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ARBUSCULAR MYCORRHIZAL FUNGI; POTENTIAL INOCULANTS FOR CULTIVATION OF AROMATIC PLANT *VETIVERIA ZIZANIOIDES* IN ALKALINE SOIL

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In order to assess the potentiality of AM fungi in improving the establishment, survival and growth performance of *Vetiveria zizanioides var*. KSI plants raised in alkaline/sodic soils of Handia, Allahabad, amended with gypsum and organic matter (compost), a pot experiment was conducted under greenhouse conditions. Data on the rate of mortality, mycorrhizal intensity, dry weight of root/shoot and essential oil content in the plants were recorded. In order to assess the potentiality of AM fungi in alleviating the stress conditions in the plants certain physiological parameters, *viz.* concentration of photosynthetic pigments chl *a*, chl *b* and carotenoids, total protein content, proline accumulation, the relative permeability of the root plasma membrane were also estimated. Reduction in mortality rate, proline accumulation, root membrane permeability in vetiver plants grown in gypsum and compost amended soil showed that, adverse effects of alkalinity were reduced to a greater extent. Further, increase in biomass and essential oil production in AM inoculated plants over non-inoculated plants showed that, AM fungi have played beneficial role for vetiver plants under alkaline soil conditions. Thus, the application of native AM fungal inoculum would be of potential use for improving the growth performance and essential oil yield in aromatic plants under alkaline soils.

Key Words
Alkaline soil,*Vetiveria zizanioides*, root/shoot dry weight, essential oil production, AMF inoculation.

Essential oils are in consistent demand all over the world as they play a vital role in our day to day living as spices, food flavoring agents, perfumes and deodorants, antibacterial and insecticidal agents, etc. In India, there is competition for land utilization, food production and it is not beneficial to use these agricultural lands for the cultivation of aromatic plants. Besides the agricultural land, there are several categories of barren lands, which could be utilized for cultivation of aromatic plants and production of essential oils. The salt-affected land is one of them. More than 7% of the earth land surface is occupied by salt-affected land and represent a major limiting factor in crop production (Ruiz-Lozano *et al.* 1996). In India approximately 6727468 ha (2.04 %) land is salt affected out of which 3770659 ha (56 %) land is alkaline (Mandal *et al.* 2010). Alkaline soil in commonly known as '*usar*' or '*reh*' in local language. These soils are characterized by the presence of Na_2CO_3 and $NaHCO_3$.

High pH, high exchangeable sodium percentage (ESP), poor infiltration capacity and poor soil structure make the soil unsupportive for crops. Only some crops resistant to drought and water logging could be grown in such soil with low productivity (Chhabra *et al.* 1979). Traditional approaches for reclamation of sodic soil for agricultural use are based on the leaching of exchangeable sodium from the soil. Amendments like gypsum ($CaSO_4.2H_2O$), calcium chloride, pyrite, farm yard manure, green manure, etc. are used for improving alkaline soils (Verma and Abrol 1980) but, these processes are too slow and costly. Hence there should be a faster and cost-effective method for management of alkaline soils.

Application of native biological inputs such as arbuscular mycorrhizal (AM) fungi for the restoration of degraded ecosystem is an innovative approach (Barea and Jeffries 1995). Plants exhibit considerable dependency on mycorrhizal association, even in salt affected lands for an adequate supply of nutrients and water, enabling them to thrive under stress conditions (Ruiz lozano *et al.* 2012). In a number of recent reports it has been well established that the AM fungi enhance the ability of plants to cope with environmental stresses, generally prevalent in the degraded ecosystems, by providing a

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number of nutritional and physiological benefits (Ruiz-Lozano 2003, Giri and Mukerji 2004, Al-Karaki 2006, Kehri *et al.* 2016).

Vetiveria zizanioides (Vetiver) is one of the most important aromatic plant commonly known as '*Khus*' has been used for various ailments. Essential oil extracted from vetiver plant is of high commercial value and utilized in cosmetics and perfumery industries. The essential oil of vetiver has antibacterial, antifungal, insecticidal and antioxidant properties (Chahal *et al.* 2015). Plant is moderately tolerant to drought and salt stress and known to grow successfully in the alkaline soil having pH up to 9.8 and ESP up to 55% as it has an ability of Na^+ exclusion and produces large biomass of root, which yield essential oil. It was hypothesized that, inoculation of native AM fungi along with other amendments could be a new biotechnological approach for cultivation of aromatic plants in alkaline soils at commercial scale and hence, the present study has been planned to evaluate the efficacy of native AM fungi in improving the essential oil production in the alkaline soils.

MATERIALS AND METHODS

Soil collection : To set the experiment alkaline soil was collected from the fallow fields of Handia site (Plate-1) 25º36'84.13 N, 82º15'66.13 E, located in the Gangetic plains of India near district Allahabad, Uttar Pradesh. The pH of the soil was 10.2 with 4.06 $m.mhos.cm^{-1}$ electrical conductivity, 0.20% organic carbon, 2.25% nitrogen, 32 kg/ha phosphorus and 125 kg/ha potassium.

Mycorrhizal inoculum production : A consortium of native AM fungi, dominant in the alkaline soil of the selected site was applied as mycorrhizal inoculum in the experiment. For mycorrhizal inoculum production, alkaline soil with root rhizosphere was collected and mixed with coarse sand (sterilized at 15 psi pressure for 30 minutes) in 1:1 ratio (v/v) and trap culture was established using *Sorghum bicolor* as host plant. The trap culture was maintained under greenhouse conditions for four months. Mycorrhizal inoculum consisted of soil having spores (35 AMF spores/10g soil), mycelia and infected root fragments (~80% root length colonization).

Identification of dominant AM fungi in the inoculum : AM fungal spores were extracted by wet sieving and decanting method (Gerdemann and Nicolson 1963). For identification spores were mounted in PVLG and PVLG + melzer's reagent. Spore morphology along with structural details of spore wall layers, subtending hyphal

Plate-1: Showing the fallow field of alkaline soil at Handia, Allahabad.

characteristics were observed. Spore morphotypes were named according to Schüßler and Walker (2010) and the species identity was confirmed with the reference culture of International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (http://invam.wvu.edu/). *Acaulospora bireticulata* F.M. Rothwell & Trappe, *Septoglomus constrictum* (Trappe) C. Walker & A. Schüßler, *Rhizophagus fasciculatus* (Thaxt.) C. Walker & A. Schüßler and *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler, were identified as the dominant AM fungal morphotypes in the mycorrhizal inoculum.

Experimental plant material : The slips of *Vetiveria zizanioides* L. (Nash.) *var.* KSI were procured from Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. All the slips were of the same size (20cm) and of same age.

Experimental Design : A pot experiment with following four different series were maintained for 12 months under greenhouse conditions.

1. Control (alkaline soil without any amendment and inoculation)
2. + Gyp (alkaline soil amended with gypsum)
3. + Gyp + OM (alkaline soil amended with gypsum and organic matter)
4. +Gyp + OM +AM (alkaline soil amended with gypsum and organic matter and inoculated with native AM fungi.

Each pot was filled with a mixture of 5 kg salt affected soil. 40g gypsum and 2% (w/w) compost was added in gypsum and organic matter amended series respectively. Single slip of aromatic plant was planted in each pot. In AM inoculated series, 200 g soil inoculum was added in each pot by layering method (Menge and Timmer 1982) before plantation of the slips. Per treatment twelve replicates were maintained.

Determination of mycorrhization : For determination of mycorrhization, root bits were stained in 0.05% trypan blue by method given by Philips and Hayman (1970) and % root bits infection was determined by slide method. 50g air dried soil was taken for spore extraction by wet sieving and decanting method given by Gerdemann and Nicolson (1963) and spore population was counted under stereo binocular microscope at 20X magnification.

Determination of growth, physiological and biochemical parameters : Dry weight of root/shoot of the plants for each treatment was determined separately. The samples were oven dried at 70^0C for 48 hours to determine the dry weight. For better tillering, the plants were cut at about 40 cm above the ground level after six months. Dry weight of the cut portion was determined and added at the time of data collection during second and third sampling.

Photosynthetic pigments were extracted in 80% acetone and concentration was determined by method given by Lichtenthaler and Wellburn (1983). Total protein content was determined by method given by Lowry *et al.* (1951). Relative permeability was measured in terms of percent electrolyte leakage as described by Wilner (1960). Proline content was estimated according to method given by Bates *et al.* (1973).

Determination of essential oil production : Freshly collected root sample was chopped and soaked in water for about half an hour and oil was extracted by steam distillation method with an essential oil extraction apparatus (Clevenger). Essential oil content and the yield were determined as follows :

$$\textit{Essential Oil Content} = \frac{\textit{Oil produced (ml)}}{\textit{Fresh weight of sample (g)}}$$

$$\textit{Essential Oil Yield (\%)} = \frac{\textit{Weight of oil (g)}}{\textit{Fresh weight of sample (g)}} \times 100$$

Statistical Analysis : Data of each parameter, except mortality and mycorrhization were expressed as the mean of three replicates. The means within variables were separated by

using Duncan's multiple range test (DMRT) where needed. The data were statistically analyzed through the SPSS 16 on Windows 10.

RESULTS AND DISCUSSION

In the present study, alkaline/sodic soil of Handia, Allahabad (Plate-1) was used for the experimental purpose. Plants raised without AM inoculation in this soil have shown maximum mortality rate where 8 plants out of 12 died. This is because of the soil characteristics which has high pH (10.2), low organic carbon content, high sodium content and electrical conductivity (4.06m.mhos.cm^{1}). High alkalinity and high exchangeable sodium percentage (ESP) of the soil rendered it inhospitable for normal bioproductivity. High pH generally affects nutrient solubility and high ESP decreases the availability of important nutrient elements, such as Ca, Mg and K. Soil dispersion due to high ESP deteriorates the physical condition of the soil and lowers the water infiltration capacity. This adversely affects root growth and development due to which seedlings often fail to germinate and establish. This explains the high rate of mortality in *Vetiveria zizanioides* under alkaline/sodic soils in the present study. The soil amendments decreased the mortality rate (Table-1) where only 3 plants out of 12 died when the soil was amended with organic matter and Gypsum and inoculated with AM fungi (Plate - 2 a, b).

Minimum percent root bits infection (34%) was recorded in control series. Previous researches have also shown reduced mycorrhizal colonization under salt stress conditions mainly by inhibiting the germination of spores (Hirrel 1981), growth of hyphae in soil and hyphal spreading after initial infection had occurred (McMillen *et al.* 1998) and reducing the number of arbuscules (Pfeiffer and Bloss 1988).

Soil amendments increased the percent root bits infection (Table-1) significantly (p <0.05). This may be through the influence of amendments on soil structure, nutrient mineralization and water holding capacity. Addition of gypsum increases the concentration of cations like Ca^{++} and Mg^{++} in the soil, which helps in reducing the soil pH and overcoming the antagonistic effect of sodium. Presence of easily degradable compost resulting in more easily available nutrients also improves the mycorrhizal growth in the plants (Harinikumar and Bagyaraj 1989). Maximum (67%) root bits infection was recorded in AM inoculated series. The spore production has also increased significantly (p <0.05) according to the treatments (Table-1). Increased AM sporulation and colonization under stressed conditions has also been reported by Aliasgharzadeh *et al.* (2001) and Giri and Mukerji (2004). This indicates that the symbiotic association was strengthened under stressed environment once the association was established. This may be a sign showing the ecological importance of AM association for survival and establishment of plants under stress conditions (Tian *et al.* 2004).

Minimum dry weights of root and shoot (15.37 g and 36.6 g respectively) were recorded in control plants. Amendments with gypsum and organic matter alone as well as in combination enhanced the root and shoot dry weight but the enhancement was not significant at p <0.05 level (Figure-4). AM inoculated plants have shown dramatic increase in the biomass production (Plate-2) and root dry weight and shoot dry weight were significantly (p <0.001 and p <0.05 respectively) increased over other series (Figure-4).

Essential oil production has increased linearly with the treatments (r^2 = 0.990 for essential oil content and r^2 = 0.999 for essential oil yield). Essential oil content and essential oil yield were minimum (0.52 ml / g FW and 0.49 % respectively) in the roots of control plants. The soil amendments have enhanced the essential oil content in other treatment series over the control (Figure-5) but the difference was not significant at p <0.05 for the essential oil content, however, this enhancement was significantly different for the essential oil yield at p <0.01 (Figure-5). The maximum and significant enhancement in essential oil

Plate-2. a : Showing the effect of different treatments on shoot growth in vetiver plants raised in alkaline soil (Bar = 25 cm). **b :** Showing the effect of different treatments on root growth in vetiver plants raised in alkaline soil (Bar = 10 cm). **Control :** Alkaline/Sodic soil, **+Gyp :** Gypsum, **+OM :** Organic Matter (compost), **+AM :** AM fungal inoculum

Table-1 : Effect of different treatments on mortality, mycorrhization and biomass production in Vetiver plants raised under alkaline soil conditions.

Treatments series	Mortality (%)[a]	Mycorrhization	
		Root bits infection (%)[b]	AM spore population (/10g air dried soil)[c]
Control	66.6	34±3.6a	38±2.6a
+Gyp	41.6	41±2.6b	51±5.5b
+Gyp +OM	33.3	45±2.5b	62±3.5c
+Gyp +OM +AM	25.0	67±4.1c	101±7.3d

Control : Alkaline/Sodic soil, **+Gyp :** Gypsum, **+OM :** Organic Matter (compost), **+AM:** AM fungal inoculum
[a] **:** n = 12 [b] **:** Mean value ±1 SD, n =100 [c] **:** Mean value ±1 SD, n =3 ***:** Dry weight.

Table-2: Showing Pearson's correlation coefficient (r^2) between the various parameters measured.

Variables	Percent root bits infection	Root DW	Shoot DW	Essential oil content	Essential oil yield
Chl a	**0.9397**	0.9499	0.9912	0.9839	0.9566
Chl b	**0.9873**	0.9996	0.9738	0.9698	0.9720
Carotenoid	**0.9831**	0.9854	0.9904	0.9803	0.9614
total protein	**0.9046**	0.9536	0.9394	0.9533	0.9812
Proline	**-0.9421**	**-0.9772**	**-0.9883**	**-0.9940**	**-0.9994**
Relative permeability	**-0.9147**	**-0.9596**	**-0.9784**	**-0.9888**	**-0.9991**

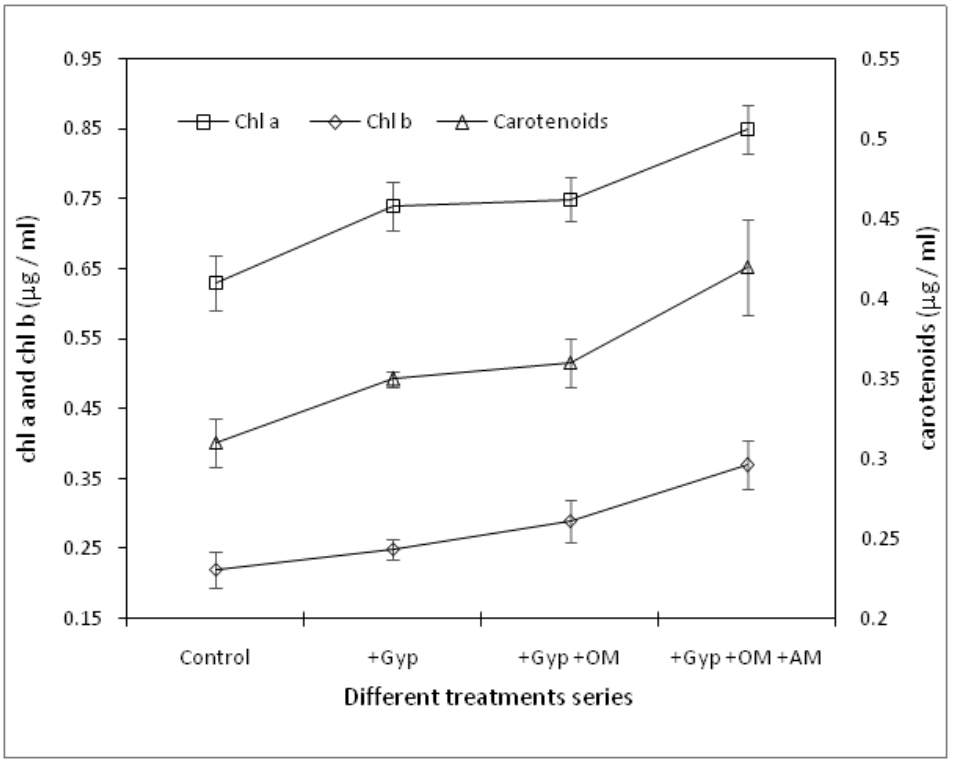

Figure-1 : Showing the effect of different treatments on photosynthetic pigments in the leaves of vetiver plants raised in alkaline soil. **Control :** Alkaline/Sodic soil, **+Gyp :** Gypsum, **+OM :** Organic Matter (compost), **+AM :** AM fungal inoculum

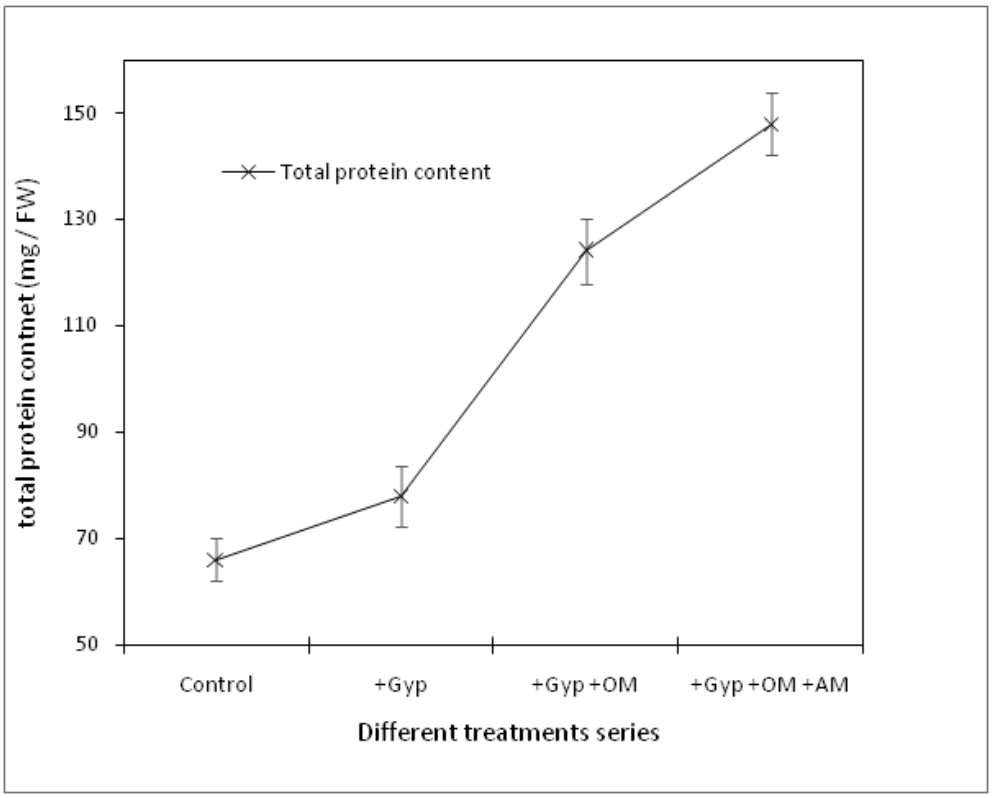

Figure-2 : Showing the effect of different treatments on total protein content in the leaves of vetiver plants raised in alkaline soil. **Control :** Alkaline/Sodic soil, **+Gyp :** Gypsum, **+OM :** Organic Matter (compost), **+AM :** AM fungal inoculum

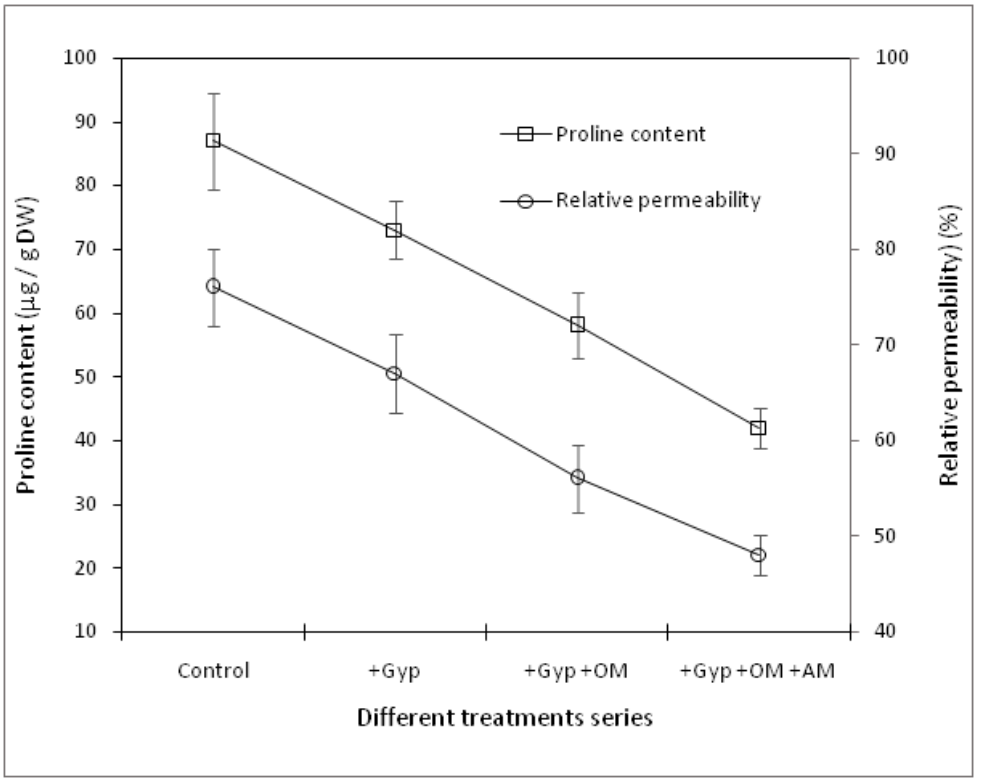

Figure-3 : Showing the effect of different treatments on proline accumulation in the leaves and relative permeability of root in vetiver plants raised in alkaline soil. **Control :** Alkaline/Sodic soil, **+Gyp :** Gypsum, **+OM :** Organic Matter (compost), **+AM :** AM fungal inoculum

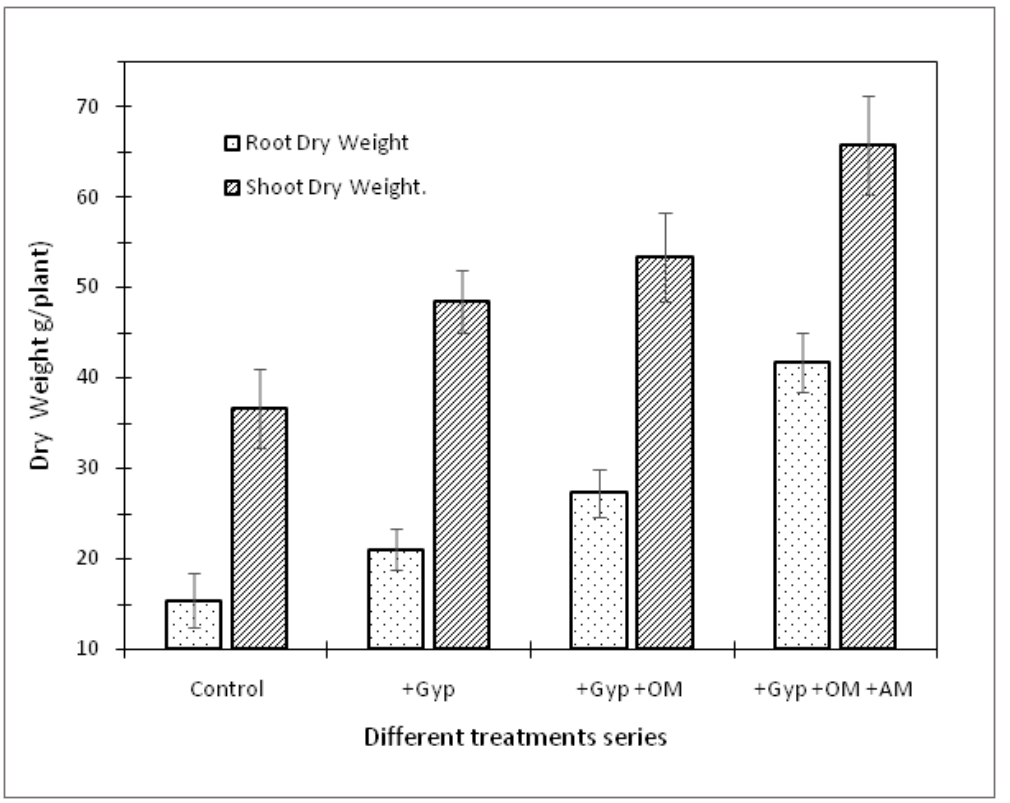

Figure-4 : Showing the effect of different treatments on biomass production in vetiver plants raised in alkaline soil. **Control :** Alkaline/Sodic soil, **+Gyp :** Gypsum, **+OM :** Organic Matter (compost), **+AM :** AM fungal inoculum

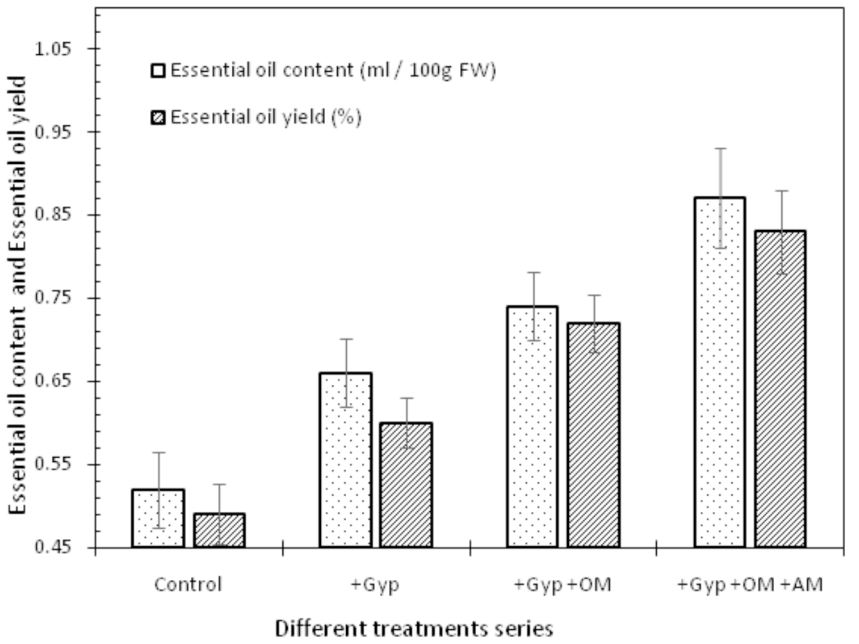

Figure-5 : Showing the effect of different treatments on essential oil production in vetiver plants raised in alkaline soil. **Control :** Alkaline/Sodic soil, **+Gyp :** Gypsum, **+OM :** Organic Matter (compost), **+AM :** AM fungal inoculum

content and essential oil yield (at p <0.05 and p <0.01 respectively) were recorded in the roots of AM inoculated plants (Figure-5). A number of workers have reported significant increase in root/shoot biomass and essential oil production in the mycorrhizal plants particularly under stressed conditions (Khare *et al.* 2008, Ratti *et al.* 2002, Copetta *et al.* 2006). The results of the present study are also in conformity with these reports.

The inhibitory effect of alkalinity on photosynthesis and protein content was pronounced in control series where minimum photosynthetic pigments (0.63 mg / ml chl a, 0.22 mg / ml chl b and 0.31 mg / ml carotenoids) and protein content (66 mg / g FW) were measured. Decline in rate of photosynthesis may be due to interference of salts with the synthesis of chlorophyll, carotenoid and other photosynthetic pigments (Alam 1994). Soil amendments with gypsum and organic matter have improved the photosynthetic pigment production (Figure-1)but, the increase was not significant (at p <0.05) while in case of proteins (Figure-2) it increased significantly (p <0.01). The significant (p <0.01) enhancement in Chl a, Chl b and carotenoids (0.85, 0.37, 0.42 mg / ml respectively) and protein content (maximum protein content (148 mg / g FW) were recorded only, when the plants were inoculated with consortium of native AM fungi. Similar results have also been reported by Ruiz-Lozano *et al.* (1996), Mathur and Vyas (2000), Feng *et al.* (2002), Beltrano and Ronco (2008).

Accumulation of proline content in the roots decreased linearly (r^2= -0.999) with the treatments (Figure-3, Table-2). Maximum proline (87 mg/g DW) was accumulated in the roots of control plants grown under alkaline soil. In the plants inoculated with native AM fungi minimum (42 mg/g DW) proline was accumulated in the roots.

In the present study also, cellular membrane dysfunction due to oxidative damage caused by alkaline/sodic stress lead to the increased permeability and ion leakage. The relative permeability decreased linearly (r^2= -0.996) with the treatments (Figure-3, Table-2). Maximum (76 %) relative permeability was recorded in the root of control plants and minimum (48 %) in AM inoculated plants. The results are in conformity with the earlier findings of Feng *et al.* (2002), Beltrano and Ronco (2008).

Reduction in proline concentration, lower permeability of the root plasma membrane and increased photosynthetic pigments and protein content in the AM inoculated plants, compared to non-AM inoculated plants, in the present study indicates that the mycorrhizal plants were less affected by the alkaline/sodic stress.

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