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LOW THIDIAZURON LEVELS PROMOTE AND SUSTAIN SHOOTLET MULTIPLICATION IN SUGARCANE

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SUMMARY

Effect of thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-ylurea; TDZ) was examined on multiplication of shootlet cultures established from buds regenerated from apical domes of sugarcane var. CoH-92 and Co-7717. A set of two shootlets taken from these cultures was inoculated on media containing varying levels of TDZ. The number of multiple shootlets formed provided a simple, quantitative test system. TDZ concentrations, as low as 10^{-9} M and 10^{-7} M, produced the highest number of shootlets in var. CoH-92 and Co-7717, respectively. These levels were up to $2x10^{-4}$ times lower than the other growth regulators tested. Greater multiplication of shootlets continued even after subculture on a medium devoid of TDZ for 24 and 48 days. This is suggestive of a high persistence of TDZ in the tissues. Cytokinin-like effects of TDZ at such low concentrations are uncommon and sustained effects of low concentrations, as in this response, unreported. Multiple advantages of TDZ in micropropagation of sugarcane are discussed.

Key words: **Shootlet multiplication, sugarcane, thidiazuron.**

INTRODUCTION

Thidiazuron, a substituted phenyl urea (N-phenyl-N'-1,2,3-thidiazol-5-ylurea;TDZ;Dropp), has been reported to mimic cytokinin activity in the release of bud-dormancy in apple (Wang *et al.* 1986), promotion of seed germination, retardation of leaf senescence, enhancement of abscission in citrus and induction of nitrate reductase activity in maple (Kerns and Meyer 1985). In *Phaseolus* callus bioassay and *Petunia* leaf test system, TDZ was more active than cytokinins (Mok and Mok 1985). It also induces callus growth in soybean, expansion of radish-cotyledons and growth in many other cytokinin dependent callus cultures (Thomas and Katterman 1986). Recently, TDZ has been reported to effectively substitute N^6-benzyl cytokinins that are commonly used in plant tissue culture (Li *et. al.* 2000, Akasaka *et al.* 2000, Hosseini and Rashid 2000, Fratini and Ruiz 2002, Qu *et al.* 2002). Since, micropropagation is being increasingly employed in sugarcane as a special purpose technique for multiplying newly developed elite materials, we examined the effect of TDZ on *in vitro* shoot multiplication of sugarcane and report its effectiveness at unusually low concentration and also persistence of its effect over two subscultures.

MATERIALS AND METHODS

Apical domes were collected from five-month-old, healthy plants of sugarcane (*Saccharum officinarum* L. x S. *spontaneum* L. hybrids) varieties CoH-92 and Co-7717 grown at experimental fields of CCS Haryana Agricultural University, Regional Research Station, Karnal. These were thoroughly washed with detergent and then with tap water and treated with 0.2% ascorbic acid +

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0.4% citric acid for 5 min. to remove phenolic substances. Finally, these were surface sterilized with 0.1% mercuric chloride for 1 min and rinsed several times with sterile water.

Apical dome explants prepared as above were inoculated in culture bottles containing "initial bud proliferation medium" comprising of MS (Murashige and Skoog 1962) basal medium, sucrose (3%), 1 mg l^{-1} benzylaminopurine (BAP), 1 mg l^{-1} kinetin (KN) and agar (0.8%) and kept in a room at 25 ± 1°C and 16 h light (100 µmol m^{-2} s^{-1}). Meristematic buds obtained after about three weeks were excised from the parent plant and transferred to MS medium supplemented with BAP (1 mg l^{-1}), KN (1 mg l^{-1}) and naphthaleneacetic acid (NAA; 0.5 mg l^{-1}). Multiple shoots obtained from these buds after 20 days were separated in sets having two shootlets each and used as source material for experiments. MS medium was supplemented with various concentrations of TDZ and other growth regulators [IAA, NAA, BAP, KN, ZEA (zeatin), PUT (putrescine) and SPM (spermidine)] as in Table 1 to 5. Five sets each containing two shootlets developed as above were cultured in each culture bottle. Three bottles were used for each treatment and observations on the number of shootlets formed recorded after 24 days.

In the pre-treatment experiment, sets of two shootlets were taken from multiple shootlets formed on TDZ containing media and transferred to fresh medium having no TDZ. Further, multiple shootlets formed after 24 days on this TDZ-free media were again sub-cultured, a second time, on medium having no TDZ. Root Growth index is the average of visual observations for root growth on a 0 to 5 scale in different cultures.

RESULTS AND DISCUSSION

Effect of TDZ on shootlet proliferation in sugarcane var. CoH-92 and Co-7717 is presented in Table 1. There was an increase in the number of shootlets formed in var. CoH-92 by 2 x 10^{-6} M and lower concentrations of TDZ, 10^{-9} M being the most effective. In sugarcane var. Co-7717, the maximum shootlets proliferation was observed at 10^{-7} M TDZ, even though 10^{-8} M also showed significant promotion. Interestingly, high concentrations of TDZ (5 x 10^{-6} and 2 x 10^{-5} M) were either ineffective or slightly inhibitory to shoot proliferation. In geranium (*Pelargonium* x *Hortorum* Bailey) and St. John's wort (*Hypericum perforatum*) also, high levels of TDZ have been reported to adversely affect shoot proliferation and rooting of regenerants (Lu 1993, Murthy *et al.* 1998).

The effects of three cytokinins (BAP, KN, ZEA), two auxins (NAA, IAA) and two polyamines (PUT, SPM) were examind in the same test system to determine the comparative efficacy of TDZ and these growth regulators. Several concentrations were tested for each of these chemicals, but the ones that produced the highest number of shootlets are included in Table 2. The data show that for shootlet proliferation of sugarcane var. CoH 92, the optimum concentration of these growth regulators was much higher compared to TDZ. While TDZ produced maximum number of shootlets at 10^{-9} M (Table 1), these growth regulators generally produce optimum effect at 10^{-5} or 2x10^{-5} M. Also, the number of shootlets produced by TDZ was comparable to those produced by NAA, but greater than those produced by other chemicals. Similarly, for var. Co-7717, the TDZ was most effective at 10^{-7} M (Table 1), while 10^{-5} or 2x10^{-5} M concentration was

Table 1. Effect of TDZ on shootlet proliferation in sugarcane var. CoH-92 and Co-7717. Sets of two shootlets each, raised as described in materials and methods, were inoculated on media containing different concentrations of TDZ for 24 days. Values are mean number of shootlets formed for 15 observations.

TDZ concentration (M)	Number of shootlets	
	CoH-92	Co-7717
0	4.2 ± 0.52	4.2 ± 0.61
1x10^{-9}	9.2 ± 0.90	4.8 ± 0.79
1x10^{-8}	7.1 ± 0.51	7.1 ± 0.43
1x10^{-7}	8.2 ± 0.60	11.6 ± 1.24
1x10^{-6}	7.7 ± 0.58	7.8 ± 0.57
2x10^{-6}	7.7 ± 0.86	8.6 ± 0.58
5x10^{-6}	5.1 ± 0.89	5.4 ± 0.68
2x10^{-5}	3.2 ± 0.40	2.9 ± 0.60
L.S.D. at 50%	2.159	1.418

required for other growth regulators, except PUT which was effective at 10^{-6} M (Table 2). Also, the number of shootlets produced by TDZ was comparable to those produced by KN and SPM but greater than other growth regulators.

TDZ-caused promotion of shoot proliferation in sugarcane at these unusually low concentrations is a significant observation. In comparative terms, TDZ produced maximum effect at concentrations that were up to $2x10^{-4}$ times lower than those of other growth regulators. Earlier, TDZ was found 20 times more effective in breaking dormancy in apple compared to cytokinins and 10^2 times more effective in soybean callus cytokinins assays, compared to the purine cytokinins (Thomas and Katterman 1986). However, in most responses only high concentrations of TDZ are effective (Preece *et al.* 1991, Eapen *et al.* 1998, Whilhelm 1999, Hosseini and Rashid 2000, Murch *et al.* 2000, Li *et al.* 2000, 2002, Puay-Koon *et al.* 2002). In our earlier work on callus initiation and proliferation from seedling explants of *Brassica juncea* also, TDZ was effective at $2x10^{-6}$ M to 2×10^{-5} M (Dhawan *et al.* 2001).

In order to examine the persistence of TDZ effect, shootlets of the two sugarcane varieties raised on TDZ containing media were sub-cultured on MS medium with no TDZ. After 24 days, shootlets derived from 10^{-8} M TDZ in var. CoH-92 and shootlets derived from 10^{-8}, 10^{-7} and 10^{-6} M TDZ in var. Co-7717 showed greater proliferation compared to control (Table 3). Thus, TDZ effect on shootlet proliferation was persistent up to 24 days. Another experiment was planned to examine the efficacy of this chemical beyond 24 days. The above-mentioned, TDZ-pretreated explants cultured on medium with no TDZ for 24 days, were again sub-cultured on medium having no TDZ and the number of shootlets formed was recorded after 24 days of second sub-culture (Table 3). Generally, there was no effect or a small decrease in the number of shootlets produced, but in var. Co-7717, shootlets originally treated with 10^{-8} M TDZ showed slightly greater proliferation even on second sub-culture without TDZ (48 days after treatment).

That the treatment with very low concentrations of TDZ continued to promote the shootlet number for several weeks after its application is another important observation. Singh and Syamal (2001) observed that a quick dip in TDZ (100 µm) caused the highest shoot proliferation from 'Sonia' and 'Raktagandha' roses as compared to normal proliferation medium containing 6-benzylamine purine, NAA and GA_3 suggesting that a short exposure of explant to TDZ can significantly enhance axillary shoot proliferation. Murch and Saxena (2001) recently observed the fate of two radiolabelled versions of TDZ [C^{14}-5-thidiazol]-TDZ and [C^{14}-U-phenyl]-TDZ in sterile

Table 2. Effect of different growth regulators on shootlet proliferation in sugarcane var. CoH-92 and Co-7717. Sets of two shootlets each, raised as described in materials and methods, were inoculated on media containing different concentrations of growth regulators for 24 days. Values are mean number of shootlets formed for 15 observations.

Treatment	Number of shootlets	Treatment	Number of shootlets
CoH-92		**Co-7717**	
Control*	4.2 ± 0.52	Control*	4.2 ± 0.61
BAP, $2x10^{-5}$ M	7.2 ± 1.10	BAP, $1x10^{-5}$ M	6.4 ± 0.49
KN, $2x10^{-5}$ M	8.0 ± 0.83	KN, $2x10^{-5}$ M	12.8 ± 1.10
ZEA, $2x10^{-5}$ M	6.2 ± 0.62	ZEA, $2x10^{-5}$ M	7.1 ± 0.84
IAA, $2x10^{-6}$ M	7.6 ± 0.93	IAA, $2x10^{-5}$ M	6.9 ± 0.69
NAA, $1x10^{-5}$ M	10.5 ± 1.30	NAA, $1x10^{-6}$ M	7.3 ± 1.14
PUT, $1x10^{-5}$ M	7.7 ± 1.07	PUT, $1x10^{-6}$ M	7.1 ± 0.83
SPM, $1x10^{-5}$ M	6.7 ± 0.60	SPM, $1x10^{-5}$ M	11.7 ± 1.67
L.S.D. at 5%	2.831		2.748

* No hormone added

Table 3. Effect of pre-treatment with TDZ on shootlet proliferation in sugarcane var. CoH-92 and Co-7717. Multiple shootlets were raised on media containing different concentrations of TDZ for 24 days. Sets of two of these TDZ-treated shootlets were then sub-cultured on a fresh media containing no TDZ for another 24 or 48 days. Values are mean number of shootlets formed for 15 observations.

Pre-treatment	Number of Shootlets			
	24 days pre-treatment		48 days pre-treatment	
	CoH-92	Co-7717	CoH-92	Co-7717
TDZ, 0	4.2 ± 0.52	4.2 ± 0.61	4.2 ± 0.52	4.2 ± 0.61
TDZ, $1x10^{-9}$ M	4.8 ± 0.60	3.8 ± 0.36	2.6 ± 0.21	4.2 ± 0.51
TDZ, $1x10^{-8}$ M	8.0 ± 0.86	6.1 ± 0.73	4.1 ± 0.51	5.8 ± 0.25
TDZ, $1x10^{-7}$ M	5.1 ± 0.49	5.8 ± 0.59	4.6 ± 0.38	3.6 ± 0.34
TDZ, $1x10^{-6}$ M	3.6 ± 0.23	5.9 ± 0.58	2.5 ± 0.18	3.8 ± 0.44
TDZ, $5x10^{-6}$ M	4.0 ± 0.23	4.2 ± 0.14	2.5 ± 0.16	3.8 ± 0.39
L.S.D. at 5%	1.586	1.324	1.108	1.219

hypocotyl explants of geranium and concluded that the TDZ molecule remains intact in both forms within the plant tissues.

Interestingly, in the pre-treatment experiment, all TDZ treated shootlets in both the sugarcane varieties showed root formation when shifted to TDZ-free media and hence observations on root multiplication and root growth were recorded. In var. CoH-92, shootlets pre-treated with 10^{-7} and 10^{-6} M TDZ showed the best response, while in var. Co-7717, all concentrations of TDZ between 10^{-9} to 10^{-6} M showed equal response (Table 4).

Table 4. Effect of pre-treatment with TDZ on root proliferation and root growth in sugarcane var. CoH -92 and Co-7717. Multiple shootlets were raised on media containing different concentrations of TDZ as in the Table for 24 days followed by inoculation on media containing no TDZ for 24 days. Sets having two of these shootlets were then sub-cultured on a fresh media containing no TDZ. The number of roots and root growth were recorded after 24 days. Values are mean for 15 observations.

Pre-treatment	Number of roots	Root growth index
	CoH-92	
TDZ, 0	0.0	0.0
TDZ, $1x10^{-9}$ M	1.9 ± 0.78	0.4 ± 0.21
TDZ, $1x10^{-8}$ M	2.5 ± 0.27	1.0 ± 0.04
TDZ, $1x10^{-7}$ M	5.4 ± 0.56	0.5 ± 0.24
TDZ, $1x10^{-6}$ M	6.0 ± 0.44	3.6 ± 0.18
	Co-7717	
TDZ, 0	0.0	0.0
TDZ, $1x10^{-9}$ M	4.5 ± 0.33	3.0 ± 0.13
TDZ, $1x10^{-8}$ M	5.0 ± 0.48	3.3 ± 0.17
TDZ, $1x10^{-7}$ M	4.7 ± 0.85	2.7 ± 0.51
TDZ, $1x10^{-6}$ M	5.3 ± 0.60	2.9 ± 0.15
L.S.D. at 5%	0.918	0.160

Experiments conducted to study the effect of TDZ in combination with other cytokinins on shootlet proliferation showed that the combination of TDZ with either BAP or KN was more effective, than any of these growth regulators alone. Thus, 10^{-7} or 10^{-6} M TDZ in combination with $5x10^{-6}$ M BAP or KN greatly enhanced shootlet proliferation in var. CoH-92, the effect being nearly additive in case of 10^{-7} M TDZ + $5x10^{-6}$ M KN (Table 5). Also, in a rare response, 10^{-6} M TDZ + 2 x

Table 5. Effect of TDZ in combination with other cytokinins on shootlet proliferation in sugarcane var. CoH-92. Sets of two shootlets each, raised as described in materials and methods, were inoculated on media containing different concentrations of growth regulators for 24 days. Values are mean number of shootlets formed for 15 observations.

Treatment	Number of shootlets
Control*	4.4 ± 0.22
BAP, $5x10^{-6}$ M	6.5 ± 1.0
TDZ, $1x10^{-7}$ + BAP, $2x10^{-6}$ M	11.4 ± 1.5
TDZ, $1x10^{-6}$ + BAP, $5x10^{-6}$ M	9.0 ± 1.5
KN, $5x10^{-6}$ M	6.5 ± 0.87
TDZ, $1x10^{-7}$ + KN, $5x10^{-6}$ M	12.4 ± 1.92
TDZ, $1x10^{-6}$ + KN, $5x10^{-6}$ M	8.5 ± 1.36
L.S.D. at 5%	3.016

*No hormones added

10^{-6} M KN showed formation of callus that subsequently formed shootlets (data not included), thereby, providing another possible method for *in vitro* multiplication in sugarcane using TDZ. Whilhelm (1999) also observed that in Sycamore maple 0.04 µM TDZ + 1.0 µM BA showed best proliferation capacity, both for shoots and callus. Similarly, combinations of BA with TDZ resulted in highest number of shootlets in *Miscanthus ogiformis* (Nielsen *et al.* 1995).

Thus, a count of shootlets formed in various media provides a simple, well-controlled and quantitative system to study effects of TDZ on shootlet proliferation *in vitro*. Also TDZ-induced high production of shootlets, directly from buds regenerated from apical domes, represents an improvement in the technique of micropropagation of sugarcane. Since it permits root formation also, satisfactory regeneration in sugarcane (yielding complete plantlets) can be realized by using low concentrations of TDZ. The persistence of TDZ effect would make it an ideal choice in plant tissue culture and offers a unique possibility of its use as a sole regulator for shootlet formation, root formation, callus initiation and regeneration of plantlets from callus in sugarcane. Incidentally, TDZ-mediated sugarcane regeneration is one of the few examples of TDZ application to monocot plants (Chen *et al.* 2000, Qu *et al.* 2002). Sustained promotion of shootlet formation at these low concentrations would suggest that it might act as a kind of signal or trigger that "switches on" processes resulting in this response. Our work on protein profiles and DNA amplification products supports this view (manuscript under preparation).

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