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Vitality of tree fine roots: reevaluation of the tetrazolium test

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Summary Reduction of triphenyltetrazolium chloride (TTC) by tissue to the red-colored insoluble triphenylformazan (TF) is directly linked to the activity of the mitochondrial respiratory chain. Thus, only living tissues should reduce TTC to TF. However, TF production can be detected when the TTC test is applied to control tissues boiled in water. This artifact is mainly the result of the hot ethanol extraction step, which is used to disintegrate the cells and solubilize the TF. We observed that cell wall materials such as cellulose and pectin interact with TTC at temperatures above 60 °C, reducing the TTC to TF. By replacing the hot ethanol extraction step with an extraction procedure that involved grinding the boiled tissues and extracting the TF with ethanol at room temperature, the formation of TF was almost entirely eliminated. Application of the modified TTC assay to fine roots of Norway spruce from forest topsoil indicated that the extent of TTC reduction was related to root morphological class as: white fine roots > brown fine roots > black fine roots > boiled fine roots, corresponding to formation of 10.8, 6.1, 0.2 and 0.1 mM TF g_{DW}^{-1}, respectively. No significant differences in TTC reduction were recorded between fresh and frozen tissues (frozen in liquid N_2) for any fine root class. Application of the modified TTC assay to seedling roots stressed either by drying or by exposure to the toxic metals Cd or Al resulted in significant decreases in TF formation that were related to both the duration of stress and the concentrations of toxic substances, indicating that the modified TTC assay can be used to assess the physiological condition of roots.

Keywords: mitochondrial respiration chain activity, Norway spruce, stress factors, toxic metals, TTC test.

Introduction

Fine roots play an important role in the nutrient cycling of terrestrial ecosystems because they constitute both sinks and sources for nutrients. In recent years, there has been increased interest in the study of fine roots of trees (roots with a diameter < 2 mm) (Cronan and Grigal 1995, Persson et al. 1995, 1998, Högberg et al. 1998, Bakker 1999). Global environmental changes, such as increases in atmospheric CO_2 concentration, increases in soil acidity, and increases in deposition of N and heavy metals, lead to considerable changes in forest ecosystems, with potential consequences for tree roots (e.g., Blaser et al. 1999, Norby and Jackson 2000, Jentschke et al. 2001).

Assessment of root quality is a fundamental part of many root studies; however, distinguishing living from dead roots is difficult because cell death occurs gradually and the resulting effects on root function are not well understood (Comas et al. 2000). Currently, root quality is assessed mainly on the basis of color, texture and brittleness (Ruess et al. 1996, Helmisaari and Hallbäcken 1999). An alternative method for determining the condition (vitality, viability) of roots is to use tetrazolium salts, which are metabolically reduced to brightly colored formazan products (Clemensson-Lindell 1994, Comas et al. 2000). Because this biochemically based assay depends on the activity of the mitochondrial respiratory chain (Altman 1976, Comas et al. 2000, Byth et al. 2001), only living tissues should reduce tetrazolium salts to formazan products.

The most frequently used tetrazolium salt in botanical research is triphenyltetrazolium chloride (TTC). A major disadvantage of the TTC assay is that dead roots and boiled roots (controls) produce a relatively high absorption at 520 nm due to the formation of triphenylformazan (TF) (Clemensson–Lindell 1994, Comas et al. 2000).

In a preliminary study, we found that cell wall materials such as cellulose and pectin interact with TTC at temperatures above 60 °C, reducing it to TF, suggesting that the hot (80 °C) ethanol extraction step of the TTC test, proposed by Clemensson-Lindell (1994) and others, may be responsible for this artifactual formation of formazan. The hot ethanol has the double function of disrupting the cells and dissolving the formazan product. We hypothesized that omitting high temperatures and using alternative methods for cell disruption would eliminate this artifact. Therefore, the main objective of our study was to improve the TTC method by minimizing the formation of formazan in dead and boiled control roots. In addition, we adapted the improved TTC test for small quantities (2-ml reaction tubes) to faciliate the processing of large numbers of samples.

Materials and methods

TTC test

Fresh fine roots weighing 100 mg and sectioned into 1–2 mm long segments were transferred to 2-ml reaction tubes, and 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing

0.6% TTC and 0.05% Tween 20 was added to each tube. One or two holes were made in the caps and the samples were placed under vacuum for 10 min to remove air from the intercellular spaces, thereby allowing tissue infiltration by the TTC solution. The samples were then incubated for 20 h at 30 °C. After incubation, the TTC solution was decanted. Three different procedures for cell disruption and TF extraction were compared: (1) adding 1 ml of ethanol and boiling at 80 °C for 15 min; (2) grinding with two steel balls (2 mm in diameter) at full speed for 3 min in a swing mill MM 2000 (Retsch, Haan, Germany), adding 1 ml ethanol at room temperature and vortexing for 10 s; and (3) adding 1 ml ethanol at room temperature followed by freezing (in liquid N_2) and thawing (at room temperature) three times. After the cells had been disrupted, the reaction tubes were centrifuged at 10,000 *g* for 2 min, and the absorbance of 0.3 ml of the supernatant was measured at 520 nm with a spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, CA). The root residues in the test tubes were dried at 105 °C for 24 h and weighed. Reduction of TTC was calculated as absorption of TF produced per g root dry mass ($A_{520}\,g_{DW}^{-1}$) and multiplied by the factor 0.1024 to obtain the concentration of TF produced (mM TF g_{DW}^{-1}). This factor was calculated from a linear calibration curve of TF (0.1 mM TF has an absorption of 0.977 at 520 nm). Fine roots that were killed by boiling in water at 100 °C for 20 min before being incubated in TTC solution served as controls.

Effects of fine root morphology and freezing

Fine roots (< 2 mm in diameter) of ~30-year-old Norway spruce (*Picea abies* (L.) Karst.) trees were collected from 0–5 cm soil depth in the Rameren forest near the WSL Institute in Birmensdorf, Switzerland. The fine roots were washed in water and classified into three morphological classes (white, brown, black) based on visual examination (cf. Clemensson-Lindell 1994, Clemensson-Lindell and Persson 1995). Fine roots with light coloring, many white and turgid root tips, and white and elastic steles were classified as white. Fine roots with darkened coloring and few white root tips were classed as brown, and fine roots with many blackened root tips that fell off and brownish and non-elastic but compressible steles were classed as black (dead). Four to five root samples of each root class were used for each TTC test, which consisted of incubating each fine root sample in 0.6% TTC solution for 20 h, followed by tissue grinding and addition of 1 ml of ethanol at room temperature, and determination of absorbance at 520 nm as described in the previous section (hereafter referred to as the modified TTC assay). White fine roots were boiled in water for 20 min to serve as controls. A series of samples from each root class was run without incubating the root samples in TTC solution to determine absorption of the root extracts.

To establish whether freezing negatively affected the TTC test, fine roots were frozen in liquid N_2 and stored for one week at –80 °C before the modified TTC assay was applied. The results were compared with the results for fresh fine roots.

Effects of temperature and organic and inorganic materials on the TTC assay and time course of TTC reduction

To determine the influence of temperature on TF formation in boiled root tissue, the ethanol extraction was performed at 20, 40, 60, 80 and 100 °C. Roots were obtained from Norway spruce seedlings. Seeds were surface sterilized for 20 min with 30% H_2O_2, then spread on autoclaved and moist vermiculite and incubated under non-sterile conditions in a growth chamber at 20 °C. The vermiculite was moistened frequently. After 2 months, the seedlings were harvested and the roots excised. The roots of five seedlings were pooled per sample (about 100 mg_{FW}, yielding about 30 mg_{DW}), boiled, ground as described above and put in a 2-ml reaction tube. Fifty µl H_2O, 100 µl 0.6% TTC solution and 1 ml ethanol were added, and the tubes incubated for 15 min in a thermomixer (Eppendorf, Wesseling-Berzdorf, Germany) at the various temperatures. The tubes were centrifuged and the absorption of the solution was measured at 520 nm. Three samples were assayed per temperature treatment.

We also determined the effects of various organic and inorganic materials (30 mg) on the TTC assay including: dried roots (from dried and ground fine roots of Norway spruce collected from forest topsoils in Switzerland), cellulose (for column chromatography; Macherey, Nagel and Co., Düren, Germany), pectin (from apples; Fluka, Buchs, Switzerland), starch (from potatoes, for electrophoresis; Fluka), tannic acid (from oaks; Fluka), agarose (Gibco, Paisley, UK), PVP (polyvinylpyrolidone K30; Fluka) and fine quartz sand (washed; Merck, Darmstadt, Germany). For the controls, either the materials were omitted, or the assay was run without the TTC solution to check for auto-absorption (only with dried roots, cellulose, agarose, tannic acid and without any materials). After incubation for 15 min at 100 °C, the solution was adjusted to the original volume with ethanol and the absorbance at 520 nm determined.

To determine the time course of TTC reduction by living and boiled control tissues, fine roots of five seedlings were pooled per sample. Half of the samples were boiled in water for 20 min. All of the samples were then incubated with 0.6% TTC solution for 1, 2, 3, 5, 8, 20 and 40 h and processed by the modified TTC assay. Three samples were assayed per treatment and time period.

Root stress treatments

Norway spruce seeds were surface sterilized for 20 min with 30% H_2O_2. The seeds were germinated aseptically on water agar in petri dishes sealed with parafilm at room temperature. After 1 month, seedlings were harvested and their roots excised either immediately after harvest or after an air drying period of 6 or 24 h at room temperature. As controls, roots were boiled in water for 20 min. The control, fresh and air-dried roots were incubated with 0.6% TTC for 20 h and then assayed by the modified TTC procedure. Roots of 40 seedlings were pooled per sample (about 100 mg_{FW}), and three samples were assayed per treatment.

One-month-old seedlings were incubated for 6 or 24 h at room temperature with 50 or 100 μM $CdCl_2$, or 0.5 or 1.0 mM $AlCl_3$, or 0.5 mM $AlCl_3$ + 0.75 mM $CaCl_2$. Seedlings treated with 0 or 100 mM $CaCl_2$ served as controls for the Cd treatments and seedlings treated with 0.75 mM or 1.5 mM $CaCl_2$ served as controls for the Al treatments. Forty seedlings were placed around the edges of petri dishes (9 cm in diameter) containing 40 ml of a treatment solution at pH 4.0, with the roots in the solution and the shoots in the air. After the treatments, the roots were excised and all roots from one petri dish were combined (about 100 mg_{FW}) and incubated in 0.6% TTC solution for 20 h and then measured for TF formation by the modified TTC assay. Three samples were assayed per treatment.

Statistical analyses

The data were subjected to one- to three-way analyses of variance (ANOVA). Least significant differences were calculated at $P < 0.05$ by Fisher's PLSD test. All tests were conducted with StatView 5.0 (SAS Institute, Cary, NC).

Results

Modified TTC test

The three cell disruption procedures tested yielded different results. The TTC test based on the hot ethanol extraction procedure indicated that the two morphological fine root classes, white and brown, had similar TF formation at around 7.2 mM TF g_{DW}^{-1}, whereas black and boiled fine roots had values of around 2.5 mM TF g_{DW}^{-1} (Figure 1A). In contrast, the TTC test based on the grinding procedure followed by ethanol extraction at room temperature to dissolve the TF formed indicated that TF formation varied among the white, brown and black fine root classes as 10.8, 6.1 and 0.2 mM TF g_{DW}^{-1}, respectively, with boiled fine roots having a value of 0.1 mM TF g_{DW}^{-1} (Figure 1A). The repeated freezing–thawing procedure resulted in relatively low values of TF formation by white and brown fine roots (0.7–1.2 mM TF g_{DW}^{-1}), even though values of TF formation by the black and boiled fine roots were similar to those obtained after the grinding procedure (Figure 1A). When incubation in 0.6% TTC solution was omitted, both the grinding and the freezing–thawing procedures yielded absorptions at 520 nm of around 1 (A_{520} g_{DW}^{-1}), which probably originated from root components (e.g., tannins), whereas the hot ethanol extraction yielded significantly higher absorptions of around 4 (A_{520} g_{DW}^{-1}) (Figure 1B). Freezing roots in liquid N_2 had no significant effect on their ability to reduce TTC in the modified TTC assay (Figure 2), indicating that fine roots can be harvested and frozen immediately in the field.

Effect of hot ethanol on TTC reduction

Use of hot ethanol in the TTC assay of boiled roots and various organic and inorganic materials induced formation of TF from TTC in most cases. Boiled and dried roots both yielded similiar absorption values at 520 nm, which increased from 1 to 19 (A_{520} g_{DW}^{-1}) with increasing extraction temperature (Table 1). Other materials, such as cellulose, pectin and starch, which also occur in roots, resulted in relatively low absorp-

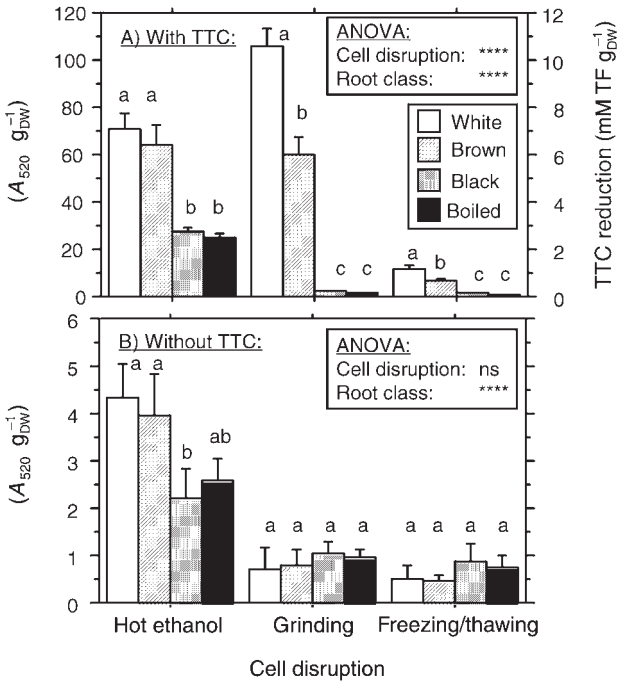

Figure 1. Absorption at 520 nm (A_{520} g_{DW}^{-1}) and triphenyltetrazolium chloride (TTC) reduction (mM TF g_{DW}^{-1}) by Norway spruce fine roots of various morphological classes after incubation in the presence (A) and absence (B) of TTC solution followed by cell disruption by hot ethanol extraction, grinding or freezing and thawing. Bars represent ± 1 SE of the mean ($n = 4$). Probability level for the two-way analysis of variance (ANOVA): **** = $P < 0.0001$; and ns = not significant. Within a treatment, different letters indicate a significant difference ($P < 0.05$).

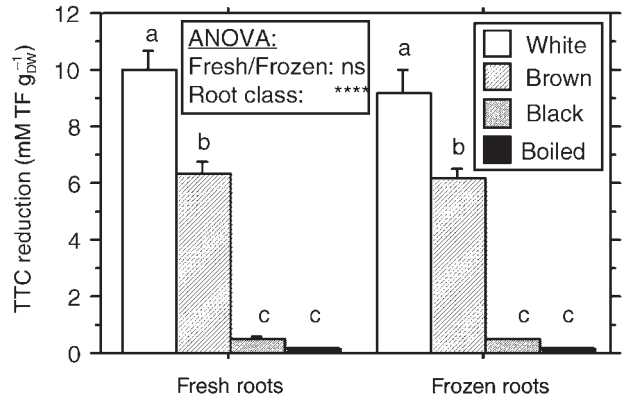

Figure 2. Reduction of triphenyltetrazolium chloride (TTC) (mM TF g_{DW}^{-1}) by fresh and frozen Norway spruce fine roots of various morphological classes. Bars represent ± 1 SE of the mean ($n = 5$). Probability level for the two-way analysis of variance (ANOVA): **** = $P < 0.0001$; and ns = not significant. Within a treatment, different letters indicate a significant difference ($P < 0.05$).

Table 1. Effect of extraction temperature on the absorption at 520 nm (A_{520} g_{DW}^{-1}) of ethanol extracts of various materials. Extracts were made in the presence and absence of triphenyltetrazolium chloride (TTC) solution ($n = 3$). Within a row, different letters indicate a significant difference ($P < 0.05$).

	Material	Extraction temperature (°C)				
		20	40	60	80	100
With TTC	Boiled roots	1.3 c	3.6 c	8.3 b	8.8 b	17.6 a
	Dried roots	1.5 d	4.8 cd	7.1 bc	8.4 b	19.0 a
	Cellulose	0.1 c	0.1 c	0.3 c	3.3 b	8.2 a
	Pectin	1.5 b	1.4 b	1.4 b	2.6 b	8.0 a
	Starch	1.4 b	1.8 b	1.5 b	1.5 b	5.3 a
	Tannic acid	10.0 a	10.7 a	14.8 a	12.3 a	14.1 a
	Agarose	0.2 d	0.7 d	3.7 c	8.9 b	17.8 a
	PVP	1.5 b	1.4 b	1.4 b	1.8 b	4.7 a
	Quartz sand	0.3 b	0.3 b	0.3 b	0.2 b	0.5 a
	No material	0.1 b	0.2 b	0.2 b	0.2 b	0.5 a
Without TTC	Dried roots	1.2 c	2.2 bc	2.4 bc	3.6 b	5.4 a
	Cellulose	0.2 a	0.4 a	0.3 a	0.3 a	0.3 a
	Tannic acid	12.7 a	11.2 a	13.5 a	13.1 a	13.5 a
	Agarose	0.2 a	0.3 a	0.3 a	0.3 a	0.5 a
	No material	0.06 a	0.01 a	–0.01 a	0.03 a	–0.01 a

tions at 520 nm except at temperatures > 60 °C (1.5–8.2 A_{520} g_{DW}^{-1}) (Table 1). In contrast, tannic acid showed a relatively high absorption at 520 nm at all temperatures (10–15 A_{520} g_{DW}^{-1}). Agarose and, to a minor extent, PVP also induced a marked reduction in TTC with increasing temperature, with agarose yielding values similar to those obtained with boiled or dried roots at 80 or 100 °C. With quartz sand or without any materials, the assay produced only slight absorptions at 520 nm with values between 0.1 and 0.5 (A_{520} g_{DW}^{-1}), even at temperatures of 100 °C (Table 1).

When TTC was omitted from the assay mixture, dried roots showed about one third the absorption at 520 nm compared with the assay with TTC (Table 1). Even at 100 °C, cellulose and agarose absorbed much less in the absence of TTC (0.2–0.5 A_{520} g_{DW}^{-1}) (Table 1) than in its presence, which suggests that these materials interacted with TTC at temperatures > 60 °C, reducing it to TF. Tannic acid had similar absorption values at 520 nm both with and without TTC, indicating that the increase in absorption at 520 nm of dried roots with increasing temperature may be due in part to the release of tannins from the root tissues.

Time course of TTC reduction

Figure 3 shows how the duration of incubation in TTC affects the time course of TF formation. If we assume that TF formation of 14 mM g_{DW}^{-1} after 40 h = 100%, then 90% of the total TF had developed after 20 h, the time period used in the modified TTC assay (Figure 3). About 40, 50 and 70% of the total TF was produced after 3, 5 and 8 h, respectively. Formation of TF by boiled roots increased with increasing incubation time in TTC, but remained below 5% even after 40 h (Figure 3). Based on these results, a 5 h incubation in TTC solution would provide a clear distinction between living roots (> 6 mM TF g_{DW}^{-1}) and boiled control roots (< 0.3 mM TF g_{DW}^{-1}).

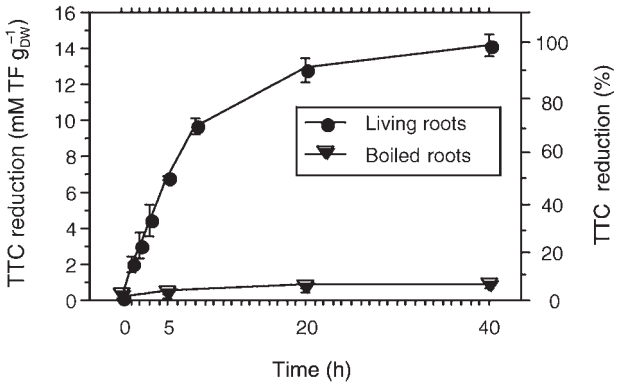

Figure 3. Time courses of triphenyltetrazolium chloride (TTC) reduction (mM TF g_{DW}^{-1}) by living and boiled roots of 2-month-old Norway spruce seedlings after incubation in TTC solution for different durations. Bars represent ± 1 SE of the mean ($n = 3$).

Effect of stress on seedling roots determined by modified TTC assay

Air drying roots of seedlings significantly decreased their capacity for TTC reduction from about 14 mM TF g_{DW}^{-1} in fresh roots to 6 and 2 mM TF g_{DW}^{-1} after 6 and 24 h of drying, respectively (Figure 4). Boiled roots yielded a TF concentration of about 0.4 mM g_{DW}^{-1}.

Cadmium significantly decreased TTC reduction by roots, and the decrease was dependent on both concentration (50 versus 100 µM) and duration (6 versus 24 h) of exposure (Figure 5). Similarly, exposure of roots to Al significantly reduced their capacity to reduce TTC; the effect was dependent on concentration (0.5 versus 1.0 mM), but not on duration (6 or 24 h) of exposure (Figure 6). Formation of TF by roots exposed to

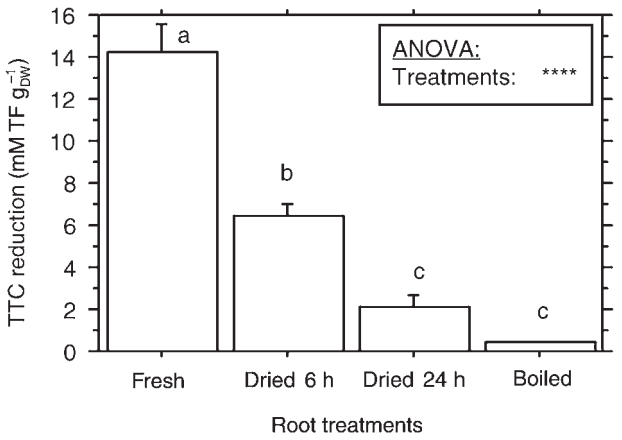

Figure 4. Reduction of triphenyltetrazolium chloride (TTC) (mM TF g_{DW}^{-1}) by roots from 1-month-old Norway spruce seedlings after drying or boiling. Bars represent ± 1 SE of the mean ($n = 3$). Probability level for the one-way analysis of variance (ANOVA): **** = $P < 0.0001$. Different letters indicate a significant difference ($P < 0.05$).

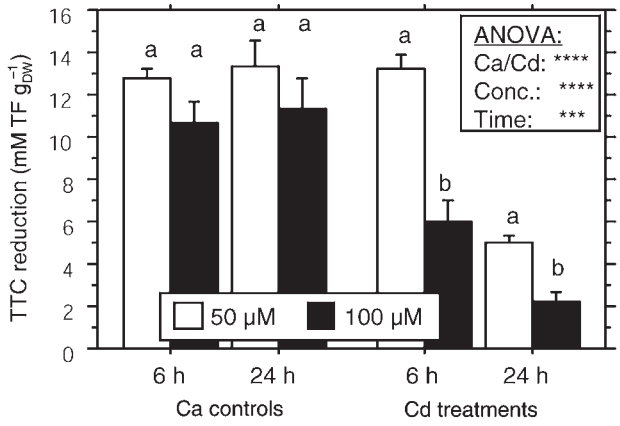

Figure 5. Reduction of triphenyltetrazolium chloride (TTC) (mM TF g_{DW}^{-1}) by roots from 1-month-old Norway spruce seedlings after exposure for 6 or 24 h to calcium (Ca) or cadmium (Cd). Bars represent ± 1 SE of the mean ($n = 3$). Probability level for the three-way analysis of variance (ANOVA): **** = $P < 0.0001$; and *** = $P < 0.001$. Different letters within a treatment indicate a significant difference ($P < 0.05$).

the 0.5 mM Al + 0.75 mM Ca treatment for 6 and 24 h was similar to that observed in the 0.5 mM Al treatment (data not shown).

Discussion

When we applied the TTC assay as proposed by Clemensson-Lindell (1994) or Clemensson-Lindell and Persson (1995), ethanolic extracts of dead or boiled fine roots of Norway spruce usually had an absorption at 520 nm of around 25 ($A_{520}\, g_{DW}^{-1}$). The reason, we found, was that the hot ethanol extraction of boiled root tissues induced TF production. By substituting alternative methods of tissue disruption for hot ethanol extraction, we obtained cold ethanolic extracts of dead or boiled roots with an absorption at 520 nm of around 2 ($A_{520}\, g_{DW}^{-1}$), which was considered acceptable.

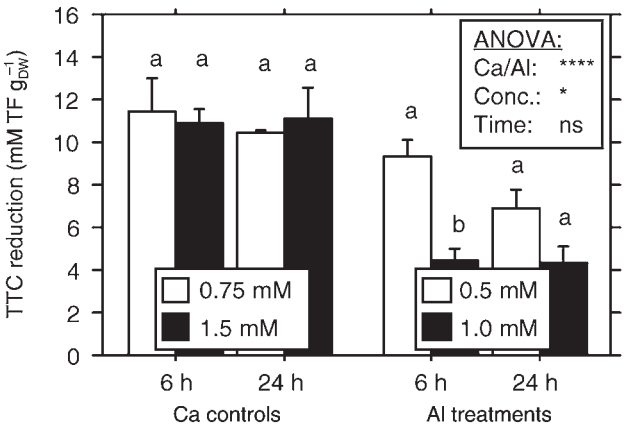

Figure 6. Reduction of triphenyltetrazolium chloride (TTC) (mM TF g_{DW}^{-1}) by roots of 1-month-old Norway spruce seedlings exposed for 6 or 24 h to calcium (Ca) or aluminum (Al) at various concentrations. Bars represent ± 1 SE of the mean ($n = 3$). Probability level for the three-way analysis of variance (ANOVA): **** = $P < 0.0001$; * = $P < 0.05$; and ns = not significant. Within a treatment, different letters indicate a significant difference ($P < 0.05$).

The TTC test has mainly been used to distinguish between living and dead tissue, or to estimate tissue vitality (Joslin and Henderson 1984, Lindström and Nyström 1987, Lassheikki et al. 1991, Clemensson-Lindell 1994, Clemensson-Lindell and Persson 1995, Coria et al. 1998, Stattin and Lindström 1999, Comas et al. 2000, Zhu et al. 2000, 2002, Brunner et al. 2002). In all of these studies, the TTC test was based on ethanol extraction of TF at temperatures of 80 °C or higher (Steponkus and Lanphear 1967, Towill and Mazur 1975), although, occasionally, TF has been extracted at lower temperatures, e.g., at 70 °C (Weidner et al. 1996), 60 °C (Symeonidou and Buckley 1999, Kalengamaliro et al. 2000), or at room temperature (Chen et al. 2000, Byth et al. 2001).

We found that various plant cell components such as cellulose, pectin, starch or agarose cause TF formation when the TTC test procedure includes ethanol extraction at 80 °C. Even when plant components were replaced by PVP, TF was formed at 80 °C, indicating that hot ethanol extraction produces artifacts. However, when inorganic quartz sand was applied, no TF was formed, indicating that TTC itself is thermostable, and that quartz sand, in contrast to organic materials, is inert and does not interact with TTC at high temperatures. Thus, we conclude that cell wall materials such as cellulose or pectin interact with TTC at 80 °C and reduce it to TF.

Therefore, we replaced the hot ethanol extraction step with a tissue grinding procedure to disrupt the cells before ethanol extraction at room temperature. The modified TTC assay gave satisfactory and reproducible results with clearly distinct values for the various fine root classes. The absorbance values at 520 nm of white, brown and black roots were > 100, 60 and about 2 ($A_{520}\, g_{DW}^{-1}$), respectively, and boiled roots had a value of about 1 ($A_{520}\ g_{DW}^{-1}$). Use of the modified TTC assay in the absence of TTC indicated that the grinding procedure resulted in absorption values at 520 nm of around 1 ($A_{520}\, g_{DW}^{-1}$), which is probably due to a release of root components such as tannins. Thus we can conclude that, in the modified TTC assay, boiled roots produce no TF and almost 100% of the measured absorption for white roots originated from the TF produced. The hot ethanol extraction, on the other hand, yielded absorptions of around 4 ($A_{520}\ g_{DW}^{-1}$), indicating that significantly more root components are released by the hot ethanol procedure than by the grinding procedure followed by ethanol extraction at room temperature. Mechanical homogenization of plant material, as recently performed by Mugai et al. (2000) and by Lin et al. (2001), can be considered equivalent to our proposed grinding method. Both these methods are superior to a repeated freezing–thawing procedure as indicated by the low TF values measured for freeze–thawed white fine roots.

Although the original TTC test overestimates TF production, many of the results remain qualitatively useful including those obtained on the effects of freezing (e.g., Lindström and Nyström 1987, Lassheikki et al. 1991, Stattin and Lindström

1999, Zhu et al. 2000, 2002) and desiccation (Symeonidou and Buckley 1999) and the studies designed to distinguish between living fine roots and dead fine roots (Clemensson-Lindell 1994, Comas et al. 2000). However, when effects associated with certain soil conditions were investigated (e.g., fertilization, nutrient deficiency, pH), the results of the TTC test were often unclear (Clemensson-Lindell and Persson 1995, Hiltbrunner and Flückiger 1996, Weidner et al. 1996, Brunner et al. 2002). It is likely that our modified TTC test would yield more accurate results under such conditions, because we found that it reliably determined the physiological conditions of roots that had been dried or treated with the toxic metals Cd or Al. Furthermore, the test is easy to use and reliable following our additional improvements that included adapting the test to 2-ml reaction tubes, reducing the incubation time in the TTC solution from 20 to 5 h, and freezing the fresh material in liquid N_2 immediately after harvest.

Assays applying tetrazolium are used not only in botanical research, but are also common in clinical research for measuring viability or vitality of animal cells after treatment with toxic substances or drugs (e.g., Khan et al. 2000, Beani 2001, Bunger et al. 2002, Huang et al. 2002, Islam et al. 2002, Kondo et al. 2002, Perry et al. 2002, Sumii and Lo 2002). Because TTC accepts electrons directly from the electron transport chain (Altman 1976, Byth et al. 2001), TTC reduction is directly linked to the mitochondrial respiratory chain (Comas et al. 2000). The TTC probably accepts electrons directly from the low potential cofactors of the NADH dehydrogenase (complex I) (Rich et al. 2001) or the cytochrome c oxidase (complex IV) (Slater et al. 1963, Musser and Oseroff 1994) (Figure 7).

We conclude that the modified TTC assay can distinguish among vital or healthy (white), stressed or diseased (brown), dead (black) and boiled tissues, with TTC reduction ranging from more than 100 ($A_{520}\,g_{DW}^{-1}$) (~10 mM TF g_{DW}^{-1}) for healthy tissues to less than 2 ($A_{520}\,g_{DW}^{-1}$) (~0.2 mM TF g_{DW}^{-1}) for dead tissues. The modified TTC assay can also be used to assess the health of tissues subject to stresses such as desiccation and exposure to heavy metal toxicity.

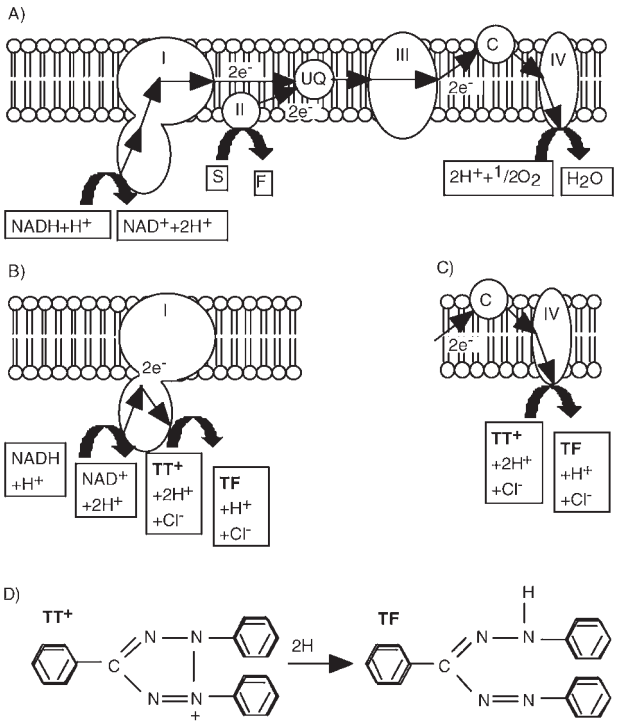

Figure 7. Simplified illustrations of (A) electron flow through the respiratory enzyme complexes in the inner mitochondrial membrane (according to Buchanan et al. 2000), (B) postulated points of coupling of the triphenyltetrazolium at complex I or (C) complex IV, and (D) the mechanism of triphenylformazan production from triphenyltetrazolium. Abbreviations: I = NADH dehydrogenase complex; II = succinate dehydrogenase complex; UQ = ubiquinone; III = cytochrome bc_1 complex; C = cytochrome c; IV = cytochrome c oxidase complex; TT = triphenyltetrazolium; TF = triphenylformazan; S = succinate; and F = fumarate.

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