ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 09, pp.19888-19892, September, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ELECTROTHERAPY TREATMENT FOR RATOON STUNTING DISEASE (*LEIFSONIAXYLI* SUB SP. *XYLI*) ELIMINATION INSUGARCANE MICROPROPAGATION

***Laureen M. Houllou, Diogo C. B. A. Torres, Maria J. L. Medeiros, Patrícia V. P. Dantas, Andrezza B. Soares, Robson A. de Souza, Paloma de F. Cavalcante and Mariana C. de M. Sobral**

Centro de Tecnologias Estratégicas do Nordeste, Phone: 55 (81) 33347200, FAX:55 (81) 33347219, Brazil

ARTICLE INFO

Article History:
Received 29th June, 2015
Received in revised form
21st July, 2015
Accepted 05th August, 2015
Published online 16th September, 2015

Key words:

Plant Tissue Culture,
Saccharum Spp.,
Polymerase Chain Reaction.

ABSTRACT

Ratoon stunting disease (RSD) is a pathology caused by *Leifsoniaxyli* subsp. *Xyli* bacterium, one of the major diseases affecting sugarcane world production. The accurate diagnosis of the disease presents some difficulties, because once noticeable, not always the symptoms are specific. Therefore, diagnostic tests should include reliable methods that ensure the health report with high sensibility, even in the asymptomatic stages of the disease in plants, such as PCR. The present study aimed to evaluate *in vitro* shoot tips of sugarcane by PCR-specific technique. Sugarcane plants (RB92579) with 8-11 months old were used as a source of explants (stem cuttings). After *in vitro* establishment, shoots were cut approximately 3 cm long and they were subjected to an electrotherapy treatment (electric currents of 20 mA and 30 mA for 15 minutes). The plant DNA material was submitted to the PCR assays with specific primers for Lxx. The PCR technique manifested itself as capable method for detection of Lxx with sensibility even in *in vitro* plants. Through this method was possible identify samples previously submitted to electrotherapy which did not PCR presented amplification using specific primers for Lxx. As so, PCR can be used to analyze *in vitro* shoot tips of sugarcane.

***Citation*:** Laureen M. Houllou, Diogo C. B. A. Torres, Maria J. L. Medeiros, *et al*. 2015. "Electrotherapy treatment for ratoon stunting disease (*Leifsoniaxyli* subsp. *xyli*) elimination in sugar cane micro propagation", *International Journal of Current Research*, 7, (9), 19888-19892.

INTRODUCTION

Sugarcane (*Saccharum* spp.) is one of the major agronomic crops cultivated in Brazil. Despite its economic importance and technology development, this crop has still been affected by pathogens such as fungi, bacteria, viruses and phytoplasmas which may lead production losses in the field. The fastidious bacterium *Leifsoniaxyli* subsp. *xyli* (Lxx) is the causal agent of ratoon stunting disease (RSD) in sugarcane and is characterized by colonize the xylem of plants, causing obstruction and hindering the transport of water and nutrients to leaves (Brumbley *et al*., 2002). The RSD has no easy external classic signs or symptoms, being easily spread to a new planting area. Although there is variation in susceptibility to RSD, has not been found yet an immune variety to the pathogen. However, the lack of genetic variation among Lxx isolates, independent of sampling conditions, suggests the worldwide spread of a single pathogenic clone (Young *et al*., 2006), but failure in previous procedures to obtain complete elimination of the pathogen generated a new source of inoculum (Benda, 1994).

****Corresponding author: Laureen Michelle Houllou,***
Centro de Tecnologias Estratégicas do Nordeste, Phone: 55 (81) 33347200, FAX:55 (81) 33347219, Brazil.

Therefore, it is essential to associate different disinfection procedures, besides the traditionally used thermotherapy practice, and perform systematic examination of plant sanity condition (Tokeshi, 2005). Heat treatment, used to eliminate Lxx, works the binomial parameters time-temperature. Stem explants are submitted to immersion in hot water in attempt to degrade proteins and enzymes of bacterial cells. Even so, is known that thermotherapy treatment (50ºC/2h) alone is not completely effective since did not completely eradicate Lxx in the treated stems (Urashima and Grachet, 2012). The alternative most widely used for the obtainment of pathogen-free plants is the combination of more than one technique. Some studies have suggested the combined use of thermotherapy and others techniques to improve the success on contamination control (Neelamathi *et al*., 2014). Electrotherapy consists in a simple method for elimination of some plant pathogens without the need for any special or expensive equipment. The technique presupposes submit plant tissue to an electric current in order to eliminate the pathogen cells by interruption or degradation of some of its nucleoprotein. Electrotherapy has been successfully applied to the elimination of potyvirus from different potato clones (Meybodi *et al*., 2011), and its efficiency has been investigated also in beans mosaic virus, in which based on the Therapy Efficiency Indices, the electric current was an effective

treatment for both cultivars tested (Hormozi-Nejad *et al*., 2010). The use of the PCR technique for Lxx sugarcane detection has been used for over a decade (Taylor *et al*., 2003; Grisham *et al*., 2007; Gao *et al*., 2008; Liu *et al*., 2013). In the work described by Taylor *et al*. (2003), two primers designed from the sequence pSKC2-800, were effective for amplifying the Lxx DNA from the vascular fluid of infected sugarcane. However, despite PCR efficiency, no work has been found describing the use of PCR to amplify Lxx specific sequences in sugarcane *in vitro* cultured plants.

The present study aimed to evaluate the efficiency of electrotherapy associated with thermotherapy for Lxx elimination on micropropagated sugarcane plants, and also its effect on explant development, as well as the PCR technique detection efficiency on micropropagated sugarcane for pathogen-free plants identification.

MATERIALS ANDMETHODS

All the studies carried out in Centro de Tecnologias Estratégicas do Nordeste (CETENE), and performerat Laboratório de Pesquisas Aplicada a Biofábricaand Laboratório de Diagnose Fitossanitária e Fidelidade Genética. Sugarcane plants (*Saccharum* spp.), from variety RB92579, with 8-11 months old were used as a source of explants (stem cuttings). The explants were collected from donor plants in the Estação Experimental de Itapirema, from the Instituto Agronômico de Pernambuco (IPA).

Thermotherapy

The stems were cut into a 7 cm long explant. The cut stems were washed with sponge and commercial detergent and rinsed in water. After superficial disinfestation, the heat treatment series (HTS) was performed through explants submersion in hot water over controlled time and temperature. The HTS was reproduced in three consecutive days, consisting in three baths with 24 h time interval between them, first 52°C / 20', second 50°C / 15', and finally 52°C / 10'. After the HTS, cut stems were treated with Kasumin® fungicide and planted in plastic trays using commercial substrate Basaplant®, and transferred to a greenhouse with daily irrigation for 45 days to promote buds development and shoots maturation. Explants were treated with Kasumin® fungicide 10 days before *in vitro* introduction, and after fungicide treatment, the stem apex was removed from the stems (for *in vitro* establishment) and leaf samples were collected for molecular diagnosis.

Once collected, the processing of explants was performed on laboratory. Oldest leaves were removed and explants were cleaned with sponge, water and benzalkonium chloride 1%. The material was deposited in pots, washed with sterile water and taken to hot water bath at 51 °C for 10 minutes (to perform another thermotherapy treatment). The disinfection was made with explants immersion in 70% ethanol for 2 minutes, followed by two immersions in 1.5 % sodium hypochlorite solution with two drops of Tween 20 with stirring for 15 min each. All explants were rinsed four times with sterile distilled water under stirring. After that, explants were kept in a sterile solution of 5 % citric acid until *in vitro* introduction.

For *in vitro* introduction, the explants were cutter, in a flow chamber, to obtain a 2 cm long explant. After this, all explants were placed inverted into a test tube containing 10 mL modified MS medium culture (MSS)for sugarcane *in vitro* establishment and a filter paper bridge. The MSS medium was composed of 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, 0.1 mg.L^{-1} KIN, 20 g L^{-1} sucrose, 2.0 g L^{-1}Phytagel™, pH 5.0.The test tubes containing the explants were kept in the dark for 10 days; then were placed in a growth chamber with temperature 25 ± 2 °C, 16 hours photoperiod and light intensity of 50 micromole m^{-2} s^{-1}, for five days. After this period, the infected explants were eliminated and the viable explants were kept for more 15 days and then were subculture on micropropagation medium (MMS). The MMS medium was composed of salts and vitamins of MS (Murashigue and Skoog, 1962) added 50 mg L^{-1} citric acid, 50 mg L^{-1}ascorbic acid,100 mg L^{-1}myo-inositol, 2.0 mg L^{-1} BAP, 1.0 mg.L^{-1} KIN, 30 g L^{-1} sucrose, 2.0 g L^{-1}Phytagel™, pH 5.8.

Eletrotherapy

Shoots tips from introduced and acclimatized sugarcane were cut approximately 3 cm long which were submitted to electric currents of 20 mA and 30 mA for 15 minutes. The plants were arranged in three groups: Group A (20 mA for 15 minutes), Group B (30 mA for 15 minutes) and Group C (Controls: 0 v), each group containing 20 shoot tips. All electrotherapy procedure was performed in a laminar flow in sterile TBE buffer (90 mMTris borate, 2 mM EDTA, pH 8), using a horizontal electrophoresis system. Soon after the treatment, the shoots were kept in flasks containing MMS medium modified in the growing room with appropriate light and temperature (25 ± 2 °C, 16 hours photoperiod and light intensity of 50 micromole m^{-2} s^{-1}). Two weeks after electrotherapy treatment, the shoots tips were evaluated for contamination, oxidation, and propagation.

Molecular diagnosis

Samples of *in vitro* sugarcane plant leaves (RB92579) were collected from shoot tips and stem segment, and 25 mg of each sample was frozen in liquid nitrogen for subsequent grind. After, processing plant material was submitted to DNA extraction using QIAamp DNA Mini Kit from Qiagen® (California, USA). PCR assay was performed. The material was submitted to PCR for Lxx genome amplification. PCR was performed using primers LxxW.1 (CAGAGCACCA TCGTGAAGAC) and LxxW.2 (AAGGACAAGTCCACCAA GGA) for RSD with 182 base pair (bp). PCR reaction mix was carried out in a volume of 50 µL containing 10 µL of DNA sample, primers at the final concentration of 0,5µM each, 1.5mM of $MgCl_2$, 1X PCR buffer, 0.2mMdNTP and 0.05 unit of Taq DNA polymerase under the conditions of a denaturation step at 94°C/5'followed by 35 cycles of denaturation at 94°C/45", annealing at 56°C/30", extension at 72°C/1', and a final extension hold of 10' at 72°C. At the end of PCR reaction, the amplicons were submitted to electrophoresis on 2% agarose gel and visualized in ultraviolet light. The generated amplicons were visualized in the form of bands and was defined as positive when observed an 182bp fragment, compared with molecular weight pattern of 100bp.

RESULTS

During plant *in vitro* establishment was observed that the main problem was a higher contamination rate caused by the incidence of fungus (Table 1). However, in subsequent subculture steps, contamination tended to be reduced. From the third subculture was no longer identified visual fungal contamination occurrence. However, the occurrence of visual bacterial contamination was identified only after the start of micropropagation. This result may indicate that the bacterial infection is present as endophyte condition in some explants and only under stress conditions the contamination is visually expressed.

A traditional parameter of evaluation for explants development is the explants necrosis (Figure 1A), which in this study did not showed as a hurdle. The occurrence of tissue necrosis can interfere with the capacity for regeneration and *in vitro* proliferation. Various antioxidant compounds can be used on *in vitro* culture, although according to Klenotičová *et al*. (2013), they can interfere with the development of explants, in the present study the use of citric acid was satisfactory to avoid death of explants by oxidative process during *in vitro* establishment of sugarcane, not being the tissue necrosis a relevant problem for micropropagation. Tissue oxidation/ necrosis was observed at the base of the explants, despite the tissue being or not in contact with the culture medium (establishment and micropropagation). This result indicates that the culture medium composition probably did not prevent phenolic compounds accumulation in the culture medium and from causing the necrosis of the tissue explant base.

Table 1. Result of each development parameter analyzed during *in vitro* culture of sugarcane (RB92759) shoot tips

*In vitro*culturestage		Evaluatinparameter*				
		Visiblefungalcontamination	Visiblebacterialcontamination	Tissuenecrosis	Mediumoxidation	Propagation/explant
Establishment		54	0	100	100	-
Subcultives	1°	17	0	100	100	5
	2°	2.4	14.6	100	100	9
	3°	0.0	9.7	100	100	20
	4°	0.0	0.0	100	100	29

(*)Values expressed in percentual(%) of sample units.

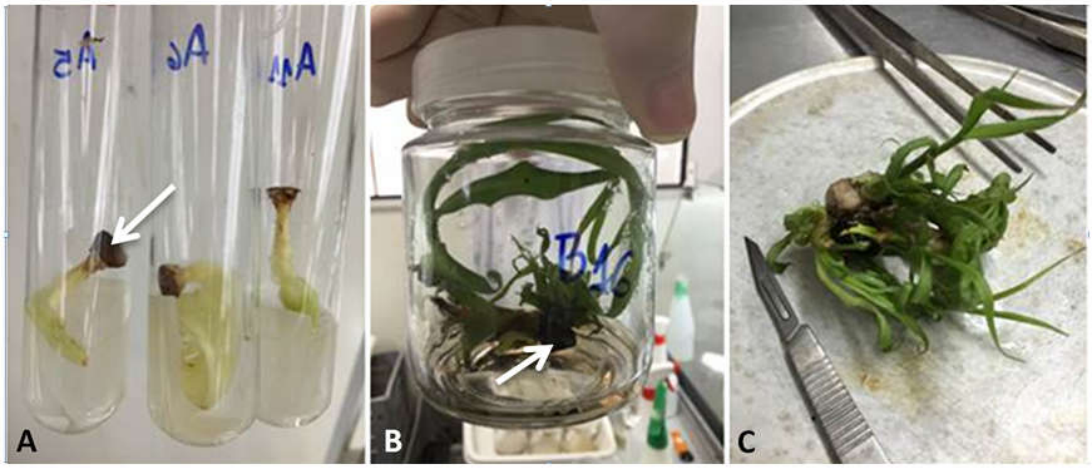

Figure 1. Aspect of *in vitro* sugarcane shoot tips (establishment and plant micropropagation). A. – Sugarcane shoot tips on establishment medium, B. –*In vitro* sugarcane whit new shoo tips and C. - Sugarcane shoot tips used to micropropagation. White arrow indicates tissue necrosis areas

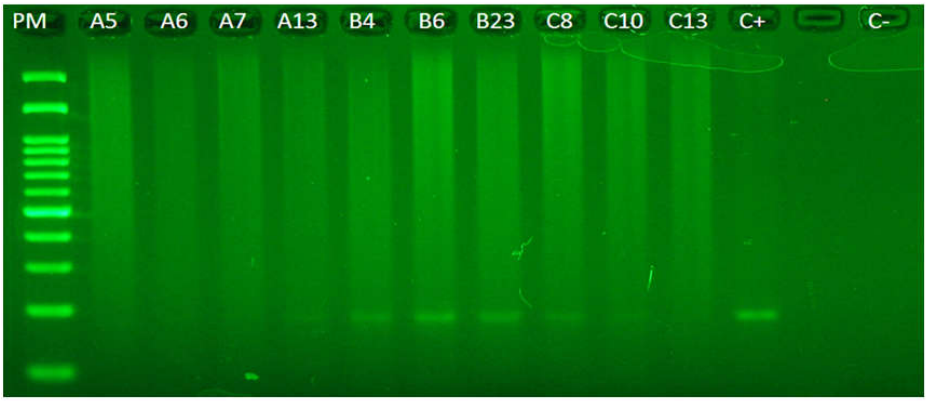

Figure 2. Example of sugarcane shoot tips DNA amplification using specific primers for Lxx. Samples A5, A6 and A7 showed no amplification. *C+ - Positive sample, C- Negative sample.

However, oxidation observed in all explants did not affect the ability to new shoots tips sprout during the micropropagation process (Figures 1B and 1C). It was observed that the electrotherapy process interfered with explants propagation rate. However, decrease in explants able to propagate was not higher than 10%. Therefore, electrotherapy does not apparently interfered on sugarcane explants viability after this treatment. All plants showed oxidation in the explant base, similar to that observed in explants that were not exposed to electrotherapy. The propagation was higher in the groups A, with 91% of shoot development, ranging between 2 to 11 new shoot tips/explant. In Group B was 90% with shoots propagating with 1 to 6 new shoot tips/explant. Already in group C was all explants initiate propagation, with 2 and 5 new shoot tips/explant.

Electrotherapy efficiency on healthy recovery of sugarcane plant was observed using PCR analysis. According to the results, it was observed that part of the explants undergoing electrotherapy showed no amplification using specific primers for the Lxx genome. The bacterial DNA was detected by conventional PCR of stems and leaves of *in vitro* sugarcane plants. All samples were retested and showed that PCR test are efficiency for Lxx detection in micro propagated sugarcane plants.

This result indicates that PCR methodology can be an efficient tool for identification of healthy *in vitro* plants of sugarcane.

In the performed analysis, it was observed that PCR assays have been shown to differentiate plants with or without amplification with specific Lxx primer. This result indicates that electrotherapy can be an auxiliary technique to help the *in vitro* obtainment of clean sugarcane plants. Although, PCR analysis can identify clean plants for large scale propagation, obtained by electrotherapy treatment or other methodology.

DISCUSSION

According to Mbah and Wakil (2012), although the culturing plant tissue is a successfully used technique for commercial production of pathogen-free plants, obtaining clean cultures is not easily achieved. It has been reported that some cultivars maintained in multiplication medium are often contaminated with endophytic bacteria. These bacteria can survive within these *in vitro* plants and hamper commercial micropropagation (Barrett and Cassells, 1994; Cassells, 2001; Wang and Valkonen, 2009). The combine use of traditional plant culture procedures, such as thermotherapy, and electrotherapy showed, in the present work, to be a promising strategy for healthy sugarcane plant production. Antibiotics are usually used to eliminate endophytic contamination. However, clean culture rates (explants free from contamination) through antibiotics use during *in vitro* establishment are not 100% (Gilbert *et al*., 1991). Part of this bacterial contamination may be latent in the plant tissues, and so the antibiotic treatment can turn poorly effective (Gilbert *et al*., 1991; Reed and Tanpraser, 1995; Kałużna *et al*., 2013). The development of the experiment showed that traditional protocols of sugarcane micropropagation onlycan possibly control visual contamination, after a few cycles of the subculture. However, molecular analysis showed that electrotherapy treatmentfavored the possible elimination of Lxx in sugarcane micropropagated plants. The results indicate that the main problem for successful establishment and *in vitro* micropropagation of sugarcane explants is the microorganism contamination (latent or not), which according to Carvalho, *et al*. (2012) can compete with the explant for nutrients from medium, with a higher growth rate, culminating in losses of plantlets through the micropragating process. In this evaluation it was observed that during explant establishment, the emergence of sugarcane shoot tips only occurred in explants whit no visible sing of contamination, showing that part of endophytic contamination can interfere with successful establishment *in vitro* of sugarcane.

Compared to different methodology (thermotherapy and meristem culture) used in others plant species (Lassois *et al*., 2013; Mishizuki and Ohki, 2015), the electrotherapy could not be the most efficient isolated technique for endophytic contamination elimination, however, electrotherapy may also be associated with other methodologies to optimize pathogen elimination (Jung-Yoon *et al*., 2003; Mahmoud *et al*., 2009; AlMaarri *et al*., 2012) and this method is simpler, faster, and requires fewer infrastructures than the others (Hormozi-Nejad *et al*., 2010), as observed during the experiments performed with sugar cane.It was observed that the nodal segments treated with electrotherapy and grown in medium with ribavirin favored to obtain a larger number of potato free PVY (Potato Virus Y) explants compared with the explant cultivated in medium without Ribavirin. Plants free of infection were obtained with electrotherapy and explant culture in medium with Ribavirin (Mahmoud *et al*., 2009). According to AlMaarri *et al*. (2012), the highest percentage virus free plantlets of PVY were observed in meristem-tips excised after electric treatments. These results may indicate that electrical stimulation works similarly as the thermotherapy and can be as efficient as thermotherapy for sugarcane Lxx elimination. The main difference between the electrical therapy and thermotherapy is how explants tissue heats. It seems that electrotherapy increases the temperature only within the explant tissue. Thus, as the plant cell is thermally insulated by the cell wall, this technique may be effective for treating endophyte in the intercellular spaces. It is believed that when the microorganism is in the intercellular spaces, electrotherapy can denature proteins present in the cell membrane of the pathogen(González *et al*., 2006). The inactivation of specific nucleoproteins which are responsible for cell to cell movement can help prevent the spread of the pathogen to healthy tissues. However, until now, the understanding of this mechanism is not fully known in any plant species already tested with this methodology(González *et al*., 2006;Hormozi-Nejad*et al*., 2010).On effort to increase explant development, in general, it is believed that the success of electric treatment could depend in part on the propagation rate and the plant development speed (AlMaarri *et al*., 2012), but in order to be used as a process to control contamination, the size of explant can be one of the most influent parameters, in which its size is related with the transference of heat through the tissue. The technique presupposes heating the tissues inside-out, and so, smaller explants apparently are much susceptible to its effect. Therefore, so as the explant tissue, Lxx cellular size (0.25 to 0.50 cells µm wide by 1.0 µm long) been smaller than that of other pathogenic bacterial species (*Xanthomonasalbilineans*

0.4 to 1.0 µm wide by 3.0 µm long), could be more sensitive to thermic and electrotherapy treatments, which can partly explain the success achieved with the use of electrotherapy in sugarcane *in vitro* plants.Another important detail is that, in some cases, the electrotherapy treatment seems to stimulate the explants development (Mahmoud *et al*., 2009). However, in the present study the electrotherapy treated explants showed a lower number of propagating explants compared with the control explants. This result suggests that sugarcane (RB92579) is more sensitive to this kind of treatment than others plant species.

REFERENCES

AlMaarri1, K., Massa, R. andAlBiski, F. 2012. Evaluation of some therapies and meristem culture to eliminate Potato Y potyvirus from infected potato plants. *Plant Biotechnol.,* 29: 237–243.

Barrett, C. and Cassells, A.C. 1994. An evaluation of antibiotics for the elimination of *Xanthomonas campestris*pv. *pelargonii* (Brown) from Pelargonium x domesticum cv. 'Grand Slam' explants *in vitro*. Plant Cell, *Tissue Organ Cult.,* 36: 169- 75.

Benda, G.T.A. 1994.Serial hot-water treatment for sugarcane disease control. In: Rao, G.P., Gillaspie Jr, A.G., Upadhyaya, P.P., BergaminFilho, A., Agnihotr, V.P. and Chen, C,T. (Eds.),Current trends in sugarcane pathology. New Delhi: *International Books and Periodicals Supply Service*, pp. 297-310.

Brumbley, S.M., Petrasovits, L.A., Birch, R.G. and Taylor P.W.J. 2002.Transformation and transposon mutagenesis of *Leifsoniaxyli* subsp. *xyli,* causal organism of ratoon stunting disease of sugarcane.Mol*. Plant-Microbe Interact*., 15:262- 268.

Carvalho, A.C.P.P., Rodrigues, A.A. de J. and Santos, E. O. 2012.Panorama da produção de mudas micropropagadas no Brasil. Fortaleza: *Embrapa Agroindústria Tropical*, 42 pp.

Cassells, A.C. 2001.Contamination and its impact in tissue culture.ActaHortic., 560: 353-59.

Gao, S.J., Pan, Y.B., Chen, R.K., Chen, P.H., Zhang, H. and Xu, L.P. 2008. Quick detection of *Leifsoniaxyli* subsp. *xyli* by PCR and nucleotide sequence analysis of PCR amplicons from Chinese *Leifsoniaxyli* subsp. *xyli* isolates. *Sugar Tech*, 10: 334-340.

Gilbert, J.E., Shohet, S. and Caligari, P.D.S. 1991. The use antibiotics to eliminate latent bacterial-contamination in potato tissue-culture. *Annals of Applied Biology,* 119: 113-120.

González, J.E., Sanchez R. and Sanchez A. 2006.Biophysical analysis of electric current mediated nucleoprotein inactivation process.*Cent.Agric.*, 2:42-47.

Grisham M. P., Pan Y.-B., and Richard-Jr. E. P.2007. Detection of *Leifsoniaxyli* subsp. *Xyli*in sugarcane leaves by Real-Time Polymerase Chain Reaction. *Plant Disease*, 91:430 – 434.

Hormozi-Nejad, M. H., Mozafari, J. andRakhshandehroo F. 2010.Elimination of bean common mosaic virus using an electrotherapy technique. *J. Plant Dis. Prot.,* 117:201–205.

Hoy, J.W., Bischoff, K.P., Milligan, S.B. and Gravois, K.A. 2003. Effect of tissue culture explant source on sugarcane yield components. *Euphytica*, 129:237-240.

Jung-Yoon, Y., Hyo-Won, S., Young-Mee, C. and Young-Eun, P. 2003. Ribavirin, electric current and shoot tip culture to eliminate several potato viruses. *Journal of Plant Biotechnology,* 5: 101-105.

Kałużna, M., Mikiciński, A., Sobiczewski, P., Zawadzka, M., Zenkteler, E.andOrlikowska, T.2013.Detection, isolation, and preliminary characterization of bacteria contaminating plant tissue cultures. *ActaAgrobot*., 66:81–92.

Klenotičová, H., Smýkalová, I., Svábová, L. andGriga, M. 2013.Resolving browning during the establishment of explant cultures in *Viciafaba* L. for genetic transformation. ActaUniv.Agric.etSilvic.MendelianaeBrun., 61:1279-1288.

Lassois, L., Lepoivre, P., Swennen, R., van den Houwe,I. and PanisB. 2013. Thermotherapy, Chemotherapy, and Meristem Culture in Banana. In:Protocols for Micropropagation of Selected Economically-Important Horticultural Plants. New York: Springer, pp. 419-433.

Mahmoud, S.Y.M., Hosseny, M.H. and Abdel-Ghaffar, M.H. 2009.Evaluation of some therapies to eliminate potato Y potyvirus from potato plants. *Int.J.ofVirol*., 5:64-76.

Meybodi, D.E., Mozafari, J., Babaeiyan, N. and Rahimian, H.2011. Application of electrotherapy for the elimination of potato potyviruses. *J. Agr. Sci. Tech.,* 13: 921-927.

Mochizuki, T. and Ohki, S. T. 2015.Detection of plant virus in meristem by immunohistochemistry and *in situ* hybridization. *Methods Mol. Biol.,* 1236:275-287.

Murashige T, Skoog F.A. 1962. A revised medium for a rapid growth and bioassays with tobacco tissues cultures. *Plant Physiol.,* 15: 473-479.

Neelamathi, D., Manuel, J. and George, P. 2014. Influence of apical meristem and chemotherapy on production of virus free sugarcane plants. *Res. J.RecentSci*., 3:305-309.

Reed, B. M. and Tanpraser, P. 1995. Detection and control of bacterial contaminants of plant tissue cultures.A review of recent literature. *Plant Tiss.Cult.andBiotechnol*, 3:137-142.

Taylor, P. W. J., Petrasovits, L. A., Van der Velde, R., Birch, R. G., Croft, B. J., Fegan, M., Smith, G. R. and Brumbley S. M. 2003. Development of PCR-based markers for detection of *Leifsoniaxyli* subsp. *xyli* in fibrovascular fluid of infected sugarcane plants.Australas. *Plant Pathol*., 32:367–375.

Tokeshi, H. and Rago, A. 2005. Doenças da cana-de-açúcar. In: Kimati, H., Amorim, L., Rezende, J.A.M., Bergamin Filho, A. and Camargo, L.E.A. (Eds.) Manual de Fitopatologia. Vol2. Doenças das plantas cultivadas. São Paulo: Agronômica Ceres, pp. 185-196.

Urashima, A.S. and Grachet, N.G. 2012. Métodos de detecção de Leifsoniaxylisubsp. xyli e efeito da termoterapia na brotação das gemas de diferentes variedades de cana-de-açúcar. *Trop. Plant Pathol*., 37(1):57-64.

Wang, Q. and Valkonen, J.P.T. 2009. Cryotherapy of shoot tips: novel pathogen 198 eradication method. *Trends in Plant Science*, 14: 119–122.

Wakil, S. M. and Mbah, E. I. 2012. Screening antibiotics for the elimination of bacteria from *in vitro* yam plantlets.AU *Journal of Technology*, 16:7-18.

Young J., PetrasovitsL. A., Croft B. J., Gillings M. and Brumbley S. M. 2006. Genetic uniformity of international isolates of *Leifsoniaxyli* subsp. *xyli*, causal agent of ratoon stunting disease of sugarcane. Australas. *Plant Pathol.,* 35:503–511.

****