ABSTRACT

MEYER, ELISABETH MARIE. Evaluation, Propagation, and Improvement of Gordonieae Trees and an Interspecific *Hypericum* Hybrid. (Under the direction of Dr. Thomas G. Ranney.)

Experiments were conducted to evaluate disease resistance of *Franklinia* Bart. ex Marshall, *Gordonia* Ellis, *Schima* Reinw. ex Blume and their hyrids to *Phytopthora cinnamomi* Rands., develop an effective vegetative propagation protocol of the three parental genera, and induce shoot regeneration and polyploidy in leaves of *Hypericum* hybrid H2003-004-016 cultured *in vitro*.

Some trees in the Theaceae tribe Gordonieae are highly susceptible to root rot caused by *P. cinnamomi*. A collection of Gordonieae taxa were evaluated for resistance to this pathogen. *Abies fraseri* (Pursh) Poir was included in the study as a positive control. Container-grown trees were inoculated with 3 isolates of *P. cinnamomi,* and symptoms were rated twice a week over an 84 day period during the summer of 2008. None of the *Schima khasiana* Dyer or *Schima wallichii* Choisy exhibited any root rot symptoms, while the rest of the taxa showed symptoms at varying levels over time. Symptoms in *F. alatamaha* and *A. fraseri* were apparent before other taxa, and mortality for both species reached 100% by the end of the experiment. Comparison of area under the disease progress curve (AUDPC) values indicated that *F. alatamaha* was the most susceptible, followed by *A. fraseri*. There was no significant difference in AUDPC among the more resistant taxa including *G. lasianthus,* both *Schima* species, and the intergeneric hybrids. Hybrid taxa were similar to their more resistant parental genus, indicating that resistance to *P. cinnamomi* is a partially dominant trait in these plants.

To develop an effective vegetative propagation protocol for 5 taxa of Gordonieae trees, hardwood, semihardwood, and softwood terminal stem cuttings were taken and treated with 0, 2500, 5000, 7500, or 10000 ppm potassium salt of indolebutyric acid (K-IBA) solution. The concentration of K-IBA affected rooting percentage in hardwood cuttings of *F. alatamaha, G. lasianthus,* and *S. remotiserrata* and had varying effects on root number and length of longest root amongst the different taxa and growth stages. *Franklinia alatamaha* and *G. lasianthus* were rooted at high percentages (>50%) at all growth stages, and *S. khasiana* rooted at high percentages (72%) from softwood cuttings. *Schima remotiserrata* Hung T. Chang and *S. wallichii* cuttings exhibited poor rooting (<25%) at all growth stages, with the highest rooting percentages for both species occurring at the hardwood stage.

Hypericum H2003-004-016 is a complex hybrid between *Hypericum frondosum* Michx., *Hypericum galioides* Lam., and *Hypericum kalmianum* L. *In vitro* shoot regeneration and application of dinitroaniline herbicide oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide) were investigated as a means of inducing allopolyploidy. Regeneration was optimized for callus and shoot production through culture of leaf segments on MS medium supplemented with benzylamino purine (BA) or *meta*-topolin (mT) (5, 10, 15 μM) in combination with indoleacetic acid (IAA) (0, 1.25, 2.5, 5 μM). Both BA and mT treatments produced regenerative callus and shoots. Maximum regenerative callus (94%) and shoot production (18 shoots per callus) were optimized at 5 μM BA + 3.75 μM IAA. The exposure of regenerative callus to oryzalin at 0, 7.5, 15, 30, 60, and 90 μM concentrations for 3, 6, and 9 days was investigated for polyploid induction. There was no survival for any of the 60 and 90 μM oryzalin treatments, but all

other treatments exhibited some survival and polyploidy induction. Duration had no effect on callus survival or ploidy level, but oryzalin concentration was a significant factor in both. The greatest percent (44.4%) of polyploids was produced at 30 μM oryzalin. Spontaneous chromosome doubling occurred in 8.3% of control explants receiving no oryzalin treatment.

Evaluation, Propagation, and Improvement of Gordonieae Trees and an Interspecific *Hypericum* Hybrid

by
Elisabeth Marie Meyer

A thesis submitted to the Graduate Faculty of
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APPROVED BY:

Dr. Anthony V. LeBude	Dr. Kelly Ivors

Dr. Thomas G. Ranney
Chair of Advisory Committee

DEDICATION

To my family, who have always supported me whole-heartedly,

Listened sympathetically, bragged on me unashamedly,

Teased me boisterously, and moreover, passed on

To me at a young age that most applicable adage:

“If at first you don’t succeed,

Try, try again.”

BIOGRAPHY

Elisabeth Marie Meyer was born to Garry and Peggy Meyer on May 5th, 1983 in Wilson, NC. She was raised in the small community of Rosewood, where she attended Rosewood Elementary and High Schools. Her parents instilled a love of growing things in her from a young age by teaching her about wildflowers, working in the garden with her, and encouraging her to explore the woods surrounding their home. After high school, Lis followed in the footsteps of her sister (Rachel) and brother (David) by enrolling at North Carolina State University, where she continued to pursue her interest in plants with a major in Botany and a minor in Horticulture. She also continued to develop her love for reading and writing with a second major in English.

In addition to the academic opportunities offered during her undergraduate years at NC State, Lis had many important learning experiences out of the classroom. She grew spiritually through her involvement with Campus Crusade for Christ, in which she led a girls' Bible study for five years and traveled to Russia to teach English for a summer. Through the Caldwell Scholars Program, she was able to further expand her knowledge of the world through a summer abroad in Eastern Europe and at Oxford University in England. Lis also spent five years developing her laboratory, field, and glassware washing skills at the Center for Applied Aquatics and Ecology in the NC State Department of Botany. Internships at the J.C. Raulston Arboretum in Raleigh, NC and the Arnold Arboretum of Harvard University in Boston, MA cemented Lis' desire to further her studies in horticulture, while an opportunity to serve as a teaching assistant to

Dr. Paul Fantz for a course in ornamental plant identification helped her discover her love of teaching.

In the fall of 2006, Lis began working towards her M.S. degree in Horticulture under Dr. Thomas G. Ranney at the Mountain Horticultural Crops Research and Extension Center in Mills River, NC. She spent semesters taking classes and teaching in Raleigh. Having learned from previous internships that working summers outdoors in eastern North Carolina was to be avoided at all costs, she gratefully spent summers doing research in Mills River. Upon completion of her degree, she will pursue a career in horticulture education.

ACKNOWLEDGEMENTS

If my acknowledgements section seems to be exceptionally long, it is only because I have had exceptional help and support, and I want to make sure I have a chance to thank everyone. Those of you who know me well also know I like to tell a good, long story, so saddle up and get ready for it. I'll start with Dr. Van Dyke, my undergraduate advisor. You may not remember it Dr. Van Dyke, but on my very first visit to N.C. State, upon hearing about my interests, you recommended that I pursue a degree in horticulture. In my typical fashion, having already set my mind on majoring in botany, I disregarded this insightful advice. Fortunately, this did not discourage you from advising me to continue on to graduate school later, and after a little resistance, I listened that time. Hopefully one day I will have the patience with my students that you had with me.

The staff and students at the Center of Applied Aquatic Ecology in the Botany department also deserve thanks. I had no idea how much my time with all of you would prepare me for graduate school. Graduate students of Dr. Burkholder, you gave me valuable advice about graduate school and choosing a graduate program. Jenny Jones and Jessie Alexander, you showed me what perseverance and hard work in research looks like. Dr. Brant Touchette (who I once told I would never study Botany) and Dr. Matthew Parrow, you may take both the credit and the blame of turning a polite, quiet girl from rural North Carolina into a sharp-tongued, sassy student. I know, I know, you only pick on me because you care. You also taught me how to work in a lab, and while I thought you were ridiculously meticulous at the time, you were right. So much of what you

taught me, whether it had to do with sterile technique or finding obscure references at the library, has been useful to me as a grad student.

To Dr. Denny Werner, thanks for sparking my interest in horticulture and the world of plant propagation during my time in your class. Also, thanks for giving a clueless undergraduate sound advice about graduate school. I would have had no idea how to proceed in the graduate school application process without you, and I probably would never have thought to look into research with Dr. Tom Ranney either. Finally, thanks for taking the time to serve as an alternate for my defense so that I could graduate on time! Dr. Paul Fantz shares the credit for encouraging my interest in horticulture and helping me realize that I wanted to go to graduate school and to teach. Dr. Fantz, not only did you teach me almost everything I know about taxonomy, but you also taught me everything I know about teaching. Your guidance, confidence in me, and without question, your many stories, over all the semesters I taught plant identification for you will not be forgotten. The new generation of horticulture undergraduates don't know what they're missing now that you're retired.

After years of vehemently resisting the idea of graduate school, I decided to pursue Masters degree under Dr. Tom Ranney, and this was possibly the best decision of my academic career. Thanks for giving me the opportunity to work with you and for ensuring that I would never become bored by providing me with a "multi-faceted" program full of backup and side projects. You made sure I had plenty of work to do, but never minded taking time out to expand my learning through field trips, research

conferences, or just discussions of other projects and ideas you had. Also, thanks for the confidence you have shown in me. When I declared I was going to graduate this semester, you never batted an eye (despite what you may have been thinking about the lack of time for thesis writing) or suggested that I might not be able to do it. This is just one example of the many times you've demonstrated trust in my ability to meet a challenge and that has meant a lot to me.

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entirety of my time here. You invited me into your home meals, helped with my numerous vehicular difficulties, did most of the heavy labor involved in building containment area for diseased plants for my project, faithfully checked on seeds that didn't germinate much, faithfully checked on me to make sure I was dealing with everything well, and reminded me when it was time for break. Joe Connor, thanks for being available any time I needed your expert and always cheerful help with rooted cuttings.

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Chapter 1

Differential Resistance of Gordonieae Trees to *Phytopthora cinnamomi*

(In the format appropriate for submission to HortScience)

Differential Resistance of Gordonieae Trees to *Phytophthora cinnamomi*

Elisabeth M. Meyer[1], Thomas G. Ranney[2], and Thomas A. Eaker[3]

Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759

Kelly Ivors[4]

Department of Plant Pathology, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759

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[1]Graduate Research Assistant

[2]Professor and corresponding author. Email address: tom_ranney@ncsu.edu

[3]Research Specialist

[4]Assistant Professor

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Differential Resistance of Gordonieae Trees to *Phytophthora cinnamomi*

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Abstract. Trees in the Theaceae tribe Gordonieae are valuable nursery crops, but some of these taxa are known to be highly susceptible to root rot caused by *Phytophthora cinnamomi* Rands. The objective of this study was to evaluate a collection of Gordonieae taxa for resistance to this pathogen. These taxa included *Franklinia alatamaha* Bart. Ex Marshall, *Gordonia lasianthus* (L.) Ellis, *Schima wallichii* Choisy, *S. khasiana* Dyer, ×*Schimlinia floribunda* Ranney & Fantz, and ×*Gordlinia grandiflora* Ranney & Fantz. *Abies fraseri* (Pursh) Poir. was also included in the study as a positive control. Container-grown trees were inoculated with three isolates of *P. cinnamomi* and symptoms were rated over an 84-day period during the summer of 2008. Disease symptom ratings from 1 (healthy) to 4 (dead) were collected twice weekly and area under the disease progress curve (AUDPC) values were calculated. None of the *S. khasiana* or *S. wallichii* exhibited any root rot symptoms or mortality, while the remaining species showed symptoms of

infection at varying levels over time. Symptoms in *F. alatamaha* and *A. fraseri* were apparent before other taxa, and mortality for both species reached 100% by the end of the experiment. Comparison of AUDPC values indicated that *F. alatamaha* was the most susceptible, followed by *A. fraseri*. There was no significant difference in AUDPC among the more resistant taxa including *G. lasianthus,* both *Schima* species, and the intergeneric hybrids. Values for AUDPC in the hybrid taxa were similar to their more resistant parental genus, indicating that resistance to *P. cinnamomi* is a partially dominant trait in these plants. These results further suggest the potential to breed improved hybrids of Gordonieae trees with substantial resistance to *P. cinnamomi*.

Introduction

Franklinia alatamaha, a member of the Theaceae tribe Gordonieae, has both ornamental and historical significance. It was first discovered by John and William Bartram in 1765, but is now considered to be extinct in the wild and is only maintained in cultivation (Fry, 2000). *Franklinia,* with its large, white flowers that bloom in the fall and bright red fall foliage, makes a unique and desirable plant in the landscape. In addition to these characteristics, *Franklinia* is cold hardy at temperatures as low as -38°C (Dirr, 1998). Despite these appealing features, *Franklinia* remains a rarity in the landscape due to its susceptibility to root and crown rots caused by pathogens including *P. cinnamomi* (Koslow and Peterson, 1980). *Franklinia* is a monotypic genus, and there is little genetic diversity within this species (Griffiths, 1994; Krüssman, 1986; Liberty

Hyde Bailey Hortorium, 1976; Prince and Parks, 2001). This narrow diversity limits the potential for selecting or breeding disease resistant cultivars within *F. alatamaha*.

Possibilities that do exist for breeding *Franklinia* include two closely allied genera in the tribe Gordonieae—*Gordonia* and *Schima*. Embryological and morphological studies showed these genera to be closely related to each other (Luna and Ochoterena, 2004; Tsou, 1997) and molecular studies have placed all three genera together in the same tribe of the Theaceae (Prince and Parks, 2001; Yang et al., 2004). Some breeding work with genera *Franklinia, Gordonia,* and *Schima* has already been conducted. Successful crosses of *Franklinia* × *Schima* produced the intergeneric hybrid ×*Schimlinia* (Ranney et al., 2003) and crosses of *Franklinia* ×*Gordonia* produced the intergeneric hybrid ×*Gordlinia* (Ranney and Fantz, 2006). However, little is known about the resistance of related species and potential parents to *P. cinnamomi*. The objective of this study was to evaluate a collection of species, clones, and hybrids of *Franklinia, Gordonia*, and *Schima* for resistance to *P. cinnamomi*.

Materials and Methods

During the summer of 2008, seven taxa of Gordonieae trees were inoculated with *P. cinnamomi* at the N.C. State University Mountain Horticultural Crops Research Station in Mills River, NC. These taxa included *F. alatamaha, G. lasianthus, S. khasiana, S. wallichii,* ×*Gordlinia* H2004-024-008, ×*Schimlinia* H2002-022-083, and ×*Schimlinia* H2002-022-084. The plants of the selected Gordonieae taxa were five-

month-old rooted cuttings collected in early February of the same year. Two-year-old seedlings of *A. fraseri* were also included in the experiment as a positive control (Frampton and Benson, 2004). Plants of all taxa were grown in a 3:1 pine bark with peat (by volume) substrate amended with 1.8 kg/m^3 dolomitic limestone and 1.0 kg/m^3 micronutrients in 3.8 L containers. For the duration of the experiment, plants were kept on a gravel container pad in an open-ended structure covered with white polyethylene providing ~40% shade. Plants were watered by drip irrigation once daily for 5 min.

Three isolates of *P. cinnamomi* were grown on sterile commercial rice grains in flasks in the dark at room temperature for ten days. The isolate accessions used, as well as their host, country of origin, and year of isolation were: 2378 (fraser fir, Avery Co., N.C., 1993), 2399 (*Callitropsis × leylandii* (A.B. Jacks. & Dallim.) D.P. Little, Ashe Co., N.C., 1999), and 2 Hundley (fraser fir, Avery Co., N.C., 2005). On June 27, 2008, ten individuals (replicates) from each taxon were inoculated with *P. cinnamomi* in a completely randomized design. Three rice grains from each of the three isolates were placed 4 cm below the surface of the media on opposite sides of the plant for each of the inoculated individuals. Five additional non-inoculated plants of each taxon were maintained in a separate, completely randomized block under the same environmental and cultural conditions as negative controls. In order to prevent the spread of the pathogen, the gravel floor of the study site was covered with polyethylene, and any water draining from the site was collected and sterilized with bleach before being discarded.

Plants were monitored for symptoms of root rot every other week until symptoms occurred, and thereafter rated twice a week. A scale from 1 to 4, based on that described by Benson (1990), was used to rate the plants: 1=healthy, 2=initial symptoms (flagging of new growth, chlorosis), 3=severe symptoms (wilting, necrotic leaves), 4=dead. Plants were rated for 84 days, at which point all plants exhibiting symptoms had died and all remaining plants appeared healthy. During the course of the study, nine root and stem segments, approximately 1.3 cm in length, were excised from at least one of the dead plants for each taxon, surface sterilized, and placed on three separate plates of selective P_{10}ARP(H) media (Erwin and Ribeiro, 1996) to confirm the presence of the pathogen. This process was also performed on at least one inoculated plant of taxa that did not show any disease symptoms at the end of the study.

Area under the disease progress curve (AUDPC) values were calculated for each replicate in the experiment using the formula of Shaner and Finney (1977):

$$\text{AUDPC} = \sum_{i=1}^{n} [(Y_{i+1}+Y_i)/2][X_{i+1}-X_i]$$

where Y_i = disease rating at the *i*th observation, X_i = days after inoculation at the *i*th observation, and *n* = the total number of observations. Disease ratings at specific dates and AUDPC values were subjected to analysis of variance and means separation using LSD (Proc GLM, SAS version 9.1; SAS Institute, Cary, NC) to compare taxa.

Results and Discussion

The non-inoculated control plants did not exhibit symptoms of infection by *P. cinnamomi* throughout the duration of the study (data not shown). Among the inoculated plants, none of the *S. khasiana* or *S. wallichii* exhibited symptoms, while the remaining taxa showed varying levels of infection (Fig. 1; Table 1). Symptoms in *F. alatamaha* and the susceptible control, *A. fraseri,* were apparent 28 and 42 days after inoculation, respectively, and mortality for both reached 100% by the end of the experiment (84 days after inoculation). Symptoms in *Gordonia*, ×*Schimlinia,* and ×*Gordlinia* were generally delayed and less severe than in *Franklinia* and were not significantly different from the two species of *Schima*. When plant parts were harvested for re-isolation of *P. cinnamomi*, roots of dead plants appeared brown and thinner in comparison with the white, fleshy roots of plants that did not exhibit any disease symptoms. *P. cinnamomi* was recovered from roots of inoculated plants in all taxa, including those taxa that appeared completely healthy at the end of the experiment, suggesting tolerance of the pathogen rather than total resistance.

The AUDPC, which reflects both rate of onset and severity of infection, also showed significant differences among taxa (Table 1). *Franklinia alatamaha* had the highest AUDPC, followed by *A. fraseri*, a species known to be highly susceptible to *P. cinnamomi*. There was no significant difference between the AUDPC of *G. lasianthus,* both *Schima* species, and the hybrid taxa. Resistance in the hybrid taxa was similar to their more resistant parental genus, i.e., *Gordonia* or *Schima*.

Although *Franklinia, Gordonia, Schima*, and their hybrids are all closely related, it is not surprising that significant differences exist in their susceptibility to *P. cinnamomi.* Differences in susceptibility to *P. cinnamomi* among closely related species have been reported in *Rhododendron* L. (Benson, 1980; Hoitink and Schmitthenner, 1974; Krebs and Wilson, 2002), *Vaccinium* L. (Clark et al., 1986), *Banksia* L.f. (McCredie et al., 1985), and *Abies* Mill. (Benson et al., 1998; Hinseley et al., 2000). Even cultivars, clones, or provenances of the same species have shown significant differences in susceptibility, as in the case of *Pinus radiata* D.Don (Butcher et al., 1984), *Persea americana* Mill. (Gabor et al., 1990), *Abies fraseri* (Frampton and Benson, 2004), and *Eucalyptus marginata* Sm. (Stukely and Crane, 1994).

Previous studies have suggested that resistance to *P. cinnamomi* may be partially recessive (Clark et al., 1986) and controlled by multiple genes (Stukely and Crane, 1994; Butcher, 1987). Resistance to the pathogen in this experiment appears instead to be at least partially dominant, as all hybrid taxa expressed a level of resistance similar to that of the more resistant parent.

The results of this study show that sources of resistance to *P. cinnamomi* do exist in the tribe Gordonieae and that resistance can be successfully transmitted to hybrid progeny. This information will aid ongoing breeding efforts to combine the desirable ornamental traits of these taxa with a high level of resistance to *P. cinnamomi*. In addition, an effective protocol for the quick screening for *P. cinnamomi* resistance in this tribe has been established and can be applied to hybrids developed in the future.

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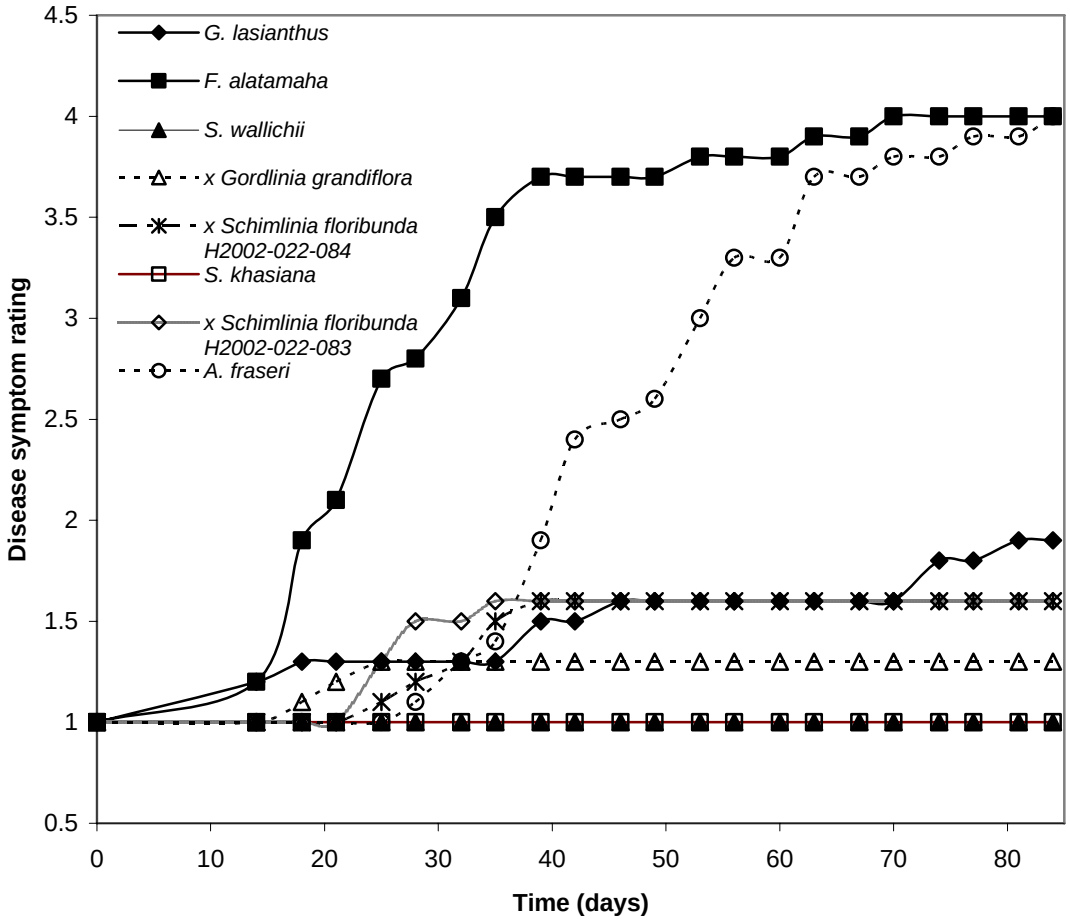

Figure 1. Development of symptoms in *Abies fraseri* and selected taxa from the tribe Gordonieae following inoculation with *Phytophthora cinnamomi*.

Table 1. Disease symptom ratings and area under the disease progress curve (AUDPC) for *Abies fraseri* and selected taxa from the tribe Gordonieae following inoculation with *Phytophthora cinnamomi*.

	Disease symptom rating[z]							AUDPC[y]
Taxa	**Day 0**	**Day 14**	**Day 28**	**Day 42**	**Day 56**	**Day 70**	**Day 84**	**(rating · days)**
F. alatamaha	1.0[x] a	1.2 a	2.8 a	3.7 a	3.8 a	4.0 a	4.0 a	255.15 a
A. fraseri	1.0 a	1.0 a	1.1 b	2.4 b	3.3 a	3.8 a	4.0 a	192.10 b
G. lasianthus	1.0 a	1.2 a	1.3 b	1.5 c	1.6 b	1.6 b	1.9 b	122.50 c
×*Schimlinia* H2002-022-083	1.0 a	1.0 a	1.5 b	1.6 bc	1.6 b	1.6 b	1.6 bc	118.85 c
×*Schimlinia* H2002-022-084	1.0 a	1.0 a	1.2 b	1.6 bc	1.6 b	1.6 b	1.6 bc	116.05 c
×*Gordlinia* H2004-024-008	1.0 a	1.0 a	1.3 b	1.3 c	1.3 b	1.3 b	1.3 bc	103.35 c
S. wallichii	1.0 a	1.0 a	1.0 b	1.0 c	1.0 b	1.0 b	1.0 c	84.00 c
S. khasiana	1.0 a	1.0 a	1.0 b	1.0 c	1.0 b	1.0 b	1.0 c	84.00 c

[z]1=healthy, 2=initial symptoms (flagging of new growth, chlorosis), 3=severe symptoms (wilting, necrotic leaves), 4=dead.

[y]AUDPC calculated using all ratings taken twice a week over a period of 12 weeks.

[x]Values are mean, n=10. Means followed by the same letter, within a column, are not significantly different, $P \leq 0.05$.

Chapter 2

Vegetative Propagation of Gordonieae Trees by Stem Cuttings

(In the format appropriate for submission to the Journal of Environmental Horticulture)

Vegetative Propagation of Gordonieae Trees by Stem Cuttings

Elisabeth M. Meyer[1], Anthony V. LeBude[2], and Thomas G. Ranney[3]

Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759

[1] Graduate Research Assistant

[2] Assistant Professor and corresponding author. E-mail address: anthony_lebude@ncsu.edu

[3] Professor

Abstract

The Theaceae tribe Gordonieae contains trees with both desirable ornamental characteristics and adaptability to a broad range of environmental conditions. In order to develop an effective vegetative propagation protocol for five taxa of Gordonieae trees, terminal softwood, semi-hardwood, and hardwood cuttings were collected from these trees and treated with either 0, 2500, 5000, 7500, or 10000 ppm of the potassium salt of indolebutyric acid (K-IBA). The concentration of K-IBA only affected rooting percentage of hardwood cuttings of *Franklinia alatamaha*, *Gordonia lasianthus*, and *Schima remotiserrata*, and the treatment had varying effects on root number and length of

longest root amongst the different taxa and cutting types. *Franklinia alatamaha* and *G. lasianthus* were rooted at high percentages (>50%) from hardwood, semihardwood, and softwood cuttings, and *S. khasiana* rooted at high percentages (72%) from softwood cuttings. Despite both poor rooting from all types of stem cuttings (<23%), *Schima remotiserrata* and *S. wallichii* exhibited the highest rooting percentages from hardwood cuttings.

Index words

potassium salt of indolebutyric acid (K-IBA), timing, vegetative propagation, Gordonieae

Species used in this study

Franklinia alatamaha Bart. ex Marshall, *Gordonia lasianthus* (L.) Ellis, *Schima khasiana* Dyer, *Schima remotiserrata* Hung T. Chang, *Schima wallichii* Choisy

Significance to the Nursery Industry

Trees in the Theaceae tribe Gordonieae have exceptional ornamental merit and considerable potential for breeding and improvement due to their broad diversity and crossability. However, some taxa in this tribe can be difficult to propagate from stem cuttings. In order for these taxa to be effectively produced by the nursery industry or included in programs for breeding and improvement, reliable protocols for their propagation must be developed. The development of adventitious roots was achieved

with varying levels of success in taxa of *Franklinia*, *Gordonia* and *Schima* using different growth stages of cuttings and concentrations of K-IBA. Treatment with K-IBA provided little benefit for improving rooting percentages for these taxa and was only beneficial at 2500 ppm for hardwood cuttings of *G. lasianthus*. All taxa could be propagated using hardwood cuttings (maximum rooting ranged from 19% to 69%), however, semi-hardwood cuttings of *G. lasianthus* rooted well at 84% and softwood cuttings of *S. khasiana* rooted well at 72%.

Introduction

The Theaceae tribe Gordonieae contains three genera of trees (7). Two genera, *Franklinia* and *Gordonia*, are native to warm temperate and subtropical regions of the New World (including the southeastern U.S.) while the third, *Schima*, is restricted to warm temperate to tropical regions of the Old World. All three genera have large white flowers that vary in bloom time from mid-summer to early fall (4, 9). Each genus has desirable foliage characteristics including the bright red fall foliage of *F. alatamaha* (4), evergreen and sometimes variegated foliage of *Gordonia lasianthus* (4, 8), and glossy, bright red new foliage of some *Schima* species (6). Gordonieae trees have also been shown to be adaptable to a wide range of environmental conditions. *Franklinia alatamaha* has been reported to be cold-hardy to temperatures as low as -36°F (-38°C) (2), and *Schima* can withstand fire (5) and tolerate soils that are dry (13), wet (6), or infertile (1).

Previous work on vegetative propagation of these genera is very limited. For *F. alatamaha*, Dirr and Heuser (3) recommended that softwood cuttings be taken from June to August and treated with a basal dip of 1000 ppm indolebutyric acid (IBA). Another study indicated that IBA solutions at concentrations lower than 1000 ppm were not as effective for rooting softwood cuttings of *F. alatamaha,* and concentrations higher than 1000 ppm actually inhibited rooting (11). For *G. lasianthus*, Dirr and Heuser (3) indicated that cuttings may be taken in March or from June until August and treated with a solution of 3000 ppm IBA. Limited research on vegetative propagation of *Schima* spp. did not achieve successful rooting of the cuttings (12), and observations at the Mountain Horticultural Crops Research and Extension Center in Mills River, NC have indicated that *Schima* spp. can be difficult to root from softwood cuttings (Dr. Thomas G. Ranney, pers. comm., June 2007). The objective of this study is to evaluate the influence of tissue type and the concentration of the potassium salt of indolebutyric acid (K-IBA) on rooting of stem cuttings of *Franklinia, Gordonia*, and *Schima* spp.

Materials and Methods

In this study, terminal stem cuttings from *F. alatamaha*, *G. lasianthus, Schima wallichii, S. khasiana,* and *S. remotiserrata* were collected at the hardwood, softwood, and semihardwood stage of growth on February 1, 2008, June 30, 2008, and September 26, 2008, respectively. Cuttings were classified as semihardwood when a resting bud had been set, regardless of the rigidity of the stem tissue. At each date, cuttings from each

taxon were trimmed to 3 to 4 in. (7.5 to 10 cm) in length and the basal 0.4 in. (1 cm) was dipped in 0, 2500, 5000, 7500 or 10000 ppm K-IBA dissolved in water. The cuttings were then inserted 0.4 in. (1 cm) in plastic flats (40 cm L x 40 cm W x 15.2 cm D) filled with a rooting substrate of 2 peat:3 perlite (v/v). Stem cuttings were misted intermittently for 8 sec every 10 minutes between 0600 and 1800 HR. The experimental design was a randomized complete block with five K-IBA treatments and 6 replicates per treatment combination. Each replicate consisted of 6 cuttings (subsamples). Each taxon was considered a separate experiment. Cuttings were harvested and data collected after twelve weeks, with the exception of data for hardwood cuttings of *S. remotiserrata,* which were collected after twenty-four weeks due to the slow rooting of this taxon. At the time of harvest, percent rooting, number of roots, and length of longest root were determined. Data were subjected to analysis of variance and regression analysis where appropriate (Proc GLM, SAS v. 9.1.3; SAS Institute, Cary, NC).

Results and Discussion

Hardwood cuttings

Rooting percentage of hardwood cuttings was significantly influenced by K-IBA concentration for *F. alatamaha, G. lasianthus*, and *S. remotiserrata,* but not for *S. wallichii* or *S. khasiana* (Fig. 1). In *F. alatamaha*, the highest rooting percentage (69%) occurred for non-treated control cuttings and decreased linearly with increasing concentrations of K-IBA (Fig. 1). *Gordonia lasianthus* demonstrated a quadratic

response to increasing concentrations of K-IBA, with a predicted maximum rooting percentage at 2500 ppm K-IBA (69%). Like *F. alatamaha, S. remotiserrata* exhibited the highest rooting percentage (19%) in the non-treated control, and expressed a negative linear relationship with increasing concentrations of K-IBA. K-IBA did not affect rooting percentage of cuttings of *S. wallichii* or *S. khasiana*, which had mean rooting percentages of 23% and 43%, respectively.

Root number was significantly affected by K-IBA concentration for *S. khasiana*, but not the other taxa (Fig. 2). Rooted cuttings of *S. khasiana* had a quadratic response to K-IBA concentration with the optimum of 22 roots per cutting occurring at 7500 ppm. Mean root number did vary among the other species. *Gordonia lasianthus* had the largest mean root number at 33, followed by *S. khasiana* (mean=15), *F. alatamaha* (mean=9), *S. wallichii* (mean=4), and *S. remotiserrata* (mean=3). Although there was a significant difference in the length of longest root for the non-treated and treated cuttings of *S. wallichii* and *S. khasiana,* there were no trends for the effect of K-IBA concentration on length of longest root in these species.

Softwood cuttings

There was no significant effect of K-IBA concentration on rooting percentage in softwood cuttings of any taxa. The taxa exhibited a wide range of mean rooting percentages from a high of 72% in *S. khasiana* to a low of 0.6% in *S. wallichii.* The mean rooting percentages of the other species were 52% for *F. alatamaha,* 51% for *G. lasianthus,* and 6% for *S. remotiserrata*. Number of roots was not significantly affected

by K-IBA concentration in any of the taxa. *Franklinia alatamaha* had the largest mean root number at 46 roots per cutting, followed by *G. lasianthus* (mean=19), *S. khasiana* (mean=7), *S. remotiserrata* (mean=2), and *S. wallichii* (mean=1).

The only case in which a rooting variable was significantly affected by K-IBA concentration for softwood cuttings was that of length of longest root in *G. lasianthus* (Fig. 3). Length of longest root in *G. lasianthus* demonstrated a quadratic response to K-IBA concentration with a predicted maximum root length of 18 cm at 7500 ppm K-IBA.

Semihardwood cuttings

There was no significant effect of K-IBA concentration on rooting percentage in semihardwood cuttings of any taxa. The highest mean rooting percentage occurred for *G. lasianthus* (84%), followed by *F. alatamaha* (62%), *S. khasiana* (43%), *S. wallichii* (3%), and *S. remotiserrata* (3%).

Mean root number was significantly affected by K-IBA concentration in *F. alatamaha, G. lasianthus,* and *S. khasiana* (Fig. 4). Rooted cuttings of *F. alatamaha* had a quadratic response to K-IBA concentration with the optimum of 12 roots per cutting occurring at 5000 ppm. In *G. lasianthus,* root number exhibited no trend in relation to K-IBA concentration, though there was a significantly higher number of roots in cuttings treated with K-IBA than in the non-treated control cuttings. The mean root number for *G. lasianthus* was 21 roots per cutting. Cuttings of *Schima khasiana* demonstrated a quadratic response to K-IBA concentration with a predicted maximum of 20 roots per cutting at 10000 ppm. *Schima wallichii* and *S. remotiserrata* did not have a significant

response to K-IBA concentration and had mean root numbers of 4 and 2 roots per cutting, respectively. Length of longest root was significantly affected by K-IBA concentration in *G. lasianthus* only, where it decreased linearly with increasing K-IBA (Fig. 5).

Based on the data presented here, *F. alatamaha* successfully rooted at 52% from softwood cuttings, 62% from semi-hardwood cuttings, and 69% from hardwood cuttings with K-IBA treatments providing no benefit. Similarly, *G. lasianthus* was successfully rooted at 51% from softwood cuttings, 84% from semihardwood cuttings, and 69% from hardwood cuttings and only benefited from treatment with K-IBA at a low rate of 2500 ppm at the hardwood stage. Studies of the related taxa *Stewartia pseudocamellia* Maxim. have shown a similar ability to root from different physiological growth stages of cuttings (10). The *Schima* species included in this study varied considerably in their rooting percentages. *Schima khasiana* rooted at 72% from softwood cuttings and 43% for both semi-hardwood and hardwood cuttings with no benefit from K-IBA treatments. *Schima wallichii* only had a mean rooting percentage of 23%, and *S. remotiserrata* never exceeded 19% rooting. Both of these maximum rooting percentages were attained from hardwood cuttings, and there was no benefit of K-IBA treatment. Other approaches, such as stock plant management (i.e., severe pruning, etiolation, or increased fertility), different formulations and strengths of growth regulators, or post-severance treatments of the cuttings may be necessary to further enhance rooting percentages.

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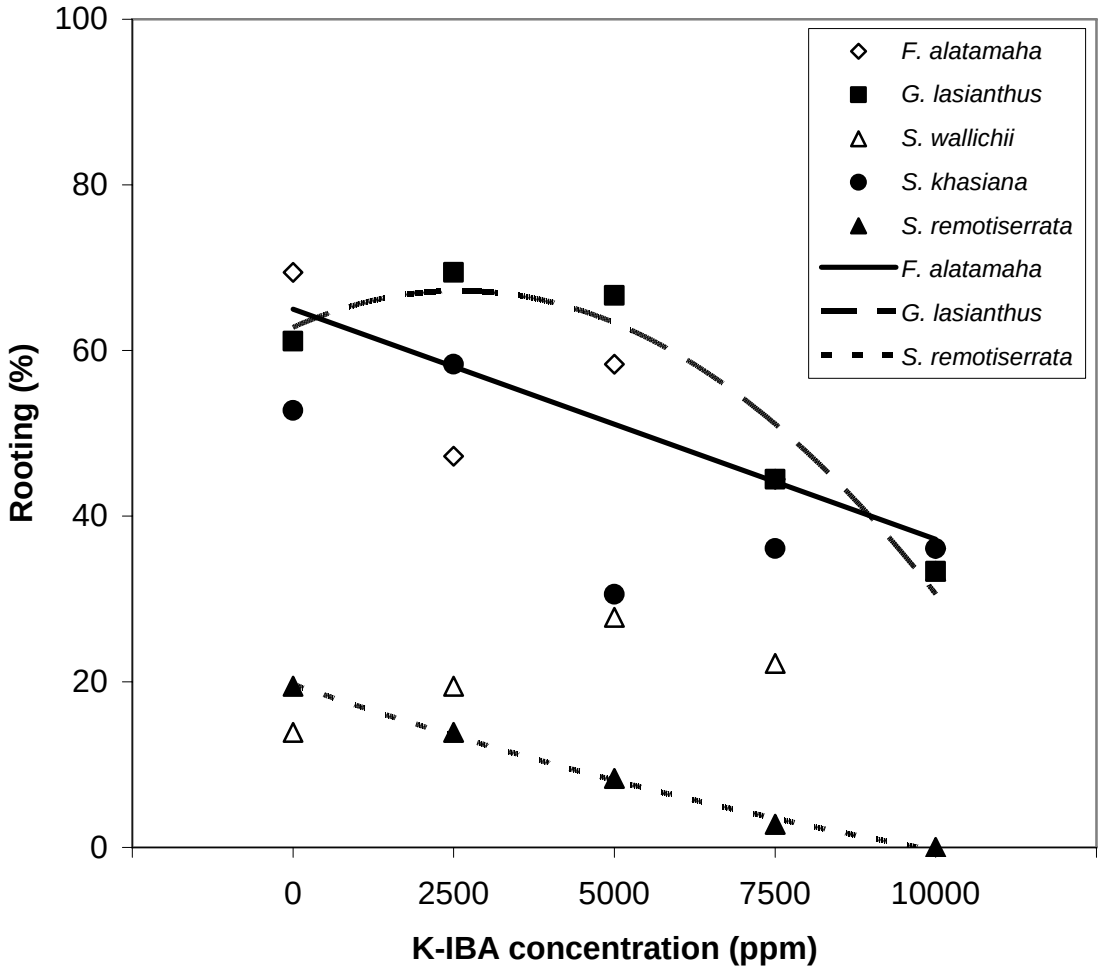

Figure 1. Effect of K-IBA concentration on rooting percentage of hardwood cuttings of *F. alatamaha* (y = 65-0.0028x, R^2=0.72, *P*=0.01), *G. lasianthus* (y = 62.78+0.0034x-(6.67*10^{-7})x^2, R^2=0.93, *P*=0.08), *S. remotiserrata* (y = 19.44-0.0022x, R^2=0.99, *P*<.0001), *S. wallichii,* and *S. khasiana*.

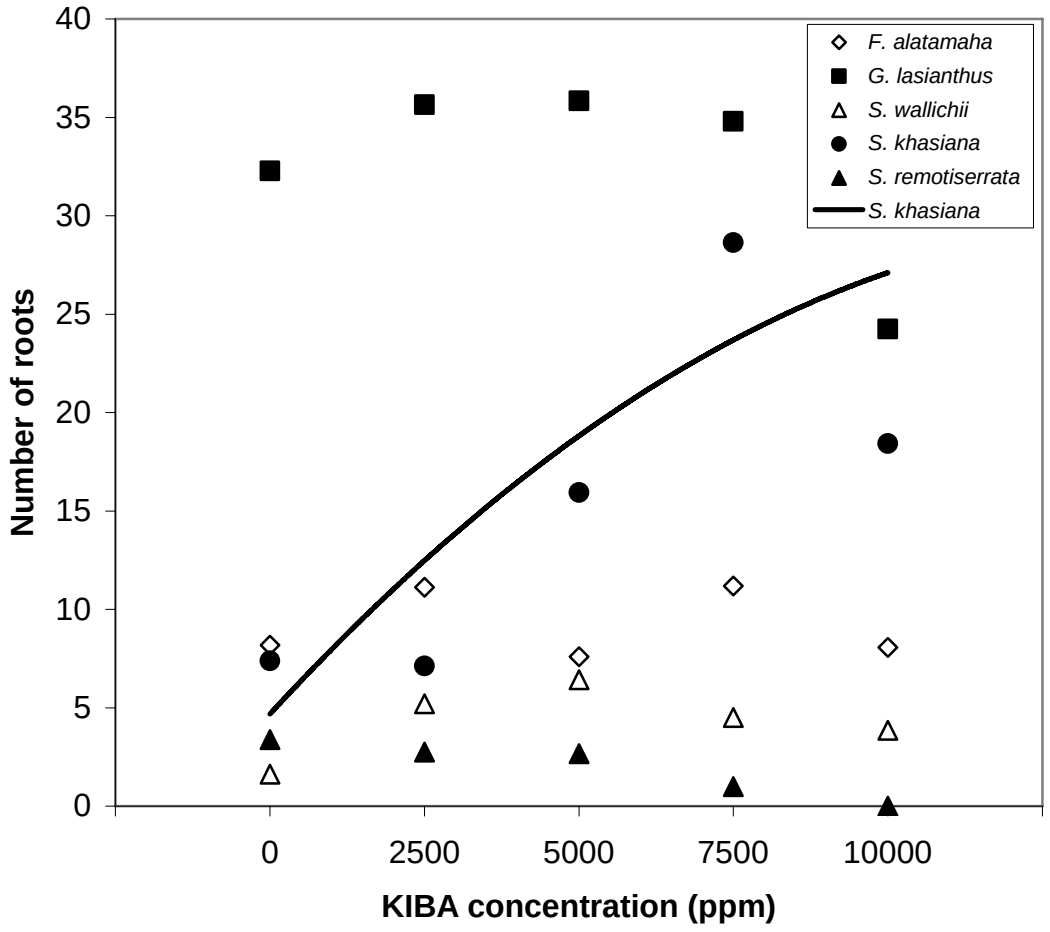

Figure 2. Effect of K-IBA concentration on number of roots of hardwood cuttings of *F. alatamaha, G. lasianthus, S. wallichii, S. remotiserrata,* and *S. khasiana* ($y = 4.68+0.0034x-(1.67*10^{-7})x^2$, $R^2=0.43$, $P< 0.05$).

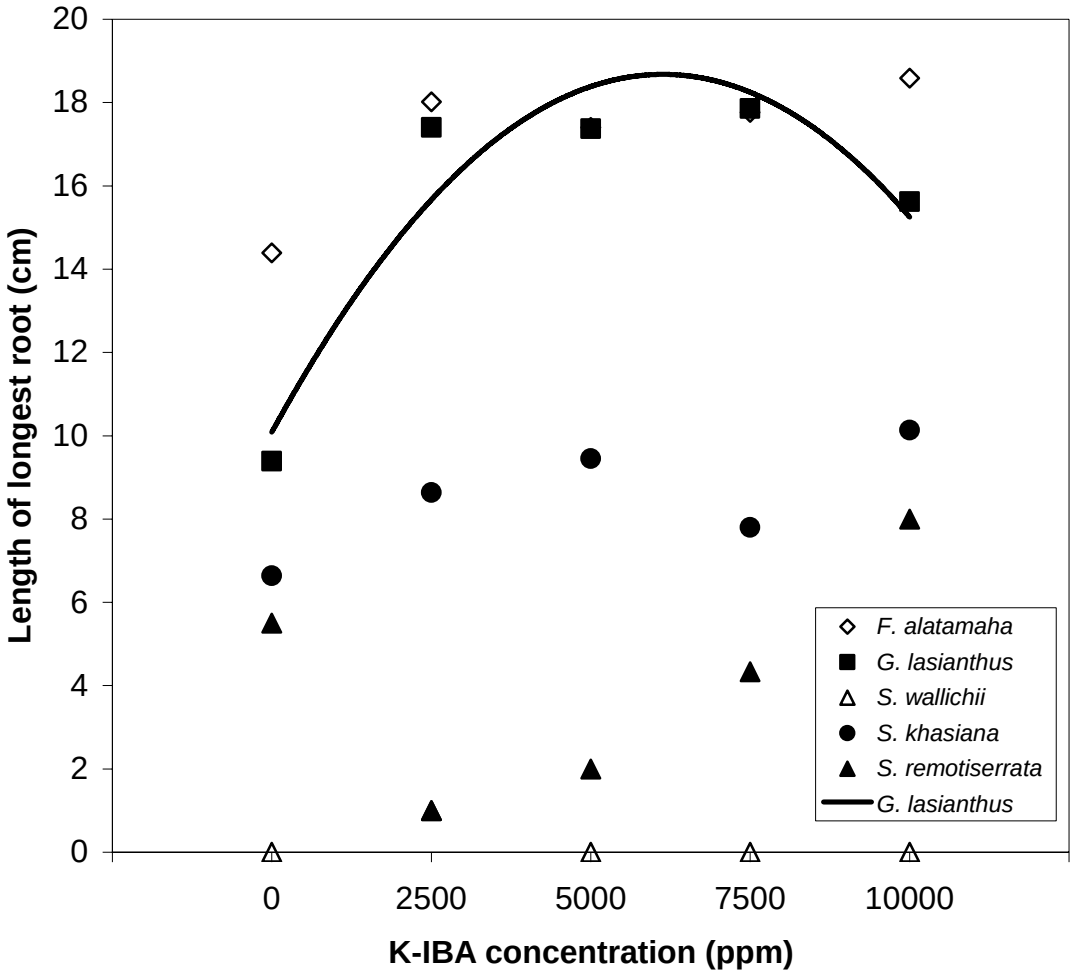

Figure 3. Effect of K-IBA concentration on length of longest root of softwood cuttings of *F. alatamaha*, *G. lasianthus* ($y = 10.09+0.0028x-(2.29*10^{-7})x^2$, $R^2=0.90$, $P=0.10$), *S. wallichii*, *S. khasiana*, and *S. remotiserrata*.

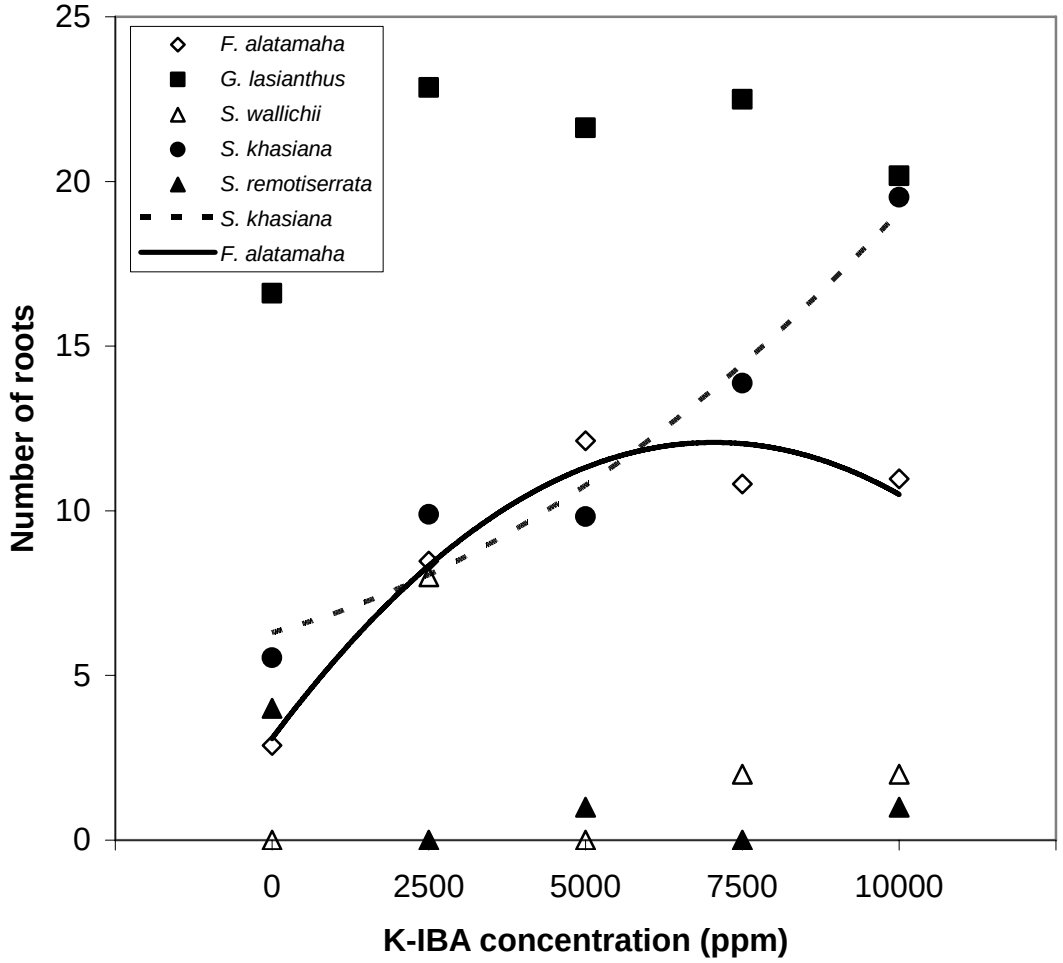

Figure 4. Effect of K-IBA concentration on number of roots in semihardwood cuttings of *F. alatamaha* (solid line—$y = 3.08+0.0026x-(1.81*10^{-7})x^2$, R^2=0.96, P=0.05), *G. lasianthus, S. khasiana* (dotted line—$y = 6.30+0.0005x+(7.67*10^{-8})x^2$, R^2=0.95, P=0.05), *S. wallichii,* and *S. remotiserrata*.

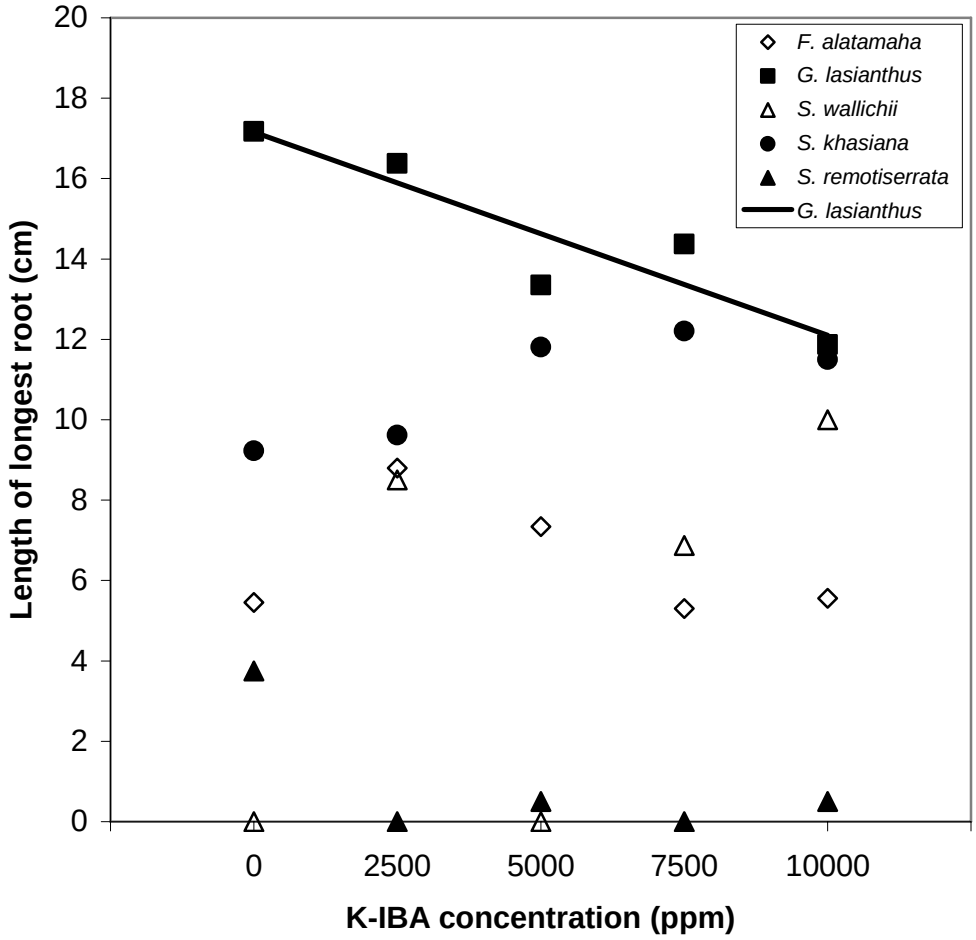

Figure 5. Effect of K-IBA concentration on length of longest root in semihardwood cuttings of *F. alatamaha*, *G. lasianthus* (y = 17.15-0.0005x, R^2=0.84, *P*=0.05), *S. wallichii*, *S. khasiana*, and *S. remotiserrata*.

Chapter 3

In Vitro Shoot Regeneration and Polyploid Induction from Leaves of *Hypericum sp.*

(In the format appropriate for submission to Plant Cell, Tissue, and Organ Culture)

In Vitro Shoot Regeneration and Polyploid Induction from Leaves of *Hypericum* sp.

Elisabeth M. Meyer, Darren H. Touchell[1], and Thomas G. Ranney

Department of Horticultural Science, Mountain Horticultural Crops Research and Extension Center, North Carolina State University, 455 Research Drive, Mills River, NC 28759-3423

[1]Corresponding author: darren_touchell@ncsu.edu

Abstract

Hypericum L. H2003-004-016 is a complex hybrid between *Hypericum frondosum* Michx., *Hypericum galioides* Lam., and *Hypericum kalmianum* L. and exhibits valuable ornamental characteristics including compact habit, bluish-green foliage, and showy flowers. Inducing polyploidy may further enhance the ornamental traits of this hybrid and provide new opportunities for hybridizing with other naturally-occurring polyploid *Hypericum* sp. In this study, *in vitro* shoot regeneration and treatment of regenerative callus with dinitroaniline herbicide oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide) were investigated as a means of inducing allopolyploidy. First, *in vitro* regeneration was optimized for callus and shoot production by culture of leaf explants on medium supplemented with benzylamino purine (BA) or *meta*-topolin (mT) at 5, 10, or 15 μM in combination with indoleacetic acid (IAA) at 0, 1.25, 2.5, or 5 μM.

Both BA and mT treatments successfully produced regenerative callus and shoots. Maximum regenerative callus (94%) and shoot production (18 shoots per callus) were predicted in medium supplemented with 5 μM BA and 3.75 μM IAA. In the second part of the study, exposure of regenerative callus to oryzalin at 0, 7.5, 15, 30, 60, or 90 μM for durations of 3, 6, or 9 days was investigated for polyploid induction. There was no survival for any of the 60 or 90 μM oryzalin treatments, but all other treatments exhibited some survival and polyploidy induction. Duration had no effect on callus survival or ploidy level, but oryzalin concentration was a significant factor in both. The greatest percentage (44%) of polyploids was produced at 30 μM oryzalin. Spontaneous chromosome doubling was observed in 8% of control explants receiving no oryzalin treatment.

Key words: *Hypericum frondosum, Hypericum galioides, Hypericum kalmianum,* meta-topolin, oryzalin, endoreduplication

Introduction

The genus *Hypericum* L. comprises approximately 370 species worldwide that are well known for their pharmaceutical qualities. Several species including *Hypericum frondosum* Michx., *Hypericum galioides* Lam., and *Hypericum kalmianum* L. also have desirable ornamental characteristics and environmental tolerances that make them promising species for breeding and improvement (Coulter 1886; Hasselkus 1998;

Touchell et al. 2008). All three species demonstrate broad adaptability and have showy, gold colored flowers. In addition to attractive flowers, *H. frondosum* 'Sunburst' also has bluish-green foliage and a compact growth form. Although generally more open in habit than the other two species, *H. kalmianum* has the desirable characteristic of being cold-hardy to USDA zone 4. *Hypericum galioides* 'Brodie' is particularly tolerant of the hot, humid conditions in the southeastern United States. At the NC State Mountain Horticultural Crops Research and Extension Center (MHCREC) in Mills River, N.C., these three species have been crossed, through multiple generations, to develop hybrid H2003-004-016. The hybrid exhibits a dense, compact growth form, narrow, bluish-green foliage, and an abundance of showy, gold colored flowers.

In addition to basic propagation, tissue culture can be a useful tool in improvement of ornamental features. Development of *in vitro* regeneration systems provides an ideal foundation for further improvements by ploidy manipulations, mutation treatments, and transgenic applications. Previous *in vitro* regeneration studies of *Hypericum* species have included *H. perforatum* L. (Franklin and Dias 2006; McCoy and Camper 2002; Murch et al. 2000; Pretto and Santarém 2000), *H. heterophyllum* Vent. (Ayan and Crak 2006), *H. brasiliense* Choisy (Cardoso and de Oliveira 1996), *H. foliosum* [Dryand.] (Moura 1998), and *H. frondosum* (Touchell et al. 2008).

In vitro regeneration from leaves of *Hypericum* sp. characteristically requires a high cytokinin:auxin ratio (Moura 1998; Santarém and Astarita 2003). Specifically, work with *H. frondosum*, a parent of H2003-004-016, shows high levels of benzylamino purine

(BA) facilitate optimum *in vitro* regeneration from leaves. Benzylamino purine is the most commonly used cytokinin for *in vitro* regeneration of *Hypericum* sp. However, BA has been associated with inducing somaclonal variation (Siragusa et al. 2007) and hyperhydricity (Rossetto et al. 1992; Bairu et al. 2007), which may negatively influence the quality of regenerated shoots. In comparison, meta-topolin (mT) is a naturally occurring cytokinin with a similar aromatic structure to BA and has not been linked with somaclonal variation or hyperhydricity, but there is a need for more extensive research of its long- and short-term effects on plant *in vitro* cultures. Meta-topolin may have potential as a highly active alternative to BA. Although mT has not been used with *Hypericum* sp., it has shown promise in regeneration of *Spathiphyllum floribundum* N.E.Br. (Werbrouck et al. 1996), *Musa* AAB L. (Roels et al. 2005), *Aloe polyphylla* Schönland ex Pillans (Bairu et al. 2007), and *Pelargonium* × *hederaefolium* 'Bonete' Salisb. (Wojtania et al. 2004).

Several auxins, including indoleacetic acid (IAA) (Ayan and Crak 2006; Cardoso and de Oliveira 1996; Franklin and Dias 2006; Touchell et al. 2008; Wang et al. 2007), α-naphthaleneacetic acid (NAA) (Cardoso and de Oliveira 1996; Moura 1998; Wang et al. 2007), and 2,4-dichlorophenoxyacetic acid (2,4-D) (Ayan and Crak 2006; Cardoso and de Oliveira 1996; Pretto and Santarém 2000; Wang et al. 2007) have been used in regeneration of *Hypericum* sp. However, recent work with one of the parents of hybrid H2003-004-016, *H. frondosum* 'Sunburst', has shown 2,4-D to be ineffective for *in vitro* shoot regeneration (Touchell et al. 2008).

Efficient *in vitro* regeneration systems provide a tool to manipulate ploidy and improve ornamental features. Polyploidy in some plants may result in desirable ornamental characteristics such as larger, longer-lasting flowers, thicker petals, and larger, thicker leaves (Kehr 1996). In addition, polyploid plants, particularly allopolyploids, can have other advantageous traits such as enhanced vigor, improved pest resistance and stress tolerance, and protection from deleterious mutations due to gene redundancy (Comai 2005; Ranney 2006).

Various mitotic inhibitors have been used to induce polyploidy in *in vitro* systems, including colchicine (N-(5,6,7,9-tetrahydro-1,2,3,10-tetra-methoxy-9-oxobenzo(a)heptalen-7-yl) acetamide) on *H. perforatum* (Wang et al. 2007). However, the dinitroaniline herbicide oryzalin (3,5-dinitro-N4,N4-dipropylsufanilamide) is often preferred to colchicine for use in polyploidy induction in plants due to its lower toxicity (van Tuyl et al. 1992), effectiveness at lower concentrations (Morejohn et al. 1987; Väinölä 2000; van Tuyl et al. 1992), and tendency to produce plants without deformed tissue or abnormal growth (van Tuyl et al. 1992). For example, in *Lilium* L., 0.005% oryzalin treatments produced more polyploids than colchine at 0.1% (van Tuyl et al. 1992). *Lilium* sp. treated with oryzalin also experienced less growth abnormalities than those treated with colchicine. Similarly, oryzalin treatments (0.001% or 0.005%) of *Rhododendron* L. yielded 45% polyploids as opposed to 15% in colchicine treatments (0.025% or 0.05%) (Väinölä 2000). Therefore, objectives of this study were to 1) develop an effective shoot regeneration protocol for *Hypericum* H2003-004-016 from

leaf explants and 2) establish a reliable procedure for *in vitro* induction of polyploidy in this hybrid using oryzalin.

Materials and Methods

Plant material and regeneration protocols

Young leaves of *Hypericum* H2003-004-016 were collected from four glass house grown stock plants at the MHCREC and surface sterilized in a laminar flow hood for 17 min. in a 20% commercial bleach solution (1.3% NaOCl), followed by three rinses of 5 min. each in sterile distilled water. Leaf explants (5 mm^2) were placed abaxial side down in petri dishes containing medium composed of Murashige and Skoog basal salts and vitamins (Murashige and Skoog 1962), 3% sucrose, and solidified with 0.8% agar. Media was supplemented with either BA or mT at 5, 10, or 15 μM in combination with IAA at 0, 1.25, 2.5 or 5 μM. Plates were incubated in the dark at 23 ± 2 °C.

Each set of cytokinin treatments was treated as a separate experiment with a completely randomized design. There were at least seven replicates (plates) per treatment and five subsamples (leaf explants) per replicate. After 5 weeks, data were collected on the percentage of leaf explants producing regenerative callus and number of shoots produced per leaf explant. Data were subjected to ANOVA and multiple regression analyses (Proc GLM, SAS version 9.1; SAS Inst., Cary, N.C.).

Polyploid induction

Regenerative calli maintained on solid MS media supplemented with 10 μM BA and 2.5 μM IAA were submerged in a liquid MS medium supplemented with 3% sucrose and oryzalin at 0, 7.5, 15, 30, 60 or 90 μM and placed on a rotating shaker (80 rpm) for a duration of 3, 6, or 9 days. An additional control, receiving no oryzalin treatment and no submersion in liquid media, was also included (0 days). Following treatment with oryzalin, calli received three rinses of 5 min. each with sterile distilled water before being transferred back onto plates of the same medium. The experiment was a completely randomized design with six replicates (plates) per treatment and five subsamples (leaf explants) per replicate. After approximately 5 weeks, survival data were recorded for each treatment and subjected to ANOVA and multiple regression analysis (Proc GLM, SAS version 9.1; SAS Inst., Cary, N.C.).

Approximately 4 months after treatment, ploidy of shoots was determined using flow cytometry. One shoot was finely chopped with a double-sided razor blade in a petri dish with 400 μL of nuclei extraction buffer (CyStain UV Precise P Nuclei Extraction Buffer, Partec, Münster, Germany). The solution was filtered through Partec CellTrics™ disposable filters with a pore size of 50 μm to remove tissue debris. Nuclei were stained with 1.2 mL 4', 6-diamidino-2-phenylindole (DAPI) staining buffer (CyStain UV Precise P Staining Buffer, Partec). Stained nuclei were analyzed with a flow cytometer (Partec PA II, Partec). Ploidy level was determined by comparing peak position of a diploid shoot of *Hypericum* H2003-004-016 with peak position of each sample. Three shoots

(subsamples) were analyzed from each replicate for each treatment, including the control. Three leaves from each of the four stock plants were also analyzed to ensure diploid homogeneity of the source material. Data were subjected to ANOVA and multiple regression analyses (Proc GLM, SAS version 9.1; SAS Inst., Cary, N.C.).

Results and Discussion

Regeneration protocols

Production of regenerative callus and shoots was achieved *in vitro* for *Hypericum* H2003-004-016. Shoots were induced in all treatments combining BA with IAA. In the mT treatments, all treatments produced shoots except for 5 μM mT + 0 μM IAA, 10 μM mT + 0 μM IAA, 15 μM mT + 0 μM IAA, and 15 μM mT + 1.25 μM IAA.

Regression analysis demonstrated BA and IAA concentrations and their interaction had a significant effect on production of regenerative callus and number of shoots ($P < 0.05$) (Figs. 1, 2). Both production of regenerative callus and number of shoots had a linear response to BA concentration and the interaction between BA and IAA, as well as a quadratic response to IAA concentration. Based on regression analysis, 18 shoots per explant were predicted as the optimum for the treatment combination 5 μM BA + 3.75 μM IAA with a predicted 18 shoots per explant based on the regression analysis (Figs. 1,2). In contrast, Touchell et al. (2008) reported the optimal treatment for regeneration of shoots of *H. frondosum* was 10 μM BA + 2.5 μM IAA.

Regression analysis of treatments utilizing mT in combination with IAA indicated mT and IAA concentrations had a significant effect on production of regenerative callus ($P < 0.05$) and number of shoots per explant ($P < 0.10$) (Figs. 3, 4). Production of regenerative callus and number of shoots per explant both decreased linearly with increasing mT concentration. In relation to IAA concentration, production of regenerative callus had a quadratic response while number of shoots decreased linearly as IAA increased. The optimal treatment in the mT experiment was 5 μM mT + 5 μM IAA and produced approximately 10 shoots per explant. Thus, mT can be used successfully as a cytokinin in an *in vitro* regeneration system for *Hypericum* H2003-004-016. Other systems utilizing mT produced plants with more larger, greener shoots, more successful rooting both *in vitro* and *ex vitro*, better acclimatization *ex vitro*, less abnormal growth, and less hyperhydricity than plantlets produced using BA (Bairu et al. 2007; Werbrouck et al. 1996). A higher number of longer, thicker shoots were also observed in mT treatments of this study when compared to BA treatments (pers. obs., data not presented).

Polyploid induction

Hypericum H2003-004-016 had a 2C DNA content of 0.8 ρg ± 0.01 (mean ± SEM, n=7) consistent with a diploid (Brutovská et al. 1998; Matzk et al. 2003). Mixoploids (2x+4x) and tetraploids (4x) were successfully induced by the use of oryzalin. Oryzalin concentration had significant effects on shoot survival and ploidy level, but there were no significant effects of duration on these variables. All calli exposed to 60 or 90 μM oryzalin died. Survival of calli exposed to 0, 7.5, 15, or 30 μM

oryzalin exhibited a quadratic response to oryzalin concentration ($P < 0.01$) (Fig. 5). The percentage of diploids had a negative linear relationship with increasing oryzalin concentration ($P < 0.01$), while percentage of homogenous tetraploids had a positive linear relationship ($P < 0.05$) (Fig. 5). Percentage of mixoploids demonstrated a quadratic response to oryzalin concentration ($P < 0.10$) (Fig. 5). The greatest percentage of homogenous tetraploids was ~35% and occurred in the 30 μM oryzalin treatment. One octoploid was also produced in the 30 μM, 3 day treatment (data not presented).

Unexpectedly, the untreated controls across all durations, including the 0 day duration control not submerged in liquid medium also produced tetraploids (8%). This spontaneous chromosome doubling may have resulted from endoreduplication taking place either in cells of the original stock plant tissue or within the *in vitro* regeneration system. During endoreduplication, multiple, uniform copies of chromosomes are produced without segregation or cytokinesis due to oscillation of cyclin-dependent kinase (CDK) activity during S-phase and lack of CDK activity during M-phase (Grafi and Larkins 1995; Larkins et al. 2001). Since analysis by flow cytometry showed the leaf tissue from the four original plants to be diploid, it is possible *in vitro* regeneration promoted spontaneous chromosome doubling. Occurrence of endoreduplication has been linked to cellular cytokinin and auxin levels (Larkins et al. 2001; Valente et al. 1998), both of which were increased in the *in vitro* system. This process has been reported in a wide variety of plants including *Solanum* L. (Chauvin et al. 2003), *Humulus lupulus* L. (Škof et al. 2007), *Rosa hybrida* L. (Moyne et al. 1993), *Doritaenopsis* Guillaumin

(Mishiba et al. 2001), and *Zea mays* L. (Antoine-Michard and Beckert 1997; Grafi and Larkins 1995). Consistent with other studies in which endoreduplication occurred *in vitro* (Chauvin et al. 2003; Škof et al. 2007), the controls in the present investigation did not produce any mixoploids.

However, it is also possible that endoreduplication occurred in the stock plant tissue before it was placed in tissue culture. The condition in which the same plant contains cells of different ploidy levels due to endoreduplication is known as endopolyploidy and occurs in 90% of angiosperms (D'Amato 1984). Not only is endopolyploidy apparent at different levels among species, but it also occurs with varying frequency among different organs and tissues within the same plant (Barow 2006). In leaves alone, endopolyploidy has been shown to vary between the base, middle, and tip of the leaf (Cionini et al. 1983; Lim and Loh 2003; Pyke et al. 1991). Although flow cytometry of leaves from the stock plants showed them to be diploid, it is feasible that if only a few polyploid cells were present, these cells may not have made a sufficiently distinguishable peak for their presence to be known. Thus, endoreduplication in the stock plant cells might also explain the presence of tetraploids in the controls.

In conclusion, effective protocols for *in vitro* regeneration of this hybrid were developed providing a platform for further studies and improvement projects including mutation treatments and transgenic applications. Protocols for development of polyploids were also established, as well as a new population of polyploids that have

potential utility in future breeding projects, for genomic studies of nascent polyploids, and as new commercial selections.

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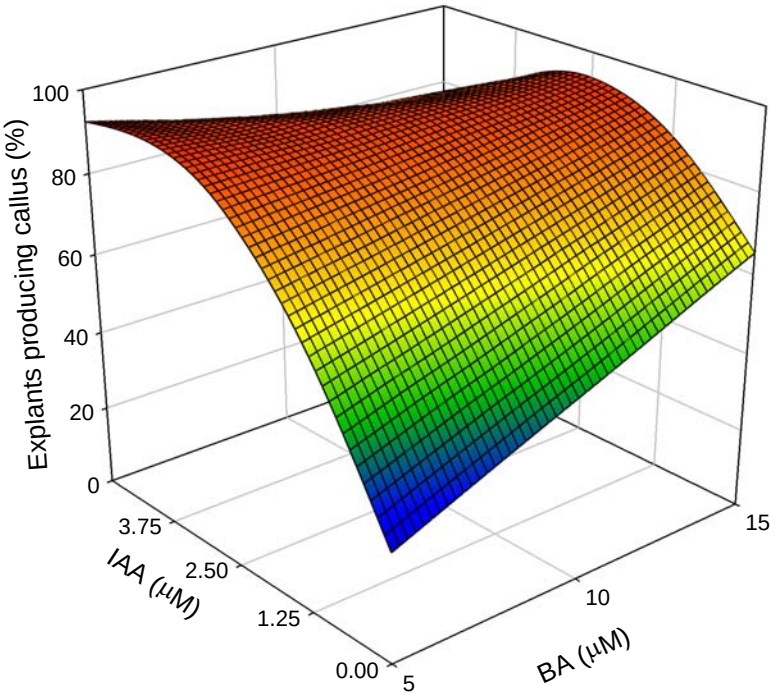

Fig. 1 Effect of BA and IAA on percentage of leaf explants producing regenerative callus. Data points are predicted percentages of regenerative callus based on a general linear model: $y = 0.082 + 0.037(BA) + 0.398(IAA) – 0.0415(IAA^2) –0.0123(BA)(IAA)$, $P < 0.001$, $r^2=0.83$

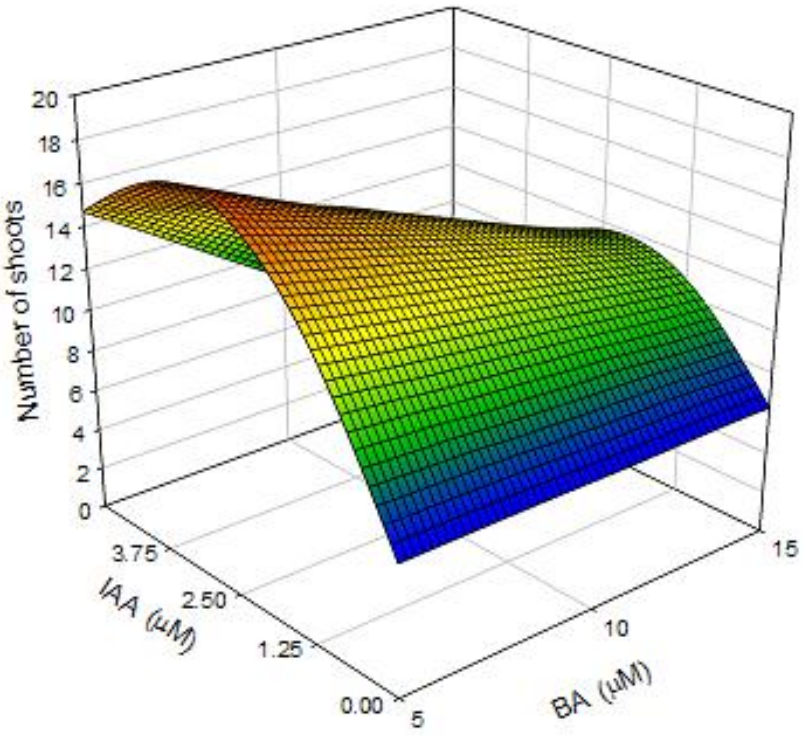

Fig. 2 Effect of BA and IAA on number of regenerated shoots per leaf explant. Data points are predicted values for number of shoots based on a general linear model: $y = 6.25 – 0.0014(BA) + 8.58(IAA) – 1.14(IAA^2) – 0.24(BA)(IAA)$, $P < 0.05$, $r^2=0.61$

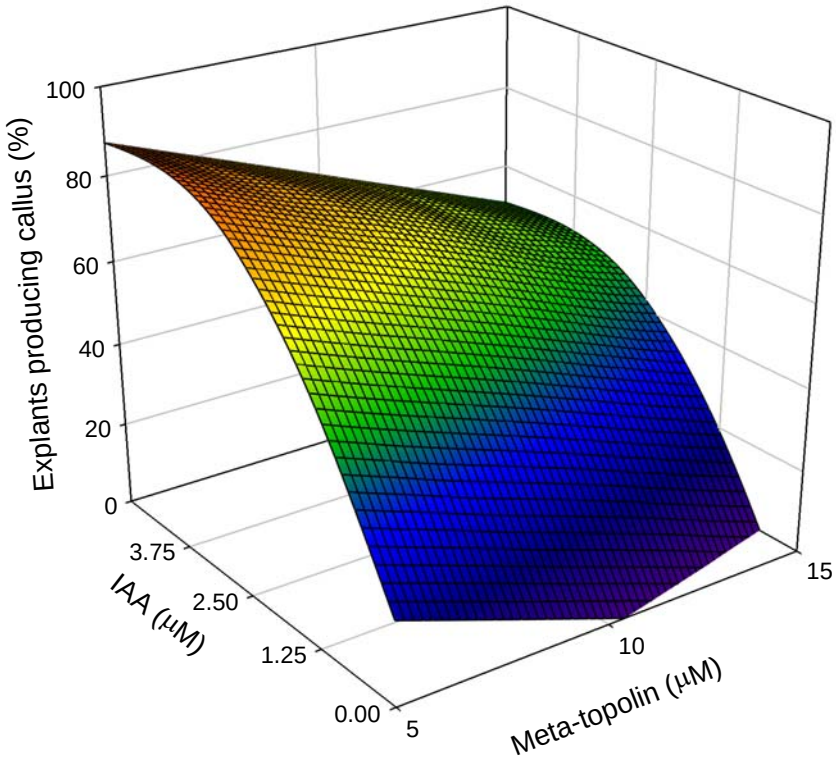

Fig. 3 Effect of mT and IAA on percentage of leaf explants producing regenerative callus. Data points are predicted percentages of regenerative callus based on a general linear model: $y = 0.38 - 0.037(mT) + 0.34(IAA) - 0.041(IAA^2)$, $P < 0.05$, $r^2=0.81$

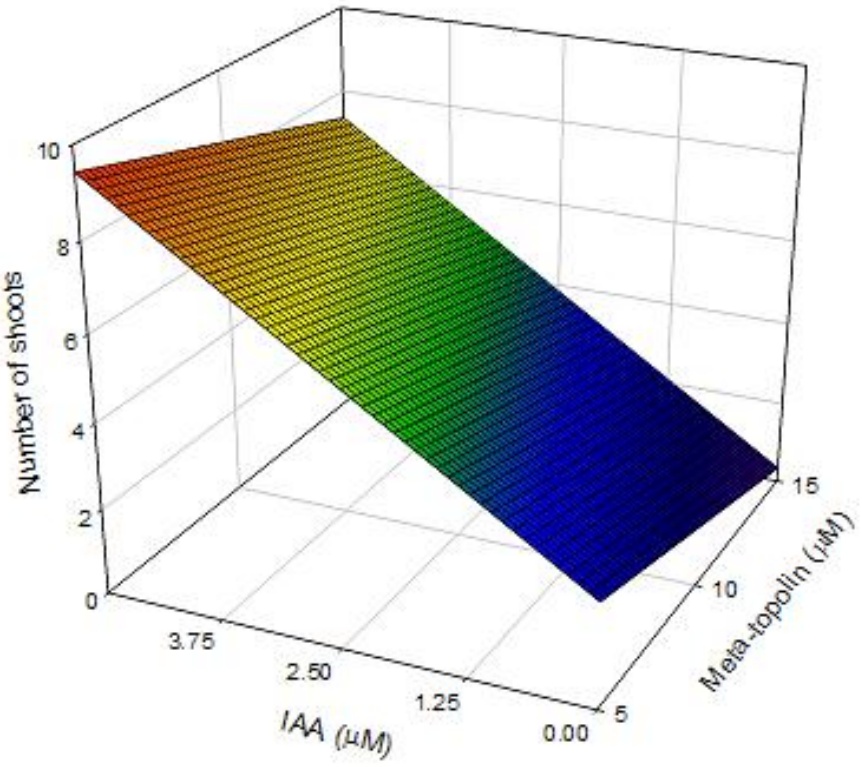

Fig. 4 Effect of mT and IAA on number of shoots per leaf explant. Data points are predicted values for number of shoots based on a general linear model:

$y = 3.49 – 0.21(mT) + 1.49(IAA)$, $P < 0.05$, $r^2=0.49$

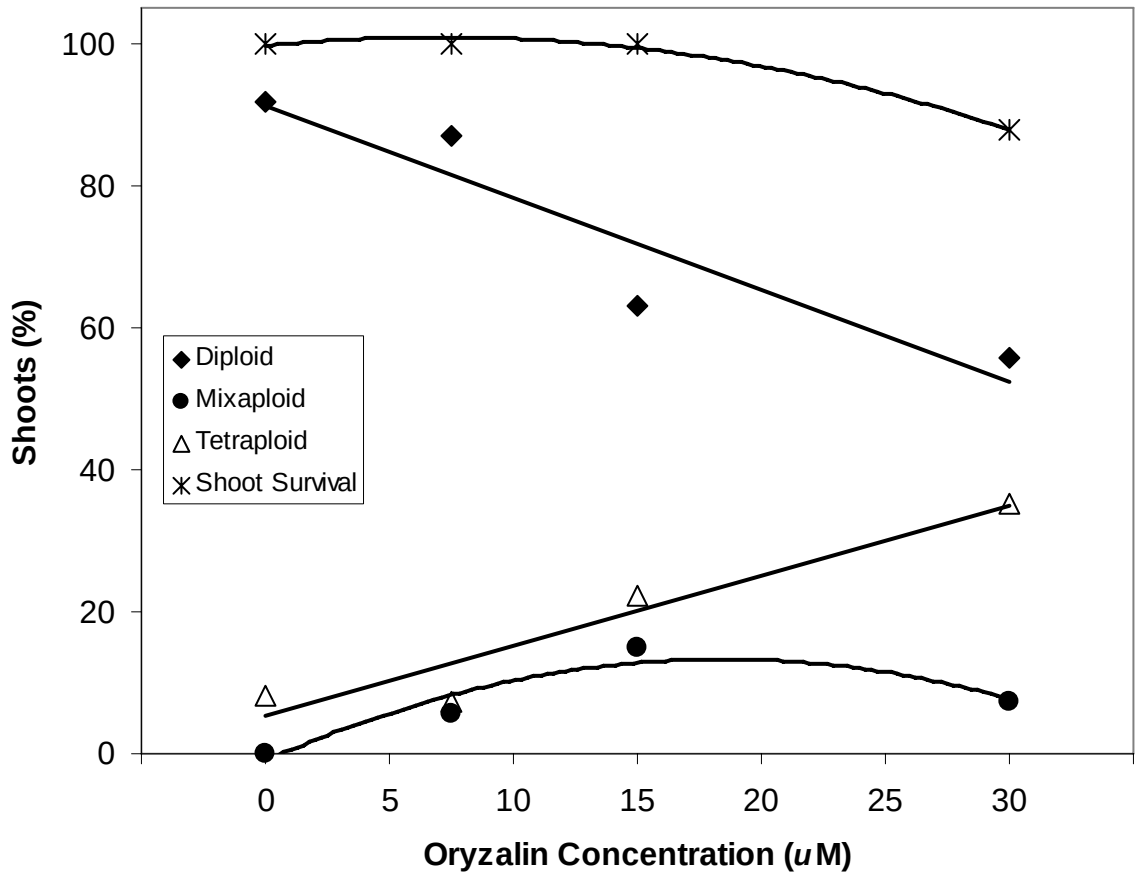

Fig. 5 Effect of oryzalin concentration on percentage shoot survival and ploidy level. Solid lines represent trends fitted using linear and quadratic regression analyses: shoot survival (*) = $1.01 - 0.00014x^2$, r^2=0.74, $P < 0.01$; diploid (♦) = $0.91 - 0.013x$, r^2=0.70, $P < 0.01$; mixoploid (•) = $-0.0077 + 0.015x - 0.0004x^2$, r^2=0.37, $P < 0.10$; tetraploid (Δ) = $0.06 + 0.0096x^2$, r^2=0.44, $P < 0.05$