Selecting alternative sterile and non-sterile substrates for mycorrhizal inoculant production

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ABSTRACT

Alternative substrates were evaluated for mycorrhizal inoculants production considering the beneficial effects of arbuscular mycorrhizal fungi (AMF) for plants and environment. The first step of the experiment aimed at evaluating the potential of substrates produced from sugarcane bagasse (SB), coconut fibre (CF) and urban waste compost (UWC) to produce AMF inoculants, by using *Brachiaria decumbens* as multiplier plant. The experiment has followed a randomized block design, at 7x3 factorial arrangement (seven substrates: SB, CF, SB+CF 1:1, SB+UWC 3:1, CF+UWC 3:1, SB+CF+UWC 3:3:2, Soil+Sand 3:1, vs. three substrate preparations: inoculated/sterilized, inoculated/non-sterilized, non-inoculated/sterilized). Inoculums produced in the first experimental stage were tested at the second stage, based on the inoculated/sterilized preparation. Seed germination, dry shoot biomass, mycorrhizal root rate and total spore production were assessed in both tests. There was significant decrease in seed emergence in organic substrates in relation to Soil+Sand, although the plants growth in organic substrates was greater. Artificial inoculation was not superior to spontaneous substrate mycorrhization or affected inoculum production. Mycorrhizal colonization of roots was equivalent in most organic substrates and in Soil+Sand. However, spore production stood out in organic substrate mixes, mainly when CF and UWC were used. This finding was confirmed at the second experimental stage.

Keywords: sugarcane bagasse; coconut fibre; urban waste compost; *Glomus* sp.*; Rhizophagus* sp.

INTRODUCTION

Beneficial effects of symbiosis between plants and arbuscular mycorrhizal fungi (AMF) have been investigated in several plant species under different conditions. According to Brundrett (2009), 90% of botanical families are colonized by mycorrhizal fungi or present other nutritional adaptations, based on which, plants nourish fungi with energy to allow their growth and maintenance via photosynthetic products, whereas fungi provide water and nutrients for host plant species (Berbara *et al.*, 2006). According to Rodrigues *et al.* (2003), another important aspect lies on the likelihood of AMF-mediated nutrient transfer between plants within and between species. The addition of pre-selected inoculum suitable to the cultivated host plant can significantly increase mycorrhization and crop yield in poor soils or in soils where the native AMF inoculum is inefficient (Jin *et al.*, 2013), as well as enhance plants' ability to adapt to adverse conditions (Garcia *et al.,* 2017).

If one takes into consideration the agricultural potential of AMF, inoculant production is a promising strategy to stimulate plant growth and development, as well as to reduce costs, and the need of using corrective mineral fertilizers. Consequently, it minimizes environmental impacts generated by agriculture. AMF are biotrophic and they cannot be artificially multiplied; thus, they must be inoculated in host plants. The main AMF inoculum multiplication methods comprise the inocula-

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tion of different host plant types grown in substrates, plastic pots or bags, or maintained in hydroponic or aeroponic culture (Ijdo *et al.*, 2011). It is possible inoculating roots induced in tissue culture; however, this technique is mostly used to isolate monosporic cultures in order to guarantee the genetic purity of mycorrhizal inoculums produced *in vitro* (Ijdo *et al.*, 2011). Arbuscular mycorrhizal multiplication in substrates is the most used method. It stands out among other methods because it can be conducted by farmers themselves in rural properties, as long as plants show satisfactory growth in the adopted substrate, as well as because it is an excellent fungal multiplier (Rodrigues & Rodrigues, 2017).

Sterile soil-sand mix is the substrate mostly used for AMF multiplication. However, the high density of this material hinders its transportation and commercialization. Thus, it is desirable using lighter and more productive substrates composed of cheaper and easily available raw materials that present the following features: low density, in order to make inoculant trade easier; physicochemical features (such as nutrients and texture capable of enabling drainage and aeration), in order to promote proper host plant and fungal development; being phytopathogen-free; undergoing sterilization process without losing their original features; as well as enabling the growth of other microorganisms with beneficial effect on mycorrhizal association (Ijdo *et al.*, 2011; Pal *et al.*, 2016; Rodrigues & Rodrigues 2017).

The viability of directly producing mycorrhizal inoculants in rural properties allows farmers and nurseries to have access to the most effective strains in order to meet their conditions, whether it is host culture, soil type and climatic conditions. The production of mycorrhizal seedlings adds value and quality and allows producers to have access to these beneficial fungi, which have positive effects on plant production (Schlemper & Stürmer, 2014; Goetten *et al.*, 2016; Chaiyasen *et al.*, 2017; Moreira *et al.*, 2019), as well as enables using substrates that can be easily obtained in rural properties.

Several agro-industrial wastes, as well as urban waste compost (UWC), have the potential to be tested in AMF multiplication processes carried out, mainly due to greater sugarcane bagasse (SB) and coconut fibre (CF) availability in the Northern and North-western Fluminense regions/RJ. Schlemper & Stürmer (2014) have shown that on-farm multiplication using *Rhizophagus clarus* and *Claroideoglomus etunicatum* grown in agro-industrial waste, such as SB, is an effective strategy that can be applied to AMF multiplication, with spore production in quantity. In addition, to be highly available in the region, the aforementioned waste has advantages such as low density, reduced cost, easy application in agriculture and the ability to minimize environmental impacts.

The current study has evaluated the potential of alternative substrates produced from different SB, CF and UWC mixes to produce mixed AMF inoculants by using multiplier plant species *Brachiaria decumbens*. Initially, the effect of substrate sterilization on plant germination and growth, as well as root colonization and spore production, were evaluated. The effectiveness of the inoculum multiplied in sterilized substrates during the first experimental stage was evaluated as inoculant in *B. decumbens* (reinoculation), during the second stage.

MATERIALS AND METHODS

The experiment was carried out in two different stages conducted in greenhouse at the Higher Institute of Technology in Agricultural Sciences in Campos dos Goytacazes (RJ), (latitude 21º72'15"S and longitude 41º34'43"W), at mean minimum and maximum temperatures ranging from 17 ºC to 27 ºC, respectively, and relative humidity ranging from 60% to 80%. Substrates comprising different sugarcane bagasse (SB), coconut fibre (CF) and urban waste compost (UWC) mixes were tested for *B. decumbens* Stapf. Prain planting and for arbuscular endomycorrhizal fungi inoculant (AMF) production.

First experimental stage

Treatments conducted in the first experimental stage have followed a randomized blocks design, with four repetitions (two pots per repetition) and seven substrates (SB; CF; SB+CF 1:1; SB+UWC 3:1; CF+UWC 3:1; SB+CF+UWC 3:3:2 and Soil+Sand 3:1). Three different methods were used to prepare the substrates before *B. decumbens* sowing, namely: inoculated/sterilized (I-S), inoculated/non-sterilized (I-NS) and non-inoculated/sterilized (NI-S).

Second experimental stage

The second experimental stage adopted a randomized block design with four repetitions (two pots per repetition). All seven substrates were tested again: SB, CF; SB+CF 1:1, SB+UWC 3:1, CF+UWC 3:1, SB+CF+UWC 3:3:2 and Soil+Sand 3:1. A sample (30 g) of each inoculated/sterilized (I-S) substrate from the first experimental stage was collected and used to inoculate the same treatments in the second experimental stage in order to evaluate the substrate as mycorrhizal inoculant.

Arbuscular mycorrhizal fungi (AMF) inoculum

AMF inoculum comprising fungal species belonging to genera *Glomus* and *Rhizophagus* (AMF04) was used – the specimens derived from the inoculum bank of

arbuscular fungi of the soil microbiology sector (COFMSOL) of UENF Soil Laboratory. It was multiplied in washed and autoclaved sand, and *B. decumbens* was used as the original host plant. The mix comprising cultivation substrate and chopped roots of the host plant was used as initial inoculant at the first experimental stage.

Organic waste and substrate preparation

Residue SB deriving from sugarcane waste was obtained at Companhia Açucareira – Usina Barcelos, which is located in São João da Barra County/RJ. It was left to dry in the shade, for five days, before it was subjected to chemical analysis (Table 1). Coconut fibre (CF) derived from coconut collected from coconut water traders in Campos dos Goytacazes County/RJ. Green coconut shell was chopped and crushed in cane chopper in order to obtain fibres with mean length of 1.7 cm. Coconut fibres were left to dry in the shade, for 30 days, before they were subjected to chemical analysis (Table 1). Urban waste compost (UWC) deriving from organic waste composting process was produced in the composting area of Miracema County/RJ. UWC was considered appropriate after stabilizing the temperature, pH and the colour of the material was dark and odourless of substances in a putrefied state. It was sieved in 4-mm mesh before it was subjected to chemical analysis (Table 1). The soil used in the Soil+ Sand substrate (washed river sand) derived from an area of Distrocohesive Yellow Argisol (Santos *et al.*, 2018a), in Campos dos Goytacazes County/RJ. Its physical and chemical features were analysed based on Claessen *et al.* (1997) (Table 1). The soil was fertilized with 20 $mg.dm^{-3}$ of P and N, in their natural phosphate and urea forms.

With respect to treatments with sterile substrate, wastes SB, CF and UWC were autoclaved twice, in separate, for 1 h, at 1atm and 24 h interval, 30 days before sowing. They were homogenized by taking into consideration the proportion and density of each waste, namely: SB (52 $g.dm^3$), CF (105 $g.dm^3$) and UWC (800 $g.dm^3$).

Conducting the experiment

New pots (0.5 dm^{-3}) disinfected with sodium hypochlorite solution (2.5% v/v) were used in seeding tests, at both experimental stages. Substrates were inoculated with 30 g of AMF, after they were added to the experiment.

Brachiaria seeds were disinfected with sodium hypochlorite solution (0.05% v/v) for 20 minutes and rinsed with deionized water, before sowing. Fifty seeds

Table 1: Chemical analysis applied to agro-industrial waste such as sugarcane bagasse (SB), coconut fibre (CF), urban waste compost (UWC) and soil used as substrates for mycorrhizal inoculum multiplication in *Brachiaria decumbens* roots

		[2]SB	[1]CF	UWC	Soil
pH		3.2	5.5	7.7	5.3
[3]P	$mg.dm^3$	48.0	65.0	462.0	5.0
K	$mg.dm^3$	156.0	1,080.7	2,366.0	100.0
Ca	$cmol_c.dm^3$	0.2	0.1	20.0	1.0
Mg	$cmol_c.dm^3$	0.5	0.2	5.3	0.5
Al	$cmol_c.dm^3$	-	-	0.0	0.0
H+Al	$cmol_c.dm^3$	-	-	1.4	1.9
Na	$cmol_c.dm^3$	0.1	2.2	4.1	-
C	%	-	-	5.5	0.9
OM	$g.dm^3$	-	-	94.8	16.6
SB*	$cmol_c.dm^3$	1.2	5.3	35.4	1.8
T	$cmol_c.dm^3$	-	-	36.8	3.7
t	$cmol_c.dm^3$	-	-	35.4	1.8
m	%	-	-	0.0	-
V	%	-	-	96.2	58.0
Fe	$mg.dm^3$	-	-	31.6	32.0
Cu	$mg.dm^3$	0.1	0.2	1.9	0.4
Zn	$mg.dm^3$	0.8	0.3	56.4	1.4
Mn	$mg.dm^3$	5.0	0.1	82.2	5.9
Cl	$mg.dm^3$	269.0	1,704.0	-	-
S	$mg.dm^3$	-	-	7.9	-
B	$mg.dm^3$	-	-	0.8	0.8
[4]SAR		0.60	20.41	-	-

[1]Extraction based on the water saturation method (97.90 g of substrate + 700 mL of H_2O); [2]Extraction based on the saturation method (45.84 g of substrate + 400 mL of H_2O); [3] North Carolina Extractor; OM = organic matter; SB* = sum of bases; T = CEC at pH 7.0; t = effective CEC; m = aluminium saturation; V = base saturation; SAR = sodium adsorption ratio.

were sown per pot and covered with 1 cm of substrate. Seeds were irrigated with deionized water, based on their daily need, throughout the experiment.

Assessments

Seed germination assessments started seven days after sowing; they were carried out every two days, for 30 days. Plant shoot was collected and dried in oven at 70 ºC, for 72 h, 58 days after sowing.

Next, ¼ of substrate presenting roots was collected from the pots and washed with the aid of water jets under 2 mm sieves. Subsequently, roots were manually collected from the substrate. This procedure was adopted to assess mycorrhizal colonization rate. Roots were placed in glass containers filled with 50% alcohol and kept in refrigerator at 8 ºC, until analysis time. Root segments were discoloured by heating in KOH (5%) at 80 ºC for 10 min, and washed with deionised water. Samples were acidified in HCl (1%) for 10 min; roots were stained with methyl blue (0.05%) diluted in acid glycerol and heated at 80 ºC until the fragments were evenly coloured. Colonization was assessed in 10 root segments (approximately 1 cm long, each) - roots presenting fungal structures such as arbuscules, hyphae, spores, or vesicles were classified as colonized (Koske & Gemma, 1989; Grace & Stribley, 1991). At the end of the first experimental stage, the structures of AMF associated with roots grown in inoculated/sterilized (I-S) substrates were analysed through optical microscopy in bright field, at 200x or 400x magnification (Nikon Eclipse E400).

Pots holding the remaining substrate were covered with paper bags to avoid contamination and deprived of water for 120 days in greenhouse. It was done to stimulate AMF sporulation in the two experimental stages after sample collection for mycorrhizal colonization evaluation. Subsequently, 50 g of substrate with roots were collected in order to extract AMF spores (Gerdemann & Nicolson, 1963; Lopes *et al.*, 1983). Twenty-five (25) g of substrate were homogenized in 1 L of water and left to rest for 5 min. The mix was transferred to a set of three sieves (opening of 850, 212, and 53 μm). The material retained in the 850 μm sieve was discarded, whereas the material that passed through the other sieves was sieved three more times. Again, the material retained in the 850 μm sieve was discarded, whereas the material retained in the 212 and 53 μm sieves was transferred to conical tube and centrifuged at 1,660.23 G (equivalent to 3,000 rpm), for 4 minutes. Supernatant was discarded and sucrose solution (45%) was added to the precipitate, which was centrifuged again at 737.88 G (equivalent to 2,000 rpm), for 3 minutes. Supernatant was recovered in 53 μm sieves and washed in abundant water. Spores were counted with the aid of magnifying glass, on Petri dish. The analysis was not performed to count the number of roots given the hard time separating and collecting them from substrates presenting coconut fibre.

Statistical analysis

Data about all variables analysed in the two experimental stages were subjected to normality, homogeneity and homoscedasticity tests, which were followed by analysis of variance (ANOVA). In the first stage, analyzed as factorial, as there was an interaction of factors, the averages were broken down by factor and compared next. Treatment means were compared to each other through Tukey test, at a 5% probability level (α), at both experimental stages. All statistical analyses were carried out in the SAEG 9.1 statistical software (Universidade Federal de Viçosa, 2007).

RESULTS

First experimental stage

There was significant interaction (Table 2) between substrate and inoculation/sterilization factors in germination rate and shoot dry biomass. Seed germination (Table 3) in inoculated/sterilized (I-S) and inoculated/non-sterilized (I-NS) substrates was higher in the Soil+Sand mix, as well as in CF, SB+CF and SB+UWC, in comparison to SB. Substrate inoculation (I-S and I-NS) did not affect seed germination in mixes, such as SB+UWC, CF+UWC, SB+CF+UWC and Soil+Sand, in comparison to the non-inoculated/sterilized substrate condition (NI-S).

The highest seed germination rates recorded for inoculated substrates (I-S and I-NS) did not correspond to the treatment with higher shoot dry biomass, except for the Soil+Sand condition, which was used as control and showed high seed germination, as well as high dry biomass production. Shoot dry biomass (Table 3) was higher in CF+UWC and Soil+Sand when the substrate was inoculated (I-S and I-NS), regardless of whether it was sterile or not. The lowest shoot dry biomass production was observed for Brachiaria grown in SB, which was followed by treatments CF and SB+CF (Table 3).

There was no significant interaction (Table 1) between substrate and inoculation/sterilization factors in variables “mycorrhizal colonization rate” and “total number of spores”. Sterile non-inoculated substrates (NI-S) have shown lower mycorrhizal colonization rate and smaller number of spores than the inoculated treatments; this outcome indicates the inoculation effect, except for substrate SB (Table 3). AMF inoculation in sterile (IS) or non-sterile (I-NS) substrate has significantly contributed to increase mycorrhizal root rates and

number of spores, in comparison to non-inoculated substrates (NI-S) (Table 3).

CF, SB+CF, SB+UWC, and SB+CF+UWC stood out among the non-sterilized (I-NS) inoculated substrates that showed larger number of spores than sterilized (IS) substrates. It may have happened due to spontaneous inoculation resulting from previous contaminating inoculum found in non-sterile substrates or in irrigation water.

Substrates added with SB, whose composition lacked UWC, recorded the lowest mycorrhizal colonization rate, which also led to smaller number of spores. Substrates CF+UWC and SB+CF+UWC recorded the highest total number of spores, both in the sterilized (I-S) and non-sterilized (I-NS) substrate (Table 3). The number of spores observed in these substrates was even larger than that observed for the conventional method using Soil+Sand (Table 3).

Structures of AMF deriving from samples subjected to sterile and inoculated treatments at the first experimental stage

Vesicles, spores (chlamydospores), and fungal hyphae associated with roots were observed in all tested inoculated and sterilized (I-S) substrates (Figure 1). Arrangements often seen inside root cortex cells were observed in the present study, which also recorded folded intracellular hyphae indicative of Paris-type colonization. These thicker hyphae that intracellularly fold into each other often develop into arbuscular hyphae (Jalonen *et al.,* 2013).

Second experimental stage – Effectiveness of the inoculant produced in sterile substrate (I-S)

Differences between substrates were observed for all the investigated variables – germination, shoot dry biomass, mycorrhizal root rate and total number of spores – at the second experimental stage (Table 4).

The lowest mean seed germination rates were recorded for substrates SB+UWC and CF+UWC, in a way similar to that of the first experimental stage. Likewise, these substrates, together with the Soil+Sand treatment, were the ones presenting the largest shoot dry biomass (Figure 2). The lowest mycorrhizal colonization rate was observed for the substrate added with SB. All other substrates recorded higher values for this parameter. CF+UWC recorded the largest total number of spores per litre of substrate; it was followed by SB+CF+UWC and Soil+Sand (Figure 2).

DISCUSSION

Different SB, CF and UWC mixes used to produce AMF inoculants had different effects on *B. decumbens* plants. Soil+Sand was the substrate presenting the best positive effect on seed germination, if one takes into consideration inoculation and sterilization at the first experimental stage. CF+UWC and Soil+Sand were the substrates presenting the highest shoot dry biomass production. The inoculum produced in substrates CF+UWC and SB+CF+UWC at the second experimental stage (reinoculation) enabled seed germination to increase by 87%, in comparison to that observed at the first experimental stage (72%); CF+UWC was the only substrate capable of increasing shoot dry biomass production by 94%.

The inoculation of substrates investigated in the current study did not increase dry matter production. Reduced shoot dry biomass was observed in substrates CF+UWC and SB+CF+UWC. Overall, substrate inoculation with different AMF species can significantly increase dry matter in host plants, since the mycorrhization process leads to increased photosynthetic, respiration, and transpiration rates in several plant species (Da Costa *et al.*, 2011). However, it is important to emphasize that the mycorrhizal colonization rate may not be directly correlated to plant growth (Chaiyasen *et al.*, 2017), as observed in the present study. Mendonça *et al*. (2019) have used fungal species *Gigaspora albida*, *R. clarum*, *C. etunicatum,* and *Acaulospora morrowiae* in tomato plants and observed colonization rate ranging from 40.3% to 83.8% during plant growth, 42 days after inoculation. However, the growth of plants

Table 2: Summary of the analysis of variance (P-value) applied to results obtained at the first experimental stage

	D.F.	Germ.	SDB	SpoT.	ColM.
Sub.	6	0.0000	0.0000	0.0000	0.0000
Ste.	2	0.0000	0.0222	0.0177	0.0000
Sub. x Ste.	12	0.0005	0.0038	ns	ns
Bl.	3	ns	ns	ns	-
Residue	60				
C.V.		29.4	36.3	43.2	30.7

Sub = substrate; Ste. = Sterilization; Bl. = Block; C.V. = coefficient of variation; D.F. = degree of freedom; Germ. = germination; SDB = shoot dry biomass; SpoT. = total number of spores; ColM. = mycorrhizal colonization; ns = not significant.

presenting mycorrhizal colonization was not affected by it in comparison to the control. This outcome shows, once again, that mycorrhizal colonization does not guarantee plant biomass increase.

Substrate SB reinoculation has increased mycorrhizal colonization rate by 23%, whereas substrate SB+CF has increased mycorrhizal colonization rate by 9%, in comparison to the first experimental stage. Substrate CF+UWC has increased the total number of spores by 48%, whereas substrate SB+CF+UWC has increased it by 39%, after reinoculation. These findings indicate the potential of substrates CF+UWC and SB++CF+UWC to be used for AMF inoculant multiplication since they produced 20,860 and 14,623 spores per L^{-1} of substrate, respectively. The other substrates recorded decrease in the total number of spores, whereas SB+UWC has also reduced mycorrhizal colonization rate by 4% at the second experimental stage.

Sugarcane bagasse and coconut fibre have been used for substrate composition to grow several plant species, which can present different results depending on the adopted substrate (Da Silva Júnior *et al.*, 2010; Da Silva Júnior *et al.*, 2012; Do Nascimento *et al.*, 2014; Tanwar *et al.*, 2013; Rodrigues *et al.*, 2016). Both SB and CF are rich in cellulose, hemicellulose, and lignin; thus, they are capable of increasing soil aeration, porosity, and water retention capacity (Kim & Day 2011). Besides, agricultural activities carried out in Brazil produce large

Table 3: Effect of the substrate-sterilization interaction on seed germination, shoot dry biomass, mycorrhizal colonization and total number of spores of *Brachiaria decumbens*

	Inoculated		Non-inoculated
Substrate	**Sterilized (I-S)**	**Non-sterilized (I-NS)**	**Sterilized (NI-S)**
	Seed germination (%)		
SB	64.5 abA	44.0 bAB	22.5 bB
CF	51.5 abcAB	63.5 abA	28.5 abB
SB+FC (1:1)	57.3 abA	70.5 abA	19.5 bB
SB+UWC (3:1)	35.5 bcA	56.0 abA	43.0 abA
CF+UWC (3:1)	25.8 cA	45.5 bA	28.5 abA
SB+CF+UWC (3:3:2)	27.5 cA	46.0 bA	45.5 abA
Soil+Sand (3:1)	65.0 aA	75.0 aA	56.0 aA
	Shoot dry biomass (g)		
SB	0.1978 cA	0.2122 bA	0.0775 dA
CF	0.5885 bcA	0.3992 bA	0.2943 dA
SB+FC (1:1)	0.4445 bcA	0.6010 bA	0.0403 dA
SB+UWC (3:1)	1.6220 bA	1.3610 bA	1.6467 cA
CF+UWC (3:1)	2.9729 aB	3.0287 aB	4.0142 aA
SB+CF+UWC (3:3:2)	1.3140 bcB	1.0435 bB	2.6435 bcA
Soil+Sand (3:1)	3.9267 aA	2.6042 aB	3.5108 abAB
	Mycorrhizal colonization (%)		
SB	35.0 cA	37.5 bA	7.3 aA
CF	100 aA	100 aA	20.0 aB
SB+FC (1:1)	55.0 bcA	62.5 abA	7.3 aB
SB+UWC (3:1)	86.5 abA	82.5 aA	42.5 aB
CF+UWC (3:1)	97.5 aA	90.0 aA	13.5 aB
SB+CF+UWC (3:3:2)	95.0 abA	92.5 aA	26. 5 aB
Soil+Sand (3:1)	95.0 abA	100 aA	40 aB
	Total number of spores (L^{-1} of substrate)		
SB	3460 gB	2480 gC	3712 fA
CF	7502 dC	12531 cA	9527 bB
SB+FC (1:1)	4130 fB	4159 fA	2939 gC
SB+UWC (3:1)	5910 eB	10310 dA	5400 eC
CF+UWC (3:1)	14073 aA	13340 bB	10570 aC
SB+CF+UWC (3:3:2)	10500 bB	16970 aA	9360 cC
Soil+Sand (3:1)	9847 cA	7080 eB	6250 dC

Equal letters did not differ from each other in the same analysis, based on the Tukey test, at 5% probability level - lowercase letters refer to comparisons between substrates, whereas uppercase letters refer to sterilization/inoculation. SB = sugarcane bagasse; CF = coconut fiber; UWC = urban waste compost.

amounts of lignocellulosic waste, although the composition of the used mix may have different effects on sporulation and colonization by AMF (Schlemper & Sturmer, 2014). The vetiver grass species *Chrysopogonzi zanioides* inoculated with *G. albida*, *R. clarum*, *C. etunicatum*, and *A. morrowia* has shown mycorrhizal colonization rate ranging from 1% to 16%, 100 days after cultivation in commercial substrate added with coconut powder (2:1) (Santos *et al.*, 2018b). Mycorrhizal propagule production in substrate added with sugarcane bagasse (dilution 1:1:1, with rice husk and sand) reached 1,620 L^{-1} in *R. clarus* and 2,400 L^{-1} in *C. etunicatus*, whereas mycorrhizal colonization rate in these species ranged from 11 to 59%, respectively (Schlemper & Sturmer, 2014). On the other hand, Do Nascimento *et al*. (2014) did not find significant differences in mycorrhizal colonization rate and in the total number of spores after the application of increasing sugarcane bagasse doses in cassava plants.

It is worth emphasizing that the use of these wastes in their pure state may be restricted to fungal or plant growth. Therefore, mix comprising fibrous materials and organic compounds rich in nutrients can produce substrates whose physical and chemical features play key role in symbiont growth. Substrate features can affect the spore germination necessary to enable symbiosis to take place (Giovannetti *et al.*, 2010).

The use of 25% UWC in substrate composition in the present experiment, mainly in association with CF, enabled greater host plant growth in the first inoculation and reinoculation, as well as increased the number of infectious AMF structures. These results were equal to, or higher than, that observed for the Soil+Sand mixture. These agro-industrial waste types have the potential to multiply AMF inoculum, mainly due to their lower density in comparison to the subsoil, low cost, easy obtainment and distribution in the field. In addition, they are capable of increasing AMF sporulation; consequently, they increase inoculum production (Carneiro *et al.*, 2008; Da Silva Jr *et al.*, 2010; Da Silva Jr *et al.*, 2012; Rodrigues & Rodrigues, 2017).

Commercial AMF inoculants are available in different formulations, based on the legislation in place, in each country. Dry products hold < 10% of their weight

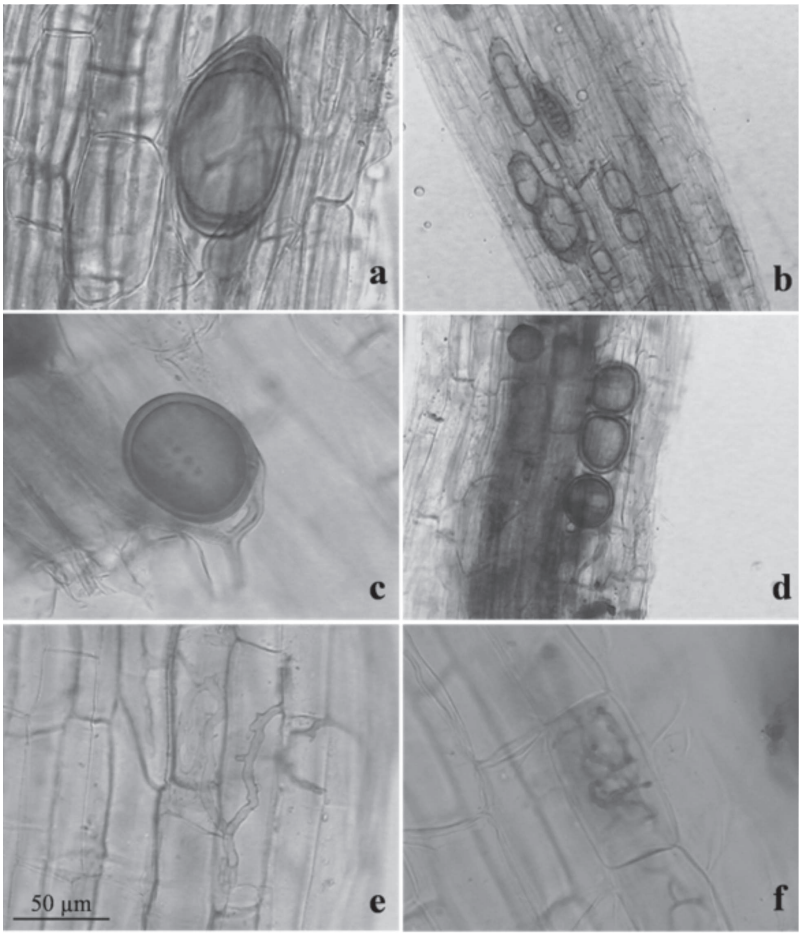

Figure 1: Structures of AMF colonizing Brachiaria roots. Vesicles a) 400x and b) 200x; chlamydospores c) 400x and d) 200x; intracellular hyphae e) 400x and hyphae in the folding process - indicative of Paris-type colonization 400x f).

composed of fungal structures, on average. These products may include inert carriers, such as clay minerals (perlite, vermiculite and bentonite), starch polymers, dry fertilizers or even ground-plant waste (Pal *et al.,* 2016) such as SB, CF and UWC.

Commercial inoculant production processes often require the substrate sterilization to reduce the likelihood of contamination with other species or even with phytopathogens. According to responses observed in sterilized inoculated substrates (I-S), the autoclaving sterilization process eliminates native inoculants, or even phytopathogens, as well as enables inoculant production based on selected AMF. However, the sterilization process must maintain substrate features that play important role in host plant growth and increase inoculant-production costs. The sterilization of inoculated substrates (I-S and I-NS) did not affect seed germination, multiplier plant growth and mycorrhizal colonization at the first experimental stage. Nevertheless, spore production - which is the most important reproductive structure for AMF inoculant composition - was lower in sterilized (I-S) substrates, except for SB, CF+UWC, and Soil+Sand, which recorded higher values. On the other hand, the increased number of spores in non-sterile substrate (I-NS), in association with high mycorrhizal colonization of host plants grown in CF (12,531 spores and 100% colonization), SB+UWC (10,310 spores and 83% colonization) and in SB+CF+UWC (16,970 spores and 92% colonization) has pointed out the potential of these substrates to be used for inoculum multiplication in rural properties (on-farm), without the need of sterilization. Besides, as observed in the current findings, although

Table 4: Summary of the analysis of variance (P-value) applied to results obtained at the second experimental stage

	D.F.	Germ.	SDB	SpoT.	ColM.
Sub.	6	0.0031	0.0000	0.0000	0.0000
Bl.	3	-	ns	-	-
Waste	18				
C.V.		19.8	27.8	41.5	42.3

Sub = substrate; Bl. = Block; C.V. = coefficient of variation; D.F. = degree of freedom; Germ. = germination; SDB = shoot dry biomass; SpoT. = total number of spores; ColM. = mycorrhizal colonization; ns = not significant.

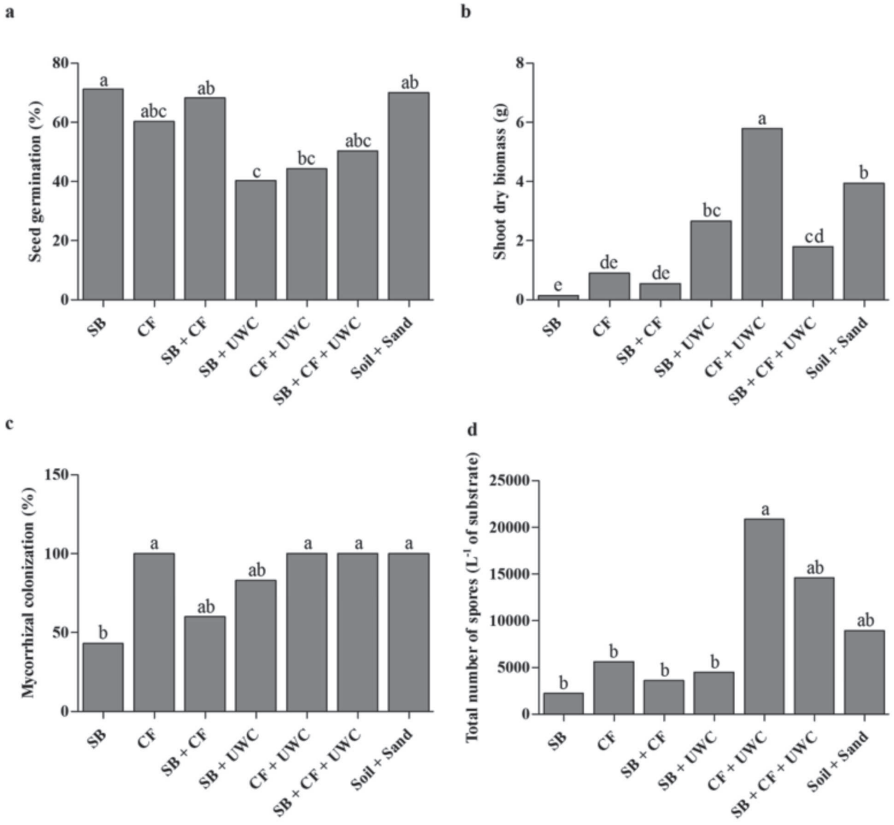

Figure 2: Effectiveness of inoculants produced in sterile substrate (I-S) at the second experimental stage in (a) seed germination, (b) shoot dry biomass, (c) mycorrhizal colonization and (d) total number of spores of *Brachiaria decumbens.* SB = sugarcane bagasse; CF = coconut fiber; UWC = urban waste compost.

substrates can present previous inoculum (contaminant), it may not be efficient in infecting host plants, due to low root colonization rates.

Besides the promising likelihood of producing inoculants on-farm (Chaiyasen *et al.,* 2017; Moreira *et al.,* 2019), AMF multiplication in non-sterile substrates does not impair such a production. The efficiency of AMF introduced on-farm depends on their competitiveness with endogenous microbiota, in terms of infective capacity, inoculum density, propagules' distribution, fungal ability to produce external hyphae, hyphae's ability to colonize roots fast, and the ability of the introduced fungi to maintain colonization rates under competitive conditions (Goetten *et al.,* 2016).

In addition to inoculum's adaptive ability, there may be great variation in AMF sporulation depending on the substrate type used for its multiplication. Moreira *et al.*, (2019) observed 5,770 spores per L^{-1} of soil, on average, after using different AMF inoculants produced on-farm. Pineapple root colonization rate reached 67.7%, after 180-day cultivation. However, the aforementioned authors have pointed out that root colonization can be influenced by different aspects such as AMF species, plant species, as well as soil type and physical-chemical and microbiological composition. Although it is necessary performing further tests to assess the physical-chemical and microbiological features improved by the use of these substrates, based on the current results, such aspects can be favoured by the CF and/or UWC addition as soil enrichers or conditioners for mycorrhization processes carried out on-farm.

CONCLUSIONS

The mixed substrate composed of CF+UWC enabled higher shoot biomass production, colonized root rates and larger number of spores. Substrate sterilization did not affect multiplier plants' growth responses or AMF propagule production. Inoculants produced in sterile substrates were capable of maintaining their infectious capacity after reinoculation. Sterilized CF+UWC and SB+CF+UWC substrates can be used as mycorrhizal inoculants, since they presented high colonization rate and spore production.

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