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Genetic Diversity, Micro Propagation, and Cold Hardiness of Ilex glabra (L.) A. Gray

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GENETIC DIVERSITY, MICRO PROPAGATION, AND COLD HARDINESS OF *ILEX GLABRA* (L.) A. GRAY

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GENETIC DIVERSITY, MICRO PROPAGATION, AND COLD HARDINESS OF *ILEX GLABRA* (L.) A. GRAY

By Youping Sun

Thesis Advisor: Dr. Donglin Zhang

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
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Ilex glabra (L.) A.Gray (inkberry) is a native evergreen shrub with dark green foliage and compact habit. This shrub has gained popularity in the northern landscapes of the United States and more nursery growers would like to produce it. To better understand genetic relationships among inkberry cultivars and breed cold-hardy cultivars for northern nursery growers and landscape specialists, the following projects were conducted.

A group of 48 inkberry accessions and two other *Ilex* species (*Ilex crenata* Thunb. and *I. mutchagara* Makino) were studied using amplified fragment length polymorphism (AFLP) markers. A total of 229 markers between 50 and 500 base pairs (bps) were produced from eight AFLP primer combinations. Eighty-seven percent of the markers were polymorphic. The markers for each primer-pair ranged from 22 to 45 and the genetic distance ranged from 0.001 to 0.349. Within the inkberry clade, 48 accessions were classified into six groups including, a wild species group and five cultivated groups, 'Densa', f. *leucocarpa*, 'Pretty Girl', 'Shamrock' and 'Viridis'. Within each group, the

legitimacy of named cultivars and distinguished clones were discussed based on both morphological and molecular data.

Inkberry was hybridized with cold-hardy male *Ilex verticillata* (L.) A. Gray (common winterberry) and *Ilex* × *meserveae* S.Y. Hu (meserve holly) to increase the cold hardiness. Cross pollination of inkberry and its five cultivars with both male plants was carried out in a greenhouse. Inkberry 'Chamzin' and 'Densa' had higher compatibility with either common winterberry or meserve holly, while inkberry wild species and its cultivar 'Compacta' were less compatible, and 'Nigra' and 'Shamrock' were almost incompatible. The pollen germination *in situ* observed with fluorescence microscope also supported the above results. Pollen germination of common winterberry and meserve holly on the stigma of inkberry wild species and inkberry 'Chamzin', 'Compacta', 'Densa' was more than that on inkberry 'Nigra' and 'Shamrock'. A dramatic reduction in the number of pollen tubes was observed as they grew along the style and into ovary. The percentage of pollen tubes reaching the ovary of inkberry wild species and inkberry 'Chamzin', 'Compacta', 'Densa' was higher than that of inkberry 'Nigra' and 'Shamrock'. Most of their fruit set were aborted and the fully developed seeds were less than 54.2% (meserve holly) and 32.4% (common winterberry). Reproduction barriers, including the inhibition of pollen germination, pollen tube growth to the style and the ovary, and lack of fertilization, resulted in the cross incompatibility of inkberry with both cold hardy species. Further cross-pollination should consider the incompatibility of cultivar variations.

Nodal segments containing one axillary bud (1-1.5 cm) of inkberry were established on a Murashige and Skoog (MS) medium without hormones. The sprouted shoots (~1.0 cm) were cultured on a MS medium supplemented with BAP, KIN or ZT at

2.3, 4.5, 9.1, or 18.2 μM. After 38 days, ZT and BAP induced multiple shoot formation with multiplication rates of 4-6, while the multiplication rate of KIN was less than 2. Shoots cultured on ZT grew significantly taller than those on BAP and KIN. The height of the longest shoots treated with ZT was 4.6 cm, 1.6-2.2 times greater than those treated with BAP or KIN. Shoots (~2 cm) were subcultured on ¼ strength MS (¼ MS) medium containing either IBA or NAA at 2.6, 5.1, or 10.3 μM. Adventitious roots formed in vitro after 2-4 weeks. IBA at 10.3 μM produced the best rooting (100%) compared to other treatments after 38 days of culture. The average number of roots per shoot for IBA was about 15, 1.6-3.1 times as many as that of other treatments. All rooted plantlets were successfully transplanted.

Cold hardiness tests of inkberry cultivars were conducted in both field trials and laboratory tests. A total of 72% and 93% of plants survived for 2007 and 2008 planting, respectively. 'Shamrock' was the most cold-hardy cultivar; f. *leucocarpa*, 'Viridis', and 'Nigra' were the least cold-hardy cultivars; while 'Compacta', 'Densa', 'Chamzin', 'Pretty Girl', and wild species had intermediate cold hardiness. Based on controlled freezing test of inkberry cultivars, the REC_{50} value of inkberry cultivars ranged from -19 to -32 ℃ for Jan. 2007 and -18 to -38 ℃ for Jan. 2008. The cold hardiness rate from field trials was significantly correlated with the REC_{50} value from laboratory tests. Laboratory test could be used to reliably predict the cold tolerance of inkberry cultivars in the field.

DEDICATION

Dedicated to my grandparents, parents and family.

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CHAPTER ONE

INTRODUCTION

Ilex glabra (L.) A. Gray

Ilex glabra (L.) A. Gray is known as inkberry to gardeners in northern parts of America because of its glossy black fruits and as gallberry in the southern parts because of the taste of its fruits. It belongs to Aquifoliaceae (holly family), which comprises more than 500 species of evergreen and deciduous trees and shrubs distributed throughout temperature and tropical regions of the world (Galle, 1997; Hume, 1953). Like other species in this family, *Ilex glabra* is dioecious, which means rudimentary pistils in staminate flowers and sterile stamens in pistillate flowers are borne on different plants. It is a native evergreen shrub with fine textured foliage.

Ilex glabra has a rounded upright habit with multiple stems. It tends to form suckers resulting in colonies. This species can grow to a mature height of 1.8 to 2.4 m and a width of 2.4 to 3.0 m. It becomes open and leggy when it reaches maturity. The flexible light green twigs are usually pubescent at first and glabrous finally. Stems contain round lenticels with vertical slits. Leaves are alternate, simple, and leathery, shiny and dark green on top and duller and lighter on the underside. The leaf blade is obovate to oblanceolate, elliptic, or narrowly oval with round or obtuse apices and acute bases. They are 1.9 to 5.1 cm long and 0.8 to 2.0 cm wide. The margin of the leaf is entire except near the tip, where there are several teeth. The petiole is 0.4 to 0.7 cm long. Tiny, creamy white flowers form in the axils of the current season's growth in late spring. On male plants, the flowers are borne in clusters on a slender stalk (Fig. 1.1A), whereas female plants bear solitary flowers

Figure 1.1. Flowers, fruits, and habit of *Ilex glabra* cultivars. A: male flowers of 'Pretty Boy'; B: female flowers of 'Compacta'; C: black berry of 'Pretty Girl'; D: white berry of f.*leucocarpa*; E: habit of 'Densa'; F: habit of 'Shamrock'.

(Fig. 1.1 B). Each flower has 6 to 8 creamy petals. Fruits are black or white berry-like drupes, 0.8 cm in diameter, which turn black as they ripen in the early fall (Fig. 1.1C-D). These fruits persist on bushes from September through May of the following year, but are too sparse to be of ornamental value. The foliage is usually dark green and often lustrous in summer, sometimes becoming light yellow-green in late summer or in excessively sunny or windy locations, and develops a plum colored cast in the winter (Dirr, 1998; Dirr and Alexander, 1991; Hume, 1953).

Native populations of *Ilex glabra* have been recorded within the state of Georgia, New Jersey, New York, Pennsylvania, North Carolina, South Carolina, Virginia, and throughout New England and as far north as Maine and west to Louisiana and Missouri, although it is confined to coastal areas at its northern and eastern limits (Fig. 1.2) (Maine Department of Conservation, 2006; United States Department of Agriculture, 2006). A disjunct population has also been found in Nova Scotia, Canada (Dirr and Alexander, 1991; Hume, 1953). However, it is imperiled in Maine because of its extreme rarity, location disjunct from principal range, and vulnerability to extirpation or low-temperature injury. The single known population of this plant grows in Maine around the perimeter of a coastal sphagnum bog in Knox County (Zone 4a) (Fig. 1.2) (Maine Department of Conservation, 2006). Unfortunately, we could not locate the Knox County plant population. This may attributed to extremely low winter temperatures of -34 ℃ (Cappiello and Littlefield, 1994).

Ilex glabra has widely environmental adaptability. It can survive conditions ranging from full sun to moderate shade, and wet to dry, clay to sandy soils that have an acidic to neutral pH. It can withstand heavy pruning and is free of disease and insects (Dirr,

Figure 1.2. Distribution of *Ilex glabra* in US and Maine (insert). (Adapted from United States Department of Agriculture, 2006; Maine Department of Conservation, 2006).

1998; Dirr and Alexander, 1991; Hume, 1953). It is, therefore, an excellent ornamental plant for foundation, hedges, and mass plantings. Because of the importance of including native plants in the landscapes and due to the plant superior geographical adaptability and admirable landscape attributes, *Ilex glabra* is an ideal species for increased landscape use. Nurseries are experiencing increased demand for inkberry and are increasing production in response to this demand. Significant variations in growth habit, foliage color and retention, and fruit color have encouraged nurseries and plant collectors to select forms for better uniformity and consumer palatability. From a commercial standpoint, the better forms must have dark green foliage that does not discolor significantly in winter, reasonably compact habit, and longer leaf retention in the production or landscape-use phases. By

now, many noteworthy cultivars of *Ilex glabra*, including clones selected for compact habit, are listed in the literature and/or nursery trade (Dirr, 1998; Dirr and Alexander, 1991). All cultivars of inkberry are much more dense and compact than the species and less inclined to spread by suckering.

Ilex glabra Cultivars

Ilex glabra 'Alba' has ivory white fruits and glossy deep green foliage. It is probably a rename of another cultivar or belongs under *Ilex glabra* f. *leucocarpa* (Dirr, 1998).

Ilex glabra 'Bob Rappleye', which is listed in collections/gardens of the Dawes Arboretum with accession No.D1991-0083, was selected and originated from cuttings introduced from the Secrest Arboretum (Wooster, OH) (The Dawes Arboretum, 2007). No other description is available.

Ilex glabra 'Bronze' is a form that is 1.5 to 1.8 m tall and coriaceous. The bright green leaves are 2.5 to 3.8 cm long and 0.8 to 1.3 cm wide, and give a pleasing bronze color in winter. Its glossy black global fruits are produced abundantly on compact plants (Hume, 1953). This form was selected by Elizabeth C. White of Whitesbog, NJ and may not remain in cultivation (Dirr, 1998; Dirr and Alexander, 1991).

Ilex glabra 'Cape Cod' is a 2.4 to 3.0 m tall female cultivar that was observed in a large planting in Eastham, MA. It was full to the ground with lustrous dark green foliage closely spaced along the stems (Dirr, 1998).

Ilex glabra 'Chamzin', also called by its trade name 'Nordic™', is a patented clone (PP 6,962) with a compact rounded form and lustrous deep green leaves (Zampini, 1988). It has a mature height of 0.9 to 1.5 m and width of about 0.9 to 1.2 m. Its leaves are larger

than other cultivars of this species and maintain their dark-green color throughout the winter. Unfortunately, the lower leaves drop very easily. Pruning improves canopy density. It was selected from seedlings by James Zampini of Lake County Nursery (Perry, OH). He noticed one plant, which was distinct from the others, among two thousand seedlings in the field. This plant had a distinct broad, pyramidal growth habit with the best foliage color (Dirr, 1998; Dirr and Alexander, 1991).

Ilex glabra 'Compacta' is notable for its compact, oval-rounded habit and fine-textured branches. The dark green leaves are 3.0 to 3.8 cm long and 0.8 cm wide. Its lustrous, jet-black fruits persist through the winter. It grows to 1.2 to 1.8 m high (even a 3.0 m tall) and 4.6 m wide. It becomes leggy at the base and loses a portion of the lower foliage, so it must be pruned properly to retain its better shape. *Ilex glabra* 'Compacta' is a female clone selected from a group of seedlings by Princeton Nursery (Allentown, NJ). Recently, more than one clone has been considered *Ilex glabra* 'Compacta'. The Princeton clone of *Ilex glabra* 'Compacta' was selected from a block of seedlings by William Flemer II in 1937. The seedlings were produced from seeds, which were collected in the New Jersey pine barrens near Whiting, NJ. It is a compact, very hardy, broadleaf evergreen shrub. Ideally the Princeton form should be called *Ilex glabra* 'Princeton Compact'. *Ilex glabra* 'Dodd Compact' is upright form with small and densely set leaves. It becomes quite leggy at an early age (Dirr, 1998). *Ilex glabra* 'Cole's Compacta' and 'Jackson Compacta' also belongs to the group of 'Compacta', listed in Western Maine Nursery Catalogue (Western Maine Nurseries, 2007).

Ilex glabra 'Densa' has a rounded upright habit. It grows to 2.4 to 3.1 m tall. The leathery dark green leaves are 3.8 cm long and 1.3 cm wide. The basal part of the stem

suffers leaf drop so that it becomes leggy with age. Flowers give way to jet-black inkberries, which mature in early fall and persist through the winter to early spring (Dirr, 1998; Dirr and Alexander, 1991). Bert Flemer at F & F Nursery selected it from a batch of 500 seedlings, which were planted in 1938 (Freserick, 1975). It is quite popular in the market (Fig. 1.1E).

Ilex glabra 'Dilatush' is an exceedingly large cultivar with lustrous dark green leaves that was collected in the New Jersey pine barrens by Tom Dilatush, Robbinsville, NJ. The leaves are somewhat concave (Dirr, 1998). No other description is available.

Ilex glabra f. *leucocarpa* ('Leucocarpa') is a distinctly broad-rounded white-fruited form with lustrous medium to dark green foliage. The leaves are 4.5 cm long and 1.3 cm wide. Frank W. Woods in Jackson County, FL discovered it in 1955 (Dirr, 1998).

Ilex glabra f. *leucocarpa* 'Ivory Queen' is another white fruit form. It grows 1.8 to 2.4 m high and wide and relatively dense in youth but opens with time. The leaves are 5.1 cm long by 1.3 cm wide and fruit is ivory white with a black dot at the apex due to the slight stylar scar. C.R. Wolf of New Jersey Silica Sand Co., Millville, NJ discovered it as a branch sport. However, it was originally considered the same as f. *leucocarpa*. But 'Ivory Queen' has leaves that are more leathery, darker green, and more densely set (Dirr, 1998).

Ilex glabra 'Georgia Wine' was discovered by Mr. Bill Craven, Twisted Oaks Nursery, Waynesboro, GA. The parent clone ranges from 0.8 to 1 m tall and 1.8 to 2.1m wide. The canopy is also somewhat leggy and open with age. The lustrous dark green leaves are 3.8 cm long and 1.9 cm wide and develop burgundy winter foliage coloration. It produces abundant black fruit (Dirr, 1998; Dirr and Alexander, 1991).

Ilex glabra ‘Green Billow’ is a patented cultivar (PP 10,678), which was discovered as a branch sport of *Ilex glabra* ‘Nigra’ by Mark Griffith in 1995 (Griffith, 1997). It has a compact, mounded, broadly spreading habit with good lower foliage retention. The foliage is dark green in summer and burnished grayed-purple in winter. Its leaves are 1.6 to 2.0 cm long and 6 to 11 mm wide (Dirr, 1998).

Ilex glabra ‘Green Magic’ is a slow-growing type with dark green leaves and retains its lower branches. It was introduced by Willoway Nurseries, Inc., Avon, OH (Dirr, 1998). No detailed information is available for this cultivar.

Ilex glabra ‘Gold Mine’ is another patented cultivar with patent number: PP14, 233. Gabriel Cesarini, Ridgely, MD, discovered it as a sport on the variety *Ilex glabra* ‘Shamrock’. This form has a bright yellow-gold band around the leaf margins, and occasionally an entire gold leaf. It exhibits a slow to moderate growth habit (Gabriel, 2002).

Ilex glabra ‘Hawksridge’ is a reasonably compact form with lustrous dark green leaves, which originated at Hawksridge Farms, Inc., Hickory, NC (Dirr, 1998). Little information on this selection is available.

Ilex glabra ‘Nigra’ is a relatively compact selection with lustrous dark green foliage colors in summer and purple foliage in winter. The leaves are 3.0 to 3.8 cm long by 0.8 to 1.9 cm wide. Dirr considers it “the best clone because of compact growth habit, increased lower leaf retention, thick, lustrous dark green leaves, and showy black fruits” (Dirr, 1998). Its origin is still unclear.

Ilex glabra ‘Nova Scotia’ was collected in 1994 from the wild in Nova Scotia by Raymond Fielding, Pleasantville, Nova Scotia (Dirr, 1998). It is a rounded compact shrub

of 0.6 to 0.9 m. Its leaves are a lustrous dark green and smaller than the species. It is a female clone that produces the standard black inkberries.

Ilex glabra 'Peggy's Cove' is a yet-to-be-registered selection from the Arnold Arboretum of Harvard University (Jamaica Plain, MA) (,personal communication).

Ilex glabra 'Pretty Boy' is a very compact form and has dark green foliage, which is 2.5 cm long but just 0.6 cm wide. Plants in this study were ordered from Rarefind Nursery (Jackson, NJ). No information is available on its origin.

Ilex glabra 'Pretty Girl' (syn. Seedling #2) is about 0.6 m high and 0.9 m wide when mature. Its leaves are small and round and set around the twigs in rosette fashion. Plants from Rarefind Nursery (Jackson, NJ) were used for study. Unfortunately, its origin was unknown.

Ilex glabra 'Red Tip' was named for the bronze-red color of its new flush growth. It has a dense form and excellent foliage throughout the growing season. This plant originated from Roslyn Nursery (Dix Hills, Long Island, NY) in 2001 (Dirr, 1998).

Ilex glabra 'Shamrock' is a popular cultivar that was selected from a block of approximately five hundred seedlings by John Tankard in 1977 (Dirr, 1998). It is compact and the bright, glistening new green foliage overlays the previous year's mature dark green foliage. The lustrous dark green leaves are approximately 3.8 cm long and 1.3 cm wide. The plants are 1.5 m tall and 1.5 m wide when mature (Fig. 1.1F).

Ilex glabra 'Squat' is a compact shrub that is approximately 0.9 to1.2 m tall with similar wide. It has small dark green leaves with decorative berries or fruit. No information is available on its origin. However, it has been listed as *Ilex glabra* 'Princeton's Compact', possibly the same as 'Compact' or 'Compacta' as listed in catalogue.

Ilex glabra ‘Steed’ (‘Stead’) is another compact form (Dirr, 1998). No other literature has been found.

Ilex glabra ‘Tin Mine’ was selected from Summer Hill Nursery, Madison, CT by Tom Dilatush (Dirr, 1998). It grows 1.2 m high and 2.4 m wide.

Ilex glabra ‘UGA’ is a relatively compact broad mound with dark green foliage. Michael A. Dirr found this cultivar in 1994 in a large planting of seedlings on the University of Georgia campus (Dirr, 1998). *Ilex glabra* ‘UGA’ is approximately 0.6 tall and 0.9 m wide.

Ilex glabra ‘UMASS’ is a female plant with smaller leaves than the other cultivars. It may grow up to 1.5 to 3.0 m tall. Leaf color changed from dark green in summer to red or brown in late summer. White flowers form in spring. It was selected from plants at the University of Massachusetts campus by Michael A. Dirr in 1991 (Dirr, 1998).

Ilex glabra ‘Viridis’ is a fast growing plant with a distinct pyramidal form and upright branches and dense foliage. This plant usually grows to 0.9 to 1.8 m high and slightly less in spread. Its leaves are 3.8 cm long and 0.8 cm wide (Dirr, 1998). No one knows from where it exactly originated.

In addition to the black and white fruited forms described above, Dr. John K. Small reported a red-fruited form of *Ilex glabra* in Florida (Hume, 1953). He gave no Latin name or further description. Unfortunately, its origin is unknown and no one knows if this selection is still in cultivation.

Most of the selections described, however, are under-utilized in the nursery trade. This is mainly because the plants lack the ability to tolerant low winter temperatures. In addition, due to lack of description and origination of many cultivars and morphological

similarities between cultivars, nomenclature and classification are unclear or even contradictory. Thus, many synonyms occur, such as *Ilex glabra* 'Princeton Compact', 'Dodd Compact', 'Cole's Compacta' and 'Jackson Compacta'; 'Steed' and 'Stead'; and so on.

Since little information is avaiable for inkberry genetic diversity, propagation and cold hardiness, it is of practical importance to: 1): investigate the genetic relationships among inkberry cultivars using amplified fragment length polymorphism (AFLP); 2) selectively breed cold hardy hybrids; 3) develop an effective in vitro propagation protocol for inkberry cultivars; 4) select cold hardy cultivars for northern nursery growers and landscape specialists.

CHAPTER TWO

GENETIC DIVERSITY AND TAXON DELINEATION OF *ILEX GLABRA* (L.) GRAY USING AFLP MARKERS

ABSTRACT

Ilex glabra (L.) A. Gray (inkberry) is a native evergreen shrub with dark green foliage and compact habit. This shrub has gained popularity in the northern landscapes of the United States and more nursery growers would like to produce it. To better understand genetic relationships among *Ilex glabra* cultivars and selectively breed superior cultivars, a group of 48 *Ilex glabra* accessions and two other *Ilex* species (*Ilex crenata* Thunb. and *I. mutchagara* Makino) were studied using AFLP markers. A total of 229 markers between 50 and 500 base pairs (bps) were produced from eight AFLP primer combinations (E-ACT/M-CAC, E-AGG/M-CTT, E-AGC/M-CAA, E-ACA/M-CAC, E-AGG/M-CAA, E-ACC/M-CAG, E-AGC/M-CAT, E-ACG/M-CAG). Among them, 87% of markers produced were polymorphic. The markers for each primer-pair ranged from 22 to 45 and the genetic distance observed ranged from 0.001 to 0.349. Within the *Ilex glabra* clade, 48 accessions were classified into six groups including, a wild species group, *Ilex glabra* and five cultivated groups, *Ilex glabra* 'Densa', *Ilex glabra* f. *leucocarpa*, *Ilex glabra* 'Pretty Girl', *Ilex glabra* 'Shamrock' and *Ilex glabra* 'Viridis'. Within each group, the legitimacy of named cultivars and distinguished clones are discussed based on both morphological and molecular data. The AFLP data generated provide useful information to researchers and growers for *Ilex glabra* cultivar identification, genetic improvement, and germplasm conservation.

INTRODUCTION

Ilex L., Aquifoliaceae (holly family) comprises more than 500 species of deciduous and evergreen trees and shrubs dispersed throughout temperate and tropical regions (Galle, 1997; Hume, 1953). *Ilex glabra* (L.) A. Gray (inkberry) is a popular ornamental evergreen shrub with glossy green foliage. It is native to the eastern region of North America, from Nova Scotia to Florida and west to Missouri, Mississippi, and Texas (Dirr, 1998). About 27 *Ilex glabra* cultivars are published in journals and nursery catalogues but their nomenclature and classification had been contradictory due to limited morphological variations, inadequate cultivar descriptions and lack of data on their origins (Dirr, 1998; Dirr and Alexander, 1991).

Traditionally, leaf and fruit characteristics have been used to divide the genus *Ilex* into evergreen black-fruited, deciduous black-fruited, evergreen red-fruited, and deciduous red-fruited groups (Dirr, 1998; Hume, 1953). Although morphological and anatomical characteristics have been reported to vary with environmental factors such as climate, light, amount of rainfall, soil, relief and altitude (Givnish, 1984), a comparative study of *Ilex paraguariensis* and the Argentinean congeneric taxa suggested that anatomical and quantitative anatomical parameters (stomata index, and palisade ratio) of these leaves could be used for *Ilex* species identification (Etile et al., 2002).

DNA fingerprinting techniques are useful, reliable and promising tools for identifying cultivars or genotypes within species (Zhang et al., 2000). Amplified fragment length polymorphism (AFLP) is often preferred over other molecular markers since the technique is rapid, highly polymorphic, highly reproducible (Jones et al., 1997) and requires no prior DNA sequence information (Vos et al., 1995). For analyzing genotypic

variability within species and cultivar identification, AFLP had been applied for many ornamental crops, such as *Cephalotaxus* (Zhang et al., 2000), *Prunus persica* (Hu et al., 2005), *Stewartia* (Nair et al., 2005). The relationships among southern South American representatives of the genus *Ilex* detected by AFLP analysis revealed that individuals in coherent clades shared the same morphology (Gottlieb et al., 2005). Golan-Goldhirsh et al. (2001) suggested that seven AFLP markers were associated with either the male *I. crenata* 'Microphylla' or female *I. crenata* 'Sky Pencil' cultivars. In this study, forty-eight *Ilex glabra* accessions, *Ilex crenata* 'Schworbels Compacta', and *Ilex mutchagara* were studied to determine the genetic relationship among cultivated *Ilex glabra* taxa.

MATERIALS AND METHODS

Plant Materials

Thirty *Ilex glabra* individuals and two other *Ilex* species [*Ilex crenata* 'Schworbels Compacta' and *Ilex mutchagara*] were collected from Arnold Arboretum of Harvard University (Jamaica Plain, MA) (AA), Griffith Propagation Nursery, Inc. (Watkinsville, GA) (GPN), Longwood Gardens (Kennett Square, PA) (LW), Worcester, MA, Rarefind Nursery (Jackson, NJ) (RN), Prides Corner Farm (Lebanon, CT) (PCF), the University of Maine campus (Orono, ME) (UMO), and Western Maine Nursery (Fryeburg, ME) (WMN). All plants were grown in the nursery at the University of Maine. Newly sprouting leaves were collected for DNA extraction. Additional leaf samples (silica gel dried leaves) were collected from the Atlanta Botanical Garden (Atlanta, GA) (ABG), the Dawes Arboretum (Newark, OH) (DA), State Botanical Garden of Georgia (Athens, GA) (SBGG) and the University of Georgia campus (Athens, GA) (UGA). Plant sources and their key characteristics are listed in Table 2.1.

Table 2.1. Accessions used in this study and their key characteristics.

Taxon	Plant name and source	Key Characteristics[z]	Suggested Name
1	♀ *Ilex glabra* (200-2005-A); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 3.5-4.5 cm,W: 1-2 cm	*Ilex glabra*
2	♀ *Ilex glabra* (652-70-Mass); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 2.5-3 cm, W: 1-1.5 cm	*Ilex glabra*
3	♀ *Ilex glabra* (929-88-A); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 2.5-4 cm, W: 1-1.5 cm	*Ilex glabra*
4	♀ *Ilex glabra*; State Botanical Garden of Georgia, Athens, GA	B,L, Broad elliptic, L: 2.5-3.5 cm, W: 1-1.5 cm	*Ilex glabra*
5	♀ *Ilex glabra*; the University of Georgia campus, Athens, GA	B,L, Narrow elliptic, L: 1.5-2.5 cm, W: 0.5-1 cm	*Ilex glabra*
6	♀ *Ilex glabra*; the University of Georgia campus, Athens, GA	B,L, Broad elliptic, L: 2.5-3.5 cm, W: 1-1.5 cm	*Ilex glabra*
7	♀ *Ilex glabra*; the University of Georgia campus, Athens, GA	B,C, Narrow elliptic, L: 1.5-2.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Pretty Girl'
8	♀ *Ilex glabra*; the University of Georgia campus, Athens, GA	B,C, Narrow elliptic, L: 1.5-2.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Pretty Girl'
9	♀ *Ilex glabra*; The Atlanta Botanical Garden, Atlanta, GA	B,C, Narrow elliptic, L: 1.5-2.5 cm, W: 0.5-1 cm	*Ilex glabra*
10	♀ *Ilex glabra* (1994-0494); Longwood Garden, Kennett Square, PA	B,C, Narrow elliptic, L: 1-2 cm, W: 0.5-1 cm	*Ilex glabra* 'Pretty Girl'
11	♀*Ilex glabra*; Worcester, MA	B,L, Broad elliptic, L: 4-5 cm, W: 1.5-2.5 cm	*Ilex glabra* 'Viridis'
12	♀ *I.g.* 'Chamzin' (1997-1435); Longwood Garden, Kennett Square, PA	B,C, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
13	♀ *I.g.* 'Compacta' (179-2005-A); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Viridis'
14	♀ *I.g.* 'Compacta'(179-2005-B); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Viridis'
15	♀ *I.g.* 'Compacta'(179-2005-C); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Viridis'
16	♀ *I.g.* 'Compacta'(745-69-D); Arnold Arboretum, Jamaica Plain, MA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1.5 cm	*Ilex glabra* 'Shamrock'
17	♀ *I.g.* 'Compacta'(745-69-G); Arnold Arboretum, Jamaica Plain, MA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1.5 cm	*Ilex glabra.* 'Shamrock'
18	♀ *I.g.* 'Compacta'(1051-70-F); Arnold Arboretum, Jamaica Plain, MA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 1-1.5 cm	*Ilex glabra* 'Shamrock'
19	♀ *I.g.* 'Compacta'; State Botanical Garden of Georgia, Athens, GA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
20	♀ *I.g.* 'Compacta'(1997-1423); Longwood Garden, Kennett Square, PA	B,C, Broad elliptic, L: 2-3.5 cm, W: 0.5-1.5 cm	*Ilex glabra* 'Densa'
21	♀ *I.g.* 'Compacta'; the University of Maine campus, Orono, ME	B,C, Broad elliptic, L: 3-4.5 cm, W: 0.5-1.5 cm	*Ilex glabra* 'Densa'
22	♀ *I.g.* 'Coles Compacta'; Western Maine Nursery, Fryeburg, ME	B,C, Broad elliptic, L: 3.5-4.5 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
23	♀ *I.g.* 'Jackson Compacta'; Western Maine Nursery, Fryeburg, ME	B,C, Broad elliptic, L: 3-4 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
24	♀ *I.g.* 'Densa'; The Atlanta Botanical Garden, Atlanta, GA	B,C, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
25	♀ *I.g.* 'Densa'(1992-0298); Longwood Garden, Kennett Square, PA	B,C, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Densa'

Table 2.1. continued

Taxon	Plant name and source	Key Characteristics	Suggested Name
26	♀ *I.g.* 'Densa'; Prides Corner Farm, Lebanon, CT	B,C, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
27	♀ *I.g.* f. *leucocarpa* (1489-82-mass); Arnold Arboretum, Jamaica Plain, MA	W,L, Broad elliptic, L: 5-7 cm, W: 1-2 cm	*Ilex glabra* f. *leucocarpa*
28	♀ *I.g.* f. l*eucocarpa* (1989-0826.001); The Dawes Arboretum, Newark, OH	W,L, Broad elliptic, L: 5-7 cm, W: 1-2 cm	*Ilex glabra* f. *leucocarpa*
29	♀ *I.g.* 'Georgia Wine'; the University of Georgia campus, Athens, GA	B,C, Broad elliptic, L: 3-4.5 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
30	♀ *I.g.* 'Gold Mine'(2005-0317-A); Longwood Garden, Kennett Square, PA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
31	♀ *I.g.* 'Green Magic'(1999-0158.001); The Dawes Arboretum, Newark, OH	B,C, Broad elliptic, L: 4-5 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
32	♀ *I.g.* 'Ivory Queen'(1992-0180.001); The Dawes Arboretum, Newark, OH	W,L, Broad elliptic, L: 3-5 cm, W: 1-1.5 cm	*Ilex glabra* f. *leucocarpa*
33	♀ *I.g.* 'Ivory Queen'(1998-1168.001); The Dawes Arboretum, Newark, OH	W,L, Broad elliptic, L: 3-5 cm, W: 1-1.5 cm	*Ilex glabra* f. *leucocarpa*
34	♀ *I.g.* 'Ivory Queen'(2000-0818-A); Longwood Garden, Kennett Square, PA	W,L, Broad elliptic, L: 3-5 cm, W: 1-1.5 cm	*Ilex glabra* f. *leucocarpa*
35	♀ *I.g.* (Longwood Trial #940494); Rarefind Nursery, Jackson, NJ	B,C, Narrow elliptic, L: 3-4 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
36	♀ *I.g.* 'Nigra' (1464-82-A); Arnold Arboretum, Jamaica Plain, MA	B,C, Broad elliptic, L: 3-4 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
37	♀ *I.g.* 'Nigra' (1464-82-B); Arnold Arboretum, Jamaica Plain, MA	B,C, Broad elliptic, L: 3-4 cm, W: 1-2 cm	*Ilex glabra.* 'Densa'
38	♀ *I.g.* 'Nigra' (1464-82-C); Arnold Arboretum, Jamaica Plain, MA	B,C, Broad elliptic, L: 3-4 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
39	♀ *I.g.* 'Nigra'; Griffith Propagation Nursery, Inc., Watkinsville, GA	B,C, Broad elliptic, L: 3-4 cm, W: 1-2 cm	*Ilex glabra* 'Densa'
40	♀ *I.g.* 'Nova Scotia'(99-0042); State Botanical Garden of Georgia, Athens, GA	B,L, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Viridis'
41	♀ *I.g.* 'Nova Scotia'; Rarefind Nursery, Jackson, NJ	B,L, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Viridis'
42	♂ *I.g.* 'Pretty Boy'; Rarefind Nursery, Jackson, NJ	B,C, Narrow elliptic, L: 2-3 cm, W: 0.5-1 cm	*Ilex glabra* 'Pretty Boy'
43	♀ *I.g.* 'Pretty Girl'; Rarefind Nursery, Jackson, NJ	B,C, Narrow elliptic, L: 2-3 cm, W: 0.5-1 cm	*Ilex glabra* 'Pretty Girl'
44	♀ *I.g.* 'Shamrock'; the Atlanta Botanical Garden, Atlanta, GA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
45	♀ *I.g.* 'Shamrock'; State Botanical Garden of Georgia, Athens, GA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
46	♀ *I.g.* 'Shamrock'(1997-0973); Longwood Garden, Kennett Square, PA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
47	♀ *I.g.* 'Shamrock'; Griffith Propagation Nursery, Inc., Watkinsville, GA	B,C, Narrow elliptic, L: 3-4.5 cm, W: 0.5-1 cm	*Ilex glabra* 'Shamrock'
48	♀ *I.g.* 'Viridis'(1488-82); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 3-5 cm, W: 1-2 cm	*Ilex glabra* 'Viridis'
49	♀ *Ilex crenata* (371-2002-D); Arnold Arboretum, Jamaica Plain, MA	B,C, Narrow elliptic, L: 1-2 cm, W: 0.5-1 cm	*Ilex crenata*
50	♀ *Ilex mutchagara* (168-97-B); Arnold Arboretum, Jamaica Plain, MA	B,L, Broad elliptic, L: 2-3 cm, W: 0.5-1.5 cm	*Ilex mutchagara*

[z] Fruit color: black (B) or white (W); habit: compact (C) or loose (L); leaf shape: broad elliptic or narrow elliptic; leaf size: length (L) or width (W).

DNA Extraction

Total genomic DNA was isolated using the DNeasy® Plant Mini Kit (QIAGEN Inc., Valencia, CA) following manufacturer's protocols. DNA concentrations were measured using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE) and DNA samples were diluted to a concentration of 80 $\mu g \cdot mL^{-1}$ and above for the AFLP work.

AFLP Procedure

Amplified fragment length polymorphism digestion, ligation, preselective amplification and selective amplification reactions were conducted according to the Perkin Elmer AFLP Plant Mapping Protocol (PE Applied Biosystems, Foster City, CA) except that 35 cycles for preselective and 40 cycles for selective amplifications. Eight primer pairs (*E*+ACT/*M*+CAC, *E*+AGG/*M*+CTT, *E*+AGC/*M*+CAA, *E*+ACA/*M*+CAC, *E*+AGG/*M*+CAA, *E*+ACC/*M*+CAG, *E*+AGC/*M*+CAT, *E*+ACG/*M*+CAG) (Golan-Goldhirsh et al., 2001; Gottlieb et al., 2005) were used for the selective PCR. The samples were run through a 48-capillary 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) equipped with 3730/3730xl Data Collection Software version 3.0 (Perkin-Elmer, Applied Biosystems, Foster City, CA).

AFLP Data Analysis

Combined data files containing size data for all DNAs for each primer combination were created using Peak Scanner™ Software v1.0 (Applied Biosystems Inc., Foster City, CA). All data files were read individually and prepared for Mesquite Software (Maddison and Maddison, 2006). This software was used to generate an unweighted pair group method using arithmetic average (UPGMA) tree based on parsimony reconstruction

methods. Bootstrap values were generated using Winboot software to show how robust the different clusters were in the tree (Yap and Nelson, 1996).

RESULTS AND DISCUSSION

A total of 229 markers between 50 and 500 base pairs (bps) were produced from the eight EcoRI/MseI AFLP primer-pair combinations. Among them, 199 (87%) markers were polymorphic. The number of markers generated by each primer-pair ranged from 22 to 45 (Table 2.2). Based on the AFLP markers generated, the genetic distance among all *Ilex* taxa

Table 2.2. AFLP markers produced from eight primer pairs.

Primer Pair	Number of Polymorphic Markers	Number of Markers
E+ACT/*M*+CAC	23	27
E+AGG/*M*+CTT	22	22
E+AGC/*M*+CAA	26	29
E+ACA/*M*+CAC	23	27
E+AGG/*M*+CAA	26	31
E+ACC/*M*+CAG	22	26
E+AGC/*M*+CAT	38	45
E+ACG/*M*+CAG	19	22
Total	199	229

was less than 0.349, while that within *Ilex glabra* accessions was less than 0.245. Identical AFLP profiles were observed in three groups of accessions including (1) two accessions of *Ilex glabra* 'Compacta' (AA745-69-D and -G), (2) three accessions of *Ilex glabra* 'Compacta' (AA179-2005-A, -B and -C) and (3) three accessions of *Ilex glabra* 'Nigra'

(AA 1464-82-A, -B and -C). Very low genetic differences were observed between two accessions of *Ilex glabra* f. *leucocarpa* (AA1489-82 and DA1989-0826), between two accessions of *Ilex glabra* 'Ivory Queen' (DA1992-0180 and DA1998-1168) and between *Ilex glabra* 'Shamrock' from the Atlanta Botanical Garden and State Botanical Garden of Georgia. Both *Ilex glabra* f. *leucocarpa* (AA1489-82 and DA1989-0826) had the highest genetic distance from *Ilex mutchagara*. This result supported the previous studies that high genetic distance was obtained among established species and low values among clones (Hu et al., 2005; Zhang et al., 2008). Genetic distance of varieties and cultivars were intermediate to values among species and clones. The robustness of the AFLP technique for determining the similarity of *Ilex glabra* cultivars was demonstrated by the observed clustering of cultivars with their maternal parents. For instance, individual plants of either *Ilex glabra* 'Compacta' (AA745-69-D and -G; AA179-2005-A, -B and -C) or 'Nigra'(AA 1464-82-A, -B and -C) clustered together; *Ilex glabra* 'Shamrock' clustered with its sport, *Ilex glabra* 'Gold Mine' and *Ilex glabra* f. *leucocarpa* also clustered with its sport, *Ilex glabra* 'Ivory Queen' (Fig. 2.1).

Two major groups were observed in the UPGMA tree. One included *Ilex crenata* 'Schworbels Compacta' and *Ilex mutchagara*, while the other consisted of all *Ilex glabra* accessions (Fig. 2.1). *Ilex crenata* 'Schworbels Compacta' (Southeastern Asia native) and *Ilex mutchagara* (South America origin) formed an outgroup to *Ilex glabra* (North America species), which suggested that they were genetically distant from other cultivars of *Ilex glabra*.

Within the *Ilex glabra* group, 48 accessions can be classified into two major groups, wild species and cultivated plants (Fig. 2.1). Seven *Ilex glabra* collected from

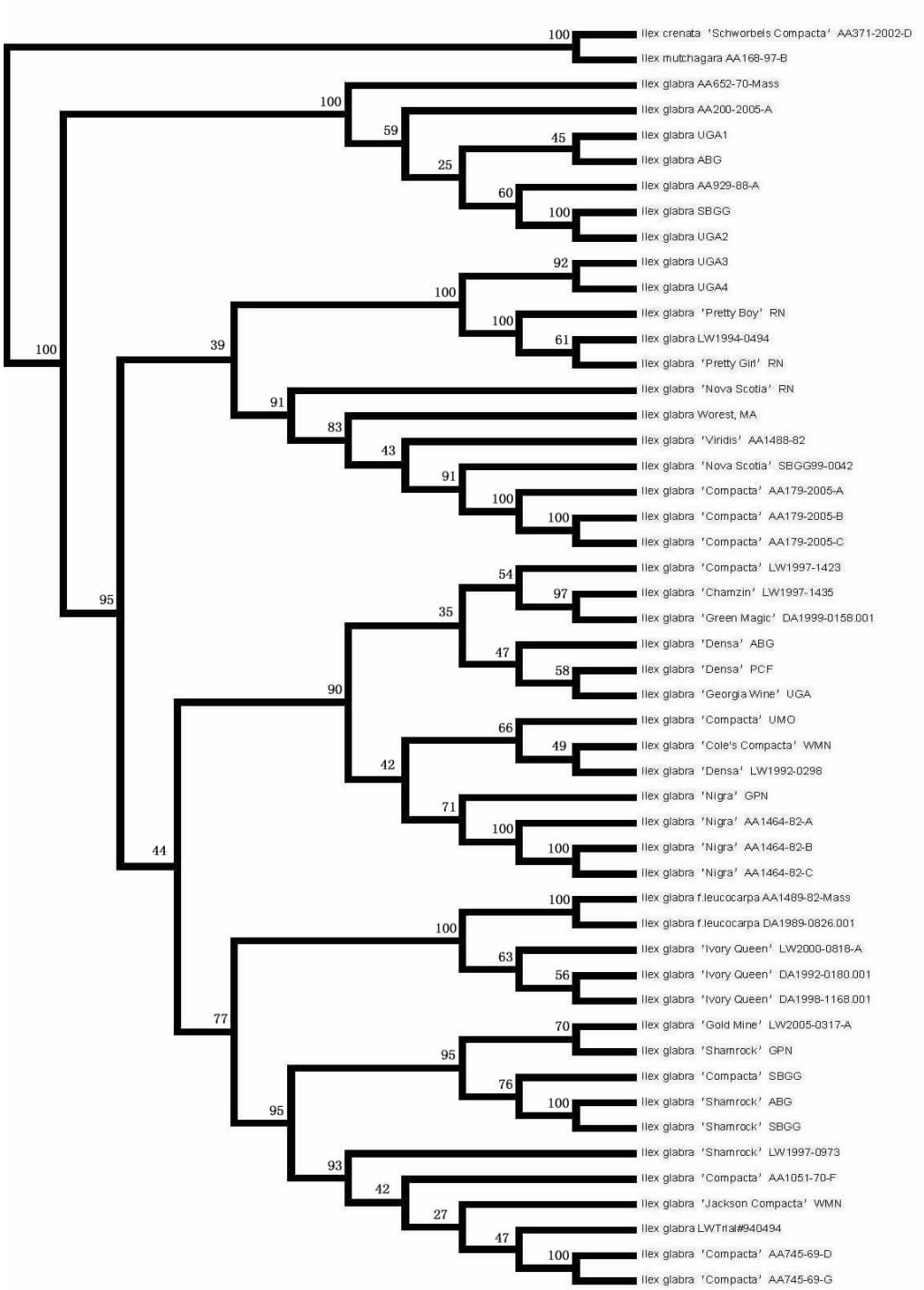

Figure 2.1. Unweighted pair group method using arithmetic average (UPGMA) tree based on AFLP markers for 50 *Ilex* accessions. Bootstrap values for clusters are indicated to the left of each node.

Georgia, Nova Scotia, Massachusetts and Rhode Island formed a wild species cluster. Three *Ilex glabra* from Georgia (SBGG, UGA1 and 2) were similar to *Ilex glabra* AA929-88 [genetic distances (GD): 0.014-0.051] than to *Ilex glabra* AA652-70 (GD 0.051-0.063) and to *Ilex glabra* AA200-2005 (GD 0.059-0.075). However, *Ilex glabra* from ABG was much closer to *Ilex glabra* AA652-70 (GD 0.084) and *Ilex glabra* AA200-2005 (GD 0.065) than to *Ilex glabra* AA929-88 (GD 0.112). *Ilex glabra* AA929-88 was originally collected in Nova Scotia, Canada, *Ilex glabra* AA652-70 from Forest Hills Nurseries, RI and *Ilex glabra* AA6200-2005 from Sylvan Nurseries, MA (The Arnold Arboretum of Harvard University, 2009). Morphologically, these accessions were loose in growth habit with black fruit and broad elliptic leaves (2.5-4 cm long, 1-1.5 cm wide) (Table 2.1).

The cultivated plants (*Ilex glabra*) could be further separated into five groups. *Ilex glabra* 'Chamzin' (LW1997-1435), 'Coles Compacta' (WMN), 'Densa' (ABG, PCF and LW1992-0298), 'Georgia Wine' (UGA), 'Green Magic' (DA1999-0158), 'Nigra' (AA1464-82-A, -B, -C and GPN) and two 'Compacta' (UMO and LW1997-1423) were clustered with a bootstrap value of 90 % (Fig. 2.1). These plants have a compact growth habit with elliptic leaves (3-4 cm long, 1-1.5 cm wide) (Table 2.1). *Ilex glabra* 'Densa' was proposed as the cultivar name for these accessions.

Ilex glabra f. *leucocarpa* (AA1489-82 and DA1989-0826) were strongly clustered with its sport *Ilex glabra* 'Ivory Queen' (DA1998-1168, 1992-0180 and LW2000-0818) (Fig. 2.1). These accessions were morphologically similar except for their leaf sizes which were 5-7 cm and 3-5 cm long for *Ilex glabra* f. *leucocarpa* and *Ilex glabra* 'Ivory Queen', respectively (Table 2.1). Leaf size is speculated to have been induced by local

environmental conditions (Givnish, 1984) because they were collected from three different arboreta. Plants in this cluster had white fruits. Since *Ilex glabra* f. *leucocarpa* was published earlier, it is a legitimate name for these plants.

Ilex glabra 'Pretty Boy' (RN), 'Pretty Girl' (RN) and *Ilex glabra* (UGA3, UGA4 and LW1994-0494) were closely placed in a cluster (Fig. 2.1). Plants in this cluster were compact form with narrow elliptic leaves (1.5-2.5 cm long, 0.5-1 cm wide) (Table 2.1). Although 15 bands distinguishing 'Pretty Boy' from 'Pretty Girl' were obtained, they were not enough to determine the gender of *Ilex glabra* cultivars in this study. Therefore, *Ilex glabra* 'Pretty Boy' and 'Pretty Girl' has to be used for the male and female plants, respectively.

Ilex glabra LW940494, *Ilex glabra* 'Compacta' (AA745-69-D and -G, AA1051-70-F, and SBGG), 'Jackson Compacta' (WMN), 'Gold Mine' (LW2005-0317) and 'Shamrock' (ABG, GPN, SBGG, and LW1997-0973) formed a cluster with a bootstrap value of 95% (Fig. 2.1). Plants in this cluster had a compact habit and narrow elliptic leaves (3-4 cm long, 1-1.5 cm wide) (Table 2.1). In this case, *Ilex glabra* 'Shamrock' should be the legitimate name because it was published first (Fig. 2.1).

Ilex glabra 'Compacta' (AA179-2005-A, -B, -C), 'Nova Scotia' (SBGG99-0042 and RN), 'Viridis' (AA1488-82), and *Ilex glabra* collected from Worcester, MA were clustered with a bootstrap value at 91% (Fig. 2.1). They grew fast and had a loose growing form and broad elliptic leaves (3-4 cm long, 1.5-2 cm wide) (Table 2.1). *Ilex glabra* 'Viridis' is the recommended name for this group (Fig. 2.1).

Limited information on origins was available for majority of the *Ilex glabra* cultivars. It is possible that these plants in the same groups were of the same genotype or

genetically similar sub-clones because they were difficult to be separated morphologically. If these cultivars were the same genotype, morphological differences that led to the selection of these cultivars could have been induced by local environmental conditions or epigenetic influences, but not by genetic mutation.

CONCLUSIONS

Based on both molecular and morphological data, forty-eight accessions could be classified into six groups which include a wild species group, *Ilex glabra*, and five cultivated groups, 'Densa', f. *leucocarpa*, 'Pretty Girl', 'Shamrock', and 'Viridis'. All cultivated *Ilex glabra* plants should belonged to the proposed five cultivars and other names should be eliminated in the trade. These AFLP data provide useful information to researchers and growers for *Ilex glabra* cultivar identification, genetic improvement, and germplasm conservation.

CHAPTER THREE

CROSS COMPATIBILITY OF INKBERRY WITH COMMON WINTERBERRY AND MESERVE HOLLY

ABSTRACT

Ilex glabra (L.) A. Gray (inkberry) is a native evergreen shrub with dark green foliage, which is of interest to nursery growers in the northern landscapes in US. Inkberry was hybridized with cold-hardy male *Ilex verticillata* (L.) A. Gray (common winterberry) and *Ilex* × *meserveae* S.Y. Hu (meserve holly) to breed cold-hardy cultivars. Cross pollination of inkberry wild species and its five cultivars ('Chamzin', 'Compacta', 'Densa', 'Nigra', and 'Shamrock') with both male plants was carried out in the greenhouses to test their compatibility. Cross compatibility of common winterberry and meserve holly with inkberry significantly varied among inkberry and its cultivars. Inkberry 'Chamzin' and 'Densa' were more compatible with either common winterberry or meserve holly, while the wild species and 'Compacta' were less compatible; 'Nigra' and 'Shamrock' were almost incompatible with them. Pollen germination observed *in situ* with a fluorescence microscope supported the above results. Pollen germination of common winterberry and meserve holly on the stigma of wild species 'Chamzin', 'Compacta', and 'Densa' was greater than that on inkberry 'Nigra' and 'Shamrock' stigma in both 2008 and 2009. Following the pollen tube growth, a dramatic reduction of developed pollen tubes was observed as they grew along the style and into the ovary. The percentage of pollen tubes reaching the ovary of the wild species and 'Chamzin', 'Compacta', or 'Densa' was much higher than that of 'Nigra' or 'Shamrock', which increased the probability of their

tubes entering into the ovule and setting fruit. Most of their embryos were aborted and less than 54.2% (meserve holly) or 32.4% (common winterberry) of seeds fully developed. Reproduction barriers, including the inhibition of pollen germination, pollen tube growth to the style and the ovary, and the lack of fertilization, resulted in the cross incompatibility of inkberry with both cold hardy species. Future cross-pollination studies should consider the incompatibility of cultivar variations.

INTRODUCTION

Ilex L. (holly) comprises at least 500 deciduous and evergreen shrubs or trees with economic importance as crops and ornamental plants (Galle, 1997; Loizwau and Spichiger, 2004). *Ilex verticillata* (L.) A. Gray (common winterberry) is a deciduous multi-stemmed shrub that grows to a height of 1.8-3.0 m. It bears bright red fruits which ripen in the fall and persist through winter (Dirr, 1998). The fruiting branches are often used considerably as a Christmas decoration. Common winterberry is a very cold-hardy plant, tolerating temperatures down to about -35 ℃ (Iowa State University Extension, 2007). Cappiello and Littlefield (1994) reported that little or no damage was found on these two species when they were planted in the Lyle E. Littlefield Ornamentals Trial Garden at University of Maine in Orono. On the other hand, *Ilex* × *meserveae* S.Y. Hu (meserve holly), a hybrid between *Ilex rugosa* F. Schmidt (prostrate holly) and *Ilex aquifolium* L. (english holly), is an evergreen tree or shrub that attains a maximum height of 1.8-3.6 m and a maximum width of 2.4-3.6 m. The leaves are glossy and bluish to dark green with prominent spiny margins. Fruits are showy red or yellow berries (Dirr, 1998). Meserve holly is a cold-hardy broadleaf evergreen shrub withstanding temperatures as low as -29 to -34 ℃ (Dirr, 1998; Rhodus, 2007). *Ilex glabra* (L.) A. Gray (inkberry) is a native evergreen shrub with glossy,

evergreen foliage and black berries. It grows to a mature height of 1.8 to 2.4 m and a width of 2.4 to 3.0 m (Dirr, 1998). It is a desirable ornamental plant for northern landscapes. However, inkberry is less cold hardy than the above two species (Cappiello and Littlefield, 1994). It is of practical importance to breed a cold hardy hybrid with shiny and high quality evergreen foliage for northern nursery growers and landscape specialists.

Interspecific hybridization and introgression could be used to tap genes of ornamental importance for horticulture improvement programs. However, reproduction barriers that make introgression difficult are common between divergent relatives and ornamental species. These barriers, such as genetic incompatibility, lack of fertilization, endosperm failure, embryo abortion and seedling lethality, play an important role in any part of the plant's reproductive cycle (Hodnett et al., 2005). Although many *Ilex* hybrids recently have been bred, patented, and marketed (US Patent and Trademark Office, 2008; USDA National Agricultural Library, 2007), the biological nature of the incompatibility system(s) that prevent hybridization and/or seed development of *Ilex* species is yet to be understood. The authors observed that fruit set of inkberry artificially cross-pollinated with meserve holly and common winterberry varied among cultivars, which suggested that some degree of incompatibility existed between them (unpublished data). Eisenbeiss (1990) reported that a wide range of compatibility between *Ilex* species resulted in full fruit set and fertile seed, to no fruit or seed at all. He also summarized the compatible *Ilex* species under garden conditions. However, there are no reports in the literature characterizing reproductive barriers between inkberry, meserve holly and common winterberry.

The objectives of this research were to: 1) investigate the cross-hybrid compatibility of inkberry cultivars with both cold hardy meserve holly and common winterberry; and 2) observe meserve holly and common winterberry pollen germination and tube growth in inkberry pistils to determine if pistil-pollen interactions are reproduction barriers to production of interspecific hybrids.

MATERIALS AND METHODS

Pollen Viability, Surface Structure and Stigma Receptivity

Pollen viability of common winterberry 'Raritia Chief', inkberry 'Pretty Boy', and meserve holly was determined *in vitro* using the protocol described by Hicks et al. (2004). Flowers were collected from the potted plants on 26 June 2007 and pollen was attached to a brush. The brush was shaken to allow the pollen fall down onto surface of a culture medium (1% Agar, 0.01% boric acid, 1mM $MgSO_4$, 2mM $CaCl_2$, 18% sucrose, and pH 6.5). Three petri dishes with the above medium were prepared for each species. All petri dishes were sealed with parafilm and cultured up-side-down in an incubator (30.4 ±0.2 ℃). After being cultured for 24 hrs, a total of 30 fields for each petri dish were observed using a dissecting microscope and the number of individual germinated and non-germinated pollen grains was recorded. Pollen germination was calculated using the following equation: (number of germinated pollen grains ÷ total number of pollen grains observed) × 100%. Their surface structures were also observed using a scanning electron microscope (SEM) (AMR 1000 A, AMRAY, USA). Pollen was mounted on a SEM stub, coated with gold in Conductavac I Sputter Coater (SeeVac Inc., Pittsburgh, PA), and examined at 5 KV. Five pollen grains for each species were observed and micrographed. Their size (length and width) and aperture were calculated according to the scale and magnification.

On 31 June 2007, stigma receptivity was evaluated in vivo by artificial cross-pollination (Dafni, 1992) under greenhouse condition. The recently opened male flowers of potted plants of common winterberry were collected and stored in clear vials in a cooler at about 4 ℃ until use. To avoid dosage interference, pollen was shaken onto clear micro-slides and then brushed onto the stigma surfaces of inkberry 'Densa' and 'Shamrock' at three subsequent stages which were 8 hrs, 16 hrs, and 24 hrs after flower opening. Four or less flowers were pollinated on each branch to eliminate the influence of nutrient competition on fruit development. The pistils were observed at three stages using a dissecting microscope. After pollination, all late opening flowers were removed using tweezers. The number of fruit that developed after pollination was recorded two months later. Percentage of fruit set was calculated using the following equation: (number of fruit ÷ number of pollinated flowers) × 100%.

Artificial Pollination

Female inkberry wild species and its cultivars 'Chamzin', 'Compacta', 'Densa', 'Nigra', and 'Shamrock' were used for pollination with male common winterberry and meserve holly in 2008 and 2009. Pollination was also conducted among wild species and cultivars of inkberry in 2009 with 'Pretty Boy' as the pollinator (control: inkberry × inkberry cultivars). All selected plants were grown in 3.3 L plastic pots with Metro-mix 560 (Scotts-sierra Horticultural Products Company, Marysville, OH) and fertilized with control released fertilizer (Peters Professional 15N-4.4P-24.9K, Scotts-sierra Horticultural Products Company, Marysville, OH). By the end of November, they were moved into cold storage at 3.9 ℃ for overwintering about 5 months. One group of plants was then moved into a glass greenhouse at University of Maine (Orono, ME) on 14 May 2008 and 8 May

2009 to induce flowering for pollination with meserve holly. Other plants stayed outside until they were moved into the greenhouse on 24 June 2008 and 22 June 2009 for pollination with common winterberry or inkberry 'Pretty Boy'. During blooming (4-14 June and 25 June-4 July 2008; 26 May-6 June and 28 June-8 July 2009), hand pollination was carried out as described above. Plants were arranged in a randomized complete block design in each of 3 glass greenhouses. Three blocks (three greenhouses) were used for hand pollination. In each block (greenhouse), each treatment had 1 to 12 plants as sub-samples based on the available plants. Plants were randomly arranged in each greenhouse. Two weeks after pollination, all pollinated plants were moved outside the greenhouses.

Pollen-Pistil Interaction

To evaluate pollen-pistil interaction for each case, ten flowers were collected 24 hr after pollination in 2008, and thirty flowers were collected 48 hr after pollination in 2009. Pistils were processed using a modified protocol described by Kho and Baer (1968). They were fixed in formaldehyde-acetic acid-alcohol (FAA, 46.3% ethyl alcohol, 5.5% formaldehyde, 3.5% methanol, 2.5% glacial acetic acid and 42.2% H_2O) (Fisher Scientific, Chicago, IL) and stored at –4 ℃ until examined. Pistils were cleared and softened in 0.8 M NaOH overnight, stained with 0.04% (w/v) aniline blue in 0.1 M KH_2PO_4 for approximately 30 min, and mounted on microscope slides in 50% 0.1 M KH_2PO_4 and 50% glycerol. The slides were kept in the dark until observation under a Leitz fluorescence microscope (K.A. Dawson Co., Belmont, MA). Fluorescence was induced using 390 to 420 nm light filtered from a mercury lamp with a 450 nm emission filter (Martin, 1959; Dumas and Knox, 1983). Images were captured with and digitally stored in an Olympus EVOLT E-330 digital camera. Pollen germination on inkberry pistils and pollen tube

growth into the style and ovary were noted. Pollen germination percentage and percentages of pollen tube growth to the style and the ovary were then calculated.

Fruit and Seed Set

On 1 Oct. 2008 and 2009, the number of developed fruits was counted. The fruit set percentage of fruit set was then calculated using the following equation: (number of fruit on plants ÷ number of flowers pollinated) × 100%. On 15 Oct. 2008, all fruits were harvested and pulped for seed collection. The number of aborted and developed seeds was recorded. Aborted seeds were shriveled and papery, while the developed seeds were plump and solid. The seed set percentage was then calculated using the following equation: (number of developed seeds ÷ total number of seeds including aborted seeds) × 100%.

Data Analysis

All data including pollen germination, percentage of pollen tube growth to style and ovary, fruit set and seed set were included in the analysis of variance (ANOVA). A two way ANOVA was performed using Statistical Analysis Systems (SAS Version 9.1, SAS Institute, Inc., Cary, NC). Student-Newman-Keuls Test at $P \leq 0.05$ was applied for means separation.

RESULTS AND DISCUSSION

Pollen Viability, Surface Structure, and Stigma Receptivity

Both common winterberry and meserve holly had larger pollen size and aperture than inkberry ‘Pretty Boy’. The length and width of their pollen grains were 25.2-32.5% and 10.5-14.9% longer than that of inkberry ‘Pretty Boy’, respectively. However, there were no difference in the the pollen size of common winterberry and meserve holly pollen (Table 3.1). The aperture length and width of common winterberry and meserve holly were

Table 3.1. Pollen germination and pollen size of *Ilex glabra* 'Pretty Boy', *Ilex* × *meserveae* and *Ilex verticillata*.

Species	Average No. of Pollen Grains Observed	Pollen Germination[z] %	Size (μm)[y]		Aperture (μm)[y]	
			Length	Width	Length	Width
Ilex glabra 'Pretty Boy'	1509	89.2 ±0.6a[x]	23.4±0.2 b	18.1±0.4 b	13.2± 1.2c	1.9±0.1 b
Ilex × *meserveae*	1285	30.1±0.8c	31.0±0.9 a	20.8±0.6 a	25.4±1.4 a	3.0±0.4 a
Ilex verticillata	1259	48.4±0.1 b	29.3± 0.8a	20.0±0.6 a	21.8±0.9 b	2.3± 0.2b

[z] Values are means ± standard error of 3 replications.
[y] Values are means ± standard error of 5 pollen grains.
[x] Different letters adjacent to means indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation.

65.2-92.4% and 21.1-57.9% longer than that of the inkberry 'Pretty Boy' (Table 3.1). Additionally, there was a significant difference between the aperture size of common winterberry and meserve holly. Pollen surface structure of three species was similar. They were all tricolporate, sunken, dumbbell shaped (Fig. 3.1). That was in accordance with the previous reports of *Ilex aquifolium* and other *Ilex* species (Halbritter et al., 2007; The University of Arizona, 2008). They have a distinctive pilate sculpturing of the heavy exine, with rod-like elements with swollen heads. They also have closely packed large clavae, which taper towards the long, kinky furrows (Fig. 3.1) (Andrew 1984, Halbritter et al., 2007; Moore et al., 1991). Pollen of common winterberry, inkberry and meserve holly

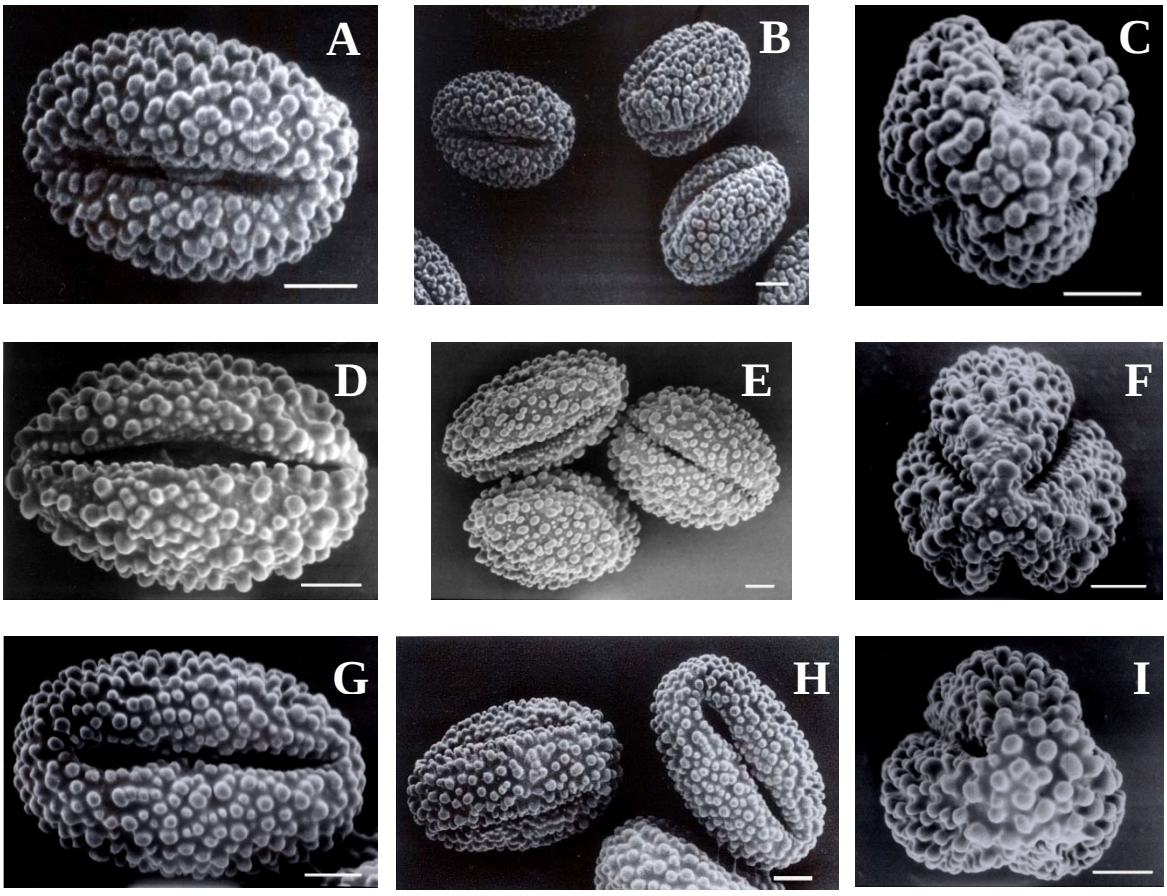

Figure 3.1. Pollen surface morphology of *Ilex glabra* 'Pretty Boy' (A-C), *Ilex* × *meserveae* (D-F), and *Ilex verticillata* (G-I). Scale = 5μm.

started to germinate after 4 hrs of incubation and the percentage of pollen germination reached the highest peak after 16 hrs of incubation (data not shown). After 24 hrs of incubation, pollen germination percentages were 89.2%, 30.1%, and 48.4% for inkberry, meserve holly and winterberry, respectively. Pollen germination of meserve holly was much lower than that of common winterberry and inkberry 'Pretty Boy'. It is not surprising since an interspecific hybrid such as meserve holly could have a high degree of pollen sterility (Eisenbeiss, 1990).

Stigma receptivity changes during maturation of the flower which may greatly influence pollination success at different stages in the flower life-cycle, the interference between female and male functions, the rate of competition via improper pollen transfer, and the chances of gametophytic selection (Dafni, 1992). Any success in breeding experiments and controlled pollination procedures should be accompanied by tests on the timing and duration of the stigma receptivity. Stigma receptivity of inkberry 'Densa' and 'Shamrock' was determined *in vivo* by controlled cross-pollination. Both 'Densa' and 'Shamrock' changed their stigma surface color from light green to yellow and finally to the brown (Fig. 3.2). Fruit set of 'Densa' significantly reduced by 27% after flowering at 16 hrs to 24 hrs ($P = 0.01$; Fig. 3.3), which suggested that stigma receptivity of 'Densa' should be 0-16 hours. However, significant reduction was observed on 'Shamrock' ($P = 0.08$) at 8 hrs to 16 hrs, which led to its stigma receptivity for only 8 hours. Since the duration of 'Densa' stigma receptivity was two times longer than that of 'Shamrock', it was understandable that 'Shamrock' had a lower percentage of fruit set. To ensure active stigma receptivity, all other cross pollinations, therefore, were done between 0-8 hrs.

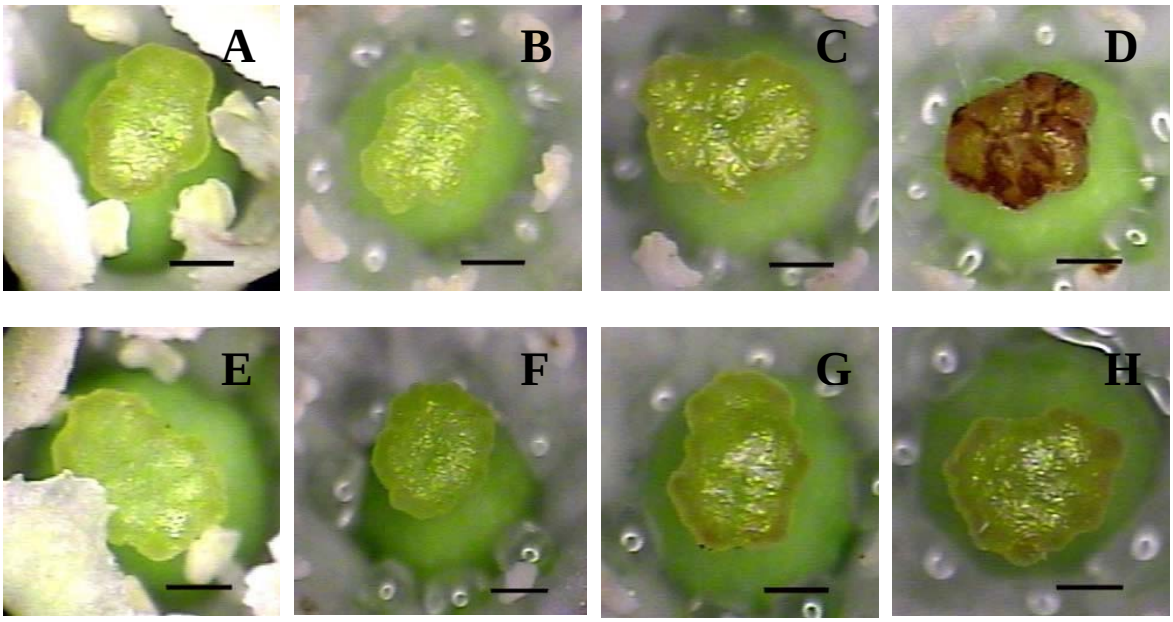

Figure 3.2. Pistil morphology of *Ilex glabra* 'Densa' (A-D) and 'Shamrock' (E-H). Stigma color of flower at unopened stage (A, E), at 8 hrs (B, F), 16 hrs (C, G), and 24 hrs (D, H) after flowers opened. Scale bar = 5 mm.

Pollen-Pistil Interaction

A significant difference of pollen germination percentage ($P < 0.0001$ for 2008 and 2009) and the percentage of pollen tube growth into the style ($P < 0.0001$ and $P = 0.0039$ for 2008 and 2009, respectively) was observed between common winterberry and meserve holly as pollinators in both years. The percentage of their pollen tube growth into the ovary was also significant between both pollinators in 2008 ($P = 0.0094$), but not in 2009 ($P = 0.67$). Pollen germination percentage ($P = 0.13$) and percentage of pollen tube growth into the style ($P = 0.86$) among inkberry cultivars were not significant in 2008; however, they differed among inkberry cultivars ($P < 0.0001$) in 2009. The percentage of pollen tubes that grew into the ovary differed among inkberry cultivars ($P < 0.0001$) in 2008, but not in 2009 ($P = 0.12$). A significant interaction between pollinators and cultivars for pollen

germination percentage ($P < 0.0001$) and percentage of its pollen tube growth into the style ($P < 0.0001$) and ovary ($P = 0.0006$) was found in 2009, but not in 2008 ($P > 0.11$).

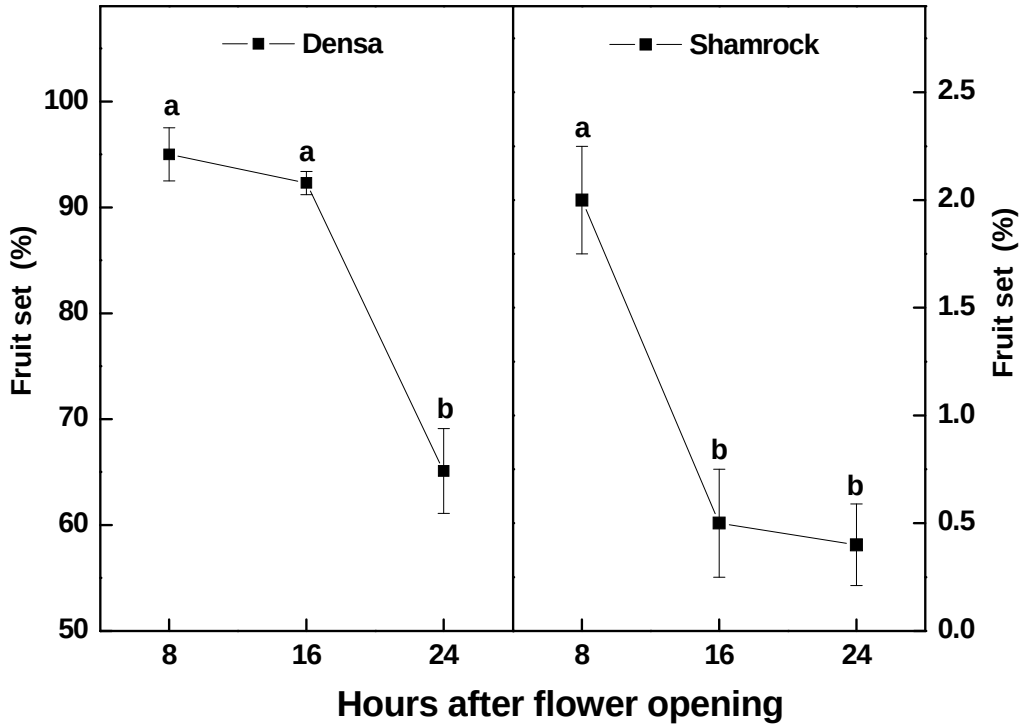

Figure 3.3. Stigma receptivity of *Ilex glabra* 'Densa' and 'Shamrock' determined in vivo by controlled cross-pollination with *Ilex verticillata*. Different letters on the top of the standard error bar indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation. Each symbol is the mean ± standard error of three replications.

Compared with 2008 pollination, pollen germination of both common winterberry and meserve holly were much lower in 2009 (Table 3.2 and 3.3). This might have resulted from higher humidity during the 2009 bloom season since higher relative humidity tends to decrease pollen viability (Dubay and Murdy, 1983; Perveen et al., 2007). In both years, pollen germination of common winterberry and the percentage of its pollen tube growth

Table 3.2. Pollen germination and tube growth in *Ilex glabra* pistils following pollination with *Ilex* × *meserveae* and *Ilex verticillata* in 2008.

Female	Male	Pollen Germination[z] (%)	% Pollen Tube Growth to the:[z] Style	Ovary
Ilex glabra		21.5 ±3.0 bcd[y]	11.3 ± 2.1 bc	1.8 ± 0.4 bc
Ilex glabra 'Compacta'		23.9 ±1.2 abcd	12.8 ± 1.5 bc	0.5 ± 0.2 c
Ilex glabra 'Densa'	*Ilex* × *meserveae*	21.3 ± 6.3 bcd	11.2 ± 4.9 bc	0.6 ± 0.4 c
Ilex glabra 'Nigra'		9.8 ± 2.7 d	5.9 ± 2.0 c	0.0 ± 0.0 c
Ilex glabra 'Shamrock'		17.7 ± 2.5 cd	12.0 ± 1.8 bc	0.0 ± 0.0 c
Ilex glabra		39.2 ± 5.5 a	21.7 ± 3.4 ab	3.3 ± 0.9 a
Ilex glabra 'Compacta'		28.2 ± 2.5 abc	17.4 ± 1.9 ab	0.9 ± 0.4 bc
Ilex glabra 'Densa'	*Ilex verticillata*	25.5 ± 2.4 abc	13.8 ± 1.8 bc	2.2 ± 0.7 ab
Ilex glabra 'Nigra'		35.5 ± 3.5 ab	23.7 ± 3.2 a	0.2 ± 0.2 c
Ilex glabra 'Shamrock'		33.1 ± 4.5 abc	20.1 ± 3.2 ab	0.0 ± 0.0 c

[z] Values are means ± standard error of 10 pistils**.**

[y] Different letters in each column indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation.

Table 3.3. Pollen germination and tube growth in *Ilex glabra* pistils following pollination with *Ilex* × *meserveae* and *Ilex verticillata* in 2009.

Female	Male	Pollen Germination[z] (%)	% Pollen Tube Growth to the:[z] Style	Ovary
Control[y]		21.8 ±1.2 a[x]	5.5 ±0.5 a	1.3 ±0.2 a
Ilex glabra		14.8 ±1.2 b	0.9±0.2 d	0.1±0.0 b
Ilex glabra ' Chamzin'		8.8 ±0.8 c	1.5±0.5 cd	0.4±0.1 ab
Ilex glabra 'Compacta'	*Ilex* × *meserveae*	15.1 ±1.5 b	3.0±0.5 bc	0.7±0.4 ab
Ilex glabra 'Densa'		18.0 ±1.0 ab	5.1±0.9 a	0.9±0.3 a
Ilex glabra 'Nigra'		6.7 ± 0.7 c	0.8±0.2 d	0.3±0.1 ab
Ilex glabra 'Shamrock'		14.7 ±0.7 b	0.8±0.2 d	0.3±0.1 ab
Ilex glabra		18.6 ±0.7 ab	2.7±0.4 cd	0.6±0.2 ab
Ilex glabra ' Chamzin'		17.9 ±1.0 ab	4.5±0.6 ab	1.0±0.2 a
Ilex glabra 'Compacta'	*Ilex verticillata*	15.8 ±1.1 b	3.0±0.4 bc	0.5±0.1 ab
Ilex glabra 'Densa'		21.1 ±1.3 a	2.9±0.5 bc	0.1±0.1 b
Ilex glabra 'Nigra'		14.6 ±0.8 b	2.4±0.3 cd	0.3±0.1ab
Ilex glabra 'Shamrock'		14.9 ±1.0 b	1.2±0.4 cd	0.3±0.2 ab

[z] Values are means ± standard error of 30 pistils.

[y] *Ilex glabra* pollinated with compatible pollen of *Ilex glabra* 'Pretty Boy' was used as control.

[x] Different letters in each column indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation.

into the style were higher than that of meserve holly regardless of the cultivars (Table 3.2 and 3.3). Pollen germination on inkberry stigma surface was 25.5-39.2% for common winterberry and 9.8-23.9% for meserve holly in 2008 (Table 3.2), and 14.6-21.1% for common winterberry and 6.7-18.0% for meserve holly in 2009 (Table 3.3). Compared with pollen of inkberry 'Pretty Boy' on inkberry stigma surfaces (control), pollen germination of common winterberry and meserve holly were 3.2-33.0% (common winterberry) and 17.4-69.3% (meserve holly) less (Table 3.3). This reduced the opportunity of their tubes growing into the ovule and then fruit and seed setting.

Following pollen tube growth, the number of pollen tubes observed in the style was dramatically reduced by 33.2-45.9% (2008) and 74.9-91.9% (2009) for common winterberry, and 32.2-47.4% (2008) and 71.7-94.6% (2009) for meserve holly (Table 3.2 and 3.3). This trend was observed in *Sorghum bicolor* pistils following pollination with other Sorghum species (Hodnett et al., 2005). At this stage, the number of pollen tubes was 18.2-78.2% (common winterberry) and 7.3-85.5% (meserve holly) less than that of inkberry 'Pretty Boy' (control). This stage further decreased the possibility of their tubes entering into ovule and thus reduced fruit setting.

In 2008, the percentage of pollen tube growing to the ovaries of inkberry wild species and its cultivars 'Compacta', 'Densa' and 'Nigra' was less than 3.3% and 1.8% when common winterberry and meserve holly were used as a pollinator, respectively (Table 3.2). However, pollen tubes were never observed in the ovaries of the inkberry 'Shamrock', which might be due to a small sample size or short period after pollination. In 2009, less than 1.0% and 0.9% of pollen tubes grew to the ovaries of inkberry when pollinated with common winterberry and meserve holly, respectively (Table 3.3).

These numbers only accounted for 76.9% and 69.2% of that when inkberry 'Pretty Boy' was used as a pollinator. The probability of fruit setting was further decreased at this stage.

Fruit Set

A significant difference in fruit set occurred between pollen sources (2008: $P = 0.005$; 2009: $P = 0.0047$), among inkberry cultivars ($P < 0.0001$ for both years) and interaction of both factors (2008: $P = 0.044$; 2009: $P < 0.0001$).

A large variation in fruit set occurred among inkberry cultivars pollinated with common winterberry in both years (Table 3.4). In 2008, fruit set for inkberry 'Densa' and 'Chamzin' was 72.3% and 65.9%, respectively, while in 2009 fruit set was about 72%. Less fruit were observed on the inkberry wild species and its cultivars 'Compacta' in both years. 'Nigra' and 'Shamrock' had 13.1% and 1.7% fruits set, respectively, in 2008, while only 2.7% and 0.2% fruits was observed in 2009. Similarly, fruit set varied to some degree among inkberry cultivars when pollinated with meserve holly (Table 3.4). Inkberry 'Densa' had the highest fruit set in both years. The fruit set of inkberry wild species, 'Chamzin', and 'Compacta' ranged from 21.2% to 47.8%, but fruit set on inkberry 'Nigra' and 'Shamrock' in 2008 was only 4.7% and 2.0%, respectively. In 2009, fruit set for inkberry wild species, 'Chamzin', 'Compacta', and 'Nigra' ranged from 12.3% to 24.1%, while fruit set for inkberry 'Shamrock' was only 5.6%.

Based on hand pollination for two years, the fruit set of inkberry differed from one cultivar to another when pollinated with common winterberry or meserve holly. This was in agreement with previous reports by Vargs et al. (2002). They found that fruit set of *Prunus dulcis* (Mill.) D.A. Webb (almond) differed considerably among cultivars. Davies and Buchanan (1979) reported the fruit set of *Vaccinium ashei* Reade (rabbiteye blueberry)

Table 3.4. Fruit set of *Ilex glabra* accessions following pollination with *Ilex* × *meserveae* and *Ilex verticillata* on 1 Oct. 2008 and 2009.

Taxa (Female)	Fruit Set (%)[z]			
	Ilex × *meserveae* (Male)		*Ilex verticillata* (Male)	
	2008	2009	2008	2009
Ilex glabra	47.8 ± 3.0a[y]	20.3 ± 5.3 ab	26.0 ± 2.5b	11.7 ± 3.5b
Ilex glabra 'Chamzin'	39.0 ± 7.8 ab	17.2 ± 6.6 ab	65.9 ± 3.2a	72.0 ± 3.8a
Ilex glabra 'Compacta'	21.2 ± 4.0bc	24.1 ± 7.8 ab	29.5 ± 1.6b	14.4 ± 2.2b
Ilex glabra 'Densa'	56.6 ± 6.7a	57.8 ± 3.3 a	72.3 ± 1.9a	72.4 ± 2.6a
Ilex glabra 'Nigra'	4.7 ± 0.8c	12.3 ± 4.4 ab	13.1 ± 1.1[x]	2.7 ± 0.7b
Ilex glabra 'Shamrock'	2.0 ± 0.6c	5.6 ± 1.0 b	1.7 ± 0.2c	0.2 ± 0.1b

[z] Values are means ± standard error of three replications.
[y] Different letters in each column indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation.
[x] Data of preliminary experiment in 2007 are presented as a reference but were excluded from the analysis

varied with its cultivars. Our results also indicated that most inkberry cultivars produced more fruits when pollinated with common winterberry than with meserve holly. This was reasonable since pollen germination of common winterberry on inkberry stigma was much higher and a higher percentage of its pollen tubes grew into inkberry ovaries. Similar results were found in *Prunus cerasus* L. (sour cherry); cultivars with higher pollen germination produced higher fruit set (Milutinovic et al., 1998). In addition, when highly-compatible intraspecific pollen was used for pollination of inkberry cultivars, there was over 85.5% fruit set of inkberry cultivars with no significant difference among cultivars ($P = 0.06$) (Fig. 3.4). This suggested no self sterility existed among cultivars. Thus, interspecific compatibility barriers among these two *Ilex* species and one

interspecific hybrid might be responsbible for the lower fruit set and large variation among cultivars.

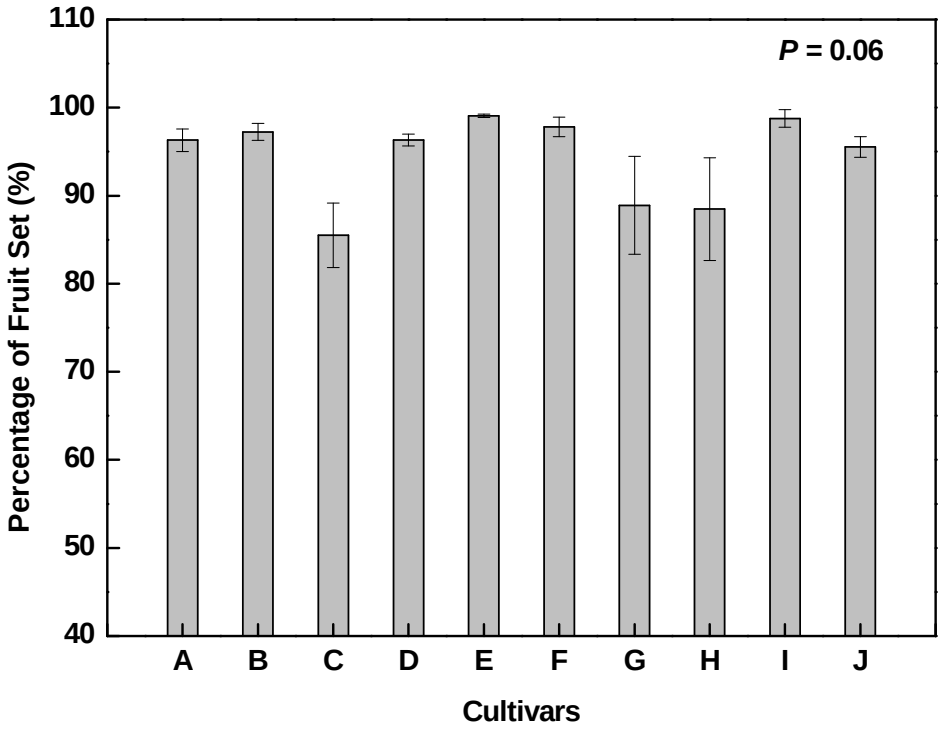

Figure 3.4. Fruit set of *Ilex glabra* accessions following pollination with *Ilex glabra* 'Pretty Boy' on 1 Oct. 2009. A) *Ilex glabra* from Worest, MA; B) *Ilex glabra* 'Chamzin'; C) *Ilex glabra* 'Compacta'; D) *Ilex glabra* 'Densa'; E) *Ilex glabra* f. *leucocarpa*; F) *Ilex glabra* 'Nigra'; G) *Ilex glabra* 'Nova Scotia'; H) *Ilex glabra* 'Pretty Girl'; I) *Ilex glabra* 'Shamrock'; J) *Ilex glabra* 'Viridis'.

Seed Set

The majority of inkberry seeds aborted, regardless of the pollen source (Table 3.5). When the pollen source was meserve holly, the highest percentage of aborted seeds, 73.3%, was observed in the fruit of inkberry 'Shamrock', while the lowest one, 45.8%, from fruits of inkberry. However, when common winterberry was the pollen source, the

Table 3.5. Seed sets of *Ilex glabra* accessions following pollination with *Ilex* × *meserveae* and *Ilex verticillata* in 2008.

Accessions (Female)	Seed Set (%)					
	Ilex × *meserveae* (Male)			*Ilex verticillata* (Male)		
	Seeds Observed	Developed Seeds (%)	Aborted Seeds (%)	Seeds Observed	Developed Seeds (%)	Aborted Seeds (%)
Ilex glabra	695	54.2	45.8	320	9.4	90.6
Ilex glabra 'Chamzin'	78	53.9	46.2	289	11.1	88.9
Ilex glabra 'Compacta'	1257	45.0	55.0	1457	7.5	92.5
Ilex glabra 'Densa'	4073	53.4	46.6	3198	13.8	86.2
Ilex glabra 'Nigra'	422	32.4	67.6	-[z]	-[z]	-[z]
Ilex glabra 'Shamrock'	30	26.7	73.3	19	25.2	74.9

[z] Data not available due to shortage of plants for cross pollination with *Ilex verticillata*

highest (92.5%) and lowest (67.6%) percentage of aborted seeds was recorded in the fruit of inkberry 'Compacta' and inkberry 'Nigra', respectively. Results also indicated that there was an average of 30% more aborted seeds with common winterberry as pollen source, which implied that common winterberry has lower probability of fertilization. Other factors, such as ovule penetration, endosperm failure and/or embryo abortion may account for lower seed set. Therefore, a histological study should be conducted to determine what results in low seed development after pollination. Seed germination would provide evidence as to whether or not fertilization is occurring and if the developing embryo aborts or the endosperm deteriorates. Both histological study and germination study are still under way.

CONCLUSIONS

In summary, cross compatibility of meserve holly or common winterberry with inkberry varied among inkberry wild species and its cultivars. The inkberry wild species, its cultivars ‘Chamzin’ and ‘Densa’ had greater compatibility with either meserve holly or common winterberry, while ‘Compacta’ and ‘Nigra’ were less compatibile and ‘Shamrock’ was almost incompatible with either meserve holly or common winterberry. Reproduction barriers, including the inhibition of pollen germination, pollen tube growth to the style and the ovary, and lack of fertilization, resulted in varying degrees of the cross incompatibility of inkberry with both cold hardy species. Further cross pollination should consider should consider the incompatibility of cultivar variations.

CHAPTER FOUR

MICROPROPAGATION OF *ILEX GLABRA* (L.) A. GRAY

ABSTRACT

Nodal segments containing one axillary bud (1-1.5 cm) were disinfected using 10% bleach and were established on a Murashige and Skoog (MS) medium without hormones at 27 ℃ and with a 16 h photoperiod. The sprouted shoots (~1.0 cm) were cultured on a MS medium supplemented with BAP, KIN or ZT at 2.3, 4.5, 9.1, or 18.2 μM. After 38 days, ZT and BAP significantly induced shoot formation with multiplication rates of 4-6, while the multiplication rate of KIN was less than 2. Shoots cultured on ZT grew much taller than those on BAP and KIN. The height of the longest shoots treated with ZT was 4.6 cm, which was 1.6-2.2 times greater than those treated with BAP or KIN. To induce rooting, shoots (~2 cm) were subcultured on ¼ strength MS (¼ MS) medium containing either IBA or NAA at 2.6, 5.1, or 10.3 μM. Adventitious roots formed in vitro after 2-4 weeks. IBA at 10.3 μM produced the best rooting (100%) compared to other treatments after 38 days of culture. The average number of roots per shoot for IBA was about 15, which was 1.6-3.1 times as many as that of other treatments. All rooted plantlets were then transplanted into a mix of peat moss and perlite (1:1 v/v) and acclimatized in a mist system. Average plantlet survival was 73.6% after 35 days. Following acclimatization, they were grown in a pot with Metro-mix under greenhouse conditions for 10 weeks where 95% of plants survived and grew up to 6.8 cm high. The protocol, i.e. nodal segments containing one axillary bud proliferated on MS with 4.5 μM ZT followed by in vitro rooting on ¼ MS plus 10.3 μM IBA, could be used for mass propagation of new inkberry cultivars.

INTRODUCTION

Ilex, a member of the Aquifoliaceae (holly family), comprises more than 500 deciduous and evergreen shrubs or trees with economic importance as crops and ornamentals in tropical and temperate regions of the world (Galle, 1997; Hu, 1989). *Ilex glabra* (L.) A. Gray (inkberry) is a native evergreen shrub with glossy green foliage and black berry-like drupes. This species grows to a mature height of 1.8 to 2.4 m and a width of 2.4 to 3.0 m (Dirr, 1998). It is a desirable ornamental plant for colder regions. Conventional seed germination and vegetative propagation are two major procedures for mass propagation. However, similar to other *Ilex* species, seed germination of inkberry is inefficient due to low germination rate, long germination time, and seedling variation. It may take 2 to 3 years to overcome the double dormancy caused by hard impermeable seed coat and immature embryos (Dirr and Heuser, 1987). In addition, vegetative propagation of inkberry by cuttings may be subject to seasonal variations.

Tissue culture is a well-known and efficient tool for mass propagation of uniform plants. In vitro culture of zygotic embryos for propagation of various *Ilex* species plants from rudimentary embryos has been reported (Hu, 1975, 1976; Hu et al., 1979; Mattis et al., 1995; Sansberro et al., 1998, 2001a). They focused on the influences of different factors on overcoming dormancy in rudimentary embryos. Hu (1979) reported that cell suspension cultures and somatic embryos were obtained from immature *Ilex aquifolium* L. (english holly) zygotic embryos; however, the conversion rate of embryos into plants was low. In general, *Ilex* species have been propagated by shoot proliferation using nodal segments, shoot tips and/or apical meristems (Bernasconi et al., 1998; Luna et al., 2003;

Majada et al., 2000; Mattis et al., 1995; Morte et al., 1991; Sansberro et al., 1999, 2000, 2001b; Zaniolo and Zanette, 2001).

As there is no reported protocol for mass propagation of inkberry using tissue culture, our evaluation of this procedure includes the study of the effects of plant growth regulators on shoot proliferation and rooting.

MATERIALS AND METHODS

Plant Material and Culture Establishment

A one-year-old plant of *Ilex glabra* 'Pretty Boy' (Rarefind Nursery, Jackson, NJ) maintained under greenhouse conditions was used as the source of explants. On 2 June 2008, young, nonlignified branches were collected, surface disinfected in 70% ethanol for 10 seconds, then immediately transferred into 10% Ultra bleach (6.0% Sodium Hypochlorite; Wal-Mart Stores, Inc. Bentonville, AR) with 7 drops of Tween 20 (ACC00528/0030, Agdia® Inc., Elkhart, IN) per 200 mL for 10 minutes, and washed with three to five rinses of sterile distilled water. The branches were dissected into 1 to 1.5 cm long segments of stem containing a single bud (Fig. 4.1A). The nodal segments were transferred to 60 mL PYREX glass tubes containing Murashige and Skoog (MS) (1962) basic medium plus 90 mM sucrose and 8 $g \cdot L^{-1}$ agar for shoot induction. The pH of the media was adjusted to 5.8 ± 0.5 with NaOH or HCl prior to adding agar (Sigma Chemical Co., St. Louis, MO). A total of 10 mL of the media was transferred via pipette into glass tubes, which were covered with caps and autoclaved at 121 °C for 30 min. All tubes with explants were loaded into tube racks and placed in a plastic bag. They were cultured in a growth room at a temperature of 27.2 ± 1.9 °C with a 16 h photoperiod (138 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) from cool white fluorescent lamps). After 2

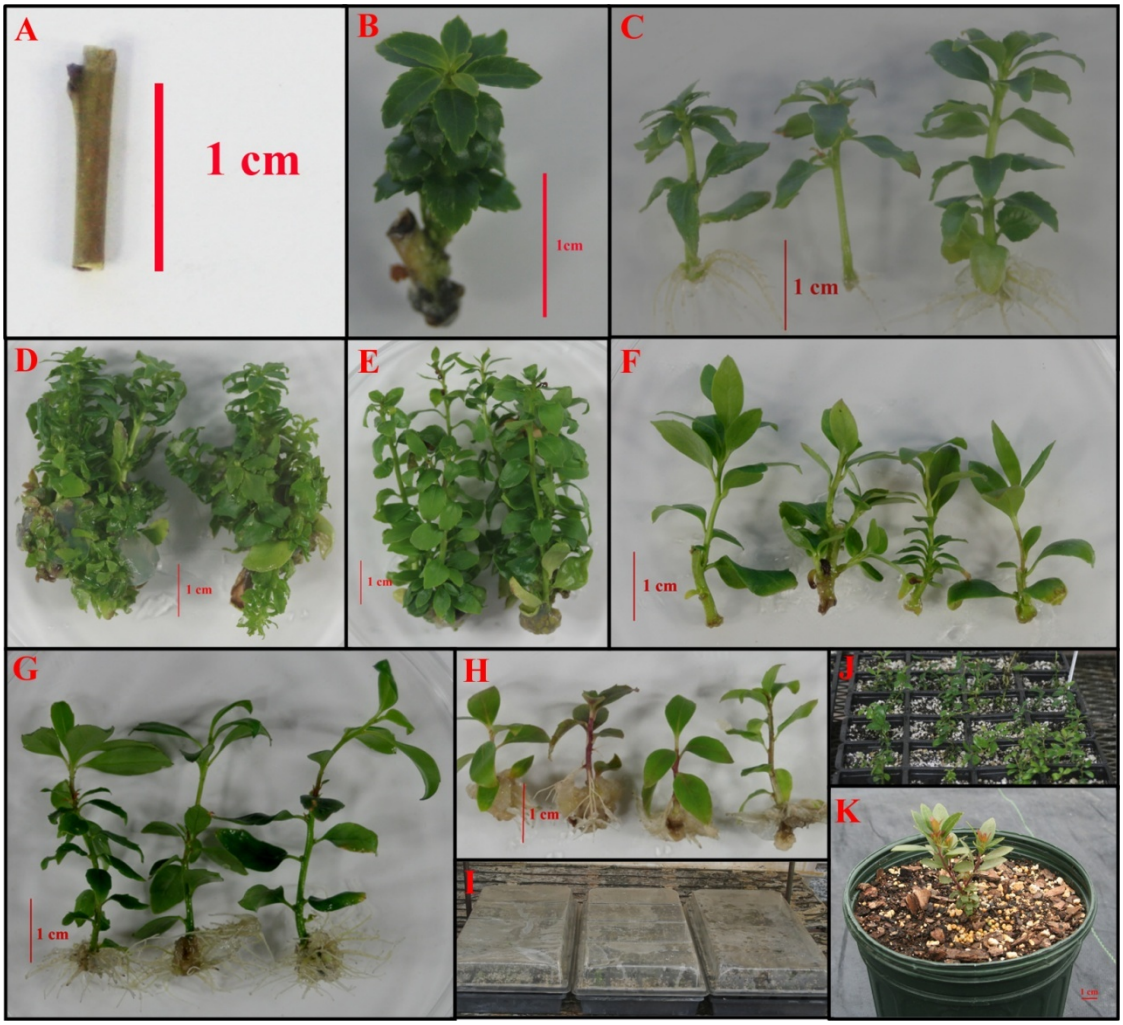

Figure 4.1. Morphogenetic responses of *Ilex glabra* during the establishment (A-B), multiplication (C-F), rooting (G-H), acclimatization (I-J), and transplanting (K) stages. A: nodal segment containing one axillary bud collected as explants; B: explants established on MS medium plus 90 mM sucrose; C: shoots cultured on MS medium without cytokinin; Shoots proliferated and elongated on MS medium supplemented with 4.5 µM BAP (D), 4.5 µM ZT (E), or 4.5 µM KIN (F); G: microcuttings rooted in vitro on ¼ MS medium plus 10.3 µM IBA; H: roots and callus tissue formed on ¼ MS medium plus 10.3 µM NAA; Plantlets rooted in vitro acclimated using dome for first two weeks (I), then kept under mist for two more weeks (J), K: Acclimated plantlet transplanted into pots.

months, axillary shoots had elongated up to 4 cm (Fig. 4.1B). Nodal segments (~1.0 cm long) were excised from these axillary shoots and used for the following experiments.

Effects of Cytokinins on Multiple Shoot Proliferation

To determine the optimal conditions for both axillary bud proliferation and shoot elongation, BAP (6-benzylaminopurine), KIN (Kinetin, 6-furfurylaminopurine), or ZT (Zeatin, 4-hydroxy-3-methyl-trans-2-butenylaminopurine) (all three obtained from Sigma Chemical Co., St. Louis, MO) at 2.3, 4.5, 9.1 or 18.2 μM were compared. Nodal segments (~1.0 cm long) were excised and cultured on an MS medium plus 90 mM sucrose and one source and rate of cytokinin. Nodal segments were also cultured on the same medium lacking any cytokinin. All cultures were incubated under the same physical conditions as described above. A total of 36 glass tubes (replications) with one explant per tube were used for each treatment. After 38 days of culture, total number of shoots (>1.0 cm long), and the length of the tallest shoot were recorded.

Root Induction

Terminal shoots (~2.0 cm long) were rooted in ¼ strength MS (¼ MS) medium plus 90 mM sucrose with either IBA (3-indolebutyric acid) or NAA (1-naphthylacetic acid) (both obtained from Sigma Chemical Co., St. Louis, MO) at 2.6, 5.1, or 10.3 μM. Shoots were also rooted on MS and ¼ MS medium without auxin. A total of 36 glass tubes with one explant per tube were used for each treatment. All cultures were incubated in the same physical conditions as described above. After 38 days of culture, the number of explants with roots, the number of roots per explant, and the length of the longest root on each explant were recorded.

At the same time, in a separate trial, terminal shoots (3-4 cm long) obtained during the multiplication stage were also dipped in a 5.1 μM IBA solution for 20 s, inserted in a flat with 5.0 × 5.0 × 6.0 cm^3 cell, which contained perlite (Whittemore Company Inc.,

Lawrence, MA) and a commercial substrate (PRO-MIX BX which contains 65-75%, by volume, of Canadian sphagnum peat moss; 35-25% of perlite, vermiculite, macronutrients and micronutrients, dolomitic and calcitic limestone, Scotts Sierra Horticulture Products Co., Marysville, OH) in a ratio of 1:1 by volume. Flats were placed under intermittent mist. Misting frequency was controlled by a timer (Phytotronics Inc., Earthcity MO) set at 20 seconds every 10 minutes for the first 2 weeks, and then reduced to 20 seconds every 20 minutes for the remainder of the experiment. Mist system was on in the morning and off in the evening. No additional light was provided. The number of surviving plantlets, the number of explants with roots, the number of roots per explant, and the length of the longest root were recorded.

Acclimatization and Transplanting

On 10 Oct. 2008, the plantlets obtained in vitro were removed from the culture tubes and their roots carefully washed under tap water to remove the agar medium. They were transferred to a tray with 32 cells ($5.0 \times 5.0 \times 6.0$ cm^3) filled with a mixture of PRO-MIX BX described as above and perlite (1:1 v/v). During transplanting, relative humidity (RH) was maintained 100% using Ultrasonic Whisper Quiet Cool Mist Humidifier (SU-2000, Sunpentown International Inc., Industry, CA) to avoid dehydration. Plantlets in a tray were covered with a clear, plastic dome and acclimated (25.4 ± 1.7 °C, 100% RH) under mist system in a greenhouse for first two weeks. The domes were removed and plantlets were then kept under the mist system described above for two more weeks. By lengthening the interval between misting, the RH was gradually decreased from 100% to about 70%. After 4 weeks of acclimatization, the percentage of surviving plants was recorded. They were transplanted into 3.3 L plastic pots with a commercial substrate

containing 35-45% bark, 20-30% coir, 10-20% Canadian sphagnum peat moss, 5-15% horticultural grade perlite, 5-15% processed bark ash, starter nutrient charge, dolomitic limestone and our long-lasting wetting agent (Metro-Mix 560 Coir, Scotts-sierra Horticultural Products Company, Marysville, OH) and grown in a glass greenhouse (22.6 ± 6.2 °C). Plant survival after transplant and the plant height (shoot length) were also recorded seven weeks later.

Experimental Design and Statistical Analysis

A completely random design was employed in all cases. A total of 36 explants were cultured per treatment. The treatments were arranged randomly on the shelves in the growth room. The results presented for multiple shoot proliferation were the means of 36 explants with the standard error (± SE), while those for rooting experiments were the means of three individual experiments with the standard error (± SE), 36 explants per experiment. Since ex vitro rooting experiments were performed with 3-4 cm shoots whereas in the other experiments 2 cm shoots were used, data of ex vitro rooting were omitted from the analysis. Data on full strength MS medium were also excluded from the analysis since auxin was not added. The square root of original data was used for analysis. A two way analysis of variance (ANOVA) was performed using Statistical Analysis Systems (SAS Version 9.1, SAS Institute, Inc., Cary, NC). Student-Newman-Keuls test at $P < 0.05$ was applied for means separation. Linear trend analysis for each cytokinin was also conducted.

RESULTS AND DISCUSSION

The effects of cytokinin source and rate on in vitro shoot proliferation of inkberry single-node explants are summarized in Table 4.1. Shoot proliferation occurred to varying

Table 4.1. Effect of cytokinin source and rate on in vitro multiple shoot production of *Ilex glabra* single-node explants after 38 days of culture on MS media supplemented with 90 mM sucrose.

Hormone[z]	Concentration (μM)	Number of shoots/explant[y]	Height (cm)[y]
Control	0	1.2 ± 0.1c[x]	2.6 ± 0.1 bc
BAP	2.3	4.2 ± 0.4 b	4.4 ± 0.2 a
	4.5	3.8 ± 0.3 b	2.6 ± 0.2 bc
	9.1	5.5 ± 0.6 a	2.8 ± 0.2 b
	18.2	4.8 ± 0.7 ab	2.1 ± 0.1 c
KIN	2.3	1.5 ± 0.1 c	2.1 ± 0.1 c
	4.5	1.7 ± 0.1 c	2.1 ± 0.2 c
	9.1	1.7 ± 0.2 c	2.5 ± 0.2 bc
	18.2	2.3 ± 0.2 c	2.5 ± 0.1 bc
ZT	2.3	2.3 ± 0.2 c	4.2 ± 0.2 a
	4.5	3.9 ± 0.3 b	4.6 ± 0.2 a
	9.1	4.4 ± 0.3 ab	4.2 ± 0.2 a
	18.2	5.5 ± 0.4 a	4.4 ± 0.2 a

[z] BAP is 6-benzylaminopurine, KIN is Kinetin, 6-furfurylaminopurine, and ZT is Zeatin, 4-hydroxy-3-methyl-trans-2-butenylaminopurine.
[y] Values are means ± standard error of 36 explants.
[x] Different letters in each column indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation.

degrees on all tested media. Both the type and concentration of the cytokinins significantly affected the number of shoots per explant and their height. ANOVA also demonstrated a significant interaction between both factors. Explants on MS medium without cytokinin

(control) produced few shoots, and developed many adventitious roots (Fig. 4.1C). BAP at all four concentrations and ZT at 4.5, 9.1 or 18.2 μM significantly increased the number of shoots per explant compared to the control (no cytokinin) (Fig. 4.1D and E). KIN at all tested concentrations and ZT at 2.3 μM did not stimulate shoot proliferations (Fig. 4.1F). The range of shoot number per explants was 3.8-5.5 on media containing 2.3, 4.5, 9.1 or 18.2 μM BAP, or on media containing 4.5, 9.1 or 18.2 μM ZT, but less than 2.3 on that with KIN or 2.3 μM ZT (Table 4.1). Our results are in general agreement with previous reports for english holly (Majada et al., 2000) and *Ilex dumosa* var. *dumosa* Reissek (Luna et al., 2003) even though they reported BAP was a better cytokinin for shoot proliferation than ZT. The response to the increasing KT and ZT concentrations was linear ($P < 0.0001$ for both KT and ZT), but not for BAP ($P = 0.11$). After 38 days of culture, the highest number of shoots per explant (5.5), was produced when nodal segments were grown on MS medium supplemented with 9.1 μM BAP or 18.2 μM ZT. This number was higher than that reported with english holly (Majada et al., 2000), *Ilex dumosa* var. *dumosa* (Luna et al., 2003), and *Ilex paraguariensis* A. St. Hil. (Sansberro et al., 1999; Zaniolo and Zanette, 2001) where an average of 4 shoots per explant were obtained with 4.5 μM BAP. ZT, at all four tested concentrations, and BAP, at 2.3 μM, increased shoot growth, which ranged from 4.2 to 4.6 cm, compared with the control (2.6 cm). However, the heights of the tallest shoot on 4.5, 9.1, or 18.2 μM BAP and all tested KINs were less than 2.8 cm (Table 4.1) and not significantly different from that of the control. Although different basic medium was employed, these results were still supported by those reported for english holly, whose (Majada et al., 2000). They reported that the length of axillary shoots on woody plant (WP, Lloyd and McCown, 1981) medium containing with 2.3 and 9.1 μM ZT was higher than

that with BAP and KT. The response to the increasing BAP ($P < 0.0001$) and KIN ($P = 0.013$) concentrations was linear, but that was not the case for ZT ($P = 0.97$). On the basis of above results and the cost of the cytokinins, a concentration of 4.5 μM ZT is most appropriate for shoot proliferation of inkberry.

Adventitious roots initiated after shoots were cultured about 2 weeks on all media supplemented with IBA or no auxin (Fig. 4.1G), the percentage of rooted explants ranged from 10% to 39% (data not shown). Similarly, Majada et al. (2000) reported that roots of english holly formed after 2 weeks of culture. However, rooting of *Ilex dumosa* var. *dumosa* took about 4 weeks (Luna et al., 2003). Shoots on all media containing NAA began to root after 3 weeks in culture (data not shown) and these shoots had high basal callus formation (Fig. 4.1H). After 38 days of culture, much higher rooting percentage was observed on all ¼ MS media compared to full strength MS media and ex vitro rooting (Table 4.2). Compared with ¼ MS media without auxin, there was no significant difference among the media with IBA or NAA with exception of the ¼ MS plus 10.3 μM IBA (Table 4.2). The highest percentage of rooted shoots (100%) was achieved using this highest concentration of IBA (Table 4.2). These results were in agreement with previous reports on the rooting of english holly (Majada et al., 2000) and *Ilex paraguariensis* (Zaniolo and Zanette, 2001), although their rooting percentages were lower. On the other hand, Morte et al. (1991) reported that a greater percentage of english holly explants produced roots when pretreated with 0.5 mM NAA (50%) compared to 0.5 mM IBA (15%). It's possible that IBA produced lower rooting percentages in their study since it was not incorporated into the media. Both the kind and concentration of auxin significantly affected the number and length of roots per explants. A significant interaction between both factors was also

Table 4.2. Effect of auxin source and concentration on in vitro rooting of *Ilex glabra* microcuttings after 38 days of culture on ¼ strength MS media plus 90 mM sucrose. In vitro rooting of *Ilex glabra* microcuttings on full strength MS media plus 90 mM sucrose and ex vitro rooting are presented as a reference but were excluded from the analysis.

Media	Hormone[z]	Concentration (µM)	% Rooting[y]	Number of Roots	Length of Roots (cm)
¼ MS	-	0	63.9 ± 1.1 b [x]	5.4 ± 0.4 c	1.4 ± 0.1 b
¼ MS	IBA	2.6	58.3 ± 2.8 b	5.3 ± 0.6 c	0.5 ± 0.1 c
¼ MS	IBA	5.1	66.7 ± 2.3 b	14.9 ± 1.1 a	1.9 ± 0.2 a
¼ MS	IBA	10.3	100 ± 0.0 a	14.8 ± 1.2 a	1.3 ± 0.1 b
¼ MS	NAA	2.6	55.6 ± 2.3 b	9.3 ± 0.6 b	0.6 ± 0.1 c
¼ MS	NAA	5.1	74.4 ± 0.8 b	7.8 ± 0.6 bc	1.3 ± 0.1 b
¼ MS	NAA	10.3	75 ± 0.6 b	6.8 ± 0.6 bc	1.1 ± 0.1 b
MS	-	-	33.3 ± 0.9	4.9 ± 0.8	1.4 ± 0.1
Ex vitro rooting[w]			37.6 ± 1.3	3.7 ± 0.2	5.0 ± 0.3

[z] IBA and NAA are 3-indolebutyric acid and 1-naphthylacetic acid, respectively.
[y] Values are mean ± standard error of three individual experiments, 36 explants per experiments, the percent is the percentage of explants with any visible roots.
[x] Different letters in each column indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation.
[w] Microcuttings were dipped in 5.1 µM IBA solution for 20 s, inserted in a medium containing perlite and PRO-MIX BX (65-75% of Canadia sphagnum peat moss; 35-25% of perlite, vermiculite, macronutrients and micronutrients, dolomitic and calcitic limestone) in a ratio of 1:1 by volume, and then kept under mist.

observed. The number of roots per explant on ¼ MS plus 5.1 or 10.3 µM IBA was significantly higher than other treatments (Table 4.2, Fig. 4.1G). Both 5.1 and 10.3 µM IBA treatments induced 1.6-3.1 times more roots than other treatments. Compared with in vitro rooting, ex vitro rooting produced fewer roots; however, ex vitro roots grew much longer. In addition, prolific root hairs were noted on the main roots of ex vitro roots.

Among all in vitro treatments, ¼ MS plus 5.1 μM IBA produced much longer roots than other treatments, while roots on ¼ MS plus 2.6 μM IBA or NAA were much shorter. However, no significant difference was observed among ¼ MS, ¼ MS plus 10.3 μM IBA, ¼ MS plus 5.1 μM NAA or ¼ MS plus 10.3 μM NAA. This may result from either different root initiation or inhibitory effects of auxin on root growth, or both. Due to higher percentage and greater number of roots forming, in vitro rooting using ¼ MS plus 10.3 μM IBA is recommended.

All in vitro rooted plantlets were pooled and transplanted into a mixture of peat moss: perlite (1:1 v/v) and acclimated in a mist system. After transplantation, some of the in vitro rooted plants died immediately, while others died during acclimatization. After 35 days, 73.6 ± 1.8% of the plantlets survived. This was much higher than ex vitro rooted plants (34.5 ± 3.0%). The original in vitro rooted plants continued to elongate after transplanting and newly formed roots had morphology similar to that of plants rooted ex vitro. Root hairs on the newly formed roots decreased the imposed stress during and after acclimatization. After 10 weeks in a greenhouse, 95 ± 2.9% of plants that survived initial acclimation survived and grew up to 6.8 ± 0.3 cm high. This yielded a final efficiency of 70%, which was much higher than ex vitro rooting (13.5%). This was also higher than those in previous reports (Majada et al., 2000; Morte et al., 1991), in which a final efficiency 64% or 40-60% was obtained.

Previous studies achieved rooting induction from shoots in two steps (Luna et al., 2003; Majada et al., 2000; Morte et al., 1991; Sansberro et al., 2001b; Zaniolo and Zanette, 2001). They treated the shoots or microcuttings with liquid hormone via quick dipping, or cultured them on a medium with rooting hormone for about one week, then cultured or

subcultured them on a medium devoid of hormones. This method is time-consuming and expensive. Majada et al. (2000) also reported that higher efficiency was achieved from ex vitro rooting of english holly (80%) than in vitro rooting (64%), and the survival rate of successfully in vitro or ex vitro rooted plants was not significantly different. However, in our study, ex vitro rooting was not efficient. The highest efficiency for propagation was obtained with in vitro rooting (70%). Less than 50% of the explants of *Ilex paraguariensis* formed roots when they were cultured on ¼ MS medium supplemented with 51.5-76.5 μM IBA (Sansberro et al., 2001b). Generally, rootless microcuttings may die in larger numbers when removed from culture, whereas rooted microcuttings have a higher survival rate. Since a great number of unrooted microcuttings were lost and low rooting percentages obtained during ex vitro rooting, in vitro rooting and acclimatization appears to be more appropriate.

In conclusion, a protocol for in vitro propagation of inkberry from juvenile explants, is: Stage I, surface sterilized single nodal segments implanted on MS medium plus 90 mM sucrose (Fig. 4.1B); Stage II, shoots proliferated and elongated on MS supplemented with 4.5 μM ZT (Fig. 4.1E); Stage III, in vitro rooting on ¼ MS plus 10.3 μM IBA (Fig. 4.1G); and Stage IV, acclimatization in a mist system (Fig. 4.1I and J) followed by transplanting into growing medium (Fig. 4.1K).

CHAPTER FIVE

COLD HARDINESS OF *ILEX GLABRA* CULTIVARS FROM FIELD TRIALS AND LABORATORY TESTS

ABSTRACT

To evaluate the cold hardiness of *Ilex glabra* (L.) A. Gray (inkberry) cultivars and provide growth and cold-hardiness data for growers as references for production and marketing, field trials and laboratory tests were conducted in 2007, 2008 and 2009. Plant survival was 72% and 93% for the 2007 and 2008 planting, respectively. *Ilex glabra* 'Shamrock' was the most cold-hardy cultivar; *Ilex glabra* f. *leucocarpa*, 'Viridis' and 'Nigra' were the least cold-hardy cultivars; while *Ilex glabra* wild species and its cultivars including 'Compacta', 'Densa', 'Chamzin', and 'Pretty Girl' had intermediate cold hardiness. Based on controlled freezing tests of *Ilex glabra* cultivars, the REC_{50} value of *Ilex glabra* cultivars ranged from -19 to -32 ℃ for Jan. 2007 and -18 to -38 ℃ for Jan. 2008. The cold hardiness rating from field trials was significantly correlated with the REC_{50} value from laboratory tests. Our results suggested that data from a combination of field trials and laboratory tests were reliable for the measurement of *Ilex glabra* cold hardiness. The factors influencing cold hardiness, including plant cultivar, plant size, temperate, and others (e.g. snow pack, mechanical injury, deaacclimation, winter desiccation and photoinhibition) should be in consideration.

SIFNIFICANCE TO THE NURSERY INDUSTRY

Ornamental plant field trials and laboratory tests provide very important growth and cold hardiness data for growers to produce and market their plants. Our results suggested that cold hardiness of *Ilex glabra* varied with cultivars. *Ilex glabra* 'Shamrock' was the most cold-hardy among tested cultivars; while *Ilex glabra* f. *leucocarpa*, 'Viridis' and 'Nigra' were the least cold-hardy cultivars. Their field performance was significantly correlated with results from laboratory tests, suggesting laboratory testing could be used to predict the cold tolerance of *Ilex glabra* cultivars.

INTRODUCTION

Ilex glabra (L.) A. Gray (inkberry) is a native evergreen shrub in the *Aquifoliaceae* (holly family). It is an important foundation and hedge plant due to its environmental adaptability, ability to withstand heavy pruning, and resistance to diseases and insects (Dirr, 1998; Dirr and Alexander, 1991; Hume, 1953). Native populations of *Ilex glabra* have been recorded along eastern US coast from Florida to Maine (United States Department of Agriculture, 2006). In Maine, the single known population of this plant grew around the perimeter of a coastal sphagnum bog in Knox County (Maine Department of Conservation, 2006). Unfortunately, we could not locate this native population. Although 27 cultivars are reported in the literature (Dirr, 1998, Dirr and Alexander, 1991), most cultivars are difficult to find or under-utilized in the nursery trade possibly because of susceptibility to winter injury.

The cold hardiness of other *Ilex* species and their cultivars had been investigated in field or laboratory studies. *Ilex opaca* Ait. (american holly), *Ilex opaca* Ait.'Greenleaf' (greenleaf american holly), and *Ilex* × *attenuata* Ashe 'Foster's #2' ('foster's #2' holly)

were hardy to –30.0 ℃; *Ilex latifolia* Thunb. (lusterleaf holly) and *Ilex* L.'Lydia Morris' (lydia morris holly) were less cold hardy, only surviving temperature as low as –17.8 ℃; while *Ilex* × 'Nellie R. Stevens' (nellie r. stevens holly) and *Ilex* × *koehneana* Loes. 'Wirt L. Winn' (wirt l. winn koehne holly) exhibited intermediate cold hardiness and survived a temperature of –21.1 ℃ (Dirr and Lindstrom, 1990). Among the *Ilex* × *attenuata* cultivars, 'Foster's #2' was more tolerant than *Ilex* × *attenuata* Ashe 'Savanna' (savannah holly) and *Ilex* × *attenuata* Ashe 'East Palatka' (east palatka holly) (Dirr and Lindstrom, 1990).

Leaves of all *Ilex* taxa were generally less cold hardy than their stems while leaves of *Ilex* × *koehneana* 'Wirt L. Winn', *Ilex latifolia* and *Ilex* 'Lydia Morris' were equal to or more cold hardy than the stems (Dirr and Lindstrom, 1990). Extent of root injury of *Ilex crenata* 'Green Island' ranged from 33-100% when the substrate temperature decreased from –1.7 to –10.0 ℃ (Regan et al., 1990). The killing temperature of *Ilex crenata* Thunb. 'Hetzi' roots, estimated using TTC (2, 3, 5-Triphenyl tetrazolium chloride), ranged from –5.6 to –7.8 ℃ (Havis et al., 1972).

Only observational data have been reported regarding the cold hardiness of *Ilex glabra* wild species and its cultivars. The white-fruited form, *leucocarpa*, which was collected from Florida could tolerate at least –26.0 ℃. 'Chamzin' survived winter temperatures ranging from –29.0 to –34.0 ℃ and 'Nigra' was not as cold hardy as 'Compacta' and 'Viridis' (Dirr and Alexander, 1991). *Ilex glabra* was more cold-hardy than *I. crenata* Thunb. at temperatures of –26.0 to –29.0 ℃ in severe winters (Dirr, 1998). *Ilex glabra* 'Compacta' and 'Nordic' survived well in Orono, ME (USDA Zone 5a) (Cappiello and Littlefield, 1994). The cold hardiness of *Ilex glabra* wild species and its

cultivars have not yet been comprehensively examined. Therefore this information would increase its use as an alternative native woody plant.

To systematically study plant response to low temperature when damaging temperatures occur in the field, more frequent assessment of low temperature tolerance rather than sporadic observations are required. Field test, the whole-plant freeze test, is the standard method used to assess plant cold hardiness (Rietveld and Tinus 1987; Ritchie 1984). Entire above-ground portions of plants are exposed to frost in the natural environment until visible evidence of injury develops. This test is reliable, but the occurrence of freezing ambient temperatures in the field may be unpredicable and variable. Moreover, field tests need a larger plant population and often require years to get results. However, tissue tests for cold hardiness, based on physiological parameters that can be conveniently measured and utilized, provide results within one to forty-eight hours (Bannister and Neuner, 2001). Use of a programmed freezer to expose plant tissues to repeatable time-temperature regimes allows sampling of large numbers of plants in a short period of time and with precise temperature control. The reliability of the results from a tissue test would then depend on whether the tissue test results match field test results. If there is a consistent relationship between the field test and one or more tissue tests, plant cold hardiness can be measured efficiently.

The short growing season and harsh winters leave gardeners in the northern United States with a relatively limited selection of broadleaf evergreen shrubs. Due to the glabrous dark green and fine textured foliage of *Ilex glabra*, it could be an important northern landscape plant if cold hardy cultivars were identified. The objective of this study was to evaluate the cold hardiness of *Ilex glabra* cultivars or accessions through a combination of

field trial and laboratory leaf tissue hardiness tests. The growth and cold-hardiness data will help growers produce and market *Ilex glabra*.

MATERIALS AND METHODS

Field Trials

Field trials were conducted in the Lyle E. Littlefield Ornamentals Trial Garden at the University of Maine (Orono, ME; lat. 44°54′05"N, long. 68°39′36"W, Ele.44 m). The dominant soil is Orthods (United States Department of Agriculture, 2009). The chemical characteristics of the soil were within the optimum range (Table 5.1). A 40 ×7.4 m^2 plot was completely tilled and prepared for planting. A total of 20 *Ilex glabra* cultivars (Table 5.2) were established on 26 July 2007. All plants were planted in a randomized complete block design (RCBD) with four rows as blocks and four plants for each cultivar. The

Table 5.1. Soil characteristics at the test site in the Lyle E. Littlefield Ornamentals Trial Garden.

Characteristic		Level[z]	Optimum Range
Cation Exchange Capacity (Me/100 gm)		8.0 ± 0.1	>5
Organic Matter (%)		7.9 ± 0.1	5-8
pH		5.7 ± 0.0	5.5-6.5
P($g \cdot m^{-2}$)		1.35 ± 0.004	0.79-1.21
	K	2.6 ± 0.1	2.8-4.0
% Saturation	Mg	7.0 ± 0.2	10-25
	Ca	67.0 ± 0.2	60-80
Sulfur ($mg \cdot L^{-1}$)		11.7 ± 0.3	>15

[z] Values are mean ± standard error, three soil samples was tested.

distance between plants was 1.5 m, with 2.4 m between rows. On 30 June 2008, another 22 *Ilex glabra* cultivars (Table 5.3) were added to the existing plants. They were also arranged in a RCBD. The whole plot was mulched with newspaper for weed control. Ambient temperature 30 cm aboveground and 25 m away from the plots was monitored using type T thermocouples attached to CR10X Datalogger (Campbell Scientific Inc., Logan, Utah). Plant height and width, measured at the east-to-west and north-to-south directions, were recorded at the beginning and end of every growing season. Plant cold hardiness rate was evaluated on 7 May 2008 and 2009, respectively, using the following rating scale: 5 = little or no damage; 4 = occasional tip dieback; 3 = regular tip dieback and occasional substantial dieback; 2 = typical moderate to severe dieback or dieback to the ground with suckering from the roots; 1= entirely winter killed (Cappiello and Littlefield, 1994). The number of dead branches and mean length of all dead branches were also recorded.

Laboratory Tests

Cold hardiness was determined by a modification of the frost-induced electrolyte leakage (FIEL) procedure (Binnie et al., 1994, Burr et al., 1990, O'Reilly et al., 2001). Twenty two-year-old plants of each *Ilex glabra* cultivar (Table 5.4) were grown in 3.3 L plastic pots with a commercial substrate containing 35-45% bark, 20-30% coir, 10-20% Canadian sphagnum peat moss, 5-15% horticultural grade perlite, 5-15% processed bark ash, starter nutrient charge, dolomitic limestone and our long-lasting wetting agent (Metro-Mix 560 Coir, Scotts-sierra Horticultural Products Company, Marysville, OH). All plants were fertilized with control released fertilizer (Peters Professional 15 N-4.4 P-24.9K, Scotts-sierra Horticultural Products Company, Marysville, OH). They were mulched during winter periods. About 10 fully developed, non-senescent leaves of each

plant were excised from the upper half of the current season's growth on 15 Jan. 2008 and 2009. Leaves were immediately placed in a sealable plastic bag with moistened wet paper towels and placed on ice. Leaves were rinsed off with distilled water to remove any electrolytes released by cells damaged during leaf excision. The detached leaves were prepared for freezing within 5 hr of collection. The leaves were cut into disk (28.7 ± 0.01 mm^2 and 0.095 ± 0.004 g) using a hole puncher. After all leaf discs (390 and 520 disks in 2008 and 2009, respectively) were pooled from each cultivar and mixed completely, ten discs were randomly sampled and transferred into one of twelve 14 mL polypropylene round bottom tubes (Becton Dickinson Labware, Franklin Lakes, NJ) containing 10 ml of deionized water. Samples were subjected to 12 temperatures (-2, -6, -10, -14, -18, -22, -26, -30, -34, -38, -42, -46 ℃) plus a control (3.9 ± 1.6 ℃). This procedure was repeated for three to four times. The test tubes with 10 ml of deionized water (0.002 ms) were prepared in advance and frozen overnight in the refrigerator (Thermo Electron Corporation, Asheville, NC). Before leaf discs were loaded, test tubes were thawed, leaving a piece of ice floating as a nucleation core. All test tubes, except the control, were kept overnight in a 40-9.4 super cold freezer (ScienTemp Corporation, Adrian, MI), preset at the temperature of 0 ℃. Controls were kept on ice in a cooler during the whole freezing program. The temperature, monitored by type T thermocouples attached to a datalogger (CR-10, Campbell Scientific, Logan, Utah), was lowered at a rate of 4 ℃·h^{-1} to each of the desired target temperatures within the tubes. Three or four test tubes corresponding to the target temperature were thawed on ice in a cooler overnight after they were held at the target temperature for 30 min. Another three or four sets of leaf discs were immersed in liquid nitrogen (LN, -196 ℃) for inducing total cell lysis. After the tissues were fully thawed, all

test tubes were agitated on a shaker at 150 rpm for 24 h at a temperature of 16.5 ± 0.9 ℃ and the electrical conductivity (EC) was measured using an EC meter (Amber Science Inc., Eugene, OR). From these data, the relative electrical conductivity (REC) was calculated by the following formula: $REC = 100 \times \left(\left(EC_{frozen} \right) - \left(EC_{control} \right) / \left(EC_{liquid-nitrogen} \right) \right)$. Plots of REC versus temperature were constructed and non- linear curve based on the growth/sigmoidal model from the Boltzmann equation were fitted using the OriginPro 7.0 (OriginLab Corporation, Northampton, MA). The Boltzmann sigmoidal equation is expressed as $y = a + (b - a)/(1 + e^{((c-x)/d)})$, where the parameters a and b represent the probability at the minimum and maximum plateaus, c represents the *X*-value when the response is halfway between a and b, and d represents the slope of the curve. From these fitted regression, the temperature that results in 50% REC was determined and denoted as REC_{50}.

Experimental Design and Statistical Analysis

A randomized complete block design was employed in the field trials. Since the influence of plant source of *Ilex glabra* cultivars on cold hardiness was not significant (2007: $P = 0.48$; 2008: $P = 0.91$), all data were pooled for the following data analysis. The results presented were the means of 4, 8, or 12 plants with the standard error. Data transformations were performed as necessary. A completely random design was employed for the laboratory tests. Results from laboratory test showed that all REC values fit well for Boatman's model and no significant differences was observed among plant sources of *Ilex glabra* (2008: $P = 0.47$; 2009: $P = 0.93$). Data from different plant sources were therefore pooled for further data analysis. The results presented were the means of 4 or 8 replications with the standard error.

All data including height, cold hardiness rating, length and percentage of dead branches, canopy size, and REC_{50} were analyzed using analysis of variance in the Statistical Analysis Systems 9.1 (SAS Institute, Inc., Cary, NC). Student-Newman-Keuls test at $P < 0.05$ was applied for means separation. Correlation and linear regression analysis was also conducted using data of both field trials and laboratory tests.

RESULTS

Field Trials

All *Ilex glabra* cultivars in the 2007 planting were fairly uniform. No significant differences were observed for their height ($P = 0.59$) and canopy size ($P = 0.19$) (Table 5.2). They grew to about 15 cm (12-17 cm) high with a canopy size of about 196 cm^2 (137 – 258 cm^2). They were covered by snow for most of the winter season (15 Jan. 2008 to 14 Mar. 2008). The lowest temperature recorded was -25 ℃ on 4 Mar. 2008 (Fig. 5.1). Plant survival was 72% (57/80) in 2008. All surviving plants were still alive in 2009 (Data not presented). There was a significant difference of cold hardiness ratings among cultivars ($P = 0.0012$). *Ilex glabra* 'Shamrock' (GPN/ LG1997-0973), *Ilex glabra* 'Compacta' (AA745-69/ 1051-70/Jackson Compacta), and *Ilex glabra* 'Densa' (LG1992-0298/ PCF) were the most cold-hardy cultivars, which corresponded to their low cold hardiness ratings of 4.0, 3.8 and 3.6, respectively. In contrast, *Ilex glabra* f. *leucocarpa* (AA 1489-82-Mass) and *Ilex glabra* 'Nigra' (AA1464-82/GPN) were clearly not cold hardy in Orono, ME. Their cold hardiness ratings were 1.5 and 1.3, respectively. But they were not statistically different from *Ilex glabra* (AA200-2005/652-70-Mass/929-88), *Ilex glabra* 'Chamzin', *Ilex glabra* 'Compacta'AA179-2005/*Ilex glabra* Worest, MA, *Ilex glabra* 'Compacta' (LG1997-1423/UME /WMN) and *Ilex glabra* (LG1994-0494), whose cold hardiness

Table 5.2. Growth characteristics and cold hardiness parameters of *Ilex glabra* accessions following the winter of 2007-2008.

Accession and Sources	Height (cm)	Canopy Size (cm^2)	Cold Hardiness Rating[z]	Length of Dead Branch (cm)	Dead Branch %
Ilex glabra AA200-2005/AA652-70-Mass/AA929-88	15.7 ± 1.1[y] a[x]	241.3 ± 26.3a	2.6 ± 0.2abc	3.6 ± 0.4b	50.6 ± 6.9ab
'Chamzin' LG1997-1435	14.8 ± 1.4a	171.5 ± 23.0a	3.3 ± 0.4ab	5.2 ± 1.5b	25.0 ± 12.5ab
'Compacta' AA-179-2005, *Ilex glabra* Worest, MA	12.2 ± 1.0a	166.6 ± 27.1a	3.1 ± 0.2abc	9.2 ± 0.9ab	33.8 ± 4.8ab
'Compacta' AA-745-69 / AA1051-70, 'Jackson Compacta'	16.6 ± 0.9a	257.9 ± 19.4a	3.8 ± 0.1a	4.0 ± 0.6b	30.8 ± 4.1ab
'Compacta' LG1997-1423/UME, 'Cole's Compacta' WMN	15.5 ± 0.7a	140.1 ± 7.3a	2.2 ± 0.2abc	7.4 ± 0.7ab	61.2 ± 5.7ab
'Densa' LG1992-0298/PCF	15.4 ± 1.3a	255.5 ± 38.5a	3.6 ± 0.2a	6.0 ± 0.9b	36.0 ± 5.6ab
f. *leucocarpa* AA1489-82-Mass	15.1 ± 1.8a	349.8 ± 52.2a	1.5 ± 0.3bc	13.7 ± 0.8a	51.2 ± 10.9ab
'Nigra' AA1464-82/GPN	14.4 ± 1.1a	147.9 ± 19.0a	1.3 ± 0.1c	9.3 ± 0.6ab	85.4 ± 6.2a
'Pretty Girl' LG1994-0494	12.7 ± 1.1a	137.3 ± 11.9a	2.8 ± 0.2abc	3.0 ± 0.5b	38.8 ± 7.7ab
'Shamrock' GPN/LG1997-0973	16.0 ± 0.8a	251.0 ± 18.8a	4.0 ± 0.2a	3.4 ± 0.8b	5.7 ± 1.1b

[z] Cold hardiness rating was defined as follows: 5 = little or no damage; 4 = occasional tip dieback; 3 = regular tip dieback and occasional substantial dieback; 2 = typical moderate to severe dieback or dieback to the ground with suckering from the roots; 1= entirely winter killed (Cappiello and Littlefield, 1994).

[y] Values are the means of 4, 8, or 12 plants with the standard error.

[x] Different letters adjacent to means indicate that they are significantly different according to Student-Newman-Keuls at $P \leq 0.05$.

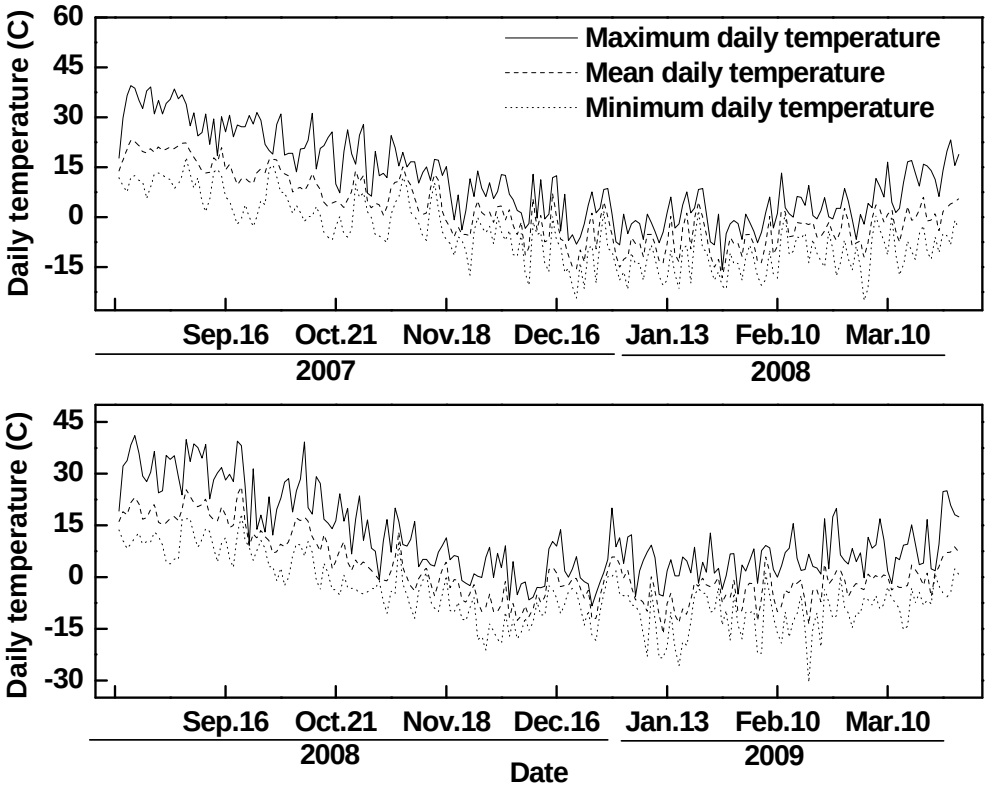

Figure 5.1. Daily temperatures recorded at 30 cm aboveground from 20 Aug. 2007 to 28 Mar. 2008 (top graph) and 20 Aug. 2008 to 28 Mar. 2009 (bottom graph).

ratings ranged from 2.2 to 3.3. The length of damaged branches was similar among most of the cultivars tested; only *Ilex glabra* f. *leucocarpa* (AA 1489-82-Mass) showed excessive damage, but it was not significantly different form *Ilex glabra* 'Compacta'AA179-2005/ *Ilex glabra* Worest, MA, *Ilex glabra* 'Compacta' (LG1997-1423/UME /WMN), 'Nigra' (AA 1464-82/GPN). There had a high percentage of dead branches, 85%, for 'Nigra' (AA 1464-82/GPN), while the percentage of the damaged branches of shamrock was less than 6%. Among the rest of the cultivars tested, there was such a large variation in percentage of dead branches (25-62%), but the values were not significantly different.

For the 2008 planting, there were much higher variations in cultivar height and canopy size (Table 5.3). Their height was about 23 cm (14–34 cm), while canopy size was about 644 cm^2 (218–1620 cm^2). These significant variations resulted from the different growth habit or different length of growth in greenhouse and/or nursery. However, all plants were still completely covered with snow starting from 21 Dec. 21 2008 until 19 Mar. 2009. The lowest temperature recorded was -30.5 ℃ on 1 Mar. 2009 (Fig. 5.1). Plant survival was 93% (81/87) during this winter. Cold hardiness ratings differed among cultivars ($P < 0.0001$) (Table 5.3). *Ilex glabra* (AA200-2005/AA652-70-Mass/AA929-88) and *Ilex glabra* cultivars including 'Chamzin', 'Compacta', 'Densa', 'Nova Scotia', 'Pretty Boy', 'Pretty Girl', and 'Shamrock' were the most cold-hardy cultivars. For these cultivars, less than 15% of their branches were damaged and the length of damaged branches was less than 3 cm. which was, on average, about 14% of the plant height. However, there was not significantly difference among each other. On the other hand, *Ilex glabra* f. *leucocarpa* (AA1489-82-Mass), *Ilex glabra* 'Nigra' (AA1464-82/GPN), and 'Viridis' (AA1488-82) were the least cold hardy among all cultivars tested. The damaged branches accounted for 24%, 48%, and 53% for *Ilex glabra* f. *leucocarpa*, 'Viridis' and 'Nigra', respectively. A length of 10.5 to 15 cm of branch was damaged, which accounted for 34-79% of the height of whole plant.

Based on data from the two growing seasons, *Ilex glabra* 'Shamrock' was the most cold-hardy cultivar, followed by *Ilex glabra* 'Compacta', *Ilex glabra* 'Densa', *Ilex glabra* 'Chamzin', *Ilex glabra* 'Pretty Girl', *Ilex glabra*, then *Ilex glabra* f. *leucocarpa*, *Ilex glabra* 'Viridis' and *Ilex glabra* 'Nigra'.

Table 5.3. Growth characteristics and cold hardiness parameters of *Ilex glabra* accessions following the winter of 2008-2009.

Accession and Sources	Height (cm)	Canopy Size (cm^2)	Cold Hardiness Rating[z]	Length of Dead Branch (cm)	Dead Branch %
Ilex glabra AA200-2005/AA652-70-Mass/AA929-88	19.9 ± 1.0cd	399 ± 25cde	5.0 ± 0.0a	0.0b	0.0b
'Chamzin' LG1997-1435	24.0 ± 1.2bc	400 ± 24cde	4.8 ± 0.1a	0.0b	0.0b
'Cole's Compacta', 'Compacta' UME	34.3 ± 0.9a	1427 ± 104a	4.8 ± 0.1a	0.0b	0.0b
'Compacta' AA-179-2005, *Ilex glabra* Worest, MA	21.0 ± 0.7bcd	771 ± 32bc	4.1 ± 0.1a	0.0b	0.0b
'Compacta' AA-745-69/AA1051-70	25.1 ± 1.3bc	676 ± 48bcd	4.9 ± 0.1a	0.0b	0.0b
'Densa' LG1992-0298/PCF	28.4 ± 0.5ab	812 ± 34b	3.9 ± 0.2a	3.1 ± 0.9b	15.3 ± 6.1b
f. *leucocarpa* AA1489-82-Mass	33.5 ± 1.0a	1620 ± 43a	3.0 ± 0.0b	11.5 ± 2.6a	24.0 ± 4.1b
'Nigra' AA1464-82/GPN	19.0 ± 1.1cd	264 ± 22e	2.3 ± 0.1b	15.0 ± 0.5a	52.9 ± 5.4a
'Nova Scotia' RN	14.7 ± 0.8d	458 ± 20b-d	4.3 ± 0.2a	0.5 ± 0.3b	4.5 ± 2.3b
'Pretty Boy' RN	20.2 ± 0.3cd	441 ± 7b-d	4.0 ± 0.2a	1.8 ± 0.9b	12.5 ± 6.3b
'Pretty Girl' LG1994-0494/RN	14.6 ± 0.2d	229 ± 8e	4.6 ± 0.1a	0.0b	0.0b
'Shamrock' GPN/LG1997-0973	14.5 ± 0.5d	298 ± 7de	4.3 ± 0.1a	2.0 ± 0.7b	8.0 ± 2.7b
'Viridis' AA1488-82	22.3 ± 0.6bcd	218 ± 13e	3.0 ± 0.2b	10.5 ± 1.9b	47.5 ± 8.5a

[z] Cold hardiness rating was defined as follows: 5 = little or no damage; 4 = occasional tip dieback; 3 = regular tip dieback and occasional substantial dieback; 2 = typical moderate to severe dieback or dieback to the ground with suckering from the roots; 1= entirely winter killed (Cappiello and Littlefield, 1994).

[y] Values are the means of 4, 8, or 12 plants with the standard error.

[x] Different letters adjacent to means indicate that they are significantly different according to Student-Newman-Keuls mean separation at $P \leq 0.05$.

Laboratory Tests

Cold hardiness based on REC_{50} values differed significantly among tested *Ilex glabra* cultivars (2008 and 2009: $P<0.0001$) (Table 5.4). Similar results had been observed for other *Ilex* species or cultivars (Dirr and Lindstrom, 1990). From the 2008 laboratory test, *Ilex glabra* 'Compacta' (AA-745-69/AA1051-70) was one of the more cold-hardy cultivar with a REC_{50} of -31.8 ℃, while *Ilex glabra* 'Cole's Compacta'/'Compacta' (Umaine) was the least cold-hardy cultivar with a REC_{50} of -19.4 ℃. Umaine accession of 'Compacta' was produced via cuttings from the plants which were located under the canopy in the Lyle E. Littlefield Ornamentals Trial Garden at University of Maine, Orono. These plants may have adapted to the specific understory microclimates. They might have been subjected to photoinhibition under low temperature and high light irradiation, and thus sensitive to the low temperature (Levitt, 1980; Rütten and Santarius, 1998). 'Cole's Compacta' came from the rooted cuttings at the Western Maine Nursery, but we don't know the exact origin. It is reasonable to speculate that they were from a more southern source. *Ilex glabra* 'Chamzin', *Ilex glabra* 'Compacta' (AA-179-2005)/*Ilex glabra* (Worest, MA), and *Ilex glabra* 'Densa' (LG1992-0298/PCF), and *Ilex glabra* 'Shamrock' (LG1997-0973/GPN) were more cold-hardy with a REC_{50} value of -28.9, -27.7, -27, and -26.1 ℃, respectively. Our experimental result with *Ilex glabra* 'Chamzin' was in agreement with the observations of Dirr and Alexander (Dirr and Alexander, 1991). They found that *Ilex glabra* 'Chamzin' survived winter temperatures ranging from –29.0 to –34.0 ℃. *Ilex glabra* 'Pretty Girl' (LG1994-0494/RN) was the intermediate cold-hardy cultivar. From 2009 laboratory test, REC_{50} values of *Ilex glabra* 'Chamzin', *Ilex glabra* 'Compacta' (AA-745-69/AA1051-70), *Ilex glabra* 'Densa' (LG1992-0298/PCF), *Ilex*

Table 5.4. Cold hardiness of *Ilex glabra* accessions based on laboratory tests on 15 Jan. 2008 and 2009.

Accessions	REC_{50} (℃)[z]	
	Jan. 2008	Jan. 2009
'Chamzin' LG1997-1435	-28.9 ± 1.6bc[y]	-37.5 ± 1.4f
'Cole's Compacta', 'Compacta' UME	-19.4 ± 0.9a	-
'Compacta' AA-179-2005, *Ilex glabra* Worest, MA	-27.7 ± 2.4bc	-26.0 ± 1.0bc
'Compacta' AA-745-69/AA1051-70	-31.8 ± 1.1c	-33.4 ± 0.6ef
'Densa' LG1992-0298/PCF	-27.0 ± 1.6bc	-32.8 ± 1.1ef
f. *leucocarpa* AA1489-82-Mass	-	-29.1 ± 0.8bcde
'Nigra' AA1464-82/GPN	-	-17.8 ± 3.5a
'Nova Scotia' RN	-	-33.3 ± 0.4ef
'Pretty Boy' RN	-	-26.9 ± 0.9bcd
'Pretty Girl' LG1994-0494/RN	-24.4 ± 1.7b	-30.7 ± 0.7cde
'Shamrock' GPN/LG1997-0973	-26.1 ± 0.5bc	-31.8 ± 0.5def
'Viridis' AA1488-82	-	-24.7 ± 2.0b

[z] Values are the means of 3 or 6 replications with the standard error for 2008, while that of 4 or 8 replications with the standard error for 2009. REC_{50} was defined as the temperature that results in 50% relative electrical conductivity (REC).
[y] Different letters adjacent to means within column indicate that they are significantly different according to Student-Newman-Keuls mean separation at $P \leq 0.05$.

glabra 'Nova Scotia', *Ilex glabra* 'Shamrock'(GPN/LG1997-0973) were higher than -31.8 ℃. They can be considered the most cold-hardy cultivars. REC_{50} values of *Ilex glabra* 'Pretty Girl', *Ilex glabra* f. *leucocarpa*, *Ilex glabra* 'Pretty Boy', *Ilex glabra* 'Compacat' (AA-179-2005, Worest, MA), and *Ilex glabra* 'Viridis' ranged from -24.7 to -30.7 ℃. They were the intermediate cold hardiness cultivars. The least cold hardy cultivar

with a REC_{50}value of -17.8 ℃ was *Ilex glabra* 'Nigra'. The 2008 and 2009 laboratory tests suggest that *Ilex glabra* 'Chamzin', *Ilex glabra* 'Compacta', *Ilex glabra* 'Nova Scotia' were the most cold hardy cultivars, followed by *Ilex glabra* 'Densa', *Ilex glabra* 'Shamrock', *Ilex glabra* 'Pretty Girl', *Ilex glabra* f. *leucocarpa*, *Ilex glabra* 'Pretty Boy', *Ilex glabra* 'Viridis', and *Ilex glabra* 'Nigra'.

The cold hardiness ratings from field trials were significantly correlated with REC_{50} value from laboratory test in 2008 (P = 0.0002, Pearson Correlation Coefficient = -0.87) and 2009 (P = 0.0011, Pearson Correlation Coefficient = -0.80) (Fig. 5.2). These results suggested that data from a combination of field trials and laboratory tests were reliable for the measurement of *Ilex glabra* cold hardiness. They support the use of laboratory tests to predict cold tolerance of *Ilex glabra* cultivars in the field. Both data were then analyzed using a linear regression model to establish the equation for the prediction. Results showed that a significant linear relationship between both methods (2008: $y = -0.17x - 1.35$ ($P = 0.05, r^2 = 0.75$); 2009: $y = -0.13x + 0.12$ ($P = 0.05, r^2 = 0.69$) (Fig. 5.2). Cold ambient temperature in Orono, ME could account for over 70% of the observed damage to field plants. In addition, snow pack, stem injury from insects, and/or physiological changes such as deacclimation, winter desiccation and photoinhibition could enhance damage in the field trials.

DISCUSSION

The extent of winter injury depends on taxa (species, cultivars, and/or individual plant) (Cappiello and Littlefield, 1994; Dirr, 1998; Dirr and Alexander, 1991; Dirr and Lindstrom, 1990). In our studies, *Ilex glabra* 'Shamrock' was the most cold-hardy cultivar,

while *Ilex glabra* f. *leucocarpa*, *Ilex glabra* 'Viridis' and *Ilex glabra* 'Nigra' were not cold hardy cultivars in Orono, ME.

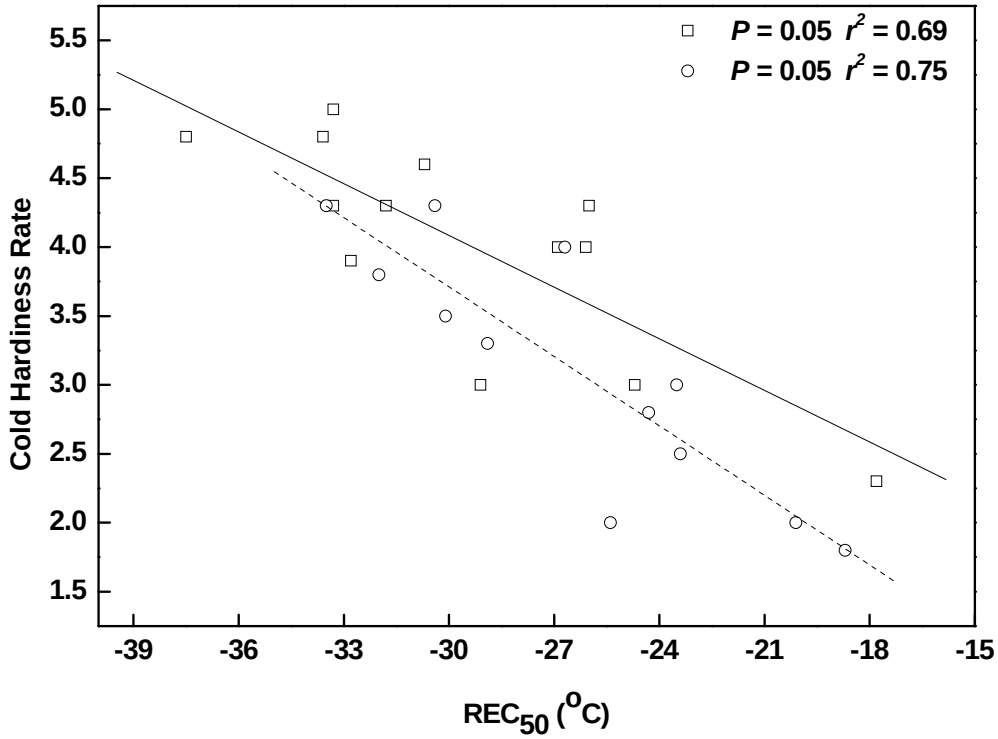

Figure 5.2. REC_{50} value of *Ilex glabra* accessions in Jan. 2008 (open circle) and 2009 (open square) relating their cold hardiness rating in 2007-2008 and 2008-2009 field trial. Linear regression analyses between both were applied and the linear equations were also showed, 2008: $y = -0.17x - 1.35$(dash line); 2009: $y = -0.13x + 0.12$(solid line).

For *Ilex*, variation in cold hardiness among tissues has been reported (Dirr and Lindstrom, 1990). During the winter season from 2006 to 2007, we observed that 100% of our *Ilex glabra* plants were killed when placed outside without any protection. However, when plants were placed in a white plastic winter house without any supplementary heat, where the lowest temperature recorded was -20.5 ℃, all roots turned brown, while the aboveground plant parts still looked healthy, suggesting that the leaves and stems of were

more cold-hardy than roots. Regan et al. (1990) reported that roots of *Ilex* were less cold-hardy than shoots and the hardiness of *Ilex* mature root tissue was -6.3 ℃ while that of shoots was -32.3 ℃ and Dirr and Lindstrom (1990) reported that leaves of *Ilex* are generally less cold hardy than stems.

Temperature has been identified as the major environmental factor that affects plant cold hardiness (Levitt, 1980). The distribution of *Ilex aquifolium* L. (english holly) and its habitat preference guggest sensitivity to low temperature (Rüand and Santarius, 1998). It is absent from areas where the mean temperature of the coldest month falls below -0.5 ℃ (Iverson, 1944). In our field studies, most *Ilex glabra* cultivars could tolerate – 25 ℃. Subzero temperatures in plants usually lead to the freezing of tissue water, which entails ice growth, cell dehydration, osmotic concentration, and complex freeze-induced cell-volume changes (Rajashekar, 2000). This can result in the death of plant tissues or even whole plants. The cellular sap concentration in current year leaves of natural stand english holly increased, while osmotic potential of the cellular sap decreased and cell volume reduced when the minimum ambient temperature decreased (Rütten and Santarius, 1998).

Plant cold hardiness varies seasonally. A gradual increase in cold resistance of english holly leaves from about -9 ℃ in late summer to -24 ℃ in mid-winter when the minimum air temperature decreased to 0 ℃ or below has been reported (Rütten and Santarius, 1998). Usually, the maximum frost hardiness occurs in the fall season when the temperature gradually decreases and subsequently it is lost when temperatures become favorable for growth in the spring. We found that the maximum frost hardiness of *Ilex glabra* ‘Densa’, *Ilex glabra* f. *leucocarpa*, and *Ilex glabra* ‘Shamrock’ occurred in mid to

late January (data not presented). This is similar to the previous studies (Dirr and Lindstrom, 1990), in which most of the tested *Ilex* species reached their maximum cold hardiness in mid-January. In midwinter, plants can quickly deacclimate to lose their cold hardiness to some extent when they are exposed to warmer ambient temperature (Quamme, 1987). Our previous experiments indicated that deacclimation significantly reduced cold hardiness of *Ilex glabra* and its effect varied with cultivars (Sun and Zhang, 2008). A total of 33.5% and 17.2% of cold hardiness reduction were observed for *Ilex glabra* 'Densa' and *Ilex glabra* 'Shamrock', respectively, when they were deacclimation for 9 days in a greenhouse (20.8 ± 1.93 ℃).

A relationship between light and plant cold acclimation and subsequent frost tolerance is well established (Levitt 1972). Changes in light intensity and day length trigger plant cold acclimation and increase frost tolerance in many species through the accumulation of cryoprotective metabolites (Levitt, 1980). During the winter, photoinhibition can significantly damage the photosynthetic systems of english holly (Groom et al., 1991). English holly exhibited significant photoinhibition under high light conditions in the field and chronic photoinhibition was most common in winter (Valladares et al., 2005). Low temperatures decrease the dissipation of absorbed light enery and increase the susceptibility of leaves to photoinhibition (Greer et al., 1986; Ögren et al., 1984).

Other factors (such as snow pack, animal and insect damage, and mechanical injury) and winter desiccation might affect to some degree the cold hardiness of *Ilex glabra*. Buried beneath snow, plants are exposed to mild temperatures close to 0 ℃ and protected from excessive irradiation and winter desiccation. In our winter protection

practice, a total of 17.7% plants experienced mechanical injury, such as branch and bark damage, in a snow pack area, 7.6% in a less snow covered area (such as a canopy), while less than 3.2% in other snow-free places (e.g. cold storage, fabric cover, microfoam and plastic winter house) (Data not shown).

CONCLUSIONS

Based on both field trials and controlled laboratory tests of *Ilex glabra* cultivars, *Ilex glabra* 'Shamrock' was the most cold-hardy cultivar; *Ilex glabra* f. *leucocarpa*, *Ilex glabra* 'Viridis' and *Ilex glabra* 'Nigra' were the least cold-hardy cultivars; while *Ilex glabra* 'Compacta', *Ilex glabra* 'Densa', *Ilex glabra* 'Chamzin', *Ilex glabra* 'Pretty Girl', *Ilex glabra* had intermediate cold hardiness. Laboratory tests were strongly correlated with winter damage in field trials and could be used to predict the cold tolerance of *Ilex glabra* cultivars in the field. Many factors contribute to cold hardiness, including: plant cultivars, tissues, temperature, deaacclimation, photoinhibition and others (e.g. snow pack, mechanical injury).

BIBLIOGRAPHY

Andrew, R. 1984. A practical pollen guide to the British flora. Technical Guide 1. Quaternary Research Association, Cambridge, UK.

Bannister, P. and G. Neuner. 2001. Frost resistance and the distribution of conifers. p. 3-21 *In*: F.J. Bigras and S.J. Colombo (Editor). Conifer cold hardiness. Kluwer Academic Publishers, Dordrecht, Netherlands.

Bernasconi, N. K., L. A. Mroginski, P.A. Sansberro, and H.Y. Rey. 1998. Micropropagation of yerba mate (*Ilex paraguariensis* St. Hil.): effect of genotype and season on the in vitro establishment of explants. Phyton Buenos Aires 62(1-2):95-99.

Binnie, S.C., S.C. Grossnickle, and D.R. Roberts. 1994. Fall accumulation patterns of interior spruce seedlings and their relationship to changes in vegetative storage proteins. Tree Physiology 14:1107-1120.

Burr, K.E., R.W. Tinus, S.J. Wallner, and R.M. King.1990. Comparison of three cold hardiness tests for conifer seedlings. Tree Physiology 6:351-369.

Cappiello, P.E. and L.E. Littlefield. 1994. Woody landscape plant cold-hardiness ratings. Maine Agricultural and Forest Experiment Station Technical Bulletin 156.

Dafni, A. 1992. Pollination ecology: a practical approach. Oxford University Press Inc., NY.

Davies, F.S. and D.W. Buchanan. 1979. Fruit set and bee activity in four Rabbiteye blueberry cultivars. Proceeding of Florida State Horticultural Society 92:246-247.

Dirr, M.A. 1998. Manual of woody landscape plants, their identification, ornamental characteristics, culture, propagation and uses. Stipes Publishing L.L.C., Champaign, IL. pp 461-464.

Dirr, M.A. and C.W. Heuser, Jr. 1987. The reference manual of woody plant propagation, from seed to tissue culture. Varsity Press, Inc. Athens, GA.

Dirr, M.A. and J.H. Alexander III. 1991. *Ilex glabra*-the inkberry holly. Arnoldia 51(2): 16-22.

Dirr, M.A. and O.M. Lindstrom, Jr. 1990. Leaf and stem cold hardiness of 17 broadleaf evergreen taxa. Journal of Environmental Horticulture 8(2):71-73.

Dubay, D.T. and W.M. Murdy. 1983. Direct adverse effects of SO_2 on seed set in *Geranium carolinianum* L.: a consequence of reduced pollen germination on the stigma. Botanical Gazette 144:376-381.

Dumas, C. and R.B. Knox. 1983. Callose and determination of pistil viability and incompatability. Theoretical and Applied Genetics 67:1-10.

Eisenbeiss, G.K. 1990. Sexual compatibilities among holly species. Holly Society Journal 8 (1):7-10.

Etile, D.S., María, T.C. Stella, M.C. and Nájera, M.T. 2002. Taxonomic determination of therapeutic Argentine species of *Ilex*. Pharmaceutical Biology 40(1):2-15.

Fresderick, W.H. 1975. 100 great garden plants. Timber Press, Portaland, OR.

Gabriel, C. 2002. Inkberry plant named 'Gold Mine'. United States Patent, PP14233.

Galle, F.C. 1997. Hollies: the genus *Ilex*. Timber Press, Portland, OR.

Givnish, T.J. 1984. Leaf and canopy adaptations in tropical forests, p.51-84. In: Medina, E. H., Mooney, A. and Vasques-Yanes, C. (eds.). Physiological ecology of plants in the wet tropics. Dr. W. Junk Publishers, The Hague.

Golan-Goldhirsh, A., Jones, R. and Rowland, L.J. 2001. AFLP markers for sex determination in an *Ilex* species. Acta Horticulturae 546:591-595.

Gottlieb, A.M., Giberti, G.C. and Poggio, L. 2005. Molecular analysis of the genus *Ilex* (Aquifoliaceae) in southern South America, evidence from AFLP and ITS sequence data. American Journal of Botany 92:352-269.

Greer, D.H., J.A. Berry, and O. Björkman. 1986. Photoinhibiton of photosysthesis in intact bean leaves: role of light and temperature, and requirement for chloroplast protein synthesis during recovery. Planta 168:253-260.

Griffith, M.P. 1997. *Ilex glabra* plant named 'Green Billow'. United States Patent, PP10678.

Groom, Q.J., N.R. Baker, and S.P. Long. 1991. Photoinhibition of holly (*Ilex aquifolium*) in the field during the winter. Physiologia Plantarum 83:585-590.

Halbritter, H., M. Weber, R. Zetter, A. Frosch-Radivo, R. Buchner, and M. Hesse. 2007. PalDat-Illustrated handbook on pollen terminology. Vienna. 66pp.

Havis, J.R., R.D. Fitzgerald, and D.N.Maynard. 1972. Cold hardiness response of *Ilex crenata* Thumb. cv. Hetzi roots to nitrogen source and potassium. HortScience 7:195-196.

Hicks, G.R., E. Rojo, S. Hong, D.G. Carter, and N.V.Raikhel. 2004. Germinating pollen has tubular vacuoles, displays highly dynamic vacuole biogenesis, and requires *VACUOLESS1* for proper function. Plant Physiology 134:1227-1239.

Hodnett, G.L., B.L. Burson, W.L. Rooney, S.L. Dillon, and H. J. Price. 2005. Pollen-pistil interaction result in reproductive isolation between *Sorghum bicolor* and divergent *Sorghum* species. Crop Science 45:1403-1409.

Hu, C.Y. 1975. In vitro culture of rudimentary embryos of eleven *Ilex* species. Journal of American Society for Horticultural Science 100:221-225.

Hu, C.Y. 1976. Light-mediated inhibition of in vitro development of rudimentary embryos of *Ilex opaca*. American Journal of Botany 63:651-656.

Hu, C.Y. 1989. Holly (*Ilex* spp.), p. 412-487. In: Y.P.S. Bajaj (eds.). Biotechnology in agriculture and forestry. V.5 Springer-Verlag, Berlin.

Hu, C.Y., F. Rogalski, and C. Ward. 1979. Factors maintaining *Ilex* rudimentary embryos in the quiescent state and the ultrastructural changes during in vitro activation. Botanical Gazette 140(3):272-279.

Hu, D.Y., Zhang, Z.S., Zhang, D.L., Zhang, Q.X. and Li, J.H. 2005. Genetic relationship of ornamental peach determined using AFLP markers. HortScience 40:1782-1786.

Hume, H.H. 1953. Hollies, The Macmillan Company, Macmillan, N.Y. pp14.

Iowa State University Extension, 2007. ISU extension news release, winterberry adds color to the winter landscape. 24 Nov. 2008. <http://www.extension.iastate.edu/newsrel/2002/dec02/dec0221.html>.

Iverson, J.1944. *Viscum*, *Hedera* and *Ilex* as climatic indicators. Geologiska Föreningens Stockholm Förhandlingar 66:463-483.

Jones, C.J., Edwards, K.J., Castiglione, S., Winfield, M.O., Sala, F., van de Wiel, C.C.M., Bredemeijer, G.M.M., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A. and Karp, A. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding 3:381–390.

Kho, Y.O. and J. Baer. 1968. Observing pollen tubes by means of fluorescence. Euphytica 17:298-302.

Levitt, J. 1972. Responses of plants to environmental stresses. Vol. 1. Chilling, Freezing and high temperature stress. Academic Press, NJ.

Levitt, J. 1980. Response of plants to environmental stresses. 2nd ed. Academic Press, NY.

Loizeau, P.A. and R. Spichiger. 2004. Aquifoliaceae, p.26-28. In: N. Smith, S.A. Mori, A. Henderson, D.W. Stevenson, and S.V. Heald (eds.). Flowering plants of the neotropics. Princeton University Press, Princeton, NJ.

Luna, C., P.A. Sansberro, L.A. Mroginski, and J. Tarragó. 2003. Micropropagation of *Ilex dumosa* (Aquifoliaceae) from nodal segments in a tissue culture system. Biocell 27 (2):205-212.

Maddison, W.P. and Maddison, D.R. 2006. Mesquite: A modular system for evolutionary analysis. Version 1.12. June 8 2009. <http://mesquiteproject.org>.

Maine Department of Conservation. 2006. Natural areas program, maine rare plant list and rare plant fact sheets, PDAQU010F0. 11 Nov. 2006. < http://www.maine.gov>.

Majada, J.P., R. Sánchez-Tamés, M.A. Revilla, and A. Casares. 2000. Micropropagation of *Ilex aquifolium* L. In Vitro Cellular Developmental Biology - Plant 36 (6):521-526.

Martin, F.W. 1959. Staining and observing pollen tubes in the style by means of fluorescence. Stain Technology 34:125-128.

Mattis, P.R., H.J. Swartz, and G. Eisenbeiss. 1995. Development of embryo rescues and shoot regeneration techniques in *Ilex*. Journal of Environmental Horticulture 13(4):164-168.

Milutinovic, M., V. Rakonjac, and Nikolic, D. 1998. Functionality of pollen and fruit set in sour cherry cultivars. Acta Horticulturae 468:591-594.

Moore, P. D., J. A. Webb, and M. E. Collinson, 1991. Pollen analysis. 2nd ed. Blackwell Scientific Publications, Oxford, UK.

Morte, M.A., E. Olmos, E. Hellin, A. Piqueras. 1991. Micropropagation of holly (*Ilex aquifolium*). Acta Horticulturae 289:139-140.

Murashige, T. and F.A. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

Nair, A., Zhang, D. and Hu, D. 2005. Application of AFLP markers on taxon discrimination of cultivated *Stewartia*. HortScience 40:1041.

Oddie, R.L.A. and J.A. Mccomb. 1998. Stigma receptivity in Eucalyptus camaldulensis DEHNH. Silvae Genetica 47(2-3):142-146.

Ögren, E. 1988. Photoinhibition of photosynthesis in willow leaves under field condition. Planta 175:229-236.

O'reilly, C., C.P. Harper, N. McCarthy, and M.Keane. 2001. Seasonal changes in physiological status, cold storage tolerance and field performance of hybrid larch seedlings in Ireland. Forestry 74:407-421.

Perveen, A., S. Alikhan, R. Abid. 2007. Maintenance of pollen germination capacity of *Carica papaya* L. (Caricaceae). Pakistan Journal of Botany 39:1403-1406.

Quamme, H.A. 1987. Low temperature stress in Canadian horticultural production-an overview. The Canadian Journal Plant Science 67:1135-1149.

Rajashekar, C.B. 2000. Cold response and freezing tolerance in plants. p.321-341. In: R.E. Wilkinson (ed.). Plant-environment interactions. 2nd ed. Marcel Dekker, Inc., NJ.

Regan, R.P., R.L. Tichnor, D.D. Hemphill, Jr., T.H.H. Chen, P. Murakami, and L.H.Fuchigami. 1990. Influence of winter protection covers on survival and hardiness of container grown *Ilex crenata* Thunb. 'Green Island' and *Euonymus fortunei* (Turcz.) 'Emerald' n Gold'. Journal of Environmental Horticulture 8(3):142-146.

Rietveld, W.J. and R.W. Tinus. 1987. A simple method for evaluating whole-plant cold hardiness. Tree Plant Notes 38:16-18.

Ritchie, G.A. 1984. Effect of freezer storage on bud dormancy release in douglas-fir. Canadian Journal of Forest Research 14:186-190.

Rhodus, T. 2006. OSU pocket gardener, shrubs, *Ilex* × *meservea*. 24 Nov. 2008. <http://hcs.osu.edu/pocketgardener/source/index.html>.

Rütten, D. and K.A. Santarius. 1998. Cold acclimation of *Ilex aquifolium* under natural conditions with special regard to the photosynthetic apparatus. Physiologia Plantarum 72:807-815.

Sansberro, P. A., H.Y. Rey, A. Bernardis, C. Luna, M. M. Collavino, and L.A. Mroginski. 2000. Plant regeneration of *Ilex paraguariensis* (Aquifoliaceae) by in vitro culture of nodal segments. Biocell 24(1):53-63.

Sansberro, P. A., H.Y. Rey, and L.A. Mroginski. 2001a. In vitro culture of zygotic embryos of *Ilex* species. HortScience 36:351-352 (abstr.).

Sansberro, P. A., H.Y. Rey, L.A. Mroginski., and M. A. Krivenki. 2001b. Plant regeneration from *Ilex* spp. (Aquifoliaceae) in vitro. Biocell 5:139-146.

Sansberro, P. A., H.Y. Rey, L.A. Mroginski, M.M. Collavina. 1998. In vitro culture of rudimentary embryos of *Ilex paraguariensis*: responses to exogenous cytokinins. J. Plant Growth Regulation 17:101-105.

Sansberro, P.A., H.Y. Rey, L.A. Mroginski, and M.M. Collavino. 1999. In vitro plant regeneration of *Ilex paraguariensis* (Aquifoliaceae). In Vitro Cellular Developmental Biology-Plant. 35(5):401-402.

Solomon, A.M., J.E. King, P.S. Martin, and J. Thomas. 1973. Further scanning electron photomicrographs of southwestern pollen grains. Journal Arizona Academy of Sciences 8 (3):135-157.

Sun, Y.P. and D.L. Zhang. 2008. Deacclimation significantly reduced cold hardiness of Densa and Shamrock inkberry. HortScience 43(4):1273-1278 (abstr.).

The Arnold Arboretum of Harvard University. 2009. Plant inventory, *Ilex glabra*. The President and Fellows of Harvard University. June 8 2009. < http://arboretum.harvard.edu/index.html >.

The Dawes Arboretum. 2007. Collections/Gardens. 19 Mar. 2007 <http://www.dawesarb.org/>.

The University of Arizona. 2008. Palynology. 24 Nov. 2008. <http://www.geo.arizona.edu/palynology/pid00045.html>.

United States Department of Agriculture. 2006. Natural Resources Conservation Service, Plants Topics. Threatened &Endangered. 11 Nov. 2006. <http://plants.usda.gov/>.

United States Department of Agriculture. 2009. Natural resources conservation service, distribution maps of dominant soil orders. 20 May 2009. <http://soils.usda.gov/technical/classification/orders/>.

US Patent and Trademark Office, 2008. Patent full-text and full-page image databases. 30 Nov. 2008. <http://patft.uspto.gov/>.

USDA National Agricultural Library, 2007. Article citation database, *Ilex* hybrids. 24 Nov. 2008. <http://agricola.nal.usda.gov/cgi-bin/Pwebrecon.cgi.>.

Valladares, F., D. Tena, F. Suarez, J.A. Pardos, D. Sanchez-Gomez, S. Arrieta, I. Aranda, and D. Lorenzo. 2005. Shade tolerance, photoinhibition sensitivity and phenotypic plasticity of *Ilex aquifolium* in continental Mediterranean sites. Tree Physiology 25:1041-1052.

Vargas, F.J., M.A. Romero, J. Clavé, J. Santos, I.Battle, and M. Rovira. 2002. Fruit set in controlled crosses of almond cultivars. Acta Horticulturae 591:365-370.

Vos, P., Hogers, R., Bleeker, M., Reijans, M. , Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper M. and Zabeau. M. 1995. AFLP: A new technique for DNA fingerprinting. Nucleic Acids Research 23:4407–4414.

Western Maine Nurseries, 2007. Conifer & woody ornamentals catalog. 19 Mar. 2007 < http://www.wmnurseries.com/ >.

Yap, I.V. and Nelson, R.J. 1996. Winboot. International Rice Research Institue, Manila, The Philippines. 25 pp.

Zampini, J.W.1988. *Ilex glabra* named ‘Chamzin’. United States Patent, PP06962.

Zaniolo, S. R. and F. Zanette. 2001. Micropropagation of the erva-mate through culture of nodal segments. Scientia Agraria 2(1-2):39-44 (abstr.).

Zhang, D.L., Dirr, M.A. and Price, R.A. 2000. Discrimination and genetic diversity of *Cephalotaxua* accessions using AFLP markers. Journal of the American Society for Horticultural Science 125:404-412.

Zhang, D.L., Hu, D.Y., Li, J.H. and Anisko. T. 2008. Relationship of *Buxus* L. accessions from Longwood Gardens based on morphological and molecular characters. Journal of Central South University of Forestry and Technology 28(4):1-7.

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