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RESPONSE OF WOODY PLANT MICROCUTTINGS TO IN VITRO AND EX VITRO ROOTING METHODS

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Abstract. Direct, side by side, comparison of *ex vitro* and *in vitro* produced roots on woody plant microcuttings reveals several important differences likely to influence the qualities that control survival of micropropagated nursery stock. In preliminary observations, *ex vitro* root systems are well-branched with normal root hair development, whereas roots initiated *in vitro* lack secondary roots and have a comparatively sparse development of root hairs. The *in vitro* roots tend to have extremely enlarged cortical cells (a false secondary thickening) and poor vascular connections. *Ex vitro* produced roots are more slender and have greater tensile strength. This research project will follow the fate of roots initiated *in* or *ex vitro* through acclimation, greenhouse growth, and in field environments, to determine if the root differences established by either root initiation method early in production continue to influence the root morphology and growth of the ultimate landscape plant.

INTRODUCTION

Micropropagation has recently become an important technique for insuring rapid, uniform delivery of new plant selections to the nursery industry. The speed by which plants can be multiplied and the reliability of the clonal nature of the plants produced is unsurpassed when compared to conventional propagation methods (3). The usual time, space, and materials restrictions on production are eliminated, and very quick release of new clonal propagules is now possible. Many woody plants are responsive to microculture propagation, although they may be recalcitrant to other clonal propagation methods (5).

Even though micropropagation is an accepted technique, there are surprisingly few standards within the industry on production methods. For example, the formulation of propagation media is determined empirically on a producer by producer basis. A wide

variety of containers, facilities, and acclimation techniques are also used. This diversity is especially surprising given the differences in quality that can result (2). Informal observations have revealed that the same propagule produced under different culture methods can vary substantially in quality, size, and degree of development. But these differences have never been well documented.

During production of a micropropagated plant, the root initiation phase is particularly critical to future plant performance. Just as is the case for softwood cuttings, the initial distribution of the microcutting root system has direct bearing on the eventual root branching pattern of the full-grown landscape plant. The integrity of the new root system can predetermine plant susceptibility to stress and ability to survive.

Yet, little research has been done to elucidate the factors that govern production of good, initial root systems on a microcutting. Growers supplying micropropagated woody shrubs and trees to the industry often root the cuttings *in vitro* (in tissue culture medium) in a 100% humid environment. For the *in vitro* method, microcuttings are transferred from shoot proliferation medium to a rooting medium (usually auxin supplemented), where rooting will occur over a 2 to 4 week period. The rooted microculture is then carefully removed from culture and transferred to the greenhouse for acclimation.

Other research and commercial labs elect to excise microcuttings, then place them directly into a rooting medium *ex vitro*. They may or may not be treated with rooting compound at this point. Depending on the individualized lab protocol and type of plant, the microcuttings might be inserted into trays of vermiculite, sand, peat and sand, or other medium, or into Techniculture plugs (Castle and Cooke, Techniculture, Inc.) in a mist or high humidity environment. Acclimation and rooting steps may be combined. Or, the supply labs may sell cuttings unrooted, leaving it to the nurseryman to perform *ex vitro* rooting.

The resultant differences in initial root system qualities can be substantial. A few scattered and isolated studies have implied, for example, that *in vitro* methods fail to stimulate normal root hair development, or that vascular connections might be discontinuous if rooting is accomplished *in vitro* (9,6). Another study suggested that *in vitro*-produced roots may die after transplanting to greenhouse conditions and are completely replaced by new *ex vitro* roots before the plants are established (1).

On the other hand, the *in vitro* method is widely practiced, and some believe it is more efficient and results in better uniformity (4). The two alternative routes for root production on microcuttings are characterized by differences in production timing, costs, and degree of required expertise (8, 10). Unfortunately, almost no side by side comparisons of roots generated *in* and *ex vitro* have been reported.

The consequences of using different rooting media or methods are largely conjecture.

One reason that this important comparative information is not available to nurserymen is that, by nature, the root system is very difficult to evaluate. Usually, it is hidden with soil or medium, so it is difficult for a grower to assess the distribution or quantity. The very act of removing the roots from the soil disrupts the root system. A woody root system can be quite extensive and complex even for a young nursery plant, making it difficult to achieve a thorough or objective evaluation. Conclusive evidence about the effect of root quality on plant performance is not available, yet the scattered reports that have appeared are certainly enough to raise serious questions, and to justify some comprehensive tests on the alternative techniques.

To recap on the background that has motivated our research project: 1) the quality of the initial root system is of critical importance to woody plant survival, stability, and performance; 2) there is little consensus in the industry on rooting standards, yet substantial differences in the product can result from different methods; 3) these differences are not well documented because roots, by nature, are difficult to examine.

The objectives of this study are to investigate and assess the effect of *in vitro* and *ex vitro* microcutting root initiation methods on root quality and distribution, for the young rooted propagule, for the acclimating plant, and for later growth stages. Anatomical sectioning and analysis of root systems at several stages in the production cycle reveals key contrasts between *ex* and *in vitro* generated roots at the cell and tissue level. Video image analysis techniques are adapted to provide a quantitative record of root system development and distribution, and explore in depth aspects of root integrity that might otherwise be overlooked due to inherent obstacles to root examination.

MATERIALS AND METHODS

Plant material. Proliferating shoot cultures of *Malus* × *zumi* 'Calocarpa', *Acer rubrum* 'Red Sunset', and *Betula nigra* provide microcuttings for rooting experiments. Shoot cultures are uniformly maintained by monthly subculture of horizontally explanted microshoots. *Malus* and *Acer* cultures are maintained on medium supplemented with 1 μM benzyladenine (BA) and 0.05 μM thidiazuron, whereas *Betula* cultures are maintained on a different salts medium with 2.2 μM BA.

All of the microcuttings are produced in Magenta GA7 vessels under standard light and temperature regimes (25°C, light intensity 45 $\mu M\ m^{-2}\ s^{-1}$, 24 h photoperiod). Uniform microshoots are excised with upper leaves intact when they are 2 to 3.5 cm long (approximately 4 to 5 weeks from the last subculture).

Rooting procedures. For *in vitro* rooting experiments, the cuttings are inserted vertically to a depth of about 1 to 1.5 cm into 20 ml of rooting medium contained in test tubes. Indole-3-butyric acid (IBA) at a concentration of 1 μM supplements *Acer* rooting medium. *Malus* and *Betula* require a slightly higher concentration of IBA. *Malus* cuttings require a brief dark pretreatment prior to rooting.

For parallel *ex vitro* rooting treatments, comparable microcuttings are excised from culture and inserted into trays containing sterile sand or a sand and peat mixture. Both *in vitro* and *ex vitro* treatments are held in identical growth chamber facilities (25 to 30 μM m^{-2} s^{-1}; 25°C day, 21°C night; growth chamber humidity 30 to 65%) for the duration of the rooting period.

In vitro rooting is usually accomplished in less than 4 weeks. After rooting, agar is gently washed away from the *in vitro* rooted plants, which are next transplanted into pots containing a standard greenhouse mix of peat/perlite/soil. The pots are placed under mist for acclimation, then moved onto a standard greenhouse bench with supplemental high intensity discharge (HID) lamps and saran cloth. Day temperature is kept at 24°C, night at 21°C, in a 16 h photoperiod. Plants are hand-misted periodically for the first few days in the greenhouse bench as they make the critical transition to the lower humidity conditions. Standard greenhouse fertilization and irrigation regimes are continued.

Treatment of the *ex vitro* cuttings is similar. Like the *in vitro* cuttings, these cuttings remain in their initial rooting medium a total of 4 weeks. Once roots are well initiated (approx. 2 weeks), the humidity in the plastic boxes is gradually reduced by cracking open the seals and later lifting up the lids. For the remainder of the root initiation phase, these plants are simultaneously becoming acclimated to lower humidity environments. Plants are then transplanted to the greenhouse bench with the same conditions as the *in vitro* rooted samples.

Plants from both the *in* and *ex vitro* rooting treatments will eventually be transplanted to larger-sized pots (in the same potting mixture) for an additional 60 to 90 days growth in the greenhouse, and then transplanted into nursery field plots in the spring. The production schedule has been designed to parallel standards used in commercial nursery operations.

Evaluation Methods. Evaluations are scheduled at root initiation, transplant and acclimation, transplant into the larger pot size in the greenhouse, transplant to the field, and after 1-year in the field nursery. Information on a variety of root parameters is collected at each observation, including number of days until root initiation, root fresh mass, tissue color, root branching patterns, sites of root initiation, and distribution of the root system.

Routine time course measurements of root system development are facilitated by a novel adaptation of microcomputerized

video image analysis. Entire cultures are staged for non-destructive observation, and root systems *in vitro* are imaged with a CCD video camera. The entire image is captured with the aid of an Imaging Technologies image analysis board housed in an IBM PC/AT microcomputer. The information is rapidly and automatically recorded (within seconds), calibrated in terms of two dimensional area, and broken down into component colors corresponding to visual density of the root system image.

This unique technique converts the irregular, complex information of a developing root system to a quantitative form. The visual data from digitized images has been previously correlated with manual measurements of fresh mass and length. Manual collection of the same root data would be extremely time consuming, tedious, and subjective. *Ex vitro* root systems are observed in the same way after gently removing any medium adhering to the roots. For each subsequent root observation during the production cycle, entire washed root systems as well as smaller subsamples are imaged to collect the required information.

Sample live root segments are transversely sectioned to approximately 80 to 100 μM (or 2 to 3 cell layers thick) at each observation with a vibrating microtome to analyze the anatomical layout of root tissues. The vibrating microtome allows thin, fresh sections to be taken from microculture tissue without prerequisite staining or embedding, which would otherwise create artifacts in observation (7). The live sections are viewed using an inverted microscope at 4x, 10x and 20x magnification. Cross sectional area, vascular system dimensions, cell types and cell sizes are determined. The presence or scarcity of root hairs is noted, and sections can be stained to determine the degree of root tissue lignification. A video camera is also mounted to the inverted microscope, and views of root sections are displayed on a video monitor for rapid, objective image analysis of anatomical measurements.

RESULTS AND DISCUSSION

Direct comparisons have helped to pinpoint clearcut differences between *in vitro* and *ex vitro*-produced roots at early stages of development. For example, root systems produced *in vitro* tend to have sparser root hair development. As *in vitro* roots develop and mature they continue to grow in length, often circling the base of a test tube if not transplanted, but branch root formation is rare. *In vitro* roots are formed at the base of microcuttings inserted into rooting medium, not at sites higher on the cut microcutting stem unless associated with callus. *In vitro* formed roots have a fat, fleshy composition. In some species the *in vitro* root has an almost "carrot like" appearance, but the roots are actually quite fragile and have poor tensile strength.

Sample *in vitro* roots readily tear (break and separate into short

pieces and different cell layers) when tested by gently applying tension along the longitudinal axis. This may signify, as some older literature has implied, that the vascular connections are incomplete or lacking. Older roots (30 to 60 days) are resistant to desiccation, and remain turgid up to 20 min. or more after removal from the agar medium.

In contrast, ex vitro-produced *root systems are profusely* branched indicating the presence of more absorptive surface area. The roots tend to be more slender and fibrous. They resist breaking and instead separate into long, continuous layers of cells when pulled apart.

In *transverse sections* of six-week-old *in vitro roots*, the most conspicuous feature is the lack of secondary growth (Fig. 1). The cortical cells develop without evident vascular cambium. Root cell size is expanded, and since *in vitro* roots are exposed to the light during initiation, they often contain chloroplasts and have a greenish cast. The cortex occupies the largest relative area in the root section. Overall root diameter is consequently much greater for *in vitro* roots, as compared to the more slender *ex vitro* roots.

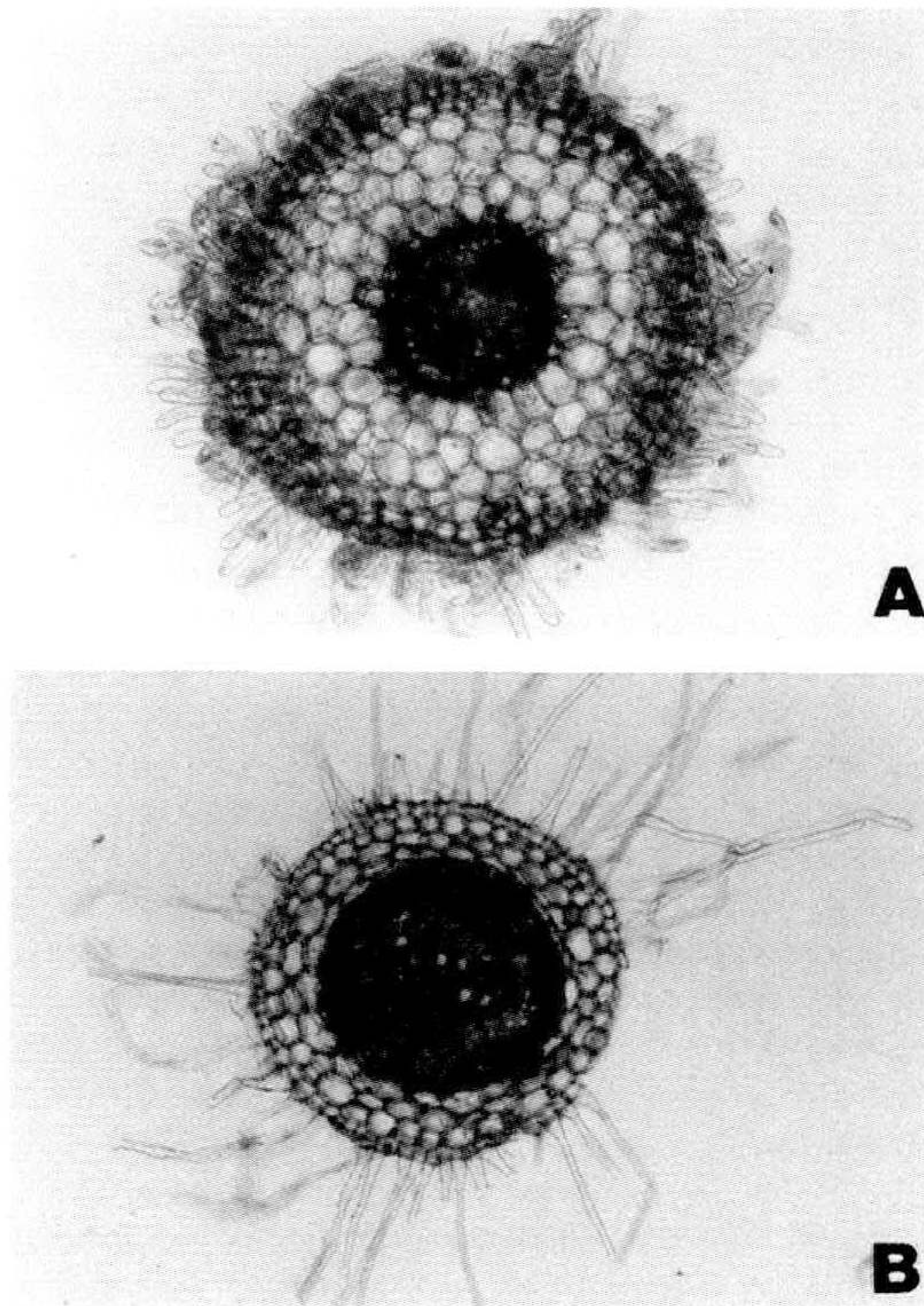

Figure 1. Comparison of transverse sections of six-week-old *in vitro* (A) and *ex vitro* (B) *Acer rubrum* 'Red Sunset' root system anatomy.

The *ex vitro* sections exhibit what could be considered normal root development, with a large amount of secondary xylem occupying the majority of the root section (Fig. 1). Comparative staining tests to confirm the presence of lignin, a substance abundant in secondary tissue, will be conducted for parallel *in* and *ex vitro* root treatments for each of the woody species. Cell size *ex vitro* is reduced, vascular systems are more compact and extensive, and cells have a dense cytoplasmic composition (Fig. 1). Some differences (distribution and texture) are apparent between *ex vitro* roots produced in sand, versus the sand and peat mixture, but these are yet to be characterized.

This preliminary comparison of initial root systems will continue throughout several subsequent stages of woody plant nursery production, with direct side by side comparisons at later stages of acclimation, transplanting, and adaptation to new environments.

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