Field Evaluation of Tissue Proliferation of Rhododendron

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Rhododendrons are an important group of nursery crops that are vegetatively propagated either from cuttings or by micropropagation. In addition to the nursery production problems associated with *Phytophthora* spp. (root rot) and *Otiorynchus sulcatus* F. (black vine weevil), a relatively new syndrome known as tissue proliferation (TP) has caused concern in the industry (American Nurseryman, 1992). TP is characterized by formation of gall-like growths (referred hereafter as galls) on woody stems, usually during the second growing season in the nursery. In some, but not all cases, small shoots are associated with stem galls (Linderman, 1993). Spherical gall-like swellings on the tips of roots within several centimeters of the root/shoot junction have also been observed, which are referred to here as root galls. Although root galls were initially considered to be another symptom associated with the TP syndrome because of the frequent occurrence of both types of galls on TP affected plants, a similar pairing of these two symptoms has not always been noted. It has been suggested that root galls may be due to herbicide application, and hence should not be considered part of the TP syndrome (J. Britt, Briggs Nursery, Olympia, Wash., personal communication), or, alternatively, that TP may be caused by herbicide application (American Nurseryman, 1992).

It is widely believed among industry and research personnel familiar with TP that it is primarily associated with elepidote (nonscaly) rather than lepidote rhododendrons, and it has been suggested that lepidote rhododendrons are not susceptible to TP (Brand and Kiyomoto, 1992). Another common generalization is that TP occurs primarily or exclusively in plants that have been propagated via tissue culture (micropropagation) rather than cuttage (Linderman, 1993). If true, this could have significant consequences because micropropagation is one of the most important technical advancements in rhododendron production in decades, making numerous cultivars available that would otherwise be difficult to vegetatively propagate (by cuttage). Indeed, ericaceous crops (rhododendron, azaleas, and mountain laurel) are among the economically more important woody ornamental crops to be commercially propagated by tissue culture in North America.

Reports from growers about the extent and seriousness of TP range widely from those who claim a high percentage of crop loss (death or unsalability) (American Nurseryman, 1992) to others who report little if any occurrence of TP and/or insignificant impact on affected plants (American Nurseryman, 1992; Wells, 1992). To some growers, the mere fact that TP galls are "abnormal" renders affected plants undesirable, and hence unsalable. In addition to cosmetic impact, TP may be responsible for brittle stems, poor root system development, enhanced susceptibility to *Phytophthora*, and to black vine weevil, as well as outright plant death. Curiously, there have been few if any reports of TP occurrence on rhododendrons in the landscape.

The objectives of the nursery survey and experiments reported here were threefold: 1) to determine the extent of TP and its relationship, if any, to propagation history by direct systematic observation of plants from commercial nurseries, 2) to determine experimentally if TP persists and if it has a negative impact on plant survival and performance in the landscape, and 3) to investigate the effect of commonly used herbicides on development of shoot and root galls.

EXPERIMENTS CONDUCTED

Nursery survey. Two- to 3-year-old containerized rhododendrons were evaluated from each of eight wholesale container production nurseries, including five in New York, two in Ohio, and one in

Connecticut. Plants surveyed at a given nursery were either propagated there or brought from another nursery and/or micropropagation laboratory for growing-on. All plants reported (Table 1) were directly observed by at least one of us, and scored for TP-related symptoms based on uniform criteria. Determination of propagation history (cuttage or tissue culture) was based on an interview with the grower. Plants of uncertain origin (method of propagation) were excluded from the survey. Our results were based on a detailed examination of plants chosen randomly from a production block. Selected plants were either transported to the Dept. of Floriculture at Cornell Univ. (Ithaca, N.Y.) for examination (nurseries A and G) or examined directly at the nursery. Evaluations performed at Cornell included examination of stem and root systems. Stem evaluation consisted of careful visual and tactile (fingers run over the trunk) examination of the main woody trunk from ≈ 10 cm above, to the surface of the potting medium, past the root/shoot junction, and down to a distance of 1 cm below the surface (potting medium pushed aside manually). Examination for root galls was performed after washing the potting medium from the root system. Root examination was omitted when plants were examined at the commercial nursery to avoid destructive sampling. Importantly, the nursery survey was not a controlled experiment since we had no control over cultural practices (potting medium, fertilization, pest and disease control, pruning, etc.), which varied from nursery to nursery. Hence, no statistical analysis with respect to possible effect of cultivar and/or propagation history was performed. The objective was to observe any trends that might be apparent to develop testable hypotheses. Although other cultivars were examined, data reported here are limited to elepidote cultivars ('Nova Zembla', 'Scintillation', and 'Montego') for which cutting and micropropagation sources were available, and the only lepidote cultivar ('Black Satin') that was available in sufficient numbers (micropropagation only).

Landscape performance experiment. This experiment was performed to test the hypothesis that the presence of TP galls would result in increased mortality or poorer performance than its absence. Five cultivars of rhododendrons with and without preexisting TP stem galls were transplanted from containers into a landscape-like field plot, and monitored for three growing seasons for survival, performance, and gall persistence. Plants were originally propagated by tissue culture (from several commercial micropropagation laboratories), and grown-on in an upstate New York nursery (nursery A, Table 1) in a 1 pine bark : 1 peat moss potting medium. We used 'Bravo', 'Scintillation', 'Rio', 'Henry's Red', and 'Lee's Dark Purple'. For each of the first three cultivars, 20 plants were selected with preexisting stem galls (+), and 20 without (-). For 'Henry's Red' and 'Lee's Dark Purple', only 12 and 11 plants, respectively, were available for each category (+ and -). For 'Bravo' only, a third treatment (n = 20), designated +/-, was included; it consisted of removal of preexisting galls at the time of planting. Field planting took place on 30 June 1993, in a completely randomized arrangement in a recently cultivated bed using 1.3-m spacing within and between rows. The soil was a Chenango gravelly loam (U.S. Dept. of Agriculture, 1961), pH 5.7. The entire bed was mulched to a depth of ≈ 10 cm with a shredded, composted hardwood bark shortly after planting and again on about the same date in 1994 and 1995. Weeding was done by hand twice each growing season. The bed was irrigated as needed with overhead sprinklers during the first growing season. Plants were evaluated at the time of planting for survival, height, and occurrence and size of stem galls (if any), as described above for the nursery survey. Measurements were repeated during the second and third growing seasons. The mortality data and gall occurrence data from 1993 and 1995 were subjected to statistical analysis using the chi square test in SAS (SAS Institute, 1982).

Herbicide experiment. The herbicides Surflan [oryzalin (4-(dipropylamino)-3,5-dinitrobenzenesulfonamide) (Dow-Elanco,

Received for publication 22 Aug. 1996. Accepted for publication 17 Dec. 1996. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

Indianapolis) and Ronstar [oxadiazon (3-[2,4-dichloro-5-(1-methylethoxy)phenyl]-5-(1,1-dimethylethyl)-1,3,4-oxadiazol-2-(3H)-one)] (Rhone-Poulenc, Princeton, N.J.) were chosen because they are commonly used in commercial rhododendron production either separately or in combination with other herbicides. 'Montego' was donated from Nursery F where it had been propagated from cuttings taken from micropropagated stock plants during the 1991 growing season. Plants were shipped in flats during Mar. 1993 and repotted into 3.8-L (17.2 × 17.8 cm) containers in a 1 pine bark : 1 sand : 1 peat moss potting medium. Twenty-six or 27 plants were assigned to each of four treatment groups: 1× and 2× Surflan, 1× Ronstar, and an untreated control. The first herbicide treatment was applied on 4 Aug. 1993 as a drench with Surflan 4AS at 6.6 kg·ha⁻¹ (1×) or 13.3 kg·ha⁻¹ (2×) active ingredient of Surflan 4AS or 6.6 kg·ha⁻¹ of Ronstar 2G. Containers were grown outdoors, on a gravel pad at the test garden of the Dept. of Floriculture at Cornell Univ. They were irrigated by overhead sprinkler as needed. Plants were transferred to a double polyethylene-covered overwintering greenhouse in October, and moved back to the gravel pad the following spring. A second herbicide treatment, identical to the first, was applied after the resumption of growth in the Spring 1994. During the latter half of July 1995, plants were evaluated for above-ground stem galls and root galls as described earlier for the nursery survey.

FINDINGS

Nursery survey. TP stem galls occurred on 0% to 100% of samples tested from a given nursery (Table 1). Galls varied in size from about 5 mm in diameter to those that surrounded the entire stem base. Twenty to 24% of plants with stem galls had gall-associated shoots ranging from <1 to several cm in length. Overall, variation among nurseries within a given cultivar/propagation history was not as great as might be expected. For example, <20% of 'Nova Zembla' propagated from cuttings exhibited TP stem galls at all four nurseries where it was evaluated (A, B, C, G). More than 50% of micropropagated 'Scintillation' exhibited TP stem galls at four nurseries (A, C, D, E), but only 2% at a fifth one (G). Ideally, to determine if propagation history was associated with subsequent TP development, it would be necessary to compare cutting-propagated plants to micropropagated plants of a given cultivar grown under identical conditions at the same nursery. In reality, most production nurseries that were contacted had either cutting-propagated or micropropagated plants, but not both. The exception was 'Nova Zembla' at nurseries A and G. For this cultivar, TP stem galls tended to occur on a lower percentage of cutting-propagated than micropropagated plants. At nursery A, this difference was substantial (5% vs. 58%), but at nursery G, TP stem galls were found on <10% of cutting-propagated and micropropagated plants (2% vs. 8%).

Based on grower interviews, some cutting-propagated plants had always been rooted from cuttings since the original cultivar was

selected from a seedling (referred to as "exclusive" cutting propagation). Others were of mixed propagation history, i.e., the current plant had been cutting-propagated, but the stock plant from which cuttings were taken (or one of an earlier propagation cycle) had been grown from a previously micropropagated stock plant. The four samples of 'Nova Zembla' from exclusively cutting-propagated plants exhibited less TP stem galls (≤26%) than the two samples (one each from 'Scintillation' and 'Montego') of cutting-propagated plants that had been propagated from previously micropropagated stock plants (66% and 100%, respectively). Overall, the data suggest an association between a history of micropropagation and TP stem galls, but it is also important to note that galls were found on exclusively cutting-propagated plants.

In the cases of the five samples for which there is data (Table 1) (plants destructively sampled at Cornell), root galls were found on plants from the same four of the five samples that exhibited stem galls, but neither root galls nor stem galls were found on plants from the fifth sample ('Nova Zembla' from nursery B/cutting source). Importantly, a given stem-galled plant within a sample did not necessarily have root galls, and visa versa. Root galls almost always occurred on root tips within a few centimeters of the root/shoot junction, and ranged in size from 1 to 15 mm in diameter. We have observed similar root galls on several other cultivars not listed in Table 1, including 'Lee's Dark Purple', 'Black Satin', and 'Henry's Red'. Root galls were absent on any plants from the one sample that was free of stem galls (nursery B, 'Nova Zembla'). Root galls were found on the roots of plants of all four other samples that exhibited stem galls. This result suggests that root galls may be another manifestation (symptom) of TP.

With the exception of 'Black Satin' (Table 1), we show data for only those cultivars for which both cutting-propagated and micropropagated plants could be found. All three ('Scintillation', 'Nova Zembla', and 'Montego') are large-leaved elepidote types, whereas 'Black Satin' is a lepidote rhododendron. We have also observed TP-like stem galls on the lepidote 'April Rain', but this cultivar was not included in the survey because too few samples were available.

Landscape performance experiment. By the end of the first growing season (Nov. 1993), some plants of each variety had died in the field plot. The relatively high level of overall mortality in this experiment may be due in part to the nature of the field site, which was neither shaded nor protected from wind. Thus, this experiment constitutes a test of rhododendron performance under more extreme environmental conditions than found at many landscape sites. For each of the five cultivars, there was a tendency for higher mortality among plants with preexisting stem galls (+) than those without (−) (Table 2). This difference was only significant at $P \leq 0.10$ for 'Scintillation' and 'Rio' on the first sampling date (Nov. 1993). With the exception of the preexisting gall (+) treatment for 'Lee's Dark Purple', there was relatively little additional mortality over the next two growing seasons. By the third growing season, 'Lee's Dark Purple' was the only cultivar with higher mortality for those originally galled (+) than for those

Table 1. Occurrence of TP shoot and root galls on cutting-propagated and micropropagated rhododendrons from several nurseries

Cultivar	Nursery	Method of propagation					
		Cuttings			Micropropagation		
		n	Stem galls (%)	Root galls (%)	n	Stem galls (%)	Root galls (%)
Nova Zembla	A	200	5	NE ²	60	58	82
	B	20	---	---	---	---	---
	C	50	26	NE	---	---	---
	G	49	2	NE	51	8	NE
Scintillation	A	---	---	---	59	59	76
	B	20	100 ³	15	---	---	---
	C	---	---	---	50	50	NE
	D	---	---	---	50	54	NE
	E	---	---	---	41	66	NE
	H	---	---	---	63	2	NE
Montego	F	56	66 ³	NE	39	62	NE
Black Satin	A	---	---	---	40	25	2.5

¹NE = not examined.

²Not available.

³Cutting from previously micropropagated stock plants.

Table 2. Effect of presence or absence of TP galls at the time of planting on landscape survival and subsequent gall status of five rhododendron cultivars.

			Plant response									
			Cumulative mortality (%)			Cumulative survivors w/galls (%)			Gall diam (cm)			
			Time of examination									
Cultivar	Gall status ^a	n	Nov. 93	Nov. 94	July 95	Nov 93	Nov. 94	July 95	June 30	Nov. 93	Nov. 94	July 95
Bravo	—	20	0	0	5	20***	10	0	---	1.7	1.5	---
	+	20	0	5	5	65	32	26	1.9	2.0	2.5	1.3
	+/-	20	15	15	15	65	29	53	1.7	2.0	2.6	1.7
Scintillation	—	20	20*	25	25	19***	33	40	---	2.0	2.0	1.5
	+	20	45	45	45	100	82	91	2.2	2.5	4.8	2.2
Rio	—	20	0*	10	15	16***	24	13	---	1.8	1.5	1.1
	+	20	20	30	30	81	36	36	2.3	2.4	1.9	1.4
Henry's R.	—	12	8	25	25	36	0	0	---	0.8	---	---
	+	12	33	33	42	75	25	29	1.0	1.6	1.9	4.4
Lee's D.P.	—	11	0	0	0*	36*	27	36	---	1.5	2.6	1.1
	+	11	9	36	36	80	14	43	1.4	1.1	2.5	1.3
All cultivars	—	83	7	13	14	0***	19	31	---	---	---	---
	+	83	21***	30	32**	67	38	34	---	---	---	---

²Gall status: +, galls present; —, galls absent; +/-, galls present but removed at the time of planting.

*, **, ***Significant at $P \leq 0.10$, 0.05, or 0.01, respectively, based on chi square comparison among treatments (+, —, or +/- galls), within a cultivar.

originally ungalled (—). Overall, the data suggest that the presence of preexisting galls contributed to plant mortality during the first growing season, but had little if any influence on mortality beyond that time.

Gall development was closely monitored on plants in the field experiment and on these and several other cultivars kept in a greenhouse (data not shown). A fairly consistent pattern of increased gall size during the early part of a growing season, followed by gall necrosis in the latter part of that season was observed. Eventually, sloughing of necrotic galls during overwintering occurred in some cases. During the next growing season, in some cases, new gall tissue regrew at the same site and the cycle was repeated. Even in the greenhouse, where plants were not subject to a cold over wintering period, this same cyclic pattern of gall development—necrosis and loss followed by replacement—occurred. It is evident that even in Nov. 1993, only several months from the time of planting (June 1993), some of the originally galled (+) plants no longer had galls, and some of the originally gall-free (—) plants had developed new galls (Table 2). In fact, in the case of 'Henry's Red', there was no longer a significant difference in the percentage of galled plants between the (+) and (—) groups. By the third growing season (1995) galls had disappeared on some of the originally galled plants, and galls that had developed on some of the originally ungalled plants progressed to the point where there was no longer a significant difference in percentage of galled plants between the (+) and (—) groups for any of the five cultivars. There was no clear trend in gall diameter among treatments, cultivars, or over time (Table 2). Although, at the end of three growing seasons, some of the originally gall-free plants had developed new galls, there were fewer galled plants across both treatments (—, +) than at the start of the experiment.

Herbicide experiment. Fifty percent of the untreated control plants in this experiment exhibited root galls. None of the herbicide treatments unequivocally increased the development of TP symptoms on 'Montego'. The 2× application of Surflan tended to do so, but the difference was not significant (Table 3). Hence, these data do not support the hypothesis that TP is a response to application of two uncommonly used herbicides in the nursery. Likewise, the hypothesis that root galls are unrelated to TP, but instead occur in response to herbicide application, is not supported. Nonetheless, the nonsignificant trend towards enhanced gall formation for the 2× Surflan treatment suggests further experimentation is warranted.

DISCUSSION

The research reported is preliminary since the survey of commercial nurseries was for information gathering and not an experiment per se, and because the landscape and herbicide experiments have been performed only once and should be repeated.

The findings from the nursery survey (Table 1) should be considered less than definitive because they are not based on a controlled

experiment, and because the propagation history could not be verified for any of the plants examined. Thus, any conclusions suggested from survey data, including the likely association of TP with micropropagation history, and the inclusion of root galls as part of the TP syndrome, should be treated as testable hypotheses. The atypically low gall incidence for 'Nova Zembla' from cutting-propagated as well as micropropagated plants from Nursery G compared to the same cultivar from several other nurseries suggests that there is nursery-to-nursery, and/or micropropagation laboratory-to-laboratory, variation in TP. Future research to determine the cause of this variation is essential if the industry is to deal effectively with TP.

A mechanistic model for the development of TP should take into consideration the finding from the nursery survey that TP is associated primarily with a history of micropropagation. However, it is very important for any such model to accommodate the fact that stem galls were found on some exclusively cutting-propagated plants, although at a lower frequency than on micropropagated plants. This finding suggests that TP may be a "normal" but infrequent occurrence in cultivated rhododendrons which can be dramatically enhanced by some unidentified aspect of the micropropagation and/or postmicropropagation process. Any experiment designed to test the association-with-micropropagation hypothesis will not be easily performed because many cultivars currently micropropagated, including 'Scintillation' and 'Montego', are difficult to find from an exclusively cutting-propagated source. Also, the long lag time between propagation and appearance of TP symptoms contributes to the difficulty of conducting such an experiment.

Another notable result from the nursery survey, that root galls tended to be associated with groups of plants that also exhibited stem galls (Table 1), is consistent with the results of the herbicide experiment in which root galls were found on 50% of untreated control plants (Table 3). These findings suggest the possibility that root galls are a component of the TP syndrome. We recommend that root systems be examined as part of any future TP-related experimentation. Further

Table 3. Effect of the herbicides Surflan and Ronstar on development of root and stem galls on the rhododendron cultivar Montego.

Herbicide	n	Gall incidence		
		Root		Shoot
		(%)	(per plant)	
None	26			31
Surflan	1×	50	3.0	23
	2×	46	2.8	30
Ronstar	27	70*	2.5	33
		48	2.2	

* $P = 0.12$ based on chi square comparison of individual herbicide treatments with the control.

Literature Cited

research is also needed to determine if Surflan exacerbates the development of TP in susceptible plants.

The results from the landscape performance experiment suggest the need for additional experimentation to verify or refute the possibility that TP does play a role in rhododendron mortality or poor performance in the early transitional period from nursery to landscape. Furthermore, absence of TP stem galls at the time of landscape planting does not guarantee that the plants will remain gall free. Given the uncertainty about the mechanism by which micropropagation appears to predispose plants to TP, the safest recommendation to minimize TP is to avoid use of micropropagated plants.

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Chromosomal Rearrangements Not Detected in Rhododendrons with Tissue Proliferation Disorder

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Tissue proliferation (TP) is a condition found on some cultivars of rhododendron (*Rhododendron* spp.), azalea (*Rhododendron* spp.), and mountain laurel (*Kalmia latifolia* L.). In rhododendron, TP is characterized by a mass of callus-like woody tissue that forms on the lower stem, crown, or roots. Although it superficially resembles crown gall, TP galls may have numerous shoots, unlike the galls associated with crown gall disease. Inoculation of plants with bacterial isolates from TP galls does not incite crown gall, either. Furthermore, the distribution of TP is usually random within production blocks, suggesting that the disorder is not contagious (Linderman, 1993).

TP can occur on plants derived from cuttings, tissue culture, seeds, or grafts, although according to claims from the industry, it occurs most often on plants from tissue culture. TP does not affect all cultivars, suggesting that some cultivars may be genetically predisposed to developing TP, while others are not. Various theories have been suggested to explain the occurrence of TP. Most researchers, however, tend to favor one hypothesis, that of epigenetic variation. According to this hypothesis, TP is a result of epigenetic changes that occur in some cultivars during micropropagation. These changes would predispose plants to develop TP if exposed to the right set of conditions. These conditions might include 1) excessive use of chemicals, such as growth regulators, or pesticides or herbicides with growth regulating activity, and 2) growing techniques that stress plants, such as severe pruning (Linderman, 1993).

Although epigenetic variation is probably the favored hypothesis, genetic mutation has not been ruled out as the cause of TP. We have been testing the hypothesis that some type of gross chromosomal rearrangement might be occurring at a higher frequency in tissue-cultured plants and be the cause of TP disorder in rhododendron. For this, we have analyzed DNA markers known as RAPD (random amplified polymorphic DNA) (Welsh and McClelland, 1990; Williams et al., 1990). For identification of RAPD markers, 10-base primers of arbitrary sequence but with GC contents of 50% or higher are used to amplify regions of genomic DNA. The DNA profiles generated are the result of the number and the size of fragments

amplified, which, in turn, depend on the nucleotide sequence of the primer and on the source of template DNA. Polymorphisms between samples arise from the absence of the homologous primer sequence in regions of one template DNA (absence of amplified fragments) or from differences in the distance between two adjacent primer annealing sites (amplified fragments of differing sizes). We have compared RAPD marker profiles from DNA of original or cutting-propagated plants of 'Montego' and 'Lee's Dark Purple' to profiles from tissue-cultured lines of these same cultivars. Plants derived from these tissue-cultured lines are known to exhibit symptoms of TP (Kenneth Mudge, personal communication).

EXPERIMENTS CONDUCTED

Plant material. Leaf material from the original hybrid 'Montego' and cutting-propagated 'Lee's Dark Purple' rhododendrons was provided by Stephen Krebs of the Holden Arboretum (Kirtland, Ohio). Tissue cultures of 'Montego' and 'Lee's Dark Purple' in the shoot proliferation stage were obtained from Kenneth Mudge of Cornell Univ., who had originally obtained these cultures from a commercial tissue culture laboratory (Herman Losely and Son, Perry, Ohio). For each cultivar, two lines identified as "old" and "new" were used. Those indicated as "old" had been in culture for more than 9 years when received in late 1993, whereas those designated "new" had been in culture for slightly more than 2 years. At this time, the "old" culture of 'Montego' was habituated and did not require a cytokinin in the medium for continued shoot proliferation and growth. Furthermore, the "old" culture of 'Montego' displayed visible swellings or nodules in vitro. The other three cultures ('Montego' "new," 'Lee's Dark Purple' "old," and 'Lee's Dark Purple' "new") were grown on medium containing 2-isopentenyladenine (2iP) at 2.5 mg·L⁻¹. The medium used for all four types was woody plant medium (WPM) (Lloyd and McCown, 1980) with (g·L⁻¹) 25 sucrose, 1 Gelrite, and 0.6 Sigma agar with 100 mL dispensed per Magenta GA-7 container. Cultures were grown at 25 ± 2 °C on a 16-h photoperiod with a photosynthetic photon flux of ≈80 μmol·m⁻²·s⁻¹.

RAPD reactions. DNA was extracted on two separate occasions from leaf tissue collected from the original hybrid known as 'Montego' and cutting-propagated 'Lee's Dark Purple', and from leaf and stem tissue collected from tissue-cultured lines of 'Montego' and 'Lee's Dark Purple'. DNA was isolated using a CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle and Doyle, 1987), as modified by Rowland and Nguyen (1993), but

Received for publication 22 Aug. 1995. Accepted for publication 30 Oct. 1996. We would like to acknowledge Soline Charles, Michael Hessling, and Ingrid Fordham for their technical assistance on this research project. This research was supported in part by grants from the Holden Arboretum and the Horticultural Research Institute. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.