Plant Cell Reports (1993) 12:154–159

Plant Cell Reports

Somatic embryogenesis in *Nicotiana tabacum* L.: induction by thidiazuron of direct embryo differentiation from cultured leaf discs

R. Gill and Praveen K. Saxena

Department of Horticultural Science, University of Guelph, Guelph, Ontario NIG 2W1, Canada

Received July 22, 1992/Revised version received October 15, 1992 – Communicated by F. Constabel

Summary. Induction of somatic embryogenesis by different growth regulators was examined in leaf disc cultures of *Nicotiana tabacum* L. Direct differentiation of somatic embryos occurred on media supplemented with naphthaleneacetic acid (NAA) and N^6-benzylaminopurine (BAP). Thidiazuron (N-phenyl-N'-1, 2,3,-thiadiazol-5-ylurea; TDZ) not only substituted for the most effective NAA-BAP combination but also induced a higher frequency of somatic embryogenesis. Regenerated somatic embryos were capable of developing into plants.

Keywords: *Nicotiana tabacum* - somatic embryogenesis - growth regulators - thidiazuron

Abbreviations: BAP = N^6-benzylaminopurine; MS = Murashige and Skoog (1962) medium; NAA = Naphthaleneacetic acid; TDZ = N-phenyl-N'-1,2,3,-thiadiazol-5-ylurea

Introduction

In their pioneering study with tobacco tissue cultures, Skoog and Miller (1957) demonstrated that a balance of growth regulators, especially those of auxins and cytokinins, determines the developmental fate of cultured cells. The overwhelming success in regenerating a wide variety of plants via organogenesis and somatic embryogenesis by *in vitro* manipulation of auxin(s) and cytokinins(s) reflects the power and general applicability of the central dogma developed by these researchers.

Thidiazuron (N-phenyl-N'-1,2,3,-thiadiazol-5-ylurea; TDZ) is a substituted phenyl urea which exhibits cytokinin-like activity (Mok *et al.* 1982; Thomas and Katterman 1986). Recently, we found that TDZ induced high-frequency regeneration in bean and peanut, two highly recalcitrant grain legumes (Malik and Saxena 1992; Saxena *et al.* 1992; Gill and Saxena 1992). In this communication, we report on the high efficiency of TDZ-induced direct somatic embryogenesis in leaf disc cultures of *Nicotiana tabacum*, a response previously shown to be regulated primarily by a balance of BAP and NAA (Stolarz *et al.* 1991 and references cited therein). The culture procedure developed in this study is expected to be of general use and interest since tobacco is used as a model experimental system in many physiological and developmental studies.

Correspondence to: P. K. Saxena

Materials and Methods

Seed germination. Seeds of three different cultivars of *Nicotiana tabacum* L. (cvs. Petit Havana SR1, Xanthi and BEL W-5) were surface sterilized by soaking in 70% ethanol for 3 min. This treatment was carried out using a steel sterilization sieve (30 micron) suspended in a Magenta box (Magenta Corporation, Chicago, Ill., USA) containing 70% ethanol. Seeds were further disinfected with 1.5% sodium hypochlorite solution for 20 min. The sterilant was removed by washing the seeds five times with sterile distilled water (each wash 50 ml) and 30 to 40 seeds were placed in a Petri dish (90 x 15 mm) containing 20 ml of medium (pH 5.8) which consisted of MS salts and vitamins, 3% sucrose and 0.25% Gelrite (Scott Laboratories, Carson, Cal., USA). All media were sterilized by autoclaving at 0.122 MPa for 20 min. Seedlings were maintained at 25 °C under light (20-25 $\mu mol \cdot m^{-2} \cdot s^{-1}$;16 hr photoperiod) provided by "cool white" fluorescent tubes (Philips Canada, Scarborough, Ont., Canada). Shoot-tips of 45-day-old plants were transferred to MS0 and shoots were subcultured every week. For all experiments, first pairs of fully expanded leaves were excised from 10-day-old shoot cultures and used as the source of explants.

Preparation and culture of explants. Leaves were cut into four to five 5x5 mm pieces. These explants were cultured in disposable Petri dishes (100 x 15 mm) containing 20 ml of MS medium with 0, 0.25, 0.50, 1.0, 5.0, 10.0, 15.0 or 20.0 μM TDZ. In one experiment, leaf explants were also cultured in the presence of NAA plus BAP or BAP alone, as described previously by Stolarz *et al.* (1991), for comparing the regeneration efficiency with these growth regulators. For regeneration of plants, the explants bearing somatic embryos or shoots

were directly subcultured in Petri dishes containing MS basal medium (MS0). After two weeks of growth, embryo-derived shoots were separated and cultured individually on MS0 for promoting root elongation. All cultures were incubated at 25°C in light (20-25 $\mu mol \cdot m^{-2} \cdot s^{-1}$;16 hr photoperiod).

Twenty five explants were used to test the effect of each treatment and all experiments were repeated at least twice. All primary cultures were examined every week for the effects of growth regulators on growth and differentiation of callus, shoots and somatic embryos.

Histology. For histology, leaf discs grown in the presence of TDZ (1 μM) were fixed and stored in 70% ethanol. These samples were dehydrated in tertiary butyl alcohol and embedded in paraffin (Paraplast, Brunswick Co., St. Louis, USA). Transverse sections of 10 μm thickness were cut using the Spencer 820 microtome (American Optical Corp., Buffalo, NY, USA). Cut paraffin sections were passed through a series of deparaffinizing solutions of Hemo-De (Fisher Scientific Co. USA), stained with Alcian Green and Safranin and permanently mounted in Permount (Fisher Scientific Co. USA) before examination under a light microscope.

Results

Seeds of *Nicotiana tabacum* L. cvs. Petit Havana, BEL W-5 and Xanthi, germinated within one week of culture on MS0 and within 30-45 days the seedlings were big enough to provide shoot apices. Leaf discs excised from axenic shoot cultures were used to examine the effect of various growth regulators.

Leaf sections of the cv. Petit Havana did not produce callus or somatic embryos when cultured on MS0 (Fig. 4A). These cultures remained green for one week but thereafter turned brown and became necrotic. A very small fraction of cultures (<1%) produced roots from wounded edges. Failure of the explants to develop shoot buds or embryos on MS0 indicated the need for the addition of growth regulators.

Different growth and differentiation responses were obtained on media supplemented with TDZ (0.25-15.0 μM) or BAP (4.4 or 44.4 μM) or BAP (4.4 or 44.4 μM) plus NAA (0.54 μM). Irrespective of the type of growth regulator(s), all explants remained greener than the control and enlarged in size within a week of culture. Wounded edges of the explants became thick compared to the central region. Explants which were cultured on NAA (0.54 μM) produced callus and roots. None of the explants produced callus at any concentration of TDZ (0.25-15.0 μM) or BAP (4.4 or 44.4 μM), with or without NAA (0.54 μM).

Somatic embryos were produced in the presence of TDZ at all concentrations in the range of 0.25-15.0 μM (Fig. 1) but the response varied in terms of the period of induction and the emergence of somatic embryos. Somatic embryos at the globular stage were seen within a week originating directly on exposed edges of the explants grown in the presence of 1 or 5 μM TDZ (Fig. 2A). At lower concentrations of TDZ (0.25-0.5 μM), the induction of somatic embryogenesis was delayed. About 110 to 135 somatic embryos were produced per leaf disc after 3 weeks of treatments with 0.25 to 0.5 μM TDZ (Fig. 1). In these cultures, somatic embryos were first seen emerging after two weeks as compared to one week with 1.0 μM TDZ. All cultures developed somatic embryos within 10-15 days except those grown at 10-15 μM TDZ which showed embryogenesis after three weeks. TDZ at 20 μM did not produce somatic embryos even after a period of one month.

Development of somatic embryos in all cultures progressed through typical globular, heart and torpedo-shaped to cotyledonary stages of embryo development (Fig. 2 A,B,C,D). The globular, torpedo and cotyledonary stages could be seen frequently in all cultures but the occurrence of fully developed heart-shaped stage was rare (Fig. 3A). Histology of the embryo producing regions of the explants further confirmed the presence of different developmental stages of somatic embryo differentiation (Fig. 3 A,B,C). The presence of anatomically discrete (closed) radicular ends separating the developing embryos from the maternal tissue (Fig. 3 A,C) further confirmed embryogenic mode of regeneration. Although differentiation of shoot axes of the somatic embryos occurred while explants were on TDZ-supplemented medium, the elongation and subsequent growth of root axes required transfer to MS0.

Post-induction development of somatic embryos was affected by the concentration of TDZ used for the induction of embryogenesis. At 0.25 and 0.5 μM TDZ the initial development of somatic embryos required two weeks but about 70-80% of these embryos rapidly developed into shoots within a week of emergence (Fig. 4 B and F, respectively).

Although somatic embryos were visible within a week on the medium containing 1 μM TDZ, their differentiation from cotyledonary stage to shoots occurred after three more weeks (Figs. 2E, 4G). About 90% of somatic embryos which were induced at 1 or 5 μM TDZ could be regenerated to plants. A fraction (5-10%) of somatic embryos developed on 1 or 5 μM TDZ appeared vitrified. TDZ at 10 μM induced a large number of well organised somatic embryos (Fig. 4C) but these did not develop beyond the cotyledonary stage on the same medium. However, 40-50% of the embryos produced shoots on transfer to MS0 after 45 days of culture. Thus, the maximum numbers of embryo-derived shoots and plants were obtained from cultures induced at 1 μM TDZ and subsequently transferred to MS0, although initial frequency of embryogenesis was higher at 0.5 μM TDZ (Fig. 1).

BAP alone at 4.4 or 44.4 μM levels induced organogenesis only. Lower concentration of BAP (4.4 μM) produced more shoots (40-60 per explant) compared with a higher concentration (44.4 μM) which suppressed the induction of shoots (5-10 per explant; Fig. 4E). Shoots which were produced at 4.4 μM developed roots on subsequent subculture to MS0 whereas those

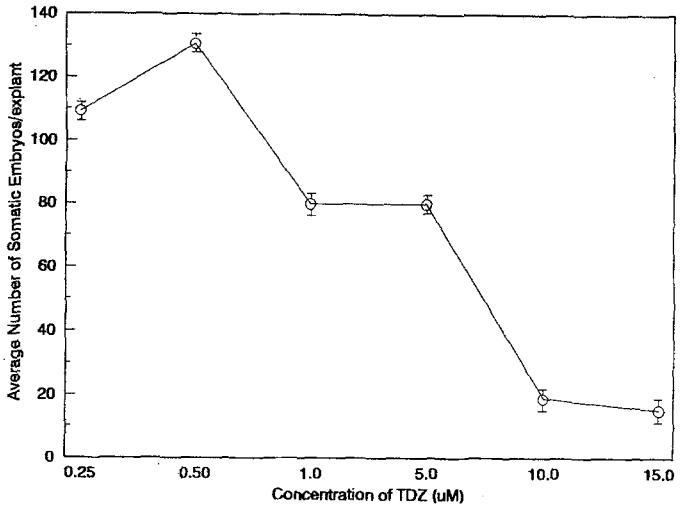

Fig. 1. The effect of thidiazuron on the induction of somatic embryogenesisin leaf disc cultures of *Nicotiana tabacum* L. Explants were cultured on MS medium containing TDZ at 0, 0.25, 0.5, 1.0, 5.0, 10.0, or 15.0 μM for 3 weeks. Each value represents mean of twenty five replicates $\pm$ S.E.

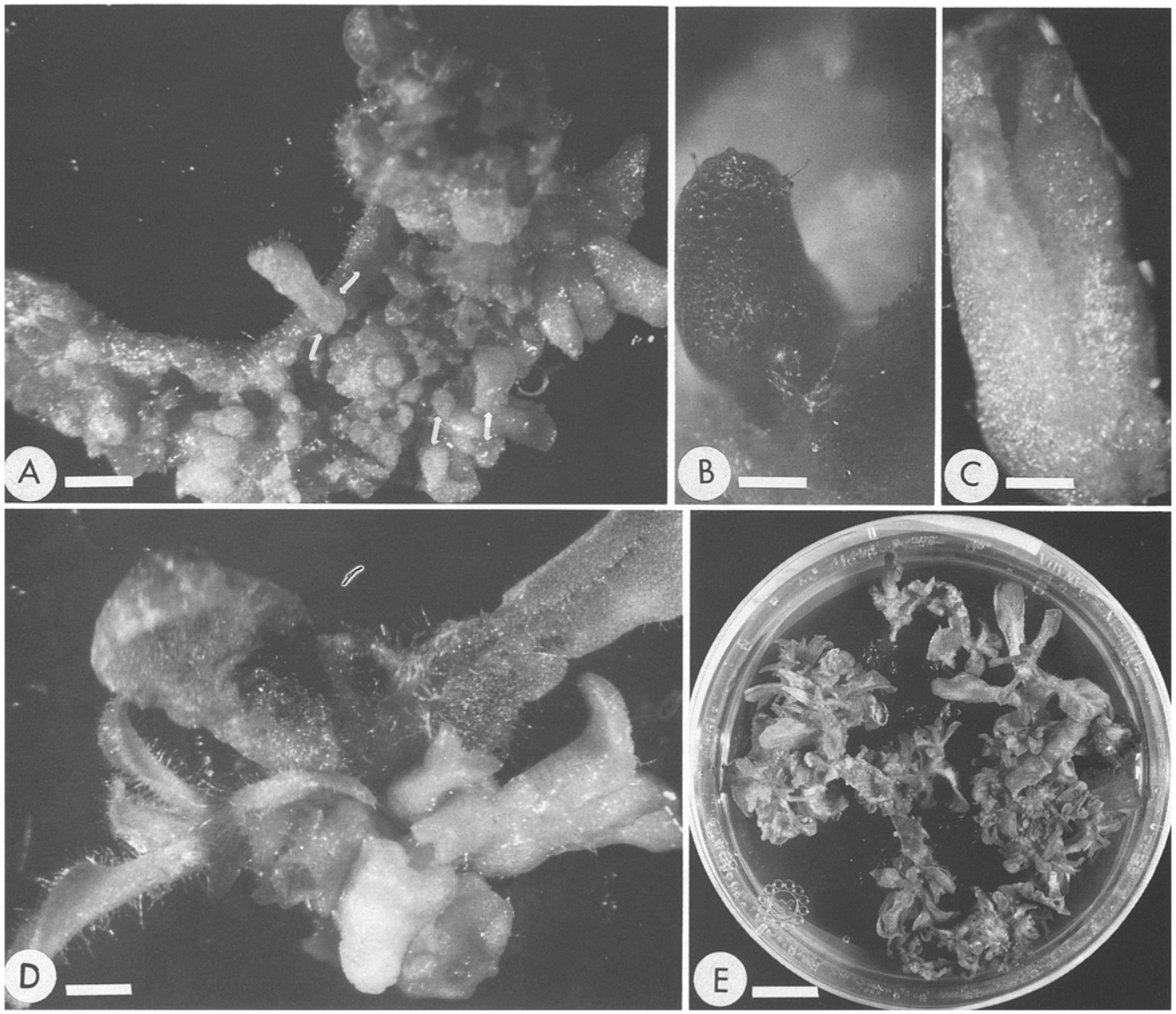

Fig. 2. Somatic embryogenesis and plant regeneration in leaf disc cultures of *Nicotiana tabacum* L. cv Petit Havana on MS medium supplemented with 1.0 μM TDZ. A. leaf disc after 2 weeks of culture on MS medium supplemented with 1.0 μM TDZ; Note the presence of globular and cotyledonary staged somatic embryos at various stages of development (arrows) (Bar = 2mm). B. A torpedo-shaped somatic embryo (Bar= 2mm). C. A somatic embryo at an early cotyledonary stage (Bar= 1.5mm). D. Development of shoot buds on mother explant without subsequent transfer to MS0 (Bar= 2mm). E. Leaf discs which were originally cultured on MS medium supplemented with 1.0 μM TDZ for two weeks and subsequently transferred to MS0. Note the proliferation of embryo-derived shoots (Bar= 82mm).

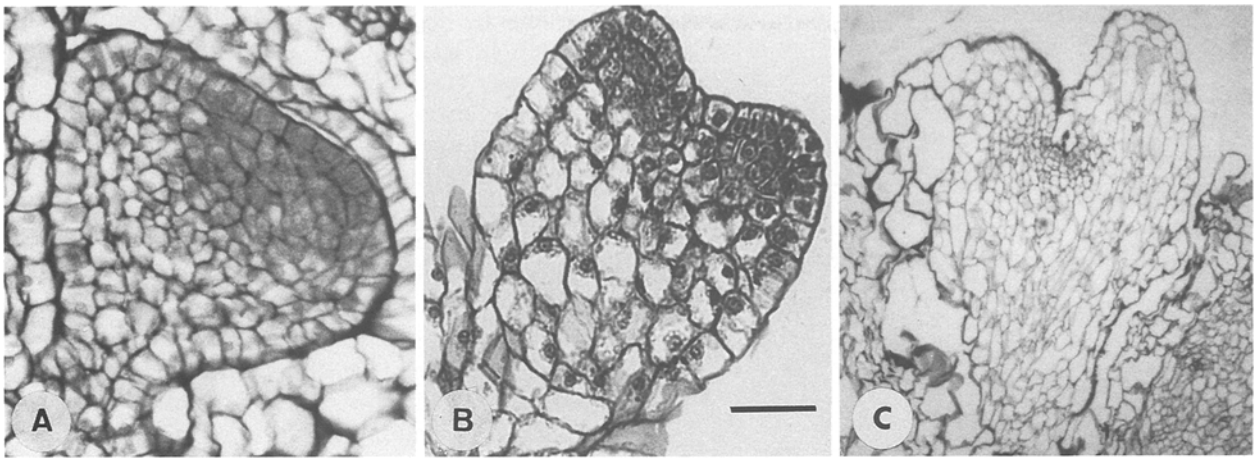

Fig 3. Histological examination of the *in vitro* development of somatic embryos from leaf discs of *Nicotiana tabacum* Petit Havana grown on MS medium supplemented with TDZ (1 μM). Transverse sections of leaf explant showing heart-shaped (A), Early and late cotyledonary stages of somatic embryo development (B,C). In all figures 11 mm bar = 100 μm.

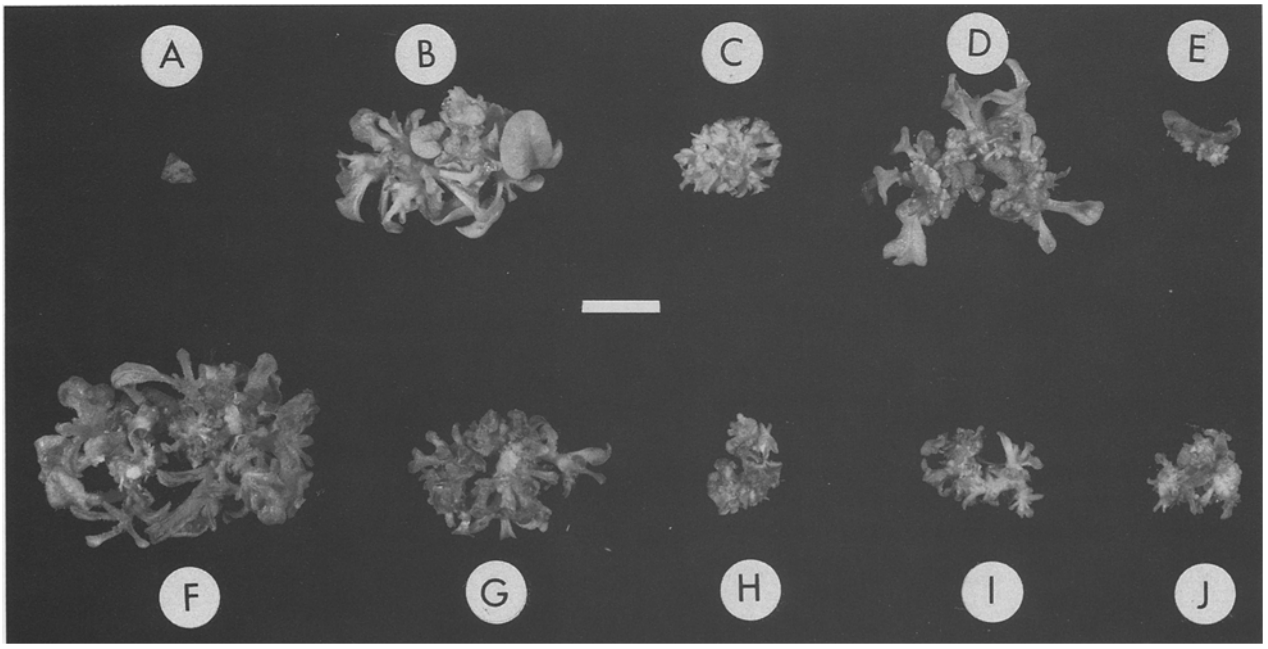

Fig. 4. The expression of the morphogenetic potential of leaf disc of *Nicotiana tabacum* cv. Petit Havana after three weeks of culture on MS medium supplemented with various growth regulators. A, leaf disc on MSO. B, A leaf disc on MS medium supplemented with 0.25 μM TDZ. Note the rapid differentiation of embryo-derived shoots. C, A leaf disc on MS medium supplemented with 10.0 μM TDZ. Note the suppressed growth of somatic embryos. D,E, Leaf discs cultured on BAP (4.4 or 44.4 μM, respectively) showing organogenesis. F,G,H, Leaf discs on MS medium supplemented with 0.50, 1.0, 5.0 μM TDZ, respectively. Note the increasing suppression of growth with higher concentration of TDZ. I,J, Somatic embryogenesis induced by 4.4 μM BAP plus 0.54 μM NAA and 44.4 μM BAP plus 0.54 μM NAA, respectively. (10 mm bar = 50mm)

developed on the medium supplemented with 44.4 μM BAP were vitrified. Inclusion of NAA (0.54 μM) with a lower (4.4 μM) or higher (44.4 μM) concentration of BAP induced the production of somatic embryos. These NAA-BAP combinations were found to be highly effective for inducing somatic embryogenesis in a previous study (Stolarz *et al.* 1991) as well as in our experiments (data not shown). A comparison of the efficacy of TDZ with that of BAP (4.4 or 44.4 μM) and NAA (0.54 μM) revealed that TDZ (0.25-15.0 μM) induced a higher number of somatic embryo-derived shoots (Table 1; Fig. 4 B,C,F,G,H). The 1 μM concentration of TDZ which produced maximum regenerants in the cv. Petit Havana was found to be equally effective for inducing somatic embryogenesis in two other cultivars (Xanthi and BEL W-5) of tobacco (data not shown).

Table 1. A comparison of the efficacy of various growth regulators in inducing somatic embryogenesis in leaf disc culture of *Nicotiana tabacum* cv. Petit Havana. Leaf explants were cultured for four weeks on MS medium supplemented with TDZ (1 μM) or two different combinations of BAP plus NAA and then transferred to MS0 for two weeks.

Growth regulators (μM)	% response[a]	No. of regenerants[b]
TDZ (1)	100	170 ± 6.3
BAP (4.4) + NAA (0.54)	100	80 ± 4.5
BAP (44.4) + NAA (0.54)	100	34 ± 3.8

a = percentage of leaf discs showing embryogenesis
b = Mean number somatic embryo-derived shoots ± S.E.

Discussion

Regeneration of plants *in vitro* is accomplished by organogenesis or somatic embryogenesis. Cytokinin-induced shoot organogenesis in tobacco is well documented (Skoog and Miller 1957; Prabhudesai and Narayanswamy 1974; Brown and Thorpe 1986). Our results of shoot induction by BAP are in agreement with these findings. Earlier, stages of somatic embryogenesis in *Nicotiana tabacum* were observed in callus cultures maintained on kinetin and under high light intensity (Haccius and Lakshmanan 1965). Later, Prabhudesai and Naryanaswamy (1974) described differentiation of shoots and embryoids from subepidermal cells of the petioles or pseudo embryonic masses resulting from proliferation of the cortical cells of the petiole. Recently, Stolarz *et al.* (1991) obtained direct somatic embryogenesis from leaf discs cultured on a medium supplemented with BAP (1 mg/l) and NAA (0.1 mg/l). Combinations of kinetin and IAA or BAP and IAA also produced somatic embryos although the frequency of embryogenesis varied from one combination to another and also among different cultivars tested. The reason why somatic embryogenesis in many previous studies with BAP-IAA or kinetin-IAA concentrations was not observed, is not clear. According to Skoog and Miller (1957), quantitative interactions for between growth factors, auxins and cytokinins, and between these and other factors, provide a common mechanism for the regulation of all types of growth ranging from cell enlargement to organ formation. These other factors that might have influenced the differentiation of shoots versus somatic embryos in different studies (Skoog and Miller 1957; Prabhudesai and Naraynaswamy 1974; Stolarz *et al.* 1991) might include both the physical and chemical conditions of culture. For instance, the basic media used in these investigations were different: a modified White's medium by Skoog and Miller (1957) and Prabhudesai and Naraynaswamy (1974) and Murashige and Skoog's medium by Stolarz *et al.* (1991). Similarly, a relatively high light intensity was shown to promote adventive embryony (Haccius and Lakshamanan 1965) but photoregulation of embryogenesis was not explored in other studies.

In our experiments with leaf disc cultures of tobacco, somatic embryogenesis was initiated effectively on MS medium containing TDZ. TDZ is chiefly used as a cotton defoliant (Arndt *et al.* 1976) but it also acts as a growth regulator in plant tissue culture systems. In assessing the potential of TDZ for substituting purine-based cytokinins, Capelle *et al.* (1983) found that it induced cytokinin autonomy in callus cultures of *Phaseolus lunatus*. The high efficiency of TDZ in stimulating cytokinin-dependent shoot regeneration from a wide variety of plants ranging from apple to common beans (Van Nieuwkerk *et al.* 1986; Malik and Saxena 1992) supports the idea of a modified cytokinin metabolism in TDZ-treated tissue (Capelle *et al.* 1983; Thomas and Katterman 1986). However, in peanut and geranium, the use of TDZ effectively replaced the requirement of auxin and (or) cytokinins for inducing somatic embryogenesis (Saxena *et al.* 1992; Gill and Saxena 1992; Visser *et al.* 1992). On the basis of the induction of embryogenesis in tobacco leaf explants by TDZ alone, together with our previous observations, it may be logically concluded that TDZ-regulated morphogenesis involves modulation of endogenous cytokinins and auxins.

As shown in Table 1, the number of regenerants recovered from explants treated with TDZ for 4 weeks and later tranferred to MSO for 2 weeks was significantly higher than those obtained with BAP plus NAA. It is noteworthy that the numbers of somatic embryos induced in the first 3-4 weeks of treatments with TDZ and NAA-BAP combinations were comparable (Fig. 1; Table 1). Increased efficiency of embryogenesis in TDZ-induced cultures (Table 1) was due to continued production of embryos even after transfer to MS0; no further embryogenesis occurred when explants induced for 4 weeks with the NAA-BAP combination were subcultured to MS0. Our studies with bean and geranium have previously shown that a continuous exposure of the tissue to TDZ is not essential for achieving maximum morphogenetic response (Malik and Saxena 1992; Visser *et al.* 1992) which is perhaps a reflection of the capacity of TDZ to induce cytokinin and auxin autonomy in differentiating tissue.

Acknowledgements. This research was supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada to PKS. We thank Nor-Am Chemical Co. for providing a technical-grade sample of thidiazuron. We thank Tannis Slimmon for assistance in preparation of the manuscript.

References

Arndt F, Rusch R, Stillfried HV (1976) SN 49537, a new cotton defoliant. Plant Physiol. 57:99.

Brown DCW, Thorpe TA (1986) Plant regeneration by organogenesis. In: Cell culture and somatic cell genetics of plants (IK Vasil ed.) pp. 49-65. Academic Press. Orlando, Florida.

Capelle SC, Mok DWS, Sandra C, Kirchner SC, Mok MC (1983) Effect of thidiazuron on cytokinin autonomy and the metabolism of N^6-(Δ^2-Isopentenyl) [8-^{14}C] adenosine in callus tissue of *Phaseolus lunatus*. Plant Physiol.73:796-802.

Gill R, Saxena PK (1992) Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (*Arachis hypogaea* L): Promotive role of thidiazuron. Can. J. Bot. 70:1186-1192.

Haccius B, Lakshmanan KK (1965) Adventive embryony in callus culture of *Nicotiana* under high light intensity. Planta 65:102-104.

Malik KA, Saxena PK (1992) Regeneration in *Phaseolus vulgaris* L. High-frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. Planta 186:384-389.

Mok MC, Mok DWS, Armstrong DJ, Shudo K, Isogai Y, Okanoto T (1982) Cytokinin activity of N-phenyl-N'-1,2,3-thidiazol-5 yl urea (thidiazuron). Phytochemistry 21:1509-1511.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant.15:473-497.

Prabhudesai VR, Narayanswamy, S (1974) Differentiation of cytokinin-induced shoot buds and embryoids on excised petioles of *Nicotiana tabacum*. Phytomorphology 23:133-137.

Saxena PK, Malik KA, Gill R (1992) Induction by thidiazuron of direct somatic embryogenesis in intact seedlings of peanut. Planta 187:421-424.

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11:118-140.

Stolarz A, Macewicz J, Lorz H (1991) Direct somatic embryogenesis and plant regeneration from leaf explants of *Nicotiana tabacum* L. J. Plant Physiol. 137: 347-357.

Thomas JC, Katterman FR (1986) Cytokinin activity induced by thidiazuron. Plant Physiol. 81:681-683.

Van Nieuwkerk, Zimmerman RH, Fordham I (1986). Thidiazuron stimulation of apple shoot proliferation *in vitro*. Horticulture Sci. 21: 516-518.

Visser C, Qureshi JA, Gill R and Saxena PK (1992) Morphoregulatory role of thidiazuron. Substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in geranium hypocotyl cultures. Plant Physiol. 99:1704-1707.