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Breeding willows for biomass

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Summary

A willow breeding programme was initiated at IACR-Long Ashton in 1996, to produce high-yielding, disease and pest-resistant clones with a growth habit that facilitates harvesting. To combat disease pressures associated with the UK's maritime climate, it is also desirable to produce clones for effective mixed clonal plantations, which are believed to help prevent damaging outbreaks of willow rust (*Melampsora* spp.). To meet this objective there is a need to broaden the species range of clones available, at present dominated by those derived from *Salix viminalis*. A number of different breeding strategies are being pursued to achieve these aims. These are described, along with the practical crossing technique and selection procedures used in the breeding cycle. In addition, the use of molecular screening techniques is discussed as a means of rapid identification and selection of desired traits.

Key words: *Salix*, Short Rotation Coppice, breeding, clone, DNA, molecular markers

Introduction

Short Rotation Coppice (SRC) for renewable energy has recently come under close scrutiny in the UK as a potential non-food crop for set-aside land. SRC involves growing fast-growing willow (*Salix spp*.) and poplar (*Populus spp*.) clones in an arable environment with short harvesting cycles of three to five years. It is hoped that, in the future, these energy crops will to some degree supplant dwindling fossil fuel reserves. By 2025, energy crops could be contributing 30 terrawatt hours per year of electricity, equivalent to 10% of the current UK power demand (Anon., 1996).

The genus *Salix* contains over 300 species worldwide and there are representatives native to all the continents except Australasia (Zsuffa, Mosseler & Raj, 1984). Stott (1984) identified 54 willow species as having potential for biomass use. The majority of these fall into the sub-genus *Vetrix* known as bushy willows. Some of these species, such as *Salix triandra*, *S. purpurea* and *S. viminalis,* have been cultivated for centuries as the raw materials for basket making. These species tend to produce many erect shoots when coppiced and exhibit rapid growth. For SRC, however, one of the main desirable aspects is the total above-ground biomass and for this reason many basket willows are unsuitable for use in arable energy plantations. To produce new genetically diverse willow clones aimed specifically at biomass use, commercial breeding

programmes have been initiated in both Sweden and the UK. The Swedish programme was established by the plant breeding company Svalöf Weibull AB in 1987 (see Larsson, this volume). The need for new willow varieties for the UK was also recognised and a complementary programme began at IACR-Long Ashton Research Station in 1996, funded by a partnership comprising Long Ashton, Svalöf Weibull AB and Murray Carter.

Breeding Objectives

The fundamental aims of both the British and Swedish programmes are to produce high-yielding, disease and pest-resistant clones with a growth habit that facilitates harvesting. These objectives are illustrated in Table 1.

Table 1. *General objectives of the UK willow breeding programme.*

	Breeding Objectives	
Yield	1	Increase dry matter yield/hectare.
	2	Select tallest clones with either few, thick or many, thin shoots per stool.
Disease and pest resistance	3	Improve resistance to willow rust (*Melampsora* spp.).
	4	Improve resistance to brassy beetles *(Phyllodecta* spp.).
	5	Improve resistance to terminalis midge (*Dasineura* spp.) and leaf roll gall midge (*Dasineura marginemtorquens*).
	6	Select clones less palatable to rabbits, deer and hares.
Harvesting ability	7	Improve growth form. Select straight rather than curved rods, with fewer side branches.

One of the prime objectives of the Long Ashton breeding programme is to widen the genetic base of the crop. This is of particular concern because of the increasing evidence that arable coppice should be grown in clonal mixtures to produce a level of host diversity which will reduce selection pressure on major pathogens and pests, such as willow rust (*Melampsora* spp.) and beetles. If planted in appropriate clonal combinations, willow plantations should require virtually no pesticide inputs. The decision of which clones to be used in a mixture should be made on the basis of their incompatibility codes (Anon, 1994). These are derived from a clone's reaction to different forms of rust compared with the known reactions of a standard set of willow clones. The selection of too many clones with similar codes is ill-advised. Farmers should thus, resist the temptation to grow only *S. viminalis* clones, because in spite of the fact that they possess the highest yield potential, they are infected by a similar range of rust isolates. Yet, until clones bred specifically for UK conditions become available, there remains an over reliance on these "A" type clones, increasing the risk in the short to medium term of damaging rust attacks. Long Ashton has the necessary resources to offset this problem within the breeding programme, as it maintains the National Willow Collection which totals 1,100 clones comprising 120

different species.

Breeding Methods

A number of strategies can be implemented to achieve the objectives summarised in Table 1. Irrespective of the crop, a goal of all plant breeders is to produce new varieties capable of making revenue that can be funnelled directly back into the breeding programme. The safest way of achieving this is to make crosses using the best available material. Intra-specific crosses, such as the cross between *S.viminalis* clones (L 830201 and L 81102) from which Jorunn was derived, are an example of this strategy. However, in this situation, gains in yield and vigour over the parental clones are limited because of the narrow gene pool involved. An alternative is to make inter-specific crosses between clones from different species. Not only does this type of cross widen the genetic base of the crop, but it is also advantageous in that the recombination of the separate genomes may produce an F_1 hybrid that is superior to both parents. This is achieved when all the desirable traits of the parental clones are assimilated in the progeny and is termed hybrid vigour or heterosis. An example of this phenomenon is illustrated by a cross performed at Long Ashton, prior to the inception of the breeding programme, between *S.viminalis* Bowles Hybrid and *S. burjatica* Korso. Amongst the 600 progeny produced, a single hybrid (at present referred to as Stott 10) was identified, which in trials has out-yielded both parents significantly.

Another option is to cross the best native willow clones with their exotic relatives from around the world. This has been done very successfully in the Swedish programme where the *S. viminalis* clone Orm was crossed with *S. schwerinii* L79069, a near relative collected from Siberia. From the progeny of this cross, the high yielding and virtually disease-free clones called Björn and Tora were identified.

A number of speculative crosses should also be attempted, for instance, inter-specific crosses involving willow species hitherto unused in SRC. Correspondingly, a breeder should never dismiss a highly speculative cross, such as those that produce triple hybrids. Examples of the latter are the clones Delamere (*S. aurita* x *S. caprea* x *S. viminalis*) and *S.* x *calodendron* Wimm. (*S. caprea* x *S. cinerea* x *S. viminalis*).

Reproductive Biology and Practical Breeding Technique

Willows are very amenable to breeding. They reach sexual maturity quicker than most trees, usually producing catkins in the second year of growth. They are also dioecious with individual plants being either male or female. This means that pollen can be transferred from a male to a female without any physical modification to the latter. This contrasts with monoecious crops, such as maize, where male components of a flower must be emasculated prior to a cross to guard against self pollination. As hundreds of crosses are made, this is a very time consuming and labour-intensive process. Willows can also be propagated from cuttings which enables the breeder to make crosses from cut material in the glasshouse.

Crosses are attempted in January and February. Male and female shoots bearing flower buds are collected in December and either kept in a cold store or, more usually, taken to a glasshouse and placed immediately into glass jars containing water. Male and female clones are isolated in

separate compartments. As a rule, rooting and flowering occur simultaneously. The time to anthesis may vary from clone to clone and between male and female. Therefore, it may be necessary to introduce males to the glasshouse a week or so prior to the females. This allows pollen to be collected and retained until the female stigmas are receptive. Sufficient male shoots should be kept and introduced in a staggered manner so that there is always a fresh supply of pollen available, in case viability of stored pollen is low. A cross is performed simply by transferring pollen directly onto the female catkin with a small brush. Seed-set normally takes 3-6 weeks. If the female appears to be bearing seeds towards the end of the second week, a perforated bag is placed over the shoot, preventing any of the maturing seeds from being dispersed. At maturity, the seeds appear to be suspended in a medium resembling cotton wool, at which time the seeds should be threshed by placing the catkins and wool into a clear polythene bag which is inflated and tapped lightly so that the seeds fall to the bottom free of the wool. Seeds are sown onto a tray containing a mixture of peat and sand, on a mist propagator bench maintained at 20°C. At a height of about 3cm, individual seedlings are pricked out from the trays and placed in peat-filled polystyrene cell modules. When the seedlings reach 10cm in height they are transferred, 24 per tray to soil containing slow release fertilizer (Osmocote). These are then transferred outside where they are arranged in double rows. The trays are watered daily by a system of irrigation tubes. There are many benefits of tray cultivation compared to growing the material in the field (see Larsson, this volume).

The Breeding Scheme

Each year, 6-7000 seedlings are raised in the nursery and 5-10% are selected on the basis of simple inherited traits such as *Melampsora* resistance and height. Clones are also discarded if they have poor form. In the first year, selections are made of the best plants within a cross rather than between crosses, although progenies lacking too many of the attributes desired may be omitted altogether. Selections from the nursery are planted in successive observation trials each lasting 2 years. These consist of non-replicated rows of 10 plants per clone (observation trial 1) and non-replicated plots of 100 plants per clone (observation trial 2). Individual clones are assessed for pest and disease incidence and rough estimates of yield are made by examining components such as height, shoot diameter and shoot number per stool. At the end of the first observation trial, 5-10% of 500-600 clones are selected, and following the second the number is further reduced to about 15-20 clones. These are planted in yield trials for 3 years, with individual plots (4 x 10m) containing 52 plants and three replicate plots of each clone. Reference clones are also included in this configuration as a means of early identification of elite clones. From the yield trials, possibly 2-3 clones are selected and named as new varieties. If a clone is found to be distinct, uniform, stable (DUS) and show good value for cultivation and use (VCU) it is added to the UK National Recommended List and the breeder will be awarded Plant Breeders Rights, a patent that ensures the breeder will receive a small royalty payment for every cutting sold on the market. From initial cross to a marketable clone can take up to 10 years, yet willows are still one of the quickest crops to breed. By comparison, breeding a new wheat variety can take up to 12 years and a potato variety up to 18. The relative swiftness of the process for willow is due to the fact that they can be multiplied by cuttings. Each cutting of a clone may yield a new plant, from which approximately 25, 20cm cuttings can be obtained, two

years after planting (Carter, pers. comm.). Multiplication is initiated at the end of the fifth year. The abandoned observation trial is maintained for an additional season in order to provide the base material for multiplication plantations. These operate in tandem with the breeding programme and ensure there is sufficient material available, once an elite clone is launched on the market.

In order to maximise the potential of SRC willows in the commercial environment, emphasis must be placed on reducing the timescale of the breeding cycle. This could be done by omitting the second observation trial, planting material directly in yield trials and multiplication plantations at the beginning of the fourth year. For this to be successful, the breeder must be sure that the clones selected for "fast-tracking" have the potential to become commercial clones. One way of gaining this degree of certainty is the use of molecular screening techniques which aid both the selection of parents and progeny. As part of an EC-AIR project (AIR2-CT94-1617) at Long Ashton, DNA marker technology has been developed in willow to produce a first-generation genome map onto which DNA markers linked to traits of interest such as rust resistance, can be placed. Once the map position of a trait is known, relative to nearby molecular markers, the presence of the trait can be tracked by screening for the markers rather than the traits themselves. This is termed marker-assisted breeding. The progress of this research is discussed below.

DNA Markers: For Identification and Rapid Selection of Desired Traits

Three Polymerase Chain Reaction (PCR)-based molecular screening techniques, which allow for the discrimination of individual genotypes by DNA markers have been developed.

A rapid and fairly inexpensive technique, known as Random Amplified Polymorphic DNA (RAPD) analysis, has been used for DNA fingerprinting (Williams *et al*., 1990). Here, short random primers are hybridised to the DNA and the intervening DNA is amplified by PCR. Products from this reaction are observed after agarose gel electrophoresis. Failure to bind a primer due to a mutation results in the loss of an amplified fragment or one of a variant size. This method is simple to perform and only small amounts of DNA are necessary. RAPD fingerprints have been used previously to differentiate species or hybrids of *Populus* and *Salix* as well as to identify individual clones (Castiglione *et al.,* 1993; Lin, Hubbes & Zsuffa, 1994). The results obtained indicated that RAPDs were able to distinguish different biomass clones but problems were experienced with obtaining reproducible profiles which necessitates the need to duplicate all experiments prior to the scoring and analysis of the data.

Another approach, known as Amplified Fragment Length Polymorphism (AFLP) combines Restriction Fragment Length Polymophism (RFLP) and RAPD analyses (Zabeau & Vos, 1992; Vos *et al*., 1995). This method identifies RFLPs, a traditional method used in plant breeding programmes, but is less expensive and incorporates PCR to survey hundreds of loci simultaneously. In AFLP, DNA is digested with two restriction enzymes (a rare and a frequent-cutting enzyme). Adapters are ligated to the ends of these fragments, where the adapter sequences which are designed to the rare-cutter ends are biotinylated. This allows for the isolation of the biotinylated fragments only using streptavidin beads. The isolated fragments are amplified by PCR using P^{33} labelled primers that are homologous to the linker and have a 3'-extension of either 1, 2 or 3 nucleotides (depending on the number of fragments required).

Products from PCR (ranging from 50-500 bp in size) are then analysed on a polyacrylamide gel and exposed to X-ray film. Although more complicated than RAPD analysis, AFLP gives a greater number of data points from a single experiment, is reproducible and is especially useful for distinguishing between closely related individuals. The only disadvantage is that it requires relatively pure DNA to ensure complete digestion by the restriction enzymes. At present, AFLP analysis is being used to produce a preliminary genetic map from a population which has been scored for rust resistance. Bulked segregant analysis (Michelmore, Paran & Kesseli, 1991), which permits the identification of markers linked to specific loci of interest, will also be used on the same population in an attempt to isolate rust-resistance markers.

We have also investigated the use of microsatellite markers in willow. Microsatellites or Simple Sequence Repeats (SSRs) are highly mutable loci comprised of short tandem repeats that are present at many sites throughout the genome. Since the flanking sequences at each of these SSR loci may be unique, if SSR loci are cloned and sequenced, primers to the flanking regions can be designed to produce a sequence-tagged microsatellite (sequence-tagged SSR). Sequence-tagged SSRs are attractive markers because they are (usually) a single locus which, because of the high mutation rate of SSRs, is often multi-allelic. SSRs are also co-dominant markers (in contrast with RAPDs and AFLPs) and are detected by a simple PCR assay. They are very robust tools that can be exchanged between laboratories and they provide highly informative data. Disadvantages are that SSRs have been difficult to retrieve from plant genomes and they often show very limited cross-transferability between different species. We have produced a small insert library, highly enriched for SSRs, from willow genomic DNA (Edwards *et al*., 1996). To date, 145 clones have been sequenced and *c*.75% were found to contain an SSR. Primer pairs have been designed for several of these sequences and, when used in PCR amplification reactions, the microsatellites show high levels of polymorphism with all sets of *Salix* clones tested. These markers will be placed on the AFLP-based genome map.

The Future: The Plant Breeding Treadmill

The potential for varietal improvement of willow from breeding is considerable. With diligence and good judgement, it should be a relatively simple task to quickly establish Long Ashton bred clones in the marketplace. In only a few years time, a plant breeding treadmill will be well established, in which new superior willow clones are released each year. This will help to combat the build-up of virulent pathogen races, increase the potential yield and profitability of the crop and improve export potential.

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