INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 16, No. 1, p. 279-293, 2020

RESEARCH PAPER OPEN ACCESS

Increased soil enzymatic reactions and soil microbial biomass by application of Lignitic humic acid

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Key words: Lignitic humic acid, Microbial biomass, Stimulation, Enzymatic reactions. CO_2-C evolution, Alkaline soils

http://dx.doi.org/10.12692/ijb/16.1.279-293 Article published on January 30, 2020

Abstract

A laboratory incubated study was conducted to evaluate the effects of lignitic humic acid addition on soil microbial activities and enzymatic reactions and indirectly improving the soil fertility level. Two types of soils (alkaline soils and sandy clay loam soils) were taken to apply lignitic humic acid at different rates (0, 2, 4, 6, 8µg g^{-1} soil). Treated and controlled soil samples were incubated at 25°C with moisture contents of 50% soil WHC for a period of 56 days. An increase in the cumulative CO_2-C release, microbial biomass C, microbial biomass N, microbial biomass P and the activities of dehydrogenase and alkaline phosphatase enzymes in the soil was recorded by the addition of lignitic humic acid as compare to control. The decrease in ratios of C/N and C/P in the microbial biomass was observed, while the ratios of dehydrogenase/microbial biomass C, and alkaline phosphatase/microbial biomass C increased significantly. Addition of 8µg g^{-1} soil humic acid was the optimum humic acid application rate for its effect on soil microbial parameters in both soil types. All the microbial biomass indices exhibited similar temporal pattern being highest on 14th day of incubation followed by a gradual decline during rest of the incubation period. It was concluded that application of humic acid at an appropriate level (8µg g^{-1} soil) positively impacted soil ecology and soil fertility by improving microbial activities and enzymatic reactions in soil.

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Introduction

Deficiency of organic matter in soil has deleterious effects on soil properties and plant growth resulting in poor crop yields (Sarwar *et al.*, 2008; Hijbeek *et al.*, 2017). This challenging situation escalates the need to achieve the healthy soil status by using environmentally-friendly inputs for sustaining crop production (Pukalchik *et al.*, 2019; Gazzola *et al.*, 2019). Humic substances form an important fraction of soil organic matter and are known to improve soil productivity by exerting favorable effects on soil properties such as aggregation, porosity, water retention, and cation exchange capacity (Hussein *et al.*, 2011; Muter *et al.*, 2015). Humic acid contribution to increase soil fertility on long term basis is highly recognized, as it helps in organic matter build up (Noroozisharaf and Kaviani, 2018), enhances uptake of macronutrients and micronutrients (Sharif *et al.*, 2002; Jones *et al.*, 2007; Eyheraguibel *et al.*, 2008), stimulates microbial growth, and promotes soil microbial activities (Kalaichelvi *et al.*, 2006, Valdrighi, 2013). Humic acid has a great tendency to involve in binding reactions which facilitates the substrate access to the active sites of the enzymes (Pukalchik *et al.*, 2019). Chen *et al.*, (2004) reported the chelating characteristics of humic acids to hold ionized nutrients to prevent the leaching losses of soil nutrients. Recently, the use of humic substances like sodium and potassium humates, are becoming more popular as an alternative to organic manures, to improve crop production in soils with low organic matter contents (Lee *et al.*, 2004; Tahir *et al.*, 2011, Arjumend *et al.*, 2015; Lyons and Genc, 2016; Kumar and Singh, 2017). Studies has shown that application of humic acid as an organic agricultural input can improve soil properties, nutrient availability and crop growth effectively, however, there is a lake of proper understanding of humic acid effects on microbial activities and the enzymatic reactions in soil, and rarely has been reported.

The activities of beneficial microbes in soil are vital for the long term sustainability of any type of soil and plant growth (García-Orenes *et al.*, 2013), as they play an integral role in the decomposition of organic matter (Wiseman *et al.*, 2012). Therefore, the status of soil microbial biomass content is considered as the most sensitive and rapid indicator of the long term soil productivity and a healthy soil ecosystem (Russell *et al.*, 2006; Smith *et al.*, 2007; Roger-Estrade *et al.*, 2010; García-Orenes *et al.*, 2013). Research has revealed that the soil microbial community can negatively be affected by soil treatments by chemical applications (fertilizers/pesticides). Being the source and sink of essential plant nutrients, soil microbial biomass is a key element behind all biochemical transformations leading to soil nutrient availability (Nannipieri *et al.*, 2003) and can assist in measuring the status of processes like nitrogen fixation, nitrification, global carbon cycle (Wiseman *et al.*, 2012), solubilization of phosphate, and production of indole acetic acid (Bashan *et al.*, 2014; Viscardi *et al.*, 2016). Therefore, presence of beneficial soil microorganisms can possibly establish sustainable systems for productive soils (Couillerot *et al.*, 2013), and can be considered as biomarkers for soil fertility status by promoting mechanisms like biological fixation of nitrogen, solubilization of phosphate, production of indole acetic acid, etc. (Bashan *et al.*, 2014; Viscardi *et al.*, 2016). So far a few studies have been conducted to evaluate humic acid application effects on soil microbial activity (Filip *et al.*, 2004; Lizarazo *et al.*, 2005; Kalaichelvi *et al.*, 2006; Muscolo *et al.*, 2007), however, contradictory results are reported from most of these studies. Additionally, there are inconsistent references found for dose applications of humic acid to agricultural soils. The lack of understanding for humic acid effects on soil microbial biomass as well as its appropriate level of application to improve soil productivity demands a holistic consideration. It is very important to evaluate the effect of humic acid on microbial communities in the light of sustainable goals to improve or maintain the soil quality and biodiversity (García-Orenes *et al.*, 2013). The present study was conducted to investigate the effects of humic acid application at an optimum level on the size and activity of soil microbial biomass in relation to improve soil productivity on long term basis.

Materials and methods

Soils

Two alkaline soils were collected from two different locations in Pakistan with varying soil physical and chemical properties.

The sandy loam soil was collected from University Research Farm, Rawalpindi (referred as Rawalpindi soil), and the sandy clay loam soil was collected from University Research Farm, Koont (referred as Koont soil). The field moist soils were brought to the laboratory, hand-picked to remove stones, soil animals etc., passed through a 2-mm sieve, mixed thoroughly and frozen at -15°C till the start of the experiment. A subsample of each soil was taken, air dried and mixed for the analysis of important physical and chemical soil properties, while the microbial parameters were measured in the field moist soil samples.

The Rawalpindi soil (sandy loam) was developed from sandstone while the Koont soil (sandy clay loam) was developed from the loess parent materials (Table 1). The Koont soil had higher sand, but lower contents of silt than the Rawalpindi soil, however both the soils had similar clay content. The Koont soil was also low in available P (< 3µg P g^{-1} soil), total N and organic C than the Rawalpindi soil, though both the soils had a similar pH and were alkaline in nature. All microbial parameters i.e., microbial biomass C, microbial biomass N, microbial biomass P, dehydrogenase and alkaline phosphatase activities were higher in the Rawalpindi soil as compared to the Koont soil.

Table 1. Physical and chemical properties of soils.

Property	Rawalpindi soil	Koont soil
Sand (%)	44.5	64.5
Silt (%)	32.5	15.0
Clay (%)	23.0	20.5
Texture	Loam	Sandy Clay Loam
pH $_{1:2.5}$	7.9	8.2
EC $_{1:2.5}$ (dS m^{-1})	0.13	0.25
Organic C (mg g^{-1})	5.8	3.8
Total N (mg g^{-1})	0.5	0.3
Olsen P (µg g^{-1})	3.6	2.0
Microbial biomass C (µg g^{-1})	130.7	89.7
Microbial biomass N (µg g^{-1})	11.3	6.9
Microbial biomass P (µg g^{-1})	3.2	1.7
Dehydrogenase activity [TPF(µg g^{-1} dwt 24 h^{-1})]	8.9	4.8
Alkaline phosphatase activity [*p*-nitrophenol (µg g^{-1} dwt 24 h^{-1})]	45.7	20.1
C/N ratio	11.6	12.7

Lignitic humic acid

The lignitic humic acid used in the study was slightly acidic in nature (pH 6.0), had high contents of organic C (57.6%), and total N (4.2%). The Olsen P contents in the humic acid were 51.2mg kg^{-1}, while the extractable Fe, Cu, Mn and Zn contents were 6.5, 5.5, 14.1 and 6.5mg kg^{-1}, respectively.

Experimental set up

The soil samples kept in the freezer were taken out, equilibrated to room temperature and the moisture contents were adjusted to 50% soil water holding capacity (WHC). The moist soil was then added to 2.0 l capacity incubation jars at the rate of 600g jar^{-1} (on oven dry weight basis) and incubated at 25°C for 7 days (pre-incubation). After pre-incubation, the humic acid was applied at 0 (control), 2µg g^{-1} soil, 4µg g^{-1} soil, 8µg g^{-1} soil and 12µg g^{-1} soil and the jars were incubated for a period of 56 days at aforementioned conditions. CO_2-C evolution from each jar was measured after day 1, 2, 3, 5, 7, 10, 14 and thereafter weekly. The soil samples were collected from each incubation jar at day 0 (immediately after treatment application), 14, 28, 42 and 56 and analyzed for microbial biomass C, microbial biomass N, microbial biomass P, dehydrogenase activity and alkaline phosphatase activity.

Soil analysis

Soils were analyzed for important physical and chemical properties following the standard methods. Particle size analysis was carried out by Hydrometer method (Gee and Bauder, 1986). Water holding capacity (WHC) was determined by preparing saturated soil paste and measuring water contents gravimetrically in the saturated soil after surplus water removal (Anderson and Ingram, 1993). Electrical conductivity of the soil saturation extract was recorded by conductivity meter (Rhoades, 1982) and the pH was measured in 1: 2.5 soil: water suspension with a standardized pH meter (McLean, 1982). Total organic C was determined by dichromate digestion method (Nelson and Sommers, 1982), total N was determined by *Kjeldahl* method (Bremner and Mulvaney, 1982), and Olsen P was determined in 0.5M $NaHCO_3$ (pH 8.5) soil extract using a spectrophotometer (Olsen and Sommers, 1982), For microbial analyses, the field moist soil samples stored at–15 °C were taken out of the freezer and equilibrated to room temperature.

Microbial biomass C and N were estimated by the fumigation-extraction method using 0.5M K_2SO_4 as extractant (Brookes *et al.*, 1984; Vance *et al.*, 1987). Microbial biomass C was calculated as E_C / k_{EC}, where E_C = (organic C extracted from fumigated soils) - (organic C extracted from non-fumigated soils) and k_{EC} = 0.45 (Wu *et al.*, 1990). Microbial biomass N was calculated as E_N / k_{EN}, where E_N = (total N extracted from fumigated soils) - (total N extracted from non-fumigated soils) and k_{EN} = 0.54 (Brookes *et al.*, 1984). Soil microbial biomass P was also measured by the fumigation-extraction method using 0.5M $NaHCO_3$ (pH 8.5) as extractant (Brookes *et al.*, 1982), as described by Joergensen *et al.* (1995). Microbial biomass P was calculated as E_P / k_{EP} / recovery, where E_P = (PO_4-P extracted from fumigated soil) - (PO_4-P extracted from non-fumigated soil) and k_{EP}= 0.40 (Brookes *et al.*, 1982). Dehydrogenase activity was measured by the reduction of 2,3,5-triphenyltetrazolium chloride in triphenylformazan (TPF) (Alef *et al.*, 1995) and alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as the substrate (Alef *et al.*, 1995; Nannipieri *et al.*, 2011). CO_2-C evolved was trapped in 1.0 M NaOH, and measured by titration against standard HCl using phenolphthalein indicator (Anderson, 1982).

Analysis of Humic Acid

The pH and EC of the humic acid was measured in a 1:2 solid/water ratio (Peters *et al.*, 2003). Total organic C was determined by the Walkley-Black method (Nelson and Sommers, 1982). For the determination of total N and total P, the humic acid was digestedin1.2:1 H_2SO_4/H_2O_2 mixture at 360 °C, and the total N and P in the digest were measured colorimetrically (Anderson and Ingram, 1993). Zinc, copper, iron and manganese contents in the digests were determined using atomic absorption spectrophotometer (GBC-932 plus).

Statistical analysis

The data obtained were analyzed statistically using Fisher's analysis of variance technique and the treatment means were compared by Tukey HSD Test at 5% level of significance (Steel *et al.,* 1997). Cumulative CO_2 release was analyzed by one-way ANOVA, while the microbial biomass C, microbial biomass N, microbial biomass P, dehydrogenase activity and alkaline phosphatase activity were analyzed by three-way ANOVA considering soils, humic acid levels and sampling days as the factors. Software "Statstix 8.1" was employed for statistical analysis Russell and Eisensmith (1983).

Results

Effect on soil microbial biomass

Application of humic acid significantly promoted all the soil microbial biomass parameters as compared to control. The maximum increase was observed in the treatment receiving humic acid at 8µg g^{-1} soil, followed by 12µg g^{-1} soil humic acid treatment, which were significantly higher than other humic acid levels. However, Microbial biomass C, microbial biomass N and microbial biomass P contents varied significantly (p< 0.0001) in the two soils (Table 2), being 1.5, 1.4 and 1.8 folds higher in the Rawalpindi soil than the Koont soil. The highest values of the above microbial parameters in both soils were measured at 14th day of incubation which declined significantly afterwards up to day 56 of incubation. This decline in microbial parameters was quite rapid during 14 to 28 days of the study period and slowed down later to become almost stable by the end of study (Fig. 1-6). Overall, the decline in microbial parameters was relatively more in Rawalpindi soil than the Koont soil.

Table 2. Properties of lignitic coal derived humic acid.

Property	Unit	Value
pH		6.1
Organic C	%	57.6
Total N	%	4.2
Olsen P	mg kg^{-1}	51.2
Zn	mg kg^{-1}	6.5
Mn	mg kg^{-1}	14.4
Fe	mg kg^{-1}	6.5
Cu	mg kg^{-1}	5.5

The ratios of microbial biomass C/ N and microbial biomass C/ P were significantly higher in the Rawalpindi soil as compared to Koont soil. Application of humic acid significantly reduced these ratios in both the soils. The maximum decline occurred with humic acid application at 8µg g^{-1} soil, followed by 12µg g^{-1} soil and 4µg g^{-1} soilhumic acid

treatments which were statistically similar to each other. The lowest microbial biomass C/N and C/ P ratios were observed at day 14, which then increased gradually until the end of 56-day incubation.

Effect on enzymatic activities

The results showed that humic acid treatments had significant effects on soil enzymatic reactions as compare to control. The activities of soil enzymes (dehydrogenase and alkaline phosphatase) were significantly ($p < 0.0001$) different in the two soils (Table 3). In the Rawalpindi soil, the dehydrogenase activity was 24% higher, and the alkaline phosphatase activity was 57% higher than in the Koont soil. Humic acid addition resulted in a significant 21% to 78% increase in dehydrogenase activity and 31% to 112% increase in alkaline phosphatase activity over the un-amended control. In both the cases, humic acid applied at 8µg g^{-1} soil showed maximum increase in enzymes activities, followed by 12µg g^{-1} soil humic acid and 4µg g^{-1} soil humic acid application rates. The highest dehydrogenase activity was noticeably affected on the 0-day soil samples i.e., soil samples taken immediately after humic acid application, and showed a consistent decline thereafter throughout the incubation period. Contrary to dehydrogenase activity, the alkaline phosphatase activity increased during the first 14 days of incubation and thereafter declined till the end of incubation.

Table 3. Main effects of soils and humic acid application on microbial biomass indices of soils during 56 days of incubation.

Main Effects	Microbial biomass C (µg g^{-1} soil)		Microbial biomass N (µg g^{-1} soil)		Microbial biomass P (µg g^{-1} soil)		Microbial biomass C/N		Microbial biomass C/P	
Soils (S)										
Rawalpindi (S1)	151.2	A	13.6	A	4.12	A	11.3	A	44.9	A
Koont (S2)	102.4	B	9.4	B	2.308	B	11.1	B	37.6	B
LSD	0.723		0.075		0.038		0.073		0.39	
Humic acid levels (HA)										
Control (T1)	111.8	D	8.9	E	2.5	E	12.7	A	46.1	A
2 µg g^{-1} soil (T2)	116.2	C	9.9	D	2.7	D	11.8	B	43.8	B
4 µg g^{-1} soil (T3)	128.1	B	11.9	C	3.3	C	10.7	C	39.8	C
8 µg g^{-1} soil (T4)	139.6	A	13.8	A	3.9	A	10.1	D	37.2	D
12 µg g^{-1} soil (T5)	138.1	A	12.9	B	3.6	B	10.7	C	39.5	C
LSD	1.603		0.166		0.083		0.163		0.876	
Sampling days (D)										
Day 0	121.8	D	12.3	B	3.1	C	10.1	E	42.5	A
Day 14	135.9	A	12.7	A	3.7	A	10.1	D	38.7	C
Day 28	129.3	B	11.7	C	3.3	B	11.2	C	40.7	B
Day 42	124.9	C	10.6	D	3.1	C	11.8	B	41.5	B
Day 56	121.9	D	10.2	E	2.9	D	12.1	A	42.9	A
LSD	1.602		0.166		0.083		0.163		0.876	
Analysis of Variance										
	p-value		*p*-value		*p*-value		*p*-value		*p*-value	
S	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
HA Levels	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
D	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
S × HA	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
S × D	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
HA × D	<0.0001		<0.0001		<0.0001		<0.0001		0.5131	
S × HA × D	<0.0001		<0.0001		<0.0001		0.0027		0.3093	
CV (±%)	1.99		2.06		3.90		2.21		3.00	

*Data are mean of three repeats. Means with different letters indicate significant difference at $P< 0.05$ according to Tukey HSD Test.

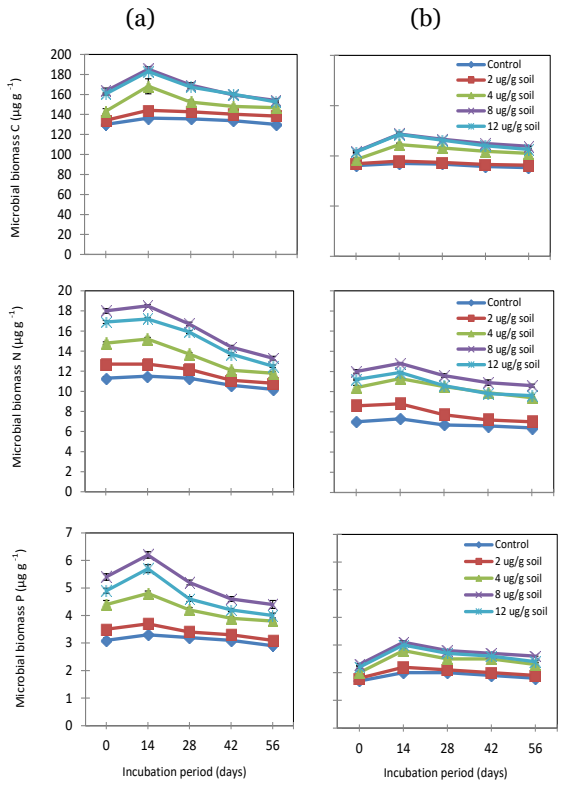

Fig. 1. Microbial biomass C, N and P in (a) Rawalpindi and (b) Koont soils as affected by lignitic humic acid application during 56 days of incubation.

(a)
(b)
Microbial biomass C (µg g -1)
Microbial biomass N(µg g -1)
Microbial biomass P (µg g -1)
Control
2 ug/g soil
4 ug/g soil
8 ug/g soil
12 ug/g soil
Incubation period (days)

Fig. 1. Microbial biomass C, N and P in (a) Rawalpindi and (b) Koont soils as affected by lignitic humic acid application during 56 days of incubation.

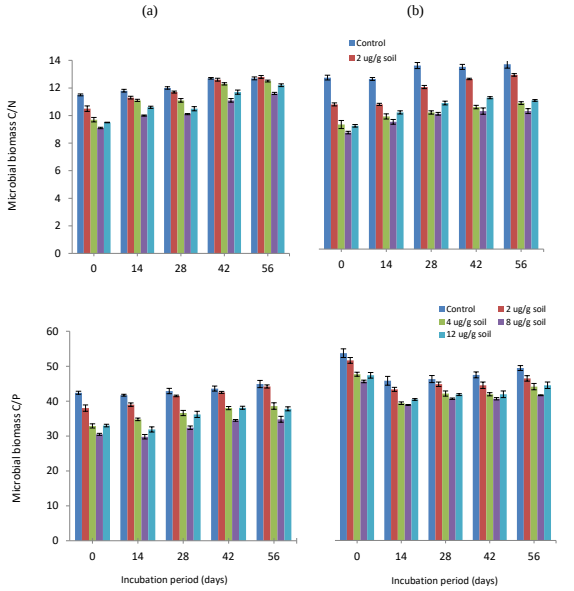

Fig. 2. Ratios of microbial biomass C/N and C/P in (a) Rawalpindi and (b) Koont soils as affected by lignitic humic acid application during 56 days of incubation.

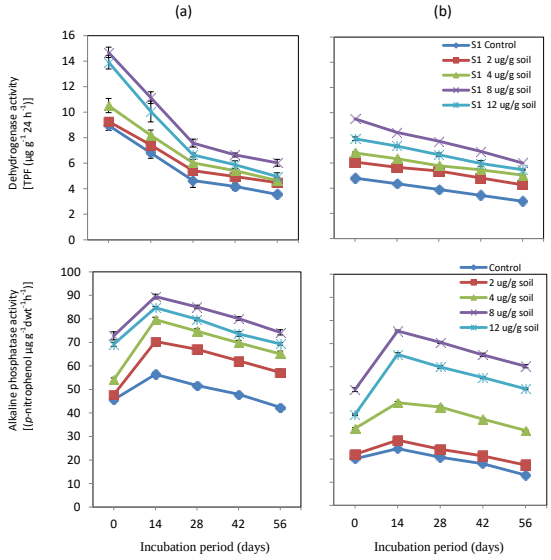

Fig. 3. Dehydrogenase and alkaline phosphatase activities in (a) Rawalpindi and (b) Koont soils as affected by lignitic humic acid application during 56 days of incubation.

The ratio of dehydrogenase/microbial biomass C was significantly lower in the Rawalpindi soil, while the ratio of alkaline phosphatase/microbial biomass C was significantly higher in the Rawalpindi soil as compared to Koont soil. Humic acid application significantly increased dehydrogenase/microbial

biomass C (18.6% to 44%) and alkaline phosphatase/ microbial biomass C (28% to 82%) ratios in both the soils, maximum at 8µg g^{-1} soilhumic acid application rate. The maximum value of dehydrogenase/ microbial biomass C was observed at day 0 of incubation, which decreased later throughout the incubation period (Fig.5). Unlike dehydrogenase/ microbial biomass C, the ratio of alkaline phosphatase/microbial biomass C ratio increased significantly from 0 to 15 days of incubation, but thereafter declined till the end of incubation.

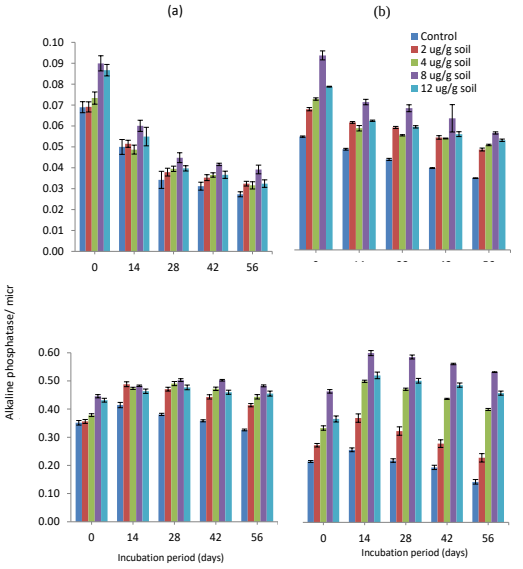

Fig. 4. Dehydrogenase/ microbial biomass C and alkaline phosphatase/microbial biomass C ratios in (a) Rawalpindi and (b) Koont soils as affected by lignitic humic acid application during 56 days of incubation.

Effect on CO_2-C evolution

The mean CO_2-C evolution rate from the soils, expressed as µg CO_2-C g^{-1} soil day^{-1}, was significantly 2.6-fold higher in the Rawalpindi soil than the Koont soil (Table 4). Addition of humic acid increased mean CO_2-C evolution from 16% to 73% in both the soils as compared to control, but maximum at humic acid application rate of 8 µg g^{-1} soil. The cumulativeCO_2-C evolution during 56 days of incubation period was also significantly (1.9-fold) higher from the Rawalpindi soil as compared to that from the Koont soil (Table 5). There was 9% to 39% increase in cumulative CO_2-C evolution from the soils at different levels of humic acid application, again being maximum at humic acid application rate of 8 µg g^{-1} soil (Fig. 6).

Table 4. Main effects of soils and humic acid application on dehydrogenase (DHA), alkaline phosphatase (APA) activities and dehydrogenase/ microbial biomass C, and alkaline phosphatse/ microbial biomass C ratios in the soils during 56 days of incubation.

Main Effects	DHA [TPF (µg g^{-1} 24 h^{-1})]		APA [p-nitro-phenol (µg g-1 dwt h^{-1})]		DHA / Microbial C		APA / Microbial C
Soils (S)							
Rawalpindi (S1)	7.3	A	66.8	A	0.05	B	0.44
Koont (S2)	5.9	B	42.5	B	0.06	A	0.40
LSD	0.173		0.757		0.001		0.01
Humic acid levels (HA)							
Control (T1)	4.8	E	34.1	E	0.04	D	0.29
2 µg g-1 soil (T2)	5.8	D	44.6	D	0.05	C	0.37
4 µg g-1 soil (T3)	6.4	C	57.6	C	0.05	C	0.45
8 µg g-1 soil (T4)	8.5	A	72.3	A	0.06	A	0.53
12 µg g-1 soil(T5)	7.5	B	64.7	B	0.06	B	0.47
LSD	0.371		1.675		0.002		0.013
Sampling days (D)							
Day 0	9.3	A	45.8	E	0.08	A	0.37
Day 14	7.6	B	63.9	A	0.06	B	0.47
Day 28	5.9	C	59.3	B	0.05	C	0.45
Day 42	5.4	D	54.6	C	0.04	D	0.43
Day 56	4.7	E	49.6	D	0.04	E	0.39
LSD	0.371		1.675		0.002		0.0133
Analysis of Variance							
	p-value		*p*-value		*p*-value		*p*-value
S	<0.0001		<0.0001		<0.0001		<0.0001
HA	<0.0001		<0.0001		<0.0001		<0.0001
D	<0.0001		<0.0001		<0.0001		<0.0001
S × HA	0.0359		0.0005		<0.0001		<0.0001
S × D	<0.0001		0.9926		<0.0001		<0.0001
HA × D	<0.0001		<0.0001		<0.0001		<0.0001
S × HA × D	<0.0001		<0.0001		<0.0001		<0.0001
CV (±%)	7.88		4.28		6.19		4.43

Data are mean of three repeats. Means with different letters indicate significant difference at *P*< 0.05 according to Tukey HSD Test.

Table 5. Main effects of soils and humic acid application on rate and cumulative CO_2-C evolution from soil during 56 days of incubation.

Main Effects	CO_2-C evolution rate (µg CO_2-C g^{-1} soil day^{-1})		∑ CO_2-C evolution (µg CO_2-C g^{-1} soil)	
Soils (S)				
Rawalpindi	7.5	A	419.7	A
Koont	4.9	B	274.1	B
LSD	0.035		1.6146	
Humic acid levels (HA)				
Control	4.4	E	207.5	E
2 µg g^{-1} soil	5.1	D	226.5	D
4 µg g^{-1} soil	6.3	C	253.5	C
8 µg g^{-1} soil	7.6	A	287.9	A
12 µg g^{-1} soil	7.5	B	280.8	B
LSD	0.0800		3.6725	
Analysis of Variance				
	p-value		*p*-value	
S	<0.0001		<0.0001	
HA	<0.0001		<0.0001	
S x HA	<0.0001		<0.0001	
CV (±%)	0.74		0.84	

Means with different letters indicate significant difference at *P*< 0.05 according to Tukey HSD Test.

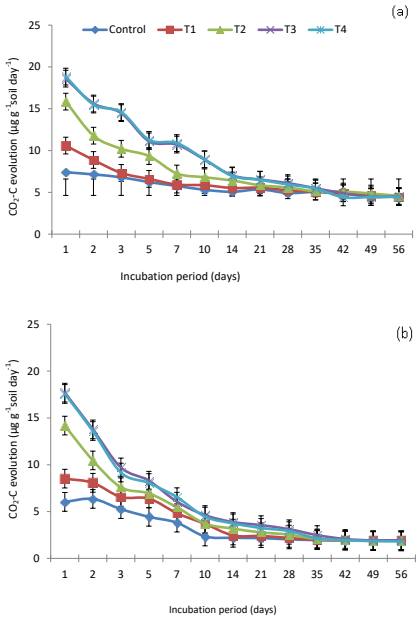

Fig. 5. CO_2-C evolution from (a) Rawalpindi and (b) Koont soils as affected by lignitic humic acid application during 56 days of incubation.

Discussion

This investigation concluded that addition of humic acid significantly improved the microbial biomass and enzymatic reactions. However, the response of the Rwalpindi soil to humic acid amendment was remarkably higher than koont soil in relation to all the soil microbial biomass parameters i.e., microbial biomass C, biomass N and biomass P. This attribute can of Rawalpindi soil cam be because of higher contents of organic C, total N and available P in the Rawalpindi soil (Tajeda *et al.*, 2006; Malik *et al.*, 2013) as compare to Koont soil. Humic acid application significantly increased soil microbial biomass C. This indicates that humic acid stimulates the growth of microbial populations present in the soils (Muter *et al.*, 2015). The above observations are in line with the findings of Turgay *et al.* (2011) and Tavares & Nahas (2014), who reported increase in microbial biomss C by the addition of different organic materials, composts and humic substances to soils. However, no effects of humic acid on soil microbial biomass were observed by Little *et al.* (2014). In the present study, microbial biomass C increased in the soils during first 14 days after humic acid application. Since both the soils had low organic matter contents, thus microbial populations in the soils were C restricted (Demoling *et al.*, 2007). Therefore, the increase in microbial biomass C can be attributed to the addition of a C source promoted the microbial populations in the soils. The decline in soil microbial biomass observed at later stages of incubation might be due to exhaustion of available carbon compounds with the time (Trevisan *et al.*, 2010; Malik *et al.*, 2013). Although humic acid is regarded as a highly decomposed and stable fraction of organic matter, yet it appears that the lignitic humic acid contains some organic components that can be utilized by the soil microorganisms as a source of energy.

Microbial biomass nitrogen is important because it controls soil organic nitrogen availability and loss, especially in high input systems (Moore *et al.*, 2000; Dong *et al.*, 2012). Humic acid application significantly increased microbial biomass N in both the soils. The maximum increase in microbial parameters occurred at 8µg g^{-1} soil application rate, which seems to be an optimum level of humic acid application in the soils under study. These findings support the conclusion that there is always an optimum level for humic acid application (Sharif *et al.*, 2002; Haroon *et al.*, 2010). Although no reference exists for microbial biomass, but it has been shown that the excessive applications of humic acid can negatively affect plant growth (Atiyeh *et al.,* 2002), possibly through reduced availability of chelated nutrients (Chen *et al.,* 2004).

The assimilation of soil N by the increasing population of soil microorganisms occurred within first few hours of the humic acid application, and reached at its peak at 14th day of incubation. This immediate increase in microbial biomass N, as observed in case of microbial biomass C as well, might be due to supplementation of C substrate as well as improving N availability to the soil microorganisms by humic acid (Tejada and Gonzalez, 2006). Microbial biomass N exhibited more temporal fluctuations than microbial biomass C. Similar temporal trend of microbial biomass N in comparison to microbial biomass C was reported by Joergensen (1995). In fact, soil microorganisms show great diversity in their N content than in their C content

depending upon their stage of growth, and thus microbial biomass N always showed great variation due to diversity of soil microorganisms.

The increase in microbial biomass P might be ascribed to the growth of microbes which resulted in more assimilation of P into the microbial cells (Gichangi *et al.,* 2009). The temporal trend for variations in microbial biomass P was similar to that observed for microbial biomass C and microbial biomass N. In fact, the peaks of microbial biomass P at 14th day of incubation and its decline thereafter could be the result of microbial P mineralization as reported by others (Kabba *et al*., 2004; Reddy *et al*., 2005; Yang *et al*., 2011; Yang *et al*., 2013). Microbial biomass P is a highly labile source of P for plants because P assimilated in microbial cells is easily mineralized on microbial turn over (Reddy *et al*., 2001; Reddy *et al*., 2005).

Various studies are in agreement that humic acid generates an stimulatory effect on microorganisms (Kirschner *et al*., 1999; Gryndler *et al*., 2005; Tikhonov *et al*., 2010; Kanaparthi and Conrad, 2015) by directly stimulation of biomass growth. This biomass growth occurs due to humic acid influence on biosynthetic activity that regulates the metabolism of a cell (Kirschner *et al*., 1999; Kulikova *et al*., 2005; Tikhonov *et al*., 2010).

The net mineralization or immobilization of nitrogen in soils can be determined primarily by carbon to nitrogen ratios of the added organic source. Soil microorganisms, particularly the bacteria and fungi, differ in their nitrogen requirements (Rashid *et al*., 2016). Thus, microbial biomass C/ N ratio is frequently used to sense changes in the microbial community structure (Khan and Goergensen, 2009). The C/N ratio of microbial biomass in the two soils under study was almost similar. Humic acid application decreased the ratios of biomass C/ N in the soils indicating the role of humic acid in making N available to soil microorganisms. However, the decrease in C/ N ratio of microbial biomass might also be linked to the changes in microbial community structure caused by the humic acid application.

Addition of humic acid resulted in increase of dehydrogenase activity which is considered as a positive effect on soil health as soil enzymes are good markers of soil fertility being the biological catalysts in specific biochemical reactions. The activity of dehydrogenase has been proposed as a measure of the overall microbial activity in soils (Lizarazo *et al*., 2005), and has also been indicated as a good index of soil microbial biomass in semi-arid Mediterranean areas (Garcia *et al*., 1997). Therefore, humic acid application in relation to increased dehydrogenase activity in the soils can be attributed to its stimulation effect on the size and activity of soil microorganisms (Liang *et al*., 2005; Haroon *et al*., 2010). On an average, dehydrogenase activity was 34% higher in Rawalpindi soil than the Koont soil, which again be linked to the variation in organic C and microbial biomass contents in both the soils. The Rawalpindi soil had 2-folds higher organic C than the Koont soil, which resulted in high dehydrogenase activity in the former. It has been reported that incorporation of organic amendments to soil influences soil enzyme activities, because the added material may contain intra- and extra-cellular enzymes and may also stimulate microbial activity in the soil (Pascual *et al*., 2000).

Phosphatase is another important soil enzyme, because it provides P for plant uptake by the mineralization of soil organic P fractions. Being an extracellular hydrolase enzyme, it catalyzes the hydrolysis of phosphate from organic monoesters required by plants and microorganisms to maintain the cellular metabolism (Trevisan *et al.,* 2010; Malik *et al*., 2013). Therefore, phosphatase is considered as an indicator of P mineralization potential of soils (Sinegani and Mahohi, 2009).In addition to dehydrogenase, the activity of alkaline phosphatase also increased in the soils due to humic acid application. The increasing demand for P by the growing microbial populations in the soils might have been responsible for the stimulation and the synthesis of phosphatase enzyme (Garcia *et al*., 1996).

Addition of humic acid increased mean respiration rate as well as the cumulative CO_2-C evolution from both the soils over the un-amended control.

These results are supported by the previous studies where humic acid application has been shown to immediately increase the soil respiration rate due to rapid promotion of the population of heterotrophic soil microorganisms. This happens due to the availability of carbon substrate following the organic amendments addition (Sarir *et al.*, 2006; Yang *et al.*, 2013). The fact that soil microbial biomass and soil respiration were promoted in lignitic humic acid amended soils indicates the ability of soil microbes to utilize some fraction of the organic compounds present in humic acid or in the soils (Cook and Allen, 1992). Additionally, the humic acid also contained essential elements like N, P and some micronutrients, therefore availability of these nutrients might have stimulated microbial populations in the soil (Piccolo, 2002). Curves representing cumulative CO_2–C with time showed that the slope at the outset was higher in the soil amended with humic acid suggesting that with the addition of this amendment, some fraction of the native soil organic carbon was also mineralized. Gilani and Behmanyar (2008) declared the availability of organic C as the most limiting factor for soil respiration. It is important to note that the CO_2 released from the soils may not merely come from the microbial respiration. The soils used in the present study were alkaline and had sufficient quantity of $CaCO_3$, therefore, CO_2 released from action of humic acid on the soil carbonates may also contribute to the CO_2 release. However, the fact that the humic acid application had a similar effect on cumulative CO_2 release and on dehydrogenase activity, which is a measure of soil microbial activity, suggests that most of the CO_2 evolved did come from microbial respiration. It was observed that Soil respiration rates declined over time following cessation of labile carbon (C) inputs which can be attributed as a reduction of the available soil organic matter (SOM) pool as labile inputs diminish. This factor is in an agreement of several studies (Six & Jastrow, 2002; Conant *et al.*, 2011; Schmidt *et al.*, 2011).

Conclusions

Application of humic acid significantly promoted the size and activity of soil microbial biomass, therefore acted as a soil biostimulant. However, the magnitude of increase in microbial biomass corresponded to the contents of native microbial biomass pool in the soils. Addition of 8µg g^{-1} soil humic acid proved to be best recommended application rate to all other humic acid application levels. Temporal trend of soil respiration rate in response to humic acid application not only indicated the effect of humic acid on soil microbial CO_2 evolution but also specifies that most of the carbon utilized by microbes came from the native organic C pool of the soils. Overall humic acid positive effect was recorded on microbial parameters including enzymatic reactions, and thus confirmed its stimulatory effect on microbial biomass. Therefore, humic acid application can be considered as an effective biotechnological tool for promotion of plant growth by developing a sustainable agriculture system.

Acknowledgements

Lubna Ayub Durrani thanks Higher Education Commission of Pakistan for the award of indigenous fellowship and research grant. We thank the technical staff of Central Research Laboratory, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi for help in analysis of soil samples.

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