Multiple Shoot Formation in *Cymbidium aloifolium* L. (Sw.) Shoot Explants under the Influence of $KMnO_4$ within the Culture Vessel in Thidiazuron Containing Medium

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ABSTRACT

It has previously been shown that solid potassium permanganate ($KMnO_4$) acts to absorb ethylene under diverse conditions. We have previously shown that thidiazuron (TDZ) induces abnormal, hypertrophied shoot buds in *Cymbidium aloifolium* L. (Sw.) at high levels, a response that may be related to production of stress ethylene. In this study, we investigated the possibility that solid $KMnO_4$, (a known ethylene absorbant) can ameliorate this hypertrophication. We estimated both, the number of explants that initiated buds after 4 weeks of culture, and the number of normal shoots with leaves formed after 8 weeks of culture. Numerous shoots were induced and almost every shoot showed proper initiation of deep green leaves on MS medium with 5 µM TDZ + 400 mg/l $KMnO_4$ as compared to the control and higher level of $KMnO_4$ (800 mg/l). This shows, as previously known, that high level of TDZ is associated with ethylene emission in the culture vessel, and leads to production of hypertrophied shoot buds in stem explants of *Cymbidium aloifolium* (L.) Sw. in vitro, but is ameliorated by $KMnO_4$.

Key words: Culture atmosphere, $KMnO_4$, shoot, thidiazuron

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Introduction

The potential for commercialization of orchids is high, as native tropical Indian species offer blooms with superior hardiness. Native orchid species will add new crops to the horticultural trade and substantially boost the returns for propagators, growers and sellers. Among all orchid species, Cymbidiums occupy the foremost position as cut flowers that fetch high prices. *Cymbidium aloifolium* L. (Sw.) is a medicinally important orchid (Radhika et al., 2013). Apart from its medicinal value, it is also valued as a parent for developing hybrids. Santa Barbara Orchid Estate (USA) developed *C. aloifolium* var. album, which is a creamy yellow, cascading flower. Tissue culture provides an efficient means to propagate such important orchid species. Choice of growth regulators is crucial to achieve this.

There are two broad groups of plant growth regulators that exhibit cytokinin activity, synthetic phenylurea derivatives (including thidiazuron, TDZ) and purine-based derivatives (including N^6 benzylaminopurine, BAP). TDZ, a cotton defoliant (Arndt *et al*., 1976), has been shown in various cytokinin bioassays to exhibit strong cytokinin-like activity similar to that of N^6-substituted adenine derivatives (Mok *et al*., 1985). There is considerable evidence that TDZ may be involved in increasing the biosynthesis or accumulation of endogenous purine cytokinins (Murthy *et al*., 1995). Murch et al. (1997) hypothesized that mode of action of TDZ is stress mediated in plant tissue culture. TDZ appears to fulfil both role of both auxin and cytokinin requirements for the induction and expression of somatic embryogenesis in peanut (Victor et al., 1999). Other additional mechanisms have been suggested for the role of TDZ in organogenesis and somatic embryogenesis in African violet (Erland et al., 2020).

Though TDZ is a proven effective and potent synthetic plant growth regulator for organogenic, regeneration and developmental pathways, including axillary and adventitious shoot proliferation, yet it has several drawbacks which are associated with its application in plant tissue culture (Hassan *et al*., 2018). TDZ is effective in induction of multiple shoot formation in several orchids, including Cymbidiums, when applied at low concentrations but with increasing concentration, TDZ treatment leads to hypertrophy (abnormality) in shoot formation (Guha, 2007). In *Cymbidium giganteum*, TDZ at higher concentrations induced more protocorm-like bodies (PLBs), but affected

further development of plantlet and root formation (Roy *et al*. 2012). Mundhara and Rashid (2006), reported triple response of *Linum* seedlings, a characteristic of ethylene action in response to TDZ treatment. This is consistent with the efficiency of TDZ, being more effective than cytokinin, in production of stress ethylene (Yip and Yang, 1986). Ethylene produced by plant tissues growing *in vitro* may be accumulated in large quantities in the culture vessels, particularly from rapidly growing non-differentiated callus or suspension cultures, and hence is likely to negatively influence growth and development in such systems (Biddington,1992). It is reported that potassium permanganate ($KMnO_4$) acts as an ethylene absorber in papaya ripening (Corrêa et al. 2005) In this communication, we report that the modification of culture atmosphere using $KMnO_4$ in TDZ-containing culture medium increases direct shoot regeneration with well differentiated leaves, and prevents callusing (hypertrophied shoots).

Materials and Methods

Seedlings derived from the seeds of *C. aloifolium* L. (Sw.), were used as donors from which shoot segments of 0.5 to 1.0 cm, were taken as explants. The mineral formulation employed was that of Murashige and Skoog's (1962) standard (MS) and the medium was prepared in reagent grade water. The reagent grade water was produced by passing distilled water through a Milli-Q water purification system (Millipore, Bedford, MA) equipped with two deionization cartridges, an Organex Q cartridge and a 0.22 µm filter. The concentration of 5 µM TDZ has been observed to produce hypertrophied shoots in several orchids including Cymbidiums (Guha, 2007). Therefore, in the current study we used that concentration of TDZ to determine whether ethylene and moisture in the culture vessel has any role in the production of hypertrophied shoots. Hence, about 5 µM of cold sterilized TDZ (Sigma) was added to the autoclaved agar-agar (0.6%) and nutrients, while 100 ml of the medium was dispensed in 250 ml Erlenmeyer flasks capped with non-absorbent cotton plugs. The cultures were kept at 25 ± 2°C, with the white fluorescent tubes providing 46 µE $s^{-1}m^{-2}$. $KMnO_4$ is a strong oxidizer and water soluble, therefore, it was weighed in solid form and placed using a sterile spatula in autoclaved 1.5 ml Eppendorf tubes under laminar flow. The Eppendorf tubes filled with solid $KMnO_4$ crystals were inserted in solid agar medium under the laminar air flow. The Eppendorf tubes were kept open inside the Erlenmeyer flasks. $KMnO_4$ was used at the levels of 200, 400 and 800 mg per 100 ml of culture medium. At higher levels two Eppendorf tubes were used to accommodate the quantity of $KMnO_4$ used. Because $KMnO_4$ eventually becomes liquified due to moisture inside the culture vessel, we used distilled water as control in this current study. We also had an overall control with not TDZ and no added $KMnO_4$. Responses were recorded in terms of (a) the number of explants induced to form multiple shoot buds (EIM) after 4 weeks of culture and (b) the number of shoots with proper leaf initials from such explants (S/EIM) after 8 weeks of culture. The experiment was conducted twice with six replicates (n = 6) with ten explants in each Ehrlenmeyer flask. Cultures were retrieved from the culture vessels for detailed observations during the experiment and photographs were taken in Reichert-Diaster Inverted Microscope.

Bottom Cooling of Culture Vessels

Culture vessels were subjected to bottom cooling as described by Vanderschaeghe and Debergh (1987), with some modifications based on locally available equipment. Bottom cooling was applied by placing the cultures over a bench of interconnected copper tubes, of which one end was inserted in an ice box containing ice cold water and the other end was connected by a pipe and inserted in the same ice box for recycling of water.Fresh ice was added from time to time in the ice box. Water was pumped into the copper tubes with the help of a water pump (Khaitan, India Ltd.), and an approximate temperature of 7°C was created inside the culture vessel.

Data Analysis

The experiments were repeated more than once, with at least six replicates (n = 6) for each treatment. Summary statistics (arithmetic mean, standard deviation and standard error) were calculated, differences among treatments tested by ANOVA and least significant difference (LSD) at $p < 0.05$ (Sigma Stat; SPSS, Chicago).

Results

Shoot explants of *C. aloifolium* growing on MS medium without TDZ formed a well-developed shoot with normal leaf development (Fig. 1B). Cultures on MS supplemented with 5 µM TDZ showed a few shoot primordia without proper initiation of leaves and were callused at the bases (Fig. 1B). Explants cultured on TDZ-supplemented medium in the presence of 200 mg/l

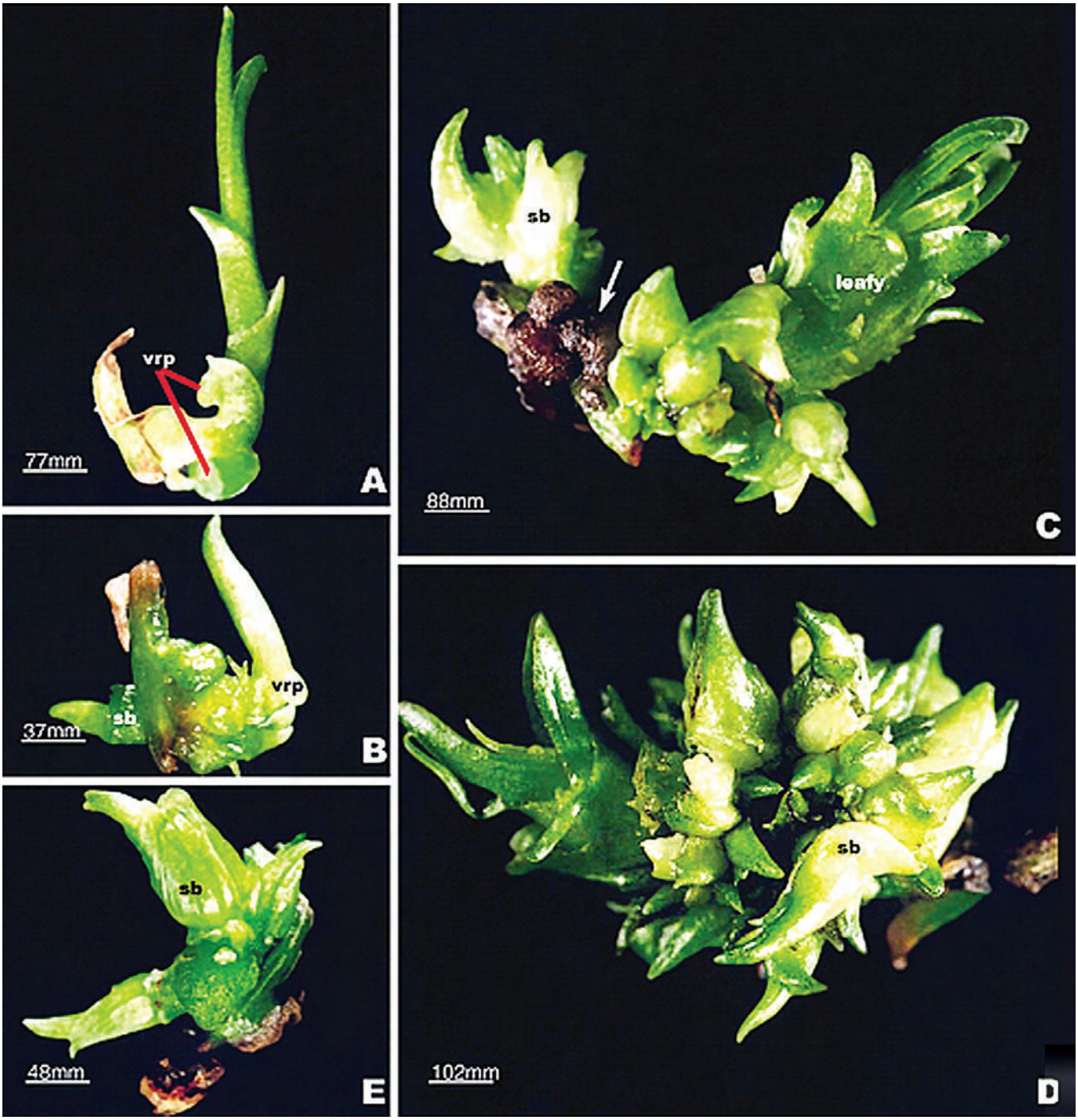

Figure 1. Induction of shoot buds and their development in shoot explants of *Cymbidium aloifolium* L. (Sw.).(A) A single shoot with deep green well-differentiated leaves and two velamenous root primordia (vrp) on MS medium; (B) One to two shoot buds (sb) emerging from the explant with callusing at the base and at the early stages of growth and development: MS medium with 5 µM TDZ and distilled water (control) as culture atmosphere modifier; (C) Formation of deep green, multiple leafy shoots on the shoot explant. Three to four shoot buds (sb) are at early stage of leaf initiation. Notemultiple leaves arising from the small shoot and necrotic globular shoot buds (arrow): MS medium with 5 µM TDZ and 200 mg/l KMnO4; (D) Formation of numerous shoots with most shoot buds showing proper initiation and differentiation of leaves. Three to four creamy shoot buds (sb) at early stages of leaf initiation are also seen: MS medium with 5 µM TDZ and 400 mg/l KMnO4. (E) Two to three deep green hypertrophied shoot buds (sb). Deep green swollen primordia initiated from shoots emerging from shoot explants: MS medium, with 5 µM TDZ and 800 mg/l KMnO4.

Table 1. Effect of TDZ and $KMnO_4$ on induction of shoot buds and their development in shoot explants of *Cymbidium aloifolium* L. (Sw.).

Treatments		Observations	
		After 4 Weeks	After 8 Weeks
TDZ (µM)	$KMnO_4$ (mg/l)	EIM ($\bar{x}$ ± S.E.)	S/EIM ($\bar{x}$ ± S.E.)
0	0	1.4 ± 0.4	1.2 ± 0.2
5	DW	4.2 ± 1.2	7.0 ± 0.9
5	200	5.0 ± 0.6	7.7 ± 0.7
5	400	4.4 ± 0.9	11.6 ± 0.9
5	800	4.0 ± 1.1	2.6 ± 0.4

($\bar{x}$ = Mean values of six replicates; **S.E.** = Standard Error; **EIM**, number of explants induced to form multiple shoot buds; **S/EIM**, number of shoots/EIM; DW, distilled water**)**

$KMnO_4$ showed most shoots with well differentiated leaves, termed here as leafy, but some shoot primordia did not grow further and turned necrotic (Fig. 1C). At a higher level of $KMnO_4$ (400 mg), the TDZ-containing medium showed many shoots with leaves (Fig. 1D). However, at the higher level of $KMnO_4$ (800 mg/l), there were fewer shoot primordia, with swollen bases (Fig. 1E).

As shown in Table 1, the number of explants that responded to TDZ by forming multiple shoot primordia (EIM) was similar in all the treatments irrespective of the concentrations of $KMnO_4$ suspended in open Eppendorf tubes as compared to the control (without TDZ and $KMnO_4$) after 4 weeks of culture. However, the treatments differed in the number of shoots induced after 8 weeks of culture: more leafy shoots were formed in 400 mg/l $KMnO_4$ + TDZ supplemented medium in comparison to DW, 200 and 800 mg/l $KMnO_4$ + TDZ medium ($p<0.05$, Table 1).

The most important finding of these experiments was that $KMnO_4$ modified the growth response of shoot buds induced by 5 µM TDZ. Besides, 5 µM TDZ was found to inhibit, the differentiation of leaves after eight weeks of culture. However, induction of multiple shoot buds was promoted in MS=TDZ at all levels after four weeks of culture.

Discussion

Thidiazuron acts like cytokinin and promotes regeneration of shoots (Thomas and Katterman, 1986). In the present study Cymbidium, 5 µM TDZ showed a dramatic decline in the frequency of direct shoot formation with increasing callus formation of the shoot bases (Fig. 1B). Putative role of ethylene in the presence of high concentration of TDZ for such retardation of direct shoot growth in *C. aloifolium* was investigated. The possibility of stress being a result of TDZ treatment was probed by application of an ethylene absorbent like $KMnO_4$ within the culture atmosphere. In the absence of $KMnO_4$ and at low level (200 mg/l), the number of leafy shoots formed was lower than at 400 mg/l. However, with further increase in the concentrations of $KMnO_4$ (800 mg/l), there was a reduction in the number of shoots even in the presence of TDZ in the medium. Our results contradict Mundhara and Rashid (2006), who reported a complete elimination of shoot formation in *Linum* at 200 mg/l $KMnO_4$ application within the culture atmosphere. In the present study, our focus was not only on the formation of multiple shoots, but also well differentiated leaves. In the present study, though the number of shoot bud initiation was not affected in the cultures, where distilled water was taken as ethylene trap (as control for $KMnO_4$), after four weeks of culture, the differentiation of leaves was compromised after eight weeks of culture. The shoot buds were in callusing phase with swollen shoot base and leaves still in the early stages of differentiation (see Fig. 1 B).

This response might be due to a combined effect of $KMnO_4$ as ethylene trap and moisture more than $KMnO_4$ alone in the culture vessel. As after eight weeks of culture there was a significant rise in the number of well differentiated shoot buds, even when distilled water was considered as control to $KMnO_4$, as ethylene trap (Table 1).

At this point, we attribute both the role of $KMnO_4$ and bottom cooling of culture for differentiation of leaves. Bottom cooling is based on the reduction of relative humidity of the vessel atmosphere by condensation on the cooled culture medium (Vanderschaeghe and Debergh,

1987), this way it reduces the relative humidity in the culture vessels and prevents vitrification. However, we assume that ethylene, in trace amount, might promote differentiation, which was retarded when $KMnO_4$ (as ethylene trap) was applied at a very high concentration (800 mg/l) in this study. The application of ethephon, an ethylene releasing compound enhanced ethylene evolution and increased leaf area of mustard at a lower concentration, while inhibited at higher concentration (Khan, 2005; Khan *et al.*, 2008).

From the current study, it is quite clear that, with the modification of culture atmosphere, by application of $KMnO_4$ and by reducing the humidity, TDZ even at high concentration brings radical change in differentiation of leaves in *C. aloifolium* (L.) Sw.

References

Arndt, F., Rusch, R. & Stillfried, H.V. 1976. SN49537, a new cotton defoliant. Plant Physiology, 57: 99.

Biddington, N.L. 1992. The influence of ethylene in plant tissue culture. Plant Growth Regulation, 11: 173-187.

Corrêa, S.F., Filho, M.B., da Silva, M.G., Oliveira, J.G., Aroucha, E.M.M., Silva, R.F., Pereira, M.G. & Vargas, H. 2005. Effect of potassium permanganate during papaya fruit ripening: Ethylene production. Journal de Physique IV, 125: 869-871.

Erland, Lauren A., Giebelhaus, Ryland T., Victor, Jerrin M.R., Murch, Susan J. & Saxena, P. K. 2020. The morphoregulatory role of thidiazuron: metabolomics guided hypothesis generation for mechanisms of activity. Biomolecules, 10: 1-28.

Guha, S. 2007. *In Vitro* Propagation of Orchids: Heterotrophy to Autotrophy. Ph.D. thesis, Department of Botany, University of Delhi, India.

Hassan, D.Y., Nurmansyah, N., Naidoo, Y. & Teixeira da Silva, J.A. 2018. Thidiazuron induced abnormalities in plant tissue cultures. Plant Cell Reports, 37: 1451-1470.

Khan, N.A. 2005. The influence of exogenous ethylene on growth and photosynthesis of mustard (*Brassica juncea*) following defoliation. Scientia Horticulturae, 105: 499-505.

Khan, N.A., Mir, M.R., Nazar, R. & Singh, S. 2008. The application of ethephon (an ethylene releaser) increases growth, photosynthesis and nitrogen accumulation in mustard (*Brassica juncea* L.) under high nitrogen levels. Plant Biology, 10: 534-538.

Mok, M.C., Mok, D.W.S., Armstrong, D.J., Shudo, K., Isogai, Y. & Okamoto, T. 1985. Cytokinin activity of N-Phenyl-N-1,2,3-thiadiazol-urea-5ylurea (thidiazuron). Phytochemistry, 21: 1509-1511.

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

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum, 15: 472-497.

Murch, S.J., Krishnaraj, S. & Saxena, P.K. 1997. Thidiazuron induced morphogenesis of Regal geranium (*Pelargonium domesticum*): A potential stress response. Physiologia Plantarum, 101: 183-191.

Murthy, B.N.S., Murch, S.J. & Saxena, P.K. 1995. Thidiazuron induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea* L.): endogenous growth regulator levels and significance of cotyledons. Physiologia Plantarum, 94: 268-276.

Radhika, B., Murthy, J.V.V.S.N. & Grace, N. 2013. Preliminary phytochemical analysis and antibacterial activity against clinical pathogens of medicinally important orchid *Cymbidium aloifolium* (L.) Sw. International Journal of Pharmaceutical Sciences and Research, 4: 3925-3931.

Roy, A.N., Sajeev, S. & Deka, B.C. 2012. TDZ induced micropropagation in *Cymbidium giganteum* Wall. Ex. Lindl. and assessment of genetic variation in the regenerated plants. Plant Growth Regulations, 68: 435-445.

Thomas, J.C. & Katterman, F.R. 1986. Cytokinin activity induced by thidiazuron. Plant Physiology, 81: 681-683.

Vanderschaeghe, A.M. & Debergh, P.C. 1987. Technical aspects of the control of the relative humidity in tissue culture containers. 68-76. In: Ducate G, Jacob M, Simeon A (eds). Plant Micropropagation in Horticultural Industries Presses Universitaires, Liege.

Victor, J.M.R., Murch, S.J., Krishnaray, S. & Saxena, P.K. 1999. Somatic embryogenesis and organogenesis in peanut: The role of thidiazuron and N^6-benzylaminopurine in the induction of plant morphogenesis. Plant Growth Regulation, 28: 9-15.

Yip, W.K. & Yang, S.F. 1986. Effect of thidiazuron, a cytokinin-active urea derivative, in cytokinin dependent ethylene production system. Plant Physiology, 80: 515-519.