The development of improved willow clones for eastern North America¹

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Efforts aimed at genetic improvement of *Salix* are increasing in North America. Most of these are directed towards developing improved clones for biomass production, phytoremediation, nutrient filters, and stream bank stabilization in the Northeast and North-central United States. Native species are of primary interest, but a small number of clones containing non-native germplasm are also being used in the breeding program to provide valuable traits. Parent combinations for controlled crosses are being selected with the hope of maximizing the probability of producing clones exhibiting heterosis for traits of interest, such as rapid early growth, pest resistance, general adaptability, etc. The present strategy is to test as many parent clone combinations as possible, and then repeat the most promising crosses to produce large families from which the best clones will be selected for further testing. Molecular fingerprinting technology will be applied to accelerate the rate of improvement. National and international cooperation would facilitate regional clone development and promotion of willow as a bioenergy crop.

Key words: *Salix*, biomass production, breeding, heterosis, molecular fingerprinting

Les efforts destinés à l'amélioration génétique du *Salix* croissent sans cesse en Amérique du Nord. La majeure partie des efforts est concentrée sur le développement de clones améliorés pour la production de biomasse, la phytoremédiation, les filtres d'éléments nutritifs et la stabilisation des rives des cours d'eau dans le nord-est et le centre-nord des États-Unis. L'intérêt porte avant toute chose sur les espèces indigènes, mais un petit nombre de clones contenant des cellules embryonnaires non indigènes sont également utilisés dans le programme de reproduction afin d'apporter des caractéristiques intéressantes. Les combinaisons parentales pour obtenir des descendants de provenance contrôlée sont choisies dans le but de maximiser la probabilité de produire des clones démontrant une hétérogénéité des caractéristiques intéressantes, comme la croissance initiale rapide, la résistance aux ravageurs, l'adaptabilité générale, etc. La stratégie actuelle est de tester autant de combinaisons parentales de clones que possible, et de reproduire par la suite les descendants les plus prometteurs pour produire des grandes familles desquelles les meilleurs clones seront choisis pour poursuivre les tests. La technologie de l'empreinte digitale moléculaire sera utilisée pour accélérer le taux d'amélioration. La coopération nationale et internationale faciliterait le développement de clones régionaux et la promotion du saule en tant que ressource bioénergétique.

Mots-clés : *Salix*, production de biomasse, reproduction, hétérogénéité, empreinte digitale moléculaire

History of Willow Genetic Improvement in the United States

The documented history of *Salix* culture in North America starts during the period from 1840–1850 when German immigrants began producing willows for basketry in New York State and Pennsylvania. Early basket makers used native species, but soon began importing and planting willows from Europe, particularly *Salix purpurea* L., to increase product uniformity. The importance of selecting clones with desirable form and wood qualities that were adapted to local soils and pests was recognized in the earliest days of willow culture (Hubbard 1904). Early willow improvement efforts in the United States included field trials of 70 European clones in the 1880s (Simpson 1898). The willow basket industry in the U.S. reached a peak during the 1870s and continued until World War II.

Interest in growing and using poplars and willows for energy and to produce high-value chemicals and other bio-based products has increased in the United States during the past two decades, due to unstable petroleum prices, a desire to decrease dependency on imported fossil fuels, and concern about the environmental impacts of burning fossil fuel. Exceptionally high productivity rates have been achieved with hybrid poplars in the Pacific Northwest, but in the Northeast and North-central United States *Septoria* canker severely limits growth of many hybrid poplar clones (Abrahamson *et al.* 1990, Ostry and McNabb 1990). However, *Septoria* is not a disease problem for willows. Because of disease problems with hybrid poplars and reports of high biomass yields achieved with willows grown under intensive culture systems in Europe (Stott 1984, Christersson 1986), *Salix* became the primary genus of interest for short-rotation woody biomass production in the Northeast United States during the late 1980s. Despite the availability of improved clones from Europe, genetic improvement of willows became necessary to obtain locally-adapted clones that express increased average production rates, improved growth form, and improved levels of pest resistance, thereby improving the economic attractiveness of growing willows for bioenergy and bio-based products.

Willows are also being planted in North America for purposes other than bioenergy with increasing frequency. Phytoremediation is an emerging technology being used for remediation of contaminated soils and filtration of water throughout North America (Isebrands 2000) and Europe

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(Perttu 1999). Phytoremediation involves the direct use of plants to decrease risks from contaminated soil, sludge, sediments, and ground water by contaminant removal, degradation, or confinement (US EPA 1998). Willows (*Salix* spp.), along with poplars (*Populus* spp.), are the most commonly used tree species for phytoremediation, due to their rapid growth rate, ease of propagation, and demonstrated contaminant uptake capability. Willows are currently under study across North America for a variety of phytoremediation and riparian buffer strip applications (Isebrands and Karnosky 2001).

Willows are widely used in riparian management in North America. Dormant willows are planted as cuttings, posts, and brush mattresses for stream bank stabilization (Schultz *et al.* 2000). Willows are also used to stabilize reservoir banks. Research on using willows for purification of waste-water and sludge has been in progress in Sweden since the 1980s, and *Salix* clones have been shown to be effective for removing large quantities of nutrients from land-applied wastes in Europe (Perttu and Kowalik 1997) and even the removal of caesium from radio-contaminated soils (Sennerby-Forsse *et al.* 1993). Nutrient filters composed of grasses have been successfully used for decades in North America (Young *et al.* 1980), but planting willows in filter strips is relatively new. Genetic improvement through selection and breeding may be necessary to develop clones specifically for planting in filter strips and clones that are more effective for stream and reservoir bank stabilization.

The *Salix* Germplasm Resource in North America

North America contains a *Salix* germplasm resource that is large, rich in genetic diversity, and virtually unexplored (Zsuffa 1988). About 70 willow species are native to North America (Fowells 1965). In addition, willow species introduced from Europe, including *S. alba* and *S. purpurea*, have become naturalized in the Northeast and North-central United States and Southeast Canada (Voss 1985). Many native, naturalized and introduced species have desirable traits for biomass production, phytoremediation, nutrient filters, and stream bank stabilization, but few of them have been tested across a wide variety of geographic areas or site types (Table 1).

A genetically diverse willow breeding population is necessary to provide sufficient variation from which clones can be developed that are adapted to a wide variety of purposes and site conditions in eastern North America. A broad genetic base in the breeding population will ensure flexibility in the breeding program and the potential to adapt to changes in types of sites desired for planting and/or pest problems. Maintaining a genetically diverse germplasm collection is critical for the long-term success of willow breeding efforts (Eriksson *et al.* 1984). From 1987 through 1999, researchers at the State University of New York College of Environmental Science and Forestry (SUNY-ESF) assembled a breeding population of willow containing more than 500 clones. Most of the clones came from Ontario, Canada (developed by co-operators at the University of Toronto) and from wild stands across New York and Pennsylvania, and are of species native or naturalized in the Northeast. However, because many of the clones are closely related and several species are represented by a small number of clones, the genetic diversity in this collection will be expanded in order to sustain a long-term breeding program.

The overall goal of our willow breeding population development effort is to assemble a large and diverse germplasm collection that will permit continuous improvements over many breeding generations. Specific goals include doubling the number of *S. eriocephala* clones from approximately 100 to 200 and expanding the number of *S. purpurea* clones from 20 to 150. Based on genetic diversity estimates for *S. eriocephala*, approximately 150 clones collected from a broad geographic range should contain most of the alleles present in the species (Aravanopoulos *et al.* 1999). Up to 75 clones of both *S. lucida* and *S. nigra*, and a small number of clones of other *Salix* species will be collected. Clones from non-native species will be obtained from colleagues in Asia and Europe to provide additional useful genes that may be incorporated into production clones through advanced-generation breeding.

Efforts to expand our willow germplasm collection began in July 2000. We collected 160 willow clones from native stands across New York, Ohio, Pennsylvania, Connecticut, and Massachusetts and propagated them in a greenhouse for field-planting during spring 2001. Trees were selected based on vigour, form, and insect and disease resistance relative to other trees of the same species in the immediate vicinity. Most of the clones collected thus far were identified as *S. eriocephala* and *S. purpurea* with smaller numbers of *S. lucida* and *S. nigra* also included. Collections will be completed in Michigan, Minnesota and Wisconsin during 2001.

All of the new clones collected for this project will be planted in observation trials on two sites, one in New York and the other in Wisconsin. The purposes of these trials are: 1) to assess the phenotypic variability in new clones collected from a wide geographic region, 2) to determine how the clones perform in locations distant from their origin, and 3) to identify clones for future breeding work with desirable traits. The trials will also serve as clonal archives duplicated on two sites to minimize the chance of losing clones. Intra- and inter-specific hybrid clones produced in our previous breeding efforts will be included in these trials to compare with clones collected from native stands. Production clones currently being planted will also be included in these trials to serve as controls ("commercial checks").

Clones will be selected for use in the first parental generation based on measurements and observations completed during the first and second growing seasons after the initial coppice. Selection criteria will vary, depending on the desired end-use of the clones. Statistical analyses will be used to determine if clonal differences exist in initial height growth, average stem diameter, stool dry weight, number of stems per stool, disease and/or insect resistance, and crown form (Table 2) and if clone-by-site interaction is large. If large clone-by-site interactions are observed, then selection of superior clones will be accomplished in a site-specific manner. Large variability will be maintained in the base breeding population to allow selection for numerous end-uses.

Background on Genetic Improvement of Willow in North America

A limited amount of willow breeding and selection has been conducted in North America. Native species were bred in the 1980s by researchers at the University of Toronto to produce F_1 intra- and interspecific hybrids for bioenergy production (Mosseler 1985). Controlled breeding of *S. eriocephala* and

Table 1. Classification of *Salix* species tested in biomass production trials in the Northeast or North-central United States.

Sectional Classification ¹	Species	Form	Native Range
Subgenus <i>Salix</i>	Humboltianae		
	<i>S. amygdaloides</i> Anderss.	tree	N.C. USA
	<i>S. nigra</i> Marsh	tree	Eastern USA
	Salix		
	<i>S. alba</i> L. <i>S. fragilis</i> L.	tree tree	Central Europe Central Europe
Subalbae	<i>S. matsudana</i> Koidz.	tree	Northern China
Salicaster	<i>S. lucida</i> Muhl.	shrub	Eastern N. America
Longifoliae	<i>S. exigua</i> Nutt.	shrub	North America
Subgenus <i>Vetrix</i>	Vetrix		
	<i>S. bebbiana</i> Sarg.	shrub	transcontinental
	<i>S. discolor</i> Muhl.	shrub	North America
	<i>S. petiolaris</i> Smith	shrub	North America
	Vimen		
	<i>S. dasyclados</i> Wimm.	tree	C. Europe to E. Siberia
	<i>S. pellita</i> Anderss.	shrub	E. Canada
	<i>S. sachalinensis</i> Schmidt.	shrub	Japan
	<i>S. viminalis</i> L.	shrub	C. Europe to E. Siberia
	Cordatae		
	<i>S. eriocephala</i> Muhl.	shrub	North America
Helix	<i>S. miyabeana</i> Seeman	shrub	E. Siberia, China
	<i>S. purpurea</i> L.	shrub	Europe

¹Sectional classification according to Dorn (1976).

S. sericea by researchers at Vassar College in Poughkeepsie, NY yielded intra- and interspecific hybrids, which were used in studies on herbivore preference and gene introgression (Roche and Fritz 1997, Hardig *et al.* 2000). Our group initiated a program of controlled willow breeding in 1998 to produce willows for bioenergy or conversion to high-value chemicals. Native and introduced species were used to produce F₁ intra- and interspecific progeny in 1998, and some of these clones were crossed in 2000 to produce F₂ progeny (Kopp *et al.* 2000). Thus, very few multiple-generation, structured *Salix* pedigrees exist today that could be useful in gene expression studies.

Interspecific hybridization of willows may yield superior clones through the combination of desirable traits, heterosis, and greater phenotypic stability in varied environments (Stettler *et al.* 1996). Many examples of successful interspecific hybridization with willows have been reported (Argus 1974, Hathaway 1977, Zsuffa *et al.* 1984, Larsson 1998). However, interspecific hybridization of willow species native to North America generally resulted in F₁ hybrid progeny, which often had decreased vigour and poor survival, though some individuals exceeded the growth of either parent (Mosseler 1990). *S. eriocephala* females failed to yield viable seed in any interspecific hybridization attempts in studies completed by Mosseler (1990) or Kopp (2000). *S. eriocephala* is known to be difficult to hybridize when it is the pistillate parent because of inhibition of pollen tube growth (Mosseler 1989). These findings suggest that *S. eriocephala* males should be used in interspecific hybridization efforts with this species.

Vigorous growth by *S. viminalis* in northern Europe is well documented (Gullberg 1993, Rönnerberg-Wästljung and Gullberg 1996, Larsson 1998), but various insect pests, par-

ticularly potato leafhoppers (*Empoasca fabae* Harris), cause substantial damage and associated poor growth of this species in the Northeast and North-central United States (Kopp 2000). Preliminary information from an unreplicated field trial suggests that progeny of crosses between *S. viminalis* and *S. miyabeana*, an Asian species, may be less susceptible to potato leafhopper attack. If substantiated, *S. viminalis* germplasm may contribute to the production of high-yielding hybrid clones for use in North America once genes for resistance to potato leafhopper can be introduced.

Role of Biotechnology in Willow Tree Improvement

Application of biotechnology to willow tree improvement may accelerate the rate of genetic gain. Recently developed molecular techniques may enable willow breeders to narrow the pool of candidate breeding trees, so that effort is not wasted on crosses that have low probability of producing desirable progeny. Molecular markers can help to characterize populations, estimate genetic variability within and among wild or generated populations, and identify individuals at a young age that will express a trait at maturity (Dinus and Tuskan 1997). Molecular fingerprinting can be used to produce genetic maps for traits of interest. A genetic map including restriction fragment length polymorphism (RFLP), sequence-tagged sites (STSs), and RAPD markers was produced for a hybrid poplar family derived from an inbred F₂ family (Bradshaw *et al.* 1994). This map was used to identify quantitative trait loci (QTLs) with large effects on growth, form, and phenology (Bradshaw and Stettler 1995). Similar techniques may be applied to willow breeding. We have generated populations of F₁ and F₂ proge-

Table 2. Traits of interest for willow biomass production.

Mensurational Traits	Physiological Traits
Biomass production ^a	Sylleptic shoot production ^a
Stem height ^a	Insect susceptibility ^a
Stem diameter ^a	Pathogen susceptibility ^a
Number of stems ^a	Mammal browse susceptibility ^a
Stool form ^a	Herbicide tolerance ^a
Survival ^a	Foliage chemistry ^b
Wood density ^b	Cuticle thickness/chemistry ^b
	Leaf area ^b
	Leaf nitrogen percentage ^b
	Leaf weight/unit area ^b
	Photosynthesis rate ^b
	Budburst date ^b
	Growth cessation date ^b

^aTraits that will be measured on trees at all sites.

^bTraits that will be measured as time and funding permit.

ny, which may be amenable to molecular mapping, and are currently developing AFLP and microsatellite markers for willow.

Genetic transformation research with *Salix* is not as advanced as for *Populus*. In Sweden, transformation of a limited number of *Salix* clones was successful using *Agrobacterium tumefaciens*, but plantlets could not be produced from transformed calli (Vahala *et al.* 1989). *Salix lucida* was transformed using *A. tumefaciens* and plantlets were produced, but the transferred genes were not stable (Xing 1995). Efforts to develop a transformation system for *Salix* are in progress at SUNY-ESF. Genes controlling flowering in *Salix* will be identified, with the objective of altering their expression in transgenic plants to produce clones that do not flower. As noted above, this strategy might then be used to essentially eliminate the possibility of introgression of genes from introduced species into natural populations of willow through cross-pollination.

Willow Breeding Strategy for the Northeast and North-central United States and Southeast Canada

We have devised a strategy for the genetic improvement of willows with the goal of rapidly producing clones that are adapted to growth in intensively cultured biomass plantings in the Northeast and North-central United States and Southeast Canada (Fig. 1). The system can be used to produce clones for other purposes by altering the selection criteria. This strategy is based on our initial results and from reports on improvement efforts in Sweden and Canada. Briefly, the strategy assumes that hybrid vigour for growth rate or other traits of interest can be obtained by controlled intra- and inter-specific mating and depends on identifying specific favourable combinations of parents. Because reliable prediction of desirable parent clone combinations is not currently possible, large numbers of crosses will be completed yielding many families with small numbers of progeny, which will be field tested (Fig. 1). Crosses that appear promising will be repeated to produce large numbers of progeny. High selection intensity will be applied to these large families and selected individuals will be vegetatively propagated, resulting in large genetic gain.

Efforts are in progress to identify crosses that have potential to generate exceptional progeny and are worth repeating

to generate large families. More than 120 relatively small full-sib families were produced by controlled pollination from 1998 to 2000 (Kopp 2000). Progeny from these crosses are currently being tested in field trials. More crosses to identify favourable family combinations (approximately 50) will be completed when flowers and juvenile growth and pest resistance data are available from clones collected or produced from 1998 to 2001. Parent clone combinations will be selected to maximize the probability of generating progeny exhibiting heterosis for biomass production or other traits of interest. Molecular fingerprint data may also be used to identify particular parent combinations that are likely to yield desirable clones based on maximizing the amount of molecular diversity among crossed genotypes.

After large numbers of families containing small numbers of progeny have been evaluated in the field, five to 10 crosses will be selected that are likely to yield progeny exhibiting outstanding performance. These selected crosses will be repeated to produce families with large numbers of progeny. Data from small families produced from 1998 to 2000 will be available in 2001, so that the first set of crosses to produce large families will be made during 2002. A second set of selected crosses to produce large families will be completed when new, promising parent combinations are identified.

Selection of exceptional individuals to be propagated for operational plantings should ideally be from families exhibiting large variability with 500 or more individuals (Tuskan 1997). Such families will be evaluated in the field in a non-replicated screening trial. The top 5% of the progeny in each family will be selected based on measurements of traits of interest completed at the end of the first coppice-growing season. Large numbers of clonal replicates from each selected clone will be produced using small one- or two-bud cuttings, then transplanted to a fertilized, irrigated cutting orchard. Dormant one-year-old stems from the cutting orchard will be harvested to provide unrooted cuttings for clone-site trials that will be established using operational spacing and cultural practices on at least two sites.

Basic genetic studies are necessary to obtain heritability estimates for traits of interest. Progeny tests containing 34 full-sib *S. eriocephala* families from an 8 x 7 factorial mating design completed during 1998 were established in 2000 to provide these estimates (Kopp 2000). Ten randomly selected seedlings from each family and the parents were vegetatively propagated and dormant, hardwood cuttings were planted in replicated tests at LaFayette and Tully, NY. These tests will permit estimates of general combining ability, specific combining ability, additive and non-additive genetic variance components and genotype-by-environment interaction for any traits of interest.

Advanced-generation breeding strategies, i.e., F₃ and beyond, are not clearly defined at this point in willow development. There are multiple, equally likely alternatives that may ultimately prove useful. It is unlikely that insights into which alternative should be pursued will come from breeding programs in any other woody species. Even with *Populus*, the generation intervals are considerably longer than with *Salix*. As a result, a willow breeding program will face decisions on advanced-generation breeding well before most tree breeding programs are into their F₂ generation. Therefore, concessions need to be made early in the formation of any willow improvement program that will 1) allow flexibility in pursu-

Fig. 1. Generalized willow breeding strategy for the Northeast and North-central United States.

ing alternative strategies and 2) quickly determine the appropriate advanced-generation approach.

Today, there is considerable debate on whether willow advanced-generations should be formed using simple recurrent selection, reciprocal recurrent selection or to abandon these strategies altogether for random wide crossing among unselected individuals. Information on field performance, intrafamily phenotypic variability, and molecular diversity will allow us to choose from among one of these three alternatives. For example, if heterosis is mainly the result of overdominance and intrafamily phenotypic variances are high, then reciprocal recurrent selection may be the most appropriate advanced-generation strategy.

Based on conservative estimates, it is expected that the strategy described above will initially result in 10 to 20% gains in biomass production each generation. Willow breeding in Sweden completed since 1987 yielded clones with 10 to 20% increases in biomass production compared with commercial check clones (Larsson 1998). Because most of the clones currently being used in operational biomass plantings in New York were selected from wild populations or from F_1 populations that had been tested in small plots on a minimal number of sites, improvements exceeding 10% to 20% are anticipated. Hybrids between *Populus trichocarpa* and *P. deltoides* showed more than twice the growth rate of either parent (Stettler *et al.* 1988). Identifying willow species combinations resulting in similar levels of heterosis may be possible.

The strategy described above includes importing non-native willow species to North America as well as making use of naturalized species. Importing non-native willows is desired, because some species or clones have unique traits and presumably unique alleles that are not present in the native species. All imports are completed under strict compliance with USDA guidelines on pollen, seeds, or dormant unrooted cuttings, so the risk of pest introduction is minimized. Many steps

are taken to reduce the risk of introduced willows becoming pests or of introgression of their genes into the native gene pool. Non-native clones or their progeny will be used in biomass plantings and not in riparian areas, so there is little chance that they could spread by natural vegetative propagation. Pollen-pistil incongruity and asynchronous flowering prevents successful natural interspecific hybridization among many willow species (Mosseler 1989). The potential for hybridization of introduced species with natives will also be evaluated prior to clone release. Trees produced by interspecific hybridization frequently do not flower (Mosseler 1990), so efforts are in progress to identify species combinations that can be bred and yield infertile progeny with desired growth characteristics. In addition, production clones may be rendered sterile through the use of genetic transformation in an effort to eliminate gene flow into native stands (Strauss *et al.* 2000).

Conclusions

Willow genetic improvement in North America is at an early stage of development. Breeding with willows native to North America plus several naturalized and non-native species has been completed, providing a starting point for future breeding activities. The breeding strategy described above may be modified as new information becomes available. Critical assumptions of the proposed strategy are that heterosis can be obtained by mating appropriate pairs of clones and controlled matings will result in families with large variability. Success of the breeding program depends on assembling a collection of genetically diverse germplasm. Collection efforts are focused on native species gathered from a broad geographic range, but a limited number of non-native clones are desired to maximize genetic diversity and breeding options. Cooperation with willow breeders around the world is desired, so that germplasm can be exchanged, although environmental stewardship will be a priority when considering large-scale deployment of non-native material.

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