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## Effect of biofertilizers on the plant height and leaf area in *Sorghum vulgare* grown under lead toxic soil

**Anmoldeep Kaur and Prasann Kumar**

**Abstract**

Heavy metal Lead is the second most toxic heavy metal after arsenic, which has zero roles in biological systems. Lead toxicity has a hazardous effect as it can damage the plant from germination stage to yield formation stage. Lead toxicity has a hazardous effect as it can damage the plant from germination stage to yield formation stage. Arbuscular mycorrhizal (AM) fungi are soil-borne species of fungi and globally universal fungi that are colonized in plant roots system and allocate supplementary pathways for the transfer and uptake of mineral nutrients from the soil particles to the plant system. *Trichoderma viride* is a fungus and also a bio fungicide. It is mainly used for the seed and soil treatment for the suppression of various diseases which are mainly caused by fungal pathogens. *T. viride* is a mould which asexually produces spores by mitosis. Along with a unique role as a biocontrol agent, it also has the capability of chemical degradation. *T. viride* is when applied, start colonizing seed/rhizosphere soil surface of the crop and then multiplies on the same. Trichoderma is used as a biopesticide, biofertilizer in agriculture. A pot experiment was set up with *Sorghum vulgare* as test plant to evaluate the role of trichoderma, rhizobium and mycorrhiza mediated mitigation of Lead toxicity on morphological parameters. It was noticed that the plant height was significantly more in control (T<sub>0</sub>) but it was noticed that plant height was significantly reduced with 18.91%, 48.89%, 46.78%, and 43.48% when exposed to lead metal stress (T<sub>1</sub>) as compared to (T<sub>0</sub>) at 30, 60, 90 and 120 DAS. The average leaf area was significantly reduced by 25.03%, 24.9%, 33.3%, and 45.4% due to the lead stress in (T<sub>1</sub>) compared to (T<sub>0</sub>) at 30, 60, 90 and 120 DAS respectively.

**Keywords:** Arbuscular mycorrhizal, biopesticide, *Kharif*, lead, trichoderma

**Introduction**

*Sorghum* (*Sorghum bicolor*), belongs to panicoideae which are a subfamily of the Poaceae (Gramineae). *Sorghum* is a C<sub>4</sub> plant grown mainly for grain production and used as a feed of animals across several continents of the world. It is distinctive due to its drought tolerance capacity and heat-stress, and it also has improved water and nutrient use efficiency (de Vries, *et al.*, 2010) [6]. It is one of the cereals that constitute a major source of proteins, calories, minerals for many people in Africa and Asia. Its rainfall requirement is 500 to 800 mm and can withstand waterlogging conditions (Balole and Legwaila 2006) [3]. It can be grown on different soil types including heavy vertisols, light sandy soils, loams and sandy loams and can tolerate a pH level from 5.0 to 8.5. Due to the global climate change sorghum is mainly grown for grain purpose with high growing interest and supporting fertilizer costs. This is only due to the higher-ranking production under dryland soils with low-fertility conditions (Assefa *et al.*, 2013, Kumar and Dwivedi 2018) [2, 14]. Heavy metals are the most important agents of contamination in the environment. Aside from the natural activities, nearly all the human activities also have an equal contribution to cause contamination of Heavy metals (Gaur and Adholeya 2004) [8]. Heavy metals are the elements with properties of metal and with an atomic number >20. Most usual heavy metal contaminants are Cd, Cr, Cu, Hg, Pb, and Zn. These are naturally present components in soil (Lasat 2000, Kumar and Dwivedi, 2018a) [19, 15]. Some of them are the micronutrients necessary for the growth of plant and development, such as Zn, Cu, Mn, Ni, and Co, some other nutrients have an unknown biological function, such as Cd, Pb, and Hg (Kumar and Dwivedi 2018b) [10]. Heavy metals resist in the soil for thousands of years and bring numerous dangers of health to higher organisms (Kumar *et al.*, 2016) [16]. Heavy metal Lead is the second most toxic heavy metal after arsenic, which has zero roles in biological systems. Lead toxicity has a hazardous effect as it can damage the plant from germination stage to yield formation stage (Kumar and Dwivedi 2018c) [11]. Lead toxicity has a hazardous effect as it can damage the plant from germination stage to yield formation stage (Kumar and Dwivedi 2014) [14].

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Lead toxicity is dependent on both time and concentration. Higher doses of this toxicity can interrupt the plant water and nutritional relations of the plant (Kumar and Harshavardhan, 2020) [13]. This metal disturbs the enzymatic activities of the plant, cell membrane damage and stomatal closure which occur due to the induction of abscisic acid and negative correlation of lead with potassium present in the plant (Kumar *et al.*, 2013a) [17]. The lead dynamics in soil rhizosphere and role physical, biological, chemical, remediation strategies used to decontaminate the lead polluted soil has been declared as harmful (Kumar *et al.*, 2013b) [18]. Every plant needs certain heavy metals for their growth and development but the excess amount of these heavy metals lead to disturbing the plant system (Shahid *et al.*, 2014) [24]. As the plants acquire the essential metals it also acquires non-essentials metals. Once the heavy metal toxicity exceeds from the optimal level, it cannot be broken down and start affecting the plant directly and indirectly (Kuma *et al.*, 2016) [16]. They play a crucial role in the movement of essential metals from their actual binding site on biological molecules as As and Cd compete with Pb and Zn in the plant. This may lead to imbalance and deficiencies of nutrients (Kumar *et al.*, 2018b) [10]. Heavy metals can generate oxidative stress due to the overproduction of reactive oxygen species (ROS) causes damage to the inherent defence system of the cell which can lead to cell's death (Kumar and Dwivedi 2014) [14]. Lead is naturally occurring metal in soils, typically having concentrations range from 10 to 50 mg/kg. These concentrations can also be ranged from 150 mg/kg to the peak of 10,000 mg/kg. Pb metal is found to be severely toxic to human beings when present in high concentrations. It is non-biodegradable, soil contaminated with lead remains for the long term (Kumar and Dwivedi 2018a) [15]. Lead has some important properties like softness, malleability, ductility, poor conductivity and resistance to corrosion which make it difficult to give up on its uses. Arbuscular mycorrhizal (AM) fungi are soil-borne species of fungi and globally universal fungi that are colonized in plant roots system and allocate supplementary pathways for the transfer and uptake of mineral nutrients from the soil particles to the plant system (Kumar and *et al.*, 2013a, b) [17]. The symbiosis between mycorrhizal fungi and sorghum plant contribute to improving the yield of sorghum and nutrition in the soils which are nutrient-deficient (Raju *et al.*, 1990) [23]. As sorghum makes use of C4 pathway of photosynthesis it shows obligate symbionts with AM fungi (Wilson and Hartnett, 1998) [25], the plants of sorghum are highly responsive to the symbiosis for growth and grain production in low fertility soils. Rhizobium is aerobic, gram-negative, symbiotic diazotrophic nitrogen-fixing bacterial symbionts of papilionaceous roots. It is a prokaryotic organism that carries out dinitrogen fixation. Rhizobium is present in the root nodules of *Sesbania rostrata* and stem nodules of aerorhizobium. Some of the species of rhizobium live in the soil but they are unable to fix nitrogen by themselves in soil. They need an association with the legumes plants to produce nitrogen. Rhizobia enhance the yield of non-leguminous plants by the production of Phyto-stimulating substances such as auxins, cytokinins, gibberellins, ACC deaminase. *Trichoderma viride* is a fungus and also a bio fungicide. It is mainly used for the seed and soil treatment for the suppression of various diseases which are mainly caused by fungal pathogens. *T. viride* is a mould which asexually produces spores by mitosis. Along with a unique role as a biocontrol agent, it also has the capability of chemical degradation. *T. viride* is when applied, start colonizing

seed/rhizosphere soil surface of the crop and then multiplies on the same. *Trichoderma* is used as a biopesticide, biofertilizer in agriculture. There are more than 100 species of *Trichoderma* recorded worldwide (Pandya *et al.*, 2011) [20], from which many are potential biocontrol agents (Kumar and Dwivedi 2018, 2018a, 2018c) [14, 15, 11].

## Materials and Methods

The experiment was laid out in a completely randomized design (CRD). There was thirteen treatment including control. Each treatment was replicated three times, therefore, total no. of pots are 39. Data collected at 30, 60, 90 and 120 DAS was assessed in SPSS V22.0 software with Descriptive statistics, multivariate test, Duncan's multiple range test (DMRT) with probability  $p < 0.05$  values. Duncan values present in analyzed homogenous sets used to make tables and graphs. The pots used for the experiment had a diameter of 30 cm and height 25 cm and each with the capacity of 10 kg soil, with a small hole at the bottom. According to the plan of work, targeted pots were inoculated with 5 gm Endomycorrhiza, Rhizobium and *Trichoderma*. Heavy metal stress was created by the exogenous application of 100ppm Lead chloride. The various measurements were taken at four stages such as 30 DAS, 60 DAS, 90 DAS and 120 DAS (Days after sowing) of the concerned pots.

## Treatments Details

The details of the treatments are:- Control (T0), Lead chloride (100ppm) (T1), *Trichoderma* fungi (*Trichoderma viride*) (5 gm) (T2), Rhizobium (*Rhizobium trifolli*) (5 gm) (T3), Endomycorrhizal fungi (*Glomuss mycorrhizae*) (5 gm) (T4), Lead chloride (100 ppm) + *Trichoderma* (*Trichoderma viride*) (5 gm) (T5), Lead Chloride + Rhizobium (*Rhizobium trifolli*) (5 gm) (T6), Lead chloride (100 ppm) + Mycorrhizae (*Glomuss mycorrhizae*) (5 gm) (T7), Lead chloride (100 ppm)+ *Trichoderma* (*Trichoderma viride*) (5 gm)+ Rhizobium (*Rhizobium trifolli*) (5 gm) (T8), Lead chlorie (100 ppm) + *Trichoderma* (*Trichoderma viride*) (5 gm) + Mycorrhizae (*Glomuss mycorrhizae*) (5 gm) (T9), Lead chloride (100 ppm) + Rhizobium (*Rhizobium trifolli*) (5 gm) + Mycorrhizae (*Glomuss mycorrhizae*) (5 gm) (T10), Lead chloride (100 ppm) + *Trichoderma* (*Trichoderma viride*) (5 gm) + Rhizobium (*Rhizobium trifolli*) (5 gm) + Mycorrhizae (*Glomuss mycorrhizae*) (5 gm) (T11), Control + *Trichoderma* (*Trichoderma viride*) (5 gm) + Rhizobium (*Rhizobium trifolli*) (5 gm) + Mycorrhizae (*Glomuss mycorrhizae*) (5 gm) (T12).

## Observation Was Recorded

### Plant Height (cm)

Plant height measured at 30, 60, 90 and 120 days after sowing. Measuring scale was used to measure the height of the plant from the ground to the top surface to the topmost leaf of the plant.

### Leaf Area (cm<sup>2</sup>)

Leaf area is measured at 30, 60, 90 and 120 days after sowing. Leaf area meter was used to measure the area of leaves by placing a leaf in the meter.

## Results and Discussion

### Plant Height (cm)

Effect of *Trichoderma*, Rhizobium, Mycorrhiza and their combination on plant height (cm) was studied in sorghum variety SSV 74, under the stress of lead. Data were recorded at 30, 60, 90, and 120 days after sowing (DAS). (Table 1, Fig

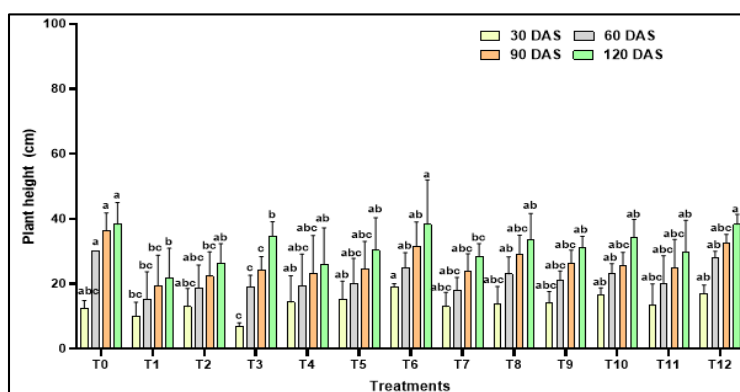
1). The average plant height was significantly reduced with 18.91%, 48.89%, 46.78%, and 43.48% when exposed to lead metal stress (T1) as compared to (T0) at 30, 60, 90 and 120 DAS. On the other hand, plant height was increased by 5.40% at 30 DAS and decreased by 37.78% to 46.78% and 31.30% at 60, 90 and 120 DAS as compared to (T0) under the Trichoderma treatment (T2). In (T3) Rhizobium application conditions, plant height was reduced by 56.75% to 66.66% at 30, 60 DAS, and 53.21% to 46.08% at 90 and 120 DAS as compared to (T0). Under The treatment of Endomycorrhizal fungi in (T4) showed increasing value of plant height with 16.21% at 30 DAS but reduced with minor changes by 35.55% to 35.78% and 32.17% as compared to (T0) on proposed dates of interval. Similarly when (T0) was compared to (T5) having Lead and Trichoderma and treatment plant height was increased by 24.32% due to the mitigating effect of on toxicity at 30 DAS and showed a reduction in height on other proposed days of the interval by 33.33%, 32.11%, and 20.87%. In comparison to T0, exogenous application of lead with Rhizobium (T6), showed mitigation by rhizobium in plant height with 54.05%, at 30 DAS then reduced to 16.66% to 12.84 at 60 and 90 DAS but remain constant at 120 DAS. When (T7) treatment was compared to (T0) mycorrhiza showed its minor mitigating effects at 30 DAS with an increase in plant height by 5.40% but reduced by 40%, 33.94%, and 26.08% on the proposed date of interval. There is minor increase by 5.1% in (T8) as compared to (T7) on 30 DAS when Trichoderma and rhizobium both are mitigating the effect of lead and on the other proposed dates of the interval, plant height was reduced by 23.33% and 20.18% when the lead is exposing its effect while on the other hand at 120 DAS there was little change by 4.3% when compared to (T0). The soil, when exposed to lead

along with Trichoderma and Mycorrhiza in (T9) plant height, was increased by 13.51% at 30 DAS and decreased in further proposed dates of the interval by 30%, 27.52%, and 19.13% compared to (T0). When (T10) compared to (T0) plant height increased by 35.14% initially for a month due to the mitigating effect of rhizobium and mycorrhiza on lead stress and reduced by 22.22%, 29.3% and 10.4% on the proposed date of intervals due to the lead stress increased its effect on the plant. Similarly, when we go for treatment (T11) where all the three biofertilizers are present with lead, plant height increased by 8.10% at 30 DAS and reduced in all other proposed date of intervals. When we compare the heights of plants at 30 DAS the maximum was obtained in (T6) and (T12) compared to (T0) by 54.04% and 48.65%. and approximately the same or reduced by 1.74% at 120 DAS compared to (T0). Kumar *et al.*, 2016 [16], suggested that arbuscular mycorrhizal can enhance the growth of the plant, protect the host plant from various stresses and mediate plant-plant interactions. They conducted a greenhouse experiment to investigate the AM functions in drought stress and mortality of forage sorghum plant. The experimental results concluded that the AM associations could reduce the retardation of growth and can prolong the lifespan of plants under the stress of drought. Moreover, the use of AM fungi can increase the forage sorghum yield under semi-arid and arid conditions. Kumar *et al.*, 2018a, b & c, they concluded that plant growth was inhibited 50% when exposed to 100ppm Pb concentration. They said that accumulation of metal was higher in roots than in shoot tissues. The maximum Pb level of concentration was noticed in roots (5.45%) followed by petiole 2.72% and leaf tissues 0.66%. they also concluded that chlorophyll content was decreased with the increase in Pb concentration.

**Table 1:** Plant Height (cm) of sorghum during *Kharif*

Treatments	30 DAS	60 DAS	90 DAS	120 DAS
T0	12.33 <sup>abc</sup> ± 2.51	30.00 <sup>a</sup> ± .00	36.33 <sup>a</sup> ± 5.50	38.33 <sup>a</sup> ± 6.65
T1	10.00 <sup>bc</sup> ± 4.35	15.33 <sup>bc</sup> ± 8.38	19.33 <sup>bc</sup> ± 9.45	21.66 <sup>b</sup> ± 9.29
T2	13.00 <sup>abc</sup> ± 5.56	18.66 <sup>abc</sup> ± 7.09	22.33 <sup>bc</sup> ± 7.50	26.33 <sup>ab</sup> ± 6.02
T3	5.33 <sup>c</sup> ± 0.577	10.00 <sup>c</sup> ± 1.00	17.00 <sup>c</sup> ± 2.64	20.66 <sup>b</sup> ± 1.15
T4	14.33 <sup>ab</sup> ± 8.14	19.33 <sup>abc</sup> ± 9.81	23.33 <sup>abc</sup> ± 11.5	26.00 <sup>ab</sup> ± 11.26
T5	15.33 <sup>ab</sup> ± 5.50	20.00 <sup>abc</sup> ± 7.81	24.66 <sup>abc</sup> ± 8.38	30.33 <sup>ab</sup> ± 10.01
T6	19.00 <sup>a</sup> ± 1.00	25.00 <sup>ab</sup> ± 4.58	31.66 <sup>ab</sup> ± 7.37	38.33 <sup>a</sup> ± 13.57
T7	13.00 <sup>abc</sup> ± 4.35	18.00 <sup>abc</sup> ± 4.00	24.00 <sup>abc</sup> ± 5.29	28.33 <sup>ab</sup> ± 4.04
T8	13.66 <sup>abc</sup> ± 5.50	23.00 <sup>ab</sup> ± 5.29	29.00 <sup>abc</sup> ± 6.00	33.66 <sup>ab</sup> ± 8.02
T9	14.00 <sup>abc</sup> ± 3.60	21.00 <sup>abc</sup> ± 3.00	26.33 <sup>abc</sup> ± 4.04	31.00 <sup>ab</sup> ± 3.60
T10	16.67 <sup>ab</sup> ± 2.08	23.33 <sup>ab</sup> ± 2.88	25.66 <sup>abc</sup> ± 4.04	34.33 <sup>ab</sup> ± 5.50
T11	13.33 <sup>abc</sup> ± 6.65	19.66 <sup>abc</sup> ± 8.96	25.00 <sup>abc</sup> ± 8.66	29.66 <sup>ab</sup> ± 9.86
T12	18.33 <sup>ab</sup> ± 2.08	23.33 <sup>ab</sup> ± 7.63	29.00 <sup>abc</sup> ± 6.55	37.66 <sup>a</sup> ± 3.05

where, DAS: Days after sowing, data are in the form of Mean±SD at  $p < 0.05$



where, DAS: Days after sowing, data are in the form of Mean±SD at  $p < 0.05$

**Fig 1:** Plant Height (cm) of sorghum *Kharif*

**Leaf Area (cm<sup>2</sup>)**

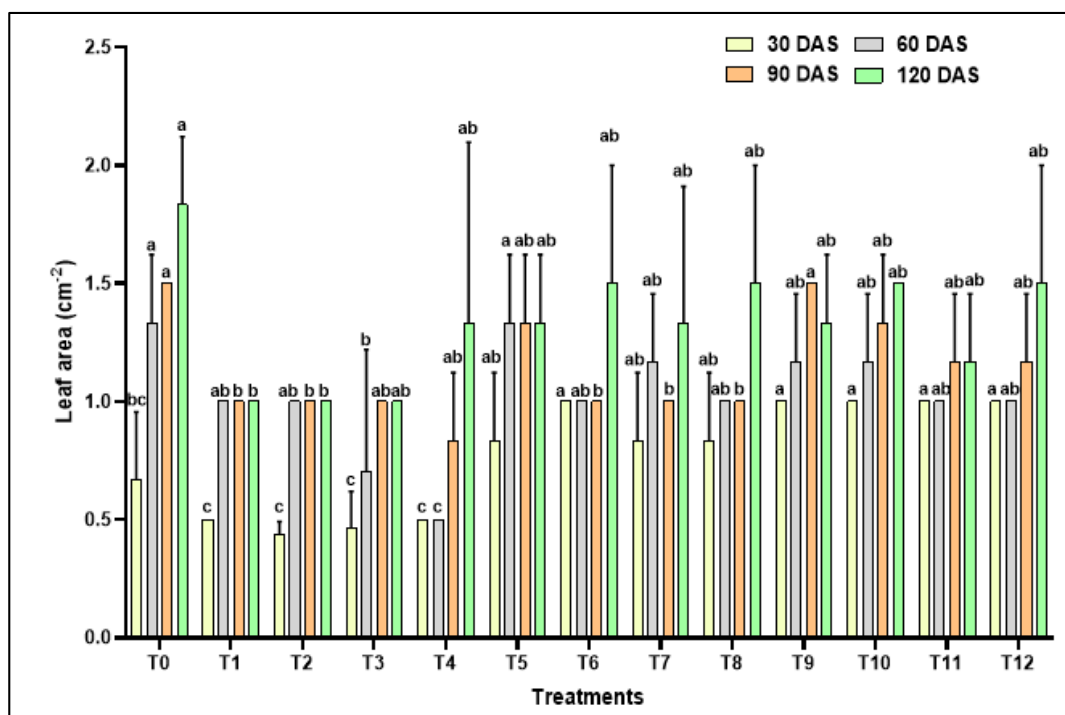
Effect of Trichoderma, Rhizobium, Mycorrhiza and their combination on leaf area (cm<sup>2</sup>) was studied in sorghum variety SSV 74, under the stress of lead. Data were recorded at 30, 60, 90, and 120 days after sowing (DAS) (Table 2, Fig 2.). The average leaf area was significantly reduced by 25.03%, 24.9%, 33.3%, and 45.4% due to the lead stress in (T1) compared to (T0) at 30, 60, 90 and 120 DAS. When we compare (T2) with (T1), we noticed that at 30 DAS leaf area reduced to 13.4% and remains the same on proposed dates of interval. Similarly, when we compare (T3) with (T0) we found that leaf area was decreased with 35.08%, 37.5%, 22.2%, and 36.3% on proposed dates of interval. Exogenous application of endomycorrhiza in soil (T4) showed the mitigation effect by lower the decreasing value of leaf area with 25.03%, 62.4%, 22.2%, and 27.2% on 30, 60, 90 and 120 DAS. In the treatment of lead with Trichoderma (T5), leaf area was increased by 24.8% at 30 DAS, and same at 60 DAS but decreased by 11.1% and 27.2% at 90 and 120 DAS. When we go for (T6) where there is an application of lead with rhizobium leaf area was increased by 130.9%, 20.0%, and 28.6% at 30, 60 and 120 DAS due to the mitigating effect of rhizobium on lead stress but the other hand at 90 DAS leaf area was reduced to 14.2% as compared to (T3). When we compare (T7) with (T4) we found that both the treatments have mycorrhizae, so we concluded that mycorrhiza mitigates the effect of lead stress and increase the leaf area up to 66.6%, and 133.3% at 30 and 60 DAS and reduced by 14.2% at 90 DAS but remain same at 120 DAS. On the other hand, (T8) when compared to (T0) lead stress was exposing its effect on leaf area initially at 30 DAS, it was increased by 24.8% and then decreased by 24.9%, 33.3%, and 18.1% on proposed dates of interval. The average leaf area was significantly increased by 49.9% at 30 DAS and same at 90 DAS but reduced by 12.5% and 27.2% at 60 and 120 DAS in (T9) compared to (T0). (T10) in comparison with (T0) increases leaf area with 49.9% at 30 DAS and reduced by 12.5%, 11.1% and 18.1% on proposed dates of interval. (T11) where

there is the application of all the three biofertilizers along with lead, leaf area was increased by 49.9% at 30 DAS and reduced by 24.9%, 22.2% and 36.3% on proposed dates of interval. When we compare (T12) with (T11) where control was there with all the three biofertilizers, leaf area was same at 30, 60, 90 DAS but there was a minor decrease in value of leaf area by 9.1% at 120 DAS. Kumar and Dwivedi 2018c<sup>[11]</sup> reported the genetic improvements in sorghum breeding and adapt trails in small-plot across multiple environments. They said that leaf are development information in the water-limited environment would provide valuable insight into water use and adaptation to the scarcity of water during the specific phenological stages of crop development. Leaf area at 30 days seems to be same in many treatments like T6, T9, T10, T11, and T12 as the lead toxicity is exposing its effect in these treatments. There is less improvement in leaf area on proposed dates of interval. The best leaf area was obtained in T0 at 120 DAS this shows that the controlled treatments have more growth than the other treatments. The treatment T4 at 60 DAS showed a mitigating effect of mycorrhiza by reduced the leaf area by 62.4%.

**Table 2:** Leaf area (cm<sup>2</sup>) of sorghum during *Kharif*

Treatments	30 DAS	60 DAS	90 DAS	120 DAS
T0	0.66 <sup>bc</sup> ± .28	1.33 <sup>a</sup> ± .28	1.50 <sup>a</sup> ± .00	1.83 <sup>a</sup> ± .28
T1	0.50 <sup>c</sup> ± .00	1.00 <sup>ab</sup> ± .00	1.00 <sup>b</sup> ± .00	1.00 <sup>b</sup> ± .00
T2	0.43 <sup>c</sup> ± .05	1.00 <sup>ab</sup> ± .00	1.00 <sup>b</sup> ± .00	1.00 <sup>b</sup> ± .00
T3	0.43 <sup>c</sup> ± .11	0.83 <sup>b</sup> ± .28	1.16 <sup>ab</sup> ± .28	1.16 <sup>ab</sup> ± .28
T4	0.50 <sup>c</sup> ± .00	0.50 <sup>c</sup> ± .00	1.16 <sup>ab</sup> ± .28	1.33 <sup>ab</sup> ± .76
T5	0.83 <sup>ab</sup> ± .28	1.3 <sup>a</sup> ± .28	1.33 <sup>ab</sup> ± .28	1.33 <sup>ab</sup> ± .28
T6	1.00 <sup>a</sup> ± .00	1.00 <sup>ab</sup> ± .00	1.00 <sup>b</sup> ± .00	1.50 <sup>ab</sup> ± .50
T7	0.83 <sup>ab</sup> ± .28	1.17 <sup>ab</sup> ± .28	1.00 <sup>b</sup> ± .00	1.33 <sup>ab</sup> ± .57
T8	0.83 <sup>ab</sup> ± .28	1.00 <sup>ab</sup> ± .00	1.00 <sup>b</sup> ± .00	1.50 <sup>ab</sup> ± .50
T9	1.00 <sup>a</sup> ± .00	1.16 <sup>ab</sup> ± .28	1.50 <sup>a</sup> ± .00	1.33 <sup>ab</sup> ± .28
T10	1.00 <sup>a</sup> ± .00	1.16 <sup>ab</sup> ± .28	1.33 <sup>ab</sup> ± .28	1.50 <sup>ab</sup> ± .00
T11	1.00 <sup>a</sup> ± .00	1.00 <sup>ab</sup> ± .00	1.16 <sup>ab</sup> ± .28	1.16 <sup>ab</sup> ± .28
T12	1.00 <sup>a</sup> ± .00	1.00 <sup>ab</sup> ± .00	1.16 <sup>ab</sup> ± .28	1.66 <sup>ab</sup> ± .28

where, DAS: Days after sowing, data are in the form of Mean±SD at  $p < 0.05$



where, DAS: Days after sowing, data are in the form of Mean±SD at  $p < 0.05$

**Fig 2:** Leaf area (cm<sup>2</sup>) of sorghum during *Kharif*



## Conclusion

From the result, it was concluded that plant height was reduced when exposed to lead toxicity as compared to control (T0). Exogenous application of biofertilizers with Lead metal was able to mitigate the Lead toxicity by enhancing the plant height as compared to Lead stress. Similarly the combined application of mycorrhiza and trichoderma along with Lead metal able to reduce the toxicity of Lead by improving the leaf area as compared to the Lead stress. It was also found that the combined application of biofertilizers was more effective in mitigating the toxicity stress of Pb contaminated soil by improving the potential production of sorghum.

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## Author Contributions

P.K. designed the study, established the biochemical protocols, A.K performed the experiments and collected the data analyzed and interpreted the data. The P.K., and A.K. wrote the paper.

## Conflict of Interest Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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