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Nodulation of beans with inoculant carriers from pyrolyzed and non-pyrolyzed sugarcane bagasse in response to different pre-planting water availability

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ABSTRACT

Over the past few decades elite strains of rhizobia have become commercially available for agricultural production. However, these elite rhizobia are often not as competitive as native strains under adverse edaphic conditions, primarily concerning tolerance to soil desiccation. Biochar has been proposed as a soil amendment to reduce water stress. The effect of biochar made from sugar cane bagasse on rhizobium survival and inoculation of beans was tested in comparison to uncharred sugarcane bagasse and an unamended control in the greenhouse using a gradient of soil-sand mixtures (0%, 25%, 50%, 75%, 100% sand in the mixture) under two watering regimes over eight weeks between inoculant addition and planting in comparison to a control where inoculants were applied at planting. For the control, shoot growth did not differ between the charred or uncharred carrier materials ($P > 0.05$). However, the number of nodules was ten- and 13-fold greater with uncharred bagasse over biochar or control carriers, respectively. When pots were allowed to dry for eight weeks between inoculant addition and planting, the bagasse carrier was the only carrier that resulted in root nodules. With intermittent between inoculant application and planting, shoot biomass with the biochar carrier was 147 and 151% greater than with the bagasse and control carriers, respectively. Under the same intermittent drying, nodule number using the bagasse carrier was 925% greater than that with the biochar carrier, while nodules were absent in the control. DNA fingerprinting of the root nodules indicated that nodule occupancy was dominated by native rhizobia and not the introduced strain. However, occupancy of the introduced CIAT899 in bean nodules (1–38%) was significantly greater than expected values based on carrier application rates (2–7%), irrespective of carrier.

1. Introduction

Legumes and biological nitrogen fixation (BNF) are fundamental components to both natural and agricultural ecosystems. BNF accounts for 50–90 Tg N yr^{-1} and around 40 Tg N yr^{-1} of N inputs in natural and agricultural ecosystems, respectively (Galloway et al., 1995). In smallholder agricultural systems in developing countries BNF is often the primary source of N inputs because of limited availability of synthetic N fertilizers due to logistic and economic constraints (Mueller et al., 2012). BNF may also reduce the dependence on external N sources in high-input agriculture (Westhoff, 2009). Efficient BNF requires intricate biological interactions between the host legume plant and the rhizobial symbiont. This symbiosis can be negatively affected by several edaphic conditions, principally nutrient availability, soil pH, temperature, and drought stress (Zahran, 2001). Of these, drought stress will be of increasing importance under the current projections of global climate change (Wang, 2005; Chadwick et al., 2016).

Survival of rhizobia under drought stress depends on both the adaptability of the rhizobia strain to desiccation and on the ability of the soil to buffer against changes in water status. While some free-living rhizobia have shown high survival rates under desiccation (Fuhrmann et al., 1986), many of the commercial strains have a much lower desiccation tolerance (Evans, 2005; Mnasri et al., 2007; Shoushtari and Pepper, 1985). Increasing the ability of soil to buffer against desiccation is a potent strategy as the benefits affect the rhizobia community irrespective of strain sensitivity.

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Table 1
Selected soil and organic amendment physicochemical properties. Each soil also was used as the control carrier. Particle size distribution of the pure soil is 8-51-39 sand, silt, and clay, respectively.

Soil	Sand	pH	Total C	Total N	CEC[a]	NO_3^{-}[b]	NH_4^{+}[b]	P[c]	K[c]	Ca[c]	Mg[c]	Mn[c]	Fe[c]	S[c]	Al[c]	Bulk density	Porosity
(%)	(%)	H_2O	(%)		($mmol_c\ kg^{-1}$)	($mg\ kg^{-1}$)										($g\ cm^{-3}$)	(%)
100	0	6.26	2.18	0.20	296	83.7	13.3	7	403	2017	277	509	61	33	969	0.84	62
75	25	5.68	1.46	0.12	134	36.1	10.4	9	274	1326	185	511	76	32	989	0.98	57
50	50	5.00	0.91	0.08	133	10.8	4.8	19	246	662	115	469	94	28	1409	1.13	53
25	75	4.48	0.40	0.03	68	3.9	3.4	31	136	434	86	457	123	33	1582	1.28	47
0	100	3.29	0.04	0.00	6	1.2	7.6	16	115	25	13	9	55	3	751	1.42	41
Biochar		9.58	68.63	2.50	43	0.0	11.0	1	6	2	1	1	12	0.3	12	0.20	86
Bagasse		5.25	43.32	2.80	–	0.7	68.8	0.7	0.8	2	0.8	0.5	8	0.5	3	0.17	97

[a] Cation exchange capacity NH_4OAc at pH 7.
[b] 2 N KCl extraction.
[c] Mehlich-3.

One proposed way to increase the resilience of soil biota against desiccation is the application of biochar to soil (Kammann et al., 2011; Karhu et al., 2011). Biochar is derived from the thermal transformation of organic materials under reduced oxygen conditions. The porosity and surface area of many plant biomass-derived biochars can be high (Enders et al., 2012; Keiluweit et al., 2010; Kloss et al., 2012), and it has therefore been hypothesized that the moisture held in these pores might reduce desiccation of microbes (Lehmann et al., 2011). Concurrently applying rhizobia with biochar as an inoculant carrier (Lehmann et al., 2011; Hale et al., 2014; Sun et al., 2016; Głodowska et al., 2017; Egamberdieva et al., 2018) may therefore provide greater drought resilience (Hale et al., 2015). Indeed, rhizobia survival was greatly enhanced under inoculant storage conditions without soil, when biochars were able to reduce water stress of the microorganism (Vanek et al., 2016). Drought effects with simultaneously added biochar to soil on rhizobia survival have only rarely been studied (Hale et al., 2015; Egamberdieva et al., 2017), and we are not aware that periodicity of drought has been investigated.

As a low-cost alternative to established, but expensive inoculant carriers, such as peat, by-products from the sugarcane industry have been widely used as inoculant carriers in many countries (Rebah et al., 2007; Dotaniya et al., 2016). Such press mud and bagasse were very effective in promoting rhizobia survival under inoculant storage conditions (Vanek et al., 2016). In soil, sugarcane bagasse may decompose much more rapidly than biochar produced from it (Jeong et al., 2016), but may at the same time promote soil biota abundance (Sharma et al., 2014). Biochars made from sugarcane bagasse are increasingly examined as a way to increase value of this waste product (Quirk et al., 2012; Bernardino et al., 2017). Whether provision of decomposable organic matter by sugarcane bagasse for rhizobia survival is more important than mitigation of soil moisture deficits in comparison to biochar is not clear.

Therefore, the objectives of this study were (i) to test the ability of pyrolyzed and unpyrolyzed sugarcane bagasse to protect rhizobia from desiccation following induced water stress; and (ii) to evaluate this effect along a simulated soil texture gradient using different proportions of sand added to a fine-textured soil. The hypotheses tested were: (i) biochar carriers will promote greater survival of introduced rhizobia and nodulation of host plants following water deficit events than the uncharred bagasse or no additions; (ii) use of biochar as a carrier will increase competitiveness of biochar-associated rhizobia than rhizobia lacking a carrier especially with a sandier texture and induced drought stress between inoculant addition and planting.

2. Methods

2.1. Experimental design

To test the hypotheses, a fully factorial randomized complete design experiment was executed in pots in a greenhouse. The treatments consisted of simulating a soil textural gradient by adding different proportions of sand by volume to a fine-textured soil, three different inoculant carrier materials, and two different soil moisture regimes between inoculant addition and planting in comparison inoculant application at planting. All treatments were replicated five times (Table S1).

2.2. Soil collection

A humic Acrisol was collected from the top 0.1 m of soil from a site in Kapsengere village in the highlands of western Kenya that had been converted to agriculture in the year 1900 (Guerena et al., 2016; Kimetu et al., 2008; Kinyangi, 2007; Ngoze et al., 2008). The soil was air-dried, passed through a 2-mm sieve, and homogenized.

A quartz-feldspar sand was collected from alluvial and lacustrine deposits in a quarry adjacent to Lake Victoria northwest of Kisumu, Kenya. The sand was washed with water four times to remove clay and silt and then acid-washed (10% HCl) and rinsed four times with water. After rinsing the washed sand was air-dried and sieved to 2 mm for use in the experiment.

Five soil textural combinations were prepared by adding different volume amounts of the sand to the Acrisol, namely: 100:0, 75:25, 50:50, 25:75, and 0:100 v:v soil:sand, generating variable mass ratios (Table S2). Physicochemical and moisture retention characteristics of these soils are listed in Tables 1 and 2.

2.3. Soil analysis

Soil mineral nutrient analysis was performed on the soil prior to inoculant carrier applications. Soil pH was measured in 1:2 (w/v) soil:deionized water. Mineral N was extracted with 2 N KCl and quantified colorimetrically using a continuous flow autoanalyzer (Braun and Luebbe Autoanalyzer, SPX, Charlotte, NC, USA). Exchangeable

Table 2
Moisture retention characteristics (water:soil w/w) using a pressure plate apparatus for the biochar, bagasse, and sand/soil carrier materials at the beginning of the experiment ($n = 1$).

Soil	Sand	Applied pressure (kPa)				
(%)	(%)	1	3	98	294	1471
100	0	0.42	0.40	0.30	0.26	0.23
75	25	0.28	0.28	0.18	0.17	0.14
50	50	0.16	0.15	0.11	0.10	0.08
25	75	0.09	0.07	0.05	0.05	0.04
0	100	0.02	0.01	0.01	0.01	0.00
Biochar		1.65	1.28	0.46	0.26	0.25
Bagasse		1.28	1.15	0.89	0.75	0.65

nutrients and plant-available P were determined by Mehlich-3 extraction (Mehlich, 1984). Cation exchange capacity was determined by ammonium acetate extraction buffered at pH 7 (Sumner and Miller, 1996). All extracts were analyzed by inductively coupled plasma mass spectrometry (ICP 61E, Thermo Electron, Waltham, MA, USA).

2.4. Biochar production

Sugarcane bagasse was collected from the Kibos Sugar factory in Kisumu, Kenya. The bagasse was air-dried and hammer-milled to pass a 200-μm sieve. Biochar was produced from this milled bagasse at 550 °C in a slow-pyrolysis kiln with continuous agitation using a motorized paddle. The kiln temperature was increased by 5 °C min^{-1} to 550 °C, followed by a 45-min dwell time at 550 °C. After the dwell, steam was injected into the kiln until the temperature had cooled to 120 °C, and then both the steam and the kiln power were shut off.

2.5. Inoculant production

Three different inoculant carriers, or carrier mixtures, were used. These include biochar (Hale et al., 2014, 2015), bagasse (Dotaniya et al., 2016), and a control using soil from each of the soil/sand mixtures as the carrier.

Thirty-three grams each of biochar or dried, sieved bagasse were placed separately into 45 × 90-mm high-density polyethylene (HDPE) bags and were heat-sealed shut. Soil from each of the soil/sand mixtures were also placed into HDPE bags at equivalent volumes to the biochar and bagasse treatments. The mass of these soils therefore varied and were 154.77 g for the pure soil, 263.72 g for the pure sand, 182.01 g, 209.24 g, and 236.48 g for the 75:25, 50:50, and 25:75 treatments, respectively. After sealing, all of the HDPE bags of biochar, bagasse, and soil were autoclaved for 25 min at 120 °C. The soil moisture retention characteristics for carrier materials were measured before autoclaving using a pressure plate apparatus (Soil Moisture Equipment Corp., Goleta, CA) and are given in Table 2.

Inoculant preparation was done at the MIRCEN lab located at the University of Nairobi, Nairobi, Kenya. An initial most probable number (MPN) of native rhizobia in the soil was taken for the pure soil (Somasegaran et al., 1985). MPN is a method used to estimate the concentration of viable microorganisms in a sample, soil or other, through growth in replicated liquid broth. In this study, the MPN was used to estimate the concentration of colony forming units (CFU) of rhizobial bacteria in the soil through counting root nodules on bean plants grown in serial dilutions of soil, in order to match this number with the number of CFUs in the inoculant carriers.

The MPN of the soil was 8.4×10^{-1} CFU cm^{-3}, which is a low value commensurate with low nodule formation found for beans grown in the same soil in a prior experiment (Guerena et al., 2015). Initial aliquots of sterile deionized water were added to each sterilized HDPE bags of soil, biochar, and bagasse using sterilized syringes through septa stuck to the bags to bring the moisture content to 20% (w/w). The bags were massaged by hand to evenly distribute the water in the bags and the bags were then allowed to rest overnight at room temperature. A liquid culture of the *Rhizobium tropici* strain CIAT 899 was grown in yeast mannitol broth (YMB). The CFU of the YMB broth was estimated via growth plates. A dilution series of aliquots of the YMB culture was created to match the CFU of each particular soil mixture. The YMB aliquots were added to each bag of inoculant material (biochar, bagasse, sand/soil) to bring the total moisture content of each bag to 60% (w/w) and to ensure the concentration of rhizobium CFU in each bag was equal to the MPN of the soil. A subsample of each inoculant mixture was reserved and used to determine the MPN of the inoculant mixture at the time of incorporation into the soil. Therefore, an increase in nodule occupancy above the natural abundance of rhizobia can be interpreted.

2.6. Greenhouse set-up and management

Air-dry soil, sand or soil/sand mixtures (Table S2) were added to plastic pots (0.17 m diameter and 0.14 m height), filling the pot to within 10 mm of the pot rim. Sufficient amounts of no-N fertilizer were added to each pot at 0.09 g pot^{-1} and homogenized, in order to alleviate any nutrient constraints other than N and account for differential nutrient additions with the carrier materials (i.e., any differences in plant growth or biological N fixation should not be a result of the availability of the added nutrients with different soil/sand mixtures and additions of carrier materials for those nutrients in the fertilizer). The added fertilizer contained 54 mg (60% w/w) of monocalcium phosphate $Ca(H_2PO_4)_2{\cdot}H_2O$, 27 mg (30% w/w) of KCl, 6.3 mg (7% w/w) of $MgSO_4$, 0.9 mg (1% w/w) $Na_2B_4O_7{\cdot}10H_2O$, 0.9 mg (1% w/w) $MnSO_4$, and 0.9 mg (1% w/w) ZnO.

One bag of prepared inoculant and its carrier was added to each of the pots containing a particular soil/sand mixture (Table S2) in a full factorial design with five replicates and homogenized. For biochar and bagasse, the 33 g of carrier material in each bag constituted an application rate by weight of 1.0–1.7% w/w or 15 t ha^{-1} as done previously on the same soil (Guerena et al., 2015). For soil/sand carriers, the variable weight of carrier material in each bag constituted an application rate by weight of 7.9–8.1% w/w (Table S2).

Two separate water regimes were implemented after addition of the inoculant with their carriers to the different soil/sand mixtures and before planting in comparison to adding the inoculant at planting. One set of soils was watered to field capacity and allowed to dry over eight weeks (WD1). A second set of soils was watered to field capacity and allowed to dry for one week, then re-watered to field capacity (WD4). The wetting and drying of this treatment continued for eight weeks (four cycles). At the end of eight weeks both WD4 and WD1 treatments were watered to field capacity. At this time a third set of fresh inoculants was made and incorporated into an additional set of soil then watered to field capacity (WP), serving as a control that underwent no moisture stress. All treatments were planted with *Phaseolus vulgaris* L., variety KK15 (Kenyan Agriculture Research Institute, Kakamega, Kenya) on 20 May 2013 and harvested on 25 June 2013. Three seeds were planted per pot and thinned to one seedling one week after emergence. Once planted, the pots were maintained at field capacity for the remaining duration of the experiment by drip irrigation (The Drip Store, Vista, CA, USA). Field capacity was gravimetrically determined.

At harvest, the roots were separated from the shoots, placed into separate paper bags and dried at 60 °C in a forced air oven. Prior to drying, the roots were washed and nodules were separated from the roots and placed in a plastic bag containing silica gel desiccant.

2.7. Rhizobium culture and DNA extraction

All of the root nodules from each experimental unit were rehydrated overnight in 10 mL of sterile deionized water. The next day the water was decanted and 10 mL of a 0.8% bleach solution was added to surface sterilize the nodules. The root nodules were left in the bleach solution for 4 min then rinsed six times with sterile deionized water. Each nodule was then transferred individually to a 1.5-mL centrifuge tube (Eppendorf, Hamburg, Germany) along with 50 μL of sterile deionized water. Once the nodules were placed into the tubes, they were gently crushed with sterilized forceps to release the inner contents and briefly vortexed (VWR, Randnor, PA, USA). The crushed nodule suspension was then streaked onto plates with yeast mannitol agar (YMA) containing congo red. The plates were incubated at 30 °C until bacterial colonies were noted. One colony from each successful rhizobia culture was streaked and grown on YMA plates without congo red at 30 °C. These cultures were transferred and incubated at 30 °C in 3 mL of YMB in sterilized glass test tubes on an orbital shaker.

DNA was extracted from the YMB cultures once the media became turbid, after approximately 24 h, using an UltraClean® microbial DNA

isolation kit (MO BIO Laboratories, Carlsbad, CA, USA). The DNA samples were stored at −20 °C prior to amplification.

Polymerase chain reactions (PCR) for DNA extracted from the YMB cells were completed using a nested approach. The primers REP 1R (5′-IIIICGICGICATCIGGC-3′) and REP 2 (5′-ICGICTTATCIGGCCTAC-3′) were used to amplify the 16S portion of the DNA. All PCRs contained nuclease-free dionized water, 10× PCR buffer, 2 μmol L^{-1} $MgCl_2$, 80 μmol L^{-1} dNTP, 700 nmol L^{-1} of each primer, one unit of Sigma REDTaq Genomic Polymerase (Sigma-Aldrich, St. Louis, MO USA), and 0.5 μL of template DNA per 25 μL reaction. DNA was amplified with a PTC-200 (MJ Research, St. Bruno, Canada) thermal cycler. The PCR conditions were as follows: (1) 95 °C for 3 min, (2) 94 °C for 60 s, (3) 55 °C for 60 s, (4) 65 °C for 8 min, (5) repeat 1–4 34 times, (6) 65 °C for 16 min, (7) hold at 10 °C.

After the PCR was completed, the PCR product was mixed with 1 μL of 1:100 SYBR green and 1 μL loading dye. Five μL of this solution were added to each well in a 1.5-% TAE agarose gel. Four μL of 1Kb DNA ladder were added to the initial and final columns of each row. A positive control of DNA extracted from a pure culture of CIAT 899 was added to each gel, as well as a negative control. The gels were imaged using an EC3 Imaging System (UVP LLC, Upland, CA, USA).

2.8. Statistical analysis

All statistical analyses were performed with JMP software (SAS, Cary, NC). All procedures were performed at $P < 0.05$, unless otherwise indicated. Significant treatment effects were determined using the Tukey's HSD or Student's-*t*-tests.

3. Results

3.1. Shoot biomass

On average, different watering prior to planting or adding the inoculant at planting did not significantly affect shoot biomass (Table 3). Shoot biomass was greatest across all water treatments in the pure soil and was lowest in the pure sand with the greatest gradient under alternate wetting and drying (WD4) (Fig. 1). Only biochar, but not bagasse, increased plant growth in only two cases that depended on both watering before planting or direct application and the proportion of sand in the soil/sand mixture (significant interactions, Table 3). When inoculants were added to pure sand immediately before planting (WP), shoot biomass increased the most with the biochar carrier by 220% over the bagasse carrier and by 190% over the control carrier (Fig. 1). With periodic drying and wetting between inoculant addition and planting (WD4) in the pure soil, shoot biomass was about 250% greater with the biochar carrier relative to the bagasse and control soil carrier materials. With 25% sand the shoot biomass was 179% greater with the biochar carrier relative to the bagasse carrier. In one case under complete drying (WD1) between inoculant addition and planting in the 50% sand-soil mixture, the use of biochar or bagasse decreased plant growth compared to the control.

Table 3
Statistical significance of the main treatment effects.

Source	Shoot biomass (g pot^{-1})	Nodules (Number)	Nodule biomass (mg pot^{-1})
	P-value		
Carrier (C)	0.0009	< 0.0001	< 0.0001
Watering (W)	0.1856	0.4146	< 0.0001
Soil/sand mixture (S)	< 0.0001	0.0213	< 0.0001
C*W	< 0.0001	0.1926	< 0.0001
C*S	< 0.0001	< 0.0001	< 0.0001
W*S	0.0007	0.0099	< 0.0001
C*W*S	< 0.0001	0.0134	< 0.0001

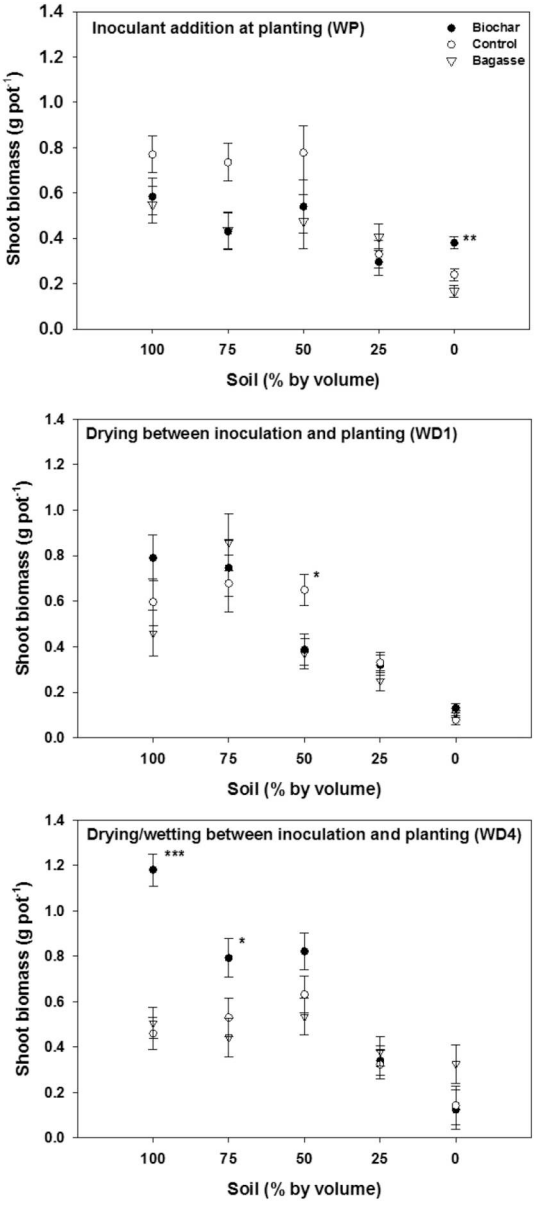

Fig. 1. Shoot biomass in response to biochar, bagasse and soil/sand inoculum carriers, to the proportion of sand mixed into soil, and to watering treatments between inoculant addition and planting in comparison to inoculant addition at planting (inoculant addition to soil adjusted to field capacity at planting: WP; drying for 8 weeks between inoculant addition and planting: WD1; alternate wetting and drying between inoculant addition and planting: WD4). Presence of asterisks indicates significant differences (Tukey's HSD, *0.05 > P < 0.01, **0.01 > P < 0.0001, ***P < 0.0001, $n = 5$). Error bars are standard error of the mean (data shown in Supplementary Tables S4–S6).

3.2. Nodulation

Varying the soil water content alone between inoculant addition and planting or adding inoculants at planting did not significantly affect nodulation and only under a few but notable conditions influenced how the different inoculant carriers improved nodulation (Table 3). Irrespective of watering between inoculant addition and planting or direct application at planting, the bagasse carrier increased nodulation several-fold over the biochar and control carriers in all soil/sand mixtures, but not in the pure sand (Fig. 2). With direct application to the pure sand at planting, nodulation with the biochar carrier was 3 and 29 times greater than with the bagasse and control sand carriers,

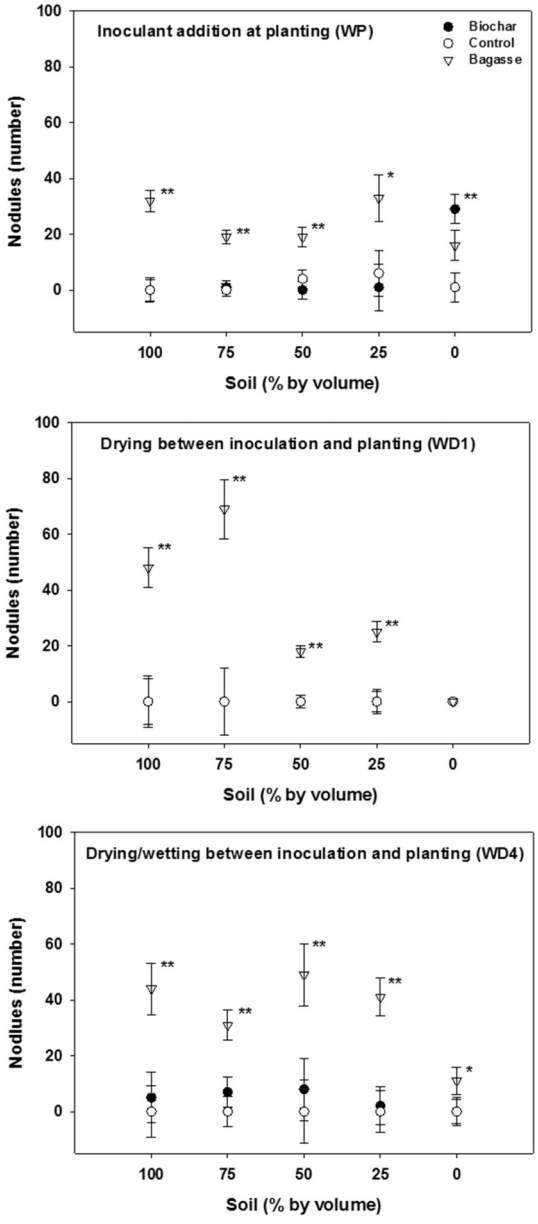

Fig. 2. Number of root nodules in response to biochar, bagasse and soil/sand inoculum carriers, to the proportion of sand mixed into soil, and to watering treatments between inoculant addition and planting in comparison to inoculant addition at planting (inoculant addition to soil adjusted to field capacity at planting: WP; drying for 8 weeks between inoculant addition and planting: WD1; alternate wetting and drying between inoculant addition and planting: WD4). Presence of asterisks indicates significant differences (Tukey's HSD, *$0.05 > P < 0.01$, **$0.01 > P < 0.0001$, ***$P < 0.0001$, $n = 5$). Error bars are standard error of the mean (data shown in Supplementary Tables S4–S6).

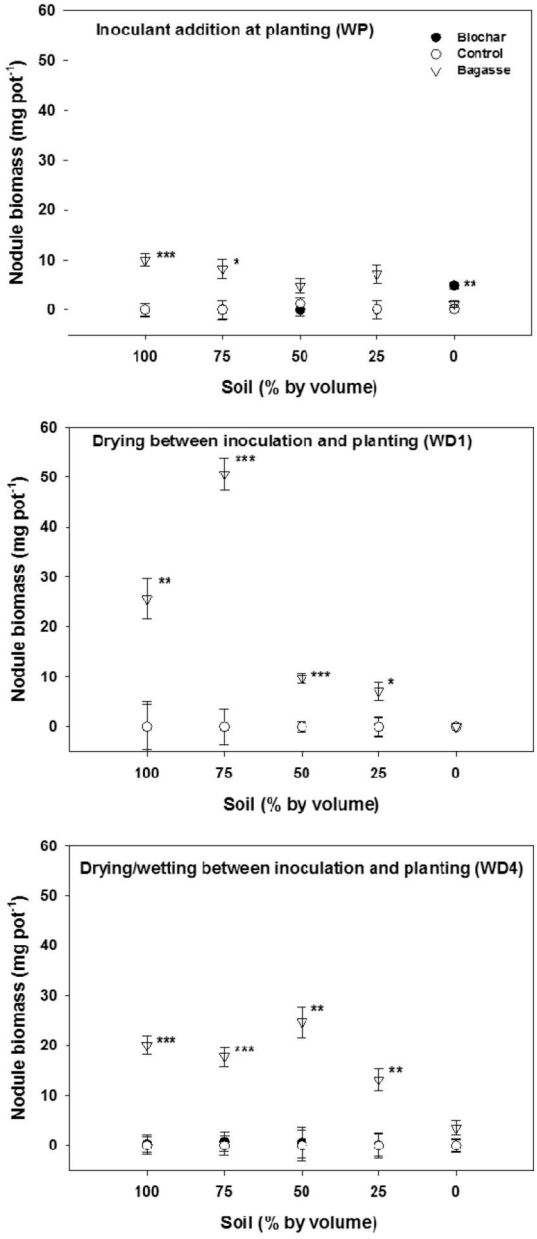

Fig. 3. Root nodule biomass in response to biochar, bagasse and soil/sand inoculum carriers, to the proportion of sand mixed into soil, and to watering treatments between inoculant addition and planting in comparison to inoculant addition at planting (inoculant addition to soil adjusted to field capacity at planting: WP; drying for 8 weeks between inoculant addition and planting: WD1; alternate wetting and drying between inoculant addition and planting: WD4). Presence of asterisks indicates significant differences (Tukey's HSD, *$0.05 > P < 0.01$, **$0.01 > P < 0.0001$, ***$P < 0.0001$, $n = 5$). Error bars are standard error of the mean (data shown in Supplementary Tables S4–S6).

respectively. No nodules were observed for the control soil/sand carriers when applied eight weeks ahead of planting with induced moisture stress in most soil/sand mixtures.

3.3. Nodule biomass

In contrast to plant growth and nodule numbers, water treatments significantly affected nodule biomass (Table 3) that was lowest when inoculants were added to moist soil at planting. Similar to nodulation, nodule biomass decreased with more sand in the mixture and was typically several-fold greater when bagasse was used as an inoculant carrier in comparison to biochar, except for in pure sand (Fig. 3). With only sand, nodule biomass was 4- and 3-fold greater with the biochar carrier over the bagasse and control carriers, respectively, yet with equal average nodule mass between the two carriers (Supplementary Table S3).

3.4. Nodule occupancy

Many of the root nodules could not be cultured and viable microbial isolates could not be obtained for ten of the treatments (Table 4). The

Table 4
Nodule occupancy assessed by DNA fingerprinting of culturable root nodules of *P. vulgaris* plants inoculated with the *R. tropici* strain 899 using biochar and bagasse carrier materials. Application of rhizobia with carriers either directly at planting with soil adjusted to field capacity (WP) or after alternate drying and wetting to field capacity for eight weeks before planting (WD4). Different letters indicate significant differences between amendments, no letters are shown when main effect is not significant (Student's *t*-test, $P < 0.05$, $n = 5$). X^2 *P*-values indicate significance of difference to expected abundance of CIAT899 of 1.7–6.7% based on applied amounts as a proportion of native rhizobia. NC = not culturable, NN = no nodules.

		Soil (%)					X^2
		100	75	50	25	0	
		Sand (%)					
		0	25	50	75	100	
Inoculants applied at planting (WP)							
Culturable nodules (%)	Biochar	NC	11	24	10	NC	–
	Bagasse	10	NC	10	10	NC	–
	P-value	–	–	0.2301	0.9739	–	
899 nodules (% of culturable)	Biochar	NC	0	14	18	NC	$P < 0.0001$
	Bagasse	34	NC	23	38	NC	$P < 0.0001$
	P-value	–	–	0.5981	0.4469	–	
Inoculants applied after 4 weeks of alternate wetting and drying (WD4)							
Culturable nodules (%)	Biochar	46	8.75	7	19A	NN	
	Bagasse	66	NC	17	10B	NN	
	P-value	0.8122	–	0.4007	0.0347	–	
899 nodules (% of culturable)	Biochar	4	25	0	2	NN	$P < 0.0001$
	Bagasse	1	NC	22	8	NN	$P < 0.0001$
	P-value	0.1515	–	0.3439	0.2790	–	

nodule occupancy (the particular strain of rhizobia cultured from each nodule) of the culturable nodules was not significantly correlated to culturability of the rhizobia ($r^2 = 0.009$, $P = 0.5115$). This reduces the concern for bias resulting from certain strains of rhizobia (either native or introduced) being more easily cultured. Nodule occupancy of CIAT899 did not significantly differ between using biochar and bagasse carriers in any of the soil/sand mixtures (Table 4). However, nodule occupancy of the introduced CIAT899 lay between 1 and 38% of all rhizobia in the nodules. This was significantly greater (x^2, $P < 0.0001$), irrespective of watering, than the proportion of CIAT899 that we added with the inoculants to soil at 1.7–6.7% of all rhizobia in the nodules.

4. Discussion

4.1. Responses of introduced rhizobia

Irrespective of watering between inoculant addition and planting or direct application at planting, both the biochar and bagasse carriers resulted in greater proportions of the introduced rhizobia strain CIAT899 in the bean nodules than expected. To our knowledge CIAT899 had not been introduced to these soils prior to our experiment. We observed a lower proportion of culturable nodules and the lower occupancy of the introduced CIAT899 strain after 8 weeks of intermittent drying and rewetting between inoculant addition and planting than direct application of the inoculant at planting. These observations may be explained by greater mortality during an initial rapid moisture decline than during later stages of slow drying (Pena-Cabriales and Alexander, 1979). As this alternating water treatment induced a greater water deficit and lower occupancy of the introduced rhizobia than direct application at planting, the observed significantly greater proportion of culturable nodules with biochar than bagasse carriers with 75% sand in the soil/sand mixture, suggests that the biochar carrier might protect the introduced rhizobium from desiccation to a greater extent than bagasse.

Surprisingly, bagasse was as effective as biochar in promoting the survival of the introduced rhizobia, except in the one case mentioned above. Several studies have found that the additions of simple sugars to soil as an energy source stimulated the populations of soil rhizobia (Acea et al., 1988; Pena-Cabriales and Alexander, 1983). As the bagasse is the byproduct of sugar production, it is possible that the residual sugars and other easily mineralizable C material found in the bagasse resulted in a similar stimulation in this experiment. This could account for the greater occupancy with the introduced CIAT899 using uncharred bagasse as a carrier than with the soil/sand carrier. This is further supported by greater effectiveness of press mud (another byproduct of the sugar processing industry) as an inoculant carrier over some biochars (Kibunja, 1984; Woomer, 2013) and of more rapidly decomposable hydrochar over pyrolysis biochars (Egamberdieva et al., 2017). Washing press mud with acetone and 1% HCl decreased the survival of rhizobia when used as a carrier material relative to unwashed press mud (Vanek et al., 2016) supporting the importance of easily available organic C.

4.2. Responses of native rhizobia

The majority of the rhizobia in the bean nodules came from native strains rather than from the CIAT899 strain introduced with the inoculant carriers. The only significant increase in total nodulation or nodule biomass (dominated by native rhizobia) with biochar over no additions was observed when the biochar inoculant was added directly at planting in the pure sand, as would be done under many common inoculant management strategies. This improvement of nodulation compared to soils with less or no sand was unlikely related to carrier-induced reduction in desiccation, since the soil moisture was adjusted to field capacity. Since there was little time between inoculant carrier addition and likely infection of the beans, enhanced growth of the rhizobia by added metabolizable organic matter, as discussed above, played a minor role with the inoculant addition at planting. This is also evident from the lower nodulation and nodule mass of total rhizobia with direct application than with induced water stress across most soil/sand mixtures. Explanations for the improved nodulation and nodule mass with biochar in pure sand may include improved habitat properties (Vanek et al., 2016) with rapid electron shuttling (Sun et al., 2017) and modulation of intra-specific (Masiello et al., 2013) or plant-rhizobia signaling (Lehmann et al., 2011), which would need to be investigated in the future.

Even though the pure sand had the lowest pH and CEC of any of the

Table 5
pH of soil amended with biochar and bagasse-based inoculum carriers in comparison to soil/sand carrier. pH was measured in 1:2 soil:deionized water w/v (field capacity before planting). Different letters indicate significant differences, no letters are shown when main effect is not significant (Student's *t*-test, $P < 0.05$, $n = 5$).

Carrier	Soil (%)				
	100	75	50	25	0
	Sand (%)				
	0	25	50	75	100
Biochar	5.84 B	5.88	5.62	5.70	5.13
Bagasse	5.97 A	6.02	5.87	5.74	4.85
Control	5.80 B	5.82	5.71	5.70	5.02
P-value	0.0033	0.2197	0.4416	0.9349	0.4660

soils (Table 1), the pH did in general not increase after incorporation of the different carrier materials (Table 5). Whether pH effects around biochar particles could explain the differences in nodulation that are undetected by bulk soil pH measurements (Lehmann et al., 2015), was not assessed here but may be a possibility.

Mitigation of water stress appeared to be a minor reason, why bagasse aided in survival of native rhizobia to a greater extent than biochar. When water stress was induced throughout or intermittently between inoculant addition and planting, the bagasse carrier resulted in greater total nodulation than either the biochar or control carriers irrespective of the proportion of sand added to soil. This may be partially explained by the greater water content of the bagasse carrier than the biochar and control carrier materials at greater applied pressure and therefore lower moisture availability (Table 2). However, if alleviation of moisture stress was the primary cause for the greater nodulation, we would expect to see this effect in the pure sand and we would also expect to see greater nodule occupancy of CIAT899 with the bagasse carrier. Neither of these was found to be the case. The data, therefore, suggest that the bagasse is also acting as a growth promoter by adding metabolizable sugars for the native strains of rhizobium (Acea et al., 1988; Pena-Cabriales and Alexander, 1983), as discussed above, in addition to possibly providing a refuge against desiccation. Hence, following lower water availability, the native soil rhizobia are responding positively to both the presence of the residual sugars in the bagasse upon rehydration and the soil moisture retained by the added bagasse carrier. This then resulted in greater nodulation by using the bagasse carrier than either the biochar or control carriers. This corroborates the observations when inoculants were added at planting to moist soil.

4.3. Shoot biomass response

Differences in plant growth were most likely unrelated to differences in biological N fixation, as rhizobial response to the biochar carrier in these soils was poor. Even though the pH of the biochar carrier was basic (Table 1), pH changes induced with the biochar carrier would not be expected to promote plant productivity as the pH of the native soil was within the optimum range for the growth of common beans. In addition, bulk pH values of soil amended with the biochar carrier did not increase (Table 5), making it unlikely that pH induced differences in plant growth. In a study that used the same soil and biochar application rates, the authors found applications of biochar manufactured from bagasse under the same production conditions also increased bean shoot growth, albeit with greater effects on nodule abundance (Guerena et al., 2015). These changes in plant growth were correlated to greater soil P uptake as a result of greater abundance of mycorrhizae (Guerena et al., 2015). It is possible that these responses also occurred in our experiment, even though with overall lower effects.

While we tried to account for nutrient deficiencies by full fertilization, it is also possible that the biochar is alleviating other nutrient deficiencies not accounted for (e.g., Mo, see Rondon et al., 2007). Nutrient additions other than N with biochar manufactured from bagasse would be greater than the equivalent additions of nutrients in uncharred bagasse. When a material is pyrolyzed the proportion of the mineral components (e.g. Ca, Mg, P, etc.) increase due to the volatilization of the H, O, and C (Enders et al., 2012). However, the effect of nutrient additions was not directly tested. Additions of mineralizable organic materials with a high C:N ratio may induce N immobilization, which would have lowered plant growth. The data do not support this explanation, because there were no significant plant growth differences between the bagasse and control carriers in these soils.

5. Conclusion

Raw bagasse was more effective at stimulating the nodulation of native rhizobium than bagasse-based biochar irrespective of whether and how water deficit was induced and led to a significant survival of an introduced strain, except in pure sand. The stimulatory effect on the native rhizobia strains was largely lost when the bagasse was pyrolyzed. However, the bagasse-based biochar carrier was more effective in improving not only nodulation or nodule biomass but also plant growth when added to pure sand directly at planting without any induced water stress. In addition, the pyrolyzed bagasse carrier was able to maintain viable introduced rhizobia to a greater extent than what was expected based on the amount of rhizobia introduced or total nodulation. This study extends previous findings and shows that over a period of eight weeks prior to planting, metabolizable organic matter was more important in promoting survival of rhizobia than alleviation of drought stress under most conditions investigated here except in pure sand. It is possible that the demonstrated efficacy of available sugars in the raw bagasse carrier diminishes quickly while there is evidence that the benefits from biochar-based materials can persist over longer periods of time. In addition, our experiments do not inform on how seed coating with inoculants would perform. Evidence from this experiment suggests that sugarcane bagasse or pyrolyzed bagasse may promote survival of introduced beneficial microorganisms in soil over monthly time scales. However, multi-season trials, either in the greenhouse or in the field, would be needed to assess the long-term potentials for either of these materials to maintain rhizobial populations through longer periods of desiccation.

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Appendix A. Supplementary data

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