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Efficacy of non-purine and purine cytokinins on shoot regeneration *in vitro* in sugarcane

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Effect of two non-purine cytokinins, TDZ [N-phenyl-N′ - (1,2,3-thidiazol-5-yl) urea] and 4-CPPU [N- (2-chloro-4-pyridyl) N-phenylurea] and meta-topolin [6 (3-hydroxybenzylamino) purine] a biologically active aromatic compound, on shoot regeneration in sugarcane var. CoS 8436 and Co 1148 and *S. officinarum* clone 'Gungera' was observed at 15, 25 and 35 d. 4-CPPU significantly enhanced shoot regeneration in var. CoS 8436, but showed no effect or caused only a slight promotion in var. Co 1148 and clone 'Gungera'. Meta-topolin was most effective in promoting shoot regeneration at 5 µ*M* in var CoS 8436, at 1 µ*M* in var Co 1148 and at 10 µ*M* in in clone Gungera. The best response to TDZ was observed at 0.01 µ*M* in vars CoS 8436 and Co 1148 and at 0.1 µ*M* in clone Gungera. Thus, there was a clear varietal response of sugarcane to these chemicals. Clone 'Gungera' responded best to meta-topolin, CoS 8436 to 4-CPPU and Co 1148 to TDZ. Results obtained in this work clearly demonstrate TDZ induced shoot regeneration in sugarcane at unusually low concentration, such as 0.01 µ*M* and 0.1 µ*M*. Further, 4-CPPU, meta-topolin and TDZ simulate cytokinin activity and may thus provide a better substitute of cytokinins like BAP or Kn that are generally used in tissue culture.

Keywords: 4-CPPU, meta-toplin, TDZ, sugarcane, shoot regeneration, *in vitro*, growth regulators

Introduction

Several purine type synthetic and naturally occurring cytokinins such as BAP, Kn, Zeatin and isopentenyladenine are commonly employed in plant tissue culture. In recent years, some non-purine compounds, notably, thidiazuron [TDZ; N-phenyl-N′-(1,2,3-thidiazol-5-yl) urea] and 4-CPPU [N-(2-chloro- 4 -pyridyl) N-phenylurea] have been reported to simulate cytokinin activity in some responses[1-3]. Also, meta-topolin [MT; 6 (3-hydroxybenzylamino) purine], first isolated from poplar leaves, has been shown to have biological activity of an active aromatic cytokinin[4]. It is pertinent to mention that with the exception of BAP, purine type cytokinins are chemically unstable, whereas these non-purine types are generally stable and hence have the obvious advantage in procedures that involve heat sterilization.

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Built up of systemic pathogens due to transmission through generations and low multiplication rates are serious problems in the propagation of vegetatively multiplied crops such as sugarcane. Therefore, micropropagation is being increasingly employed in sugarcane especially for multiplying newly developed elite materials. Lee[5] reported micropropagation of sugarcane using shoot tip culture; also *in vitro* regeneration of sugarcane cultivar CoC 671 has been reported by Dhumale *et al*.[6]. In the present study, effect of different concentrations of TDZ, 4-CPPU and MT on the *in vitro* shoot regeneration in sugarcane var. CoS 8436 and Co 1148 and *S. officinarum* clone 'Gungera' was examined and compared with commonly used cytokinins such as BAP, Kn and Zeatin.

Materials and Methods

Apical domes were collected from 5-month-old healthy plants of sugarcane (*S. officinarum* L. × *S. spontaneum* L. hybrids) varieties CoS 8436 and Co 1148 and *S. officinarum* clone 'Gungera' grown at the experimental fields of CCS Haryana Agricultural University, Regional Research Station, Karnal. These were thoroughly washed with tap water and treated with 0.2% ascorbic acid and 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.

BAP and NAA were obtained from Central Drug House, Mumbai; Kinetin (Kn) from SISCO Research Laboratory, Mumbai; 4-CPPU, Meta-topolin and TDZ from Haartem, Netherlands and MS Media from Hi-Media, Mumbai. MS media was supplemented with test regulators and *p*H adjusted to 5.8 followed by the addition of agar agar (0.8%) and autoclaving at 121°C, 15 psi for 20 min.

Apical dome explants, sterilized as above, were inoculated in culture bottles containing initial bud proliferation medium comprising MS^7+5 µ*M* BAP+5 µ*M* Kn and kept in a room at 25±1°C and 16 h light (100 µmol m^{-2} s^{-1}). After two weeks, lateral buds, which appeared on the explants, were excised from the parent plant and transferred to MS medium supplemented with 5 µ*M* BAP+5 µ*M* Kn+2.5 µ*M* NAA. Multiple shoots obtained from these buds after 20 d were separated in sets having two uniform shoots each and used as source material for 'regeneration efficacy tests'.

'Regeneration efficacy test' involved supplementing MS media with various concentrations of test chemicals (Figs 1 to 3) to examine the effect of these chemicals in three sugarcane varieties. Also, for comparison three concentrations each of BAP, Kn and zeatin were used (Table 1). Five sets, each containing two shoots raised as above, were cultured in various media containing these chemicals. Three bottles were used for each treatment. Each set of two shoots that was inoculated in these culture bottles, started proliferating under the effect of test chemicals and thus, the number of shoots formed in these sets in different media after 15, 25 and 35 d provided a measure of the 'regeneration efficacy' of the chemical.

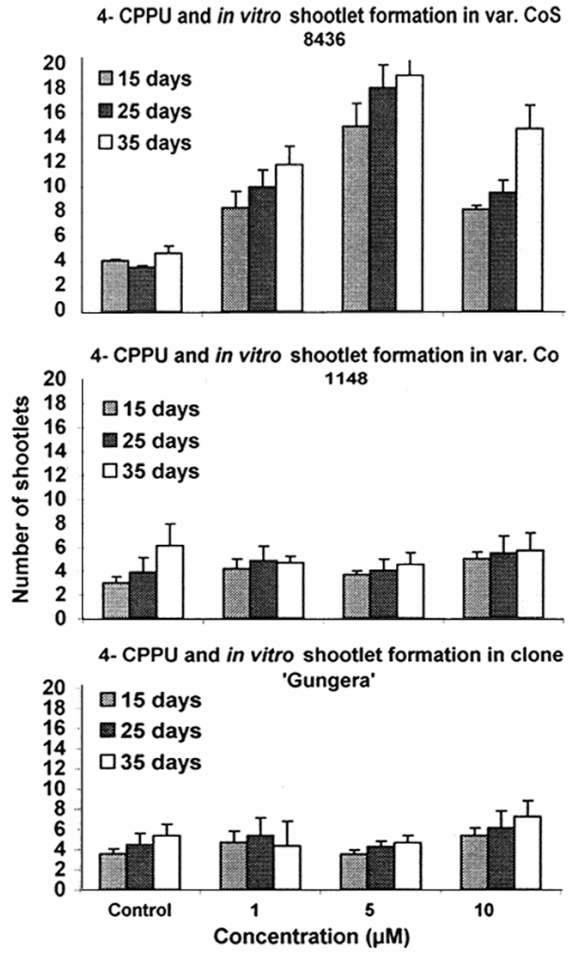

Fig. 1—Regeneration efficacy of 4-CPPU as measured by the number of shootlets formed on 15, 25 and 35 d after culture of an initial set of 2 shootlets of different sugarcane genotypes. Lines above the bars represent standard error.

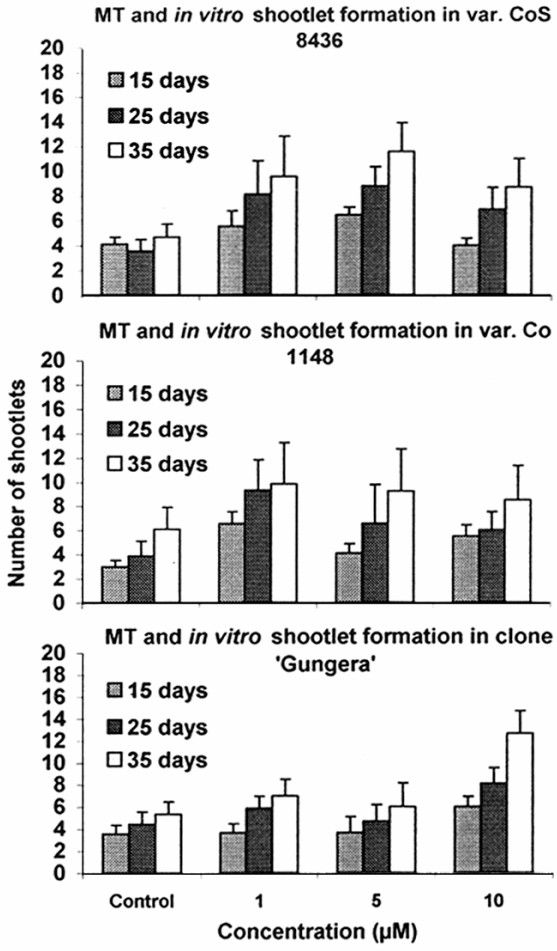

Fig. 2—Regeneration efficacy of MT as measured by the number of shootlets formed on 15, 25 and 35 d after culture of an initial set of 2 shootlets of different sugarcane genotypes. Lines above the bars represent standard error.

The growth of shoots was also recorded visually on 0 to 5 scale as below: 0 = shoots died; 1 = no growth; 2 = poor growth; 3 = medium growth; 4 = good growth; 5 = very good growth. The visual growth ratings of 0 to 5 were averaged to provide growth index (Table 2).

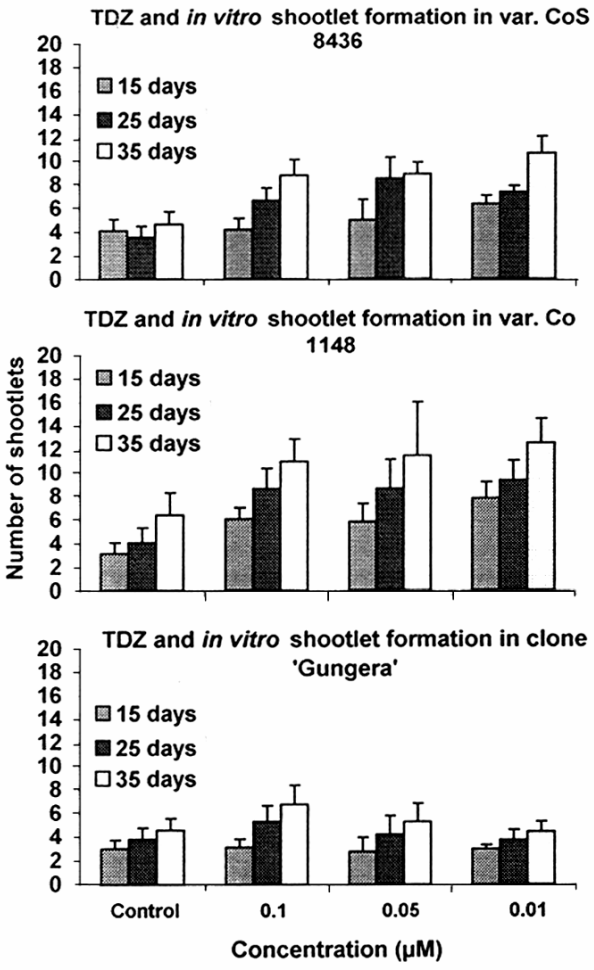

Fig. 3—Regeneration efficacy of TDZ as measured by the number of shootlets formed on 15, 25 and 35 d after culture of an initial set of 2 shootlets of different sugarcane genotypes. Lines above the bars represent standard error.

Table 1—Regeneration efficacy of BAP, Kn and zeatin as measured by the number of shootlets formed after 15, 25 and 35 d of culture of an initial set of two shootlets of sugarcane var. CoS 8436

Cytokinin treatment (µ*M*)	Number of shootlets		
	15 d	25 d	35 d
Control	3.7±0.96	3.6±0.95	4.7±1.05
BAP- 2	2.4±0.12	4.4±0.65	6.2±0.91
BAP- 5	2.6±0.12	4.9±0.67	6.3±0.38
BAP- 20	3.0±0.19	2.9±0.17	4.9±0.37
Kn- 2	4.7±0.24	5.7±0.38	5.9±0.39
Kn- 5	5.2±0.40	5.4±0.43	6.2±0.51
Kn- 20	4.7±0.42	6.2±0.28	7.1±0.63
Zeatin- 2	4.4±0.42	5.5±0.48	7.2±0.34
Zeatin- 5	5.7±0.44	7.5±0.67	8.9±0.53
Zeatin- 20	5.1±0.42	7.6±0.65	8.5±0.29

Values in table are mean ± S.E. for 15 observations.

Results and Discussion

Regeneration efficacy of 4-CPPU, MT and TDZ for the three sugarcane genotypes as measured by the number of shoots formed in different media is presented in Figs 1 to 4. At 5 µ*M*, the 4-CPPU caused enhancement of shoot regeneration in var. CoS 8436, which was nearly four times greater than the control. However, there was no effect or only a slight promotion in var. Co1148 and in clone 'Gungera' by the addition of 4-CPPU (Fig. 1). Addition of MT to the media resulted in nearly twice the number of shoots compared to the control in each of the three genotypes, though the most effective concentrations were 5 µ*M* for var. CoS 8436, 1 µ*M* for var. Co 1148 and 10 µ*M* for Gungera (Fig. 2). The best response to addition of TDZ was observed at 0.01 µ*M* in var. CoS 8436 and Co 1148 and at 0.1 µ*M* in clone Gungera (Fig. 3). The results clearly show that *Saccharum* clone 'Gungera' responded best to MT, CoS 8436 to 4-CPPU and Co1148 to TDZ and there was a distinct varietal response of sugarcane to these chemicals.

The results on the effect of BAP, Kn and zeatin on shoot regeneration in var CoS 8436 are presented in Table 1 for comparison. The highest number of shoots formed with these cytokinins was 8.9 in media containing 5 µ*M* zeatin, while 4-CPPU containing media formed upto 19 shoots in this variety (Fig. 1).

None of 4-CPPU concentrations had significant effect on shoot growth in any of the sugarcane varieties, except a slight reduction in growth by 10 µ*M* in CoS 8436 and 1 µ*M* in Gungera. Similarly, MT had no significant

Table 2—Effect of different concentrations of 4-CPPU, MT and TDZ on *in vitro* growth of sugarcane varieties CoS 8436 and Co 1148 and clone 'Gungera'

Cytokinin treatment (µ*M*)	Growth index		
	CoS 8436	Co 1148	Gungera
Control	4.66±0.27	2.55±0.62	3.44±0.48
4-CPPU, 1.0 *M*	4.11±0.43	3.50±0.29	2.33±0.72
4-CPPU, 5.0 *M*	4.00±0.38	3.66±0.94	3.33±0.64
4-CPPU, 10.0 *M*	2.50±0.80	3.88±0.18	3.11±0.51
MT, 1.0 *M*	3.44±0.40	3.11±0.33	3.66 ± 0.54
MT, 5.0 *M*	3.33±0.67	3.00±0.38	2.66±0.72
MT, 10.0 *M*	2.55±0.40	4.16±0.40	4.44±0.29
TDZ, 0.10 *M*	3.44±0.48	3.00±0.27	3.33±0.64
TDZ, 0.05 *M*	4.33±0.47	3.22±0.36	2.11±0.88
TDZ, 0.01 *M*	3.33±0.27	2.77±0.36	3.22±0.36

Explants raised were inoculated and the shootlet growth recorded after 15 d.
Values in the table are mean ± S.E. for 15 observations.

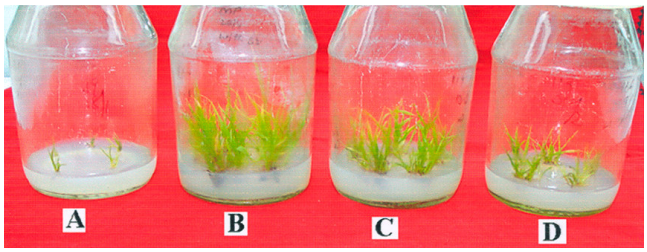

Fig. 4—Shoot regeneration in sugarcane var. CoS 8436. Five sets of two shoots each (A) were inoculated in different media containing 5 µ*M* 4-CPPU (B), 5 µ*M* MT (C), & 0.01 µ*M* TDZ (D) for 35 d.

effect on shoot growth in any of the three sugarcane varieties except a decrease caused by 5 µ*M* in Gungera. Also, shoot growth was generally not affected by TDZ. Enhancement in growth observed by different concentrations of 4-CPPU in var. Co 1148, and by 10µM MT in 1148 and Gungera was not observed at 25 and 35 d (data included only for 15d, Table 2).

The number of shoots formed in a test medium provides a well-controlled and quantitative test system to study the effect of a chemical on shoot regeneration *in vitro*. Thus, the number of shoots formed after 15, 25 and 35 d of treatment from an initial set of two provides a direct measure of 'regeneration efficacy' of a test chemical.

The effect of 4-CPPU, MT and TDZ on three sugarcane genotypes suggests that these chemicals may provide a better substitute to purine cytokinins like BAP or Kn that are generally used in plant tissue culture. Indeed 4-CPPU at 5 µ*M* produced up to 19 shoots from initial set of two shoots in sugarcane var. CoS 8436, a number not observed with other growth regulators. Earlier, 4-CPPU has been reported to increase fruit size and yield of 'Spadona' and 'Costia' pear (*Pyrus communis* L.)[8].

In the present study, TDZ was effective at concentrations as low as 0.01 µ*M*, which is nearly 1000 times lower than the concentrations generally used for cytokinins. Earlier, TDZ was found 20 times more effective in breaking dormancy in apple compared to other cytokinins and 100 times more effective in soyabean callus assay compared to purine cytokinin[9]. In our earlier work on callus initiation and proliferation from seedling explants of *Brassica juncea*, TDZ was effective at 2 to 20 µ*M*. However, in sugarcane var. CoH 92 and Co 7717 TDZ, concentrations as low as 0.001 µ*M* and 0.1 µ*M*, produced highest number of shoots[10]. The effect of TDZ, has also been reported for many plant species including several recalcitrant woody species like *Quercus robur*[11] and red silver hybrid maples[12-14]. Singh and Syamal reported that a quick dip in TDZ (100 µ*M*) caused high shoot proliferation in rose var. 'Sonia' and 'Raktagandha'[15].

MT has been reported as an active aromatic compound. Palavan *et al* showed that the treatment at 0.25, 0.5 and 1.0 m*M* MT concentration on radish cotyledon resulted in 44, 50 and 57 per cent increment in cotyledon growth[3]. Escalona *et al* showed that root and shoot explants of banana cultivar CEMSA when cultured *in vitro* on MS medium supplemented with various concentrations of benzyladenine or MT showed significant increase in multiplication[16] Also, in a study of *in vitro* shoot multiplication of apple var. Jonagold on media containing different concentrations of cytokinins (0 to 5.0 mg L^{-1}), the highest multiplication rate was observed at 5.0 mg L^{-1} MT [17].

Results presented in this work clearly demonstrate a distinct varietal response of sugarcane genotypes to growth regulators, 4-CPPU, MT and TDZ, and may be useful in determining variety specific protocols. *Saccharum* clone 'Gungera' responded best to MT, CoS 8436 to 4-CPPU and Co1148 to TDZ. Shoot regeneration in var. CoS 8436 by 5 µ*M* 4-CPPU was 2 to 4 times greater compared to any other tested growth regulator. Further, 4-CPPU, MT and TDZ simulate cytokinin activity suggesting thereby that these chemicals provide an effective substitute to cytokinins like BAP or Kn that are generally used in plant tissue culture.

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