

# Assessing root death and root system dynamics in a study of grape canopy pruning

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## SUMMARY

Defining root death in studies of root dynamics is problematic because cell death occurs gradually and the resulting effects on root function are not well understood. In this study, metabolic activity of grape roots of different ages was assessed by excised root respiration and tetrazolium chloride reduction. We investigated changes in metabolic activity and patterns of cell death occurring with root age and changes in root pigmentation. Tetrazolium chloride reduction of roots of different ages was strongly correlated to respiration ( $R^2 = 0.786$ ). As roots aged, respiration and tetrazolium chloride reduction declined similarly, with minimum metabolic activity reached at six weeks. Tetrazolium chloride reduction indicated that the onset of root browning corresponded to a 77% reduction in metabolic activity ( $P < 0.001$ ). Anatomical examination of roots at each pigmentation stage showed that even though some cells in brown roots were still alive, these roots were functionally dead. The effect of using different definitions of root death in relation to root survivorship was determined in a study of 'Concord' grapes with two pruning treatments, using three criteria for root death: browning, blackening or shriveling, and disappearance. There was no effect of vine pruning on root life span when life span was defined as the time from first appearance to the onset of browning. However, if death was judged as the point when roots either became black or shriveled or disappeared, vine pruning decreased root life span by 34% and 40%, respectively ( $P < 0.001$ ), and also increased the decay constant for root decomposition by about 45% ( $P < 0.001$ ). We conclude that the discrepancy among determinations of root life span assessed with different definitions of death might be partly caused by the latter evaluations of root life span incorporating a portion of root decomposition in definitions of root death.

**Key words:** root death, root senescence, root survivorship and life span, minirhizotron, triphenyltetrazolium chloride (TTC), age-related root respiration, grape root decomposition, *Vitis labruscana* cv. Concord.

## INTRODUCTION

Identifying a stage to designate time of root death is not straightforward. Death of a portion of a root might not result in loss of root function. For example, the cortical region of a root might die, but with a healthy stele the root can still function as a pipeline for other roots and might retain a functional pericycle to produce new laterals (Spaeth & Cortes, 1995; Dubrovsky *et al.*, 1998; Lo Gullo *et al.*, 1998). Root senescence, even in the most distal roots that do not support laterals, is poorly understood. In leaves, individual cells can live for some time after leaf

abscission. Depending on the pattern of cell death, various root functions can cease before every cell dies (e.g. North & Nobel, 1996).

Distinguishing live and dead roots is a fundamental part of many root studies. Definitions of root death differ, making it difficult to compare results of studies of root dynamics. For example, in minirhizotron studies, assessment of root life span is dependent on assessment of root death from root images (Wang *et al.*, 1995). Studies of root dynamics could be improved by relating changes in root appearance to changes in root physiology and function. In our study, grape roots appeared to have phenotypically distinct stages of development, but the functional states of these stages were unknown.

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In a larger study of the effects of canopy manipulation on grape-root dynamics, we became interested in how different definitions of root mortality affected conclusions about the effects of treatments on root life span.

Triphenyltetrazolium chloride (TTC) reduction has been widely used as a general measure of vitality in plant tissue (Steponkus & Lanphear, 1967; Caldwell, 1993; Takeda, *et al.*, 1993; Porter *et al.*, 1994; Hiltbrunner & Fluckiger, 1996; Coria *et al.*, 1998; Schrazi & Muir, 1998; Sulpice *et al.*, 1998). TTC is colorless until reduced to formazan, a bright red pigment that is easily extracted and photometrically measured (Steponkus & Lanphear, 1967). In plant tissue, TTC is mainly reduced by dehydrogenase enzymes, most of which are associated with mitochondrial function. Although TTC reduction is an established test for vitality, the full significance of the capacity of a root to reduce TTC is not entirely clear. Respiration is a standard evaluation of metabolic activity, but to our knowledge the relationship between respiration and TTC reduction has not been investigated.

TTC reduction has been used to separate live and dead fine roots (Knievel, 1973; Joslin & Henderson, 1984; Clemensson-Lindell 1994). Wang *et al.* (1995) used TTC to ascertain whether roots classified as dead in video images based on color had the ability to reduce TTC. These researchers, studying roots of tree species, found that in 12–28% of those that were dark brown to black some portion of the root was able to reduce TTC. Thus, it was concluded that 12–28% of roots were mistaken for dead when root death was assessed from appearance. This study, however, did not establish when roots were alive or dead. From a functional perspective, it is unclear whether roots in which only a small portion of cells is able to reduce TTC should be categorized as alive. For example, these roots may be in the late stages of senescence and, in terms of water and nutrient absorption, functionally dead.

In our study, TTC reduction was compared with respiration in roots of different ages in order to corroborate measures of root metabolic activity and to determine the cessation of root activity with root age. To investigate further the necrosis of root tissue we examined TTC reduction in stained root cross sections. We then explored the consequences in the evaluation of root life span of judging root death by changes in root phenology.

## MATERIALS AND METHODS

### *Study site and plant material*

Soil at the study site at Cornell University's Vineyard Laboratory in Fredonia, NY was a well drained, Chenango gravelly loam. Mature, 25-yr-old *Vitis*

*labruscana* Bailey cv. Concord grapevines with high permanent arms 1.8 m above the ground and growing 2.4 m apart in rows 2.7 m apart were used to compare two pruning treatments, balanced pruning and minimal pruning. The treatments were initiated in 1991 and conducted November–December after leaf fall. In balanced pruning, 44 buds per kg of stems pruned during the previous winter pruning are left; this the historically recommended method in the Lake Erie region because it maintains shoot vigor. Minimal pruning consists only of undercutting the hanging vines to 1 m above the ground to keep shoots off the ground. In all other respects, cultural practices were those standard for grape production.

### *Measurements of respiration and tetrazolium chloride (TTC) reduction in roots of different age*

In early spring 1998, in order to access roots of known age, root boxes 60 × 60 cm and 30 cm deep, with acetate windows, were installed under eight minimally pruned vines and eight balanced-pruned vines (1 box per vine). Each window was insulated with a 2.5-cm-thick sheet of styrofoam and boxes were covered in order to maintain bulk soil temperature. From May–August the age of roots was tracked by marking their extent on the windows at weekly intervals. Roots growing in the windows were harvested in early August, 9 wk after the first root appeared (nine ages in total). Two roots of each root age in each root box constituted a sample. Respiration of each sample was measured 30 min–1 h of excision with either a Clark-type O<sub>2</sub> electrode (Hansatech, King's Lynn, UK) or a micro-oxygen electrode (Microelectrodes Inc., Bedford, NH, USA) in aerated 1 mM CaSO<sub>4</sub> + 5 mM MES solution (pH 5.5) at 25°C.

Samples were then assayed by a modified TTC test procedure (Steponkus & Lanphear, 1967). Control roots were boiled for 10 min in distilled water to insure that enzymes were denatured. Maximum ability of tissue to reduce TTC has been found at a TTC concentration of 0.5% (Steponkus & Lanphear, 1967). All roots were cut into 1-cm pieces, submerged in 3 ml of 0.6% (w/v) 2,3,5-triphenyltetrazolium chloride in 0.05 M Na<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub> (pH 7.4) + 0.05% wetting agent (Triton X-100), and vacuum-infiltrated for 5 min to insure infiltration of TTC. Samples were incubated at 30°C for 24 h, rinsed twice with distilled water, and extracted four times in 4 ml of 95% (v/v) ethanol for 5 min in a waterbath at 85°C. The total solution extracted was brought up to a volume of 25 ml and measured with a spectrophotometer (Shimadzu UV160U, Kyoto, Japan) at 490 nm. Spectral analyses had been previously made of root extractions in 95% ethanol to verify that plant pigment would not interfere with the absorption of formazan.

*Tetrazolium chloride (TTC) reduction in roots of different appearance*

In September 1997, fine roots (<1 mm) were collected from 16 locations in the vineyard, eight from balanced-pruned and eight from minimally pruned vines. Collections were made from the top 20 cm of soil, 50–60 cm from the base of the vines, then divided into white, brown, and black/shriveled classes. Roots in the white and brown classes were turgid whereas those in the black class were shriveled and limp. One collection lacked white roots and another lacked brown, leaving 15 samples of white and brown classes and 16 of the black/shriveled. Each sample was composed of two to three roots. Five control samples were formed by pooling roots of all pigmentation classes from the collections and boiling them for 10 min in distilled water. All samples were stained with the TTC procedure previously described. Four of the samples of each pigmentation class were sectioned four times. Each section was viewed with a dissecting microscope to determine whether the cells in the epidermis, cortex and stele were red, indicating that they were alive. Dye from the control and remaining samples of each pigmentation class was extracted and measured as previously described, except that samples were extracted twice and extraction volume was brought up to 10 ml. A natural log transformation was used to correct for heterogeneity of variances among the pigmentation and control classes. ANOVA was used to test for effects among classes, followed by Fisher's protected multiple range test (LSD,  $\alpha = 0.05$ ) to compare means between the different classes.

*Measurements of root life span*

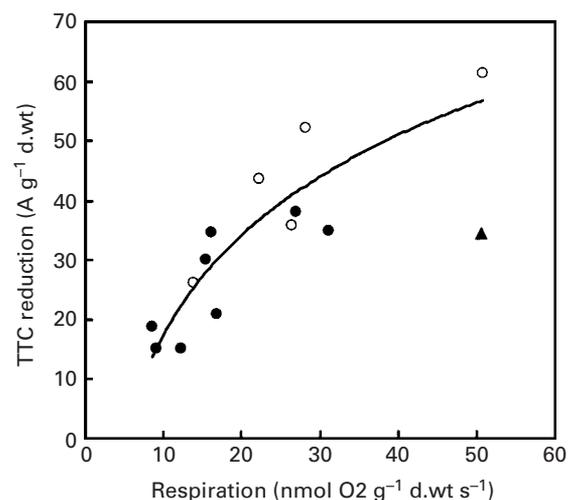
Root populations were monitored through 183-cm-long butyrate minirhizotron tubes of 5.7 cm diameter, installed in fall 1996. Tubes were etched with a column of 127 numbered windows  $1.0 \times 1.5$  cm. From March 1997, images of the windows were collected every 2 wk with a microvideo camera system (Bartz Technology, Santa Barbara, CA, USA). Eight tubes were examined for each pruning treatment. Dates that individual roots were born, became pigmented, turned black or shriveled, and disappeared were recorded. Roots were divided into two diameter classes, <0.6 mm and >0.61 mm. Data on the smaller-diameter class in the top 30 windows were included in the analyses of root survivorship and decomposition presented here. Root survivorship was analysed as the percentage of a population that was viable at each root age. The start date of a cohort was determined as the median birth date so that a seasonal reference could be given. Root decomposition was analysed as the percentage of a population persisting with time beyond a visible

change in root viability. Two independent analyses of root decomposition were made, one using browning and one using blackening as the stage indicating the end of root viability. Effects of treatment on root survivorship and decomposition were analysed with the Wilcoxon test (SAS Institute, Cary, NC, USA).

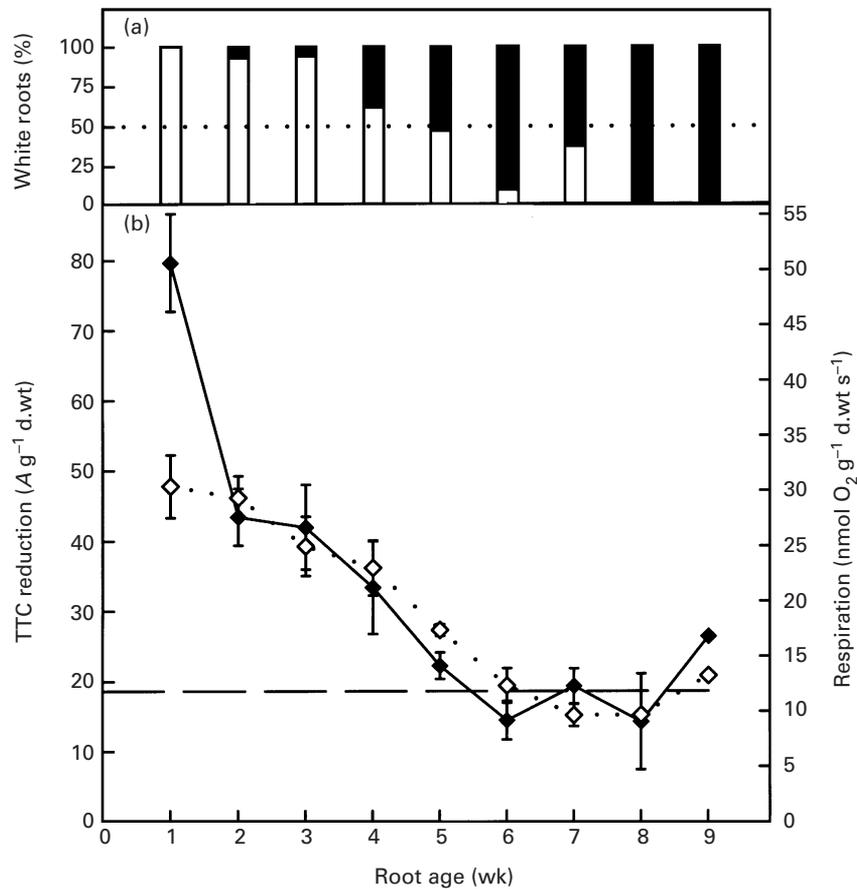
## RESULTS

When 1-wk-old roots of minimally pruned vines were excluded, TTC reduction for roots of various ages was highly correlated with root respiration in a logarithmic fashion ( $R^2 = 0.786$ ; Fig. 1). In roots of minimally pruned vines, TTC reduction was generally lower than in balanced-pruned vines, especially when respiration was high (Fig. 1). Metabolic activity assessed by TTC reduction and respiration declined with root age, with minimum metabolic activity reached at 6 wk (Fig. 2b), when TTC reduction of roots was equal to that of boiled roots. Median functional root life span of roots growing against root box windows corresponded to approx. 5 wk, at which time *c.* 50% of the roots were brown or black (Fig. 2a).

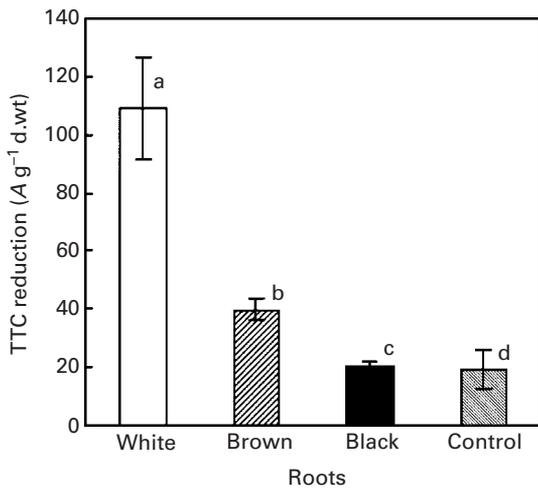
Significant differences in TTC reduction levels were found between control and root pigmentation classes ( $P < 0.001$ ,  $df = 3$ ). In brown roots TTC reduction was only 23% of that in white roots after accounting for boiled-root absorption ( $P < 0.001$ ,  $df = 35$ ). In black roots TTC reduction was not significantly different from that in boiled control roots. In white roots TTC was reduced in all anatomical regions (Fig. 4a). Brown roots either



**Fig. 1.** The relationship between tetrazolium chloride (TTC) reduction and respiration for 'Concord' grape roots of various ages. Open circles, balanced-pruned vines; filled circles and triangles, minimally pruned vines. Closed triangle, 1-wk-old roots of minimally pruned vines.  $y = 24.144 \text{ Log}_e(x) - 38.1$ .  $R^2 = 0.786$  without 1-wk-old roots of minimally pruned vines.  $R^2 = 0.633$  with all roots. For clarity, SE omitted.



**Fig. 2.** (a) The proportion of root samples in each age class that were white (open bars) vs the proportion that were brown or black (filled bars). Dotted line, 50% of the roots sampled were brown or black. (b) Metabolic activity with root age estimated by tetrazolium chloride (TTC) reduction (open diamonds) and respiration (filled diamonds) for ‘Concord’ grape roots undergoing both balanced and minimal pruning ( $\pm$  SE). Dashed line, absorption determined from control roots. Number of samples in age classes 1–9 were 14, 14, 16, 16, 16, 4, 3, 2 and 1, respectively.

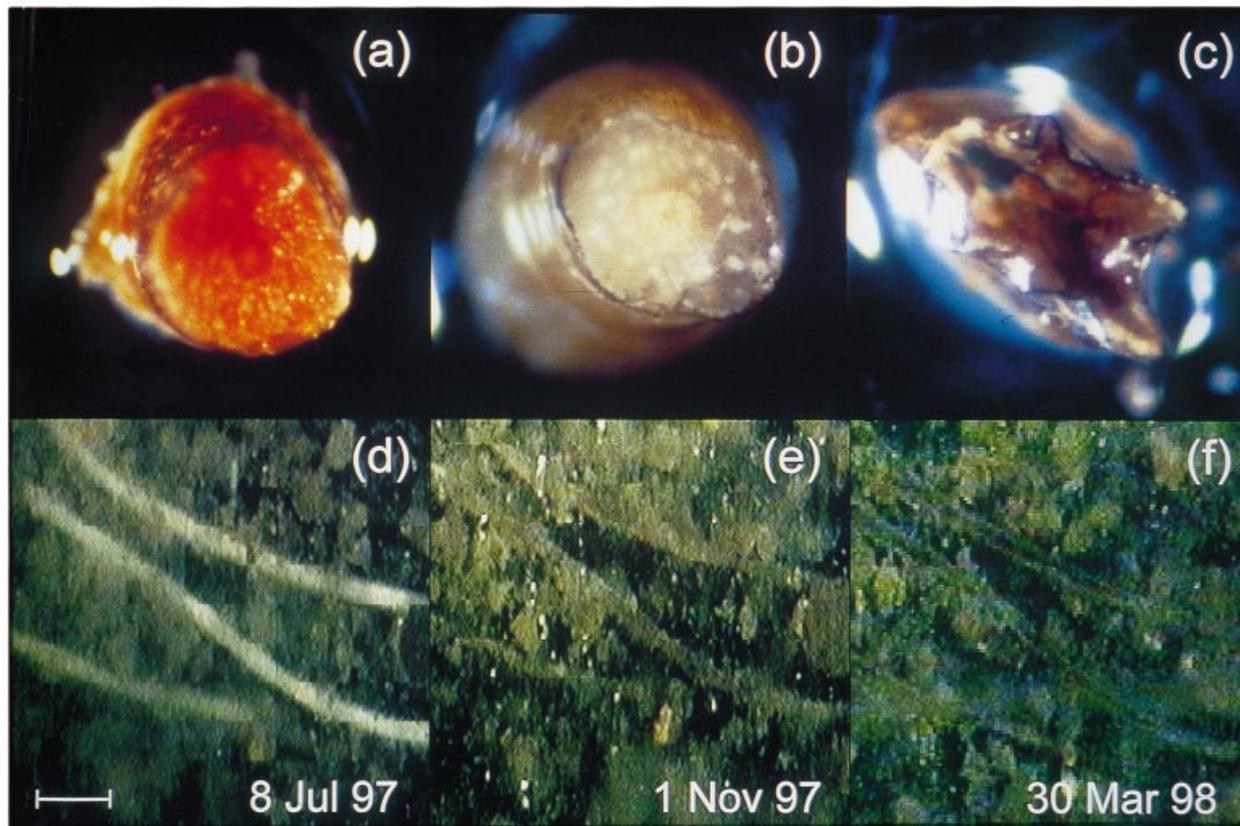


**Fig. 3.** Metabolic activity estimated by tetrazolium chloride (TTC) reduction of ‘Concord’ grape roots in each pigmentation class ( $\pm$  SE). Bars that do not share letters are significantly different ( $P \leq 0.05$ ). Numbers of samples in white, brown, black, and control classes were 11, 11, 12 and 5, respectively.

displayed trace amounts of formazan throughout a cross section (Fig 4b) or strong color only in pockets of cells in a section. Occasionally, brown roots

exhibited TTC reduction in the stele without showing any activity in the cortex, indicating that, while certain root functions requiring an intact cortex might have ceased, the root was functional as a pipeline and possibly still had a healthy pericycle to initiate new laterals. At least once, however, a root section with an active stele was preceded by one with no activity in the stele, indicating that the entire root was no longer functional, either in transport or as a source for new laterals. Occasionally, brown roots exhibited TTC reduction in a few cells of the cortex and no activity in the stele, also indicating that they were no longer functional organs. Black roots exhibited no activity in any region (Fig. 4c). Consequently, we concluded that, among roots of diameter  $< 0.6$  mm, brown roots were ‘senescing’ and black roots were ‘dead’.

When root survivorship curves were formulated by assessing the time from root birth to root browning, median root life spans of balanced-pruned and minimally pruned vines were not significantly different ( $P = 0.777$ ; Fig. 5a, Table 1). However, when root life span was evaluated as the time from root birth to roots turning black or shriveled, or roots disappearing, median root life spans of



**Fig. 4.** (a–c) Freehand cross sections of tetrazolium chloride (TTC)-incubated ‘Concord’ roots in three pigmentation classes; (a) white, (b) brown, and (c) black. Root diameters of white and brown roots, approx. 0.4 mm. White roots showed TTC reduction in all anatomical regions (red stain). Brown roots showed trace amounts, while black roots exhibited no staining. (d–f) Minirhizotron images illustrating critical changes in visual appearance of roots; (d) new white roots; (e) the same roots having turned brown and (f) having turned black and shriveled. Bar, 1 mm.

balanced-pruned vines were 34–40% shorter than those of minimally pruned vines ( $P < 0.001$ ; Fig. 5b,c, Table 1).

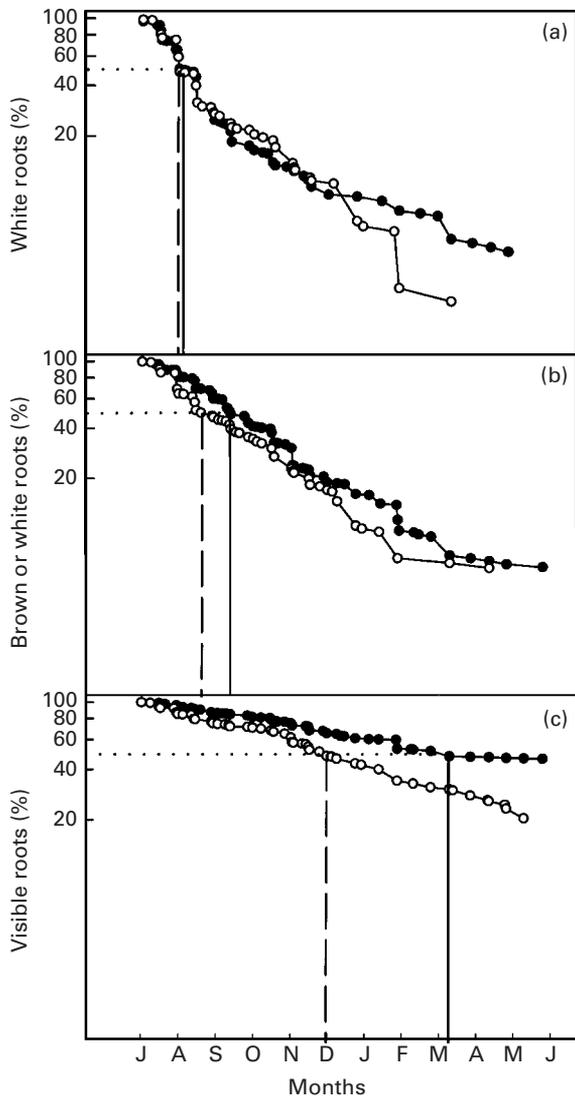
Decomposition rates determined from the disappearance of brown and black roots was 42% and 45% higher in balanced-pruned vines than in minimally pruned vines, respectively ( $P < 0.001$ ,  $df = 1$ ; Fig. 6). Decomposition of brown and black roots of balanced-pruned vines closely followed an exponential decay function, but decomposition in minimally pruned vines appeared to diverge from the exponential function after 200 d (Fig. 6). The average time from roots browning to turning black or shriveled was 56.1 and 57.1 d in balanced and minimally pruned treatments, respectively, and highly variable (data not shown).

#### DISCUSSION

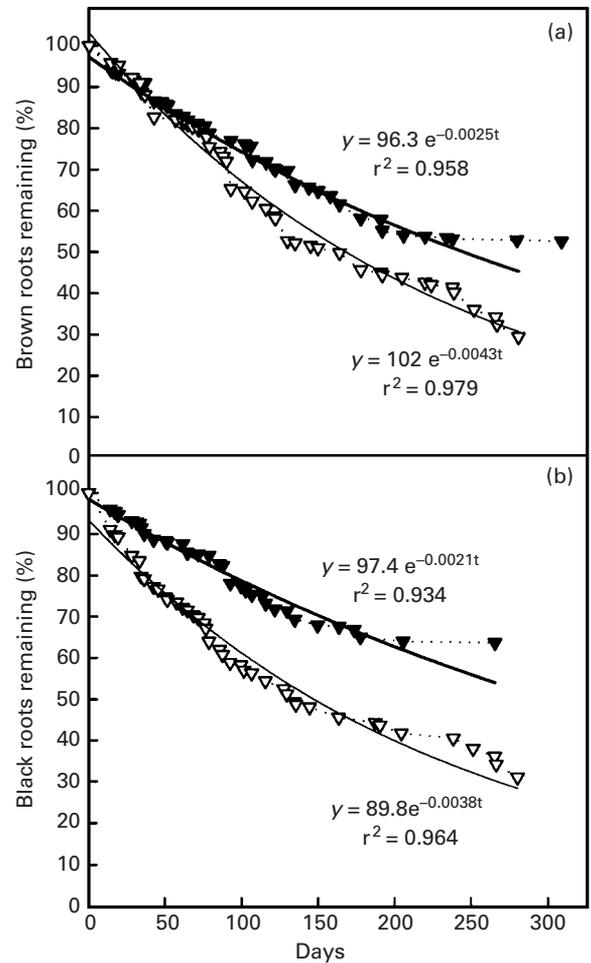
Canopy pruning in grape dramatically increased root decomposition but did not affect ‘functional’ root life span, defined as time to root browning. However, when root death was assessed more conservatively, by the criteria of a later stage of senescence or root

disappearance, canopy pruning greatly decreased root life span. In grape, the onset of root browning generally indicated death of the root as a ‘functional’ organ. Metabolic activity of brown roots was greatly reduced and patterns of cell death indicated that root function was compromised. Examination of survivorship curves based on different stages of root senescence gave greater insight into the dynamics and decomposition of roots in grape.

Implicit in any study of root dynamics is examination of the survivorship of functional roots. Normally considered a dichotomous variable, root death actually represents a progression of cell and tissue death. In grape, root browning and turning black/shriveled represented two stages on the continuum of root senescence. Most minirhizotron studies are conservative and identify roots as dead when dark and shriveled (Pregitzer *et al.*, 1995; Steele *et al.*, 1997). Inevitably, evaluations of death such as blackening or shriveling, or disappearance of roots include a portion of decomposition in the assessment of death. If a treatment, such as pruning in this study, affected decomposition, then estimates of root life span when evaluated by these indicators can also be affected.



**Fig. 5.** Root-survivorship curves for minimally (filled circles) and balanced-pruned (open circles) ‘Concord’ grape vines representing: (a) time from root appearance to turning brown; (b) time from root appearance to blackening or shriveling; (c) time from root appearance to disappearance. Root populations for each treatment included all roots <0.6 mm diam. born in the 1997 season in the top 30 cm of soil ( $n = 390$ , minimally pruned;  $n = 240$ , balanced-pruned). At  $t_0$ , 100% of the population was present. To give a seasonal reference,  $t_0$  was assigned the median appearance date of roots. Solid vertical line, median life span, minimally pruned vines; dashed vertical line, median life span, balanced-pruned vines.



**Fig. 6.** Decomposition curves of brown and black roots of minimally (solid triangles) and balanced-pruned (open triangles) ‘Concord’ grape vines. (a) Decomposition curves formulated from the length of time between root browning and disappearing. (b) Decomposition curves formulated from the length of time between roots turning black/shriveled and disappearing.

The correlation between pigmentation and metabolic activity that occurred in grape might not, however, occur in all other woody species. For example, root pigmentation in *Picea abies* (Norway spruce) and *Pinus sylvestris* (Scots pine) did not consistently correlate with a significant decrease in TTC reduction (Clemensson-Lindell, 1994). Unlike the finest roots of grape, those of pine often have

**Table 1.** Median root life spans and decomposition half-lives ( $t_{1/2}$ ) of roots in balanced- and minimally pruned ‘Concord’ grape vines

Pruning treatment	Functional lifespan <sup>1</sup> (d)	Total lifespan <sup>2</sup> (d)	Root duration <sup>3</sup> (d)	Brown root disappearance <sup>4</sup> (d)	Black root disappearance <sup>5</sup> (d)
Balanced	29 a <sup>6</sup>	47 a	149 a	161 a	182 a
Minimal	33 a	71 b	248 b	277 b	330 b

<sup>1</sup>Root birth to turning brown. <sup>2</sup>Root birth to turning black or shriveled. <sup>3</sup>Root birth to disappearing. <sup>4</sup>Time between root browning and disappearing. <sup>5</sup>Time between roots turning black/shriveled and disappearing. <sup>6</sup>Within each column, medians followed by a different letter are significantly different ( $P = 0.001$ ) based on a Wilcoxon test of whole survivorship curve.

white, actively growing tips after turning darkly pigmented. The generality of the relationship between pigmentation and loss of metabolic activity is unclear.

In apple (T. Bouma & D. M. Eissenstat, unpublished), P uptake and root respiration declined with root age similarly to grape in this study. Generally, however, the manner in which different root functions decline with root age is poorly understood. In this study we used metabolic activity as a broad measure of root function because declines in specific root functions, such as the uptake of different nutrients and water, can occur at different times. The slight rise in TTC reduction and respiration at 9 wk was possibly due to small sample size or to increased microbial activity (Fig. 2b). The maximum TTC reduction in Fig. 2b ( $48 \text{ A g}^{-1} \text{ d. wt}$ ) was about half that in Fig. 3 ( $109 \text{ A g}^{-1} \text{ d. wt}$ ), probably because of differences in environmental conditions between the two years of sampling. In 1998 (Fig. 2b), the growing season was considerably drier than in 1997 (Fig. 3), which probably influenced overall metabolic activity of the roots. It should also be noted that roots born at various times over the season were harvested at the same time to compare the metabolic activity of roots of different age. While environmental factors occurring over the course of the season affect root physiology, mitochondrial activity is probably most influenced by current photosynthate production and current environmental conditions (Lambers *et al.*, 1995; Fitter *et al.*, 1998) making it necessary to compare measurements taken at one point in time. However, the relationship of root metabolic activity as a function of root age illustrated in Fig. 2b might differ somewhat from year to year, throughout the growing season, and among locations as a result of varying environmental conditions. For example, we would expect that the effects of ageing on root metabolic activity would be dampened in cold soil and accelerated in drying soil. The important points are that TTC seems to describe this relationship as well as direct measurement of respiration, and that we found a close correspondence between pigmentation level and decline in root activity.

Overall, respiration and TTC reduction in grape roots were closely correlated and appeared to be equally good estimators of root metabolic activity (Fig. 1). The youngest roots of the minimally pruned treatment, having low TTC reduction and high respiration, deviated from this correlation. The reason for this is unclear. A logarithmic function best described the relationship between TTC reduction and respiration (Fig. 1). The declining increase in TTC reduction with increase in respiration might be due to limitations on the amount of TTC infiltrating roots because TTC in solution was in excess and extracted formazan was diluted to insure that color was unsaturated. Also, the extension

of the correlation between TTC reduction and respiration to high values was dependent on one point; however, removal of this point from the correlation does not affect the regression line through the remaining values.

While respiration clearly evaluates metabolic activity, TTC reduction is a more rapid method and has the additional benefit of allowing for the observation and identification of active anatomical regions. Quantifying TTC reduction by extraction of formazan is less subjective than sectioning of stained roots, but both evaluations were valuable in assessing root senescence. To our knowledge, the extent to which TTC reduction mirrors root respiration has not been investigated, although both have been used independently to evaluate root metabolic activity. The correlation between respiration and TTC reduction, however, should be tested in other species before a general relationship is assumed.

Few studies have examined the effects of pruning or defoliation on root dynamics. In citrus, heavy pruning has been associated with apparent reductions in root life span (Eissenstat & Duncan, 1992). A unique aspect of our study was the 7-yr duration of the pruning treatments. Presumably the C balance of the vines would be in equilibrium after pruning for this length of time, and might explain why a reduction in functional root life span was not observed in this study. Canopy pruning of grapevines is known to reduce total dry-matter productivity of vines and to invigorate the remaining shoot growth (Winkler *et al.*, 1974). After pruning, more shoots tend to be grown in order to re-establish an equilibrium in root : shoot ratio. Differences in root composition related to C status, such as cell-wall lignification, might explain why roots of heavily pruned vines decompose more rapidly than those of minimally pruned vines.

A better understanding of root death can provide valuable insight into patterns of root dynamics. TTC reduction was a relatively simple and quick method of relating root function to root appearance in grape. The effect of pruning on root decomposition in our study might have been missed without examination of the stages of root senescence, death and disappearance. Conclusions about the effect of canopy pruning on root dynamics were strongly influenced by different definitions of root death. Although we have not resolved all the problems of defining root death, we were able to identify stages of root senescence that were valuable in assessing patterns of root dynamics in response to canopy pruning.

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