SI : TISSUE CULTURE

Tissue proliferation condition in micropropagated ericaceous plants

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Abstract Tissue proliferation (TP) is an abnormal tumor-like growth produced at or near the crown of the plant, 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. Genera exhibiting TP include *Rhododendron*, *Kalmia* and *Pieris* of the Ericaceae family. Development of TP symptoms in a plant is highly correlated to a history of micropropagation and also to genetic background of the genotype. Similarities of TP symptoms to crown gall by *Agrobacterium tumefaciens* led many to initially believe TP to be a form of crown gall, but all evidence suggests that a pathogen is not involved in the TP disorder. Abnormal lignotuber formation is another possible cause for TP for which little supportive evidence could be developed. An epigenetic condition, possibly cytokinin habituation, is the possible cause of TP that is best supported by the majority of research evidence. It is likely that TP is induced in vitro by adventitious shoot formation, resulting from high cytokinin concentrations used to rapidly proliferate shoots. Some nursery production practices may result in increased TP symptoms development post-propagation. TP is well managed now due to greater awareness and adoption of sound micropropagation practices for ericaceous plants by tissue culture labs.

Keywords Epigenetic · Habituation · Tumor · Rhododendron · Tissue culture · Somaclonal variation

Introduction

Tissue proliferation (TP) is a disorder primarily found in micropropagated rhododendrons that is characterized by the formation of abnormal callus-like growths, or tumors at or near the crown or base of the plant (Brand and Kiyomoto 1992). This condition was first observed in the mid-1980s and its superficial similarity to crown gall, caused by the bacterium *Agrobacterium tumefaciens*, has created considerable concern among micropropagators, nursery professionals and nursery inspectors (Zimmerman 1997). The first published report of TP appeared in 1992 (Anonymous 1992) and several other reports on the TP condition soon followed (Brand 1992a, b; LaMondia et al. 1992; Linderman 1993).

In early 1993 a workshop was convened to bring together growers, micropropagation laboratory operators, extension personnel, research scientists and nursery inspectors. The goal of the workshop was to exchange experiences with TP, understand the magnitude and complexities of the problem and come to a consensus about the research needed to understand and correct the TP condition. A second meeting was organized in 1996 to summarize the findings of research planned in the first workshop and to see if efforts to curtail TP were having an effect on the incidence of the disorder.

Description of tissue proliferation symptoms

Tissue proliferation can be characterized by the development of gall-like or tumor-like growths on the trunk and lower stems of some ericaceous plants (Fig. 1). TP symptoms typically become apparent on 2–5 year-old plants, but close inspection of young micropropagated plants reveals

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the TP tumors and symptoms can be evident well before plants reach mature nursery size (Brand and Kiyomoto 1997). The surface of TP growths resembles callus, but the growths are highly lignified and woody. TP galls can grow to be 40 mm in diameter or larger (McCulloch and Britt 1997). Some TP growths are easily snapped off and appear as distinct woody galls attached to the stem by relatively small connections. Other TP growths are integrated into the stem and are swollen areas along the stem rather than separate growths. Stem girdling can occur when a TP growth completely or largely encircles the stem. In such cases, stems are prone to breaking off below the TP growth. Root systems below large TP growths are often small and poorly developed, compromising the entire plant or part of the plant. Large TP growths may be substantial sinks, preventing the root system below from receiving adequate photosynthate or hormone supplies to provide for adequate growth.

TP growths may or may not develop numerous small shoots arising from the swellings. TP shoots have small leaves, whorled leaf arrangement and shortened internodes (Fig. 1). They frequently die off, only to be replaced by new shoots (Brand and Kiyomoto 1997). Some lepidote rhododendrons produce only symptoms of broomed shoots with thickened stems, but no tumor-like growths (Keith and Brand 1995).

TP has been reported in Connecticut, Georgia, Massachusetts, Michigan, North Carolina, New York, Ohio, Oregon, Pennsylvania, Rhode Island, Virginia, Washington and West Virginia (McCulloch and Britt 1997). It has also been found in Canada and the United Kingdom. Rhododendron has been the genus most often found with TP symptoms, but TP has also been observed on other genera of the Ericaceae including Kalmia and Pieris (Linderman 1993). Over 35 cultivars of Rhododendron have been found with TP (Zimmerman 1997). TP most often occurs on elepidote rhododendrons, but it has also been found on lepidote rhododendrons and azalea cultivars as well.

Possible causes of tissue proliferation

Pathogens

Due to the superficial similarities between TP and crown gall, the initial concern was that TP may be caused by *Agrobacterium tumefaciens*. While crown gall disease has been reported on *Rhododendron* (Moore 1986) it has not been a major problem in nurseries or landscapes (LaMondia et al. 1997). TP tumors differ from *Agrobacterium* tumors in several ways (Brand and Kiyomoto 1992). TP produces tumors with adventitious shoots (found on many genotypes), organized vascular tissue, nodular, semi-organized, meristematic regions and the tumors rarely form on roots. In contrast, *Agrobacterium* tumors produce no adventitious shoots, have unorganized internal tissues, fail to form meristematic nodules and are present on roots.

Attempts to inoculate rhododendrons with various pathogenic strains of *Agrobacterium tumefaciens* have been unsuccessful for different researchers (Linderman 1993; LaMondia et al. 1997). In addition, *Agrobacterium* isolated from gall-like growths on *rhododendron* failed to induce galls on *rhododendron* test plants following reinoculation (Linderman 1993; McCulloch and Britt 1997). Furthermore, these isolates did not react to T-DNA probes used for detecting pathogenic strains of *Agrobacterium*. In my lab, stab inoculation tests of *Rhododendron* 'Scintillation' with *A. tumefaciens*, *A. radiobacter* and *A. rhizogenes* produced negative results. Stab inoculations of *Nocardia vaccinii*, which infects *Vaccinium* species (Ericaceae) and causes basal galls and adventitious shoots (Demaree and Smith 1952), also produced negative results following stab inoculations in my lab. McCulloch and Britt (1997) also reported no gall formation on *Rhododendron* 'Nova Zembla' following inoculation with *Nocardia*. They also report that *Erwinia herbicola*, a gall forming bacterium, was not found in TP *rhododendron* tissues. While no amount of negative data can definitively prove that TP is not the result
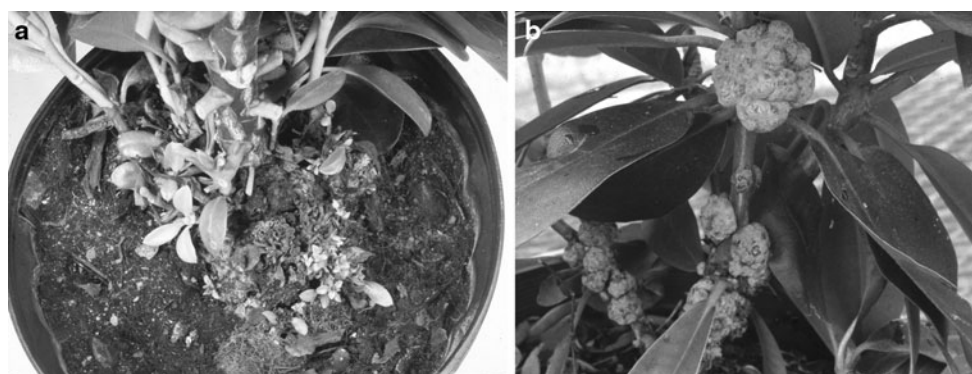

Fig. 1 **a** *Rhododendron* 'Scintillation' exhibiting basal tissue proliferation with numerous associated dwarf shoots. Note clusters of necrotic and living shoots and various shoot sizes. **b** *Rhododendron* 'Solidarity' exhibiting non-shooty aerial tissue proliferation tumors at nodes

of a pathogenic organism, the findings to date suggests that the common gall-forming diseases are not involved in the development of TP.

Lignotubers

The morphology of TP bears some similarities to lignotubers and it has been theorized that TP is the result of enhanced expression of naturally occurring lignotubers or burls triggered upon passage through tissue culture (Linderman 1993; McCulloch and Britt 1997). Lignotubers are permanent, woody extensions of the crown embedded with dormant axillary buds (James 1984). Several genera in the Ericaceae family, including *Arctostaphylos*, *Kalmia* and *Rhododendron* form natural lignotubers as survival structures against forest fires and catastrophic canopy damage. Several differences exist between TP and lignotubers. TP tumors from some *Rhododendron* cultivars are poorly connected to the stem and are easily removed, unlike lignotubers, which are integrated into the stem (Brand and Kiyomoto 1994). TP growths can develop in as little as one growing season, while lignotubers develop very slowly over many growing seasons. The dormant buds in lignotubers regenerate a new canopy only after the main stem is removed, such as by fire (James 1984), but TP tumors produce shoots while the main stem is intact. In addition, when the main stem of TP-affected rhododendrons is removed, the TP tumors at the crown fail to regenerate permanent canopies (Brand and Kiyomoto 1994) as would be expected for lignotubers. Furthermore, regenerated shoots from some TP tumors are abnormal in appearance (LaMondia et al. 1992), and axillary buds are not present in TP tumors on many *rhododendron* cultivars (Linderman 1993). In *Kalmia*, Del Tredici (1992) found that tissue-cultured plants had one-third as many bud clusters in basal burls after 7 years as did the basal burls on 4-year-old nursery grown seedlings, so there are significant limitations in the ability of micropropagated *Kalmia* to form lignotubers. It seems unlikely that lignotuber formation is the explanation for TP.

Nursery and landscape cultural practices

Variation exists between growers in reporting or recognizing TP development even when the liner plants have come from the same supplier, suggesting that cultural triggers may exist that stimulate TP development (Linderman 1993; Maynard 1995). There seems to be a link between high plant vigor and the development of TP symptoms. Container-grown plants have a higher incidence of TP than field-grown plants (Zimmerman 1997). Container production uses softwood bark-based porous media that requires frequent watering and high levels of fertility which promotes vigorous growth of plants. Growth of ericaceous plants under field production is usually much more controlled.

Micropropagated *Rhododendron* 'Solidarity' grown in containers for two growing seasons at two fertility rates exhibited different levels of TP symptom development (McCulloch and Britt 1997). Only 23% of plants receiving the low fertilizer rate developed TP symptoms, while 62% of the plants receiving a higher fertilizer rate developed TP. In addition, there was a significant correlation between plant size and TP development, further suggesting that rapid plant growth stimulates TP symptom development.

Herbicides and fungicides were studied as possible inducers of TP. McCulloch and Britt (1997) examined the use of several common herbicides and two fungicides, but could not implicate any of the tested products as triggers for TP development. Mudge et al. (1997) found that use of the herbicide oryzalin at normal rates did not affect TP incidence in *Rhododendron* 'Montego'.

Genetics, epigenetics and habituation

The genetic background of a plant may determine if a particular genotype is highly prone to developing TP. *R. yakushimanum*, *R. griffithianum* and the cultivar 'The Honorable Jean Marie de Montague' were frequently found to be parents of the *Rhododendron* cultivars which often develop TP symptoms (McCulloch and Britt 1997). Elepidote rhododendrons seem to be more prone to developing TP than lepidote rhododendrons or azaleas. There are many *Rhododendron*, *Kalmia* and *Pieris* genotypes that are routinely micropropagated, but which have never been observed to develop TP.

The occurrence of TP on *Rhododendron* was not reported until the widespread use of plants originating from micropropagation. Almost all instances of TP can be traced back to propagation by tissue culture (Linderman 1993; Zimmerman 1997). From nursery surveys, Mudge et al. (1997) determined that TP symptoms developed on cutting propagated plants when the cuttings were collected from previously micropropagated stock plants. The connection between tissue culture and TP has led to speculation that TP is the result of mutations or somaclonal variation. However, Rowland et al. (1997) was unable to detect chromosomal rearrangements in plants with TP symptoms using RAPD markers. Given that so many different genotypes, and even different genera, have developed similar TP symptoms, it is difficult to understand how the same mutation could have occurred so many times to produce similar phenotypic changes in such a wide range of plants.

The theory that epigenetic changes, brought about by the tissue culture process, are the cause of TP has received the greatest support from those familiar with the disorder

(Linderman 1993; Brand and Kiyomoto 1997). Epigenetic variation is a common occurrence in micropropagated plants, resulting from a change in the timing of gene expression (Zimmerman 1997). Also known as developmental variation, epigenetic variation includes persistent changes in phenotype resulting from changes in gene expression in the absence of mutation (Skirvin et al. 1994). One of the best known epigenetic changes is phase change in woody perennials. It has been demonstrated that tissue culture can cause a form of rejuvenation in woody plants (Webster and Jones 1989; Marks 1991; Brand and Line-berger 1992). In vitro rejuvenation, therefore, is an example of tissue culture induction of an epigenetic change and altered phenotype. Micropropagated plants also commonly branch more freely than cutting propagated forms of the same genotype (Zimmerman 1997).

Habituation, or the loss of auxin, cytokinin or vitamin requirements in vitro, is another well-known epigenetic variation triggered by tissue culture (Jackson and Lyndon 1990). It is probable that cytokinin habituation plays a significant role in the development of TP, since cytokinin-habituated *Rhododendron* cultures have been demonstrated (Brand and Kiyomoto 1997). Shoot cultures of *Rhododendron* 'Montego' were maintained on hormone-free medium for a 5 year period, but continued to proliferate rapidly in the absence of exogenous cytokinin (Fig. 2). The shoots that were produced were densely branched, had very small leaves and often produced tumors or swellings at nodes on the stems (Fig. 2). I have also grown habituated *Rhododendron* 'Besse Howells' cultures in vitro with similar morphology to that found on 'Montego'.

Non-habituated 'Montego' shoot cultures [TP(−)] can be initiated and require medium containing 10 μM 6-(γ,γ-dimethylallylamine)purine (2iP) to grow and proliferate (Brand and Kiyomoto 1997). TP(−) 'Montego' cultures can be maintained indefinitely in vitro by careful removal of callus and adventitious tissue at each subculture and they also maintain their requirement for exogenous 2iP in the culture medium. To see what effect cytokinin concentration has on TP development in vitro, TP(−) shoot cultures were placed on either 10 or 50 μM 2iP for three 5-week culture periods (Brand and Kiyomoto 1997). Cultures grown on both cytokinin concentrations developed in vitro TP symptoms, but cultures maintained on 50 μM 2iP had a 5 times higher rate of conversion to the TP(+) phenotype than those grown on 10 μM 2iP. Conversion to the TP(+) phenotype in vitro occurred as a result of basal callus and adventitious shoot development that was not removed in this study (Fig. 3). While the higher cytokinin level may not have directly induced the TP condition, it allowed for extensive adventitious shoot formation that can lead to TP induction. It is important to point out that not all adventitious shoot formation events lead to the development of TP or cytokinin habituation. The findings of Brand and Kiyomoto (1997) conflict with those of McCulloch and Britt (1997) who did not find a significant or consistent effect of cytokinin concentration on the incidence of TP. However, in the McCulloch and Britt work no information was provided about the prior history of the cultures used in the cytokinin concentration experimentation. It is possible that the cultures used may have already been TP(+) since this work was conducted in a commercial micropropagation laboratory.

Brand and Kiyomoto (2000) demonstrated that within TP(+) 'Montego' tissue cultures there can be differing levels of cytokinin habituation in different portions of the culture. TP(+) cultures grown on hormone-free medium can contain very compact, small-leaved, highly habituated shoots with associated tumors, as well as elongated shoots with larger leaves and partial habituation. When elongated shoots from TP(+) cultures growing in hormone-free medium are placed back on even low levels of 2iP, they

Fig. 2 **a** Cytokinin habituated shoot clusters of *Rhododendron* 'Montego' grown in vitro. **b** In vitro *Rhododendron* 'Montego' shoots exhibiting stem tumors (*black arrows*)
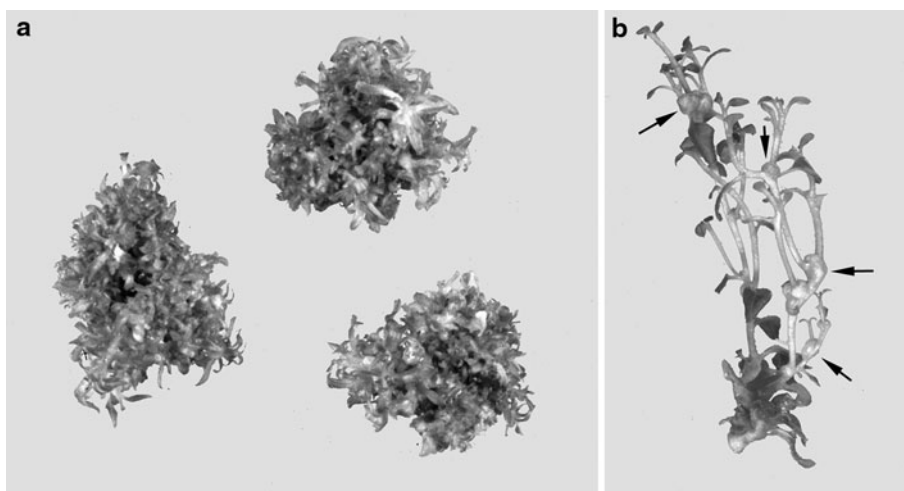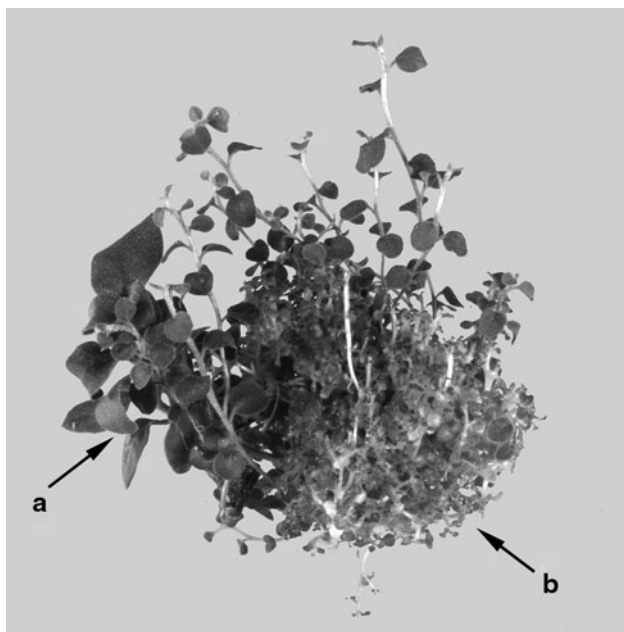
Fig. 3 In vitro *Rhododendron* 'Montego' shoot cluster showing the original large shoot of axillary origin (**a**) and adventitious shoots of with varying degrees of dwarfness and tissue proliferation (TP) induction (**b**) TP tumors are visible along the stems of the TP-induced portion of the culture

quickly become fully habituated and redevelop the very compact, dwarf shoot morphology and associated tumors. In a complementary study, Brand and Kiyomoto (2000) found that cultures of *Rhododendron* 'Montego' established from nursery plants exhibiting TP symptoms adapted to in vitro culture conditions much more rapidly than cultures established from TP(−) nursery plants. Cultures started from TP(+) nursery plants quickly became habituated, were able to proliferate on cytokinin-free medium and developed dwarf, highly-branches shoot clusters with small leaves and stem tumors. Cultures initiated from TP(−) nursery plants required cytokinin in the culture medium to grow and proliferate, but maintained normal leaf size, shoot expansion and did not produce stem tumors.

Persistence of tissue proliferation in landscape plants and cutting propagation

While many rhododendrons, especially new cultivars, are micropropagated, standard stem cuttings are also used for large scale vegetative propagation. Studies were conducted to determine if the TP condition will reoccur in plants grown from normal-appearing cuttings collected from plants with TP tumors at the base of the plant. Conventional stem cuttings from TP(+) and TP(−) plants of seven *Rhododendron* cultivars were rooted and plants were grown in containers for 2 years (Brand and Kiyomoto

1999). None of the plants developed TP tumors, but all plants from TP(+) cuttings had more leaves per flush than plants from TP(−) cuttings. Some of the plants from TP(+) cuttings had shorter stems and narrower leaves than their TP(−) counterparts. Plants from TP(+) cuttings of two cultivars had shorter, smaller leaves than TP(−) plants. To further test the influence of plant age and TP status of source plants used for cutting propagation, 'Montego' plants were grown from cuttings collected from the following sources: (1) in vitro shoot cultures know to produce TP plants; (2) 3-year-old plants with TP; (3) 6-year-old plants with TP and (4) TP(−) plants. Eighty-three percent of plants from microcuttings and 74% of plants from cuttings of 3-year-old TP(+) plants formed tumors, whereas no plants grown from 6-year-old TP(+) or TP(−) cuttings did so (Brand and Kiyomoto 1999). Large tumors surrounding the stem were more likely to be found on plants grown from microcuttings than from 3-year-old TP(+) stock plants. In summary, as micropropagated TP(+) plants age, the apical portions of the plant seem to lose their tumor forming capacity first, but retain altered leaf and stem characteristics for a significantly longer period of time. This relatively slow, stepwise loss of TP characteristics is similar to other epigenetic conditions such as phase change in woody plants.

There are few controlled studies examining the fitness of plants with TP for landscape use. Anecdotal observations have found that plants with large TP growths involving the majority of the circumference of the stem are susceptible to breaking off below the growth and have weak root systems, while plants with smaller growths seem relatively unaffected and often grow larger and more rapidly than plants without TP growths (Zimmerman 1997). In one controlled study, five cultivars of rhododendrons, with and without TP, were transplanted from containers to a field where they grew for 3 years under typical, but challenging landscape conditions. Plants with TP had a significantly higher mortality after one season than plants without tumors (Mudge et al. 1997). In another study, *Rhododendron* 'Montego' and 'Lee's Dark Purple', with and without TP symptoms, were installed in two landscape sites in Connecticut. No differences in mortality, black vine weevil damage or *Phytophthora* infection could be detected between plants with and without TP (LaMondia et al. 1997). Kiyomoto and Brand (1994) also did not find differences in landscape mortality between *Rhododendron* 'Besse Howells' and 'Holden', with and without TP.

Management of tissue proliferation

Tissue proliferation was a substantial problem for micropropagators and growers of ericaceous plants during the

1980s and 1990s. Since that time, TP has become less common and does not seem to be presenting significant problems for producers any longer. Despite initial rejection by tissue culture laboratories that micropropagation practices could be inducing TP development, adjustments were made by commercial micropropagators to dramatically reduce the induction of TP. Micropropagators began to strictly adhere to micropropagation procedures that were suggested by Brand (1992b) and others for ericaceous plants. These recommendations included regular initiation of cultures from non-tissue-cultured, correctly identified stock plants, use of minimal cytokinin concentrations to stimulate modest levels of shoot multiplication, avoidance of adventitious shoot production and rooting of only the distal tip of shoots to avoid bases with clustered nodes. Laboratories must also carefully select genotypes to micropropagate that are not prone to TP development. Cultivars know to be susceptible to TP must be handled very carefully in culture or be propagated by conventional stem cuttings. Refrigeration of cultures can also be used to extend the life of a culture by retarding cell division and potentially "resetting" many physiological conditions within the plant tissue. At the second, 1996 TP workshop, growers and micropropagators reported that changes in micropropagation procedures and production techniques were working to limit the incidence of TP.

While the cause(s) of TP is still not fully understood, the majority of information suggests that it is most likely the result of an epigenetic or habituated condition brought on by adventitious shoot formation in vitro. The use of proper micropropagation techniques and nursery production practices appears to be effective at eliminating or significantly reducing the incidence of a once common malady of ericaceous plants.

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