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Quantifying Apoplastic Flux through Red Pine Root Systems Using Trisodium, 3-hydroxy-5,8,10-pyrenetrisulfonate[1]

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ABSTRACT

The fluorescent compound trisodium, 3-hydroxy-5,8,10-pyrenetrisulfonate (PTS) was used to quantify the apoplastic flux through red pine (*Pinus resinosa* Ait.) root systems—that portion of the total water flux reaching the xylem without ever crossing a semipermeable membrane. Flow was induced by pressure through detopped root systems, and by transpiration through intact seedlings. Apoplastic flux was determined by multiplying total flux by the ratio of PTS concentration in the xylem exudate to PTS concentration in the bathing medium.

Under aeration, apoplastic flux was less than 1% of total flux. Under anaerobic conditions, up to 50% of total flux was apoplastic suggesting that anaerobic conditions change the pathway of water flow into root xylem. The change under anaerobic conditions was reversible. Detopped root systems under pressure and intact seedlings under transpiration gave similar results. In detopped root systems, the magnitude of the pressure gradient may alter the apoplastic contribution to total flux.

Water flow through root systems may be expressed by the following relationship:

$$Q_t^4 = Q_a + Q_s \quad (1)$$

where Q_t is the total water flux; Q_a is the exclusively apoplastic flux, involving water which reaches the xylem without ever crossing a semipermeable membrane; and Q_s is the symplastic flux, involving water that travels most of its pathway either in the symplast or apoplast but which must cross a membrane at least once during its passage to the xylem.

Apoplastic flux is considered relatively unimportant since Q_t is severely inhibited by metabolic inhibitors, and ion movement into roots appears to function as if it were crossing a membrane (5, 13). However, quantitative estimates of the apoplastic contribution to total flux were not found. A need to quantify the components in flux models is frequently indicated (1, 2, 10). A major objective of this study was to quantify the apoplastic component of water flux through root systems.

The existence of an exclusively apoplastic pathway is demonstrated by the appearance in the xylem of materials totally excluded by membranes (15). In roots without secondary cambium, breaks in the endodermis in the emergence zone around lateral roots (15, 16), wounds, and the root tips where the casparian strip is incompletely developed (4) all provide potential apoplastic pathways. The pathway across the endodermis has been traced with electron opaque elements, radioactively labeled substances, and apoplastic dyes. PTS was used as the marker of apoplastic water flux in our study. PTS is fluorescent, highly water soluble, nontoxic, not adsorbed onto cell walls, and is totally excluded from the symplast (14, 15, 19). The proportion of uptake which was exclusively apoplastic was calculated using an equation modified from one used by Mees and Weatherley (11) to calculate leaks in their root system under pressure:

$$Q_a = \frac{k(C_e)}{C_b} Q_t \quad (2)$$

Q_a and Q_t are measured in cm^3 of water per cm of root length per second ($cm^3\ cm^{-1}\ s^{-1}$); C_e and C_b are the PTS concentrations ($\mu g\ ml^{-1}$) in the xylem exudate and bathing media, respectively; and k (greater than or equal to 1) is the constant that accounts for any difference in the rate with which PTS *versus* water moves through the exclusively apoplastic pathways. For PTS, we assumed that k was equal to 1. Equation 2 was used to quantify Q_a of red pine roots exposed to hydrostatic pressure gradients under aerobic and anaerobic conditions.

MATERIALS AND METHODS

Plant Materials and Culture. Three-year-old red pine (*Pinus resinosa* Ait.) seedlings were transplanted in April from the nursery to the greenhouse where they were grown for 6 months (1982 transplants), or 1.5 months (1983 transplants) in 20 cm pots. Greenhouse conditions included sandy loam soil watered to saturation every 2 to 3 d, 14.5 to 16.0 h photoperiod, and day/night temperatures of 24 to 29/18 to 24°C. Two to 4 weeks before measurement, the seedlings were transferred to aerated half-strength Hoagland solution where they were cultured for a minimum of 2 weeks to heal any wounds inflicted during transfer. Growth in solution was not allowed to continue beyond 4 weeks in order to maintain the morphological characteristics of a soil-grown root system.

Measurements Using Applied External Pressure. A single root system was sealed in a solution-filled pressure chamber with the stem protruding. The system is described generally by Mees and Weatherley (11) and for pine by Sands *et al.* (17). Early in the morning, a seedling was transferred to the pressure chamber containing either deionized H_2O or an aqueous 0.02% (w/v) PTS solution made with deionized H_2O. When deionized H_2O was used alone, the PTS was injected at a later time. The solution

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[4] Abbreviations: Q_t, total flux; Q_a apoplastic flux; Q_s, symplastic flux; PTS, trisodium, 3-hydroxy-5,8,10-pyrenetrisulfonate; C_e, PTS concentration of the exudate; C_b, PTS concentration of the bathing medium; k, a constant; PPFD, photosynthetic photon flux density.

was held at 24 ± 1°C. Compressed nitrogen or air was gradually added to the chamber to raise the hydrostatic pressure to the desired levels, usually 0.40 ± 0.002 MPa. Exudation rates were monitored at 10 min intervals by recording the changes in weight of an exudate collection bottle to the nearest 0.001 g. One hundred μl samples of xylem exudate and bathing medium were taken periodically for the analysis of PTS concentration. The exudate collection tube was attached only to the xylem tissue of the stem, which protruded above the pressure chamber seal, to exclude the possibility that PTS from the bathing medium might contaminate the xylem exudate.

Measurements Using Transpiration. Ten 1983 transplant seedlings were transferred from half-strength Hoagland solution in the greenhouse to aerated flasks containing the same solution in a growth chamber at PPFD of 240 $\mu E\ s^{-1}\ m^{-2}$, 30% RH, and 24 ± 1°C. Twenty-four h later, the Hoagland solution was replaced with 0.02% (w/v) PTS in deionized H_2O, and five of the flasks were bubbled with nitrogen.

Total transpiration was measured under continuous light for the next 24 h, a period giving total flow equivalent to that required to approach steady state in the pressure system. Evaporation, measured in flasks without seedlings, was subtracted from the total flow. After transpiration, the stem was cut at the root collar and the water potential of the shoot measured (18). Shoot water potential was used as a measure of the effective gradient across the roots. Following the water potential measurements, the pressure was increased to 1.03 MPa for root systems and 2.07 MPa for shoot systems and the first 100 μl of exudate from the cut surface was collected for the analysis of PTS concentration. Analysis showed that the PTS concentration of the exudate collected from the root equalled that from the shoot.

Measurement of PTS Concentration. The 100 μl samples of exudate and bathing medium were diluted to 2 and 3 ml, respectively. PTS concentrations of the samples were determined on a Turner 430 spectrofluorometer using an excitation wavelength of 403 nm and emission wavelengths of 513 nm for the low concentration exudate samples and 540 nm for the higher concentration bathing media samples. The use of the 540 nm emission wavelength for the bathing media samples eliminated the need for large sample dilution necessary in order to measure them at 513 nm.

Measurement of Root Length. Root system lengths were measured after each experiment using a technique similar to that of Voorhees *et al.* (20). They applied the line intersect method of evaluating root length (12) to a computer-controlled digital scanning microdensitometer system.

Calculation of Apoplastic Flux. When the ratio C_e/C_b did not increase or decrease more than 0.01 ratio units in 100 min and observations were at least 100 but not greater than 200 min apart, the C_e/C_b value was considered to be at steady state. Steady state C_e/C_b combined with the flow rate over the corresponding time period was then used to calculate Q_a from equation 2.

RESULTS

In initial experiments with 1982 transplants total flux (Q_t) from root systems exposed to a 0.4 MPa pressure gradient was constant during the first 600 min and then increased to a second steady rate from 900 to 1300 min (Fig. 1B). C_e/C_b decreased rapidly during the first 400 min of the experiment from a peak of 0.77 to a level of 0.02 by 600 min and to 0.004 by the end of the experiment (Fig. 1A).

Anaerobic conditions significantly affected both Q_t and C_e/C_b (Fig. 2; Table I). Immediately after the replacement of air with nitrogen, Q_t decreased, reaching a new steady state after 400 min. When the bubbling gas was switched back to air, the Q_t recovered back to initial levels (Fig. 2B). Exposure to nitrogen caused an increase in C_e/C_b from 0.004 to 0.52 in about 200 min. The

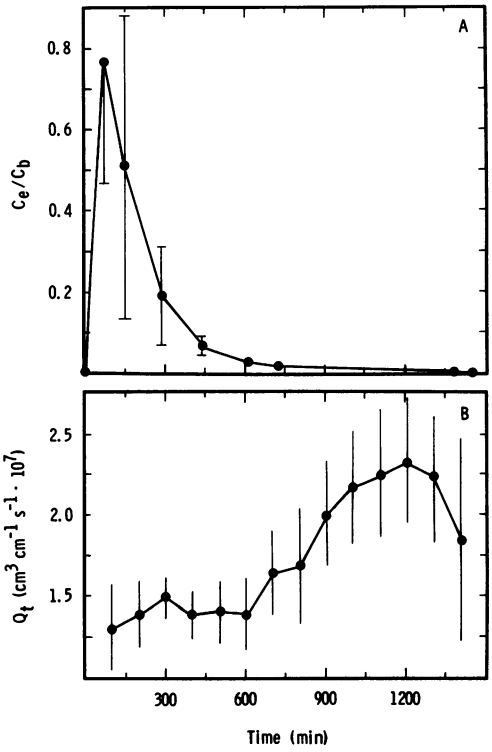

FIG. 1. C_e/C_b and Q_t over time under aeration and a constant pressure gradient of 0.40 MPa. Data points represent the mean and SE of three 1982 root systems.

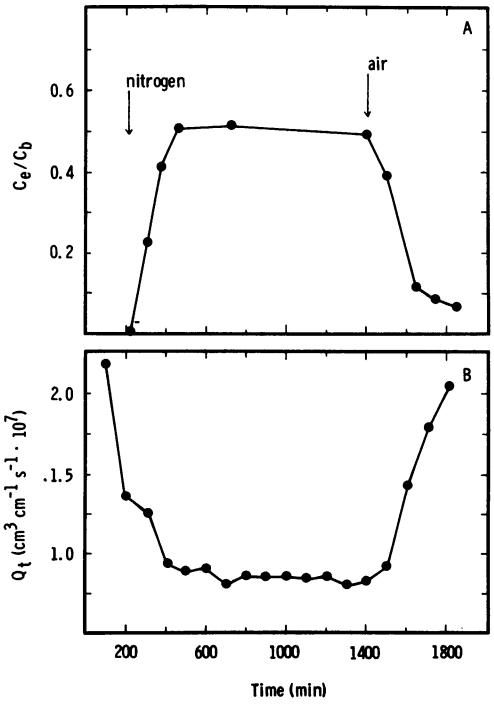

FIG. 2. C_e/C_b and Q_t under anaerobic conditions. Pressurization began under compressed air, was changed to nitrogen at 210 min, and was switched back to air at 1400 min. The pressure gradient was 0.40 MPa throughout the experiment. PTS was added just prior to the nitrogen. The experiment shown is an example of a typical result on 1982 root systems.

Table I. *Fluxes at Steady State under Aerobic and Anaerobic Conditions for Detopped Roots Exposed to External Pressure and for Intact Transpiring Plants*

The means and SE are based on five plants for the pressure and transpiration experiments except for the pressure data under anaerobic conditions which is for three plants.

Experimental Conditions	Effective Gradient	Total Flux (Q_t)	C_e/C_b	Apoplastic Flux (Q_a)
	MPa	$cm^3\ cm^{-1}\ s^{-1}\ 10^8$		$cm^3\ cm^{-1}\ s^{-1}\ 10^{10}$
Pressure				
Aerobic	0.40	22.6 ± 3.0	0.003 ± 0.0007	6.76 ± 1.70
Anaerobic	0.40	4.21 ± 1.39	0.448 ± 0.028	196 ± 78
Transpiration				
Aerobic	0.90 ± 0.02	141.0 ± 18.0	0.0013 ± 0.0003	16.9 ± 9.3
Anaerobic	1.32 ± 0.04	28.2 ± 6.3	0.023 ± 0.0058	66.0 ± 43.2

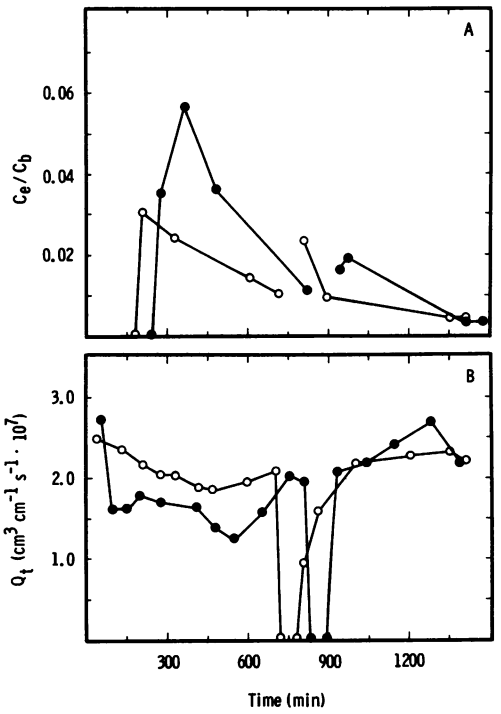

FIG. 3. The effect of pressure relaxation on C_e/C_b. The pressure gradient was reduced to 0 MPa at 830 min (●——●) and at 720 min (○——○) for 60 min. The pressure gradient was constant at 0.40 MPa at all other times. Data points of similar type represent individual values for a specific 1982 root system. Data for 1983 root systems responded similarly.

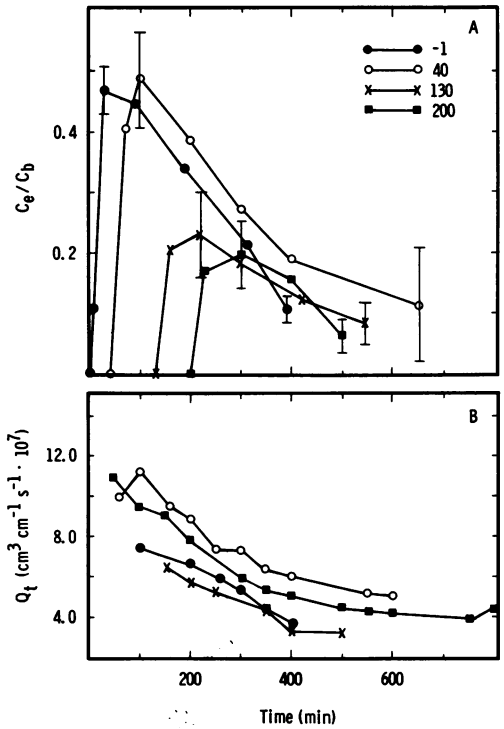

FIG. 4. The effect of injecting PTS at 1 min prior to (−1), or 40, 130, or 200 min after pressurization on Q_t and C_e/C_b. Data points are the mean of three (40, 130, 200 min injections), or two (−1) 1983 root systems. The SE bars included represent the variation typical of the entire curve.

switch back to air resulted in a rapid decrease of C_e/C_b (Fig. 2A).

To determine if the initial peak in C_e/C_b was due to root system damage during preparation, root systems were pressurized to 0.4 MPa for approximately 800 min and then the pressure was slowly reduced to 0 MPa for 60 min. The pressure was then slowly returned to 0.4 MPa. The depressurization had no effect on the Q_t as the flow rate returned to the level prior to the pressure drop (Fig. 3B). C_e/C_b, however, was significantly greater after the pressure drop than before, but rapidly dropped back to levels consistent with systems that had no relaxation of the pressures (Fig. 3A).

To investigate the nature of the C_e/C_b peak further, a set of 1983 root systems was used where PTS was injected into the pressurized vessel at 1 min prior to (−1) or 40, 130, or 200 min after pressurization. Q_t behaved somewhat differently than with the 1982 plants; decreasing during the first 300 to 400 min after pressurization to a value that remained constant to at least 800 min (Fig. 4B). The maximum value of the C_e/C_b decreased with the time of injection. The peaks for the −1 and 40 min injections were similar, and greater than the 0.23 and 0.20 peaks for the 130 and 200 min injections (Fig. 4A).

Several experiments were performed with intact transpiring plants. Results summarized in Table I indicate that, in general, detopped root systems exposed to a positive pressure gradient responded similarly to intact plants under a transpiration induced tension gradient. Anaerobic conditions decreased Q_t and increased C_e/C_b. Absolute comparisons between the two sets of experiments are inappropriate because the intact plants were younger than the detopped plants, the pressure gradients differed in magnitude between the two experiments, and the intact plants yielded only one measurement of Q_t and C_e/C_b per plant.

DISCUSSION

Previous researchers have reasoned, without direct measurement, that exclusively apoplastic pathways would contribute only a small percentage to the total water flux through a root system (4, 8, 9). In our experiments at steady state C_e/C_b in either intact plants or detopped roots, the proportion of flow that is apoplastic is on average approximately 0.2% (Table I). This calculation is based on a k value of one in equation 2. Because PTS moves slower than water (6), k is likely greater than 1 resulting in an underestimation of the relative contribution of apoplastic flow. Correction would not, however, raise Q_a above 1% of Q_t.

The high C_e/C_b ratio early in the time course experiments, followed by the general decline, could have been due to damage to the root during preparation. The increased C_e/C_b ratio during the 60 min pressure relaxation (Fig. 3) suggests that the elevated ratio is not due to tissue damage. Alternatively, the high ratio could be due to an initial accumulation of PTS during preparation which then is washed out as the mass flow of solution is driven through the root system. If this were the case, then when the PTS was added at different times after pressurization the C_e/C_b would rise from zero to a steady state value. Figure 4 shows that this is not the case.

A third possibility is a gradual change in the relative contribution of the symplastic and apoplastic pathways with time after pressurization. This is supported by the decrease in the initial C_e/C_b peak height with increased time of injection after pressurization (Fig. 4A). The increase in C_e/C_b following a pressure relaxation (Fig. 3A) confirms that the change in pathway is not rapid and is only partially reversed after 60 min. This interpretation suggests that at lower applied pressures, the Q_a to Q_t ratio could be higher than at larger applied pressures.

Changes in Q_t and C_e/C_b under anaerobic conditions provide additional insight regarding the effect of nitrogen on root system properties. The decrease in Q_t under nitrogen has been reported by others (7, 21) and interpreted as a decrease in the hydraulic conductance of the rate limiting membrane. Direct microscopic examination of anaerobic onion epidermis showed no penetration of PTS into the cells suggesting that the low oxygen levels did not increase the permeability of root membranes to PTS. Root tissue proved intractable to observation. The increase in C_e/C_b indicates that as the resistance to total flow increases, the relative contribution of the apoplastic pathway increases (Table I). This supports the idea that anaerobic conditions decrease the permeability of the rate limiting membrane to water (3).

In addition to the proportional increase in apoplastic flow, calculations using equation 2 show an absolute increase in the amount of apoplastic flow (Table I). A decrease in the apoplastic resistance would account for the absolute increase in apoplastic flow; however, it is unclear how the change in resistance might occur. Alternatively, Hanson (6) showed that the increase in Q_a could be explained by keeping apoplastic resistance constant and increasing symplastic resistance, provided that stellar resistance was at least 95% of total resistance across the root. Using a simple electrical analog model having a large stellar resistance in series with the parallel resistances of the symplast and the apoplast, Hanson showed that the increase in apoplastic flux under anaerobic conditions resulted from an increase in the water potential difference across the apoplastic pathway. The simplification of the complex distribution of resistances across the root by this model make these conclusions somewhat speculative. The large increase in Q_a under anaerobic conditions suggests that root systems may lose much of their control over the solutes that enter the root system since a considerable portion of the flow bypasses any membrane control. This could explain the observation that, over the short term, the solute concentration of xylem sap increases under anaerobic conditions (E. Fiscus, personal communication).

The results presented here demonstrate that PTS is effective in estimating the amount of apoplastic water flow through root systems, and that under aerobic conditions in a rapidly transpiring plant the apoplastic flow is less than 1%. The results also suggest that the pathway for water movement through the root may change as a result of anaerobic conditions and pressure gradients.

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