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Short communication

Protocol optimization for *in vitro* sugarcane establishment from stem cuttings shoot tips

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An optimized protocol for sugarcane (variety RB92579) *in vitro* establishment was developed by using stem cuttings shoot tip. The study revealed that success on aseptically inoculated shoot tips of sugarcane can be influenced by the appropriate plant growth and development stage. Sugarcane plants with 8-11 months old were used as a source of stem cuttings. The shoots collections from the stem cuttings were made at three different ages, 103 days (S1), 115 days (S2) and 145 days (S3) after planting in the greenhouse. After explant inoculation on MS medium, all explants were evaluated with 15 days of *in vitro* culture. The analysis of *in vitro* explant development showed that the S3 explants (145 days of age) were superior to the S1 and S2 explants (103 and 110 days respectively). The best *in vitro* explant viability was observed in S3 explants (47%), compared with 4% and 17% observed in S1 and S2 explants respectively. The *in vitro* explant discard, by microorganism contamination, were highest on S1 explants (76%) than in S2 (17.4) and S3 (29%) explants. These results indicate that the shoot tips age of development can influence the success on *in vitro* sugarcane establishment.

Key words: *Saccharum*, Tissue culture, Sugar cane setts, Shoot tips

Micropropagation is considered one of the most efficient biotechnological alternatives to replace the conventional method of vegetative propagation of sugar cane stalks. The adoption of *in vitro* culture have numerous advantages over field multiplication as: Large scale seedling production, higher plant quality, less time and space for the propagation of seedlings **(**IAEA, 2004). In general, the propagation protocols on a large scale *in vitro* sugarcane are based organogenesis processes and / or somatic embryogenesis (Khan and Khatri, 2006, Dibax et al., 2011). However, the morphogenic response can be significantly influenced by various factors such as the plant genotype, the plant health (phyto-sanitation), the age of the donor plant and*in vitro* culture conditions.

In sugarcane commercial plantations, the seedlings are obtained by vegetative propagation from stem segments. However, this form of seedling production favors the spread to various pathogens, which accumulate in the plant during the cultivation cycle. According to Mims and Mims (2004), many fungal spores in smoke can be spread considerably farther than surrounding fields.

As so, the development of a large scale protocol for healthy sugarcane plant propagation could help to minimize disease spread in commercial field,

The establishment of a commercial protocol for *in vitro* propagation depends in

part on the control and prevention of endophytic contamination (Toledo, 2011), caused by bacteria, yeasts and filamentous fungi (Toledo, 2011; Tam and Diep, 2014). In general, some endophytic microorganisms compete with *in vitro* explants for culture medium nutrients. Some of these micro-organisms also produce toxic metabolites that accumulate in the culture medium, compromising the viability of the explants (Khafagi et al., 2001; Pereira et al., 2003).

As so, the micropropagation success depends upon the explant successful introduction in the culture medium (establishment step). As so, in the cultivation establishment phase, the endogenous contamination can cause loss of time, financial resources and genetic material (Odutayo et al., 2007). The endophytic contamination control can be made by different protocols. Among the most common substances used are antibiotic and fungicides added to the culture medium (Altan et al., 2010, Danso 2011). However, these substances can interfere with *in vitro* regenerative response of some plant species (Bhau and Wakhlu, 2001).

The objective of this study was to evaluate if the developmental stage of young shoots of sugarcane explants can interfere with endophytic contamination levels and *in vitro* development.

Material and methods

The study was carried out in the CETENE (Northwest Strategic Technology Center) Plant Tissue Culture Laboratory. Sugarcane plants (RB92579) with 8-11 months old were used as a source of explants (stem cuttings).The explants were collected from donor plants in the Itapirema Experimental Station of IPA (Agronomy Institute of Pernambuco). For the experiment were used 300 stem cuttings heat treated. In the laboratory, the plant material was cleaned with a commercial detergent and rinsed under running water. Then the explants were subjected to a heat treatment, using the following procedure: 52 °C for 20 minutes on the first day; 50 °C for 15 min on the second day and 52 °C for 10 minutes on the third day.

Next, the stem cuttings were maintained on a fungicidal solution (1.5 mL $L^{-1}$of Kasumin®) for 20 min and were planted in plastic trays with commercial substrate Basaplant®. The trays containing the explants were kept in the greenhouse of CETENE under irrigation to promote the development of shoots.

The collections of the shoots of the stem cuttings were made at three different ages, 103 days (S1), 115 days (S2) and 145 days (S3) after planting in the greenhouse. Irrigation was suspended for 48 h before explant collection. The shoots were isolated and washed with water and benzalkonium chloride 1%. Then the explants were placed in a hot water bath with distilled water having a temperature of 51°C for 10 minutes. Then, in a laminar flow, the explants were surface sterilized with 70% ethanol (2 minutes with stirring), two immersions in sodium hypochlorite solution (1.5% active chlorine) for 15 min / each, follow by washes with sterile distilled water to remove any hypochlorite residue.

After rinsing with sterile distilled water, the explants were transferred to a Derosal solution (2.0 g L^{-1}) for twenty minutes and, finally, transferred to a sterile solution of citric acid (50.0 mg L^{-1}), where they remained until the *in vitro* introduction of shoot tips.

In laminar flow, leaves were removed and the explant size was reduced to about 2.0 cm long. Then the explants were inoculated in test tubes with filter paper bridges 80 g, containing 10 ml of culture medium for ***in vitro*** establishment of sugarcane. The nutrient medium was prepared with salts and vitamins of MS (Murashigue and Skoog, 1962) added 50 mg L^{-1} citric acid, 100 mg L^{-1} myo-inositol, 0.2 mg L^{-1} BAP (benzylaminopurine), 0.1 mg L^{-1} Kin (Kinetin) and 20 g L^{-1} sucrose and pH 5.8.

The test tubes containing the explants were kept in the dark for eight days, and

eight days in a growth chamber with temperature of 25 ± 2° C, 16 hours photoperiod and light intensity of 50 micromol m^{-2} s^{-1}. After 15 days of *in vitro* culture, the explants were evaluated for the presence of contamination (fungus and/or bacteria) and the explant development (Explant viability, leaf expansion, oxidation, necrosis, shoot tips emission). All the data were subjected to analysis by Kruskal-Wallis test using the BioEstat software (version 5.3).

Results and discussion

Almost all explants (stem cuttings) kept in the green house started shoots development (98%) after about 30 days. Shoots containing the apical meristem emerged from each node. This result indicates that thermotherapy methodology did not affect the viability of the explants maintained in greenhouse. During *in vitro* establishment phase were observed a significant difference in some of the parameters analyzed. The incidence of endophytic contamination was higher in younger explants, treatment S1 and S2 (103 and 110 days respectively). This result indicates that older explants have a lower incidence of bacteria (gram positive and gram negative) during *in vitro* establishment (Table 1). In general, for the *in vitro* cultivation of plants, it is suggested use younger explants, which have better plant health (phytosanitation) and better *in vitro* viability rate (Basto et al., 2012).

In general, the main characteristics that interfere with the sugarcane ***in*** *vitro* response are the genotype, the physiological state of the donor plant and explant used to initiate *in vitro* culture (Gandonou et al., 2005; Nawaz et al., 2013; Tolera et al., 2014).

Thermotherapy is one of the most adopted methodologies to control *Leifsonia xyli* subs. *xyli* in sugarcane. However, it may also be responsible for the elimination of other endophytic contaminants (Balamuralikrishnan et al., 2003; Wagih et al., 2009).

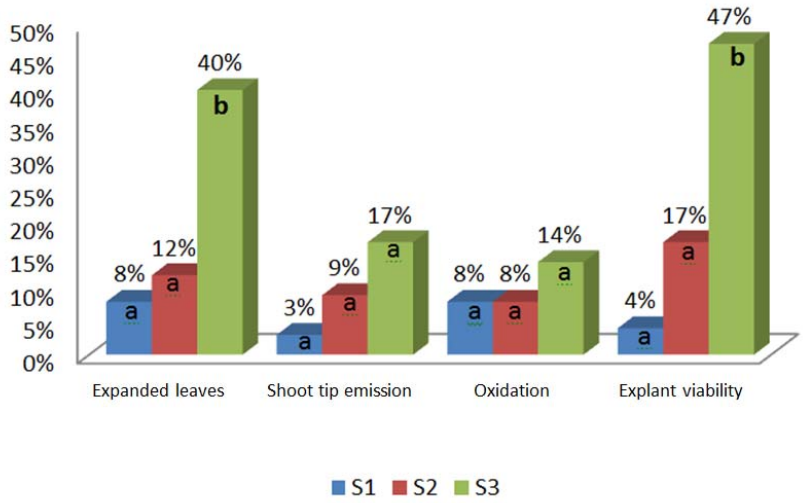
The result obtained in this study indicates that the explant physiological state can influence both the development of shoots and the endophytic microorganism populations. Thus, the identification of an ideal shoots age of development can help to maximize the efficiency of thermotherapy process, making *in vitro*establishment a more effective step.Explants with different ages (103, 110 and 145 days) were not significantly different in the incidence of contamination by the fungus (*Fusarium* and *Penicillium*). However, the occurrence of contamination by bacteria appears to be significantly influenced by the age of the explant. Younger explants (103 days) have a high incidence of endophytic bacteria contamination. This result indicates that the endophytic bacteria contaminants (gram-positive and gram-negative) are the main problems that interfere with successful sugarcane *in vitro* establishment. Part of an efficient establishing strategy for sugarcane, which minimizes the loss of the explants by endophytic bacteria contamination, can be correlated to age and development stage of shoots from stem cuttings.

Table 1. Incidence of microorganisms contamination in sugarcane shoot tips established *in vitro*

Contamination rate analysis of sugarcane after *in vitro* establishment				
Explantages	Microorganism			
	Fusarium	*Penicillium*	Bacteria	UM
S1 (103 days)	0.0% a	3.0% a	**70.0% a**	3.0% a
S2 (110 days)	2.2% a	2.2% a	**13.0% b**	0.0% a
S3 (145 days)	2.0% a	5.0% a	**20.0% b**	2.0% a

*UM – Unidentified microorganisms

Also, a higher rate of explants viability, during *in vitro* establishment stage, was observed on shoots tips collected with 145 days of culture in a greenhouse (Graph 1). The analysis of *in vitro* explants development (Graph 1 and Figure 1) showed that, despite the tissue oxidation levels were higher in the S3 explants(145 days of age), the development of the explants (expanded leaves and shoot development) was superior to the S1 and S2 explants (103 and 110 days respectively).



Graph 1.Analysis of Sugarcane *in vitro* propagationusing shoot tip explants. Explant ages used: 103 (S1), 110 (S2) and 145 (S3) days.

Among the evaluated parameters (fungal, bacterial contamination, oxidation, leaves expansion and explant viability) after 15 days of *in vitro* culture, the most relevant criteria for determining the best age and developmental stage for explants collection for *in vitro* culture were: Contamination (yeast / bacteria), leaf expansion and explants viability (Figure 1). The contamination rate seem to be the most use parameter to protocol efficiency evaluation during explant *in vitro* establishment.

The young shoot tips (S1 and S2) showed the lowest oxidation indices and the lowest viability rates after 15 days of *in vitro* culture. A progressively higher rate of explants viable was observed on elder shoot tips explants. This result may be due to better nutritional status and maturity of the explants collected with 145 days.

For explants with 103 (S1) and 110 (S2) days of age, high infection rates and lower *in vitro* viability rates resulted in a loss over 40 % of *in vitro* explants. Despite endophytic contamination level, all viable materials developed new shoots with leaves and tillers. The proliferation of endophytic microorganisms in explant already established on *in vitro* conditions is rare. Liu et al., (2005) eliminate *Pasteurella multocida*, *Stenotrophomonas maltophilia*, and *Alcaligenes* sp. endophytic contamination of *Limonium sinuatum* L. Mill. by using antibiotic during all micropropagation process. Dias et al., (2009) also isolate twenty endophytic bacteria (genera *Bacillus* and *Sphingopyxis*) from the meristematic tissues of three varieties of strawberry cultivated *in vitro*. As so, microorganismpresences on *in vitro* plant tissue are commonly found in many plant species.

The most common strategy for microorganism controls in sugarcane micropropagation is the use of hot water treatment and antibiotic addition on medium culture (Dash et al., 2011; Meena et al., 2014). However, the plant tissue culture medium with antibiotic can interfere on

plant development (Mittal et al., 2009). As so, an alternative strategy for minimize endophytic contamination can be helpful ona large scale propagation of this culture.

During micropropagation of these viable explants, no repeated incidence of endophytic contamination was observed in sugarcane of S1, S2 and S3 treatment.The observed oxidation rate was lower than those previously described for sugarcane (Molina et al., 2005 Shimelis, 2015). The rates observed in this work did not exceed 15%. However, this result may be attributed to the culture medium used antioxidant and the fact that one of the processing steps, for *in vitro* establishment, involves an incubating stage in antioxidant solution. Similar procedures have not been described for sugarcane *in vitro* establishment.

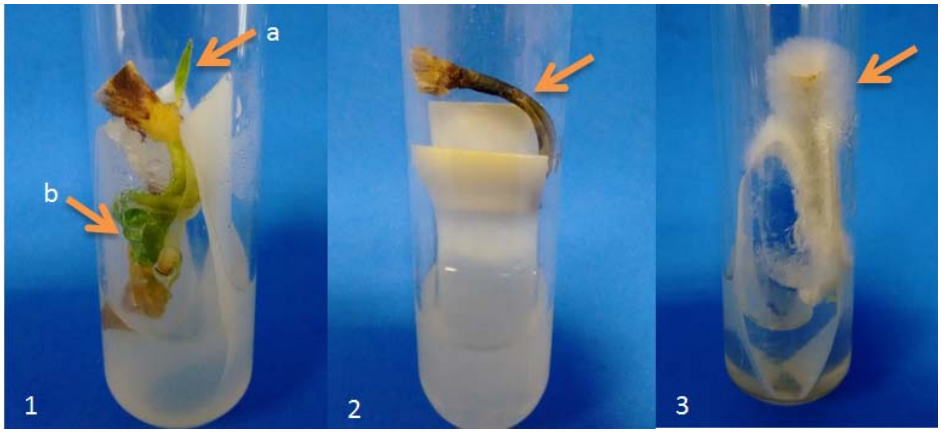


Figure 1.Viable sugarcane shoot tips appearance after 15 days of *in vitro* establishment. 1. New shoot tip emission (a) and expanding sheet (b), 2. Shoo tips with tissue necrosis and 3. Shoot tip with fungus contamination.

The large loss of explants by oxidation and contamination are compensating by a high organogenic response of each explant that remains viable, with the potential ability to produce thousands of *in vitro* plants. However, the formation of a large population of plants, from single meristems, can generate some epigenetic and somaclonal variants that are considered a serious problem in tissue culture (Zucchi et al., 2002; Sobhakumari, 2012). Thus, the optimization of *in vitro* establishment process, identifying the most appropriate explant physiological stage development can allow increasing the rate of viable explants and, hence, be an excellent alternative to reduce the incidence of somaclonal variation.

Conclusion

The age of sugarcane explants can interfere significantly on *in vitro* success establishment of this culture. As so, the correct determination of the best age, plant growth and development stage can significantly contribute to *in vitro* establishment of sugarcane protocol for large scale micropropagation.

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