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Co-addition of humic substances and humic acids with urea enhances foliar nitrogen use efficiency in sugarcane (*Saccharum officinarum* L.)

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ABSTRACT

Humic substances (HS) and humic acids (HA) are proven to enhance nutrient uptake and growth in plants. Foliar application of urea combined with HS and HA offers an alternative strategy to increase nitrogen use efficiency (NUE). The objective of this study was to understand the effects of foliar application of HA and HS along with urea on NUE and response of different biometric, biochemical and physiological traits of sugarcane with respect to cultivar, mode of foliar application, geographic location and intervals of foliar application. To study this, two different independent Experiments were conducted in green house facilities at two different agro-climatic zones (USA and Brazil) using two different predominant varieties, modes and intervals of foliar applications. The three different foliar applications used in this study were (1) urea (U), (2) mixture of urea and HS (U+HS) and (3) HA (U+HA). In both Experiments, ^{15}N (nitrogen isotope) recovery or NUE was higher in U+HS followed by U+HA. However, magnitude of NUE changed according to the differences in two Experiments. Results showed that foliar application of U+HS and U+HA was rapidly absorbed and stored in the form of protein and starch. Also induced changes in photosynthesis, intrinsic water use efficiency, protein, total soluble sugars and starch signifying a synergistic effect of U+HS and U+HA on carbon and nitrogen metabolism. These results showed promising use of HS and HA with urea to improve NUE in sugarcane compared to using the urea alone. Simultaneously, mode, quantity, and interval of foliar application should be standardized based on the geographic locations and varieties to optimize the NUE.

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the important C4 crop which is mainly cultivated in the warm and humid tropical and sub-tropical regions (Elsheery et al., 2020). Sugarcane is important crop with versatile use such as sugar and bioenergy (alcohol) production, residues or foliage as manure for alkaline and saline soil, fodder for cattle, and paper production (Weijde et al., 2013). Brazil is the largest producer of sugarcane which covers approximately 41% of the global production followed by India and China (FAO, 2017).

Among the essential plant nutrients, nitrogen (N) is important and required in large quantity for sugarcane for normal growth, leaf expansion, root growth, biomass production and tiller and sucker development (Calcino, 1994; Salter and Bonnett, 2000; Saleem et al., 2012; Otto et al., 2014). Meanwhile, N deficiency results in early flowering, early transition of vegetative to reproductive stage and reduced growth, yield and sugar quality, which directly impact on the economic stability of sugarcane farmers and sugarcane related industries (Innes, 1960; Fageria and Baligar, 2005; Witte, 2011). On the other hand, conditions such as soil pH, sandy soil, low organic matter in soil, drought and high rainfall make the N deficiency more critical for the sugarcane production (Saleem et al.,

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2012) and these conditions results in soil N loss through leaching, de-nitrification, volatilization, surface runoff, and microbial consumption (Fageria and Baligar, 2005; Dawar et al., 2011).

Global requirement of N fertilizer for sugarcane production is approximately 45–300 kg ha^{-1} yr^{-1} and demand for N is higher in plants as it is an essential component of many organic compounds ranging from proteins to nucleic acids (Srivastava and Suarez, 1992; Acquisti et al., 2009; Saleem et al., 2012). Furthermore, N is also a constituent of compounds such as chlorophyll and enzymes involved in biochemical and physiological processes, which in turn helps in growth and development of plants (Fageria and Baligar, 2005; Kingston, 2014). The plants assimilate N mainly in the form of minerals (nitrate [NO^{3-}] and ammonium [NH^{4+}]). At the same time, organic compounds with lower molecular weights (urea, amino acids and peptides) are also assimilated by plants as source of N from soil (Jones and Kielland, 2002; Fageria and Baligar, 2005). However, N fertilizers are produced from ammonia synthesis reaction *via.,* Haber-bosch process, but higher energetic cost to produce ammonia makes N fertilizers expensive. Among N fertilizers, urea is widely used in agriculture (Galloway et al., 2004; Witte, 2011).

Efficient management of N fertilizer application and utilization (minimizing N loss form soil and maximizing N assimilation by plants) can reduce the environmental pollution, greenhouse gas emissions, and fertilizer costs; and more over it can increase the crop growth and yield. Hence, it is crucial to develop new methods to increase nitrogen use efficiency (NUE); and it is estimated that, even a 1% increase in NUE could save 1.1 billion US dollar per annum (Kant et al., 2011; Stuart et al., 2014). To achieve this goal, some strategies have already been proposed and implemented such as reducing rates of N fertilizer application based on the existing soil N and requirement of crops, timing and split application of N fertilizer, finding alternative sources for N fertilizer and coating of N fertilizer granules with different compounds for slow release of N (Fageria and Baligar, 2005; Cantarella et al., 2008; Dawar et al., 2011; Roberts, 2008; Chien et al., 2009).

Another feasible and excellent alternative is the use of humic substance (HS), humic acid (HA), and fulvic acid (FA) along with N fertilizers which have proven records to increase plant growth and assimilation of major plant nutrients such as N, phosphorous (P) and potassium (K). HS and HA are naturally available substances and are effectively used in cultivation of different crops. HS are organic compounds and formed by the chemical and biological humification of plant and animal matter by the biological activities of soil microorganisms. Humin, HA and FA are the major factions of HS which are most complex and biologically active organic matter compounds in the soil. HA is natural acidic organic polymer. HS is identified to fuel the plants and microbial activities through several mechanisms (Canellas and Oliivares, 2014; Klucakova, 2018; Ekin, 2019). In maize (*Zea mays* L.), HS and HA applications changed carbon and nitrogen metabolisms and photosynthetic rate (Canellas et al., 2013). The application of HS and HA showed higher plant growth in several crops such as potato (*Solanum tuberosum* L. Suh et al., 2014; Ekin, 2019), tomato (*Solanum lycopersicum* L.; Olivares et al., 2015), maize (Puglisi et al., 2013; Canellas et al., 2013), hungarian vetch (*Vicia pannonica* L.; Esringu et al., 2016), durum wheat (*Triticum durum* L.; Delfine et al., 2005) and blueberry (*Vaccinium corymbosum*; Schoebitz et al., 2016). Furthermore, application of HS positively influences root growth, especially lateral root emergence and root hair initiation which are involved in plant nutrient uptake (Canellas and Olivares, 2014; Puglisi et al., 2013). At the same time, capacity of HS and HA to stimulate the uptake of nitrate [NO_3-] (Quaggiotti et al., 2004; Canellas et al., 2010; Muscolo et al., 2013; Rose et al., 2014) is not tested to mitigate the N starvation in sugarcane.

Previous studies on sugarcane with isotopes techniques (^{15}N) to measure the NUE had confirmed low N recovery (5–40%) in plants grown in soils with N fertilizer (Basanta et al., 2003; Franco et al., 2011) and urea applied as foliar sprays at a rate of 15 kg N ha^{-1} has shown increased NUE (>50%) in sugarcane (Trivelin et al., 1988). Hence, the objective of this study was to understand the effects of foliar application of HA and HS along with urea on NUE and response of different biometric, biochemical and physiological traits of sugarcane with respect to cultivar, mode of foliar application, geographic location and intervals of foliar application.

2. Materials and methods

2.1. Preparation of humic substances (HS), humic acid (HA) and foliar applications

The HS and HA were extracted from peat as described by the International Humic Substance Society (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC166705/, Schnitzer and Skinner, 1982; Canellas et al., 2002). In current Experiment, peat was collected from the Agrolatino Minerals Administration coal mine, Rincao-SP, Brazil. Subsequently, three foliar applications were prepared using urea, HA and HS; (1) Urea (Urea; 120 mg) labeled with ^{15}N (2.08 atom %) was dissolved in distilled water (1.5 L), (2) Urea with HA (U+HA): Urea-^{15}N (2.08 atom %) (120 mg) and carbon (30 mg) derived from HA were dissolved in distilled water (1.5 L) and; (3) Urea with HS (U+HS): Urea-^{15}N (2.08 atom %) (120 mg) and carbon (30 mg) derived from HS were dissolved in distilled water (1.5 L).

2.2. Characterization of humic substances (HS) and humic acid (HA)

The elemental composition of HS and HA was evaluated using CHNS elemental analyzer (Fisons, LECO CNS-2000, MI, USA) (Table 1). The oxygen content was determined based on the difference of carbon (C), Hydrogen (H) and N. The ash content was measured by incinerating HS and HA (50 mg) at 700 °C for 8 h in a furnace (CM Furnaces Inc., NJ, USA). The total acidity and carboxylic groups in HA and HS were analyzed (Purmalis and Klavins, 2012) (Table 1). Further characterization of HS and HA was carried out using Fourier Transform Infrared Spectroscopy (Cary 630 FTIR-ATR spectrometer, Agilent Technologies, CA, USA) and $_{13}C$ Nuclear Magnetic Resonance (600MHz WB Advance III HD NMR system, Bruker corporation, Germany) (Table 1; Figure 1). Interpretation of FTIR data was based on published literature (Pospisilova and Fasurova, 2009; Stevenson, 1982).

2.3. Experiment 1

2.3.1. Growth conditions and plant materials

The Experiment was conducted using greenhouse facility under controlled microclimatic conditions (Photoperiod [12 h], day and night temperatures [30/22 °C], photosynthetic flux density [600 μmol m^{-2} s^{-1}] and relative humidity [60–70%]) at Agrolatino experimental station (21°35′59.6″S, 47°55′55.4″W) at Rincao, SP, Brazil during 2014. The cv. RB855453, one of the vastly cultivated sugarcane cultivar in Brazil was used for this study. The plants were grown in pots (width 25 cm x height 38 cm) filled with growth medium (N free and washed sand). Water was added till 100% pot capacity and maintained throughout the Experiment. Except N, other macro and micro nutrients (P [120 mg L^{-1}]; K [150 mg L^{-1}]; Ca [200 mg L^{-1}]; Mg [50 mg L^{-1}]; S [40 mg L^{-1}]; B [0.5 mg L^{-1}]; Cu [1.3 mg L^{-1}]; Fe [2 mg L^{-1}]; Mn [3 mg L^{-1}]; Mo [0.1 mg L^{-1}]; Ni [0.05 mg L^{-1}]; Zn [4 mg L^{-1}]) were added per pot.

2.3.2. Foliar application of urea, U+HS and U+HA

After 60 days after planting (DAP), plants with similar physiological maturity were selected for foliar application of urea, U+HA and U+HS. Fertilizer solutions were applied on upper and lower surface of leaves with a brush. Volumes of fertilizer solutions (6% [w/v]; 1.45 mL) with surfactant Triton x100 (0.1%) which contains 100 mg of N was applied per plant. After foliar application, the flask and the brush used for fertilizer application were weighed with left over residue inside and washed and weighed again after drying to estimate the applied quantity and residual loss. After one day, leaves were rehydrated using a hand sprayer to facilitate the absorption of dried fertilizer residues (Trivelin et al.,

Table 1. Characterization of humic substances (HS) and humic acid (HA).

	C (%)	H (%)	O (%)	N (%)	S (%)	Carboxylic acid (mmol g^{-1})	Total acidity (mmol g^{-1})	*Carboxyl (%)	*Aromatic (%)	*O-alkyl (%)	*Alkyl C (%)
HA	15.7	3.4	79.5	1.4	0	3.37	5.9	13	35.9	27.9	23.2
HS	20.3	3.2	74.9	1.6	0	4.05	8.3	27.1	51.1	19.2	2.6

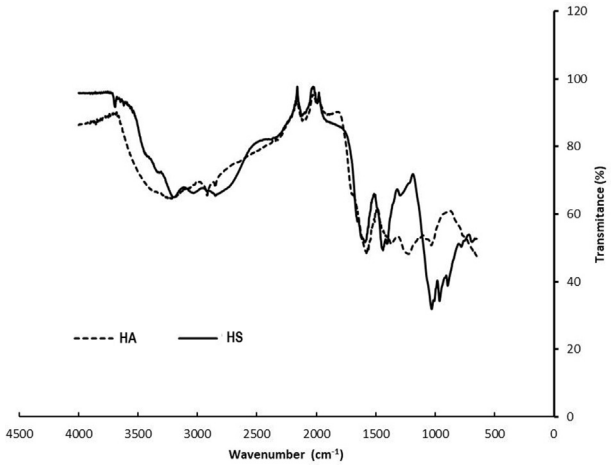

Figure 1. Fourier Trasform Infrared (FTIR) spectra of humic substance (HS) and humic acid (HA).

1988) and plants were allowed to grow for 360 h (15 days). The plants with applied with urea considered as the absolute control for plants with sprayed with U+HA and U+HS applications.

2.4. Experiment 2

2.4.1. Growth conditions and plant materials

The second Experiment was conducted using greenhouse facility at Kansas State University (39.1974°N, 96.5847°W), Manhattan, KS, USA in 2015. Single-buds stalk section (4 cm) of the cv. CP78-1628, a vastly cultivated sugarcane cultivar in USA was used for this Experiment under controlled microclimatic conditions (Photoperiod [12 h], day and night temperatures [30/22 °C], photosynthetic flux density [600 μmol m^{-2} s^{-1}] and relative humidity [60–70 %]). The plants were grown in pots (width 22cm x height 21cm) and filled with Metro Mix 360 growing medium (Hummert International, Topeka, KS, USA) and sand in a proportion of 2:1. Except N, other macro and micro nutrients (P [120 mg L^{-1}]; K [150 mg L^{-1}]; Ca [200 mg L^{-1}]; Mg [50 mg L^{-1}]; S [40 mg L^{-1}]; B [0.5 mg L^{-1}]; Cu [1.3 mg L^{-1}]; Fe [2 mg L^{-1}]; Mn [3 mg L^{-1}]; Mo [0.1 mg L^{-1}]; Ni [0.05 mg L^{-1}]; Zn [4 mg L^{-1}]) were added per pot. Water was added to 100% pot capacity and maintained till the end of Experiment.

2.4.2. Foliar application of urea (U), U+HS and U+HA

Preparations and compositions of urea, U+HS and U+HA were similar to Experiment 1. However, in this Experiment, mode of foliar application was changed by using a hand spray instead of brush. To calculate the applied quantity and residual loss after the foliar application of each plant, sprayer was weighed with residue inside and washed and weighed again after drying. Unlike Experiment 1, water was not applied to rehydrate the dried fertilizer residues in the leaves and plants were allowed to grow for 720 h (30 days). In Experiment 2, plants with urea application was considered as the absolute control for plants sprayed with U+HA and U+HS.

2.4.3. Biomass, total N, nitrogen in the plant derived from the fertilizer (NDFF) and NUE

In both Experiments, leaves and stems were collected at different time intervals (1, 3, 6, 24, 192, and 360 h) after foliar applications. In addition, an additional time point of 720 h was used in Experiment 2 to study the long-term effect of foliar applications. The collected samples of leaves and stems were dried at 65 °C in a hot air oven till the weight was stable and dry weights were recorded. Subsequently, samples were powdered using grinding mill equipped with a mesh sieve of 1mm mesh size (WS, TYLER, USA). These powdered samples were used to measure total N and ^{15}N abundance (%^{15}N atoms) by an automated mass spectrometer (Hydra 20-20 coupled with an auto analyzer ANCA-GSL, Sercon Limited, Crewe, UK). Total N and $^{15}N/^{14}N$ isotope ratio were calculated according to the method of Barrie and Prosser (Barrie, 1996). The N in the plant derived from the fertilizer (NDFF) and N use efficiency (NUE) were calculated.

The following equation (Eq. 1) was used for the NDFF calculation:

$$NDFF = (^{15}Np - ^{15}Nn) / (^{15}Np - ^{15}Nn) \text{ x } Total\ N \quad \text{(Eq.1)}$$

where NDFF is the amount of N in the plant tissue derived from the fertilizer. ^{15}Np is the amount of ^{15}N in the plant tissue. ^{15}Nn is the natural ^{15}N abundance (values of plant tissues collected from control plants were considered for this). ^{15}Nf *is* the quantity of ^{15}N in the fertilizer and *total N* is the amount of N present in biomass of the plant.

The following equation (Eq. 2) was used for the NUE calculation:

$$NUE = NDFF \,/\, Rate\ N \text{ x } 100 \quad \text{(Eq.2)}$$

where NUE is the percentage of applied N recovered from the plant (%) and *Rate N* is the amount of N applied to the leaves.

2.4.4. Leaf gas exchange

In Experiment 2, leaf photosynthetic rate (P_N, μmol m^{-2} s^{-1}) and stomatal conductance (*gs*, mol m^{-2} s^{-1}) were recorded from the fully expanded and physiologically matured second leaf using a portable photosynthesis system (LI-6400 XT; LiCor. Lincoln, NE, USA). The gas exchange traits were recorded between 1000-1200 h at different time intervals (24, 192, 360 and 720 h), except for the initial three-time intervals (1, 3, and 6 h) after foliar application. A minimum of six observations were taken from each treatment using an external light of photosynthetic active radiation (PAR) 1000 μmol m^{-2} s^{-1} supplied by red-blue LED light source and with a set level of 400 ppm CO_2 concentration inside the leaf chamber. Intrinsic water use efficiency (iWUE) was calculated by taking the ratio of photosynthetic rate and stomatal conductance (P_N/gs) (Rymbai et al., 2014).

2.4.5. Total soluble sugars, starch and protein concentrations

In Experiment 2, total sugars, starch and total protein concentrations were estimated from the same leaves used for recording gas exchange traits. Tissue samples were collected from the middle portion of the leaves (without midrib) immediately after recording gas exchange, and wrapped with aluminum foil, immersed in liquid nitrogen and stored at -80 °C until further analysis. Sugars were extracted from leaf samples (1 g) using ethanol (70%). The samples were ground into a powder using liquid nitrogen, homogenized thoroughly with ethanol (70%), incubated at 70 °C in a water bath for 30 min and filtered through Whatman No. 1 filter paper. The filtrate was used for the estimation of total soluble sugars (Dubois et al., 1956). After filtration, the filter paper with the solid residue was dried in a hot air oven at 50 °C. The dried residue was carefully

collected and used for the estimation of starch by following (Hedge and Hofreiter, 1962; Sunoj et al., 2016).

Total protein was extracted and estimated following Sunoj et al. (2014) and Bradford (1976), respectively. For that, plant tissue samples (0.5 g) were homogenized with a pestle and mortar using sodium phosphate buffer (15 mL; 0.1 M; pH 7.6) which contains insoluble polyvinyl polypyrrolidone (PVPP; 1 g) and β-mercaptoethanol (200 μL). The mixture was then centrifuged at 12,000 rpm at 4 °C for 15 min using refrigerated centrifuge (Beckman J2-HC, USA) and supernatant was used to estimate total protein.

2.5. Statistical analysis

Two independent Experiments were conducted, and experimental design was split plot for both Experiments. The significance of the effects of different foliar applications and their interactions were evaluated by analysis of variance (ANOVA) using generalized linear model in SPSS (SPSS Inc. Ver.16, Chicago, USA). The traits with significant difference were compared using Duncan Multiple Range Test (DMRT).

3. Results

The results from both Experiments have showed similar trends, but the magnitude of response was different because of the differences in geographic locations, cultivars (origins of genotypes) and modes of foliar application. Except biomass and total soluble sugars, all the studied traits were showed significant ($P < 0.05$) changes among different foliar applications. At the same time, U+HS and U+HA applications showed increase in total nitrogen (total N), nitrogen derived from fertilizer (NDFF), nitrogen use efficiency (NUE), starch, protein, photosynthesis (P_N) and instantaneous water use efficiency (iWUE) with a non-significant increase in biomass was observed at last two time points of Experiment 2 (720 h) as compared to urea (U) application (Table 3). Same trend was observed in Experiment 1 (360 h), except iWUE in U+HA application (Table 2). On the other hand, interaction effect of foliar application and different time points were significant for NDFF, NUE, total protein, P_N and iWUE. The response of plants to foliar applications by means of biomass were higher in the last time points of two Experiments. The results were explained and discussed by comparing U+HA and U+HS application with U application.

3.1. Effect of foliar application on biomass, total nitrogen (total N), nitrogen derived from fertilizer (NDFF) and nitrogen use efficiency (NUE)

Either Experiment, gradual increase in biomass and total N was observed after applying foliar spray till the last time point across all the foliar applications (Tables 2 and 3). Meanwhile, total N, NDFF and NUE was increased in U+HS and U+HA applications at the last time point of both Experiments as compared to U application. On the other hand, U+HS application showed high total N, NDFF and NUE as compared to the other foliar applications, while the biomass was higher in U+HA application, but not significant in both Experiments and except total N, same trend was observed in Experiment 2 (Tables 2 and 3).

3.2. Effect of foliar application on photosynthesis (P_N) and intrinsic water use efficiency (iWUE)

In Experiment 2, P_N and iWUE didn't showed any significant change along with the gradual increase of time, while P_N significantly higher at last two time points (360 and 720 h) of U+HA and U+HS applications as

Table 2. Effect of foliar application of urea (U), urea with humic acid (U+HA) and urea with humic substance (U+HS) on biomass, total nitrogen (Total N), nitrogen derived from fertilizer (NDFF) and nitrogen use efficiency (NUE) of sugarcane variety (RB855453) in Experiment 1. Each value is mean of four replications.

Hours after foliar application	Foliar application	Traits			
		Biomass (mg plant^{-1})	Total nitrogen (mg plant^{-1})	NDFF (mg plant^{-1})	NUE (%)
1	U	10.7 (±0.9)	96.5 (±5.2)^{b}	15.3 (±1.6)^{ab}	19.2 (±1.2)^{a}
	U+HA	11.4 (±1.2)	99.3 (±6.1)^{a}	17.3 (±2.5)^{ab}	21.2 (±2.2)^{a}
	U+HS	11.5 (±0.8)	101 (±5.4)^{a}	19.0 (±2.1)^{b}	22.1 (±1.5)^{a}
3	U	11.3 (±1.0)	84.2 (±6.2)^{c}	11.3 (±1.6)^{c}	18.6 (±2.1)^{b}
	U+HA	11.1 (±1.1)	94.1 (±5.4)^{b}	14.2 (±1.7)^{b}	20.2 (±2.5)^{a}
	U+HS	12.2 (±1.1)	101 (±5.1)^{a}	20.1 (±2.1)^{a}	21.8 (±1.6)^{a}
6	U	11.8 (±0.9)	82.4 (±3.2)^{a}	9.9 (±2.3)^{c}	15.4 (±1.6)^{c}
	U+HA	9.8 (±0.7)	72.9 (±7.8)^{ab}	14.7 (±2.1)^{ab}	21.4 (±1.9)^{b}
	U+HS	8.3 (±0.8)	69.7 (±3.5)^{b}	16.2 (±1.6)^{a}	25.4 (±1.5)^{a}
24	U	10.5 (±0.9)	81.3 (±5.4)^{b}	22.2 (±3.2)^{c}	35.2 (±2.2)^{c}
	U+HA	13.3 (±1.1)	111 (±5.63)^{a}	40.2 (±2.5)^{a}	47.4 (±3.2)^{a}
	U+HS	10.6 (±1.0)	82.2 (±3.1)^{b}	26.1 (±1.2)^{b}	44 (±3.4)^{ab}
96	U	13.5 (±0.6)	98.9 (±3.36)^{b}	32.9 (±3.5)^{b}	46.3 (±3.6)^{bc}
	U+HA	12.1 (±1.2)	92.8 (±5.6)^{bc}	31.7 (±2.4)^{b}	49.3 (±3.4)^{b}
	U+HS	13.6 (±2.0)	106 (±6.4)^{a}	38.3 (±2.7)^{a}	60.3 (±3.5)^{a}
192	U	14.8 (±1.9)	125 (±5.7)^{b}	38.6 (±3.6)^{b}	49.6 (±2.5)^{c}
	U+HA	15.9 (±1.8)	119 (±6.4)^{c}	39.5 (±2.5)^{b}	53.6 (±2.6)^{b}
	U+HS	18.6 (±2.1)	151 (±5.6)^{a}	58.3 (±4.1)^{a}	69.2 (±2.9)^{a}
360	U	22.4 (±2.0)	148 (±8.7)^{c}	46.5 (±4.1)^{bc}	58.1 (±2.4)^{b}
	U+HA	24.8 (±1.9)	157 (±5.9)^{b}	50.3 (±3.5)^{b}	64.5 (±2.9)^{ab}
	U+HS	23.1 (±1.8)	163 (±4.6)^{a}	54.4 (±2.6)^{a}	67.3 (±2.7)^{a}
LSD					
H		3.4**	22.9**	7.7**	9.2**
F		NS	11.3*	5.0**	6.0**
F x H		NS	NS	9.9*	16.0*

Hours after foliar application (H); foliar application (F); non-significant (NS); Significant values according to the LSD followed by* and ** corresponding to $P < 0.05$ and $P < 0.01$ respectively. Values followed by different letters are significantly different according to Duncan's multiple range test ($P < 0.01$; the test was conducted independently for each interval hour after foliar application). Values in the parenthesis are ±standard deviation of respective mean values.

Table 3. Effect of foliar application of urea (U), urea with humic acid (U+HA) and urea with humic substance (U+HS) on biomass, total nitrogen (Total N), nitrogen derived from fertilizer (NDFF) and nitrogen use efficiency (NUE) of sugarcane variety (CP78-1628) in Experiment 2. Each value is mean of four replications.

Hours after foliar application	Foliar application	Traits			
		Biomass (mg plant^{-1})	Total nitrogen (mg plant^{-1})	NDFF (mg plant^{-1})	NUE (%)
1	U	9.8 (±1.2)	184 (±7.8)	6.6 (±0.9)^{c}	12 (±1.2)^{c}
	U+HA	10.4 (±1.6)	230 (±10.2)	13.4 (±1.4)^{a}	25 (±1.4)^{a}
	U+HS	10.4 (±2.3)	217 (±12.4)	10.0 (±0.7)^{b}	19 (±2.1)^{b}
3	U	8.9 (±2.1)	171 (±6.7)	7.9 (±1.4)^{b}	15 (±1.5)^{b}
	U+HA	9.1 (±2.4)	191 (±5.4)	11.3 (±1.2)^{a}	21 (±1.6)^{a}
	U+HS	8.7 (±3.1)	192 (±7.8)	7.6 (±1.1)^{b}	14 (±0.9)^{b}
6	U	9.5 (±1.4)	207 (±12.3)	12.6 (±1.0)^{b}	23 (±2.1)^{b}
	U+HA	8.9 (±2.3)	200 (±10.2)	14.5 (±1.3)^{a}	27 (±2.0)^{a}
	U+HS	9.3 (±3.1)	197 (±11.5)	12.4 (±0.9)^{b}	23 (±1.2)^{b}
24	U	10.8 (±1.4)	229 (±9.5)	15.6 (±1.0)^{a}	29 (±1.4)^{a}
	U+HA	10.5 (±3.2)	222 (±8.7)	14.9 (±1.2)^{b}	28 (±1.7)^{a}
	U+HS	9.2 (±0.9)	215 (±9.7)	15.6 (±0.9)^{a}	29 (±1.4)^{a}
96	U	11.1 (±1.2)	235 (±10.2)	10.4 (±0.9)^{b}	19 (±2.4)^{bc}
	U+HA	10.9 (±2.1)	230 (±12.5)	11.4 (±0.8)^{b}	21 (±11)^{b}
	U+HS	9.8 (±1.5)	230 (±11.0)	14.3 (±1.0)^{a}	26 (±2.1)^{a}
192	U	13.8 (±1.4)	254 (±13.2)	11.4 (±0.9)^{b}	21 (±2.4)^{bc}
	U+HA	13.3 (±1.6)	255 (±10.7)	12.7 (±1.1)^{b}	24 (±2.4)^{b}
	U+HS	12.2 (±2.1)	261 (±11.6)	14.7 (±0.9)^{a}	27 (±1.4)^{a}
360	U	15.6 (±2.0)	263 (±10.2)	14.1 (±1.0)^{a}	22 (±1.4)^{b}
	U+HA	22.6 (±3.1)	305 (±9.9)	15.7 (±0.9)^{a}	29 (±1.9)^{a}
	U+HS	23.2 (±1.5)	337 (±9.7)	14.9 (±1.2)^{a}	28 (±1.7)^{a}
720	U	52.6 (±1.4)	329 (±10.7)	9.3 (±0.8)^{c}	17 (±2.9)^{c}
	U+HA	59.3 (±1.5)	353 (±11.4)	11.3 (±11)^{b}	21 (±1.8)^{b}
	U+HS	56.5 (±3.2)	348 (±10.2)	16.8 (±1.5)^{a}	31 (±1.3)^{a}
LSD					
H		1.8**	25.1**	1.5**	2.7**
F		NS	NS	NS	NS
F x H		NS	NS	2.6**	4.8**

Hours after foliar application (H); foliar application (F); non-significant (NS); Significant values according to the LSD followed by* and ** corresponding to $P < 0.05$ and $P < 0.01$ respectively. Values followed by different letters are significantly different according to Duncan's multiple range test ($P < 0.01$; the test was conducted independently for each interval hour after foliar application). Values in the parenthesis are ±standard deviation of respective mean values.

compared to the U application (Figure 2). Among the foliar applications, U+HS showed higher P_N and iWUE. At the same time, there was only a minor difference in P_N was found among U+HS and U+HA applications.

3.3. Effect of foliar application on total soluble sugars, starch, and total protein

In Experiment 2, concentrations of starch and total protein were significantly higher in U+HS and U+HA than urea application. Simultaneously, total soluble sugars were not changed, while starch concentration was higher in U+HA followed by U+HS. Meanwhile increase in total protein showed an opposite trend (Table 4).

4. Discussion

4.1. Nitrogen utilization and biomass accumulation

There were promising responses observed in both Experiments after the foliar application of urea with HS and HA. At the same time, there was no significant effect of foliar applications on biomass, which is important for the sugarcane production and quality. However, a progressive increase observed over time in biomass and total N in both Experiments which can be due to the growth and developmental processes of plants; and moreover, gradual increase of total N is also due to the increased NUE. Meanwhile, in Experiment 1, NUE and NDFF were progressively enhanced over the time after foliar application (Table 2), while in Experiment 2, this trend was not observed even after 720 h (Table 3). This large difference in the trend of NUE and NDFF between both Experiments can be attributed to the mode of fertilizer application or differential genotypic response. As described previously, in Experiment 1, water was sprayed to rehydrate the dried fertilizer residues on the leaves after one day of foliar application and it was not done in Experiment 2.

Earlier studies indicated that, foliar urea application penetrates the leaf surface *via.*, both the cuticle and stomata. The penetration of fertilizer through the cuticle is a passive process driven by the concentration differences between the surface and the leaf interior (Oosterhuis, 2009; Eichert and Fernández, 2012). Hence, rehydrating the dried fertilizer residue can decrease the solute concentration and increase the permeability of polar solutes through the cuticle (Oosterhuis, 2009; Eichert and Fernández, 2012; Riederer and Schneider, 1990; Popp et al., 2005). Furthermore, difference between varieties and geographic locations can also be other reasons, because of the variation in leaf cuticle thickness due to the epicuticular wax deposition on leaves which can change according to the climatic condition of geographic location (Rymbai et al., 2014). Earlier studies show an inverse relationship between epicuticular wax concentration and ^{15}N uptake (Bondada et al., 1997). These differences in the trend of magnitude of NDFF and NUE between both Experiments directing towards the importance of standardizing intervals, concentrations and mode of foliar application according to differences in varieties and geographic locations to achieve the best result of foliar application. Furthermore, increasing the intervals of foliar applications in each growth stages can further increase the biomass.

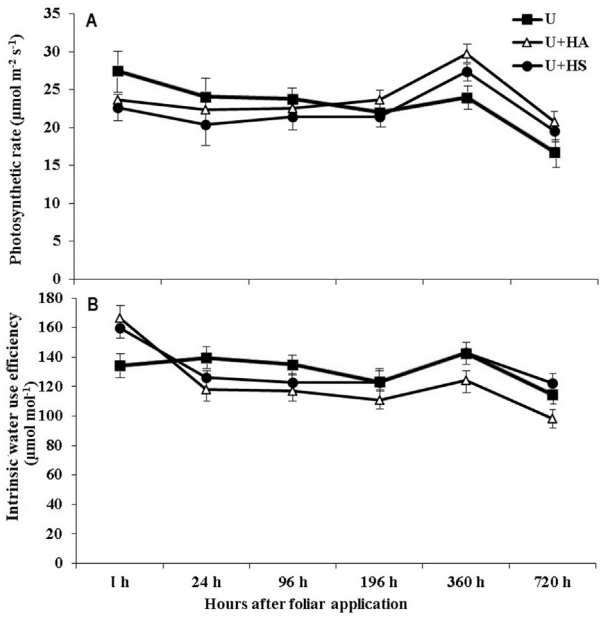

Figure 2. Effect of foliar application of urea (U), urea with humic acid (U+HA) and urea with humic substance (U+HS) on photosynthetic rate (P_N) and instantaneous water use efficiency (iWUE) of sugarcane variety (CP78-1628) in Experiment 2. Significant values according to LSD (Photosynthetic rate: hours after foliar application [H] = nonsignificant, foliar application [F] = $P < 0.01$ and HxF = $P < 0.05$; Intrinsic water use efficiency: H = non-significant, F=$P < 0.01$ and HxF = $P < 0.01$).

Concurrently, among the different foliar applications, significant increase in NDFF and NUE was observed in U+HS followed by U+HA application across both Experiments (Tables 2 and 3). This can be attributed to conversion of urea into ammonium (NH^{4+}) by the activity of urease enzyme present in the HS and induction of plasma membrane H^+-ATPase (PM H^+-ATPase) enzyme activity, which increases the entry of ammonium (NH^{4+}) in to the leaves. Simultaneously, size of NH^{4+} is smaller than urea and that allow NH^{4+} to enter easily into the leaves through cuticle (Eichert and Fernández, 2012; Popp et al., 2005). Earlier studies in maize showed presence of urease enzyme in HS and HA (Canellas et al., 2013; Borghetti et al., 2003; Busato et al., 2010). The principle activity of PM H^+-ATPase enzyme is to increase the ion uptake by providing an electrochemical gradient necessary to energize ion transport for cell uptake and induce cell growth by a mechanism know as acid growth (Canellas et al., 2013; Nardi et al., 2002). In this study, 70% of the urea was modified into NH^{4+} after mixing with HS (data not shown). At the same time, conversion rate of urea into NH^{4+} is higher in U+HS than U+HA treatment, because the purification process of HA reduced or inactivated the urease activity (Krajewska, 2009).

4.2. Gas exchange, water use efficiency, and primary metabolites

Furthermore, gas exchange traits and metabolism of C and N were measured in Experiment 2 to study the physio-chemical responses sugarcane after foliar applications. The P_N and iWUE was significantly improved in U+HS as compared with other applications (Figure 2). There are several field studies indicates the positive correlation between P_N, iWUE and foliar nitrogen concentration (Mitchell and Hinckley, 1993; Guo et al., 2016). The current study revealed that increase in P_N and higher iWUE is due to the increased mesophyll conductance, higher carboxylation efficiency and decreased stomatal conductance associated with increase in foliar nitrogen concentration. Similar results were reported after foliar application of HS in bacteria-inoculated maize plants (Canellas et al., 2013).

The concentrations of starch and total protein were significantly higher in U+HS and U+HA than urea application. Contrastingly, concentration of total soluble sugars was not changed (Table 4). An increase in total protein and decrease nitrate content was reported in maize treated with HS extracted from different residues and results from similar studies showed an increase in rubisco and glutamine synthetase activities in maize plants (Ertani et al. 2011, 2013). In another study, application of HS increased the activities of nitrate reductase and glutamine synthetase which in turn increased total protein concentration (Canellas et al., 2013). The protein is the form of N used for the storage; and increased protein concentration in current study is an indicator of better assimilation of N from the foliar applications through leaves. At the same time, no change in total soluble sugars even at higher photosynthetic rate indicates that synthesis of protein is in the expense of total soluble sugars (Bi et al., 2004). Moreover, mobilization of total soluble sugars for the starch synthesis also caused increased starch accumulation and no change in sugar (Sunoj et al., 2016) which is an indicator of higher

Table 4. Effect of foliar application of urea (U), urea with humic acid (U+HA), urea with humic substance (U+HS) on total soluble sugars, starch and total protein concentrations of sugarcane (CP78-1628) in Experiment 2. Each value is mean of six replications.

Hours after foliar application	Foliar application	Traits		
		Total soluble sugars (mg g^{-1} DW)	Starch (mg g^{-1} DW)	Total protein (mg g^{-1} DW)
1	U	43.8 (±3.5)^{b}	36.7 (±3.5)^{a}	27.9 (±2.5)^{a}
	U+HA	60.9 (±4.5)^{ab}	37.4 (±4.5)^{a}	27.7 (±3.5)^{a}
	U+HS	63.0 (±5.2)^{a}	29.5 (±5.5)^{b}	21.4 (±3.1)^{b}
96	U	66.0 (±6.4)^{a}	43.2 (±6.4)^{c}	28.1 (±3.6)^{b}
	U+HA	46.7 (±4.7)^{c}	51.1 (±2.1)^{a}	31.6 (±4.6)^{a}
	U+HS	57.4 (±6.5)^{b}	48.0 (±4.5)^{b}	30.1 (±4.1)^{ab}
720	U	70.7 (±4.7)^{a}	56.1 (±5.7)^{c}	7.2 (±5.4)^{c}
	U+HA	68.3 (±6.5)^{a}	73.4 (±6.4)^{a}	13.1 (±5.6)^{b}
	U+HS	70.8 (±5.7)^{a}	63.7 (±5.7)^{b}	14.2 (±2.4)^{a}
LSD				
H		7.9**	7.8**	3.2**
F		13.6**	7.9**	2.4*
F x H		NS	1.2*	5.6**

Hours after foliar application (H); foliar application (F); dry weight (DW); non-significant (NS); Significant values according to the LSD followed by* and ** corresponding to $P < 0.05$ and $P < 0.01$ respectively. Values followed by different letters are significantly different according to Duncan's multiple range test ($P < 0.01$; the test was conducted independently for each interval hour after foliar application). Values in the parenthesis are ±standard deviation of respective mean values.

growth. On the other hand, starch has little influence on protein synthesis (Bi et al., 2004). This specifies the positive influence HS on carbon and nitrogen metabolism by improving protein and starch content by utilizing total soluble sugars and N on the leaves (Rose et al., 2014; Bi et al., 2004; Canellas and Olivares. 2014).

5. Conclusions

In summary, results from two Experiments showed that foliar application of U+HS and U+HA was rapidly absorbed by sugarcane leaves which increased NDFF and NUE than urea alone. Among different foliar fertilization, U+HS application is a great strategy for complementing the sugarcane nitrogen management. From our Experiments, results pointing the influence of the U+HS on carbon and nitrogen metabolism in sugarcane plants. Better NDFF and NUE were obtained when the water was applied on leaves to rehydrate the residues after fertilizer application specifies the further requirement of standardization of intervals, mode and concentration of foliar application according to the varieties and geographic locations. The detected changes in P_N, iWUE, ^{15}N uptake and total soluble sugars, starch and total protein concentrations were related to physiochemical responses of plants to the foliar applications and these progressions can promote plant growth. Furthermore, foliar application of U+HS has the benefit of lowering fertilizer cost and input, maximum assimilation and quicker response in plants that in turn results in increased economic sustainability of farmers, decreases the wastage of fertilizer, environmental pollution and greenhouse gas emission. Furthermore, higher NUE capacity of U+HS can also be used in the nitrogen deficient soil along with additional nutritional and growth advantages from HS.

Declarations

Author contribution statement

Jose M. Leite, S.V. John Sunoj: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pavithra S. Pitumpe Arachchige: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ignacio A. Ciampitti, Paulo. C. O. Trivelin, P.V. Vara Prasad: Conceived and designed the experiments.

Ganga M. Hettiarachchi: Analyzed and interpreted the data.

Leila Maurmann: Performed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

Acquisti, C., Kumar, S., Elser, J.J., 2009. Signatures of nitrogen limitation in the elemental composition of the proteins involved in the metabolic apparatus. Proc. Biol. Sci. 22 (276), 2605–2610 (1667).

Barrie, A., Prosser, S.J., 1996. Automated analysis of light-element stable isotopes by isotope ratio mass spectrometry. In: Boutton, T.W., Yamasaki, S. (Eds.), Mass Spectrometry of Soils. Marcel Dekker, New York, pp. 1–46.

Basanta, M.V., Dourado-Neto, D., Reichardt, K., Bacchi, O., Oliveira, J., Trivelin, P., Timm, L., Tominaga, T., Correchel, V., Cássaro, F., 2003. Management effects on nitrogen recovery in a sugarcane crop grown in Brazil. Geoderma 116, 235–248.

Bi, G., Scagel, L., Fuchigami, L.H., 2004. Soil and foliar nitrogen supply affect the composition of nitrogen and carbohydrates in young almond trees. J. Hortic. Sci. Biotechnol. 79, 175–181.

Bondada, B.R., Oosterhuis, D.M., Norman, R.J., 1997. Cotton leaf age, epicuticular wax, and nitrogen-15 absorption. Crop Sci. 37, 807–811.

Borghetti, C., Gioacchini, P., Marzadori, C., Gessa, C., 2003. Activity and stability of urease-hydroxyapatite and urease-hydroxyapatite-humic acid complexes. Biol. Fertil. Soils 38, 96–101.

Bradford, M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Busato, J.G., Zandonadi, D.B., Dobbss, L.B., Façanha, A.R., Canellas, L.P., 2010. Humic substances isolated from residues of sugar cane industry as root growth promoter. Sci. Agric. 67, 206–212.

Calcino, D., 1994. Australian Sugarcane Nutrition Manual. SRDC/BSES Limited, Brisbane, Queensland, Australia, pp. 1–60.

Canellas, L.P., Balmori, D.M., Médici, L.O., Aguiar, N.O., Campostrini, E., Rosa, R.C., Façanha, A.R., Olivares, F.L., 2013. A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). Plant Soil 366, 119–132.

Canellas, L.P., Olivares, F.L., Okorokova-Facanha, A.L., Facanha, A.R., 2002. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+-ATPase activity in maize roots. Plant Physiol. 130, 1951–1957.

Canellas, L.P., Olivares, F.L., 2014. Physiological responses to humic substances as plant growth promoter. Chem. Biol. Tec. Agric. 1, 1–11.

Canellas, L.P., Piccolo, A., Dobbss, L.B., Spaccini, R., Olivares, F.L., Zandonadi, D.B., Façanha, A.R., 2010. Chemical composition and bioactivity properties of size-fractions separated from a vermicompost humic acid. Chemosphere 78, 457–466.

Cantarella, H., Trivelin, P.C.O., Contin, T.L.M., Dias, F.L.F., Rossetto, R., Marcelino, R., Coimbra, R.B., Quaggio, J.A., 2008. Ammonia volatilisation from urease inhibitor-treated urea applied to sugarcane trash blankets. Sci. Agric. 65, 397–401.

Chien, S.H., Prochnow, L., Cantarella, A.H., 2009. Recent developments of fertilizer production and use to improve nutrient efficiency and minimize environmental impacts. Adv. Agron. 102, 267–322.

Dawar, K., Zaman, M., Rowarth, J., Blennerhassett, J., Turnbull, M., 2011. Urea hydrolysis and lateral and vertical movement in the soil: effects of urease inhibitor and irrigation. Biol. Fertil. Soils 47, 139–146.

Delfine, S., Tognetti, R., Desiderio, E., Alvino, A., 2005. Effect of foliar application of N and humic acids on growth and yield of durum wheat. Agron. Sustain. Dev. 25, 183–191.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356.

Eichert, T., Fernández, V., 2012. Uptake and release of mineral elements by leaves and other aerial plant parts. In: Marschner, P. (Ed.), Mineral Nutrition of Higher Plants. Academic Press, San Diego, pp. 71–84.

Ekin, Z., 2019. Integrated use of humic acid and plant growth promoting rhizobacteria to ensure higher potato productivity in sustainable agriculture. Sustainability 11, 3417.

Ertani, A., Francioso, O., Tugnoli, V., Righi, V., Nardi, S., 2011. Effect of commercial lignosulfonate-humate on *Zea mays* L. metabolism. J. Agric. Food Chem. 59, 11940–11948.

Elsheery, N.I., Sunoj, V.S.J., Wen, Y., Zhu, G.J.J., Muralidharan, G., Cao, K.F., 2020. Foliar application of nanoparticles mitigates the chilling effect on photosynthesis and photoprotection in sugarcane. Plant Physiol. Biochem. 149, 50–60.

Ertani, A., Pizzeghello, D., Baglieri, A., Cadili, V., Tambone, F., Gennari, M., Nardi, S., 2013. Humic-like substances from agro-industrial residues affect growth and nitrogen assimilation in maize (*Zea mays* L.) plantlets. J. Geochem. Explor. 129, 103–111.

Esringu, A., Kaynar, D., Turan, M., Ercisli, S., 2016. Ameliorative effect of humic acid and plant growth-promoting rhizobacteria (PGPR) on Hungarian vetch plants under salinity stress. Commun. Soil Sci. Plant Anal. 47, 602–618.

Fageria, N., Baligar, V.C., 2005. Enhancing nitrogen use efficiency in crop plants. Adv. Agron. 88, 97–185.

FAO, 2017. Report, Food and Agricultural Organization. Economic and Social Department," The Statistical Division, FAO 2017 FAOSTAT, 2018, United Nations. http://faostat3.fao.org/home/index.html#DOWNLOAD.

Franco, H.C.J., Otto, R., Faroni, C.E., Vitti, A.C., Almeida, E.C.O., Trivelin, P.C.O., 2011. Nitrogen in sugarcane derived from fertilizer under Brazilian field conditions. Field Crop. Res. 121, 29–41.

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Cleveland, C.C., Green, P., Holland, E.A., 2004. Nitrogen cycles: past, present, and future. Biogeochemistry 70, 153–226.

Guo, R., Sun, S., Liu, B., 2016. Difference in leaf water use efficiency/photosynthetic nitrogen use efficiency of Bt-cotton and its conventional peer. Sci. Rep. 6, 33539.

Hedge, J.E., Hofreiter, B.T., 1962. In: Whistler, R.L., Be Miller, J.N. (Eds.), Carbohydrate Chemistry 17. Academic Press, New York.
Innes, R.F., 1960. The nitrogen, phosphorus and potassium requirements of sugarcane. J. Sci. Food Agric. 11, 299–309.
Jones, D.L., Kielland, K., 2002. Soil amino acid turnover dominates the nitrogen flux in permafrost-dominated taiga forest soils. Soil Biol. Biochem. 34, 209–219.
Kant, S., Bi, Y., Rothstein, S.J., 2011. Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. J. Exp. Bot. 62, 1499–1509.
Kingston, G., 2014. Mineral nutrition of sugarcane. In: Moore, P.H., Botha, F.C. (Eds.), Sugarcane: Physiology, Biochesmetry, and Functional Biology. John Wiley & Sons, Oxford, pp. 85–120.
Klucakova, M., 2018. Size and charge evaluation of standard humic and fulvic acids as crucial factors to determine their environmental behavior and impact. Front. Chem. 6, 235.
Krajewska, B., 2009. Ureases I. Functional, catalytic and kinetic properties: a review. J. Mol. Cat. Enz. 59, 9–21.
Mitchell, A.K., Hinckley, T.M., 1993. Effects of foliar nitrogen concentration on photosynthesis and water use efficiency in Douglas-fir. Tree Physiol. 12 (4), 403–410.
Muscolo, A., Sidari, M., Nardi, S., 2013. Humic substance: relationship between structure and activity. Deeper information suggests univocal findings. J. Geochem. Explor. 129, 57–63.
Nardi, S., Pizzeghello, D., Muscolo, A., Vianello, A., 2002. Physiological effects of humic substances on higher plants. Soil Biol. Biochem. 34, 1527–1536.
Olivares, F.L., Aguiar, N.O., Rosa, R.C.C., Canellas, L.P., 2015. Substrate biofortification in combination with foliar sprays of plant growth promoting bacteria and humic substances boosts production of organic tomatoes. Sci. Hortic. 183, 100–108.
Oosterhuis, D., 2009. Foliar Fertilization: Mechanisms and Magnitude of Nutrient Uptake. Paper for The Fluid Fertilizer Foundation Meeting in Scottsdale, Arizona, pp. 15–17.
Otto, R., Franco, H.C.J., Faroni, C.E., Vitti, A.C., Cantidio, E., Oliveira, A.D., Sermarini, R.A., Trivelin, P.C.O., 2014. The role of nitrogen fertilizers in sugarcane root biomass under field conditions. Agri. Sci. 5, 1527–1538.
Popp, C., Burghardt, M., Friedmann, A., Riederer, M., 2005. Characterization of hydrophilic and lipophilic pathways of Hedera helix L. cuticular membranes: permeation of water and uncharged organic compounds. J. Exp. Bot. 56, 2797–2806.
Pospisilova, L., Fasurova, N., 2009. Spectroscopic characteristics of humic acids originated in soils and lignite. Soil Water Res. 4, 168–175.
Puglisi, E., Pascazio, S., Suciu, N., Cattani, I., Fait, G., Spaccini, R., Crecchio, C., Piccolo, A., Trevisan, M., 2013. Rhizosphere microbial diversity as influenced by humic substance amendments and chemical composition of rhizodeposits. J. Geochem. Explor. 129, 82–94.
Purmalis, O., Klavins, M., 2012. Formation and changes of humic acid properties during peat humification process within ombrotrophic bogs. Open J. Soil Sci. 2, 100–110.
Quaggiotti, S., Ruperti, B., Pizzeghello, D., Francioso, O., Tugnoli, V., Nardi, S., 2004. Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L.). J. Exp. Bot. 55, 803–813.
Riederer, M., Schneider, G., 1990. The effect of the environment on the permeability and composition of Citrus leaf cuticles. Planta 180, 154–165.
Roberts, T.L., 2008. Improving nutrient use efficiency. Turk. J. Agric. For. 32, 177–182.
Rose, M.T., Patti, A.F., Little, K.R., Brown, A.L., Jackson, W.R., Cavagnaro, T.R., 2014. A meta-analysis and review of plant-growth response to humic substances: practical implications for agriculture. Adv. Agron. 124, 37–89.
Rymbai, H., Laxman, R., Dinesh, M., Sunoj, V.J., Ravishankar, K., Jha, A., 2014. Diversity in leaf morphology and physiological characteristics among mango (*Mangifera indica*) cultivars popular in different agro-climatic regions of India. Sci. Hort. 176, 189–193.
Saleem, M.F., Ghaffar, A., Anjum, S.A., Cheema, M.A., Bilal, M.F., 2012. Effect of nitrogen on growth and yield of sugarcane. J. Amer. Soc. Sugarcane Tech. 32, 76–93.
Salter, B., Bonnett, G.D., 2000. High soil nitrate concentrations during autumn and winter increase suckering. Proc. Aust. Soc. Sugar Cane Technol. 22, 322–327.
Schnitzer, M., Skinner, L., 1982. Method of Soil Analysis, Part 2. Chemical and Mineralogical Properties. Agronomic Monograph. ASA/SSSA Publishers. Organic Matter Characterization, Madison, WI, pp. 581–597. American Society of Agronomy/ Soil Science Society of America.
Schoebitz, M., López, M.D., Serrí, H., Martínez, O., Zagal, E., 2016. Combined application of microbial consortium and humic substances to improve the growth performance of blueberry seedlings. J. Soil Sci. Plant Nutr. 16, 1010–1023.
Srivastava, S.C., Suarez, N.R., 1992. Sugarcane. In: Wichmann, W. (Ed.), World Fertilizer Use Manual. BASF AG, Germany, pp. 257–266.
Stevenson, F., 1982. Extraction, Fractionation, and General Chemical Composition of Soil Organic Matter. Humus Chemistry. Genesis, Composition, Reactions, second ed. Wiley Pub, pp. 26–54.
Stuart, D., Schewe, R., McDermott, M., 2014. Reducing nitrogen fertilizer application as a climate change mitigation strategy: understanding farmer decision-making and potential barriers to change in the US. Land Use Pol. 36, 210–218.
Suh, H.Y., Yoo, K.S., Suh, S.G., 2014. Tuber growth and quality of potato (*Solanum tuberosum* L.) as affected by foliar or soil application of fulvic and humic acids. Hortic. Environ. Biotechnol. 55, 183–189.
Sunoj, J.V.S., Kumar, N.S., Kumar Muralikrishna, K.S., 2014. Effect of elevated CO_2 and temperature on oxidative stress and antioxidant enzymes activity in coconut (*Cocos nucifera* L.) seedlings. Indian J. Plant Physiol. 19, 382–387.
Sunoj, J.V.S., Shroyer, K.J., Jagadish, S.V.K., Prasad, P.V.V., 2016. Diurnal temperature amplitude alters physiological and biochemical response of Maize (*Zea mays*) during the vegetative stage. Environ. Exp. Bot. 130, 113–121.
Trivelin, P., Carvalho, D.J., Silva, A., Primavesi, A., Camacho, E., Eimore, I., Guilherme, M., 1988. Adubacao foliar de cana-de-açucar (*Saccharum spp.*) absorção e translocação de uréia-N. Energ. Nucl. Agric., Piracicaba. 9, 52–65.
Weijde, T.V.D., Kamei, C.L.A., Torres, A.F., Vermerris, W., Dolstra, O., Visser, R.G.F., Trindade, L.M., 2013. The potential of C4 grasses for cellulosic biofuel production. Front. Plant Sci. 4, 107.
Witte, C., 2011. Urea metabolism in plants. Plant Sci. 180, 431–438.