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Full Length Article

Copper Toxicity Affects Seed Emergence, Stand Establishment and Copper Accumulation of Soybean and its Mitigation through Biogas Slurry

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Abstract

The present study was aimed at investigating the toxic effect of copper on seed emergence, seedling growth, chlorophyll contents and copper concentration in different parts of soybean [*Glycine max* (L.) Merr.]. Four soybean cultivars viz Walliam-82, NARC-2, Rawal-1 and Ajmeri-2104 were stressed with six Cu concentrations i.e., 50, 100, 150, 200, 250 and 300 mg Cu L^{-1} in two factor factorial completely randomized design with three replications. It was noted that shoot and root weights decreased gradually as the concentration of Cu increased for all cultivars especially Rawal-1 while Ajmeri-2014 was found as the most tolerant. Moreover, with the increase in the concentration of Cu, the drastic effect on growth and chlorophyll contents was recorded in Rawal-1 followed by NARC-2 > William-82 while Ajmeri-2014 showed tolerance under Cu toxic impacts. The addition of biogas slurry (BGS) improved plant growth, total chlorophyll contents and Cu concentration in soybean seedlings. Different levels of BGS (0, 2, 3, 4, and 5%) were applied but 4% significantly mitigated the adverse effect of Cu stress. It was concluded that Rawal-1 was more sensitive to Cu stress followed by Walliam-82, NARC-2 and Ajmeri-2104. Among all cultivars, Ajmeri-2014 is a suitable cultivar for Cu affected soils and BGS could be used an amendment under Cu contaminated soil to relieve the stress in soybean. © 2018 Friends Science Publishers

Keywords: Copper stress; Soybean; Emergence; Growth; Chlorophyll content; Biogas slurry

Introduction

Phytotoxic effects of Cu on plant are higher than most of other heavy metals (Kramer *et al.*, 2007; Gür and Topdemir, 2008). The normal range of Cu in many plants is usually from 5−20 mg kg^{-1}; above this range, toxicity occurs in plants (Nafees *et al.*, 2009). Excess in soil Cu concentration affects germination and plant growth through the prevention in uptake of other essential elements like Fe, K and Ca causing metabolic disorders and growth inhibition in different crops like maize, wheat and psyllium (Singh *et al.*, 2007; Rahoui *et al.*, 2010; Mohammadi *et al.*, 2013). Previously, Cu effect on germination of alfalfa seed has been reported when soil was contaminated with 20 mg kg^{-1} and further increase in concentration (Aydinalp and Marinova, 2009). In other reports, it caused reduction in seed germination, embryonic growth, root and shoot length, and vigor of *A. elongatum* (Saberi and Shahriari, 2011).

Pakistan economy is characterized by dire need to explore hidden potential of oil seed crops such as soybean [*Glycine max* (L.) Merr.] (Government of Pakistan, 2015). Usually the research and investigations have paid attention on estimating the possible health risks of vegetables, corn, rice and wheat grains in contaminated soils (Huang *et al.*, 2008; Hao *et al.*, 2009). As other agronomic crops are affected by Cu toxicity, soybean is not an expectation. Least attention has been paid to health risk via consumption of soybean (Angelova *et al.*, 2003; Shute and Macfie, 2006). However, soybean is a staple food of many people worldwide and it has been found that soybean like other bean crops accumulate high amount of heavy metals. It has been reported that soybean produces oil for human consumption and as supplement dietary protein containing 40−42% good quality protein and 18−22% oil which might be affected by heavy metal contamination (Khan *et al.*, 2013). Cu toxicity in excess was demonstrated stronger in dividing and elongation of root meristematic cells, cause breaks on roots tips and change morphology of soybean plant. It also reduced the biomass accumulation and linear plant growth (Kulikova *et al.*, 2011). Therefore, it is dire need to evaluate the effect of Cu on seed emergence and growth of different varieties of soybean to find out the level of tolerance for different varieties against Cu toxicity.

The fate of heavy metals contaminated soil is inclined by numerous chemical, physical and biological reactions. The retention of heavy metals in soil occurs through

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sorption, complexation and precipitation. All these reactions cut off metal mobility and its bioavailability (Clemente *et al.,* 2005; Mora *et al.,* 2005). But these ordinary processes of reduction can be supplemented by *in situ* application of different technologies such as use of organic and inorganic amendments. Organic amendments consist of high amount of humified organic matter and could decrease heavy metals bioavailability in soil, even though temporally and thus allowing the restitution of flora (Castaldi and Melis, 2004). The addition of organic amendments effectively immobilizes Cu by increased formation of Cu–organic matter complexes (Cao *et al.,* 2007). Biogas slurry (BGS) also known as fermented slurry, is a product of anaerobic fermentation (in biogas digester) of animal excreta and an excellent source of organic fertilizer which is an important input to better crop yield and soil fertility (Maqbool *et al*., 2014). The organic contents of biogas slurry quickly decompose, breakdown and provide fast acting nutrients source that enter the soil solution and instantly become available to plants (Islam *et al.,* 2010).

In view of above mentioned facts this study was aimed to better understanding the effect of Cu stress on seed germination and seedling growth of different varieties of soybean. Moreover, biogas slurry was also investigated to alleviate Cu stress in soybean varieties.

Materials and Methods

Seed Material

Seeds of four soybean cultivars (Walliam-82, NARC-2, Rawal-1, Ajmeri-2014) were provided by the National Agricultural Research Centre (NARC) Islamabad, Pakistan. The healthy and vigorous seeds of each cultivar were surface sterilized with 5% sodium hypochlorite solution for 10 min followed by three washing with ethanol and five washing with deionized water (Ahmad *et al.,* 2012).

Copper Treatment

Copper sulphate ($CuSO_4 . 5H_2O$) of high purity (98%) was purchased from Merck chemicals, Germany and used to prepare required Cu concentrations in distilled water. Different levels of Cu i.e., 0, 50, 100, 150, 200, 250, 300 mg L^{-1} were used in the experiment (Waseem *et al.,* 2014). For second step to check the effect of BGS on Cu alleviation five levels of Cu (0,100,150, 250, 300 mg kg^{-1} soil) were used.

Experimental Conditions

The effect of Cu on soybean cultivars was evaluated in growth room of Institute of soil and environmental sciences, University of Agriculture Faisalabad, Pakistan under controlled conditions. Sand was washed with distill water, air dried and then sieved through 2 mm sieve before filling in plastic jars. Each plastic jar with dimensions 15″×7″×5.5″ length, top and bottom diameter contained 400 g sand, Cu levels were developed in sand by adding 50 mL of each Cu solution of above mentioned concentrations in individual plastic jars before sowing. Seeds of each cultivar were soaked in aerated distilled water for 24 h at 28ºC in incubator (Ahmad *et al.,* 2012) due to the short growth period and to increase the emergence. Ten hydroprimed seeds of each cultivar were placed in sand uniformly. These jars were placed in growth room at 25ºC under 14 h. photoperiod with humidity 42 ± 10.2%. Seeds emergence data were recorded from first day of sowing till seven days. After seven days, thinning of plants was done and one plant was kept and after 25 days the plant was harvested and data regarding plant growth was recorded.

In second step the effect of BGS on soybean cv. Rawal-1 was investigated due to its sensitivity and poor growth in Cu contaminated soil. The healthy seeds of cultivar were surface sterilized as already mentioned in seed material and same experimental conditions were followed as mention above. The BGS was collected from the biogas plant located at Agriculture Engineering Department, air dried and crushed in the crushing unit to the final grain size of 5 mm (properties shown in Table 6). Biogas slurry was weighted according to its levels (BGS: 0, 2, 3, 4, 5%) and thoroughly mixed in sand.

Seed Emergence Evaluation

Emerged seeds were counted daily until a constant count according to rules of Association of Official Seed Analysts (AOSA, 1990). The amount of time taken to reach 50% emergence (E50) was calculated according to the formula (Coolbear *et al.,* 1984). Mean emergence time (MET) was calculated according to the equation of Ellis and Roberts (1981). Emergence index (EI) and final seed emergence (%) were calculated by the formulae given in the Association of Official Seed Analysts (1983).

Total Chlorophyll Contents

Total chlorophyll contents were measured with the help of portable SPAD chlorophyll meter (SPAD-502 plus). After 22 days of emergence, third fully expanded leaf of plant was selected for chlorophyll measurement. All the readings were done at 10:00 to 11:00 am.

Seedling Vigor Evaluation

After 25 days of final emergence, data regarding shoot and root length were measured with the help of scale while root and shoot weight was measured with the help of analytical weighting balance.

Quantitative Analysis of Cu in Seedlings

Wet digestion for Cu determination: Plant samples were oven dried at 70ºC and then ground in rotary mill into powder form passing 2 mm sieve. About 1 g ground sample was taken in 100 mL Pyrex digestion flasks. Ten mL of concentrated HNO_3 and HCl in 2:1 ratio were added and left over night at room temperature in fume hood followed by heating up to 350ºC on hot plate till the material became colorless. The deionized water was added in the flasks, filtered through Whatman filter paper No. 42 and the volume was made up to 50 mL. Resultant extract was stored and used for determination of Cu (AOAC, 2003). Copper concentrations in digested samples were quantified with an atomic absorption spectrophotometer (Model: Z-8200).

Statistical Analysis

Statistical analysis was performed using the Statistix 8.1. A CRD two factor factorial design was used for analysis of variance (ANOVA) (Steel *et al.*, 1997). LSD test was used to compare treatment means at $p < 0.05$.

Results

Effect of Cu on Seed Emergence

Excess Cu concentration in plant growth media has shown inhibitory effect on seed emergence while degree of inhibition for emergence varied for different varieties of soybean. Ajmeri-2014 and NARC-2 showed significantly higher emergence (%) under Cu contaminated (300 mg Cu L^{-1}) media and % of seed emergence (20, 23%) and index (50, 48%) as compared to other cultivars. Similarly, under same conditions these parameters were reduced in Rawal-1. The reduction was recorded in seed emergence (44%) and index (64%) respectively. The inhibition of emergence was higher at high level of Cu (250 and 300 mg Cu L^{-1}) in Walliam-82 and Rawal-1 (50 and 60%, respectively) (Table 1, 2 and 3).

Mean emergence time (MET) of seeds was also affected under Cu stress as in case of seed emergence and emergence index. It was observed that MET significantly ($p \leq 0.05$) increased in soybean cultivar Ajmeri-2014 at 250 and 300 mg Cu L^{-1} as compared to control, it was statistically increased with control (Table 3). Mean emergence time of Walliam-82 (17%), NARC-2 (14%), Rawal-1 (17%) and Ajmeri-2014 (17%) was increased respectively, at 300 mg Cu L^{-1} compared to control. However, all these cultivators were non-significant with respect to each other. The results revealed that Cu stress significantly increased the half time for emergence (E_{50}) for soybean cultivars as shown in Table 4. Maximum time for half seed emergence was observed for Rawal-1 (32%) under stress of 300 mg Cu L^{-1}. While minimum increase in half emergence time was observed for Ajmeri-2014 (25%), NARC-2 (26%) and Walliam-82 (29%) at 300 mg Cu L^{-1}. Among all treatments Rawal-1 showed poor performance under Cu stress.

Table 1: Effect of various concentrations of Cu on emergence index (EI) of soybean seeds

	Emergence Index			
mgL^{-1}	Walliam-82	NARC-2	Rawal-1	Ajmeri-2014
Cu-0	11.19±0.9a	11.31±1.0a	11.44±1.1a	11.31±0.8a
Cu-50	9.49±0.8bc	9.62±0.8bc	9.09± 1.2bc	9.28±0.6bc
Cu-100	7.82±0.7de	8.36±0.6cd	7.10± 0.8e-g	7.54±0.8de
Cu-150	7.23±0.8de	5.30±0.9h-k	6.12±0.8f-h	5.13±0.9h-i
Cu-200	5.54±0.6h-j	6.01±0.5gh	5.16±0.8h-i	5.67±0.7hi
Cu-250	4.27±1.1k-m	5.14±0.7k-l	3.93±1.3lm	4.57±0.4i-m
Cu-300	4.45±1.2j-m	5.84±0.6h	4.06±0.6m	5.55±0.8h-j

The mean values sharing different alphabet let0.8ters are significantly different by LSD test ($p \leq 0.05$). The data presented average of three replicates (n=3)

Table 2: Effect of various concentrations of Cu on final seed emergence (%) of soybean seeds

	Final seed emergence			
mgL^{-1}	Walliam-82	NARC-2	Rawal-1	Ajmeri-2014
Cu-0	93±0.8a	100±0.7bc	90±1.5ab	100±0.8a
Cu-50	83±1.0ab	93±1.4c-d	83±1.6c-e	93±0.6ab
Cu-100	77±2.7ab	93±1.4fg	73±1.7e-g	93±0.5ab
Cu-150	70±2.8g	90±0.7g	70±1.7g	87±0.8b-d
Cu-200	73±3.3fg	87±1.4g	70± 1.1fg	87±1.0b-d
Cu-250	53±3.3e-g	77±0.2h	60±1.5hi	83±1.2c-e
Cu-300	53±3.3e-g	77±0.8i	50±1.8hi	80±0.8df

The mean values sharing different alphabet letters are significantly different by LSD test ($p \leq 0.05$). The data presented average of three replicates (n=3)

Table 3: Effect of various concentrations of Cu on mean emergence time (days) of soybean seeds

	Mean emergence time			
mgL^{-1}	Walliam-82	NARC-2	Rawal-1	Ajmeri-2014
Cu-0	5.01±0.6g	5.04±0.5g	5.00±0.8g	5.04±0.5g
Cu-50	5.21±0.6fg	5.26±0.6e-g	5.2±0.6e-g	5.26±0.5e-g
Cu-100	5.33±0.5d-g	5.41±0.7b-g	5.31±0.6c-g	5.40±0.7c-g
Cu-150	5.66±0.6a-f	5.75±0.8a-g	5.65±0.9a-d	5.74±0.7a-d
Cu-200	5.73± 0.6a-f	5.77±0.7a-d	5.70±0.8a-d	5.77±0.5a-d
Cu-250	5.86±0.3a-c	5.93±0.6a	5.90±0.6a-d	5.95±0.7a
Cu-300	5.88±0.5a	5.91±0.5a	5.87±0.8a	5.92±0.7a

The mean values sharing different alphabet letters are significantly different by LSD test ($p \leq 0.05$). The data presented average of three replicates (n=3)

Chlorophyll Contents

Total chlorophyll contents of soybean were drastically decreased in Cu contaminated media at 50, 100, 150, 200, 250 and 300 mg Cu L^{-1} as shown in (Fig. 2). Under normal conditions, total chlorophyll contents of seedling were maximum as compared to the Cu contaminated treatments. The concentration of Cu also had adverse effect on the total chlorophyll contents of soybean plant. Ajmeri-2014 had minimum loss (26%) of chlorophyll contents under maximum concentration of Cu (300 mg Cu L^{-1}solution) as compared to NARC-2, Walliam-82 and Rawal-1 (33, 32 and 41%).

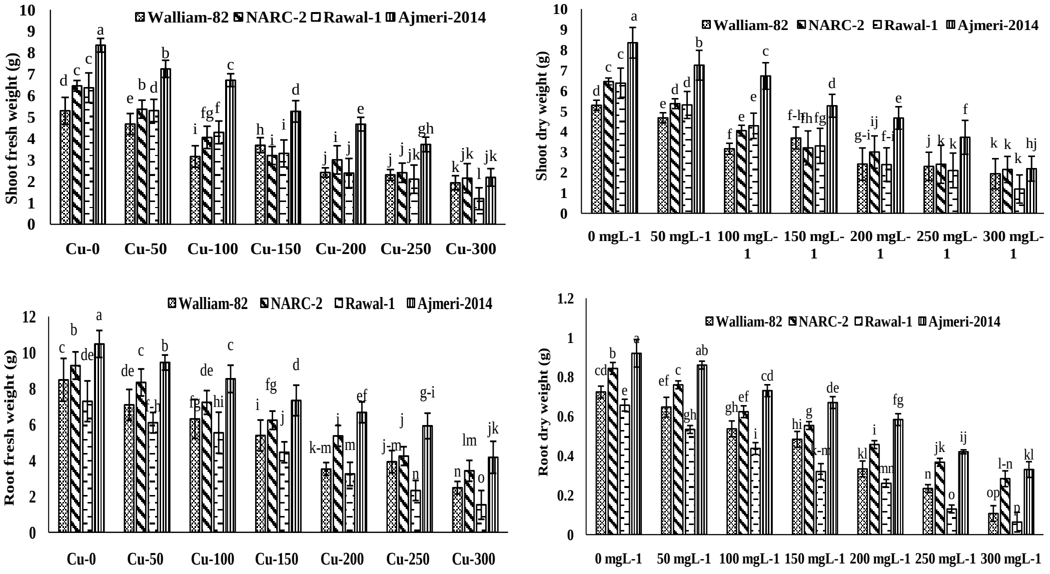

Fig. 1: Effect of Cu (mg L^{-1}) on shoot fresh weight, shoot dry weight, root fresh and dry weight of soybean cultivars. Walliam-82, NARC-2, Rawal-1 and Ajmeri-2014. Bars show means (n=3). Bars having similar letters differ non-significantly at ($p \leq 0.05$) according to LSD test

Table 4: Effect of various concentrations of Cu on half time for emergence of soybean seeds

	E50			
mgL^{-1}	Walliam-82	NARC-2	Rawal-1	Ajmeri-2014
Cu-0	2.31± i	2.21±ij	2.31± i	2.15± i
Cu-50	4.69± fg	5.02± f	4.36± gh	4.17± fg
Cu-100	7.67± a	6.97± b-d	7.47± ab	6.67± a
Cu-150	7.17± a-c	6.59± de	7.00± b-d	6.45±a-c
Cu-200	1.43± l	1.37± l	1.43± l	1.35± l
Cu-250	1.47± l	1.30± l	1.51± l	1.35± l
Cu-300	1.69± kl	1.56± l	1.72± kl	1.45± j-l

The mean values sharing different alphabet letters are significantly different by LSD test ($p \leq 0.05$). The data presented average of three replicates (n=3)

Seedling Growth

The growth of root and shoot of all verities was significantly affected under Cu stress. But maximum reduction in shoot length was observed in Rawal-1 (39%) as compared to control. Similarly, the root length was also reduced in all varieties under Cu stress i.e., maximum reduction in root was observed in Walliam-82 (55%) followed by Rawal-1 (54%) at 300 mg L^{-1} (Table 5). As the concentration of Cu reduced the length of both shoot and root, it also caused reduction in fresh and dry weight of seedling in all cultivars (Fig. 1). The maximum reduction in shoot fresh weight was found in Rawal-1 which was decreased with increase in Cu concentration i.e., from 50 to 300 mg L^{-1}. The maximum reduction in shoot dry weight was recorded in Rawal-1 and Walliam-82 at 300 mg L^{-1} (90 and 80%, respectively) in comparison to control. As far as reduction in root dry weight, NARC-2 and Ajmeri-2014 had less reduction (63 and 60%) in root dry weight as compared to control. It was observed that maximum reduction in plant biomass was observed in Rawal-1 > Walliam-82 > NARC-2 > Ajmeri-2014.

It was observed from results that root and shoot lengths were amplified in soil amended with BGS as compared to non-amended soil under normal as well as Cu stressed conditions (Fig. 3.). The efficacy of biogas slurry (BGS) (2, 3, 4 and 5%) was reduced with increase in Cu concentration (100 to 300 mg Cu kg^{-1} soil). Maximum increase in root shoot length (68%) under normal conditions was recorded at 5% BGS as compared to its respective control but under Cu stress (300 mg Cu kg^{-1} soil). Shoot length (81, 64, 44 and 42%) was achieved with the application of 4% BGS in soil having 100, 150, 250 and 300 mg Cu kg^{-1} soil respectively. As far as Cu contamination is concerned it decreased root length by 66, 69, 74 and 80% in treatments which received 100, 150, 200 and 300 mg Cu kg^{-1} soil, with respect to their control.

In soil contaminated with 100 and 150 mg Cu kg^{-1} soil the shoot dry weight was reduced significantly by 28 and 37% respectively. The soil amended with BGS (2, 3, 4 and 5%) with no Cu improved shoot dry weight by 15, 37, 62 and 82% increase as compared to their respective control respectively. Sudden reduction in root dry weight in Cu contaminated soil at the level of 100, 150, 250 and 300 mg Cu kg^{-1} soil was 10, 30, 55 and 60% respectively as compared to their respective controls. Where as in a soil contaminated with 300 mg Cu kg^{-1} soil and amended with 4% BGS the increase in root dry weight was increased 14% as compared to the soil contaminated with Cu and without addition of BGS (Fig. 3).

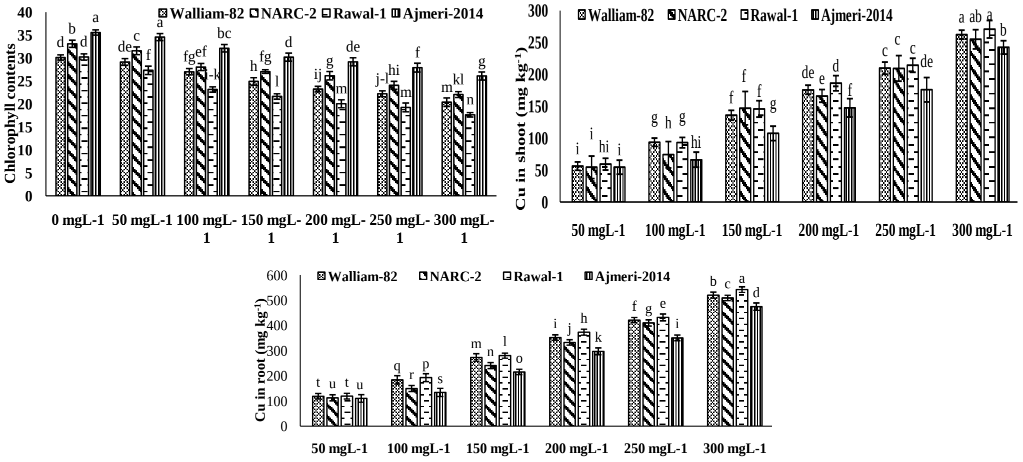

Fig. 2: Effect of Cu (mg L^{-1}) on chlorophyll, shoot and root concentration of Cu in soybean cultivars. Walliam-82, NARC-2, Rawal-1 and Ajmeri-2014. Bars show means (n=3). Bars having similar letters differ non-significantly at (p≤ 0.05) according to LSD test

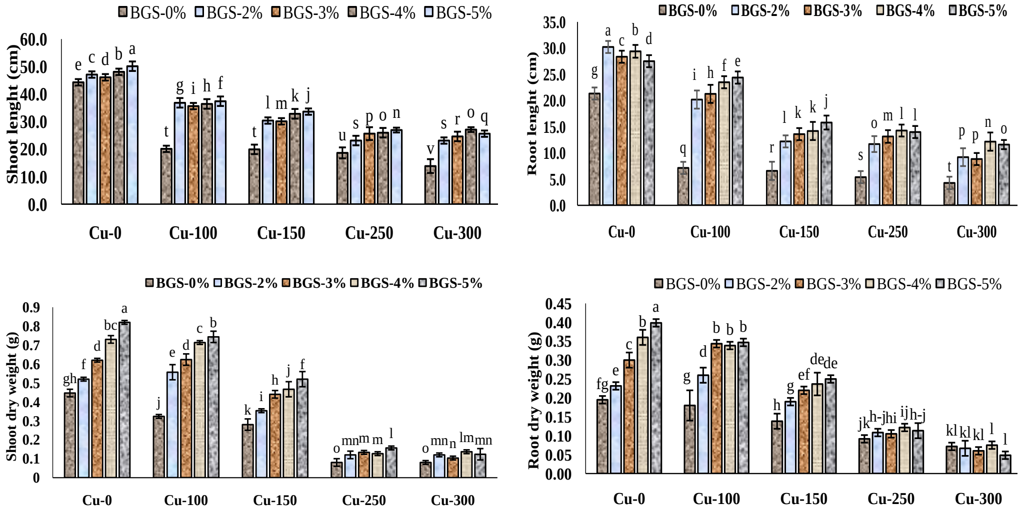

Fig. 3: Copper stress alleviation on shoot, root length and dry weight of soybean by the application of biogas slurry (BGS). Bars show means (n=3). Bars having similar letters differ non-significantly at (p≤ 0.05) according to LSD test. Cu concentrations were in mg kg^{-1} soil

Concentration of Cu in Root and Shoot

The concentration of Cu in both root and shoot was revealed maximum in Rawal-1 and Walliam-82 and minimum was found in Ajmeri-2014 and NARC-2. The concentration of Cu in roots was more as compared to the shoot (Fig. 2). The increase in Cu concentration in roots of Rawal-1 was maximum (25%) where Cu was applied at 300 mg L^{-1} as compared to control while decrease in concentration was observed in roots of NARC-2 (6%) and Ajmeri-2014 (12%) as compared Rawal-1. While the decrease in concentration of Cu in shoots was found in Walliam-82 (3%), NARC-2 (5%) and Ajmeri-2014 (10%) as compared to Rawal-1 (26%) at 300 mg Cu L^{-1} solution. The concentration of Cu was increased in both root and shoot of all cultivars of soybean as the level of Cu was increased from 50 to 300 mg Cu L^{-1} solution.

The concentration of Cu in plant shoot increased with the exogenous addition of Cu at the rate of 300 mg Cu kg^{-1} soil of Cu in soil. The biogas slurry limited its uptake in root and shoot however it depends upon the soil applied Cu (Fig. 4.). Under Cu stress (250 mg Cu kg^{-1}) the increase in concentration of Cu in shoot was observed 98% and in case of root the concentration increase was 103% as compared to control. In this condition the application of BGS reduced the root Cu concentration and decrease (45% and 7%) in concentration was observed more where BGS applied at the rate of 3 and 4%. Similarly, In case of shoot the application

Table 5: Effect of various concentrations of Cu on shoots and root length of soybean

	Shoot length (cm)				Root length (cm)			
mgL^{-1}	Walliam-82	NARC-2	Rawal-1	Ajmeri-2014	Walliam-82	NARC-2	Rawal-1	Ajmeri-2014
Cu-0	52.23± 0.6c	54.83± 0.8b	47.06± 1.3ef	56.81±2.0a	12.66±1.1c	14.93±1.6b	12.33±1.2c	16.29±2.0a
Cu-50	48.58± 0.8d	46.43±0.8ef	44.60±1.5gi	54.28±2.3b	11.03±1.5de	12.36±0.8 c	11.36±1.5d	15.49±1.1ab
Cu-100	47.04± 1.1ef	44.36± 1.1h-j	43.03± 1.3jk	47.81±1.0de	9.17±1.4gh	10.63±1.7de	10.46±1.7d	12.43±1.0c
Cu-150	45.85± 0.8fg	43.00± 1.5jk	39.40±1.0n	45.70±0.8f-h	8.60±1.8h	10.43±1.0ef	9.59±1.8fg	11.21±1.0de
Cu-200	44.09±0.9ij	42.46± 0.8kl	35.06±0.9p	44.05±0.9ij	7.33±0.9i	10.46±1.6e	6.73±1.6 ij	10.43±1.2ef
Cu-250	40.30±1.1mn	41.32± 0.8m	33.20±1.2q	43.25±1.7i-k	7.50±1.1i	9.38±1.3gh	6.16± 1.8jk	9.28±0.9gh
Cu-300	37.70±1.0o	40.33±1.0mn	28.70± 2.3r	41.16±1.4lm	5.31±0.9k	7.57±1.4i	5.62±1.0k	8.56±1.1h

The mean values sharing different alphabet letters are significantly different by LSD test ($p \leq 0.05$). The data presented average of three replicates (n=3)

Table 6: General characters of biogas slurry (BGS)

Characteristics	Unit	value
Organic matter	%	42.0
Total Nitrogen	%	1.65
Extractable Potassium	mg kg^{-1}	1.38
Total Copper	mg kg^{-1}	0.12
Total Calcium	mg kg^{-1}	7.70
Total Magnesium	mg kg^{-1}	3.50
Total iron	mg kg^{-1}	2.12
pH		7.50
EC$_{1:2}$	dS m^{-1}	2.80

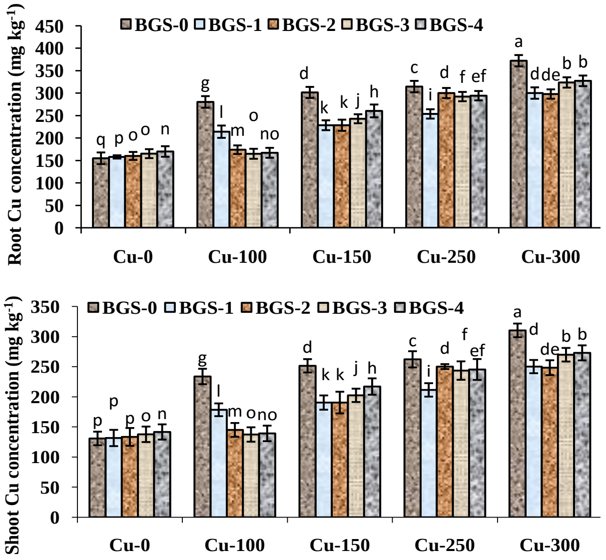

Fig. 4: Copper stress alleviation on root and shoot concentration of soybean by the application of biogas slurry (BGS). Bars show means (n=3). Bars having similar letters differ non-significantly at ($p \leq 0.05$) according to LSD test. Cu concentrations were in mg kg^{-1} soil

of BGS at the rate of 3 and 4% the reduction in Cu concentration was 3 and 7%.

Discussion

Copper is an imperative plant micronutrient, while in elevated concentration it has toxic effect on plants (Jeliazkova and Craker, 2001). The results showed that higher Cu concentrations had adverse effects on parameters related to emergence of soybean. For plant toxicity in soil, emergence index can be used as an indicator to evaluate plant performance (Ahmad *et al.*, 2013). The adverse effect of Cu on emergence index showed its phytotoxicity to soybean seedlings (Table 1). Emergence was indorsed non-significantly by the application of Cu up to 100 mg L^{-1} in Ajmeri-2014 whereas it was decreased in all other soybean cultivars at this Cu concentration. Seed emergence in Ajmeri-2014 and NARC-2 increased at lower level of Cu which might be due to different genetic makeup of these cultivars. At higher concentration of Cu, MET and E$_{50}$ increased while FEP and EI decreased in all cultivars of soybean. Similar results were observed by Gowayed and Almaghrabi (2013). This decrease might be due to some metabolic changes in the seed which leads towards reduced abilities of the seed to germinate as Houshmandfar and Moraghebi (2011) stated that decrease in seed emergence after application of heavy metals might be due to quick breakdown of stored food in seed and change in selective permeability of cell membrane. Related verdicts have been detected by different scientists like Bhardwaj *et al.* (2009) when working on green gram; the growth of seedling was affected because metal contamination interrupted the plant metabolism because they replace the important metals from enzyme surface and biochemical responses occur inside the plant body. Adhikari *et al.* (2012) reported that above 200 ppm copper sulphate solution restricted the germination of soybean seeds and it is due to high level of salinity.

The growth parameters like biomass have been shown to very sensitive to heavy metals (Arun *et al.*, 2005). The increase in Cu concentration reduced the growth of root and shoot and similar results were reported by Liu *et al.* (2001) the root growth of maize plant was gradually reduced when the solution concentration of Cu ion increased and Dey *et al.* (2014) in *Camellia sinensis*. Toxicity due to metal increased enzymatic activity which involved in digestion of stored food in seed and converted into sugar though an enzyme known as amylase after its transportation to roots and shoots. The reason behind root and shoot retarded growth was diversion in actions of hydrolytic enzymes and due to this disturbance root and shoot are unable to utilized reserved food and ultimate result was stunted growth (Jun-yu *et al.*, 2008). Cu-stress induced the retardation of soybean seedling growth which is linked with disturbance of organization and proper functioning of cytoskeleton as a result cell and organs stop their growth and root morphology changed due to injury of auxin transport and distribution mechanism (Kulikova *et al.,* 2015).

The chlorophyll contents of all the cultivars of soybean reduced under Cu stress. It might be due to as the concentration of heavy metals increase the photosynthetic enzymes denatured due to which the chlorophyll contents reduced (Thapar *et al.,* 2008). Negative linear relationships were observed between total chlorophyll contents and Cu concentration (Dey *et al.,* 2014). Metal stress causes decrease in rate of photosynthetic and chlorophyll contents which could also reduce shoot growth (Khan *et al.,* 2006). Zengin and Kirbag (2007) reported that the increase in Cu concentration remarkably reduce the concentration of chlorophyll in sunflower. The Cu toxicity reduced chlorophyll, inactivate enzymes and proteins linked to photosynthesis and modified thylakoid membrane. Due to Cu induced chlorosis the inhibition of pigment accumulation and also decreased in chlorophyll integration into photosystems occur. Overall it caused damage in the structure and function of chlorophyll (Kupper *et al.,* 2003).

The Cu tissue concentration in root and shoot of plants presented a linear response for all cultivars, the concentration of Cu in root and shoot increased with increase in Cu dosages. Maximum increase was found in Rawal-1 followed by Walliam-82, NARC-2 and Ajmeri-2014. It might be due to increase in Cu ion contents in roots, hypocotyls, cotyledons and leaves of sunflower which were increased with increasing Cu ion concentration in solution (Lin *et al.,* 2003). Similarly, the Cu contents in roots of maize plants treated with high amount of Cu in solution are greater than that of roots of control plant (Liu *et al.,* 2001). In present study, amount of Cu in shoot of plants was less as compared to the root concentration of Cu it might be the plants transported and concentrated only a small amount of Cu in their shoots (Liu *et al.,* 2001). Das *et al.* (1997) also found that plants accumulated higher amount of metals in thin roots. The development and morphology of roots mainly affected under Cu stress. It assimilates in the roots of plants and movement towards shoot is very low (Marschner, 1995).

Organic matter addition in soil contaminated with heavy metals not only improves soil fertility but also reduces the bio-availability of heavy metals to plants. Biogas slurry is produced under anaerobic conditions therefore it consists of lump of dead bacterial biomass (Gerardi, 2003). Biogas slurry may also help in availability of metals to plants because it contains several molecules of biological origin which help in bio-sorption of metals (Odlare *et al.,* 2008) it is also helpful in plant disease prevention (Yu *et al.,* 2006). Tiwari *et al.* (2000) reported similar results to present investigation that addition of BGS increased crop performance it might be due to increase in concentration of NH4¬N in digested BGS. Cu-organo complexes association is slightly higher as compared to other materials treatments (Lagomarsino *et al.,* 2011).

In contaminated soil the data showed that Cu was mostly present in acid soluble and reducible portions but in case of amended soil with organic matter the soluble and exchangeable Cu fraction was decreased. It might be the possible reason behind this reduction is due to precipitation and complexation of Cu at stabilized organic matter like humic acid and lignin (Cao *et al.,* 2007; O'Dell *et al.,* 2007). Ali *et al.* (2004) revealed that Cu metal has more attraction for soil organic matter and as a result it became unavailable to plants. The results had shown that organic matter plays a functional role in controlling and reducing heavy metal adsorption to soil especially for Cu which is associated with organic fraction of soil (Dragovi´c *et al.,* 2008).

Conclusion

Cu at high concentration severely reduced seed emergence of soybean cultivars. The order of reduction in seedling growth cultivars under Cu stress was; Ajmeri-2014 < NARC-2014 < Walliam-82 < Rawal-1, germination index, shoot and root length were important parameters for observing Cu toxicity. It could be concluded that Rawal-1 is sensitive cultivar to Cu among other cultivars of soybean (*Glycine max* L.). The present *in vitro* study described preliminary results of BGS application in Cu contaminated soil to improve soybean growth, and was found successful. Further, it was concluded that BGS both under natural and Cu contaminated soils improved growth of soybean.

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