MICROPROPAGATION OF THE RELICT GENUS *CERCIDIPHYLLUM*

(CERCIDIPHYLLACEAE)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Focusing on various aspects of micropropagation with respect to well represented genotypes within *Cercidiphyllum*, this study is an attempt to broaden the experimental knowledge of the genus. The Tertiary relict *Cercidiphyllum* (Cercidiphyllaceae) is endemic to Japan and China and consists of two deciduous tree species, common katsura, *C. japonicum* Sieb. & Zucc. and the hiro-ha-katsura, *C. magnificum* Nakai. To date, there exists no literature on the *in vitro* requirements of *C. magnificum* and limited information on *C. japonicum*. Prized as specimen trees, the importance of *Cercidiphyllum* extends beyond its ornamental merit and regarded a multifaceted genetic resource. Subsequent protocols and analysis will not only be useful for nursery production and cultivar improvement but aid in ongoing conservation efforts of this rare genus.

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DEDICATION

I especially thank my dad for always being a positive force in my life as well as my brother for all of the "brother projects" that have made us so very close. My propensity to dig deeper into the science of horticulture can be attributed to my long-time mentor, Neal Holland. Many interesting topics within this thesis have been hashed-out with my best friend, Adam Ogden (over a decade!). Arête! Axios! Alethes! To Ken Parker for the companionship, Josh Silbernagel for the danger, the Echevaria clan for loyalty, Ian Johnson for the Beam, Shane Ocshner for the riffs, Michael Dosmann for the wit, Hannah Passolt for the goofiness, Grady Reed for the shenanigans, and to Ana and Baird Heilman, Whitney Harchencko, Veronica Brotons, and all the other NDSU PLSc Latinos for your passion. Without my lab mate, Juan Ramon Franco-Coronado, I would have never heard Illmatic. You kept my head to the grindstone and taught me about the good things in life. You are a good man and I hope to be your friend and colleague for many years to come. Next time I see you, I hope we're Stillmatic. Adios, mi amigo. To you, Lo.

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Budbreak (BB)...……the mean individual treatment sample percentage of buds broken, defined as the presence of one fully expanded leaf derived from bud primordia and scored binary, 0 = budbreak or 1 = budbreak, and measured as a mean percentage of buds broken by the total number of buds per stem.

Shoot length (SL)…….……..the mean individual treatment sample somatic shoot length, measured from the explant shoot apical meristem primordia to the developing microshoot terminal apical meristem.

Shoot number (SN)…….……..the mean individual treatment sample somatic shoot of at least 4 mm in length, derived from the apical meristem of explant bud primordia and measured proximally from explant bud primordia to the elongating shoot apical meristem.

Leaf surface area (LA)……the mean individual treatment sample leaf length, from the attachment of the distal petiole and proximal leaf base, and the leaf width, from the widest point, perpendicular to the leaf midrib, of 3 random, fully expanded leaves, which show no marginal curling, as well as omission of the precocious axial leaf after budbreak from preformed bud primordia.

Bud number (LN)……..….the mean individual treatment sample bud number, which consists of fully expanded leaves and is derived from microshoot terminal and axial bud primordia.

Root number (RN)……..the mean individual treatment sample root number of at least 1 mm in length, from adventitiously derived root primordia located at the proximal base of an explant microshoot.

Root length (RL)……the mean individual treatment sample root length, from adventitiously derived root primordia located at the proximal base of an explant microshoot, basipetally to the distal end.

Plant Growth Regulator (PGR)…….commonly referred as plant hormones, these compounds occur as signal molecules within the plant at low levels, such as: the cytokinins, 6-benzylaminopurine (BA) and thidiazuron (TDZ), the auxins, indol-3-butyric acid (IBA), napthaleneacetic acid (NAA) 2,4-Dichlorophenoxyacetic acid (2,4-D) and the gibberellin, gibberellic acid (GA_3).

Phenolic Acids (PA)……..a group of vast and highly diverse secondary plant metabolites consisting of an aromatic ring bearing one or more hydroxyl groups, derived from the phenylpropanoid pathway, and considered to be a main component of allelopathic, seed dispersal, and structural adaptations of plants.

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CHAPTER 1. INTRODUCTION

The genus *Cercidiphyllum* (Cercidiphyllaceae) is endemic to Japan and China (Brown, 1939) and consists of two deciduous dioecious tree species, common katsura (*C. japonicum* Sieb. & Zucc.) and the hiro-ha-katsura (*C. magnificum* Nakai). Prized as specimen trees, nearly all genotypes of the genus have potential for commercial nursery development (Haag, 1982; Andrews, 1998). To date, there exists no literature on the micropropagation of *C. magnificum* and limited information on *C. japonicum*. This study focuses on various aspects of micropropagation with respect to well-represented genotypes within *Cercidiphyllum*. The micropropagation of woody ornamentals has steadily increased in the last decade, providing the industry a stepping-stone to biotechnological approaches to develop tools for further plant improvement and aid in conservation efforts.

Taxonomy

Common katsura tree (*C. japonicum* Sieb. & Zucc.) was first described in Japan (Hoffman and Schultes, 1853) and is the most well-known species of the genus. Nakai (1919) published a second variety, the hiro-ha-katsura (*C. japonicum* var. *magnificum* Nakai), which was given specific status (*C. magnificum* (Nakai) Nakai) the following year. The hiro-ha-katsura exists naturally in diminutive mountain regions in Japan (Lancaster, 1997; Lindquist, 1954). *Cercidiphyllum* typically grows 5-15 m in height and similar width. Young plants generally undergo rapid growth, owing to the predominance of apical shoots. This predominance diminishes after the onset of sexual maturity (10-20 years) until a prominent long shoot is to be found only at the terminal portion of any particular branch and is never associated with an inflorescence (Swamy and Bailey 1949; Titman, 1955).

The generic epithet *Cercidiphyllum* is partially derived from the Greek word *kerkis*, meaning "shuttle," a flat weaver's tool that carries thread across a loom, referring to the flat, woody seed pods of the Redbud tree, *Cercis* (subfamily Caesalpinioideae), which is morphologically akin to katsura. Flowers of the genus are inconspicuous and anemophilious, maturing to small 1-2 cm long dehiscent pods containing brown trapezoidal seeds (Bailey, 1979), with germination rates ranging from 8% to 34% (Dosmann, 2000). Seed pods begin to split in late fall (Dirr, 1987), shedding most seeds within a few days.

Leaves of the genus are opposite, rounded, simple, and petiolate (Hoffman and Schultes, 1853; Swamy, 1949). Additionally, leaves are heterophyllous, whose shoots concurrently undergo long apically dominant and short axial growth, depending on environmental and physiological conditions (Titman, 1955). The slight downward-cupped margins and rugose texture of katsura foliage is enhanced by its seasonal display of color. In the spring, single precocious leaves appear along shoots, emerging light green to wine-red, then fading to dark blue-green mid-summer, and variably to a delicate reddish-purple or golden-yellow in the fall. Leaf emergence is often accompanied by a citrus-like fragrance, similar to the warm musk of mango flesh; whereas, senescence is known to bring about hints of cinnamon and vanilla.

The broader-leaved hiro-ha-katsura subtly differs morphologically from its cogener, *C. japonicum*; and has subsequently been in and out of taxonomic discussion (Spongberg, 1979; Koller, 1987; Li, 2002), along with a number of weeping katsura cultivars (i.e., 'Morioka Weeping', 'Amazing Grace', 'Pendulum' and 'Tidal Wave') that recently have become popular in the ornamental nursery industry as an alternative to the upright, tree-form. The vegetative growth of weeping varieties tend to be more vigorous than the straight species; demonstrated by an often erratic nature of bud placement along stems, the longer distance between internodes, and

'whorled' axial and terminal multi-nodal meristems. Although these two species are quite similar, the characteristic morphological difference is the number of 'wings' that proliferate from the seed capsules; common katsura bears only one and its cogener, two. Nevertheless, any existing debate as to the status of *C. magnificum* and the weeping cultivars of katsura has been resolved. Based on the results of nuclear ribosomal sequences, Li et al. (2002) found weeping varieties *C. japonicum* forma *pendulum* (designated the 'Weeping Group') to be phylogenetically derived from *C. japonicum*, whereas, *C. japonicum* and *C. magnificum* to be genetically distinct, supporting the recognition of *C. magnificum* as a separate species; data which is additionally supported by a larger molecular phylogenetic study (Qi, et al., 2012).

Habitat and Genetics

Cercidiphyllum is considered to be a 'living fossil' of the temperate East Asian Northern Hemisphere Tertiary period, similar to montotypic relicts; *Metasequoia* (Merill, 1948), *Eupetela* (Wei, 2010), and *Ginkgo* (del Tredici, 2000), whose shared native distribution range spans from southwest China to south Japan (Liu, 1988; Qi, 2012). The native habitat of *Cercidiphyllum* is generally thought to be in riparian forests, found near forest margins and streams (Chien, 1992; Dosmann, 2000; Fu, 2001). Light-demanding, *C. japonicum* populations prefer a riparian habitat on gentle slopes, whose species composition varies relating to physical (altitude, slope, soil temperature) and chemical (available K, NH_4, pH) properties (Gunkel, 1949a; 1949b; Wei, 2010). Seedling establishment conditions of *C. japonicum* are sparse and also thought to be influenced by topography and light availability (Dosmann, 1999; Kubo, 2000). Though seedlings are rarely found in forests, *C. japonicum* maintains its populations over long periods by sprouting, which compensates for sparse seedling regeneration (Titman, 1955; Kubo, 2005, 2010; Wei, 2010).

The importance of *Cercidiphyllum* extends beyond its ornamental merit and should be regarded a multifaceted genetic resource. In Japan, the wood of *C. japonicum* var. *sinense* is used as a common timber source for construction, as it is generally of taller, fastigiate habit (30-40 m) and single trunked (Bailey, 1979). Additionally, knowledge of katsura population dynamics have given climate researchers insight into the complex adaptive patterns of plant movement and evolution, relative to our current understanding, both during and after the Cenezoic (Brown, 1939; Wolfe, 1997; Stockey, 1983; Krassilov, 2010; Chien, 2012).

The Tertiary is a geologic period from 65 to 2.6 mya, a timespan that lies between the Secondary and Quaternary periods, now considered to be of the Cenezoic Era. It was in this time frame, history experienced the adaptive radiation of angiosperms throughout the globe (Brown, 1939; Wolfe, 1997), rapidly evolving to adjust to a myriad of catastrophic environmental effects of the upper Cretaceous (98 to 65 mya). Conversely, the persistence of these relictual species can be attributed to relatively minimal environmental changes (Krassilov, 2010) and a suite of locally well-adapted response mechanisms to exogenous stimuli. For example, on exposed sites or limits of its range, vegetative basal resprouting capacities of *C. japonicum* (Wei, 2010) allowed the species to spread into adjacent areas, likely circumventing the difficult process of seedling establishment (del Tredici, 2001), and can be assumed continual resprouting plays a key role in maintaining low genetic diversity of this species within populations. Similar evidence for intrapopulation genetic isolation via clonal propagation was also described in shrub willow (*Salix purpurea*) (Lin et al., 2009), correlating this with the ability to become successfully naturalized in a new environment.

Focusing on the evolution of temperate hardwoods, DeVore et al. (2013) suggests, on the basis of *C. japonicum* and other Tertiary relicts, temperate deciduous forest trees' seasonal

morphological heterophylly can be detected in the fossil record. Heterophyllous leaves on branches of extant *Cercidiphyllum* trees were measured and used as a profile with which to compare fossil leaves of a morphologically similar family, Trochodendraceae (Brown, 1939). The frequency ratios of leaves from short and long shoots on branches of living *C. japonicum* (Cercidiphyllaceae) trees were compared with fossil leaves of late Paleocene *Zizyphoides flabellum* of Almont, North Dakota, comparing seasonal dormancy and the correlative distribution of leaf phenology and wood porosity type. Similar work on the development of long and short shoots in the relict, *Ginkgo biloba* (Gunkel, 1946a; 1946b; 1949a; 1949b) has also been refereed (del Tredici, 2000) with respect to temperate hardwood evolution.

Devore et al. (2013) speculate specific triggers, such as photoperiod and changing temperature, were pivotal to the evolution of modern temperate deciduous trees, agreeing with past research (Kubo, 2000). For example, in the case of katsura, the development of preformed leaves and diffuse-porous wood, consisting of spongy pith and cortex in the spring (Titman, 1955), allowed for early leaf emergence in the temperate environment. These particular responses may have been first to the trigger of photoperiod change in high-latitude plants and later combined with lower-temperature regimes of upland regions (Devore et al., 2013).

Efforts have been made to develop microsatellite markers for both *C. japonicum* and *C. magnificum* (Li, 2002; Isagi, 2005; Sato, 2006) at the population level across the species' range for phylogeographic molecular analysis of the genus. A multidisciplinary approach (Qi, 2012) integrating fossil-calibrated molecular phylogenies was used to clarify the temporal origin of *C. japonicum* and *C. magnificum*, their evolutionary genetic relationships, and to identify the species' last glacial maximum (LGM) distributions and postglacial colonization routes.

Phylogeographic analyses indicate during the LGM, *C. japonicum* experienced massive habitat losses in north-central China and northern Japan, but simultaneously expanded its range northward within three major refugia (southwest China, southeast China, and south Japan). This model raises the possibility that the postglacial range expansion of *C. japonicum* to the very north of Japan may have been facilitated by the introgressive hybridization with its cool-temperate cogener, *C. magnificum*, found almost exclusively in central Honshu, Japan.

It is also worthwhile to note that common katsura is found primarily in deciduous forests and the hiro-ha-katsura in higher altitude, alpine forests. Whether allopatry or sympatry occurs within *Cercidiphyllum*, is unclear. Qi et al. (2012) alluded by molecular lineage sorting, that the genus is largely sympatric throughout its range but particularly allopatric in the northern regions of Japan, where it is separated altitudinally. Plants of different genome sizes are often reproductively isolated by strong post-zygotic barriers and can be used as one of the basic factors demarcating infragenic taxa (Ohri, 1998); however, quantitative genome variation is not a prerequisite of species divergence (Darlington, 1963). Beyond molecular analysis, little is known about the cytogenetics of the genus but the mentions of a chromosome count of *C. japonicum* root-tips, 2n=38 (Zhao, 1988). Notwithstanding, molecular work on *Cercidiphyllum* has experienced no issues in marker development (Li, 2002; Chen, 2010; Qi, 2012); thus it can be assumed to some extent that collected populations of *C. japonicum* and *C. magnificum* behave in a diploid manner. Curiously, the vigorous *C. japonicum* forma *pendulum* (Weeping Group) seems to exhibit the characteristic traits of a cultivated polyploid. It was previously thought that the popular Weeping Group member, 'Morioka Weeping,' was a result of interspecific hybridization (Koller, 1987). Any further cytogenetic analysis may provide some use to the currently limited knowledge of reproductive strategies and chromosomal variation of the genus.

The nature and success of open pollination, pollen viability, phenology, controlled cross compatibility, and genome size of *C. japonicum* and *C. magnificum* will aid in determining the value of improving useful traits for the nursery industry. Though cytological and embryological techniques required to examine these phenomena are well developed, they are not commonly practiced by modern biologists and may find use in woody ornamental breeding programs. Further experiments involving these factors should undoubtedly reveal mechanisms that promote reproductive isolation, if any do exist.

Cultivation

Cercidiphyllum has been a popular ornamental in the United States since seed was first shipped to the Arnold Arboretum of Harvard University in the late 19th century by William Penn Brooks, a Massachusetts native as well as a teacher and administrator of the Sapporo Agricultural School, in Japan. Brooks often surveyed the land around Hokkaido for interesting plants, sending collected seed to the Arnold Arboretum; for instance, the exceptional ornamentals, Japanese clethra (*Clethra barbinervis*) and hardy kiwi (*Actinidia arguta*) (Schulof, 2009). In 1998, katsura (*C. japonicum*) was commended as "Tree of the Year" (Andrews, 1998), and since continues to garner the respect of propagators, horticulturists, and researchers from around the world.

While accessions of *C. japonicum* and its' corresponding Weeping Group have cultivars, true *C. magnificum* is scarcely mentioned in nursery catalogs, and if so mentioned, a majority of these selections are now considered to be *C. japonicum* (Li et al., 2002); and are subsequently rarely found in cultivation. Moreover, *ex situ* accessions of *C. magnificum* are underrepresented in North American arboreta; of which, are reproductively immature and present in low genetic

diversity (Dosmann, correspondence) as a result of recent molecular acceptance of the species opposed to variety or subspecies nomenclature.

Nearly all selections of *Cercidiphyllum* have ornamental characteristics worthy of cultivation. The colorful medley its' foliage undergoes throughout the growing season is complimented by its continually evolving fragrance. After leaves abscise, the winter months bring a new show. When sexually mature, there are certain genotypes that develop large, glossy, droplet-like, reddish-mauve dormant buds, most notably those in *C. magnificum*. Previous years' shoot growth is muted olive grey, yet there are those which resemble the coral-red branches of the red twig dogwood (*Cornus alba* 'Sibirica'), a popular ornamental contrast to a backdrop of winter snow. Furthermore, cultivars of katsura are selected based on overall plant size, habit and architecture, foliage size and color, and the duration and density of bloom – a common set of traits regarded necessary for production in the woody ornamental industry.

Traditionally, *C. japonicum* is asexually propagated by softwood vegetative cuttings (Dirr, 1987) or by budding compatible rootstocks in the early-spring (Morgenson, correspondence). Commercially, it is almost exclusively propagated sexually by seed (Dosmann, 1999). Although stratification is not required for seed germination (Dirr, 1987), it improves germinability of *C. japonicum* and *C. magnificum* from 34% to 52% and from 8% to 15%, respectively (Dosmann, 2000).

Plants produced by these described techniques may not be desirable because of several factors. Plant populations grown from open pollinated seed are typically not genetically uniform, a consequence of crossing-over via meiosis (Acquaah, 2010). The resulting seed may necessitate extensive care during seedling stages. Bacterial, viral, and fungal pathogens are problems in seedling leaf and root necrosis and potentially disastrous to developing seed lots. In addition,

shoot-tip cuttings may not be readily available at a particular life stage for propagation or there may be limited stock plants. These propagation limitations can be overcome with the use of micropropagation. With micropropagation, cultivar multiplication can be exponentially increased at any stage of the parent, opposed to traditional methods, which require copious amounts of plant material and time to obtain similar results, as well as reduce the occurrence of pathogens in a sterile environment.

Micropropagation

Tissue culture plays an important role in basic and applied biological studies. The application of tissue culture technology as a central tool or as an adjunct to other methods and is central in plant modification and improvement for horticulture and forestry crops (Brown, 1995). Micropropagation involves the clonal propagation of plants in a sterile environment. A clone is a genetically uniform population of plants, identical to the donor plant, produced via mitosis not meiosis.

In vitro regeneration of whole plantlets from plant tissues require specific chemical and physical supplements. Taking advantage of the phenomenon of totipotency, plant regeneration can be accomplished by employing callus, organ, cell, and protoplast cultures. Once the explant source has been selected, the micropropagation and deployment of micropropagules include four general stages: Stage I – initiation and establishment of explants, Stage II – elongation and proliferation of microshoots, Stage III – rooting, and Stage IV – subsequent acclimatization (Ahuja, 1993). Each stage may require multiple combinations of plant growth hormones, nutrient basal salts, vitamins, carbon source, or matrix binding agents, depending on the species or intraspecific genotype. In addition, the physiological state or stage, source, age, and *in vitro* conditions all affect the morphogenic response.

With respect to the influences of plant growth hormones, the concentrations and proportions of hormones made available to *in vitro* plant cultures is a prime factor in shoot expression. The primary auxin present in most plants is indole-3-acetic acid (IAA). The IAA content of plants is synthesized in many tissues, and regulated by various conjugation and catabolic pathways, via the basipetal polar transport system (Buchanan et al., 2000). This auxin is involved in a variety of physiological processes, including apical dominance, tropisms, cambial cell division, and root initiation.

Synthetic auxins, such as indole-3-butyric acid (IBA), are used extensively in horticulture to induce rooting of cuttings and microshoots and are classified as a plant growth regulator (PGR). In conjunction with auxins, plant growth hormones or PGRs, called cytokinins, induce plant cell division, influence differentiation and at high cytokinin/auxin ratios, promote shoot production. The first cytokinin hormone isolated from plants was zeatin (Z), defined as an adenine derivative with an isopentenyl side chain attached to the N^6 amino group. The primary synthetic cytokinin used in plant tissue culture is the structurally similar analog, 6-benzylaminopurine (BA) and is classified as a PGR. Cytokinins are known to induce opening of stomata, suppress auxin induced apical dominance, inhibit senescence of plant organs (Buchanan et al., 2000), and *in vitro* promotion of axillary and adventitious shoot production (Acquaah et al., 2010). Axillary shoot production requires the presence of preformed meristems, whereas adventitious shoot production originates from non-meristems, induced to form plant organs (Preece, 2008).

Titman et al. (1955) experimented with the curious nature of long and short shoot growth; finding *C. japonicum* fails to react normally to external stimuli (excision, decapitation, etc.) or produce noticeable amounts of auxin in shoot apical meristems; suggesting an auxin-inhibiting

agent present in the uppermost axillary buds of the long shoots since higher auxin yields were only achieved by removal of leaves and apices after the axillary buds were also removed.

In order to expand awareness and accessibility of *Cercidiphyllum* to the producer and consumer, the effects of *in vitro* conditions must first be examined. Preliminary shoot elongation experiments with *C. japonicum* (Chapter 2) revealed heightened vigor of microshoots when one or more pairs of axial leaves remained during transfer. A seemingly temporary solution, microshoots eventually declined in vigor and health within a week. Non-hormonal supplemental solutions to the difficulties of woody plant micropropagation are preferred, as many woody plants tend to be rather recalcitrant, demanding diverse hormone and nutrient requirements. Experimenting with continued long shoot growth *in vitro* may elucidate, whether induced or preformed, the nature of this unusual behavior.

Information regarding the clonal micropropagation of *Cercidiphyllum* is limited (Mai et al., 2005; Fu et al., 2012), omitting several important steps of a broad-spectrum analysis of the species, which focused only on hormone requirements of a single *C. japonicum* tree of Chinese origin. There is currently no information on the micropropagation requirements of *C. japonicum* Weeping Group or *C. magnificum*.

Micropropagation of this genus must be based on preliminary experiments that investigate the extent secondary metabolites have on *C. japonicum* nutrient uptake and organogenesis as it is extremely recalcitrant. Subsequent solutions will provide a platform for the development of further *in vitro* protocols.

Carbon Source

Sucrose is the most common fixed carbon source used in plant cell tissue culture systems. It is a non-reducing disaccharide which consists of the monosaccharide moieties, fructose and

glucose, both reducing sugars, linked by an O-glycosidic bond (Thorpe et al., 2008). Common table sugar is usually of high enough purity to be used in tissue culture; however, the main source of glucose is D-(+)-glucose (dextrose), and fructose, D-(−)-fructose (fruit sugar) are made less available.

One or more sugars are typically required for regular plant metabolism (Buchanan et al., 2000). D-(+)-glucose has been known to outperform sucrose in the multiplication of *Alnus crispa*, *A. cordata*, and *A. rubra* (Tremblay and Lalonde, 1984) and shoot formation in *Capsicum annum* (Phillips and Hubstenberger, 1985); whereas, fructose gave better results in orchid culture (Ernst, 1967) and the production of adventitious shoots in *Glycine max* cotyledonary nodes (Wright et al., 1986).

The uptake of sugar molecules into plant tissue appears to be partly through passive permeation and active transport. In culture, available sucrose from the phloem can be imported from the apoplast via direct sucrose transporters (DSTs) or hydrolyzed to glucose and fructose, by cell wall-bound invertases and taken up via monosaccharide transporters (MSTs) (Williams et al., 2000). Typically, sucrose is hydrolyzed completely (Thorpe et al., 2008).

Besides their role as carbon and energy sources, sugars synthesized during photosynthesis can act as regulatory signals that affect gene expression. These transporters have been shown to be influenced by light (Stadler et al., 2003) and responses to biotic and abiotic factors (Truernit et al., 1996). The ability to sense altered sugar concentrations is important in the context of resource allocation, allowing the plant to tailor its metabolism in source tissues to face the demands in sinks. Because sugar transporters play such a key role in source-sink interactions, it is likely that their expression and activity are tightly regulated by sugar concentration and type (Conde et al., 2006) made available to the plant.

Secondary Metabolites

Plants produce a vast and diverse assortment of simple and complex organic compounds, a majority of which do not appear to participate directly in growth and development (Buchanan et al., 2000). Although these natural products have been perceived as biologically insignificant, there are numerous studies that show there is an adaptive significance, ranging from allelopathy in *Ailanthus altissima* (Heisey, 1997) to facilitating supercooling capacities in *C. japonicum* (Wang et al., 2012).

These metabolites, known as plant phenolics, can range from simple molecules to highly polymerized compounds and are characterized by possessing one or more acidic hydroxyl group attached to an arene (phenyl) ring (Buchanan et al., 2000). Ubiquitous and structurally diverse, these products arise from the shikimate-phenylpropanoids-flavanoid and related biochemical pathways, accounting for about 40% of the organic carbon circulating in the biosphere (Lattanzio, 2006).

Generally, phenolic acids are either derivatives of benzoic acid, such as gallic acid, or derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Phenolics usually accumulate in the vacuoles of guard and epidermal cells as well as subepidermal cells of leaves and shoots. Some are found covalently linked to the plant cell wall; others occur in waxes or on the external surfaces of plant organs (D'Archivio, 2001).

Although the majority of these substances assume structural roles, there is a vast array of nonstructural qualitative constituents, with such roles as flower color (Buchanan et al., 2000), resistance to pathogens (Felton and Duffey, 1991; Down and Norton, 1995; Hammerschmidt, 1999; Ikonen et al., 2001; 2002), nutrient bioavailability (Haslam, 1989; Hu et al., 2006; Johnson et al., 2013), tastes, and odors. Phenolics have several industrial applications as well. Recently,

interest in food phenolics (mainly flavonoids) has increased, owing to their antioxidant capacity (free radical scavenging and metal chelating activities), supposed anticancer benefits (Bravo, 1998; Fresco, 2006; Aguilera, 2010), as well as enzyme and receptor modulation (Dai and Mumper, 2010).

Lattanzio et al. (2006) divides plant phenolics into two classes: a) preformed phenolics that are synthesized during the normal development of plant tissues, and b) induced phenolics that are synthesized by plants in response to physical injury, infection or when stressed by suitable elicitors such as heavy-metal salts, UV-irradiation, temperature, etc. (phytoalexins). Induced phenolics may be constitutively synthesized but their synthesis is often enhanced under biotic or abiotic stress.

Phenolics

Alternatively, these compounds have plagued plant scientists for years by interfering with experimental methods. For example, when exposed to air, plant phenolics readily oxidize and turn brown, generating products that form complexes with proteins and inhibit enzyme activity (Buchanan et al., 2000). Phenols can also form complexes with metal cations through their carboxylic and hydroxylic groups (chelation), and thus interfere with nutrient absorption and bioavailability (Johnson et al., 2013). Numerous studies have shown that polyphenols strongly inhibit iron absorption, like monomeric flavonoids in coffee (Brune, 1989), tea (Hurrell, 1998), and wine (Cook, 1995). In its native state, iron can be an initiator of hydroxyl radical production by the Fenton and Haber-Weiss reactions (Haber, 1934).

With respect to plant tissue culture, phenolic compounds which exude from excised organs of woody plants and vegetables (Julkunen, 1985; Laurila et al., 1998) cause serious problems initiating and maintaining *in vitro* cultures. This blackening can be prevented by

chemical, enzymatic and physical treatments (Whitaker and Lee, 1995); however, these treatments often cannot be used *in vitro*. Pizzocaro et al. (1993) recommended that oxidizing and stabilizing agents such as activated charcoal, ascorbic acid, citric acid, sodium chloride, or silver nitrate added to culture medium to limit *in vitro* tissue blackening. It has also been noted that the degree of explant blackening is related to phenolic concentration and enzyme activity in plant tissues, particularly the presence of polyphenoloxidase (PPO) (Kahn, 1975), a catalyst of phenolic oxidation. Reduced peroxidase and PPO activity is said to increase the ability of tissues to initiate growth *in vitro* (Andersone and Ivenish, 2002) with similar treatments as those previously stated.

It is possible that wound-induced suberization leads to explant decline during culture and maintenance. Suberized tissues are formed as multilamellar domains consisting of alternating aliphatic and aromatic layers, providing a means to limit water loss by forming an impenetrable barrier. The aromatic domain is said to form before the aliphatics, particularly from monomeric building blocks that contain hydroxycinnamate-derived substances (Whitaker and Lee, 1999). This process has been an important evolutionary adaptation to living on land and even thought to precede lignification (Buchanan et al., 2000).

Some polyphenol-protein complexes originate during damage and senescence of plant tissues where phenolics stored in the vacuole come into contact with cytoplasmic proteins (Lattanzio et al., 2003a; 2003b); the most visible symptom of *in vitro* phenolic exudation and a key component of explant decline. Preliminary shoot initiation and elongation experiments of *C. japonicum* showed high concentrations of phenolic compounds suspended in culture medium at the cut base of *C. japonicum* explants (Carlson, unpublished). Explant stems and petioles typically appeared oxidized immediately after *in vitro* transfer. Repeated transfer slightly

reduced noticeable phenolic compounds; however, shoots were stunted and less vigorous than typical explant donor shoots.

Somaclonal Variation

A common problem with adventitious regeneration in plant tissue culture systems is the occurrence of somaclonal variation (Preece, 2008). Undifferentiated tissue is more susceptible to deterioration than actively growing, determined somatic tissue. There are a variety of techniques to test for genetic uniformity after plants have gone through de-differentiation. The development of simple genetic markers (Cuesta et al., 2010), chromosome counts (Anamthawat-Jonsson, 2003) and flow cytometrics (Ishii et al., 2000) are common. Chromosome counts are the most efficient way to test for large chromosomal aberrations in mitotic metaphase cells and can be conducted with a compound light microscope and conventional carmine staining (Feulgen) or with fluorochromes such as (DAPI) and chromomycin.

Alginate Encapsulation

There is a growing effort to conserve the genetic diversity of temperate forest tree species (Webb et. al, 2010). Conservation of plant germplasm can be accomplished by techniques such as seed storage, *ex situ* collections, *in vitro* plant cell tissue cultures, and DNA libraries (Acquaah et al., 2010). In the 1980's, a technique was developing into a very useful plant tissue culture tool for germplasm preservation; a concept first devised by Murashige (1977). It focused on the alginate encapsulation of somatic embryos to form "synthetic seed" (Redenbaugh and Ruzin, 1987; Preece et al., 1994). Later, in the 1990's, non-embryogenic tissues were examined, such as nodal explants (Bapat and Rao, 1990).

Synthetic or encapsulated artificial seed can be defined as somatic tissue encapsulated inside a coating and considered to be analogous to a zygotic seed. Nodal or somatic embryo

alginate bead encapsulation is a low-input, effective tool to store plant germplasm as an alternative to seed storage. Encapsulation offers the quick clonal and economical propagation of plants that are difficult to propagate by seed, and easy genetic storage and maintenance (Patel et al., 2000). Contrary to *in vitro* cultures and *ex vitro* plantings (Preece and Triggiano, 2001), alginate coating protects disease-free plants from any possible infestation that may occur.

This technique involves suspending plant material (i.e. plant cells, tissues, organs, shoot tips, somatic embryos) in a stirred alginate (alginic acid) solution and then dripping it into a calcium chloride ($CaCl_2$) solution for hardening (Patel et al., 2000). Encapsulated plant material can then be stored at 4-5°C in refrigerator, far easier and less costly than cryopreservation (Preece and West, 2006). At this time, encapsulated plant material is not necessarily in stasis but has an extremely reduced metabolism. There is a shelf-life to encapsulated synthetic seed and should be tested periodically to ensure germination after long-term storage (West et al., 2006).

Beyond seed stratification experiments, there have been limited efforts pertaining to *in vitro* conservation techniques for *Cercidiphyllum*. The development of micropropagation protocols for *Cercidiphyllum* will not only be useful for nursery production and cultivar development, but will aid in ongoing conservation efforts of the genus. Although katsura is not difficult to propagate by seed, the establishment of a reliable, broad-spectrum, plant hormone and nutrient analysis is necessary for further high-throughput clonal *in vitro* studies. Understanding the affinity of nutrient and hormone treatments of *Cercidiphyllum* will elucidate physiological requirements as well as provide useful insight into further *in vitro* techniques within the genus.

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CHAPTER 2. DEVELOPMENT OF THE MICROPROPAGATION PROTOCOL

Abstract

A micropropagation method for the woody ornamental genus *Cercidiphyllum* (Cercidiphyllaceae), was demonstrated using diverse accessions within common katsura (*C. japonicum* Sieb & Zucc.), the corresponding 'Weeping Group,' (*C. japonicum* forma *pendulum* E.H. Wilson), and the broader-leaved hiro-ha-katsura, (*C. magnificum* Nakai). *In vitro* nodal explant initiation was examined with a single *C. japonicum* accession and three others with the basal salt formulations, WPM, MS, LP, and DKW, in combination with 0, 1.1, 2.2, and 4.4 µM BA or 0, 0.05, 0.1, and 0.5 µM TDZ with 0 or 0.05 µM IBA. Initial nodal explant response to subculture was analyzed after budbreak on the same factorial as microshoot proliferation. Microshoots were proliferated on WPM basal salts, with 0, 2.2, 4.4, and 10µM BA, in combination with 0 or 5 µM GA_3, and 0, 0.05, or 0.1 µM IBA or NAA. To induce *in vitro* rooting, microshoots were subcultured on WPM basal salts with 0, 0.5, 1.0, or 2.0 µM IBA or NAA, or 1:1 auxin mixtures of the same concentrations. In general, the highest nodal explant segment-growth initiation rate for all genotypes analyzed was on the low basal salt, WPM or medium basal salt, LP, and low concentrations BA (1.1 or 2.2 µM) with or without the presence of 0.05 µM IBA; whereas, subcultured nodal segments elongated on 4.4 µM BA alone or in combination with 0.05 µM IBA, on either MS, WPM or LP, and IBA+BA significantly promoted higher bud and shoot number, and proliferation rates were highest on high concentrations of BA, 10 µM or 4.4 µM, respectively, with the presence of 5 µM GA_3 and 0.1 µM NAA. Induction of longer and more fibrous *in vitro* roots was highest on 2 µM IBA and number on either 2 µM IBA or NAA alone. This micropropagation analysis will prove useful for

commercial clonal micropropagation of new *Cercidiphyllum* varieties and conservation techniques of this relict tree.

Introduction

Cercidiphyllum (Cercidiphyllaceae) has been an underutilized ornamental in the United States since seed was first shipped to the Arnold Arboretum of Harvard University in the late 19th century by a Massachusetts native, William Penn Brooks, whom at that time was administrator of the Sapporo Agricultural School, in Japan (Andrews, 1998). Endemic to Japan and China (Brown, 1939), *Cercidiphyllum* represents the two dioecious tree species, common katsura (*C. japonicum* Sieb. & Zucc.) and the hiro-ha-katsura (*C. magnificum* Nakai). Common katsura was first described in Japan (Hoffman and Schultes, 1853) and is the most well-known and abundant species of the genus. Nakai (1919) published a second variety, *C. japonicum* var. *magnificum* Nakai, which was given specific status as *C. magnificum* Nakai the following year.

Considered a 'living fossil' of the temperate East Asian Northern Hemisphere Tertiary period (65 to 2.6 mya) (Brown, 1939; Devore et al., 2013), *Cercidiphyllum* shares a similar phylogenetic history as other relictual species, e.g., *Metasequoia* (Merill, 1948), *Eupetela* (Wei, 2010), and *Ginko* (del Tredici, 2000), whose native distribution range spans from southwest China to south Japan (Liu et al., 1988; Qi et al., 2012). The native habitat of *Cercidiphyllum* is generally thought to be riparian forests near forest margins and streams (Chien, 1992; Dosmann, 2000; Fu and Endress, 2001), whose species composition varies relating to physical (altitude, slope, soil temperature) and chemical (available K, NH_4^+, pH) properties (Gunkel and Thimann, 1949a; 1949b). Seedling establishment conditions of *C. japonicum* are sparse and also thought to be influenced by topography and light availability (Dosmann, 1999; Kubo et al., 2000; Seiwa, 2007). Though seedlings are rarely found in forests, *C. japonicum* maintains its populations over

long periods by sprouting, which compensates for sparse seedling regeneration (Titman and Wetmore, 1955; Kubo et al., 2005, 2010; Wei et al., 2010).

Leaves of the genus are opposite, rounded, simple, and petiolate (Hoffman and Schultes, 1853; Swamy and Bailey, 1949). Interestingly, its leaves are heterophyllous, whose shoots concurrently undergo long apically dominant and short axial shoot growth, depending on environmental and physiological conditions (Titman and Wetmore, 1955). The slight downward-cupped margins and rugose texture of katsura foliage is enhanced by its seasonal display of color. In the spring, single precocious leaves appear along shoots, emerging light green to wine-red, fading to dark blue-green mid-summer, and variably to a delicate reddish-purple or golden-yellow in the fall. The colorful medley its' foliage undergoes throughout the growing season is complimented by its continually evolving fragrance. Leaf emergence is often accompanied by a warm citrusy fragrance, similar to the musk of mango flesh, whereas, senescence often brings about hints of cinnamon and vanilla (Dosmann, 1999).

The broader-leaved hiro-ha-katsura subtly differs morphologically from its cogener, *C. japonicum* and has subsequently been in and out of taxonomic discussion (Spongberg, 1979; Li et al., 2002), along with a number of weeping katsura varieties, i.e., 'Morioka Weeping,' 'Amazing Grace,' and 'Pendulum' (Koller, 1987), popular in the nursery industry as an alternative to the upright, tree-form *C. japonicum*. Typically, weeping varieties (*C. japonicum* forma *pendulum*) are distinguishably more vigorous than the straight species, demonstrated by an erratic nature of axillary bud placement along stems, longer internodes and leaf petioles, and often occurrence of 'whorled' multi-nodal meristems . Conversely, differences between species are subtle; oftentimes, *C. magnificum* is excluded from mention as part of *Cercidiphyllum* and generally thought monophyletic (Fu et al., 2012). Although these two species are quite similar, a

characteristic morphological difference is the number of 'wings' attached to seed capsules; common katsura bearing only one and the hiro-ha, two (Swamy, 1949; Dosmann, 2000).

Nevertheless, any existing debate as to the taxonomic status of *C. magnificum* and weeping varieties has been resolved. Based on the results of nuclear ribosomal sequences, Li et al. (2002) found weeping varieties, *C. japonicum* forma *pendulum* (designated the 'Weeping Group') to be phylogenetically derived from *C. japonicum*, whereas, *C. japonicum* and *C. magnificum* genetically distinct, supporting the recognition of *C. magnificum* as a separate species; a distinction sustained by more recent phylogeographic (Wei et al., 2010) and molecular phylogenetic analysis (Chen et al., 2010).

While accessions of *C. japonicum* and its' corresponding Weeping Group have cultivars, true *C. magnificum* is scarcely listed in nursery catalogs, and if so mentioned, a majority are considered *C. japonicum* or *C. japonicum* var. *magnificum* (Li et al., 2002) and thus rarely in cultivation. Moreover, *ex situ* accessions of *C. magnificum* are underrepresented in North American arboreta, are reproductively immature, and present in low genetic diversity (Dosmann, correspondence).

Traditionally, *Cercidiphyllum japonicum* is clonally propagated by softwood cuttings, wedge grafted (Dirr, 1987), or by chip budding on to compatible rootstocks in the early spring (Morgenson, correspondence), and commercially is almost exclusively propagated asexually by seed (Dosmann, 1999). Although stratification is not required for germination (Dirr, 1987), it improves germinability of *C. japonicum* and *C. magnificum* from 34% to 52% and from 8% to 15%, respectively (Dosmann, 2000). Information regarding the clonal micropropagation of *Cercidiphyllum* is limited to a single *C. japonicum* tree of Chinese origin (Fu et al., 2012) and no literature on the tissue culture of *C. japonicum* 'Weeping Group' or *C. magnificum*. For a lack of

information, this micropropagation procedure includes the study of the effects of plant growth regulators and basal salt formulation on shoot growth and rooting and their affinity to diverse *Cercidiphyllum* accessions. Physiological elucidation of *in vitro* requirements will provide useful insight into further techniques within the genus.

Materials and Methods

Source of Explant Material

Ex Vitro Initiation

Nodal Explants of three *Cercidiphyllum* accessions, *C. japonicum* (Acc. No. 200-48, bulked half-sibs of Japanese origin), *C. japonicum* cv. 'Pendulum' (Acc. No.72-84 of Japanese origin), and *C. japonicum* var. *sinense* (Acc. No. 464-84, of Chinese origin), were acquired from the Morton Arboretum in Lisle, IL., as well as half-sib *C. japonicum* family (Acc. No. TS9821-6)[1] consisting of 6 bulked individuals, collected from North Dakota State University evaluation planting in Fargo, ND. Accessions arrived as dormant winter cuttings, wrapped in a moist paper towel, sealed in a plastic bag, and stored in the dark at 4±1°C.

In Vitro Initiation

Nodal explants from one half-sib family (Acc. No. TS9821-6), represented *C. japonicum* (Sieb. & Zucc.). Two genotypes of *C. magnificum*, a bulked half-sib pair, Acc. No. 270-2003*A*C, of garden origin and wild collected in Hokkaido, Japan, and Acc. No. 291-2008*A, of Chinese origin, obtained from the Arnold Arboretum of Harvard University, Boston, MA, as well as the weeping cultivar, *C. japonicum* cv. 'Amazing Grace' Acc. No. 113-2002*A. The Acc.

[1] Bulked individuals of Acc. No.'s TS9821-6 are progeny collected from the parent Acc. No. 943, germinated in 1949 at the Lesny Zaklad Doswiadczalny W. Rogowre Arboretum, Poland, which derived from seeds obtained from an unknown *C. japonicum* accession at the Warsaw University Botanical Garden, Poland (Banaszczak, 2013; communication).

No.'s 270-2003*A*C, 291-2008*A, and 113-2002*A were selected to represent their respective taxon group based on molecular typification by Li et al. (2002). Accessions arrived as softwood cuttings, wrapped in a moist paper towel, sealed in a plastic bag, and stored in the dark at 4±1°C.

Proliferation and Rooting

Plant material used for proliferation and rooting experiments were microshoots excised from elongated Stage I and II cultures of Acc. No. TS9821-6. Initiates were excised from elongating microshoots maintained on Lloyd & McCown Woody Plant Medium (WPM) (Lloyd, 1980) basal salts and nutrients, with the addition of 2.2 µM 6-benzylaminopurine[1] (BA), 0.05 µM indole-3-butyric acid (IBA)[2], 3.0% (w/v) D-(+)-glucose[3], 7.0 g L^{-1} agar[4], with the pH adjusted[5] to 5.8 ± 0.01 with 1.0 N KOH.

Nutrient Media and Hormones

Ex Vitro Initiation

All liquid media was composed of WPM basal salts, 0% or 3% (w/v) sucrose, with either 5 or 10 µM BA, in combination with 15 or 30 µM gibberellic acid (GA_3)[6], and a treatment control consisting of ddH_2O, with the pH adjusted[7] to 5.8 ± 0.01 with 1.0 N KOH.

In Vitro Initiation

Initial nodal explants of *C. japonicum* (Acc. No.'s TS9821-6) were placed on medium containing the basal salt and nutrients of either WPM, Juglans Basal Medium (DKW) (Driver, 1984), Long and Preece Medium (LP) (Preece et al., 1994), or Murashige & Skoog (MS)

[1] No. B800, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

[2] No. I5386, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.
[3] No. G7520, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

[4] No. A111, gel strength: 1080 g/cm^2, *Phyto*Technology Laboratories®, P.O. Box 12205 Shawnee Mission, KS 66282, USA.
[5] No. AB15, Accumet® Basic pH meter, Thermo Fisher Scientific Inc. 81 Wyman Street Waltham, MA 02454.
[6] No. G500, *Phyto*Technology Laboratories®, P.O. Box 12205 Shawnee Mission, KS 66282, USA.
[7] Accumet® Basic No. AB15 pH meter

(Murashige and Skoog, 1962), with or without the addition of 0.05µM IBA, in combination with 0, 1.1, 2.2, or 4.4 µM BA, or 0, 0.05, 0.1, 1.0 µM thidiazuron (TDZ)[1].

Further, nodal explants of *C. magnificum* Acc. No.'s 291-2008*A and 270-2003*A*C and *C. japonicum* Acc. No. 113-2002*A were placed on WPM with or without the addition of 0.05µM IBA, in combination with 0, 1.1, 2.2, or 4.4 µM BA, and further analyzed on the basal salts and nutrients of MS, DKW, or LP with 2.2 µM BA, 0.05µM IBA. Accession No. TS9821-6 was also analyzed for shoot growth after initiation on hormone-free media consisting of WPM salts and nutrients. All treatments included 3.0% (w/v) sucrose and 7.0 g L^{-1} agar with the pH adjusted to 5.8 ± 0.01 with 1.0 N KOH.

Proliferation and Rooting

Microshoots were excised from Stage I *C. japonicum* Acc. No. TS9821-6 were placed on medium containing the basal salt and nutrients of WPM, with or without the addition of 0.05 µM IBA and 1-napthaleneacetic acid (NAA)[2], in combination with 0, 2.2, 4.4, or 10 µM BA, with or without the addition of 0.1 µM TDZ. Additionally, treatments for microshoot elongation consisted of WPM basal salts with 0, 4.4, or 10 µM BA, in combination with or without the addition of 0.2 µM NAA, and 5 µM GA_3 with the same media compositions. All treatments included 3.0% (w/v) D-(+)-glucose[3] and 7.0 gL^{-1} agar with the pH adjusted to 5.8 ± 0.01 with 1.0 N KOH.

Initiates were excised from elongated Stage II microshoots and placed on medium containing WPM basal salts and nutrients and the addition of either 0, 0.5, 1, or 2 µM IBA or 0, 0.5, 1, or 2 µM NAA or 1:1 mixtures of the same concentrations, 0.5:0.5, 1:1, and 2:2µM IBA:NAA. All treatments included 3.0% (w/v) D-(+)-glucose[1] and 7.0 g L^{-1} agar with the pH

[1] No. P6186 Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.
[2] No. 0640, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.
[3] No. G7520, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

adjusted to 5.8 ± 0.01 with 1.0 N KOH. Concurrent work (Chapter 3) has significantly shown D-(+)-glucose to be an effective carbon replacement source to sucrose and why it was incorporated in the rooting experiment.

Procedures

Ex Vitro Shoot Forcing

Dormant stems 20 cm in length having at least 10 nodes were disinfested by first shaking in 70% (v/v) ethanol for 2 min, then rinsed in distilled deionized water (ddH_2O)[1] (18.2 MΩ) at 25ºC for 10 min and decanted under non-sterile conditions. Shoot sections were then excised from stems and cut proximally with a pruner at a 45-degree angle to 18-20 cm in length. Shoots were used to puncture Parafilm®[2] covering baby food jars to prevent desiccation, then incubated in an illuminated incubator[3] under cool white florescent lamps that provided a photon flux of approximately 40µmol $m^{-2}s^{-1}$ for a 16-hour photoperiod at 28°C. Shoots were transferred 3 d to fresh media consisting of the same media compositions for the 2w duration of this study.

In Vitro Initiation

All axial nodal explants were excised and cut to 1-2 cm; distally, 3-5 mm above distal portions of bud primordia and proximally 1-2 cm below primordia, and disinfested in bulk. Explants were vigorously shaken in ddH_20 for 3 min (repeated three times, 3x), placed in a 70% (v/v) ethanol solution for 2 min, decanted and rinsed with ddH_20 (3x), then lightly shaken for 10

[1] Milli-Q Water System, Millipore, Milford, MA, USA
[2] No. "M", Peachiney Plastic Packaging, Chicago, Ill.
[3] No. 818, The Precision Scientific Co. Palaniyappan, Precision Plaza No 397, Anna Salai, Teynampet, Chennai.

minutes in a 12% (v/v) Clorox® solution[1] and 0.1% (v/v) Tween 20®[2] solution, rinsed with ddH_2O[3] (3x) (18.2 MΩ) at 25ºC and subsequently decanted under sterile conditions.

After disinfestation, all explants were freshly cut under sterile conditions using a surgical-grade scalpel. Using a long-tipped forceps, explants were placed in 15 mL x 150 borosilicate culture tubes[4] vertically, 5 mm below axillary buds. Explants were incubated 30 cm below cool white florescent lamps that provided a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16 h photoperiod at 25ºC for 4 weeks.

Proliferation and Rooting

Maintained Stage I elongated microshoots were excised under sterile conditions with a surgical-grade scalpel. Leaves were removed from each microshoot. Using a long-tipped forceps, microshoots were placed in culture tubes, containing treatment compositions, vertically, 5 mm below axillary buds. Culture tubes were incubated 30 cm below cool white florescent lamps that provided a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16 h photoperiod at 25ºC for 4 weeks.

Data Collection and Statistical Analysis

All experiments were arranged as a completely random design (CRD) and conducted twice with a minimum of 3 replicates per run of every experiment. All data were analyzed using the General Linear Model (GLM) of SAS (SAS Institute, Inc.) because of the potential for unequal replication within treatments as a result of potential contamination or explant death. Shoot growth was measured as mean shoot number and length (>5 cm), mean bud number and

[1] 6% NaClO solution.
[2] No. P9416, Polyethylenesorbitan momolaurate, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.
[3] Milli-Q Water System, Millipore, Milford, MA, USA.
[4] No. 9820, Pyrex®, 836 North Street Building 300 Suite 3401 Tewksbury MA 01876, USA.

surface area, as well as mean root number and length, scored across respective treatments over 4 weeks of incubation (23±1ºC in light).

Results and Discussion

Ex Vitro Initiation

To enhance ease of tissue culture, *ex vitro* initiation was analyzed for potential use as a tool for providing cleaner dormant winter material, bypassing extensive explant disinfestation protocols. However, treatment samples excluding the controls declined shortly after 2 w incubation, likely a result of the presence of sucrose and/or basal salts and nutrients in media solutions at 23±1 ºC in light which served as a medium for fungal growth. Treatment controls, though slow to initiate, were less affected and continued to flush single axial leaves from dormant buds following the emergence of inflorescence. In addition, controls were the only treatment across genotypes to form terminal shoots and forced shoots of all accessions developing from axillary buds were not witnessed, i.e., whether the excised stem was a shoot-tip or decapitated segment, and no treatments yielded shoots from preformed axillary buds.

Calculated analysis of variance (ANOVA) showed that the two-way interaction, BA x GA_3, significantly (α=0.05) promoted initiation of excised dormant *Cercidiphyllum* stems (Appendix Table A-1) as well as BA x Sucrose (Table 2-1). The significant two-way interaction, BA x GA_3, can be dissected as being inversely related, whose positive interaction was found at high concentrations of BA coupled with low GA_3 or low BA with high GA_3 concentrations, both promoting significantly higher shoot growth than other treatments but not differing from the other. Both 72-84 and 464-84 responded similarly to treatments, however, 200-48 consistently responded to all treatments at higher budbreak percentages, excluding the control (Figure 2-1; Table 2-1).

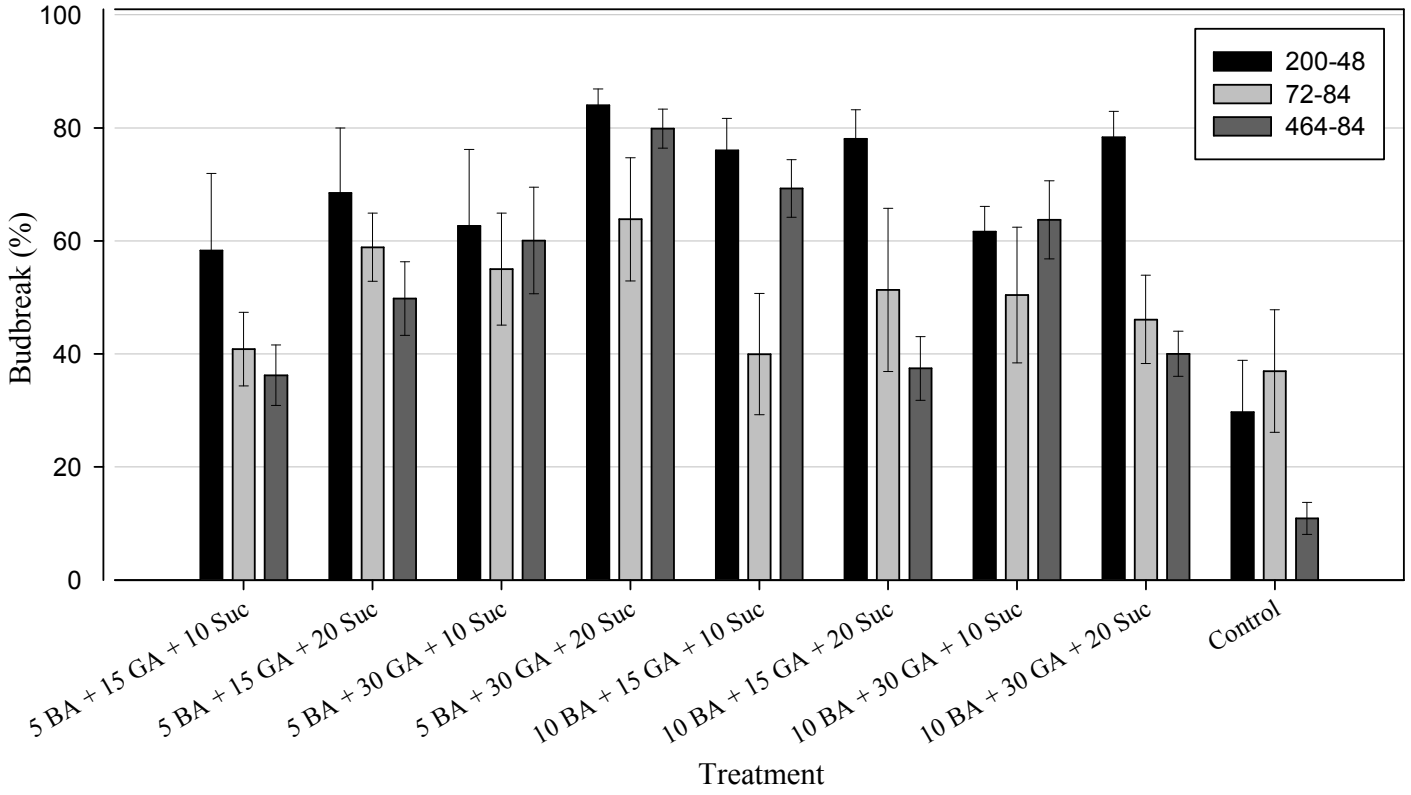

Figure 2-1. Treatment means (±SE) of *C. japonicum* accessions 200-48, 72-84, and 464-84 for ex-vitro budbreak (%) after 4 weeks incubation (23±1ºC in light).

Table 2-1. Effects of BA and presence of 3% (w/v) sucrose on percent *ex vitro* initiation of dormant buds of three *C. japonicum* accessions after 2 w incubation (23±1 ºC in light).

Plant Growth Regulator (μM)[1]	Carbon Source[2]	Initiated Dormant Buds (%)[3]		
		Accession[4]		
BA	Sucrose	200-48	72-84	464-84
5	-	36.2	58.3	40.9
5	+	49.8	68.5	58.9
5	-	60.1	62.6	55.0
5	+	79.9	84.0	63.9
10	-	69.3	76.0	40.0
10	+	37.4	78.1	51.4
10	-	63.8	61.6	50.4
10	+	40.0	78.3	46.1
Control[5]		10.9	29.7	37.0
$LSD_{0.05}$[6]		15.9	25.5	ns

[1] BA, 6-benzylaminopurine in 10^{-3} mol/L^3 molar (μM) concentration.
[2] +/-; with/without 3.0% weight/volume (w/v) sucrose included.
[3] Mean sample stem percentage of buds broken, scored binary as a mean percentage of subsample buds broken by the total number of buds per stem.
[3] 200-48, 72-84, 464-84; half-sib *C. japonicum* (Japanese origin), *C. japonicum* cv. 'Pendulum' (Japanese origin), and *C. japonicum* var. *sinense* (Chinese origin) accessions, respectively.
[4] Control; ddH_2O, WPM basal salts and nutrients only.
[5] $LSD_{0.05}$, least significant difference for paired comparisons; ns, non-significant (α=0.05) according to the *F*-test.

Accessions were significant in shoot forcing (Appendix Table A-1). inferring from least squared difference ($LSD_{0.05}$), Acc. No. 464-84 did not differ in mean initiated dormant buds across PGRs, Acc. No. 200-48 significantly differed among treatments which included 5 or 10 μM BA and 15 or 30 μM GA_3. Similarly, in relation to Acc. No. 72-84, the treatment composition 5 μM BA, 30 μM GA_3, and without sucrose, was the only combination that significantly different from all others.

In Vitro Initiation

The effects of plant growth regulator on the *in vitro* initiation Acc. No. TS9821-6 microshoot growth occurred to varying degrees on all media. TDZ significantly interacted with

IBA and salts to the leaf dimensions, leaf length and leaf width. Lower TDZ concentrations (0.05 μM), with or without the presence of auxin, contributed to significantly longer and thinner leaves compared to lower concentrations, BA (1.1μM), which promoted leaves morphologically akin to *ex vitro* katsura (Appendix Table A-2). Higher TDZ concentrations had significantly negative effects on leaf size, a factor in reducing leaf length and width as concentrations increased from 0.05 μM; a similar correlation also observed with increasing concentrations of BA. The presence of IBA was a contributor to higher shoot growth when in conjunction with BA, but not with TDZ (Table 2-2). Moreover, treatments including TDZ within the factorial were excluded from the overall ANOVA (Appendix Table A-3; Table 2-3) on the basis of its poor performance and confounding effects on analysis of cytokinin x auxin x salt interactions, reducing cytokinin factors to only BA main effects and interactions, opposed to combined analysis.

PGR concentrations did not differ significantly within treatments with WPM basal salts, compared to those with different salt formulations but the same hormone concentrations, e.g., 2.2μM BA with the presence of IBA, which were significantly different (Table 2-2). Shoot number was only different between cytokinin treatments with BA with or without the presence of auxin. In particular, the interaction, BA x IBA x Salts, was significant for shoot growth of Acc. No. TS9821-6 (Appendix Table A-3). Salt formulation WPM induced significant effects on shoot initiation and elongation (Table 2-2) compared to the basal salt formulations, LP, MS, and DKW, which did not significantly vary.

The factors BA and Salts were found related orthogonally linear to shoot length (Appendix Table A-4). Among basal salts, the quadratic relationship of BA to shoot growth was consistent of individual basal salt formulations, whereas, the significant effects of basal salt formulation increased inversely to its salt concentration. To extrapolate, the basal salt

Table 2-2. Effects of basal salt and nutrient formulation, cytokinin concentration, and presence of IBA on mean shoot length, leaf length and width of *C. japonicum* Acc. No. TS9821-6 after 4w of incubation (23±1ºC in light).

Plant Growth Regulator (μM)[1]			Nutrient Salt Formulation[2]											
			Shoot Length[3]				Leaf Length				Leaf Width			
BA	TDZ[4]	IBA	MS	DKW	WPM	LP	MS	DKW	WPM	LP	MS	DKW	WPM	LP
1.1		0.05	2.25	2.05	9.90	3.90	7.25	9.15	11.00	7.95	4.50	7.25	8.00	5.05
2.2		0.05	2.85	2.05	6.47	2.25	8.10	8.00	8.33	7.85	3.75	4.45	5.19	5.25
4.4		0.05	2.10	2.15	4.30	3.10	9.35	9.30	10.3	8.25	5.40	6.15	6.55	5.90
1.1			2.00	8.25	8.25	8.80	12.05	8.60	8.60	6.25	7.45	6.95	6.95	7.00
2.2			1.05	0.25	3.40	3.40	9.70	5.80	8.35	8.35	7.75	3.90	5.50	5.50
4.4			1.80	1.00	4.65	3.10	9.20	8.35	6.40	8.90	5.90	5.50	3.70	5.85
	0.05	0.05	2.20	1.50	0.80	0.95	10.45	4.95	5.10	8.20	7.30	3.20	3.20	5.80
	0.1	0.05	1.20	0.00	5.25	1.15	9.55	5.30	11.70	9.00	5.90	4.15	7.15	6.05
	0.5	0.05	1.90	1.20	0.40	0.40	5.30	5.20	7.75	1.75	3.15	3.30	5.00	0.85
	0.05		2.90	2.85	0.55	2.70	0.00	15.55	8.45	10.10	3.85	11.35	5.45	7.30
	0.1		2.30	2.30	0.45	0.00	2.20	6.15	11.85	8.25	6.55	3.75	8.20	6.00
	0.5		0.55	0.40	0.35	1.10	5.75	3.65	4.65	6.45	4.15	2.20	3.50	4.90
		0.05	0.50	0.50	0.0	0.50	0.50	6.35	7.35	8.95	8.35	4.65	6.4	6.10
Control[5]			0.00	0.00	0.50	1.05	5.00	8.10	7.45	5.75	3.45	5.30	4.00	2.75
$LSD_{0.05}$[6]			0.71 (2.31)				ns (3.69)				ns (2.85)			

[1] BA, TDZ, IBA; 6-benzylaminopurine, thidiazuron, and indole-6-butryic acid, respectively, in 10^{-3} mol/L^3 molar (μM) concentrations.

[2] MS, DKW, WPM, and LP; Murashige & Skoog, Juglans Basal Medium, Woody Plant Medium, and Long and Preece macro and micronutrient salt formulations, respectively.

[3] Treatment means in millimeters (mm).

[4] TDZ treatment means present but excluded from ANOVA analysis; $LSD_{0.05}$ with TDZ shown in parentheses.

[5] Control; no plant growth regulators present.

[6] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

formulations, WPM, LP, MS, and DKW, which range from low to high salt concentrations, respectively, salt formulations initially were significant on shoot growth by interaction with PGRs then normalized after a single subculture, where BA x IBA was significant (Appendix Table A-5; Table 2-3). Other formulations were significantly more effective in promoting Acc. No. TS9821-6 shoot number after subculture (Appendix Table A-6), i.e., salts MS, LP, and DKW significantly differed from WPM (Table 2-4). However, both shoot length and bud number did not differ with respect to salt formulation.

The inclusion of leaf width and length was an attempt to capture a morphological feature which could be referenced not only as surface area but additional variables to compare micropropagules to *ex vitro* plants to different nutrient and hormone concentrations.

Table 2-3. Effects of cytokinin type and concentration, and presence of auxin on mean shoot length, shoot number, and leaf length, for elongation of C. *japonicum* Acc. No. TS9821-6 from budbreak after 4 w of incubation (23±1ºC in light).

Plant Growth Regulator (μM)[1]		Shoot Length[2]	Shoot Number	Bud Number
BA	IBA			
1.1	0.05	4.50	1.00	6.67
2.2	0.05	6.17	0.67	6.83
4.4	0.05	9.50	1.33	10.7
1.1		4.83	1.00	7.00
2.2		5.33	0.83	8.33
4.4		12.7	1.33	7.83
	0.05	6.67	1.83	8.17
Control[3]		1.50	0.33	4.50
$LSD_{0.05}$[4]		4.64	0.72	6.14

[1] BA, IBA; 6-benzylaminopurine and indole-6-butryic acid, respectively, in 10^{-3} mol/L^3 molar (μM) concentrations.
[2] Treatment means in millimeters (mm).
[3] Control; WPM basal salts only.
[4] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

Table 2-4. Effects of basal salt on mean shoot length, shoot number, and bud number of Acc. No. TS9821-6 from budbreak after 4w of incubation (23±1ºC in light).

Basal Salt[1]	Shoot Length[2]	Shoot Number	Bud Number
WPM	6.50	1.00	7.40
MS	10.6	2.20	12.2
DKW	6.00	1.80	10.8
LP	10.4	2.00	11.2
$LSD_{0.05}$[3]	ns	0.39	ns

[1] MS, DKW, WPM, and LP; Murashige & Skoog, Juglans Basal Medium, Woody Plant Medium, and Long and Preece macro and micronutrient salt formulations, respectively.
[2] Treatment means in millimeters (mm).
[3] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

However, these morphological characteristics proved to be extremely variable, as both replication and run was significant for leaf width and replication, leaf length, and when only BA was analyzed, both leaf with and length were significant for replication and leaf length was also significant in run (Appendix Table A-3). Although this significance is also attributable to the recalcitrance of *Cercidiphyllum* as a result of high polyphenolic content (discussed in Chapter 3), leaf area is not a reliable morphological feature to measure.

It is imperative to investigate mineral nutrients as a component to micropropagation to further enhance and expedite organogenesis, especially for *Cercidiphyllum*, where success is particularly dependent upon genotype, maturity, and physiological stage. Historically, Dirr et al. (1987) notes that rooted katsura cuttings from seedlings have only been successful donors, with no success rooting cuttings from mature trees. This is also observed *in vitro*; especially microshoots derived from newly established node or shoot cultures (Preece, 2008). Often times, protocols only use one medium formulation, focusing on only growth responses, which may not

be optimal for the various stages of the micropropagation process, overlooking the role of minerals and nutrients as morphogenic elicitors. Some insight may be gained by the varying chemical and physical requirements of katsura in a natural setting (Gunkel and Thimann, 1949a; 1949b; Wei et al., 2010) for it is often tissue culture requirements between and within a species varies considerably (Edwin et al., 2008).

The interaction of BA x IBA significantly promoted greater mean shoot number and shoot length of the weeping *C. japonicum* Acc. No. 113-2002*A and with increasing concentrations of BA alone promoted greater bud numbers (Appendix Table A-7; Table 2-5).

Table 2-5. Effects of cytokinin type concentration and auxin on mean shoot length, shoot number, and bud number of three *Cercidiphyllum* accessions after 4w of incubation (23±1ºC in light).

Plant Growth		Accession[2]								
Regulator (μM)[1]		270-2003*A*C			291-2008*A			113-2002*A		
BA	IBA	SL[3]	SN	BN	SL	SN	BN	SL	SN	BN
1.1	0.05	29.67	2.00	11.50	3.00	1.50	3.33	1.67	0.33	2.83
2.2	0.05	2.50	0.67	3.83	3.83	1.33	3.50	5.50	1.50	4.17
4.4	0.05	1.50	0.33	2.00	2.50	0.67	2.50	2.67	0.50	3.17
1.1		7.17	1.50	7.83	2.67	1.00	2.33	2.00	0.67	3.17
2.2		4.50	1.00	5.50	2.33	0.50	2.67	1.50	0.50	2.83
4.4		3.17	1.67	4.00	2.83	0.83	3.33	6.00	1.33	5.00
	0.05	2.16	0.67	3.17	2.83	0.80	3.30	3.00	0.67	2.33
Control[4]		1.50	0.50	2.00	1.33	0.33	2.17	0.67	0.17	2.00
$LSD_{0.05}$[5]		2.00	0.80	3.40	ns	ns	ns	2.90	0.71	1.62

[1] BA, IBA; 6-benzylaminopurine and indole-6-butryic acid, respectively, in 10^{-3} mol/L^3 molar (μM) concentrations.
[2] 270-2003*A*C, 291-2008*A, 113-2002*A; *Cercidiphyllum magnificum* (Japanese origin), *C. magnificum* (Chinese origin), and *C. japonicum* cv. 'Amazing Grace' (unknown garden origin) respectively.
[3] SL, SN, LN; mean shoot length, shoot number, and bud number, respectively, in millimeters (mm).
[4] Control; WPM basal salts only.
[5] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

Additionally, the effects of salt formulation on shoot growth significantly impacted shoot length and number (Table 2-6) performing best on WPM salts, but required higher

concentrations of BA (4.4 µM) without the presence of IBA or medium BA (2.2 µM) concentrations with the addition of IBA. Effects of cytokinin type concentration and auxin on mean shoot length, shoot number, and bud number of *C. magnificum* Acc. No. 291-2008*A was not significant (Appendix Table A-8); however, shoot length was greater on DKW basal salts than any other formulation (Appendix Table A-11; Table 2-6). Accession No. 270-2003*A*C shoot length and number was significantly affected by the interaction BA x IBA and bud number was significantly greater with low concentrations of BA (Appendix Table A-9). Further, salt formulation was significant (Appendix Table A-10) for shoot length and bud numbers, with MS segregating from all other formulations. The Acc. No.'s TS9821-6 and 270-2003*A*C, representing the species *C. japonicum* and *C. magnificum*, respectively, were both bulked half-sib families. Though divergent species, these accessions significantly performed better on the same basal salts and PGR concentrations within all shoot growth variables (Table 2-2; Table 2-6; Figure 2-1).

Table 2-6. Effects of basal salt on mean shoot length, shoot number, and bud number of three *Cercidiphyllum* accessions after 4w of incubation (23±1ºC in light).

Basal Salt[1]	Accession[2]								
	270-2003*A*C			291-2008*A			113-2002*A		
	SL[3]	SN	BN	SL	SN	BN	SL	SN	BN
WPM	11.0	1.67	3.83	3.83	1.33	3.50	5.50	1.50	4.17
MS	24.8	1.60	9.00	3.60	1.00	3.00	1.60	0.60	2.40
DKW	5.60	1.80	5.80	7.60	2.00	5.60	2.80	0.80	3.20
LP	6.40	1.60	8.20	1.60	0.40	2.60	3.00	1.20	3.00
$LSD_{0.05}$[4]	13.0	ns	2.74	2.99	0.93	1.78	2.89	0.59	ns

[1] MS, DKW, WPM, and LP; Murashige & Skoog, Juglans Basal Medium, Woody Plant Medium, and Long and Preece macro and micronutrient salt formulations, respectively.
[2] 270-2003*A*C, 291-2008*A, 113-2002*A; *Cercidiphyllum magnificum* (Japanese origin), *C. magnificum* (Chinese origin), and *C. japonicum* cv. 'Amazing Grace' (unknown garden origin) respectively.
[3] SL, SN, LN; shoot length, shoot number, and bud number, respectively, in millimeters (mm).
[4] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

In the *in vitro* initiation and elongation experiments, no axillary growth from nodal bud primordia of elongating stem segments or microshoots was observed. Removal of leaves did not improve axillary shoot growth nor did excision of shoot apical meristems. After one month of incubation on initiation media, microshoots were excised from donor explants and subcultured to establishment medium. Interestingly, decapitated microshoots underwent characteristic stages of pre-winter dormancy. Visually, the health of microshoot stems did not decline; all stems were green, and seemingly healthy. Within a month of incubation, leaves began to senesce from the distal shoot apical meristem, then along the microshoot stem to proximal bases, then leaf abscission, followed by axial bud swell.

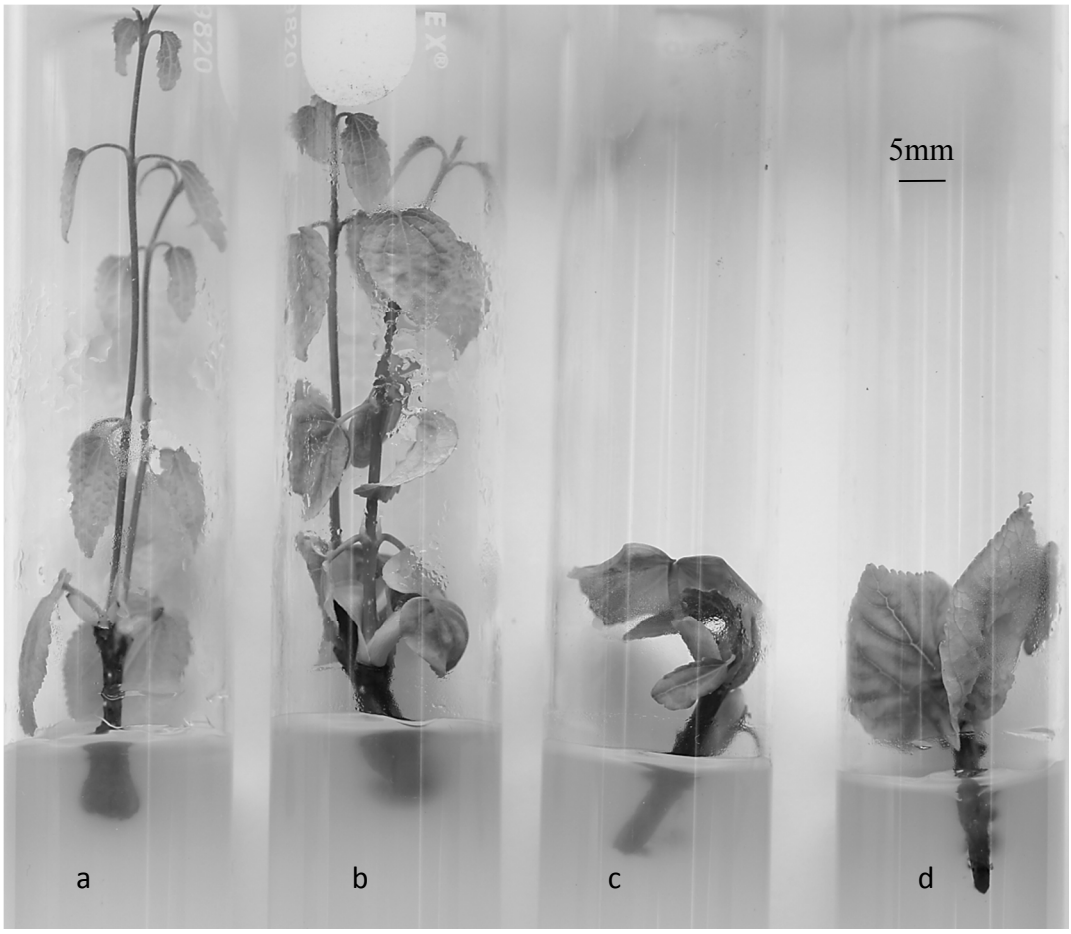

Figure 2-2. Axillary shoot initiation and development of *C. magnificum* Acc. No. 270-2003*A*C a) WPM b) MS c) DKW and d) LP nutrient salt formulations after 4 w incubation (23±1 ºC in light).

Proliferation

The gibberellin, GA_3, had a significant impact on shoot length, shoot number, and bud number (Appendix Table A-13) for the *C. japonicum* Acc. No. TS9821-6. However, treatment combinations with the presence of GA_3 and NAA were not significantly different in stimulating shoot growth. Although gibberellin application positively impacted shoot growth, after subculturing, microshoots were less vigorous than those initiated from nodal explants and may be a symptom of growth-inhibiting endogenous compounds or programmed cell death as a response to wounding. Repeated subculturing intensified this response and any stimulation of shoot growth was only amenable to a reversion to bud dormancy then transfer to a medium containing lower concentrations of salt and cytokinin.

In Vitro Rooting

Treatment effects were significant to both mean root length and number; however, 2 µM concentrations of IBA and NAA were not significantly different in relation to root number (Appendix Table A-14). NAA did segregate from all other treatments with lower concentrations of auxin or treatments composed of a mixture of both auxin types with overall higher concentrations (Table 2-7). The significant effect of auxin type on *in vitro* rooting may be a result of the presence of a mixture in this experiment and its failure to stimulate a heightened response, as a result of auxin interaction at different concentrations. Enhanced root growth was observed only at high concentrations of IBA, NAA, and mixture classes, respectively. The effects of auxin concentration on root length were significantly enhanced with the presence of 2 µM IBA (Figure 2-7); a common response to type and concentrations in woody plants (Ahuja, 1993).

Table 2-7. Treatment effects of auxin type and concentration on root growth of *C. japonicum* Acc. No. TS9821-6 after 4w incubation (23±1ºC in light).

Plant Growth Regulator (μM)[1]		Root Number[2]	Root Length
IBA	NAA		
0.5		0.0	0.0
1.0		0.5	0.8
2.0		2.5	4.5
	0.5	0.0	0.0
	1.0	0.0	0.0
	2.0	2.8	1.3
0.5	0.5	0.0	0.0
1.0	1.0	0.0	0.0
2.0	2.0	0.2	0.8
Control[3]		0.0	0.0
$LSD_{0.05}$[4]		1.19	1.29

[1] IBA, NAA; indole-6-butryic acid, naphthaleneacetic acid, respectively, in 10^{-3} mol/L[3] molar (μM) concentrations.
[2] Treatment means in millimeters (mm).
[3] Control; WPM basal salts only.
[4] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

Conclusion

In general, *Cercidiphyllum* reacts well with low concentrations of BA+IBA during *in vitro* initiation (Table 2-2; Table 2-5) and elongation (Table 2-3); however, between genotypes, these requirements differ among treatments; a response further shown by *ex vitro* initiation (Table 2-1). Elongation of *C. japonicum* subcultured initiates performed significantly better on high BA concentrations without the presence of an auxin (Table 2-5), whereas, axillary proliferation and elongation was significantly enhanced by the interaction GA_3 x BA x NAA (Table 2-7).

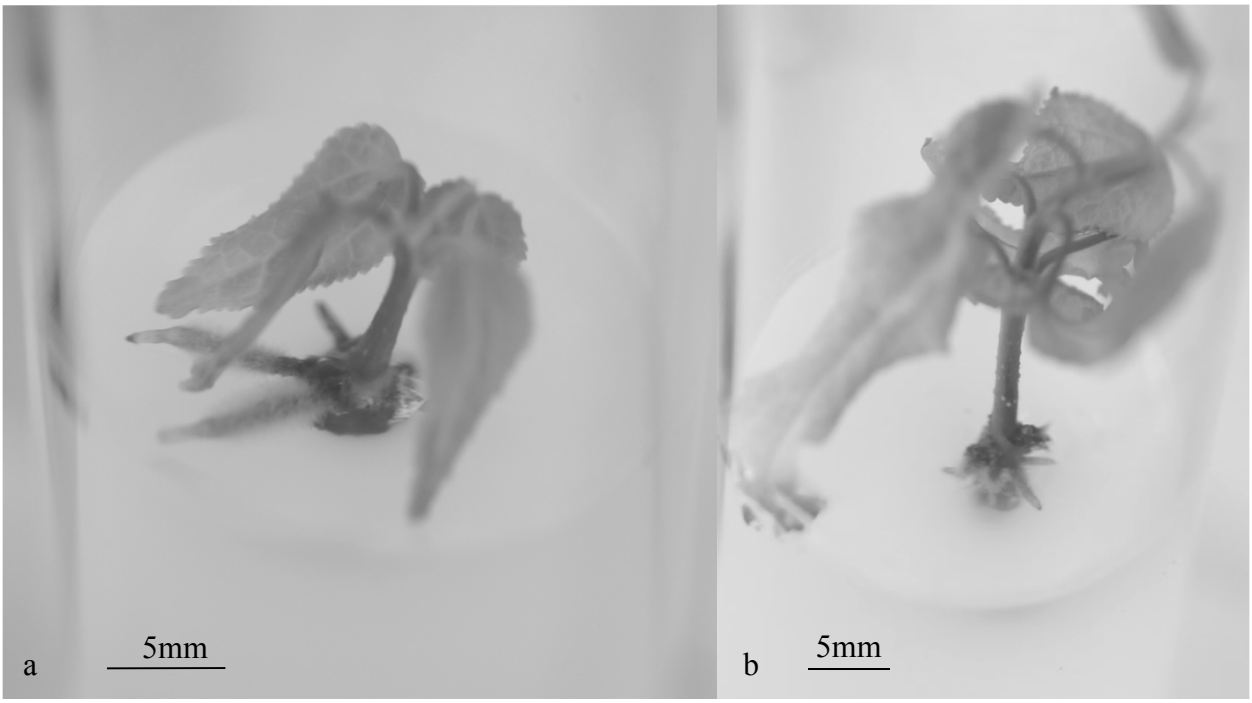

Figure 2-3. *In vitro* rooted microshoots on a) 2 µM IBA and b) 2 µM NAA after 4 w incubation (23±1 ºC in light) supplemented with WPM salts and nutrients, 0.3% (w/v) D-(+)-glucose, and 7.0 g L^{-1} agar.

Rooting of microshoots performed well on high auxin concentrations and significantly in the case of root length (Table 2-7) but root number was not significantly different from NAA at the same concentrations, i.e., concentration of auxin had a greater effect, opposed to auxin type. During these experiments, the presence of endogenous secondary compounds was found to be the limiting factor of nodal explant initiation and elongation, and microshoot proliferation, establishment, and rooting (Figure 2-1).

The micropropagation procedure, i.e., nodal segments containing two axillary buds initiated on WPM or LP with 2.2µM BA with or without 0.05µM IBA, subcultured nodal segments elongated on 4.4µM BA alone or in combination with 0.05µM IBA, on either MS, WPM or LP (whereas IBA+BA significantly promoted higher bud and shoot number), proliferation on WPM, 10µM BA, 0.1µM NAA, and 5µM GA_3, followed by *in vitro* rooting on WPM with 2.2µM IBA, could be used for commercial clonal production of new *Cercidiphyllum*

cultivars. Further experimentation forming quadratic relationships of growth variables and treatment factors and interactions may be accomplished using more extreme PGR concentrations and replicates, thus developing an optimized protocol via threshold.

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CHAPTER 3. REDUCING PHENOLICS IN *CERCIDIPHYLLUM JAPONICUM* CULTURES

Abstract

An approach for reducing the negative effects of endogenous phenolic compounds in *Cercidiphyllum japonicum* Sieb. & Zucc. was developed to optimize the mass clonal nursery production of the species. Cultures were compared by presoaking nodal explants in anaerobic conditions for 12 or 24 h, as well as incorporating the oxidizing and sequestering agents, PVP, L-ascorbic acid, citric acid, and silver nitrate at concentrations of 0, 10, 20, or 40 μM into initiation media, and comparing the carbon sources, sucrose and D-(+)-glucose at concentrations of 0%, 1.5%, 3%, 5% (w/v), and 3% (w/v) 1:1 mixture of D-(+)-glucose : sucrose over 4 w on a bulked half-sib *C. japonicum* family. Presoaking solutions of 1% (w/v) L-ascorbic acid and citric acid significantly outperformed all other treatments, whereas, incorporation of L-ascorbic acid at 40 μM showed similar effects. Additionally, D-(+)-glucose at 3% (w/v) is shown to be an effective carbon source compared to types and level.

Introduction

Preliminary micropropagation experiments with *Cercidiphyllum japonicum* Sieb. & Zucc. have shown the need for a protocol to reduce the negative effects of phenolics, as they inhibit the vigorous initiation and establishment of nodal explants. Tissue necrosis caused by the oxidation of phenolic compounds which exude from *C. japonicum* organs cause serious problems in establishing and maintaining *in vitro* cultures. Preliminary shoot initiation and elongation experiments showed a high degree of phenolic exudate suspended in culture medium at the cut proximal base of *C. japonicum* nodal explants (Chapter 2, Fig. 2-1). It was observed that explant stems and petioles typically oxidize after *in vitro* transfer. Repeated transfer reduces noticeable

phenolic compounds suspended in the culture medium; however, shoots are stunted and less vigorous than typical *ex vitro* shoots.

The compounds that give katsura its brilliant foliar color, anthocyanins, are grouped with structurally similar secondary metabolites, known as phenolics. These metabolites can range from simple molecules to highly polymerized compounds. Phenolic acids are characterized as aromatic metabolites possessing one or more acidic hydroxyl group attached to an arene (phenyl) ring (Buchanan et al., 2000). These ubiquitous and structurally diverse products arise from the shikimate-phenylpropanoids-flavanoid and related biochemical pathways.

Generally, phenolic acids are either derivatives of benzoic acid, such as gallic acid, or derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Phenolics usually accumulate in the vacuoles of guard and epidermal cells as well as subepidermal cells of leaves and shoots. Some are found covalently linked to the plant cell wall; others occur in waxes or on the external surfaces of plant organs (D'Archivio, 2001).

Phenolics in plant tissues have plagued plant scientists for years, interfering with experimental methods. For example, phenolics of woody plants, fruits, and vegetables when exposed to air, readily oxidize and turn brown (Julkunen, 1985; Laurila, 1998), generating products that can form complexes with proteins and chelate metals (Walker, 1998), inhibit enzyme activity (Buchanan, et. al., 2000), nutrient bioavailability (Johnson et al., 2013) or modulation of receptors (Dai and Mumper, 2010). This reaction to phenolic oxidation can be prevented by chemical, enzymatic, or physical treatments (Whitaker and Lee, 1995); however, these treatments often cannot be used *in vitro*. Pizzocaro et al. (1993) recommended sequestering agents be added to culture medium to limit *in vitro* tissue blackening. It has also been noted that the degree of explant blackening is related to phenolic concentration and enzyme activity in plant

tissues, particularly the presence of polyphenoloxidase (PPO) (Kahn, 1975), a catalyst of phenolic oxidation; reduced peroxidase activity is said to increase the ability of tissues to initiate growth *in vitro* (Andersone and Ivanesh, 2002).

In theory, incorporating oxidizing and sequestering agents in culture media will chemically reduce phenolics or form phenol-conjugates, preventing oxidization and necrosis of plant tissues, and subsequently increase the availability of nutrients to explant organs. Reduction of phenolic exudation and their effects on *in vitro* explants during the initiation and elongation phase of *C. japonicum* using different carbon sources, oxidizing and sequestering agents, and anaerobic conditions were used improve explant vigor on the initiation, elongation, and establishment of *in vitro* cultures.

Materials and Methods

Source of Explant Material

Plant material used in this study were fresh, softwood nodal explants of *Cercidiphyllum japonicum*, bulked as one half-sib family, Acc. No.'s TS9821-6, consisting of 6 individuals[1] originating from the North Dakota State University research planting in Fargo, ND.

Disinfestation and Preparation of Plant Material

Incorporation

Nodal explants were placed on an initiation medium consisting of Lloyd & McCown Woody Plant Medium (WPM) salts and vitamins (Lloyd, 1980), 2.2 µM 6-benzylaminopurine[2] (BA), 0.05 µM indole-3-butyric acid[1] (IBA), 3% sucrose, 7.0 g L^{-1} agar[3].

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[1] Bulked individuals of Acc. No.'s TS9821-6 are progeny collected from the parent Acc. No. 943, germinated in 1949 at the Lesny Zaklad Doswiadczalny W. Rogowre Arboretum, Poland, which derived from seeds obtained from an unknown *C. japonicum* accession at the Warsaw University Botanical Garden, Poland (Banaszczak, 2013)

[2] No. B800, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

[3] No. A111, gel strength: 1080 g/cm^2, *Phyto*Technology Laboratories®, P.O. Box 12205 Shawnee Mission, KS 66282, USA.

The pH was adjusted[1] to 5.8 ± 0.01 with 1.0 N KOH, with either 0% or 0.1% (w/v) polyvinylpyrrolidone-4[2] (polymerizer/sequestering agent), and 0, 10, 20, or 40 µM of L-ascorbic acid[3] (oxidoreductase), citric acid[6] (chelator), or silver nitrate[4] (oxidizer) and dispensed at 15 mL per 25x150 mm borosilicate glass culture tube [5], capped with autoclavable plastic caps[6], and autoclaved for 20 min at 121 ºC with 1.0-kg cm^{-2} pressure.

Pretreatment

Nodal explants were presoaked in treatment solution concentrations of 0% or 1% (w/v) in distilled and deionized water (ddH_20)[7] (18.2 MΩ). Explants were presoaked in the same compounds in the dark for 12 or 24 h at 4-5 ºC and placed on an initiation medium consisting of Woody Plant Medium (WPM) salts and vitamins, 2.2 µM BA, 0.05 µM indole-3-butyric acid (IBA), 3.0% sucrose, with the pH adjusted to 5.8 ± 0.01 with 1.0 N KOH, and 7.0 gL^{-1} agar.

Carbon Source

Additionally, treatments including a non-reducing disaccharide, sucrose, and the reducing monosaccharide, D-(+)-glucose [8]were analyzed at concentrations of 0%, 1.5%, 3%, or 5% (w/v) and 1:1 mixtures of D-(+)-glucose: sucrose over 4 w to determine an efficient carbon source.

All axial nodal explants were excised and cut to 1-2 cm; distally, 3-5 mm above distal portions of bud primordia and proximally 1-2 cm below primordia, and disinfested in bulk. Explants were vigorously shaken in ddH_20 for 3 min (3x), placed in a 70% (v/v) ethanol solution for 2 min, decanted and rinsed with ddH_20 (3x), then lightly shaken for 10 min in a 12% (v/v)

[1] No. AB15, Accumet® Basic pH meter, Thermo Fisher Scientific Inc. 81 Wyman Street Waltham, MA 02454.
[2] P727, PVP-40 Povidone, *Phyto*Technology Laboratories®, P.O. Box 12205 Shawnee Mission, KS 66282, USA.
[3] A4544, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.
[4] *Phyto*Technology Laboratories®, P.O. Box 12205 Shawnee Mission, KS 66282, USA.
[5] No. 9820, Pyrex®, 836 North Street Building 300 Suite 3401 Tewksbury MA 01876, USA.
[7] Kim-Kap®
[7] Milli-Q Water System, Millipore, Milford, MA, USA
[8] No. G386, *Phyto*Technology Laboratories®, P.O. Box 12205 Shawnee Mission, KS 66282, USA.

NaClO solution[1] and 0.1% (v/v) Tween 20®[2] solution, rinsed with ddH_20 (3x) (18.2 MΩ) at 25ºC, and subsequently decanted under sterile conditions.

Nodal explants were freshly cut under sterile conditions using a surgical-grade scalpel. With a long-tipped forceps, explants were placed in 25 mL x 150 borosilicate culture tubes containing corresponding treatments, upright and proximally inserted 5 mm below axillary buds. Cultures were incubated 30 cm below cool white florescent lamps that provided a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16-h photoperiod at 25 ºC for 4 w.

Data Collection and Statistical Analysis

All experiments were arranged as completely random designs (CRD) and conducted twice. A minimum of 5 replicates were used in each treatment of every experiment. All data were analyzed using the General Linear Model (GLM) of SAS (SAS Institute, Inc.) because of the potential for unequal replication within treatments as a result of potential contamination or explant death. Mean shoot number and length, as well as mean bud number and surface area were scored across all treatments over 4 w.

Results and Discussion

Micropropagation of *C. japonicum* Acc. No. TS9821-6, showed high concentrations of phenolic compounds suspended in culture medium at the cut base of *C. japonicum* explants (Figure 3-1). Fu et al. (2012) also noted the 'browning' of *C. japonicum* explants. Repeated transfer slightly reduced noticeable phenolic compounds suspended in the medium; however, shoots are stunted and less vigorous than explant donor shoots.

The incorporation of oxidizing and sequestering agents in culture media and presoaking time had significant effect on explant response (Appendix Table A-15). Effects on shoot growth

[1] 6% NaClO.
[2] No. P9416, Polyethylenesorbitan momolaurate, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

for the 24 h presoak were significant, shown by $LSD_{0.05}$ segregation of the citric acid treatment from all other treatments, whereas, effects of the 12 h presoaking treatments over a period of 4w were not significant for any variables (Table 3-1). The 24 h presoaking treatment after 4 w weeks was significant for shoot length and treatment significant for shoot and bud number compared to the 12 h presoaking treatment for the same incubation time (Table 3-1).

Table 3-1. Effects of 1% (w/v) presoaking treatment solutions and presoak time on shoot length, shoot number, and bud number on *C. japonicum* Acc. No. TS9821-6 after 4 w incubation (23±1 ºC in light).

Solution 1 % (w/v)[1]	12 h			24 h		
	Shoot Length[2]	Shoot No.	Bud No.	Shoot Length	Shoot No.	Bud No.
ddH_2O	6.8	1.5	4.8	3.6	1.1	4.0
PVP	4.7	1.2	3.9	3.4	0.8	3.0
Citric Acid	8.3	1.8	5.5	11.3	1.8	5.3
L-Ascorbic Acid	8.5	1.5	4.8	4.9	1.3	3.8
$AgNO_3$	5.4	1.4	4.4	7.7	1.6	4.8
$LSD_{0.05}$[3]	ns	ns	ns	3.42	0.69	1.01

[1] ddH_2O, PVP, $AgNO_3$; deionized distilled water, Povidone-40, and silver nitrate, respectively.
[2] Treatment means in millimeters (mm).
[3] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

The citric acid treatment solution was significantly different from all other treatments for shoot number, which induced better shoot growth. Ascorbic acid was not significantly different from silver nitrate but did segregate from the other treatments, whereas, silver nitrate did not segregate from the treatments with lower mean shoot length. Shoot number segregated similarly over presoaking treatments and significant treatment effects on bud number after 4w incubation (Table 3-1), with citric acid and silver nitrate outperforming PVP and the control. For the presoaking experiment, replication was significant over all variables but not run; a likely result of the uniformity of single level treatments between runs.

Incorporation treatment effects were significant for silver nitrate on mean shoot length and bud number (Appendix, Table A-16). Significant treatment effects on bud number also were a result of high concentrations of citric acid (Table 3-2). The incorporation of a single level of 1% PVP was not significantly different from these treatments and was excluded from Table 3-2 and the overall ANOVA based on concentration comparison differences.

Table 3-2. Effects of incorporated treatment concentrations on shoot length, shoot number, and bud number on *C. japonicum* Acc. No. TS9821-6 after 4 w of incubation (23±1 ºC in light).

Treatment (μM)[1]					
Citric Acid	Ascorbic Acid	$AgNO_3$[2]	Shoot Length[3]	Shoot Number	Bud Number
10			2.3	0.6	3.7
20			3.5	0.9	3.3
40			5.2	1.4	4.6
	10		2.9	1.2	3.4
	20		4.1	1.2	3.8
	40		3.0	0.8	3.4
		10	1.9	0.8	2.6
		20	3.0	1.1	3.4
		40	7.2	1.2	4.8
Control[4]			2.4	0.6	2.0
$LSD_{0.05}$[5]			3.1	ns	1.7

[1] 10^{-3} mol/L^3 molar.
[2] $AgNO_3$; silver nitrate.
[3] Treatment means in millimeters (mm).
[4] Control; incorporated salts and nutrients only.
[5] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

Carbon source treatments were represented by D-(+)-glucose and sucrose at varying concentrations and mixtures. The source of carbon did have a significant effect on shoot growth; further, D-(+)-glucose performed significantly better as a carbon source compared to sucrose (Appendix Table A-16; Table 3-3).

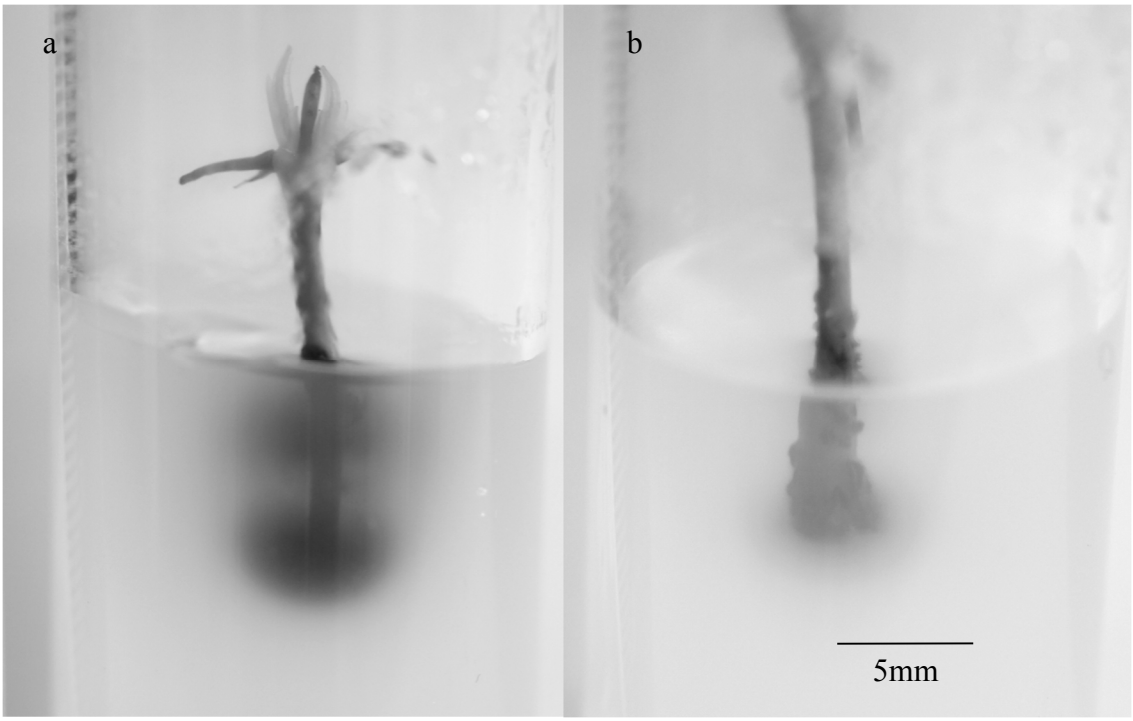

Figure 3-1. Reduced phenolic exudate with presoaking for a) 12 h and b) 24 h in a 1 % (w/v) citric acid solution after 2 w incubation (23±1 ºC in light)

However, beyond main effects, there was no significant interaction in regards to D-(+)-glucose and sucrose mixtures (Appendix, Table A-16). D-(+)-glucose at 3% (w/v) significantly outperformed all treatments in mean shoot length and bud number, and not significantly different from the 1.5%:1.5% (w/v) D-(+)-glucose : sucrose mixture for mean shoot number, but from all other treatments (Table 3-3). Medium concentrations of 3.0% (w/v) of both carbon sources performed better in mean bud number and mean shoot length than low or high concentrations within their type; significantly, in the case of D-(+)-glucose. Lower concentration (1.0% w/v) of sucrose did outperform the same concentration of D-(+)-glucose, which yielded no shoot formation, but was significantly different from 5% (w/v) D-(+)-glucose in mean shoot length. High concentration (5% w/v) of sucrose or D-(+)-glucose was not significantly different.

Table 3-3. Effects of carbon source and concentration on leaf and shoot growth of *C. japonicum* Acc. No. TS9821-6 after 4 w of incubation (23±1 ºC in light).

Carbon Source % (w/v)		Bud	Shoot	Shoot
Sucrose	D-(+)-glucose	Number[1]	Number	Length
1		3.73	0.93	4.36
3		3.88	1.13	5.08
5		3.00	0.55	4.03
	1	3.50	0.0	0.0
	3	7.80	2.00	11.3
	5	2.50	0.25	1.38
1	1	5.33	0.20	0.90
1.5	1.5	4.80	1.78	7.33
3	0.5	5.00	1.10	2.30
Control[2]		3.10	0.94	4.57
$LSD_{0.05}$[3]		1.92	0.68	3.56

[1] Treatment means in millimeters (mm).
[2] Control; incorporated salts and nutrients only.
[3] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

The mixture concentrations (Table 3-3) 1.5%:1.5%, 1.0%:1.0%, and 3.0%:3.0% (w/v) of sucrose and D-(+)-glucose, respectively, differed from one another based on the concentrations of carbon present, similar to the trend within carbon type. The control, which excluded any carbon source, was not significantly different from any treatments in shoot length but the highest performing treatment, 3.0% (w/v) D-(+)-glucose, and the lowest treatments, 1:1 (1% w/v) mixture and the 1.0% (w/v) D-(+)-glucose treatment.

The signature symptoms of untreated *C. japonicum* explants after microshoot transfer is the stunted nature of developing meristems, darkening of the explant stem submerged in culture medium, and the pooling of exudate suspending at the proximal base of the explant shoot. The magnitude and density of exudate is significantly related to presoaking and incorporation treatment concentrations (Figure 3-1). Exudate of untreated explants is present within minutes of culture and stabilizes in particle density within 48 hours. At this time, it can be assumed

concentrations of native endogenous phenolics in microshoots exhaust their osmotic potential in the culture matrix.

It is possible that wound-induced suberization following excision from the parent donor plant leads to explant decline during the initiation stage and subsequent microshoot transfer. Suberized tissues are formed as multilamellar domains consisting of alternating aliphatic and aromatic layers, providing a means to limit water loss by forming an impenetrable barrier. The aromatic domain is said to form before the aliphatics, particularly from monomeric building blocks that contain hydroxycinnamate-derived substances (Buchanan et al., 2000).

This relationship, between auxin and xylem regeneration, may be a result of the auxin-transport capacity of vascular tissues rather than the presence of endogenously produced auxin in plant tissues or available concentrations *in vitro*. The amount of xylem that can be regenerated during suberization after excision may be limited by the amount of auxin which can reach the regenerating area from the internodes of *Cercidiphyllum* explants.

Titman and Wetmore (1955) made similar observations while investigating auxin yields in *Cercidiphyllum* using the *Avena* bioassay developed by Went and Thimann (1948). Regardless of the material tested, auxin yields were so inappreciable that modification of the assay was necessary with the inclusion of potassium cyanide (KCN); an inhibitor of oxidative phosphorylation. The elongating stem in *Cercidiphyllum* was determined to be the major auxin production center of shoots with the use of KCN but still yielded miniscule concentrations. They postulated that either auxin destruction occurs at the cut surface or is a result of indole-3-acetic acid (IAA) oxidase or another enzyme unbeknownst to them.

Concurrent work profiling ten diverse genotypes within *Cercidiphyllum* using high performance liquid chromatography with diode array detection (HPLC-DAD) identified high

concentrations of hydroxycinnamate-derived phenolics in stem and leaf tissues of Acc. No. TS9821-6, such as catechin, chlorogenic, and protocatechuic acids, as well as other related phenolic compounds (Chapter 4, Table 4-3). In the case of mechanical damage to plant tissues, phenolic compounds are released from vacuoles, free to oxidize with O_2. Upon oxidization, electrophilic quinones are formed via diphenol oxidase, and stabilized by conjugating with available amino acids and proteins (Walker and Ferrar, 1998). These phenolics have also been shown to inhibit IAA transport and amino acid transport and protein formation (Walker, 1998) necessary for seed germination.

With respect to herbivory, this polymerization leads to a reduced bioavailability of amino acids to phytopathogens as well as the host plant. However, the levels of oxidizable phenolics in foliage are not necessarily a prooxidant. The net oxidative balance depends on the predominant types of plant phenolics and phenolases present in the pathogen, which can vary substantially among species. In willow (*Salix* sp.), the response of different beetles on chlorogenic acid was dependent on willow species (Ikonen et al., 2001; 2002). For example, in some lepidopteron species, initial oxidation of chlorogenic acid to chlorogenoquinone expresses toxicity, while further oxidation considerably decreases it due to formation of phenolic oligomers present in the midgut (Felton and Duffey, 1991).

Further, the diversity of phenolic compounds are well documented in *Centaurea maculosa* (Broz, 2006), providing evidence of the allelopathic nature of catechin. When applied to plant roots, cytoplasmic condensation initiates at the root tip and spreads in an upward wave. This wave of cell death was thought to be accompanied by reactive oxygen species (ROS) and a spike in intracellular calcium ions (Ca^{2+}), both considered hypersensitive responses to pathogen invasion. Catechin is one of the building blocks of proanthocyanins (condensed tannins) and

often exists in the form of a glycoside (Chumbalov, 1976) and may be highly upregulated by physical excision from *Cercidiphyllum* microshoots.

One or more types of sugars are typically required for regular plant metabolism. Sucrose is the most common fixed carbon source used in plant cell tissue culture systems. It is a non-reducing disaccharide which consists of the monosaccharide moieties, fructose and glucose, both reducing sugars, linked by an O-glycosidic bond (Thorpe et al., 2008). Glucose has been known to outperform sucrose in the multiplication of *Alnus crispa*, *A. cordata*, and *A. rubra* (Tremblay and Lalonde, 1984) and shoot formation in *Capsicum annum* (Phillips and Hubstenberger, 1985); whereas, fructose gave better results in orchid culture (Ernst, 1967), and the production of adventitious shoots in *Glycine max* cotyledonary nodes (Wright et al., 1986).

A number of these phenolic compounds bind with sugars. Sugar conjugates of phenolic compounds have been known to decrease the toxicity or reactivity and increase the solubility of the compounds to make it easier for them to be transported or stored without harm to the plant producing them (Vickery, 1981). During sugar polymerization, the anomeric carbon of one sugar molecule is joined to the hydroxyl group of another sugar, hydroxylamino acid, or a phenylpropanoid compound in a glycosidic linkage. These compounds can be linked to D-(+)-glucose at the hydroxyl oxygen's on C-2, 3, 4, or 6. Many proteins have binding sites that are highly specific to glucose. For example, a majority of the known phenolic compounds in *Cercidiphyllum* appear to show an affinity to glucose polymerization (Nonaka, 1989; Kasuga, 2007b; 2008; 2010; Wang and Kasuga, 2012).

These observations can be compared to the carbon source, sucrose, which is only hydrolyzable by endogenous plant invertases. Unless these enzymes are present, uninhibited by phenolic compounds, sucrose molecules cannot pass through cell membranes via diffusion

(Buchanan et al., 2000). When sucrose is present as the carbon source, phenolics are likely oxidizing and chelating with basal salt macro and micronutrients and vitamins (amino acids).

Conclusion

High concentrations of presoaking solutions of ascorbic acid and citric acid outperformed all other treatments, with particular emphasis on citric acid (Table 3-1). Ascorbic acid has a lower redox potential than the quinones formed by diphenyl oxidase action so it is oxidized and the quinones reduced back to their parent dihydroxyphenols, whereas, citric acid acts as a chelator as well as decreasing the pH below optimum for catecholase activity (Walker and Ferrar, 1998). These treatments were significantly better at reducing the effects of phenolic compounds in *C. japonicum*. With regards to the presoaking experiment, exudate seizure after transfer (Figure 3-1) is partially a result of the anaerobic reducing atmosphere of the presoaking solution, in which oxidation of phenolic compound exudate to quinones is prevented through the removal of oxygen and other oxidizing gases which may contain actively reducing gases such as hydrogen, carbon monoxide and gases that would oxidize in the presence of oxygen (Janeiro, et al. 2004), demonstrated by the control outperforming the sequestering agent, silver nitrate, which was the most effective incorporation treatment (Table 3-1). D-(+)-glucose was a superior carbon source at 30 μM, compared to the typical carbon source, sucrose. The propensity of phenolics to appear in conjugated forms is fitting the presence of D-(+)-glucose in the medium serves as a powerful reducing agent in the presence of oxidized quinones converse to non-reducing sucrose. Understanding the relationship of phenolic compounds and sequestering agents, as well as their use in plant trait improvement and disease resistance (Broz et al. 2006).

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CHAPTER 4. IDENTIFICATION AND QUANTIFICATION OF MAJOR PHENOLIC ACIDS IN *CERCIDIPHYLLUM* (CERCIDIPHYLLACEAE) USING HPLC-DAD

Abstract

A quantitative phenolic profile of *Cercidiphyllum* spp. was undertaken to provide further evidence of metabolic diversity within and among species. Both cultivated and wild collected leaf and stem tissues of *C. japonicum* Sieb & Zucc. and *C. magnificum* Nakai were analyzed for concentrations of 11 common pure (>99%) phenolic standards for peak identification and quantification using high-performance liquid chromatography with diode array detection (HPLC-DAD). Wavelengths for detection were within 250 and 360 nm; covering most phenolic acid absorbance levels. Of the phenolic standards, 8 were identified in *Cercidiphyllum* in varying concentrations and tissue type. The hydroxycinnamic derived phenolic, chlorogenic acid (5-caffeoylquinic acid), and the hydroxybenzoic phenolics, protocatechuic acid (3,4-dihydroxybenzoic acid) and gallic acid (3,4,5-trihydroxybenzoic acid; 3-O-gallate) shared presence and relative concentrations in leaves and stems of all samples, whereas, the flavan-3-ol, (+)-catechin was found in *C. japonicum* and *C. magnificum* leaf tissues but not in the Weeping Group.

Introduction

The East Asian Tertiary relict *Cercidiphyllum* (Cercidiphyllaceae) is endemic to Japan and China and consists of two dioecious tree species, common katsura (*C. japonicum* Sieb. & Zucc.) and the hiro-ha-katsura (*C. magnificum* Nakai). Prized as specimen trees, all genotypes of the genus have potential for commercial nursery development (Haag, 1982; Andrews, 1998). The broader-leaved hiro-ha-katsura subtly differs morphologically from its cogener, *C. japonicum*; and has subsequently been in and out of taxonomic discussion (Spongberg, 1979; Koller, 1987;

Li et al., 2002) along with a number of weeping cultivars (the "Weeping Group") that have become popular in the ornamental nursery industry as an alternative to the upright, tree-form *C. japonicum*.

Traditionally, *C. japonicum* is asexually propagated by softwood vegetative cuttings (Dirr, 1987) or budding compatible rootstocks in the early-spring (Morgenson, correspondence). Commercially, it is almost exclusively propagated sexually, by seed (Dosmann and Widrlechner, 2000). Nearly all genotypes of *Cercidiphyllum* have ornamental characteristics worthy of cultivation. The colorful medley the foliage undergoes throughout the growing season is complimented by its continually evolving fragrance.

The compounds that give katsura its brilliant fall foliar color, anthocyanins, are grouped with structurally similar secondary metabolites, known as phenolics. These metabolites can range from simple molecules to highly complex polymerized compounds. Woody plants produce a vast and diverse assortment of phenolics, a majority of which do not appear to participate directly in growth and development (Buchanan et al., 2000). Although these natural products have been perceived as biologically insignificant, there are numerous studies that show there is adaptive significance of these compounds; ranging from allelopathy in *Ailanthus altissima* (Heisey, 1997) to facilitating supercooling capacities in *C. japonicum* (Wang et al., 2012).

Generally, phenolic acids are either derivatives of benzoic acid, such as gallic acid, or derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid (D'Archivio, 2001). Phenolics usually accumulate in the vacuoles of guard and epidermal cells as well as subepidermal cells of leaves and shoots. Some are found covalently linked to the plant cell wall; others occur in waxes or on the external surfaces of plant organs. Although the majority of these substances assume structural roles, there is a vast array of nonstructural qualitative constituents

(Buchanan et al., 2000), with such roles as color (Duenas, 2003), resistance to pathogens (Heisey, 1997; Hammerschmidt, 1999), nutrient bioavailability (Hu, 2006; Johnson et al., 2013), tastes and odors (Dai and Mumper, 2010). Phenolics have several industrial applications as well. Recent interest in food phenolics (mainly flavonoids) has increased, owing to their antioxidant capacity (free radical scavenging and metal chelating activities), supposed anticancer benefits (Bravo, 1998; Fresco et al., 2006), as well as enzyme and receptor modulation (Dai and Mumper, 2010).

Takasugi et al. (1986), noted the biphenyl phytoalexin, magnolol (5,5'-diallyl-2,2'-dihydroxybiphenyl) was present in *Cercidiphyllum japonicum* cortical tissue after prescreening for potential antifungal compounds (*Fusarium solani* f. sp. *mori* as inoculum) with a thin layer chromatography (TLC) bioassay. Unusual ellagitannin-glucose conjugates were isolated from fresh bark of *C. japonicum*, characterized as cercidinin A and B (2,3´-(R)-hexahydroxydiphenoyl glucoses), on the basis of chemical and spectroscopic evidence (Nonaka, 1989). With the use of silica gel column chromatography, crude xylem parenchyma cell (XPC) extracts in *C. japonicum* dormant winter bark samples revealed the presence of four supercooling-facilitating (SCF; anti-ice nucleation) glycosides (Kasuga et al., 2007b; 2008) and 12 compounds that had similar structures to flavinol glycosides (Kasuga et al., 2010). Further, Wang and Kasuga (2012) identified four hydrolyzable gallotannins, showing additional diversity of SCF secondary metabolites.

Quantitative measurements of phenolic acids in plant material are commonly accomplished with high-performance liquid chromatography (HPLC) systems (Olkowski, 2003). The use of HPLC with diode array detection (DAD) to identify and quantify major phenolics of

10 diverse genotypes within *Cercidiphyllum* and the potential of resulting profiles as a conservation tool are discussed.

Materials and Methods

Source of Explant Material

Plant material used in this study consisted of three *Cercidiphyllum* accessions, *C. japonicum* (Acc. No. 200-48, bulked half-sibs of Japanese origin), one weeping variety, *C. japonicum* 'Pendulum' (Acc. No. 72-84), and *C. japonicum* var. *sinense* (Acc. No. 464-84, of Chinese origin), acquired from the Morton Arboretum in Lisle, IL. Two *C. japonicum* accessions (Acc. No. 232-2000*A of Japanese origin and 12-2007A of Chinese origin), two weeping cultivars *C. japonicum* cv. 'Amazing Grace' (Acc. No. 113-2002*A of garden origin) and *C. japonicum* cv. 'Morioka Weeping' (Acc. No.698-81*A of Japanese origin), and two *C. magnificum* accessions (Acc. No.'s 270-2003*A*C of cultivated Japanese origin and 291-2008*A, wild collected in Japan) were acquired from the Arnold Arboretum of Harvard University in Boston, MA. A bulked, half-sib *C. japonicum* family (Acc. No.'s TS9821-6), consisting of 6 individuals[1] originating from the North Dakota State University research planting in Fargo, ND. All accessions arrived as spring cuttings (leaves attached), wrapped in a moist paper towel, sealed in a plastic bag, and stored in the dark at 4±1°C.

Standards

A total of 11 pure (>99%) phenolic standards[2] were used to create calibration curves for each analyte and for peak identification and quantification: caffeic acid, catechin, chlorogenic

[1] Bulked individuals of Acc. No.'s TS9821-6 are half-sib progeny collected from the parent Acc. No. 943, germinated in 1949 at the Lesny Zaklad Doswiadczalny W. Rogowre Arboretum, Poland, which derived from seeds obtained from an unknown *C. japonicum* accession at the Warsaw University Botanical Garden, Poland (Banaszczak, communication).

[2] Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

acid, ferulic acid, gallic acid, 4-hydroxybenzoic acid, protocatechuic acid, sinapic acid, syringic acid, isovanillic acid, and vanillic acid. Standard HPLC retention time was regressed against both leaf and stem samples of *Cercidiphyllum* genotypes (Table 4-1).

Phenolic Extraction and Quantification

Extraction and quantification of major phenolic acids was carried out using a method by Johnson et al. (2013). Phenolic compounds were extracted from dried (40 °C for 48 h) and finely ground *Cercidiphyllum* softwood shoot and leaf samples (0.3 g) with 3 mL of extraction solvent [Methanol:1M HCl (85:15 v/v)]. Individual vials were vortex mixed and shaken in an incubator for 3 h. Samples were centrifuged for 10 min at 3000 rpm. The supernatant was passed through Nylon Chromospec syringe filters (0.45 μm) before the extracts were injected into the Agilent high-performance liquid chromatography (HPLC)[1] system with diode array detection (DAD). Wavelengths for DAD were 250, 270, 300, 320, 340 and 360 nm; covering most phenolic acid absorbance levels. Chemicals were separated using a Prodigy 5u ODS3 100A column[2]. A gradient mobile phase with a flow rate of 1.0 mL min^{-1} was applied using the following eluents: (A) water: acetic acid (98:2 v/v), and (B) water: acetonitrile: acetic acid (78:20:2 v/v).

The linear solvent gradient parameters used was as follows: 0-30 min linear gradient from 100% to 20% A (v/v) and from 0% to 80% B; 30-40 min linear gradient from 20% to 10% A (v/v) and from 80% to 90% B (v/v); 40-50 min linear gradient from 10% to 10% A (v/v) and from 90% to 90% B (v/v); 50-55 min linear gradient from 10% to 5% A (v/v) and 90% to 95% B (v/v); 55-57 min linear gradient from 5% to 0% A (v/v) and 95% to 100% B (v/v); 57-58 min linear gradient from 0% to 100% A (v/v) and 100% to 0% B (v/v); and 58-60 min linear gradient from 100% to 100% A (v/v) and 0% to 0% B (v/v).

[1] Agilent® 1100 series No. G1323-90005, 5301 Stevens Creek Blvd. in Santa Clara, CA
[2] Phenomenex® 411 Madrid Avenue, Torrance, CA

Table 4-1. Genotype accession number, species, origin, and tissue type of 10 *Cercidiphyllum* genotypes for HPLC-DAD analysis.

Sample ID[1]		Accession Number	Taxonomy	Notes	
Leaf	Stem			Origin	Source[3]
1	11	270-2003*A	*C. magnificum* Nakai	Japan	AA
2	12	291-2008*A	*C. magnificum* Nakai	Japan	AA
3	13	232-2000*A	*C. japonicum* Sieb. & Zucc.	Japan	AA
4	14	12-2007*A	*C. japonicum* Sieb. & Zucc.	China	AA
5	15	113-2002*A	*C. japonicum* forma *pendulum* 'Amazing Grace'	Cultivated	AA
6	16	698-81*A	*C. japonicum* 'Morioka Weeping'	Japan	AA
7	17	200-48	*C. japonicum* Sieb. & Zucc.	Japan	MA
8	18	72-84	*C. japonicum* forma *pendulum* 'Pendula'	Japan	MA
9	19	464-84	*C. japonicum* var. *sinense*	China	MA
10	20	TS9822	*C. japonicum* Sieb. & Zucc.	Japan[2]	NDSU

[1] Sample identifiers for peak identification tables.

[2] Half-sib progeny collected from the parent Acc. No. 943, germinated in 1949 at the Lesny Zaklad Doswiadczalny W. Rogowre Arboretum, Poland, which derived from seeds obtained from an unknown *C. japonicum* accession at the Warsaw University Botanical Garden, Poland.

[3]AA, MA, NDSU; the Arnold Arboretum of Harvard University, Morton Arboretum, and the North Dakota State University research trial plantings, respectively.

Data Collection and Statistical Analysis

Pure phenolic standards were used to create calibration curves for each analyte and for peak identification and quantification. Standards were regressed against both leaf and stem samples of all *Cercidiphyllum* genotypes. A laboratory reference and pure standards[1] (Figure 4-3) were periodically used to ensure consistency and <1% error.

Results and Discussion

Phenolic acids (PAs) identified in leaf and stem tissue of the Japanese *C. japonicum* accession 232-2000*A (Table 4-2) was catechin at 598.4 ppm in leaf, chlorogenic acid at 1340.4 ppm in leaf and 172.1 ppm in stem, gallic acid at 9.3 ppm stem, and protocatechuic acid 196.2 ppm in stem. PA identified in leaf and stem tissue of the Chinese *C. japonicum* accession 12-2007*A was chlorogenic acid at 1251.3 ppm in leaf and 133.4 in stem, gallic acid at 11.4 ppm in stem, and isovanillic acid in leaf at 36.8 ppm. PA identified in leaf and stem tissue of the Japanese *C. japonicum* accession 200-48 was chlorogenic acid at 248.8 ppm in leaf and 0.9 ppm in stem, ferulic acid at 10.9 ppm in leaf, gallic acid at 14.8 ppm in leaf, and protocatechuic acid at 94.6 ppm in leaf and 2.4 ppm in stem. PA identified in leaf and stem tissue of Chinese *C. japonicum* var. *sinense* accession 464-84 was chlorogenic acid at 433.0 ppm in leaf and 1.2 ppm in stem, gallic acid at 10.6 ppm in leaf, and protocatechuic acid at 148.2 ppm in leaf and 3.0 in stem. PA identified in leaf and stem tissue of the Chinese *C. japonicum* accession TS9822 was chlorogenic acid at 1366.9 ppm in leaf and 145.2 ppm in stem, ferulic acid at 5.6 in stem, and gallic acid at 12.2 ppm in leaf and 11.3 in stem.

PAs identified in leaf and stem tissue of the cultivated *C. japonicum* cv. 'Amazing Grace' (Weeping Group) accession 113-2002*A (Table 4-3) was chlorogenic acid at 761.6 ppm in leaf and 82.7 in stem, gallic acid at 7.5 ppm in stem, and protocatechuic acid at 219.9 ppm in leaf and

[1] Sigma Aldrich, 3050 Spruce St. St. Louis, MO

94.4 in stem. PAs identified in leaf and stem tissue of the cultivated Japanese *C. japonicum* cv. 'Morioka Weeping' (Weeping Group) accession 698-81*A was chlorogenic acid at 870.3 ppm in leaf and 85.2 ppm in stem, gallic acid at 13.0 ppm in stem, and protocatechuic acid at 402.5 ppm in leaf and 170.5 ppm in stem. PAs identified in leaf and stem tissue of the cultivated *C. japonicum* f. pendula 'Pendulum' (Weeping Group) accession 72-84 was chlorogenic acid at 615.3 ppm in leaf and 2.1 ppm in stem, ferulic acid at 39.7 ppm in leaf, gallic acid at 11.4 ppm in leaf, protocatechuic acid at 195.1 ppm in leaf and 7.2 ppm in stem, and isovanillic acid at 19.7 ppm in leaf.

PAs identified in leaf and stem tissue of the Japanese *C. magnificum* accession 270-2003*A (Table 4-3) was chlorogenic acid at 225.7 ppm in leaf and 57.9 ppm in stem, gallic acid at 10.3 ppm in leaf and 35.6 ppm in stem, protocatechuic acid at 1.1 ppm in leaf, sinapic acid at 28.7 ppm in leaf and 28.4 ppm in stem, and vanillic acid at 402.9 ppm in leaf. PAs identified in leaf and stem tissue of the Chinese *C. magnificum* accession 291-2008*A was catechin at 610.3 ppm in leaf, chlorogenic acid at 1258.0 ppm in leaf and 101.3 ppm in stem, gallic acid at 7.3 in leaf and 11.0 ppm in stem, and protocatechuic acid at 23.8 in leaf and 60.0 ppm in stem.

Utilizing molecular evidence based on nuclear ribosomal ITS data, Li et al. (2002) found weeping varieties (Weeping Group) to by phylogenetically-derived from *C. japonicum*, whereas, *C. japonicum* and *C. magnificum* were found to be genetically distinct, supporting the recognition of them as a separate species. However, there is still a degree of confusion as to whether Cercidiphyllaceae is monospecific (Qi et al., 2012). The inclusion of *C. magnificum* (Nakai) seems to meet inadvertent resistance (Wei et al., 2010), as the species is often associated with other East Asian monotypic tertiary relicts (c. 65-2.6 million years ago), such as *Davidia*, *Euptelea*, *Metasequoia*, and *Ginko*.

Table 4-2. The identification of phenolics in dried leaf and stem tissue samples of *Cercidiphyllum*, phenolic taxonomy, and chromatographic characteristics.

Peak	t_R (min)[1]	λ_{max} (nm)[2]	Polyphenol Taxonomy			Sample Tissue[5,6]	
			Common Name	Class[3]	Sub-class[4]	Leaf	Stem
1	33.640	320	Caffeic acid	PA	HC	-	-
2	38.087	270	(+)-Catechin	FLV	F-3	2,3	-
3	30.944	320	Chlorogenic acid	PA	HC	1-10	11-20
4	48.146	320	Ferulic acid	PA	HC	8,7	9
5	10.400	270	Gallic acid	PA	HB	1,2,7-10	1-6, 10
6	26.373	250	*p*-Hydroxybenzoic acid	PA	HB	-	-
7	18.735	250	Protocaechuic acid	PA	HB	1,2,5-9	2,3,5-9
8	49.121	320	Sinapic acid	PA	HC	1	1
9	35.378	270	Syringic acid	PA	HB	-	-
10	34.964	250	isoVanillic acid	PA	HB	4,8	-
11	32.421	270	Vanillic acid	PA	HB	1	-

[1] Retention time obtained by HPLC-DAD.
[2] Lambda max (absorbing maximum).
[3] PA, FL; phenolic acid and flavonoid polyphenol classes, and respectively.
[4] HC, F-3, HB; hydroxycinnamic acid, flavan-3-ol, and hydroxybenzoic acid polyphenol subclasses, respectively.
[5] Leaf, Stem; polyphenolic compound identified in *Cercidiphyllum* leaf and stem tissue, respectively.
[6] Accession identification (ID) numbers are tissue samples from respective genotype. A dash (-) denotes phenolic standards were not detected in tissue.

Table 4-3. Quantification of of catechin, chlorogenic, ferulic, gallic, protocaechuic, sinapic, isovanillic, and vanillic acid concentrations (ppm) in leaf and stem tissue of *Cercidiphyllum* spp.

	Polyphenol Peak Concentrations (ppm)[1]										
Accession	1	2	3	4	5	6	7	8	9	10	11
	Leaf										
270-2003*A	-	-	225.7	-	10.3	-	1.1	28.7	-	-	402.9
291-2008*A	-	610.3	1258.0	-	7.3	-	23.8	-	-	-	-
232-2000*A	-	598.4	1340.4	-	-	-	-	-	-	-	-
12-2007*A	-	-	1251.3	-	-	-	-	-	-	36.8	-
113-2002*A	-	-	761.6	-	-	-	219.9	-	-	-	-
698-81*A	-	-	870.3	-	-	-	402.5	-	-	-	-
200-48	-	-	248.8	10.9	14.8	-	94.6	-	-	-	-
72-84	-	-	615.3	39.7	11.4	-	195.1	-	-	19.7	-
464-84	-	-	433.0	-	10.6	-	148.2	-	-	-	-
TS9822	-	-	1366.9	-	12.2	-	-	-	-	-	-
	Stem[2]										
270-2003*A	-	-	57.9	-	35.6	-	-	28.4	-	-	-
291-2008*A	-	-	101.3	-	11.0	-	60.0	-	-	-	-
232-2000*A	-	-	172.1	-	9.3	-	196.2	-	-	-	-
12-2007*A	-	-	133.4	-	11.4	-	-	-	-	-	-
113-2002*A	-	-	82.7	-	7.5	-	94.4	-	-	-	-
698-81*A	-	-	85.2	-	13.0	-	170.5	-	-	-	-
200-48	-	-	0.9	-	-	-	2.4	-	-	-	-
72-84	-	-	2.1	-	-	-	7.2	-	-	-	-
464-84	-	-	1.2	-	-	-	3.0	-	-	-	-
TS9822	-	-	145.2	5.6	11.3	-	-	-	-	-	-

[1] Peak number refers to standard peak numbers listed in Table 2.

[2]Stem tissue samples of Acc. No.'s 200-48, 464-84, and 464-84 yielded only 50 mg for extraction and HPLC-DAD analysis, as shown by comparatively reduced phenolic yields.

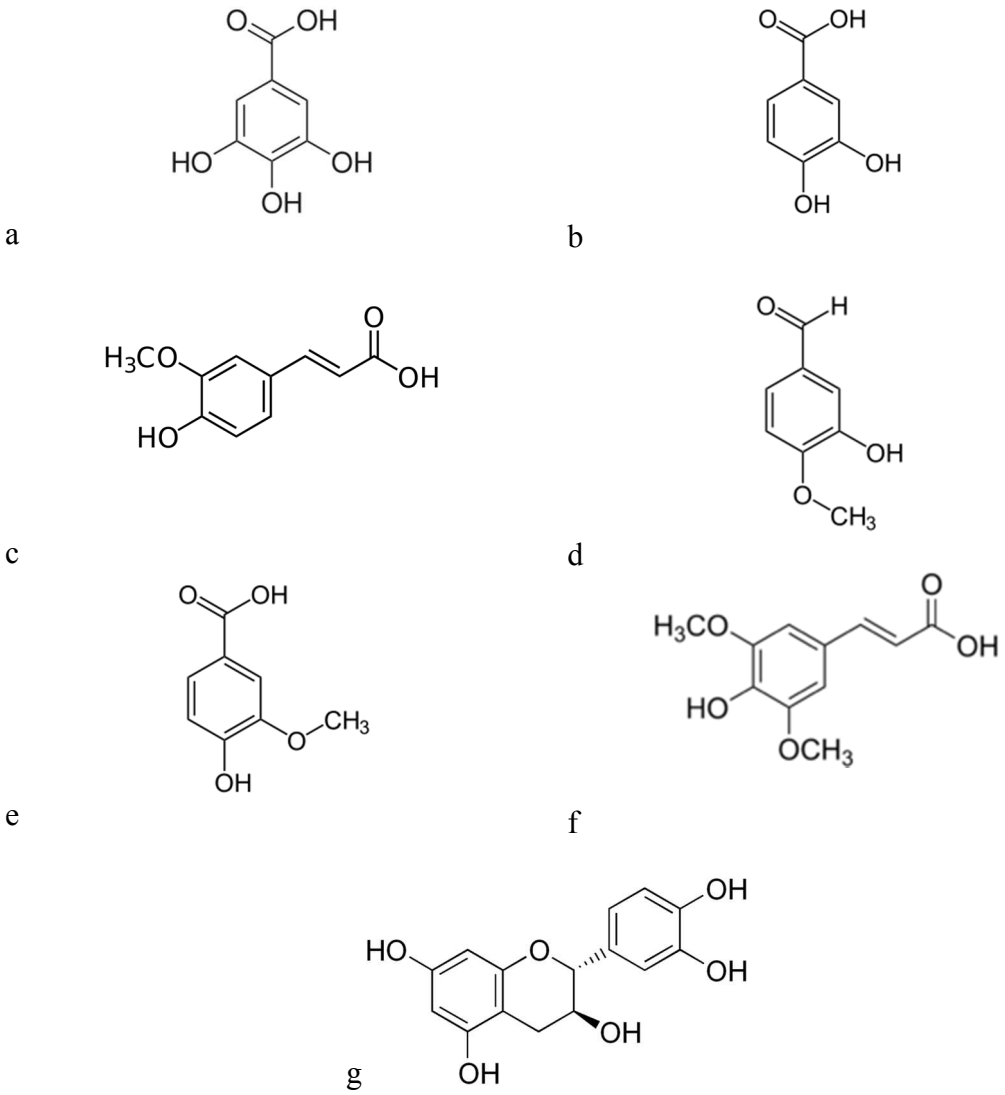

Figure 4-1. Common structures[1] of major phenolic acids found in *Cercidiphyllum* a) gallic acid, b) protocatechuic acid, c) ferulic acid, d) isovanillic acid, e) vanillic acid, f) sinapic acid, and g) catechin.

[1] eMolecules, 11025 N. Torrey Pine Rd Suite 140 La Jolla, CA 92037 USA

Natural populations of *Cercidiphyllum* are scattered and fragmented (Chien, 1992; Fu and Endress, 2001; Wei et al., 2010), which is partially a result of poor seed-set and seed germination of *C. japonicum* and *C. magnificum*, ~34 and 8%, respectively (Dosmann and Widrlechner, 2000). These populations are maintained over long periods of time by sprouting, which compensates for sparse seedling regeneration (Kubo et al., 2005; Kubo et al., 2010; Wei et al., 2010) and can be considered a factor in the production of many secondary metabolites as a defense mechanism, in lieu of primary quantitative differences, with respect to intra-population sexual reproduction. Moreover, in the case of this genus, a high degree of intra-population sexual reproduction may not have been completely necessary within this group, compared to LGM-affected species undergoing rapid, post-glacial evolution.

It may be as relatively inconsequential local environmental changes occur, major genes controlling *Cercidiphyllum* architecture and primary metabolic processes are less affected through time, whereas, plant defense mechanisms, such as secondary metabolites, persist to evolve through exposure to niche herbivory. With further research, major phenolic profiles of *Cercidiphyllum* are likely to be comparatively more diverse than respective morphology (Fig. 4-2), which can be attributable to common ancestry or convergent evolution.

Lattanzio et al. (2006) notes closely related plants often have phytoalexins more closely chemically related than plants phylogenetically more distant. Floyd, et al. (1980) found consistency between populations of *Pelea anisata* Mann and unique TLC assay fingerprints of eight species within the genus by fractioning phenolic compounds. Classification of *Cercidiphyllum* may further be separated by utilizing statistical clustering techniques, i.e., principle components analysis (PCA), to model retention data among and within particular genotypes. This type of analysis has been used to classify pharmaceutical drugs by applying

molecular modeling structural descriptors and HPLC retention data, allowing drugs to be segregated based on their pharmacological properties by PCA (Bober et al., 2011).

In general, chemotaxonomy may be useful in classifying relictual species with particular emphasis on monotypic genera. Today, the common philosophy of systematics is reliant on reproductive systems, cytogenetics, and molecular computation. However, phenolic profiling may provide additional tools for uncovering elements of speciation, population dynamics, plant defense mechanisms, and useful data for conservation that may be applied to rare monotypes, like those previously mentioned. Clustering analysis and morphology are very useful in demarcating species and genera, but quantitative mechanisms that have provided the persistence of taxa through time may serve some use to the conservationist partitioning the importance of collection sites across a species range.

Supplementary experiments which include additional phenolic standards may provide information as to whether particular phenolics are preformed or induced phytoalexins. An interesting study may include *in vitro*, *ex situ*, and greenhouse-grown plant material, collected at different seasons, to compare mechanically injured and non-injured plants before being oven-dried and macerated for HPLC analysis. Induced phenolics may also be constitutively synthesized but, additionally, their synthesis is often enhanced under biotic or abiotic stress (Lattanzio et al., 2006). This study would partition preformed and induced phenolics as well as provide the researcher with information as to whether there is a difference in magnitude of induced and preformed phenolics after mechanical injury.

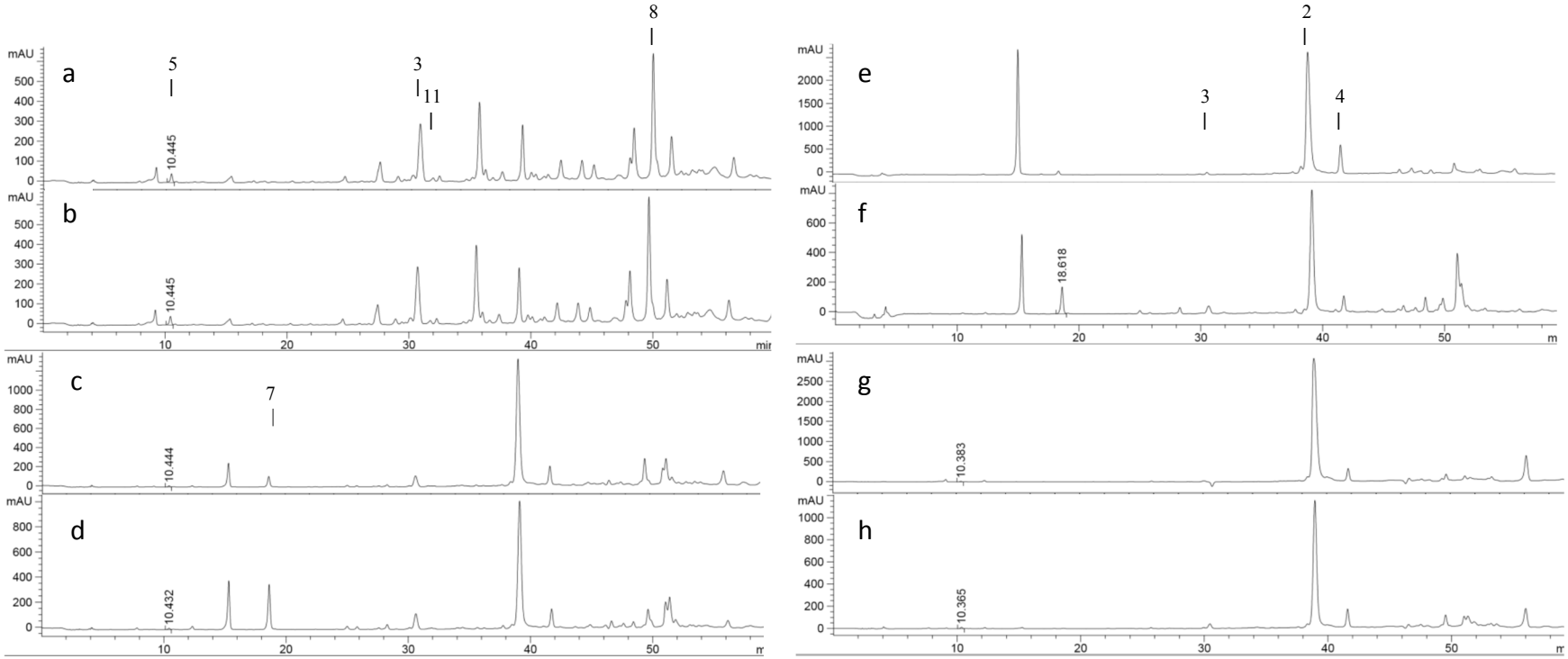

Figure 4-2. Phenolic diversity represented by peaks between and among HPLC-DAD chromatograms of the genotypes a) *C. magnificum* Acc. No. 270-2003*A leaf and b) stem tissues, c) *C. japonicum* Acc. No. 232-2000*A leaf and d) stem tissues, e) *C. magnificum* Acc. No. 291-2008*A leaf and f) stem tissues, g) and *C. japonicum* Acc. No. TS9821 leaf and h) stem tissues.

In relation to micropropagation, an experiment to determine if macronutrient bioavailability is affected by concentrations in basal media or by the phenolic interference of metal cations, parent plants from field conditions can be compared to respective *in vitro* propagated plants treated with or without phenolic-inhibiting agents (citric and ascorbic acid, etc.) using HPLC-DAD and inductively coupled plasma mass spectrometry (ICPMS). Correlating major phenolic compounds to mass macronutrient concentrations of treated and untreated *in vitro*-grown plants may provide direct answers to their inhibitory affects.

Conclusion

The major phenolic acids identified in this survey of *Cercidiphyllum* were chlorogenic, protocatechuic, and gallic acids, as well as others that differ among genotypes (Table 4-2; Figure 4-2). Concentrations of chlorogenic acid were not consistent between leaf and stem samples of accessions (Table 4-3), exhibiting 10x higher concentrations in leaf than stem tissue; this 10:1 ratio was consistent throughout all genotypes analyzed. Protocatechuic acid was similarly present at higher concentrations in leaf than in stem tissues (~2x). Gallic acid was identified in all accessions of *Cercidiphyllum*, present in 7 of the 10 accession's dried leaf samples and 7 of the 10 accession's dried stem samples. However, the presence of gallic acid and protocatechuic acid in one type of sample did not necessarily correspond with the presence of the same compound in the other (Table 4-3).

There were a number of unknown peaks present in chromatograms (Figure 4-2), among genotypes and sample types, likely representing larger tannin constituents. While all leaf samples were from actively growing stems, samples used in *C. magnificum* Acc. No. 270-2003*A were from woodier stems, bearing the "short shoot" morphology, and were taken from their single, rugose leaves. Comparing the Acc. No. 270-2003*A chromatogram to the actively growing *C.*

japonicum Acc. No. TS9821, in which a single compound, chlorogenic acid, was identified at appreciable concentrations, differences in *leaf type* may additionally play a factor in these profiles; particularly the extent in which plants store sequestered compounds.

Phenolic profiles of *Cercidiphyllum* may improve upon plant culture techniques by correlating compound type and concentration with complementary sequestering compounds. In addition, a deeper phylogenetic understanding of the correlation between the phenolic profiles of relictual species and the metabolic changes that reflect divergence between environments may well be used as 'metabolic' markers in absence of dense genomic marker coverage. It is likely that an even greater degree of phenolic variation as well as other nursery traits exist within this genus and is indicative of even greater improvements through intra- and interspecific breeding efforts.

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CHAPTER 5. ALGINATE ENCAPSILATION OF *CERCIDIPHYLLUM* (CERCIDIPHYLLACEAE)

Abstract

A nodal encapsulation method for the ornamental relict tree, *Cercidiphyllum* (Cercidiphyllaceae), was demonstrated using a bulked, half-sib *C. japonicum* Sieb & Zucc. family and a garden variety *C. magnificum* Nakai. The focus was to refine the range of sodium alginate concentrations by comparing 0%, 2.5%, 2.7%, or 3.0% (w/v) alginate over cold storage incubation (5±1ºC) in darkness for periods of 4, 8, and 12 w. Species, length of cold storage, and alginate concentration were analyzed factorially after 4 w incubation (23±1 ºC) under light to determine an appropriate encapsulation procedure for the genus. Both species responded similarly to alginate concentration and cold storage periods, significantly producing greater shoot growth at 3.0% sodium alginate and further enhanced survivability with increasing cold storage time. To our knowledge, this is the first attempt at nodal encapsulation of *Cercidiphyllum*.

Introduction

Cercidiphyllum (Cercidiphyllaceae) is considered to be a 'living fossil' of the East Asian Northern Hemisphere Tertiary period (c. 65-2.6 mya) (Wolfe, 1997; Liu, 1988) whose natural range extends from southwest China to south Japan (Brown, 1939; Liu, 1988; Krassilov, 2010; Devore et al., 2013), consisting of two dioecious tree species, common katsura (*C. japonicum* Sieb. & Zucc.) and the hiro-ha-katsura (*C. magnificum* Nakai). Common katsura was first described in Japan (Hoffman and Schultes, 1853) and is the most well-known and abundant species of the genus. Nakai (1919) published a second variety, *C. japonicum* var. *magnificum* Nakai, which was given specific status as *C. magnificum* Nakai the following year.

Phylogenetically akin to East Asian Tertiary relicts, i.e., *Metasequoia* (Merill, 1948), *Eupetela* (Wei et al., 2010), and *Ginko* (del Tredici, 2000), *Cercidiphyllum* is oftentimes considered monospecific (Dosmann, 1999); neglecting the subtle but morphologically distinct hiro-ha-katsura. Nevertheless, any existing debate as to the taxonomic status of *C. magnificum* has been resolved. Based on the results of nuclear ribosomal sequences, Li et al. (2002) found *C. japonicum* and *C. magnificum* genetically distinct, supporting the recognition of *C. magnificum* as a separate species; a distinction sustained by recent phylogeographic (Wei et al., 2010) and molecular phylogenetic analysis (Qi et al., 2012) which has subsequently brought *C. magnificum* (Nakai) to a particularly rare status (Dosmann, 1999).

The native habitat of *Cercidiphyllum* is generally thought to be riparian forests near forest margins and streams (Chien et al., 1992; Dosmann, 1999; Fu, 2001), whose species composition varies relating to physical (altitude, slope, soil temperature) and chemical (available K, NH_4, pH) properties (Gunkel, 1949a; 1949b; Wei, 2010). Seedling establishment conditions of *Cercidiphyllum* are sparse and also thought to be influenced by topography and light availability (Dosmann, 1999; Kubo, 2005, 2010). Though seedlings are rarely found in forests, katsura maintains its populations over long periods by sprouting, which compensates for sparse seedling regeneration (Titman and Wetmore, 1955; Kubo, 2005, 2010; Wei et al., 2010).

Prized as specimen trees, *Cercidiphyllum* is admired for its colorful foliage, evolving fragrance, and diverse habit (Haag, 1982; Andrews; 1998). Its leaves are simple, rounded, petiolate (Hoffman and Schultes, 1853; Swamy, 1949), and heterophyllous, whose shoots concurrently undergo long apically dominant and short axial shoot growth, depending on environmental and physiological conditions (Titman and Wetmore, 1955).

Unlike its popular cogener, accessions of the hiro-ha katsura are underrepresented in North American arboreta' living collections, reproductively immature, and present in low genetic diversity (Dosmann, correspondence). Further, the importance of *Cercidiphyllum* extends beyond its ornamental merit. These once widely distributed but now narrowly restricted eastern Asian endemic genera provide links between the modern flora of eastern Asia and the Tertiary floras of other continents (Qian et al., 2006). Population dynamics of the genus have proven important in understanding patterns of plant evolution and movement both during and after glaciations (Brown, 1939; Wolfe, 1997; Stockey, 1983; Krassilov, 2010; Qi et al., 2012) and should therefore be regarded a multifaceted genetic resource, worthy of conservation.

Conservation of germplasm can be accomplished by techniques such as seed storage, *ex situ* collections, *in vitro* cultures, and cDNA libraries. Synthetic or encapsulated artificial seed can be defined as somatic tissue encapsulated inside a coating and considered to be analogous to a zygotic seed (Redenbaugh and Ruzin, 1989). Nodal or somatic embryo alginate bead encapsulation is a low-input, effective tool to store plant germplasm as an alternative to seed storage. Encapsulation offers quick clonal propagation, economical propagation of plants that are difficult to propagate by seed, and easy genetic storage and maintenance (Patel et al., 2000). Contrary to *in vitro* cultures and *ex vitro* plantings, the alginate coating protects disease-free plants from any possible infestation that may occur.

This technique involves suspending plant material (i.e. plant cells, tissues, organs, shoot tips, somatic embryos) in a stirred sodium alginate solution and then dripping it into a calcium chloride solution for hardening (Patel et al., 2000). Encapsulated plant material can then be stored at 4-5 °C in a common refrigerator, far easier and less costly than cryopreservation. At this time, encapsulated plant material is not necessarily in stasis but has an extremely reduced

metabolism. Alginate concentration and length of cold storage can have a significant effect on auxiliary tissue viability and survival (West et al., 2006). Moreover, there is a shelf life to encapsulated synthetic seed and should be tested periodically to ensure long-term germination. This study focuses on the development of an encapsulation protocol in regards to sodium alginate concentration and length of cold storage of these cogeners. To date, there is no published information on the alginate encapsulation of *Cercidiphyllum japonicum* or *C. magnificum*.

Materials and Methods

Source of Explant Material

Plant material used in this study was Stage II nodal microshoots of *Cercidiphyllum japonicum* Seib. & Zucc., bulked as one half-sib family Acc. No. TS9821-6 consisting of 6 individuals[1] originating from the North Dakota State University research trial plantings in Fargo, ND and a single accession of *C. magnificum* Nakai Acc. No. 1998-104 of garden origin, originating from the Hoyt Arboretum in Portland, Oregon.

Aseptic Nutrient Media

Stage II culture medium for stock cultures consisted of Lloyd & McCown Woody Plant Medium (WPM) nutrients and vitamins (Lloyd,), 4.4 μM 6-benzylaminopurine[2] (BA), 0.2 μM 1-napthaleneacetic acid[3] (NAA), 7.0 g L^{-1} agar [4] with the pH adjusted[5] to 5.8 ± 0.01 with 1.0 N

[1] Bulked individuals of Acc. No.'s TS9821-6 are half-sib progeny collected from the parent Acc. No. 943, germinated in 1949 at the Lesny Zaklad Doswiadczalny W. Rogowre Arboretum, Poland, which derived from seeds obtained from an unknown *C. japonicum* accession at the Warsaw University Botanical Garden, Poland (Banaszczak, communication).

[2] No. B800, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

[3] No. 0640, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.

[4] No. A111, gel strength: 1080 g/cm^2, *Phyto*Technology Laboratories®, P.O. Box 12205 Shawnee Mission, KS 66282, USA.

[5] No. AB15, Accumet® Basic pH meter, Thermo Fisher Scientific Inc. 81 Wyman Street Waltham, MA 02454.

KOH and placed in 15 mL per 25x150 mm borosilicate glass culture tubes[1], capped with autoclavable plastic caps[2] and autoclaved for twenty minutes at 121 ºC with 1.0-kg cm^{-2} pressure. Stored cultures were incubated 30 cm below cool white florescent lamps that provided a photon flux of approximately 40 µmol $m^{-2}s^{-1}$ for a 16-hour photoperiod at 25 ºC.

Nutrient Media and Hormones

Stage II nodal microshoot segments were coated with sterile concentrations of 2.5%, 2.7%, and 3.0% (w/v) high viscosity sodium alginate[3] agar-less medium with supplemental WPM nutrients and vitamins, 2.2 µM BA, 0.1 µM NAA, 3% (w/v) sucrose, with the pH adjusted to 5.8 with 1.0 N KOH, and autoclaved for twenty minutes at 121ºC with 1.0-kg cm^{-2} pressure.

Alginate Encapsulation

The focus of this experiment was to refine the range of sodium alginate concentrations by comparing 0%, 2.5%, 2.7%, or 3.0% (w/v) alginate concentrations for encapsulation of a bulked, half-sib *Cercidiphyllum japonicum* family Acc. No. TS9822 and a garden variety *C. magnificum* Acc. No. 1998-104, over cold storage incubation periods of 4, 8, and 12 w. Species, length of cold storage, and alginate concentration were analyzed to determine an appropriate encapsulation procedure.

Stage II microshoots were excised from parent cultures and coated with 0%, 2.5%, 2.7%, or 3.0% (w/v) sodium alginate medium concentrations. All Stage II microshoots were 4±1 mm and contained 2 axillary buds. Coated microshoots were picked up using a pair of curve-tipped forceps and dropped in to a sterile 50 µM $CaCl_2$ (2 H_20) solution for 30 min for hardening. The encapsulated nodes were then transferred to 5 min sterile deionized water (ddH_20) [4] rinses,

[1] No. 9820, Pyrex®, 836 North Street Building 300 Suite 3401 Tewksbury MA 01876, USA.
[2] Kim Kap®
[3] No. A7128, Sigma-Aldrich, Co., 3050 Spruce Street St. Louis, MO 53103, USA.
[4] Milli-Q Water System, Millipore, Milford, MA, USA

placed in to sterile baby food jars (125 mL vessel), 5 encapsulated nodes per jar, and capped with autoclavable plastic caps, with the edges wrapped in Parafilm®[1] to maintain a high-humidity environment.

All alginate encapsulated Stage II microshoots were placed in 15 mL per 25x150 mm borosilicate glass culture tubes, and capped with autoclavable plastic caps consisting of WPM nutrients and vitamins, 2.2 μM BA, 0.1 μM NAA, 3% (w/v) sucrose, 7 g L^{-1} agar, with the pH adjusted to 5.8 with 1.0 N KOH, and autoclaved for twenty minutes at 121 ºC with 1.0-kg cm^{-2} pressure. Encapsulated microshoots were incubated at 4, 8, and 12 w at 5±1 ºC in darkness. After respective cold storage incubation periods, the encapsulated nodes were removed from storage, placed in Stage II multiplication medium, and incubated under the same environmental conditions as Stage II cultures for 4w.

Data Collection and Statistical Analysis

All experiments were arranged as completely random designs (CRD) and conducted twice. A minimum of 3 replicates with 5 samples per treatment were used in each run. All data were analyzed using the General Linear Model (GLM) of SAS (SAS Institute, Inc.). Shoot number, shoot length, bud number, and survival were scored across all treatments after 4 w.

Results and Discussion

Both alginate concentration and storage time significantly affected shoot growth of *C. japonicum* Acc. No. TS9821-6 (Appendix Table A-18; Table 5-1), and can be expressed as an interaction between alginate concentration and storage time on shoot length, shoot and bud numbers. Survival of the explant was based on the control, which was non-encapsulated and quickly desiccated in cold storage. When treatments are taken into account over time, higher alginate concentrations stimulate significantly more shoot numbers, bud numbers, and greater

[1] No. "M", Peachiney Plastic Packaging, Chicago, Ill.

shoot length compared to those at the lowest concentrations of alginate (Figure 5-1). The effects of 12 w cold storage periods were not significantly different from 8 w on shoot growth; however, 12 w cold storage was significantly different when compared to 4 w cold storage.

Table 5-1. Effects of alginate concentration and storage time on shoot length, shoot number, and bud number of *C. japonicum* Acc. No. TS9821-6 after removal from cold storage (5±1 ºC in darkness) and incubated 4w (23±1 ºC in light).

Weeks in 5ºC Dark Storage	Alginate Conc. (%)[1]	Shoot Length[2]	Shoot Number	Bud Number
4	2.5	3.50	1.00	4.67
	2.75	3.83	1.00	4.00
	3.0	5.33	2.00	7.50
8	2.55	3.50	1.17	4.33
	2.75	5.50	1.33	7.33
	3.0	6.50	1.67	10.00
12	2.5	4.67	1.17	6.67
	2.75	5.50	1.50	10.17
	3.0	6.50	3.33	14.17
$LSD_{0.05}$[3]		1.87	0.69	4.55

[1] Controls (0% alginate) are excluded from this table as all completely desiccated over all time periods.
[2] Treatment means in millimeters (mm).
[3] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

Similar effects of treatment and storage time on *C. magnificum* Acc. No. 1998-104 (Table 5-2) was significant to shoot growth compared to the control, which desiccated shortly after transfer. The interaction of alginate concentration and cold storage time was significant to shoot length and bud number, whereas, shoot number and bud number were positively affected by alginate concentration (Figure 5-2b; Appendix, Table A-19). Treatments under cold storage for 12 w with 3% and 2.7% alginate concentrations had significantly longer shoots and greater bud

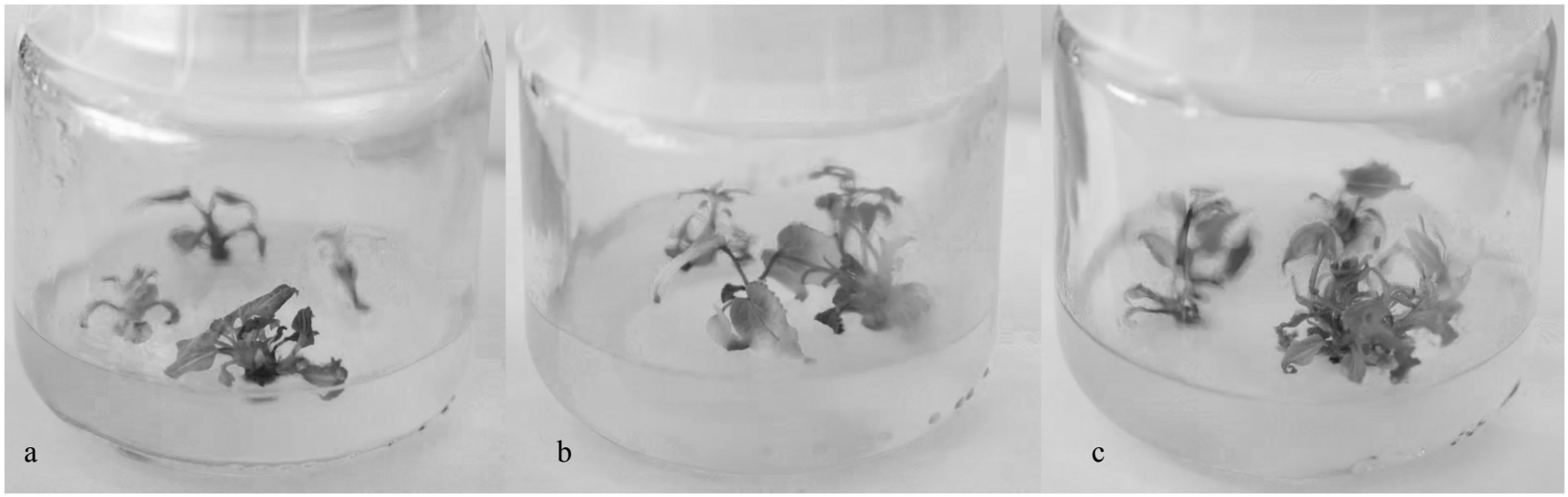

Figure 5-1. Effects of alginate concentration on shoot growth of *C. japonicum* Stage II nodal explants after 12 w cold storage (5±1 ºC in darkness) in sterile concentrations of a) 2.5, b) 2.7, and c) 3.0% (w/v) high viscosity sodium alginate agar-less medium with supplemental 3% (w/v) sucrose, WPM salts and vitamins, 2.2 µM BA, 0.1 µM NAA, after 6 w incubation (23±1 ºC in light).

numbers compared to those encapsulated with 2.5% concentration sodium alginate, different from 8 w periods on shoot growth, and like *C. japonicum*, 12 w storage promoted significantly better shoot growth when compared to 4 w cold storage.

Table 5-2. Effects of alginate concentration and storage time on shoot length, shoot number, and bud number of *C. magnificum* Acc. No. 1998-104 after removal from cold storage (5±1 ºC in darkness) and incubated 4w (23±1 ºC in light).

Weeks in 5ºC Dark Storage	Alginate Conc. (%)[1]	Shoot Length[2]	Shoot Number	Bud Number
4	2.5	4.17	1.00	3.67
	2.75	4.67	1.00	3.50
	3.0	5.33	1.67	6.00
8	2.5	4.67	1.00	5.00
	2.75	5.50	1.00	4.17
	3.0	5.33	1.50	6.50
12	2.5	4.00	1.33	4.17
	2.75	7.17	1.17	6.33
	3.0	6.83	1.50	9.67
$LSD_{0.05}$[3]		1.29	0.38	1.78

[1] Controls (0% alginate) were excluded from this table as all completely desiccated over all time periods.
[2] Treatment means in millimeters (mm).
[3] $LSD_{0.05}$, least significant difference (α=0.05) for paired comparisons; ns, non-significant, according to the *F*-test.

Shoot growth significantly increased with 3% alginate concentrations and longer storage periods of both species (Figure 5-3; Appendix Table A-19). Although explants reacted positively to encapsulated cold storage, they were slow to develop, compared to non-encapsulated nodal segments. Interestingly, the propensity for *Cercidiphyllum* explants to develop greater shoot and bud numbers after longer periods of cold storage encapsulation, which, when placed on the same medium, normal explants only form axillary shoots from preformed bud primordia, could further

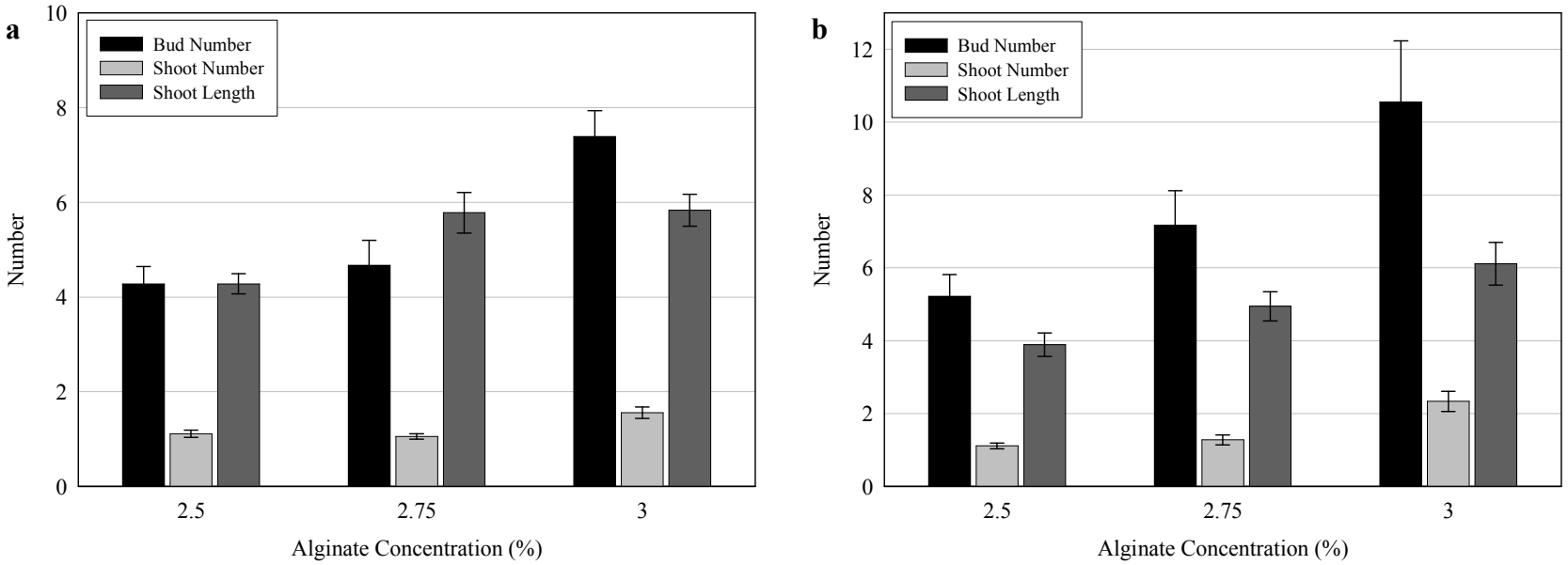

Figure 5-2. Mean comparisons (±SE) of alginate concentration (%) of a) *C. japonicum* Acc. No. TS9821-6 and b) *C. magnificum* Acc. No. 1998-104 bud number, shoot number, and shoot length (in cm) after removal from cold storage (5±1 ºC in darkness) and incubated 4w (23±1 ºC in light).

be developed into a protocol for enhanced proliferation of *Cercidiphyllum.* The positive reaction to the cold storage may not necessarily be a result of higher alginate concentrations but the humid environment or reduction of growth-inhibiting phenolic compounds from accumulating in reduced temperatures.

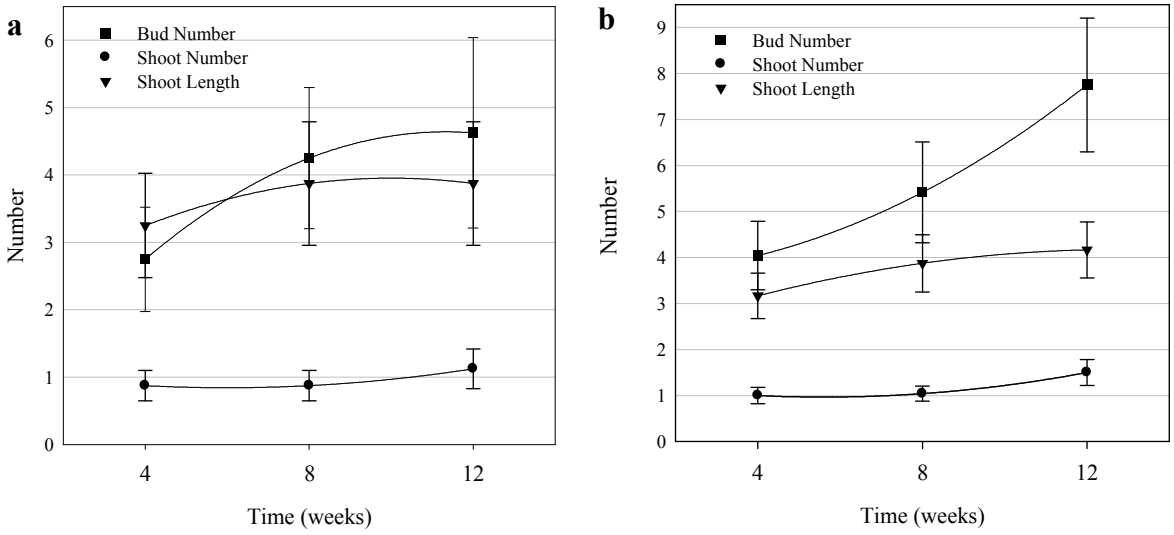

Figure 5-3. Mean comparisons (±SE) of a) *C. japonicum* Acc. No. TS9821-6 and b) *C. magnificum* Acc. No. 1998-104 over 4, 8, and 12 weeks on bud number, shoot number, and shoot length (in cm) after removal from cold storage (5±1 ºC in darkness) and incubated 4w (23±1 ºC in light).

Conclusion

It is not typically common for clonal nodal segments to improve vigor after long cold storage periods (Appendix Table A-20), for decline in vigor and survivability of encapsulated material is commonly expressed in a linear fashion, decreasing with time (West et al., 2006; Preece, 2008). Although shoot growth was improved in these storage conditions, prolonged storage greater than 12 w is likely to produce different results, as 8 and 12 w were not significantly different compared to the control. Additional experiments involving different

chemical formulations and concentrations of alginate, longer storage periods, would likely demarcate the threshold this genus has in relation to long-term cold storage preservation.

There is a growing effort to conserve the genetic diversity of forest tree species (Webb, 2010; Liu, 1988). Nodal encapsulation is a novel method that can be used an inexpensive germplasm storage technique for *Cercidiphyllum* to reduce labor requirements, somaclonal variation, and costs of serial transfer. This data suggests both *C. japonicum* and *C. magnificum* can be effectively stored for up to 12 w utilizing a 3.0% concentration of sodium alginate, which provides evidence for the similarities within the genus in relation to long-term cold storage preservation and its utility in plant production systems and conservation techniques.

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APPENDIX. ANOVA OUTPUT

Table A-1. Analysis of variance for the effects of accession, BA and GA_3 concentration, and the presence of sucrose on the mean *ex vitro* dormant bud initiation (%) of three *C. japonicum* accessions after 2 w incubation (23±1 ºC in light).

Variable: Bud Initiation

Source of Variation	df	MS	F	Pr > F
Run	1	882.7	2.07	0.1299
Rep	2	172.0	0.40	0.5262
BA	1	164.6	0.39	0.5351
GA	1	1634.7	3.84	0.0522
Sucrose (SUC)	1	961.4	2.26	0.1353
Accession (ACC)	2	3568.8	8.38	0.0004***
BA*GA	1	2738.8	6.43	0.0124*
BA*SUC	2	3691.5	8.67	0.0038**
BA*ACC	2	521.3	1.22	0.2973
GA*SUC	1	58.4	0.14	0.7116
GA*ACC	2	390.3	0.92	0.4025
BA*GA*SUC	1	345.3	0.81	0.4467
BA*GA*ACC	2	345.3	0.8	0.4507
BA*SUC*ACC	2	1329.2	3.12	0.0474*
GA*SUC*ACC	2	530.8	1.25	0.2909
Error	132	425.8		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-2. Analysis of variance for the effects of basal salt, presence of auxin, BA and TDZ concentration on shoot length, leaf length, and leaf width on the initiation and elongation of *C. japonicum* (TS9821-6) after 4 w of incubation (23±1 ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	72.29	3.84	0.0507
Rep	9	320.46	17.00	<.0001***
Salt	3	255.09	15.33	<.0001***
IBA	1	20.7	1.25	0.2642
BA	3	458.43	27.56	<.0001***
TDZ	3	18.39	1.11	0.3526
Salt*IBA	3	38.06	2.29	0.0769
Salt*BA	9	75.89	4.56	<.0001***
Salt*TDZ	9	6.02	0.36	0.6966
IBA*BA	3	20.26	1.22	0.2941
Salt*IBA*TDZ	9	73.31	4.41	0.0015**
Salt*IBA*BA	9	21.27	1.28	0.2249
Error	1216	16.64		

Table A-2. Analysis of variance for the effects of basal salt, presence of auxin, BA and TDZ concentration on shoot length, leaf length, and leaf width on the initiation and elongation of C. japonicum (TS9821-6) after 4 w of incubation (23±1 ºC in light) (continued).

Variable: Leaf Length

Source	df	MS	F	Pr > F
Run	1	31.10	1.44	0.2301
Rep	9	45.69	2.12	0.0266*
Salt	3	112.44	2.95	0.0317*
IBA	1	0.31	0.01	0.9279
BA	3	302.96	7.95	<.0001***
TDZ	3	128.10	3.36	0.0095**
Salt*IBA	3	64.91	1.70	0.1645
Salt*BA	9	74.44	1.95	0.0062**
Salt*TDZ	9	15.59	0.41	0.6643
IBA*BA	3	96.07	2.52	0.0198*
Salt*IBA*TDZ	9	216.21	5.67	0.0002***
Salt*IBA*BA	9	41.15	1.08	0.3732
Error	1216	38.10		

Table A-2. Analysis of variance for the effects of basal salt, presence of auxin, BA and TDZ concentration on shoot length, leaf length, and leaf width on the initiation and elongation of C. japonicum (TS9821-6) after 4 w of incubation (23±1 ºC in light) (continued).

Variable: Leaf Width

Source	df	MS	F	Pr > F
Run	1	84.88	5.12	0.0241*
Rep	9	39.34	2.37	0.0123*
Salt	3	29.68	1.31	0.2709
IBA	1	44.63	1.96	0.1614
BA	3	149.31	6.57	<.0001***
TDZ	3	45.30	1.99	0.0932
Salt*IBA	3	63.10	2.78	0.0401*
Salt*BA	9	45.39	2.00	0.0048**
Salt*TDZ	9	30.40	1.34	0.2628
IBA*BA	3	65.52	2.88	0.0086**
Salt*IBA*TDZ	9	124.66	5.49	0.0002***
Salt*IBA*BA	9	15.74	0.69	0.7596
Error	1216	22.72		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-3. Analysis of variance for the effects of basal salt, presence of auxin, and cytokinin concentration on shoot length, leaf length, and leaf width on the initiation and elongation of *C. japonicum* (TS9821-6) after 4 w of incubation (23±1 ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	72.29	3.84	0.0508
Rep	9	320.1	17.0	<.0001***
Salt	3	566.67	30.07	<.0001***
IBA	1	2.09	0.11	0.7395
BA	3	869.09	46.11	<.0001***
Salt*IBA	3	51.96	2.76	0.0418*
Salt*BA	9	123.25	6.54	<.0001***
IBA*BA	3	54.45	2.89	0.0350*
Salt*IBA*BA	9	42.17	2.24	0.0185*
Error	552	23.76		

Variable: Leaf Length

Source	df	MS	F	Pr > F
Run	1	31.18	1.44	0.2301
Rep	9	45.69	2.12	0.0266*
Salt	3	18.69	0.87	0.4587
IBA	1	53.78	2.49	0.1152
BA	3	28.47	1.32	0.2676
Salt*IBA	3	2.34	0.11	0.9552
Salt*BA	9	34.67	1.61	0.1105
IBA*BA	3	18.37	0.85	0.4666
Salt*IBA*BA	9	21.98	1.02	0.4242
Error	552	23.76		

Table A-3. Analysis of variance for the effects of basal salt, presence of auxin, and cytokinin concentration on shoot length, leaf length, and leaf width on the initiation and elongation of C. japonicum (TS9821-6) after 4 w of incubation (23±1 ºC in light) (continued).

Variable: Leaf Width

Source	df	MS	F	Pr > F
Run	1	80.75	4.87	0.0278*
Rep	9	39.34	2.37	0.0123*
Salt	3	4.90	0.30	0.8286
IBA	1	0.96	0.06	0.8096
BA	3	27.27	1.64	0.1782
Salt*IBA	3	28.19	1.70	0.1660
Salt*BA	9	9.73	0.59	0.8084
IBA*BA	3	21.89	1.32	0.2672
Salt*IBA*BA	9	29.34	1.77	0.0713
Error	552	16.96		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-4. Linear (L) and quadratic (Q) contrasts for the effects of basal salt (s) and cytokinin (b) concentration on shoot length, leaf length, and leaf width on the initiation and elongation of *C. japonicum* (TS9821-6) after 4 w of incubation (23±1 ºC in light).

Variable: Shoot Length

Contrast	df	MS	F	Pr > F
bL linear	1	172.73	7.27	0.0072**
bQ quadratic	1	1140.41	47.99	<.0001***
sLbL linear	1	0.04	0.00	0.9669
sQbQ quadratic	1	200.31	8.43	0.0038**
sLbQ linear & quadratic	1	265.07	11.15	0.0009***
sQbL linear & quadratic	1	19.71	0.83	0.3629

Variable: Leaf Length

Contrast	df	MS	F	Pr > F
bL linear	1	43.27	1.97	0.1612
bQ quadratic	1	12.36	0.56	0.4537
sLbL linear	1	46.16	2.10	0.1480
sQbQ quadratic	1	64.48	2.93	0.0874
sLbQ linear & quadratic	1	15.15	0.69	0.4069
sQbL linear & quadratic	1	20.71	0.94	0.3322

Variable: Leaf Width

Contrast	df	MS	F	Pr > F
bL linear	1	86.48	5.10	0.0243*
bQ quadratic	1	0.058	0.00	0.9535
sLbL linear	1	2.52	0.15	0.6998
sQbQ quadratic	1	9.48	0.56	0.4551
sLbQ linear & quadratic	1	0.71	0.04	0.8382
sQbL linear & quadratic	1	5.36	0.32	0.5744

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-5. Analysis of variance for the effects of basal salt, presence of auxin and cytokinin concentration on shoot length, shoot number, and bud number on the elongation of *C. japonicum* Acc. No. TS9821-6 4 w incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	27.00	1.57	0.2186
Rep	2	20.69	1.22	0.3085
BA	3	19.50	1.15	0.3438
IBA	1	19.50	1.15	0.3438
IBA*BA	3	18.39	1.09	0.3695
Error	30	16.91		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	1.33	2.51	0.1239
Rep	2	0.02	0.04	0.9616
BA	3	0.69	1.31	0.2908
IBA	1	1.33	2.51	0.1239
IBA*BA	3	1.83	3.45	0.0289*
Error	30	0.53		

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	143.52	5.98	0.0206*
Rep	2	37.77	1.57	0.2241
BA	3	122.91	5.12	0.0056**
IBA	1	4.69	0.20	0.6618
IBA*BA	3	35.97	1.50	0.2353
Error	30	24.01		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-6. Analysis of variance for the effects of basal salt on shoot length, shoot number, and bud number on the elongation of *C. japonicum* Acc. No. TS9821-6 after 4 w incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	0.74	0.03	0.8660
Rep	2	19.49	0.77	0.4783
Salt	3	34.39	1.36	0.2888
Error	17	25.31		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.09	0.57	0.4613
Rep	2	0.03	0.18	0.8341
Salt	3	1.42	8.34	0.0013**
Error	17	0.17		

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	0.81	0.05	0.8315
Rep	2	12.27	0.71	0.5055
Salt	3	28.27	1.64	0.2182
Error	17	17.26		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-7. Analysis of variance for the effects of auxin and cytokinin concentration on shoot length, shoot number, and bud number on the initiation and elongation of *C. japonicum* (113-2002*A) after 4 w of incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	1.33	0.21	0.6493
Rep	2	6.94	0.96	0.3943
BA	3	18.75	2.95	0.0444*
IBA	1	5.33	0.84	0.3651
IBA*BA	3	30.89	4.86	0.0057**
Error	47	6.35		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.33	0.91	0.3468
Rep	2	0.64	1.70	0.1992
BA	3	1.03	2.80	0.0528
IBA	1	0.08	0.23	0.6366
IBA*BA	3	2.03	5.52	0.0030**
Error	47	14.33		

Variable: Bud Number

Source	DF	MS	F	Pr > F
Run	1	1.02	0.52	0.4749
Rep	2	2.44	1.16	0.3276
BA	3	7.91	4.03	0.0137*
IBA	1	0.19	0.10	0.7588
IBA*BA	3	5.29	2.70	0.0587
Error	47	117.31		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-8. Analysis of variance for the effects of auxin and cytokinin concentration on shoot length, shoot number, and bud number on the initiation and elongation of *C. magnificum* (291-2008*A) after 4 w of incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	10.083	1.79	0.1889
Rep	2	2.77	0.41	0.6669
BA	3	2.17	0.38	0.7649
IBA	1	6.75	1.20	0.2806
IBA*BA	3	2.47	0.44	0.7268
Error	47	5.63		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.33	0.43	0.5165
Rep	2	0.19	0.21	0.8128
BA	3	0.97	1.25	0.3048
IBA	1	2.08	2.68	0.1098
IBA*BA	3	0.53	0.68	0.5705
Error	47	0.78		

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	7.52	4.61	0.0381*
Rep	2	2.08	1.52	0.2355
BA	3	0.52	0.32	0.8114
IBA	1	2.52	1.54	0.2213
IBA*BA	3	2.24	1.37	0.2648
Error	47	1.63		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-9. Analysis of variance for the effects of auxin and cytokinin concentration on shoot length, shoot number, and bud number on the initiation and elongation of *C. magnificum* (270-2003*A*C) after 4 w of incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	11.05	0.12	0.7300
Rep	2	80.08	1.49	0.6415
BA	3	660.70	7.19	0.0004***
IBA	1	749.65	8.16	0.0060**
IBA*BA	3	330.17	3.59	0.0189*
Error	57	91.85		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.24	0.43	0.5137
Rep	2	0.39	0.62	0.5456
BA	3	1.59	2.84	0.0456*
IBA	1	0.12	0.22	0.6432
IBA*BA	3	2.89	5.15	0.0032**
Error	57	0.56		

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	0.05	0.00	0.9447
Rep	2	42.27	5.0	0.0133*
BA	3	113.14	11.37	<.0001***
IBA	1	23.86	2.40	0.1270
IBA*BA	3	24.97	2.51	0.0678
Error	57	9.94		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-10. Effects of basal salt on mean shoot length, shoot number, and bud number of *C. magnificum* (Acc. No. 270-2003*A*C) after 4 w of incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	187.7	2.07	0.1736
Rep	2	114.3	1.26	0.3155
Salt	3	367.5	4.06	0.0307*
Error	13	90.5		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	1.10	3.56	0.0816
Rep	2	0.005	0.02	0.9833
Salt	3	0.54	1.77	0.2025
Error	13	0.30		

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	0.02	0.01	0.9414
Rep	2	1.99	0.50	0.6205
Salt	3	24.8	6.17	0.0077**
Error	13	4.02		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-11. Effects of basal salt on mean shoot length, shoot number, and bud number of *C. magnificum* (Acc. No. 291-2008*A) after 4 w of incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	0.05	0.01	0.9225
Rep	2	0.03	0.01	0.9937
Salt	3	30.5	6.37	0.0069**
Error	13	4.79		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.18	0.38	0.5483
Rep	2	0.07	0.14	0.8690
Salt	3	2.19	4.72	0.0194*
Error	13	0.46		

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	2.75	1.62	0.2254
Rep	2	3.95	2.33	0.1367
Salt	3	9.88	5.81	0.0095**
Error	13	1.69		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-12. Effects of basal salt on mean shoot length, shoot number, and bud number of *C. japonicum* cv. 'Amazing Grace' (Acc. No. 113-2002*A) after 4 w incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	1.18	0.26	0.6170
Rep	2	10.82	2.40	0.1293
Salt	3	17.60	3.91	0.0342*
Error	13	4.49		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.006	0.03	0.8584
Rep	2	0.77	4.09	0.0420*
Salt	3	0.82	4.35	0.0249*
Error	13	0.18		

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	2.53	1.31	0.2729
Rep	2	0.24	0.12	0.8856
Salt	3	6.49	3.36	0.0520
Error	13	1.93		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-13. Analysis of variance for the effects of BA, GA_3, and NAA concentration on shoot length, shoot number, and bud number on the proliferation of *C. japonicum* Acc. No. TS9821-6 after 4w incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	0.08	0.02	0.8929
Rep	2	2.89	0.63	0.5402
BA	1	0.33	0.07	0.7878
NAA	1	3.00	0.66	0.4210
GA	1	30.08	6.63	0.0139*
BA*NAA	1	0.08	0.02	0.8929
BA*GA	1	5.33	1.18	0.2849
BA*NAA*GA	2	6.04	1.33	0.2757
Error	47	4.53		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.19	0.73	0.3972
Rep	2	0.33	1.32	0.2783
BA	1	0.02	0.08	0.7769
NAA	1	0.02	0.08	0.7769
GA	1	6.02	23.53	<.0001***
BA*NAA	1	0.19	0.73	0.3972
BA*GA	1	0.02	0.08	0.7769
BA*NAA*GA	2	0.52	2.04	0.1443
Error	47	0.26		

Table A-13. Analysis of variance for the effects of BA, GA_3, and NAA concentration on shoot length, shoot number, and bud number on the proliferation of *C. japonicum* Acc. No. TS9821-6 after 4w incubation (23±1ºC in light) (continued).

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	0.52	0.04	0.8387
Rep	2	29.02	2.55	0.0920
BA	1	1.02	0.08	0.7757
NAA	1	6.02	0.49	0.4901
GA	1	157.6	12.72	0.0010***
BA*NAA	1	6.02	0.49	0.4901
BA*GA	1	7.52	0.61	0.4408
BA*NAA*GA	2	33.77	2.72	0.0782
Error	47	0.14	0.05	0.8252

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-14. Analysis of variance for the effects of type of auxin treatment on root number and root length on the *in vitro* rooting of *C. japonicum* Acc. No. TS9821-6 after 4 w incubation (23±1ºC in light).

Variable: Root Length

Source	df	MS	F	Pr > F
Run	1	3.26	2.69	0.1077
Rep	2	4.65	3.18	0.0502
Treatment	9	5.26	4.33	0.0004***
Error	47	1.21		

Variable: Root Number

Source	df	MS	F	Pr > F
Run	1	2.02	2.12	0.1522
Rep	2	2.87	3.01	0.0588
Treatment	9	3.12	3.28	0.0036**
Error	47	0.95		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-15. Analysis of variance for the effects of presoaking treatment over time on shoot length, leaf length, and leaf width on the initiation and elongation of *C. japonicum* Acc. No. TS9821-6 after 4w incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	10.89	0.94	0.3353
Rep	4	107.86	9.30	<.0001***
Hrs	1	10.89	0.94	0.3353
Trt	4	84.99	7.33	<.0001***
Hrs*Trt	4	42.82	3.69	0.0081**
Error	85	11.59		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.04	0.07	0.7863
Rep	4	2.30	4.25	0.0035**
Hrs	1	0.64	1.18	0.2797
Trt	4	1.70	3.14	0.0184*
Hrs*Trt	4	0.34	0.63	0.6433
Error	85	0.54		

Variable: Bud number

Source	df	MS	F	Pr > F
Run	1	0.25	0.11	0.7374
Rep	4	9.57	4.33	0.0031**
Hrs	1	6.25	2.83	0.0962
Trt	4	9.74	4.41	0.0028**
Hrs*Trt	4	1.75	0.79	0.5335
Error	85	2.21		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-16. Analysis of variance for the effects of incorporation treatment and concentration on shoot length, leaf length, and leaf width on the initiation and elongation of *C. japonicum* Acc. No. TS9821-6 after 4w incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	7.29	0.59	0.4434
Rep	4	18.63	1.51	0.2051
ddH_2O	1	14.69	1.20	0.2774
Citric Acid	3	14.16	1.15	0.3332
Ascorbic Acid	3	5.41	0.44	0.7252
$AgNO_3$	2	78.23	6.36	0.0027**
Error	85	12.29		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.16	0.18	0.6683
Rep	4	0.82	0.94	0.4441
ddH_2O	1	1.60	1.85	0.1770
Citric Acid	3	1.14	1.31	0.2761
Ascorbic Acid	3	0.36	0.42	0.7411
$AgNO_3$	2	0.43	0.50	0.6079
Error	85	0.86		

Table A-16. Analysis of variance for the effects of incorporation treatment and concentration on shoot length, leaf length, and leaf width on the initiation and elongation of *C. japonicum* Acc. No. TS9821-6 after 4w incubation (23±1ºC in light) (continued).

Variable: Bud Number

Source	df	MS	F	Pr > F
Run	1	6.76	1.84	0.1786
Rep	4	6.08	1.65	0.1685
ddH_2O	1	25.00	6.80	0.0107*
Citric Acid	3	3.56	0.97	0.4119
Ascorbic Acid	3	0.378	0.10	0.9582
$AgNO_3$	2	12.40	3.37	0.0389*
Error	89	3.67		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-17. Analysis of variance for the effects of carbon source and concentration on shoot length, shoot number, and bud number on the initiation and elongation of *C. japonicum* TS9821-6 after 4w incubation (23±1ºC in light).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	3.14	0.15	0.9628
Rep	4	5.12	0.24	0.6234
Sucrose	3	38.96	1.85	0.1458
Glucose	3	203.71	9.67	<.0001***
Glucose*Sucrose	2	196.94	9.35	0.0002***
Error	73	21.06		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.13	0.25	0.9072
Rep	4	0.21	0.40	0.5279
Sucrose	3	0.25	0.49	0.6907
Glucose	3	6.12	11.85	<.0001***
Glucose*Sucrose	2	2.12	4.11	0.0203*
Error	73	0.52		

Variable: Bud number

Source	df	MS	F	Pr > F
Run	1	1.09	0.27	0.8991
Rep	4	0.57	0.14	0.7109
Sucrose	3	10.55	2.56	0.0617
Glucose	3	58.51	14.17	<.0001***
Glucose*Sucrose	2	10.75	2.60	0.0807
Error	73	4.13		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-18. Analysis of variance for the effects of alginate concentration (0, 2.5, 2.7, and 3.0%) and storage time on shoot length, shoot number, bud number and survival of *C. japonicum* Acc. No. TS9821-6 4w after removal from cold storage (5±1ºC in darkness).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	7.35	2.79	0.1004
Rep	2	2.51	0.95	0.3911
Alginate Conc. (AC)	3	126.5	48.03	<.0001***
Weeks in Storage (WS)	2	6.35	2.41	0.0989
AC*WS	6	1.55	0.59	0.7378
Error	57	2.63		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.35	0.99	0.3235
Rep	2	0.43	1.23	0.3000
Alginate Conc. (AC)	3	16.42	46.90	<.0001***
Weeks in Storage (WS)	2	1.85	5.28	0.0079**
AC*WS	6	1.09	3.11	0.0106*
Error	57	0.35		

Variable: Bud number

Source	df	MS	F	Pr > F
Run	1	66.13	4.26	0.0435*
Rep	2	15.09	0.97	0.3840
Alginate Conc. (AC)	3	350.64	22.60	<.0001***
Weeks in Storage (WS)	2	84.35	5.44	0.0069**
AC*WS	6	16.81	1.08	0.3832
Error	57	15.5		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-19. Analysis of variance for the effects of alginate concentration (0, 2.5, 2.7, and 3.0%) and storage time on shoot length, shoot number, bud number and survival of *C. magnificum* Acc. No. 1998-104 4w after removal from cold storage (5±1ºC in darkness).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	<0.001	<0.001	0.9999
Rep	2	1.68	1.36	0.2656
Alginate Conc. (AC)	3	135.57	110.11	<.0001***
Weeks in Storage (WS)	2	5.68	4.61	0.0149*
AC*WS	6	3.09	2.51	0.0349*
Error	57	6.79		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.013	0.12	0.7258
Rep	2	0.056	0.50	0.6111
Alginate Conc. (AC)	3	7.83	70.00	<.0001***
Weeks in Storage (WS)	2	0.097	0.87	0.4247
AC*WS	6	0.079	0.70	0.6478
Error	57	0.34		

Variable: Bud number

Source	df	MS	F	Pr > F
Run	1	3.56	1.50	0.2261
Rep	2	0.88	0.37	0.6934
Alginate Conc. (AC)	3	167.87	70.69	<.0001***
Weeks in Storage (WS)	2	18.88	7.95	0.0009***
AC*WS	6	6.91	2.91	0.0152*
Error	57	9.195		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.

Table A-20. Analysis of variance for the effects of species, alginate concentration (0, 2.5, 2.7, and 3.0%) and storage time on shoot length, shoot number, bud number and survival 4w after removal from cold storage (5±1ºC in darkness).

Variable: Shoot Length

Source	df	MS	F	Pr > F
Run	1	3.67	0.54	0.4628
Rep	2	0.64	0.09	0.9117
Alginate Conc. (AC)	1	45.32	6.69	0.0108*
Weeks in Storage (WS)	2	5.26	0.78	0.4618
Species (SP)	1	2.01	0.30	0.5871
AC*WS	2	59.86	8.84	0.0003***
AC*SP	1	1.98	0.29	0.5900
SP*WS	2	0.58	0.09	0.9183
AC*SP*WS	2	3.11	0.46	0.6329
Error	131	6.77		

Variable: Shoot Number

Source	df	MS	F	Pr > F
Run	1	0.11	0.17	0.6847
Rep	2	0.34	0.46	0.6303
Alginate Conc. (AC)	1	10.35	15.43	0.0001***
Weeks in Storage (WS)	2	0.48	0.72	0.4883
Species (SP)	1	2.25	3.35	0.0693
AC*WS	2	4.09	6.09	0.0030**
AC*SP	1	2.13	3.18	0.0770
SP*WS	2	0.28	0.42	0.6587
AC*SP*WS	2	0.57	0.84	0.4323
Error	131	0.67		

Table A-20. Analysis of variance for the effects of species, alginate concentration (0, 2.5, 2.7, and 3.0%) and storage time on shoot length, shoot number, bud number and survival 4w after removal from cold storage (5±1ºC in darkness) (continued).

Variable: Bud number

Source	df	MS	F	Pr > F
Run	1	19.51	1.03	0.3128
Rep	2	4.47	0.22	0.8000
Alginate Conc. (AC)	1	251.88	13.26	0.0004***
Weeks in Storage (WS)	2	48.78	2.57	0.0806
Species (SP)	1	98.34	5.18	0.0245*
AC*WS	2	58.30	3.07	0.0498*
AC*SP	1	15.58	0.82	0.3668
SP*WS	2	7.92	0.42	0.6599
AC*SP*WS	2	9.97	0.52	0.5931
Error	131	18.99		

Variable: Survival

Source	df	MS	F	Pr > F
Run	1	17.36	0.01	0.9196
Rep	2	121.52	0.07	0.9306
Alginate Conc. (AC)	1	11.42	0.01	0.9347
Weeks in Storage (WS)	2	22.75	0.01	0.9867
Species (SP)	1	434.03	0.26	0.6138
AC*WS	2	23419.4	13.81	<.0001***
AC*SP	1	11.42	0.01	0.9347
SP*WS	2	22.75	0.01	0.9867
AC*SP*WS	2	14.48	0.01	0.9915
Error	131	1695.67		

*, **, *** Significant at the 5, 1, and .1% levels, respectively.