Short report

A simple method for dehydrogenase activity visualization of intact plant roots grown in soilless culture using tetrazolium violet

Eyal Kurzbaum[1], Felix Kirzhner[1] and Robert Armon[1,2]

[1]Faculty of Civil & Environmental Engineering, Division of Environmental, Water & Agricultural Engineering, Technion-Israel Institute of Technology, Haifa 32000, Israel
[2] Member of Grand Water Research Institute, Technion, Haifa, Israel
Corresponding author: R. Armon, E-mail: cvrrobi@tx.technion.ac.il, Phone: +97248292377, Fax: +97248293309

Received on November 17, 2009; Accepted on February 15, 2010

Abstract: A simple method for the evaluation of respiration activity of root cells of intact plants grown hydroponically and/or in agar medium was developed. The novelty of the present method is based on visual detection of dehydrogenase activity of plant roots by use of tetrazolium violet dye without destructive steps, allowing follow up of living and photosynthetically active growing plants and the impact of inhibitors such as sodium azide and cycloheximide. The results of this approach demonstrated that root tip cells comprise the highest dehydrogenase activity compared to other root parts. The non-expensive assay is easy to perform and allows to experiment a large variety of chemical compounds with potential inhibitory characteristics for plants.

Keywords: cells vitality, dehydrogenase activity, formazan, hydroponics, tetrazolium violet, *Zea mays*

Introduction

Tetrazolium based dyes were tested in various research fields and had become a standard method for visualization and quantification of cells vitality based on redox activity. Tetrazolium based dyes as a qualitative redox indicator is based on the principle that all living tissues and cells contain active dehydrogenase enzymes that catalyze chemical reductions (Steponkus and Lanphear 1967).

Various studies reported tetrazolium dyes use on plant seeds (Cottrell 1947, Porter et al. 1947, Bennett and Loomis 1949, Lakon 1949), plant tissues (Gall 1948, Dufrenony and Pratt 1948, Waugh 1948, Roberts 1950), human and animal tissues (Straus et al. 1948, Black and Kleiner 1949, Parker 1953), mitochndrial localization of oxidative enzymes (Pearse 1957), and microorganisms (Fred and Knight 1949, Gunz 1949, Currier and Day 1954). Since then, the use of tetrazolium salts in different research fields has been studied intensively due to its wide potential applications and research opportunities. In plant research the use of tetrazolium dyes to identify and quantify metabolically active cells and enzyme activity is well documented. In particular, it is used for microscopic imaging of certain enzymes activity localization in plant tissues (Šebela et al. 2001, Galuszka et al. 2005), for the quantification of cells viability and metabolic activity in excised tissues and organs (Collet et al. 2002, Chen et al. 2006), and in cell culture suspensions (Xu et al. 2004).

Although tetrazolium salts have been extensively used in visualization and quantification of plant cells vitality, these methods always hold a destructive step prior to dye reduction measurements, consequently terminating the growth of the studied plant. In the present study we investigated the colored formazan formation in root cells of intact active plants growing on soilless transparent media, with emphasis on non destructive steps.

Our aim was to develop a novel method for dehydrogenase activity assessment using tetrazolium violet on roots of intact growing plant grown hydroponically and on agar media. The dehydrogenase activity is visualized through the development of purple color of the reduced formazan on and around the intact plant root cells over a period of 2-96 hours (Fig. 1). In order to envision color development during growth of plants, sterile *Zea mays* young plants were transferred into sterile vessels containing hydroponic solution or agar media supplemented with 0.005% (w/v) tetrazolium violet (Fig. 2 and 3).

Kurzbaum E, Kirzhner F, Armon R 2010 A simple method for dehydrogenase activity visualization of intact plant roots grown in soilless culture using tetrazolium violet. Plant Root 4: 12-16. doi:10.3117/plantroot.4.12

Fig. 1 Sterile seedling roots of *Zea mays* in agar medium supplemented with tetrazolium violet after 96 hours. Scale bar represents 10 mm length.

Materials and Methods

Zea mays (Gedera Seeds Co., Gedera, Israel) seeds were surface sterilized for 20 min in 70% ethanol and 60 min in 2% sodium hypochlorite following another 10 min in 70% ethanol and 60 min in 1% sodium hypochlorite (Medina et al. 2000). The sterile seeds were rinsed five times with sterile half-strength Hoagland's minimal medium (HMM) and transferred into a sterile hydroponic chamber as previously described supplemented with the HMM solution (Burdman et al. 1996). Sterility of the seeds and seedlings was tested on nine different seeds and seedlings by placing each one onto nutrient agar (Difco, Israel) followed by incubation at 36°C for 48 hours. The sterile conditions were kept during the experiment in order to neglect the respiration and dehydrogenase activity of microorganisms.

Sven-day-old sterile seedlings (with ~ 5 cm long roots) were transferred to 20 mL sterile test tubes and to 50 and 250 mL tissue culture flasks containing solution or solid HMM media supplemented with 0.005% tetrazolium violet. While the roots were submerged into the solution and the solid HMM medium of each test tube or tissue culture flask, the shoot emerged through the experimental vessel mouth sealing. Sealing was done using five sterile parafilm layers wrapped tightly around the stem and the vessel's mouth in order to prevent microbial contamination and to hold the plant in its position. In order to keep the plant roots under darkness the test tubes were warped with aluminum foil. The experimental plants were maintained vertically in a growth chamber at 22°C with 18-h light/6-h dark photoperiod.

All experimental procedures were performed under laminar hood and all glassware and media were autoclave sterilized (at 121^{o}C for 30 min). Growth medium was based on half-strength Hoagland's minimal medium (HMM) (Hoagland and Arnon 1950) adjusted to pH 6.5 using sterile NaOH (1M). HMM solution and solid HMM consisted with 0.5% agar (Bacto agar, Difco, Israel) supplemented with 0.005% tetrazolium violet were used to grow young plants. Tetrazolium violet stock solution (0.1%) was filter sterilized (0.22 μm syringe filter, Millipore, USA) and added to previously autoclaved solution and solid HMM media at 50^{o}C. Both media are highly transparent, therefore suitable for permanent visualization of roots growth and development. Growth media containing tetrazolium violet was sterile transferred into sterile test tubes and tissue culture flasks (Greiner Bio-One, Israel).

Several control treatments were conducted in order to track the formazan formation in dead or chemically inhibited root cells. Control treatments contained plants grown in experimental solution/solid HMM

Table 1. Visualization of root dehydrogenase activity under different experimental treatments as a function of formazan color intensity

Treatment	Color development		
	0 h	24 h	48 h
Hydroponically grown seedlings	-	+	+++
Agar grown seedlings	-	++	+++
Control − Agar + cycloheximide (200 mg/l) grown seedlings	-	-	+
Control − Agar + sodium azide (0.2%) grown seedlings	-	-	-
Control − Previously boiled seedlings	-	-	-

Symbols (+++; ++; +) indicate the intensity of formazan color formation

Fig. 2: Purple color development on sterile seedling roots of *Zea mays* grown in HMM medium (250 mL tissue culture flask supplemented with 0.005% (w/v) tetrazolium violet) at 0, 24 and 48 hours. Scale bars represent 10 mm length.

media (supplemented with 0.005% tetrazolium violet) with 0.2% sodium azide or 200 mg/l cycloheximide as respiration inhibitors. In addition, 10 min boiled plants (as dead control plants) were also tested in the above media (Fig. 4). Negative control consisted of plants grown in media without tetrazolium violet in order to monitor any change in root color not as a result of formazan precipitation (data not shown). Color development on roots, as a result of reduced formazan formation, was captured using a digital camera (DMC LZ10, Panasonic, Japan) at different time intervals.

Results and Discussion

The results showed that plants grown with supplemented tetrazolium violet formed a purple formazan color on the roots, especially on the fast growing root tips. On contrary, control treatments (chemically inhibited by sodium azide and cycloheximide or boiled) showed faint or no color formation (Table 1). No difference in plants growth (measured as root elongation) with and without tetrazolium violet was observed (data not shown).

Plants grown hydroponically or in solid HMM media, had a slight purple color formation on the root tips after two hours from experimental onset. After two days, the purple color of the roots tips intensified and expanded to the rest of the root showing the accumulation of formazan as a function of time (Fig. 2 and 3). The color on roots grown in solid HMM (stagnant medium) stained brighter and further from the root tip compared to the roots grown in the hydroponic culture. It is assumed that under stagnant conditions (in agar supplemented medium) the reduced form of the stain (formazan) remains around the roots while in liquid medium it may disperse/wash into the volume. Another feature observed with *Zea mays* seedlings was related to root morphology: in HMM medium (hydrophonic) lateral roots were more developed compared to agar grown seedlings. Weather this root morphology difference is stressed controlled it is not clear, however formazan was formed on both root types.

Tetrazolium violet transformation to formazan in the presence of 200 mg/l cycloheximide decreased revealing a much weaker purple color formation in comparison to untreated plants. Comparatively,

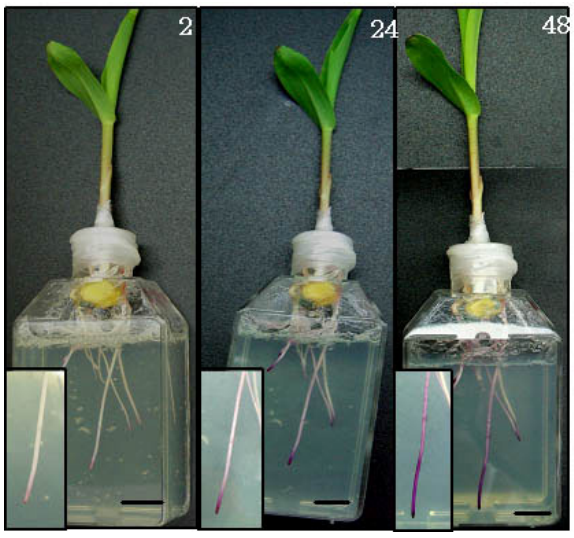

Fig. 3: Purple color development on sterile seedling roots of *Zea mays* grown in HMM agar medium (50 mL tissue culture flask supplemented with 0.005% (w/v) tetrazolium violet) at 2, 24 and 48 hours. (Insets: magnification of a single root showing formazan formation). Scale bars represent 10 mm length.

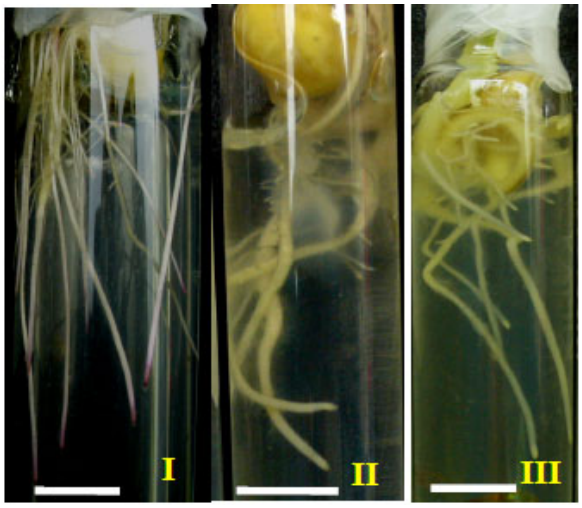

Fig. 4: Controls of sterile seedling roots of *Zea mays* grown in HMM agar medium supplemented with 0.005% (w/v) tetrazolium violet (10 mL borosilicate tubes) exposed to 3 treatments for 48 hours; **I**. with cycloheximide (200 mg/l) incorporated into growth medium; **II**. 0.2% (w/v) sodium azide incorporated into growth medium and **III**. 10 minutes previously boiled seedlings in sterile HMM. Each picture is a representative of at least three replications. Scale bars represent 10 mm length.

sodium azide exposed or boiled plants did not develop purple color at any stage of the experiment, revealing complete respiratory inhibition of root cells (and a slight folding) (Fig. 4). Cycloheximide extensively used in biological research to inhibit protein synthesis in eukaryotic cells caused a marked reduction in the overall formazan formation as noticed by the much weaker color in comparison to plants grown without cycloheximide. Sodium azide as a respiratory inhibitor revealed no formazan formation with time during the experiment, similarly to the treatment with boiled plants, proving complete respiratory activity inhibition.

In botanical research, tetrazolium based dyes are commonly used in two major applications: 1) visualization of viable cells or localization of specific enzymes activity for histological purposes and 2) quantification of the degree of cell vitality/metabolism (including experimental tissue section).

The present study describes a novel, non-destructive method for the assessment of plant root vitality under different treatments. Monitoring color development on plant roots indicate uninhibited growth and can be used directly to test a large variety of pollutants that may inhibit plant growth.

In summary, visualization of formazan formation as a result of tetrazolium violet reduction by root cells of hydroponically grown plants or in any transparent media (such as agar) can be used as an indicator of plant cell vitality exposed to a variety of physico-chemical treatments (nutrients, herbicide pollutants, light regime, temperature, transgenic plants metabolism, oxygen supply, etc.).

Acknowledgements

This research was partially supported by a fund from the Faculty of Civil & Environmental Engineering and Grand Water Research Institute at Technion. The authors would like to thank Prof. Shimon Gepstein (Faculty of Biology, Technion, Israel Institute of Technology, Israel) for helpful advice and expertise.

References

Bennett N, Loomis WE 1949 Tetrazolium chloride as a test reagent for freezing injury of seed corn. Plant Physiol. 24: 162-174.

Black MM, Kleiner IS 1949 The use of triphenyltetrazolium chloride for the study of respiration of tissue slices. Science 110: 660-61.

Burdman S, Volpin H, Kigel J, Kapulnik Y, Okon Y 1996 Promotion of nod gene inducers and nodulation in common bean (*Phaseolus vulgaris*) roots inoculated with *Azospirillum brasilense* Cd. Appl. Environ. Microbiol. 62: 3030-3033.

Chen CW, Yang YW, Lur HS, Tsai YG, Chang MC 2006 A novel function of abscisic acid in the regulation of rice (*Oryza sativa* L.) root growth and development. Plant Cell Physiol. 47: 1–13.

Collet L, de Leon C, Kollmeier M, Schmohl N, Horst WJ 2002 Assessment of aluminum sensitivity of maize cultivars using roots of intact plants and excised root tips. J. Plant Nutr. Soil Sci. 165: 357–365.

Cottrell H 1947 Tetrazolium salt as a seed germination indicator. Nature 159: 748-748.

Currier HB, Day BE 1954 The tetrazolium reaction in yeast. Science 119: 817-817.

Dufrenony J, Pratt R 1948 Histo-physiological localization of the site of reducing activity in stalks of sugar cane. Amer. J. Bot. 35: 333-334.

Fred RB, Knight SG 1949 The reduction of 2,3,5-triphenyltetrazolium chloride by *Penicillihnnit chrysogenurn*. Science 109: 169-170.

Gall HJF 1948 Some effects of 2,4-dichlorophenoxyacetic acid on starch digestion and reducing activity in bean tissue cultures. Bot. Gaz. 110: 319-323.

Galuszka P, Frébortová J, Luhová L, Bilyeu KD, English JT, Frébort I 2005 Tissue localization of cytokinin dehydrogenase in maize: Possible involvement of quinone species generated from plant phenolics by other enzymatic systems in the catalytic reaction. Plant Cell Physiol. 46: 716-728.

Gunz FW 1949 Reduction of tetrazolium salts by some biological agents. Nature 163: 98-98.

Hoagland DR, Arnon DI 1950 The water-culture method for growing plants without soil. Circular 347. Agricultural Experiment Station, University of California, Berkeley.

Lakon G 1949 The topographical tetrazolium method for determining the germinating capacity of seeds. Plant Physiol. 24: 389-394.

Medina VF, Jeffers P, Larson SL, Perez W 2000 Sterilization of

plants for phytoremediation studies by bleach treatment. Int. J. Phytorem. 2: 287–295.

Parker J 1953 Some applications and limitations of tetrazolium chloride. Science 17: 77-79.

Pearse AGE 1957 Intracellular localization of dehydrogenase systems using monotetrazolium salts and metal chelation of their formazans. J. Histochem. Cytochem. 5: 515-527.

Porter RH, Durrel M, Romm HJ 1947 The use of 2,3,5-triphenyltetrazolium chloride as a measure of seed germinability. Plant Physiol. 22: 149-159.

Roberts LW 1950 A survey of tissues that reduce 2,3,5-triphenyltetrazolium chloride in vascular plants. Bull. Torrey Bot. Club 77: 372-381.

Šebela M, Luhová L, Brauner F, Galuszka P, Radová A, Pec P 2001 Light microscopic localization of aminoaldehyde dehydrogenase activity in plant tissues using nitroblue tetrazolium-based staining method. Plant Physiol. Biochem. 39: 831–839.

Steponkus PL, Lanphear FO 1967 Refinement of the triphenyl tetrazolium chloride method of determining cold injury. Plant Physiol. 35: 543–548.

Straus FH, Cheronis ND, Straus F 1948 Demonstration of reducing enzyme systems in neoplasms and living mammalian tissue by triphenyltetrazolium chloride. Science 108: 113-115.

Waugh TD 1948 Staining of the stem tissues of plants by triphenyltetrazolium chloride. Science 107: 275-275.

Xu Q, Cheng J, Ge Z, Yuan Y 2004 Effects of organic solvents on membrane of *Taxus cuspidata* cells in two-liquid-phase cultures. Plant Cell Tissue Organ Cult. 79: 63–69.