Effects of Fly Ash Amended Soil on Biochemical Attributes and Antioxidant Responses of Medicinally Potent Plant *Calendula Officinalis*

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Research Article

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Abstract

Phytoremediation of fly ash (FA) deposits using metal tolerant plant species has become an important eco-friendly technique for reclamation nowadays. The present study was carried out to determine the impact of FA application on photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids), biochemical parameters (soluble protein, reducing sugar, nitrate and nitrate reductase (NR) activity), metal accumulation (Fe, Zn, Cu, Co, Cd, Cr, and Mn) and antioxidant defense activity (SOD, CAT, POD, and APX) of *Calendula officinalis*. With this aim in mind, under pot culture conditions, *Calendula officinalis* was grown in different combinations of FA and soil which include: Control, 10%, 20%, 40%, 60%, 80%, and 100% FA. The results from the study indicated that the addition of FA (40%) in soil not only improved the physico-chemical properties of soil but also increased the photosynthetic pigment and other biochemical parameters in plants, however, these parameters declined under high FA applications. On the other hand, antioxidant enzyme activities (SOD, CAT, APX and Peroxidase) of Calendula increased with increasing FA application to combat heavy metal stress from fly ash. At high FA applications, antioxidant enzyme levels increased in leaves thereby reflecting heavy metal stress and mitigation of reactive oxygen species.

Research Highlights-

The present study was carried out to determine the impact of FA application on photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids), biochemical parameters (soluble protein, reducing sugar, nitrate and nitrate reductase (NR) activity), metal accumulation (Fe, Zn, Cu, Co, Cd, Cr, and Mn) and antioxidant defense activity (SOD, CAT, POD, and APX) of *Calendula officinalis*. ANOVA, Correlation analysis and Duncan's multiple range test were also applied to the results obtained using SPSS (11.5) software.

The research highlights are as follows:

- Safe utilization of Fly ash (FA) as soil amendment in agriculture
- Impact of FA application in soil and on growth status of *Calendula officinalis*
- FA (40%) increased chlorophyll, carotenoids, sugar, protein, nitrate and nitrate reductase activity.
- However, at high FA application, there was a marked reduction in these parameters.
- Antioxidant activity (CAT, SOD, APX and peroxidase) of Calendula increased with increase in FA application
- Negative correlation was found between biochemical parameters and antioxidant activity

Introduction-

Coal is used as an essential fossil fuel to produce electricity. Many million tons of FA is produced every year from nearly 100 thermal power stations in India which is one of the prime solicitudes (Usmai and Kumar 2017). Therefore, considerable need for research is required for the optimum utilization of FA in different fields. On one hand, there are diverse management troubles of FA as well as the demand of land for deposition and storage is an immense task, but on the other hand, FA is proven valuable useful material for variety of applications including agriculture (Dahiya and Budania 2018). Thus, there lies a major challenge to convert a major threat into a tremendous opportunity. FA has a remarkable use as a nutrient supplement when amended with soil which further depicts improved growth and yield of the plants in agriculture (Jambhulkar et al. 2018). Even though FA has many advantages as an ameliorant for agricultural soil applications, but due to presence of some amounts of heavy (toxic) elements in FA, balanced use of FA in agriculture is highly important (Gorai 2018).

The utilization of FA as an amendment in soil for better growth and yield has been studied for different plant species. Mahale et al. 2012 observed that use of FA in agriculture at 60% concentration enhances the wheat yield (*Triticum aestivum* L.), 20% was found better for mung bean (*Vigna radiata*) (Gupta and Sinha 2009) and 50% gave good results for *Jatropha curcas* (Raj and Mohan 2018). This proves that the concentration of suitable FA application in the soil differs from species to species. FA application in soil at 180 tons/ha upgraded the soil physical properties and resulted in better growth and yield of brinjal plants (Gond et al. 2013). FA when amended with rice husk ash (RHA), paper factory sludge, farmyard manure (FYM) and chemical fertilizers (CF) not only enhanced the soil strength but also resulted in the substitution of chemical fertilizers to a considerable range in rice production (Karmakar et al. 2010).

When heavy metals in FA are susceptible to plants, a broad variety of plant biological and metabolic activities gets altered. (Srivastava et al. 2017). This causes hindrance with respect to the antioxidant system of the plants and overproduction of reactive oxygen species (ROS) in the plants. The frequent occurring modifications due to metal toxicity result in reduced plant growth, inhibition in the seed germination percentage, photosynthetic activities, suppression of antioxidant defense system and continued aging process as well as cell death in plants (Nita & Grzybowski 2016). To counteract this kind of cellular damage, plant possesses self-defense antioxidative system which includes some enzymatic antioxidants like catalase, superoxide dismutase, ascorbate peroxidase, peroxidase or non-enzymatic antioxidants such as GSH, tocopherol, carotenoids, ascorbate which scavenge free radical production (Bisoi et al. 2017, Varshney et al. 2020). The phytoremediation by plants is a highly recommended technique that owes success due to the tolerance and accumulation mechanism for toxic metals which helps to combat heavy metal stress (Bhaduri and Fulekar 2012). Thus, the efficacy of antioxidant defense system in plants may be vital for explicating its resistance mechanisms to heavy metals present in soil environment (Nadgorska-Socha et al. 2013).

Calendula officinalis (pot marigold) is an important ornamental, medicinal and aromatic plant that belongs to the Asteraceae family and is known to grow on diverse soils without any input material or fertilizer. This plant species has been reported to contain various phytochemicals including saponins, alkaloid, triterpenoids, flavonoids, coumarins, quinones, etc (Shahrbabaki et al. 2013). It has been cultivated extensively by Egyptians, Arabs, Greeks, and Hindus for its importance in herbal medicine. The calendula extracts develop a broad range of pharmacological activities (anti-inflammatory, antipyretic, antimicrobial, antifungal, antiviral, and anti-genotoxic properties) and are utilized as antiseptic, tinctures, ointments, stimulant, anti-spasmodic, and diaphoretic agents (Izadi et al. 2020).

Considering these facts and the importance of Calendula in medicine, the present venture was designed to elucidate growth, biochemical as well as antioxidant responses of *Calendula officinalis* grown in different FA-soil treatments. The relationship between antioxidant defense mechanism of Calendula grown in FA amended soil with respect to photosynthetic activity and other biochemical parameters has not yet been studied so far. The analysis was undertaken with a view to develop data from field experiments on the beneficial effects of FA with reference to the changes in growth, metal accumulation, biochemical parameters (chlorophyll, carotenoids, sugar, protein, nitrate and nitrate reductase activity) and antioxidant responses of Calendula.

Material And Methods-

Plant material and Growth condition-

FA was brought from NTPC Dadri, Uttar Pradesh to the experimental site. The garden soil was collected from Sector 18 Nursery, Noida. The FA and garden soil were mixed in various w/w ratios. The pot culture experiments were performed under natural conditions in winter season. For *Calendula officinalis* growth, FA and soil were mixed in seven different concentrations. The seven different treatments with 20 sample size (20 pots for each treatment) were prepared. These are as follows-

T1 = Control = 100% garden soil

T2 = 10% FA + 90% garden soil

T3 = 20% FA + 80% garden soil

T4 = 40% FA + 60% garden soil

T5 = 60% FA + 40% garden soil

T6 = 80% FA + 20% garden soil

T7 = 100% FA only

Certified seeds of Calendula were collected from Botanical Garden, Sector-37, Noida. The seeds were sown in the winter season in the month of October. Plant performance was evaluated during the complete growth cycle of *Calendula officinalis* for various growth stages including Pre-flowering stage (45 days after sowing (DAS), Flowering stage (90 DAS) and Post- Flowering stage (135 DAS) from October to April. Leaves were detached and gathered for the analysis of the biochemical parameters like chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, reducing sugar, protein, nitrate and nitrate reductase activity and further evaluated in the continuous process at three different developmental stages of the plant.

Physico-chemical properties of soil and FA-

Both FA and soil were tested for its different physico-chemical properties. Standard protocols were used for the analysis of pH, Electrical Conductivity, Bulk Density, Moisture Content, and Water Holding Capacity. pH was measured by adding known volume of FA and soil in distilled water and the readings were noted consequently with digital pH meter, Electrical conductivity was determined by conductivity meter using the prepared soil suspension, Bulk Density (g/cm^3) was measured by the method of Black (1965), Water Holding Capacity (%) was determined using the method of Pandaya et al. 1968, Moisture content (%) of soil and FA in different treatment was measured by taking the difference in the initial and final weight of the admixture after keeping the sample for drying in oven at 100^0 C for 24 hours (Reeb 1999).

Heavy metal analysis-

For estimation of various metals (Fe, Cu, Mn, Ni, Co, Zn and Cr), 0.5 g dried plant samples (leaf) and 1 g air dried crushed soil were powdered with mortar and pestle and mixed in 3 ml HNO_3 at 120^0 C for 2 hours and 80^0 C for 4 hours (Tripathi et al. 2012). The concentration of these metals was quantified by Inductively coupled plasma mass spectrophotometer (ICPMS, Agilent 7900). The determination of metal transfer from soil to plant can be accessed by bioaccumulation factor (BAF) values and is defined by the following equation (Kananke et al. 2018).

$$BAF = \frac{C_{plant}}{C_{soil}}$$

Where, C_{plant} = metal concentration in the plant leaf (dry weight basis) and C_{soil} = concentration of the alike metal in the admixture of soil and FA (dry weight) in which Calendula was grown.

The maximum value for this factor indicates increase availability of a particular metal to the plants. Consequently, high BAF values represent the potential health risk to the plants (Kananke et al. 2018).

Biochemical Parameters-

Pigment content- The photosynthetic pigment (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) content of fresh leaf samples was calculated at four different absorbance- 480 nm, 510 nm, 645 nm and 663 nm following the methods of Hiscox and Israelstam 1979 and the results were determined in mg/g of fresh weight.

Sugar content- Reducing sugars in the fresh leaf samples from all the soil- FA treatments was estimated following the Dinitrosalicylic acid method (DNS) (Miller 1972).

Protein content- The protein concentrations in the leaf samples for all treatments was estimated using Bovine Serum Albumin (BSA) method following the standardized protocol given by Bradford (1976) with 0.2 M phosphate buffer (pH 7.0), 0.1 N NaOH, TCA and Bradford reagent in the reaction mixture.

Nitrate content- Samples were evaluated for nitrate concentrations by using Potassium Nitrate method standardized by Grover et al. 1978, which involved the addition of 0.5 gm of crushed leaves in charcoal, $CuSO4–ZnSO4$ solution, hydrazine sulphate, 0.1 N NaOH and distilled water and lastly chilled acetone to stop further reaction. Later, sulphanilamide and NEDD were added for color development. It was determined spectrophotometrically by taking OD at 540 nm. Standard curve of sodium nitrate solution was used to determine the concentration of individual leaf samples.

Nitrate Reductase (NR) Activity- NR activity was estimated in the fresh leaf samples by using Klepper et al. 1971 method. The equivalent concentration of nitrite was assessed by nitrite standard curve of Sodium Nitrate solution.

Antioxidant enzyme activity measurement- After estimation of biochemical parameters of fresh leaf samples from different FA-soil treatments, leaves were washed, and air dried and dried leaf powder were further used for extraction. Plant leaves were used for making ethanol extracts by using Soxhlet extraction. These extractions were used for the estimation of antioxidative enzyme activity. A small amount of these extracts from different treatments were used for estimation of protein by the method of Lowry et al. 1951. Concisely, superoxide dismutase (SOD) activity was evaluated by the standard method of Gainopolitis and Ries (1977). Catalase (CAT) activity was estimated by Cakmak and Marschner (1992). The CAT activity was designated as µmoles of H_2O_2 oxidized /min/mg of protein. Ascorbate Peroxidase (APX) activity was calculated by the method of Nakano and Asada (1981). Peroxidase activity was evaluated following the procedure of Kar and Mishra (1976) by observing an increase in absorbance at 420 nm for 5 min.

Statistical analysis- Differences between various plant growth attributes, biochemical parameters, and antioxidant enzyme activities under various treatments were evaluated using one-way ANOVA to determine the significant difference between treatments by using SPSS (11.5) software. Experimental data were determined in triplicates and results were obtained as mean ± SD. Correlation analysis and Duncan's multiple range test were performed using SPSS (22) version software.

Result And Discussion-

Physico-chemical properties of soil and FA-

The physico-chemical properties of soil and FA samples (Table-1) revealed that pH of soil was significantly ($P < 0.05$) improved from 7.48 in the control (T1) to 8.17 in 100% FA (T7) as we increased the concentration of FA in soil. The upsurge in pH of soil may be attributed to the counteraction of H^+ ions by basic salts and solubilization of alkali metallic oxides of FA in the amended soils (Skousen et al. 2013). It has also been reported that an upsurge in soil pH resulted in the condensation of soluble cations in the FA and soil amendments (Sheoran et al. 2014). EC value significantly ($P < 0.05$) improved from T1 (0.001) to T7 (0.0047) with respect to increased application of FA. The maximum EC (0.0047 dS /m) was found in T7 (100% FA) while in T1 (control), 0.0012 dS /m was reported. FA addition to soil improved the Electrical conductivity by enhancing the concentration of soluble major and minor inorganic components (James et al. 2014). The same drift was observed in WHC (%)

indicating a significant ($P < 0.05$) improvement from 14.28% in T1 to 43.72% in T7 at higher FA application with respect to control. The present study represented a gradual increase in Water holding capacity from control to 100% FA that may be due to the variation in particle size distribution and increases porosity (Panda and Biswal 2018). The similar trend was also spotted in moisture content (%) as it is significantly ($P < 0.05$) improved with FA application from control (1.931%) to 100% FA (3.971%). The enrichment in moisture content might be attributable to the binding potential of the FA-soil mixture to carry out the cation exchange reaction and with application of compaction effort the voids are employed by more water (Dixit et al. 2016). TDS was noticed to be improved by 66.6%, 75.91%, 76.8%, 84.09%, 84.81% and 85.06% at 10%, 20%, 40%, 60%, 80% and 100% respectively as compared to control. The bulk density of control soil (T1) was maximum at 1.19 g/cm^3 and declined significantly ($P < 0.05$) with the addition of FA and hence was lowest (0.86 g/cm^3) at 100% FA (T9). The bulk density however decreased with the increase in FA concentration in soil which may be attributed to an increase in clay particles (Mishra et al. 2017). This indicates a significant boost in the porosity and improved the water retention capacity of the soil.

Table 1
represents Physico-chemical properties of soil and FA amended soil. The values of all the treatments are means with S.D. of all the replicate measurement (n = 3)

Treatments	pH	WHC (%)	Moisture Content (%)	EC (Ms/m)	TDS (PPM)	Bulk Density (g/ml)
T1 (Control)	7.483 ± 0.27^a	48 ± 3.27^a	1.931 ± 0.43^a	0.1 ± 0.14^a	34.7 ± 4.47^a	1.19 ± 0.137^b
T2 (10%)	7.549 ± 0.26^b	56 ± 5.66^a	2.223 ± 0.23^a	0.19 ± 0.15^a	104.1 ± 3.28^b	1.18 ± 0.107^b
T3 (20%)	7.559 ± 0.22^b	58.6 ± 3.98^a	2.452 ± 0.14^b	0.23 ± 0.1^a	144.1 ± 8.18^b	1.16 ± 0.08^b
T4 (40%)	7.709 ± 0.23^c	63 ± 2.16^b	2.531 ± 0.56^b	0.3 ± 0.20^b	149.6 ± 5.46^b	1.08 ± 0.23^b
T5 (60%)	7.785 ± 0.23^c	64 ± 3.27^b	2.865 ± 0.60^b	0.35 ± 0.28^b	218.2 ± 4.7^c	1.03 ± 0.01^b
T6 (80%)	8.115 ± 0.18^d	68 ± 8.64^b	3.875 ± 0.67^c	0.39 ± 0.26^b	228.5 ± 7.2^c	0.99 ± 0.107^a
T7 (100%)	8.171 ± 0.30^d	85.3 ± 3.77^c	3.971 ± 0.44^c	0.47 ± 0.25^c	232.4 ± 2.08^c	0.86 ± 0.11^a

Different letters represent significant differences at $P < 0.05$ from Duncan’s multiple range test.

Effect of FA on heavy metal accumulation in Calendula-

Heavy metal concentration in different treatments of soil and FA and plant grown in the same treatments is represented in Table 2. The significant pattern for metal accumulation was found, when Calendula plants were grown on different treatments of FA amended soil. Metals concentration in the leaves of Calendula were found in the trend- Ni > Co > Fe > Mn > Zn > Cr > Cu, though, in fly ash treated soil the concentration was in the trend- Ni > Fe > Co > Mn > Zn > Cr > Cu. Surprisingly, the accumulation of heavy metals (Cu, Cr, Ni, Mn, Fe, Zn, and Co) was noticed higher in plants grown on FA amended soil (100% FA) than control soil. This increase in level of lethal heavy metals (As, Cd, Cr, Pb and Hg) was also reported in rice plant grown in FA amended soil (Padhy et al. 2016) rely upon the physio-chemical properties of soil mixture. The bioaccumulation factor (BAF) of soil to plant is presented in Fig. 1. The data indicated that BAF differs from one metal to another metal and also confirmed that the BAF factor of the metals (Fe, Cu, Ni and Co) from soil to plant was higher in FA mixed soils as compared to unamended garden soil except for Cr, Mn and Zn. The bioaccumulation of metals relies upon several environmental aspects such as solubility, salinity, mineral uptake, pH, texture, chemical composition of the metal ions. The decrease in nutrient uptake in grain rice might be consequent of reduced availability of nutrients (elements) at high fly ash application was observed (Singh et al. 2016).

Table 2
Heavy metal concentration in different treatments of soil and FA and in the leaves of the plants grown in these treatments

Treatment	*Cr (ppm)*		*Mn (ppm)*		*Fe (ppm)*		*Cu (ppm)*		*Zn (ppm)*		*Ni (ppb)*		*Co (ppb)*	
	Soil	Leaf	Soil	Leaf	Soil	Leaf	Soil	Leaf	Soil	Leaf	Soil	Leaf	Soil	Leaf
Control	0.18	0.01	0.83	0.24	65.33	2.28	0.07	0.03	0.19	0.13	66.86	4.88	28.00	1.33
10%	0.21	0.01	1.12	0.42	78.37	4.48	0.07	0.03	0.22	0.13	75.28	4.02	31.18	2.88
20%	0.21	0.01	1.16	0.43	76.88	2.88	0.07	0.03	0.21	0.16	81.18	5.66	31.27	3.13
40%	0.22	0.02	1.58	0.50	89.45	5.54	0.07	0.03	0.23	0.18	95.75	6.95	39.87	4.87
60%	0.21	0.02	1.93	0.64	91.81	6.36	0.07	0.04	0.25	0.19	103.46	9.08	44.47	4.07
80%	0.26	0.02	1.82	0.55	99.42	6.50	0.08	0.04	0.28	0.20	110.74	7.64	45.38	4.81
100%	0.26	0.02	2.15	0.60	104.22	6.89	0.08	0.04	0.33	0.22	120.16	11.13	51.08	7.54

Biochemical Parameters-

Effect of FA on Pigment content- Chlorophyll is the essential component of photosynthesis and occurs as a green pigment in chloroplast of plant tissues (Rao 2015). Generally, photosynthetic pigment content depends upon the leaf area, stomatal response, and plant growth performance. Plant response to heavy metals in FA is shown in the results of pigment concentration viz chl a, chl b, total chl and carotenoids. Chlorophyll and carotenoids content exhibit environmental stress induced impairment to the photosystem (Pandey 2013). The data on photosynthetic pigment content (chl a, chl b, total chl and carotenoids) of Calendula leaves as influenced by FA and soil mixture are interpreted in Table 3. In this study, chl a, chl b, carotenoids and total chl content was noted to get enhanced under low doses of FA but reduced under high FA application. A significant ($P < 0.05$) boost in pigment content was found from control to 40% and then reduced from 60% to 100% of FA in soil. The constant increase from control to 40% FA amended soil was noticed in flowering stage but declined as the FA concentration increased above 60%. In pre-flowering stage alike observations were recorded however in post flowering stage, all three photosynthetic pigments depicted a gradual decline in comparison to the pre-flowering as well as the flowering stage. The maximum increase ($P < 0.05$) in chlorophyll a (32.03%) was recorded with 40% FA in the flowering stage followed by pre-flowering stage (22.76%) and finally in the post flowering stage (15.9%) respectively. However, the maximum upsurge ($P<0.05$) for chlorophyll b was recorded for the flowering stage of T4 Treatment (50%) followed by the pre-flowering stage (46.66%) and lastly in the post-flowering stage (40%). The highest upsurge ($P < 0.05$) of total chlorophyll content was observed at 40% FA treatment during flowering stage (28.98%) then by pre-flowering (16.66%) and post flowering stages (9.99%). Similar observations were also reported on pigment content of *Solanum nigrum* L. and *Solanum melongena* grown on different FA doses (Robab et al. 2010 and Gond et al. 2013). Accumulation of heavy metals at high concentration of FA resulting in the decline of chlorophyll content (Qurratul et al. 2014). The disintegration of photosynthetic pigment leads to replacement of Mg^{2+} ions in chlorophyll molecules by certain metal ions like Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} and Ni^{2+} (Qurratul et al. 2014).

Carotenoids are the commonly occurring antioxidants found in the plants which help in protecting the chlorophyll molecule in various environmental conditions (Praveena and Murthy 2014). The results demonstrated that the carotenoids content was significantly ($P < 0.05$) increased at 40% FA treatment in flowering stage (70.58%) then by pre-flowering (41.93%) and post-flowering stages (40.74%). The increased level of carotenoids at 40% FA amendment may ascribe to its role in the fortification of the plant cell against toxic effects of free radical species. Similar results were obtained on Chickpea and *Helianthus annus* grown in different concentrations of FA (Pandey et al. 2010 and Pani et al. 2015b).

Table 3

represents effect of FA on Chl a, Chl b, Total Chl and Carotenoids at different stages of growth of *Calendula officinalis* L. Data is Mean value ± S.D (n= 3).

PARAMETERS/ STAGES	T1 (Control)	T2 (10%)	T3 (20%)	T4 (40%)	T5 (60%)	T6 (80%)	T7 (100%)
	0% FA	10% FA	20% FA	40% FA	60% FA	80% FA	100% FA
A) Chlorophyll a (mg/g of fresh weight)							
Pre- Flowering	0.95 ± 0.004 a	0.98 ± 0.013 a	1.11 ± 0.007 b	1.23 ± 0.035 ba	1.15 ± 0.003 b	0.92 ± 0.002 a	0.88 ± 0.002 abc
Flowering	1.03 ± 0.016 a	1.17 ± 0.139 b	1.25 ± 0.026 b	1.36 ± 0.142 b	1.28 ± 0.031 b	1.01 ± 0.003 a	0.92 ± 0.002 bc
Post-Flowering	0.88 ± 0.021 a	0.93 ± 0.002 a	0.95 ± 0.034 a	1.02 ± 0.066 b	0.98 ± 0.005 a	0.85 ± 0.002 ab	0.81 ± 0.045 ab
B) Chlorophyll b (mg/g of fresh weight)							
Pre- Flowering	0.30 ± 0.021 a	0.34 ± 0.028 a	0.39 ± 0.032 b	0.47 ± 0.009 bc	0.43 ± 0.046 bc	0.26 ± 0.004 abc	0.21 ± 0.003abc
Flowering	0.36 ± 0.034 a	0.45 ± 0.042 a	0.49 ± 0.012 ab	0.54 ± 0.024 ab	0.51 ± 0.047 ab	0.34 ± 0.026 abc	0.28 ± 0.021 abc
Post-Flowering	0.23 ± 0.052 a	0.26 ± 0.003 a	0.31 ± 0.004 b	0.36 ± 0.035 b	0.33 ± 0.077 a	0.21 ± 0.005 ab	0.17 ± 0.002 ab
C) Total Chlorophyll (mg/g of fresh weight)							
Pre- Flowering	1.26 ± 0.004 a	1.29 ± 0.052 a	1.32 ± 0.073 a	1.47 ± 0.054 b	1.38 ± 0.014 ab	1.25 ± 0.006 a	1.21 ± 0.005 abc
Flowering	1.38 ± 0.051 a	1.42 ± 0.072 a	1.68 ± 0.054 b	1.78 ± 0.074 b	1.70 ± 0.068 b	1.36 ± 0.043 a	1.25 ± 0.021 ab
Post-Flowering	1.21 ± 0.007 a	1.25 ± 0.002 a	1.27 ± 0.028 a	1.33 ± 0.068 a	1.29 ± 0.003 a	1.19 ± 0.007 ab	1.12 ± 0.003 ab
D) Carotenoids (mg/g of fresh weight)							
Pre- Flowering	0.31 ± 0.008 a	0.35 ± 0.005 a	0.39 ± 0.032 a	0.44 ± 0.065 b	0.42 ± 0.005 b	0.28 ± 0.002 a	0.23 ± 0.004 ab
Flowering	0.34 ± 0.029 a	0.39 ± 0.043 a	0.47 ± 0.056 a	0.58 ± 0.041 b	0.51 ± 0.054 b	0.32 ± 0.023 a	0.29 ± 0.037 ab
Post-Flowering	0.27 ± 0.024 a	0.30 ± 0.005 a	0.34 ± 0.043 a	0.39 ± 0.038 b	0.36 ± 0.006 a	0.25 ± 0.041 ab	0.21 ± 0.002 ab

T = Treatment, Values signify mean ± SD where the sample size (n=10)

Values presented in various letters differ significantly (P < 0.05) from the control values for different stages of growth as per the Duncan's Multiple Range Test (DMRT).

Effect of FA on sugar, protein, nitrate content and nitrate reductase activity-

Effect of fly ash on reducing sugar- The observations on reducing sugar content (Fig 2A) in the leaves of *Calendula officinalis* showed that in the flowering stage, the significant (P < 0.05) enhancing sugar content (78.94%) was observed in T4 (40% FA amendment) then pre-flowering (73.91%) and post flowering stages (72.58%). However, T7 (100% FA) showed the lowest reducing sugar content (26.31%) at flowering, (23.91%) at pre-flowering and (19.35%) at post flowering stage. The rise in sugar content was noticed with FA application up to 60% FA soil ratio, further application (80% and 100%) causes decline in the sugar content in leaves. The trend of decline in sugar content at high fly ash application was directly correlated with the decline in the photosynthetic pigments. Similar results were obtained in *Helianthus annus* grown in FA amended soil (Pani et al. 2015a). It was inferred that the harmful effect of heavy metals (Pb, Cd, Ni, Cu) at higher FA applications reduces the sugar content in leaves however at low FA application in soil, these metals are within threshold limits which leads to an upsurge in the sugar content. The decline in the sugar content in heavy metal stressed plant leaves was due to the inhibition of photosynthetic activities was also noticed (Dash and Sahoo 2017).

Effect of fly ash on soluble protein- Data on protein concentration in the Calendula leaves as influenced by FA and soil is depicted in Fig-2B. The significant ($P < 0.05$) upsurge in protein content of *Calendula officinalis* leaves was observed in 40% FA amended soil at flowering stage (31.08%) then pre-flowering (28.38%) and post-flowering stages (22.22%) in comparison with control in all growth stages of plant. Further high FA application (80% and 100%) causes a significant ($P < 0.05$) drop in protein content in leaves in a growth-dependent manner. It has been observed that the high protein content occurs due to the synthesis of additional stress shock proteins and stimulating biochemical changes in plant cells because of increased heavy metal stress (Sharma and Singh 2019). But this study revealed that at high application of FA in soil, the protein level was found to be decreased in the calendula leaves (Fig-2B). The decline in the amount of protein might be due to the increase in the protease activity and further catabolic enzymes (Pani et al. 2015a). Similar observations were also reported in *Pisum sativum* and *Brassica juncea* respectively by Sharma et al. 2010 and Gautam et al. 2012. It was also noticed that FA contains high amount of heavy metals which induces the production of ROS which may further harm the photosynthetic system and damage the proteins (Shahid et al. 2014).

Effect of fly ash on nitrate content- Nitrate content in Calendula leaves is graphically determined in Fig 2C. The nitrate content was found significantly ($P < 0.05$) increased at 40% FA amended soil ratio in pre-flowering (78.23%), flowering (87.11%) and post-flowering stages (50.63%) with respect to control. The post-flowering stage shows a gradual decline in all concentrations compared to pre-flowering and flowering stages. Nitrate content was noticed as lowest in flowering stage (44.27%) then pre-flowering (32.15%) and post flowering stage (29.44%) of T7 treatment. Nitrate content has a strong correlation with protein content and photosynthetic pigment rate as plant uses nitrates as a source of nitrogen which is required for protein building which is an important criterion for healthy growth of plants (Hikosaka and Osone 2009). As nitrate content decreases, the pigment concentration in the leaves of the plant decreases significantly (Chaturvedi et al. 2013). In this study, the decrease nitrate content was observed (Fig-2C) at higher FA application as FA alone does not contain nitrogen which is responsible for the reduced plant growth.

Effect of fly ash on Nitrate Reductase (NR) Activity- Results of NR activity (Fig 2D) demonstrated a significant increase ($P < 0.05$) at T4 (40% FA amendment) in comparison to all treatments in all stages of plant growth. The maximum upsurge was observed for the flowering stage (91.6%) then pre-flowering (51.31%) and post-flowering stages (48.50%). Higher level of FA amendments (80% and 100%) showed reduced NR activity in a growth-dependent manner at all stages of plant growth. Nitrate reductase serves as the first enzyme catalyzing the assimilation pathway and is involved in the reduction of nitrate to nitrite (Karwat et al. 2019). In this study, the NR activity (Fig- 2D) was found to be significantly ($P < 0.05$) reduced at high FA application, possible due to the disintegration of the enzyme molecules (Gupta et al. 2009). The decrease in Nitrate reductase activity may be due to either the decline in the protein content, or because of the availability of the substrate. Similar results on NR activity inhibition were also reported in *Prosopis juliflora* L. at high FA application (Rai et al. 2004). As FA encompasses heavy metals, the inhibition of activity of Nitrate reductase might be attributed to the constraining ability of the heavy metals as they can bind to the SH- groups of certain enzymes (Sahay et al. 2015).

In the present study, all the biochemical parameters were found significant ($P < 0.05$) at flowering stage, pre-flowering stage and post-flowering stage for all FA-soil treatments. The sequence of the results indicates that due to aging of plant at post-flowering stage, it has poorer metabolic events that resulted in lessened biochemical activity compared to younger leaves in pre-flowering stage however maximum biochemical activity was reported in the leaves of the Calendula plant for the flowering stage suggesting the highest rate of metabolic activities (Parween et al. 2011).

Level of antioxidant enzyme activity-

The leaf samples of *Calendula officinalis* grown in different FA and soil treatments were analysed for enzyme activity by using certain antioxidant enzymes (SOD, CAT, APX, and Peroxidase) as shown in Fig. 3. In this study, the antioxidant enzyme activities of *Calendula officinalis* displayed significant changes under various treatments of FA and soil mixture compared to control. From ANOVA results, various antioxidative enzymes (SOD, APX, CAT and Peroxidase) were significantly ($P < 0.05$) improved at high fly ash application. At 100% fly ash application, these activities were found to be highest in comparison with control (garden soil). The percent increase in SOD, CAT, APX and Peroxidase activity was 202.9%, 310.4%, 432.1% and 153.74% respectively, in 100% FA treatment compared to the control. Results indicated that low FA application in soil did not trigger oxidative stress whereas higher level of metals in FA amended soil causes disturbance in the cell membrane, cell disruption, interrupt various metabolic processes and causes inhibition in plant growth (Panda et al. 2018). Plant tolerance to heavy metal stress is directly associated with an upsurge in antioxidant enzymes to detoxify reactive oxygen species (Bisoi et al. 2017). This elevation in enzymatic activity might be due to the plant defense against ROS species generated by metal ions present in FA. The same drift was observed in Chickpea grown in various treatments of FA with soil and reported an upsurge in antioxidant enzyme activities (POD, CAT and MDA) in the roots, shoots and leaves of the plant at high FA concentration in soil. Similar observations were also recorded in lemongrass where an increase in antioxidant enzyme activity (APX, SOD, and GPX) was noticed to be improved under all FA treatments over control. In addition, the growth morphology viz shoot, root, total biomass and metal tolerance index were amplified at low FA (25%) as compared to control plants followed by decrease at higher concentration of FA (50%, and above) (Panda et al. 2018). Alteration in antioxidant enzyme activity rely upon different levels of stress, plant species, period of stress acquaintance, tolerance degree and predominant ecological conditions (Zouari et al. 2016).

Correlation between antioxidant enzymes, photosynthetic pigments and growth parameters-

The relationship between photosynthetic pigments, growth parameters and antioxidant enzymes of leaves of calendula grown in different fly ash soil treatments is presented in Table 4. Results in this study show positive correlations between photosynthetic pigments and growth parameters whereas negative correlations were observed amongst antioxidant enzyme activities (SOD, CAT, APX and Peroxidase) with growth, photosynthetic and biochemical parameters in calendula leaves. These results indicated that the antioxidant enzyme activities of calendula are incapable to detoxify the ROS species triggered by metal ions due to increased FA concentration in soil which negatively affects growth and biochemical parameters of plant.

Table 4

Correlation analysis among biochemical parameters and antioxidant enzyme responses of *Calendula officinalis*

	Protein	Chl a	Chl b	Total Chl	Carotenoids	Sugar	Nitrate	NR	SOD	CAT	APX	Peroxidase
Protein	1											
Chl a	.909**	1										
Chl b	.936**	.984**	1									
Total Chl	.878**	.922**	.928**	1								
Carotenoids	.909**	.943**	.966**	.938**	1							
Sugar	.870**	.905**	.928**	.971**	.906**	1						
Nitrate	.903**	.960**	.951**	.977**	.934**	.954**	1					
NR	.931**	.897**	.924**	.925**	.961**	.890**	.915**	1				
SOD	− .489*	-0.147	-0.195	-0.169	-0.202	-0.142	-0.181	-0.365	1			
CAT	− .552**	-0.282	-0.324	-0.198	-0.29	-0.206	-0.264	-0.389	.896**	1		
APX	− .645**	-0.422	− .452*	-0.338	− .456*	-0.288	-0.37	− .564**	.821**	.779**	1	
Peroxidase	− .553**	-0.315	-0.318	-0.218	-0.288	-0.155	-0.275	-0.379	.823**	.804**	.838**	1

Chl a- Chlorophyll a; Chl b- Chlorophyll b; Total Chl- Total Chlorophyll; NR- Nitrate Reductase; SOD- Superoxide dismutase; APX- Ascorbate Peroxidase; CAT- Catalase

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Conclusion-

The major limiting aspects for Calendula growth under fly ash application are high pH, high Electrical Conductivity, high water holding capacity, increased moisture content and elevated level of Co, Mn, Fe, Zn, Cu, Cr and Ni. *Calendula officinalis* grown in 40% fly ash amended soil not only improved soil physiochemical properties but also increases the plant biochemical responses in comparison with control plant. Plant growth was significantly reduced at high fly ash application (80 to 100%), but also all the test plants endured at all concentration of fly ash. This variation to fly ash and heavy metal stress can be ascribed by the enzymatic antioxidation mechanism in *Calendula officinalis*. Under higher fly ash application, induction of antioxidative enzyme activities in studied plant shows heavy metal tolerant ability to oxidative stress. Due to the plant adaptation to heavy metal stress from fly ash, this plant can also be used as hyperaccumulators to alleviate oxidative damage. These finding indicates remediation of Cr, Mn, Fe, Zn, Co, Cu and Ni from fly ash and is largely encouraging for medicinal plant species such as *Calendula officinalis* for probable remediation of metal contaminated soil.

Abbreviations-

FA- Fly ash,

CAT- Catalase,

SOD- superoxide dismutase,

POD- Peroxidase,

MDA- Malondialdehyde,

APX- Ascorbate peroxidase,

ICPMS- Induced coupled plasma mass spectrophotometry,

RHA- Rice Husk Ash,

FYM- Farmyard Manure,

CF- Chemical fertilizer,

NTPC- National Thermal Power Corporation,

DAS- Days after sowing,

BAF- Bioaccumulation factor,

DNS- Dinitrosalicylic acid method,

TCA- Trichloroacetic acid,

BSA- Bovine serum albumin,

NBT- Nitroblue tetrazolium chloride,

NEDD- n-(1-Naphthyl)-1,2-ethanediamine,

ANOVA- Analysis of Variance,

SPSS- Statistical Product and Service Solutions,

LSD- Least significant difference,

EDTA- Ethylene Diamine Tetrachloride,

EC- Electrical Conductivity,

TDS- Total Dissolved Salts,

DMRT- Duncan’s Multiple Range Test,

NADPH- Nicotinamide adenine dinucleotide phosphate

Declarations-

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- Consent for publication- NA
- Availability of data and materials- NA
- Competing interests- The authors declare that they have no competing interests
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- Authors contribution- SM designed the study as well as the project and analyzed the biochemical parameters of Calendula with respect to the fly ash amended soil. PD contributed to the second objective of the venture pertaining to examination of antioxidant responses in Calendula due to heavy metal uptake from fly ash. AV is a significant contributor in performing the experiments and writing the manuscript. All authors read and approved the final manuscript.

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References-

1. Bhaduri MA, Fulekar MH (2012) Antioxidant enzyme responses of plants to heavy metal stress. Rev Environ Sci Biotechnol 11: 55–69.
2. Bisoi SS, Mishra SS, Barik J, Panda D (2017) Effects of different treatments of fly ash and mining soil on growth and antioxidant protection of Indian wild rice. Int J Phytoremediat 19: 446–452. doi:10.1080/15226514.2016.1244164.
3. Black GR (1965) Bulk density. In: Black CA, editor. Methods of soil analysis. Part I. Agronomy. Madison: American Society of Agronomy. 9: 374-377. https://doi.org/10.2134/agronmonogr9.1.c30.
4. Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Analytical Biochemistry 72: 248-254. https://doi.org/10.1016/0003-2697(76)90527-3.
5. Cakmak I, Marschner H (1992) Magnesium deficiency and highlight intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. Plant Physiol 98: 1222–1227. 10.1104/pp.98.4.1222.
6. Chaturvedi RK, Prasad S, Rana S, Obaidullah SM, Pandey V, Singh H (2013) Effect of dust load on the leaf attributes of the tree species growing along the roadside. Environ. Monit. Assess 185: 383–39. 110.1007/s10661-012-2560-x.
7. Dahiya HS, Budania YK (2018) Prospects of fly ash application in agriculture: A Global Review Int. J. Curr. Microbiol. App. Sci 7(10): 397-409. https://doi.org/10.20546/ijcmas.2018.710.043.
8. Dash S, Sahoo S (2017) Effect of fly ash amendment on growth of mustard. International Journal of Applied Environmental Sciences 12(9): 1617-1629. DOI: 10.13005/bbra/1164.
9. Dixit A, Nigam M, Mishra R (2016) Effect of fly ash on geotechnical properties of soil International Journal of Engineering Technologies and Management Research 3(5): 7-14.
10. Gautam S, Singh A, Singh J, Shikha (2012) Effect of fly ash amended soil on growth and yield of Indian mustard (*Brassica juncea*) Adv Bioresearch 3(4): 39-45.
11. Giannopolitis CN, Ries SK (1977) Superoxide dismutase: occurrence in higher plants. Plant Physiol 115: 159–169. https://doi.org/10.1104/pp.59.2.309.
12. Gond DP, Singh S, Pal A, Tewary BK (2013) Growth, yield and metal residues in *Solanum melongena* grown in fly ash amended soils. J Environ Biol 34: 539-544.
13. Gorai S (2018) Utilization of fly ash for sustainable environment management. J. Mater. Environ. Sci 9(2): 385-393. 10.26872/jmes.2018.9.2.42.
14. Grover HL, Nair TVR, Abrol YP (1978) Nitrogen metabolism of the upper three leaf b1ades of wheat at different soil nitrogen levels. I. Nitrate reductase activity and contents of various nitrogenous constituents *Plant. Physiol* 42: 287-92. https://doi.org/10.1111/j.1399-3054.1978.tb04084.x.
15. Gupta AK Sinha S (2009) Growth and metal accumulation response of *Vigna radiata* L. var PDM 54 (mung bean) grown on fly ash - amended soil: Effect on dietary intake. Candian Journal of Public Health 31(4): 463-473.
16. Hikosaka K, Osone Y (2009) A paradox of leaf-trait convergence: why is leaf nitrogen concentration higher in species with higher photosynthetic capacity. J. Plant. Res 122: 245–251. 10.1007/s10265-009-0222-z.
17. Hiscox and Israelstain (1979) A method for extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot 57: 1332-1334. 10.1139/b79-163.
18. Izadi Z, Nejad RA, Abadia J (2020) Physio-morphological and biochemical responses of pot marigold (*Calendula officinalis* L.) to split iron nutrition. Acta Physiol Plant 42: 6. https://doi.org/10.100/s11738-020-3011-x.
19. Jambhulkar HP, Shaikh SMS, Kumar S (2018) Fly ash toxicity, emerging issues and possible implications for its exploitation in agriculture; Indian scenario: A review. Chemosphere 213: 333–344.
20. James A, Thomas T, Kumar S (2014). Effect of fly ash on the physico-chemical properties of soil health and mustard crop. Internat. J. Agric. Sci 10(2): 576-582.
21. Kananke TC, Wansapala J, Gunaratne A (2018) Estimation of Bioaccumulation, Translocation and Distribution Patterns of Cadmium and Lead in Commonly Consumed Green Leafy Vegetables in Colombo District, Sri Lanka. MATTER: International Journal of Science and Technology 4(2): 93-112. 10.20319/mijst.2018.42.93112.
22. Kar M, Mishra D (1976) Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiol 57(2): 315–319. https://doi.org/10.1104.

23. Karmakar S, Mittra BN, Ghosh BC (2010) Enriched coal utilization for augmentic production of rice under acid laterite soil. Coal combustion and gasification product 2: 45-50. 10.4177/CCGP-D-10-00002.1.
24. Karwat H, Sparke MA, Rasche F, Arango J, Nunez J, Rao I, Moret D, Cadisch G (2019) Nitrate reductase activity in leaves as a plant physiological indicator of in vivo biological nitrification inhibition by *Brachiaria humidicola.* Plant Physiology and Biochemistry 137: 113-120. https://doi.org/10.1016/j.plaphy.2019.02.002.
25. Klepper L, Flesher D, Hageman RH (1971) Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. *Plant Physiol* 48: 580–90. 10.1104/pp.48.5.580.
26. Lowry OH, Rosebrough NL, Farr AL, Randall R.I (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 265–275.
27. Mahale K, Patil N, Sarode, SB, Attarde D, Sanjay B (2012) Effect of fly ash as an add mixture in agriculture and the study of heavy metal accumulation in wheat, mung bean and urad bean. Pol. J. Env. Stud: 21(6): 1713-1719.
28. Miller GL (1972) Use of dinitro salicylic acid reagent for determination of reducing sugars. Anal Chem 31:426-8.
29. Mishra S, Prasad SVK, Kanungo VK (2017) Impact of coal fly ash on soil amendment on physicochemical properties of soil. *Indian J. Sci. Res* 13(2): 15-20.
30. Nadgorska-Socha A, Kafel A, Kandziora-Ciupa A, Gospodarek J, Zawisza-Raszka A (2013) Accumulation of heavy metals and antioxidant responses in *Vicia faba* plants grown on monometallic contaminated soil. Environ Sci Pollut Res 20: 1124–1134. 10.1007/s11356-012-1191-7.
31. Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22: 867–880. https://doi.org/10.1093/oxfordjournals.pcp.a076232.
32. Nita M, Grzybowski A (2016) The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults. Oxidative Medicine and Cellular Longevity 1–23. 10.1155/2016/3164734.
33. Padhy RN, Nayak N, Dah-Mohini RR, Sahu RK (2016) Growth, metabolism and yield of rice cultivated in soils amended with fly ash and Cyanobacteria and metal loads in plant parts. Rice Science 23(1): 22-32.
34. Panda D, Panda D, Padhan B, Biswas M (2018) Growth and physiological response of lemongrass (*Cymbopogon citratus* (D.C.) Stapf.) under different levels of fly ash -amended soil International Journal of Phytoremediation 20(6): 538-544. https://doi.org/10.1080/15226514.2017.1393394.
35. Panda RB, Biswal T (2018) Impact of fly ash on soil properties and productivity. International Journal of Agriculture, Environment and Biotechnology 11(2): 275-283. 10.30954/0974-1712.04.2018.8.
36. Pandey VC (2013) Suitability of *Ricinus communis* L. Cultivation for phytoremediation of fly ash disposal sites. Ecol Eng 336-341. https://doi.org/10.1016/j.ecoleng.2013.04.054.
37. Pandey VC, Singh JS, Kumar A, Tiwari DD (2010 Accumulation of heavy metals by Chickpea grown in fly ash treated soil: Effect on antioxidants. Clean- Soil, Air, Water 38(12): 1116-1123. https://doi.org/10.1002/clen.201000178.
38. Pandeya SC, Puri GS, Singh JS (1968) Research methods in plant ecology. Asia Publishing House: Bombay, India.
39. Pani NK, Samal P, Das R, Sahoo S (2015a) Effect of fly ash on growth and yield of Sunflower (*Helianthus annus* L.). Int. J. Agri. & Agri. R 7(2): 64-74.
40. Pani NK, Samal P, Das R, Sahoo S (2015b) Effect of fly ash on soil properties, change in biochemical parameters and heavy metal uptake in sunflower (*Helianthus annus* L.). International Journal of Agronomy and Agricultural Research 4(7): 174-184.
41. Parween T, Jan S, Mahmooduzzafar, Fatma T (2011) Alteration in nitrogen metabolism and plant growth during different developmental stages of green gram (*Vigna radiata* L.) in response to chlorpyrifos. Acta Physiol Plant 33: 2321-2328. 10.1007/s11738-011-0772-2.
42. Praveena B, Murthy SDS (2014) Role of photosynthetic pigments in protection against oxidative damage. International Journal of Plant, Animal and Environmental Sciences 4(1): 167-171.
43. Qurratul JS, Khan R, Mahmooduzzafara, Siddiqi TO (2014) Soil amendments of fly ash: effects on function and biochemical activity of *Carthamus tinctorius* L. plants. *Israel Journal of Plant Sciences* 1-13. https://doi.org/10.1080/07929978.2014.945313.
44. Rai UN, Pandey K, Sinha S, Singh A, Saxena R, Gupta DK (2004) Revegetating fly ash with *Prosopis juliflora* L.: impact of different amendments and Rhizobium inoculation. Environment International 30: 293-300. DOI: 10.1016/S0160-4120(03)00179-X.
45. Raj S, Mohan S (2018) Influence of metal uptake from fly ash on the growth of *Jatropha Curcas* plant: Bulk utilization approach. Int J Pharm Bio Sci 9(2): 154-159. 10.22376/ijpbs.2018.9.2.b154-159.
46. Rao SN (2015) Effects of fly ash of on certain biochemical parameters of *Coleus forskohlii.* Int. J. Appl. Pure Sci. Agri. 1(12): 81-84.
47. Reeb JE, Milota MR (1999) Moisture content by the oven-dry method for industrial testing. Proceedings from the Western Dry Kiln Association Meeting. Portland. https://ir.library.oregonstate.edu/concern/conference_proceedings_or_journals/fq977v782.

48. Robab MI, Hisamuddin, Azam T (2010) Impact of fly ash on vegetative growth and photosynthetic pigment concentrations of *Solanum nigrum* L. Nanobiotechnica universal 1(2): 133-138.
49. Sahay S, Inam A, Inam A, Iqbal S (2015) Modulation in growth, photosynthesis and yield attributes of black mustard (*B. nigra* cv. IC247) by interactive effect of wastewater and fly ash under different NPK levels. Soil and Crop Sciences 1: 1-18. https://doi.org/10.1080/23311932.2015.1087632.
50. Shahid M, Pourrut B, Dumat C, Nadeem M, Aslam M, Pinelli E (2014) Heavy metal induced reactive oxygen species: phytotoxicity and physicochemical changes in plants. Rev Environ Contam Toxicol 232: 1-44. 10.1007/978-3-319-06746-9_1.
51. Shahrbabaki SMA, K, Zoalhasani S, Kodory M (2013) Effects of sowing date and nitrogen fertilizer on seed and flower yield of pot marigold (*Calendula officinalis* L.) in the Kerman Adv Environ Biol 73925-3929
(PDF) *Calendula Officinalis*-An Important Medicinal Plant with Potential Biological Properties.
52. Sharma B, Singh RP (2019) Physiological, Biochemical, Growth, and Yield Responses of Radish (*Raphanus sativus* L.) Plants Grown on Different Sewage Sludge–FA Mixture (SLASH) Ratios. Waste Valorisation and Recycling 2: 539-552.
53. Sharma S, Sharma P, Mehrotra P (2010) Bioaccumulation of heavy metals in *Pisum sativum* L. growing in fly ash amended soil. J American Sci 6(6): 43-50.
54. Sheoran HS, Duhan BS, Kumar A (2014) Effect of fly ash application on soil properties: A review. Journal of Agroecology and Natural Resource Management 1(2): 98-103.
55. Singh PK, Tripathi P, Dwivedi S *et al* (2016) Fly-ash augmented soil enhances heavy metal accumulation and phytotoxicity in rice (*Oryza sativa* L.); A concern for fly ash amendments in agriculture sector. *Plant Growth Regul* 78: 21-30. https://doi.org/10.1007/s10725-015-0070-x.
56. Skousen J, Yang JE, Lee JS, Ziemkiewicz P (2013) Review of fly ash as a soil amendment. J. Geosystem Engineering 16(3): 249-256. https://doi.org/10.1080/12269328.2013.832403.
57. Srivastava V, Sarkar A, Singh S, Singh P, de Araujo ASF, Singh RP (2017) Agroecological Responses of Heavy Metal Pollution with Special Emphasis on Soil Health and Plant Performances. Frontiers in Environmental Science 5. https://doi.org/10.3389/fenvs.2017.00064.
58. Tripathi P, Mishra A, Dwivedi S, Chakrabarty D, Trivedi PK, Singh RP, Tripathi RD (2012) Differential response of oxidative stress and thiol metabolism in contrasting rice genotypes for arsenic tolerance. Ecotoxicol Environ Safe 79: 189–198. 10.1016/j.ecoenv.2011.12.019.
59. Usmani Z, Kumar V (2017) Characterization, partitioning, and potential ecological risk quantification of trace elements in coal fly ash. *Environ. Sci. Pollut Res* **24**(18): 15547–15566
60. Varshney A, Mohan S, Dahiya P (2020) Growth and antioxidant responses in plants induced by heavy metals present in fly ash. Energ. Ecol. Environ. https://doi.org/10.1007/s40974-020-00191-1.
61. Zouari M, Ben Ahmed, Ch, Zorrig W, Rabhi M, Delmail D, Ben Rounia B, Labrousse P, Ben Abdallah F (2016) Exogenous proline mediates alleviation of cadmium stress by promoting photosynthetic activity, water status and antioxidative enzymes activities of young date palm (*Phoenix dactylifera* L.). Ecotoxicol. Environ. Saf. 128: 100–108. http://dx.doi.org/10.1016/j. ecoenv.2016.02.015.

Figures

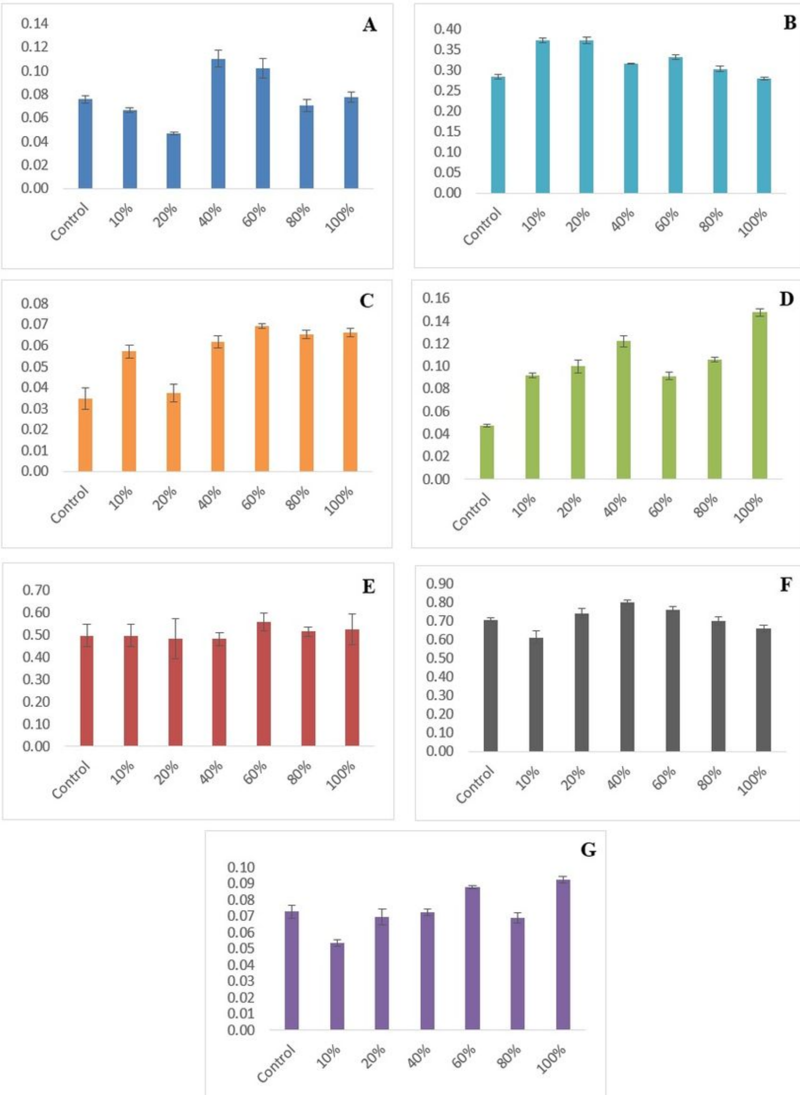

Figure 1

1A, 1B, 1C, 1D, 1E, 1F, 1G represents the bioaccumulation factor of Cr, Mn, Fe, Co, Cu, Zn, Ni metals in Calendula officinalis grown in different combination of soil and FA. All the values are the means of three replicates (n=3).

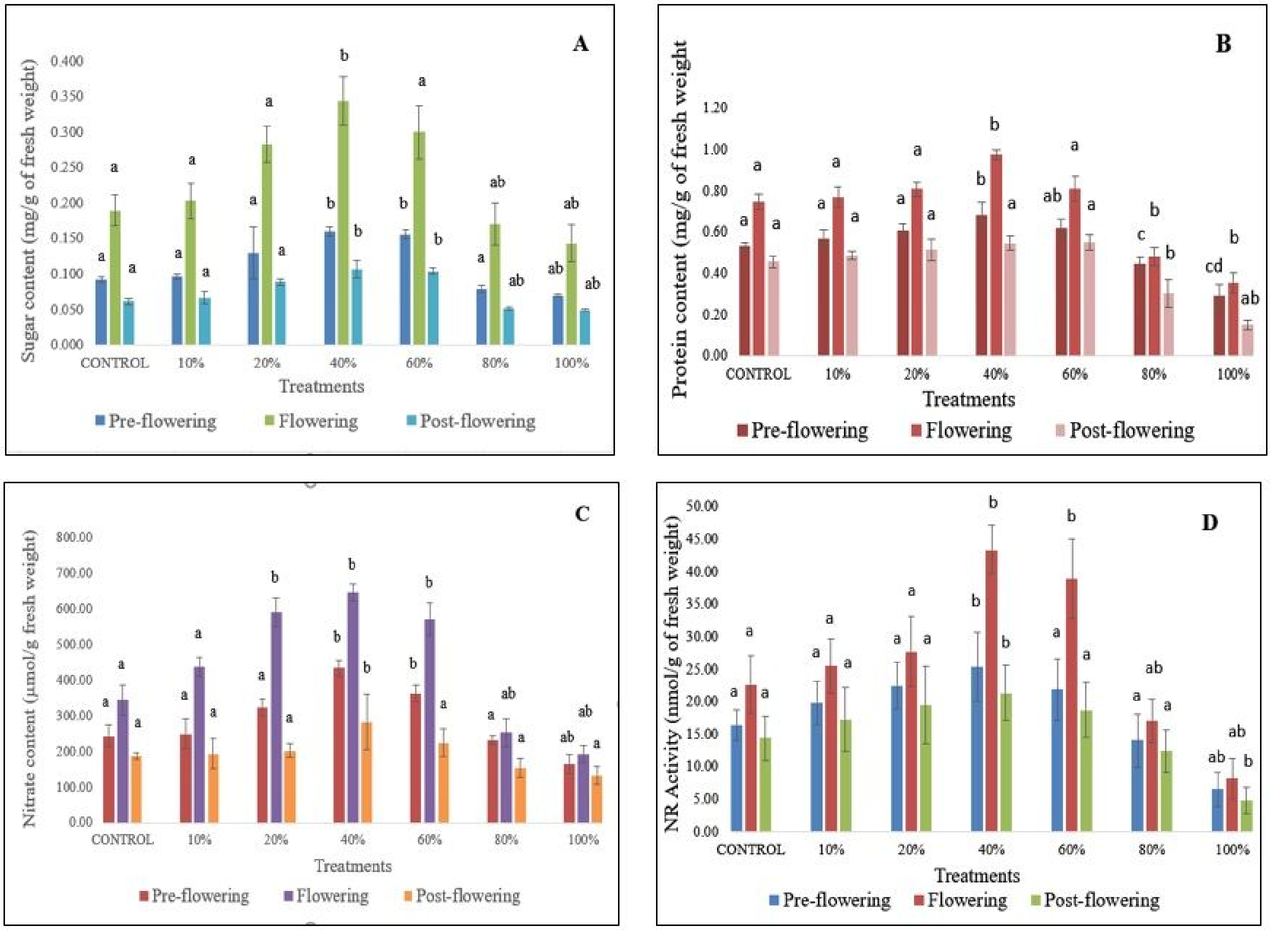

Figure 2

2A, 2B, 2C and 2D represents effect of FA on Sugar content, Protein content, Nitrate content and Nitrate Reductase (NR) Activity at three different developmental stages of Calendula officinalis L. Data is Mean value ± S.D (n= 3). Values presented in various letters differ significantly (P < 0.05) from the control values for different stages of growth as per the Duncan's Multiple Range Test (DMRT).

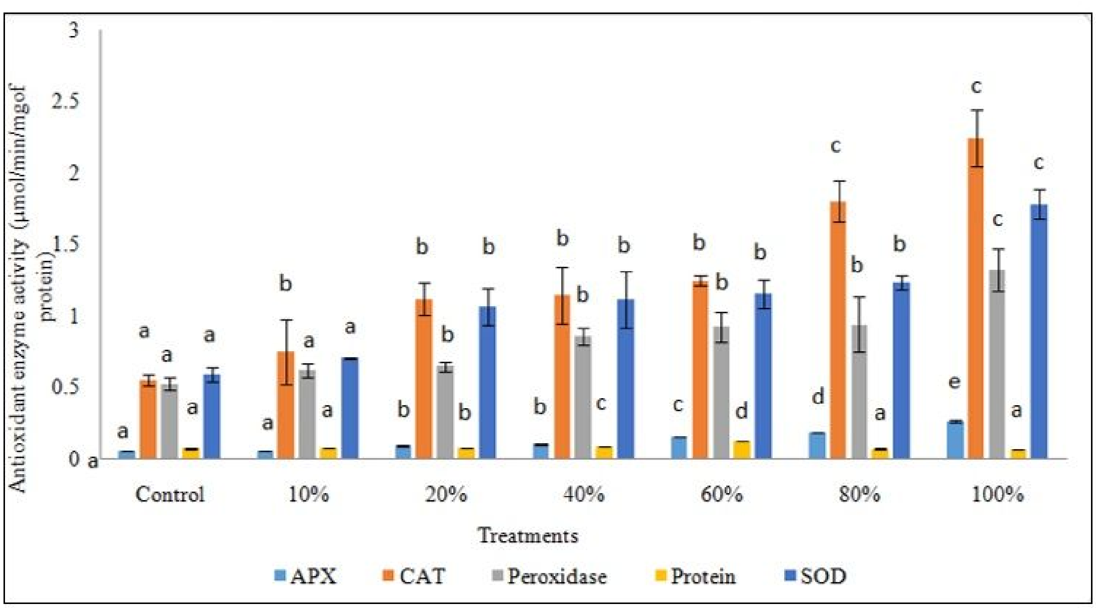

Figure 3

Changes of different antioxidant activities of Calendula officinalis grown under various treatments of FA and garden soil. Data are the means of three replicates and bars denotes for SD values. Different letters are significantly different at $P < 0.05$ from DMRT test. APX- Ascorbate peroxidase; CAT- Catalase; SOD- Superoxide Dismutase.