of cadmium-stressed spinach and fenugreek. The study employed a completely randomized design with three replicates assigned to each treatment. Two cadmium concentrations (35 mg L^{-1} and 70 mg L^{-1}) were applied to both leafy vegetables two weeks after sowing. After 10 dwere of stress, the impact of cadmium stress on the biomass attributes (root length, shoot length, root fresh weight, shoot fresh weight, root dry weight, shoot dry weight, number of leaves, and leaf area) of fenugreek and spinach varieties was assessed. The results indicate that cadmium stress decreased these attributes, with the maximum decrease observed under 70 mg L^{-1} Cd stress compared to the control and 35 mg L^{-1} Cd stress. Cattle manure demonstrated a more pronounced positive influence on these parameters, particularly at the 35 mg L^{-1} concentration, compared to the 70 mg L^{-1} concentration in both species. Thus, the use of animal manure shows promise as a treatment against heavy metal contamination.

Keywords: Cd, stress, heavy metals, spinach, fenugreek, animal manure, biochemical attributes

Introduction

Spinach (*Spinacia oleracea* L.) possesses a remarkable capacity to absorb heavy metals owing to its extensive surface area (Naz *et al*., 2013; Azad *et al*., 2017). Despite its short growth cycle, typically requiring only 30-35 days from sowing to harvest (Ninfali & Bacchinioca, 2004), spinach is highly esteemed by nutritionists for its rich nutritional profile. This leafy green vegetable is packed with essential nutrients such as iron and vitamin B9 (folate), crucial for preventing anemia and recommended for pregnant women by the World Health Organization (Benoist, 2008; Domingo *et al*., 2017), as well as an array of minerals and mineral salts (Chopra & Pathak, 2013; Waseem & Nadeem, 2001). The optimal sowing periods for spinach are late winter or early spring (Krorup & Moreira, 1998; Vossen, 2004), ensuring ideal growing conditions for its lush, dark green leaves, the part of the plant most sought after for consumption. Interestingly, while spinach leaves are rich in nitrogen, they absorb relatively little nitrogen from the soil (Smolders *et al*., 1993; Biemond *et al*., 1996), highlighting the unique physiological characteristics of this vegetable. Fenugreek (*Trigonella foenum-graecum* L.) is another invaluable herbaceous plant, belonging to the leguminoseae family. Its leaves are commonly utilized as a vegetable due to their mineral and vitamin content, while the seeds serve as a potent source of protein, doubling as both a culinary spice and a remedy for various ailments such as menstrual pain, diabetes, and heart disease (Sinha *et al*., 2004; Kumar *et al*., 2005; Erum *et al*., 2011). Fenugreek's medicinal value is further underscored by the presence of nitrogen compounds, volatile constituents, amino acids, and polyphenolic compounds (Rosengarten, 1969; Mehrafarin *et al*., 2010), making it a versatile and highly regarded botanical resource.

Industrial expansion and anthropogenic activities contribute significantly to the proliferation of environmental pollutants, including heavy metals, presenting a global

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concern (Michalak, 2006; Gerhardt *et al*., 2009). Heavy metals, located in the 4th row of the periodic table, are characterized by having high densities, typically exceeding 5g cm^{-3}, and high atomic numbers, typically greater than 20 (Gupta *et al*., 2013; Ali *et al*., 2018). Cadmium, for instance, derives primarily from sources such as phosphate fertilizers, industrial waste, and sewage sludge (Gomes *et al*., 2013; Robert, 2014). The use of phosphate fertilizer aims to improve soil quality and enhance crop productivity. However, the presence of more than 300 mg kg^{-1} of cadmium in such fertilizers renders them a significant source of heavy metal contamination (Alloway & Steinne, 1999). Elevated levels of heavy metals in plants can lead to various physiological disruptions, including disturbances in water uptake, enzymatic activities, seed germination, photosynthesis, stomatal movement, membrane stability, protein synthesis, hormonal balance, and transpiration (Nazar *et al*., 2012).

Cadmium directly impacts plant nutrients such as potassium, phosphorus, magnesium, calcium, and enzymes, leading to nutritional imbalances that ultimately decrease photosynthesis and respiration rates (Sandalio *et al*., 2001; Clemens, 2006). Furthermore, cadmium exerts indirect effects on plants by generating reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH-), and superoxide dismutase (SOD), which disrupt the metabolic processes of the plants (Gratao *et al*., 2005; Nada *et al*., 2007; Raza *et al*., 2015). To mitigate metal toxicity in plants, both organic and inorganic approaches are utilized (Rizwan *et al*., 2016a). Micronutrients, phosphorus, organic matter, carbon, nitrogen, and potassium constitute animal manure, which stands as an optimal source for facilitating plant growth and maximizing productivity (Arshad *et al*., 2014). Animal manure, enriched with soil organic matter (SOM), enhances soil porosity, moisture retention, and nutrient holding capacity, thereby augmenting soil fertility (Abbas *et al*., 2020a). The nutrients within animal manure exhibit prolonged storage in the soil, releasing slowly over time, which proves beneficial for root development and crop yield (Sharma & Mittra, 1991). The objective of the study was to check the effect of two cadmium concentrations and cattle manure amendment on physiological attributes of spinach and fenugreek varieties.

Material and Methods

Experimental design

A pot experiment was conducted in the botanical garden of the Government College University Faisalabad to investigate the efficiency of cattle manure on the physiological and morphological parameters of spinach and fenugreek under cadmium stress. Seeds of spinach varieties (Desi & Lahori Palak) and fenugreek (Kasuri methi + Guj-1 methi) were obtained from Ayub Agricultural Research Institute (AARI) Faisalabad. Seeds were sown on 1st week of August 2021 in pots and the dimensions of pots were (25 × 35 cm) with 5 kg of soil in each were used to grow seeds in their natural environment. Pots were modified for soil leachate. After 2 weeks of sowing, Cd stress (cadmium sulphate ($3CdSO_4.8H_2O$)) was applied to the seedlings of spinach and fenugreek at different concentrations, 0 mg L^{-1}, 35 mg L^{-1}, and 70 mg L^{-1}. After 10 days of stress, cattle manure was applied at 50 g per pots. Completely randomized design (CRD) was used in the experiment with three replicates of each treatment. The treatment crop was harvested after the eighth week, and initial morphological data was collected. The climate of Faisalabad is semi-arid which is very hot and damp in summer and dry and cool in winter and weather data during course of study is presented in Figure 1.

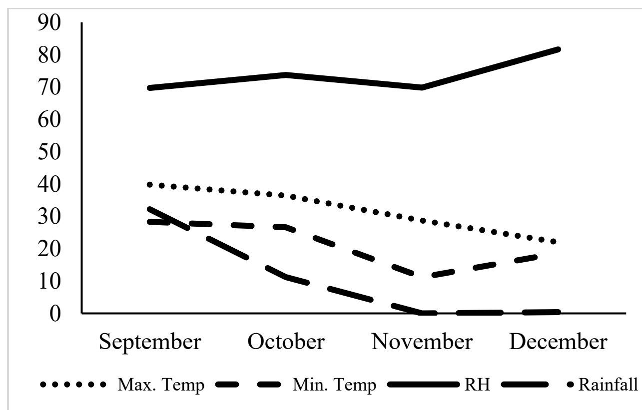

Figure 1: Maximum, Minimum Temperature, Relative Humidity (%) and Rainfall (mm) during August-21 to December-21

Morphological attributes

The crops were harvested at the end of December 2021 for the study of different morphological attributes. All the plants of both species were uprooted for the study of shoot length, leaf area, fresh weight, number of leaves, root length and dry weight of both specie's genotypes. Fresh plant material was stored at -40°C for further analysis of biological molecules in the laboratory of Stress Biology, Department of Botany, Government College University Faisalabad.

Photosynthetic pigments

Arnon method (1949) was used to measure the chlorophyll contents while carotenoids were determined by the Davis (1976) method. A homogenized mixture of leaves was prepared in 80% Acetone and readings were taken at 663, 645, and 480nm using a UV- visible spectrophotometer.

Chl a = [12.7(OD 663) – 2.69(OD 645)] ×V/1000×W [1]

Chl b = [22.9(OD 645) – 4.68 (OD 663)] ×V/1000×W [2]

Total Chl = [20.2(OD 645) + 8.02(OD 663)] ×V/100×W [3]

Carotenoids (g mL^{-1}) = Acar / Em ×100 [4]

When Acar = OD 480 + 0.114 (OD 663) – 0.638 (OD 645), Em ×100 = 2500,

OD= Optical density

V= Volume of sample extract

W= Sample weight

Determination of non-enzymatic antioxidant and bio-molecules

The phenolic content was measured by Bray & Thorpe (1954) and the reading was obtained at 750 nm. The aluminium chloride calorimetric method was used for flavonoid content and the reading was taken at 510 nm using a spectrophotometer (Pekal & Pyrzynska, 2014). The Ninhydrin technique was used to determine the free amino acid (Hamilton & Slyke, 1943) and the reading was taken at 570 nm with the help of UV- visible spectrophotometer. A spectrophotometer was used to measure the total soluble proteins (TSP) absorbance according to the Bradford method (Bradford, 1976).

Cd metal concentration in the root

The plant digestion process involved placing 1 g of dried plant material, 2 mL of H_2SO_4, and 4 mL of H_2O_2 in a digestion flask on a hot plate for 30 minutes. The flask was removed from the hot plate once fumes ceased to evaporate from it. To ensure complete digestion, an additional 2 mL of H_2O_2 was added, and the flask was reheated on the hot plate. This procedure was repeated until a clear solution was obtained. Upon removal, the sample was diluted twice with distilled water to a final volume of 50 mL, then filtered through Whatman filter paper. Subsequently, the solution was transferred to plastic bottles, labeled, and stored for ICP-OES (Inductively Coupled Plasma - Optical Emission Spectroscopy) (Ugulu *et al*., 2016).

Determination of cadmium in soil sample

In the methodology for quantifying cadmium in soil samples, the collected samples were initially dissolved using a mixture of hydrofluoric and hydrochloric acids to break down the soil matrix and release the cadmium. Subsequently, the resulting solution was digested with nitric-perchloric-sulfuric acids to further break down organic matter and ensure complete release of cadmium ions. Cadmium was then extracted from the digested solution as the dithizone complex, which is subsequently destroyed to liberate the cadmium ions. These ions were dissolved in dilute hydrochloric acid, and their concentration was determined using atomic absorption spectroscopy, facilitating accurate quantification of cadmium levels in the soil samples. This methodology enabled the assessment of cadmium contamination (Chen *et al*., 2006).

Soil analysis

Experimental soil samples were collected and then analyzed from Department of Soil Sciences, Ayyub Agricultural Research Institute, Faisalabad.

Table 1: Physiochemical properties of soil used for experiment

Soil Attribute	Value	Unit
Soil texture	Sandy Loam (Hydrological group A)	-
Slit	16.6	(%)
Clay	15.9	(%)
Sand	63.5	(%)
Electrical conductivity	0.58	dS m^{-1}
Soil PH	8.3	-
Organic matter	0.6	%
Phosphorus	7.2	mg kg^{-1}
Potassium	145	mg kg^{-1}
Nitrogen	0.041	%
Saturation	32	%

Statistical analysis

The three-way data analysis was studied using analysis of variance (ANOVA) with computer-based Co-stat version Cohorts Software 6.451. Fisher's LSD was used to examine the differences between the treatments at a value of less than 0.05. The data was normalized using inverse or logarithmic transforms before analysis. The relationships between different variables analyzed by principal component analysis are used to count (PCA). Additionally, the principal

Table 2: Mean sum of square v from a three -way examination of the data's variance for growth and biochemical attributes of spinach (*Spinacia oleracea*) after application of cattle manure under different Cd stress levels

SOV	DF	Root Fresh weight	Shoot Fresh Weight	Shoot Dry weight	Root Dry Weight	No. of Leaves	Root length	Shoot length	Leaf area	F.A. A
Varieties	1	13.056 ***	196.65***	9.97***	1.32***	584.02***	158.42***	92.48*	0.03ns	905.979 ***
Stress	2	7.039 ***	364.68***	0.72***	3.78***	199.69***	9.06ns	190.83***	36.45ns	2478.700***
Treatment	1	20.070***	102.01***	7.10***	10.69***	367.36***	125.14***	82.50*	410.55**	955.592***
Interaction										
V × S	2	6.682 ***	118.60***	2.03***	0.24ns	111.36***	8.91ns	27.72ns	36.37ns	14.367ns
V × T	1	0.332 ns	114.34***	1.17***	0.02ns	1 61.36***	5.19ns	86.80*	21.29ns	5.399ns
S × T	2	4.944 ***	270.61***	1.65***	1.93***	77.86***	39.85***	351.99***	170.18*	24.042*
V x S x T	2	7.299 ***	60.13***	3.23***	4.10***	186.86***	36.10***	72.54*	24.65ns	0.450ns
Error	24	0.130	196.65	0.04	0.09	6.55	3.55	16.22	41.45	6.478
Total	35									

SOV	DF	Chll *a*	Chl *b*	Caro	T.chl	Chl *a/b*	Phen	Flav	T.S. P
Varieties	1	0.071**	0.011ns	0.001ns	0.139***	1.584ns	156.91***	1.466***	118315.82***
Stress	2	1.096***	0.203***	1.560***	2.238***	3.440ns	944.44***	86.96***	40814.53***
Treatment	1	1.912***	0.030*	1.040***	2.426***	13.320ns	395.87***	8.022***	7346.08***
Interaction									
V × S	2	0.023ns	0.002ns	0.057**	0.015ns	4.028ns	4.182ns	3.020ns	480.14**
V × T	1	0.207***	1.693ns	0.078**	0.207***	6.481ns	2.050ns	7.500ns	187.50ns
S × T	2	0.261***	0.009ns	0.437***	0.325***	4.081ns	9.908ns	0.372ns	22.30ns
V x S x T	2	0.074***	0.006ns	0.097***	0.080**	4.655ns	4.848ns	1.285ns	97.58ns
Error	24	0.007	0.005	0.007	0.009	3.726	9.318	14.217	61.60
Total	35								

SOV= Source of variation, ns = nonsignificant *, ** and *** indicates the level of significance at 0.05, respectively. Where S.L= Shoot length, RFW= Root fresh weight, RDW=Root dry weight, SFW= Shoot fresh weight, SDW= Shoot dry weight, R.L= Root length, L.A= Leaf area, FAA= Free Amino Acid, Chl a= Chlorophyll a, Chl b= Chlorophyll b, T.chl= Total Chlorophyll, Caro= Carotenoids, Chl a/b= Chlorophyll a/b, Phen= Phenolics, Flav= Flavonoids, TSP= Total soluble Protein.

component analysis among variables for different genotypes of both crops (*Trigonella foenum-graecum* & *Spinacia oleracea* L.) and Pearson correlation coefficients were performed by using R Studio software.

Results

Effect of cadmium stress and cattle manure on root shoot length and root shoot fresh weight of fenugreek varieties

Results regarding root and shoot length of two fenugreek varieties showed significant variation under different cadmium stress levels and cattle manure amendment in (Table 2) while their mean comparisons is depicted in (Table 4).Results showed that the longest root length (18.67cm) was found in Kasuri variety under (No Cd+ CM 50g) of cattle manure whereas the shortest root length (4.77 cm) was taken in Guj variety where 35 mg L^{-1} cadmium was employed and no cattle manure was given as a treatment. For shoot length, the maximum (15.57 cm) was recorded in Kasuri where no cadmium or cattle manure was employed. On the other hand, the minimum shoot length (9.03 cm) was observed in treatment where 35 mg L^{-1} Cd along with no cattle manure was employed.

Effect of cadmium and cattle manure on root and shoot fresh weight of two fenugreek varieties

Shoot fresh weight of fenugreek genotypes (Table 3) showed that the increase of cadmium stress significantly ($\alpha \leq 0.05$) decreased the shoot fresh weight of both fenugreek genotypes. Maximum decrease of shoot fresh weight (63.04%) was found in Guj-1 and 60.80% decrease in Kasuri under 70 mg L^{-1} and for root fresh weight, the maximum decrease (53.33%) was found in Guj-1 and 56.77% decrease in Kasuri fenugreek under 70 mg L^{-1} Cd application.

Effect of cadmium and cattle manure on root and shoot dry weight (g) of two fenugreek varieties

Data regarding shoot and root dry weight of fenugreek depicted that increase of cadmium stress significantly decreased the shoot and root dry weight of both fenugreek genotypes and decreasein shoot and root dry weight (16.32% and 63.18%) was found in Guj-1 while 3.31 and 60.26% decrease was recorded in

Kasuri fenugreek genotypes under 70 mg L^{-1} cadmium stress without any application of cattle manure (Table 4).

Effect of cadmium and cattle manure on number of leaves and leaf area (cm^2) of two fenugreek varieties

For number of leaves of two fenugreek genotypes, increase of cadmium stress significantly ($\alpha \leq 0.05$) decreased the number of leaves of both fenugreek genotypes with maximum decreased (59.52% in Guj-1 and 55.62% decrease in Kasuri fenugreek genotype) in number of leaves was found under 70 mg L^{-1} cadmium without adding any cattle manure as a treatment. Moreover, leaf area of both varieties of fenugreek was also influenced due to addition of cadmium stress and cattle manure and results are presented in (Table 3). Data showed that increase of cadmium stress decreased the leaf area of both fenugreek genotypes with maximum decrease in leaf area of 21.48% in Guj-1 and 88.2% in Kasuri fenugreek under 70 mg L^{-1}. With the addition of cattle manure as a treatment, maximum increase of the leaf area was found in 46.45% in Guj-1 and 27.9% increase in Kasuri fenugreek genotypes at 35 mg L^{-1} cadmium stress and 50 g cattle manure.

Effect of cadmium and cattle manure on root shoot length and root shoot fresh weight of two spinach varieties

Mean sum of square for root and shoot length, root and shoot fresh weight marked significant difference of two spinach varieties under different cadmium stress and cattle manure (Table 3). Mean comparisons also revealed significant effects and presented in (Table 5). Results regarding root length of two spinach varieties indicated that root length decreased by 18.7% and 14.14% in lahori and desi genotype of spinach under 70 mg L^{-1} cadmium was applied. Moreover, the application of cattle manure influenced positive effect on both varieties of spinach and results showed that 50.86% and 27.17% increase was noted in desi and lahori palak under 70 mg L^{-1} cadmium stress with 50g cattle manure application. For shoot length under different concentration of cadmium metal, the shoot length of cadmium stress at 70 mg L^{-1} concentration in desi palak decreased by 8.19% and 17.66% in Lahori palak while, 0.45% in desi palak and 31.28% in lahori palak under 35 mg L^{-1} Cd stress. After application of cattle manure in stress induced treatment the shoot length was increased by 24.37 and 33.87% in desi and lahori palak, respectively, under 70 mg L^{-1} cadmium stress along with 50g cattle manure (Table 5).

Table 3: Mean sum of squares from a three-way examination of the data's variance for growth and biochemical attributes fenugreek (*Trigonella foenum-graecum*) after application of cattle manure under different Cd stress levels

SOV	DF	R.F.W	S.F.W	S.D.W	R.D.W	No. of Leaves	L.A	R. L	S.L
Varieties	1	3.82***	19.93***	0.31^{ns}	0.03^{ns}	93.444^{ns}	938.56^{ns}	42.90***	909.02***
Stress	2	3.64***	3.05*	0.25^{ns}	0.26**	321.02***	440.8^{ns}	34.65***	964.87***
Treatment	1	0.38^{ns}	3.36*	0.34^{ns}	0.06^{ns}	469.44***	1763.86^{ns}	73.10***	748.02***
Interaction									
V × S	2	1.55***	14.83***	0.06^{ns}	0.14*	1339.19***	2145.71^{ns}	51.68***	958.20***
V × T	1	0.85*	2.26^{ns}	0.13^{ns}	0.38**	58.77^{ns}	2773.91^{ns}	2.83^{ns}	440.30***
S × T	2	0.72*	10.01***	0.51*	0.01*	762.02***	237.17*	37.42***	894.60***
V x S x T	2	0.05*	4.86**	0.47*	0.19**	63.19*	734.28^{ns}	39.82***	872.69***
Error	24	0.12	0.56	0.09	0.02	31.33	745.51	2.20	3.63
Total	35								

SOV	DF	Chl *a*	Chl *b*	Caro	Total chl	Chl *a*/*b*	Phen	Flavo	TSP	FAA
Varieties	1	0.08***	0.003**	0.01***	0.05***	1.10***	1900.37***	343.48***	59251.34**	84.001**
Stress	2	0.69***	0.37***	0.79***	3.65***	0.30***	6345.18***	2956.81***	102061.13**	3177.28***
Treatment	1	0.86***	0.16***	0.52***	1.79***	0.13***	2117.23***	1874.89 ***	42773.96**	1819.37***
Interaction										
V × S	2	0.01***	0.01***	0.01***	0.06***	0.13***	207.00***	33.56*	1825.21**	23.50*
V × T	1	0.00***	9.99^{ns}	0.007***	0.002^{ns}	0.01^{ns}	1 1.08^{ns}	13.20^{ns}	1222.01**	3.29^{ns}
S × T	2	0.27***	0.004***	0.10***	0.34***	0.47***	171.59***	31.86*	1307.10***	156.33***
V x S x T	2	0.00***	0.001*	0.004***	0.007**	0.05***	0.96^{ns}	22.31^{ns}	89.53^{ns}	11.38^{ns}
Error	24	3.04	3.34	3.22	8.004	0.005	14.636	343.48	100.73	6.80
Total	35									

SOV= Source of variation, ns = nonsignificant *, ** and *** indicates the level of significance at 0.05, 0.010, and 0.001 respectively. Where RFW= Root fresh weight, S.L= Shoot length, L.A= Leaf area, SDW= Shoot dry weight, RDW=Root dry weight, SFW= Shoot fresh weight, R.L= Root length, FAA= Free Amino Acid, Chl b= Chlorophyll b, Caro= Carotenoids, Chl a/b= Chlorophyll a/b, Phen= Phenolics, T.chl= Total Chlorophyll Flav= Flavonoids, TSP= Total soluble Protein, Chl a= Chlorophyll a.

Table 4: Effect of Different level of cadmium stress and manure amendment treatment of two different varieties of Fenugreek (*Trigonella foenum-graecum* L.) on root and shoot length (cm), root shoot fresh and dry weight (g), number of leaves and leaf area (cm^2)

Variety	Treatment	SL	RL	RFW	SFW	RDW	SDW	NL	LA
Guj	T0= No cd+ CM 0	11.73±0.46cd	5.97±0.35 e	0.65±0.28 c	1.60±0.05 e	0.05±0.01 f	0.33±0.03 e	41±1.16 cd	10.47±0.75 c
	T_1= Cd 35 mg L^{-1} +CM 0	9.03±0.58 e	4.77±0.15 f	0.05±0.02 d	0.42±0.03 f	0.16±0.01 d	0.16±0.01 f	35±1.16 d	7.93±0.41 e
	T_2= Cd 70 mg L^{-1} + CM 0	12.20±1.16 c	7.33±0.35 d	0.40±0.05 c	4.20±0.15 b	0.43±0.02 c	0.38±0.02 cd	57.0±1.73 b	15.67±1.07 ab
	T_3= No cd+ CM 50g	14.00±0.78 b	9.57±0.58 c	0.08±0.01 d	1.40±0.01 ef	0.53±0.09 b	0.28±0.01 ef	30.67±1.20 e	9.23±0.18 cd
	T_4= Cd 35 mg L^{-1} + CM 50g	14.07±1.27 ab	7.40±0.26 e	0.07±0.01 e	1.27±0.06 ef	0.13±0.01 ef	0.76±0.02 b	43.67±2.34 c	12.97±0.95 bc
	T_5= Cd 70 mg L^{-1} + CM50 g	15.33±1.20 a	11.33±1.20 b	0.64±0.07 c	3.22±0.08 c	0.86±0.07 a	0.78±0.03 b	44.67±2.03 c	17.20±1.42 a
Kausri	T0= No cd+ CM 0	15.57±1.27 a	8.77±0.79 cd	0.54±0.09 c	6.35±0.21 a	0.19±0.04 c	0.30±0.01 e	53.33±3.53 bc	16.17±1.30 a
	T_1= Cd 35 mg L^{-1} +CM 0	11.43±0.98 c	8.33±0.44 c	0.16±0.03 d	2.55±0.23 d	0.19±0.04 d	0.47±0.05 c	53.33±3.39 bc	10.67±0.75 cd
	T_2= Cd 70 mg L^{-1} + CM 0	13.60±0.87 bc	9.20±1.32 c	1.42±0.21 b	3.30±1.00 c	0.50±0.13 b	1.02±0.28 a	43.67±3.29 c	13.33±1.59 bc
	T_3= No cd+ CM 50g	11.33±1.45 cd	18.67±1.45 a	0.55±0.22 c	2.34±0.50 d	0.21±0.14 d	0.58±0.37 c	37.33±5.82 d	10.47±0.54 c
	T_4= Cd 35 mg L^{-1} + CM 50g	11.57±1.27 bc	9.57±1.05 c	0.54±0.10 c	4.55±0.43 b	0.33±0.24 cd	1.16±0.37 a	66.33±5.90 a	9.30±1.22 cd
	T_5= Cd 70 mg L^{-1} + CM50 g	11.33±0.88 cd	4.93±0.96 ef	2.58±0.57 a	1.96±0.83 e	0.16±0.04 e	0.27±0.13 ef	17.33±2.34 f	8.53±0.51 d
	LSD value ≤ 0.05	**1.60**	**1.25**	**0.30**	**0.63**	**0.14**	**0.25**	**4.71**	**1.03**

Cd= cadmium; CM= cattle manure; SL= Shoot length; RL= Root length; RFW= Root fresh weight; SDW= Shoot fresh weight; RDW= Root dry weight; SDW= Shoot dry weight; NL= Number of leaves; LA= Leaf area

Table 5: Effect of Different level of cadmium stress and manure amendment treatment of two different varieties of Spinach on root and shoot length (cm), root shoot fresh and dry weight (g), number of leaves and leaf area (cm^2)

Variety	Treatment	SL	RL	RFW	SFW	RDW	SDW	NL	LA
Desi Palak	**T0= No cd+ CM 0**	39.6±0.44 a	11.27±0.31 b	3.41±0.25 a	12.41±0.06 a	2.15±0.02 b	7.41±0.02 a	45.1±1.11 a	29.41±0.65 a
	T_1= Cd 35 mg L^{-1} +CM 0	36.4±0.55 b	8.61±0.19 cd	3.12±0.03 ab	9.67±0.03 b	1.68±0.03 c	6.41±0.05 b	39.2±1.13 b	24.21±0.31 b
	T_2= Cd 70 mg L^{-1} + CM 0	34.23±0.51 c	7.69±0.31 d	2.41±0.04 c	8.41±0.12 c	0.78±0.02 f	5.29±0.04 c	38.12±1.53 b	21.14±1.17 c
	T_3= No cd+ CM 50g	37.21±0.69 b	13.57±0.55 a	3.16±0.01 ab	8.12±0.05 c	2.41±0.08 a	5.34±0.02 c	34.21±1.10 c	27.21±0.08 a
	T_4= Cd 35 mg L^{-1} + CM 50g	29.7± 0.81 d	9.63±0.21 c	2.41±0.05 c	11.41±0.04 a	1.58±0.04 c	5.21±0.03 d	32.56±1.24 c	25.21±0.45 b
	T_5= Cd 70 mg L^{-1} + CM50 g	26.41±1.11 e	8.75±1.08 cd	2.19±0.06 d	9.41±0.07 bc	1.36±0.06 d	4.25±0.04 e	44.67±1.03 a	19.52±0.42 d
Lahori Palak	**T0= No cd+ CM 0**	34.15±1.19 bc	10.68±0.7 bc	3.18±0.09 a	11.74±0.19 a	2.05±0.05 b	7.12±0.05 a	43.21±1.53 a	27.41±0.30 a
	T_1= Cd 35 mg L^{-1} +CM 0	28.12±0.91 d	8.33±0.39 d	3.08±0.02 b	9.51±0.21 b	1.45±0.07 d	6.36±0.06 b	38.41±1.29 b	23.41±0.75 b
	T_2= Cd 70 mg L^{-1} + CM 0	24.12±0.84 f	9.20±1.15 cd	2.37±0.24 c	8.54±0.97 c	0.63±0.11 f	5.19±0.27 c	37.23±1.19 bc	19.58±0.69 d
	T_3= No cd+ CM 50g	32.14±0.49 cd	14.21±1.21 a	3.19±0.20 ab	8.05±0.55 c	2.48±0.13 a	5.37±0.33 c	31.12±1.72 d	24.12±0.44 b
	T_4= Cd 35 mg L^{-1} + CM 50g	30.15±1.29 cd	9.63±1.01 c	2.45±0.09 c	9.67±0.44 b	1.46±0.21 d	5.11±0.37 d	34.26±1.80 c	18.41±0.22 e
	T_5= Cd 70 mg L^{-1} + CM50 g	29.14±0.81 d	6.93±0.93 e	2.17±0.51 d	9.53±0.81 b	1.26±0.05 e	4.39±0.14 e	30.14±1.24 d	19.41±0.45 d
	LSD value ≤ 0.05	**2.04**	**1.58**	**0.30**	**1.72**	**0.20**	**0.18**	**3.15**	**2.42**

Cd= cadmium; CM= cattle manure; SL= Shoot length; RL= Root length; RFW= Root fresh weight; SDW= Shoot fresh weight; RDW= Root dry weight; SDW= Shoot dry weight; NL= Number of leaves; LA= Leaf area

Effect of cadmium and cattle manure on root and shoot fresh weight of two spinach varieties

For fresh weight of root and shoot exhibited also significant difference for different concentration of cadmium stress and cattle manure. Results highlighted that). Largest increase of root fresh weight was found under 35 mg L^{-1} in lahori palak followed by 70 mg L^{-1} in desi palak. Maximize increase of root fresh weight was found 1.78% in desi palak and 10.6% in lahori palak under 70 mg L^{-1} while, 13.5% in desi palak and 78.9% in lahori palak under 35 mg L^{-1} Cd stress. Utilization of animal manure perform highly significant increase in root fresh weight of both varieties. Maximize increase of shoot dry weight was found 70.27% in desi palak and 33.49% in lahori palak under 70 mg L^{-1} while, 41.77% in desi palak and 12.18% in lahori palak under 35 mg L^{-1} however, 24.83% increase in desi palak and 14.04% increase of root dry weight was found in lahori palak under control condition (Table 5).

Effect of cadmium stress and cattle manure on root and shoot dry weight (g) of two spinach varieties

Results regarding root dry weight under different concentration of cadmium stress and cattle manure exposed significant difference. The root dry weight of cadmium stress under 70 mg L^{-1} concentration in desi palak decreased by 145% and 14.5% in lahori palak whereas, 170% in desi palak and 60.1% in lahori palak under 35 mg L^{-1} Cd stress. Under cattle manure application, the maximum increase of root dry weight (61.1%) was found in desi palak and 41.0% in lahori palak under 70 mg L^{-1} along with 50 g cattle manure application. Moreover, 68.8% increase was recorded in desi palak and 83.08% in lahori palak under 35 mg L^{-1} however under 50 g cattle manure application (Table 5).

Effect of cadmium and cattle manure on number of leaves and leaf area (cm^2) of two spinach varieties

Mean sum of square showed that metal (Cd) stress could cause highly significant differences. As a result of 70 mg L^{-1} concentration of Cd stress, the number of leaves of desi palak cultivars underwent decrease of 50% and 12.5% in Lahori palak whereas, 54.4% in desi palak and 25% lahori palak under 35 mg L^{-1} Cd stress. Treatment of animal manure showed high positive significant differences in both varieties. Maximum increase in the number of leaves of 28.26% in desi palak and 51.35% in lahori palak under 70 mg L^{-1} along with 50g cattle manure application was found while 29.03% in desi palak and 52% in lahori palak under 35 mg L^{-1} when 50 g animal manure was used (Table 5).

Effect of cadmium and cattle manure on photosynthetic and biochemical attributes of fenugreek and spinach genotypes

Attributes of photosynthetic pigments (chlorophyll a, b, carotenoids, a/b, and total chlorophyll) exposed significant results under different applications of manure. Varieties of spinach behaved similarly in control without application of animal manure. The same trend was noticed in fenugreek varieties. Two levels of stress (35 & 70 mg L^{-1}) showed a gradual decrease in photosynthetic pigments but animal manure had positive statistical improvement in both species. Similar trend was observed in both varieties of spinach and fenugreek concerning to stress and application of animal manure. Results indicated that the maximum chlorophyll a content (1.6 mg g^{-1} Fw.) was recorded in Guj variety of fenugreek where 50 g cattle manure was applied without inducing any cadmium stress whereas the lowest value of chlorophyll a was recorded in that treatment where 70 g L^{-1} cadmium was applied and 50 g cattle manure was also mixed. Similar results were found for other attributes like chlorophyll b, carotenoid, total phenolic, free amino acid, flavonoid and total soluble protein. For spinach varieties, the highest chlorophyll a content (1.8 mg g^{-1} FW) was accumulated in Desi Palak where 50 g cattle manure was applied, whereas, the lowest value (0.7 mg g^{-1} FW) was recorded in Desi Palak where 70 g L^{-1} cadmium was applied and 50 g cattle manure was also mixed. Similar trend was also recorded for chlorophyll b, carotenoid, total phenolic, free amino acid, flavonoid and total soluble protein. (Figure 2 and Figure 3).

Relationship among Cd uptakes and certain plant characteristics

The association shown by the Pearson's correlation graph is the influence of animal manure on morpho-physiological attributes of *S. oleracea* and *T. foenum-graecum* L. under different levels of Cd stress. Chlorophyll contents positively correlated with total soluble protein, flavonoids, phenolics, free amino acids and root length while negatively correlated with root fresh weight, shoot dry weight and root dry weight. The same trend was observed in flavonoids, phenolics, free amino acids and total soluble protein. Shoot length, leaf area and number of leaves negatively correlated with biochemical parameters except shoot fresh weight which positively correlated with phenolics, root length, total soluble protein, root fresh weight and shoot and root dry weight (Figure 4 and Figure 5).

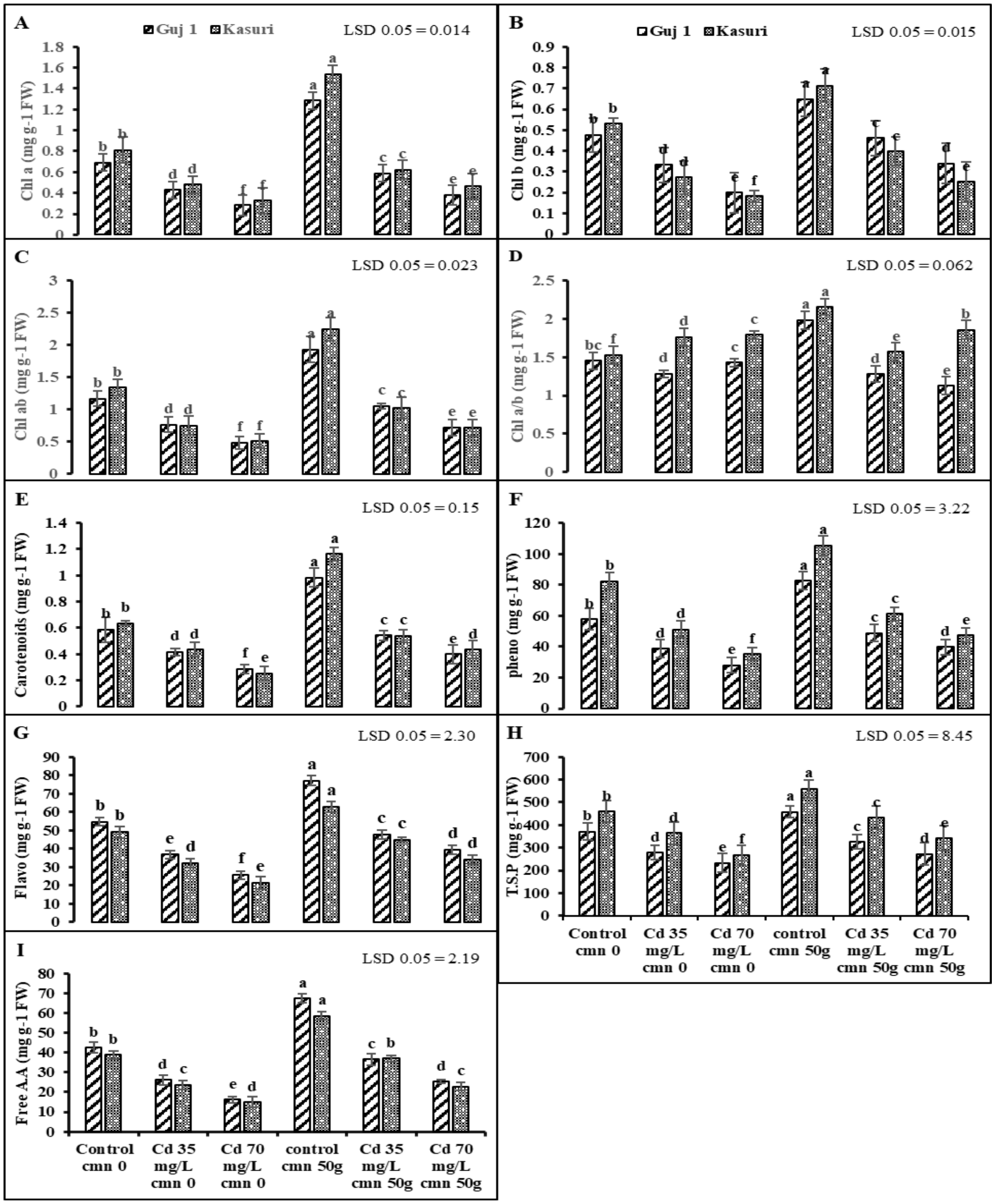

Figure 2: Effect of animal manure on cadmium stress-related variations in the antioxidant concentrations of two genotypes of fenugreek (*Trigonella foenum-graecum* L.) including photosynthetic and non-enzymatic antioxidants (A=chlorophyll a, B=chlorophyll b, C=total chlorophyll, D=chlorophyll a/b, E=carotenoids, F=Phenolic, G=Flavonoids, H=Total soluble proteins, I=Total free amino acids, mg g^{-1} F.W. Cmn stand for cattle manure (Control cmn 0, 35mg L^{-1} cmn 0, 70 mg L^{-1} cmn 0, control cmn 50g, 35 mg L^{-1} cmn 50g, 70 mg L^{-1} cmn 50g). Each statistic is the mean (SD) of three replicates. The LSD test finds a significant difference between different letters on bars at $p \leq 0.05$

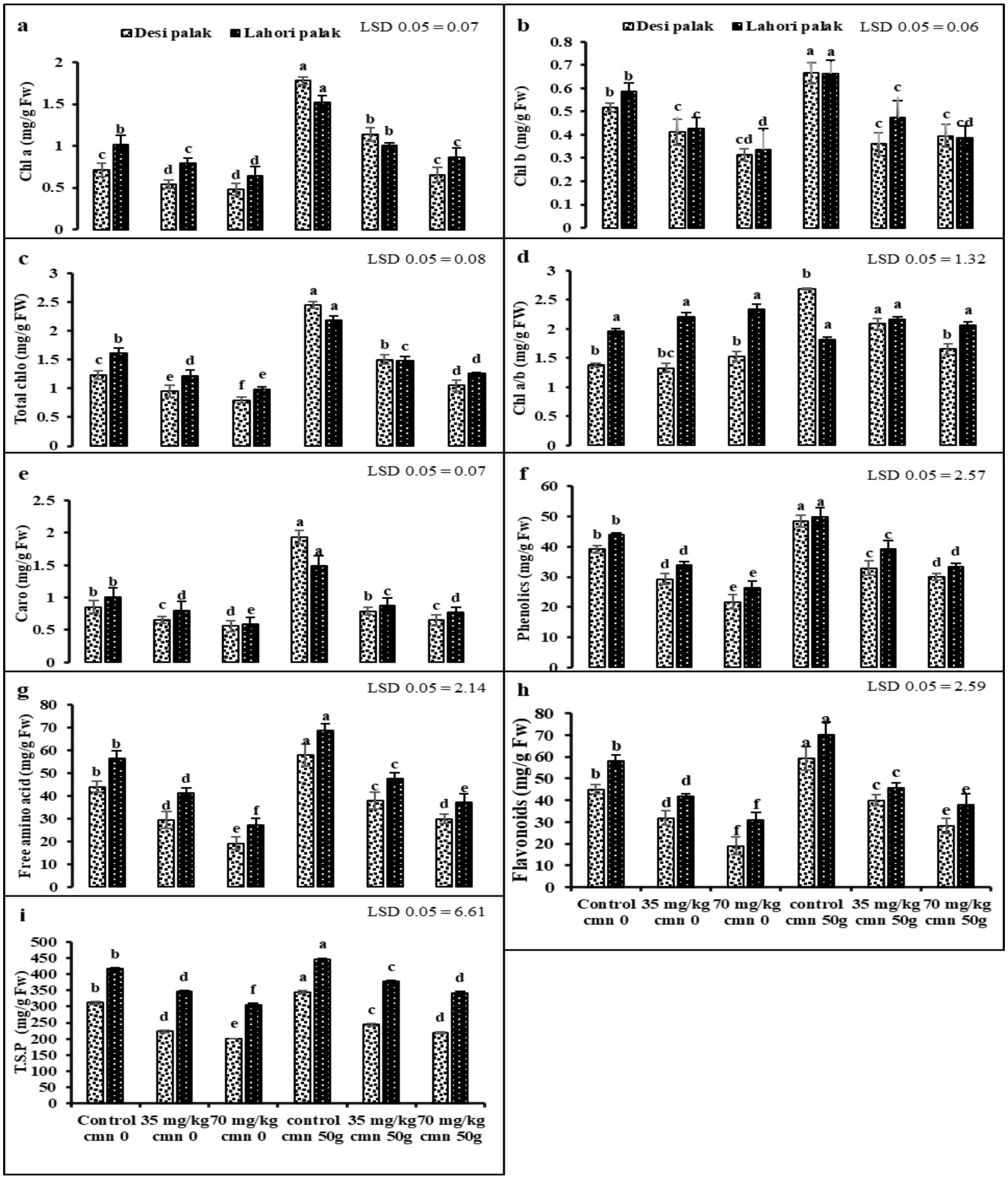

Figure 3: Effect of animal manure on photosynthetic and non-enzymatic antioxidant contents (a=chlorophyll a, b=chlorophyll b, c=total chlorophyll, d=chlorophyll a/b, e=carotenoids, f=Phenolic, g=Total free amino acids, h=Flavonoids, i=Total soluble proteins, mg g^{-1} F.W) in two genotypes of spinach (*Spinacia oleracea* L.) under varying levels of cadmium stress. Cmn stands for cattle manure (Control cmn 0, 35 mg L^{-1} cmn 0, 70 mg L^{-1} cmn 0, control cmn 50g, 35 mg L^{-1} cmn 50g, 70 mg L^{-1} cmn 50g). Each statistic is the mean (SD) of three replicates. The LSD test finds a significant difference between different letters on bars at *p*≤ 0.05

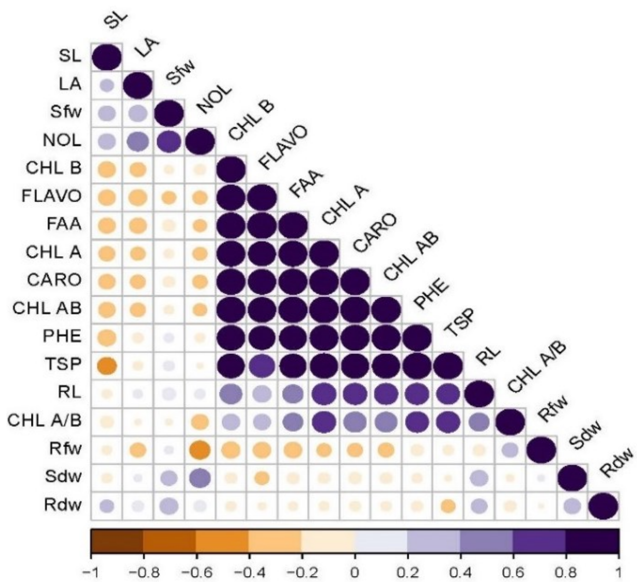

Figure 4: Relationship between morphological and physiological attributes of *Spinacia oleracea* and *Trigonella foenum-graecum* L. under the different levels of Cd-concentration. The following are various abbreviations that are used in the figure: Chl-a (chlorophyll a content), TSP (Total soluble proteins), SL (shoot length), Chloro-a/b (chlorophyll a/b content), Phen (phenolic content), RFW (root fresh weight), LA (leaf area), Flav (flavonoid content), NOL (number of leaves), SDW (shoot dry weight), FAA (free amino acids), RL (root length), TC (total chlorophyll content), RDW (root dry weight), Carot (carotenoid content), SFW (shoot fresh weight) and Chloro-b (chlorophyll b content)

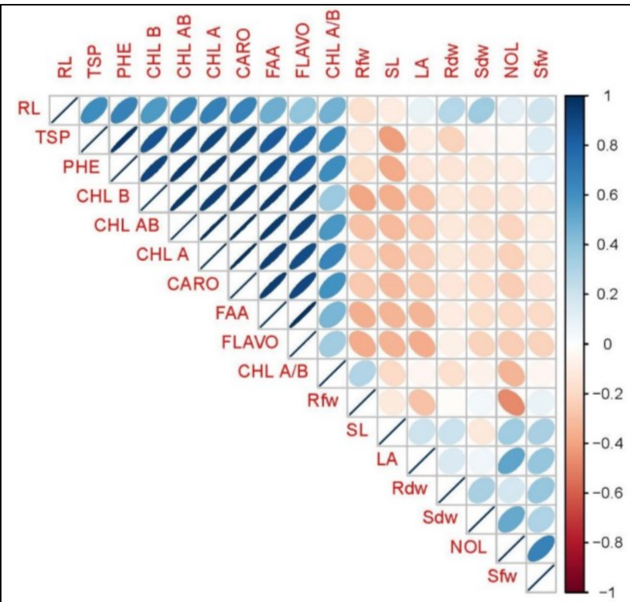

Figure 5: Root length positively relate with root fresh weight, chlorophyll content and biochemical parameters. Shoot fresh weight positively relate with shoot length, number of leaves, root & shoot dry weight, except leaf area and root fresh weight. Morphological attributes (shoot length, leaf area, root fresh & dry weight, number of leaves and shoot dry weight) show negative relationship with chlorophyll content and free amino acids and flavonoids. Shoot fresh weight show same trend in all except root length, phenolics and total soluble protein

Principal component analysis

Influence of animal manure on spinach and fenugreek was displayed by means of the principal component analysis (PCA) (Figure 6). In both dimensions, (Dim1 and 2) exhibited great influence and displayed more than 69% of differences. Dimension 1 and 2 exhibits (44.2 %) and (25 %), respectively . Successful distribution of all analyzed factors in the record is a glaring indicator that cadmium stress has a major impact on physiology and growth of *S. oleracea* and *T. foenum-graecum*. Recorded results showed that biochemical parameters (chl a, b, total chl, carotenoids, chl a/b, phenolics, free amino acids, flavonoids and total soluble protein) were positively correlated with Cd concentration in spinach but negatively correlate in fenugreek species compared to all other experimentally-tested factors. While, morphological parameters including shoot fresh weight, leaf area, shoot & root dry weight, no. of leaves, root fresh weight, and shoot length were negatively correlated in spinach while positively correlated in fenugreek species with all other studied parameters except root length. In fenugreek, Dimension 1, 2 revealed more than 66% of the differences are filled by the maximum contribution. Among which Dimension 1 and 2 exhibits (50.4 %) and (16.3 %), respectively.

Cd uptake by plants

Cadmium uptake by both leafy vegetables (fenugreek & spinach) was visibly shown in (Figure 7. A, B). Reduction in Cd uptake was recorded in plants by utilization of animal manurein both cultivars, Desi palak exhibited a highly significant reduction in uptake of Cd at 35 mg L^{-1} and trend was the same at 70 mg L^{-1}. Lahori palak showed same trend in both levels of Cd stress. Animal manure showed better

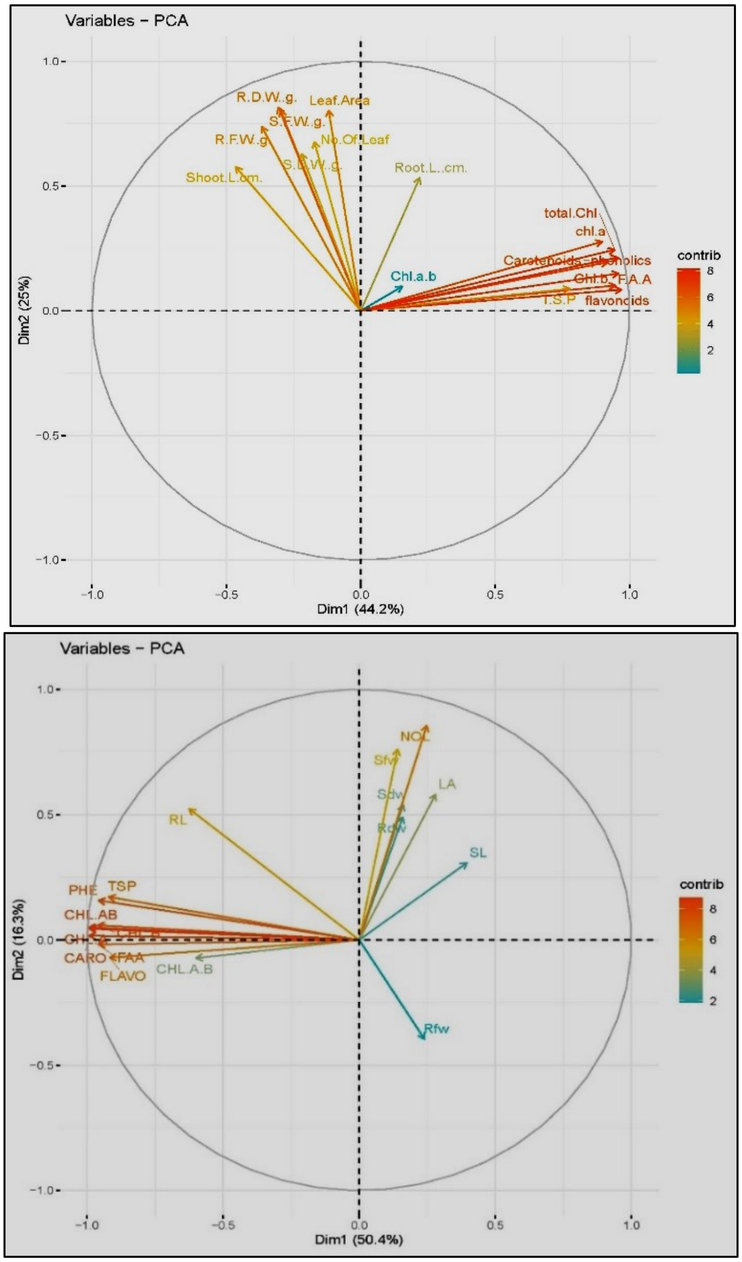

Figure 6: Principal component analysis (PCA) loading plots on several features (morpho-physiological) of *T. foenum-graecum* L. and *S. oleracea* L. at various Cd-concentration levels. The following are various acronyms that are used in the figure: Flav (flavonoid content), SL (shoot length), RDW (root dry weight), FAA (free amino acids), SDW (shoot dry weight), RL (root length), Chl-a (chlorophyll a content), SFW (shoot fresh weight), Chl-b (chlorophyll b content), Carot (carotenoid content), NOL (number of leaves), RFW (root fresh weight), TC (total chlorophyll content), TSP (Total soluble proteins), Phen (phenolic content), Chl-a/b (chlorophyll a/b content), LA (leaf area)

improvement in desi palak as compared to Lahori palak concerning Cd uptake. The same trend was observed in both varieties of fenugreek. Kasuri exhibited better results with animal manure at 35 mg L^{-1} Cd stress as compared to Guj-1methi (Figure 7).

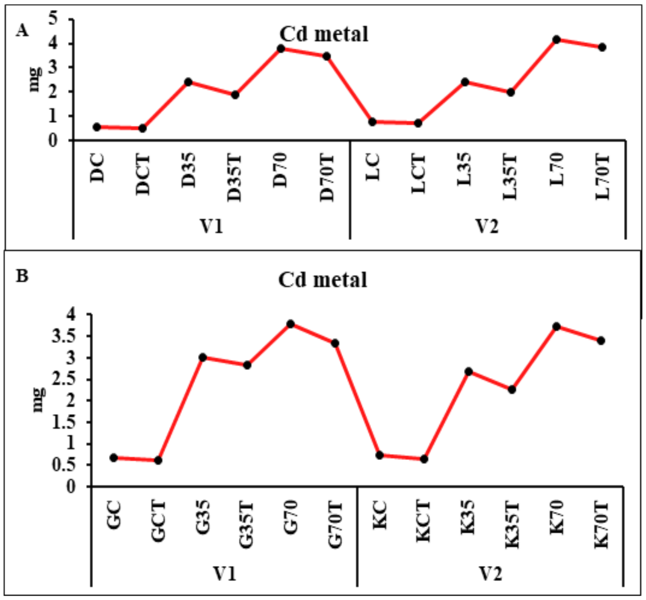

Figure 7: Effect of cattle manure on Cd uptake at shoot surface of spinach (a) (*S. oleracea* L.) and fenugreek (b) (*T. foenum-graecum* L.) grown under different levels of Cd stress. The graph represents mean values of three replicates

Discussion

For the plants to grow and flourish, normal conditions are needed. However, a lot of Cd in the soil throws off the plants' overall system. The current study showed that when there was a high concentration of Cd in the soil, the morphological and biomass characteristics of spinach and fenugreek decreased. The soil's highest level of Cd content caused the hypertonic condition in the soils and ultimately less availability of water for uptake, as a result decreased the plant's morphological attributes. Hina *et al*. (2019) also discovered a similar outcome to the effects of Cd metal on spinach. Our results positively correlate with Boysan *et al*. (2018) who reported different absorption of heavy metal (Cd) in spinach and decrease in the biomass and morphological parameters (plant length, number of leaves and fresh and dry weight of plants) was recorded. Utilization of animal manure enhances the morphological attributes of spinach and fenugreek under stress conditions (Boysan *et al*., 2018). Hina *et al*. (2019) studied similar results by application of charcoal and animal manure. Treatment of animal manure stops the mobilization of Cd metal or makes it less available or converts the metal into a less soluble form.

The current investigation indicated that the maximum quantity of Cd stress in the soil decreased the chlorophyll contents of the plants. Increased Cd uptake by plants caused amplification of cation production in leaves, high energy cation production in plants inhibits the synthesis of chlorophyll contents. Ali *et al*. (2013) and Younis *et al*. (2016) reported Cd toxicity effects on spinach and mustard plants and concluded similar results. Previous studies on spinach showed that the presence of Cd in the soils decreased the chlorophyll and carotenoid contents in different plants such as wheat (Rizwan *et al*., 2016b), spinach (Bagheri *et al*., 2013; Rezakhani *et al*., 2013; Alia *et al*., 2015) and *Brassica napus* (Ehsan *et al*., 2014). The application of animal manure enhances the photosynthetic activity of plants due to the synthesis of chlorophyll contents. Similar results were reported by Younis *et al*. (2016) that biochar increased the activity of photosynthetic pigment by increasing the adsorption of nutrients by the plant from soil (Uzoma *et al*., 2011; Sun *et al*., 2013; Ok *et al*. 2015; Rizwan *et al*., 2017). The current investigation is that cadmium stress mitigates the concentration of free amino acids and contents of total soluble proteins in both varieties of spinach and fenugreek. Cadmium stress inhibited the uptake of nitrogen from the soil by plant protease enzymes resulting in lessened amount of protein in plants observed by the researcher. The structural and functional component of amino acids that are also the constituents of plant proteins is nitrogen (Balestrasse *et al*., 2003). Our result are like the previous work done by Bavi *et al*. (2011) who revealed that cadmium stress decreased the uptake of protein content in pea plants.

In the present work, cadmium stress showed various outcomes on biochemical parameters in both cultivars of spinach and fenugreek plants. Cadmium stress decreased the effect of soluble protein content in both varieties of spinach and fenugreek. Our result correlate with Han *et al*. (2020) who studied the uptake and translocation of different level of Cd metal on rape (*Brassica napus* L.) Soluble proteins in plants show the heavy metal presence in plants. Total soluble proteins maintain the osmotic potential of cells (Ali *et al*., 2023). Continuous uptake of cadmium metal reduced the effect of soluble proteins in rape plants to disturb the transportation pathway of sugar molecules that are helpful in photosynthetic systems (Wong *et al*., 1997; Boo *et al*., 2012).

Application of animal manure improved the biochemical parameters like total soluble proteins and amino acid content in both varieties of spinach and fenugreek. Animal manure reduced the de novo enzyme production and lowered the production of proteins in sunflower, maize, and soybean (Sharma and Dhiman, 2013). Our result correlates with Younis *et al*. (2015) who studied the biochar impact on physiological and biochemical attributes of spinach (*S. oleracea* L.) in nickel-contamination soil. Biochar application increases the total soluble proteins and amino acid content in S. oleracea (L.) that are helpful in carbon and nitrogen metabolism. It was because of the promotion of de novo synthesis of enzymes. The promotion of these enzymes raised the levels of metal-binding complexes containing heavy metals (metallothionein and phytochelatins) (Inouhe, 2005; Li *et al*., 2023).

The present study showed that secondary metabolites like flavonoids, and phenolics decreased by increasing cadmium stress in both varieties of spinach and fenugreek plants. The metabolites present in plant tissue or organs decline the metal toxicity and increase the efficiency of signal transduction that is helpful to prevent the membrane alteration (Rehman *et al*., 2019; Javed *et al*., 2020; Saleem *et al*., 2020;Shen *et al*., 2023). Our findings were consistent with earlier research by Kisa *et al*. (2016), who described how the phenolic compounds responded to heavy metal stress in *Zea mays*. Secondary metabolites like phenolics and flavonoids decreased by the heavy metal in tea leaves (*Camellia sinensis* L.). This affected the membrane permeability or water-stressed like conditions (Pesci and Reggiani, 1992; Hung *et al*., 2023).

Utilization of animal manure improved the biochemical parameters like flavonoids, and phenolics in both varieties of spinach and fenugreek . Animal manure declines the effect of oxidant (ROS) and improves the uptake of nutrients produced by the Cd metal (Naveed *et al*., 2021). A similar result was also found by Yaseen *et al*. (2021) who examined the influence of biochar and ascorbic acid applied topically on the physio-biochemical characteristics of barley (*Hordeum vulgare* L.) grown in cadmium-contaminated soil. Treatment of biochar improves the plant vigour by reducing the toxic effect of cadmium metal because minerals and other micronutrients found in biochar act as osmoprotectants (Noreen *et al*., 2020).

Conclusion

Based on the study's findings, it was determined that metal (Cd) stress reduced fenugreek (*T. foenum-graecum* L.) and spinach (S. oleracea L.) growth and yield by reduction in the photosynthetic pigments and increasing the activity of ROS. Morphological and biochemical parameters were reduced in 70 mg/L cadmium concentration than 35 mg/L and control. Animal manure decreased the mobilization of cadmium metal and increased the surface charge that decreased the cadmium metals into the soil that is available to the plant. Cadmium mobility and accumulation from soil to root and leaves depend on cation channels or calcium transporters but no visible chlorotic or necrotic symptoms were found because leafy vegetables also have tolerance against metal toxicity. Treatment of animal manure was a good sorbent to alleviate the harmful effect of cadmium on morpho-physiological attributes by increasing antioxidant enzyme activities which ultimately stabilized the chlorophyll pigments and mobility of mineral nutrients. Animal manure is inexpensive and more research is needed to discover the added values of this natural sorbent in industrial applications.

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