In Vitro and ex Vitro Adventitious Root Formation in Asian Jasmine (*Trachelospermum asiaticum*)

II. Physiological Comparisons

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Additional index words. micropropagation, ³²P, tissue culture

Abstract. In vitro tissue-cultured (TC) and macropropagated (MACRO) 18-day old adventitious roots of Asian jasmine [*Trachelospermum asiaticum* (Siebold & Zucc.) Nakai] were compared for their ability to absorb and translocate radiolabelled P from a nutrient solution. Samples were taken at 1, 2, 4, 8, 12, and 24 hours after the initial dosage of the nutrient solution with 7.4×10^{-2} MBq $\text{KH}_2^{32}\text{PO}_4$ /liter. TC roots were capable of absorbing P, but at significantly lower levels than MACRO roots. Greater P absorption occurred in MACRO roots within the first hour and continued for the duration of the experiment. However, there was no significant difference in the rate of P translocation from roots to shoots between treatments. Root systems formed in vitro survived acclimation and had developed into well-branched root systems after 13 weeks. Reduced P absorption by TC roots did not limit either P translocation or survivability during and after acclimation.

Roots formed in vitro in the tissue-culture (TC) environment are hypothesized to be nonfunctional and, consequently, must be replaced once the rooted plantlet is transferred to ex vitro environmental conditions (Maene and Debergh, 1983; Pierik, 1987; Read and Fellman, 1985). Possible causes for nonfunctional TC-formed roots include 1) anatomical influences on root physiology such as poor vascular development between the shoot and root (Pierik, 1987; Ziv, 1986), 2) poor development of root hairs (Pierik and Steegmans, 1975; Yie and Liaw, 1977), 3) inhibition of functional root development due to the physical characteristics of agar-solidified medium (George and Sherrington, 1984), and 4) the effect of high medium hormone concentrations on the root-to-shoot vascular junction and associated callus-wound tissue (Dunstan, 1981).

Leaf anatomy and physiology are affected in vitro. Ziv (1986) concluded that normal leaf anatomical development in vitro is inhibited by conditions that promote vigorous shoot proliferation and growth. Donnelly and Vidaver (1984) observed that in vitro-initiated leaf primordia of red raspberry (*Rubus idaeus* L.) are unable to completely escape the effects of the TC environment; leaves ultimately developing from these primordia under glass-house conditions still showed residual aftereffects. What is not clear, however, is whether these observations with leaves are applicable to in vitro-formed roots.

There is a lack of systematic research on the functionality of TC-produced roots under ex vitro conditions. Therefore, the objectives of this research were to 1) compare absorption and translocation of radiolabelled P (³²P) between in vitro- and ex vitro-formed adventitious roots and 2) trace new root development and survival of in vitro-formed adventitious roots of Asian jasmine under two acclimation systems.

Materials and Methods

³²Phosphorus absorption and translocation. To determine root system functionality, ³²P uptake and translocation were compared between adventitious roots of macropropagated (MACRO) and in vitro-formed TC plants. One-node cuttings and explants were rooted ex vitro or in vitro, respectively, for 18 days; the ³²P uptake study began on Day 19. Neither cuttings nor explants were treated with root-promoting substances. Stock-plant management and TC and MACRO systems were as previously reported (Apter et al., 1993).

³²Phosphorus equipment and methodology. On Day 19, 28 rooted cuttings from each propagation system were removed from the media and their roots washed. Plants were inserted in a 1.3-cm-thick polystyrene foam support grid (Dow Chemical, Midland, Mich.), which was floated in a 100-liter Nalgene tank containing 10 liters of full-strength woody plant medium (WPM) (Lloyd and McCown, 1980) adjusted to pH 5.5. WPM without sucrose and agar was aerated with two aquarium pumps using aeration stones placed on the tank floor. The top of the tank was covered with clear 3.2-mm plexiglass, which created a high-humidity atmosphere to prevent the desiccation of TC-produced plantlets. A 12-h equilibrium period was allowed to elapse before the ³²P was introduced.

On Day 20, following equilibration, the solution was dosed to contain 7.4×10^{-2} MBq ³²P/liter in the form of $\text{KH}_2^{32}\text{PO}_4$ (ICN Biomedicals, Costa Mesa, Calif.). This concentration was estimated to give $\approx 4 \times 10^6$ disintegrations/min (dpm), assuming an approximate uptake of $5.0 \mu\text{mol} \cdot \text{g}^{-1}$ fresh weight per day. The $\text{KH}_2^{32}\text{PO}_4$ was introduced (time 0) 3 h after the beginning of the light period (0600 HR); samples were taken at 0, 1, 2, 4, 8, 12, and 24 h (n = 4 per time of sampling per propagation system). The design was completely randomized, with sampling time-propagation system combination randomly assigned within the tank.

Sample collection and preparation. After the cuttings were removed from the radiolabelled solution, the entire plant was rinsed by submersion in two, 500-ml distilled water baths for 15 sec each, blotted, and root and shoot fresh weights were determined. Roots were placed in 20-ml polyethylene scintillation vials and dried at 65°C for 12 h (Coltman et al., 1986). The dried roots were left intact (Brownell and Luchli, 1969; Chapin and Holleman, 1974; Fric and Finocchiaro, 1976) and 12 ml of 2.5 mM 7-amino-

Received for publication 12 Aug. 1992. Accepted for publication 25 Mar. 1993. We greatly appreciate the technical assistance of Sharon A. Duray and the review of this manuscript by M.C. Drew and D.W. Reed. We thank Delores Soto for typing the manuscript. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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1,3-naphthalene-disulfonic acid (ANDA) (Aldrich Chemical, Milwaukee) was added to the vials. ANDA acts as a wavelength shifter to overcome self-absorption (Chapin and Holleman, 1974) and is recommended for use with hard beta emitters (i.e., $E_{\max} \geq 1.0$ MeV) (Läuchli, 1969). ANDA was not observed to leach pigments from intact roots nor cause color quenching (Läuchli, 1969).

Stems and leaves were combined in glass vials, dried as above, and ashed in a muffle furnace at 575C for 11 h (Drew and Läuchli, 1985). The basal 1 cm of stem was excised and discarded before drying to avoid inclusion of tissue (excluding roots) that had direct uptake potential for ^{32}P . The resulting ash was dissolved in 3 ml of 0.1 N HCl, transferred to plastic scintillation vials, and adjusted to volume with 12 ml ANDA. Root and shoot samples were counted for Cerenkov radiation in a liquid scintillation counter (model 7500; Beckman Instruments, Irvine, Calif.) that was programmed to correct for color quenching.

Root development under two acclimation systems. In a separate experiment, 44 rooted plantlets were removed from their culture tubes and adherent agar was carefully washed from the roots under running, distilled water. The root systems of 22 plantlets were dipped for 15 sec in a 1% solution of methylene blue (Arnold and Young, 1990); the remaining root systems were not stained and these plants were treated as controls. All 44 plantlets were potted in 5.7-cm (75-ml) plastic pots containing Redi Earth Peat Lite mix (W.R. Grace, Cambridge, Mass.) and placed in a glasshouse under 80% shade in a completely randomized design with a 2×2 factorial arrangement of treatments. Half of the plants in each treatment was either enclosed in clear plastic cups or placed under intermittent mist ($n = 11$ for each of the four treatments). To reduce relative humidity, cups were gradually raised after 2 weeks and later removed. Concurrently, mist frequency was slowly reduced for plantlets under mist. Shadecloth was removed after 5 weeks. Glasshouse conditions included a 2-h night interruption with incandescent lighting to maintain long-day conditions; maximum

daytime irradiance [photosynthetic photon flux (PPF)] was $900 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Night temperatures in the glasshouse were $23 \pm 3\text{C}$, with a daytime minimum and maximum of 25 and 30C, respectively. After 13 weeks, plantlets were harvested, the soil was washed from their roots, and observations were made to determine if the stained root systems survived or were replaced by a new root system initiated in situ. Root and shoot dry weights (65C for 48h), total leaf area (measured with a leaf area meter, model LI-3000; LICOR, Lincoln, Neb.), root : shoot ratio, and leaf area ratio (total leaf area : total plant dry weight) were recorded to determine if any adverse effects occurred with the staining procedure.

Results and Discussion

^{32}P Phosphorus absorption and translocation. TC-formed adventitious roots of Asian jasmine absorbed P, but at statistically reduced levels compared to MACRO adventitious roots (Table 1, Fig. 1). Greater absorption of P occurred with MACRO roots within the first hour and continued for the duration of the experiment (24 h). Root fresh weight also differed between treatments; TC roots weighed 2.5 times more than MACRO roots.

It is unlikely that poor root-hair development reduced P uptake by TC roots. Root hairs have little or no effect on nutrient uptake in vigorously mixed, aerated solution culture (Clarkson, 1974); consequently, the potentially rate-limiting diffusion step existing in soils was eliminated.

While not quantified, it is unlikely that the entry of P into TC roots was restricted by reduced root permeability relative to MACRO-formed roots. Emmert (1972) observed that initial cellular uptake of ions in the overall process of ion passage through the root could constitute the primary rate-limiting step in the process. The TC environment could have affected root metabolism, thereby limiting the initial uptake of P into the symplast. One possible cause could be carbohydrate-sugar metabolism, since oxidation of

Table 1. Root absorption and shoot uptake of ^{32}P by macropropagated (MACRO) and tissue-cultured (TC) Asian jasmine.

Time (T) (h)	Propagation (P) system	Root fresh wt (mg)	Root ^{32}P absorption ($\mu\text{mol}\cdot\text{mg}^{-1}$ root fresh wt)	Shoot fresh wt (mg)	Shoot ^{32}P content ($\mu\text{mol}\cdot\text{mg}^{-1}$ shoot fresh wt)
<i>Mean $\pm$ SE</i>					
0	MACRO	11.0 ± 1.1	0.0 ± 0.0	175.8 ± 8.2	0.0 ± 0.0
	TC	77.8 ± 16.3	0.0 ± 0.0	286.0 ± 34.8	0.0 ± 0.0
1	MACRO	24.0 ± 6.1	2.3 ± 0.4	184.3 ± 17.9	0.1 ± 0.0
	TC	50.0 ± 14.8	1.3 ± 0.1	253.8 ± 35.0	0.3 ± 0.2
2	MACRO	25.8 ± 4.3	3.5 ± 0.8	191.0 ± 23.0	0.3 ± 0.1
	TC	74.5 ± 15.5	1.7 ± 0.2	292.8 ± 22.8	0.2 ± 0.0
4	MACRO	25.3 ± 5.1	6.2 ± 0.3	203.0 ± 7.0	0.7 ± 0.3
	TC	78.5 ± 12.3	2.7 ± 0.2	270.0 ± 18.3	0.4 ± 0.1
8	MACRO	35.8 ± 5.8	11.2 ± 0.1	186.8 ± 11.1	0.6 ± 0.1
	TC	79.8 ± 15.6	5.8 ± 0.5	272.8 ± 36.1	1.7 ± 0.3
12	MACRO	28.8 ± 6.9	18.4 ± 0.7	224.8 ± 24.9	2.2 ± 0.3
	TC	114.3 ± 33.1	7.9 ± 0.9	278.3 ± 23.6	2.2 ± 0.8
24	MACRO	32.5 ± 8.8	15.1 ± 1.6	185.5 ± 11.0	2.8 ± 0.9
	TC	79.0 ± 22.3	9.0 ± 1.3	304.8 ± 29.1	4.7 ± 0.7
<i>ANOVA F values</i>					
P		46.56**	99.06**	47.14**	3.81
T		1.12	93.96**	0.42	22.97**
P $\times$ T		0.84	11.32**	0.54	2.06
r^2		0.58	0.95	0.56	0.79

**Significant at $P \leq 0.01$.

Fig. 1. Uptake of P by adventitious roots of tissue-cultured (TC) plantlets and macropropagated (MACRO) cuttings of Asian jasmine. Vertical bars represent $\pm$ SE.

Fig. 2. Phosphate translocated to shoot systems of macropropagated (MACRO) cuttings and tissue-cultured (TC) plantlets of Asian jasmine.

sugars is the source of energy for active uptake and transport of ions (Epstein, 1972).

Translocation of P from the root and accumulation in the shoot increased in both treatments during 24 h (Fig. 2). However, there was no significant difference in P translocation between the two treatments (Table 1). This observation is surprising in light of reduced P absorption by TC roots. The extent of xylem connections at the root-to-shoot junction could have played a role in these observations. Several researchers have hypothesized that vascular connections in vitro are underdeveloped (Pierik, 1987; Ziv, 1986). However, in a related study, we observed continuous root-to-shoot vascular connections in micropropagated Asian jasmine (Apter et al., 1993).

Once absorbed, P is a highly mobile element that tends to move to metabolically active areas such as rapidly expanding meristematic tissues (Bielecki, 1973). TC plantlets had higher shoot fresh weights (Table 1) and much more rapidly expanding shoot systems than the MACRO cuttings. Therefore TC plantlets conceivably had stronger shoot system sinks that overcame initial differences in root P absorption between treatments.

Root development during acclimation. Root dry weight and other growth components during acclimation in cups exceeded those under mist (Table 2). Staining had no adverse effect on either root or shoot dry weight accumulation, total leaf area, root : shoot ratio, leaf area ratio, or the number of basal buds elongated during acclimation. Methylene blue-stained roots continued axial growth and initiated new lateral branching (Fig. 3). No plantlets in either acclimation system died during or after acclimation.

In summary, the in vitro-formed adventitious root systems proved to be fully capable of continued expansion and growth during acclimation (Fig. 3). This conclusion conflicts with the hypothesis that in vitro roots are not capable of survival under ex vitro conditions and must be replaced during acclimation (Maene and Debergh, 1983; Pierik 1987; Read and Fellman, 1985). Ziv (1986) concluded that the processes associated with in vitro leaf initiation are irreversibly altered by the TC environment; apparently, the process of Asian jasmine root development in vitro is not affected in an analogous manner and cannot, therefore, be compared to leaf primordia development in vitro. Hence, TC-formed roots of Asian jasmine are viable and fully capable of continued growth subsequent to transfer from the in vitro environment to conventional glasshouse conditions, despite their reduced 32 P absorption.

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Table 2. Acclimation of tissue culture plantlets of Asian jasmine contrasting methylene blue-stained (ST) and nonstained (NST) roots and two methods of glasshouse acclimatization (mist and cup).

Method	Stain	Root dry wt (g)	Shoot dry wt (g)	Root : shoot ratio	Total leaf area (cm ²)	Leaf area ratio (cm ² ·g ⁻¹)	Basal budbreak
Cup	NST	0.17 a ²	0.18 a	0.9 a	14.2 a	40.7 a	2.2 a
	ST	0.17 a	0.18 a	0.9 a	14.4 a	41.0 a	2.1 a
Mist	NST	0.10 b	0.06 b	1.7 b	3.8 b	23.9 b	0.6 b
	ST	0.09 b	0.05 b	1.8 b	3.7 b	26.6 b	0.6 b

²Mean separation within columns by Duncan's new multiple range test at $P \leq 0.05$ ($n = 11$ for each treatment).

Fig. 3. A portion of a single-branched TC root of Asian jasmine following acclimation and 1 week of glasshouse growth. Nonstained portions of roots (exterior to arrows) represent new root development during acclimation.

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