Histochemical Assessment of Tissue Viability and Herbicide Damage in Woody Stems of *Lonicera maackii*

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ABSTRACT

As non-native woody vegetation continues to be released into the United States, the introduction of invasive plant species capable of altering the natural balance of forest ecosystems is increasing. Since its introduction, Amur honeysuckle has contributed to the decline in native plant biodiversity of forests in much of the eastern United States. Often involving laborious and time-consuming measures, the assessment of ecological damage and subsequent control of these plants can be overly demanding. Pragmatic measures for discerning the effectiveness of control efforts in this species should help assist land management decisions and aid environmental restoration projects. In this regard, several recognized biological stains such as Evan's blue, phenosafranine, tetrazolium, fluorescein, ethidium bromide, acridine orange and rhodamine B were assessed for their ability to distinguish living tissue within intact stem sections. Through this selection process the visible light stain 2,3,5-triphenyltetrazolium chloride (TTC) and the fluorescent dye 6-carboxyfluorescein diacetate (CFDA) were found capable of being taken up quickly, reacting with living cells and being retained within honeysuckle stem tissue. Comparison of sections stained with TTC or CFDA with similar unstained tissue viewed under

white light, fluorescence and scanning electron microscopy confirmed the effectiveness of the method for identifying living and metabolically active tissues within woody stems.

Combined with conventional dyes, the location and identification of structure, metabolic activity and living tissues was additionally verified in 9 non-native invasive and 8 native species common to the eastern and mid-western United States. Anatomical measurements of structure and starch levels, though characteristic, varied among the species examined. Despite these differences, the finding of living cells within the xylem, xylem rays and phloem was similar in all the species studied. Moreover, analysis with TTC or CFDA made it possible to determine the extent of tissue death in honeysuckle shrubs treated with foliage sprays of Roundup. Under these conditions, glyphosate was shown to lethally damage phloem and vascular cambium tissues within the stem. As invasive woody plants continue to disrupt the natural systems in North American forests, investigative procedures that commonly incorporate techniques to systematically evaluate the effectiveness of herbicidal control methods should become increasingly valuable.

ACKNOWLEDGEMENTS

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CHAPTER I

BACKGROUND INFORMATION

STATEMENT OF THE PROBLEM

What is an Invasive Species?

Composed of animals, plants, fungi, microbes and the abiotic physical environment, the biosphere consist of complex ecosystems that provide the necessary links needed to sustain life (Tansley 1935). Ecosystems provide a plethora of natural services, such as regulating atmospheric gas composition, influencing weather patterns, maintaining water cycles, generating soils, disposing of wastes, cycling of nutrients and controlling of pests, that provide a diverse and sustainable habitat for plants and animals (Ehrlich and Ehrlich 1981). During the mid-1950's scientists started to realize the overwhelming influence that human activities, such as global movement and transport and trade, had on the function and composition of ecosystem processes. The human capacity to alter landscapes, exterminate and disperse organisms from area to area started to be viewed as an exponentially influencing force that could disrupt natural ecological processes (Bates 1956). Altered habitats promote the introduction of foreign organisms non-indigenous to a particular landscape as well as the dispersal of established organisms into novel regions of the world (Fox and Fox 1986, Hobbs and Huenneke 1992, Anderson 1999, Lake and Leishman 2004).

Elton (1958), recognizing the shifting balance of organisms within the environment as a direct result of human influence, pioneered the idea that dislocated populations of microorganism's, plants and animals can become

explosive as ecological pests. Almost fifty years later, the extensive exchange of organisms between geographic areas that were historically biologically inaccessible continues to proceed at an alarming rate. It has been estimated that some 50,000 non-native species have already been introduced into the United States since European settlement (Pimentel *et al.* 2000). Moreover, at any given moment a calculated 10,000 marine species are currently being carried in ballast tanks (Carlton 1999) providing a starting point for new infestations. For this reason, environmental ecology has increasingly become global. In a global ecology (Bright 1995, Vitousek *et al.* 1996, Vitousek *et al.* 1997, Mack *et al.* 2000, Mooney and Hobbs 2000, Sala *et al.* 2000, Bax *et al.* 2003) any explosion of organisms that ecologically change the structure and dynamics of an ecosystem is considered to be a serious environmental threat (Pimentel *et al.* 2001, Reichard and White 2003).

An invasive species is generally defined as any organism that is non-native to the present landscape and whose introduction causes or is likely to cause economic or environmental harm (Pyšek 1995). Generally, the consequences of invasive species are not initially evident, due to the fact that they usually begin gradually and develop slowly over time. However, when they explode the effects can be tremendously destructive (Elton 1958, Cousens and Mortimer 1995, Kowarik 1995, Renaud 1996, Sakai *et al.* 2001, Rilov *et al.* 2004). By that time, feasible eradication of the invading species becomes complicated, problematic and generally costly. Examples of introduced organisms that have overrun an environment are numerous. Zebra mussels,

Dreissena polymorpha, accidentally introduced into North America in the late 1980's, are choking out native bivalve populations, fouling substrates and shifting food webs in the Great Lakes (Haag *et al.* 1993, Macisaac 1996). The brown tree snake, *Boiga irregularis*, which after being introduced into Guam from New Guinea is responsible for eradicating more than 80 percent of the native forest bird populations, decimating two-thirds of the native mammal populations and destroying nearly one-half of the native reptile populations (Fritts and Rodda 1998 and references within). Water hyacinth, *Eichhornia crassipes*, an aquatic plant transported from South America into Africa and parts of North America, form dense mats that physically clog, transform nutrient cycles and impact the macroinvertebrates and aquatic biodiversity in waterways (Masifwa *et al.* 2001, Hill 2003). One of the best-known and tragic examples of ecosystem damage in the eastern United States was the introduction of a species of exotic bark beetle from Europe in the 1920's. Unbeknownst to science, these beetles were carriers of *Ophiostoma ulmi*, a fungal pathogen of elm trees, which caused Dutch elm disease. As a consequence, native populations of American elm (*Ulmus americana*) trees in America have been devastated along the entire eastern coast (Gibbs and Wainhouse 1986, Hubbes 1999). Although a number of alternatives, such as better sanitation and the production of hybrid elm trees (Smalley and Guries 1993) are being employed to combat the disease, the once abundant tree species has been essentially extirpated from much of the United States.

Although the term is used commonly, the concept of what is an invasive species remains quite ambiguous (Binggelli 1994, Richardson *et al.* 2000, Shrader-Frechette 2001). For instance, species occurring in regions where they are not known to exist have been termed as alien, invaders, exotics, introduced, translocated, neophytes, adventive, weeds, newcomers, naturalized, colonizers, non-native, non-indigenous, imported, endemic and immigrants (Pyšek 1995, Davis and Thompson 2000). Yet, not all invasive species are non-native. Under the right circumstances any organism, native or non-native, is capable of increasing its range and/or abundance and exerting detrimental effects upon its environment. For example, a number of naturalized species such as sagebrush, *Artemisia tridentate*, (West *et al.* 1979), honey mesquite, *Prosopis glandulosa*, (Archer *et al.* 1988), creosote bush, *Larrea tridentate*, (Hastings and Turner 1980) and various groups in the genus *Juniperus* (Smeins and Merrill 1988, West 1993) have increased in range and/or density to the point in which they are becoming problematic. On the other hand, it is important to realize that just as some native species can become environmentally harmful if put in the correct context, not all non-native species become so. In fact, most introduced species ultimately fail to become established in a new environment. Williamson and Fitter (1996) found that among some angiosperms, terrestrial vertebrates, fishes, certain invertebrates and plant pathogens only one in 1000 species that are introduced into a new region ever develop into environmental pests. These researchers pioneered the tens rule where 1 in 10 species that are introduced into a new region will take hold in that new environment. Out of those that

initially take hold, only 1 in 10 will successfully become established. Of those that become established only 1 in 10 will transform into an invasive pest. Similarly, Kowarik (1995) found a 10:2:1 ratio of species invasiveness, where less than 10 percent of the introduced species successfully took hold, two percent become established and only one percent demonstrated invasive qualities. The majority of species that did get established possessed long lag periods, up to 147 years, before they became invasive. Generally speaking, it is the rule rather than the exception that most organisms must be re-introduced multiple times before becoming established within a new environment (Sax and Brown 2000). European starlings, for example, took at least 8 different introductions into North America before they became established and ultimately spread (Lever 1987).

A few authors have set out to develop more universal and descriptive schemes for defining invasion ecology terminology (Davis and Thompson 2000, Colautti and MacIsaac 2004), though none of these proposed schemes appear to have been widely adopted. In practice, only a few terms are necessary to define invasive origins: native or indigenous; non-native or exotic; introduced, naturalized or invasive (Webb 1985, Binggeli 1994). A native or indigenous species refers to one that evolved in an area or arrived by means independent of human activity, whereas, a non-native or exotic species is one that establishes itself in an area outside its original range through human activity. Native organisms have naturally evolved, genetically and morphologically, to an area while non-native organisms gain access to an area solely through accidental or

purposeful human introduction. Determination of the native status of a species can only be verified by fossil or historical evidence. On the other hand, habitat, geographical distribution, ease of naturalization elsewhere, genetic diversity, reproduction success and means of introduction can support an organisms non-native status in absence of other information (Webb 1985).

Richardson *et al.* (2000) provides a thorough and comprehensive review of invasive ecological concepts, nevertheless, the literature remains full of terminology, some of which is contradictory (Mack *et al.* 2000, Richardson *et al.* 2000 and references within, Davis and Thompson 2000 and 2001, Colautti and MacIsaac 2004). For the purpose of this thesis the subsequent definitions and concepts will be used **(Table 1)**. In this regard, an introduced species represents any organism that has been transported or displaced outside its previous geographical range in relation to human activity. Due to environmental constraints, the introduced species will either perish or survive and establish populations of adults capable of reproducing and increasing in number. With the establishment of self-perpetuating populations, capable of dispersing, these introduced species can reach a state of naturalization within the environment. In this state, the introduced species forms self-sustaining populations incorporated within the resident landscape. These populations are capable of living, growing, reproducing and dispersing within environmental limits while maintaining a non-destructive status within that particular environment. In a sense, these species have acclimated or adapted to achieve a state of equilibrium within the new range. Generally speaking then, an invasive species is one that, after it gets

Table 1. Summary of the basic terminology related to environmental invasion ecology and the associated definitions used in this report.

Term	Definition	Synonyms
Native Species	An organism that evolves naturally, developing genetically and morphologically to an area where it arrived by means independent of human activity.	Indigenous, Endemic
Non-Native Species	An organism that has establishes itself in an area outside its original/historical range through human introduction or activity.	Exotic, Alien, Translocated, Non-indigenous, Newcomer
Introduced Species	An organism that is transported by accident or on purpose outside its previous geographical range due to human activity.	Translocated, Imported, Immigrant, Colonizer
Naturalized Species	An introduced organism that becomes established in a new range to form self-sustaining and self-perpetuating populations capable of living, growing, reproducing and dispersing within the introduced site. Having acclimated or adapted to the point at which they achieve a state of equilibrium within their new range, these organisms continue to maintain a non-destructive status within that particular environment.	Acclimated, Adapted
Invasive Species	General: An organism whose introduction causes or is likely to cause economic or environmental harm. Environmental: An organism whose introduction and successful establishment in a new range leads to the point that they proliferate, spread and overcome established ecosystems. These organisms persist to drive the system towards simplification by disrupting the habitat and displacing naturalized species and processes previously established within that environment.	Invaders, Weeds

introduced and successfully established in a new range, continues to proliferate and persist to the point that it spreads and impacts established ecosystem properties. Disrupting the habitat and displacing the established naturalized species present within that community, this assault eventually drives the system towards simplification **(Figure 1)**.

Encompassing all biological trophic levels and all classes of communal interactions from viruses to microbes to fungi, plants and animals (Muzika and Liebhold 1997), invasive species are having a negative effect on local and national economies (Pimentel *et al.* 2000, Perrings *et al.* 2002). More importantly, invasive species are helping to accelerate evolutionary change (Palumbi 2001, Sakai *et al.* 2001) and in one way or another they are altering ecosystems, destroying habitats and disturbing established populations (Vitousek 1990, Lodge 1993, McDonald 1999, Mack *et al.* 2000, Pimentel *et al.* 2000, Simberloff and Strong 2000, Shrader-Frechette 2001). While historically, little concern was directed toward the negative effects of exotic species introductions (Colton and Alpert 1998), within the last few decades, this form of "biological pollution" has been realized more and more by ecologists as equal to if not more threatening than chemical pollution (McKnight 1993). In fact, exotic plant and animal invasions are second only to habitat destruction as a cause for the endangerment and extinction of native species (Wilcove *et al.* 1998).

Economically, invasive species on a whole costs the United States approximately $137 billion annually (Pimentel *et al.* 2000) with damage by invasive plants accounting for about one-half this amount (Morin 1999). Although

Figure 1. Theoretical path an introduced non-native organism can take to eventually become invasive. In this example, an organism native to Asia (yellow) is introduced into the eastern United States (red). The introduced species has three possible routes: (1) The organism may never become established and die. (2) The organism may become established and eventually become a non-invasive functioning unit of the landscape (naturalization). If a disruption occurs this organism may spread and become invasive. (3) The organism, finding a new novel habitat or having to out-compete the new competition, may spread to eventually become invasive.

much of the economic aspect of invasive alien species focuses on losses to agriculture, grazing, forestry and human health, the cost to the natural environment is equal if not more detrimental. As monetary consequences are intimately connected with invasions that threaten ecosystem-level processes (Mack *et al.* 2000), it is becoming more and more obvious that economies are ultimately dependent on the services provided by the ecosystem. For example, removal of the water loving tamarisk, *Tamarix*, shrub from riparian areas in arid and semiarid regions of the western United States would free-up more water from lakes, rivers and groundwater. In arid environments where water consumption is tightly split between municipalities, agriculture, hydropower and fisheries, the natural services provided by a tamarisk free ecosystem would be much more cost effective than finding alternative sources of water (Zavaleta 2000). It has been estimated that ecosystem services, things such as fresh water, waste and nutrient recycling, climate regulation, pollination and fertile soil, if assigned a monetary value, would cost 33 trillion dollars a year (De Marco, Jr. and Coelho 2004).

Whereas any native or non-native species can become invasive, non-native plants generally pose one of the greatest threats to the global ecology (Binggeli 1996, Mullin *et al.* 2000). Making up one of the lower tiers of most food webs and capable of interacting with both the above and below ground biota, plants help drive ecosystem processes. The introduction of invasive plants capable of disrupting these processes can result in the loss of ecosystem stability

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and functionality, thus shifting the composition of the systems dependent on their services.

Invasive Plants - Effects on Ecosystem Stability

Invasive non-native plants enter habitats through accidental or intentionally means. Traditionally, European settlers imported most non-native plants to North America for cultivation, aesthetic pleasure and horticultural trade. It has been estimated that 85 and 14 percent of woody invasive plants currently in North America were introduced for landscape purposes or agriculture and forestry, respectively (Reichard 1997). Currently, about 12 percent of plant species (2,100 species out of nearly 18,000) within the continental United States and Canada are termed as "weeds" by the Weed Science Society of America. Out of these 2,100 weedy species 65 percent (1,365) are non-native (Morin 1999). In Ohio, out of nearly 3000 total plant species, about 800 of these species are non-native and nearly 100 of these are invasive (Ohio Department of Natural Resources 2003).

In general, invasive plants are characterized by their fast growth, prolific fruit production, rapid vegetative spread, efficient seed dispersal and elevated germination rates (Baker 1974, Rejmánek and Richardson 1996, Mack *et al.* 2000). Coupled with the lack of natural predators, parasites or diseases these plants have the ability to exploit resources, out-compete and even displace established species (Blossey and Notzold 1995, Wolfe 2002, Mitchell and Power 2003, Torchin *et al.* 2003). If given the chance, invasive plants have the capacity to overtake communities, vastly impacting the amount of biodiversity and

ecosystem functionality (Vitousek *et al.* 1996, Parker and Reichard 1998, Wilcove *et al.* 1998, Cox 1999, Mack 2001). To identify the effects non-indigenous plants were having on ecosystem processes in Florida, Gordon (1998) discovered that 40 to 60 percent of the species measured were capable of altering ecosystem properties through disruptions in the geomorphologic, hydrologic and biogeochemical processes. Disruptions were observed at both the community and ecosystem scale.

Ehrlich and Ehrlich (1981) introduced the idea that non-disturbed ecosystems tend to function like well-oiled machines. But what happens when parts or components of that machine are removed or lost? Will the machine still function as it was designed to? In this same context, is it reasonable to ask to what extent an environment can withstand invasions from non-native plants before it loses functionality? Being composed of both above and belowground biota, plants link terrestrial and subsurface landscapes together (Wardle *et al.* 2004). Consequently, plants sustain community processes by influencing and being influenced from interactions both above (shoots) and below the surface (roots). The interplay between population and ecosystem level ecology depends on the feedback loops between these two subsystems (Wardle *et al.* 2004). Therefore, shifts in the plant community ultimately influence both the subsurface and terrestrial biota.

An answer can be seen in the introduction of purple loosestrife, *Lythrum salicaria*. In the early 1800's this wetland species was introduced to North American from Europe. Since escaping cultivation, it is now present in all 48

continental states, parts of Canada and continues to spread into new environments at a rate of more than a hundred thousand hectares per year (Thompson *et al.* 1987). By out-competing and reducing the biomass of native plants, purple loosestrife is singly transforming once lush wetlands into virtual monocultures. The displacement of native wetland plants, such as cattails, has resulted in the loss of rodent, reptile and waterfowl populations dependent on the native vegetation (Thompson *et al.* 1987, Gaudet and Keddy 1988, Blossey *et al.* 2001). As this example illustrates, the effects that even a single invasive species can have on the dynamics, composition and genetics of a landscape is an ecological concern.

Are certain plant species inherently invasive or are they naturally selected for developing invasive populations (Anonymous 2004)? It is uncertain whether introduced plants become invasive because they simply discover a new niche in a novel environment void of predators, parasites and diseases (Keane and Crawley 2002, Shea and Chesson 2002, Clay 2003, Mitchell and Power 2003, Torchin *et al.* 2003) or because they directly out-compete and replace native established species (Gentle and Duggin 1997, Weihe and Neely 1998, Deering and Vankat 1999, Callaway and Aschehoug 2000, Bais *et al.* 2003). Depending on the habitat and type of plant that is invading, it may be that both processes can transpire. It has been observed that hybridization events (Ellstrand and Schierenbeck 2000) and distinctions in genetic traits, such as photosynthesis rates (Schierenbeck and Marshall 1993, McDowell 2002), seed germination (Huenneke and Thomson 1995) and shoot architecture (Luken *et al.* 1995,

Meekins and McCarthy 2000), can provide an introduced plant with a means for becoming invasive (Lee 2002). Conversely, shifts in ecosystem processes such as pollination (Cox and Elmqvist 2000, Ghazoul 2002) fire regimes (Van Wilgen and Richardson 1985, Holmes and Cowling 1997) and herbivory (Schierenbeck *et al.* 1994, Higgens *et al.* 1999, Rogers *et al.* 2000) may benefit an introduced plant, thereby allowing it to become invasive. For instance, Stout *et al.* (2002) demonstrated how the introduced bush lupine, *Lupinus arboreus*, population exploded with the arrival of exotic bee pollinators. In these instances, the introduced plant is able to thrive without having to modify the new environment.

On the other hand, invasiveness is possible in the course of landscape transformation. Through allelopathy (Hierro and Callaway 2003), parasitism (Klironomos 2002, Bray *et al.* 2003, Carey *et al.* 2004) or interference competition (Amarasekare 2002) some plants have the ability to displace the native vegetation and modify the environment. In a well-known example, Bais *et al.* (2002) discovered root secreted allelopathic effects in *Centaurea maculosa* or knapweed. Interestingly, depending on the enantiomer produced, the chemical could be used either as a phytotoxin or antibacterial agent for inhibiting the growth of plant roots or pathogens, respectively, in the surrounding rhizosphere. It is theorized that the plant uses this substance as a weapon to invade a landscape and displace neighboring plant communities (Vivanco *et al.* 2004).

By whatever mechanism, once in a new environment, an invasive plant has the ability to reduce the biodiversity and productivity of the region (Luken 1988, Jackson *et al.* 2002) by drastically altering hydrological cycles (Pearce *et*

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al. 1985), light regimes, fire frequency and other biogeochemical processes (Scott *et al.* 2001). In fact, Whittaker (1970), found the primary productivity of invasive stands of Amur honeysuckle, *Lonicera maackii*, by itself, comparable in magnitude to that of an entire woodland community. In regions composed of little to no shrub layer, this shift could have drastic widespread effects. In general, when the type of vegetation changes, the processes that depend on that vegetation change as well.

An important and often overlooked means by which invasive plants can transform ecosystems is through the disruption of soil stability. Soils are themselves a complex ecosystem, rich in diversity and responsible for sustaining larger systems. Ehrlich and Ehrlich (1981) estimated that one square yard of pasture soil contains in excess of 45,000 worms, 10 million nematodes and 48,000 insects and mites. Likewise, in one gram of agricultural soil they discovered 30,000 single celled animals, 50,000 algae, 400,000 fungi and over 2.5 billion bacteria. Interacting directly with the soil substrate, these organisms control many of the processes, cycles and dynamics of the above ground vegetation. In a bottom up approach, shifts in soil fauna directly affect the plant and animal communities, while, in a top down approach transformations in the soil biota result from changes in the above ground plant community (Ehrenfeld and Scott 2001). Either way, introduced plants can substantially influence the composition of the soil fauna by altering decomposition cycles, energy dynamics, soil food webs (Wardle *et al.* 2004) and nutrient cycling (Ehrenfeld 2003).

The soil represents a heterogeneous mixture of different organisms responsible for recycling organic and inorganic material and energy back into the system. These systems have co-evolved complex associations comprised of a diverse plant community and their associated rhizosphere microbial populations (Swift and Anderson 1994, Brimecombe *et al.* 2001, Pinton *et al.* 2001). Many abiotic factors, such as temperature, pH, water content, soil type and porosity, and biotic factors, such as root exudates from the plant community, directly influence the diversity and activity of these microbial populations (Bais *et al.* 2004, Callaway *et al.* 2004). The health of the plant community is governed by the balance between beneficial and detrimental microorganisms (Brimecombe *et al.* 2001). The ability of an invasive species to affect this balance can generate changes in soil processes and community structure (Ehrenfeld and Scott 2001). In undisturbed temperate terrestrial ecosystems, organic plant leaf litter accumulates on the forest floor and provides a reserve, particularly nitrogen, phosphorous and moisture, of essential plant resources (Berg and Tamm 1994). Recycling of this litter, through decomposition by soil organisms, contributes to the formation of organic matter that maintains soil productivity (Berg and Matzner 1997). It is generally assumed that the rate of nutrient release by decomposition of the litter layer is due to the amount of available nitrogen (Berg and Tamm 1994). By supplying different types, quality and amounts of leaf litter, invasive species can influence the rates of nitrogen alteration, soil pH levels (Binkley and Valentine 1990), cation exchange capacity (Homann *et al.* 1992) and overall composition of organic matter (Saggar *et al.* 1999).

Studying the effects of two invasive understory species, *Berberis thunbergii* (Japanese barberry) and *Microstegium vimineum* (Camus grass), Ehrenfeld *et al.* (2001) discovered that plant invasions were causing soil ecosystem processes to change. Furthermore, these changes were attributing to the continued advancement of the invasion. Examination of litter dynamics showed that soil under invasive species generally had higher pH, nitrification rates and leaf litter quantity compared to soil under native plants. Moreover, the litter layer around barberry plants was found to be much higher in nitrogen, decomposed at a higher rate and had little to no nitrogen immobilization compared to native plant litter, whereas, the invasive grass species litter decomposed more slowly than native species and was able to immobilize nitrogen. Though the mechanisms of invasion may be different in the invasive shrub and grass, both of these species had the ability to out-compete the native plants for resources. Changes in soil chemistry, nutrient availability or litter quality have the effect of inducing different activities in the decomposing litter. This in turn may result in an increased or decreased rate of litter decomposition (Carreiro *et al.* 2000). By adapting to differences in litter chemistry, Kourtev *et al.* (2002) demonstrated that patterns of enzyme activity in decomposing litter were affected by the dominant above ground species. Through the stimulation of bacterial growth, nutrient renewal rates, substrate availability or moisture levels, invasive species are influencing the composition and diversity of surrounding plant communities (Pimm 1982, Zak and Freckman 1991, Polis and Winemillar 1996). This ability to manipulate fundamental soil properties may be an

important mechanism by which invasive species overrun intact communities (Ehrenfeld *et al.* 2001).

In the western United States, invasions of cheatgrass, *Bromus tectorum*, into stands of two different native grass species affected the soil organisms differently. In a community of galleta, *Hilaria jamesii*, invasion by cheatgrass increased the number of soil invertebrates, such as the flagellates, ciliates, mites and springtails. In contrast, in a community of speargrass, *Stipa comata*, cheatgrass reduced the number of these same soil invertebrates. Though opposite in affect, the invasion of *B. tectorum* drastically caused a shift in the soil biota from that of native grass communities (Belnap and Phillips 2001). Work by Walker and Smith (1997) have shown this species capable of manipulating a number of soil properties including soil moisture, temperature, nutrient cycling, primary productivity and litter quality. Coupled with these observed changes in soil dynamics, Belnap and Phillips (2001) further found that cheatgrass, in comparison to non-invaded sites, affected the architecture of native plant roots. These short-term changes are causing food web implications, which over time could radiate out through the rest of the ecosystem. It is yet to be proven if such affects are reversible. What is evident are the effects that invasive plants have on the composition and genetics of the native biota. Taken as a whole, the disruption by invasive species is a serious matter of ecological concern, but what can be done about it? "Where do we go from here" (White and Schwarz 1998)?

Physiological Ecology and the Balanced Design of Control Measures

The most obvious strategy of managing invasive species starts with predicting their invasiveness and either preventing their introduction or exterminating their spread before gaining a foothold (Mack *et al.* 2000, Allendorf and Lundquist 2003). Incorporating everything from population biology, taxonomy, geography, biological traits, genetics, evolution and history, the process of identifying and monitoring plant invasions is the greatest and generally most expensive line of defense (Reichard and Hamilton 1997, Goodwin *et al.* 1999, Reichard 1999, Sakai *et al.* 2001, Allendorf and Lundquist 2003). The policy on invasive species introductions is overwhelmingly a "shoot first, ask questions later" attitude (Ruesink *et al.* 1995). Yet, with over 2000 invasive plant species already present in the United States and an estimated 26,000 as possible invaders (Rapaport 2001), the introduction of additional plant species is inevitable. What then can be done about the invasions that are already or will become established?

Eradication of a widespread invasion is generally impractical, economically unfeasible and ecologically unstable (Simberloff 1997, Mack *et al.* 2000, Zavaleta *et al.* 2001, D'Antonio and Meyerson 2002). Yet, the control of invasive plants is a critical and integral part of ecosystem stewardship (Gaskin 2003). The act now, ask questions later mentality, needed to prevent initial introductions, seems unwarranted when devising management plans for controlling invasives already established. More appropriate is the need for what Schardt (1997) terms "maintenance control," where an established invasive is

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maintained at a level below where it is causing damage to the environment. Yet, all too often control measures fail due to the inability of incorporating scientific based knowledge into management expertise (personal observation). For instance, it has been observed that the effectiveness of herbicides, such as glyphosate (the active ingredient in the herbicide Roundup), on woody shrubs varies with changes in plant development, carbon allocation and metabolism (Fuchs and Geiger 2004). To use herbicides effectively, the "spray and pray" technique of trial and error is not always the most efficient approach. For that reason, it is important that strategies for managing plant invasion be based on sound science and embrace a wide range of disciplines.

To address why invasive species may be more or less responsive to control efforts, techniques should systematically include not only the information about the biology of the plant and its life history (Thompson *et al.* 1995, Meekins and McCarthy 2001, Schmitt and Riviere 2002) but also incorporate knowledge of basic plant physiology (Mooney 1991, Morris *et al.* 2002, Myers and Anderson 2003), phenology (Owens and Madsen 1998, Caffrey 1999, Zotz *et al.* 2000) and metabolism. Ultimately, control practices must include everything about the plants life history, physiological processes, environmental limitations, phenology, population biology, habitat, molecular systematics, genetics and evolution. In combination with appropriate information about the invaded ecosystem (With 2002), this approach will provide an informed, practical and integrated method for controlling invasive plants (Hobbs and Humphries 1995). Once the invasion is subdued, long-term restoration strategies must be employed. Generally, this is

an adaptive management program that returns the damaged system back to a self-sufficient approximation of its natural potential (Throop 2000, SER 2002). The basic tenants of such a project involve both a scientific and social endeavor (Davis and Slobodkin 2004) and include the re-establishment of ecological integrity and ecosystem function (USEPA 2000).

SIGNIFICANCE, OBJECTIVES AND GOALS OF RESEARCH

As more and more invasive plants dominate pristine environments, better schemes are needed to control their spread. Ecological based science together with control efforts and long-term restoration projects need to be both practical and effective. As a means to understanding and ultimately controlling woody plant invasions, this research project aimed to assess the location of herbicide-induced tissue damage. Research was undertaken because the vast majority of attempts to control invasive woody shrubs with herbicides have not fully acted upon the knowledge gained from studies on physiology. Furthermore, the project avoids the common error of trying to apply control measures without a clear understanding of plant processes, such as seasonal development, phenology and carbohydrate partitioning. The overall research goal was to determine how glyphosate damages woody plant stem tissue, but before examining the mechanisms of herbicidal damage in stem tissue, research techniques had to be developed to (1) identify and locate living cells within woody stems and (2) observe the effects that foliar applications of glyphosate have on these tissues. The ability to combine plant anatomy and physiology with chemical control

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measures was central in formulating a systematic control plan. Topics to be addressed by this study include:

(1) Determination of the feasibility of using histochemical techniques to study woody stem cell structure, metabolism and viability in relation to herbicidal control methods.

(2) Development of a practical and reliable procedure to use woody stem anatomy and physiology as an indicator of plant integrity.

(3) Determination of the effects of glyphosate on cell metabolism and viability in Amur honeysuckle stem tissue.

(4) Identification of the tissues affected by glyphosate in Amur honeysuckle stems.

(5) Assessment of the practicality of these techniques and their usefulness in the design and implementation of environmental control efforts.

Research promises to better reveal the anatomical basis of progressive herbicidal damage in woody plant stems. Locating possible physiological vulnerabilities, the project focuses and learning how to effectively apply control methods that take advantage of these vulnerabilities. By understanding the basis of herbicidal damage, this research lays the foundation for identifying developmental stages when glyphosate inflicts maximum damage to plant processes. Results should yield more precise methods on how to effectively apply herbicides for controlling invasive woody plant species. Using Amur honeysuckle as the test system, the following two chapters investigate rational designs for verifying herbicidal damage in plant stems using a combination of histochemistry, scanning electron microscopy and fluorescent microscopy. Chapter II specifically focuses on developing methods based on histochemical,

cytochemical and fluorescent techniques for assessing stem tissue viability. In Chapter III, the practicality of incorporating these techniques is observed in glyphosate treated tissue. Lastly, a rational design for herbicidal control of woody plant species that incorporate these techniques is formulated and discussed.

CHAPTER II

TOOLS FOR ASSESSING TISSUE VIABILITY IN NON-NATIVE INVASIVE AND NATIVE WOODY PLANT STEMS

INTRODUCTION

In the course of displacing or adversely affecting native populations, an invasive species is defined as any organism whose introduction causes or is likely to cause economic or environmental harm (Pyšek 1995, Mack *et al.* 2000). Responsible for approximately $137 billion annually in damage to both the economy and the environment (Pimentel *et al.* 2000), an increasing number of non-native specie introductions are both transforming the natural environment and devastating biodiversity (Harty 1986, McKnight 1993, Vitousek *et al.* 1997, Wilcove *et al.* 1998). Characterized by their fast growth, prolific fruit production, rapid vegetative spread, efficient seed dispersal, high germination rates and lack of predators (Rejmánek and Richardson 1996, Mack *et al.* 2000), invasive plants are negatively affecting the dynamics of the communities they invade. Altering processes both above and within the substrate, invasive plants have the capacity to completely overtake communities and alter ecosystem properties and function (Walker and Smith 1997, Mack *et al.* 2000, Belnap and Phillips 2001, Ehrenfeld and Scott 2001, Ehrenfeld *et al.* 2001, Mack *et al.* 2001, Evans *et al.* 2001, Kourtev *et al.* 2002, Kourtev *et al.* 2003).

Woody plants, with their numerous attributes, such as superior pollination, seed production, seed dispersal, lifespan and fruit diversity, are a constant invasion threat to any natural community (Binggeli 1996). Constituting about

one-half of the plants that frequently occur on most states invasive species lists (Bowen *et al.* 2002, Swearingen *et al.* 2002, Miller 2003) non-native woody plants are common invaders of forests (Binggeli 1999). With the majority of woody plants being introduced for ornamental (Binggeli 2001), landscape (Reichard 1997) or forestry purposes (Richardson 1998), an estimated 138 known tree and shrub species have invaded native forests in the United States within the last century (Campbell 1998). In many forest ecosystems, the emergence of a new woody layer in areas historically devoid of one is altering the dynamics of the entire forest system (Wyckoff and Webb 1996, Laatsch and Anderson 2000).

Many of these problem species have been studied and their invasiveness documented in detail. For instance, the Asiatic shrub Amur honeysuckle, *Lonicera maackii*, was introduced into North America in the early twentieth century (Luken and Thieret 1996). In Ohio, early vegetation surveys of the forest fauna made no mention of this species (Gordon 1969). Pollen records, dating back many millennia, reveal a remarkably open forest dominated by oak trees. With the exception of riparian areas, surrounding streams and waterways, these early forests contained little Underwood vegetation (Hutchinson *et al.* 2003). In the early 1960's this shrub was first observed as becoming plentiful in pastures and woodlands (Braun 1961). Within the last four decades, it has broadened its range and now is the dominant understory plant not only in Ohio but also in much of the eastern United States (Trisel 1997, Hutchinson and Vankat 1997). In areas where Amur honeysuckle has never invaded or been removed, the physical architecture of the forest is much different from invaded landscapes

(Figure 2). On the other hand, where the shrub is established, it negatively impacts sapling emergence, recruitment (Gorchov and Trisel 2003) and the growth of native herbaceous plants (Collier and Vankat 2002). Similarly, other exotic shrubs now common to the northeastern United States such as Japanese barberry, *Berberis thunbergii*, and glossy buckthorn, *Rhamnus frangula*, also are responsible for displacing native understory shrub communities, affecting soil ecosystem processes and altering the composition of the herbaceous plant community (Ehrenfeld *et al.* 2001, Frappier *et al.* 2003). For maintaining the integrity and diversity of natural forest ecosystems, the design of practical methods to control the spread of these woody species is essential.

In deciduous woody plants, stem tissues play a critical function not only for the movement and translocation of water and nutrients but also as living storage areas for substances such as amino acids, protein, minerals and nutrients (Kozlowski 1992, Sauter and van Cleve 1992, Sauter and Neumann 1994). Furthermore, stem tissues are metabolically active, in retaining and breaking down carbohydrates, and photosynthetically capable of fixing carbon (Witt and Sauter 1994, Aschan and Pfanz 2003). The ability to locate and discern living tissue is crucial for verifying the effectiveness of herbicidal control methods. For instance, in the aquatic plant hydrilla, *Hydrilla verticillata*, carbohydrate allocation patterns shift throughout the year between shoot and tuber growth (Owens and Madsen 1998). The researchers discovered that these seasonal shifts in growth and carbohydrate storage offered optimal opportunities for administrating control efforts. In a similar manner, woody deciduous plants annually cycle through

Figure 2. Photographs comparing the physical architecture of a forest woodland invaded with and absent of an Amur honeysuckle layer. An invaded site in Beavercreek, OH depicts a dense honeysuckle shrub layer. **(A)** Notice how the shrub dominates the understory vegetation by shading out and **(B)** preventing the growth of a herbaceous ground layer. In comparison, the architecture of a non-invaded forest in nearby Hamilton, OH depicts **(C)** a landscape devoid of a shrub layer but with a **(D)** diverse herbaceous ground layer. Similar to historical accounts the non-invaded site has an open forest with little intermediate vegetation and a large amount of ground cover, whereas, the invaded site has an abundant intermediate layer and little to no ground cover.

(Photographs by Mark A. Fuchs)

periods of bud break, leaf development, bud set, nutrient reclamation and dormancy (Coleman and Chen 1996, Kozlowski and Pallardy 1997). In fact, between 50 and 90 percent of nitrogen from senescing leaves is translocated to storage sites, primarily stem bark, during autumn (Kang and Titus 1980, Chapin and Kedrowski 1983, Wetzel *et al.* 1989) and used for new leaf growth the following spring (Deng *et al.* 1989, Millard and Neilsen 1989). For this reason, it is logical to assume that for effective control of invasive woody plants, techniques are needed that unite invasion management with an understanding of metabolism and nutrient availability.

To track the transport of materials through the phloem and xylem, dyes as probes have been successfully incorporated into plant research (Oparka 1991, de FaŸ *et al.* 2000). Utilizing a fluorescent dye as a tracer, Fisher and Cash-Clark (2000), were able to measure the physiology of sieve element unloading and post-phloem translocation of organic molecules in wheat plants. Tattar and Tattar (1999) using a variety of xylem-mobile dyes were able to study the movement of material through the xylem vessels of plants. Similarly in fungus, Stewart and Deacon (1995) revealed a number of dyes that could be used as possible markers for tracing translocation, growth and differentiation. Many of these dyes are actively taken up, excluded or altered by cells and can be employed directly as a way for locating viable tissues. Fluorescein based fluorescent stains have been used as both a tracking dye to measure translocation of phloem sap throughout plants (Grignon *et al.* 1989) as well as a vital dye for identifying living plant tissue (Widholm 1972, Popov and Vysotskaya

1996). Carboxyfluorescein diacetate (CFDA) by itself is a non-fluorescent molecule capable of passing through cellular membranes. As a vital fluorescent dye, endogenous esterases within functioning cells cleave the acetate group and release carboxyfluorescein within the cell. The newly activated fluorescein molecules now trapped within the cell fluoresce green when excited by blue light. Thus, only living cells capable of biochemically activating the dye emit green light (Ruzin 1999).

Studies incorporating reactive viability dyes such as CFDA or tetrazolium (TTC) have primarily focused on cultured plant cells (Widholm 1972, Steward *et al.* 1999, Amano *et al.* 2003), apical meristems (Tanino and McKersie 1985, Popov and Vysotskaya 1996), roots (McKersie *et al.* 1999) or buds (Busso *et al.* 1989, Becker *et al.* 1997, Hendrickson and Briske 1997). Their practical utilization in intact plant stem tissue, pursued in the present study, has not been extensively demonstrated (Steponkus and Lanphear 1967, Nesbitt *et al.* 2002). Swain and De (1994), using plant protoplast tissue, evaluated 7 vital dyes and concluded that some produced better results than others under varying conditions. In their study, acridine orange and Evan's blue were found to be suitable indicators of protoplast activity whereas phenosafranine and neutral red were not.

Because stems play such a major role in the development of woody plants, research was undertaken to construct and assess a procedure capable of detecting cellular and tissue level viability in woody stem sections. Techniques to evaluate success of control methods could benefit the management of woody

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plant invasions. For example, herbicides, when applied at an appropriate time throughout the growing season, have been used to successfully control a number of woody species, such as the tree of heaven, *Ailanthus altissima* (Burch and Zedaker 2003). However, for a number of reasons, attempts to design effective herbicide programs to control woody invasions have been sporadic (Lanini and Radosevich 1982, Danieri and Zedaker 1990, Zedaker 1990, Netzer and Hansen 1992). A detailed examination of the location and extent of stem tissue damage in response to herbicide treatment could identify times when a control method is most effective. Employing cytochemistry, light and fluorescent microscopy, eight vital stains were surveyed for their ability to precisely locate living tissues within stems. When vital stains were able to delineate areas of living cells in fresh stem sections, the type of tissue along with its metabolic activity were further evaluated through histochemical identification and scanning electron microscopy (SEM), respectively. The integration of techniques provides an opportunity for assessing shoot damage in response to herbicide applications. Such techniques could assist in the development of more effective land management strategies for controlling invasive woody plants.

MATERIALS AND METHODS

To design and evaluate a procedure for assessing viability in woody plant stems, the study was separated into two associated components **(Figure 3)**. The first objective involved refining a method for sectioning, maintaining and testing live woody stem segments for tissue viability. The second objective involved choosing the most effective viability stains and verifying the location and metabolic activity of living tissues against standard histochemical techniques and scanning electron microscopy. Amur honeysuckle, *Lonicera maackii*, stems were used as the initial test species but was later confirmed in a variety of other plants. Unless otherwise noted, all chemicals, reagents and pigments were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Sampling Site

Stems of Amur honeysuckle (*Lonicera maackii*) along with eight other introduced invasive, consisting of Japanese honeysuckle (*Lonicera japonica*), common privet (*Ligustrum vulgare*), autumn olive (*Elaeagnus umbellate*), winged burning bush (*Euonymus alata*), multiflora rose (*Rosa multiflora*), oriental bittersweet (*Celastrus orbiculatus*), winter creeper (*Euonymus fortunei*), common buckthorn (*Rhamnus cathartica*) and Japanese knotweed (*Polygonum cuspidatum*) (Heidorn 1991, Possessky 2000, Miller 2003), and eight historically documented native, consisting of American bittersweet (*Celastrus scandens*), blackhaw (*Viburnum prunifolium*), gray dogwood (*Cornus racemosa*), American hazelnut (*Corylus Americana*), ninebark (*Physocarpus opulifolius*), spice bush (*Lindera benzoin*), swamp rose (*Rosa palustris*) and Virginia creeper

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Figure 3. Flow chart depicting the methods used to develop a simple, effective and practical stem tissue viability test for assessing woody plant stems.

Assessment of Stem Viability

Objective 1

→ collect stem tissue

methods to section woody tissue

assemble a staining system to observe viability

Most Effective Systems →

Objective 2

→ verify location and type of tissue

Metabolism → Starch
Chlorophyll

Structure → Histochemistry
Cytochemistry
Scanning Electron Microscopy

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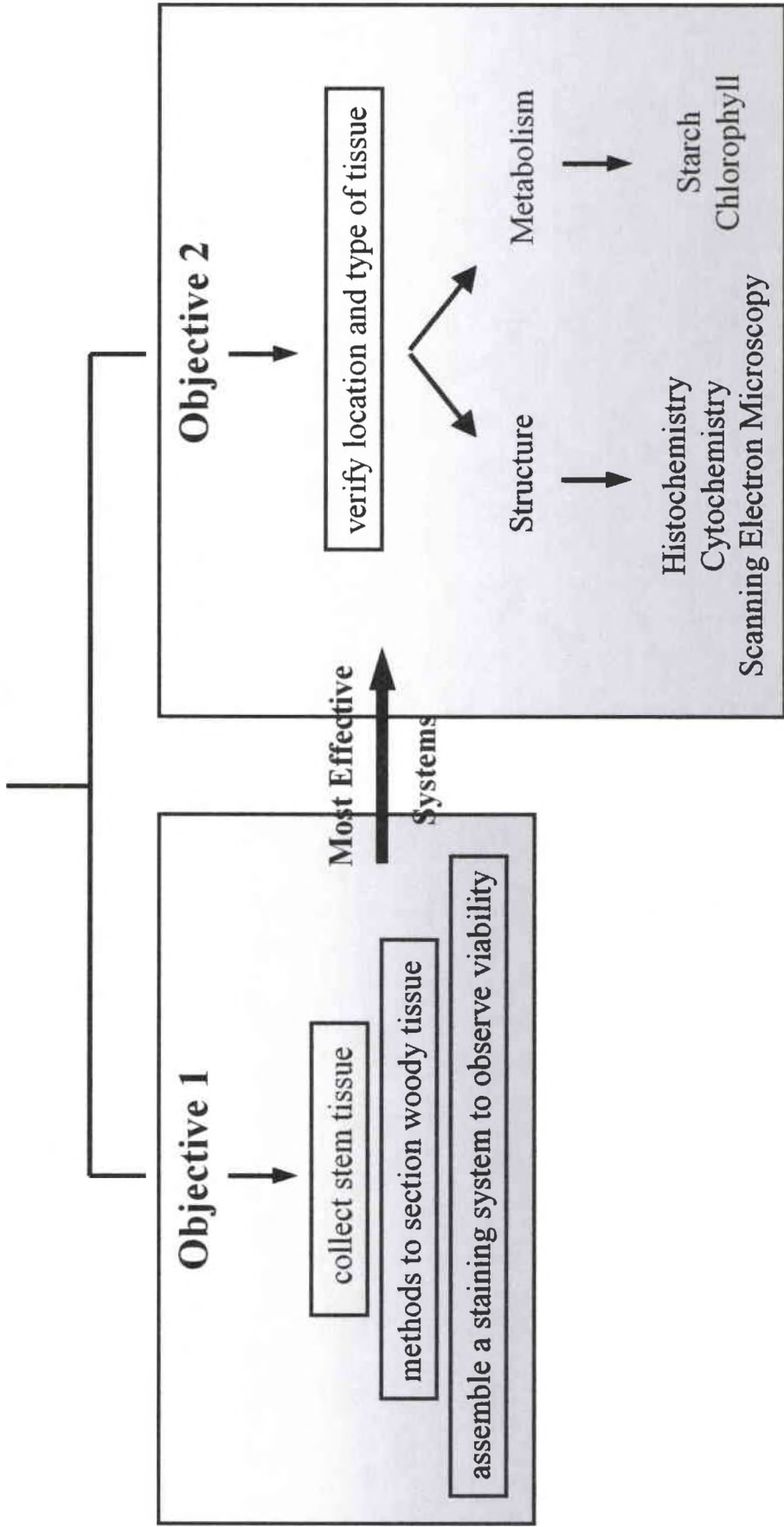
(*Parthenocissus quinquefolia*), woody plants common to eastern North America (Gordon 1969, Cooperrider *et al.* 2001) were identified and samples collected at Mt. St. John in Beavercreek, OH. Where non-invaded or managed for woody invasives, the well-drained upland site consists of a temperate forest canopy layer dominated by oak (*Quercus*), elm (*Ulmus*), ash (*Fraxinus*), hickory (*Carya*) and maple (*Acer*), an open intermediate shrub layer composed of redbud (*Cercis canadensis*), spice bush (*Lindera benzoin)* and pawpaw (*Asimina triloba*) and a diverse ground layer consisting mainly of white snakeroot (*Eupatorium rugosum*), violet (*Viola*), greenbrier (*Smilax*), Virginia creeper (*Parthenocissus quinquefolia*), wood nettle (*Laportea canadensis*), clearweed (*Pilea pumila*), and avens (*Geum*). Management of invasive species and restoration efforts has been on going at this site since 1986. Techniques to control invasive plants and reverse their harm to the environment are currently being implemented and include activities to integrate history, science and technology (Geiger *et al.* 2004).

Tissue Collection and Sectioning

First or second year stems were collected from mature plants with hand clippers during winter (January thru March 2004). Cut ends were wrapped in wet paper towels for transport from the field to the laboratory and subsequently submerged in water at room temperature until being removed for analysis. Stems stored for longer than 48 hours were discarded for fresh samples. Living stems were cut 1-2 cm from the base and the internodal tissue was cross-sectioned by hand using a modified rotary microtome (Ernst Leitz Wetzlar) adapted with a non-moveable flat aluminum plate **(Figure 4)**. The plate provided

a hole for tissue to pass through as well as a base for guiding the hand sectioning of tissue. Using the microtome's feeding mechanism to control section thickness, live stems were sectioned by hand with a steel double-edged microtome blade (Fisher Scientific, Pittsburg, PA, USA) to a selected thickness between 40-120μm. Cut sections were quickly placed into an appropriate buffer for corresponding live tissue analysis or fixed in a solution containing formalin, acetic acid and ethanol (FAA) as prepared by Ruzin (1999).

Viability Screening

For developing an optimal buffer and dye system by which to observe stem tissue viability, nine historic buffering systems **(Table 2)** and ten recognized vital dyes **(Table 3)** were examined. Buffer solutions consisting of 50 mM, pH 6.9 PIPES (Chaffey and Barlow 2001), 50 mM, pH 7.4 HEPES (Tanino and McKersie 1985), 50 mM, pH 7.4 MOPS and 50 mM, pH 5.5 MES (Good *et al.* 1966, Ferguson *et al.* 1980) with and without supplemental medium (10 mM MgSO4, 10mM KCL, 10mM CaCl2, 10 mM NaCl, 3 mM DTT and 200 mM sucrose; modified from Gill *et al.* 1999) were prepared and tested for buffering capacity by visual comparison against DI water. Sections were incubated in each buffer solution and analyzed for color, turgidity and structural integrity every hour for 24 hours. The buffering system that performed the best was employed in the *in-vitro* viability studies.

The general properties, wavelength requirements, viability functions, mode of action and associated costs for each dye examined in this study are listed in

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Figure 4. Photograph of hand-sectioning aid constructed from a rotary microtome. Insert depicts the aluminum slab used to hand section living woody stem tissue. Using this set-up, stems could be sectioned reproducibly between 40-120μm, depending on the hardness of the wood.
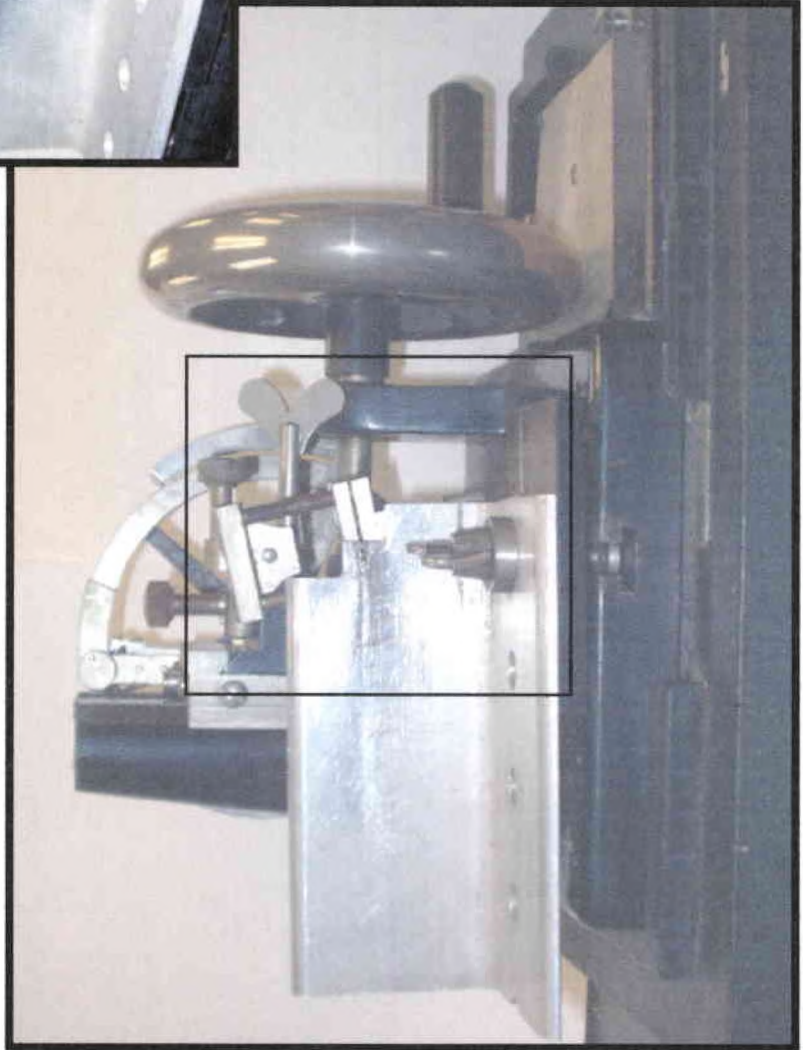
Table 2. List of the various biological buffers used in this study and their useful pH ranges. Buffers were used to maintain cellular integrity of woody stem cross sections during *in-vitro* incubation. All buffer solutions used were at a concentration of 50mM.

Buffer	pK_a	Useful pH Range	Formula Wt.	Working pH[*]
HEPES	7.5	6.8 – 8.2	238.3	7.4
MES	6.1	5.5 – 6.7	213.2	5.5
MOPS	7.2	6.5 – 7.9	209.3	7.4
PIPES	6.8	6.1 – 7.5	302.4	6.9

[*] final pH adjusted with KOH

Table 3. List of the vital dyes used in this study and information pertaining to their fluorochrome status (if available), use as a living or dead stain, associated cost and general mode of action.

Stain	Ex* λ	Em λ	Detects	Cost	Mode of Action
Acridine Orange	blue	Green	living cells	low	• acid organelle • DNA binding
Ethidium Bromide	green	Orange	dead cells	moderate	• cell membrane impermeability • DNA binding
Evans Blue	visible light		dead cells	low	• cell exclusion stain
Fluorescein-Derived CFDA FDA Calcein-AM	blue	green	living cells	high low expensive	• cleaved and retained by esterases within the cell
Phenosafranin	visible light		dead cells	moderate	• cell exclusion stain
Propidium Iodide	green	red	dead cells	high	• nuclear stain
Rhodamine B	green	red	living cells	low	• mitochondria stain
Tetrazolium	visible light		living cells	low	• dehydrogenase enzymes in respiration

*Ex   = excitation wavelength
 Em   = emission wavelength

Table 3. Visible-light stains examined in this study consisted of tetrazolium, Evan's blue and phenosafranin whereas the fluorescence dyes consisted of fluorescein derivatives, ethidium bromide, propidium iodide, phenosafranin and acridine orange. Depending on the type of stain, the stained result will either indicate living or dead tissue. As staining times and concentrations differed, a list of the specific staining procedures is given in **Appendix A**. To verify that the appropriate tissue was being stained, stem sections were killed by boiling in 50mM HEPES buffer for 10 minutes and compared against fresh tissue. Boiled tissue was cooled to room temperature and stained as above. Boiled and non-boiled tissues were compared for amount and location of coloration (Gaff and Okong'O-Ogola 1971, Busso *et al.* 1989). Observable differences between the two sets of tissue represented a positive result and further examination, whereas, those that showed inconclusive or ambiguous results were removed from further testing.

Determination of Tissue Autofluorescence

Because many plant substances and tissues such as flavins, NADH, elastin, collagen, lignin and chlorophyll are naturally autofluorescent **(Table 4)** it can be difficult to decipher between fluorescently stained tissue and natural background excitation (Ruzin 1999, Knight and Billinton 2001). To decipher between these, the location, intensity, pattern and sensitivity of unstained (autofluorescent) and stained sections were compared using an image processing and analysis program (Scion Image for Windows, Scion Corporation, version Beta 4.0.2, 2000). With the use of built-in color look-up tables (LUT), this

Table 4. Intrinsic fluorescence or autofluorescence of various plant derived materials and their corresponding excitation and emission wavelengths.

Structure	Excitation Wavelength (nm)	Emission Wavelength (nm)
Chlorophyll	430-550	685, 740
Flavins	445	520
Lignin	365, 488	450-480, 510-530
Protolignins	415-445	>470
Suberin	365	570
Guard Cells	450	520
NADH & NADPH	360-390	440-470
Elastin & Collagen	440-480	470-520

[Information attained from Ruzin (1999) and Knight and Billinton (2001)]

program transforms a digital image by assigning each pixel a unique color range between 0 (white) and 255 (black). Based on the micrograph, a three-dimensional surface plot profile was constructed. Patterns of emission for unstained (autofluorescent) and stained sections were compared for location, amount and intensity of fluorescence within the tissue.

Identification of Tissue Type and Metabolism

Stem sections were fixed overnight at room temperature in FAA, washed in FAA without fixative, dehydrated or re-hydrated through an ethanol graded series and identified histochemically with conventional stains, such as toluidine blue (TBO), phloroglucinol, safranin-fast green and aniline blue **(Table 5)**, capable of differentiating tissue based on structural composition (Ruzin 1999). Additional information about the specific procedures used for preparing the fixative and stains are presented in **Appendix B**. Stained sections were visualized under brightfield light microscopy with a Nikon inverted microscope (Eclipse TS100). Digital images were taken with a Nikon Coolpix 950 digital camera (Nikon Inc., USA).

For locating metabolically active tissues, stem sections were evaluated for the presence of chlorophyll and starch. Chlorophyll, which naturally emits red wavelengths (685nm) when excited with blue light (430nm up to 550nm), was located in freshly cut stem sections using a microscope equipped with a broadband emission filter and capable of epifluorescence (Olympus BHTU biological microscope with BH2-RFL fluorescent attachment equipped with a FITC filter cube - excitation filter = 490nm and barrier filter = 515nm). Digital

Table 5. List of conventional histochemical stains and autofluorescent capabilities of materials used in this study for visualizing tissue types and metabolic activity. Information regarding the stain and associated tissue specificity and target sites is shown.

Stain	Tissue Specificity	Target Tissues
Phloroglucinol	Xylem	• lignin in xylem and phloem fiber cells stain purple
Aniline Blue	Phloem	• callose in phloem vessels stain blue
Toluidine Blue	Vascular Tissues	• lignin in xylem, phloem fiber cells and periderm bark stains blue • phloem tissues stain purple
Safranin + Fast Green	Vascular Tissues	• lignin in xylem, phloem fiber cells and periderm bark stain red-pink • xylem ray cells stain red • phloem tissues stain light blue or green
Iodine	Starch	• stains starch grains stain black
Blue λ Autofluorescence	Chlorophyll	• chlorophyll fluoresces red

images were captured with a real life camera attachment and processed to a PC. Starch deposits were located by conventional histochemical means by incubation in iodine (Ruzin 1999). Stained sections were visualized under brightfield light microscopy with a Nikon inverted microscope (Eclipse TS100) and images captured with a digital camera.

Visualization of Tissue Structure with Scanning Electron Microscopy (SEM)

For obtaining three-dimensional surface images of stem cross sections on the SEM, a compilation of procedures from Robards (1978), Falk (1984), Jansen *et al.* (1998) and Ruzin (1999) were used and evaluated. Fresh stem tissue was hand sectioned as described above and either air dried at room temperature (23°C) or fixed. For the latter, sections were fixed and preserved for 48 hours in either cold FAA (50% EtOH) or 4% glutaraldehyde in 50mM HEPES buffer. Fixed sections were washed twice to remove excess fixative, dehydrated into absolute EtOH through an ethanol-graded series and critically point dried (AUTOSAMDRI-814B; Tousimis) with liquid CO_2.

Under a dissecting microscope, undamaged dried sections were transferred and mounted onto an aluminum stub coated with conductive tape and sputter coated (Denton Vacuum, LLC Desk II Cold Sputter/Etch Unit) with gold to a thickness of 100Å (50mTorr at 45ma for 30 seconds). Secondary electrons were imaged in a JEOL 5800LV scanning electron microscope (JEOL technics LTD, Tokyo, Japan) at 15 kV using a high-resolution 20μm objective lens aperture. Digital images were stored and processed for contrast and brightness in Adobe Photoshop (Adobe Systems Inc., San Jose, CA). For enhanced image

resolution, a combination of adjacent images were processed at high magnification and combined in Adobe Illustrator to form a photomontage.

Confirmation and Application of Viability Technique

To determine the location and type of tissue that was structurally intact and metabolically active in woody stems, similar chlorophyll, starch and structural micrographs were compared against viability micrographs. Reproducibility and confirmation of the technique, initially developed in Amur honeysuckle stem tissue, was then applied to 16 other non-native invasive and native woody species for verification of its use as a wide-ranging application.

RESULTS

Tissue Collection and Sectioning

Since studies of tissue viability require that the sections remain intact and functional, the collecting and sectioning of tissue was a critical step. The incorporation of the modified microtome was crucial for two reasons. First, it kept the tissue to be sectioned stationary, thus providing the user with complete control of the blade. This allowed for hand sectioned to be performed without damage to the woody stem. Second, the feeding mechanism could be adjusted to advance the tissue at desired thickness between 40-120μm while providing accurate and reproducible sections. This sectioning technique proved to be a more effective method than simply cutting the sections by free hand.

Buffer System and Vital Dye Screening Evaluation

For testing the ability of the buffer to maintain sectioned stem cross-sections alive and functional, 50mM HEPES at pH 7.4 greatly outperformed the other buffers (HEPES > PIPES = MOPS > MES > water). Stem sections placed in HEPES buffer were intact and functional for more than 24 hours after initial incubation. Under these testing conditions, the buffers without additional supplemental growth factors performed better than those with it **(Table 6)**.

The fluorescein-based fluorochromes greatly outperformed the rest of the fluorescent dyes **(Table 7)** in their ability to detect viable woody stem tissue. Of the fluorescent dyes tested, only fluorescein produced a noticeable difference when compared against boiled tissue **(Figure 5)**. In addition, CFDA, due to its more rapid uptake ability and greater retention time, performed noticeable better

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Table 6. The results of the buffering capacity experiment are presented. The investigation was designed to evaluate various buffer systems for their ability to retain color, turgidity and viability of stem tissue placed *in-vitro*. Sections were incubated in different buffer systems and visually assessed every hour for 24 hours. At the end of this time, a value between 1 and 5 was given to each system based on characteristics in appearance and integrity. At the end of 24 hours an overall score was tallied. The higher the value the better the system performed.

Buffer	Color Retention	Turgidity	Viability Specificity	Duration	Overall
HEPES	5	5	5	5	20
HEPES + Medium	4	4	5	4	17
MOPS	4	4	4	4	16
PIPES	4	4	4	4	16
PIPES + Medium	3	3	4	3	13
MOPS + Medium	3	3	3	3	12
MES	3	3	3	2	11
MES + Medium	3	3	3	2	11
Water	2	3	1	1	7

Table 7. Comparison of the results obtained from *in-vitro* stem viability experiments incorporating fluorescent and visible light staining systems. Woody stem sections were examined for the amount of autofluorescence (where available), differences in staining between fresh and boiled tissue and overall performance.

Stain	Autofl[a]	Fresh vs Boiled Tissue	Overall Performance
Fluorochromes			
Acridine Orange	high	same	• ambiguous staining of lignified tissue (xylem and phloem fiber cells)
Ethidium			
Bromide	high	same	• ambiguous staining over all tissue
Homedimer-1	nd [b]	nd	• not tested due to high cost
Fluorescein-Derived			
CFDA	minimal	differed	• fluorescence of xylem, xylem rays
FDA	minimal	differed	and phloem; not in boiled tissue
Calcein-AM	nd	nd	• not tested due to high cost
Propidium Iodide	Nd	nd	• not tested due to high cost
Rhodamine B	high	same	• ambiguous staining over all tissue

Table 7. (Continued from page 50)

Stain	Autofl	Fresh vs Boiled Tissue	Overall Performance
		Visible Light	
Evan's Blue	n/a [c]	same	• stained phloem tissue blue
Phenosafranin	n/a	same	• stained lignified cell walls (xylem, pith, phloem fiber cells and periderm) red; phloem colorless
Tetrazolium TTC	n/a	differed	• stained primary xylem, xylem rays and phloem red; stain not present in boiled tissue
Nitroblue	n/a	same	• stained phloem tissue blue, periderm pink

[a] Autofluorescence or intrinsic fluorescence of unstained control tissue
[b] nd = not determined
[c] n/a = test not applicable

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Figure 5. Cross section of Amur honeysuckle stem tissue illustrating the difference between fresh and boiled tissue incubated in CFDA. Micrograph images depict **(A)** fresh and **(B)** boiled tissue viewed under brightfield microscopy and **(C)** fresh and **(D)** boiled tissue stained with CFDA and viewed under blue actinic light. Notice the lack of chlorophyll and fluorescence in the boiled tissue under visible light and when incubated in CFDA (arrows). All images were taken at 100x magnification. Scale bar represents 100μm.
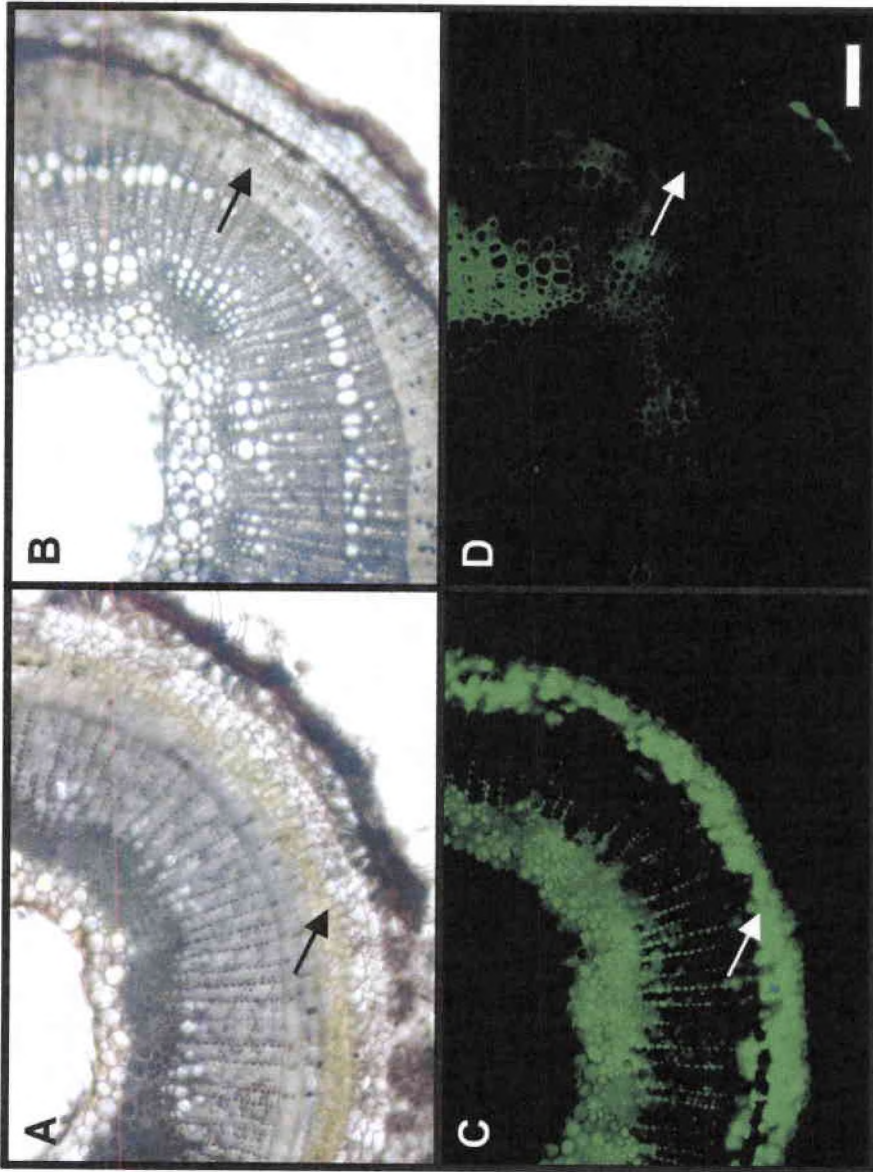
than its molecular cousin, fluorescein diacetate (FDA). On the other hand, ethidium bromide, acridine orange and rhodamine B did not show any clear distinctions between living and dead tissue. It is worthwhile to note that ethidium homedimer-1, propidium iodide and the fluorescein-based dye, Calcein-Am were not tested due to their high overall cost. It is believed that the specificity and price of these dyes would anyway be unrealistic for general use.

Of the visible light stains, only TTC-based dyes produced results unlike that of control tissue **(Table 7)**. Within the TTC-based stains just 2,3,5-triphenyl tetrazolium chloride showed a marked difference when comparing against boiled stem sections **(Figure 6)**. Interestingly, stems that were cross-sectioned before being incubated produced ambiguous results. Only when longitudinally slices were incubated did TTC stain for viability. Evan's blue, phenosafranin and nitroblue tetrazolium showed no discrimination in staining for dead or living tissue; rather they reacted with the phloem and xylem more like that of a structural stain.

The systems containing CFDA or TTC in HEPES buffer performed the best and were chosen as the optimal systems under the conditions provided in this study. Although not a totally exhaustive screening, both CFDA and TTC showed promise as a practical stain for observing living tissue in woody stems. Therefore, these systems warranted further examination.

Comparison of Autofluorescence and Location of Living Tissues

Before a fluorescent dye can be accepted, it must be viewed against natural autofluorescence. Under blue actinic light, autofluorescence, when

Figure 6. Amur honeysuckle stem sections illustrating the difference between unstained and TTC stained living and dead tissue. **(A)** Photograph of longitudinally sectioned mature stems incubated either in buffer (top photo) or buffer with TTC (bottom photo). Notice the deep red staining of the stem tissue incubated in the presence of TTC. Scale bar represents 50mm. The remaining micrographs show stem cross sections of **(B)** non-treated fresh tissue **(C)** TTC stained fresh tissue and **(D)** TTC stained boiled tissue observed under visible light. Notice how just living fresh tissue colors red. Coloration is most prominent in the xylem ray cells and phloem band. All images were taken at 100x magnification. Scale bar represent 60μm.
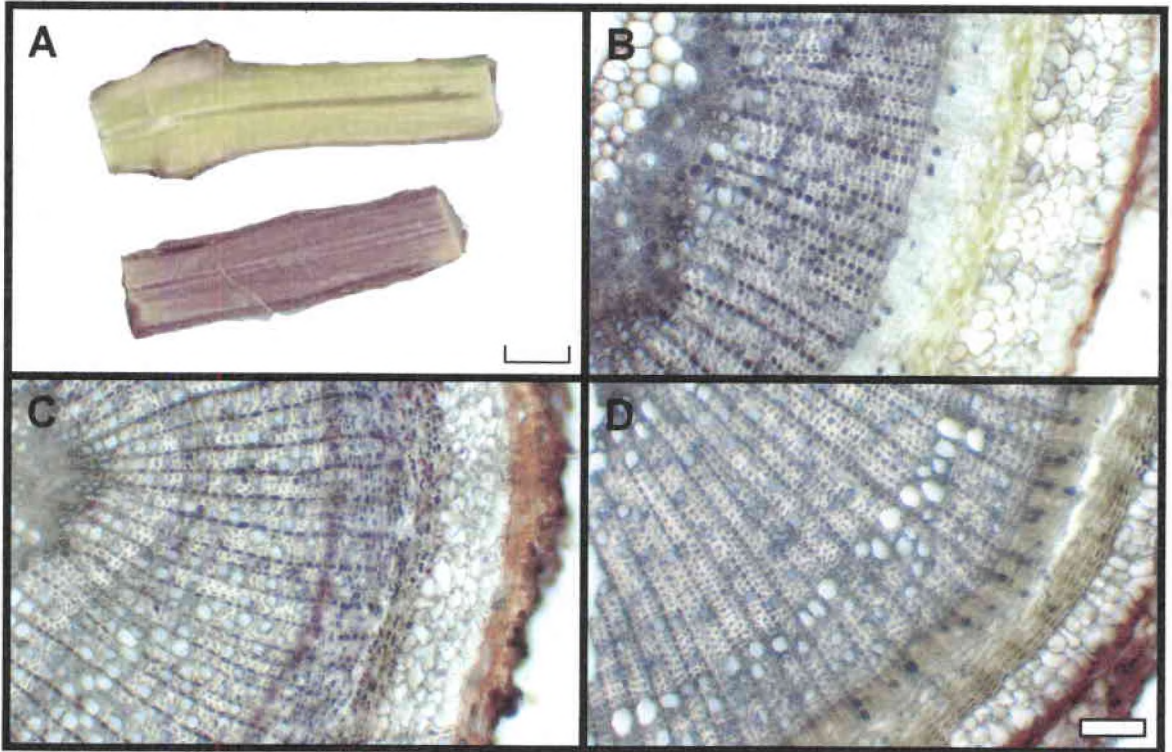
noticeable, was observed most prominently in lignified tissues of the xylem, periderm and outer pith. In fact, all one-year-old woody stem sections observed in this study showed some sort of autofluorescence in these regions, irregardless of the species being examined. Yet, in comparison to stem tissue incubated in CFDA, autofluorescence was minimal. CFDA treated stem tissue fluoresced intensely green in the xylem, xylem ray and phloem tissues **(Figure 7)**. When compared with untreated autofluorescent tissue viewed at equal sensitivity, CFDA treated stems fluoresced much brighter with distinct and recognizable cellular arrangement. When analyzed for location within the stem, CFDA treated tissue showed distinct spikes in the primary xylem, xylem rays and the phloem whereas untreated tissue viewed under autofluorescence had peaks in tissues that corresponded to cells within the pith and periderm **(Figure 8)**.

Identification of Tissue Type and Stem Metabolism

Phloroglucinol, stained lignified tissues red and was used to identify xylem and phloem fiber cells, whereas, aniline blue turned callose particles bright blue was used to identify the phloem band. Solutions of toluidine blue O and safranin-fast green stained lignified xylem tissue blue or red and pectinated tissues of the phloem purple or green, respectively, were further used to differentiate between vascular tissue types.

Starch grains, when present, turned black in the presence of iodine and were commonly observed in the primary xylem and xylem ray cells of woody stems. Similarly, the characteristic red autofluorescence of chlorophyll was

Figure 7. Comparison of Amur honeysuckle stem cross sections viewed under autofluorescence **(A-D)** or incubated in CFDA **(E-H)**. Micrographs show stem sections under **(A and E)** visible light, **(B and F)** blue wavelength actinic light, enhanced color-imaging software and presented as a **(C and G)** spectrum of different intensity colors or as a **(D and H)** three-dimensional surface plot. Micrographs were taken at 100x magnification. Scale bar represents 200µm. Color imaging software assigned colors based on micrograph intensity and producing an enhanced color image and three-dimensional plots. Notice the distinct pattern of fluorescence in CFDA treated stems. P = Pith, PX = Primary Xylem, SX = Secondary Xylem, R = xylem Ray cell, PH = Phloem and PD = Periderm.
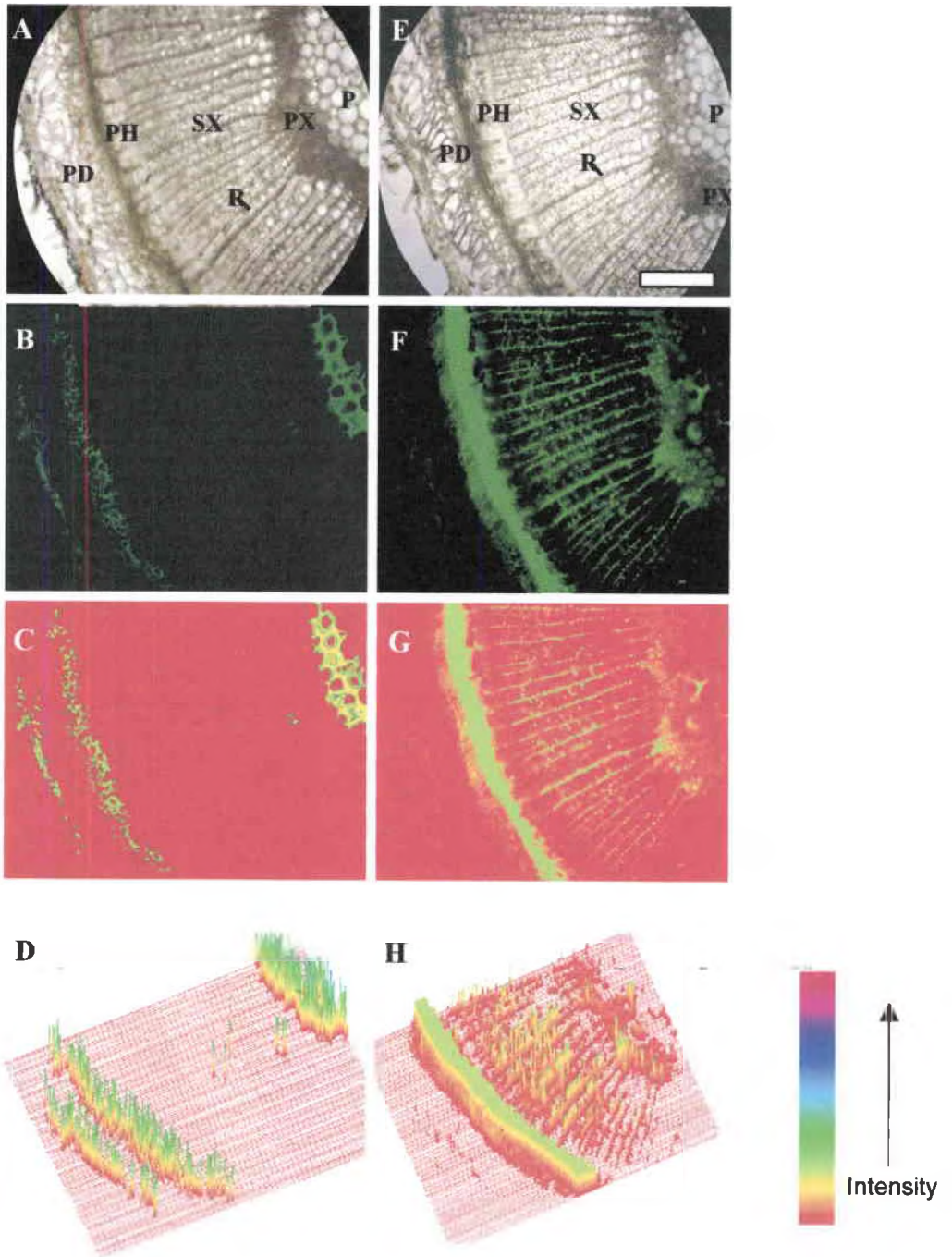
Figure 8. Comparison of the location of fluorescence in autofluorescent and CFDA incubated tissue in Amur honeysuckle stems. Fluorescent micrographs (from Figure 7) were enhanced with color imaging software, assigned intensity values (LUT) and fitted with a line plot through the center of the micrograph. The resulting intensity values were plotted against known tissue location. Normally dimly fluorescent, autofluorescent images were viewed at higher image sensitivity for illustrating the details in tissue location. Notice how the bands indicative of tissue autofluorescence (black) do not overlap those of CFDA stained tissue (red). P = Pith, PX = Primary Xyiem, Sx = Secondary Xylem, R = xylem Ray cell, PH = Phloem and PD = Periderm.

PD   PH   SX + R   PX   P
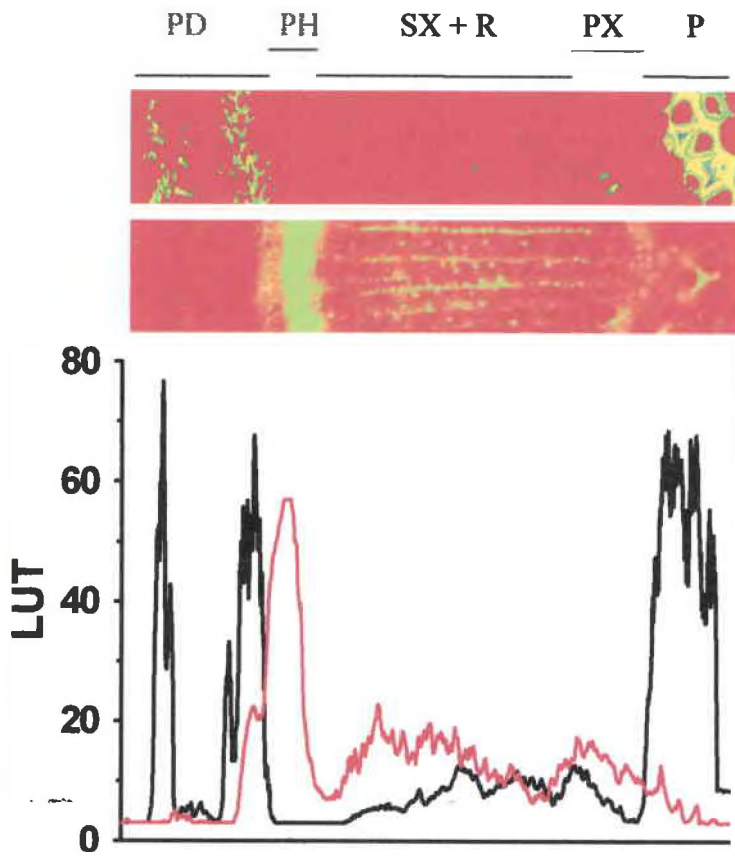
observed in the tissues that compose the primary xylem, xylem ray cells and the phloem **(Figure 9)**.

Visualization of Tissue Structure with Scanning Electron Microscopy

Using unfixed specimens, SEM under low vacuum mode was capable of construction a three-dimensional image of the surface structure of woody stem cross sections. However, the resulting images were highly distorted and lacked important tissue detail. Following a time course of sections artificially dried while viewed under light microscopy **(Figure 10)** and corresponding sections of dried sections viewed under high vacuum SEM **(Figure 11)** demonstrated the extent of tissue damage. As the cells desiccated during the air-drying process, they pulled apart from adjacent cells, rapidly losing cellular integrity. The band of softer non-lignified collenchyma and parenchyma cells of the phloem were especially damaged during this process.

For preserving tissue integrity mild fixatives of FAA and 4% glutaraldehyde in 50mM HEPES buffer were rapidly incorporated into the tissue after sectioning. As shown in **Figure 12**, both FAA and glutaraldehyde were capable of clearly revealing the cellular make-up of the phloem band. When multiple images at higher magnification were combined to form a three-dimensional mosaic **(Figure 13)**, the hollowed pith, primary and secondary xylem, xylem ray cells, phloem, periderm and in some cases even the vascular cambium and individual starch granules could be distinguished in cellular detail in Amur honeysuckle stems.

Figure 9. Histochemically stained micrographs of Amur honeysuckle stem cross sections viewed under visible light or blue actinic light. Micrographs represent **(A)** aniline blue stained callose within the phloem band, **(B)** phloroglucinol stained lignified tissue of the xylem, **(C)** safranin-fast green stained tissue showing lignified tissue in red and cellulose in green, **(D)** toluidine blue O stained tissue revealing lignified tissue (blue) and pectin (purple), **(E)** iodine stained starch granules (black) and **(F)** chlorophyll autofluorescence (red) when viewed under blue wavelength light. Using this method, lignified tissue intrinsically autofluoresces and appears yellow. Micrographs were taken at 100x magnification. Scale bar represent 100μm.
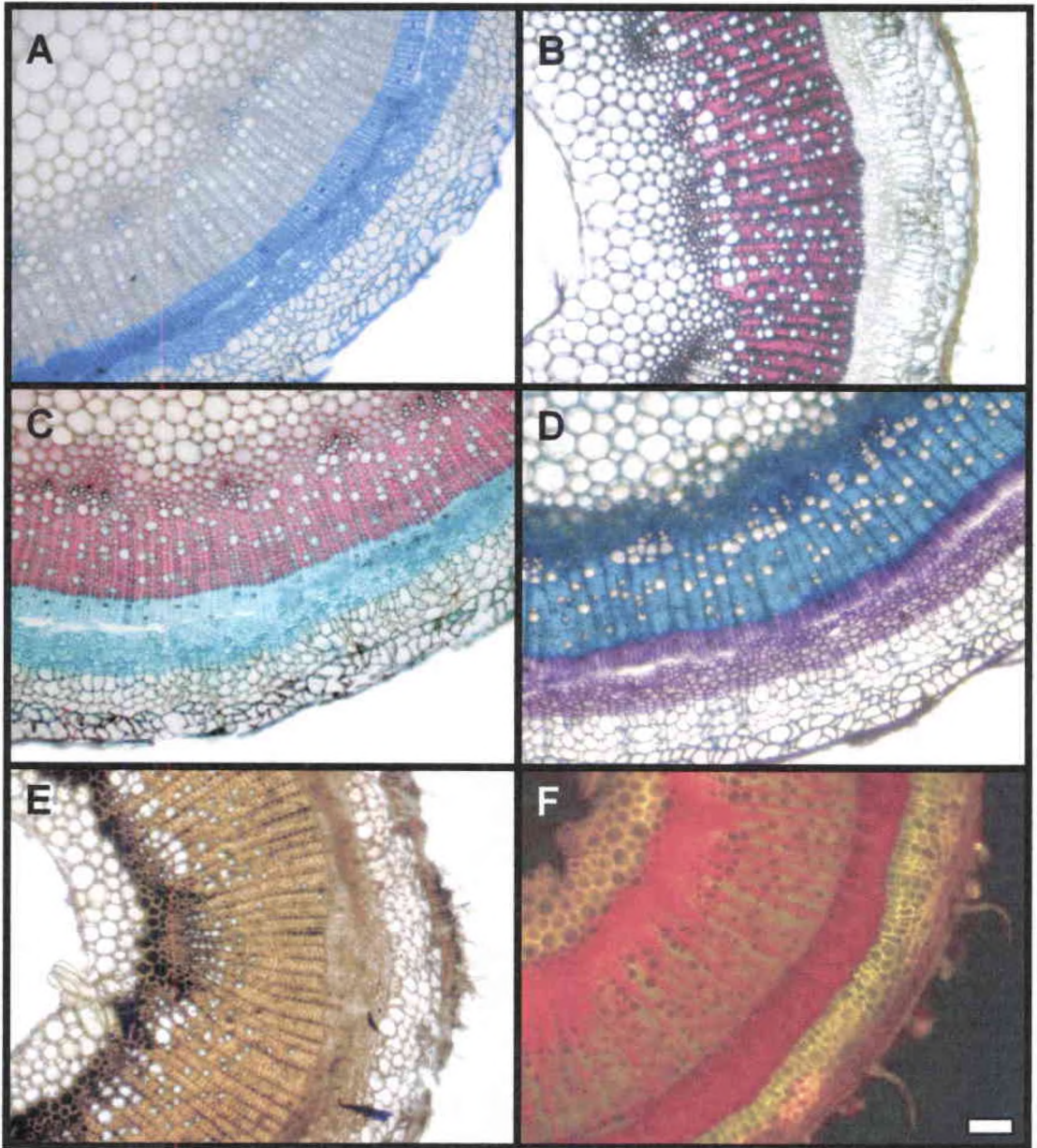
Figure 10. Light micrographs illustrating a time course of Amur honeysuckle stem cross section under an accelerated drying regime. Micrographs depict the deterioration of the phloem vascular tissue (arrows) over time as the tissue dries. Micrographs show tissue **(A)** before application of heat, **(B)** 1 minute, **(C)** 2 minutes and **(D)** 3 minutes after heat application. Images were taken at 100x magnification. Scale bar represent 100μm.
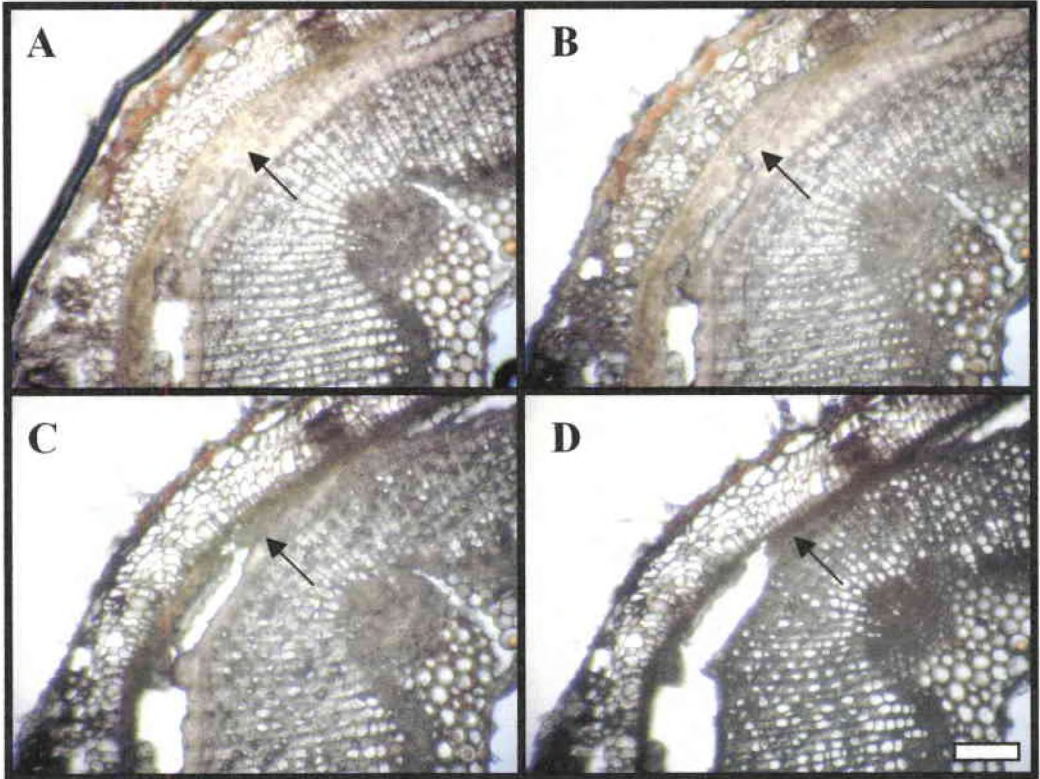
Figure 11. Scanning electron micrographs showing fresh air-dried cross
sections of first year stems of Amur honeysuckle. Both an **(A)**
overview of the stem tissue illustrating the pith, vascular tissue and
periderm at 90x magnification (scale bar represent 200μm) and **(B)**
a close-up of the phloem tissue at 400x magnification are shown
(scale bar represent 50μm). Note the deterioration and
disorganization of the cells within the phloem band (arrow).
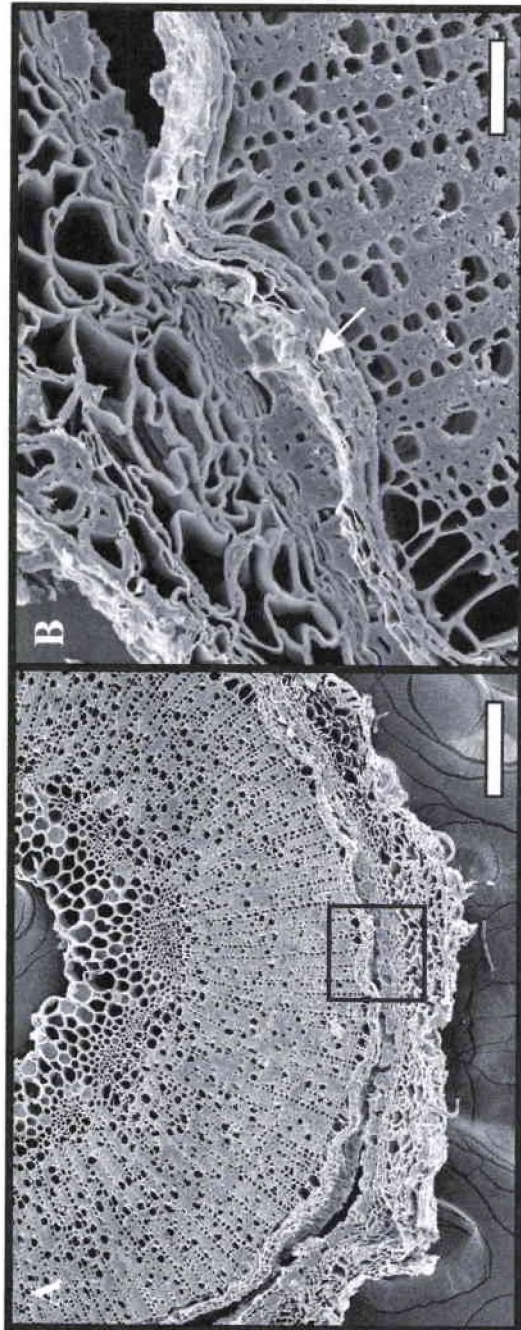
Figure 12. Scanning electron micrographs showing cross sections of Amur honeysuckle stem tissue after incubation in a fixative, dehydration through an ethanol series, critically dried and gold sputter-coated. Images show an overall view of stem tissue fixed in **(A)** FAA (55x magnification) or **(B)** glutaraldehyde (90x magnification) and corresponding close-up micrographs depicting the phloem band in tissue fixed with **(C)** FAA (300x magnification) or **(D)** glutaraldehyde (400x magnification). Note the retention of cellular structure compared to that in Figure 11. Scale bars represent 200μm for A and B and 50μm for C and D.
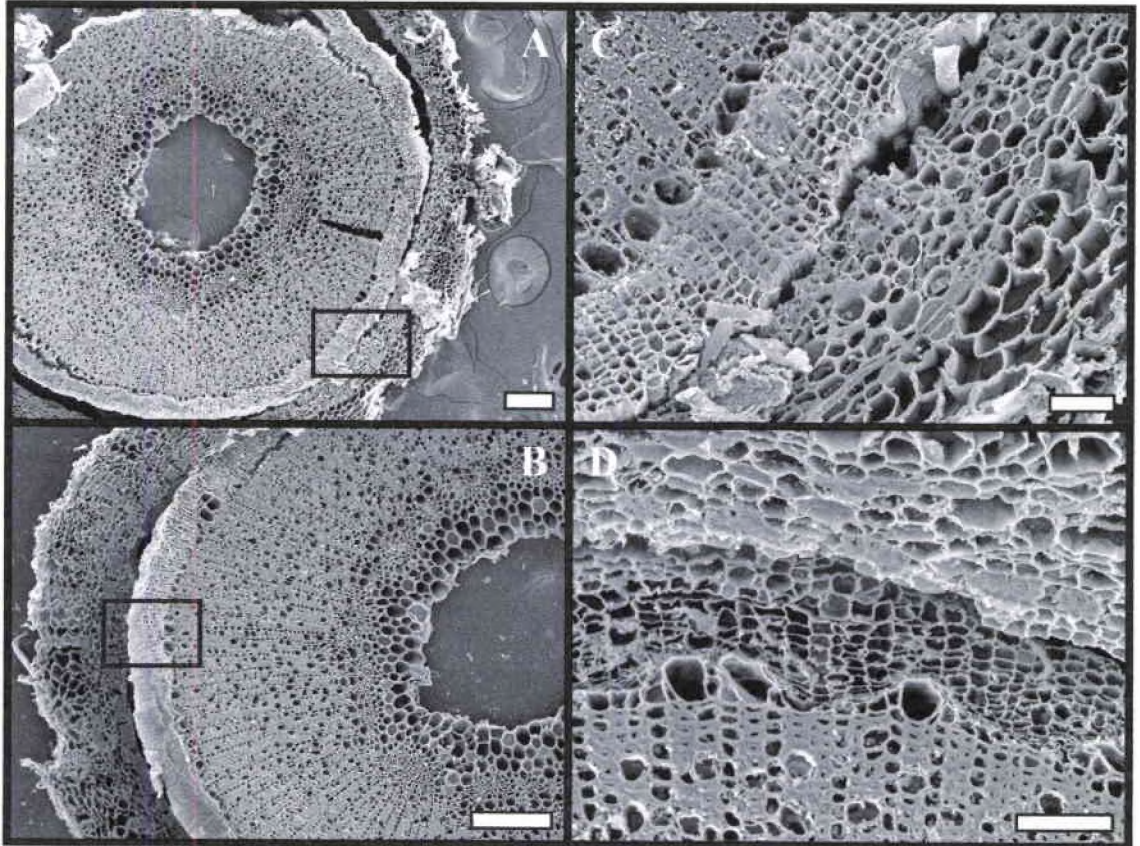
Figure 13. Scanning electron micrograph revealing a cross section of Amur honeysuckle stem tissue after being fixed, dehydrated, critically point dried and gold sputter-coated. The image represents a montage composed of multiple adjacent images at increased magnification (400x). Tissue structures are represented by P = Pith, PX = Primary Xylem, SX = Secondary Xylem, R = xylem Ray cells, PH = Phloem, PF = Phloem Fiber cells, PE = Periderm. Scale bar represents 50μm.
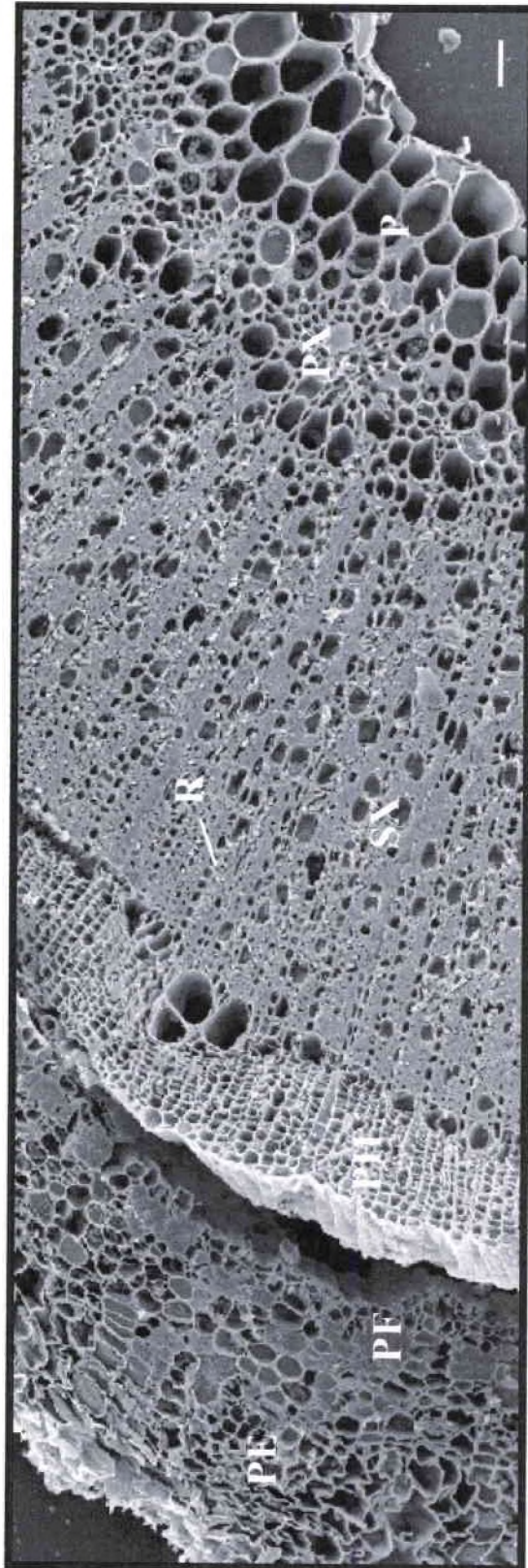
Confirmation and Application of Viability Technique

The testing of tissue viability, structure and metabolism using TTC and CFDA was confirmed for use as a broad-ranging technique in 16 other non-native invasive and native woody plant species. Except for slight changes in general stem anatomy, the location of tissue viability was fairly consistent throughout the species examined **(Appendix C)**. SEM images were correspondingly deduced for all species studied and in most cases revealed superior structural and metabolic information than light microscopy in stem cross sections **(Appendix D)**.

DISCUSSION

As a functional group, woody plants constitute one of the largest percentages of invasive plant found in North America. Notorious invaders of habitats, better methods are needed for effectively monitoring control efforts. Anatomically complex, most woody plants do not generally lend themselves to easy examination. Due to this there are relatively few methods available for examining the status of woody stem tissues. As a way to assess the condition of these cells, this study describes a novel sectioning and histochemical vital staining technique based on incorporation of CFDA and TTC for locating living tissue *in-vitro* in a variety of non-native invasive and native woody plants common to the eastern United States. Correlation among data from traditional histochemistry, structural SEM and metabolic techniques with vital staining methods, demonstrated the location of living and metabolically active cellular tissues within woody stems. The bringing together of procedures in plant anatomy and physiology with chemical control techniques was crucial in formulating a systematic approach that could be used for locating damaged tissues. Techniques such as these may ultimately be incorporated as a tool for formulating the rational design of control methods on invasive woody plants.

Method for Sectioning and Maintaining Woody Stems *In-vitro*

For obtaining cross sections needed for microscopic analysis of viability, it was critical that tissue be maintained alive during the sectioning process and subsequent incubation period. Two principle barriers were overcome during the development of a method aimed at obtaining, sectioning and preserving cross

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sections of live woody plant tissue *in-vitro*. The first allowed fresh woody stem tissue to be precisely, uniformly and reproducibly hand-sectioned at any increment between 40-120µm for observation of cellular detail under brightfield microscopy. Whereas, the second resulted in the discovery of a simple buffer system capable of maintained woody tissue sections viable *in-vitro* for periods exceeding 24 hours.

Consisting of a heterogeneous mixture of thick and thin walled cells of variable strength and hardness, woody plant tissues are notoriously difficult to work with (Chaffey 2002a). Over the years, a number of techniques that specifically examine wood anatomy (Jansen *et al.* 1998 and references within, Chaffey 2002b) have been developed. These methods generally involve softening the wood with steam or chemicals (for instance, boiling it in solutions of glycerine or treating it with acid, hydrogen peroxide or ethylenediamine) and sectioning it with a sliding or rotary microtome. Cut sections are further bleached, stained, dehydrated, cleared and finally permanently mounted on a glass slide (Jansen *et al.* 1998, Ruzin 1999). Excellent for viewing the anatomy of non-living woody tissue, these techniques cannot be used for observing processes in living tissue. A method described in this study was therefore devised to provide a means for sectioning and maintaining live woody stems *in-vitro* for subsequent examination of tissue type, metabolism and viability.

The small size and hardness of Amur honeysuckle stems made the use of both the sliding and rotary microtome impractical. Attempts to section fresh tissue by either method resulted in a shattered of the tissue during the sectioning

process. Based on these criteria, a method was invented that combined both the precision of the rotary microtome and the cutting strength of the sliding microtome. Modifying an old hand-cranked rotary microtome it was possible to make an instrument that kept the tissue stationary while allowing the tissue to be precisely advanced at a set thickness. Incorporation of an aluminum backing provided a base where stem tissue could be accurately hand-sectioned with a sharp double-sided microtome blade. It was important that the cutting blade rather than the material being sectioned had the freedom to move (similar to slicing a loaf of bread). Depending on the species, stem tissue could be sectioned at reproducible increments between 40-120μm. This was more than adequate for observing tissue structure under brightfield microscopy. Incorporation of this technique provided more precision than sectioning by free hand alone.

Because biological reactions are sensitive to even small changes in pH, buffers that stabilize pH change in solution within their proper physiological range, generally between a pH of 6 to 8, must be utilized (van Slyke 1922). Additionally, the biological buffer chosen must be water soluble, non-toxic, non-interfering with biological processes and capable of maintaining cellular processes (Ferguson *et al.* 1980). For maintaining fresh tissue *in-vitro*, the choice of buffer is critical. The Zwitterionic biological buffers developed for biological applications by Good *et al.* (1966) and extended by Ferguson *et al.* (1980) were found in this study to possess minimal physiological effects with woody stem sections. The use of these buffer solutions either by themselves or

with supplemental nutrient medium have effectively been incorporated by a number of researchers for maintaining such things as apical meristem tissue and cells in culture (Good *et al.* 1966, Ferguson *et al.* 1980, Tanino and McKersie 1985, Gill *et al.* 1999, Chaffey and Barlow 2001). Under the conditions present in this study, freshly cut Amur honeysuckle sections incubated in 50mM HEPES buffer at a pH of 7.4 was found to reliably maintain color, turgidity and viability for over 24 hours.

Evaluation of Vital Stains for Identifying Living Tissue in Woody Stems

Viability dyes function by either being excluded from cells or passing into and reacting with living cells to produce a metabolic reaction (Ruzin 1999, Hassan *et al.* 2002). A number of visible light and fluorescent dyes have been shown as effective stains for locating viable tissue in plants. Gaff and Okong'O-Ogola (1971) found that Evan's blue could be used as a survival stain by specifically staining damaged cells. Similarly, Widholm (1972) showed that phenosafranin and FDA could be used together for differentiating dead and living cells, respectively. Throughout the years, viability dyes have been successfully employed in cultured cells (Widholm 1972, Steward *et al.* 1999, Amano *et al.* 2003), pollen (Heslop-Harrison and Heslop-Harrison 1970), apical meristems (Tanino and McKersie 1985, Popov and Vysotskaya 1996), buds (Busso *et al.* 1989, Becker *et al.* 1997, Hendrickson and Briske 1997) and seeds (Vankus 1997). However, as presented in this study, they have been rarely used for examining mature root and stem tissues (Steponkus and Lanphear 1967, McKersie *et al.* 1999, Nesbitt *et al.* 2002).

Evidence confirms the fact that vital stains have specific application requirements, limitations, advantages and disadvantages. Out of 27 dyes tested for viability, Widholm (1972) found that only two that produced specific and consistent results for staining either live or dead cells. Likewise, Swain and De (1994) testing the viability of plant protoplasts through both fluorescent and non-fluorescent means, noticed that some of the dyes produced unsuitable or indefinite results. As a way to assess the condition of cells within woody stem tissue, this study describes the development and evaluation of a simple, cheap and quick system for locating living cells within complex tissues of the stem. Out of five fluorescent dyes and three visible light stains tested only TTC and CFDA were specific for locating living cells *in-vitro* within woody plant stems.

Identification of TTC as a Visible Light Stain for Locating Living Tissue

Of the visible light stains tested in woody stem tissue only TTC (2,3,5-triphenyl tetrazolium chloride) was effective in producing localized staining that substantially differed from control tissue. Evan's blue, phenosafranine and nitro-blue tetrazolium stains, although widely regarded as specific dead tissue stains (Gaff and Okong'O-Ogola 1971, Widholm 1972, Popov and Vysotskaya 1996, Li *et al.* 1999) were unable to differentiate between dead and living tissue in woody plant stems. The incorporation of tetrazolium as a viability stain is not a new concept. Tanino and McKersie (1985) and McKersie *et al.* (1999) were able to positively identify different viable stages of freezing stress in winter wheat and alfalfa with tetrazolium-based stains. Similarly, tetrazolium has been employed successfully in dual stains with Evan's blue for identification of metabolic activity

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(Busso *et al.* 1989, Becker *et al.* 1997, Hendrickson and Briske 1997). In fact, a recent study by Nesbitt *et al.* (2002) found TTC to be the best predictor of living tissue and thus plant survival. However, since staining effectiveness varies with the type and physiological state of the tissue (Popov and Vysotskaya 1996), its effectiveness as a vital stain has been mainly limited to apical meristems and cultured cells. The procedure described in this report further identifies its ability to be used as a stain for locating and identifying living cells within mature woody stems.

As a metabolic stain TTC is widely used in seed viability tests (Kuo *et al.* 1996, Vankus 1997, Ayala *et al.* 2002, Phartyal *et al.* 2003). In actively respiring cells, TTC is reduced to a permanent and insoluble formazan precipitate by dehydrogenase enzymes (Smith 1951, Parker 1953, Steponkus and Lanphear 1967). In this study, the location of metabolically active cells within woody stem sections incubated in TTC resulted in the formation of red precipitation in the primary xylem, xylem ray cells and phloem tissue. Therefore, tissues that turned red in the presence of TTC indicated the existence of metabolically active living tissue. Interestingly, the reaction only occurred when using unsectioned stems. Tissues that were incubated in TTC after being thinly cross-sectioned did not show color formation, whereas stems that were longitudinally cut in half incubated in TTC solution and then cross-sectioned did. It is likely that the initial cross sectioning of the tissue disrupted cellular respiration. In a similar manner, Roberts (1951) reported that TTC applied to homogenized tissues gave a weaker reaction and reducing amount of color formation. Therefore, to achieve effective

staining more time was needed for the dye to diffuse into the stem and react with respiring cells. Since the formation of pink formazan is proportional to the rate of tissue respiration, the amount of time needed to adequately stain stem tissue was reduced by incubated the solution at a slightly elevated temperature (40°C). Even with the increase in reaction rate, larger stems took considerably longer to stain. In this regard it was found that TTC acts as a simple and effective stain for locating living tissue in woody stems but it takes time to diffuse into and react with the tissue.

Identification of CFDA as a Fluorescent Stains for Locating Living Tissue

Of the fluorescent dyes tested only the fluorescein-based dyes, particularly CFDA, showed distinct staining patterns different from control tissue. Although, reported as effective by others (Johnson *et al.* 1980, Gambier and Mulcahy 1994, Swain and De 1994, Gliozzi *et al.* 2003), none of the other dyes tested in this study were able to differentiate living from dead cells when assessed against control tissue. A number of studies have successfully measured cell viability by combined fluorescein with ethidium bromide as part of a double stain (Papadopoulos *et al.* 1994, Yang *et al.* 1998, Lopes *et al.* 2003), yet these studies are largely specific only for use with cultured cells.

The incorporation of CFDA as a viability dye is not unique. It has been used successfully for distinguishing living from dead tissue in cultured cells and apical meristems (Heslop-Harrison and Heslop-Harrison 1970, Steward *et al.* 1999, Amano *et al.* 2003). Compared to other vital stains, such as TTC, Evan's blue and phenosafranine, Popov and Vysotskaya (1996) found FDA to be the

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most reliable. Similarly, Widholm (1972) used fluorescein as a reliable indicator for deciphering living from dead tissue. Applied as a colorless non-fluorescing dye CFDA passes through cell membranes. Upon entry into functional cells the molecule is cleaved of the acetate group by non-specific endogenous esterases present in all metabolically active cells **(Figure 14)**. Only living cells capable of cleaving the molecule fluoresce green when excited by blue wavelength light (Gahan 1984). Out of 17 woody plant species examined, all stem sections incubated in CFDA and viewed under fluorescent microscopy showed intense coloration of cells within the primary xylem, xylem rays and phloem. As a universal dye of woody stem tissue viability, the implications of this technique being incorporated into practical uses did not go unnoticed. Although CFDA is moderately expensive, once taken up by living cells and cleaved of esterase, it is nicely retained within the cell. Furthermore, being less pH sensitive than FDA it has a wider range of efficiency (Breeuwer *et al.* 1994). From the present study, with respect to effectiveness, cost and retention of the dye within the target tissue, CFDA outperformed other fluorescein-based dyes for determining living tissue within woody stems.

The intensity and more importantly the location of autofluorescence is a factor that must be accounted for when working with any fluorescent dye. Due to their inherent composition, plant stems intrinsically contain a number of compounds that autofluoresce at different excitation wavelengths. In these instances, interpreting the results achieved from fluorescent microscopy can be a painstaking ordeal (Knight and Billinton 2001). Much of the ambiguity observed

Figure 14. Structural conversion of 6-carboxyfluorescein diacetate (CFDA) to carboxyfluorescein. CFDA is used to detect cell viability via the release of free carboxyfluorescein by endogenous esterases within the cell. The non-fluorescent CFDA molecule is taken up by intact cells and when metabolically cleaved emits green fluorescence under blue wavelength excitation light.
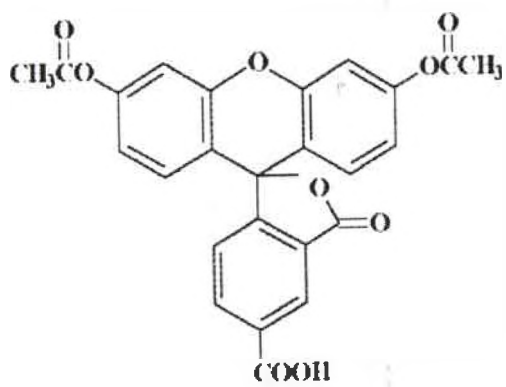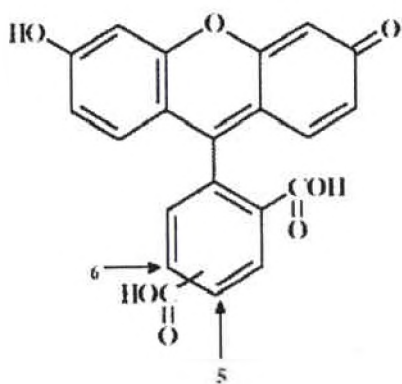
in the other dyes evaluated resulted from the fact that autofluorescence differed considerable under green and blue wavelength excitation light. Under green wavelength light the entire stem autofluoresced orange, making it impossible to distinguish between the dye and background autofluorescence. On the other hand, under blue wavelength light, woody stem sections showed only minor autofluorescence. Although some of the tissues that make up the pith and periderm portion of the stem did slightly autofluoresce, these tissues differed immensely in intensity and location from CFDA stained tissues of the primary xylem, xylem rays and phloem. Furthermore, any observable autofluorescence that was present in control tissue was not visible in CFDA stained micrographs. On the whole, the autofluorescence observed was not a problem for evaluating living tissues in stems incubated in CFDA and excited under blue wavelength light.

Comparison of TTC and CFDA in Woody Plant Stems

As specific indicators for observing living tissue in woody stems both TTC and CFDA in HEPES buffer were found in this study to be highly effective. However, when compared against each other there were notable advantages and disadvantages. The use of visible stains for evaluating fresh tissue can be somewhat harder based on the natural compounds, such as chlorophyll, found in all stems. These intrinsically colored natural products sometimes interfere with and produce false readings to visible light stains whereas fluorescent dyes are generally not influenced as much by their presence. On the other hand, many plant substances and tissues such as flavins, NADH, elastin, collagen, lignin and

chlorophyll are naturally autofluorescent, thus making it difficult or even impossible to decipher between fluorescently stained tissue and natural background excitation (Knight and Billinton 2001). Although CFDA was found to contain only a minimal amount of autofluorescence, unlike visible light stains any fluorescent dye must first be rigorously tested for autofluorescence interference. Although TTC systems are cheaper, generally produce a more permanent record and require less sophisticated equipment (light vs fluorescent microscope) than CFDA systems, they took longer and tended to show incomplete uptake of the dye into the stem tissue during the staining process.

Identification of Tissue Type by Histochemistry

Assembled of a multitude of heterogeneous cell types, woody stems provide the plant with both structure and function (Gahan 1984, Chaffey 2002a). As mixtures of parenchyma, collenchyma and sclerenchyma cells combine to form complex tissues (Esau 1953, Raven *et al.* 1999), histochemical techniques were employed in the present study to locate and identify the main tissue types within woody stem sections. Testing a variety of solvent-dye combinations, tissue fixed in FAA was found to work the best with stains specific for each tissue type.

Located in the center of the stem is a group of soft, non-lignified thin walled undifferentiated parenchyma cells. Retaining the ability to divide these meristematic derived cells, interspersed throughout the secondary xylem as axial rays, connect the primary xylem to the secondary phloem. Comprised mainly of cellulose and pectin the incorporation of fast green, as a counterstain to safranin,

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was used to locate tissues that make-up the primary xylem and xylem ray cells (Ruzin 1999, Alkio and Grimm 2003). Ray parenchyma cells serve as important connecting pathways for transporting nutrients between the primary xylem and secondary phloem throughout a woody plant's life (Chaffey and Barlow 2001). Near the outer end of the stem, callose tissues that form the sieve plates of the phloem (Altamura et al. 2001) were easily identified by their knack of turning blue in the presence of aniline blue. Utilized in transport, phloem tissues have the important function of moving photosynthates and other organic molecules and nutrients throughout the plant (Raven et al. 1999).

Thick walled lignified sclerenchyma cells of the secondary xylem and phloem fiber cells located within the secondary phloem stained red in the presence of phloroglucinol. Lignin, a complex polymer, is primarily deposited in cells with thick secondary walls such as xylem vessels, tracheids and fibers (Vallet et al. 1996, Zhong et al. 2000) and was easily identified in woody stem cross sections with such stains as phloroglucinol or TBO. The majority of these lignified hollow cells are dead at maturity and function only in transporting water and providing mechanical support to the plant (Raven et al. 1999). Adjacent to the secondary xylem is the vascular cambium. This thin band of cells arose from early procambium tissue, and continuously divides to produce new functional xylem and phloem (Chaffey and Barlow 2001). Although not distinguishable by histochemical methods used in this study, differentiation of the vascular cambium is a pivotal process in the life of a woody plant (Plomion et al. 2001). Making-up the outer portion of the stem, outside the secondary phloem and directly

underneath the epidermis contains a jumble of thick walled heavily subarized

collenchyma cells. Together identified as the periderm, these tissues make-up

the cork, cork cambium and phelloderm and provide the plant stem with flexibility

for both structural support and protection.

Identifying of Tissue Type by SEM

Stem cross sections fixed in either FAA or glutaraldehyde and processed

for high resolution SEM gave superior quality images of cellular structure and

tissue type. Providing a detailed three-dimensional surface image of the stem

tissue, high-resolution SEM images better identified cellular structures that were

hard or unable to view with traditional brightfield microscopy. Simple air-drying of

tissue, although possible when observing hardened wood (Jansen *et al.* 1998),

was not capable of adequately preserving the soft thin walled cells of the living

phloem and vascular cambium. Attempts to view these tissues directly using this

method resulted in structural deformation and gave poor image quality. Time-

courses of artificially heated tissue showed severe lose of structure and distortion

to the point of non-recognition that occurred as the stem section dried over time.

The incorporation of fixatives and critical point drying preserved tissue integrity

and countered the harmful effects of tissue desiccation.

The structure of many plant tissues, especially wood, has been explored

in great depth since the introduction of electron microscopy (Robards 1978, Falk

1984). Composed of lignified cell walls, woody tissue is intrinsically solid and

generally requires little preparation for viewing under SEM (Jansen *et al.* 1998).

However, non-lignified tissues of the phloem and vascular cambium present a

problem when trying to examine under pressure with a high-energy electron beam (Fuchs observation). Using alfalfa stems, Hamm *et al.* (2002) discovered that even with the less damaging technique of environmental scanning electron microscopy (ESEM), distortion still occurred in phloem tissue as the tissue was subjected to lower pressures. As important sites of photosynthesis (Maksymovych *et al.* 1993) carbohydrate storage and metabolic activity, detailed observations of the cellular structure of the phloem and vascular cambium was important for measuring changes in plant metabolism and anatomy.

Identification of Metabolic Activity

Not only do woody stems provide strength and structure, they also serve the plant as an area of active photosynthesis and as sites of carbohydrate and nutrient storage (Sauter and Wellenkamp 1998, Pfanz *et al.* 2002). Areas of the primary xylem, xylem ray cells and secondary phloem were found in this study to contain high levels of starch and chlorophyll. It is known that many cells within the stem are involved in a number of important metabolic and biosynthetic processes (Witt and Sauter 1994, Sauter and Wellenkamp 1998, Chaffey and Barlow 2001). Functional chlorophyll has been reported to occur not only in the outer layers of young stems but also within the xylem ray wood and in some instances even in the tissues that make up the pith (van Cleve *et al.* 1993). Chlorophyll containing parenchyma cells consisting of the primary xylem, xylem rays, secondary phloem and chlorenchyma cells located below the outer periderm layer have been found capable of assimilating carbon dioxide for the production of sugars and starch through corticular (stem) photosynthesis (Pfanz

et al. 2002, Aschan and Pfanz 2003). This process has been reported in a number of woody tree and shrub species and is thought to serve a role in the reclamation and refixation of carbon dioxide lost through respiration (Foote and Schaedle 1976a, 1976b, Cernusak and Marshall 2000, Aschan and Pfanz 2003, Damesin 2003). Interestingly, in studies of stem photosynthesis in Scotch broom, *Cytisus scoparium*, Bossard and Rejmanek (1992) reasoned that corticular photosynthesis might play a role in the success of certain shrubs to becoming invasive (see also Nilsen *et al.* 1993). It is evident that areas within the stem containing chlorophyll or starch are metabolically active and can be used as an indicator for identifying living tissues. Furthermore, sites of metabolically activity should be considered as a possible target site for evaluating herbicide control methods in woody invasive plants.

Verification of Tissue Location with Structural and Metabolic Analysis

Combining the results gathered from histochemistry, brightfield illumination, fluorescent microscopy and SEM, the structure, tissue type and metabolic activity of stems in a variety of woody plant species was determined. Correlating this information with vital staining provided by CFDA and TTC, the location of living tissues within the primary xylem, xylem ray cells and phloem band was conclusively ascertained as viable. Furthermore, the cells that had reacted with the viable stains matched those areas of the stem where chlorophyll and starch deposition were greatest. This finding was conclusive evidence for the presence of metabolic activity and verified the accuracy and sensitivity of the viability staining technique.

Although not likely practical as a technique for direct observation in the field, it is useful as a relatively rapid and effective indicator of tissue viability within woody plant stems. Requiring annual metabolic regulation, stem tissue serves as an important physiological site within a plant. Since most woody plants do not generally lend themselves to easy examination, this staining technique is currently being evaluated as a method for monitoring the effectiveness of management efforts for controlling invasive woody plants with herbicide. Better scientifically based techniques for monitoring control efforts are likely going to be an important aspect of invasive species management, therefore, the rational ability to identify the condition of stem tissue should help to better evaluate the effectiveness of control measures.

CHAPTER III

PRACTICALITY OF TRACING AND EVALUATING HERBICIDE DAMAGE IN THE NON-NATIVE INVASIVE SHRUB, AMUR HONEYSUCKLE[1]

INTRODUCTION

Traditionally, the overwhelming majority of non-native invasive woody plants have been intentially introduced into North America by horticulturalists, botanists, foresters and gardeners for landscape, agriculture, forestry, horticulture and simple aesthetic pleasure (Binggeli 2001, Reichard and White 2001). Comprising a characteristic set of reproductive attributes, such as diverse fruit types and seed dispersal mechanisms, invasive woody plant species possess the ability to invade most natural communities (Binggeli 1994, Rejmánek and Richardson 1996). Highly invasive woody species are becoming more common in natural woodlands (Binggeli 1994) disrupting ecosystem processes and threatening the biodiversity of native flora (Mack *et al.* 2000, Sala *et al.* 2000). Over the past several decades, one such woody species, Amur honeysuckle, *Lonicera maackii*, has been steadily infesting forests in much of the eastern United States (Luken and Thieret 1996, Williams 1999). This species generally ranks among the top plant invaders of natural areas targeted for eradication (Ohio Department of Natural Resources 2003).

The progression towards an Amur honeysuckle dominated forest is a relatively recent trend. According to historic surveys, written accounts and pollen

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[1] Portions of this chapter were presented at the 2004 Ohio Invasive Plant Research Conference in Columbus, OH and have been accepted for publication in the *Proceedings of the 19th North American Prairie Conference*

analysis, pre-settlement Ohio forests were composed of a diverse herbaceous layer, a moderate to thick canopy layer and a minimal shrub and sapling cover (Gordon 1969, Hutchinson *et al.* 2003, Sutherland *et al.* 2003, Yaussy *et al.* 2003). Frequent burning by Native Americans for hunting maintained a strong oak dominated canopy that kept understory shrub growth to a minimum. Historic accounts from early surveyors and trappers described the forests as being primarily open with little underwood vegetation. With the arrival of Euro-American settlers, forests were either converted to open fields for raising livestock or destroyed for agriculture, timber and the iron industry (Hutchinson *et al.* 2003). Once again, the woodlands are facing a new threat, however, this time it's from an introduced woody shrub.

Originally from Manchuria, cultivars of Amur honeysuckle were introduced into the eastern United States between 1898 and 1927 (Luken and Thieret 1996). Disregarding early warnings that the species could become a nuisance (Luken and Thieret 1995, Luken and Thieret 1996), plants were widely distributed as a means to control soil erosion, improve bird habitat and serve as an ornamental landscape shrub. A little more than a century later, its ability to dominate woodlands has become apparent. Today, invasive in more than 24 states (Trisel 1997) it plays a major role to the decline in biodiversity in many natural areas (Collier and Vankat 2002, Gorchov and Trisel 2003). Using competitive growth experiments, Miller and Gorchov (2004) found that over the long term, Amur honeysuckle shrubs considerably reducing the growth and seed output of forest herbs. Similarly, Hartman and McCarthy (2004) experimentally showed that

abundant shrub cover directly impacts the recruitment of canopy tree seedlings. When introduced into pristine environments, honeysuckle has been shown to impact native vegetation by out-competing herbaceous plants and emerging saplings (Collier and Vankat 2002, Gorchov and Trisel 2003).

In the absence of biological controls, curtailing the spread of this species into new habitats is extremely laborious and time-consuming. Effective on a small scale, many control methods are generally not feasible for curbing a large established population. In recent years, low-dose foliar applications with Roundup have been employed as a generally safe and effective method for controlling this shrub (Conover and Geiger 1999, Burch and Zedaker 2003) in heavily infested areas. Applied either as a foliar spray (Conover and Geiger 1999, Batcher and Stiles 2001), through stem injection (Franz and Keiffer 2000) or directly to a cut stump (Conover and Geiger 1999, Hartman and McCarthy 2004) Roundup has been used as an effective control method on this species. Yet, for generally unknown reasons efficiency of control depends in part on various abiotic, such as environmental conditions and timing of application, and biotic factors, such as plant metabolism and phenology (Wendel and Kochenderfer 1982, Zedaker 1990).

Glyphosate, [N-(phosphonomethyl)glycine] is a non-selective, broad spectrum, postemergent herbicide discovered in 1971 and introduced in 1974 (Baird *et al.* 1971). Used to control weeds in both commercial and residential applications (Franz *et al.* 1997), this herbicide is essentially nontoxic to most animals (Giesy *et al.* 2000, Williams *et al.* 2000) and readily broken down in soil

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to ammonia, carbon dioxide and inorganic phosphate (Franz *et al.* 1997).

Applied to foliage, the herbicide is taken up through the leaf and transported

throughout the plant (Gougler and Geiger 1981). As a systemic herbicide,

glyphosate moves throughout the plant in phloem and accumulates in regions of

high metabolic activity and growth (Sprankle *et al.* 1975, Gougler and Geiger

1981, Devine 1989, Schulz *et al.* 1990). Within the plant, the herbicide functions

as a highly specific inhibitor of the shikimate pathway enzyme 5-

enolpyruvylshikimate 3-phosphate synthase, EPSP synthase (Steinrücken and

Amrhein 1980, Duke 1990, Schmid and Amrhein 1995, Sikorski and Gruys 1997;

Figure 15). Estimated to be involved in processing 60% or more of a plant's dry

weight (Jensen 1986, Bentley 1990), blockage of this pathway in plants disrupts

a number of essential processes including chlorophyll (Kitchen *et al.* 1981),

protein and nucleic acid synthesis (Foley *et al.* 1983), growth, photosynthesis

and carbon metabolism (Geiger *et al.* 1999, Geiger and Fuchs 2002, Fuchs *et al.*

2002). In contrast with contact herbicides that only act locally, glyphosate works

not only at the point of application but also in regions to which it is translocated

(Devine 1989, Schulz *et al.* 1990). For complete plant death, these tissues must

be metabolically active as translocation sinks at the time of herbicide application.

Yet, to date a detailed mechanism illustrating herbicide-induced tissue death in

temperate forest deciduous woody shrubs is not known.

The focus of this study was to examine the living cells associated with the

phloem and xylem in an attempt to gain a better understanding of the science

behind glyphosate damage in woody plant stems. By identifying the

Figure 15. Consisting of a series of seven biochemical reactions, this illustration depicts the steps of the shikimate pathway. Through a number of enzymatic-induced reactions, the starting products of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) are eventually converted to chorismate. Chorismate then gives rise to a plethora of side chains and branches including the production of three aromatic amino acids, phenylalanine, tyrosin, and tryptophan, lignin, flavanoids and alkaloids. The site of action of glyphosate is generally accepted to be inhibition of 5-enolpyruvylshikimate 3-phosphate synthase.

Chorismate

⑦ ↑

5-Enolpyruvylshikimate 3-P

⑥ ⊠ ←

Shikimate 3-P

⑤ ↑

Shikimate

④ ↑

3-Dehydroshikimate

③ ↑

3-Dehydroquinate

② ↑

Deoxy-D-*arabino*-heptulosonate 7-P

① ↑

Erythrose 4-P + PEP

GLYPHOSATE

$$HO-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-\underset{\underset{\displaystyle H}{|}}{N}-CH_2-\underset{\underset{\displaystyle OH}{|}}{\overset{\overset{\displaystyle O}{\|}}{P}}-OH$$
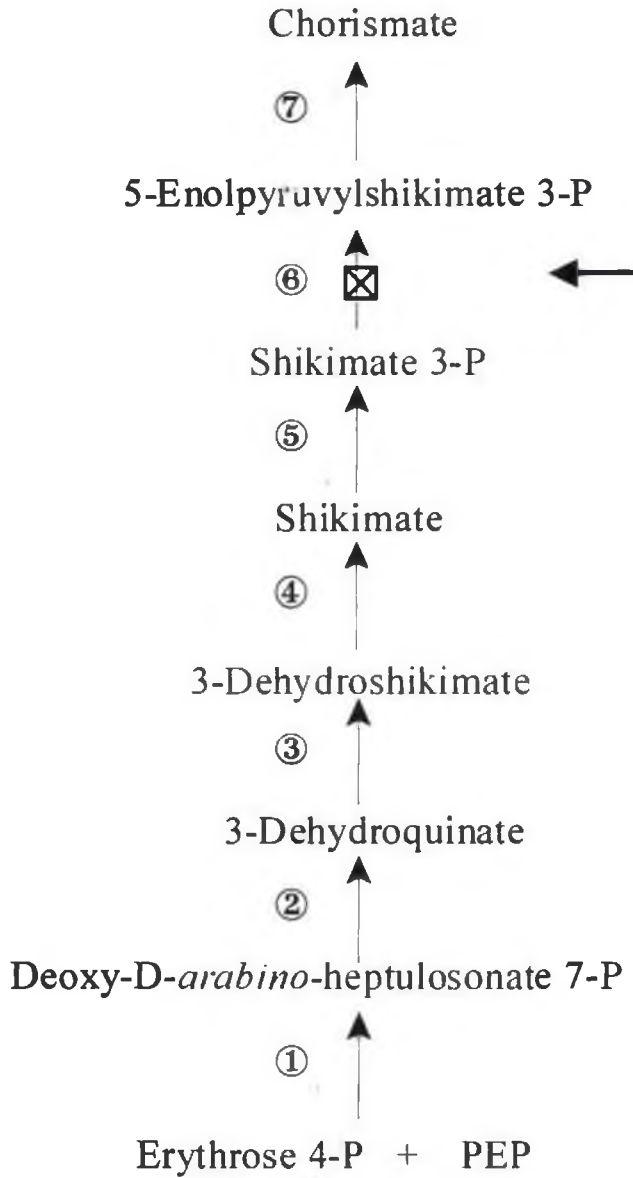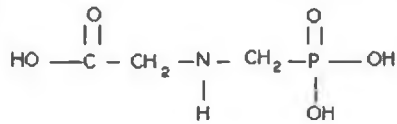
mechanism(s) in which foliage sprays of glyphosate interact with Amur honeysuckle stem tissues, the goal of the project was to learn how to more successfully and efficiently apply herbicides to control the spread of this species. Damage to the stem tissue was assessed with a combination of uptake, cytologic and physiologic techniques designed to visualize tissue structure, metabolism and viability following glyphosate application. By revealing the anatomical basis of herbicidal damage in woody plants, this research could help explain the why woody species are less susceptible to glyphosate applications. Through understanding the location and extent of stem tissue damage in detail, this project ultimately focuses on conducting research to discover possible "windows of opportunity" where the herbicide inflicts the most damage to the plant and the least amount to the environment.

MATERIALS AND METHODS

Sampling Site

Research was conducted on 3-5 year old stands of Amur honeysuckle, *Lonicera maackii*, plants **(Figure 16)** growing within the understory of a temperate deciduous oak-hickory-maple forest in Beavercreek, Ohio, USA. Management of invasive species and restoration efforts have been on-going at this site since 1986 and contain areas that were never invaded, removed or still highly infested with honeysuckle. Where non-invaded or managed for Amur honeysuckle, the site consists of a canopy over top a diverse herbaceous layer. However, areas invaded with Amur honeysuckle contain a dense shrub monoculture covered by a canopy and having little herbaceous ground cover **(Figure 17)**.

Tissue Collection and Sectioning

First or second year stems were collected in the field from mature non-treated Amur honeysuckle plants and ones treated with foliage sprays of 1.3% Roundup Pro-Dry to run-off. Stems of glyphosate treated shoots were collected from the field before and one month after herbicide application (MAA) and rapidly cross-sectioned by hand with the aid of a modified microtome to a thickness between 40-120µm. Fresh cross sections were quickly placed into 50mM HEPES buffer (pH 7.4) for live tissue analysis or fixed with solutions containing formalin, acetic acid and ethanol (FAA) for histochemistry or 4% glutaraldehyde for scanning electron microscopy (SEM; Ruzin 1999).

Figure 16. Photograph of an Amur honeysuckle shrub depicting the characteristic oblong leaflets and red berries.

Photograph by Mark A. Fuchs (2001)

Figure 17. Research site of Amur honeysuckle project at Mt. St. John in Beavercreek, Ohio. Photographs show **(A)** an aerial view of the research site (indicated by an asterisk), **(B)** a restored area on the site **(C)** and one invaded by honeysuckle. In this picture, note the density and early leafing out of the Amur honeysuckle shrub layer compared to the rest of the trees (arrow).

Determination of Tissue Structure with Histochemistry

For visualization of tissue type sections were fixed in FAA, dehydrated or rehydrated through an ethanol graded series and histochemically stained with phloroglucinol or aniline blue (**refer to Appendix B**). These stains are capable of differentiating vascular tissues, xylem and phloem, within woody stems based on the structural composition of compounds within the cell walls (Ruzin 1999). Stained sections were visualized under brightfield light microscopy with a Nikon inverted microscope (Eclipse TS100) and images captured with a Nikon Coolpix 950 digital camera (Nikon Inc., USA).

Visualization of Cellular Structure with SEM

Further verification of tissue type was gathered from SEM using a combination of procedures from Robards (1978), Falk (1984), Jansen *et al.* (1998) and Ruzin (1999). Stem sections were fixed and preserved for 48 hours in cold 4% glutaraldehyde in 50mM HEPES buffer. Fixed tissue was washed in buffer, dehydrated into absolute EtOH through an ethanol graded series and critically point dried (AUTOSAMDRI-814B; Tousimis) with liquid CO_2. Dried tissue sections were finally sputter coated (Denton Vacuum, LLC Desk II Cold Sputter/Etch Unit) with gold to a thickness of 100Å (50mTorr at 45ma for 30 seconds). Stem surface features were captured in a JEOL 5800LV scanning electron microscope (JEOL technics LTD, Tokyo, Japan) at 15kV using secondary electrons and a 20µm objective lens aperture. Digital images were stored on a PC and processed for contrast and brightness in Adobe Photoshop (if needed). For enhanced cellular detail, an assortment of adjacent images at

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higher magnification (400x) was taken and combined in Adobe Illustrator to form a single montage. This allowed for a complete section of the stem from the pith to the outer epidermis to be captured with higher image resolution.

Location of Metabolic Activity

The presence of chlorophyll and starch in living stem tissue was used as an indicator for identifying the amount and location of metabolic activity. For revealing chlorophyll, stem sections were examined for autofluorescence with fluorescent microscopy (Olympus BHTU biological microscope with BH2-RFL fluorescent attachment equipped with a FITC filter cube – excitation filter = 490nm and barrier filter = 515nm). Chlorophyll, when excited by blue wavelength light (430nm) naturally fluoresces red (685nm). For capturing chlorophyll autofluorescence, a long band emission filter was necessary (Ruzin 1999). The detection of starch within stem sections was examined histochemically with iodine. Thin stem cross-sections were stained in solutions of iodine for 5 minutes and examined for the presence of blue-black granules under brightfield illumination. Images were captured with a real life digital camera and processed to a PC (Ruzin 1999).

Identification and Location of Tissue Viability

The location of cell viability in Amur honeysuckle stems was investigated with the fluorescent dye 6-carboxyfluorescein diacetate (CFDA). Freshly cut stem sections were incubated in a 100mM CFDA buffered solution (50mM HEPES, pH 7.4) for 5 minutes (**refer to Appendix A**) and viewed under

fluorescent microscopy (Nikon Eclipse TS100 with epifluorescent attachment) using a Nikon FITC filter cube (B-2 E/C FITC) with an excitation wavelength of 465-495nm and a narrow band barrier filter wavelength of 515-555nm. The narrow band emission filter was necessary to remove interfering chlorophyll autofluorescence. Images were captured with a Nikon Coolpix 950 digital camera.

As a vital dye, non-fluorescent CFDA molecules pass through membranes and are cleaved of the acetate group by endogenous esterases found only in living cells (refer to **Figure 14**). The newly released fluorescein molecules, trapped within living cells, fluoresce green (514nm) when excited by blue light (489 nm). The visualization of green fluorescence is therefore a positive indicator of cell viability (Heslop-Harrison and Heslop-Harrison 1970, Widholm 1972, Papadopoulos *et al*. 1994, Ruzin 1999).

[^{14}C]Glyphosate Uptake and Translocation

For measuring the amount of herbicide taken up by a plant, tracer amounts of [^{14}C]glyphosate was applied as droplets to Amur honeysuckle leaf tissue and analyzed for uptake and translocation following a procedure by Geiger *et al*. (1999). After 24 hours, treated leaves were washed with 50% EtOH, and along with the adjacent stem were collected, separated, oven dried and ground into a fine powder. Being water soluble, [^{14}C]glyphosate was separated from the powder through multiple extractions in 5% EtOH at room temperature. Extraction was deemed complete when scintillation counts were less than three times normal background levels. Supernant from the washes was pooled and

scintillation counted (Beckman LS-3801 Liquid Scintillation System) for activity. Similar measurements were collected from plants growing in the field each month from April thru September. Data from 10 plants was averaged for the amount of glyphosate recovered in the leaf wash, taken up by the leaves and translocated throughout the stem. Amounts are expressed as either a percentage of the total amount of radioactivity applied or as the total amount recovered.

RESULTS

Structure, Metabolism and Viability of Amur Honeysuckle Stems

The combination of brightfield and scanning electron microscopy provided detailed information about the structural and cellular organization of Amur honeysuckle stems, respectively **(Figures 18)**. Images show the hollowed pith, lignified primary and secondary xylem and the phloem band and associated periderm in fine detail. Histochemical stains identified each tissue type within the stem and provided locations that were unable to be acquired through SEM for those types. For instance, aniline blue and phloroglucinol specifically identified the location of the phloem and the xylem, respectively. Together, the histochemical stains identified and located the specific type of tissue while corresponding SEM images revealed their cellular detail.

Iodine staining and consequent observations under blue wavelength light showed the primary xylem, xylem ray cells and phloem tissues as viable tissues packed with starch and chlorophyll. Capable of storing carbohydrates and partaking in photosynthesis, this was a strong indication that these cells were metabolically active.

Effects of Glyphosate on Cellular Processes Within Stem Tissue

The confirmation of techniques for identifying tissue type and structure made it possible to precisely ascertain areas within the stem that are being damaged by glyphosate applications. Comparison of glyphosate treated tissue one month after application with non-treated tissue reveals the primary location of damage to be within the soft parenchyma cells of the phloem and vascular

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Figure 18. Micrograph depicting a cross section of a fresh Amur honeysuckle stem viewed under both brightfield and scanning electron microscopy. The visible light image **(large image to left)** depicts the location of structure and identification of the various types of tissue within a stem [100x magnification]. Inserts represent SEM images of the **(top insert)** xylem-phloem interface and **(bottom insert)** pith-primary xylem interface [both at 400x magnification]. Tissue types are represented by P = Pith, PX = Primary Xylem, SX = Secondary Xylem, R = xylem Ray cells, PH = Phloem and PE = Periderm. Note the formation of new spring wood at the xylem-phloem interface.
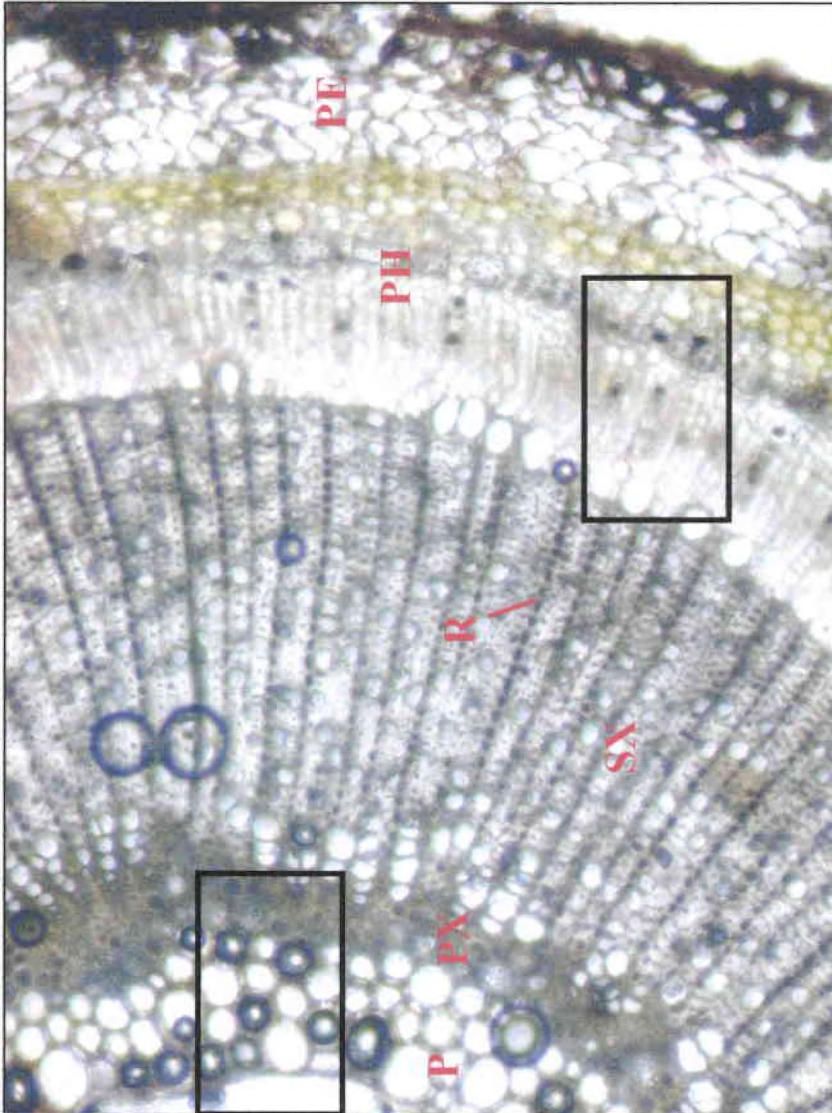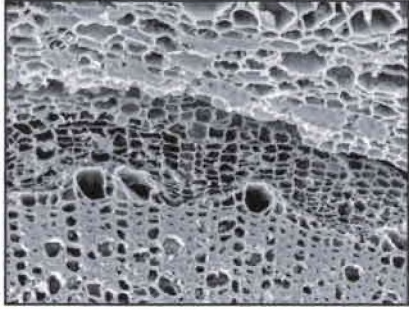
cambium tissue. Compared to the non-treated stems, glyphosate treated ones showed a significant loss of structure, integrity and organization of the cells within the phloem band **(Figure 19)**. Visible light, CFDA analysis and tests of tissue autofluorescence revealed a loss of structural integrity, viability and metabolic activity, respectively, in glyphosate treated stems when compared against non-treated ones **(Figure 20)**.

Analysis of [^{14}C]Glyphosate Uptake and Translocation Experiments

Measurements of radiolabeled glyphosate uptake and translocation throughout the middle part of the season (April thru September) suggested a season-based pattern when the herbicide was taken up and translocated more effectively **(Figure 21)**. By and large, the results indicate that glyphosate when applied to the leaf was taken up most effectively in early spring and in late summer/early fall. During these months, the plant absorbed nearly 25% of the amount of radioactive glyphosate applied to the leaf. On the other hand, during the summer months almost all of the applied glyphosate was recovered in the leaf wash (99%), unable to be taken up and absorbed by the leaf tissue. Similarly, the amount of glyphosate taken up by the treated leaf and translocated to the adjacent stem was generally the greatest in early spring and late summer.

Figure 19. Scanning electron micrographs comparing non-treated and glyphosate treated Amur honeysuckle stem cross sections. Images show overviews of **(A)** non-treated [90x magnification] and **(B)** glyphosate treated stems [120x magnification] with corresponding close-ups of the xylem-phloem band interface in a **(C)** non-treated [400x magnification] and **(D)** glyphosate treated stem [800x magnification]. The white line represents the phloem band. Note the cellular distortion and reduced band size of the phloem in the stem treated with glyphosate. Sections were fixed with glutaraldehyde, dehydrated through an ethanol series, critically dried and gold sputter coated. Stems from plants foliage sprayed with glyphosate were collected one month after application.
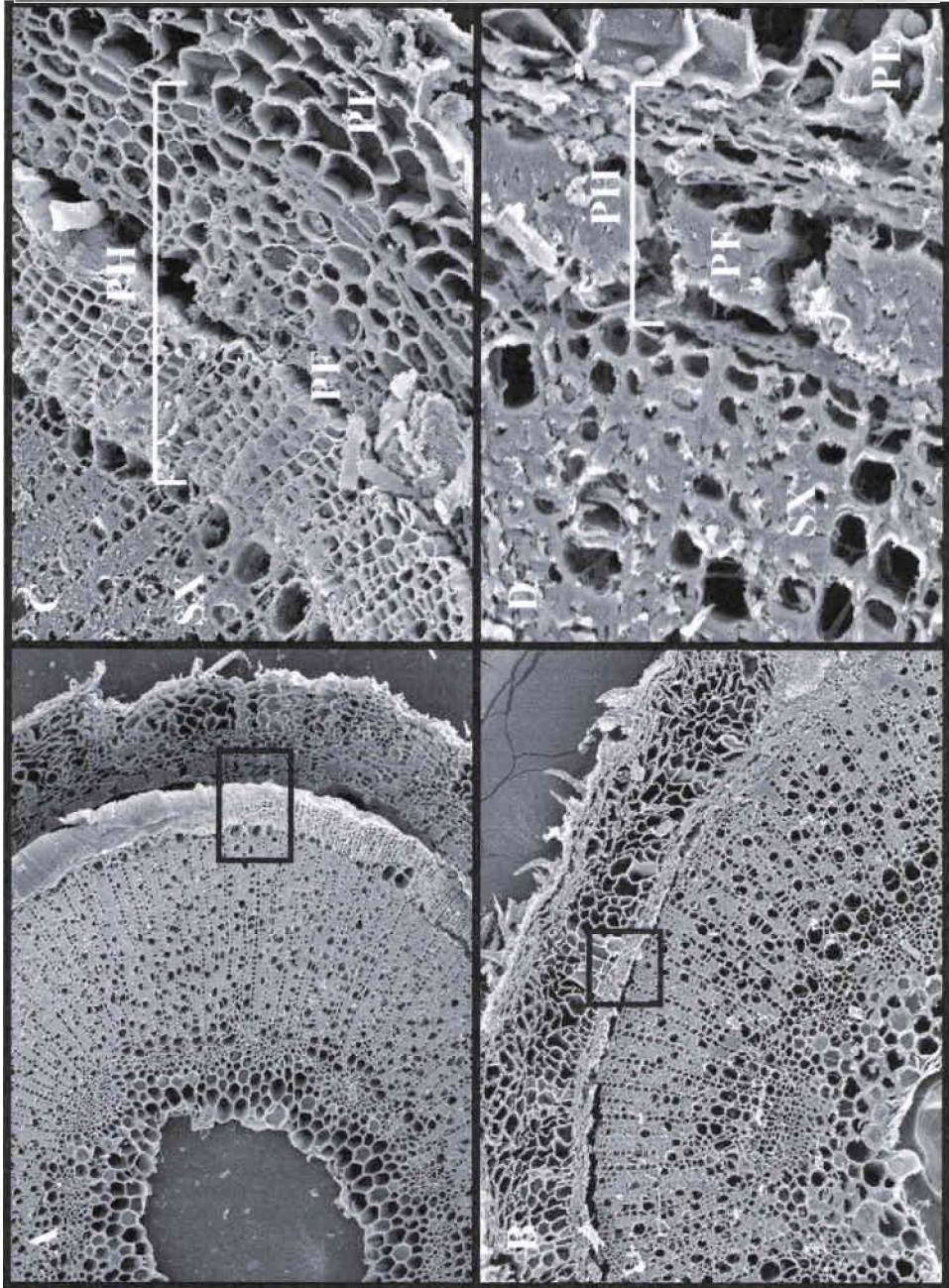
Figure 20. Visible light micrographs comparing non-treated and glyphosate treated Amur honeysuckle stem cross sections for structural and metabolic damage. Images put non-treated (top row) and glyphosate treated (bottom row; collected one month after application) stem tissue side by side when viewed with **(A and B)** brightfield illumination for identification of structure, **(C and D)** CFDA under fluorescent light for identification of living cells and **(E and F)** fluorescent light autofluorescence for identification of chlorophyll. The arrows represent the location of the phloem band. Note the lack of this band in stems treated with glyphosate [all images taken at 100x magnification].
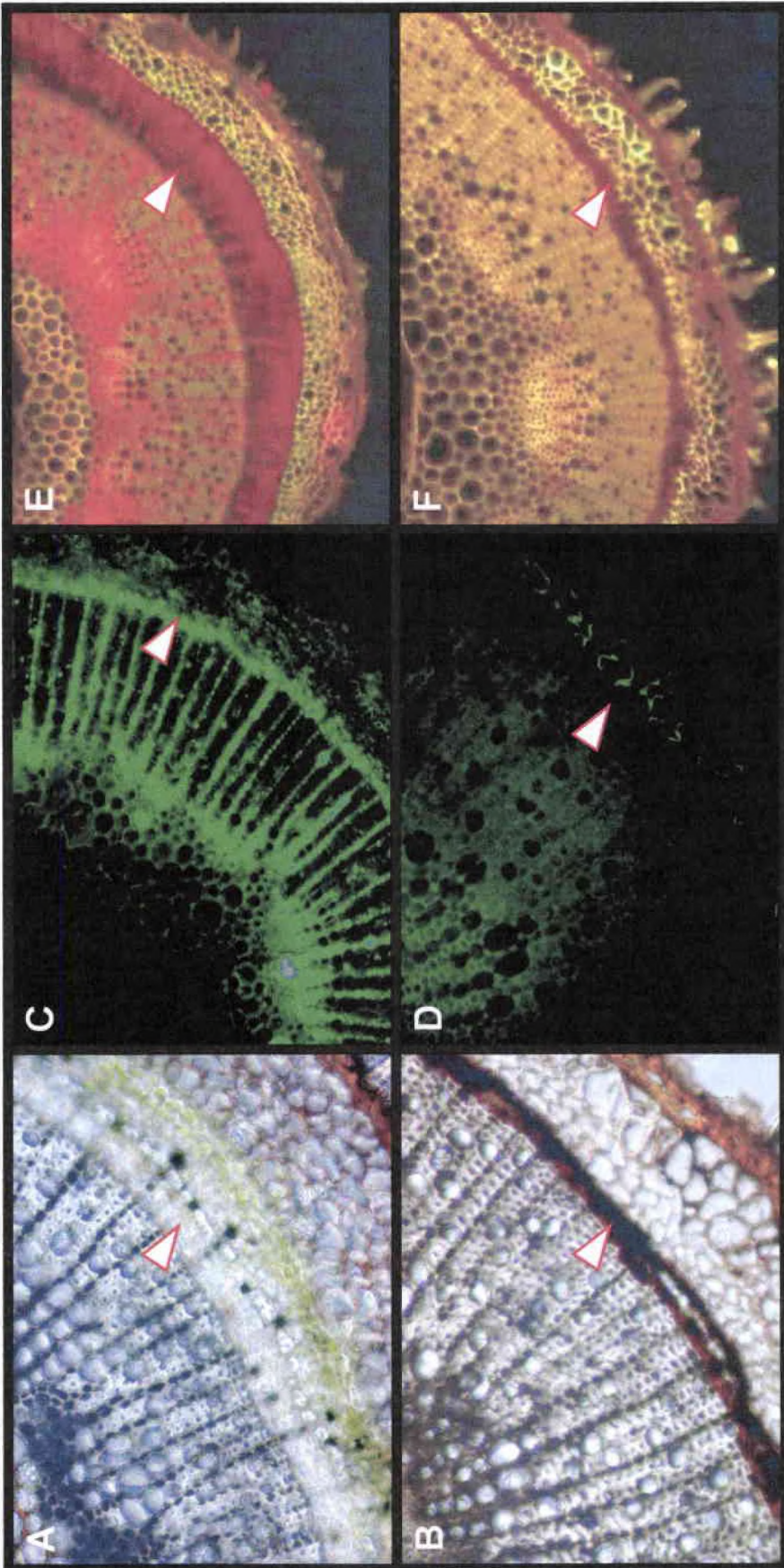
Figure 21. Seasonal measurement of [^{14}C]glyphosate uptake and translocation in Amur honeysuckle plants. **(A)** This picture illustrates the droplet technique used in applying radioactive glyphosate to the leaf tissue. Bar graph represent the **(B)** amount of glyphosate recovered in the leaf wash a day after application, **(C)** amount of glyphosate taken up by the plant and **(D)** amount of glyphosate translocated to the adjacent stem. Box B values are represented as a percentage of the total amount applied to the leaf, whereas, boxes C and D values are represented as the percentage of the total amount of glyphosate recovered from the plant.

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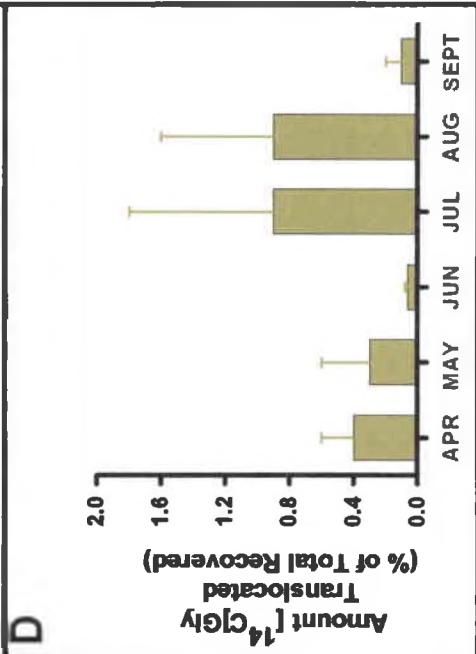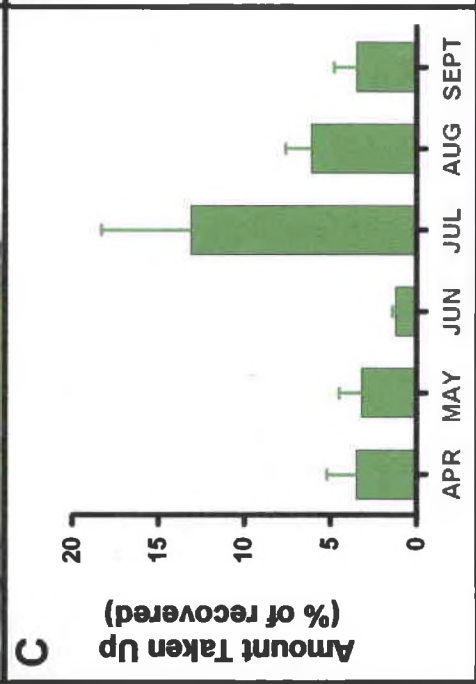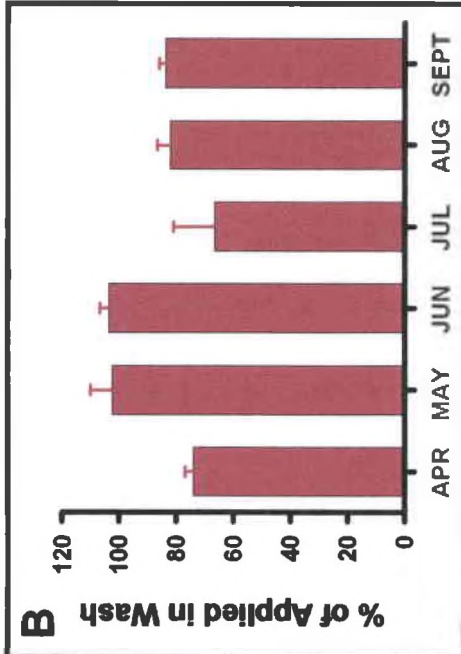
DISCUSSION

There is no simple, convincing and high-quality method for examining localized herbicide action over time in stems of woody plants. Xylem and phloem mobile dyes have been applied as tracking probes for measuring such things as water movement and phloem uptake and translocation (Oparka 1991, Tattar and Tattar 1999, de FaŸ et al. 2000, Fisher and Cash-Clark 2000), but evaluation of tissue viability is not addressed in these studies. Studies that have employed viability stains for locating living plant tissue generally focus on either simple cell types of the apical meristem or cultured cells (Widholm 1972, Tanino and McKersie 1985, Popov and Vysotskaya 1996). Rarely do these methods evaluate a complex heterogeneous tissue like a woody stem. Incorporating a wide range of anatomical and physiological based methods, the present study directly examined herbicidal damage in woody stems of Amur honeysuckle. Combining scanning electron microscopy, fluorescent microscopy and histochemical techniques, glyphosate treated stems was analyzed in detail for changes in structure, cellular viability and metabolic activity.

Glyphosate Effects on Stem Integrity

Linking tissue specific histochemical stains with standard SEM techniques (Ruzin 1999, Alkio and Grimm 2003), it was possible to locate, identify and describe the boundary cells and tissue types affected by glyphosate. Analysis of Amur honeysuckle stems following foliage sprays of Roundup revealed a complete collapse of structural integrity to the soft parenchyma cells of the phloem and vascular cambium. Hidden within the parenchyma, collenchyma and

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sclerenchyma tissues of the stem, the vascular cambium functions to ensure the perennial life of trees (Plomion *et al.* 2001) by continuously dividing to produce new secondary xylem and phloem (Esau 1953, Esau 1969, Raven *et al.* 1999, Savidge 2000, Chaffey and Barlow 2001). The accumulation of secondary phloem, along the outside of the stem, forms new cork cambium tissue from non-functional parenchyma to separate the mature phloem from immature phloem. Glyphosate, by disrupting this process, is essentially stopping the renewal of functional xylem and phloem in Amur honeysuckle stems.

Effect of Glyphosate on Stem Metabolism

Not only do woody stems provide strength and structure to the plant, they also serve as sites of active photosynthesis and play a major role in the storage of carbohydrates and other essential nutrients (Nelson and Dickson 1981). Examination of Amur honeysuckle stems found viable cells containing starch and chlorophyll to be located within the primary xylem, xylem ray cells and phloem tissues. Following foliage sprays with Roundup, xylem ray cells and phloem tissues demonstrated a total loss of metabolic activity. Stems exposed to glyphosate were non-functional and metabolically inactive. Losses in tissue viability and metabolic activity, induced by the herbicide, appeared to correspond with the deterioration of structural organization.

As important sites of photosynthesis (Maksymovych *et al.* 1993), capable of assimilating carbon dioxide directly through corticular photosynthesis (Pfanz *et al.* 2002), and carbohydrate storage (Witt and Sauter 1994, Sauter and Wellenkamp 1998, Chaffey and Barlow 2001) xylem ray cells and phloem tissue

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are critical indicators of overall plant survival (Nesbitt *et al.* 2002). Damage to

woody plant stems likely has a detrimental effect on overall plant metabolism.

Injure to living ray parenchyma cells disrupts the important connecting pathway

between the primary xylem and phloem (Chaffey and Barlow 2001). Apparently,

unable to transport nutrients to living cells within the phloem and vascular

cambium and incapable of recapturing and refixation carbon dioxide lost through

cellular respiration (Foote and Schaedle 1976a, 1976b, Cernusak and Marshall

2000, Aschan and Pfanz 2003, Damesin 2003), cells within the stem of

glyphosate treated plants essentially starve to death.

Seasonal Effects of Glyphosate on Amur Honeysuckle

In contrast with contact herbicides that act locally, glyphosate acts both at

the point of application and in actively growing tissues to which it is translocated

(Devine 1989, Schulz *et al.* 1990). Herbicide-induced plant death occurs when

glyphosate is translocated together with sucrose to metabolically active areas

within the plant (Gougler and Geiger 1981). In temperate climates, woody plants

experience a characteristic pattern of bud break, growth and nutrient reclamation.

During fall, vascular cambium activity ceases (Oribe *et al.* 2003) as plants begin

the process of reclaiming nutrients for winter dormancy. Based on these

observations, it was hypothesized that seasonal differences in plant metabolism

and allocation of carbon could make Amur honeysuckle plants more susceptible

to glyphosate at different times throughout the season.

Results from [^{14}C]glyphosate uptake and translocation studies, although

not conclusive, do indicate a pattern of seasonality where the herbicide is taken

up and translocated more effectively. It is not unreasonable to assume from this that woody plants, with their seasonal patterns of carbohydrate allocation and annual dormancy (Witt and Sauter 1994), are more susceptible to herbicide damage at certain times of the year. In fact, in hardwoods it has been shown that the most effective time to apply glyphosate to woody plants is at a time later in the growing season before leaf senescence (Wendel and Kochenderfer 1982, Becker and Fawcett 1998). If seasonal differences are a factor then, to be effective, control methods must be developed that utilize these seasonal mechanisms.

Practicality of a Physiology Based Method for Identifying Tissue Damage

To integrate herbicides effectively as environmental control agents, essential plant processes involving the seasonal timing of shoot development and nutrient allocation must be further established. By examining these processes it is believed that optimal "windows of opportunity" for the most effective and least environmentally damaging times to apply herbicides can be discovered. It is anticipated that practical techniques for elucidating the mechanisms of herbicidal damage based on sound science will help eliminate the spray and pray approach prevalent in many control efforts. The overall goal is to provide more effective land management strategies for controlling other woody invasive species including Japanese honeysuckle (*Lonicera japonica*), European privet (*Ligustrum vulgare*), autumn olive (*Elaeagnus umbellate*), winged burning bush (*Euonymus alata*), multiflora rose (*Rosa multiflora*), oriental

bittersweet (*Celastrus orbiculatus*), winter creeper (*Euonymus fortunei*), glossy

buckthorn (*Frangula alnus*) and Japanese knotweed (*Polygonum cuspidatum*).

CONCLUDING REMARKS AND IMPLICATIONS FOR FURTHER RESEARCH

Applied as a foliage treatment, glyphosate was found to lethally damage the phloem and vascular cambium tissue in stems of the woody invasive plant, Amur honeysuckle. Experimentation with distinguishing living cells in woody plant tissue led to the development of a system based on a visible (TTC) and a fluorescent dye (CFDA). Compared with methods to locate metabolism and tissue type, both stains were found capable of specifically identifying living metabolically active cells in woody stem cross sections. As more and more non-native woody plants are released into the United States, the ability to develop techniques to discern the effectiveness of control methods on these plants is becoming increasingly important. One problem related to controlling invasive woody plants is a simple lack of fundamental knowledge of their basic characteristics (Binggeli 1999). Identifying physiological life characteristics can provide insight and introduce vulnerabilities that are likely critical in formulating management plans by which to control invasive woody plants (Mooney 1991, Drake *et al.* 2003).

Since all woody plants go through a series of seasonal metabolic and developmental changes (Dickson and Nelson 1982), herbicide susceptibility is also presumed to vary with seasonal shoot development. It has been noted that the most effective time to apply glyphosate to woody plants is at a time later in the growing season before leaf senescence (Wendel and Kochenderfer 1982, Becker and Fawcett 1998). During this time, when plants are storing

carbohydrates in anticipation of winter dormancy, it is presumed that glyphosate can be effectively taken up through the leaf cuticle and distributed throughout the plant. Even though tissue dormancy and seasonal changes in metabolism (Dickson and Nelson 1982, Kozlowski 1992, Scarascia-Mugnozza *et al.* 1999) are important aspects of the life cycle of all temperate woody plant species, attempts to eradicate honeysuckle generally do not address these basic issues. Understanding physiologicial processes throughout the season may help to identify optimal "windows of opportunity" for the most effective and least environmentally damaging times to focus herbicidal control efforts.

The next major task stemming from this research is to elucidate the mechanism by which glyphosate acts in woody plants. In an ecological effort to control invasive woody plants species, the aspiration of future research is to unite the knowledge gained through studies of plant physiology with the accomplishments of invasion ecology. The integration demonstrated by this research represents an attempt to back away from the dependence on subjective judgments and replace it with sound science based assessment procedures. Continued progress to illustrate the mechanisms responsible for tissue death combined with seasonal studies, should significantly aid land managers in developing practical plans to eliminate Amur honeysuckle and other similar woody invasive plants from woodlands.

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Appendix A

Tabulation of Vital Dye Staining Procedures

Stain / Dye Name	Fluorescent	Visible-Light
Acridine Orange	X	
Ethidium Bromide	X	
Evan's Blue		X
Fluorescein	X	
Phenosafranine		X
Propidium Iodide	X	
Rhodamine B	X	
Tetrazolium		X

Acridine Orange

Chemical Form:	3,6-bis[dimethylamino]acridine
Mode of Action:	Acridine orange intercalates into deoxyribonucleic acid (DNA) and emits green fluorescence under blue excitation light. It is a vital stain used in determining the number of cells within a given population that have undergone apoptosis.
Stock solution:	10mg per mL in distilled water, stored in the dark at −20°C
Working solution:	Stock solution diluted 1:20 with 50mM HEPES buffer (pH 7.4)
Incubation Time:	Submerged for 5 min in working solution at room temperature
Wash Time:	5 min in 50mM HEPES buffer
View:	Stem sections were mounted on a glass slide in HEPES buffer and examined under blue excitation light (490nm). Living cells fluoresce bright green (590nm). Thus, a living cell will have a green nucleus and may have a red-orange cytoplasm. Stained sections were viewed with an Olympus BHTU biological microscope coupled with a BH2-RFL fluorescent attachment containing a FITC filter cube (wide band excitation filter = 490nm and barrier filter = 515nm). Digital images were captured with a real life camera attachment and processed to a PC.
Notes:	This fluorochrome has been used in combination with ethidium bromide and propidium iodide as a live/dead cell staining complex.
References:	Morris et al. 1997, Mascotti et al. 2000, Foglieni et al. 2001, Hassan et al. 2002

Ethidium Bromide

Chemical Form:	3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide
Mode of Action:	Ethidium bromide is only taken up by nonviable cells. It intercalates into DNA and appears bright orange when excited with green wavelength light. By penetrating intact cells slowly, it is essentially cell membrane impermeability and is thus excluded from live cells but rapidly enters damaged cells with non-functional or dead membranes and binds to nucleic acids. Therefore, only chromatin of compromised membranes of dead cells will appear as bright orange.
Stock solution:	10mg per mL in distilled water, stored in the dark at −20°C
Working solution:	Stock solution diluted 1:20 with 50mM HEPES buffer
Incubation Time:	Submerged for 5 min in working solution at room temperature

Wash Time:	5 min in 50mM HEPES buffer
View:	Stem sections were mounted on a glass slide in HEPES buffer and examined under green excitation light (545nm). Dead cells fluoresce orange (605nm) when viewed under fluorescent microscopy (Nikon Eclipse TS100 with epifluorescent attachment) using Nikon FITC filter cube (G-2A) with an excitation filter of 510-560nm and a wide band emission filter of 590nm. Digital images were captured with a Nikon Coolpix 950 digital camera.
Notes:	Stain is similar in function to acid dye trypan blue. Often used in conjunction with acridine orange or fluorescein as a dye-exclusion test for detecting cell viability. Ethidium Homedimer-1 has also been used in studying viability. This dye provides a strong red coloration and gives better retention than ethidium bromide but is extremely expensive to purchase (1mg for $124)
References:	Edidin 1970, Papadopoulos *et al.* 1994, Yang *et al.* 1998, Lopes *et al.* 2003, Gliozzi *et al.* 2003

Evan's Blue

Chemical Form:	Direct Blue 53
Mode of Action:	Evan's blue is a well-known non-toxic, water soluble, non-permeating inclusion pigment that is frequently used to reveal cells that have lost their cell membrane integrity. The dye is excluded and unable to penetrate intact plasmalemma or intact cellular membranes of living cells, but penetrates the plasmalemma of damaged cells where it accumulates and permanently stains the protoplasm of dead tissue intensely blue under light microscopy.
Stock solution:	0.25% in distilled water, stored in the dark at room temperature
Working solution:	Stock solution diluted to 0.05% with 50mM HEPES buffer
Incubation Time:	Submerged for 15 min in working solution at room temperature
Wash Time:	10 min in 50mM HEPES buffer
View:	Stem sections were mounted on a glass slide in HEPES buffer and examined under light microscopy using an Olympus BH-2 biological microscope and images captured with an Olympus Digital Camera (DP12). Blue stained tissue is indicative of damaged cells.
Notes:	Often times used in conjunction with tetrazolium as a live/dead cell double stain.

References: Gaff and Okong'O-Ogola 1971, Busso et al. 1989, Popov and
                Vysotskaya 1996, Becker *et al.* 1997, Hendrickson and Briske
                1997, Young and Gallie 1999

Fluorescein

Chemical Form: Fluorescein diacetate (FDA), 6-Carboxyfluorescein diacetate
                   (CFDA) and Calcein AM

Mode of Action: FDA, CFDA and Calcein-AM are capable of measuring cell
                   viability through the inclusion of fluorescein within living cells.
                   Colorless non-fluorescent diacetate substituted fluorochromes
                   diffuse across cell membranes and react in functioning cells.
                   Upon entry into the cell, non-specific endogenous esterases
                   cleave (release) the acetate rendering fluorescein and restoring
                   fluorescence. The newly released fluorescein molecules are
                   trapped within the cell and when excitation by blue light the
                   molecule emits green light.

Stock solution: 1 mg CFDA in 1 mL acetone, stored in the dark at
                   −20°C

Working solution: Stock solution diluted 1:20 with 50mM HEPES buffer for a final
                   concentration of 100mM

Incubation Time: Submerged for 5 min in freshly prepared working solution at
                   room temperature

Wash Time: 1 min in 50mM HEPES buffer

View: Stem sections mounted on glass slide in HEPES buffer and re-
                   washed with buffer under cover slip to remove free fluorescein
                   leachate. Examine using epifluorescence microscopy (Nikon
                   Eclipse TS100 with epifluorescent attachment) using Nikon FITC
                   filter cube (B-2 E/C FITC) with an excitation wavelength of 465-
                   495nm and a narrow band barrier filter wavelength of 515-
                   555nm. This emission filter is needed to remove chlorophyll
                   autofluorescent interference. Images were captured with a
                   Nikon Coolpix 950 digital camera. Under blue light (489 nm) the
                   molecule is "energized" and emits green light (514 nm).
                   Therefore, tissues with intact and functional cellular membranes
                   that retain the stain fluoresce green in CFDA and are useful as a
                   positive indicator of cell viability.

                   Superimposing the fluorescent images to those obtained under
                   ordinary light microscopy enables the specific structures of the
                   tissue and possible even individual cells to be identified.

Notes: The carboxy derivatives, such as CFDA and Calcein AM, once
                   cleaved, fluorescence is retained in the cell longer than FDA.

                   Calcein AM is also less pH dependent, however, it is
                   100 times more expensive (1mg for $122)

References: Heslop–Harrison and Heslop–Harrison 1970, Widholm 1972, Oparka 1991, Breeuwer *et al.* 1994, Papadopoulos *et al.* 1994, Lopes *et al.* 2003

Phenosafranine

Chemical Form:	3,7-diamino-5-phenylphenazinium chloride
Mode of Action:	The exclusion viability dye, phenosafranine, rapidly penetrates non-functioning cell membranes and stains the cellular contents dark red. Unable to enter living cells with functional membranes the stain does not accumulate within the cells and the tissue remains unstained, making this dye specific for dead tissue.
Stock solution:	1% in distilled water, stored in the dark at room temperature
Working solution:	Stock solution diluted to 0.1% with 50mM HEPES buffer
Incubation Time:	Submerged for 2 min in working solution at room temperature
Wash Time:	5 min in 50mM HEPES buffer
View:	Stem sections were mounted on a glass slide in HEPES buffer and examined under light microscopy using an Olympus BH-2 biological microscope and images captured with an Olympus Digital Camera (DP12). Red stained cells are indicative of cellular damage.
Notes:	The stain reacts quickly and samples could be viewed almost immediately after staining.
References:	Widholm 1972, Sakai *et al.* 1991, Popov and Vysotskaya 1996, Li *et al.* 1999

Propidium Iodide

Chemical Form:	3,8-diamino-5-[3-(diethylmethylammonio) propyl]-6-phenyl,-diiodide
Mode of Action:	Propidium iodide is unable to penetrate intact cellular membranes and is thus excluded from living cells. This stain enters cells with damaged or non-functioning cellular membranes, intercalates into DNA molecules and fluoresces bright red when excited with green wavelength light. Therefore, only compromised cells accumulate the solution and stained chromatin transmits a bright red coloration.
Stock solution:	10mg per mL in distilled water, stored in the dark at –20°C
Working solution:	Stock solution diluted to 5µg per mL with 50mM HEPES buffer
Incubation Time:	Submerged for 5 min in working solution at room temperature

Wash Time:	5 min in 50mM HEPES buffer
View:	Stained stem sections were mounted on a glass slide in HEPES buffer and examined under green excitation light (536nm). Dead cells fluoresce red (617nm) when viewed under fluorescent microscopy (Nikon Eclipse TS100 with epifluorescent attachment) using Nikon FITC filter cube (G-2A) with an excitation filter of 510-560nm and a wide band emission filter of 590nm. Digital images were captured with a Nikon Coolpix 950 digital camera.
Notes:	Used in conjunction with fluorescein as a live/dead method for its ability to stain dead cells. Similar to ethidium bromide but has replaced this dye in some circumstances due to its ability to provide a more intense red fluorescence.
References:	Dive *et al.* 1990, Mascotti *et al.* 2000, Foglieni *et al.* 2001, Watanabe *et al.* 2002

Rhodamine B

Chemical Form:	Basic Violet 10
Mode of Action:	A popular mitochondrial fluorescent dye, penetrates cellular membranes and accumulates and stains active mitochondria of living tissues through a electrical potential-dependent fashion. Only living mitochondria accumulate the stain that fluoresces red when exposed to green wavelength light.
Stock solution:	10mg per mL in distilled water, stored in the dark at −20°C
Working solution:	Stock solution diluted to 10µg per mL with 50mM HEPES buffer
Incubation Time:	Submerged for 10 min in working solution at room temperature
Wash Time:	5 min in 50mM HEPES buffer
View:	Stained stem sections were mounted on a glass slide in HEPES buffer and examined under green excitation light (555nm). Cells with functional mitochondria fluoresce red (580nm) when viewed under fluorescent viewed under fluorescent microscopy (Nikon Eclipse TS100 with epifluorescent attachment) using Nikon FITC filter cube (G-2A) with an excitation filter of 510-560nm and a broad band emission filter of 590nm. Digital images were captured with a Nikon Coolpix 950 digital camera. Red fluorescence indicates a cell with active mitochondria whereas dead cells do not accumulate the dye and do not fluoresce.
Notes:	This stain is widely used in flow cytometry studies involving mitochondrial membrane potential.
References:	Johnson *et al.* 1980, Gahan 1984, Gambier and Mulcahy 1994, Li *et al.* 1996

Tetrazolium

<u>Chemical Form</u>:	2,3,5-triphenyl tetrazolium chloride (TTC)
<u>Mode of Action</u>:	Tetrazolium is a metabolic stain and whose color is reduced in the presence of dehydrogenase enzymes of metabolically active cells. When TTC diffuses into actively respiring tissues it accepts electrons from the mitochondrial electron transport chain, reducing it to the insoluble pink compound formazan. The accumulation of this pink compound stains the tissues permanently red. The intensity of the red is proportional to the rate of respiration in the tissues.
<u>Stock solution</u>:	None (freshly prepared each time)
<u>Working solution</u>:	100 mg per 20mL (5%) 50mM HEPES buffer
<u>Incubation Time</u>:	Longitudinally sectioned intact stems were submerged in freshly prepared working solution in an air-circulated convection oven at 40°C for 24-72 hours
<u>Wash Time</u>:	None required
<u>View</u>:	Longitudinal sections were cross-sectioned and viewed under light microscopy using an Olympus BH-2 biological microscope and images captured with an Olympus Digital Camera (DP12). A positive result is the formation of a red coloration that corresponds with metabolically active tissues of living cells.
<u>Notes</u>:	Used in conjunction with Evan's blue as a live/dead stain in measuring seed viability.
	Stems had to be cut longitudinally and stained before cross sections were taken. Stained directly, cross sections showed no sign of activity.
	Nitro blue tetrazolium was also tried but gave inconclusive results.
<u>References</u>:	Smith 1951, Parker 1953, Steponkus and Lanphear 1967, Tanino and McKersie 1985, Busso *et al.* 1989, Becker *et al.* 1997, Hendrickson and Briske 1997, Yang *et al.* 1998, McKersie *et al.* 1999, Šebala *et al.* 2001, Nesbitt *et al.* 2002

Appendix B

Summary of Histochemical Procedures

Fixatives

FAA

Glutaraldehyde

Stains

Aniline Blue

Iodide Solution

Phloroglucinol

Safranin-Fast Green

Toluidine Blue (TBO)

Fixatives

FAA (formalin, alcohol, acetic acid) for Structural Analysis

Solution: 50 mL 95% EtOH
5 mL glacial acetic acid
10 mL formalin (37% formaldehyde)
35 mL 50 mM HEPES buffer (pH 7.4)

Procedure:

(1) Freshly sectioned stem cross-sections were immediately transferred into adequate amounts of FAA solution (50% EtOH prepared in HEPES buffer).

(2) Sections were fixed and de-colored in this solution for at least 24 hours (can be kept in this solution for weeks if needed).

(3) Wash sections in FAA (50% EtOH) without fixative for at least 30 minutes to remove any remaining fixative that may be in the section.

(4) Dehydrate or re-hydrate sections through an ethanol series depending on the stain

      (a) For dehydration of tissue: place washed tissue into an increasing ethanol graded series of 70, 90, 95% EtOH and absolute EtOH for at least 30 minutes each

      (b) For rehydration of tissue: place washed tissue into a decreasing ethanol graded series of 30, 10% EtOH and then into two washes of DI water for at least 30 minutes each

(5) Stain with appropriate dyes

Notes: Remember to make sure that the dye and tissues are in the same solvent system. If the dye is in an aqueous solution, it is necessary to have the tissue section in water before placing it in the dye solution. Likewise, if the dye is in an alcohol solution the tissue needs to be put into alcohol before it can be stained.

Most fixatives require a washing step in the fixative solution minus the fixative before proceeding with the dehydration step.

References: Jensen 1962, Ruzin 1999, Kitin *et al.* 2000, Chaffey and Barlow 2001 Chapman *et al.* 2001

Glutaraldehyde Preparation for SEM

Solution: 4% glutaraldehyde

Procedure: Incubate tissue for 24 – 48 hours, wash in buffer without fixative, dehydrate to absolute EtOH through an EtOH graded series, critically point dry and sputter coat. View under SEM for high resolution.

Notes: A "gentler" fixative than FAA, generally yields better structure with less deformation of tissue. Commonly used in SEM.

References: Robards 1978, Falk 1984, Jansen *et al.* 1998, Ruzin 1999

Histological and Cytochemical Structural Stains

Aniline Blue

Description: Callose molecules of the phloem preferentially stain blue in the presence of aniline blue.

Procedure:
(1) Use FAA fixed tissue and de-hydrate to 95% EtOH
(2) Incubate sections in 0.5% aniline blue prepared in 95% EtOH for 2 min
(3) Rinse sections twice in Absolute EtOH for 5 minutes
(4) Rinse sections once in a 1:1 solution of Absolute EtOH : xylene for 5 minutes
(5) Do a final rinse in xylene for 5 minutes
(6) Mount in Permount and view under light microscopy

Notes: Lignified tissues, such as that comprising the xylem, remains unstained.

References: Jensen 1962

Iodine Solution

Description: To determine the presence and location of starch, stem sections were stained with iodine solution. Starch granules turn blue to black (newly formed starch may appear red to purple) in the presence of iodine.

Procedure:
(1) Use FAA fixed sections re-hydrated in water
(2) Incubate sections in I-KI solution dissolved in water for 2 min (dissolve 2g potassium iodide, 0.2g iodine in 100mL H_2O)
(3) Wash briefly in water to remove any excess dye that was not absorbed by the tissue
(4) Mount in water and view under light microscopy

Notes: Lignified tissues may turn yellow to light orange or brown.

References: Jensen 1962

Phloroglucinol-HCl

Description: In acidic conditions, phloroglucinol reacts with cinnamaldehyde groups present in lignins and sclerenchyma cells to produce a red stain. Phloem tissue is always unstained in the presence of phloroglucinol.

Procedure:
(1) Use FAA fixed tissue re-hydrated to water
(2) Incubate 30 minutes to 1 hour in 0.1% phloroglucinol in 4% HCl prepared in water
(3) Mount in same 0.1% phloroglucinol solution without washing
(4) View under light microscopy

Notes: This stain is not permanent and will fade with time and wash out if stored in water

References: Jensen 1962, Vallet *et al.* 1996, de FaŸ *et al.* 2000,

Safranin / Fast Green

Description: Safranin is a cationic dye that reacts with various materials in the cytoplasm and cell walls and stains cutin, chromatin, lignin, phenolics, tannins and chloroplasts red. Fast Green, used as a counterstain to safranin, stains cellulose and most other structures green. Is useful as a vascular stain as safranin stains lignified cell walls of the xylem red while fast green stains cellulose in phloem tissue green.

Procedure: (1)   Use FAA fixed sections re-hydrated in water
              (2)   Incubate for 10 minutes in 0.1% safranin
              (3)   Wash in DI water until no more dye is removed
              (4)   Dehydrate tissue in 30, 50, 70, and 95% EtOH, 2 minutes each step
              (5)   Stain for 30 seconds in 0.1% Fast Green prepared in 95% EtOH
              (6)   Rinse twice in Absolute EtOH for 2 minutes
              (7)   Rinse in a 1:1 mixture of Absolute EtOH : xylene for 2 minutes
              (8)   Do a final rinse in xylene for 2 minutes
              (9)   Mount in Permount and view under light microscopy

Notes: Produces a permanent stain that nicely differentiates vascular tissue from each other.

References: Jensen 1962

Toluidine Blue O (TBO)

Description: Toluidine Blue O Is a metachromatic stain, i.e. produces different colors depending on the polymer to which it adheres, that is used as a differential staining of vascular tissues in plant tissues. TBO stains secondary cell walls impregnated with lignin, tannin or other polyphenols, xylem tracheids and vessels and schlerenchyma tissues blue-green while primary walls rich in polysaccharide carboxyl groups (pectin) or sulphate groups consisting of ground tissue and phloem are stained pink, red or purple.

Procedure: (1)   Use FAA fixed sections re-hydrated to water
              (2)   Incubate sections in 0.05% TBO for 45 seconds
              (3)   Wash in water briefly
              (4)   Mount in water and view under light microscopy

References: Jensen 1962

# Appendix C

Color Plates Depicting Stained Micrographs

Scientific Name	Common Name	Native	Invasive
Celastrus orbiculatus	Oriental Bittersweet		X
Celastrus scandens	American Bittersweet	X	
Cornus racemosa	Gray Dogwood	X	
Corylus American	American Hazelnut	X	
Elaeagnus umbellate	Autumn Olive		X
Euonymus alata	Winged Burning Bush		X
Euonymus fortunei	Winter Creeper		X
Ligustrum vulgare	Common Privet		X
Lindera benzoin	Spicebush	X	
Lonicera japonica	Japanese Honeysuckle		X
Lonicera maackii	Amur Honeysuckle		X
Parthenocissus quinquefolia	Virginia Creeper	X	
Physocarpus opulifolius	Ninebark	X	
Rhamnus cathartica	Common Buckthorn		X
Rosa multiflora	Multiflora Rose		X
Rosa palustris	Swamp Rose	X	
Vibumum prunifolium	Blackhaw	X	

Micrographs of stem cross sections depicting **(A)** tissue under visible light **(B)** TTC stained tissue under visible light **(C)** tissue autofluorescence under blue light **(D)** CFDA stained tissue under blue light **(E)** chlorophyll autofluorescence under blue light **(F)** iodine stained starch granules under visible light **(G)** xylem tissue as stained by phloroglucinol viewed under visible light **(H)** phloem tissue as stained with aniline blue viewed under visible light **(I)** the differentiation of vascular tissue stained with toluidine blue viewed under visible light and **(J)** the differentiation of vascular tissue double stained with safranin-fast green viewed under visible light for nine non-native invasive and eight native woody plants common to Ohio. P = Pith, PX = Primary Xylem, SX = Secondary Xylem, R = xylem Ray cells, PH = Phloem, PF = Phloem Fibers and PD = Periderm. Scale bar represents 100μm.

Corylus americana — American Hazelnut

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Euonymus alata   Winged Burning Bush
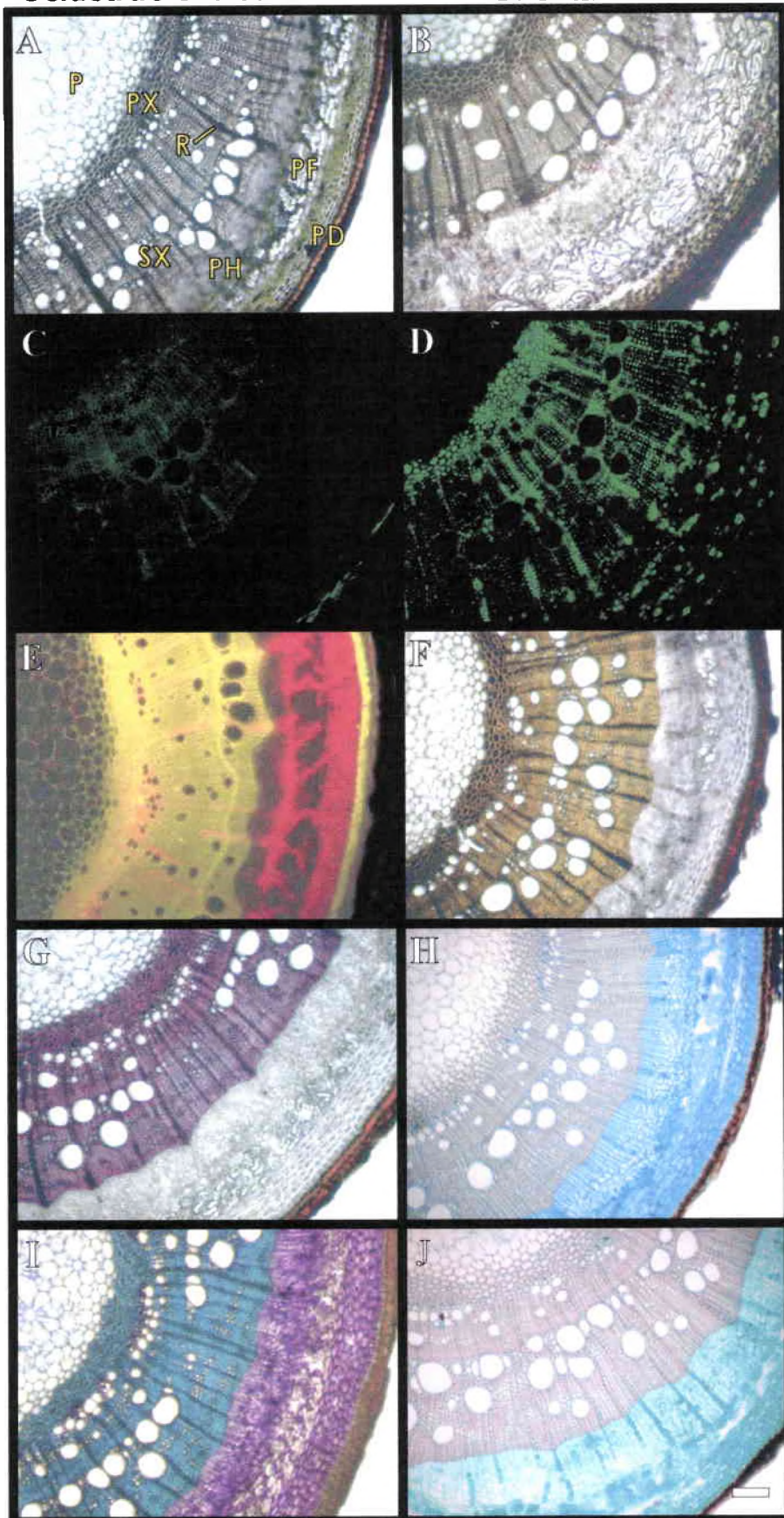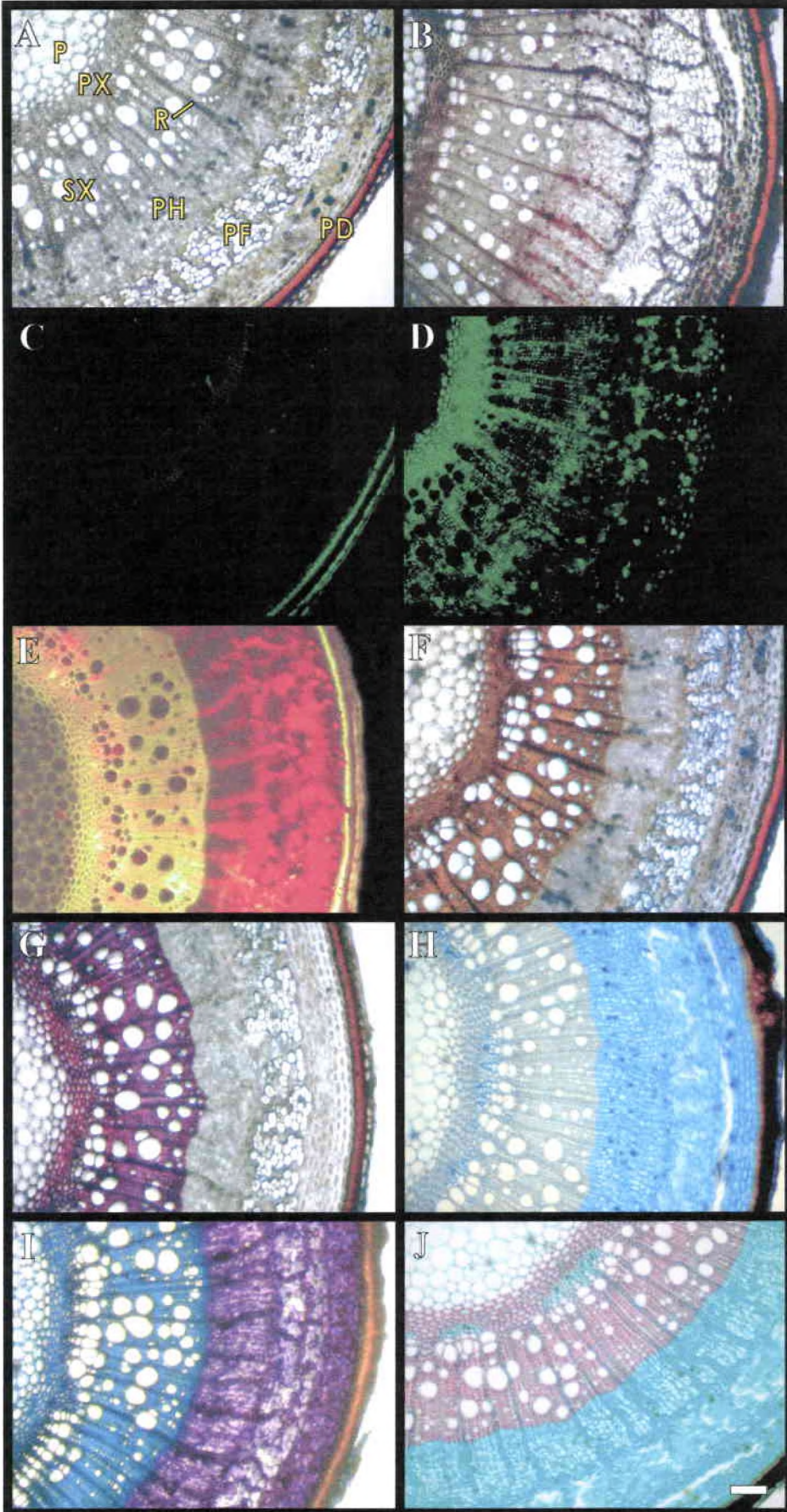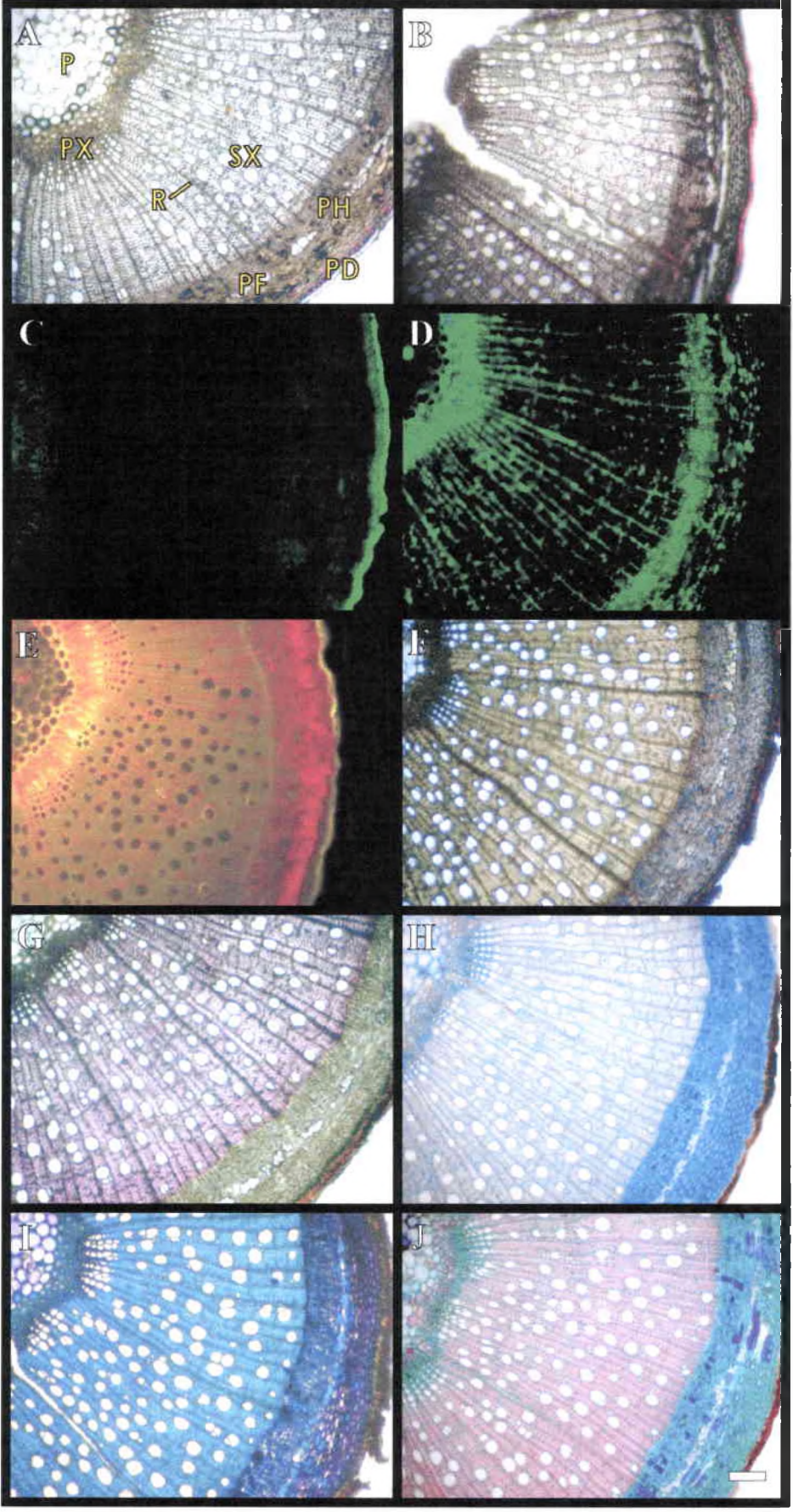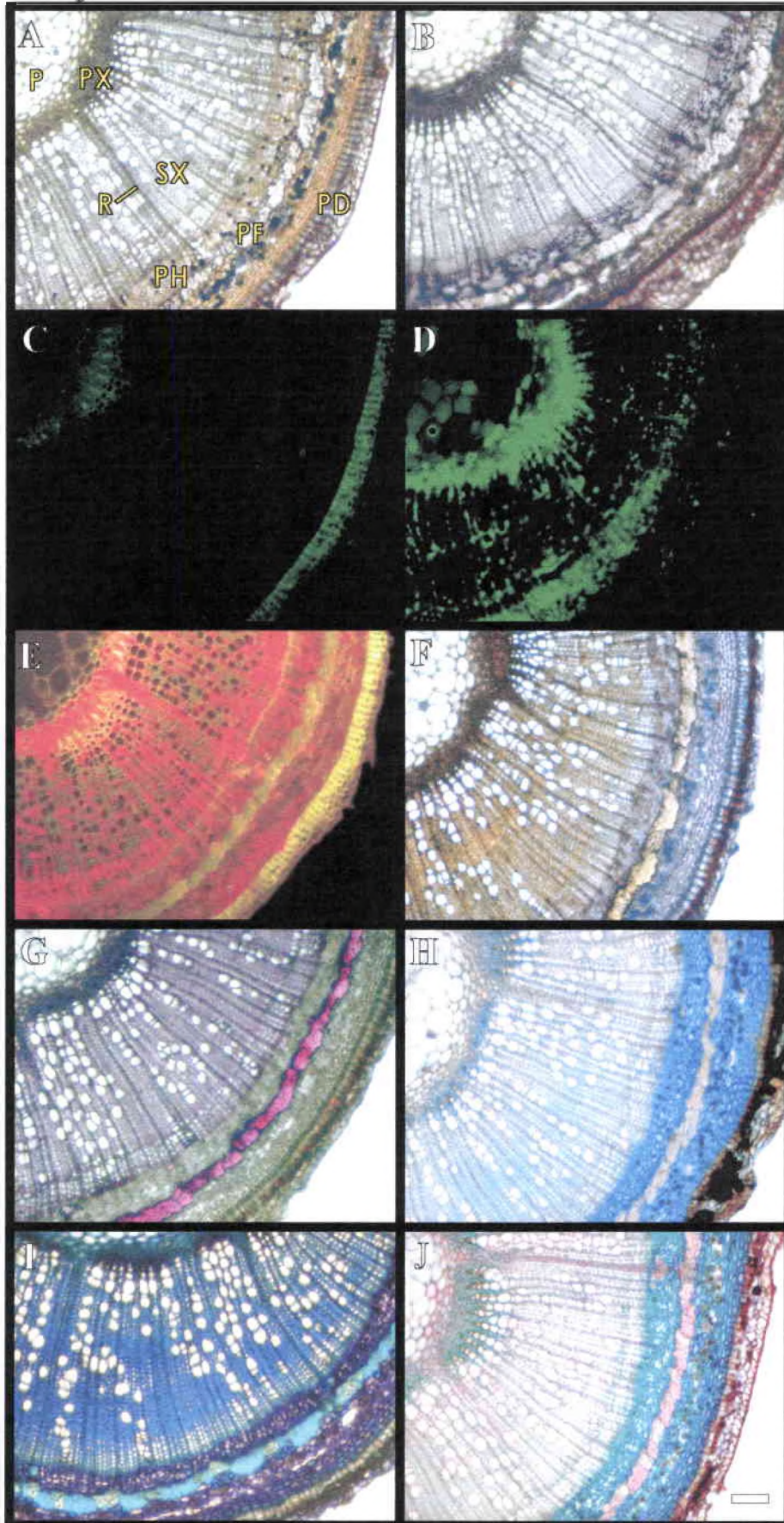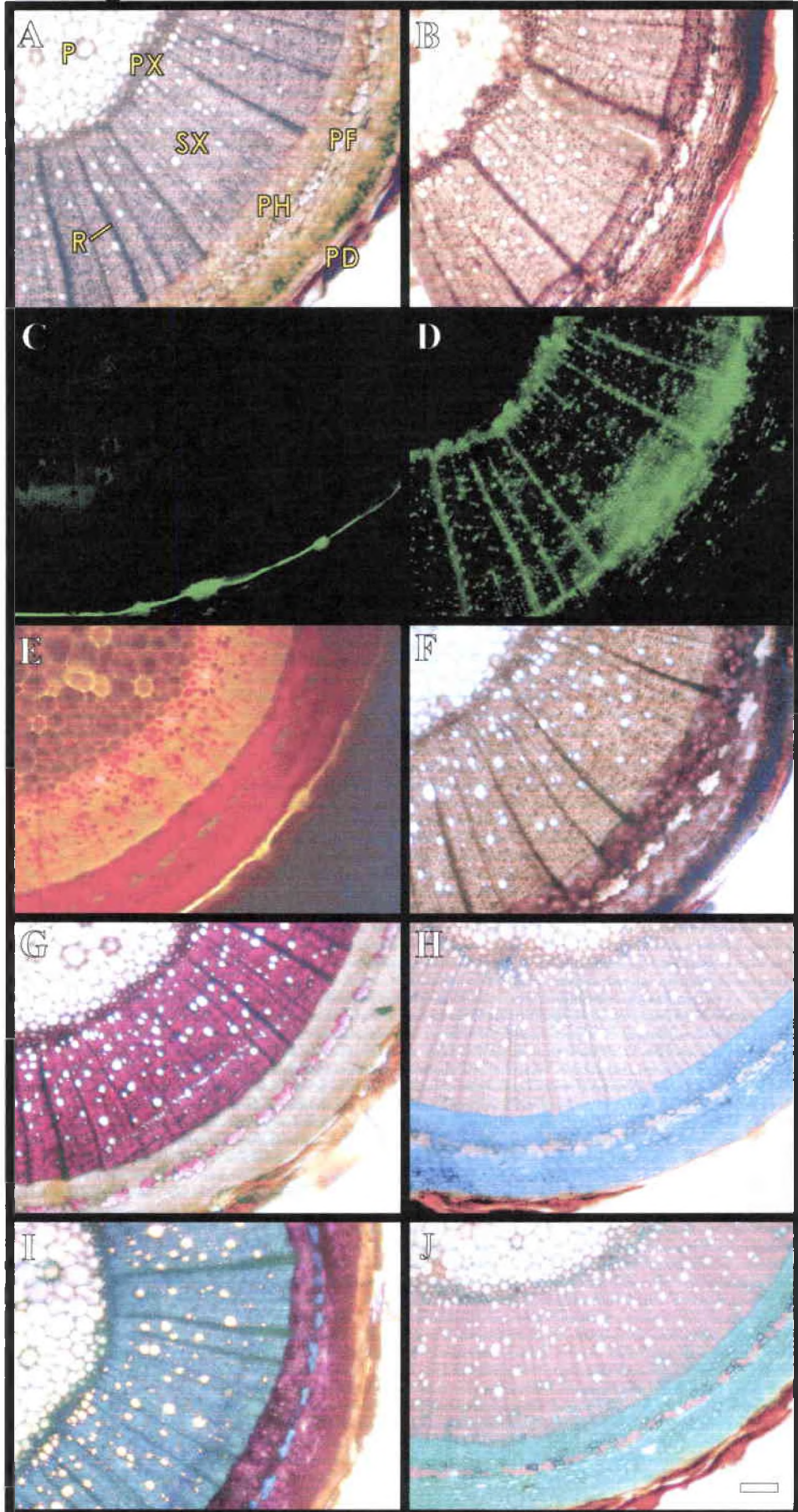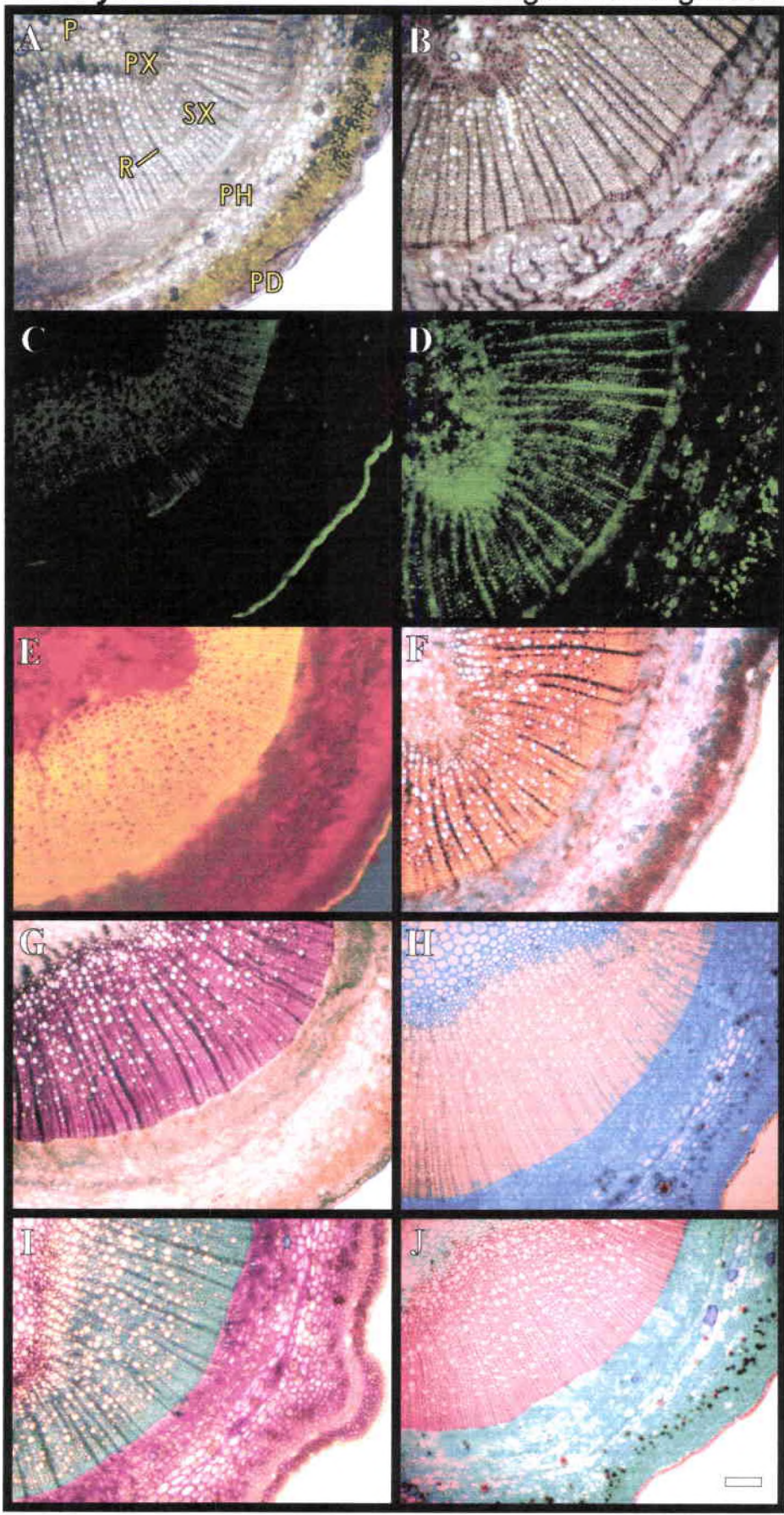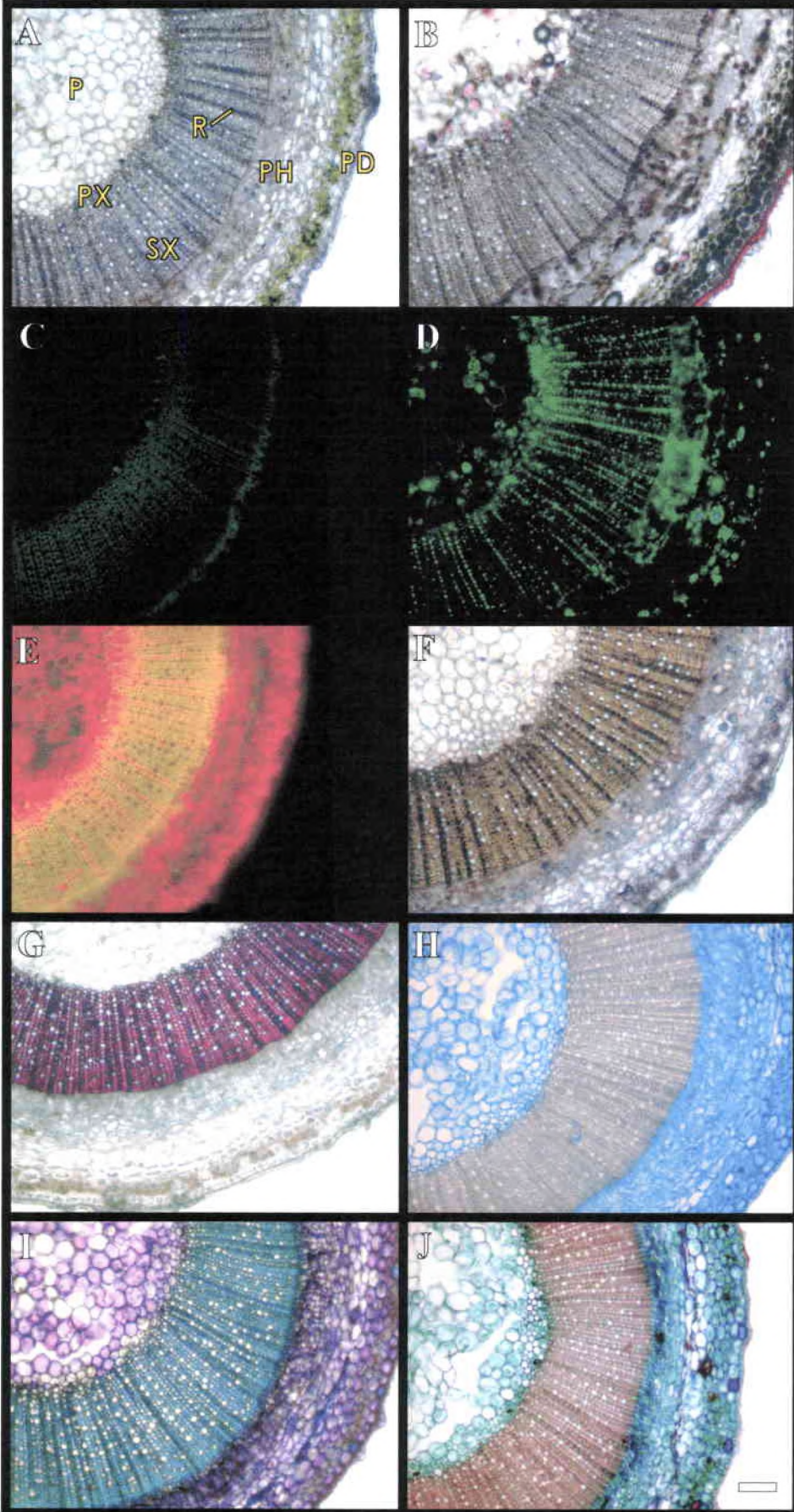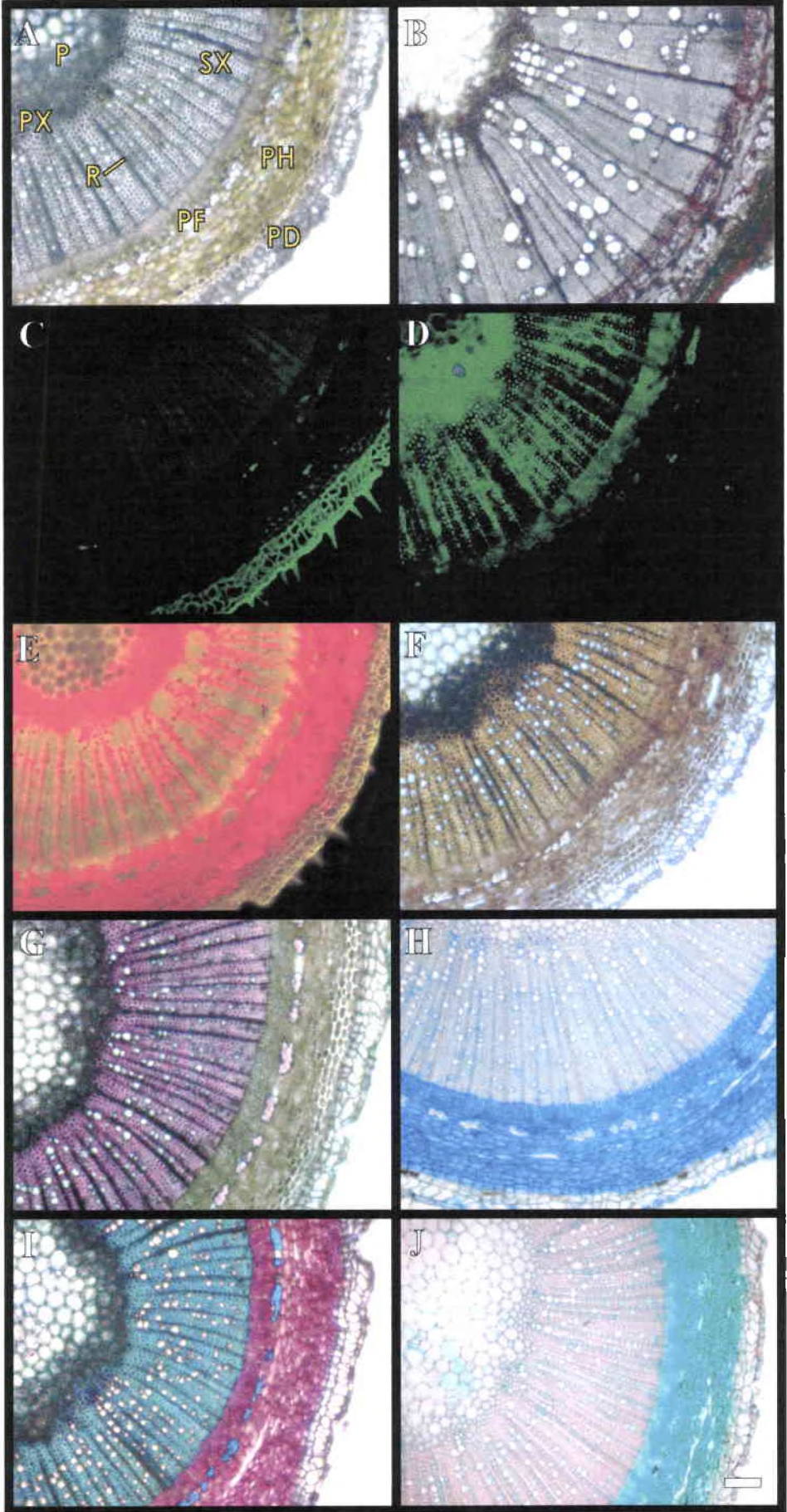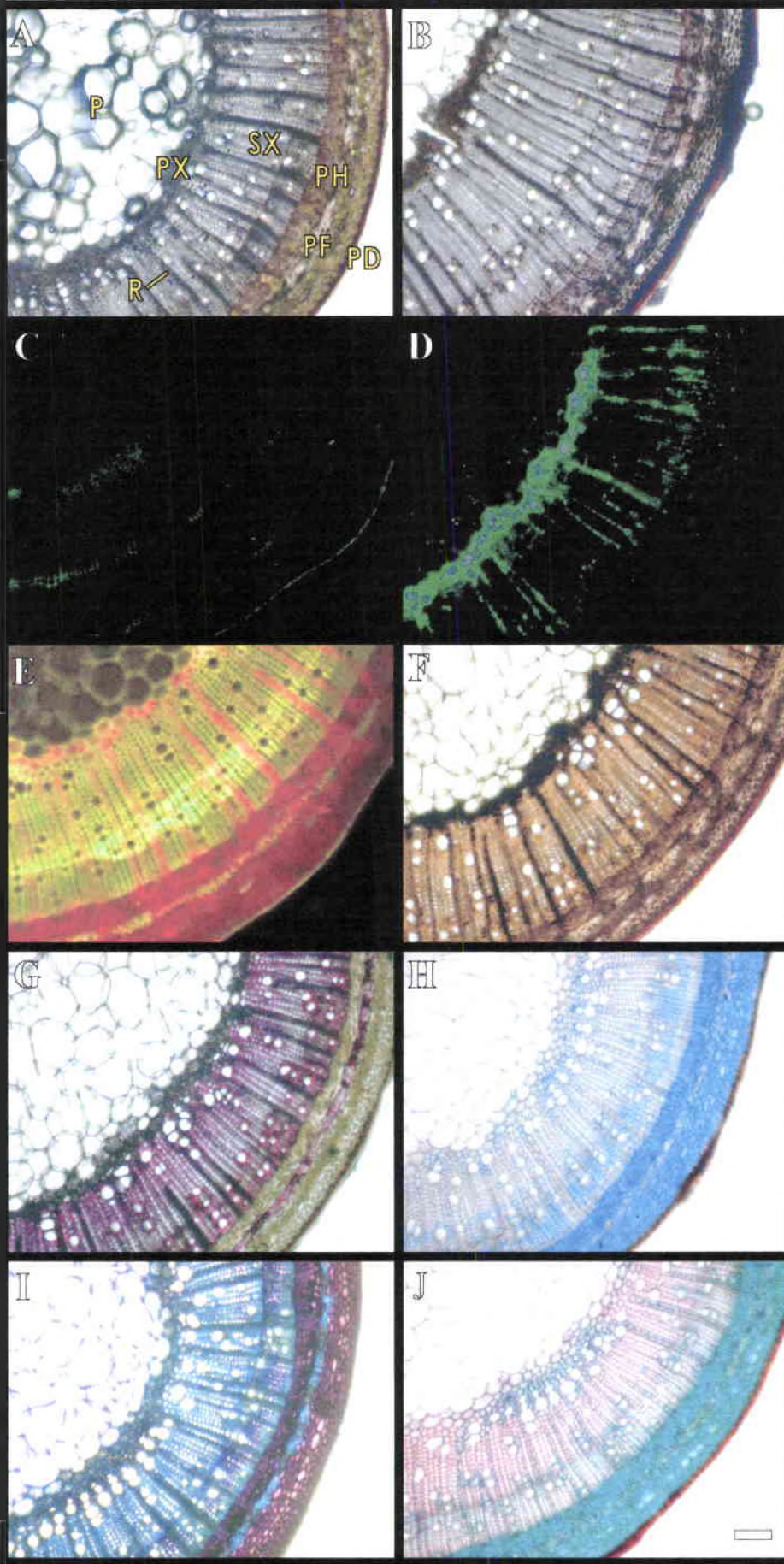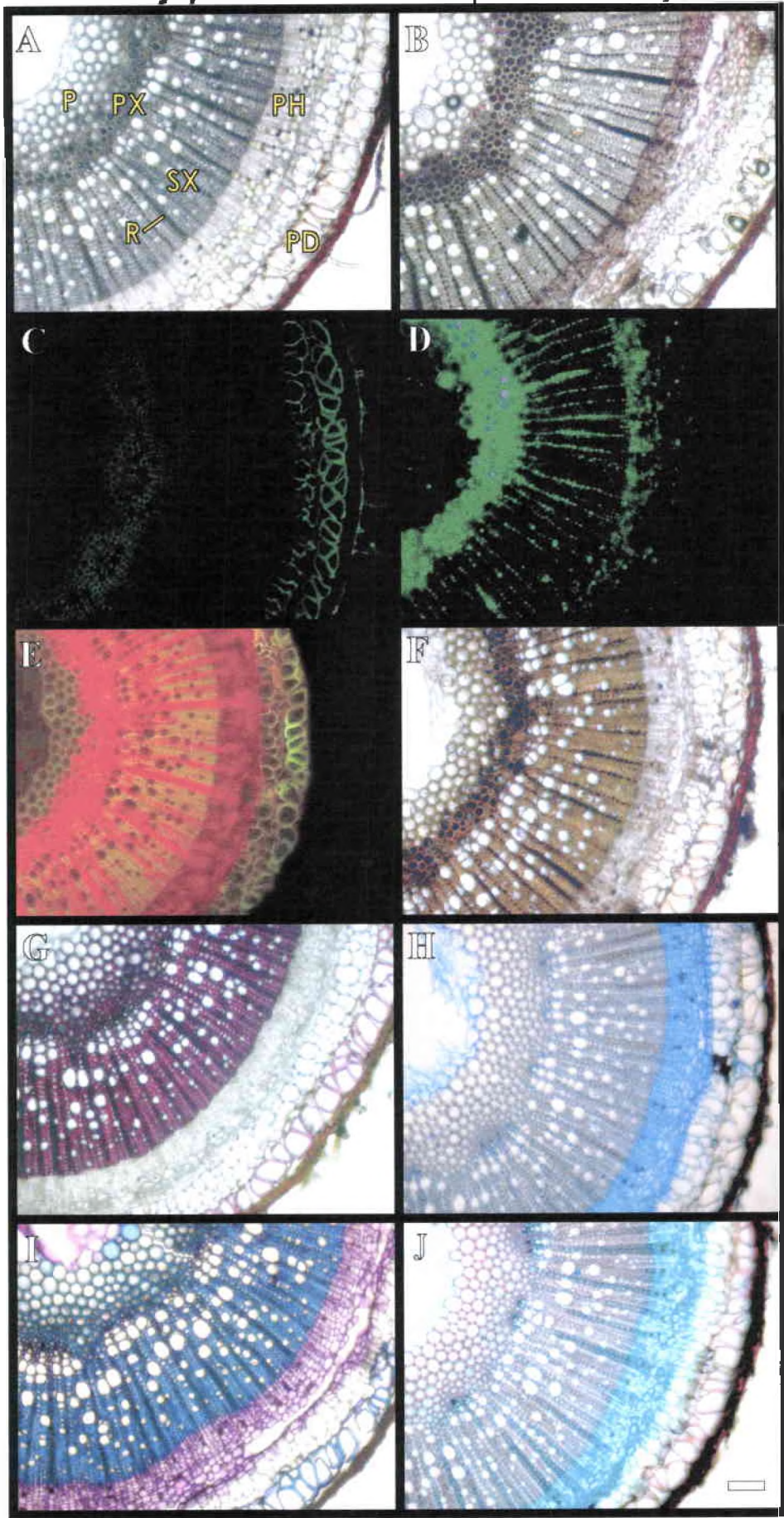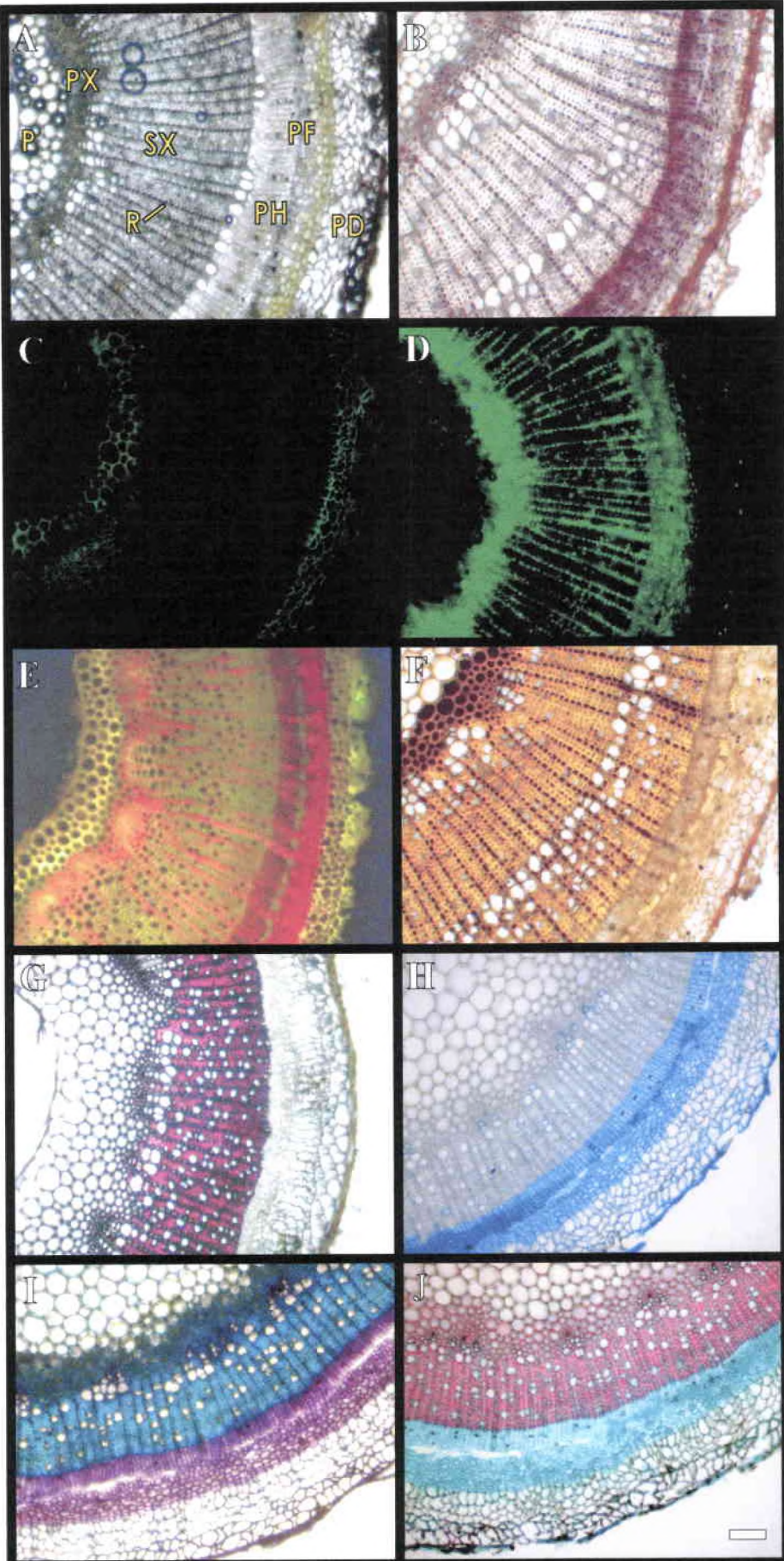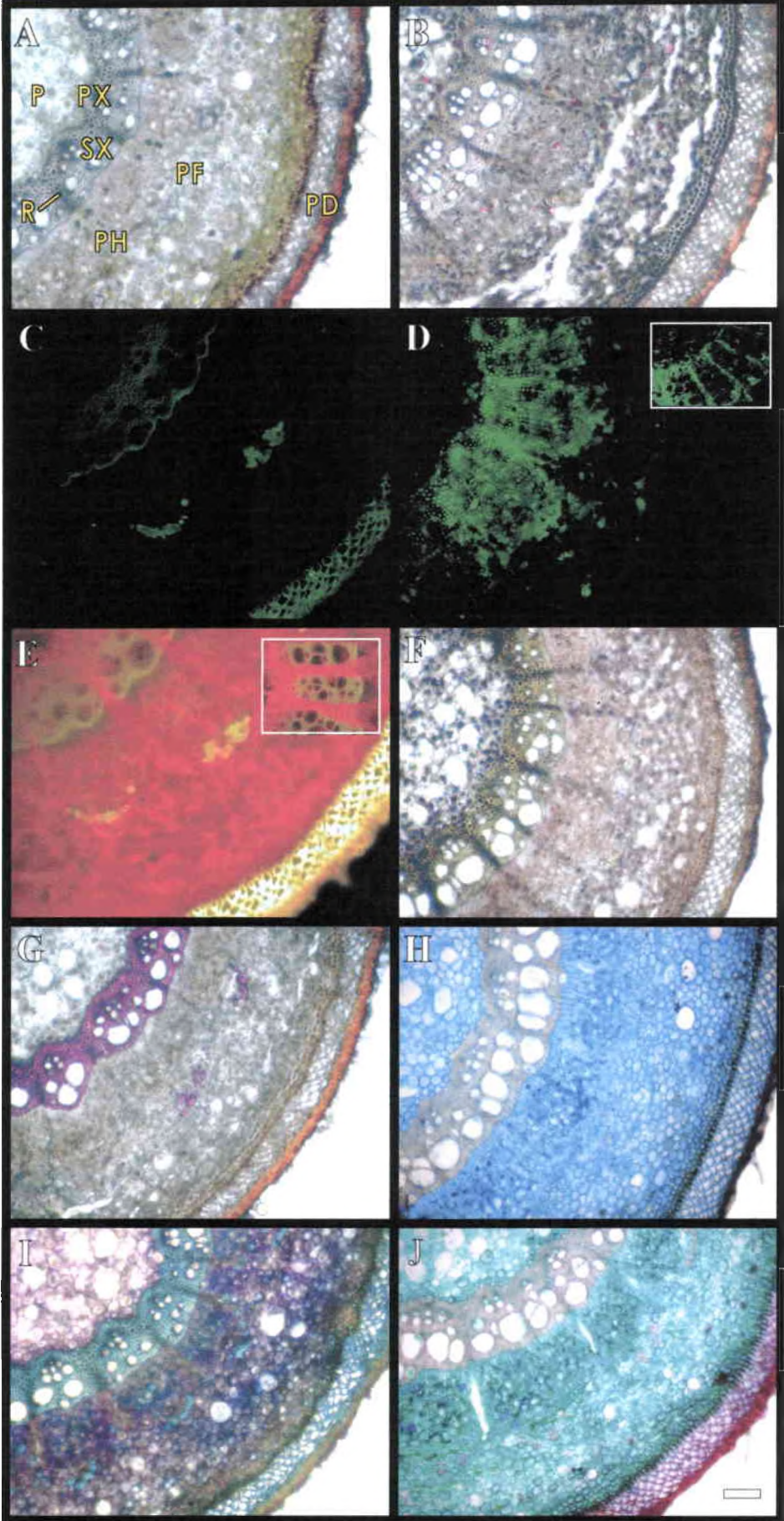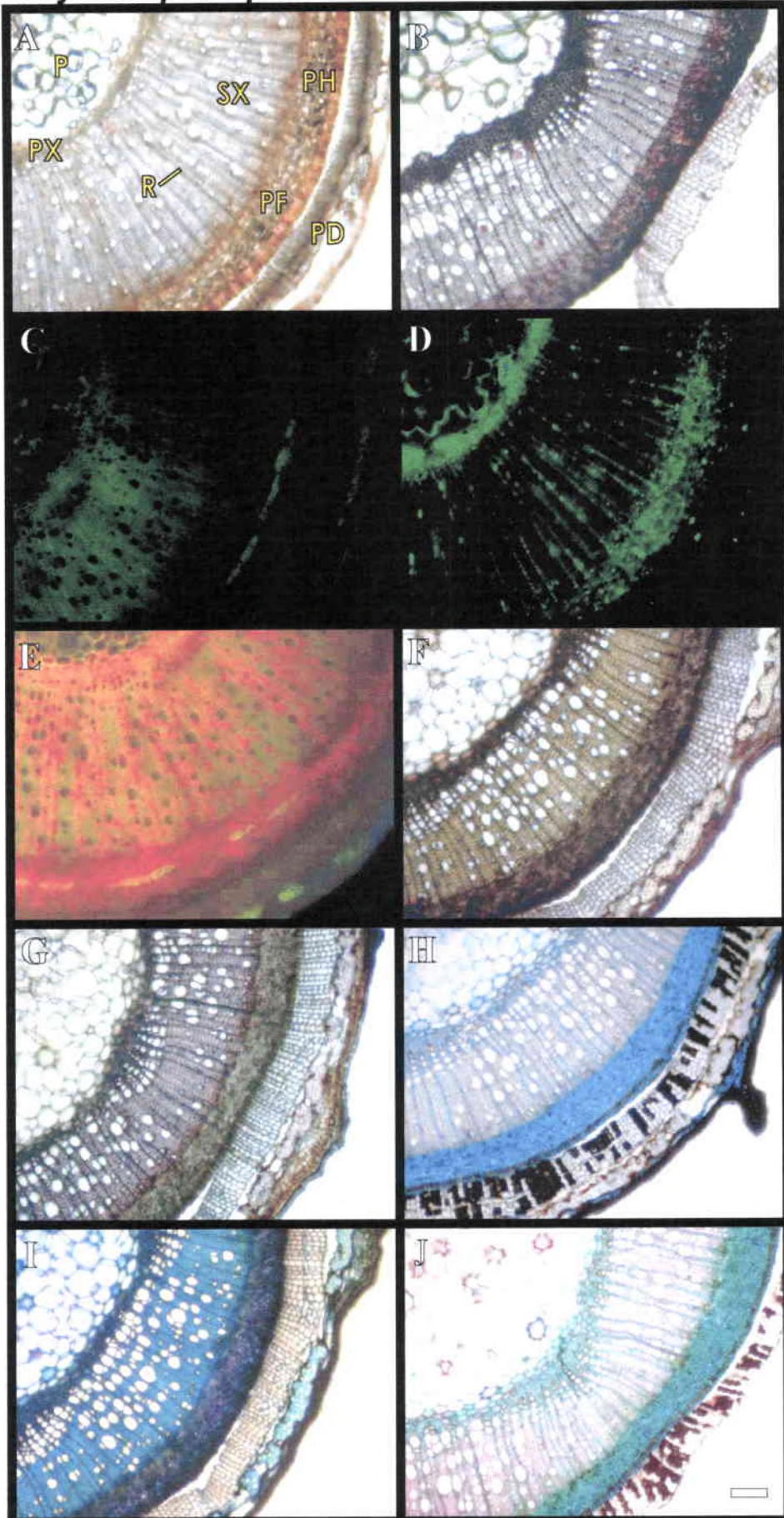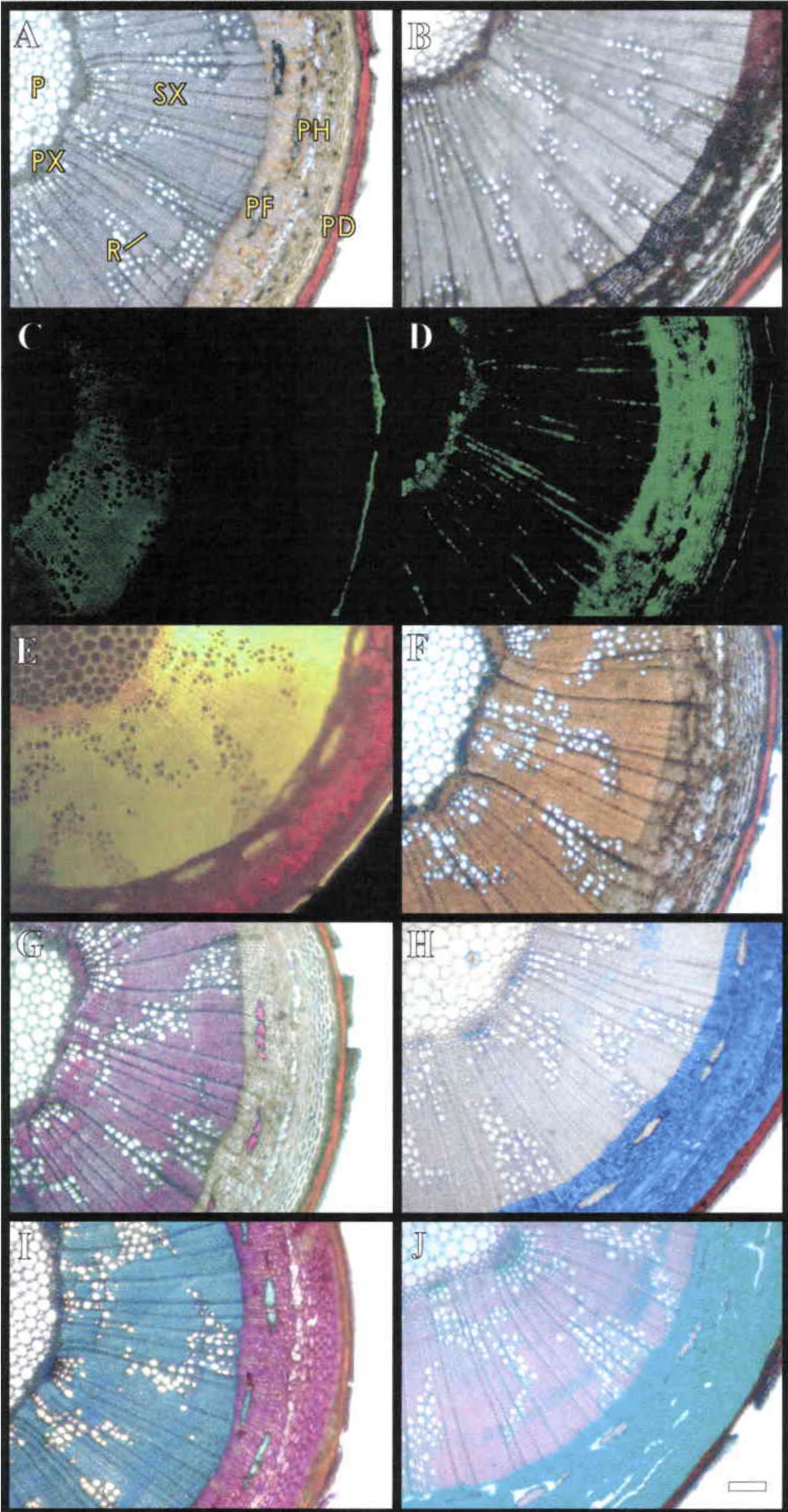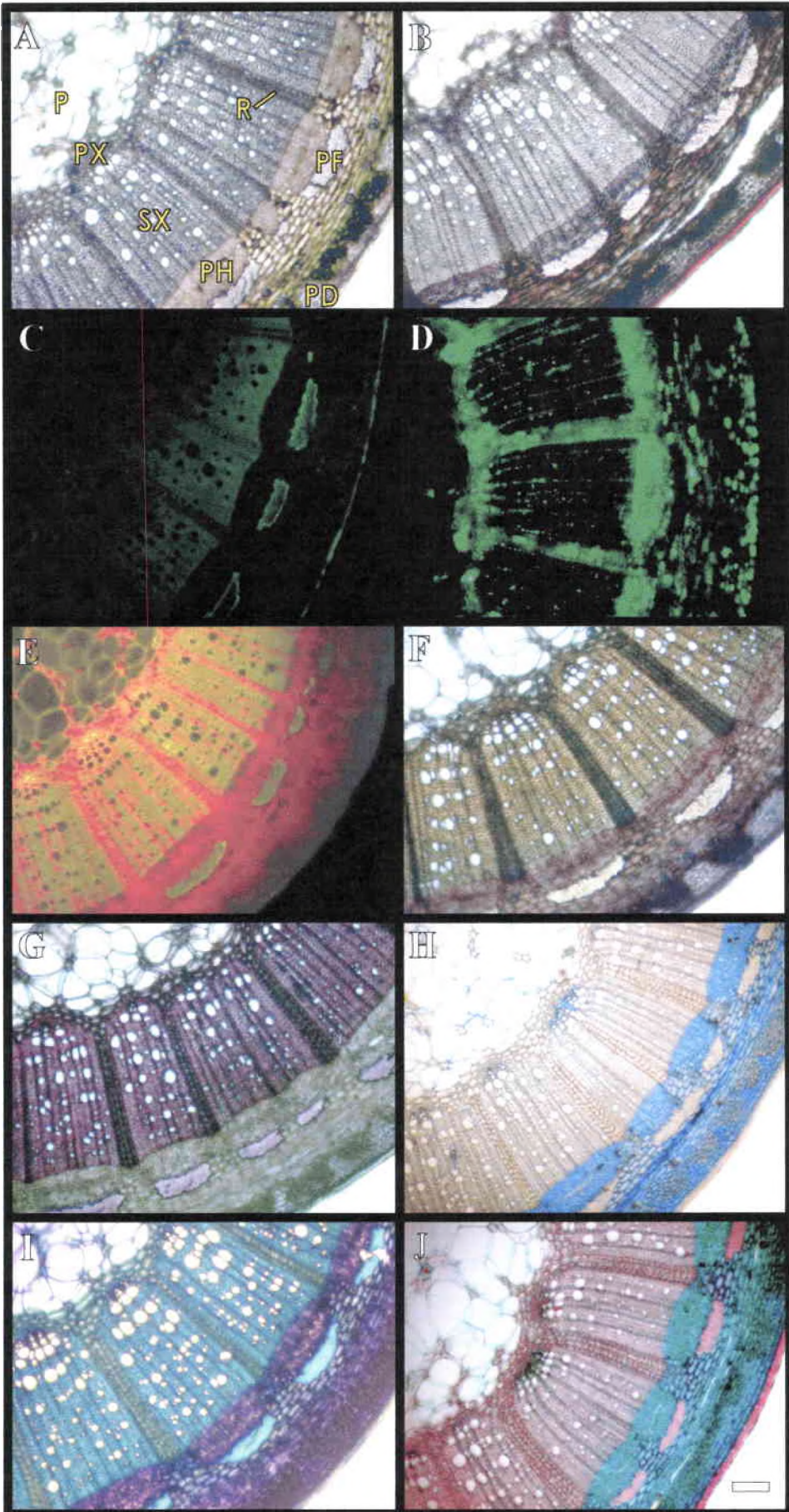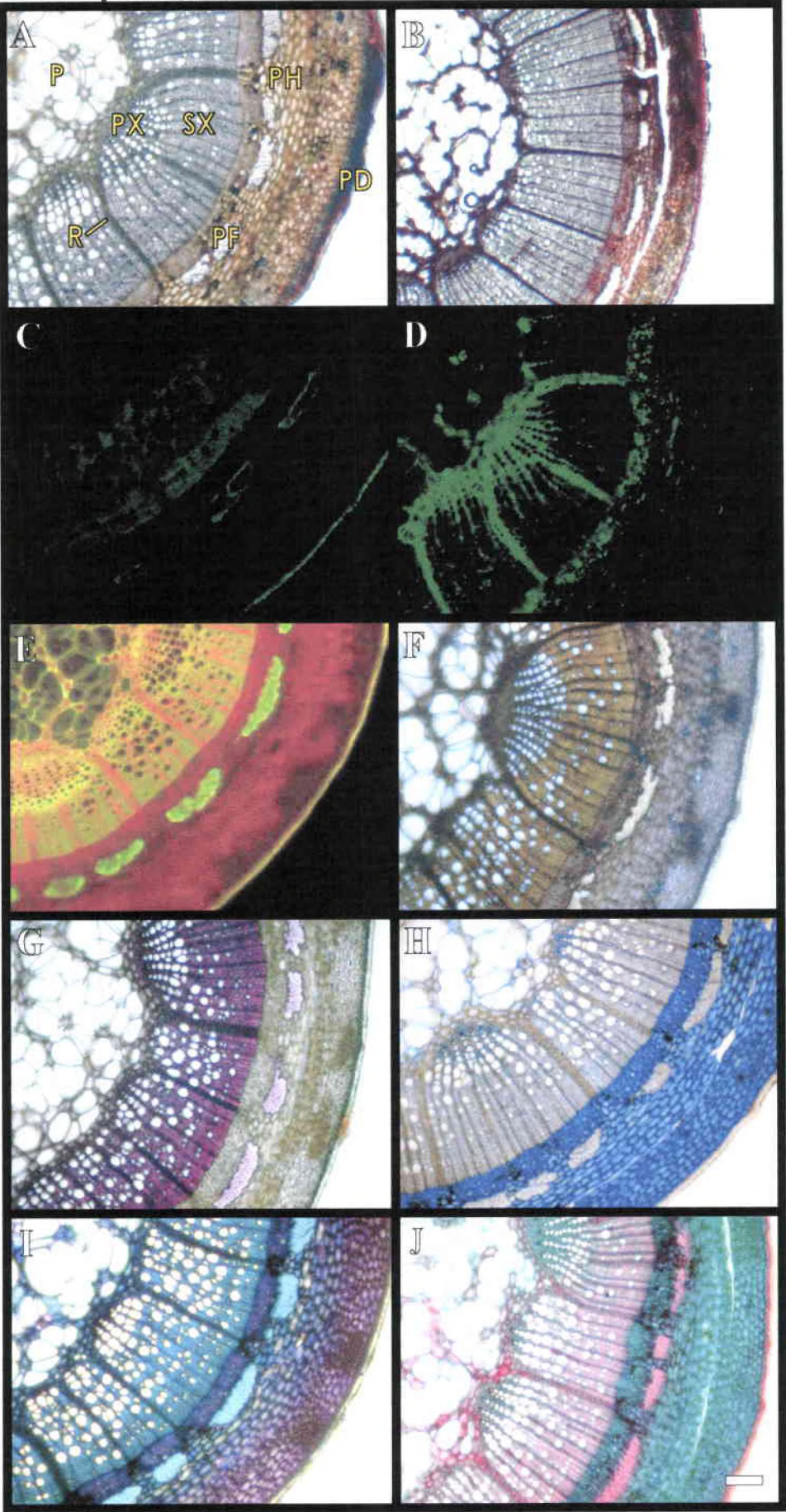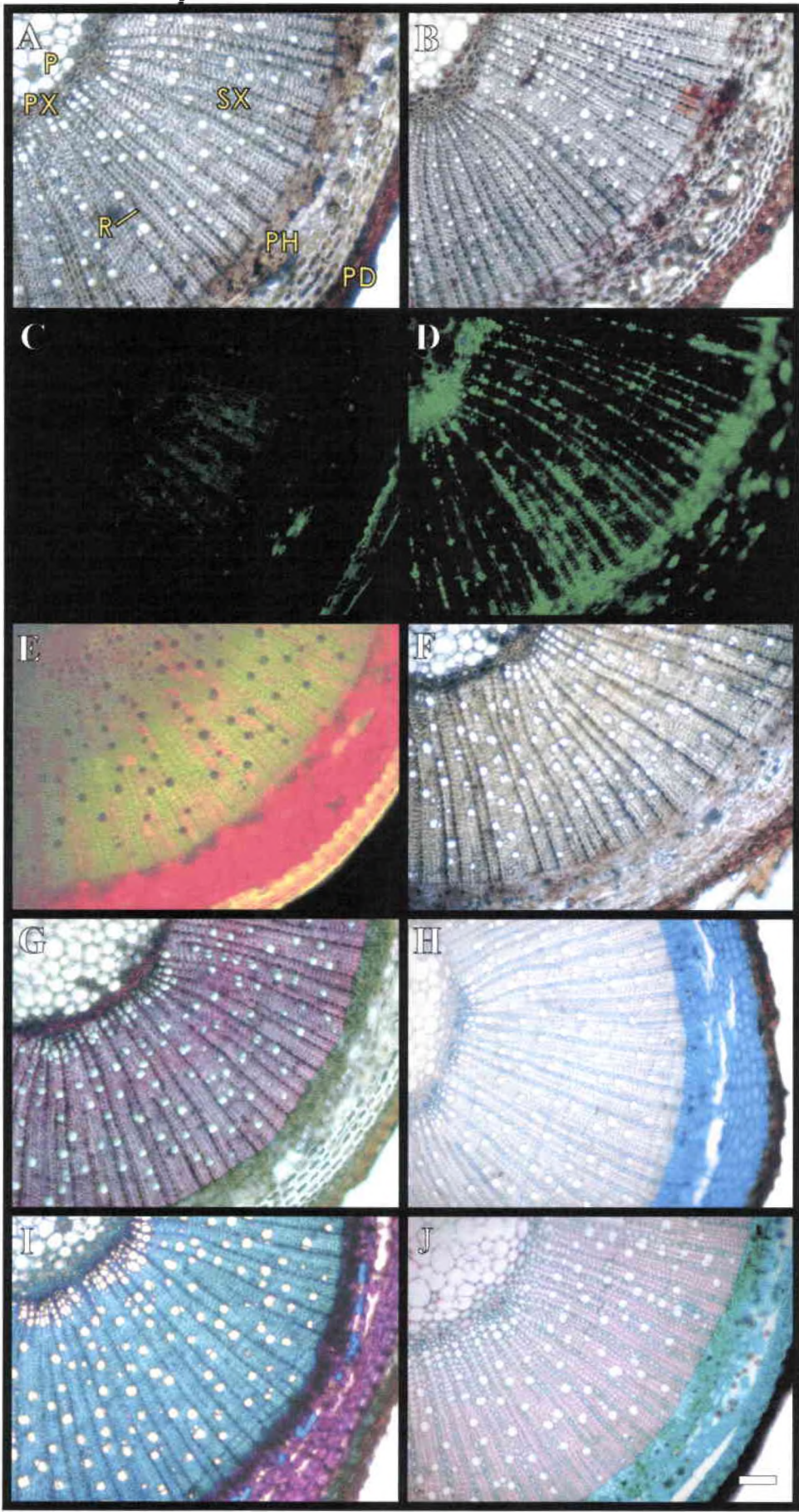
Appendix D

SEM Micrographs of Stem Cross Sections

| Scientific Name	Common Name	Native	Invasive
Celastrus orbiculatus	Oriental Bittersweet		X
Celastrus scandens	American Bittersweet	X	
Cornus racemosa	Gray Dogwood	X	
Corylus american	American Hazelnut	X	
Euonymus alata	Winged Burning Bush		X
Ligustrum vulgare	Common Privet		X
Lonicera japonica	Japanese Honeysuckle		X
Lonicera maackii	Amur Honeysuckle		X
Physocarpus opulifolius	Ninebark	X	
Rhamnus cathartica	Common Buckthorn		X
Rosa palustris	Swamp Rose	X	
Viburnum prunifolium	Blackhaw	X	

Scanning electron micrograph depicting cross section of woody stem tissue after being fixed in glutaraldehyde, dehydrated through an ethanol series, critically dried and gold sputter coated. The image shows a montage composed of multiple smaller images put together for six non-native invasive and seven native woody plants common to Ohio. Tissue structures are represented by P = Pith, PXy = Primary Xylem, SXy = Secondary Xylem, R = xylem Ray cells, VC = Vascular Cambium (when present), Ph = Phloem, PhF = Phloem Fiber cells (when present), PD = Periderm and E = epidermis. Scale bar represents 50μm

Celastrus orbiculatus

Oriental Bittersweet

American Bittersweet

Celastrus scandens

Gray Dogwood

Cornus racemosa

American Hazelnut

*Corylus americana*

Winged Burning Bush

Euonymus alata

Common Privet

Ligustrum vulgare

188

Japanese Honeysuckle

Lonicera japonica

E

PD

PhF

Ph

VC

R

SXy

PXy

P

189

Amur Honeysuckle

Lonicera maackii

190

Ninebark

Physocarpus opulifolius

Common Buckthorn

Rhamnus cathartica

Labels visible in figure: E, PD, Ph, PhF, VC, SXy, PXy, R, P

Swamp Rose

Rosa palustris

Blackhaw

Viburnum prunifolium