Enhanced micropropagation and tiller formation in sugarcane through pretreatment of explants with thidiazuron (TDZ)

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Abstract An efficient, simple and commercially applicable protocol for rapid micropropagation of sugarcane has been designed using variety Co 05011. Pretreatment of shoot tip explants with thidiazuron (TDZ) induced high frequency regeneration of shoot cultures with improved multiplication ratio. The highest frequency (80%) of shoot initiation in explants pretreated with 10 mg/l of TDZ was obtained during the study. Maximum 65% shoot cultures could be established from the explants pretreated with TDZ as compared to minimum 40% establishment in explants without pretreatment. The explants pretreated with 10 mg/l of TDZ required minimum 40 days for the establishment of shoot cultures as compared to untreated explants which required 60 days. The highest average number of shoots per culture (19.1) could be obtained from the explants pretreated with 10 mg/l of TDZ, indicating the highest multiplication ratio (1:6). Highest rooting (over 94%) was obtained in shoots regenerated from pretreated explants on ½ strength MS medium containing 5.0 mg/l of NAA and 50 g/l of sucrose within 15 days. Higher number of tillers/clump (15.3) could be counted in plants regenerated from pretreated explants than untreated ones (10.9 tillers/clump) in field condition, three months after transplantation. Molecular analysis using RAPD and DAMD markers suggested that the pretreatment of explants with TDZ did not adversely affect the genetic stability of regenerated plants and maintained high clonal purity.

Keywords TDZ (Thidiazuron) · Tissue culture · Sugarcane · Clonal fidelity

Introduction

Thidiazuron (TDZ), a phenyl urea-type plant growth regulator, was first described as a cytokinin in 1982. Initially, it was used for micropropagation of dicots but later it was tried in cereals (Schulze 2007). Among monocots, it was used for raising callus culture of sugarcane (Chengalrayan and Gallo-Meagher 2001). Recently, enhancement in culture establishment and shoot multiplication have been reported in *Harpagophytum procumbens*, *Nyctanthes arbor-tristis* and *Vitex negundo* following pretreatment of nodal explants with TDZ (Jahan et al. 2011; Grabkowaska et al. 2014; Ahmad and Anis 2007). Information on the use of TDZ for pretreatment of explants of sugarcane is not available so far.

During the past decades, several protocols for in vitro micropropagation of sugarcane have been suggested (Lakshmanan et al. 2006; Pathak et al. 2009; Kavita et al. 2015; Lal et al. 2015). Despite several advantages of applying micropropagation in sugarcane such as quick spread of newly released varieties, rejuvenation of old deteriorated varieties, easy transportation of seed material, and higher cane and sugar productivity. This technique is not gaining popularity up to the desired extent due to some constraints, especially low multiplication rates and high cost of production (Tiwari et al. 2010; Lal et al. 2015). It is experienced that the existing protocols need to be improved for increasing the explants survival and rate of multiplication to reduce the cost of production. The establishment of shoot cultures is the most crucial and important step of

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in vitro micropropagation which depends on factors such as crop age, explanting season, hormonal requirement and the genotype. The present work was undertaken to study the effect of pretreatment of explants with various concentrations of TDZ on in vitro morphogenetic responses of sugarcane using a newly released high yielding and high sugar variety Co 05011.

Materials and methods

Fresh tops of sugarcane variety Co 05011 were collected from 10-month-old crop grown at the farm of U.P. Council of Sugarcane Research, Shahjahanpur (U.P.) India. Approximately 6-cm long spindle segments were dissected out from the freshly collected tops and washed under running tap water for about 30 min. The segments were rinsed with 1% detergent solution for 5 min and washed several times with water. The segments were finally surface sterilized with 0.1% (w/v) aqueous mercuric chloride (HgCl_2) solution for 10 min followed by several washings with sterile distilled water. The sterilized segments were pretreated by incubating in liquid MS medium (Murashige and Skoog 1962) containing various concentrations of thidiazuron (5, 10, 15 and 20 mg/l) for 24 h. After pretreatment, about 1.0-cm long shoot tip explants comprising apical meristems along with 1–2 leaf primordia were carefully excised out from the segments and immediately inoculated on agar-gelled MS medium containing BAP (0.5 mg/l). The explants pretreated with MS medium without TDZ served as control. After establishment, the shoot cultures were sub-cultured on MS liquid medium containing BAP, Kinetin and NAA (0.5 mg/l each) for further multiplication. Shoots were transferred on MS medium nourished with NAA (5.0 mg/l) for root induction. The pH of the medium was adjusted to 5.8 and 3% sucrose was added as the carbon source before autoclaving in all experiments. The cultures were incubated at $25 \pm 1^\circ\text{C}$

under 16-h photoperiod with a photosynthetic photon flux density of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps (Philips, India) and $60 \pm 5\%$ relative humidity. All the experiments were performed thrice with a minimum of 15 replicates for each treatment.

Scanning electron microscopy Shoot tip explants were fixed in 5% w/v glutaraldehyde for 4 h at pH 7.0 in 0.05 M sodium cacodylate buffer. Fresh fixative was added after 2 h followed by rinsing in buffer and serial dehydration in ethanol (Busse et al. 2005). Samples were dried up to critical point and sputter-coated with palladium. Samples were viewed with JEOL 6490LV scanning electron microscope with an accelerating voltage of 15 kV. Three samples per treatment were taken on day 0, 1, 3 and 5.

Total genomic DNA was extracted from fresh young leaves of ten randomly selected in vitro raised plants following the CTAB method with minor modification (Doyle and Doyle 1987). The quality of extracted DNA after RNase treatment was evaluated on 0.8% agarose gel and finally the DNA was quantified using spectrophotometer. Fidelity analysis of plants regenerated from TDZ pretreated shoot tip was performed. Randomly selected ten RAPD and four DAMD primers were tested for genetic fidelity assay.

Results

The results revealed enhanced morphogenetic responses in explants pretreated with various concentrations of TDZ in comparison to control. Swelling of shoot bud primordia followed by shoot initiation was observed in TDZ pretreated explants within 3–8 days of inoculation. The initiated shoots grew further, developed several side shoots (tillers) and subsequently established in the shoot cultures within next 4–5 weeks. Results revealed the highest frequency (80%) of shoot initiation in explants pretreated with 10 and 15 mg/l of TDZ. Maximum 65% shoot cultures could be established from the explants pretreated with

Table 1 Effect of pretreatment with various concentrations of TDZ on responses of shoot tip explants of sugarcane variety Co 05011

Responses	Pretreatment of shoot tip explants with various concentrations of thidiazuron (mg/l)				
	Control	5	10	15	20
Shoot initiation (%)	60	60	80	80	75
Culture establishment (%)	50	50	65	50	50
No. of shoots per culture	9.6 ± 0.8	12.7 ± 1.3	19.1 ± 1.7	16.4 ± 1.5	14.3 ± 1.5
Multiplication ratio	1: 3	1:4	1:6	1:5	1:4.5
No of tillers in field 3 months after transplantation	10.9	–	15.3	–	–
Shoot growth and vigor	Moderate	Moderate	Good	Good	Moderate
Days required for establishment	60	50	40	45	45

Mean $\pm$ SD

10 mg/l of TDZ as compared to minimum 40% establishment in explants without pretreatment (Table 1). The explants pretreated with 10 mg/l of TDZ required minimum 40 days for the establishment as compared to untreated explants which required 60 days. In another set of experiment, shoot tip explants were pretreated with 10 mg/l of TDZ for various durations (12, 24, 36 and 48 h) to optimize the pretreatment duration. The highest frequency (80%) of shoot initiation was recorded in explants treated with 10 mg/l of TDZ for 24 h.

MS medium supplemented with BAP, Kinetin and NAA (each 0.5 mg/l) was used for shoot multiplication. The established shoot cultures were separated in smaller clumps containing 3–4 shoots and transferred on fresh medium of the same composition for further multiplication. The highest number of shoot per culture (19.1) could be obtained from the explants pretreated with 10 mg/l of TDZ, indicating the highest multiplication ratio (Table 1). Highest rooting (over 94%) was obtained in shoots regenerated from pretreated explants on $\frac{1}{2}$ strength MS medium containing 5.0 mg/l of NAA and 50 g/l of sucrose within 15 days.

The rooted plants were thoroughly washed under running tap water and transferred to the soil mixture comprising soil:sand:compost (1:1:2) and kept in green house for acclimatization where they survived successfully (>96% survival). Higher number of tillers/clump were obtained in plants regenerated from pretreated explants (15.3) than those from untreated ones (10.9 tillers/clump) in field condition, three months after transplantation.

Randomly selected RAPD primers (OPA-08, OPC-11, OPC-20, OPD-14, OPE-15, OPE-20, OPF-12, OPG-17, OPH-08 and OPI-12) and DAMD (33.6, HBV, HVR and M 13) primers were used for amplification of monomorphic bands. Ten RAPD primers produced a total of 47 bands

with an average of 4.7 bands per primer while four selected DAMD primers yielded 27 clear distinct bands with an average of 6.75 bands per primer. Each RAPD and DAMD primer generated a unique set of amplification products that were monomorphic across all the regenerants except one polymorphic band in RAPD primer OPA08.

Discussion

Thidiazuron has been reported to possess cytokinin-like activity similar to that of N6 substituted adenine derivatives (Mok et al. 1982). In the present study, an effort was made to get the optimum responses with an early establishment of explants. A SEM analysis of shoot tip explants showed that not only the mother shoot developed earlier but side shoot also emerged earlier (at 5th day of inoculation) in pre-treated explant (Fig. 1a) than in control (Fig. 1b), indicating that TDZ induced growth of shoot primordia along with lateral shoot formation in sugarcane. The results were in accordance with the previous observation made on rose (Singh and Syamal 2001), *Curcuma longa* (Prathanturug et al. 2005) *Vitex negundo* (Ahmad and Anis 2007) and *Ocimum basilicum* (Wang et al. 1986). The present results showed that 10 mg/l of TDZ was the most effective concentration in inducing the shoot initiation with the highest frequency of establishment of shoot cultures. The highest rate of multiplication in terms of number of shoots per culture was also observed at 10 mg/l of TDZ, however, it gradually decreased with the increasing concentration of TDZ beyond 10 mg/l, the minimum being in case of untreated explants (Table 1).

The best rooting was observed in shoots regenerated from pretreated explants on $\frac{1}{2}$ strength MS medium containing 5.0 mg/l of NAA and 50 g/l of sucrose. Over 94%

Fig. 1 Scanning electron micrograph of shoot tip explant pretreated with 10 mg/l of TDZ showing early emergence of tiller (arrow) at 5th day of inoculation (a), and control explant showing swelling of shoot primordium (b)

shoots rooted within 15 days. Shoots continued to grow during the rooting phase giving rise to longer plantlets than those obtained from untreated explants. This finding was in accordance with previous finding on *H. procumbens* (Grabkowaska et al. 2014). Highest survival (>96%) of regenerated plantlets was observed in the soil mixture comprising soil:sand:compost (1:1:2). Molecular analysis suggested that the pretreatment of explants with TDZ did not adversely affect the genetic stability of regenerated plants and maintained high clonal purity. Similar observation was recorded by Grabkowaska et al. (2014) in case of *Harpagophytum procumbens*.

Conclusion

The present study emphasizes the importance of thidiazuron in commercial scale micropropagation of sugarcane. This is the first observation made on effect of TDZ on enhanced multiplication of sugarcane using shoot tip explants; however, further studies are needed to understand the mechanism of action of TDZ on the responses of sugarcane tissues in vitro.

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Authors' contributions Kavita Kumari carried out the experiment for in vitro regeneration of shoot tip explants of Sugarcane Co 05011. Madan Lal and Sangeeta Saxena designed the experiment and were responsible for verification of this paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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