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The biostimulant manufactured using diazotrophic endophytic bacteria and humates is effective to increase sugarcane yield

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Abstract

Background: The use of biostimulants in agriculture has demonstrated great potential but more consistent field results are required for wider farm acceptance. We evaluated different delivering methods for the biostimulant produced with plant growth-promoting bacteria mixed with humic acid-like substances isolated from vermicompost in the commercial sugarcane crop yield during 3 consecutive years.

Results: Foliar spray had a better performance than furrow application and the best result was obtained when the biostimulant was applied at 60 days after emergence, thus enhancing 37% of the stem yield when compared to control. In the first and second ratoons, the productivity increases 5 and 24%, respectively. The first ratoon was marked by severe drought stress that hit all the southeastern of Brazil. Moreover, the assay using strip plot design with a large parcel area confirms the promotion of sugarcane yields by biostimulant during two consecutive ratoons increasing 19 and 18% that represent 11 and 13 tons ha^{-1} more than the control. The use of biostimulant did not change soluble solid content and polarizable sugars in the sugarcane juice obtained from both experiments.

Conclusions: The biostimulant formulated with endophytic diazotrophic bacteria and humic acids represents a low-cost technology that increases the sugarcane yield with economic use of fertilizers to enhance crop yield.

Keywords: Field response, Humic acids, Diazotrophic endophytic bacteria, Ecological intensification of crops

Background

The use of biostimulants in agriculture has grown steadily from the last decade around 10% or more at year whatever indicator is used (sales, treated hectares, number of users) [1]. Biostimulants are defined as the materials that contain one or more substances and/or microorganisms able to stimulate nutrient uptake and use efficiency by plants, increase plant tolerance to abiotic/biotic stress, and improve crop quality when applied in small amounts [2]. The main materials used in the manufacture of biostimulants are protein hydrolysates and other N-containing compounds, seaweed extracts, chitosan, humic and fulvic acids, and plant growth-promoting bacteria. We proposed the manufacture of biostimulant using plant growth-promoting bacteria mixed with humic substances [3].

Plant growth-promoting bacteria can stimulate plant growth by replacing soil nutrients through biological N_2 fixation, making nutrients more available (solubilization of phosphates) or increasing plant access to nutrients (increasing root surface area or modulating ion transporters) [4]. Humic substances can affect directly the plant physiology [5] increasing the growth of both plant root and shoot around 20% [6]. Humic substances are highly chemically reactive yet recalcitrant with respect to biodegradation according to the definition of International Humic Substances Society [7]. Humic substances can be considered as a complex mixture of microbial and plant-derived compounds in a supramolecular assembly where their hydrophobic components like lipid and lignin derivatives can protect available compounds against enzymatic degradation [8]. Humic substances can be

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mixed with bacteria at low carbon concentration without major changes in the supramolecular arrangement.

The use of beneficial microorganisms in agriculture has been demonstrating great potential [9–11]. The nature of chemical composition of humic substances was revealed [12] allowing the relationship between chemical properties of humic matter and their physiological effects [13, 14]. However, there is a disagreement between results accumulated in the laboratory and the field response of the biostimulants.

The biostimulant produced with humic acids and plant growth-promoting bacteria was used with success on short-cycle plants like maize and tomato [15, 16], but for perennial plants under field condition, there are many open questions related to the management and proper use of this biostimulant. The objective of this study was to evaluate the best way and application time of the biostimulant produced with the mixture of plant growth-promoting bacteria and humic acids in sugarcane during the cane plant cycle and two consecutives ratoon crops.

Methods

Microorganisms and humic substances

Herbaspirillum seropedicae strain HRC54, *Herbaspirillum rubrisubalbicans* strain HCC103, and *Gluconacetobacter diazotrophicus* strain PAL 5 were isolated and characterized as endophytic diazotrophs from sugarcane [17]. From pure culture, the bacterial strains were grown separately in 5 mL liquid DYGS medium with the following composition (g L^{-1}): glucose, 2.0; malic acid, 2.0; peptone, 1.5; yeast extract, 2.0; $MgSO_4 \cdot 7\ H_2O$, 0.5; L-glutamic acid, 1.5; and pH 6.0. The growth condition was 30 °C for 36 h at 150 rpm in a rotary shaker. After that, an aliquot of 20 µL of each bacterial species was inoculated into a 2000-mL flask of liquid DYGS medium at the same growing conditions for 48 h. Next, the bacterial biomass was centrifuged at 2000*g* for 10 min, resuspended in sterilized water, and adjusted to 10^9 cells mL^{-1} using the optical density at 496 nm. Humic acids were extracted from 10 L of vermicompost with 100 L of 0.1 mol L^{-1} KOH solution overnight. The extract was siphoned and acidified with 6 M HCl to pH 2.0 and left to decant for 12 h. The soluble fulvic acids were separated by siphonation from the precipitated HAs. The HAs were washed with 500 mL of distilled water followed by centrifugation at 2760*g* for 15 min. All HAs were gathered and the pH was adjusted to 7.0 with 0.1 mol L^{-1} KOH and freeze dried. Total organic carbon content was analyzed by dry combustion using an automatic CHN analyzer (Perkin Elmer series 2400, Norwalk, USA).

Inoculum and inoculation

The inoculant was prepared by mixing equal volumes of the suspensions of *H. seropedicae* strain HRC54, *H. rubrisubalbicans* strain HCC103, and *G. diazotrophicus* strain PAL 5 to produce a final bacterial concentration of 2×10^8 cells mL^{-1} and 20 mg carbon L^{-1} of K^+ humate.

Experiment localization, soil samples, and climate conditions

The experimental field was located at commercial farm of sugarcane production at Campos dos Goytacazes, Rio de Janeiro, Brazil (41°142W, 21°442S; altitude 12 m). The soil was classified as fine clayey Fluventic Eutrochrepts according to U.S. Soil Survey. Ten subsamples were taken at 0–20 cm soil depth for soil analysis. The soil pH was determined for a 1:2.5 soil:water mixture agitated for 1 h. Exchangeable Ca, Mg, and Al were determined for a 1:10 soil:(1 mol L^{-1} KCl) mixture agitated for 10 min. Aluminum was analyzed by titrating this mixture with 0.015 mol L^{-1} NaOH and bromothymol blue indicator, and Ca and Mg were analyzed using an atomic absorption spectrophotometer. Exchangeable P and K were determined for a 1:10 soil:Mehlich-1 mixture (0.05 mol L^{-1} HCl and 0.0125 mol L^{-1} H_2SO_4) agitated for 10 min. Concentrations of K were analyzed using a flame photometer and P was analyzed using the colorimetric method with molybdenum blue and ascorbic acid as the reducing agents. Carbon was determined by oxidation with dichromate. The results of chemical analysis from soil samples are shown in Table 1.

The climate is tropical savannah (Aw) according to Köppen, with a mean temperature of 23.1 °C (mean of daily maximum 29 °C; mean of daily minimum 19 °C). Mean annual precipitation is 885 mm, with 70% of this rain concentrated from October to March. The annual precipitation values during the experimental period are shown in Table 2. The experimental site was amended with phosphorous (120 kg ha^{-1}) and potassium (64 kg ha^{-1}) fertilizers as recommended for sugarcane based on soil analysis in each growth season. The soil received one application of dolomite 1 month before

Table 1 Chemical analysis of the soil samples (0–0.20 m) at experimental sites

pH (water)	Al (Cmolc dm^{-3})	Ca + Mg	Ca	K	P (mg dm^{-3})	C (%)	N	Sand	Silt	Clay
5.4	0.4	6.6	4.3	0.2	6	1.2	0.1	20	37	43

Table 2 Annual precipitation (mm) in the experimental field. *Source*: Meteorological station of Federal Rural University of Rio de Janeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil

Years	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2013	193.9	9.4	300.2	57.8	34.4	13.0	53.6	82.6	50.5	30.8	194.0	274.0	1294.6
2014	10.0	7.8	68.8	123.8	4.40	35.4	147.4	22.2	12.0	17.6	90.6	35.6	575.4
2015	0.0	11.0	111.0	26.6	64.8	57.8	4.4	29.4	103.4	43.6	174.0	99.6	725.6
2016	153.6	38.6	12.6	27.4	14.40	44.6	12.4	27.20	24.6	72.4	148.8	188.0	764.6

planting at a rate of 2000 kg ha^{-1}. The nitrogen fertilizer (urea) was applied at 15 kg N ha^{-1} that corresponds to 1/3 of nitrogen dose as recommended by COPERSUCAR, a Brazilian cooperative association of 91 companies of industrial producers and sugarcane suppliers in each growth season.

Split plot completely randomized block design field experiment

The sugarcane variety RB 96 7515 was sown in March of 2013. The field trial was set up in factorial using a split plot on complete randomized block design with five replications. In the main plot, we compare the two delivering methods by the application of biostimulant in the furrow against foliar spray and in the split plot we compare the foliar application at three different times (60, 90, and 60 + 90 days after the emergence). The total parcel area was 1200 m^2 with five rows of 20 m with 1.5 m distance between rows. The biostimulant application on furrow in the parcel was done manually using a water can at a rate of 1 L per linear m. In the sub-parcel with 5 m, the foliar application of biostimulant at different times was done using a costal sprayer at a rate of 400 L ha^{-1}. The protective sheet of plastic material was used to avoid wind derivation during foliar application. In the control parcels or split plots just water was applied. The harvest was performed at August of 2014 (18 months of sugarcane plant growth) and August of 2015 and 2016 (ratoons after 12 months of growth).

Strip plot design field experiment

In the field strip plot experiment, we compare only the use of biostimulant after 60 days of planting by foliar spraying against the control. The plot was constituted by a 25-m-wide strip containing 15 rows of 50 m with 1.5 m distance between the rows. The application of the biostimulant at a rate of 400 L ha^{-1} was done using a tractor at constant pressure and velocity in an alternate strip using five replicates. Tem central rows were used for manual harvesting of the stems which are transferred to the truck for weighing in the commercial balance used on the farm.

The quality of sugarcane juice was evaluated by soluble solid content (BRIX) and polarizable sugars (POL). The Brix values were obtained directly, using a digital densimeter with a precision of 0.01 °BRIX. The POL measurements were obtained in a digital saccharimeter with a precision of 0.01. The samples of cane juice were initially cleared with lead sub-acetate ($Pb(CH_3COO)_2{\cdot}Pb(OH)_2$) and filtered before the measurements. The degree of polarization of the sample, expressed as % of juice, was calculated based on the saccharimeter reading (SR) using the equation POL = SR (0.2605 − 0.0009882 BRIX).

Results

The results of the analysis of variance (ANOVA) of both field experiments are shown in Additional files 1, 2, and 3. The plant cane cycles were harvested at 18 months after planting and the foliar application of the biostimulant showed better performance than furrow deliver (Table 3). The stalk yield increased 29% when compared to control representing an average of 21 tons ha^{-1}. The results of different times of foliar application of the biostimulant are shown in Table 4. The plant crop productivity increase 37% in comparison with control when the biostimulant was used by foliar spray as a single application at 60 days after planting (dap). As expected, the stalk production decreases in the following ratoons. No differences were observed among the treatments in the first ratoon while in the second ratoon the foliar application (60 dap) the sugarcane crop yield enhances 24% when compared to control representing 16 ton ha^{-1} more than the control area. The absence of positive results from the first ratoon harvest may be mainly attributed to severe drought stress that impacted the North of Rio de Janeiro state during the rainy season from November to March (Table 2). No differences were observed in the industrial quality of sugarcane in the plant crop or in the following ratoons measured by total solid content in the juice as well as in the N content in the leaves (Tables 3, 4; Additional file 1: Table S1, Additional file 2: Table S2).

The strip plot experiment was carried out in parallel using a large plot to confirm the results obtained in the split plot experiment simulating the farm conditions. Sugarcane stem yield from foliar application of

Table 3 Effect of the method of biostimulant application on sugarcane yield, total solid content in the juice (Brix), and N content of leaves in plant growth

	Plant growth		
	Control	Furrow	Foliar spray
	Stalk yield (Mg ha^{-1})		
	72.0b	68.4b	92.7a
Increase in relation to the control (%)	–	–	28.8
C.V. (%)	4.5		
	Brix (°Bx)		
	22.2	22.0	22.5
C.V. (%)	3.6		
	Total N content in leaves (kg ha^{-1})		
	208	202	211
C.V. (%)	6.3		

The harvest was done at 18 months after planting

Average values followed by different letters are distinct by Fisher's LSD test ($p < 0.05$). The increase in relation to the control was calculated by 100 * ($x - y$)/y, where x is the average of treatment and y is the average of control. C.V. (%) is the coefficient of variation of the experiment

biostimulant at 60 dap was higher than control in both subsequent ratoons with an average increase of 19 and 18% in yield, respectively, representing 11 and 13 tons ha^{-1} more than the control (Table 5). Again, no changes were observed in the juice quality and in the N content of leaves (Additional file 3: Table S3). The biostimulant increased the sugarcane yield without any reduction in their industrial quality.

Table 4 Effect of time of foliar application of biostimulant on sugarcane yield of plant growth and the first and second ratoons

	Stalk yield (Mg ha^{-1})			
	Control	60 dap	90 dap	60 + 90 dap
	Plant growth			
	72.0c	98.5a	86.7b	93.0a
Increase in relation to the control (%)	–	37%	20%	29%
C.V. (%)	6.6	–	–	–
	1st ratoon			
	63.9	66.9	66.2	65.0
C.V. (%)	6.15	–	–	–
	2nd ratoon			
	67.1c	83.4a	78.1b	82.3a
Increase in relation to the control (%)	–	24%	16%	23%
C.V. (%)	6.2	–	–	–

The treatments consist of the application of water (control) and biostimulant at a rate of 450 L ha^{-1}, applied one time at 60 and 90 days after planting and two times (60 plus 90 days after planting, dap)

Average values followed by different letters are distinct by Fisher's LSD test ($p < 0.05$). The increase in relation to the control was calculated by 100 * ($x - y$)/y, where x is the average of treatment and y is the average of control. C.V. (%) is the coefficient of variation of the experiment

Table 5 Effect of foliar application of biostimulant at 60 days after planting in sugarcane yield

	Stalk yield (Mg ha^{-1})	
	Control	Foliar application
	1st ratoon	
	55.2b	65.7a
Increase in relation to the control	–	19%
C.V. (%)	8.0%	–
	2nd ratoon	
	72.2b	85.2a
Increase in relation to the control (%)	–	18%
C.V. (%)	4.5	

The experiment was carried out using strip design with a large plot to simulate the crop conditions

Average values followed by different letters are distinct by Fisher's LSD test ($p < 0.05$). The increase in relation to the control was calculated by 100 * ($x - y$)/y, where x is the average of treatment and y is the average of control. C.V. (%) is the coefficient of variation of the experiment

Discussion

In this work, we observed the influence of the biostimulant manufactured with three strains of endophytic diazotrophic bacteria and humic acid-like substances isolated from vermicompost in the yield response of the sugarcane (plant cane and two ratoon cycles). Among the way and time application tested, we demonstrated that the best biostimulant delivering method was one single foliar spray at 60 dap per crop cycle (Table 4). The first attempt to use plant growth-promoting bacteria in sugarcane crop field in Brazil was made by Embrapa Agrobiologia, which recommended the immersion of stalks for 30–60 min inside containers filled with inoculant suspension before planting [18]. They observed an average increase of 13% (equivalent to 24 tons ha^{-1}) on crop yield without changes in ^{15}N balance, indicating that the plant growth-promoting benefits may have resulted from a better nutrient use efficiency than significant nitrogen input by biological nitrogen fixation in the soil–plant system. We also did not observe any changes in the nitrogen content in leaves treated with biostimulant produced with humic acids mixed with plant growth-promoting bacteria (Additional file 1).

Another way to apply beneficial bacteria in sugarcane crop field was proposed using farm yard manure (FYM) as vehicle sprinkling it over the sugarcane setts in furrows at plant crop initiation [19]. The application of inoculated FYM not only brought economy in

the use of mineral fertilizer N but it also increased the cane yield by 6 tons ha^{-1} over control. Another interesting approach results from the inoculation of micropropagated sugarcane under in vitro conditions in plantlets and after 5 days they were transferred to a nursery for additional 60 days for acclimation after field transplantation. In such method, positive inoculation effects by inoculation with diazotrophic bacteria were limited to low natural soil fertility and low N fertilizer application [20]. The large-scale operation of these three forms of the introduction of beneficial microorganisms is obviously limited, compromising the crop management and adding cost to the sugarcane production chain. We found just one paper on peer review scientific journal reporting the effect of humic acids on sugarcane yield at field conditions [21]. The cane setts dipped in 0.3% HA solution for 30 min before planting and soil application at 6 g m^2 increase the sugarcane yield from 3 to 9% when compared to control. We did not find any differences in the time of HA application in the yield of cane [22].

The application via foliar spray is a simple and easy practice to introduce beneficial microorganisms with similar or greater results than the other delivering methods for microbes in agriculture. We observed about 37% of increment in plant crop productivity at the first year representing 26 tons ha^{-1} more when compared to control. Being placed in a historical perspective, when compared over the long term, sugarcane productivity was observed to reach a plateau in Brazil and its performance over this period has lagged far behind the other major crops. Taking 1977/8 as a baseline until 2000s, maize in Brazil has increased productivity by 164%, wheat by 318%, and soybean by 149%, whereas sugarcane has only increased by 46% [22]. In addition, these enhancements were based in high-cost technologies like irrigation systems, land systematization, and harvest mechanization. Here we observed an average increase of 24% when compared to control considering both the experiments representing the half of the increase in productivity over 38 years [22]. This argument supports the use of humic substances together with plant growth-promoting bacteria as a very promising technology under economic and environmental perspectives. Furthermore, the sugarcane sector in Brazil has really undergone the greatest crisis in their history, with dozens of mills closing their doors and a growing number of mills entering "judicial recuperation" or facing rising debt levels with unemployment of thousands of workers [23]. The first immediate decision is crop field abandonment including crop fertilization. In this context, the use of biostimulant can be a low-cost technology to maintain or increase the production level since such an approach is more effective under low soil fertility and low N fertilizer rates.

At the second year of the experiment, the sugarcane producers from the southeastern of Brazil suffered the impact of severe drought that hit the region and the productivity falls. The large size of parcels in the strip plot design simulates the field conditions, and the yield increase remained even with the drought when compared to control although obviously at a lower level (Table 5). We observed previously in a controlled greenhouse experiment that sugarcane improves its drought recovery when treated with biostimulants produced with humic acids and plant growth-promoting bacteria [24]. Humic acids activated the antioxidative enzymes, while the plant growth-promoting bacteria induce metabolic changes that drive the cell osmoprotectant mechanisms. Both effects are combined when humic acids and plant growth-promoting bacteria were used together. These protective mechanisms against drought stress may have been responsible for a significant increase of sugarcane yield and have not been considered for researches and farmers. Finally, in addition to stress response probably induced by biostimulant application the increase of sugarcane yield is a consequence of the well-known effect of humic substances and plant growth-promoting bacteria in plant physiology. Nardi et al. [25] summarize the effect of humic substances on plant growth as a result of the enhancement of nutrient use efficiency, aiding the assimilation of both macro- and microelements and the induction of carbon, nitrogen, and secondary metabolism. Plant growth-promoting bacteria can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), quorum sensing (QS) signal interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance, promoting beneficial plant–microbe symbioses, interference with pathogen toxin production, etc. [26]. Here we showed that a mixture of plant growth-promoting bacteria and K^+ humates is a technological innovation that increases the sugarcane stalk production.

Conclusions

The use of the biostimulant manufactured by humic acids and plant growth-promoting bacteria was effective to increase sugarcane production at crop fields. This innovative technology base of chemical properties of humic matter and biological effect of beneficial bacteria results in both economic and environmental advantages when used with lower nitrogen fertilization.

Additional files

Additional file 1: Table S1. Analysis of variance (anova) from factorial experiment using complete randomized blocks with split plot arrangement to sugarcane plant growth (18 months after planting).

Additional file 2: Table S2. Analysis of variance (anova) from factorial experiment using complete randomized blocks with split plot arrangement to first and second ratoon both with 12 months after harvest.

Additional file 3: Table S3. Analysis of variance (anova) from strip plot experiment.

Authors' contributions

SFL carried out the field experiments that consist part of his master thesis. LPC and FLO were responsible for the concept and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Supplemental Tables were attached in the Additional files 1, 2, and 3. Other data and materials could be requested from the corresponding author.

Consent for publication

The authors agreed to the publication of the manuscript in this journal.

Ethics approval and consent to participate

This manuscript is an original paper and has not been published in other journals. The authors agreed to keep the copyright rule.

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