academicJournals

Vol. 8(21), pp. 932-939, 4 June, 2013
DOI 10.5897/SRE12.737
ISSN 1992-2248 © 2013 Academic Journals
http://www.academicjournals.org/SRE

Scientific Research and Essays

Full Length Research Paper

Mechanism(s) of humic acid induced beneficial effects in salt-affected soils

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Accepted 22 February, 2013

There is enough evidence that humic acid (HA) helps to enhance crops yield by promoting certain physical, chemical and biological activities in soil-plant system. Indigenously produced coal derived, HA was added to saline-sodic silty clay soil to determine its effects on activities of alkaline phosphatase, urease, microbial activities, cation exchange capacity and moisture retention of soil. Results indicated that HA treatments significantly ($p<0.05$) increased alkaline phosphatase activities. Urease activities increased by 2.84, 5.71 and 20.73% with application of 0.5, 1.0 and 2.0 mg HA kg^{-1} respectively, in normal soil and by 16.7 and 33.6% with addition of 1.5 and 3.0 mg kg^{-1} in salt affected soils as compared with control (HA0). Microbial activities, measured in terms of CO_2 evolved, increased by 15.7, 36.7 and 78.8% at no NPK, 14.8, 37.1 and 66.8% at half NPK, and 15.4, 40.9 and 51.2 at full NPK with addition of 0.3, 0.6, and 1.0 mg HA kg^{-1}soil, respectively as compared to control soil (HA0, NPK0). Cation exchange capacity (CEC) enhanced by 12.3 and 20.7% over the control with additions of 1.5 and 3.0 mg kg^{-1} HA to saline-sodic soil. Moisture retention of soil increased significantly ($p < 0.05$) with 1.0 mg HA kg^{-1} at 1.5 MPa. It is concluded that physico-chemical and biological properties of soil are promoted by HA, which explains for the reported increases in crop yield.

Key words: Humic acid, alkaline phosphatase, urease, cation exchange capacity, microbial activities, water retention of soil.

INTRODUCTION

Judicious exploitation of natural resources is imperative for sustainable economic growth and development of a country. Costly chemical fertilizers are used as a major source of nutrients supply for soil fertility maintenance and to optimize crop productivity. Humic acid can be used as a supplement to chemical fertilizers and various mechanisms have been advocated to explain its role in increasing crop yield. Humic acid has a complex macromolecular structure and is considered as an alkali soluble, polymeric organic acid of aromatic subunits attached to carboxyl, phenolic, hydroxyl and alkyl groups interlinked via ether linkages (Gaines and Yilmaz, 1983; Sposito, 1989; Spark et al., 1997). The physico-chemical and molecular structure (Sutton and Sposito, 2005) and the mechanism of its stimulating effect on various crops and soil conditions have been envisaged by various workers (Schnitzer and Khan, 1972; Malik et al., 1979; Brannon and Sommers, 1985; Sharif et al., 2005). The properties of base exchange capacity and complexing ability of HA are important in soil stability, transport of metal ions in the soil through plant tissues and stabilization of soil organic matter against microbiological attack and because of its ability to form complexes (Khan, 1969; McBride, 1978). It is believed to convert elements into forms suitable for assimilation by plant (Vaughan and Donald, 1976). Humic acid contains many trace elements in its structure and various micronutrients are further complexed with to form chelates (Barron and Wilson, 1981). These chelates can regulate the supply of micronutrients needed for plant growth and development (Mackowiak et al., 2001).

Plants show more active metabolism and increased

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respiratory activity due to HA and these activities are attributed to the intervention of the quinone groups of HA (Petronio et al., 1982). It is believed that HA serves as a catalyst in promoting the activity of microorganisms in soil and reduces the adverse effects of chemicals on environment (Bhardwaj and Guar, 1970). A wide variety of enzymes have been detected in HA, among these, urease is one of the most abundant and agronomically significant enzyme. The importance of the interaction of urease with HA as a fundamental gateway for extra cellular urease stabilization in soil was confirmed (Skujins, 1967; Marzadori et al., 2000). Malcolon and Vaughan (1979) suggested that phosphatase activity is inhibited by HA fraction by combining with the enzyme but not at the most active site of enzyme activity in plant tissue. Acid Phosphatase activity showed that 156% increases with sonication over non-sonicated control, which was attributed to the release of extra-cellular enzyme fraction complexed / immobilized on humic collods and not due to cell lysis (De Cesare et al., 2000). In a very fine study using CP/MAS NMR, Spectroscopy, Zancani et al. (2009) investigated the role of natural humic substances in plant cell phosphate level and metabolism, whereby tobacco By-2 suspension cell culture were grown in the presence of humic samples with different chemical composition and size fraction. It was observed that fraction, III most hydrophilic and smallest in molecular size, induced a partial relief from Pi starvation, increased total cell phosphate amount, ATP and glucose-6-phosphate levels and as well as the activity of acid phosphatase.

Addition of HA to soil increases the rate of adsorption of mineral ions on root surfaces and their penetration into the cells of plant tissues, and this activity is highly correlated with biologically available carbon pool (Stroo and Jenckson, 1982). Hanafi and Salwa (1998) evaluated the effect of HA addition on chemical properties of the soils in an incubation study. Soil pH increased with increasing levels of HA addition and the same trend was also observed for organic C and CEC of the soils by Sharif et al. (2002).

In this study, laboratory experiments were conducted on activities of alkaline phosphatase and urease, microbial activities, cation exchange capacity and moisture retention of soil as influenced by HA to understand the mechanisms of HA induced beneficial effects in salt affected soils, whereby substantial increases in crop yield were observed by this group in normal soil (Sharif et al., 2002, 2005).

MATERIALS AND METHODS

Extraction of humic acid from coal

Humic acid was extracted from brown coal collected from Hyderabad, Pakistan in the laboratory of Soil and Environmental Sciences, KPK Agricultural University Peshawar, Pakistan following the modified procedure of Hai and Mir (1998). A 10 kg ground coal was treated with 3 L conc. HNO_3, 1 L H_2SO_4 (instead of only HNO_3) and 40 L water in a stainless steel 100 L reaction vessel. Flow of water was maintained regularly so that temperature of reaction may not exceed 40°C. Acid and water were added in four intervals. The reactants (mixture of acid and coal) were allowed to stand for 24 h for settling down of the suspended particles. The supernatant was decanted. The reactants were then made alkaline by adding 0.5 N NaOH gradually until pH of the solution was adjusted to 11.5. The solution was allowed to stand for overnight and then centrifuged. The filtrate was acidified using 0.5 N HCl until pH was dropped to 2.0. The solution containing HA suspended particles was again centrifuged. The semi solid HA was oven dried at 50°C and HA was collected. The HA contents was determined as described by Lamar and Talbot (2009), which was about 70% pure. The HA composition conformed to the typical composition reported (Hai and Mir, 1998; Sposito, 1989).

Alkaline phosphatase assay in soil treated with humic acid

Alkaline phosphatase (phosphomonoesterase) assay was conducted using the procedure as described by Tabatabai and Bremner (1996). Fresh soil samples were treated with 0, 0.25, 0.5, 1.0 mg HA kg^{-1}. One g of HA treated soil was taken in triplicate in 50 mL volumetric flask from each treatment. A 4 mL of MUB (modified universal buffer) adjusted to pH 11.0 for alkaline phosphatase, and 1.0 mL of p-nitrophenyl phosphate (PNP) solution made in the same buffer, was added to the soil. Contents of the flask were mixed by swirling the flask gently for a few seconds. Flasks were then caped and incubated for 1 h at 37°C in incubator (Shel Lab, USA). The phosphatase activity was then stopped by adding 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH and mixed gently. The contents were filtered through a Whatmann no. 2v and the yellow color intensity was measured using spectrophotometer Lambda 35 (Perkin Elmer). A calibration curve was developed by measuring intensity in 0, 20, 30, 40 and 50 ug p-nitrophenol mL^{-1} standard solutions. A blank was run each time to correct the color intensity induced by soil.

The activity of alkaline phosphatase was tested in normal soil (pH 7.96, EC_e 0.3 dS m^{-1}) and saline-sodic soils (pH 8.32, ECe 10.18 dS m^{-1} and SAR 17.53) simultaneously.

The same procedure was repeated to evaluate the effect of HA treatments (0, 1.5 and 3.0 mg kg^{-1}) and incubation time of two weeks on alkaline phosphatase activity in salt affected soil.

Urease assay in soil treated with humic acid

Urease assay was conducted by ammonium-nitrogen (NH_4-N) method as described by Tabatabai and Bremner (1972). Fresh saline-sodic soil sample was treated with 0, 1.5 and 3.0 mg, HA kg^{-1} soil. Five g soil from each treatment was taken in triplicate in 50 mL volumetric flasks. A 0.2 mL toluene and 9 mL of THAM (Tris hydroxymethyl amenomethane) were added to the flasks followed by gentle swirling to mix the contents. One ml of 0.2 M urea solution was added to the flask and mixed gently. Flasks were then caped and incubated at 37°C for 2 h in incubator (Shel Lab, USA). After removal of flasks from incubator, 35 mL of KCl-Ag_2SO_4 solution was added and swirled gently for a few seconds to stop the urease activity and to extract NH_4-N from soil. The contents were then allowed to cool down to room temperature and the volume was adjusted to 50 mL by adding KCl-Ag_2SO_4 solution. The NH_4-N in the resulting soil suspension was determined by taking 20 mL aliquot according to the procedure as described by Mulvaney (1996). Control was run simultaneously to correct for NH_4-N not derived from urea through urease activity. The same procedure was repeated for non-saline normal soil except that HA treatments were maintained as 0, 0.5, 1.0 and 2.0 HA kg^{-1}. The rate of urease activity was expressesd as mg NH_4-N kg^{-1} soil h^{-1}.

Soil microbial activities as affected by humic acid

Microbial activity as influenced by humic acid application was measured as rate of CO_2 (mg kg^{-1} day^{-1}) evolution from soil. Fresh soil collected from saline fields was amended with 0, 0.3, 0.6 and 1.0 mg HA kg^{-1} soil and with either 0, 30 - 22.5 - 15 or 60 – 45 - 15

Table 1. Effect of HA on alkaline phosphatase activity as nitrophenole production in normal and salt affected soil.

Humic acid (mg kg^{-1})	Alkaline phosphatase	
	Normal soil	Salt affected soil
	----------mg kg^{-1} soil h^{-1}----------	
0	299.24^{b}	154.56^{b}
0.25	303.47^{b}	200.54^{a}
0.5	323.93^{ab}	200.74^{a}
1.0	345.28^{a}	212.02^{a}
LSD (P < 0.05)	24.95	19.24
CV %	3.93	5.02

Mean values in a given column followed by same letters are not statistically different at P<0.05 by LSD.

Table 2. Effect of HA on alkaline phosphatase activity as measure of Nitrophenole production in soils at d0 and by making complexes with colloid (De Cesare et al., 2000) after 2 weeks of incubation.

Humic acid mg kg^{-1}	Alkaline phosphatase mg kg^{-1} soil h^{-1}	
	Day 0	day14
0	475.37^{b}	336.07
1.5	477.81^{b}	350.37
3.0	588.33^{a}	443.17
LSD (P < 0.05)	29.95	Ns
CV%	2.57	2.97

Mean values in a given column followed by same letters are not statistically different at P<0.05 by LSD.

mg NPK kg^{-1} soil. The CO_2 evolution was measured according to the procedure adapted by Shah and Saleemullah (1995). A 50 g fresh soil sample was taken into 500 mL conical flask. A glass vial containing 10 mL of 0.3 M NaOH solution was suspended carefully in each flask. Flasks were sealed using rubber bung and then incubated at 30°C for 20 day, while the CO_2 evolved and subsequently absorbed in NaOH was determined by titrating the NaOH solution against 0.1 M HCl. Readings were taken on 1, 2, 4, 7, 10, 15 and 20 day of incubation

Cation exchange capacity of soil treated with humic acid

Cation exchange capacity (CEC) of soil was determined by the procedure described by Rhodes (1982). Fresh silt loam saline soil was amended with three levels of humic acid (0, 1.5 and 3.0 mg kg^{-1} fresh soil). A 4 g soil was taken in centrifuge tubes, 100 mL of 1.0 M Na-acetate was added to soil in three intervals of 33 mL following 5 min shaking and then centrifuging the solution until the supernatant was clear. The aliquot was discarded each time. The soil was washed with 100 mL of ethanol in the same three intervals following shaking, centrifuging and then discarding the clear supernatant each time. Soil was then treated with 33 ml ammonium acetate solution following shaking centrifuging and collecting the supernatant solution in 100 mL flasks three times. The supernatant was then passed though a Whatmann 42 and the solution was analyzed for Na using flame photometer (Jenway, PFP-7) to calculate CEC of the soil.

Water retention by humic acid treated soil

The capacity of water retention as influenced by humic acid application was measured by the procedure of Cassel and Nielson (1986). Silty clay saline sodic soil was treated with 0, 0.25, 0.5, and 1.0 mg HA kg^{-1} soil. About 10 g soil was put in cores (plastic rings) and was allowed to absorb water for 24 h. The saturated soil was then subjected to 0.03 and 1.5 M Pa pressure for 24 h using pressure membrane apparatus (Santa Barbara, CA, USA). Soil samples were then transferred to Petri dishes carefully and weighed before and after oven drying at 105°C for 48 h (Shel Lab, USA).

Statistically analysis

All observations were replicated three times for a given treatments and least significant difference (LSD 0.05) was applied to compare means using SAS package for analysis of variance.

RESULTS

Alkaline phosphatase activities

The activity of alkaline phosphatase, ranged from 299.24 to 345.28 and from 154.56 to 212.02 mg nitrophenole kg^{-1} h^{-1} in normal and salt affected soil, respectively. Irrespective of HA levels, the activity of alkaline phosphatase was greater by a factor of 2 in normal soil as compared to salt affected soil.

The treatments of HA increased the activity of alkaline phosphatase consistently and the differences in nitrophenole activity observed with HA were significantly higher than control but statistically similar to each other in salt affected soil. The magnitude of the increases with HA addition was more with 0.5 and 1.0 mg kg^{-1}, HA as compared to 0.25 mg kg^{-1}. In normal soil the value of 345.28 mg nitrophenole kg^{-1} soil h^{-1} at 1.0 mg kg^{-1} HA was significantly higher than the 0 and 0.25 mg kg^{-1} HA but statistically similar to 0.5 kg ha^{-1} HA in normal soil.

In saline soils, all HA levels significantly ($p < 0.05$) increased alkaline phosphatase activities as compared to control. Results suggested that addition of HA induced increases in activities of alkaline phosphatase with low level (0.25 mg kg^{-1}) was statistically similar to higher levels (0.5 and 1.0 mg kg^{-1}) (Table 1).

Effect of HA and incubation time on alkaline phosphatase activity measured as nitrophenole production in salt affected soils

Table 2 shows data regarding the activity of alkaline phosphate in saline soil treated with HA levels of 0, 1.5 and 3.0 mg kg^{-1} soil and incubated for two weeks (d14) under laboratory condition. The activity of alkaline phosphatase increased with HA and decreased by 29.30, 26.67 and 41.67% with incubation time (day 14) at the given level of HA as compared to the activities measured at day 0. The effect of 3.0 mg HA kg^{-1} was significant ($p < 0.05$) at day 0 and non-significant at day 14.

Urease activities

The NH_4-N production increased by 2.84, 5.71 and

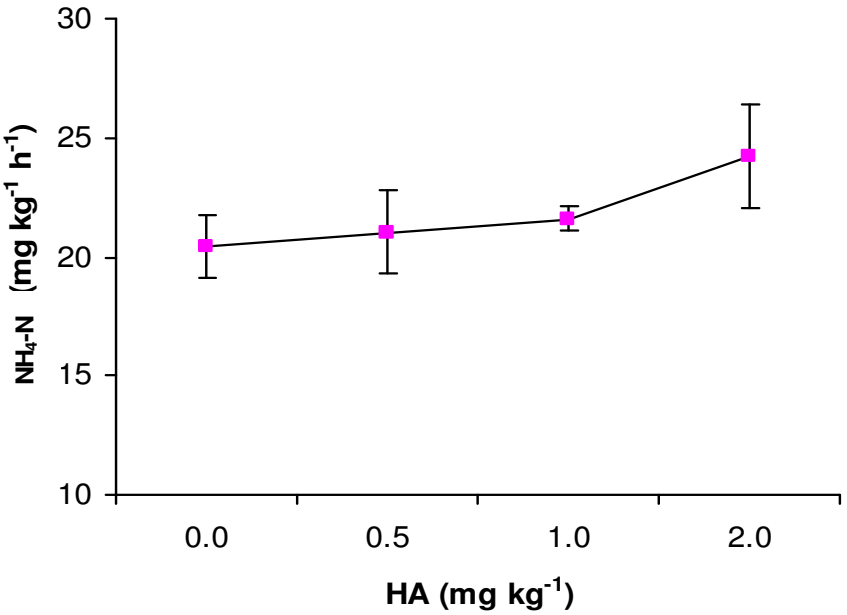

Figure 1. Effect of HA on urease activity as NH_4-N production (mg kg^{-1} soil h^{-1}) in fresh non-saline silty clay soils. (Error bar shows MSE).

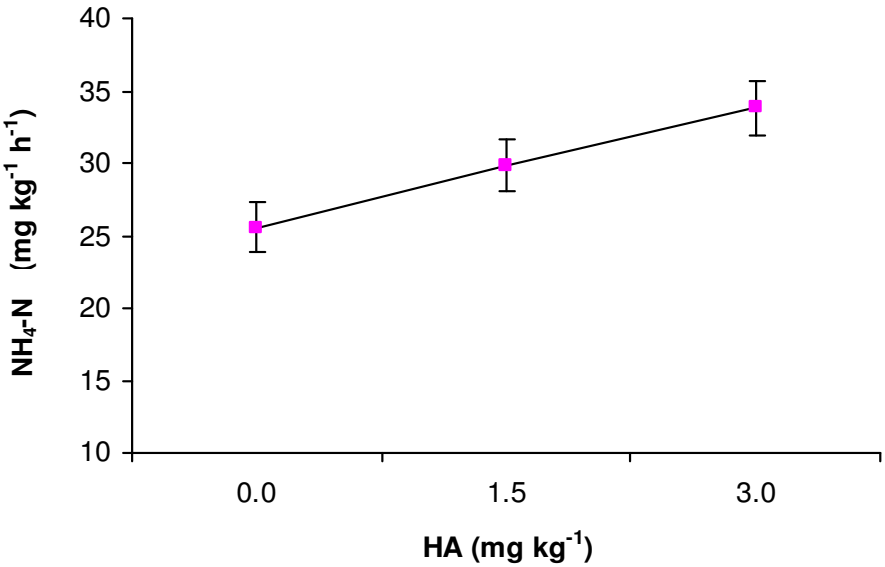

Figure 2. Effect of HA acid on urease activity as NH_4-N production (mg kg^{-1} soil h^{-1}) in salt affected silty clay soils. (Bars Shows MSE) (F0-NPK0; F1= ½ NPK; F2= Full NPK equal to recommended doses).

20.73% with application of 0.5, 1.0 and 2.0 mg HA kg^{-1}, respectively in normal soil and by 16.7 and 33.6% with addition of 1.5 and 3.0 mg kg^{-1} to salt affected soils showing linear increase as compared with control (Figures 1 and 2). The linearity observed in Figure 2 appears to be associated with distinctly spaced HA levels.

Soil microbial activities treated with HA

The cumulative CO_2 evolution in 20 days of incubation is presented in Figure 3 and CO_2 evolution at 4^{th} day of incubation is presented in Figure 4. The CO_2 evolution consistently increased with levels of HA alone and with NPK up to 4^{th} day of incubation. The increase in net CO_2 evolution at day 2 and day 4 was 3 to 4 times greater than day 0 at all levels of HA. On 4^{th} day of incubation, CO_2 evolution reached to maximum values for all treatments. After 4^{th} day the net CO_2 evolution showed a decreasing trend till the day 20, and apparently came to steady state.

The CO_2 evolution and hence microbial activities enhanced with increasing levels of HA from 0 to 1.0 with

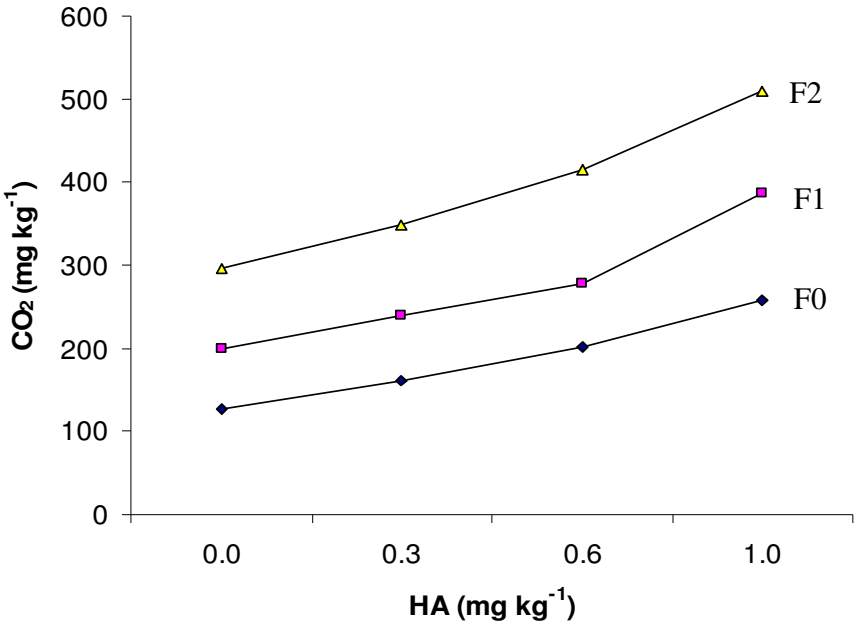

Figure 3. Effect of HA and NPK on cumulative CO_2 production in 20 days of incubation in salt affected soil (F0-NPK0; F1= ½ NPK; F2= Full NPK equal to recommended doses).

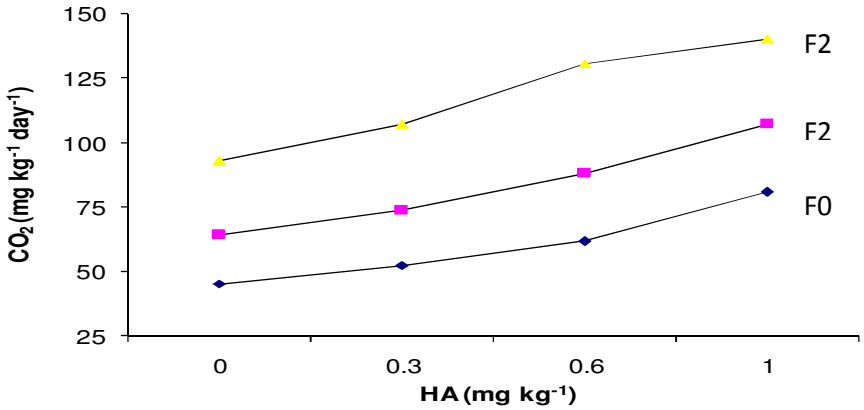

Figure 4. Effect of HA and NPK on CO_2 evolution measured at 4th day of incubation (F0= NPK0; F1= ½ NPK; F2 = Full NPK equal to recommended doses).

and without NPK levels as compared to control (HA:0 and F0). The rate of CO_2 at 1.0 mg kg^{-1} at F0 was equal or higher than the half dose of NPK alone (F1) during incubation. Both levels of NPK (F1 and F2) alone and in combination with different doses of HA stimulated microbial activities (Figures 3 and 4) in a similar fashion observed for the HA+NPK effect on crop yield (Sharif et al., 2002, 2005).

As compared to control (HA0) at 4th day of incubations, CO_2 evolution increased by 15.7, 36.7 and 78.8% at F0, 14.8, 37.1 and 66.8% at F1, and 15.4, 40.9 and 51.2 at F2 with addition of 0.3, 0.6, and 1.0 mg kg^{-1}, respectively as compared to respective NPK levels. The data clearly indicated that CO_2 evolution increased with all doses of HA and NPK alone and with combined application in salt-affected soil.

Soil cation exchange capacity treated with HA

With addition of 1.5 and 3.0 mg kg^{-1} HA to saline soil, the cation exchange capacity (CEC) increased by 12.28 and 20.7% over control (Figure 5). These increases were observed with addition of HA in a solution form to a known mass of soil and the CEC was measured immediately. Results indicate that HA contributes to the CEC of the soil which can promote cations retention capacity of the soil that regulate nutrient supply to plants.

Moisture retention/adsorption of soil treated with HA

Moisture retention tended to increase non-significantly at 0.03 MPa with increasing HA from 0 to 1.0 mg kg^{-1} and

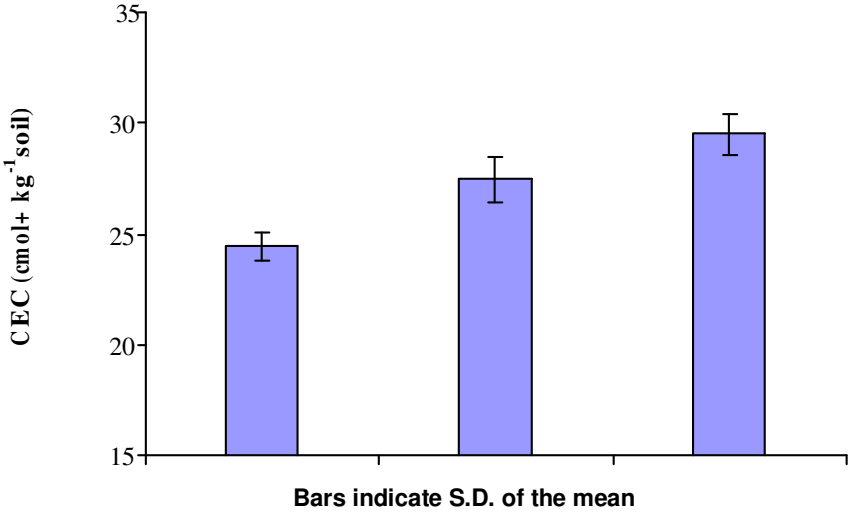

Figure 5. Effect of HA acid on cation exchange capacity ($cmol_{(+)}$ kg^{-1}) in salt affected soils.

Table 3. Effect of HA on moisture retention in silty clay loam saline sodic soil.

HA (mg kg^{-1})	0.03 M Pa	1.5 M Pa
0	18.22	8.81[b]
0.25	18.36	9.13[b]
0.5	18.50	9.18[b]
1.0	18.79	9.54[a]
LSD (P < 0.05)	Ns	0.4
CV %	2.49	2.12

Mean values in a column followed by similar letters are statistically similar at P<0.05, using LSD.

increases were significantly ($p < 0.05$) higher at 1.5 MPa and at 1.0 mg kg^{-1} (Table 3). The increase of 4.2 and 8.3% in water retention by 0.5 and 1.0 mg HA kg^{-1} soil over control, might be important in water stress conditions.

DISCUSSION

Addition of HA to salt affected and normal soil increased the phosphatase activity under laboratory conditions, which may partially explain for beneficial effect of HA on crop growth (Brannon and Sommers, 1985; Sposito, 1989). Other researchers like Blagodatsky and Richter (1998) and Liang et al. (2003) have also reported that organic matter increases soil microbial biomass and some soil enzymatic activities such as urease, alkaline phosphatase and β-glucosidase.

The saline soil showed values of enzymatic activities lower than those observed in other non-saline areas (Tejada et al., 2005), which indicated that biochemical quality 'salting-out' effect which involves a decrease in enzyme release through dehydration, thus altering the enzyme 'catalytic site'. The effect depends on the concentration of the salts and on the chemical composition of the enzymes itself (Garcia et al., 2000). Moreover, if it is accepted that soil salinity disperse the clays contained therein, the extra-cellular enzyme would be less protected and perhaps denatured by proteolysis (Garcia et al., 2000). In this study, this seems the most likely cause of the decrease in enzymatic activities observed in the saline soils as compared to normal soil. Humic acid probably protected enzymes from prophylatic effect, by forming complexes with colloid-HA (De-Cesare et al., 2000).

Interaction of HA with urease activities has been reported by various researchers. Marzadori et al. (2000) confirmed the importance of the interaction of urease with HA as a fundamental gateway for extra cellular urease stabilization in soil. A wide variety of enzymes have been detected in HA, among these, urease is one of the most abundant and agronomically significant. Organic matter induced increases soil microbial biomass and some soil enzymatic activities such as urease, alkaline phosphatase and β-glucosidase have been frequently reported by Blagodatsky and Richter (1998) and Liang et al. (2003).

Salt-affected soils are low in organic matter and

therefore microbial activities are also low as compared to fertile soil with high in organic matter; therefore addition of HA and NPK fertilizers increases in microbial activities as compared to control (untreated soils) (Sharif et al., 2002). HA is a vital constituent of soil organic matter which may serve as a biological catalyst to promote the population and activity of microorganisms in soils (Bhardwaj and Guar, 1970; Vaughan, 1976). HA improves biological conditions of soils and population density of microbes is a determining factor in the rhizosphere (Malcolm and Vaughan, 1979).

In laboratory at temperature 30°C, the CO_2 evolution rate reportedly ranged from 5 to 50 mg CO_2 kg^{-1} soil day^{-1}. Wagnar (1975) reported that under field conditions the rate of CO_2 evolution may be as low as 0.5 to 10 mg CO_2 kg^{-1} day^{-1} which also includes CO_2 from respiration of roots and soil microorganisms. Khan (1987) observed 8.6 to 50.6 mg CO_2 kg^{-1} soil $week^{-1}$ in 9 different agricultural soils at incubation temperature of 10°C. Jenkinson and Powlson (1976) recorded 31.5 to 192.5 mg CO_2 kg^{-1} $week^{-1}$ at 25°C. Pulford et al. (1988) noted 16.5-181. This brief review shows that rate of CO_2 evaluation varies from soil to soil (depending upon the experimental conditions).

Hanafi and Salwa (1998) evaluated the effect of HA addition on chemical properties of soils in an incubation study. Soil pH increased with increasing levels of HA addition and the same trend was also observed for organic C and CEC of the soils. Harper et al. (2000) found that soluble soil organic components including FA and HA have the ability to complex cations. HA is known to have high CEC and surface area (Bohn et al., 2001; Sposito, 1989) as compared to soil. This property of HA is considered to have positive role in nutrient uptake by crops (Fortun and Lopez-Fando, 1982; Stevenson, 1982; Aiken et al., 1985; Sharif et al., 2002).

Water holding capacity of soils especially that of sandy soils is raised by the addition of organic materials (Khaleel et al., 1981). The increases in water retention in HA amended soil could help promote microbial growth and sustain root development under moisture stress environment in salt affected arid zone soils.

Conclusions

It can be concluded from the results of these experiments that beneficial effects of HA on plant growth and nutrients uptake are mainly associated with the potential of HA to improve biochemical environment of soil by promoting soil enzymatic activities, microbial activities and population, cation exchange capacity and water retention of soil. Given the improvement in plant growth and nutrients uptake, and beneficial effects on soil biochemical environments, lignitic coal extracted HA can be used as low cost supplement to chemical fertilizers for crop yield.

ACKNOWLEDGEMENTS

The authors would like to thank the administration of Agricultural Linkage programme (ALP) NARC, Islamabad, Pakistan and USAID for financial support of this study.

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