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RESEARCH ARTICLE

Influence of Sugarcane Genotype and Soil Moisture Level on the Arbuscular Mycorrhizal Fungi Community

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Abstract The relationship between sugarcane genotype and symbiosis with arbuscular mycorrhizal fungi (AMF) remains poorly understood, especially regarding different soil moisture levels. Our objective was to evaluate the effect of soil moisture on the AMF community structure, spore abundance and colonization ratio in a plantation with eight sugarcane genotypes (CTC15, CTC17, RB867515, RB92579, RB931011, RB966928, IAC5000 and NCo376). The study was carried out in Piracicaba, São Paulo and Brazil in an experimental plot setup in a randomized block design, with three replicates (blocks). We collected soil and root samples in a greenhouse experiment under two water replenishment levels: 100 and 50% of soil moisture at field capacity (θ_{FC}). We extracted spores and assessed the AMF root colonization ratio by using specific dyes and determining the percentage of root length colonized in the different sugarcane genotypes. In addition, we evaluated the AMF community structure by PCR and denaturing gradient gel electrophoresis. In general, the spore abundance and root colonization ratio were higher in all varieties at 100% θ_{FC}. However, the IAC5000 and RB966928 genotypes showed higher colonization levels even at 50% θ_{FC}. The AMF community structure was also influenced by soil water levels with group separations across 100 and 50% θ_{FC}. Sugarcane productivity as measured by stalk plus root dry mass was positively correlated with AMF colonization rates in 100% θ_{FC}. Thus, the water replenishment levels used in sugarcane cultivation can influence spore abundance, colonization ratios and AMF community structure in the soil. The selection of a sugarcane genotype with greater AMF association under low water replenishment levels may be a primary factor in growing sugarcane in areas with low water availability.

Keywords Soil biology · Fine roots · AMF · Symbiosis · Soil water · DGGE

Introduction

Brazil is the world's largest sugarcane producer with around nine million planted hectares (Conab 2017). Sugarcane is one of the most productive crops in terms of biomass and sucrose accumulation per unit of area (Inman-Bamber 2004; Waclawovsky et al. 2010). However, the productivity is reduced in regions with soil water deficiency (Viana et al. 2015). The need for food, fiber and energy production is expected to increase substantially over the coming years (Wang et al. 2013; Ercin and Hoekstra 2014) and studies on crop productivity pinpoint water stress as one of the main factors in reducing yield. Irrigation has, therefore, become an indispensable practice in the expansion of sugarcane production (Olayide et al. 2016).

The adoption of new genotypes resistant to the diverse climatic and soil conditions in several regions in Brazil is a

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key factor for increased productivity and expansion of sugarcane as a lucrative crop (Liu et al. 2016). In addition, genotypes with high yield under manual or mechanized harvesting systems have been sought, with resistance to pests, diseases and other desirable characteristics (Galvão et al. 2005). Few breeding programs have focused on traits promoting soil microbiota, however, and the association of arbuscular mycorrhizal fungi (AMF) with these new sugarcane genotypes may prove of great importance in increasing crop yield.

Belonging to the *Glomeromycota* phylum, AMF performs symbiosis with more than 80% of all terrestrial plants, including sugarcane (Smith and Read 2008a; Brundrett 2009). This interconnection of plant roots with the numerous and extensive hyphal networks in the soil provides innumerable benefits to the host plant (Friese and Allen 1991; Cardoso et al. 2013) by expanding the root system in the soil (Smith and Read 2008b). There is a vast and consolidated literature that demonstrates the beneficial effects of the AMF–plant root association, mainly by facilitating the translocation of mineral nutrients and water (Finlay and Read 1986; Wu et al. 2012). However, there are few studies on AMF symbiosis in different sugarcane genotypes, under tropical soil conditions and at different soil moisture levels, using molecular techniques to evaluate AMF colonization and communities.

Our aims were to determine spore abundance, measure AMF root colonization length and to unravel the AMF community structure in a sugarcane plantation with eight genotypes grown under two water replenishment regimens: 100 and 50% of soil moisture at field capacity (θ_{FC}). We hypothesized that both the sugarcane variety and the water replacement level influence spore abundance, colonization and AMF community structure in the soil. Our data show that higher water replenishment levels resulted in greater AMF root colonization with consequent changes in the AMF community structure in the soil.

Materials and Methods

Experimental Site

The experiments were carried out at the Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil (22°43′S; 47°38′W, 546 m above sea level). The sugarcane plants were grown in a greenhouse with a total area of 400 m^2, in concrete pots with a capacity of 310 L, measuring 1.04 × 0.41 × 0.75 m width, length and depth, respectively. Seedlings were grown in a sandy soil (Typic Ustox, FAO soil classification) under typically dystrophic conditions. The different sugarcane genotypes were produced by the CTC, RIDESA and IAC breeding stations and two sugarcane seedlings were planted in each experimental unit. The soil was fertilized according to the recommendations of "Boletim 100"—Instituto Agronômico de Campinas, for the sugarcane crop in Brazil (Teixeira et al. 1997).

Soil Water Retention Curves

Soil water retention curves were constructed using a tensile table (− 1, − 2, − 4 and − 6 kPa) and Richard's extractor with a porous plate (− 10, − 30, − 50, − 100, − 500, − 1000 and − 1500 kPa). The global density (Ds), the density of the particles (Dp), the total porosity (TP) and the chemical characterization of the soil were also determined (Table S1).

Irrigation and Water Management

Watering was by drip irrigation with individual controls for each experimental unit. An automatic data acquisition system (datalogger) was set up, consisting of vacuum transducers coupled to tensiometers installed immediately after planting the seedlings. Vacuum levels were transformed into soil water potentials, and moisture estimation was performed using the retention curve equation proposed by van Genuchten (1980).

Treatments and Experimental Design

Evaluation of the plants was carried out in their first ratoon cycle, in the pre-harvest phase. Water replacement levels were calculated to raise soil moisture to 100 and 50% of the total water holding capacity (100 θ_{FC} and 50% θ_{FC}, respectively). We analyzed eight sugarcane genotypes (CTC15, CTC17, RB867515, RB92579, RB931011, RB966928, IAC5000 and NCo376), and plants were grown in experimental units in a greenhouse with three replicates in a complete block design.

Sugarcane Productivity

Fresh stalk productivity per hectare (Mg ha^{-1}) was calculated for the average canopy area occupied by plants in each experimental unit according to the following formula (Eq. 1):

$$SPH = \frac{\left(\frac{SP}{0.43}10\right)+\left(\frac{SP}{0.81}10\right)}{2} \quad (1)$$

SPH, stalk productivity per hectare, Mg ha^{-1}; SP, stalk phytomass in kg; 0.43 and 0.81, the soil area of the experimental unit for the canopy of the plants in m^2; and 10, the factor for conversion of kg m^{-2} to Mg ha^{-1}.

Soil and Root Sampling

Soil and roots were sampled for the evaluation of spore abundance, AMF colonization rates and community structure. The samples were collected using a soil probe at 0–20 cm deep. Monthly samplings were performed throughout the first ratoon cycle. The AMF attributes did not vary significantly from month to month during the sugarcane cycle in any of the treatments. Because there was no time effect, our monthly sampling data were combined.

AMF Spore Extraction and Counting

Spores were extracted by wet sieving and soil decantation (Gerdemann and Nicolson 1963). Sieves of defined sizes (0.71 and 0.045 mm) were used in the wet sieving process after which the spores were centrifuged in a 70% sucrose solution for 3 min at 3500 rpm (LABCARE - Jouan BR4i) and stored at − 20 °C until evaluated (Bonfim et al. 2016). Spores were counted at 40× using a stereoscopic microscope (Redecker et al. 2013) and the total number of spores in a 50 g sample of soil was determined (Bonfim et al. 2016).

AMF Root Colonization

Roots were rinsed in continuously flowing water and segments were diaphanized in 10% KOH solution for 30 min at 90 °C (Brundrett et al. 1996). The segments were stained with Parker ink (Quink®) for 15 s in a water bath at 90 °C. Segments were mounted on microscope slides and fixed with lacto-glycerol solution (1:1:1 lactic acid, glycerol and water). (Brundrett and Kendrick 1990). Five slides were prepared for each sample and ten root segments were evaluated on each slide (Giovannetti and Mosse 1980; Melloni and Cardoso 1999; de Araujo Pereira et al. 2018).

For the root colonization data, normality assumptions of variance were tested using the Shapiro and Wilk test (1965) and homoscedasticity by Levene's test. The comparison of means was performed by the Tukey test at a significance level of 5%. The analyses were carried out with the R software (R Core Team 2016), v.3.3.1 using ExpDes.pt and ggplo2 packages (Wilkinson 2011).

AMF Community Structure Analysis by PCR-DGGE

Soil (400 mg) DNA was extracted using MoBio Power Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific®). The DNA integrity was also confirmed by electrophoresis in a 1.5% agarose gel using 1x TAE buffer.

NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS8 (5′-TCCGCAGGTTCACCTACGGA-3′) primers were used in an initial PCR reaction targeting the 18S rRNA small-subunit (SSU) gene region of AMF (White et al. 1990). The first reaction products were then used in nested-PCR reactions with the AMF-specific primers: AM1 (5'-GTTTCCCGTAAGGCGCGCAA-3′) and GC-NS31 (5′ "GC-Clamp"-TTGGAGGGCAAGTCTGGTGCC-3′) (Simon et al. 1992; Helgason et al. 1999).

All PCR reactions were performed in a thermocycler (Viriti®, Applied Biosystems) programmed to operate with the initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s followed by a final extension at 72 °C for 5 min. In all reactions, the amplification specificity was confirmed by electrophoresis (1.5% agarose gel) and the amplicon size was determined by comparison with the GeneRuler™ DNA Ladder Mix (Fermentas Life Sciences). PCR-DGGE analysis was performed with the PhoU2 system (Ingeny, Goes, Netherlands) following a methodology adapted from Muyzer et al. (1993). PCR reaction products were run on an 8% polyacrylamide gel (30:1, acrylamide:bis-acrylamide). The denaturing solution contained urea and formamide which have specificity for fungal DNA fragments, and the gradient ran from 25 to 65%. For electrophoresis, 12 µL of amplified DNA and 5 µL of 6x loading buffer were loaded on a DGGE gel. Samples were run at 60 °C and 100 V for 16 h. Afterward, the gel was stained with SYBR-Green (Invitrogen, Breda, Netherlands) in 0.5 × TAE for 15 min in the absence of light and scanned in a Storm 845: New phosphorimager/gel scanner. The DNA fragments observed in the gel constituted the AMF community profile of the soil samples, and each band was considered a distinct operational taxonomic unit (OTU). The marking of the DNA bands was performed by Image Quant® (GE Healthcare Life Sciences) software. Differences in the AMF community structure were quantified using principal coordinate analysis (PCoA) based on the Bray–Curtis algorithm, and similarities between the groupings were verified by the ANOSIM test, both performed using the statistical software Primer- E 6+ (Ramette 2007).

Results

AMF Spore Abundance

The number of AMF spores ranged from 2 to 15 per 50 g of soil from the two soil moisture levels and the eight sugarcane genotypes (Fig. 1). In general, the abundance of AMF spores in the soil was significantly higher for

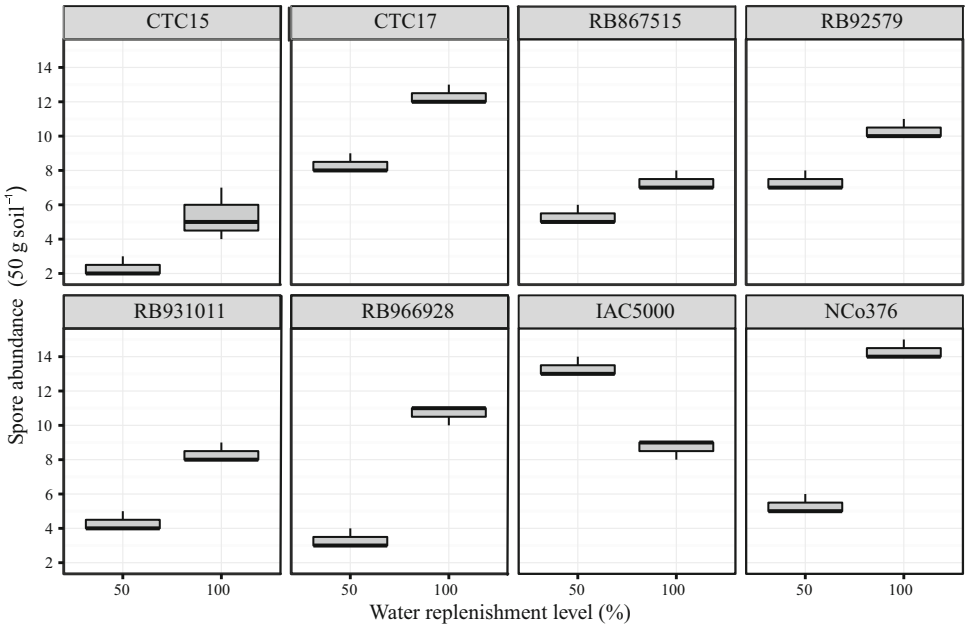

Fig. 1 Abundance of AMF spores in soil (50 g^{-1}) cultivated with eight sugarcane genotypes irrigated to water volume of 100 and 50% θ_{FC}

genotypes irrigated at 100% θ_{FC}. However, IAC5000 was the only variety that showed a higher abundance of AMF spores (13 spores per 50 g of soil) at the lower soil moisture level of 50% θ_{FC}. The mean AMF spores count in the soil cultivated with IAC5000 differed significantly from all other genotypes at both soil moisture levels (50 and 100%), except when compared with the NCo376 variety (South Africa) that had the greatest spore abundance in soil at 100% θ_{FC} (Fig. 1).

Root AMF Colonization

Root colonization rates ranged from 10 to 39% among sugarcane genotypes at both soil moisture levels. The CTC15, RB867515 and RB931011 genotypes showed the greatest colonization, with averages of 31% and 39, 21 and 36%, and 23 and 31% at levels of 50 and 100% θ_{FC}, respectively (Fig. 2). AMF root colonization was significantly higher in genotypes irrigated at 100% θ_{FC} than in those irrigated at 50% θ_{FC}. The RB966928 and IAC5000 genotypes differed from this pattern, thereby, presenting higher values of root colonization at 50% soil moisture (Fig. 2). In fact, the root colonization of IAC5000 at 50% θ_{FC} was higher than that for CTC17, RB92579 and NCo376 at 100% θ_{FC} (Fig. 2).

AMF Community Structure Analysis by PCR-DGGE and PCoA

The first two axes generated by principal coordinate analysis (PCoA) revealed 57% dissimilarity in the AMF community. Water level played an important role in the dissimilarity of the AMF community (R_{ANOSIM} = 0.65; $p < 0.006$) (Table S2). Analysis of the AMF community resulted in strong separations with segregation of the samples into four distinct AMF groups: one at 50% θ_{FC}, another at 100% θ_{FC}, and one group including the sixth and seventh genotypes at both moisture levels (Fig. 3).

Correlation Between Colonization Ratio and Productivity

The production of sugarcane stalks and root dry mass (RDM) was correlated with the AMF colonization rate. In general, stalk and root mass showed a greater correlation with colonization ratio in soils irrigated at the 100% moisture level (r^2 0.42 and 0.52, $p < 0.01$) (Fig. 4b, d), while at the 50% soil moisture level, we found a lower correlation between the ratio of colonization and yield of stalks and RDM (r^2 0.006 and 0.05, $p < 0.01$) (Fig. 4a, c).

Discussion

Abundance of AMF Spores

In general, the AMF spore count was the highest at the 100% irrigation level, whereas at the 50% irrigation level, only the IAC5000 variety had a higher spore count. Similar results were reported by Sousa et al. (2015). These authors evaluated the effect of AMF inoculation on the initial growth and development of the sugarcane variety RB 857515, and tested the effect of irrigation with 50 and 100% soil moisture levels. Their data showed a higher

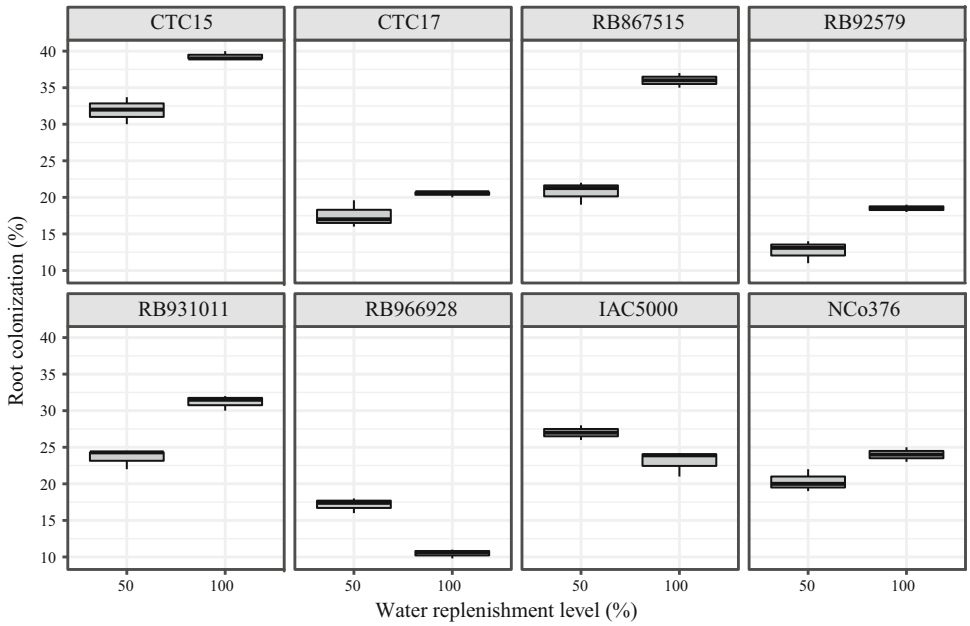

Fig. 2 Percentage of root colonization by AMF in eight sugarcane genotypes irrigated with 50 and 100% of field capacity

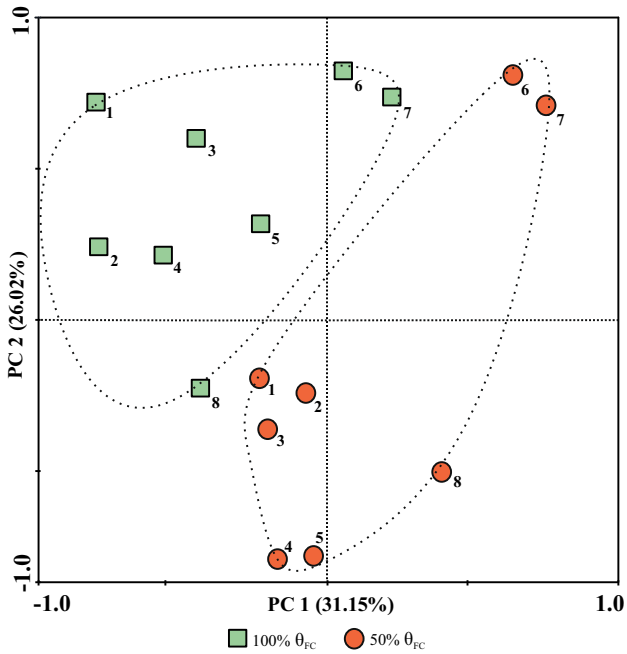

Fig. 3 Principal coordinate analysis (PCoA) obtained by the band profile of the 18S region. Genotypes: 1 = CTC15; 2 = CTC17; 3 = RB867515; 4 = RB92579; 5 = RB931011; 6 = RB966928; 7 = IAC5000 and 8 = NCo376

AMF spore count at 100% θ_{FC}. Other studies have reported a greater number of AMF spores in soils with an adequate water management regimen, probably because there is a greater stimulus to root colonization and formation of fungal propagation structures such as spores and hyphae when soil moisture levels are high (Bowles et al. 2018).

On the other hand, Bonfim et al. (2010) reported higher counts of AMF spores in forest systems and coffee plantations during the dry season. These authors suggest that lower levels of soil moisture can induce colonization and stimulate mechanisms of adaptation in fungi, such as increase in sporulation rate. This fact may explain our result in which a greater number of spores was found in soil in which the sugarcane variety IAC5000 was cultivated at 50% θ_{FC}. Such soil water stress may mimic the effects of short periods of drought. The establishment of the AMF–plant symbiosis is known to be related to edaphoclimatic factors such as availability of water and nutrients as well as aspects of the fungus–plant interaction (Zhang et al. 2011; Asrar et al. 2012).

The abundance of AMF spores, the growth and branching of extra-radical hyphae in the soil and mycorrhizal structures in the roots are the main attributes responsible for the success of root colonization (Schalamuk and Cabello 2010). Moreover, increased AMF colonization of plants because of a greater number of spores in the soil can facilitate the acquisition of resistance to adverse environmental conditions such as drought (Verzeaux et al. 2017). Our results demonstrate that sporulation is influenced by both the sugarcane variety and the soil moisture level. In this scenario, the IAC5000 variety was exposed to greater spore abundance and thus presented higher tolerance to water stress.

AMF Root Colonization

Our results demonstrate that root colonization ratio may be more dependent on the plant variety than on moisture level. IAC5000 apparently has a greater intrinsic drought resistance because this variety had a higher colonization ratio

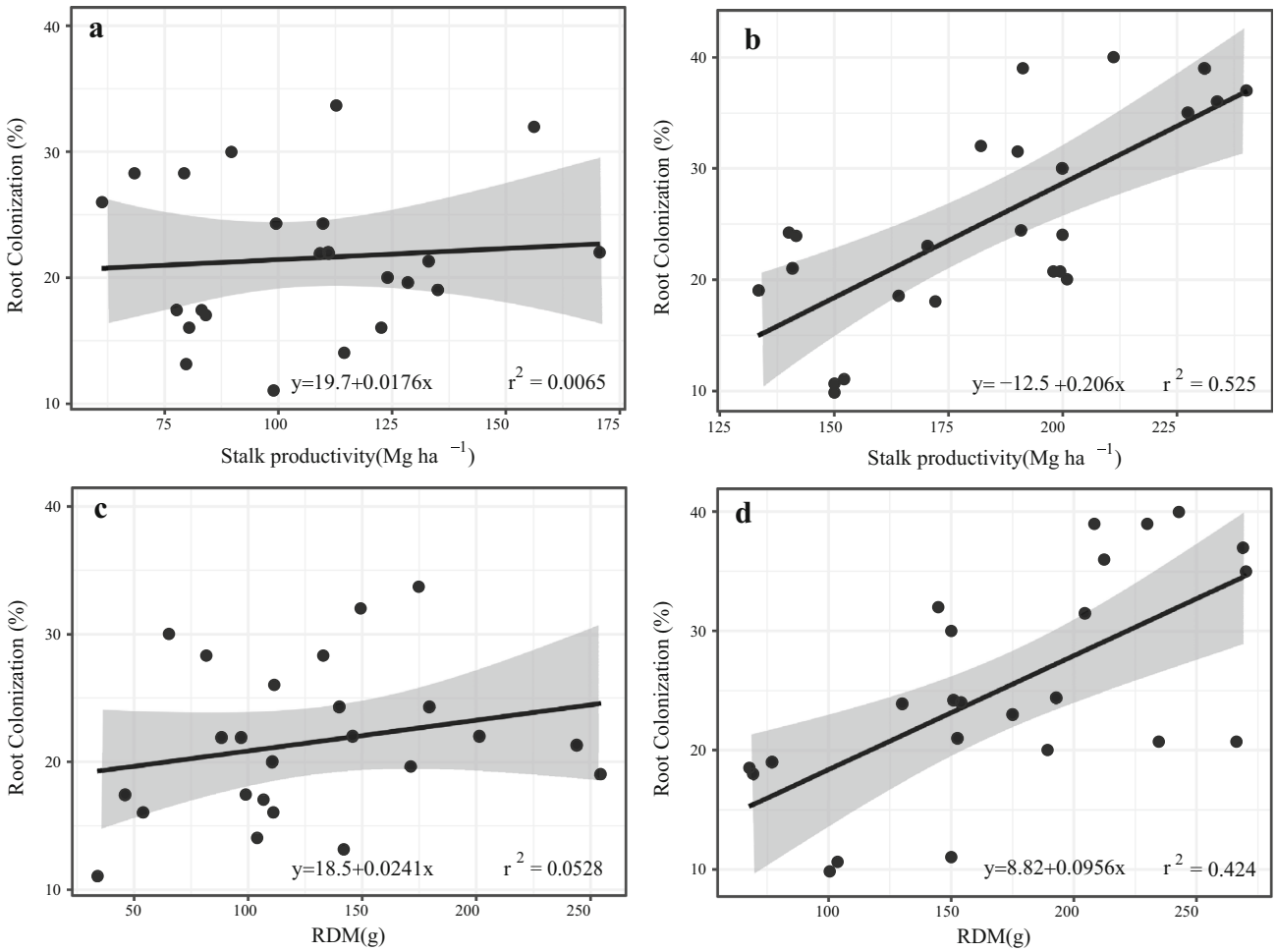

Fig. 4 Relationship between stalk productivity and root colonization, $p < 0.01$, for sugarcane genotypes submitted to the 50% replacement level θ_{FC} (**a**), and to 100% θ_{FC} (**b**)

than the CTC17, RB92579, RB966928 and NCo376 genotypes, even when they were grown at 100% θ_{FC}. In contrast, Symanczik et al. (2015) evaluated the effect of water regimen on AMF colonization of sorghum plants and verified a strong reduction in colonization ratio and the length of AMF hyphae in the soil of plants grown at 35–55% θ_{FC}. These results corroborated other studies, in which the effect of water stresses on AMF colonization ratios in dandelion (*Taraxacum officinale*) and clover (*Trifolium alexandrinum* L.) (Asrar et al. 2012; Saia et al. 2014) were determined. Bonfim et al. (2010) and Sousa et al. (2015) also found a higher AMF colonization ratio under low soil moisture conditions and concluded that greater root colonization by AMF was an important strategy for resistance to water stress.

For the production of sugarcane in Brazil, enhancement of AMF colonization may constitute an effective strategy for overcoming climatic adversity, like drought. However, more studies are needed that explore the interaction of AMF with sugarcane plants, especially considering the effect of sugarcane genotype on AMF colonization and the subsequent resistance of plants to abiotic stresses, as discussed in this paper.

The effect of AMF inoculation on the growth of sugarcane plants (RB857515 genotype) under conditions of 50 and 100% θ_{FC} soil moisture levels was studied by Sousa et al. (2015). They observed a reduction in stem diameter and aerial fresh biomass and roots under conditions of water stress, regardless of whether the plants had been inoculated with AMF. In addition, AMF colonization ratios in the soil were also lower for 50% θ_{FC}, indicating better efficiency in AMF colonization in soils managed with 100% WHC eθ_{FC}. In a study of the effects of water stress on AMF-inoculated and non-inoculated tomato cultivars, Subramanian et al. (2006) found greater AMF dependence in tomato plants submitted to water stress, similar to what we observed for the IAC5000 sugarcane variety, which presented higher spore counts and higher colonization rates when grown under the 50% water replacement level.

Structure of the AMF Community

The PCR bands resulting from amplification of the 18S rRNA genes were distinct for each sugarcane genotype and soil moisture level. The AMF community profiles in the CTC15, CTC17, RB857515, RB92579, RB931011 and NC0376 genotypes were sorted into two major groups corresponding to the 100 and 50% soil water levels. Two other similar AMF groups were found for genotypes RB966928 and IAC5000, one for each of the two water regimens. Changes in AMF community structure under field conditions and at the host level due to changes in precipitation were reported by Martínez-García et al. (2012). AMF communities also presented strong structure variations when evaluated under different land use conditions (forest, pasture, coffee plantations and no-till systems) and for rainy and dry periods (Fernandes et al. 2016). Other studies have reported a succession of AMF groups under extreme soil moisture conditions. For example, Querejeta et al. (2009) identified a change in the dominance of *Glomaceae* to *Gigasporaceae* in oak forests during dry and rainy years. In addition to soil moisture, other factors may modulate AMF communities, such as soil type (Landis et al. 2004), soil management and use (Jansa et al. 2003; Al-Yahyaei et al. 2011; Fernandes et al. 2016) and the interaction of host plant and AMF (Bever et al. 1996; Helgason et al. 1999; Verzeaux et al. 2017).

In our study, the interactions between genotype, water level and the AMF community were the strongest in RB966928 and IAC5000. The IAC5000 variety is characterized by its resistance to drought and this may explain why AMF interactions were more marked in IAC5000 under conditions of water stress (50% of field capacity). The RB966928 and IAC5000 genotypes may favor a community of specific AMF that are more adapted to conditions of water stress. This selection pressure may be associated with maintenance and growth of the root system, since the genotypes RB855453, RB92579, RB965917 and RB965902 had a drastic reduction in root dry mass under the 50% water replacement level (Holanda et al. 2014).

Correlation Between Colonization Ratio and Productivity Parameters

The correlation between root colonization ratio, fresh stalk productivity and root dry mass (RDM) was higher under 100% θ_{FC} (Fig. 4d). Although root colonization was also high at 50% irrigation, there was no significant increase in stalk production or RDM. Similar results were observed by Sousa et al. (2015), demonstrating that under water stress plants tended to experience greater colonization ratios by AMF; but lack of water can equally inhibit the plant–AMF association because it interferes with development of the host plant and its capacity for mycorrhizal fungal colonization (Antolín et al. 2010; Torres et al. 2018). Candido et al. (2015) verified that a high efficiency of AMF colonization promoted tomato plant growth and increased fruit yield in soil irrigated with 0, 50 and 100% water replacement. However, they did not find any synergy between colonization ratio and irrigation levels. Conversely, we found that the AMF colonization ratio presented synergism, with fresh stalk and RDM production under 100% water replacement conditions.

In the IAC5000 sugarcane variety, there was a quick increase in RDM even in 50% irrigated soils. This increase in sugarcane RDM is often related to symbiotic interaction with AMF which results in a greater ability to overcome water stress and to exploit a greater soil volume through the AMF network (Rapparini and Peñuelas 2014). There is still a critical need, however, to seek new ways to increase AMF colonization of sugarcane plants for the development of sugarcane plantations, especially in those marginal regions where water restrictions or drought are increasing.

Conclusions

Here we report a strong influence of the sugarcane variety and the soil moisture levels on the abundance of AMF spores, the host colonization level and the AMF community structure in the soil. When the soil moisture was at 100% afield capacity, 100% θ_{FC}, all the sugarcane genotypes we tested responded with a greater soil spore abundance and higher AMF colonization than under conditions of 50% θ_{FC}. The RB966928 and IAC5000 genotypes were the most sensitive to this effect, exhibiting increased AMF colonization even under relative dry levels of 50% soil moisture. Different soil moisture levels also modified the AMF community structure in the soil. In spite of great advances in scientific knowledge about AMF, their interactions with the whole range of factors that regulate the development of sugarcane are still far from being fully understood. Hence, more efforts are needed to explore how these interactions occur as well as to develop and characterize specific genotype varieties adapted to a wider range of agricultural conditions.

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