Response of *Rhododendron* ‘Montego’ with “Tissue Proliferation” to Cytokinin and Auxin In Vitro

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Abstract. To characterize the in vitro behavior of *Rhododendron* ‘Montego’ with tissue proliferation (TP) to cytokinin and auxin, comparisons were made of normal [TP(–)], dwarf TP [TP(+) dwarf], and long TP [TP(+) long] shoot cultures. On basal medium TP(–) and TP(+), long shoots failed to multiply and had a low relative growth rate (RGR) of 0.1, whereas TP(+) dwarf shoots produced 31.8 shoots per tip, with most shoots being <5 mm long, and RGR was 0.3. Addition of 15 μ M 2iP to basal medium induced the production of more than six shoots per TP(–) tip and doubled their RGR; TP(+) long shoots produced 16.8 shoots, most <5 mm long, and had an RGR of 0.3; TP(+) dwarf shoots produced only 16% as many shoots as on basal medium, but still exhibited an increase in RGR. Leaves from TP(–) and TP(+) sources failed to produce shoots on basal medium, but 74% of TP(–) leaves formed shoots when cultured on 1 μ M IBA and 30 μ M 2iP. TP(+) leaves were able to form shoot meristems on media containing only 5 μ M 2iP (26% of explants), but these meristems failed to elongate into shoots. Calli from TP(–) leaves, TP(+) leaves, and TP(+) tumors grown on medium containing 10 μ M NAA and 15 μ M 2iP had higher RGRs than the same calli on basal medium during the first 8 weeks of culture. Over time, RGR decreased in both TP(–) and TP(+) leaf calli, but increased in TP(+) tumor callus. The increased RGR resulted from differentiation of shoot meristems on 85% of the calli between week 4 and week 8. Our results suggest that TP(+) tissues have altered hormone metabolism or sensitivity that leads to dramatic differences in in vitro behavior and probably contributes to tissue proliferation observed in whole plants. Chemical names used: 6-(γ , γ -dimethylallylamino) purine (2iP); indole-3-butyric acid (IBA); α -naphthaleneacetic acid (NAA).

Tissue proliferation (TP) is an abnormal tumor-like growth usually produced at or near the crown of the plant (LaMondia et al., 1997), but may also be found on aerial plant parts of some genotypes. Basal tumors may or may not be accompanied by proliferation of compact shoots with short internodes and a whorled leaf arrangement (Brand, 1992; Brand and Kiyomoto, 1992; Linderman, 1993). Genera exhibiting TP include *Rhododendron*, *Kalmia*, and *Pieris* (Linderman, 1993). Tissue proliferation is most problematic in commercial production of *Rhododendron*, because the symptoms superficially resemble crown gall disease incited by the pathogen *Agrobacterium tumefaciens*. Numerous *Rhododendron* culti-

vars, mostly elepidotes, have been observed with TP symptoms (Brand and Kiyomoto, 1992; Linderman, 1993; Zimmerman, 1997). Symptoms are most commonly observed on micropropagated plants, but some reports indicate that they can occur at low frequency on cutting-propagated (McCulloch and Britt, 1997; Mudge et al., 1997) and seedling (McCulloch and Britt, 1997) plants.

Tissue proliferation may be caused by a pathogenic organism, epigenetic changes, or physiological changes. *Agrobacterium tumefaciens* isolated from TP growths failed to induce TP symptoms upon inoculation of *Rhododendron*, and did not react to T-DNA probes used for detecting pathogenic strains of *Agrobacterium* (Brand and Kiyomoto, 1992; Linderman, 1993; McCulloch and Britt, 1997). Stab inoculations with pathogenic strains of *A. tumefaciens* have not induced TP symptoms in eight cultivars of *Rhododendron* (unpublished data). The involvement of other pathogens, such as mycoplasma-like organisms and *Nocardia vaccinii* in TP (Linderman, 1993; McCulloch and Britt, 1997), has not been ruled out, but there is no evidence that TP is contagious (American Nurseryman, 1992).

Several genera in the Ericaceae family, including *Arctostaphylos*, *Kalmia*, and *Rhododendron*, form natural lignotubers (swollen, woody structures containing latent buds) as survival structures (Del Tredici, 1992). Linderman (1993) speculated that TP in *Rhododendron* might be a result of enhanced expression of naturally occurring lignotubers or burls triggered upon passage through tissue culture. However, Del Tredici (1992) found that micropropagated *Kalmia latifolia* L. was less likely to produce burls than were seedlings. In addition, when tops were removed from rhododendrons with TP, vegetative regrowth from basal proliferations did not occur or failed after a few months (Brand and Kiyomoto, 1993), which suggested that TPs were not functional equivalents of normal lignotubers.

There is evidence that TP in *Rhododendron* ‘Montego’ results from epigenetic changes occurring during the tissue culture process. Brand and Kiyomoto (1997) observed that *Rhododendron* ‘Montego’ shoot cultures from plants with TP behave differently from cultures established from plants without TP. ‘Montego’ shoot cultures initiated from plants with TP rapidly become cytokinin-habituated, proliferate rapidly and form nodal tumors in the absence of an exogenous cytokinin. Most shoots in these cultures are compact, with small leaves, and are highly branched. Few long shoots with large leaves are observed; these branch infrequently, fail to form tumors, and appear to be nonhabituated. Cultures established from plants without TP require cytokinin for shoot elongation and for proliferation of shoots from axillary buds. Even in the presence of exogenous cytokinin, multiplication is slow with cultures initiated from plants without TP. However, the habituated state, characterized by rapid proliferation and nodal tumors, can be induced in cultures established from plants without TP by elevated levels of exogenous cytokinin, which stimulates the production of adventitious shoots (Brand and Kiyomoto, 1997). In addition, Brand and Kiyomoto (1999) have shown that the ability to transmit TP symptoms through rooted shoot cuttings is dependent on the age of the mother plant. The incidence of TP symptoms in ‘Montego’ declines with time after removal from in vitro culture, suggesting that epigenetic changes are involved. Collectively, both in vitro and vegetative transmission studies (rooted cuttings) support the theory that epigenetic changes are associated with adventitious shoot formation occurring in vitro.

The type of hypertrophic growth observed on adult TP plants suggests an alteration in hormone metabolism, or sensitivity to cytokinins or auxins, or both. The differential response of tissue explants to cytokinins can be used as a phenotypic indicator of TP. The objectives of this study were to determine if: 1) TP(+) rhododendron responds differently to growth regulators in vitro than does TP(–) rhododendron; 2) callus from TP(+) tumors behaves differently than that from TP(+) leaves; and 3) TP(+) long shoots arising in TP(+) dwarf shoot cultures have returned to the normal, TP(–) condition.

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Materials and Methods

Stock plants for culture initiation. Shoots from the original 'Montego' plant were received from David Leach of the Holden Arboretum, Mentor, Ohio. These shoots were referred to as TP(-), indicating their origin from plants with no history or evidence of TP, and were used to initiate TP(-) shoot cultures in vitro. A commercial nursery provided shoots from 'Montego' plants exhibiting obvious TP symptoms and these were used to initiate in vitro shoot cultures referred to as TP(+). The commercial nursery plants had been micropropagated from explants taken from the original 'Montego' plant. Figure 1 summarizes the origins of the source material used.

Culture initiation. Recently expanded shoots were cut from TP(+) and TP(-) plants using ethanol-sterilized knives. Shoots were stripped of leaves, trimmed to 4 cm, washed in soapy water, and rinsed under running tap water for 5 min. They were then disinfested by agitation for 15 min in 10% commercial bleach (5.25% sodium hypochlorite) containing five drops of Tween 20, per 500 mL. Following three rinses in sterile distilled water, shoots were cut to 2 cm and placed on culture medium in individual vessels.

The basal woody plant (WP) medium (Lloyd and McCown, 1980) (pH 5.2 prior to autoclaving) containing 3% sucrose, 0.3% agar (Sigma Chemical Co., St. Louis), and 0.1% Phytigel (Sigma Chemical Co.) was used for all studies. For shoot multiplication, the basal medium was supplemented with 10 μ M 2iP.

During the establishment phase, explants were grown for the initial two subculture periods on 15 mL of medium in 25 $\times$ 150-mm culture tubes sealed with clear polypropylene caps. Cultures were thereafter maintained on 30 mL medium in 200-mL glass jars sealed with B-caps (Magenta Corp., Chicago). Six explants were transferred to each jar at subculture. Cultures were incubated under 40 μ mol·m⁻²·s⁻¹ light intensity provided by cool-white fluorescent tubes for 16 h·day⁻¹ at 24 $\pm$ 2 $^{\circ}$ C in all studies. TP(+) and TP(-) cultures were grown for a minimum of three subcultures prior to initiation of treatments.

Culture maintenance. Following establishment, TP(-) cultures were maintained continuously on WP medium containing 10 μ M 2iP. At transfer, apical and multi-nodal pieces (1–2 cm long) were used to perpetuate the cultures. Established TP(+) cultures were maintained on WP basal medium. TP(+) shoot cultures maintained on basal medium produced shoots of two types (Fig. 1). Most of the shoots were highly branched, had short internodes, small leaves (2–3 mm long) and nodal tumors (Mercure et al., 1998). These shoots are referred to as TP(+) dwarf shoots. Some shoots elongated, produced large leaves (6–8 mm long), branched infrequently, and appeared similar to TP(-) shoots. These shoots are referred to as TP(+) long shoots. TP(+) long shoots were segregated from the TP(+) dwarf shoots and grown separately for at least one 5-week passage before being used for studies. During maintenance, all cultures were trans-

Fig. 1. Origins of the various TP(+) and TP(-) *Rhododendron* 'Montego' material used.

Table 1. Comparison of the growth and multiplication rates of shoot tips of *Rhododendron* 'Montego' from plants with and without tissue proliferation (TP) after 12 weeks of growth on woody plant medium with and without exogenous 2iP.

Source of shoot tip ²	2iP concn (μ M)	RGR ³	Shoots per shoot tip ⁴	Shoot no. per shoot tip by length		
				2–5 mm	6–10 mm	>10 mm
TP(-) shoots	0	0.10 d ^w	1.0 d	0.0 c	0.0 c	1.0 c
	15	0.22 c	6.5 c	0.9 c	2.9 c	2.7 b
TP(+) long shoots	0	0.11 d	1.6 d	0.0 c	0.2 c	1.4 c
	15	0.30 b	16.8 b	14.8 a	1.7 bc	0.4 d
TP(+) dwarf shoots	0	0.32 b	31.8 a	17.1 a	8.6 a	5.9 a
	15	0.39 a	5.0 cd	4.3 b	0.6 c	0.0 d

²TP(-) = from shoot cultures initiated from plants without tissue proliferation; TP(+) long = from long shoots of shoot cultures initiated from plants with tissue proliferation; TP(-) dwarf = from dwarf shoots of shoot cultures initiated from plants with tissue proliferation.

³RGR = relative growth rate.

⁴Shoot number data were subjected to SQRT(x + 0.5) transformation before analysis, but nontransformed data are presented.

^wMean separation within columns by Tukey's HSD, $P \leq 0.05$.

ferred to fresh media every 5 weeks.

Expt. 1. Shoot tip growth and multiplication. Shoot tips (5 mm) were excised from TP(+) long, TP(+) dwarf, and TP(-) shoots, weighed and then transferred into 25 $\times$ 150-mm culture tubes containing 15 mL of either basal medium or medium supplemented with 15 μ M 2iP. Whole shoot cultures were transferred to fresh medium every 4 weeks for a total experimental period of 12 weeks. Following the first culture period, shoot tips were transferred to 30 mL of medium in 200-mL glass bottles that were sealed with B-caps. Culture fresh weight, number of shoots (>2 mm) per initial shoot tip, and individual shoot length were recorded at the conclusion of the experiment. The relative growth rate (RGR) of the shoot cultures was calculated as $RGR = [\ln(\text{weight } 2) - \ln(\text{weight } 1)] / (\text{time } 2 - \text{time } 1)$. A completely random design was used with 20 shoot tips of each type per treatment and two replications in time.

Expt. 2. Shoot organogenesis from leaves. Fully expanded leaves were excised from

shoots of TP(-) and TP(+) long shoot cultures and placed abaxial side down in 100 $\times$ 15-mm plastic petri dishes containing 30 mL medium supplemented with 1 μ M IBA plus 5 or 30 μ M 2iP. Except for the cut at the proximal end of the leaf, the lamina was not wounded. A randomized complete-block design was used with five leaves per petri dish and five dishes per leaf type $\times$ growth regulator combination. The experiment was replicated once in time. Cultures were transferred to fresh medium in petri dishes at 4-week intervals for a total of 12 weeks. At the conclusion of the study, the numbers of organogenic explants and shoots longer than 2 mm produced by each leaf explant were counted.

Expt. 3. Callus growth. Leaves from TP(+) long and TP(-) shoot tip cultures and nodal tumors from TP(+) cultures were placed on 50 mL medium with 10 μ M NAA and 15 μ M 2iP in 100 $\times$ 15-mm disposable plastic petri dishes for callus initiation. Calli were allowed to develop for 12 weeks with one 6-week subculture before being used for experiments. Ac-

tively growing TP(–) leaf, TP(+) leaf, and TP(+) nodal tumor calli were selected and cut into small pieces of equal size (≈ 30 mg). Each callus piece was weighed before placement into 25×150 -mm culture tubes containing 15 mL of either basal medium or medium supplemented with $10 \mu\text{M}$ NAA and $15 \mu\text{M}$ 2iP. Cultures were transferred onto fresh medium at 4-week intervals for 12 weeks. After the first culture period, calli were transferred to 30 mL fresh medium in 140-mL glass jars sealed with B-caps. Fresh weight was recorded for each callus piece on subculture, and RGR was calculated as described previously. A completely random design was used with 20 calli of each type per treatment and three replications in time.

Statistical analysis. Data for all three studies were analyzed using SAS PC, version 6.11, and its GLM procedure. Data for shoot tip growth from Expt. 1 and for leaf organogenesis in Expt. 2 were analyzed using analysis of variance (ANOVA) with mean separation by Tukey's HSD procedure. Shoot number data were transformed to square roots transformation. Arcsin transformation was used on percentage data. For callus growth, treatment effects were separated using single-degree-of-freedom orthogonal polynomial contrasts, and regression equations were generated using the significant linear component of the ANOVA. Data transformations used to stabilize variance before analysis were chosen based on the methods of Box and Cox (1964) as described by Fernandez (1992).

Results

Expt. 1. Shoot tip growth and multiplication. Initial shoot tip weight was ≈ 20 mg for TP(+) dwarf shoots, 50 mg for TP(+) long shoots, and 70 mg for TP(–) shoots. On basal medium, TP(+) dwarf shoots exhibited significantly greater RGR than did TP(–) shoots or TP(+) long shoots, which did not differ significantly from each other (Table 1). The addition of $15 \mu\text{M}$ 2iP to the medium significantly increased the RGR of all three sources of shoot tips, with TP(+) dwarf shoots still exhibiting significantly greater RGR than the other types of shoot tips.

On basal medium, TP(+) dwarf shoots produced significantly more shoots than TP(+) long shoots or TP(–) shoots, which failed to multiply and only elongated (Table 1). Addition of $15 \mu\text{M}$ 2iP significantly reduced TP(+) shoot proliferation while increasing it in TP(–) and TP(+) long shoots, with TP(+) long shoots multiplying significantly more than TP(–) or TP(+) dwarf shoots. The addition of 2iP to the medium inhibited elongation of TP(+) shoots (Table 1, Fig. 2).

On basal medium, TP(+) long shoots produced well-expanded leaves and appeared similar to shoots from TP(–) shoot tips (Fig. 2). However, when grown on medium containing $15 \mu\text{M}$ 2iP, cultures from TP(+) long shoot tips resembled TP(+) dwarf shoot cultures growing on basal medium, exhibiting reduced leaf size, excessive branching, minimal shoot elongation, and nodal tumors. The addition of 15

Fig. 2. *Rhododendron* 'Montego' shoot cultures grown for 12 weeks on basal woody plant medium, or medium supplemented with 2iP. Shoot types and 2iP concentrations are as follows: **a, b** = TP(–); **c, d** = TP(+) long; **e, f** = TP(+) dwarf; **a, c, e** = no 2iP; **b, d, f** = $15 \mu\text{M}$ 2iP. Line at bottom is 1 cm.

Table 2. Effects of exogenous IBA and 2iP on shoot organogenesis in *Rhododendron* 'Montego' leaf explants from shoot cultures initiated from plants with and without tissue proliferation (TP) after 12 weeks on woody plant medium.

Source of leaves ^a	Growth regulator concn (μM)		Cultures with:	
	IBA	2iP	Shoot meristems (%) ^y	Shoots $\geq 2\text{mm}$ (%) ^y
TP(–)	0	0	0 c ^x	0 b
	1	5	0 c	0 b
	1	30	82 a	74 a
TP(+)	0	0	0 c	0 b
	1	5	26 b	0 b
	1	30	68 a	0 b

^aTP(–) = from shoot cultures initiated from plants without tissue proliferation; TP(+) = from shoot cultures initiated from plants with tissue proliferation.

^yData were subjected to arc sine transformation before analysis, but nontransformed data are presented.

^xMean separation within columns by Tukey's HSD at $P \leq 0.05$.

μM 2iP to the medium resulted in nodular, green masses comprised of numerous tumors and significantly reduced shoot expansion in TP(+) dwarf shoot cultures.

Expt. 2. Shoot organogenic response. Shoot meristems were obtained from leaves cultured on media containing growth regulators, regardless of whether leaves were obtained from TP(–) or TP(+) cultures (Table 2). However, shoots were only obtained from leaves of TP(–) plants in the presence of $1 \mu\text{M}$ IBA and $30 \mu\text{M}$

2iP. The TP(–) leaves on the medium containing both $1 \mu\text{M}$ IBA and $5 \mu\text{M}$ 2iP failed to form adventitious shoot meristems. Although 26% of TP(+) leaves formed meristems on this medium, these meristems failed to develop into shoots during the 12-week period when they were maintained on medium containing $1 \mu\text{M}$ IBA and $5 \mu\text{M}$ or $30 \mu\text{M}$ 2iP. Some of these meristems were transferred to basal medium to stimulate meristem elongation into shoots. Following three passages (4 weeks each),

meristems developed into shoots similar to those derived from TP(+) explants.

Expt. 3. Callus growth. On basal medium, RGR was greatest for TP(+) tumor callus (0.17) and least for TP(-) leaf callus (0.09) during the first 4 weeks, with TP(+) leaf callus having an intermediate RGR (0.13) (Fig. 3). The RGR of callus from TP(+) and TP(-) leaves decreased on basal medium over time. The RGR of TP(+) and TP(-) leaf callus, when calculated over the entire 12 weeks, was half that for the initial 4 weeks of the study. At all evaluation times, RGR was less for TP(-) than for TP(+) leaf callus. The RGR of TP(+) tumor callus on basal medium remained constant during the first 8 weeks, then increased between weeks 8 and 12. The maintenance and eventual increase in RGR can be attributed to morphological changes in the TP(+) tumor callus cultures during the study. Between week 4 and week 8, differentiation of shoot meristems in or on the TP(+) tumor calli was observed for 85% of calli, and shoots were evident by week 12 (Fig. 4). Shoots that developed from the meristems on TP(+) tumor calli grew rapidly, exhibiting morphology and behavior similar to those of TP(+) shoots (Expt. 1, Fig. 2). Shoots had short internodes, nodal tumors, and small leaves, and were free branching. Shoot differentiation occurred on TP(+) tumor calli only when grown on basal medium. All other tissue source $\times$ medium combinations produced only callus.

During the first 4 weeks, the RGR of callus growing on 10 μ M NAA and 15 μ M 2iP was significantly greater than that of callus grown on basal medium, regardless of the callus source (Fig. 3). The RGR of TP(-) leaf callus grown on 10 μ M NAA and 15 μ M 2iP was 337% that of the same callus cultured on basal medium. The TP(+) leaf callus RGR also was stimulated on this medium, but was only 195% of that achieved on basal medium. Interestingly, this medium increased the RGR of TP(+) tumor callus by 33% during the first 4 weeks of culture. This clearly indicates that TP(+) tumor callus, prior to the point when differentiation occurs, responds positively to exogenous cytokinin, although not to the same degree as do the other two callus types. When evaluated over the entire 12-week culture period, the RGR of TP(-) leaf callus on medium containing 10 μ M NAA and 15 μ M 2iP was 515% of that of the same callus on basal medium.

Discussion

The TP(-) shoot cultures require exogenous cytokinin to multiply, but dwarf TP(+) shoot cultures do not. Even in the absence of exogenous 2iP, TP(+) shoot cultures branch freely and produce small leaves and short internodes, all of which are indicative of the influence of endogenous cytokinin. Furthermore, when exposed to levels of exogenous 2iP that normally support slow or moderate proliferation in *Rhododendron*, TP(+) shoots exhibit behavior and morphology similar to those of normal cultures on high levels of exogenous 2iP. This indicates that TP(+) shoots

Fig. 3. Relative growth rate (RGR) of *Rhododendron* 'Montego' callus derived from in vitro TP(+) tumors, TP(+) leaves and TP(-) leaves when grown on basal woody plant medium, or medium containing 10 μ M NAA and 15 μ M 2iP over a 12-week period. RGR was calculated as $[\ln(\text{weight } 2) - \ln(\text{weight } 1)] / (\text{time } 2 - \text{time } 1)$. Regression equations for basal medium are: $RGR = 0.1067 - 0.005625 \times \text{weeks}$, $r^2 = 0.99$ [TP(-) leaf]; $RGR = 0.16 - 0.00875 \times \text{weeks}$, $r^2 = 0.94$ [TP(+) leaf]; and $RGR = 0.15 + 0.00375 \times \text{weeks}$, $r^2 = 0.75$ [TP(+) tumor]. Regression equations for medium containing NAA and 2iP are: $RGR = 0.324 - 0.010125 \times \text{weeks}$, $r^2 = 0.98$ [TP(-) leaf]; $RGR = 0.288 - 0.009125 \times \text{weeks}$, $r^2 = 0.99$ [TP(+) leaf]; and $RGR = 0.25 - 0.005875 \times \text{weeks}$, $r^2 = 0.99$ [TP(+) tumor].

Fig. 4. *Rhododendron* 'Montego' callus initiated from TP(-) leaves, TP(+) leaves or TP(+) tumors after 12 weeks on basal woody plant medium, or medium containing 10 μ M NAA and 15 μ M 2iP.

may possess altered cytokinin metabolism or hypersensitivity to exogenous 2iP. In other studies on *Rhododendron* tissue culture (Hsia and Korban, 1997; Meyer, 1982; Preece and Imel, 1991), increasing concentrations of exogenous cytokinin enhanced branching, induced smaller leaves, and reduced stem elongation, even to the point where cultures consisted of only shoot meristems. Similarly, plants transformed with the *ipt* gene, which overproduce cytokinin, exhibit morphology resembling that of TP(+) shoot cultures, such as loss of apical dominance, reduced stem elongation, reduced leaf size, and dwarfing (Li et al., 1992; Medford et al., 1989). Although TP(+) tissues probably have altered cytokinin metabolism and/or sensitivity, auxin also may be involved. The observed differences in growth

and morphology between TP(+) and TP(-) tissues could conceivably result from stable levels of active cytokinin combined with altered auxin levels or activity. The development of nodal tumors on TP(+) shoots might also indicate a role for auxin in the TP(+) phenotype. However, cytokinin nucleotides are about five times as high in normal phenotypes of tumor-prone hybrids of *Nicotiana* as in the parental species (Nandi et al., 1990). Therefore, cytokinins, as well as auxins, can play a major role in tumor development.

Habituating is an epigenetic condition, typically observed in callus cultures, where the cells have lost their requirement for growth regulators (Jackson and Lyndon, 1990; Meins, 1989). The TP(+) tissues may be cytokinin-habituating since they can grow indefinitely on

cytokinin-free medium. Even though TP(+) shoot cultures no longer require exogenous cytokinins to multiply, they are still responsive to exogenous cytokinin, and shoot meristems fail to elongate into shoots under only low to moderate (10 μ M) concentrations of 2iP. Kevers et al. (1996) found that growth regulator-independent cultures retained sensitivity to growth regulators, and Everett (1981) determined that some auxin-independent *Acer pseudoplatanus* L. cell cultures exhibited increased sensitivity to auxin.

Habituation is persistent, but sometimes reversible (Hartmann and Kester, 1983; Poethig, 1990), and occurs at differing levels and/or degrees (Gaspar et al., 1991). Long shoots of TP(+), which appeared to be similar morphologically to TP(-) shoots, may demonstrate this reversibility in *Rhododendron*. Such shoots arose spontaneously from TP(+) dwarf shoot cultures and could be maintained as such on basal media. However, the "normal" morphology of TP(+) long shoots reverted rapidly to dwarf morphology with re-exposure to cytokinin. Therefore, TP(+) long shoots may represent a partial reversal of habituation, but certainly cannot be considered to be equivalent to TP(-) shoots. Working with tumor-prone, interspecific hybrids of *Nicotiana glauca* R.C. Grah. x *N. langsdorfii* Weinm., Ames (1972) found that exogenous cytokinin triggered tumor development in otherwise normal plants. Feng et al. (1990), also working with the same *Nicotiana* hybrids, showed that exogenous cytokinin, or endogenous cytokinin produced in *ipt*-transformed plants, restored the shooty and tumorous phenotype of a nontumorous mutant, whereas exogenous auxin from *iaaM* or *iaaH* transformants did not.

In the leaf organogenesis study, TP(-) leaves did not produce shoots when placed on medium containing 1 μ M IBA and only 5 μ M 2iP, while TP(+) leaves on the same medium did form shoot meristems. This would be expected if TP(+) tissues possessed altered cytokinin metabolism and/or cytokinin hypersensitivity, and supports the findings of the shoot multiplication study. Perrin et al. (1997) found that enhanced in vitro organogenic capacity of *Hevea brasiliensis* Muell. Arg., brought about by rejuvenation, was accompanied by an increase in the concentration of the active cytokinin zeatin riboside. Interestingly, shoot meristems formed on TP(+) leaves influenced by 5 μ M 2iP failed to elongate, suggesting that 5 μ M was supra-optimal. However, TP(+) leaves exposed to 30 μ M 2iP produced 2.5 times as many shoot meristems as did those exposed to 5 μ M 2iP, showing that, within limits, meristem organogenesis responds positively to an increased 2iP concentration.

On basal media, TP(+) tumor callus grew more than did TP(+) leaf callus, which grew more than TP(-) leaf callus. This suggests that

TP(+) tumors exhibit a greater level of habituation than do TP(+) leaves. Furthermore, TP(+) tumor callus was the only callus that formed shoots.

The results of the three studies suggest that TP(+) cultures are cytokinin-habituated and may be overproducing plant hormones, probably cytokinins. The development of nodal tumors, inhibition of shoot elongation, free branching habit and disposition toward shoot organogenesis observed in TP(+) cultures are consistent with cytokinin overproduction by shoot meristems. Further study to quantify endogenous active cytokinins and the rates of cytokinin metabolism in TP(+) and TP(-) material are necessary to confirm our contention that TP in *R. 'Montego'* is a condition of altered cytokinin metabolism.

Literature Cited

- American Nurseryman. 1992. Temporary ill mars rhododendron crops. Amer. Nurseryman 176:15-18.
- Ames, I.H. 1972. The influence of cytokinins on genetic tumor formation. Can. J. Bot. 50:2235-2238.
- Box, G.E.P. and D.R. Cox. 1964. An analysis of transformation. J. Amer. Stat. Assn. 39:357-365.
- Brand, M.H. 1992. Tissue culture variations: Problems. Amer. Nurseryman 175:60-65.
- Brand, M.H. and R. Kiyomoto. 1992. Abnormal growths on micropropagated elepidote rhododendrons. Proc. Intl. Plant Prop. Soc. 42:530-534.
- Brand, M.H. and R. Kiyomoto. 1993. Tissue proliferation is not lignotubers. Yankee Nursery Quart. 3:5-6.
- Brand, M.H. and R. Kiyomoto. 1997. The induction of tissue proliferation-like characteristics in in vitro cultures of *Rhododendron 'Montego'*. HortScience 32:989-994.
- Brand, M.H. and R. Kiyomoto. 1999. Redevelopment of tissue proliferation symptoms in rooted rhododendron cuttings. HortScience 34:723-726.
- Del Tredici, P. 1992. Seedling versus tissue-cultured *Kalmia latifolia*: the case of the missing burl. Proc. Intl. Plant Prop. Soc. 42:476-482.
- Everett, N.P. 1981. 2,4-D independent cell cultures of sycamore (*Acer pseudoplatanus* L.): Isolation, responses to 2,4-D and kinetin, and sensitivities to antimetabolites. J. Expt. Bot. 32:171-182.
- Feng, X.H., S.K. Dube, P.J. Bottino, and S.K. Kung. 1990. Restoration of shoot morphology of a nontumorous mutant of *Nicotiana glauca* x *N. langsdorfii* by cytokinin and the isopentenyltransferase gene. Plant Mol. Biol. 15:407-420.
- Fernandez, G.C.J. 1992. Residual analysis and data transformations: Important tools in statistical analysis. HortScience 27:297-300.
- Gaspar, Th., D. Hagege, C. Kevers, C. Penel, M. Crevecoeur, I. Engelmann, H. Greppin, and J-M. Foidart. 1991. When plant teratomas turn into cancers in the absence of pathogens. Physiol. Plant. 83:696-701.
- Hartmann, H.T. and D.E. Kester. 1983. Plant propagation. Prentice-Hall, Englewood Cliffs, N.J.
- Hsia, C-N. and S.S. Korban. 1997. The influence of cytokinins and ionic strength of Anderson's medium on shoot establishment and proliferation of evergreen azalea. Euphytica 93:11-17.
- Jackson, J.A. and R.F. Lyndon. 1990. Habituation: Cultural curiosity or developmental determinant? Physiol. Plant. 79:579-583.
- Kevers, C., M. Filali, G. Petit-Paly, D. Hagège, M. Rideau, and T. Gaspar. 1996. Habituation of plant cells does not mean insensitivity to plant growth regulators. In Vitro Cell. Devel. Biol.-Plant 32:204-209.
- LaMondia, J.L., V.L. Smith, and T.M. Rathier. 1997. Tissue proliferation in rhododendron: Association with disease and effect on plants in the landscape. HortScience 32:1001-1003.
- Li, Y., G. Hagen, and T.J. Guilfoyle. 1992. Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Devel. Biol. 153:386-395.
- Linderman, R.G. 1993. Tissue proliferation. Amer. Nurseryman 178:56-67.
- Lloyd, G. and B. McCown. 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Combined Proc. Intl. Plant Prop. Soc. 30:421-426.
- McCulloch, S.M. and J.L. Britt. 1997. Industry experiences and research with tissue proliferation. HortScience 32:986-989.
- Medford, J.I., R. Horgan, Z. El-Sawi, and H.J. Klee. 1989. Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. Plant Cell 1:403-413.
- Meins, Jr., F. 1989. Habituation: Heritable variation in the requirement of cultured plant cells for hormones. Annu. Rev. Genet. 23:395-408.
- Mercure, E.W., C.S. Jones, M.H. Brand, and C.A. Auer. 1998. Anatomy of shoots and tumors of in vitro habituated *Rhododendron 'Montego'* (Ericaceae) cultures with tissue proliferation. Amer. J. Bot. 85:616-628.
- Meyer, Jr., M.M. 1982. In vitro propagation of *Rhododendron catawbiense* from flower buds. HortScience 17:891-892.
- Mudge, K.M., J.P. Lardner, H.K. Mahoney, and G.L. Good. 1997. Field evaluation of tissue proliferation of rhododendron. HortScience 32:995-998.
- Nandi, S.K., G.J. M. de Klerk, C.W. Parker, and L.M.S. Palni. 1990. Endogenous cytokinin levels and metabolism of zeatin riboside in genetic tumor tissues and non-tumorous tissues of tobacco. Physiol. Plant. 78:197-204.
- Perrin, Y., P. Doumas, L. Lardet, M.C. Carron. 1997. Endogenous cytokinins as biochemical markers of rubber-tree (*Hevea brasiliensis*) clone rejuvenation. Plant Cell Tissue Organ Culture 47:239-245.
- Poethig, R.S. 1990. Phase change and the regulation of shoot morphogenesis in plants. Science 250:923-930.
- Preece, J.E. and M.R. Imel. 1991. Plant regeneration from leaf explants of *Rhododendron 'P.J.M. Hybrids'*. Scientia Hort. 48:159-170.
- Zimmerman, R.H. 1997. A review of tissue proliferation of rhododendron, p. 355-362. In: A.C. Cassells (ed.). Pathogen and microbial contamination management in micropropagation. Kluwer Academic, Dordrecht, Netherlands.