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Septoria musiva

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Abstract

Septoria leaf spot and canker are serious diseases of many hybrid poplar clones in plantations established for biomass production. Developing resistant clones through breeding is the best long-term strategy to minimize tree damage caused by this disease. Tissue culture and somaclonal selection techniques may reduce the time needed to develop disease resistance in poplars. We used a single source clone of a hybrid *Populus* to determine the influences of explant tissue source and regeneration method on the frequency of somatic variation in disease resistance. Plants were regenerated adventitiously via shoots and somatic embryos from callus derived from several tissue sources and from axillary buds. The resulting plants expressed somatic variation in disease resistance in different frequencies, except for the plants regenerated from hardwood cuttings. Several regenerated plants from various tissue sources exhibited variant morphological phenotypes, providing further evidence of the instability of this clone when cultured in vitro. Although mutagenic effects of the culture regimes alone cannot be ruled out, results of this study suggest that the explant source and culture method influenced how frequently variant plants were recovered.

Key words: Organogenesis; Somatic embryogenesis; Protoplasts; Micro-cross sections; Root organ cultures; Disease resistance; Hybrid poplar; Intraclonal variation; Somaclonal variation

1. Introduction

Plant tissue culture lines have been shown to be inherently unstable at the karyotypic, morphological, biochemical and molecular levels [1–9]. The

number of examples of in vitro isolation of somatic mutants, a phenomenon commonly referred to as somaclonal variation [10], is rapidly growing [11–16]. The recovery of variant plants of scented geraniums was one of the first reports of variation arising among plants regenerated from tissue cultures, and it also demonstrated that the amount of

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variation depended upon the explant tissue source [17]. Somatic variation in *Populus* spp. has been recovered for morphological traits [18–20], disease resistance [21,22] and herbicide tolerance [23].

The origins of somatic variation and the factors influencing the frequency of its occurrence are not fully understood, but many authors have suggested that different processes are involved in different plant species. The source plant genotype, explant tissue source, media components and duration of the culture cycle may all be involved to some degree [5,14,16,24–26].

Somatic variation in important traits has promising potential for use in forest tree breeding. However, before tissue culture and somaclonal selection can be used as an efficient strategy, more needs to be learned about the origin of somatic variation and the factors controlling it. We conducted a study to determine how the type of explant tissue from one *Populus* source clone and the method of in vitro culture and regeneration affected the frequency of recovery of plants exhibiting increased resistance to the foliar disease caused by the fungus *Septoria musiva* Peck.

2. Materials and methods

2.1. Greenhouse stock plants

A single dormant hardwood cutting of NE 299 (*Populus nigra* var. *betulifolia* Torr. X *Populus trichocarpa* Torr. & Gray), a clone susceptible to *S. musiva* from which we previously recovered somatic variants [21], was collected from a stool bed, placed in water in the dark until root initials were visible, and then placed in a peat-perlite potting medium in the greenhouse. The stock plant was hedged every 4–6 weeks to stimulate lateral branching. This plant served as the source clone for all of the shoot cultures used in this study.

Additional hardwood cuttings from the same stool were potted and maintained as hedged plants in the greenhouse to serve as controls in the bioassay used to detect resistance to the *Septoria* leaf spot disease.

2.2. Sterile shoot cultures

Stem node sections (1 cm) were harvested from the greenhouse stock plant; disinfected in 0.5%

(w/v) sodium hypochlorite for 10 min; rinsed three times in sterile, purified water; and plated horizontally in culture vessels onto Murashige and Skoog (MS) [27] medium containing 100 mg/l myoinositol, 200 mg/l casein hydrolysate, 2% (w/v) sucrose and 0.44 μM 6-benzylaminopurine (BA), solidified with 0.6% (w/v) Difco Bacto agar (pH 5.8). The resulting shoot cultures were grown in a growth room (cool-white fluorescent tubes, 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 18 h photoperiod per day, 26°C). Shoot cultures were subcultured every 3–4 weeks onto the MS medium described above.

2.3. Plantlet differentiation from embryogenic cell suspensions

A callus initiated using 1-cm internode explants from sterile shoot cultures was cultured in the dark to obtain an embryogenic callus that was used to initiate liquid suspension cultures as described for leaf explants [28]. Following 16–17 subcultures at 7-day intervals, cells from 250 ml of cell suspension were allowed to settle for harvest, and were washed with 200 ml of liquid MS medium containing 3% (w/v) sucrose. Cells were resuspended, and 1-ml aliquots were plated onto No. 1 Whatman filter paper (7 cm) in petri plates on MS medium with 0.22 μM BA, 3% (w/v) sucrose, and solidified with 0.5% (w/v) Difco Bacto agar. Cultures were grown under the environmental conditions described for shoot cultures. Developing embryos were transferred on the original filter paper to fresh basal medium at 30-day intervals for 3–4 culture periods. Germinated embryos were planted in foil trays containing RediEarth™, covered with plastic lids, and acclimated to greenhouse conditions once growth resumed.

2.4. Adventitious shoot formation from protoplast derived callus

Leaf protoplasts were isolated from sterile shoot cultures and cultured using the techniques of Russell and McCown [29] to obtain callus cultures. After adventitious shoot formation on solidified MS medium, microcuttings were excised and placed into Techniculture™ peat plugs enclosed in a plastic rooting chamber. Rooted shoots were acclimated in a continuously lighted (cool-white fluorescent tubes, 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 22–28°C) growth room, potted in a 2:1 (v/v) peat-perlite medium, and transferred to the greenhouse.

2.5. Adventitious shoot formation from micro-cross sections

Young, fully expanded leaves from a potted source plant grown in the greenhouse were used as explant sources of midveins for preparing micro-cross sections on which adventitious shoot induction and proliferation were obtained [30]. Shoots were excised and rooted in a 2:1 (v/v) peat-perlite medium under 100% humidity in a continuously lighted growth room (cool-white fluorescent tubes, $15 \mu\text{mol m}^{-2} \text{s}^{-1}$, 20–25°C). Rooted plants were acclimated and potted in the greenhouse (18-h photoperiod per day, high pressure sodium vapor lamps, 80–140 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 18–30°C).

2.6. Adventitious shoot differentiation from stem callus cultures

Callus cultures were initiated from stem internode explants plated onto woody plant medium (WPM) [31] with 2% (w/v) sucrose, 2.3 μM (2,4-dichlorophenoxy) acetic acid (2,4-D) and solidified with 0.6% (w/v) Difco Bacto agar [21]. After 3–4 weeks proliferating callus at the cut ends of the explants was excised and plated onto WPM with 0.45 μM 2,4-D. Callus cultures were divided and subcultured onto fresh medium every 3–4 weeks for 2–3 culture cycles. Adventitious shoot proliferation was induced by transferring callus cultures to WPM containing 1.1 μM BA and 0.27 μM 1-naphthaleneacetic acid (NAA). Shoot cultures were incubated under a 16-h photoperiod per day (cool-white fluorescent tubes, 68 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 25°C). Elongated shoots were excised and rooted in a 2:1 (v/v) peat-perlite medium under 100% humidity in a continuously lighted growth room (cool-white fluorescent tubes, 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 20–25°C). Acclimated plants were potted in the greenhouse (18-h photoperiod per day, high pressure sodium vapor lamps, 80–140 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 18–30°C). Plants were fertilized with slow release nutrients (Osmocote, 17-9-13).

2.7. Adventitious shoot differentiation from root organ cultures

Roots were removed from sterile shoot cultures, cut into 1-cm segments, and placed two each into 40-ml screw cap vials containing 2–3 ml liquid WPM with 1.0 μM NAA. Vials were placed on their sides and rolled at approximately 1 rev./min

on a roller bottle apparatus in complete darkness at 30°C. Newly proliferated root segments were removed from the medium after 3 weeks, cut into 1-cm segments, and placed onto WPM medium containing 0.89 μM BA and 0.053 μM NAA, and solidified with 0.7% (w/v) Difco Bacto agar. The segments were incubated under conditions described for shoot proliferation from callus to induce adventitious shoot formation and proliferation. Elongated shoots were rooted and transferred to the greenhouse as described above.

2.8. Axillary shoot culture

Single node explants excised from the greenhouse-grown donor clone and disinfected in 0.5% (w/v) sodium hypochlorite were plated onto the same solidified WPM medium, and rooted under the same conditions as used for adventitious shoot proliferation from callus cultures.

2.9. Screening regenerated plants for somatic variation and data analysis

An in vitro bioassay [32] using excised leaf disks from plants propagated by hardwood cuttings of the source clone, and plants regenerated in vitro from various tissue sources, was used to detect somatic variation in resistance to the Septoria leaf spot disease. Plants of NE 299 previously tested using this bioassay displayed disease resistance reactions similar to those recorded in field trials. Plants from which leaf disks were taken had been growing in the greenhouse for 1–3 months. In each test, 12 leaf disks of each plant were inoculated with 0.1 ml of a spore suspension (1×10^6 conidia per ml of water) of *S. musiva*. Distilled deionized water was applied to leaf disks that served as controls. Disease progression was recorded by measuring the necrotic area on each inoculated disk every 2 days using a dot grid (25 dots/1.8 cm²). Measurements continued until the uninoculated control leaf disks started to become necrotic. A regression analysis of the pooled leaf disk measurements for all regenerates from a single explant source was performed using percent green leaf area and elapsed time as the dependent and independent variables, respectively. From this, the time elapsed to 50% necrosis and the rate at which 50% necrosis occurred were estimated. All plants testing resistant were re-tested using the leaf disk

bioassay. A chi-square analysis was used to compare the frequency of regenerated plants testing resistant from each tissue source. When significant differences were found among tissue sources, multiple comparison tests were used to describe how they differed.

3. Results

Somatic variation in disease resistance was detected in differing frequencies in all tissue culture-regenerated plants, but not in the source plants regenerated from hardwood cuttings (Table 1). Of the 1065 plants regenerated from all explant sources, 10.2% were highly resistant ($t_{50} > 32$ days) to the *Septoria* leaf spot disease, and the frequency of variant plants from each source and culture method was significantly different ($P < 0.0001$) from the source plant. The greatest frequency of variants was obtained from stem callus cultures ($P = 0.0004$) followed by somatic embryos, and the lowest was obtained from plants regenerated from roots. The disease reactions of the remaining plants regenerated from all sources could not be distinguished from those of the donor plant.

By comparing the variant plants using the estimated elapsed time to 50% necrosis (t_{50}) and the rate of necrosis at the time 50% necrosis was reached (r_{50}), we found that plants from stem

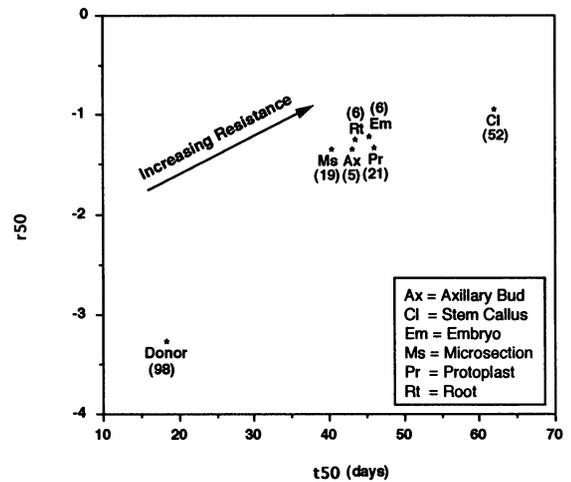


Fig. 1. Plot of the level of disease resistance of the donor clone and disease resistant plants regenerated from various explant sources of *Populus* clone NE 299 based on estimates of t_{50} (elapsed time to 50% necrosis) and r_{50} (rate of necrosis at t_{50}) in the leaf disk bioassay. The numbers in parentheses are the number of donor plants tested or the number of regenerated plants expressing a significant increase in resistance to *S. musiva*.

callus cultures were more resistant than plants obtained from any other explant sources (Fig. 1). This indicates that explant source tissue and culture method may influence not only the frequency

Table 1
Frequency of intracultural variation in morphology and increased resistance to *Septoria musiva* in plants regenerated in vitro from various tissue sources derived from *Populus* clone NE 299

| Source of regenerated plants | No. of regenerates tested | Morphological variants | | Septoria resistance variants | |
|---|---------------------------|------------------------|-----|------------------------------|---------------------|
| | | No. | % | No. | % ^a |
| Hardwood cuttings | 98 | 0 | 0 | 0 | 0 ^a |
| Axillary bud culture | 51 | 0 | 0 | 5 | 9.8 ^{b,c} |
| Adventitious buds | | | | | |
| Roots | 134 | 3 | 2.2 | 6 | 4.5 ^b |
| Stem callus | 341 | 2 | 0.6 | 52 | 15.2 ^c |
| Leaf protoplast callus | 260 | 7 | 2.7 | 21 | 8.1 ^b |
| Leaf microsections | 236 | 0 | 0 | 19 | 8.1 ^b |
| Somatic embryos from stem cell suspension | 43 | 0 | 0 | 6 | 14.0 ^{b,c} |

^aSources having the same letter were not significantly different; ^b $P = 0.34$; ^c $P = 0.56$.

of variation in disease resistance, but also the level of that resistance expressed in individual plants.

Three types of mutant morphological phenotypes were recovered among the regenerated plants. Four plants, one from stem callus and three regenerated from protoplast callus, exhibited thickened leaves resembling those characteristic of polyploid plants and vine-like growth resulting from the lack of strong apical dominance. Two plants regenerated from root culture were chimeras with variegated foliage. Six plants, one each from stem callus and root cultures and four from leaf protoplast callus, were dwarf plants with small, pale green leaves. These morphological variant plants remained stable in the greenhouse, but none were resistant to *S. musiva*. No morphological variation was observed among plants regenerated from hardwood cuttings or axillary bud cultures.

4. Discussion

Regeneration of plants adventitiously via shoots and somatic embryos from callus derived from several tissue sources resulted in recovery of plants with greater levels of disease resistance than the source plant. The percentage of regenerated plants exhibiting increased resistance to *S. musiva* was similar from both of the stem sources and from both of the leaf sources. Culture regimes of these regenerated plants varied widely, perhaps indicating that explant tissue source contributed more to the recovery of variant plants than the culture methods used. The possibility exists that some source tissues are more susceptible than others to the culture stresses that induce somatic variation.

We recovered, from axillary bud cultures, five plants that had increased disease resistance. Morphological somatic variants arising from aspen bud cultures have been reported previously [33]; they may be caused by the tissue culture cycle itself or may be a pre-existing cryptic variation in the source plant that is expressed in the regenerated plant. We previously found that among the poplar clones we tested, the frequency of variation differed with genotype [21]; the clone used in this present study exhibited the highest degree of instability in tissue culture. Recovery of 12 plants

exhibiting various morphological variations among the 1065 regenerated plants is further evidence of the instability of NE 299 in tissue culture.

As in previous work [20,29], protoplasts were the least stable source with regard to the recovery of morphological variants. This result was not unexpected because individual cell lines (protoplasts) were isolated from each other and inherent within-source variation may also have been isolated. These cell lines were differentiated independently of each other, eliminating cell line competition and interaction during the callus growth/differentiation phase, thus maximizing the recovery of variant lines. Additionally, the protoplast culture regime was the most complex and stressful, possibly inducing greater variation than under the other culture regimes.

The exact basis of the intraclonal variation in disease resistance of poplar plants recovered is currently under study. However, based on our results, two controlling factors for frequency of variation may be the explant source tissue and the level of cellular organization of the cultures from which plants are regenerated. The poplar clone NE 299 is completely stable only if propagated vegetatively by hardwood cuttings, and it is unstable to differing degrees if propagated *in vitro* from various tissue sources via two adventitious processes and an axillary bud process. However, mutagenic effects of the auxins used in the tissue culture media cannot be ruled out for inducing variation [5] because plants regenerated from stem callus growing on a medium containing 2,4-D resulted in the greatest frequency of variant recovery.

Somaclonal selection may be an efficient strategy in tree improvement. However, additional research is necessary to understand the various controlling factors so that somatic variation can either be enhanced or avoided. Additionally, because cell and tissue culture systems are used in most gene transfer systems, random somatic variation may complicate interpretation of resulting transformations. We have examined our regenerated plants only for disease resistance to one pathogen and obvious morphological differences. Other subtle changes may also have taken place

[7]. More importantly, we need to determine the stability of the trait in the field and ensure that the growth characteristics and the resistance to other pathogens of the source clone have not changed. To be truly useful, increased disease resistance must be heritable or at least stable through vegetative propagation.

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