

Chapter 5

Somaclonal Variation in *Populus*: An Evaluation¹

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Introduction

Substantial genetic and phenotypic variation occurs within plant cell, protoplast, anther, or tissue cultures. This variation also occurs among plantlets regenerated from these cultures, and it is assumed that this variation is genetically based (Michler 1993). Larkin and Scowcroft (1981) proposed that the variation occurring within cultures, or recoverable from cultures following plantlet regeneration, be termed somaclonal variation. They believed that the term described the variation among calliclones and protoclonal variation a separate category (Serres et al. 1991) and use the word gametoclonal to denote variation recoverable from haploid cultures. Ahuja (1988) proposed that somatoclonal variation encompass variability among genetically transformed plant materials.

Demonstrating this capacity for variation is dependent upon developing culture protocols for the species in question, but some genera, species, or varieties are recalcitrant to micropropagation techniques and have yet to show genetic variation within culture. Most micropropagation protocols were developed for herbaceous plants, but successful culture and regeneration was achieved in diverse woody perennial species (Dhawan 1993; Thorpe et al. 1991). Dhawan (1993) lists 7 culture systems for *Populus* and 3 for *Salix*; although, cell, protoplast, and anther culture systems exist for many species of the Salicaceae, including some poplars that are difficult to propagate (Ahuja 1987b;

Cheema 1989; Chen 1987; Chun 1993; Douglas 1989; Gebhardt 1989; Lu and Liu 1990; Rutledge and Douglas 1988). The first clonally propagated forest trees were from juvenile aspen cultures (Winton 1970). *Populus* was the first genus used for *in vitro* production of haploid plants (Lu and Liu 1990). Ahuja (1987b) and Chun (1993) recorded plantlet regeneration from tissue cultures of at least 29 *Populus* species, hybrids, or clones.

One purpose of micropropagation was to obtain genetic and morphological stability in culture and fidelity of the regenerants to the parental phenotype. Therefore, initially, acknowledgment of somaclonal variation in the literature probably depended upon the researchers' paradigm; particularly whether they viewed variation as an experimental failure. *Populus* and *Salix* species are generally easy to stabilize in culture (Thorpe et al. 1991) and were used for early experimental tissue culture systems. However, the genetic stability of regenerants was frequently doubted and callus culture was usually replaced by organ culture for propagation work (Ahuja 1987a, 1987b, 1993).

Somaclonal variation is recorded among regenerants of widely ranging species and is particularly well documented for herbaceous plants; reflecting their ability for adventitious shoot regeneration and micropropagation, and their pre-eminence among species of economic interest. Frequently, variation was a concern, but it is not universal among regenerants of successfully cultured species. Diverse semi-woody and woody perennials also exhibited somaclonal variation (table 1). Gymnosperms (especially the genus *Pinus*) are considered more genetically stable in culture than angiosperms (Diner and Karnosky 1987), but somaclonal variants were recovered in *Pinus*, *Abies*, and *Picea* (table 1). Among perennial angiosperms, somaclonal variants were recovered in woody monocots including *Musa* (Skirvin et al. 1994), oil palm, and bamboo; in dicot fruit crops, it was observed in semi-woody *Rubus* species, *Olea*, and various species of *Citrus*, *Malus*, *Prunus*, *Pyrus*, and *Carica* (Hammerschlag 1992; Manshardt 1992); in timber yielding hardwoods, excluding the Salicaceae, it was observed in *Ulmus* sp., *Eucalyptus* sp., *Albizia lebeck*, and *Santalum album*, and in ornamentals such as *Paulownia tomentosa* (Marcotrigiano and Jagannathan 1988) (table 1).

¹ Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Table 1. Range of woody perennial species that showed somaclonal, protoclonal or gametoclonal variation.

Genus/species	Reference
<i>Abies</i>	Gajdosova and Vookova 1991
<i>Picea</i>	Isabel et al. 1995
<i>Pinus</i>	Berlyn et al. 1986
<i>Musa</i>	Hammerschlag 1992
<i>Elaeis guineensis</i> (oil palm)	Benbadis 1992
<i>Bambusa</i>	Huang and Hunag 1993
<i>Rubus</i>	Swartz et al. 1983 Skirvin et al. 1994
<i>Olea</i>	Hammerschlag 1992
<i>Citrus</i>	Gmitter et al. 1992
<i>Malus</i>	Hammerschlag 1992
<i>Prunus</i>	Hammerschlag 1992
<i>Pyrus</i>	Hammerschlag 1992
<i>Carica</i>	Manshardt 1992
<i>Coffea arabica</i>	Söndahl and Lauritius 1992
<i>Santalum album</i> (sandal wood)	Sita 1992
<i>Eucalyptus</i>	Haines 1994b
<i>Albizzia lebbeck</i>	Varghese and Kaur 1991
<i>Ulmus</i>	Lineberger et al. 1990 Domir and Schreiber 1993
<i>Paulownia tomentosa</i> (foxglove tree)	Marcotrigiano and Jagannathan 1988

Poplar Somaclonal Variation

In the Salicaceae, somaclonal variation is well documented among *Populus* species, but apparently has not been recorded for *Salix*. This is probably due to the relative ease in culturing *Populus*, the general recalcitrance of *Salix* spp. *in vitro*, and the economic encouragement for working with *Populus*. Retrospectively, one of the earliest demonstrations of recoverable somatic variation in poplar was a semi-evergreen mutation on a branch of *P. nigra* cv. 'Italic' in Chile before 1900 (Pryor and Willing 1965). Somaclonal variation was also apparently observed in the 1960s when concern was expressed that regenerants from poplar cultures might be abnormal or genetically unstable (Ahuja 1987b), but no significant survey was initiated. Lester and Berbee (1977) first published the systematic demonstration of within-clone variation (somaclonal variation) in *P. nigra* cv. 'Italica' (*P. nigra* var. *italica*) and *P. x euramericana*. Since then, somaclonal variation has been repeatedly demonstrated within the genus (table 2), as have protoclonal (Serres et al. 1991) and gametoclonal variation (Chen et al. 1981; Chen 1987; Liu et al. 1992; Wu et al. 1981). Ignoring duplications at the clonal level,

somaclonal variation was demonstrated in cultures of at least 21 poplar species or hybrids (table 2; table 3). Furthermore, DNA analyses by Keim et al. (1989) demonstrated natural intra specific polymorphism in overlapping populations of *P. angustifolia*, *P. fremontii*, and their F₁ (first generation) hybrid progeny. Like the semi-evergreen *Populus nigra* cv. 'Italica', this might reflect *in vivo* somatic mutation, particularly since the hybrid genome was apparently perpetuated by natural vegetative propagation.

The use of callus culture (especially repeated subculturing) to rejuvenate hardwood tissues might be similar to somaclonal variation since it attempts to reverse the physiological change accompanied by dramatic alterations in cell DNA (Berlyn et al. 1986; Bonga and von Aderkas 1993; Franclet et al. 1987). Rejuvenation by repeated subculturing also was achieved with *Salix babylonica* (Franclet et al. 1987); but the technique is not widely applied to the Salicaceae because juvenile tissues from these species are regenerated by other means.

From the research perspective, somaclonal variation is considered highly undesirable since it may complicate studies on genetic transformation and transmission. Horticultural or silvicultural applications are of further concern because variation raises doubts about the stability of any micropropagated perennial plant over a long cropping cycle or rotation (Berlyn et al. 1986; Hammerschlag 1992; Skirvin et al. 1994). Alternatively, it has long been argued that somaclonal production is useful to uncover variants that could combine new desirable traits with the favorable characteristics of the existing variety or vegetative clone (Larkin and Scowcroft 1981; Phillips et al. 1990). The potential application of biotechnology to selective tree improvement and the drawbacks and potential benefits of somaclonal variation was generally reviewed (Ahuja and Libby 1993; Haines 1994a, 1994b; Huang et al. 1993; Zimmerman 1988); in relation to selection and improved disease resistance (Michler 1993; Ostry 1994); and for individual groups or species including conifers (Berlyn et al. 1986; Dunstan 1988) and poplars (Nelson et al. 1984; Ostry and Michler 1993).

Cultures or regenerants within the genus *Populus* exhibit the full range of genetic, physiological, anatomical, and morphological variation suggested by Parfitt and Arulsekhar (1987). Lester and Berbee (1977) observed variation in root cell chromosome counts; Noh and Minocha (1990) detected isozyme variation in 4 of 5 enzymes tested; and Son et al. (1993) recorded correlations between growth rates and specific SDS-PAGE protein banding patterns. At the physiological level, differences were recorded and/or selected for disease resistance (Antonetti and Pinon 1993; Ostry and Skilling 1988; Prakash and Thielges 1989; Valentine et al. 1988); herbicide tolerance (Michler and Haissig 1988); and gas exchange characteristics (Saieed et al. 1994a, 1994b). In some cases, variability is only detectable at the physiological level because regenerants are morphologi-

Table 2. Taxonomic distribution of *Populus* species, hybrids, or clones that exhibited somaclonal (calliclone or protoclonal) variation for one or more features.

Species/hybrid	Section ¹	Author(s)
<i>Populus alba</i>	L	Iordan-Costache et al. 1991
<i>P. alba</i> ; cl. '5972-S38'	L	Antonnetti and Pinion 1993
<i>P. alba</i> x <i>P. grandidentata</i>	L	Son et al. 1993
<i>P. alba</i> x <i>P. grandidentata</i> ; cv. 'Favorit'	L	Iordan-Costache et al. 1991
<i>P. alba</i> x <i>P. grandidentata</i> ; cv. 'Crandon' ('NC5339')	LxL	Michler and Haissig 1988
<i>P. alba</i> x <i>P. grandidentata</i> ; cv. 'Crandon' ('NC5339')	LxL	Serres et al. 1991
<i>P. alba</i> x <i>P. tremuloides</i> ; cl. '802-36-7'	LxL	Antonnetti and Pinion 1993
<i>P. angustifolia</i>	T	Keim et al. 1989 ²
<i>P. angustifolia</i> x <i>P. fremontii</i>	TxA	Keim et al. 1989 ²
<i>P. deltoides</i> cl. 'K417'	A	Prakash and Thielges 1989
<i>P. x euramericana</i>	AxA	Lester and Berbee 1977
<i>P. x euramericana</i> cv. 'Ogy'	AxA	Antonnetti and Pinion 1993
<i>P. x euramericana</i> cv. 'Robusta'	AxA	Antonnetti and Pinion 1993
<i>P. fremontii</i>	A	Keim et al. 1989 ²
<i>P. x interamericana</i> cv. 'Barn'	AxT	Antonnetti and Pinion 1993
<i>P. x interamericana</i> cv. 'Rap'	AxT	Antonnetti and Pinion 1993
<i>P. maximowiczii</i> x <i>P. trichocarpa</i> ; cv. 'Androskoggin'	TxT	Ostry and Skilling 1988
<i>P. maximowiczii</i> x <i>P. trichocarpa</i> ; cl. 'NC11390'	TxT	Michler and Haissig 1988
<i>P. nigra</i> var. <i>italica</i>	A	Lester and Berbee 1977
<i>P. nigra</i> cl. 'R1029'	A	Iordan-Costache et al. 1991
<i>P. nigra</i> x <i>P. laurifolia</i> ; cl. 'NC5272'	AxT	Michler and Haissig 1988
<i>P. nigra</i> var. <i>betulifolia</i> x <i>P. nigra</i> ; cv. 'Volga'	A	Ostry and Skilling 1988
<i>P. nigra</i> var. <i>betulifolia</i> x <i>P. trichocarpa</i>	AxT	Ostry and Skilling 1988
<i>P. nigra</i> var. <i>betulifolia</i> x <i>P. trichocarpa</i> ; cl. 'NE299'	AxT	Ostry et al. 1994
<i>P. nigra</i> var. <i>betulifolia</i> x <i>P. trichocarpa</i> ; cl. 'NC5331'	AxT	Michler and Haissig 1988
<i>P. nigra</i> var. <i>charkowiensis</i> x <i>P. deltoides</i>	AxA	Ostry and Skilling 1988
<i>P. nigra</i> var. <i>charkowiensis</i> x <i>P. nigra</i> var. <i>caudina</i>	A	Ostry and Skilling 1988
<i>P. tremula</i>	L	Ahuja 1983
<i>P. tremula</i> x <i>P. alba</i> ; cl. '717-1-B4'	LxL	Antonnetti and Pinion 1993
<i>P. tremula</i> x <i>P. alba</i> ; cl. '706-8'	LxL	Antonnetti and Pinion 1993
<i>P. tremuloides</i>	L	Valentine et al. 1988
<i>P. tremuloides</i>	L	Noh and Minocha 1990
<i>P. trichocarpa</i> x <i>P. deltoides</i> ; cv. 'Hunnegem'	TxA	Jehan et al. 1994
<i>P. trichocarpa</i> x <i>P. balsamifera</i> cl. 'TT32'	TxT	Saieed et al. 1994a/b
<i>P. hybrid?</i> -protoclone	?	McCown (In: Michler 1993)
<i>P. hybrid?</i> -shoot culture	?	Michler (In: Michler 1993)

¹A = AigeirosL = Leuce (currently termed *Populus*)

T = Tacamahaca

²natural intra specific polymorphism

? = unavailable

cally similar to the parental lines (Michler and Haissig 1988; Ostry and Skilling 1988; Prakash and Thielges 1989). Differences also were noted in various leaf characteristics including albinism, variegation, color, and chlorophyll content (Ahuja 1983; Douglas 1989; Michler 1993; Noh and Minocha 1990; Saieed et al. 1994a, 1994b; Serres et al. 1991; Son et al. 1993); and marginal serration and pubescence (Michler and Haissig 1988; Serres et al. 1991). Leaf

morphotype and thickness also vary (Antonnetti and Pinion 1993; Lester and Berbee 1977; Saieed et al. 1994a, 1994b; Serres et al. 1991; Son et al. 1993) with the morphotype and its ratio of leaf length to width, showing apparent correlation with other characteristics (Lester and Berbee 1977; Saieed et al. 1994a, 1994b; Serres et al. 1991). In addition, gross morphological variation is recorded for root form, stem girth, stem height, degree of branching, and overall

Table 3. Taxonomic distribution of *Populus* species, hybrids, or clones that exhibited gametoclonal or somatoclonal variation for one or more features.

Species/hybrid	Section ¹	Author(s)
Gametoclonal variation		
<i>P. x berolinensis</i>	TxA	Lu et al. (In: Chen 1987)
<i>P. simonii</i> x <i>P. nigra</i>	TxA	Wu et al. 1981
<i>P. xiaohei</i>	T	Liu et al. 1992
<i>Populus</i> sp.	?	Chen et al. 1981
Somatoclonal variation		
<i>P. tremula</i> x <i>P. alba</i>	LxL	Michel et al. 1988

¹A = Aigeiros

L = Leuce (currently termed *Populus*)

T = Tacamahaca

? = unavailable

growth rates (Ahuja 1983; Antonetti and Pinon 1993; Lester and Berbee 1977; Saieed et al. 1994a, 1994b; Serres et al. 1991).

Somaclonal Variation Basis and Frequency

Having demonstrated somaclonal variation, scientific and practical questions remain. Where does the variation come from, how is it evoked, and can it be avoided? Does somaclonal variation have practical use, and if so, how can it be evaluated and selected for? Once selected, will the variation remain stable throughout the life of a tree crop?

Ensuring culture stability is a crucial first step to maintaining genetic and phenotypic fidelity of regenerants. This applies to mass-propagation protocols and to culture systems used for germ plasm preservation (Ahuja 1993; Chun 1993; Wang et al. 1993). In the latter case, it is reassuring that prolonged below- or above-zero tissue cold storage apparently does not induce somaclonal variation (Cyr et al. 1994; Hausman et al. 1994). However, culture systems that produce somaclonal variation or rejuvenation involve presumed genetic changes, and these are occasionally observed at a physical level. With somaclonal variation, separate studies on the same parental material indicate that specific culture conditions can induce genetic and somaclonal variation that was not produced by gamma radiation (Douglas 1986; Saieed 1994a). Suggested causes of variation include minor point mutations in nuclear or organelle DNA, activation of transposable ele-

ments, polyploidy, aneuploidy, and epigenetic change (Michler 1993; Ostry and Michler 1993). Point mutations could have caused some albino variants that Son et al. (1993) correlated with the loss of 2 proteins, and other minor rearrangements may have caused the isozyme variation reported by Noh and Minocha (1990). Nevertheless, genetic analysis suggests that herbaceous somaclones principally change at the chromosome level rather than single genes (Michler 1993). Poplar somaclonal variation also has been correlated with changes at the chromosome level, but this is not universal. Native poplars are generally diploid ($2n = 38$), although some naturally occurring triploids and tetraploids exist (Ahuja 1993; Chen and Ahuja 1993; Särkilähti et al. 1988).

In contrast, poplar callus cultures often demonstrate variable ploidy levels (Son et al. 1993), and several poplar somaclones were fully or partially tetraploid or exhibited a leaf morphology characteristic of polyploid plants (Antonetti and Pinon 1993; Jehan et al. 1994; Lester and Berbee 1977; Saieed et al. 1994b; Serres et al. 1991). However, some desirable variants from the parental type were either diploid or similar to the parental leaf phenotype, suggesting that ploidy levels were unchanged (Antonetti and Pinon 1993; Lester and Berbee 1977; Saieed et al. 1994a, 1994b). Again, this reinforces evidence from herbaceous culture systems where most beneficial mutations are single trait characteristics (Michler 1993).

Somaclonal variation occurs inconsistently; a variability that presumably relates to tissue source and culture conditions. In most systems where somaclonal variation is undesirable and detectable by gross morphological changes, the variant poplars constitute less than 25 percent of the total regenerant population (Ahuja 1983; Antonetti and Pinon 1993; Noh and Minocha 1990; Son et al. 1993). Nevertheless, after observing the distribution of inter- and intra-treatment variation within 166 regenerants from 18 differing culture regimes that were subsequently aggregated into 4 morphotype groups, Saieed et al. (1994a, 1994b) concluded that each regenerant should be regarded as a separate somaclone. Further evidence for a significantly higher and subtler level of variation is provided by Noh and Minocha (1990) who showed a 25 to 34 percent variation for isozyme changes among tested regenerants. A considerably higher variation percentage among successful regenerants is expected when somaclonal selection is employed (Michler 1993) and variants are actively selected in culture (e.g., in systems to produce disease resistance or herbicide tolerance) (Michler and Haissig 1988; Ostry et al. 1990; Prakash and Thielges 1989; Riemenschneider et al. 1988; Valentine et al. 1988). The frequency of variation also is high among haploid plants, because some anther culture regenerants originate somatically and because of late chromosomal adjustments; the percentage of haploid cells decreases with age (Lu and Liu 1990).

Induction of Somaclonal Variation in Culture

Variability in the apparent level of induction suggests certain predisposing conditions. These conditions include differences in the inherent genetic variability within the parental material, variation with the type of culture, age-related changes amplified by the duration of culture or repeated subculturing, and dosage-related effects associated with the use of potentially mutagenic chemicals (Parfitt and Arulsekhar 1987; Skirvin et al. 1994).

Genotype significantly influences the ability to micropropagate poplars (Rutledge and Douglas 1988), and evidence from herbaceous systems and *Musa* (Hwang and Ko 1986) suggests that intra generic differences affect culture stability and the level of somaclonal induction. In a broad survey of poplar regenerants, Antonetti and Pinon (1993) examined 1,092 regenerants from 13 clones and identified 44 morphological and disease-resistant somaclones representing 8 parental lines. They suggested that somaclonal induction might occur more frequently in species from the Leuce (currently termed *Populus*) section than from Aigeiros or Aigeiros x Tacamahaca hybrids. Circumstantial support for this hypothesis is that Leuce calli can be grown on modified MS (Murashige and Skoog 1962) medium without potentially mutagenic growth regulators, and that only 1 of their 5 stable clones was from Leuce (table 4). Furthermore, although the calli were heteroploid or tetraploid and most somaclones were tetraploid, the more pronounced Leuce variants were diploid. This suggests that Leuce somaclones might be induced without undergoing major chromosomal change.

In contrast, Ostry et al. (1994) suggested that the Aigeiros x Tacamahaca (cl. 'NE299') (*P. nigra* var. *betuifolia* x *P. trichocarpa*) clone is particularly unstable in culture. In addition, in a limited study of herbicide tolerance in 4 different clones, 2

Aigeiros x Tacamahaca clones produced the lowest numbers of tolerant microplants, although they yielded more microshoots than either the Leuce or Tacamahaca lines (Michler and Haissig 1988). Unfortunately, the separate studies cannot be fully compared since culture and regeneration conditions were not identical for all lines.

Ignoring duplications, a survey of published reports on somaclonal variation at the species or hybrid level (table 2) indicates the following: Leuce (6), Aigeiros (6), Tacamahaca (2), Aigeiros x Tacamahaca hybrids (4). Given that Tacamahaca is the largest section of the genus and that most cultivated poplars are in section Aigeiros, Leuce does seem to dominate. However, Leuce species may be over represented because they are prime candidates for micropropagation due to difficulties in stem cutting propagation (Ahuja 1987b; Ernst 1993; Zsuffa 1992).

As in herbaceous systems, studies of poplar link somaclonal induction to aspects of culture history (Ahuja 1983; Jordan-Costache et al. 1991; Jehan et al. 1994; Ostry et al. 1994). Lester and Berbee (1977) were concerned that their observed variation was associated with incomplete elimination of culture viruses. This was not observed in subsequent poplar studies, but it has been suggested that the explant source may be a contributing factor. Ostry et al. (1994) produced calli from several tissue sources and showed that only regenerants from hardwood cuttings were resistant to somaclone formation. The cultural stability of tissue derived from hardwood cuttings may correlate with the general clonal fidelity of poplars propagated by rooted stem cuttings. While studying somaclonal variation, Saieed et al. (1994a) demonstrated that propagation by stem cuttings ensured phenotypic stability among individual somaclones and the parental ('TT32') clone from which the original callus was derived.

Evidence from herbaceous plants suggests that somaclones are more likely when protocols involve callus formation. Poplar micropropagation systems designed to ensure clonal stability generally involve little or no callus formation because it is assumed that callus or suspension

Table 4. Taxonomic distribution of *Populus* species, hybrids, or clones that seemed stable in culture.

Species/hybrid	Section ¹	Author(s)
<i>Populus ciliata</i>	T	Cheema 1989
<i>P. x euramericana</i> cv. 'Guariento'	AxA	Antonnetti and Pinion 1993
<i>P. x euramericana</i> cv. 'L. Avanzo'	AxA	Antonnetti and Pinion 1993
<i>P. x euramericana</i> cv. 'Carpaccio'	AxA	Antonnetti and Pinion 1993
<i>P. x interamericana</i> cv. 'Beaupré'	AxT	Antonnetti and Pinion 1993
<i>P. tremula</i> x <i>P. tremuloides</i> ; cl. '333-4'	LxL	Antonnetti and Pinion 1993

¹ A = Aigeiros

L = Leuce (currently termed *Populus*)

T = Tacamahaca

cultures have an inherent risk of somaclonal induction (Ahuja 1987a, 1987b; Ahuja et al. 1988; Jehan et al. 1994; Ostry et al. 1994; Park and Son 1988). Saieed et al. (1994a) compared S_2 plants (stem cuttings from primary regenerants) from suspension culture with those from short- and long-term callus culture. They suggested that factors related to callus culture duration or repeated subculturing, rather than callus formation, contribute to instability. Degenerative changes (i.e., the ability of cells to proliferate or regenerate) were shown to increase with poplar callus age. For example, 2,4-D/BA (2,4-dichlorophenoxyacetic acid/benzyladenine) combinations induced regeneration or protoplast division in six-month old *P. alba* suspension cultures, but not in cultures over one-year old (Sasamoto et al. 1995). Other poplar studies were similarly explained or interpreted (Chen 1987; Jehan et al. 1994; Ostry and Skilling 1988), and studies on other diverse woody species offer further support (Ahuja 1987a; Domir and Schreiber 1993; Donovan et al. 1994; Gmitter et al. 1992; Hammerschlag 1992).

Conversely, Cheema (1989) demonstrated that plants can be regenerated by either organogenesis or somatogenesis from long-term subculture systems with no immediately obvious somaclonal variation. Similarly, micropropagated apple shoots were repeatedly subcultured for up to 9 years without inducing discernible changes in the bark or leaf characteristics of regenerants (Webster and Jones 1992). This suggests that other factors, such as specific concentrations of certain growth regulators, may have roles as mutagenic agents (Ahuja 1987a; Bayliss 1973).

Some Leuce cultures were maintained without regulators (Antonetti and Pinon 1993), but controlling culture maintenance and regeneration usually required concentrations of potentially mutagenic regulators such as BA, NAA (α -naphthaleneacetic acid), and 2,4-D (Cheema 1989; Douglas 1982) that were greater than endogenous amounts. Auxins in particular are known to enhance DNA methylation and their elimination from culture systems might reduce the incidence of somaclonal variation (Phillips et al. 1990). In attempts to reverse tissue maturation changes, rejuvenation in shoot apices of diverse tree species was achieved by repeated subculturing in the presence of cytokinins (Thorpe et al. 1991).

Some studies attempted to assess the long-term implications of using particular regulators or regulator combinations. Following a three-year study of regenerants from calli exposed to varying combinations of 2,4-D and BA, Saieed et al. (1994a) observed a simple negative relationship between final growth performance and the applied BA concentration range (0.25, 0.50, or 1.00 mg/l). The response to the 2,4-D concentration range (0.1 to 0.6 mg/l) in callus culture was more complex, but superior lines were associated with either 0.1 or 0.4 mg/l 2,4-D. These results suggest that the chemical environment of final calli stages may have amplified or exerted a secondary screening af-

fect on potential variation. Comparable screening of aneuploid cells by cytokinins was demonstrated in polymorphic, herbaceous callus cultures (Dolezel and Novak 1985; Lavania and Srivastava 1988).

Poplar Somaclonal Stability

Many somaclones are nonadaptive and should probably be screened out during post-regeneration steps. Some recorded variation would be beneficial if it becomes stably fixed in somaclones exhibiting otherwise normal characteristics (Michler 1993; Ostry and Michler 1993). Heritable somaclonal stability must be confirmed by genetic analysis following sexual reproduction. Nevertheless, in an asexually propagated genus such as *Populus*, the demonstration of stability following vegetative propagation may allow practical exploitation of beneficial traits (Ostry and Skilling 1987; Skirvin et al. 1994).

Several authors report a variation in disease resistance or growth potential in somaclones that are superior or inferior to the parental line (Liu et al. 1992; Ostry and Skilling 1988; Prakash and Thielges 1989; Riemenschneider et al. 1988; Saieed et al. 1994a, 1994b; Son et al. 1993). These individual traits are often identified or selected in culture and are clearly sustained through the S_1 (primary regeneration) phase. However, questions of long-term stability arise; whether the desirable S_1 features will be continually expressed throughout the plant's life span under varying environmental conditions, and whether they persist into the S_2 stage following further vegetative or sexual propagation. A degree of instability might be expected in haploid regenerants as the growing points change to approach the more normal diploid condition (Chen and Ahuja 1993). Wu et al. (1981) observed a mixture of stability and late developing changes in leaf characteristics of haploid *P. simonii*. Such random changes might produce further material for screening and selection purposes (Chen 1987; Ernst 1993; Lu and Liu 1990).

In contrast, diploid or tetraploid plants are expected to exhibit greater levels of long-term stability. Some protoclonal lines identified by Serres et al. (1991) expressed morphological variances in the greenhouse but not under field conditions, and vice versa. Variation in resistance to *Melampsora* rust identified by Antonetti and Pinon (1993) was not immediately detectable, but appeared in 2 somaclones after a growing season in the field. Alternatively, the variant leaf morphology of many individual somaclones appeared stable over periods ranging from 4 months to 3 years and persisted through a secondary propagation phase (Lester and Berbee 1977; Michler and Haissig 1988; Saieed et al. 1994a). These observations are reconcilable and the differences are probably due to nor-

mal adjustment to environmental change or internal maturation processes. For example, the leaf length to width ratio appears to show an age-related decline even in a stable poplar clone (Pieters 1974).

Long-Term Field Stability

Silvicultural applications of clonal forestry indicate that field trials are required for several years to fully evaluate the relative performances of nonsomaclonal poplar lines (Zsuffa 1992). Somaclones are a strong argument for long-term trials due to the need to prove field stability. Some studies have been promised (Jordan-Costache et al. 1991), and our own study of stability among poplar somaclones was extended to 8 years from regeneration.

Saieed (1990) investigated 166 ('TT32') poplars from calli after 16-months culture with 6 different 2,4-D concentrations, then regenerated in 1985 with 3 different BA concentrations. Regenerated plantlets from 15 of these 18 treatment combinations (designated as lines A - O) survived to potting. The variation in several morphological, physiological, and leaf gas exchange traits was evaluated

among the primary regenerants in 1986, and in their S_2 vegetative propagules in 1987 and 1988. Qualitative differences among lines were detected by the end of 1986, most of which were statistically confirmed within the three-year period. Significant correlations were detected between gas exchange parameters and morphological characters, with some lines (especially C, F, and G) performing better than the parental reference clone over a large range of parameters, while others (especially A, H, and M) performed worse. The relative ranking among the experimental lines remained consistent in successive years despite significant changes in individual parameters induced by annual growth changes (Saieed et al. 1994a).

In 1989, the 105 surviving S_2 plants (3 to 5 per somaclone) were planted outdoors in a semi-randomized block at Kinsealy Research Centre, Dublin, and investigated for morphological growth characteristics between August 1992 and January 1993. Some previously investigated parameters were reassessed, such as height, stem diameter, and characteristics of fully-expanded, primary sun leaves. Leaf thickness was investigated for the first time and displayed an inverse correlation with leaf length or area. The comparative performances of these lines are in table 5, where the accumulated data (except leaf thickness) are ranked in descending numerical order. The ranking among

Table 5. Ranked tabulation in descending numerical order (except leaf thickness) of mean values¹ for morphological measurements on field-grown plants of 15 poplar somaclonal lines A-O² and the ('TT32') parental reference line³.

Stem characters				Leaf characters				
Height	Diameter	FW	DW	%H ₂ O ⁴	Length	Width	Area	Thickness ⁵
F	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
G	G	G	G	O	F	F	F	F
Ref	F	B	F	K	C	G	G	C
C	C	F	C	F	G	C	C	G
B	B	A	H	A	O	E	E	E
E	E	H	A	G	E	H	O	B
O	O	O	E	H	B	A	B	O
N	N	C	B	B	M	B	H	N
A	A	M	O	E	H	O	A	H
H	H	E	M	C	A	K	M	I
K	K	N	I	M	K	M	K	M
M	J	L	L	N	L	N	L	J
L	I	I	N	L	N	L	N	L
I	D	K	K	I	D	I	I	A
J	L	J	D	J	I	J	J	D
D	M	D	J	D	J	D	D	K

¹ Parameters measured August 1992 - January 1993 (3-5 plants per line).

² Lines regenerated from fifteen 2,4-D/BA regimes, designated as treatments A - O.

³ Reference line produced by vegetative propagation from the parental 'TT32' clone.

⁴ %H₂O = % leaf water content

⁵ Leaf thickness ranked in ascending numerical order.

lines is fairly consistent across the range of parameters, with the reference and somaclonal lines C, G, and F dominating. No significant differences were apparent in stem height or diameter among lines F, G, and the reference; these lines significantly outperformed line A and all lower ranking lines for these parameters ($P > 0.999$). This overall performance pattern is comparable to that obtained by Saieed (1990) and further demonstrates the long-term stability of the somaclones. Lineberger et al. (1990) used a comparable ranking technique as evidence of the fixation of somaclonal variation following a secondary propagation phase in elm somaclones. The original growth advantage of lines F and G over the reference appears to have eroded and it is unknown whether these somaclones will retain superior performance in respect to gas exchange or total biomass. No gas exchange measurements were conducted in 1992, but estimates of abaxial stomatal frequency were obtained for 5 lines. Frequencies for the reference and somaclonal lines C and G were essentially comparable, and were twice as high as those of lines K and H. This corresponds with a previous demonstration of high photosynthetic performances in lines C and G, and poor gas exchange by lines K and H (Saieed et al. 1994a). These results provide continuing evidence of stability within the somaclonal lines and reinforce the earlier belief that morphological and gas exchange measurements of newly regenerated plants are useful to predict final growth and yield.

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