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Characterization of commercial humic acid samples and their impact on growth of fungi and plants

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Abstract

Naturally occurring humates like leonardite and brown coal or lignite are marketed under different brand names e.g. Pak Humates, Humate Fertilizer, Pak Humax, Humkara and Humide etc. However, their efficacy is needed to be confirmed before their use. Different studies were conducted for the comparison of four commercial humates for their physico-chemical, optical properties, plant growth promoting ability in terms of seed germination and seedling vigour in wheat (cv Sehr), mung bean (Mung-54), maize (C-12) and sesbania and their effect on growth of some fungi. Moisture content of four humates varied from 0.52 to 71.11%, while solubility in water varied from 30.2 to 98.2% and density differed from 1.67 to 4.17. A 2% solution of humates had pH and EC varying from 5.39 to 10.11 and 3.140 to 1.143 mS cm^{-1}, respectively. Carbon and nitrogen concentrations varied from 22.95 to 36.56% and 0.658 to 1.183, respectively with a C/N ratio of 30.91 to 44.16. Humates dissolved in 0.1N NaOH were partitioned into humic acid and fulvic acid fractions. Of the total C in humates, 40.3 to 77.5% was ranged in humic acid and 22.5 to 59.7% in fulvic acid fraction. The HA was also studied for optical properties at 400, 500, 600, and 700 nm besides that at 465 and 665 to calculate E4/E6 (extinction coefficient); the later varied between 3.64 and 5.48. Optical density of the humic acid decreased at increasing wavelength and was correlated significantly with the carbon contents of humic compounds. Three fungi, Trichoderma harzianum, T. hamatum and Alternaria alternata showed maximum growth at 0.025% HA in the growth medium on the basis of colony diameter. Humates inhibited seed germination in wheat, maize and mung bean except for sesbania. Root length and shoot dry matter increased in wheat and maize but no effect was found in mung bean and sesbania. The studies revealed that humates available in the market vary widely and therefore some sort of quality monitoring is required for the benefit of end users.

Keywords: *Humic acid, optical density, Fungi, plant growth*

Introduction

Fertility of nutrient deficient soils can be improved either through fertilizer inputs or by organic residue amendments. Soil microbes especially the fungi play a key role in the release of plant-available nutrients through organic matter decomposition and transformations of the organic carbon compounds crucial to soil fertility and productivity. Among different microbial degradation products, humic compounds occupy a key position because of their multifarious roles in maintaining/improving soil fertility and positively affecting physiological functions (both of soil biota as well as plants). Plenty of information is available on the beneficial effect of organic matter and especially humic compounds in the soil-plant system (Azam and Malik, 1983; Malik and Azam, 1985; Piccolo *et al*., 1993; Nardi *et al.,* 2002; Arancon *et al*., 2006; Campitelli *et al.,* 2006; Steinberg *et al*., 2008; Khaled and Fawy, 2011).

The humic compounds or humates are synthesized through microbial activities (Felbeck, 1971). However, these have also resulted from physical changes over millions of years in the plant biomass buried in the deeper soil layers and are found in abundance in different parts of the world as brown coal or lignite. In Pakistan, Thar area is claimed to have an estimated lignite reserves of >170 billion tons (Anonymous, 2012). These geological humates have been found of immense use in agriculture over the past few decades and multiplicity of formulations are found in the market as pure humates or products therefrom (Anonymous, 2011). However, in view of the diversity of the plants from which these humates would have originated, their chemistry and physiological functioning is deemed to vary. It is pertinent therefore to evaluate the marketed products for their effectivity in agriculture. A potentially good avenue of research that remains relatively un-explored is the use of humates in improving microbial growth/activity and functions vis-à-vis decomposition/

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transformation of organic matter in soil and during the process of composting.

In Pakistan, soils are very low in organic matter. It is reported that soil carbon ranged from 0.52 to 1.38% in different soil series. Most of them have less than 1% (Azam *et al.*, 2001), of which about 10% occurs in alkali soluble humic fractions (humates). Soils in Pakistan are generally low in organic matter (<1%) firstly because of low returns and secondly because of the rapid decomposition of any plant residues that are left in/on soil or applied as organic amendment. Green manuring is a good option to augment soil organic matter and to improve soil biological activity under some cropping systems like rice-wheat rotation (Shah *et al*., 2011) but organic manures still remain a good option if available in sufficient quantities (Tahir *et al.,* 2011). In floriculture, however, organic manures are the major option for improving and maintaining soil fertility and biological activity. These manures are generally available as farmyard manure or composts prepared from leafy plant residues. Good quality and mature compost is generally obtained in 8-12 weeks (Misra and Roy 2003). However, the composting period can be reduced to even 2 weeks through interventions like microbial inoculations. The process can further be enhanced through improvement in microbial growth and proliferation. In general this has been achieved through nutrient supplementation but hardly any emphasis has been given to the role of humates in affecting microbial growth particularly the fungi that play a major role in organic matter decomposition, composting, and humification (Ahmad *et al*., 2009; Sohail *et al*., 2011). Some studies suggest, however, that humic substances increase the growth rate of many forms of beneficial microorganisms (Pouneva, 2005; Burkowska and Donderski, 2007). Chen and Wang (2008) proposed that humic substances assist in the nutrition of microorganisms by complexing and delivering trace elements like iron to microbial cell surfaces.

Humates of different venders are being sold in the commercial market. Studies were carried out to compare four different humates for their Physico-chemical properties (pH, electrical conductivity of aqueous solution, solubility in water and density), optical properties including extinction coefficient, carbon and nitrogen content, effect on growth of four fungal species, and effect on seed germination and seedling growth of wheat, maize, mung bean and sesbania.

Materials and Methods

Quantification and optical properties of humic acid

In general, the so-called humic acid available in the market is alkali treated lignite and thus contains both humic acid and fulvic acid fractions. Therefore the term humates used in this presentation refers to both. Four samples of humic acid (humates) were collected from the market and designated as A, B, C and D. Known volume (50 mL) of humates was weighed and density was calculated as W/V. For studying relative solubility of four humates, 10 g samples in triplicate were dissolved in 500 mL of deionized water by shaking for 60 minutes on a rotary shaker. The contents were centrifuged for 30 minutes at 4000 rpm and the residue was washed twice with 50-mL deionized water. The original supernatant and that from washings were collectively subjected to pH and electrical conductivity measurements.

For studying optical properties and C and N content of humic acid fraction, 2% solution of humates was prepared in 0.1 N NaOH solutions by shaking for one hour on a rotary shaker followed by centrifugation for 30 minutes at 4000 rpm. A portion of the supernatant (containing both humic acid and fulvic acid fractions) was acidified to pH 1.5-2.0 with concentrated sulfuric acid and heated at 90^{o}C for 30 minutes for precipitating humic acid fraction followed by cooling to room temperature and centrifugation (Kononova, 1961). The residue (humic acid fraction) was re-dissolved in 0.1 N NaOH. An aliquot of humic acid solution and the supernatant from which humic acid was obtained was subjected to carbon and nitrogen determination using wet digestion/colorimetric (Rhiem and Ulrich, 1954) and micro-Kjeldahl (Bremner and Mulvaney, 1982) methods, respectively. The humic acid solution was diluted 50 times for determining optical density at 400, 465, 500, 600, 665 and 700 and the extinction co-efficient (E465/E665) was calculated (Kononova, 1961).

Effect of humates on colony growth of some fungi

Four species of fungi namely *Alternaria alternnata*, *A. citri*, *Trichoderma harzianum*, and *T. hamatum* were isolated from composted materials, sub-cultured on malt extract agar medium in test tubes and preserved for use. For studies on colony growth as affected by humic acid, fungi were inoculated on malt extract agar plates and a 5 mm disc cut from the actively growing colony margins used as inoculum. Humic acid (A) was added to malt extract agar medium at 0.015, 0.025, 0.040 and 0.05% and pH of the medium adjusted to 5.6 because the humic acid (A) showed the best physiocochemical properties, so only Humic acid A was tested as carbon source in growth medium of fungi.

The medium was poured into Petri plates and allowed to solidify after which inoculum discs were placed upside down on the plated growth medium. Colony diameter was measured seven days after incubating the inoculated plates at 30°C.

Effect of humates on seed germination and seedling growth of some plants

Two hundred gram portions of acid washed sand was taken in Petri plates (4 inch diameter) and moistened to 25% with water containing 0, 0.015, 0.025 and 0.035% of 4 types of humates. Before addition to sand, the humic acid solutions were brought to pH 7 by using dilute H_2SO_4. Ten seeds each of wheat (cv Sehr), mung bean (Mung-54), maize(C-12) and sesbania were planted per plate; triplicate plates were used for each humate treatment. Data on seed germination was recorded. Seedlings were removed from plates after ten days of growth and data on root length, shoot length and shoot weight were recorded.

Statistical Analysis

Data were subjected to Analysis of Variance (Steel & Torrie, 1980) and significance of mean values at 5% significance level was tested by Duncan's Multiple Range test (Duncan, 1955) by programme MSTAT.

Results and Discussion

Four samples of commercially available humic acid (humates) varied widely in all characteristics (Table 1). Physical appearance of the humates varied from fine crystalline to thick paste (Figure 1) with moisture content of 0.52 to 71.1%; the humate C showed the highest moisture percentage due to its paste- like consistency. Solubility of humic acid samples in water varied from 30 to 98% while colour of the aqueous solution varied from light to medium brown (Table 1, Figure 2). In general, the humates marketed abroad or imported are claimed to be 100% soluble (Patil *et al*., 2011). Apparently no quality control measures are being adopted for synthesis of humates in Pakistan where the general practice is to treat raw lignite with 8-10% NaOH or KOH and the resultant mixture dried, packaged and marketed as humic acid. Therefore, the product will contain different proportions of mineral matter which may or may not be properly removed from the so-called humic acid product. This contention is also supported by the variation in density (1.67 and 4.17) which is caused mainly by the mineral matter content of the feedstock i.e. lignite which is variable in origin.

The pH of humic acid solutions varied from 5.39 to 10.11 while EC was 1.14 to 3.14 mS cm^{-1} (Table 1). There are very strong differences between the four tested humic acid products. The humic acid "A" showed its physico-chemical properties (Solubility, pH, EC and HA-C) better over the other three and it was the main reason to study its effect on growth of fungi. Generally it is assumed and claimed that the pH of the humic solution will be near neutral. Carbon concentration of humates (humic + fulvic acid) was 22.95 to 36.56%, while N concentration was 0.658 to 1.183% with the resultant C/N ratios of 30.90 to 44.14 (Table 2). Humate D showed lowest value for N concentration. These are relatively strange values as most of the reports show C and N concentration of humic acid to be around 50% and >3%, respectively, yielding a C/N ratio of 10-15 (Campitelli *et al*., 2006; Fasurova and Pospisilova 2011). Kucerik *et al.* (2008), however, reported C concentration of 9 humic acid samples from lignite to be around 57% and N concentration of around 1.3% with a resultant C/N of around 40. Previously, Kučerík and Klucakova (2003) reported 73% C and 1% N in lignite i.e. the source of most commercial humates.

Partitioning of humate samples into humic acid (HA) and fulvic acid (FA) fractions (C content was taken as the measure) after dissolving in 0.1N NaOH showed that out of total Carbon, 40.3 to 77.5% was attributable to humic acid and 22.5 to 59.7% in fulvic acid fraction with a HA/FA ratio of 0.68 to 3.45 (Table 2). The lowest ratio of HA/FA was recorded for humate - C and the highest for humate - A. Thus the range obtained in the present studies is fairly in line with results reported by Fasurova and Pospisilova (2010) who reported ratio 0.5 to 3.0 for HA/FA. The authors also suggested that a wider ratio is a direct indicator for greater humification, Fasurova and Pospisilova (2011).

Table 1: Some physico-chemical characteristics of humates A, B, C, and D

Characteristics	Humate A	Humate B	Humate C	Humate D
Physical appearance*	Coarse crystals	Fine crystals	Thick paste	Fine crystals
Moisture content, %	7.31b***	2.13c	71.11a	0.52d
Solubility in water, %	83.0b	61.8c	98.2a	30.2d
Colour of aqueous solution**	Medium brown	Medium brown	Light brown	Medium brown
Density, (g ml^{-1})	1.67d	2b	4.17a	1.79c
Electrical conductivity (EC),mS/cm	1.143a	2.627b	3.140a	2.010c
pH	5.39d	9.49b	6.92c	10.11a

*Please see Figure 1; **Please see Figure 2, ***Figures sharing a similar letter in a row are not significantly different from each other at 5% level of probability according to DMRT

Table 2: Carbon and Nitrogen analysis of humates

Humates	Humate - C	Humate - N	Humate C/N	HA-C	FA-C	HA-C/FA-C
	mg g^{-1}			mg g^{-1}		
A	329.91b*	7.91b	41.72a	255.75a (77.52)**	74.16c (22.48)	3.45a
B	365.60a	11.83a	30.91c	210.89b (57.68)	154.71b (42.32)	1.36b
C	357.54a	8.10b	44.16a	144.19d (40.33)	213.36a (59.67)	0.68c
D	229.46b	6.58c	34.87c	177.66c (77.42)	51.80d (22.58)	3.43a

*Figures in a column sharing a similar letter are not significantly different from each other at 5% level of probability according to DMRT

**Figures in parentheses indicate percent distribution of humates C in humic acid (HA) and fulvic acid (FA) fractions; C. carbon, N, nitrogen

Out of four humates, humate - C had the lowest HA/FA ratio suggesting its lower humification and thus younger nature (Zsolnay, 2003). The optical density of humate samples could be used to differentiate humic acids from fulvic acids. The quantity of unknown humic samples could be determined by taking the absorbance and making its comparison to standards varying in E4/E6 ratios for each type of source material like lignite, peat, organic soils, etc. The ratio E4/E6 of humic samples has inverse relationship with the average molecular weight of humic substances, (Chen *et al.,* 1977). It is substantially higher for fulvic acids (~8+) than humic acids (3-5). Moreover, the higher E4/E6 of any humic acid samples will be known as good indicator for bioactivity.

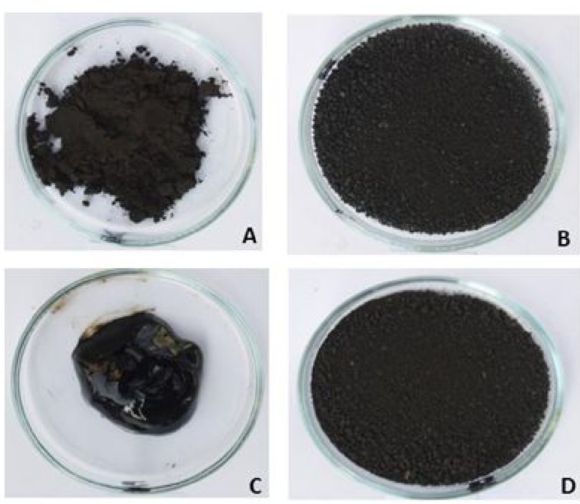

Figure 1: Physical appearance of different humates

Studies on optical properties of humic acid fraction dissolved in 0.1 N NaOH showed a consistent decrease in absorption with increase in wave length from 400 to 700 nm (Figure 3). The humate, designated as C was distinct because of least change in absorbance with increased wavelength. The remaining three humates were almost comparable. Similar observations have been reported by Kučerík and Klucakova (2003) and Fasurova and Pospisilova (2011). Extinction coefficient i.e. E4/E6 (also termed as index of humification) of the humic acid samples studied varied between 3.64 and 5.48 (Figure 4). Minimum value of 3.64 was observed for humate C and maximum (5.48) for humate B. Ratios calculated for other wavelengths showed trends similar to those for E4/E6 with a correlation coefficient of >0.92. Kučerík and Klucakova (2003) reported the value as 3.9 which are similar to those obtained in the present studies. However, Campitelli *et al.* (2006) reported an average E4/E6 of 5.1 for humic acid extracted from 3 soils while a greater variation of 5.2 to 9.1 has been reported by Fasurova and Pospisilova (2011) and is attributed to the relative maturity of the humates; higher the value of E4/E6 suggesting lesser maturity.

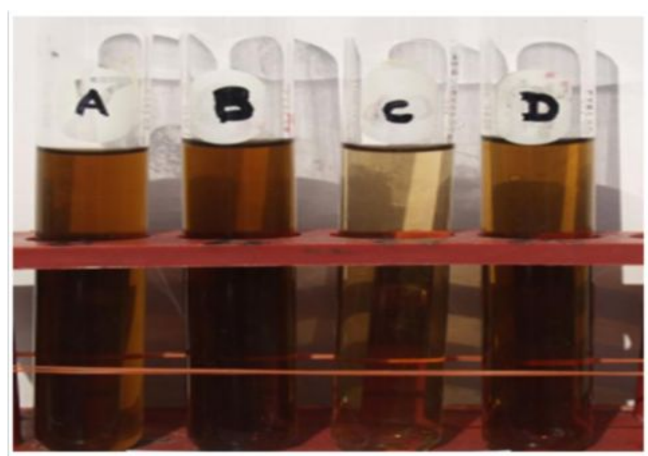

Figure 2: Color variation of 2% aqueous solution of different humates

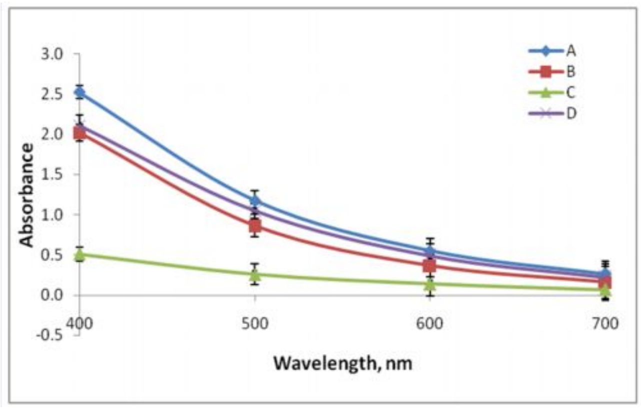

Figure 3: Optical density of humic acid dissolved in 0.1 N NaOH at 400, 500, 600 and 700 nm

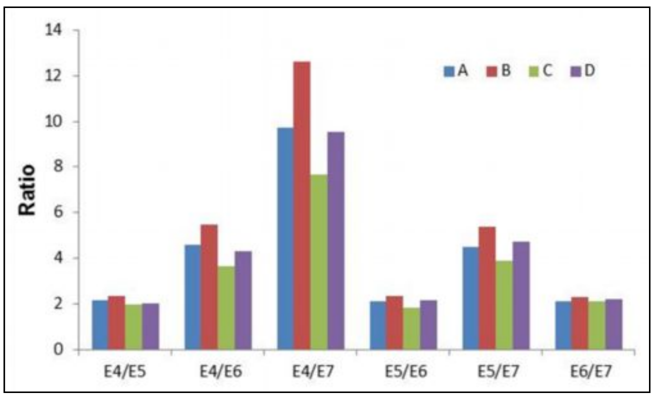

Figure 4: Extinction ratios for wavelength 400, 500, 600, and 700 nm

The cellulose degrading property of *Alternaria* sp. and *Trichoderma harzianum* sp. is well documented and is useful in composting process (Ahmed *et al.,* 2009; Sohail *et al.,* 2011).

Of the four studied fungi, *Trichoderma harzianum*, *Alternaria alternata*, and *A. citrae* showed maximum growth at 0.025% HA in the growth medium; colony diameter was taken as a measure (Figure 5, 6). At higher concentrations the colony diameter (colony growth) was retarded. In *T. hamatum*, however, growth promotion was recorded at 0.05% HA. Interestingly, a species of *Trichoderma* i.e. *T. viridae* is also known to solubilize lignite and release humic compounds (Prakash *et al.,* 2010). Reports available in the literature do such an enhancement of microbial growth at optimum concentrations of humic compounds in the growth medium (Vissera, 1985; Hartung, 1992; Pouneva, 2005; Burkowska and Donderski, 2007). Stimulation of fungal (mycorrhizal) growth has been reported by Gryndler *et al.* (2005). As observed in the present study, growth promotion is observed at optimum levels (0.2%) of humates while higher (>0.5%) amounts are reported to be inhibitory (Prakash *et al.* 2011).

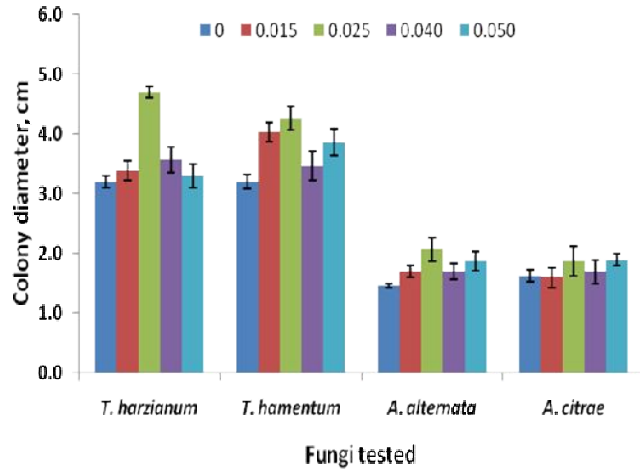

Figure 5: Colony diameter of four fungi grown on agar medium supplemented with 0.00, 0.015, 0.025, 0.040, and 0.050 % humic Acid "A"

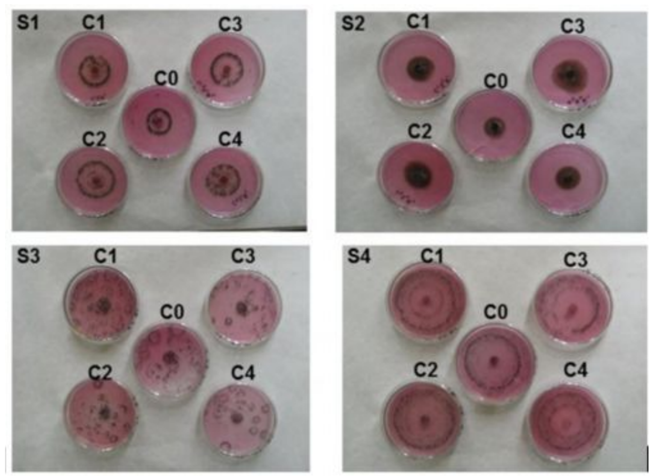

Figure 6: Colony growth of different fungi (S1, *Trichoderma harzianum*; S2, *T. hamatum;* S3, *Alternaria alternata*; S4, *Alternaria citri*) on growth medium supplemented with 0.00, 0.015, 0.025, 0.040 and 0.050% of humate "A" (C0 - C4, respectively)

The response of plant types to humic acid application in the growth medium was variable. Of the four plant types i.e. wheat, maize, mung bean and sesbania, seed germination was improved by humates only in sesbania (4.71-16.7% increase over control) while there was a mixed response in terms of root and shoot growth with no specific trends as regards concentrations of humates in the medium (Table 3). Maximum root and shoot length was recorded in

Table 3: Seed germination and seedling growth in wheat, maize, mung bean and sesbania as affected by supplementation of growth medium with humates

Humate	% seed germination	Shoot length (cm)	Root length (cm)	Shoot dry Weight (mg)
		Wheat		
Control	100.0a**	16.2ab	14.6d	41.0b
A	93.3ab	15.9ab	25.0a	49.1a
B	90.0bc	15.7bc	24.0b	50.9a
C	96.7ab	16.1ab	21.2c	44.7ab
D	96.7ab	16.8a	23.4b	52.2a
Avg*	94.2ab	16.1ab	23.4b	49.2a
		Maize		
Control	100.0a	4.6c	8.2c	210.8b
A	86.7b	6.6a	10.7a	209.4b
B	83.3b	5.7ab	8.9bc	187.2c
C	86.7b	5.5b	9.4b	215.6ab
D	93.3c	6.3a	8.5c	230.9a
Avg.	87.5bc	6.0a	9.4b	210.8b
		Mungbean		
Control	80.0b	11.5a	9.5b	32.1a
A	73.3c	9.6b	11.0a	33.7a
B	76.7c	7.9d	9.6b	28.4b
C	86.7a	8.0d	9.5b	28.8b
D	73.3c	9.7b	9.6b	20.9c
Avg	77.5c	8.8bc	9.9b	28.0b
		Sesbania		
Control	70.0b	4.8b	4.0b	15.3b
A	83.3a	4.8b	3.5c	13.9c
B	63.3c	6.2a	4.4a	17.5a
C	80.0a	3.3d	3.3cd	11.1d
D	73.3b	3.1d	3.1d	15.7b
Avg	75.0b	4.3c	3.5c	14.5bc

*Avg, average of A-D to simplify the comparison with control (Nil) where no humate was added to the growth medium. The values tabulated against A-D are average of 3 observations obtained at 3 levels of humate addition i.e. 0.015, 0.025 and 0.035%

** Figures sharing a similar letter in a row do not differ significantly different from each other at 5% level of probability according to DMRT

wheat and maize respectively, while the minimum increase was found in sesbania. However, maximum shoot weight was found in wheat. Root length and shoot dry matter increased due to humates in wheat and maize but not in mung bean and sesbania. These observations are contrary to many reports where seed germination, seedling vigour and plant productivity are reported to increase substantially due to the application of humates (Azam and Malik, 1983; Malik and Azam, 1985). Patil (2010) reported improvement in seed germination and seedling vigour due to humic acid addition to the rooting medium. However, Piccolo *et al.* (1993) did not observe seed germination enhancement but seedling vigour was improved in *Lactuga sativa and Lycopersicum esculentum*. The variation reported and observed in the present study may be due to the difference in source and chemistry of humates (Piccolo *et al.*, 1993). Apparently, the humates marketed in Pakistan do not undergo proper quality control at the production facility, leading to unpredictable results in the field.

Conclusion

In summary, the results of the present study suggest that the humates available in the market are different in all respects including their impact on microorganisms and plants.

However, these are merely exploratory studies to demonstrate that some sort of quality control of humates is required by the concerned agencies so that their well-documented positive role in agriculture could be realized consistently and on sustainable basis in Pakistan. Merely mentioning the percentage humates on commercial products may not be sufficient. It will also be pertinent to characterize in detail and evaluate humates obtained from different lignite sources of Pakistan.

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