External Leaf Features of Tissue-cultured 'Silvan' Blackberry

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Abstract. 'Silvan' blackberry (Rubus sp.) has 3 types of leaf hairs: multiserate stalked, multiloculated head colloters; thick-walled unicellular hairs; and setose hairs (multiserate trichomes that taper from a stout base). In culture, 'Silvan' blackberry leaves were unifoliolate, smaller, and thinner, with less cuticle and a decreased number of trichomes compared to mature leaves of greenhouse-grown plants, which were tri- or unifoliolate. Leaves of shoots in multiplication medium were half as large as those of plantlets in rooting medium.

Investigations of foliar anatomy and morphology of plantlets and/or transplant shock have revealed structural differences in culture that lower tolerance to water stress. Ex vitro transplant shock has been ascribed to reduced stomatal control (1, 2, 5, 18, 19), poor stomatal or trichome distribution (6), and other foliar anatomical effects related primarily to development under conditions of elevated relative humidity and low light intensity in culture. Tissue culture propagation of several blackberry cultivars has been described (3, 10, 17, 20) and summarized by Caldwell (4).

Studies of ex vitro red raspberry transplants have indicated that the culture-induced phenotype is permanent in leaves harvested soon after transplant are transitional in their anatomy and physiology, and that with time in soil, newly developing organs approach the control phenotype (6, 7). In red raspberry, filiform hairs densely cover and obscure the abaxial surfaces of control leaves and are present in plantlets incubated at high light intensity (6). As 'Silvan' blackberry (13) lacks these hairs, it is more convenient than red raspberry for such investigations. The purpose of this study was to characterize features of the leaf morphology of shoots and plantlets of 'Silvan' blackberry growing in multiplication and rooting media.

Cultures were initiated from 1-5 mm shoot tips from lateral and apical leaf buds of actively growing outdoor-potted plants. Shoot tips were explanted to Murashige-Skoog (MS) (15) medium containing 1% thiamine hydrochloride (1.20 ppm; 0.4 mg/liter) (12), myoinositol (550 ppm; 100 mg/liter), sucrose (87.6 ppm; 30 g/liter) N-(phenylmethyl)-1H-purin-6-amine (BA) (4.4 µM; 1 mg/liter) and 1H-indole-3-butyric acid (IBA) (0.49 µM; 0.1 mg/liter). After 2 months, cultures had outgrown their 10-cm test tubes and were transferred to 400-ml jars containing 40 ml of medium. The shoots were inserted between the glass and filter-paper support strip that held them above the medium. At one-month intervals, proliferating shoots were separated into 3-5 leaf cuttings for subculture to new multiplication medium or for rooting. Leaf samples were taken at this time. Shoots from multiplication medium were rooted in vitro in medium with IBA increased to 2.46 µM (0.5 mg/liter) and BA omitted. Leaf samples from plantlets in rooting medium were taken one month after subculture.

Cultures were exposed to temperatures of 27°C to 28°C and cool-white fluorescent lighting (25 µmol·s^-1·m^-2; 16 hr daylength). In the greenhouse, 'Silvan' control plants were grown at ambient temperatures and natural and fluorescent lighting at 100 µmol·s^-1·m^-2.

Fresh leaf samples of mature greenhouse-grown or in vitro 'Silvan' to be used for scanning electron microscopy (SEM) were fixed in 4% glutaraldehyde in cacodylate buffer (pH 7.0) for at least 2 hr, washed in distilled water, soaked for 1.5 hr in 1% osmium in 0.1 m cacodylate buffer (pH 7.0), again washed in distilled water, and dehydrated through an ethanol series. Samples were critical-point-dried, mounted on aluminum stubs with silver epoxy paste, sputter-coated with gold, and examined in a Hitachi S500.

Stomatal index (SI) and sizes (length, breadth) were calculated from photomicrographs of no fewer than 10 fields of view, on 5 leaves, from detached epidermal leaf strips or cleared leaves (6). At least 100 stomata were measured on both leaf surfaces of mature greenhouse-grown 'Silvan' leaves and those in multiplication and in rooting medium. Within each group, leaf areas were obtained by photocopying 30 leaves, cutting them out, weighing them, and calculating areas based on known-area paper weights. Student's t tests were used to compare SI, stomatal size measurements, and leaf areas among treatments.

Shoot multiplication in vitro was prolific (Fig. 1a). A 5- to 10-fold increase in shoot numbers per monthly cycle was established by the 3rd month after initial explantation, which was the end of the first month in large culture jars. Some rooting was seen in multiplication medium. Cuttings with 3 to 5

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leaves, rooted in vitro, had an extensive root system after one month (Fig. 1b). Leaves of cultured shoots and plantlets were predominantly unifoliolate, and only 1% to 2% of the area of mature, usually tri- or pentaloate leaves of outdoor or greenhouse-grown plants (Fig. 1c, Table 1). The mean leaf area of plantlets in rooting medium was significantly larger than that of shoots in multiplication medium (Table 1). Based on leaf area and SI, leaves of plantlets had roughly twice as many abaxial stomata as shoots, and greenhouse-grown plants roughly 200 times this number (Table 1).

Leaves are hypostomatous if adaxial stomata are fewer than 10% of abaxial numbers, and amphistomatous when adaxial stomata are more numerous (14). Although adaxial stomata are uncommon among the Rosaceae (14), in vitro adaxial SI was 5-50, similar in multiplication and in rooting media. Abaxial SI was similar in cultured and control plant leaves, and between shoots and plantlets in culture (Table 1). 'Silvan' control leaves were hypostomatous, while leaves in culture varied from hypostomatous to amphiotomatous. Guard cells were prominently raised in vitro, but flush with the epidermal surfaces in control plant leaves. Stomata were wider in vitro than in control leaves. Those in rooting medium were open wider than those in multiplication medium. While stomatal length was not different in greenhouse plants and plantlets in rooting medium, it was significantly reduced in shoots in multiplication medium (Table 1). Stomata on both leaf surfaces were similar in size within treatments. Stomata in vitro were open to such an extent that subtending mesophyll cells with chloroplasts could be seen (Fig. 2d). These stomata did not close at night. The cuticle was smooth in leaves from culture, with isolated areas of irregular epicuticular accretion (Fig. 2b and d). Hydathodes were present at the leaf tips and on leaf serrations (Fig. 2b). Little is known of these structures in the genus Rubus (11).

Three types of leaf hairs were found on 'Silvan': 1) Large numbers of glandular, multisieriate stalked, multicelled head collers (16) were present in all stages of development on leaf primordia and expanding leaves in culture (Fig. 2a). They appeared on adaxial and abaxial venal areas and at the leaf periphery. Some were freestanding while others formed secretory leaf teeth. They were dense and tall on the petioles. In control leaves (relative to treatment leaves) collers were less often found on the leaf peripheries and were fewer on both leaves and petioles. Collers may be ephemeral as fewer were seen on mature control leaves. 2) Unicellular, thick-walled straight hairs alternated with collers on leaf peripheries of cultured shoots and plantlets and were distributed in the same way as collers. In control leaves their numbers were increased on leaf peripheries and on venal areas; they also occurred interveinally and on the petioles. 3) Setose hairs [multisieriate, erect, straight, stiff trichomes tapering from a stout base; (16)], present infrequently on adaxial veins and on petioles of leaves of cultured shoots and plantlets, occurred in greater numbers in control plants.

Although no differences were apparent in the distribution or numbers of leaf hairs between leaves of shoots or plantlets in vitro these were significantly different in appearance from leaves of control plants.

Techniques are in demand that will optimize the environmental conditions required for successful hardening-off, maximizing the survival of transplants from culture. Transplant protocols can be improved through an understanding of the culture-induced phenotype. The relationship of anatomical differences in cultured shoots and plantlets (such as those described in this study) to physiology and hence functional competence must ultimately be addressed for tissue-cultured products to gain acceptance by the horticultural industry.

Literature Cited

Fig. 2. Micropropagated 'Silvan' blackberry-rooted plantlets. a. Leaf primordia and expanding leaves with developing glandular hairs (G). b. Adaxial leaf surface showing open stomata (S), hydathode (H), and epicuticular wax accretions (E). c. Abaxial leaf surface showing distribution of glandular hairs (G), unicellular thick walled trichomes (T), and open stomata (S). d. Open adaxial stoma showing subtending palisade cells containing chloroplasts (C). Scale bar = 500 µm (a, b, and c); 5 µm (d).