Cumulative effect of thidiazuron and 1-naphthylacetic acid in massive root proliferation of micropropagated sugarcane plantlet

Kavita Kumari1,2, Madan Lal1 and Sangeeta Saxena2

1Sugarcane Research Institute, UPCSIR, Shahjahanpur-242 001 (UP) India
2Babasaheb Bhimrao Ambedkar University, Lucknow (UP) India

Abstract: Thidiazuron (TDZ), a well known plant growth regulator is used in tissues culture as a supplement to the basal MS medium. Several reports suggest the role of TDZ in promoting plant organogenesis and plant regeneration. Our experiments to micropropagate the sugarcane using various growth regulators reveal the role of TDZ in rooting and survival of a high yielding new sugarcane variety Co 05011. Various permutation combinations were tried using TDZ as the main regulator to rapidly increase the number of plantlets. Shoot cultures were repeatedly multiplied for 10 cycles and then transferred to MS medium augmented with various concentration of TDZ along with 1-naphthylacetic acid (NAA) and sucrose for rooting. Results of present study indicate optimum concentration of TDZ (0.002 mg/L) in combination with NAA (0.5 mg/L) resulted in 93.6% root formation. Moreover, when these shoots were re-cultured in the same media for another two cycles it produced 100% rooting and had almost 100% survival during acclimatization. These results indicate that TDZ and NAA combination enhance the activity of NAA and resulting in massive proliferation of roots. TDZ increases the frequency and proliferation of healthy and vigorous roots in micropropagated shoots thus enhancing their survival rate in field and during hardening process. This work can be helpful in developing successful and cost effective tissue culture of elite sugarcane varieties.

Keywords: in vitro rooting, micropropagation, sugarcane, survival, thidiazuron

Abbreviations: MS medium, Murashige and Skoog medium; NAA, naphthaleneacetic acid; TDZ, thidiazuron

Introduction

The success of sugarcane production program depends on the rate of multiplication of seed cane of new varieties as it not only helps in rapid spread but also in quick adoption of new varieties over a large area. In sugarcane, the production of seed material of a newly released variety in adequate amount generally takes 8-12 years if multiplied through conventional sett planting method of seed multiplication, by the time the varieties start deteriorating in yield and quality traits. There are also chances for development of sett-borne diseases. In vitro micropropagation techniques are now promising tool to overcome such problems. Several investigators have suggested various protocols during the past decades for in vitro micropropagation of sugarcane varieties (Gallo-Meagher et al. 2000, Hendre et al. 1983, Lee 1987, Lal and Singh 1994, Lal et al. 2015, Kavita et al. 2015, Kavita et al. 2016). The previous reports indicate that the in vitro morphogenetic responses of cultured explants of sugarcane depend on hormonal requirements and vary from cultivar to cultivar.

During micropropagation the shoot cultures are sub-cultured for several cycles on shoot multiplication medium in order to produce maximum shoots. However, if the shoots are multiplied beyond 8-10 cycles, the rate of multiplication and the frequency of root formation gradually decrease resulting in lower production of...
plants. TDZ has been reported to possess a cytokinin-like activity similar to that of N6-substituted adenine derivatives (Mok and Mok 1985). It also supports various morphogenetic responses like somatic embryogenesis, multiple shoot formation and breaking of bud dormancy (Wang et al. 1986, Chennalrayan and Gallo-Meagher 2001, Grabowska et al. 2014, Kumari et al. 2017). Though the mechanism of action of TDZ is not yet understood, previous reports indicate that it possibly induces the synthesis and/or accumulation of endogenous hormones (Capelle et al. 1983, Mok and Mok 1985). Visser et al. (1982) have reported that in vitro morphogenetic responses are associated with TDZ induced auxin expression or accumulation.

The present study was therefore undertaken to assess the role of TDZ in rooting of shoots micropropagated for several cycles using sugarcane variety Co 05011. The variety Co 05011 was selected for experiment as it is high sugar variety identified in 2011.

Materials and Methods

Plant material

Sugarcane (Saccharum spp. Hybrid) variety Co 05011, released from Sugarcane Breeding Institute, Research Centre, Karnal, was selected for experimentation in this study. It is a high yielding and high sugared mid-late maturing variety.

Preparation of explants, culture media and culture conditions

Fresh tops of 8-10 month-old field grown plants of sugarcane variety Co 05011 were collected from research farm of U.P. Council of Sugarcane Research, Shahjahanpur. The leaves of collected tops were peeled out and approximately 8 cm long spindle segment containing the growing point was carefully dissected out. The segments were kept under running tap water for 30 min followed by rinsing with 1% detergent solution for 3-5 min. Segments were surface sterilized with 0.1% (w/v) aqueous mercuric chloride (HgCl2) solution for 10 min and thoroughly washed with sterile distilled water. Shoot tip explant were dissected to excise 1.0 cm apical segment containing apical meristem and 1-2 leaf primordia and immediately inoculated on MS medium (Murashige and Skoog 1962) augmented with 6- benzylaminopurine (BAP) and kinetin (0.5 mg/L). Established culture (mother shoot along with tillers) were transferred to MS medium supplemented with BAP, kinetin and NAA (0.5 mg/L each) for shoot multiplication. After 10 multiplication cycles, shoots were transferred into rooting medium (1/2 strength MS medium) with NAA (5 mg/L) and sucrose (30 and 50 g/L) and in other set of experiment to MS medium augmented with varying concentration of TDZ (0.001 mg/L to 0.02 mg/L) along with NAA (0.5 mg/L) and sucrose (30 g/L). In second set of experiment, similar old cultures were transferred into MS medium supplemented with TDZ (0.002 mg/L) along with NAA (0.5 mg/L) and compared with medium containing TDZ (0.002 mg/L alone) regarding rooting responses. After root formation plantlet were thoroughly washed and transferred to green house for acclimatization in the polybags containing soil:sand:compost mixture. The survival rate of plantlet was recorded after 30 days of hardening.

The culture medium consisted of MS formulation with 30 g/L sucrose. Shoot induction was performed on MS medium gelled with 8 g/L agar (Qualigens, India). The pH of the medium was kept to 6.0 using 1 N NaOH or HCl prior to autoclaving at 121°C (1.06 kg cm−2) for 20 min. All the cultures were incubated at 25 ± 1°C under 16-h photoperiod with a photosynthetic photon flux density of 50 μmol m−2 s−1 from fluorescent tubes (Phillips, India) under 80 ± 5% relative humidity. All the experiments were performed thrice with a minimum of 15 replicates for each treatment. The difference between the treatments means were calculated by using Duncan’s multiple range test (P = 0.05).

Result

Shoot cultures were raised from shoot tip explants on MS medium. The established cultures were multiplied through repeated separation of shoot clumps and sub-culturing on fresh multiplication medium. After multiplication of shoots up to 10 cycles, the shoot cultures were transferred to rooting medium comprising 1/2 MS salts, sucrose (30 or 50 g/L) and 5 mg/L of NAA. Root growth was visualized after 7 days of incubation into the medium. Data on percentage of rooting, number of roots per shoot and root length (in centimeters) were recorded 2 weeks after transfer of shoot cultures. In the medium containing sucrose 50 g/L and NAA (5 mg/L), 68.3 ± 2.8% rooting was observed with 3.7 ± 0.3 roots per shoot having average root length of 1.6 cm (Table 1). The plantlets were transferred to the green house after hardening for acclimatization in the polybags containing soil:sand:compost mixture. The survival rate of plantlet was 81.2 ± 6.5% recorded after 30 days of hardening (Table 1).

Thidiazuron was also added to the MS medium
The medium containing either TDZ (0.002 mg/L) alone or NAA (5.0 mg/L) alone (Table 1) did not induce better rooting than that containing both TDZ and NAA together.

Several experiments were standardized using TDZ also for optimum results and found 0.002 mg/L concentration of TDZ optimum for root formation. This was an additional help as the shoots regenerated through micropropagation had poor survival rate due to underdeveloped formed root structure. TDZ stimulated the massive root formation in micropropagated shoots and when these plants/shoots were potted and further hardened in fields they showed higher survival rate which can be attributed to strong root support. Cost of plants raised through tissue culture is one major problem of micropropagation. In the normal rooting medium sucrose is required in higher amount (50 g/L) for better rooting while in presence of TDZ (0.002 mg/L), better root proliferation could be obtained at lower amount of sucrose (30 g/L). This not only improves the rate of survival of plants during greenhouse hardening but also ensures early establishment of plants in the field after transplantation. Consequently the cost of plant production is also reduced to some extent because more number of plantlet is produced at the same laboratory expenses.

### Discussion

Our initial experiments to micropropagate sugarcane plantlet in tissue culture using MS medium were based on the regulated use of usual growth hormones to direct the developmental pathway towards the

| Growth regulators (mg/L) | Sucreose g/L | % rooting | No. of roots per shoot | Av. length of root (cm) | % survival in greenhouse | Remarks |
|------------------------|-------------|-----------|------------------------|-------------------------|--------------------------|---------|
| NAA 0.5 | TDZ 0.001 | 30 | 45.3 ± 3.6<sup>c</sup> | 4.2 ± 0.4<sup>a</sup> | 1.1 | 56.7 ± 4.3<sup>c</sup> | Thick, healthy roots |
| NAA 0.5 | TDZ 0.002 | 30 | 93.6 ± 6.3<sup>b</sup> | 8.3 ± 0.7<sup>b</sup> | 3.2 | 97.2 ± 1.4<sup>b</sup> | Thick, healthy and vigorous roots |
| NAA 0.5 | TDZ 0.01 | 30 | 81.7 ± 2.1<sup>d</sup> | 5.2 ± 0.4<sup>d</sup> | 2.1 | 78.3 ± 6.1<sup>b</sup> | Thick, healthy roots |
| NAA 0.5 | TDZ 0.02 | 30 | 78.3 ± 4.3<sup>b</sup> | 4.4 ± 0.3<sup>b</sup> | 1.5 | 66.3 ± 5.7<sup>b</sup> | Thin roots |
| NAA Nil | TDZ 0.002 | 30 | 18.3 ± 2.6<sup>d</sup> | 2.2 ± 0.2<sup>e</sup> | 0.7 | 52.6 ± 4.2<sup>e</sup> | Thin roots |
| NAA 0.5 | TDZ Nil | 30 | 45.7 ± 1.5<sup>c</sup> | 2.2 ± 0.5<sup>c</sup> | 1.2 | 77.4 ± 3.9<sup>b</sup> | Thick, healthy roots |
| NAA 5.0 | TDZ Nil | 50 | 68.3 ± 2.8<sup>b</sup> | 3.7 ± 0.3<sup>b</sup> | 1.6 | 81.2 ± 6.5<sup>b</sup> | Thick, healthy roots |

Data represent means ± SE.

Means followed by the same letter within columns are not significantly different (P = 0.05) using Duncan’s multiple range test.
shoot or root formation. In short we were aiming to regulate the development pathway with the help of plant hormones. However the rooting in case of sugarcane using routine protocol (5.0 mg/L NAA and 30 mg/L sucrose) was not up to the mark (Table 1), hence the survival rate of the micropropagated plantlets in the field was poor. Many reports suggest the role of TDZ on organogenesis in case of several other plants (Grabkowaska et al. 2014, Jahan et al. 2011) and we also could initiate shoot regeneration using the same in case of sugarcane multiple shoot regeneration, however we found a prolific result on root development too.

The aforesaid results indicate that TDZ in conjunction with NAA stimulates and enhance the activity of roots proliferation. The results also suggested that an optimum concentration of TDZ is also necessary for induction and enhancement of auxin role. TDZ is a substituted phenyl urea (N-phenyl-1,2,3-thidiazol-5-y lurea) plant growth regulator, and already described as cytokinin but other reports suggest the role of TDZ in enhancing both the endogenous auxin and cytokinin levels (Kou et al. 2016, Visser et al. 1992). Our present results are also in accordance with the results of Ramanayake et al. (2006) who found 100% root formation in Bambusa Valgaris pretreated with TDZ for two to three cycles. Similarly, rooting in all plants of soybean developed from callus of different explants collected from soybean seed was observed on B5 medium containing TDZ (Radhakrisnan et al. 2009). TDZ influence on regeneration frequency depend on different factor like choice of media composition, type of cultivar, explant source, developmental stage and age of plant (Radhakrisnan et al. 2009, Sainikhani et al. 2006). There are reports (Jaiswal and Sawhney 2006) on different species indicating root inhibition in the presence of TDZ, while other investigations support TDZ acting as inducer of auxin actions. Inhibition of root development followed by multiple shoot regeneration on hypocotyl seedlings of Linum usitatissimum was observed in a medium supplemented with TDZ (Mundhara and Rashid 2006). Jaiswal and Sawhney (2006) investigated that TDZ has influences similar to 2,3,5- triiodobenzoic acid (TIBA) in inhibiting root differentiation and shoot elongation in Kalanchee pinnata. TDZ in combination with NAA has been examined in a plant Kalanchee blossfeldiana for optimizing shoot regeneration (Sainikhani et al. 2006). TDZ treated plants has better survival rate had been reported in various plant (Grabkowaska et al. 2014, Ramanayake et al. 2006).

Conclusion
In conclusion our results indicate that TDZ (0.002 mg/L) in combination with NAA (0.5 mg/L) massively proliferate root in sugarcane Co 05011. TDZ without NAA is not effective in root induction. Conclusively, the findings of present study can be useful in enhancing the frequency of rooting and proliferation of healthy and vigorous roots in micropropagated shoots of sugarcane using TDZ. This will lead to better survival of plantlets during acclimatization in green houses and in fields after transplantation. The present protocol is cost effective so as the new varieties could reach to common farmers.

Fig. 1. Growth and morphology of root during in vitro culture. Root induction was performed on medium containing NAA (0.5 mg/L) + TDZ (0.002 mg/L) (A) or NAA (5.0 mg/L) alone (B).
Acknowledgements: Authors are thankful to the Director, U.P. Council of Sugarcane Research, Shahjahanpur for providing the laboratory facilities.

Authors contributions: All the authors contributed equally to this paper.

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

References

Capelle SC, Mok DWS, Kirchner SC, Mok MC 1983 Effects of thidiazuron on cytokinin autonomy and the metabolism of N^-(A^2-Isopentenyl)[R-^14C]adenosine in callus tissue of Phaseolus lunatus L. Plant Physiol. 73: 796-802.

Chengalrayan K, Gallo-Meagher M 2001 Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cell. Dev. Biol.-Plant 37: 434-439.

Gallo-Meagher M, English RG, Abouzid A 2000 Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. In Vitro Cell. Dev. Biol.-Plant 36: 37-40.

Grabkowaska R, Sitarek P, Wysokiniska H 2014 Influence of thidiazuron (TDZ) pretreatment of shoot tips on shoot multiplication and ex vitro acclimatization of Harpagophytum procumbens. Acta Physiol. Plant. 36: 1661-1672.

Hendre RR, Iyer RS, Kotwal M, Khupse SS, Mascarenhas AF 1983 Rapid multiplication of sugarcane by tissue culture. Sugarcane 1: 5-8.

Jahan AA, M Anis, Aref IM 2011 Preconditioning of axillary buds in thidiazuron-supplemented liquid media improves in vitro shoot multiplication in Nyctanthes arbor-tristis L. Appl. Biochem. Biotechnol. 163: 851-59.

Jaiswal S, Sawhney S 2006 Modulation of TDZ-induced morphogenic responses by anti-auxin TIBA in bud-bearing foliar explants of Kalanchoe pinnata. Plant Cell Tissue Organ Cult. 86: 69-76.

Kavita, Lal M, Saxena S 2016 Propagating sugarcane in vitro: An opportunity for Indian sugar industry. Agrica 5: 7-19.

Kavita, Saxena S, Anand A, Lal M 2015 Use of antibiotics to control bacterial contamination during in vitro micropropagation of sugarcane. Agrica 4: 41-44.

Kou Y, Yuan C, Zhao Q, Liu G, Nie J, Ma Z, Cheng C, Teixeira da Silva JA, Zhao L 2016 Thidiazuron triggers morphogenesis in Rosa canina L. Protocorm-like bodies by changing incipient cell fate. Front. Plant Sci. 7: 557.

Kumari K, Lal M, Saxena S 2017 Enhanced micropropagation and tiller formation in sugarcane through pretreatment of explants with thidiazuron (TDZ). 3 Biotech 7: 282.

Lal N, Singh HN 1994 Rapid clonal multiplication of sugarcane through tissue culture. Plant Tissue Cult. 4: 1-7.

Lal M, Tiwari AK, Gupta GN, Kavita 2015 Commercial scale micropropagation of sugarcane: constraints and remedies. Sugar Tech. 17: 339-347.

Lee, TSG 1987 Micropropagation of sugarcane (Saccharum spp.). Plant Cell Tissue Organ Cult. 10: 47-55.

Mok MC, Mok DWS 1985 The metabolism of [^14C]-tMDiazirino in callus tissues of Phaseolus lunatus. Physiol. Plant. 65: 427-432.

Mundhara R, Rashid A 2006 TDZ-induced triple response and shoot formation on intact seedlings of Linum, putative role of ethylene in regeneration. Plant Sci. 170: 185-190.

Murashige T, Skoog F 1962 A revised method for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 472-497.

Radhakrishnan R, Ramachandran AK, Ranjitha BD 2009 Rooting and shooting: dual function of thidiazuron in in vitro regeneration of soybean (Glycine max. L). Acta Physiol. Plant. 31: 1213-1217.

Ramanayake SMSD, Meemaduma VN, Weerawardene TE 2006 In vitro shoot proliferation and enhancement of rooting for the large-scale propagation of yellow bamboo (Bambusa vulgaris ‘Striata’). Sci. Hortic. 110: 109-113.

Sanikhani M, Frello S, Serek M 2006 TDZ induces shoot regeneration in various Kalanchoe blossfeldiana Poelln. cultivars in the absence of auxin. Plant Cell Tissue Organ Cult. 85: 75-82.

Visser C, Qureshi JA, Gill R, Saxena PK 1992 Morphoregulatory role of thidiazuron. Plant Physiol. 99: 1704-1707.

Wang SY, Steffens GL, Faust M 1986 Breaking bud dormancy in apple with a plant bioregulator, thidiazuron. Phytochemistry 25: 311-317.