

Tissue Proliferation in Rhododendron: Lack of Association with Disease and Effect on Plants in the Landscape

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Tissue proliferation (TP), the occurrence of abnormal tissue overgrowth at the crown often associated with an increase in small adventitious shoots, has been a recent subject of concern in the nursery industry (American Nurseryman, 1992; Brand and Kiyomoto, 1992; LaMondia et al., 1992; and Linderman, 1993). TP, primarily on elepidote rhododendrons, came to national attention as a diagnostic problem in 1991 (LaMondia et al., 1992). Because of symptoms such as tissue overgrowth at the crown, plants with TP were initially confused with crown gall-affected plants, resulting in condemnation of nursery shipments intended for out-of-state markets. In addition, field-grown or potted nursery plants with TP growths were claimed to grow poorly, die of unknown causes, or were associated with increased incidence of disease and injury caused by *Phytophthora* spp. and *Otiorynchus sulcatus* F. (black vine weevil) (American Nurseryman, 1992; Brand and Kiyomoto, 1992).

While crown gall, caused by *Agrobacterium tumefaciens* (E.F. Sm. & Town.) Conn, has been reported on rhododendrons, it has not been a major problem and neither cell transformation by bacterial plasmid nor infection resulting from artificial inoculation has been shown to date (Linderman, 1993; Moore, 1986). TP has been associated largely with micropropagated plants and TP formation may be influenced by genetic susceptibility and/or epigenetic changes (Brand and Kiyomoto, 1992). In addition, nursery cultural conditions, such as increased fertility, the use of herbicides, and water stress, may influence the incidence and expression of TP (Brand and Kiyomoto, 1992; Linderman, 1993).

Our research objectives were to determine if: 1) TP is associated with the presence of a plant pathogen, 2) affected plants are weakened or less vigorous than nonaffected plants, and 3) TP is associated with increased incidence of *Phytophthora* infection or black vine weevil feeding.

EXPERIMENTS CONDUCTED

Attempted isolation of pathogens from TP tissues. Young, intact TP tissues were removed from the crowns of 'Chinoides' and 'Scintillation' rhododendron (*Rhododendron catawbiense* Michx. and *Rhododendron* x Dexter hybrid) plants and either surface sterilized in 0.525% NaOCl for 1 min and rinsed in sterile distilled water (SDW) or rinsed for several minutes in tap water prior to rinsing in SDW. A range of tissues from just under the epidermis to the center of the overgrowths were macerated and placed into SDW, mixed, and serial dilutions made into tubes of SDW. Aliquots were transferred to water agar (WA), nutrient agar (NA), and nutrient broth yeast extract (NBY) agar and spread with a sterile bent glass rod to isolate bacteria. Additionally, gall pieces were placed directly onto WA or potato dextrose agar (PDA) to isolate fungi. Over 100 isolations of fungi and bacteria were made in two laboratories.

Fungal colonies were transferred to PDA for identification. Individual bacterial colonies were transferred to NBY agar and Kings B agar (Schaad, 1988). Black light was used to detect fluorescence on Kings B. Gram reaction of bacterial cells was determined by staining (Schaad, 1988) or by the KOH method (Gregerson, 1978), and cell shape was determined by microscopic observation.

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After axenic cultures of bacteria were obtained, colonies that had been subcultured twice on NA for 48 h were touched with a sterile needle and scratch-inoculated at the crown into 3-week-old 'Better Boy' tomato (*Lycopersicon esculentum* Mill.) or 6-month-old conventionally propagated rooted stem cuttings of 'Boursault' rhododendron. Plants were evaluated for TP or galling after 4 weeks in the greenhouse for tomato, and after 3 months for rhododendron. *Agrobacterium tumefaciens* strains isolated from blueberry (*Vaccinium corymbosum* L.) and raspberry (*Rubus strigosus* Michx.) were inoculated into tomato in a similar manner and served as positive controls.

Tissues from the interior of TP growths were also mass inoculated to wounds at the crown of six conventionally propagated 6-month-old rooted stem cuttings of 'Boursault' rhododendrons.

Inoculation of rhododendron with Agrobacterium. Six strains of *Agrobacterium*, actively growing on NA after two subcultures for 48 h, were suspended in sterile tap water to result in $\approx 1 \times 10^8$ cells/mL and inoculated to 40 replicate plants each of conventionally propagated, newly rooted stem cuttings of 'Scintillation' rhododendron. Bacteria were inoculated by syringe injection at three points per plant, just at the soil line. *Agrobacterium* strains were isolated from *Euonymus fortunei* Turcz. Hand.-Mazz., blueberry and raspberry in Connecticut, or provided by T.J. Burr Cornell-Geneva (CG 939 A. *tumefaciens* biovar 1; R-3 A. *tumefaciens* biovar 2; and CG 49 A. *vitis* biovar 3). Injections of sterile water alone served as controls. The recently rooted cuttings were inoculated on 20 Jan. 1993 and maintained in the greenhouse. After 6 months, the root ball was wounded by stabbing with a sterile scalpel and roots drenched with a bacterial suspension of the same strain previously inoculated to the stem or sterile water. On 13 May 1994, plants were washed free of soil and roots and stems visually evaluated for galling.

Interaction of TP-affected plants with Phytophthora. To evaluate the influence of TP on the development of disease by *Phytophthora cactorum* (Leb. & Cohn) Schroet., 2-year-old tissue culture-propagated 'Montego' and 'Lee's Dark Purple' rhododendron plants with and without TP were inoculated with *P. cactorum* in a greenhouse experiment. Three isolates of *P. cactorum* (NY411, NY568, and NY570, obtained from W.F. Wilcox, Cornell-Geneva, and originally isolated from apple [*Malus sylvestris* (L.) Mill.], peach (*Prunus persica* Batsch) and strawberry (*Fragaria x ananassa* Duchesne) were grown on cornmeal agar (CMA) for 2 weeks in the laboratory at room temperature. Builders sand and food-grade cornmeal were mixed (9:1, v/v) with 200 mL SDW per L in autoclavable bags and autoclaved for 1 h on 2 successive days. Colonies from six 9-cm-diameter petri dishes, two of each isolate, were mixed in a sterile Waring blender with 100 mL SDW and added to individual bags of cornmeal/ε and (CMS). Bags of CMS were incubated on the laboratory bench for 3 to 4 weeks with periodic shaking before use. CMS was similarly prepared with sterile dishes of CMA for use as a control.

Plants in 4.5-L pots were inoculated with 150 mL of CMS on 2 Dec. 1993. Inoculum was uniformly spread on the soil surface and incorporated to 2 cm deep with a wooden pot label. Separate labels were used for each pot. Pots were individually placed in plastic bags and flooded for 72 h to induce zoospore release and infection. Plants were inoculated and flooded for a second time on 27 Jan. 1994. Plants were maintained in the greenhouse to allow symptom development.

On 21 Sept. 1994, plants were destructively sampled and evaluated for root and crown rot by *P. cactorum*. Plants exhibiting symptoms of infection by *P. cactorum* (flagging or death of terminal leaves, as well as distinctive red lesions on the stems) were dissected and symptomatic tissue plated on P5ARPH media to confirm *Phytophthora* infection (Jeffers and Martin, 1986).

Table 1. Influence of rhododendron cultivar and tissue proliferation (TP) on *Phytophthora* crown rot symptoms and re-isolation after 9 months under greenhouse conditions.

Cultivar	TP	<i>Phytophthora</i> symptomatic ^c plants (% and n)	<i>P. cactorum</i> re-isolated (%)
Montego	+	13.5 (37) ^y	0.0
	-	30.4 (23)	0.0
Lee's Dark Purple	+	32.1 (28)	33.3
	-	46.4 (28)	30.8
Mann-Whitney nonparametric test			<i>P</i>
With TP vs. without			0.31
Montego vs. Lee's Dark Purple			0.10

^yPlants with flagging or death of terminal leaves and distinctly bordered red-brown lesions on the crown or stem.

^zRaw data used in analyses.

Effect of TP on plants in the field. Two-year-old tissue-culture propagated 'Montego' and 'Lee's Dark Purple' rhododendron plants with and without TP were planted in two locations and subjected to high- or low-maintenance conditions with 10 replications per treatment combination. Plants were placed in a 1 × 1-m grid at the Connecticut Agricultural Experiment Station Valley Laboratory in Windsor, Conn., (Windsor fine loamy sand) and at the Lockwood Farm in Hamden, Conn. (Cheshire fine sandy loam), on 21 Apr. 1993. High-maintenance plots received isoxaben (*N*-[3-(1-ethyl-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide) and oryzalin (4-(dipropylamino)-3,5-dinitrobenzenesulfonamide) herbicide (Snapshot 80DF, 2.9 kg·ha⁻¹) on 29 Apr. 1993 and 12 Apr. 1994, fluvalinate (*N*-[2-chloro-4-(trifluoromethyl)phenyl]-D-valine (±)-alpha-cyano-(3-phenoxyphenyl)methyl ester) insecticide (Mavrik, 21 mL·ha⁻¹) on 20 June 1993, 2 July 1993, and 14 June 1994, and fertilizer [cottonseed meal-based 10:8:10 (N at 17.8 kg·ha⁻¹)] on 12 Nov. 1993 and 15 Apr. 1994. Low-maintenance plots did not receive herbicide, insecticide, or fertilizer. Plants were also pinched back in high-maintenance plots each season after flowering. All plants were evaluated for size (canopy volume determined by height and width in two directions); number of stems, meristems, and flowers were counted; and plants were rated for black vine weevil (*Otiorynchus sulcatus* F.) damage on 22 July 1993, 12 July 1994, and 27 June 1995 (rating: 0 = none; 1 = <5 notches per plant; 2 = 5 or more notches per plant). In 1995, plants were again measured and evaluated as before. In addition,

plants were dug, root galls rated (0 = none; 1 = present), and the diameter of TP growth measured.

FINDINGS AND INTERPRETATION

Over 100 isolations of potentially pathogenic organisms from TP tissues were made. Few fungi were isolated, including yeasts. Most fungal cultures were identified as saprophytes or facultative parasites such as *Alternaria*, *Fusarium*, or *Penicillium*, and no fungi were consistently isolated. About 100 strains of bacteria were isolated and maintained on NA, slightly over half of which were gram negative. About 2% of isolated strains were fluorescent. Rod-shaped gram negative bacteria inoculated to tomato or rhododendron did not result in the formation of TP or galls. Because none of the inoculated strains caused TP or disease, further attempts at identification were discontinued. Inoculation of rhododendron plants with TP pieces or TP extracts did not result in TP development. While no amount of negative data can definitively prove that a pathogen does not exist, these results are consistent with those of other researchers (Linderman, 1993), and indicate that TP was not the result of infection by commonly recognized pathogens.

Virulent, gall-forming strains of *Agrobacterium tumefaciens* [biovars 1, 2, and 3, as well as additional strains of *Agrobacterium* spp. from ericaceous plants (that caused galls on tomato control plants)] did not result in gall formation on rhododendron roots or crowns after two inoculations to roots and crowns and a year or more of incubation. Confusion between crown gall and TP may continue to result in the rejection of TP-affected plants diagnosed as having crown gall. Very little information exists concerning crown gall of rhododendron (Moore, 1986; Sinclair et al., 1987). Our results, as well as negative results from other researchers (Linderman, 1993; L. Moore, T. Burr, personal communication) call into question whether crown gall caused by *Agrobacterium* occurs on rhododendron. Future research should examine whether *Agrobacterium* infects rhododendron, if only to differentiate crown gall anatomy from TP.

Phytophthora cactorum infected roots, crowns, and in some cases, TP tissues of 'Montego' and 'Lee's Dark Purple' rhododendron (Table 1). However, there were no significant differences in incidence of infection between TP-affected or unaffected plants or between cultivars. TP plants with *Phytophthora* symptoms were not preferentially infected at the TP site. The mean number of *Phytophthora*-symptomatic plants was lower for 'Montego' than for 'Lee's Dark Purple'. We

Table 2. The effect of cultivar, tissue proliferation, maintenance intensity, and location on rhododendron growth, extent of TP growth and black vine weevil damage in field plots in Connecticut from 1993 to 1995.

Cultivar	TP (+/-)	Intensity of ^z maintenance	Location	Canopy size cm ³	TP size cm-d	Dead/total meristem no.	No. flowers	Black vine ^y weevil rating	Root TP	New ^x TP	
Montego	+	High	Windsor	0.01	6.6	9.9/14.4	0.8	0.4	1.0 ^w	1.0	
			Hamden	0.07	7.6	0.0/13.6	5.2	1.4	0.8	0.7	
		Low	Windsor	0.01	5.7	5.5/13.6	0.1	0.4	1.0	1.0	
	-	High	Hamden	0.10	7.4	0.0/15.5	3.1	1.4	0.7	0.8	
			Windsor	0.01	3.3	8.4/14.7	2.2	0.3	1.0	1.0	
		Low	Hamden	0.06	4.0	0.0/12.8	6.1	1.1	0.8	0.6	
Lee's Dark	+	High	Windsor	0.02	3.1	4.9/11.6	0.7	0.3	0.8	0.8	
			Hamden	0.11	7.3	0.0/22.4	5.5	1.4	0.9	0.9	
		Low	Windsor	0.02	1.7	5.0/18.0	2.3	1.0	0.8	0.8	
			Hamden	0.13	1.7	0.0/32.0	2.7	0.7	0.2	0.4	
		-	High	Windsor	0.03	2.7	3.2/19.9	1.9	0.9	0.7	0.7
				Hamden	0.19	0.7	0.0/42.8	4.7	0.9	0.4	0.3
	-	High	Windsor	0.02	0.0	6.3/25.4	2.9	0.7	0.1	0.1	
			Hamden	0.16	0.4	0.0/33.0	8.5	0.7	0.1	0.1	
		Low	Windsor	0.03	0.0	3.3/20.5	2.5	0.7	0.0	0.0	
			Hamden	0.22	0.0	0.0/50.6	4.9	1.0	0.2	0.1	
		Source of variation (analysis of variance) (main effects)				<i>P</i>					
		Cultivar				0.000	0.000	0.016/0.000	NS	NS	0.000
TP				NS	0.000	NS/NS	0.019	NS	0.020	0.004	
Maintenance				0.004	NS	0.005/0.022	NS	NS	NS	NS	
Location				0.000	0.030	0.001/0.000	0.001	0.001	NS	0.040	

^xFertilizer, insecticide, herbicide, and pruning intensity.

^yBlack vine weevil rating: 0 = none, 1 = ≤5 notches per plant, 2 = >5 notches.

^zTP rating: 0 = absence; 1 = presence.

^wRating data analyzed by the Mann-Whitney nonparametric test.

were unable to re-isolate *Phytophthora* from 'Montego' after 9 months under greenhouse conditions, perhaps suggesting that 'Montego' was a somewhat less suitable host for the isolates of *Phytophthora* used.

The presence or absence of TP on both cultivars did not significantly affect plant growth or the number of actively growing or dead meristems, or influence leaf damage associated with black vine weevil feeding (Table 2). Kiyomoto and Brand (1994) obtained similar mortality rates in 'Holden' and 'Besse Howells' rhododendron with or without TP. In our experiments, TP did decrease the number of flowers present in 1995. Location and cultivar effects existed for plant size, the diameter of TP growths, and numbers of meristems in the plant canopy. 'Lee's Dark Purple' was consistently larger and had more flowers, stems, and meristems than 'Montego'. 'Montego' had larger TP growths and more dead meristems than 'Lee's Dark Purple'. 'Montego' plants had more root TP and new TP than did 'Lee's Dark Purple'.

Previous surveys differed concerning the association of root overgrowths or galls with TP (Brand and Kiyomoto, 1992; LaMondia et al, 1992). The presence of root TP may be a function of cultivar. In addition, plants were more likely to develop new TP growths on roots or at the crown if TP was already present at planting. Initial TP growth size, the presence of root TP, or the formation of new TP growths were not influenced by high- or low-maintenance practices. TP growths were larger on larger, actively growing plants. All plants were larger, healthier, had more flowers, and more black vine weevil damage on foliage at Lockwood farm in Hamden than those in Windsor. *Phytophthora* infection was absent on all plants at either location.

These results indicate that the presence of TP galls on rhododendron was not associated with increased *Phytophthora* infection, increased attraction and damage due to black vine weevils, or significant negative effects on plant growth regardless of the intensity of maintenance.

Literature Cited

- American Nurseryman Publishing Co. 1992. Temporary ill mars rhododendron crops. Amer. Nurseryman 175:15-18.
- Brand, M.H. and R. Kiyomoto. 1992. Abnormal growths on micropropagated elepidote rhododendrons. Intl. Plant Prop. Soc. Comb. Proc. 42:530-534.
- Gregerson, T. 1978. Rapid method for distinction of gram-negative from gram-positive bacteria. European J. Appl. Micro. and Biotechnol. 5:123-127.
- Jeffers, S.N. and S.B. Martin. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Dis. 70:1038-1043.
- Kiyomoto, R.K. and M.H. Brand. 1994. Tissue proliferation in elepidote rhododendrons. HortScience 29:516.
- LaMondia, J.A., T.M. Rathier, V.L. Smith, T.M. Likens, and M.H. Brand. 1992. Tissue proliferation/crown gall in rhododendron. Yankee Nursery Quarterly 2:1-3.
- Linderman, R.G. 1993. Tissue proliferation. Amer. Nurseryman 178:57-67.
- Moore, L.W. 1986. Diseases caused by bacteria: Crown gall, p. 29-30. In: D.L. Coyier and M.K. Roane (eds.). Compendium of rhododendron and azalea diseases. APS Press, St. Paul, Minn.
- Schaad, N.W. (ed.). 1988. Laboratory guide for identification of plant pathogenic bacteria. 2nd ed. APS Press, St. Paul, Minn.
- Sinclair, W.A., H.H. Lyon, and W.T. Johnson. 1987. Diseases of trees and shrubs. Comstock Publishing Assoc., Ithaca, N.Y.