Plant Cell Reports (1995) 15:205–211

Plant Cell Reports

Morphoregulatory role of thidiazuron: morphogenesis of root outgrowths in thidiazuron-treated geranium (*Pelargonium x hortorum* Bailey)

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Received 23 November 1994/Revised version received 12 June 1995 – Communicated by F. Constabel

Summary. Root outgrowths formed on the root tissue of geranium (*Pelargonium x hortorum* Bailey cv. Kim and cv. Shone Helena) plants in response to treatment with the phenylurea derivative, thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5'-ylurea; TDZ). Treatment with the cytokinin N^6-benzylaminopurine (BAP) or the auxin α-naphthaleneacetic acid (NAA) did not result in stimulation of similar abnormal structures on the root tissue. Significantly more outgrowths developed on roots of plants treated with 10 μM and 20 μM TDZ than on control plants or those treated with 1 μM TDZ for the eight-week treatment period. Some outgrowths produced shoots and plantlets while still attached to roots, and regenerants were easily separated from the root tissue and transferred to soil in the greenhouse where they grew to maturity. Histological observations suggested these outgrowths originated from the vascular cambium region of the root.

Keywords: thidiazuron, TDZ, geranium, regeneration, root outgrowths, histology

INTRODUCTION

Morphogenesis *in vitro* is primarily effected by two factors: selection of an appropriate explant and determination of optimum levels of phytohormones, mainly auxin and cytokinins. In our previous work with *Pelargonium*, *Phaseolus* and *Lathyrus* species, we demonstrated that thidiazuron (TDZ), a phenylurea derivative with high intrinsic cytokinin activity (Mok et al. 1982), stimulated a rate of regeneration much higher than that obtained with other phytohormones including auxin and cytokinins (Visser *et al.*, 1992; Malik and Saxena, 1992). It has also been shown that excision of a tissue explant is not essential for inducing regeneration and that high-frequency regeneration occurs from tissues of intact seedlings germinated on TDZ-supplemented medium (Malik and Saxena, 1992).

Based on these observations, we hypothesized that adventitious plant formation may occur in the plants treated with TDZ. Interestingly, the present study conducted to test this hypothesis revealed yet another regulatory role of TDZ. Treatment of vegetatively propagated geranium with TDZ stimulated the formation of outgrowths on the root tissue and several of these outgrowths differentiated into normal plants. Structural evidence on the site of origin of root outgrowths at the light microscopic level, is also provided.

MATERIALS AND METHODS

Rooted geranium (*Pelargonium x hortorum* Bailey) plantlets of the cultivars Kim and Shone Helena were purchased from Belgian Nurseries (Breslau, Ont.) and transplanted into 13 cm standard pots (Kord Products, Toronto, Ont.) containing potting mix (Promix BX, Plant Products, Brampton, Ont.). Treatments began four weeks after transplanting of plantlets and continued for eight weeks. Five plants of each cultivar were watered with 300 ml of growth regulator solution a total of 15 times over the 8 week treatment period. Treatment solutions included the auxin α-naphthaleneacetic acid (NAA) at 1, 10 and 20 μM, the cytokinin N^6-benzylaminopurine (BAP) at 1, 10 and 20 μM, N-phenyl-N'-1,2,3-thiadiazol-5'-ylurea (thidiazuron, TDZ, Nor-Am Chemical Co., Willmington, DE., U.S.A.) at 1, 10 and 20 μM and a combined solution of 10 μM NAA and 10 μM BAP. Control plants were watered with the same amount of deionized water. Plants were fertilized twice over the treatment period with 80 mg of Nitrogen: Phosphorous: Potassium (20:8:20) in 300 ml of water. All flower buds were removed during the treatment period to avoid physiological variations resulting from quantitative and qualitative differences in flowering. Plants were harvested 12 weeks after the treatments commenced. For regeneration, root outgrowths and developing shoots (as shown in Figs. 2D, 3D) were transferred to potting mix in 6 cm pots and were fertilized twice during the treatment period. Rooted plantlets were transplanted after four weeks of growth to 13 cm pots and maintained in the greenhouse as normal cutting-propagated plants. The methods of sample preparation and histology of root outgrowths were as described earlier (Malik and Saxena, 1992)

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RESULTS AND DISCUSSION

The cultivars Kim and Shone Helena responded in very similar ways to treatment with the auxin and cytokinin. Control plants were well established with thick hard roots. The root systems of plants treated with 1 μM NAA or 1 μM BAP (Fig. 1A) were comparable to the control plants but at higher concentrations of NAA the roots became much more numerous and fibrous than the roots watered with either BAP or deionized water (Fig. 1B). Although no major differences between cultivars were observed, Kim appeared to develop a more fibrous root system at high concentrations of BAP than did Shone Helena. Treatment of either cultivar with a combined solution of NAA and BAP resulted in the formation of mostly fibrous root tissue (similar to those shown in Fig. 1B).

In plants treated with increasing concentrations of TDZ, there was a trend towards stunted plant growth (Fig. 2A). Watering with 20 μM TDZ solutions resulted in a bright pinkish tinge on the leaf margins (Fig. 2A: light regions indicated by arrows). Examination of the root systems of plants treated with the TDZ solutions revealed the formation of root outgrowths (Fig. 2B, arrowheads) and proliferation of adventitious shoots at the crown region (Fig. 2C and 3C, arrow). There were significantly more root outgrowths found on the root tissue of plants treated with 10 or 20 μM TDZ than those treated with 1 μM TDZ (Table 1; Fig. 2B, extreme right). Root outgrowths were not observed in control plants. At 10 μM TDZ, the cultivar Kim produced significantly more root outgrowths than Shone Helena. Proliferation at the crown region appeared in several forms including callusing tissues, root outgrowths, shoots and structures resembling somatic embryos at different stages of development (Fig. 2B, C, D, and 3D). Newly formed regenerants emerged above the soil due to continued proliferation and growth (Fig. 2D arrow).

Root outgrowths appeared as globular or elongated structures and some appeared to have associated shoot-like proliferation (Fig. 3A, B). When treatment with TDZ was suspended, plants resumed growth and flowering, but the proliferation of root outgrowths continued on the root and crown tissue (Fig. 3C, D). The regenerants (Fig. 3D arrowhead, G, arrow) resemble *in vitro* induced shoots and somatic embryos. A few root outgrowths removed from treated roots and transplanted to soil developed roots and shoots (Fig. 3E, F) under greenhouse conditions and produced apparently normal flowering plants. Regenerated plantlets continued to develop additional root outgrowths in the crown region without any treatment with TDZ (Fig. 3F, G) but their further growth was inhibited.

Table 1. The appearance of outgrowths on the root tissue of two geranium cultivars in response to thidiazuron. Means within each column followed by the same letter are not significantly different ($P = 0.05$)

Concentration of TDZ (μM)	Number of root outgrowths	
	Shone Helena	Kim
0	0[a]	0[a]
1	18[a]	23[a]
10	232[b]	489[c]
20	231[b]	267[b]

Structural organization of outgrowths developing on root tissues of Shone Helena are shown in Fig. 4. A distinct vascular connection was evident even at the early stages of outgrowth development (not shown). Root outgrowths at later stages of development (as in Fig. 4) were characterized by frequent cell divisions, both anticlinal and periclinal, in the vascular cambium region (Fig. 4A, C). These actively dividing cells were distinct from the adjacent differentiated cells (larger in size and highly vacuolated). The root stele was found to extend into the outgrowth and form a well organized vasculature, with tracheids and vascular initials (Fig. 4A, B). Well organized meristematic zones, composed of characteristic isodiametric cells with dense cytoplasm and prominent nuclei were also seen (at the distal end of the root outgrowth in Fig. 4A, D). Based on our observations on the root outgrowth ontogeny, we presume that these outgrowths originate from the vascular cambium adjacent to the protoxylem of the root.

TDZ is a diphenylurea derivative and is commonly used as a cotton defoliant. The mode of action of TDZ is yet unknown, but there is evidence that TDZ increases the biosynthesis and accumulation of endogenous purine cytokinins in tissue cultures (Capelle *et al.* 1983; Thomas and Katterman 1986). Recently, we have shown that TDZ can substitute for both the auxin and cytokinin requirement for inducing somatic embryogenesis in geranium and tobacco tissue cultures (Visser *et al.* 1992; Gill *et al.* 1993) and it also induces somatic embryogenesis in peanut explants or seedling cultures which otherwise respond primarily to an auxin for somatic embryo differentiation (Saxena *et al.* 1992; Gill and Saxena 1992). In the present investigation, TDZ induced root outgrowths and shoot morphogenesis whereas BAP and NAA stimulated the fibrous texture of the root tissue. It is possible that the concentration of exogenous cytokinin and auxin used was not the most appropriate to cause changes similar to those stimulated by TDZ. Alternatively, TDZ may act in a manner different than NAA and BAP.

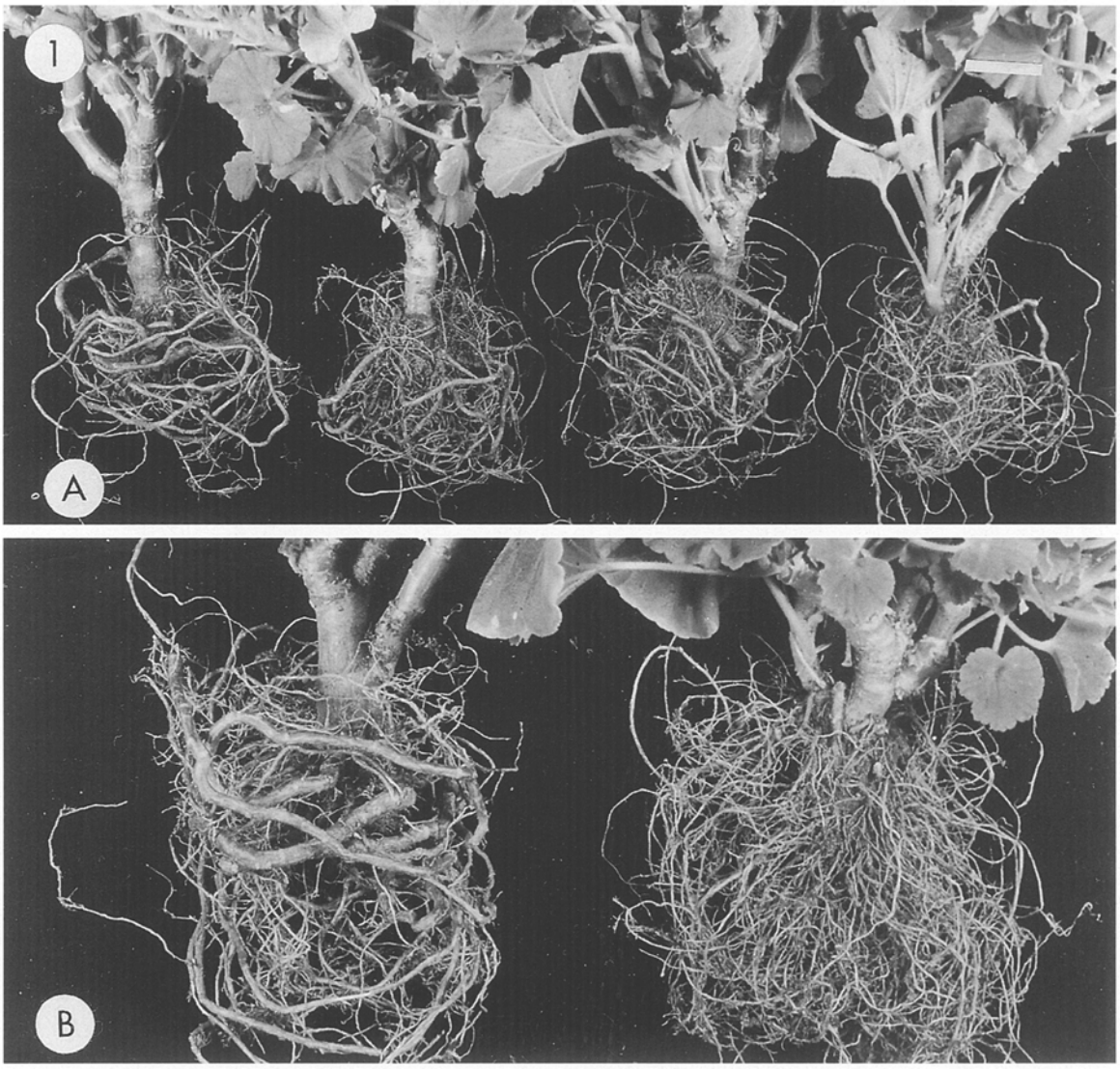
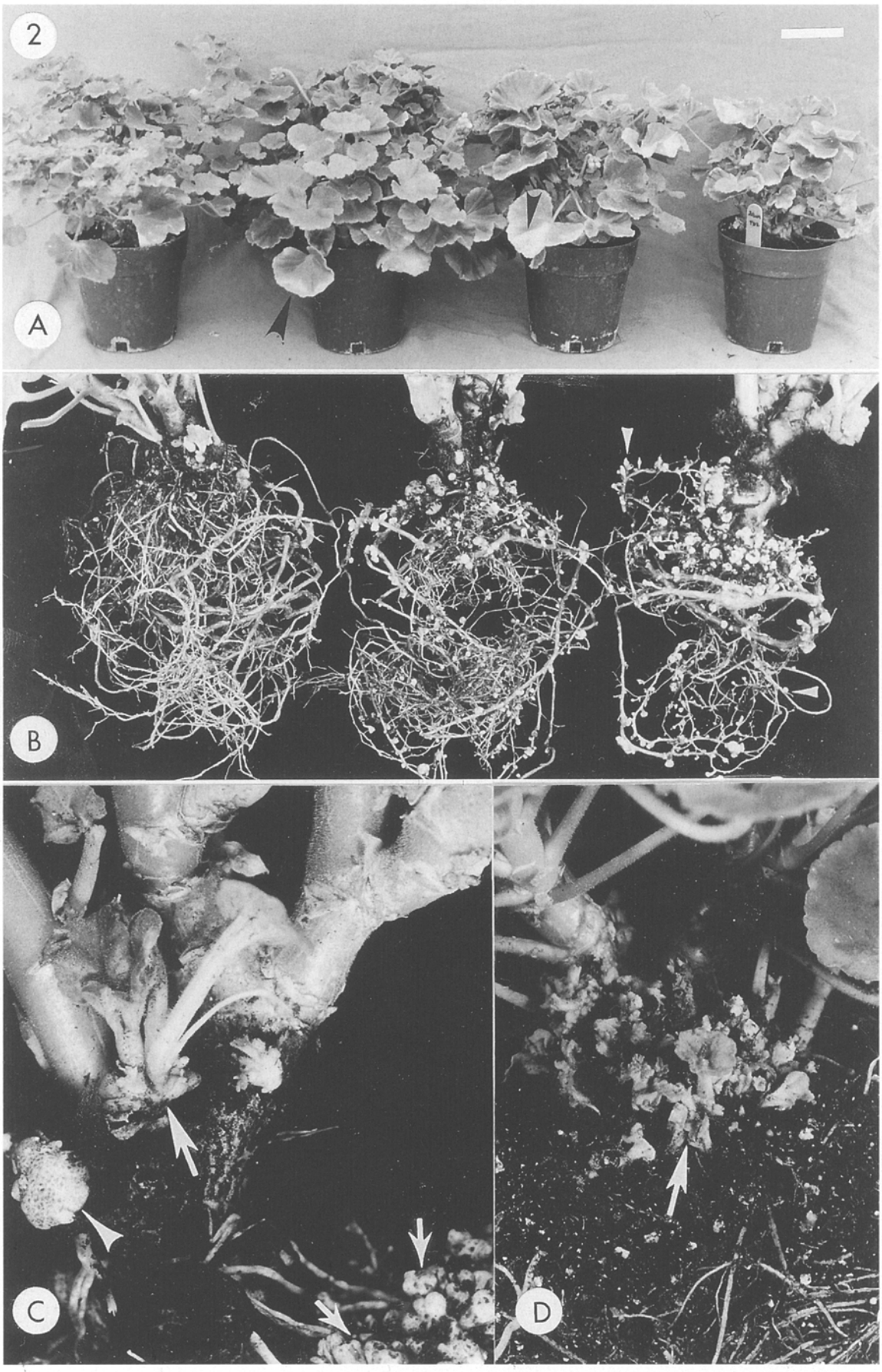


Figure 1A-B. Effect of watering with NAA and BAP on root systems of geranium (*Pelargonium x hortorum* Bailey). **A.** Cultivar Kim watered with 0, 1, 10 and 20 μM BAP, respectively (Bar = 4.5 cm). **B.** Cultivar Shone Helena watered with 0 and 20 μM NAA (Bar = 2.6 cm).

Figure 2A-D. Effect of watering with TDZ solutions on geranium (*Pelargonium x hortorum* Bailey). **A.** Cultivar Shone Helena watered with 0, 1, 10 and 20 μM TDZ, respectively (Bar = 6.0 cm). Note the suppression of growth with increasing concentrations of TDZ. Arrowheads show areas on leaf margins that had a pink tinge. **B.** Root tissue of Shone Helena plants watered with 1, 10 and 20 μM TDZ (Bar = 3.2 cm). Note the formation of outgrowths on the root tissue (arrowheads). **C.** Proliferation at the crown region of Shone Helena treated with 10 μM TDZ showing callus (white arrowhead), root outgrowths (small arrowheads) and shoot formation (arrow) (Bar = 0.9 cm). **D.** Crown proliferation on the cultivar Kim treated with 1 μM TDZ showing shoot formation (Bar = 1.0 cm). Note the emergence of regenerated shoots and plantlets above the soil (arrow).

Figure 3A-G. Plant regeneration from root outgrowths developed on TDZ treated roots of geranium (*Pelargonium x hortorum* Bailey). **A.** Globular root outgrowth with shoot primordium (Bar = 0.18 cm). **B.** Elongated root outgrowth (Bar = 0.2 cm). **C.** Flowering plant with proliferation in the crown and root region following the termination of TDZ treatment (Bar = 5.7 cm). **D.** Magnified view of root region in Fig. **C** with root outgrowths (arrows) and developing regenerants (arrowheads), some of which resemble somatic embryos (Bar = 0.44 cm). **E.** Plantlet grown from a single root outgrowth (Bar = 1.3 cm). **F.** Plant regenerated from a root outgrowth and beginning to show proliferation in the crown region without subsequent treatment with TDZ (Bar = 1.3 cm). **G.** Magnification of crown proliferation (arrow) (Bar = 0.27 cm).

Figure 4. A. Light micrograph of a median cross section of root outgrowth illustrating the stele of the main root (black arrows) and the origin of root outgrowths (white arrows) and the vascular tissue of the outgrowth (white arrowheads). Meristematic regions are located at the distal face of the root outgrowths (arrowheads) (Bar = 0.28 mm). **B-D.** Magnifications of Fig. 4A showing structural details of tissues of the stele and the root outgrowth. **B.** Enlarged view of the vascular tissue of the outgrowth; arrows point to tracheids (Bar = 0.12 mm). **C.** Cell division in the vascular cambium region; note the increase in cell size as they become differentiated (Bar = 0.07 mm). **D.** Meristematic region showing dividing cells with dense cytoplasm, and distinct nuclei (Bar = 0.12 mm).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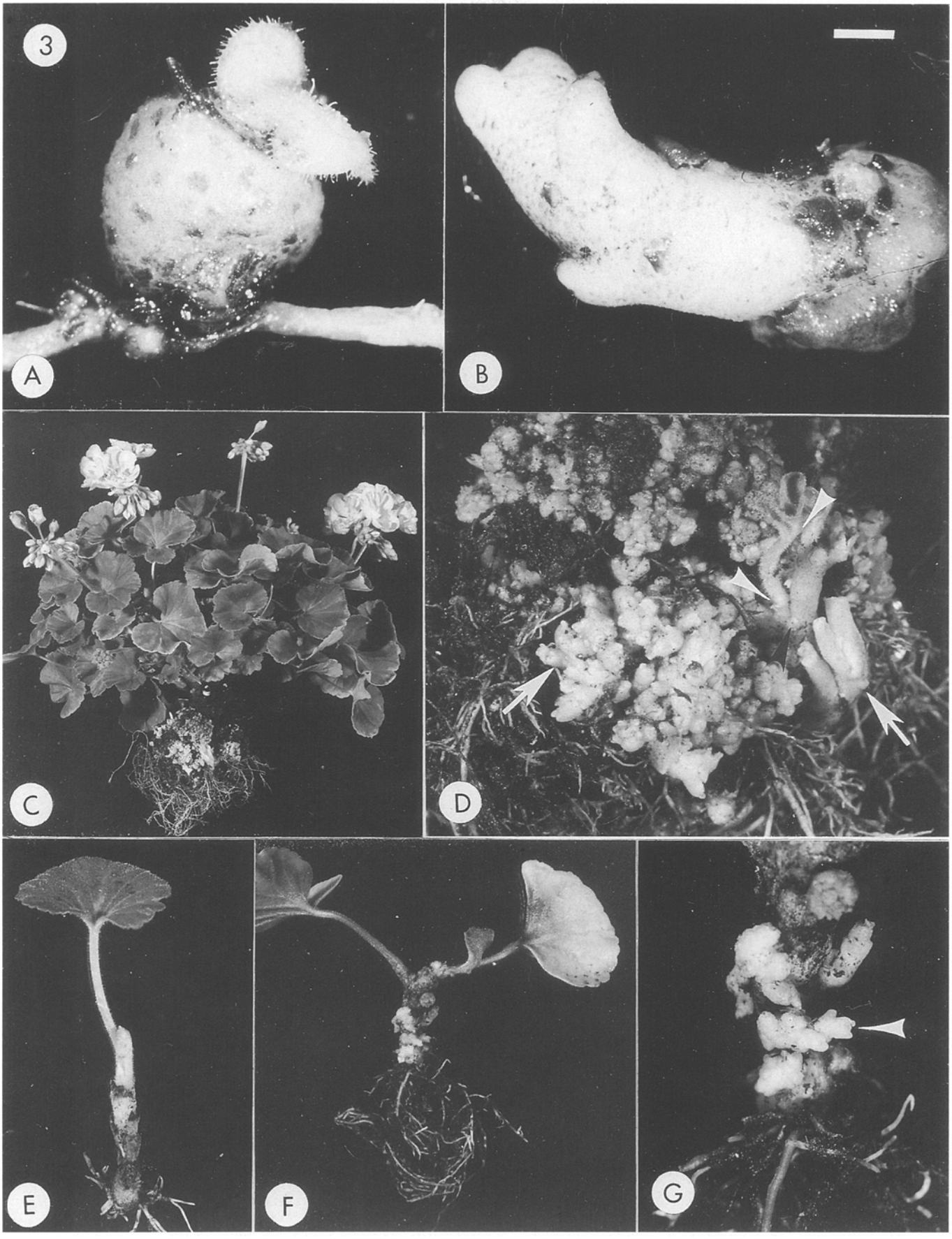
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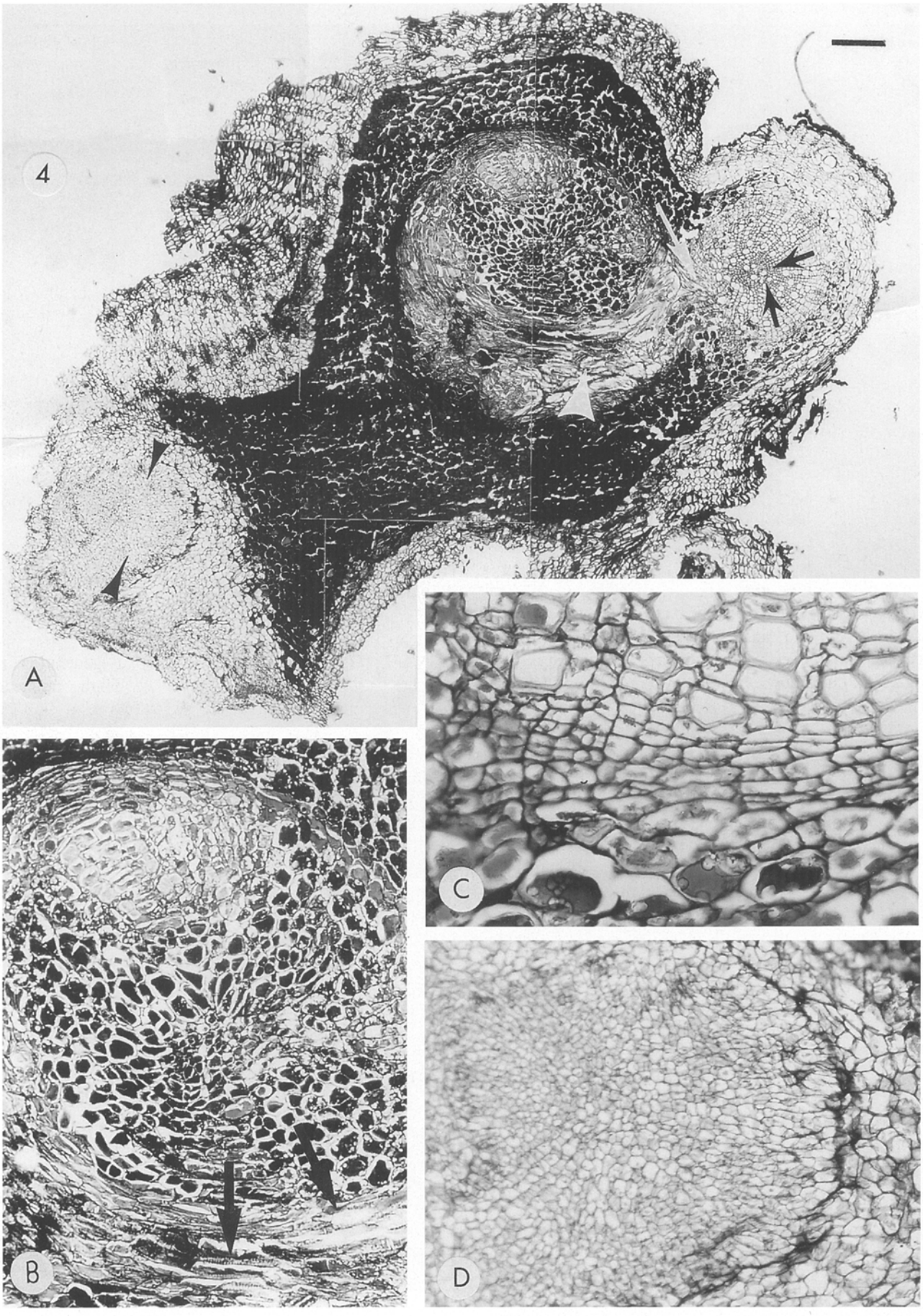
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The system reported in the present study offers the opportunity for further investigation into whole plant metabolism under the influence of TDZ and the regulatory role of TDZ in morphogenesis. For instance, it seems logical to assume that direct differentiation of shoots at the crown region (Fig. 2C, D, 3D) and extensive development of root outgrowths (Fig. 2B) may result from different concentrations of endogenous growth substances particularly auxins and cytokinins. Growth and morphogenesis in tissue culture is known to be regulated by a delicate balance of auxins and cytokinins and their interaction with other nutrients (Skoog and Miller 1957). It is possible that there is a differential gradient or perhaps the modulation of an existing gradient due to the synthesis and transport of cytokinins and auxins in different regions of the plant. Continued development of the root outgrowths and their differentiation into plants (Fig. 3C, D) after the conclusion of two months of TDZ treatment is indicative of the possible residual effects in TDZ-treated tissue. This speculation gains further support from the observation that some of the plantlets regenerated from root outgrowths continued to produce similar structures (Fig. 3F, G). In addition to phytohormones, several other metabolic pathways, such as nitrogen and carbohydrate assimilation and transport, may also be involved in the development and differentiation of root outgrowths. The analysis of endogenous growth regulators and metabolic pathways is currently in progress in our laboratory to determine the cellular and physiological basis of the mode of development of the root outgrowths.

A noteworthy aspect of this study is the resemblance of root outgrowths with nodules of leguminous plants which are induced in response to the infection by *Rhizobium* strains and are capable of nitrogen fixation. Several researchers have attempted to stimulate nodulation on non-leguminous plants. Nodule-like root outgrowths (also referred to as pseudonodules or para-nodules) have also been induced on non-leguminous plants using growth regulators such as 2,4-D (Ridge *et al.* 1992, and references therein). TDZ stimulated root outgrowths of geraniums were different than rhizobium-induced nodules. The major differences were the presence of largely unorganized tissue and the ability of root outgrowths to differentiate into plants.

The regeneration of plantlets from root outgrowths opens the possibility for using this technique for micropropagation. A common problem inherent in current micropropagation techniques is the adaptation of plants grown in tissue culture to greenhouse conditions due to inefficient photosynthesis, poor gaseous exchange and high transpiration (Kozai, 1991). Plantlets grown from root outgrowths are already adapted to a natural environment. In addition, *in vivo* micropropagation does not require aseptic conditions or expert technical support which together constitute about 70-80% of the cost of commercial plant production by micropropagation *in vitro*. However, further work will be required to optimize the conditions for developing micropropagation systems for commercial use.

Acknowledgement. This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada and Agriculture Canada Partnership Programs.

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