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RESEARCH REPORT

Supplementation of fly ash improves growth, yield, biochemical, and enzymatic antioxidant response of chickpea (*Cicer arietinum* L.)

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Abstract

This research was investigated to assess the effect of fly ash (FA) on the growth, yield, biochemical attributes, and enzymatic antioxidant response of chickpea (*Cicer arietinum* L. cv. Avrodhi). After evaluating soil and FA nutrient status by energy-dispersive X-ray spectroscopy (EDX), different concentrations of FA with soil were applied (5, 10, 15, 20, 25, and 30%). Chickpea plants grown at all FA-amended soil concentrations demonstrate significant improvement ($p \leq 0.05$) in growth and yield biomass. Exhibition of enhancement in biochemical attributes such as photosynthetic pigments (chlorophyll a and b, total chlorophyll, and total carotenoid by 16.83, 24.50, 19.13, and 21.74%), total protein (48.54%), leghemoglobin (42.39%), nitrate reductase activity (18.35%), proline content (28.16%), and antioxidant enzymes activities. Moreover, an improvement in the stomatal pore of the FA treated plant also noticed by a scanning electron microscope (SEM). Overall, the findings of this study indicate that the supplementation of FA contributed to increment in these attributes at all the concentrations compared with control (without FA) 'but maximally ~ up to FA 20%.' However, plant growth and yield biomass decreased above the concentration of FA 20% because chickpea can balanced nutrients efficiency up to FA 20%.

Keywords Antioxidants · EDX · FA · Growth · SEM · Sustainable

1 Introduction

Fly ash (FA) is an environmentally harmful and problematic waste produced by coal-burned thermal power plants, and its environmental disposal is one of the biggest issues worldwide, particularly in developing countries. The continuous massive production of FA causes various dumpsites requirements. FA globally recognized as a significant environmental risk due to different harmful effects on the environment. The generation rate of FA was reached up to 196.44 million tonnes from 624.88 million tonnes of coal over the period 2017–2018 in India (CEA 2017–2018). Usually, FA is discharged in different ways, for example in the FA basin, landfills, and washed with water in the pond ash. Although its usage in the sanitary, cement, and bricks manufacturing industries but huge amounts remain unutilized and are dumped (Verma et al. 2014). Besides, the multi-nutrient composition of FA provides considerable possibilities for sustainable and environment-friendly use in the farming sector, as well as a viable option for mitigating harmful effects.

In the agriculture sector, the application of FA has been shown to serve as manure that supplies macro and micronutrients for crop plants (Haris et al. 2019). Low-rate addition of the FA amended with soil has been observed to ensure higher water holding capacity, soil conductivity, texture, pH, and organic carbon (Pandey and Singh 2010; Haris et al. 2019) and provides preferable conditions for growth performance and yield biomass of the plants (Pandey et al. 2009). Generally, the supply of nutrients considered as the essential regulatory constituent for plant growth and productivity. Thus, FA often improves soil nutrient content and uptake plant beneficial nutrients, which enhance the crop yield biomass (Thind et al. 2012; Ukwattage et al. 2013). Numerous studies have been investigated to assess the beneficial impact of FA on plant growth and biomass production of different crops as seen in wheat (Ochecova et al. 2014),

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rice (Bisoi et al. 2017), carrot (Haris et al. 2019), radish (Sharma and Singh 2019), and safed musli (Hadke et al. 2019).

Chickpea, *Cicer arietinum* L., is the third most important legume crop in the world which belongs to the family Fabaceae. It is a commercially significant crop grown worldwide and a good source of proteins, carbohydrates, and fats (Jukanti et al. 2012). It widely used in the human diet and animal feed. Chickpea also a rich source of minerals and β-carotene, its protein content is higher than that of the other leguminous crops (Jukanti et al. 2012; Siddique et al. 2012). Due to its high nutritional values, chickpea is one of the most important in Indian, Middle Eastern, and Mediterranean cuisine. India accounted for 66% of the world's production of chickpea in 2018 (FAOSTAT 2019). Given the above, the present study aims to evaluate the growth performance, yield biomass, biochemical attributes, and enzymatic antioxidant response of chickpea in different concentrations of soil modified with FA.

2 Materials and methods

2.1 Treatment and plant growth conditions

FA was collected from the Harduaganj Thermal Power Station which situated 14 km away from AMU, Aligarh, U.P., India and soil collected from the agriculture field. Then, different concentrations prepared in the rations of soil and FA (w/w): Control (without FA), FA 5, 10, 15, 20, 25, and 30%. After appropriate mixing of soil with FA, each pot was filled with 1 kg mixture in earthen pots (12 cm). The experiment was laid with five replicates of each treatment ($n=5$) in randomized block design. A total of 35 pots (7 treatments × 5 replicates) were prepared of soil and FA. Compatible size of chickpea seeds was surface-sterilized with 1% NaOCl for 10 min and then washed six times with the help of distilled water. Subsequently, five seeds were planted in each pot containing a mixture of soil-FA. After seven days of seedling germination, thinning was done to accommodate only one plant in each pot. Plants were grown under natural conditions of day/night temperature of mean 20 ± 5 ℃/15 ± 2 ℃. The experiment was started in the 1st week of November 2019 and terminated at the end of January 2020. Analysis of different biochemical parameters was done after 40 days of seed sowing. On day 80th, plants were harvested for determination of leghaemoglobin content, growth, and yield biomass.

2.2 Soil and FA analysis

Before the formulation of soil with FA, physicochemical properties of soil and FA were examined with the help of EDX (Fig. 1) and ultrastructure was also observed (Fig. 2) by scanning electron microscope (JSM-6510 LV, JEOL Ltd., Tokyo, Japan).

2.3 Assessment and bioassays

2.3.1 Growth and yield biomass

At the end of this experiment, all plants were removed from the pots and washed properly through running water. The length of the shoots and roots was measured by using a scale. For the determination of dry weight, plant samples were oven-dried at 80 °C for 24 h and then weighed with the help of weighing balance. The number of ~ nodules and yield parameters (Number of flowers, pods, and leaves) counted manually. Leaf area was estimated with the help of the graph paper method.

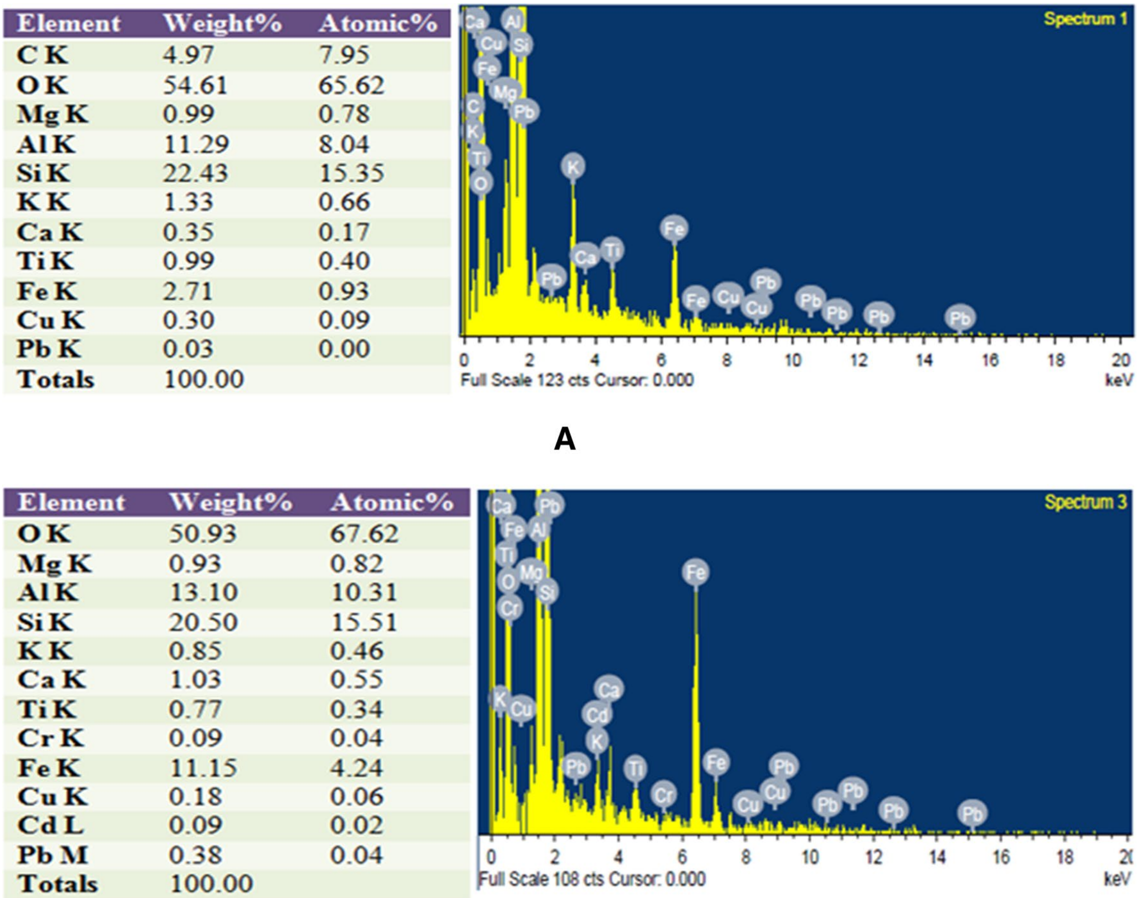
2.3.2 Photosynthetic pigments and relative water content

The photosynthetic pigments (chlorophyll a and b, total chlorophyll, and total carotenoid) were determined by the procedure of Maclachlan and Zalik (1963). Absorbance was recorded at 663, 645, 510, and 480 nm by a spectrophotometer (VS-3305 EI, EE Ltd., Kerala, India) against the solvent (80% acetone) as blank. Relative water content (RWC) of plant leaf was used to assay the water status of the plant, based on Yamasaki and Dillenburg's (1999) method. RWC was calculated by the following formula:

$$\text{RWC}(\%) = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

2.3.3 Protein and leghemoglobin

Protein content in fresh leaves was estimated by the protocol of Bradford (1976). Absorbance was recorded at 595 nm by spectrophotometer with bovine serum albumin used as a blank. Leghemoglobin content in fresh nodules was determined according to the Sadasivam and Manickam (1992) method. Absorbance was read at 539 and 556 nm against a blank reagent through a spectrophotometer. Leghemoglobin content (mM) was measured by using the following formula:



Element	Weight%	Atomic%
C K	4.97	7.95
O K	54.61	65.62
Mg K	0.99	0.78
Al K	11.29	8.04
Si K	22.43	15.35
K K	1.33	0.66
Ca K	0.35	0.17
Ti K	0.99	0.40
Fe K	2.71	0.93
Cu K	0.30	0.09
Pb K	0.03	0.00
Totals	100.00	

Element	Weight%	Atomic%
O K	50.93	67.62
Mg K	0.93	0.82
Al K	13.10	10.31
Si K	20.50	15.51
K K	0.85	0.46
Ca K	1.03	0.55
Ti K	0.77	0.34
Cr K	0.09	0.04
Fe K	11.15	4.24
Cu K	0.18	0.06
Cd L	0.09	0.02
Pb M	0.38	0.04
Totals	100.00	

Fig. 1 Energy-dispersive X-ray (EDX) profiling of soil (**a**) and only FA (**b**)

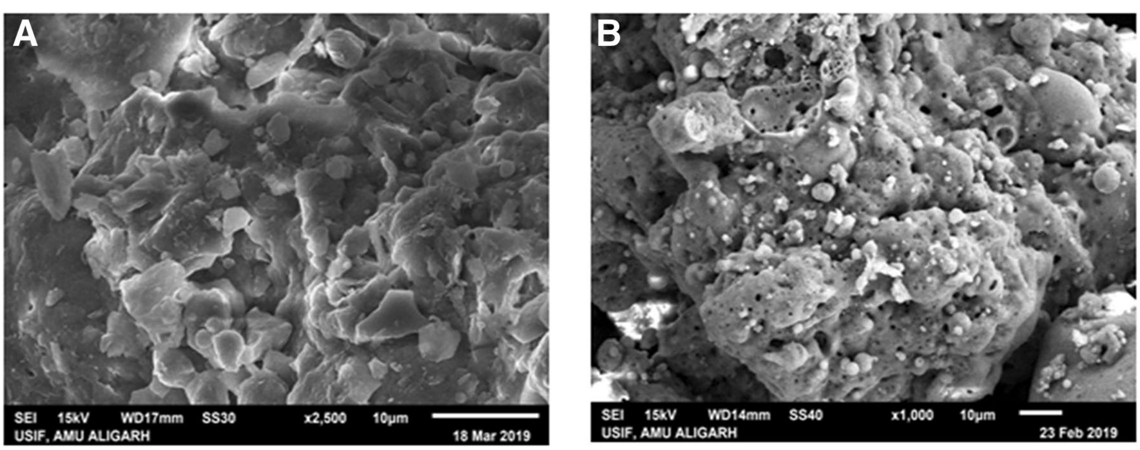


Fig. 2 Scanning electron micrograph (SEM) of soil (**a**) and FA (**b**)

$$\text{Leghemoglobin content (mM)} = \frac{A_{556} - A_{539}}{23.40} \times 2D$$

where D = initial dilution.

2.3.4 Nitrate reductase activity and proline

Nitrate reductase (NR) activity was quantified by the method of Jaworski (1971). The absorbance was recorded at 540 nm with the help of a spectrophotometer. Proline content in chickpea leaves was described by Bates et al. (1973). Absorbance at 520 nm was recorded by using a spectrophotometer and toluene served as a blank.

2.3.5 Assessment of antioxidant enzymes activity

For the determination of the activity of antioxidant enzymes, leaf tissue of 0.5 g was homogenized in 5 ml of 50 mM phosphate buffer (pH 7.0) with 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15,000 rpm at 4 °C for 10 min. The supernatants were accumulated for the determination of SOD by using the method of Beauchamp and Fridovich (1971). CAT and POX were determined by the method of Chance and Maehly (1955).

2.3.6 Leaf characteristics

For the estimation of leaf characteristics**,** leaf surface was visualized through scanning electron microscope (JSM-6510 LV, JEOL Ltd., Tokyo, Japan). Before the analysis, the specimen was coated with gold.

2.3.7 Statistical analysis

Data analysis was presented statistically by using SPSS, 17 software (SPSS Inc., Chicago, IL, USA). Mean values ± standard error of five replicates ($n = 5$). Duncan's Multiple Range Test (DMRT) and analysis of variance (ANOVA) were utilized on the data to consider as significant at $p \le 0.05$.

3 Results

3.1 Physicochemical properties of soil and FA

EDX was applied to summarize the elemental analysis of soil and FA which showed that some beneficial plant nutrients (Mg, Al, K, Ca, Fe, and Cu) present in the FA (Fig. 1b). SEM was used to analyze the morphology of soil and FA particles (Fig. 2). FA made up of mainly spherical particles and small in size.

3.2 Effects of FA on plant growth and yield biomass

Plants of chickpea under soil amended with FA resulted in the enhancement of growth and yield biomass at all the

Table 1 Effect of FA treatments on growth of chickpea plants

Treatments	Shoot length (cm/plant)	Root length (cm/plant)	No. of nodules (/plant)	Dry weight (g/plant)		
				Shoot	Root	Nodule
Control	41.44 ± 2.94 e	18.35 ± 1.36 e	44.39 ± 3.45 e	6.68 ± 0.58 f	1.44 ± 0.13 f	0.29 ± 0.021 f
FA 5%	44.19 ± 3.13 d	19.73 ± 1.36 de	47.48 ± 3.52 d	7.05 ± 0.59 e	1.64 ± 0.16 e	0.30 ± 0.023 ef
FA 10%	46.94 ± 3.32 c	21.41 ± 1.42 cd	50.57 ± 3.43 c	7.64 ± 0.59 c	1.95 ± 0.16 c	0.32 ± 0.023 cd
FA 15%	49.69 ± 3.41 b	23.44 ± 1.47 b	53.66 ± 3.66 b	8.10 ± 0.61 b	2.20 ± 0.18 b	0.34 ± 0.027 b
FA 20%	52.48 ± 3.61 a	25.57 ± 1.53 a	56.78 ± 3.19 a	8.61 ± 0.65 a	2.47 ± 0.19 a	0.36 ± 0.024 a
FA 25%	47.57 ± 3.39 c	22.64 ± 1.41 bc	51.63 ± 3.47 c	7.69 ± 0.63 c	2.00 ± 0.16 c	0.33 ± 0.022 bc
FA 30%	45.82 ± 3.28 cd	20.44 ± 1.39 d	48.53 ± 3.48 d	7.23 ± 0.63 d	1.75 ± 0.14 d	0.31 ± 0.023 de

Data represent mean values of five replicates ± show standard error (SE). Mean with different letters in the same column differ significantly at $p \le 0.05$

Table 2 Effect of FA treatments on yield of chickpea plants

Treatments	No. of flowers (/plant)	No. of pods (/plant)	No. of leaves (/plant)	Leaf area (cm^2/plant)
Control	25.63 ± 1.55 f	22.37 ± 1.45 g	176.81 ± 10.72 f	1.34 ± 0.109 f
FA 5%	26.82 ± 1.58 e	23.56 ± 1.45 f	189.28 ± 10.87 e	1.46 ± 0.112 e
FA 10%	28.46 ± 1.61 c	25.31 ± 1.54 d	201.75 ± 11.69 cd	1.59 ± 0.118 c
FA 15%	29.79 ± 1.61 b	27.28 ± 1.62 b	214.22 ± 11.71 b	1.61 ± 0.119 bc
FA 20%	32.13 ± 2.46 a	29.26 ± 1.62 a	227.69 ± 12.07 a	1.73 ± 0.122 a
FA 25%	28.52 ± 1.66 c	26.29 ± 1.57 c	207.61 ± 11.69 bc	1.65 ± 0.019 b
FA 30%	27.16 ± 1.58 d	24.43 ± 1.67 e	195.32 ± 11.82 de	1.52 ± 0.013 d

Data represent mean values of five replicates ± show standard error (SE). Mean with different letters in the same column differ significantly at $p \le 0.05$

Fig. 3 Effect of FA on the morphology of chickpea show maximum at FA 20%

concentrations of FA from 5 to 30% as compared to control (Tables 1, 2). The maximum improvement in growth (Fig. 3) such as shoot length, root length, number of nodules, dry weight of shoot, root, and nodules by 26.64, 39.35, 27.91, 28.89, 41.53 and 24.14% respectively were found at FA 20%. Similarly, the best increment of yield biomass in terms of the number of flowers, pods, leaves, and leaf area was about 25.36, 30.80, 28.78, and 29.10% respectively at FA 20% over the control. However, when FA concentrations increased above 20%, all the growth and yield parameters of chickpea reduced significantly compared to the FA 20%.

3.3 Effects of FA on photosynthetic pigments and RWC

Photosynthetic pigments were observed in chlorophyll a and b, total chlorophyll, and total carotenoid which enhanced significantly through the application of FA at FA 5–30% in comparison to control. However, the maximum increase of chlorophyll a and b, total chlorophyll, and total carotenoid by 16.83, 24.50, 19.13, and 21.74% respectively were recorded at FA 20% (Fig. 4). FA significantly improved the RWC over the control as shown in Fig. 5a and the maximum improvement was 14.07% recorded at FA 20%. But, the higher concentrations of FA (25% and 30%) decreased photosynthetic pigments and RWC.

3.4 Effects of FA on protein and leghemoglobin content

Protein content was increased when plants were treated with different concentrations of FA in comparative relation to control. FA 20% proved to be the best improvement of protein by 48.54% (Fig. 5b). Similarly, the leghemoglobin content of the nodule was increased by 42.39% at FA 20% (Fig. 5c). Moreover, protein and leghemoglobin content decreased at higher FA concentrations (25% and 30%).

3.5 Effects of FA on NR activity and proline content

The improvement was noted at FA 05% to FA 30% compared to control but the maximum was 18.35% recorded at FA 20% (Fig. 5d). However, when FA concentrations increased above 20%, NR activity of chickpea reduced significantly compared to the FA 20%. Data presented in Fig. 6a showed that FA triggered the induction of proline biosynthesis and its increment favored stress tolerance. FA amendment with soil caused continuous improvement of proline content from FA 5% to FA 30%. The maximum increase was observed in the plants 28.16% that were grown at FA 30%.

3.6 Effects of FA on antioxidant enzymes activities

FA treated chickpea plants boosted antioxidant enzyme activities in terms of SOD, CAT, and POX. All the FA concentrations were showed a continuous and significant increase in SOD, CAT, and POX as compared to control. However, the highest values were observed at FA 30% in chickpea plants (Fig. 6b, c, d).

3.7 Effects of FA on stomatal response

Analysis of the number of stomata and stomatal pore showed a clear difference in the presence and absence of FA (Fig. 7). SEM images of stomata also exhibit several stomata which increase at FA 20% as compared with control (Fig. 7a, b). Availability of FA to plants increases the length by 8.107 µm, 9.028 µm and width by 1.846 µm, 2.132 µm of stomatal pore at the control and FA 20% respectively (Fig. 7c, d).

4 Discussion

The agricultural soils are continuously degraded by rapid urbanization and industrialization, leading to global yield production losses. Various harmful substances show toxic

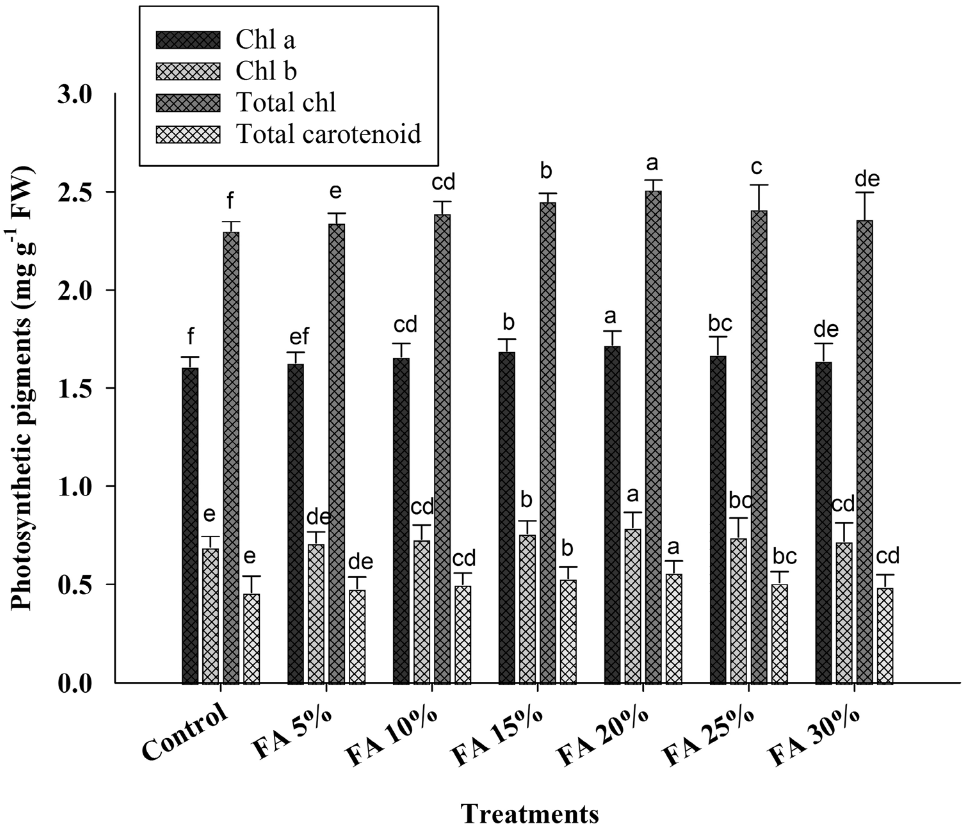


Fig. 4 Effect of different FA concentrations amended to the soil on the photosynthetic pigments of chickpea. Different letters indicate significant differences between treatments at $p \leq 0.05$

effects in crop plants and decrease the fertility of the soil. Nowadays, it becomes a significant challenge for researchers to improve the agronomic properties of the soils. The soil can retain water, depending on the pore space, surface area, and pore space continuity (Panda and Biswal 2018). The lower bulk density, volume, and higher FA porosity contribute to a significant porous soil area. The absolute hollow silt-sized particles allow a broader scale to supply water molecules (Skousen et al. 2013). Thus, Fly ash (FA) has the potential to augment the fertility of agricultural soil provides better conditions for the growth and yield of the plants (Pandey et al. 2009). It contains beneficial plant nutrients as shown in the EDX profiling of FA (Fig. 1b) and increases water holding capacity, texture, pH, organic carbon, and soil conductivity (Pandey and Singh 2010; Haris et al. 2019). To determine the physicochemical properties, EDX is one of the most recent techniques to determine the elemental analysis of soil and FA (Shakeel et al. 2020) (Fig. 1). FA has shown benefits in the utilization of agricultural soils due to the availability of a variety of important as well as beneficial nutrients such as Cu, Fe, Al, Si, P, S, K, Mg, and Ca, etc. (Haris et al. 2019). So, the present investigation reported that the enhancement of the growth including higher production of nodules (nodulation is an important growth parameter of the leguminous plant), dry biomass (Table 1), high yield of flowers, pods, leaves, and leaf area (Table 2) of chickpea plant. Our results are consistent with Marschner (2012) reported that a high amount of Ca present in the FA enhanced signal transduction and acted as a secondary messenger. Likewise, K also performs a role in the eliminating of negative consequences of stress on the plants, since it acts as a catalyst for the different enzymatic activity that is important for the plant production and the growth (Cakmak 2005).

In this study, the application of FA enhanced the photosynthetic pigments (Fig. 4). This finding has been confirmed by the outcome of Moorby and Besford (1983) because the Mg enrichment in FA makes a major improvement and it is the key part of chlorophyll that necessary for chloroplast's structural integrity. FA also enhanced RWC in the leaves

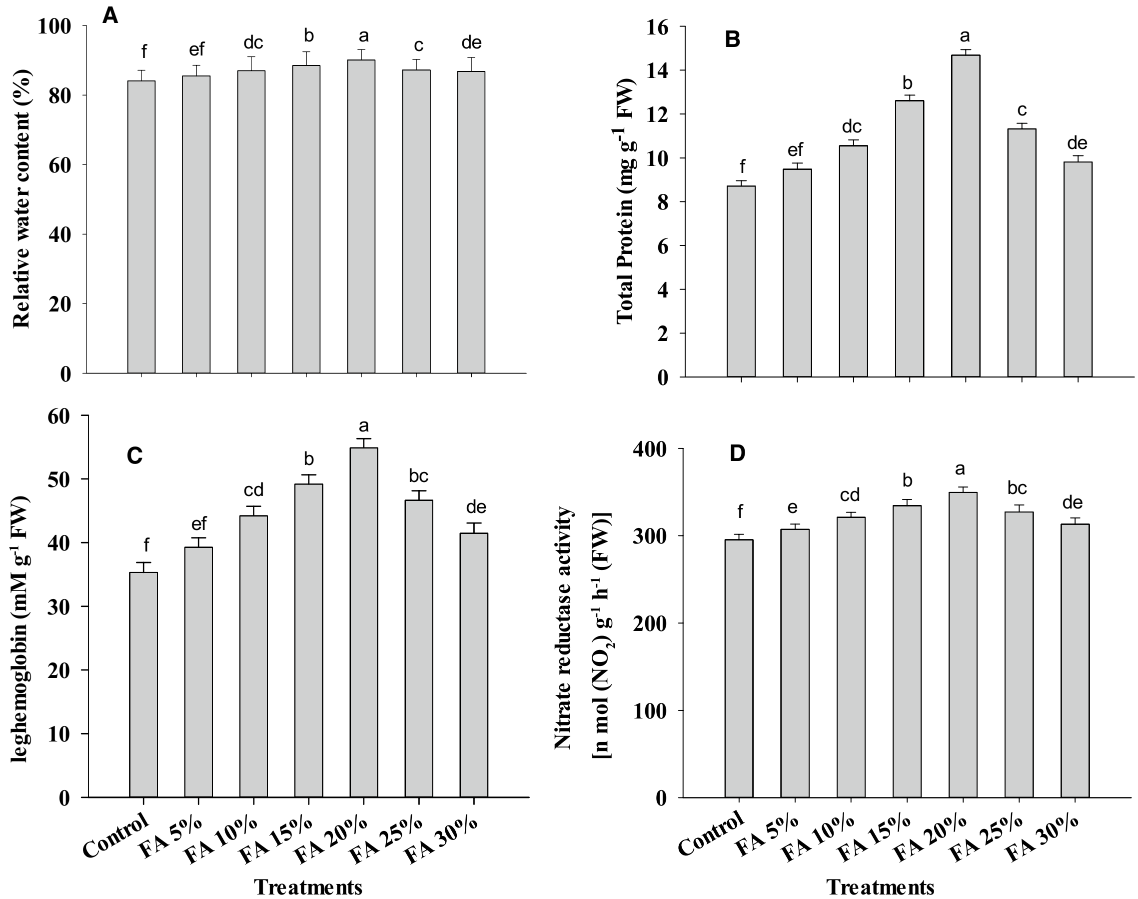


Fig. 5 Effect of different FA concentrations amended to the soil on relative water content (**a**), total protein (**b**), leghemoglobin (**c**) and nitrate reductase activity (**d**) in chickpea. Different letters indicate significant differences between treatments at $p \leq 0.05$

of the chickpea plant (Fig. 5a). Our data regarding protein content in chickpea showed increment (Fig. 5b) due to S. It performs the distinct function as seen in the Fe–S cluster formation of the photosynthetic apparatus. S also play a vital role in protein disulfide-bridge, electron transport, secondary metabolism, and sulfur-containing amino acid such as methionine (Saito 2000; Hell and Hillebrand 2001). Leghemoglobin content was shown to increase by the supplementation of the FA amendment to soil (Fig. 5c). It plays a physiological role in the supply of oxygen to the nitrogen-fixing bacteria. NR is one of the most crucial enzymes responsible for the conversion of nitrate (NO_3^-) to nitrite (NO_2^-). FA supplementation through amended with soil improved NR activity, but a decrease was observed at higher concentrations of FA (Fig. 5d). Qurratul et al. (2013) also reported the same findings. We observed that different concentrations of FA continuously enhance proline content and the maximum was found on the higher concentration at FA 30% (Fig. 6a). Our results are consistent with Wang et al. (2008) reported that proline involved in the scavenging of ROS and is capable of preventing the oxidative stress caused by higher FA concentrations.

The present investigation found that antioxidant enzymes (SOD, CAT, and POX) improved through FA's application (Fig. 6b–d) in the chickpea plant. The activities of these enzymes increased significantly as well as an increment in FA concentrations. Similar results demonstrated in

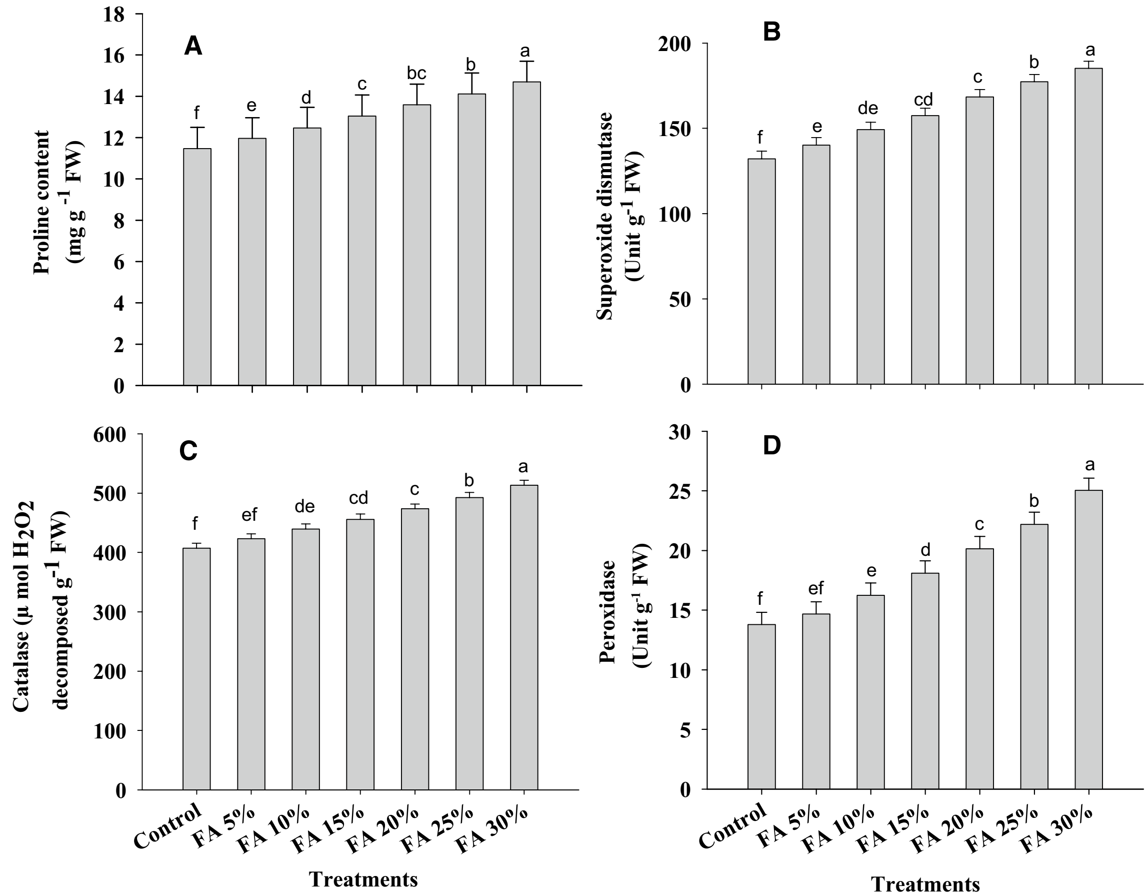


Fig. 6 Effect of different FA concentrations amended to the soil on the proline (**a**), superoxide dismutase activity (**b**), catalyase activity (**c**) and peroxidase activity (**d**) in chickpea. Different letters indicate significant differences between treatments at $p \leq 0.05$

lemongrass (Panda et al. 2018), where enzymes activity increases and can be considered a plant response against reactive oxygen species. Moreover, according to the results of Shamsi et al. (2008), K presents in the FA play various essential roles such as the improved activity of antioxidant enzymes and activates more than 60 enzymes. These enzymes used in different processes like photosynthesis, enhancement in starch synthesis, and regulating stomatal opening and nutrient-translocation. The present study also reported the increased size of the stomatal pore (Fig. 7). However, higher FA concentrations (FA 25 and 30%) reduced growth and yield. It is possible by the imbalance of nutrients due to the low N and P availability (Wong and Wong 1990). Accumulation of heavy metals (Cd, Cr, and Pb) also reduces plant growth, yield, and biochemical parameters at higher FA concentrations. But FA 20% has been ensured and substantially observed the heavy metal accumulation below toxic rates.

5 Conclusions

Our study demonstrates that the supplementation of FA is effective for chickpea at FA 20% to improve the growth, development, and economic yield in plants/crops growing in FA amended to soils. The induction of some antioxidant enzymes in chickpea under elevated FA concentration indicates the metal tolerance ability of this plant. Therefore, the utilization of FA in a sustainable and eco-friendly manner might be a promising approach in the era of climatic changes.

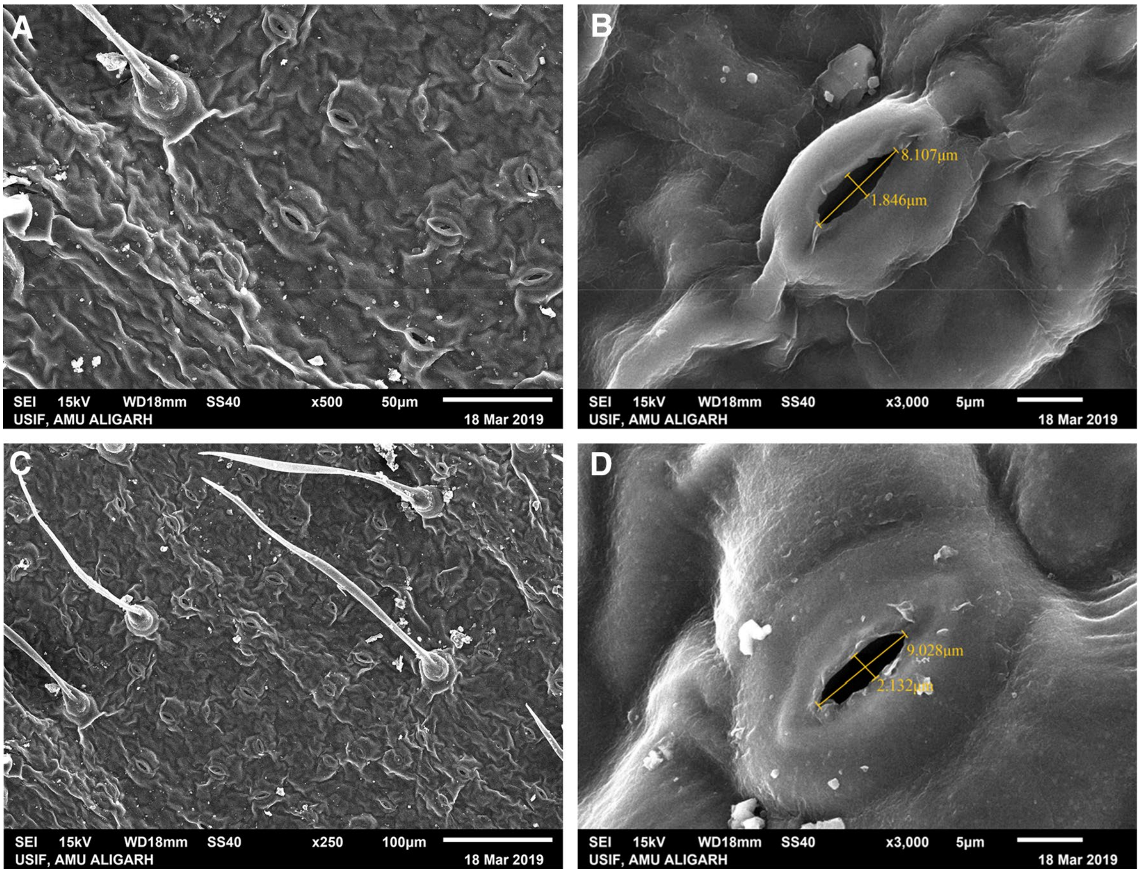


Fig. 7 Scanning electron micrograph showing the effect of different FA concentrations amended to the soil on the stomatal pore of leaf surface at control (**a**, **b**) and FA 20% (**c**, **d**)

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Author's contributions MH and AAK designed the experimental work. MH and MSA performed the experimental work. MH has done the statistical analysis, and formatting of the manuscript. AAK has done the final editing of the manuscript. The manuscript has been read and approved by all the authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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