Effect of Fly Ash Application on the Chlorophyll and Proline Content of Pea (Pisum Sativum L) Plant

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Abstract:
The present study has been undertaken to see the effect of fly ash application on the physiological parameters of Pea plant like Chlorophyll content and Proline content of plant. Photosynthetic pigments (Chlorophyll a, Chlorophyll b) content increased with addition of 10% fly ash, while decreased with addition of fly ash, without any amendment while Carotenoids content increased with addition of 30% fly ash, without any amended. It was also observed that Proline accumulation was more in 20% and 30% fly ash series as well as in combination with Cynodon in comparison to control. All other series showed a significant decrease in the Proline content. However the minimum Proline content was recorded in a series where soil was amended with Cynodon, 10% fly ash and inoculated with AM fungi, PSF and nitrogen fixer (Rhizobium).

Keywords: *AM fungi, Carotenoids, Chlorophyll, Pea, Cynodon, Proline, Rhizobium*

I. INTRODUCTION

Fly ash, a waste by-product of coal combustion, has been noted for its potential use as a soil amendment. Its potential benefits include altering soil texture and enhancing water-holding capacity, increasing soil pH (depending on source), and enhancing soil fertility. In order to nullify the adverse effects of fly ash and to improve the N and P status of soils and crops there is a need to explore the potentialities of bio-inoculants, especially the nitrogen fixers, phosphate solubilizers and P scavengers; the arbuscular mycorrhizal fungi. The most fascinating and key role of AM fungi played for hosts in fly ash is the amelioration of toxic effects of heavy metals. In fact, it is the AM fungi which protect the host plants from heavy metal toxicity in the fly ash. In view of the above facts present study has been undertaken to see the effect of fly ash on the physiological parameters of plant.

II. MATERIALS AND METHODS

Site description- For conducting the experiments in the present study, both fly ash and agriculture soil were used. Fly ash was collected from the fly ash dumping sites situated at IFFCO, Phulpur, 25°33'N, 82°6'E near Allahabad, Uttar Pradesh and the agriculture soil from Ganga basin region of Allahabad.(Plate 1,2) Characteristics of agriculture soil and fly ash used in the experiments are presented in Table-1.

A. Collection of Soil Samples:

The rhizospheric soil samples were collected from the root region of the plants growing in the vicinity of fly ash damping site of IFFCO, Phulpur, Allahabad. Samples were brought to the laboratory in polythene bags and stored at 5°c until processed.

B. Isolation of AM Fungi:

AMF spores were isolated by wet sieving and decanting method of (Gerdemann and Nicolson 1963). A known amount of soil was dissolved in water. After through shaking, it was left for some time for the soil particles to settle down. The clear solution was passed through sieve of 500, 350, 210, 150, 90 and 60 micro meters in descending order. The AM spores retained on various sieves were transferred on filter papers. Filter papers were examined under binocular microscope.

C. Identification of AM Fungi:

Different AM spores present in the soil were recovered and AM spores were mounted in PVLG and identified to the species level using the synoptic keys of (Trappe 1982), (Schenck and Parez 1990) and INVAM species guide (*http//: invam.caf.wvu.edu*).

The most dominant indigenous AM fungi were the species of Acaulospora and Glomus viz. Acaulospora denticulata, Acaulospora scrobiculata, Glomus deserticola, Glomus fasiculatum, Glomus tortosum, Glomus clarum, Glomus multicaule, Glomus intraradices, Glomus mosseae, ,Gigaspora sp. etc.

D. Maintenance of Trap culture:

To obtain abundant and healthy spores of different AMF species rhizospheric soils from the plants growing in the vicinity of fly ash dumping site were collected. Shoots were removed at crown and roots were chopped into small fragments. These root segments along with rhizospheric soil were mixed with autoclaved coarse sand soil mixture 1:1 ratio (v/v). These mixtures were then transferred to sterilized earthen pots and seeds of *Trifolium repens* (L.) were sown in each pot. Cultures were grown under greenhouse conditions for three months. After three months spore population was determined in trap

cultures. Another set of trap cultures was prepared on *Sorghum bicolor* (L.) using the soil of first set. Mycorrhizal inoculum consisted of soil having 50 AM spores/10 gm. soil, mycelia and infected root fragments (95% root length colonization). This consortium was used as inoculum for the experimental work.

E. *Isolation and Maintenance of Phosphate Solubilizing Microbes:*

Soil dilution and plate count method of (Timonin 1940) was used for isolating/counting of phosphate solubilizing microbes from the rhizospheric soil of the plants growing in the vicinity of fly ash dumping site IFFCO, Phulpur, Allahabad.
All the colonies of phosphorus solubilizing microbes which appeared on the Petri plates and exhibited zone of solubilization were examined carefully, dominant species were *Aspergillus niger*, *Cladosporium* sp., *Fusarium oxysporum*, *Penicillium* sp. and sub-cultured these dominant species in Pikovskaya's broth media. They were re-examined critically, identified with the help of specific monographs and their phosphate solubilizing potentiality was estimated. *Aspergillus niger* highest phosphate solubilizing potentiality.

F. *Isolation and Maintenance of N_2- Fixing Bacteria, Rhizobium leguminosarum:*

Rhizobium leguminosarum was isolated from the nodules of Pea growing in the fields near the fly ash dumping site, IFFCO, Phulpur, Allahabad on Yeast extract Mannitol Agar plates. Large gummy colonies of bacteria that emerged within four or five days were selected, isolated and subsequently transferred on fresh nutrient plates and sub cultured.

G. *Experimental Setup:*

The seeds of Pea (*Pisum sativum* var. AP3) were procured from registered seed shop of Allahabad, which served as the unit of propagation during the experiments.

H. *Experimental Design:*

An experiment was setup in pots under greenhouse condition to assess the performance of both the crops raised in agriculture soil of Allahabad amended with organic matter (*Cynodon* 2% w/w), different concentration of fly ash (10, 20, 30%) and inoculated with consortium of AM fungi, PSF and *Rhizobium* alone as well as in combination.

The experiment had a complete randomized design in three blocks, eight treatment / block and three replicates / treatment. The treatment were as follows

Block I
- Agriculture soil (Control)
- Agriculture soil + Phosphate solubilizing fungi (*Aspergillus niger*) (PSF)
- Agriculture soil + AM
- Agriculture soil+ *Rhizobium* (RHZ)
- Agriculture soil + AM+PSF
- Agriculture soil+ PSF+RHZ
- Agriculture soil+ AM+RHZ
- Agriculture soil + PSF+ AM+RHZ

Block II
- Agriculture soil + Organic matter (*Cynodon* 2% w/w) (CN)
- Agriculture soil + CN + Phosphate solubilizing fungi (*Aspergillus niger*) (PSF)
- Agriculture soil + CN + AM
- Agriculture soil + CN + RHZ
- Agriculture soil + CN + AM +PSF
- Agriculture soil + CN+PSF+RHZ
- Agriculture soil + CN+ AM + RHZ
- Agriculture soil + CN + AM +PSF+RHZ

Block III
- Agriculture soil +CN+10% Fly ash
- Agriculture soil + Organic matter (CN) + 10% Fly ash + PSF
- Agriculture soil + CN + 10% Fly ash + AM
- Agriculture soil + CN + 10% Fly ash + RHZ
- Agriculture soil + CN + 10% Fly ash + AM + PSF
- Agriculture soil + CN+ 10% Fly ash + PSF + RHZ
- Agriculture soil + CN + 10% Fly ash + AM + RHZ
- Agriculture soil + CN + 10% Fly ash + AM + PSF + RHZ

Block IV
- Agriculture soil + CN + 20% Fly ash
- Agriculture soil + CN + 20% Fly ash + PSF
- Agriculture soil + CN + 20% Fly ash + AM
- Agriculture soil +CN + 20% Fly ash + RHZ
- Agriculture soil + CN + 20% Fly ash + AM + PSF
- Agriculture soil + CN + 20% Fly ash + PSF + RHZ
- Agriculture soil +CN + 20% Fly ash + AM + RHZ
- Agriculture soil +CN + 20% Fly ash + AM + PSF + RHZ

Block V
- Agriculture soil +CN +30% Fly ash
- Agriculture soil + CN + 30% Fly ash + PSF
- Agriculture soil + CN + 30% Fly ash + AM
- Agriculture soil +CN + 30% Fly ash + RHZ
- Agriculture soil +CN + AM + 30% Fly ash + PSF
- Agriculture soil +CN + 30% Fly ash + PSF + RHZ
- Agriculture soil + CN + 30% Fly ash + AM + RHZ
- Agriculture soil +CN + 30Fly ash + AM+ PSF + RHZ

Earthen pots were filled with 4 kg soil amended with 2% (w/w) organic matter. All series were supplemented with organic matter except control series. Some sets of experiments were provided with

microbial inoculations singly as well as in dual and triple combination

J. Physiological Analysis:

Estimation of Photosynthetic pigments Chlorophyll a, Chlorophyll b and Carotenoids :

Concentration of photosynthetic pigments in leaves of plants was determined after extraction with acetone using the equation given by Lichtenthaler and Welburn (1983). In this method fresh leaves (.05g) were cut into small pieces and crushed in 80% acetone for extracting photosynthetic pigments. The extract was centrifuged and the leaf pieces were again treated with 80% acetone and centrifuged. The process was repeated till the leaf pieces became colourless, final volume of the supernatant was maintained to 5 ml with 80% acetone and the absorbance of this solution was recorded at 663, 646 and 470nm through calorimeter (Spectronics-20, Bausch & Lomb). The amount of chlorophyll a, chlorophyll b and carotenoids was calculated using the equation given by Lichtenthaler and Welburn (1983).

K. ***Estimation of Proline content :***

Proline content in the leaves of plants was estimated according to the method given by Bates *et al*., (1973). In this method fresh leaves (0.5g) were crushed in 10ml 3% aqueous sulfosalicylic acid and centrifuged at 10,000g for 10 min. 2 ml tissue extract was reacted with 2 ml 3% glacial acetic acid and 2 ml acid ninhydrin solution. The reaction mixture was incubated for 1hr in water bath at 95°c. after proper cooling 4ml toluene was added to the reaction mixture. The tolune layer was extracted after vortexing for 15 sec. with a test tube mixer and its absorbance was read at 520nm through calorimeter (Spectronics-20, Bausch & Lomb), the proline content in the sample was estimated against the standard curve.

The above mentioned series were set up in five blocks. In first block soil in the pots was without any amendments and maintained as control for the experiment. In the second block, soil was amended with *Cynodon*, in the third block soil was amended with 10% fly ash, whereas in fourth and fifth blocks with 20 and 30% fly ash respectively.

Crops were raised in earthen pots. Seeds were surface sterilised by 3 % (v/v) sodium hypochloride solution for 2-3 minutes and rinsed in sterilized distilled water 2-3 times and dried in shade for 10-15 minutes. In single inoculation series with AM, before sowing the seeds, the mycorrhizal inoculum of AM fungi was separately placed below the seeds by the layering method (Menge *et al*. 1977). The inoculum was spread as a layer at a depth of 3-5 cm in the pot and the seeds were sown just above the inoculum layer. The seeds were covered with a layer of soil to ensure an efficient host fungus association. The inoculum consisted of a mixture of infected root pieces and soil with extrametrical spores from cultures of different AM fungi maintained on *Sorghum vulgare* (L.). In single inoculation series with *Rhizobium*, before sowing, the seeds were soaked for 4 hrs in culture suspensions of the isolate of *Rhizobium* (containing approximately 108 cells / ml) prepared from its 8 days old cultures on YEMA liquid medium. For single inoculation series with PSF (*Aspergillus niger*) the seeds were soaked for four hrs in culture suspension (containing approximately 108 conidia / ml) prepared from the 10 days old culture on Pikovskayas liquid medium. For dual inoculation series involving *Rhizobium* and PSF, the crops were raised from seeds treated with a mixture of an equal amount of culture suspensions containing 108 cells or conidia/ml. On the other hand, in dual inoculation series involving *Rhizobium* or PSF and AM fungi, the crops were raised from *Rhizobium* / PSF treated seeds in soil supplemented with inoculum of AM fungi. In triple inoculation series involving *Rhizobium*, PSF and AM fungi, the crops were raised from the seeds treated with Rhizobium and PSF supplemented with inoculum of AM fungi. The seeds treated with *Rhizobium* or PSF in single, dual or triple inoculated series were then dried in shade and shown at 10 seeds per pot. Ten seeds per pot were sown and after finally emergence and establishment only five seedlings per pot were maintained. Five plants from each treatment series were carefully uprooted at different stages of plant growth *viz*; vegetative, flowering and fruiting. Samples of roots along with adhering soil were collected and processed for determining the mycorrhizal intensity in the roots and population of AM spores.

III. RESULTS AND DISCUSSION

In the present study photosynthetic pigments (Chlorophyll a, Chlorophyll b) increased with addition of 10% fly ash while carotenoid content decreased. In comparison to control chlorophyll ‘a’and ‘b’ content increased up to 52.5% and 83.8% respectively in pea, in a series where agriculture soil was amended with *Cynodon* and 10% FA and inoculated with all the three microbial inoculants (Table 3, 4, 5) (fig. 1,2)

Application of high amount of fly ash causes decrease in photosynthetic pigments and total protein content due to presence of metal ions or degradation of chlorophyll by free radical generated by metals (Pandey *et al.*, 2010, Gupta *et al*., 1999).

The breakdown of photosynthetic pigment may be due to substitution of Mg^{2}+ ion in chlorophyll molecules by metal ions such as Cu^{2+}, Zn^{2}+, Cd^{2}+, Pb^{2}+, Ni^{2}+ (Kupper *et al*., 1998). Metals possess a potential to alter the photosynthesis rate by disturbing the structure of chloroplast leading to the changes in the fatty acid composition, inhibiting photosynthetic

pigment and enzymes of Calvin cycle (Vazquez *et al.*, 1987).

In the present study proline accumulation was more in a series where soil was amended with 20% and 30% fly ash alone as well as in combination with *Cynodon*. In comparison to control decrease in proline content was recorded in pea (Table 6) (fig. 2). Similar results were reported by several authors (Khan and Khan, 1996; Gupta and Sinha, 2009; Pandey *et al*., 2009, 2010). According to Pandey *et al*. (2010) accumulation of high amount of proline may be because of heavy metals present in fly ash such as Fe, Cu, Ni, Cr, Pb, Cd etc. All other series showed a significant decrease in the proline content. However, the minimum proline content was recorded in a series where soil was amended with *Cynodon*, 10% fly ash and inoculated with AM, PSF and nitrogen fixer.

The results clearly indicated that the addition of organic matter and inoculation of microbes reduce the toxic effects of fly ash in Pea the leguminous crop.

Fly ash dumping site

Plate 2

Ganga Basin Area

Plate 1

Plate 2

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Table 1: Physico-Chemical Characteristics of Agriculture and Fly Ash

	Agriculture soil	Fly ash
Physical		
BD (g cm^{-1})	1.5	<1.0
W.H.C (%)	22-25	35-40
Chemical		
pH	8.1	7.4
Al_2O_3 (PPm)	1.0	18.7
Fe_2O_3 (PPm)	0.37	3.4
CaO (PPm)	0.39	1.54
MgO (PPm)	0.24	0.53
Na_2O (PPm)	0.008	0.05
K_2O (PPm)	232	2.5
SO_3 (PPm)	0.19	0.1
Organic carbon, %	1.2	0.42
Nutrient		
Nitrogen	24	0.030
Phosphorus	29	0.035

Table 2: The Phosphate Solubilizing Potentiality of the Isolated Phosphate Solubilizers

P solubilizing Microbes	P solubilization (ppm)
Aspergillus niger	**0.197**
Cladosporium sp.	0.024
Curvularia sp.	0.072
Fusarium oxysporum	0.124
Penicillium sp. 1	0.091
Penicillium sp. 2	0.026
Penicillium sp. 3	0.062
Penicillium sp. 4	0.026

Table 3: Chlorophyll 'B'content of Pea Plant Raised in Agriculture Soil Amended with 10 %, 20% And 30% Fly Ash and *Cynodon* and Provided with Consortium of AM Fungi, PSF and N_2 Fixer Alone as Well As in Combination, At Fruiting Stage.

Treatment	Chlorophyll 'b' content (µg / ml)				
	AgS	CN	10% FA + CN	20% FA + CN	30% FA + CN
Control	0.31	0.40	0.50	0.43	0.27
+PSF	0.33	0.42	0.52	0.50	0.20
+VAM	0.37	0.39	0.55	0.47	0.34
+RHZ	0.31	0.40	0.38	0.34	0.23
+PSF+VAM	0.33	0.42	0.59	0.56	0.28
+PSF+RHZ	0.37	0.39	0.58	0.48	0.25
+VAM+RHZ	0.39	0.43	0.57	0.47	0.36
+PSF+VAM+RHZ	0.40	0.45	0.63	0.57	0.39

Table 4: Chlorophyll 'A' Content of Pea Plant Raised in Agriculture Soil Amended with 10 %, 20% and 30% Fly Ash and *Cynodon* and Provided with Consortium of AM Fungi, PSF and N_2 Fixer Alone as Well as in Combination, at Fruiting Stage.

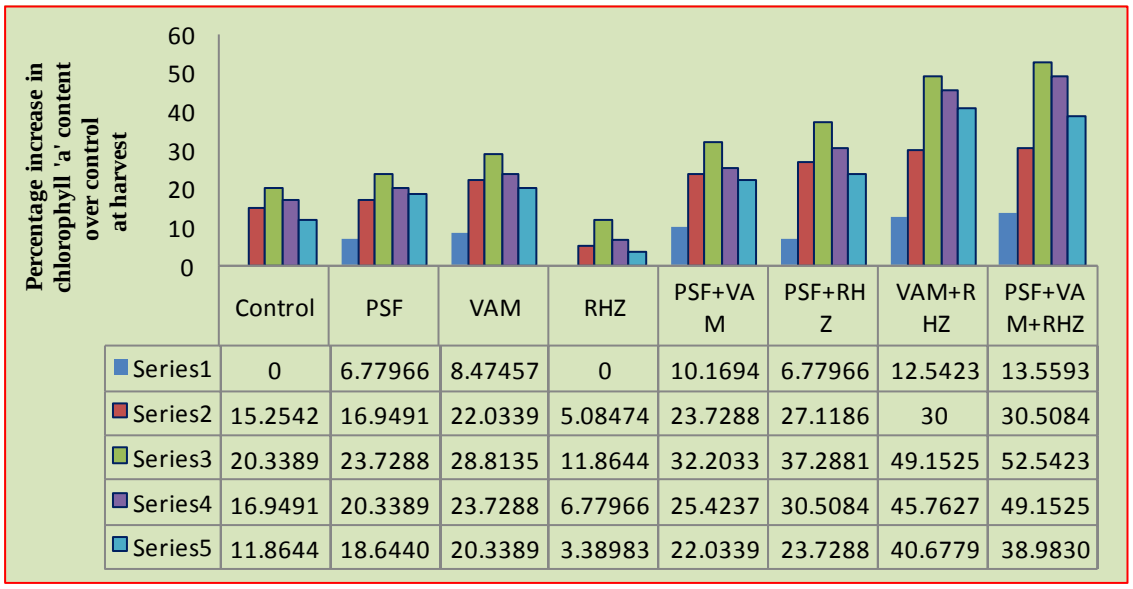
Treatment	Chlorophyll 'a' content (µg / ml)				
	AgS	CN	10% FA + CN	20% FA + CN	30% FA + CN
Control	0.590	0.680	0.710	0.690	0.660
+PSF	0.630	0.690	0.730	0.710	0.700
+VAM	0.640	0.720	0.760	0.730	0.710
+RHZ	0.590	0.620	0.660	0.630	0.610
+PSF+VAM	0.650	0.730	0.780	0.740	0.720
+PSF+RHZ	0.630	0.750	0.810	0.770	0.730
+VAM+RHZ	0.664	0.767	0.880	0.860	0.830
+PSF+VAM+RHZ	0.670	0.770	0.900	0.880	0.820

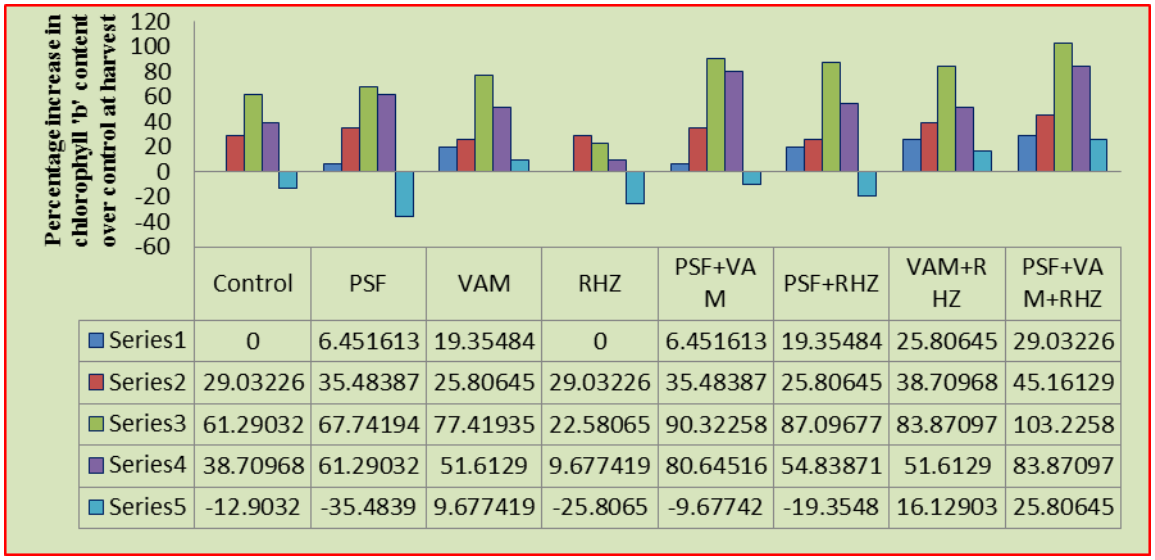
Table 5: Carotenoid Content of Pea Plant Raised in Agriculture Soil Amended with 10 %, 20% and 30% Fly Ash and *Cynodon* and Provided With Consortium of AM Fungi, PSF and N_2 Fixer Alone as Well as in Combination, at Fruiting Stage.

Treatment	Carotenoid content (µg / ml)				
	AgS	CN	10% FA + CN	20% FA + CN	30% FA + CN
Control	1.233	1.428	1.890	1.421	1.289
+PSF	1.236	1.445	1.940	1.492	1.332
+VAM	1.239	1.515	1.840	1.790	1.387
+RHZ	1.133	1.228	1.834	1.752	1.665
+PSF+VAM	1.236	1.445	2.320	1.810	1.389
+PSF+RHZ	1.219	1.415	1.560	1.420	1.310
+VAM+RHZ	1.242	1.536	2.380	1.920	1.286
+PSF+VAM+RHZ	1.246	1.539	2.440	1.870	1.423

Table 6: Proline Content of Pea Plant Raised in Agriculture Soil Amended with 10 %, 20% and 30% Fly Ash and *Cynodon* and Provided with Consortium of AM Fungi, PSF and N_2 Fixer Alone as Well as in Combination, at Fruiting Stage.

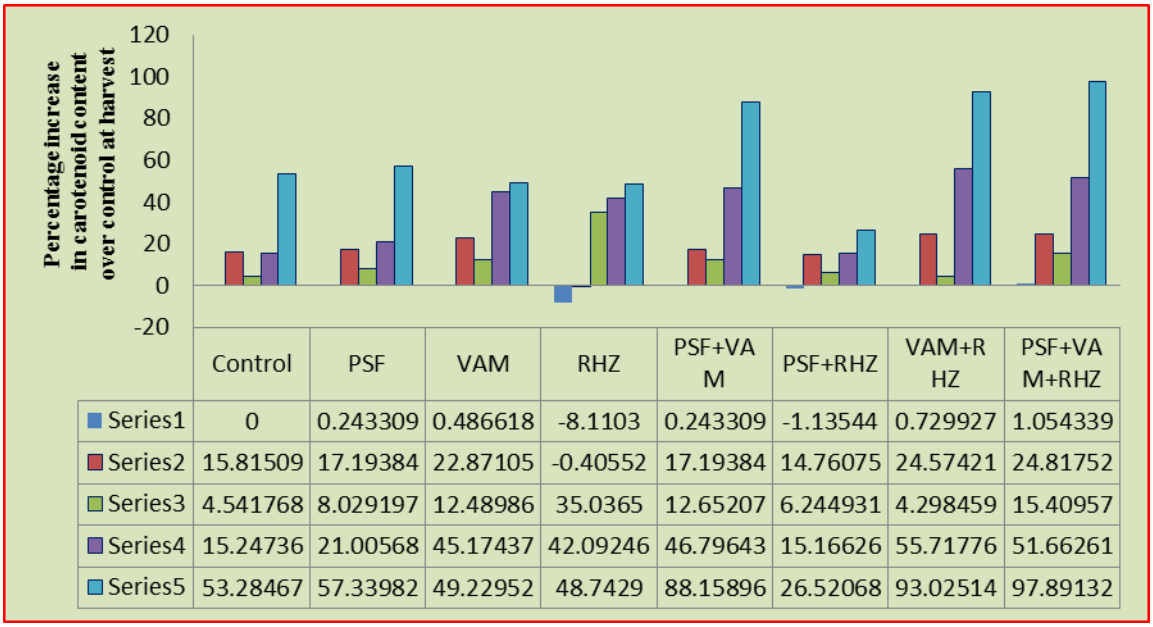
Treatment	Proline content (µg)				
	AgS	CN	10% FA + CN	20% FA + CN	30% FA + CN
Control	0.020	0.016	0.042	0.044	0.046
+PSF	0.015	0.013	0.040	0.041	0.043
+VAM	0.010	0.012	0.021	0.024	0.028
+RHZ	0.018	0.015	0.029	0.033	0.039
+PSF+VAM	0.012	0.010	0.014	0.020	0.023
+PSF+RHZ	0.019	0.015	0.027	0.033	0.036
+VAM+RHZ	0.013	0.010	0.017	0.016	0.021
+PSF+VAM+RHZ	0.011	0.008	0.009	0.010	0.012



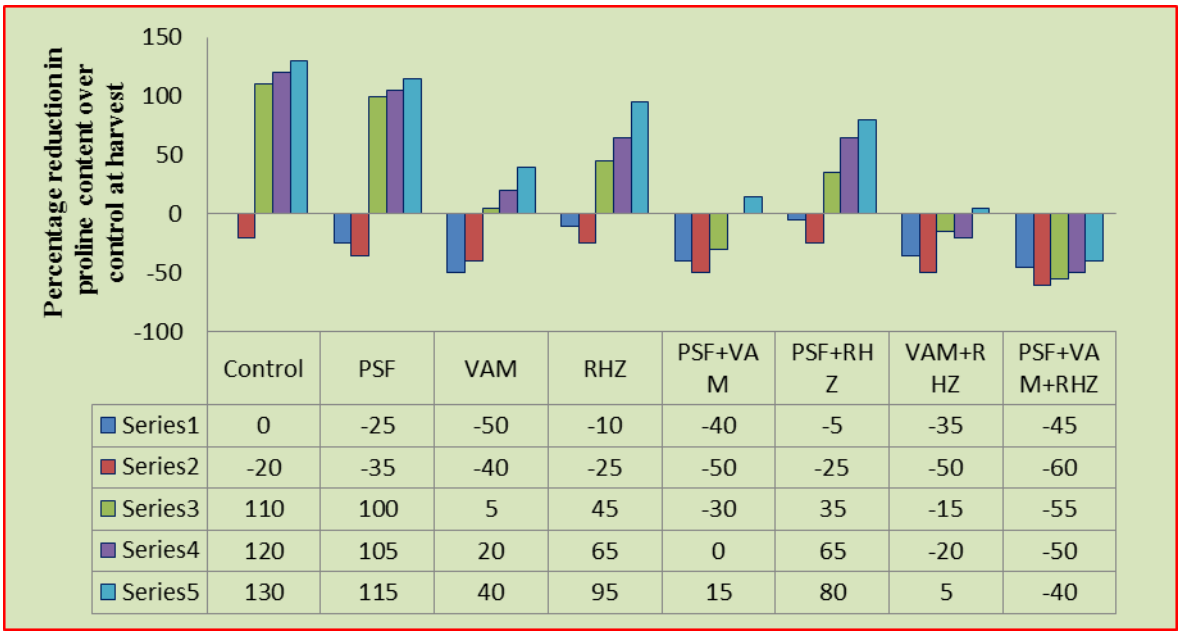


Series1 : AgS (Agriculture soil)
Series2 : AgS+CN (*Cynodon*)
Series3 : AgS+CN+10%FA (fly ash)
Series4 : AgS+CN+20%FA
Series5 : AgS+CN+30%FA

Figure 1



	Control	PSF	VAM	RHZ	PSF+VAM	PSF+RHZ	VAM+RHZ	PSF+VAM+RHZ
Series1	0	0.243309	0.486618	-8.1103	0.243309	-1.13544	0.729927	1.054339
Series2	15.81509	17.19384	22.87105	-0.40552	17.19384	14.76075	24.57421	24.81752
Series3	4.541768	8.029197	12.48986	35.0365	12.65207	6.244931	4.298459	15.40957
Series4	15.24736	21.00568	45.17437	42.09246	46.79643	15.16626	55.71776	51.66261
Series5	53.28467	57.33982	49.22952	48.7429	88.15896	26.52068	93.02514	97.89132



	Control	PSF	VAM	RHZ	PSF+VAM	PSF+RHZ	VAM+RHZ	PSF+VAM+RHZ
Series1	0	-25	-50	-10	-40	-5	-35	-45
Series2	-20	-35	-40	-25	-50	-25	-50	-60
Series3	110	100	5	45	-30	35	-15	-55
Series4	120	105	20	65	0	65	-20	-50
Series5	130	115	40	95	15	80	5	-40

Series1 : AgS (Agriculture soil)
Series2 : AgS+CN (*Cynodon*)
Series3 : AgS+CN+10%FA (fly ash)
Series4 : AgS+CN+20%FA
Series5 : AgS+CN+30%FA

Figure 2