

COMPARATIVE STUDY OF VIABILITY MEASUREMENT METHODS IN CROP PLANTS

O. K. GONDOR, T. JANDA and G. SZALAI

AGRICULTURAL INSTITUTE, CENTRE FOR AGRICULTURAL RESEARCH, HUNGARIAN
ACADEMY OF SCIENCES, MARTONVÁSÁR

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The aim of the present study was to find the best way of measuring the viability of root and leaf samples from various plant species (pea, wheat and maize) exposed to different concentrations of the heavy metal Cd. A comparison was made of three viability tests, namely electrolyte leakage measurements, and TTC and NBT reduction. The results suggested that electrolyte leakage was the most useful method for measuring leaf viability, being simple, fast, reliable and reproducible. The TTC reduction measurement proved the most useful for maize roots, while NBT reduction was the best method for detecting the viability of pea and wheat roots.

Key words: cadmium, conductivity, NBT, TTC, maize, pea, viability test, wheat

Introduction

Various physiological, morphological and biochemical effects of biotic and abiotic stress factors have been studied in plants. Abiotic stressors induce growth inhibition, the inhibition of photosynthesis and changes in enzyme activities (Pál et al., 2006). Abiotic stresses also reduce the chlorophyll content and increase lipid peroxidation in the leaves (Zawoznik et al., 2007).

Heavy metals have received considerable attention over the years as a result of increased environmental pollution from industry or agriculture. It was found that Cd treatment caused a reduction in root growth, as demonstrated for example in *Pisum sativum* L., where it was directly related to a reduction in apex length (Fusconi et al., 2007). Morphological measurements (length, biomass) and the structure of the root system are important for the characterisation of the level of stress. Although morphological measurements are able to show the general condition of the plant, they are not able to give information about root viability.

Fundamentally, all viability measurements ask the same question: are the cells alive or dead? One rapidly occurring response to stress is the increased leakage of ions from chilled tissue, presumably due to increased membrane permeability (Creencia and Bramlage, 1971), so this parameter can be used to characterise membrane damage to the plants during stress. Several viability assays have been used for estimating the level of stress tolerance. The compound 2,3,5-triphenyl-tetrazolium chloride (TTC), a reduced tetrazolium salt, is a redox indicator used to differentiate between metabolically active and inactive tissues. It is enzymatically reduced to red 1,3,5-triphenylformazan in living tissues due to the activity of various dehydrogenases. The most sensitive test for assessing heat tolerance was regrowth, followed by fluorescein diacetate staining and the TTC test. When estimating salt tolerance, TTC assays were in close agreement with regrowth measurements (Ishikawa et al., 1995).

Another method used to test viability involves nitroblue tetrazolium chloride (NBT), which is used in immunology for the detection of alkaline phosphatase. It is also useful for colorimetric and spectrophotometric measurements, because the reduced form of NBT has a distinct colour (Kulikova et al., 2011). NBT has also been used for viability measurements, for example in pea roots (Fusconi et al., 2007).

The aim of the present study was to find the best way of measuring the viability of root and leaf samples from various plant species (pea, wheat and maize) exposed to different concentrations of the heavy metal Cd. In order to achieve this, a comparison was made of three viability tests, namely electrolyte leakage, and TTC and NBT reduction.

Materials and methods

Plant material and growth conditions

Seeds of pea (*Pisum sativum* L. Kelvedon), wheat (*Triticum aestivum* L. Mv Emese) and maize (*Zea mays* L. Mv Norma) were germinated for 3 days at 26°C and grown at 22/20°C with 16/8-h light/dark periodicity in a Conviron PGR-15 plant growth chamber in modified Hoagland solution containing 0.3125 mM KNO₃, 0.45 mM Ca(NO₃)₂, 0.0625 mM KH₂PO₄, 0.125 mM MgSO₄·7H₂O, 11.9 µM HBO₃, 4.57 µM MnCl₂·4H₂O, 0.191 µM ZnSO₄·7H₂O, 0.08 µM CuSO₄·5H₂O, 0.24 µM (NH₄)₆Mo₇O₂₄·4H₂O, 15.02 µM FeSO₄·7H₂O and 23.04 µM Na₂EDTA·5H₂O. The photosynthetic photon flux density was 340 µmol m⁻² s⁻¹, provided by metal halide lamps, with a relative humidity of 75%. The maize plants were treated with 25 µM and 50 µM Cd(NO₃)₂ for one day on the 7th day, the wheat plants with 1 mM and 2 mM Cd(NO₃)₂ for one day on the 14th day and the pea plants with 10 µM and 20 µM Cd(NO₃)₂ for one day on the 21st day, and the leaves and roots were used for the measurements.

Membrane damage measurements

The leaves were washed in distilled water and the roots in 0.1 mM EDTA to eliminate all traces of the nutrient solution ions. Leaf discs 1 cm in diameter were cut from pea and maize leaves, while the wheat leaves and the pea, maize and wheat roots were cut into 1 cm long pieces. Five leaf or root pieces were put in each 20 ml vial with 2 ml ultra-pure water (distilled water cleaned with Milli Q 50 from Millipore, USA). The vials were closed and shaken for one hour,

after which the conductivity of the water was measured with an Automatic Seed Analyzer (ASA610, Agro Science Inc., USA). The water was then returned to the vials and the samples were frozen at -80°C for 10 hours. In the next phase, the vials were thawed to room temperature again and the conductivity was measured again with the same equipment. These data represented membranes suffering 100% damage. The percentage of membrane damage was calculated as the conductivity of the stressed plants divided by the conductivity after freezing (100% damage).

Viability testing by means of TTC assay

The leaves were washed in distilled water and the roots in 0.1 mM EDTA to eliminate all traces of nutrient solution ions. The samples were put into 10 ml vials containing 3 ml 0.6% TTC in 50 mM phosphate buffer (pH 7.5) and then incubated at room temperature in the dark for 24 h. The formazan generated was extracted with 3 ml ethanol at 60°C for 30 minutes. The ethanol solution was measured at 485 nm with a UV-160A spectrophotometer (Shimadzu, Japan). Plants kept either in liquid N_2 or boiling water for 5 minutes were used as a control (100% killed plants). Viability was calculated as $A_{485} \text{ g}^{-1} \text{ FW}$.

Viability testing by means of NBT assay

The leaves were washed in distilled water and the roots in 0.1 mM EDTA to eliminate all traces of nutrient solution ions. In the next step 0.05 g samples were put into 1 ml vials and 100 μl 0.1% hot NBT solution, 50 μl 0.05 M hot Na_2 -succinate solution, 50 μl 0.05 mM Tris-HCl solution, 50 μl 0.5 mM MgCl_2 solution and 50 μl phosphate buffer (pH 7.3) were added. The samples were shaken at room temperature for 200 minutes, after which the samples were washed with distilled water. After adding 1 ml ethanol the solution was measured at 510 nm using a UV-160A spectrophotometer (Shimadzu, Japan). Viability was calculated as $A_{510} \text{ g}^{-1} \text{ FW}$.

Statistical analysis

The results were the means of at least three measurements and were statistically evaluated using the standard deviation and *t*-test methods.

Results

Electrolyte leakage measurements

In order to detect differences in membrane stability, electrolyte leakage from the tissues was measured. Different relative initial conductivity values were detected in the different plant species. These were of the same order of magnitude for the leaves and roots in pea and maize, but in the case of wheat the values were 3-fold higher in the roots than in the leaves (Fig. 1).

In the case of pea and maize leaves, Cd treatment caused a slight but statistically significant increase at the highest Cd concentration. In contrast, the treatment caused no changes in wheat leaves. Cd stress is well tolerated by wheat, so it is possible that the concentration applied was not high enough. Cd treatment caused a slight increase in electrolyte leakage in all the plant species tested.

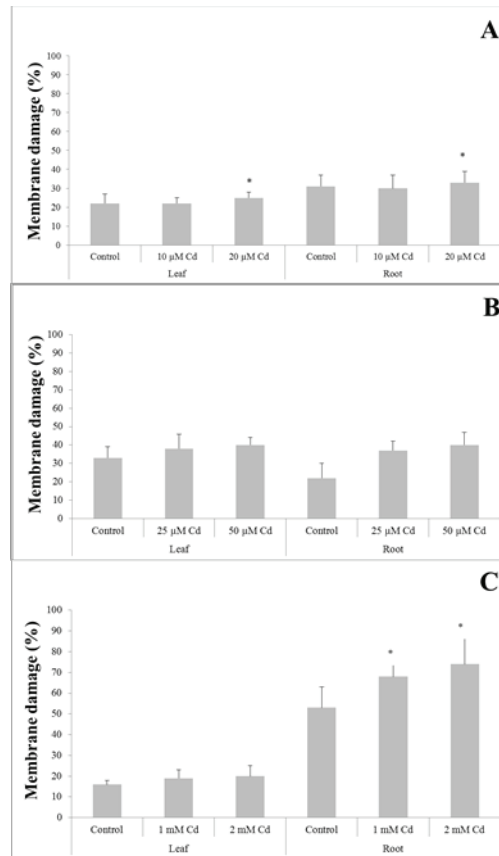


Fig. 1. Membrane damage measured in pea (A), maize (B) and wheat (C) leaf and root tissues after treatment with different concentrations of Cd. *, **: significant differences from the control values at the $p \leq 0.05$ and 0.01 levels, respectively ($n=10$)

TTC measurements

In order to make the TTC test more quantitative, some of the plants were killed using liquid nitrogen. The initial level of absorbance measured at 485 nm (A_{485}) in maize leaves was relatively high compared to pea or wheat, and was also high in those damaged by liquid N_2 (Fig. 2). The A_{485} value of the control maize leaves did not differ from that of plants either treated with Cd or killed in liquid N_2 . The initial level of A_{485} in wheat leaves was much lower than in pea or maize. The A_{485} value decreased significantly in 100% injured wheat leaves, but the difference between the control and the 100% injured leaf A_{485} was not great enough, to make the method sufficiently sensitive to differentiate the injury caused by different Cd concentrations. In pea plants the A_{485} value decreased significantly at high Cd concentration and in the 100% injured leaves.

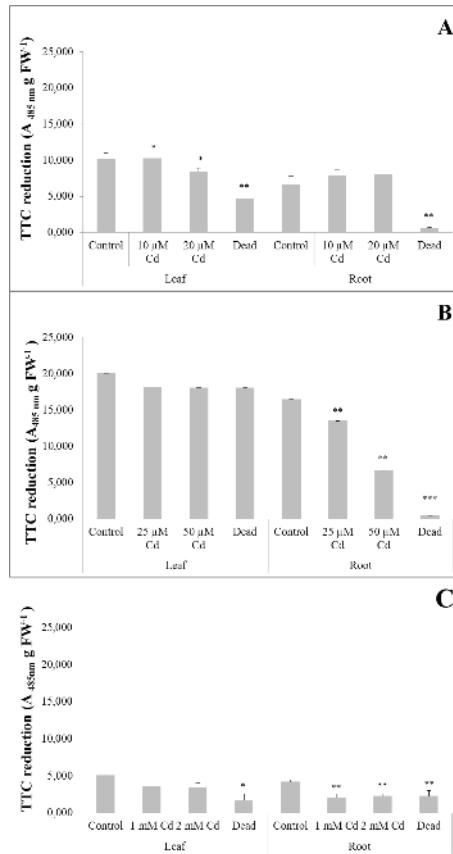


Fig. 2. TTC measurements on pea (A), maize (B) and wheat (C) leaf and root tissues after treatment with different concentrations of Cd. *, **, ***: significant differences from the control values at the $p \leq 0.05$, 0.01 and 0.001 levels, respectively (n=5)

The A_{485} level decreased significantly in killed pea roots, but there was no change after Cd treatment. There was also a great difference between the control maize roots and those completely killed, while the viability decreased in a concentration-dependent manner in Cd-treated plants. Both killing in liquid N_2 and Cd treatment significantly reduced the A_{485} level in wheat roots; however, due to the low control value it was difficult to differentiate between the treatments.

NBT measurements

In general, there was no change in the quantity of the reduced form of NBT measured in the leaves; the only significant difference was observed between the control and the completely killed pea leaves (Fig. 3). In contrast, killing the plants in liquid N_2 substantially decreased the reduction of NBT in the roots of all the plants tested, and Cd treatment also caused a concentration-dependent decrease in wheat roots.

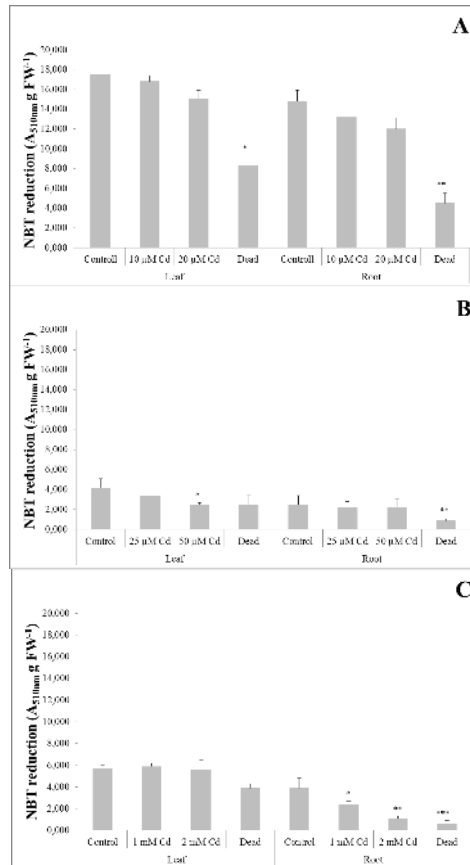


Fig. 3. NBT measurements on pea (A), maize (B) and wheat (C) leaf and root tissues after treatment with different concentrations of Cd. *, **, ***: significant differences from the control values at the $p \leq 0.05$, 0.01 and 0.001 levels, respectively (n=5)

Discussion

It is important to assess plant viability following stress. In the present work the applicability of three methods, namely membrane damage measurements and TTC and NBT tests, was compared during Cd stress in three plant species with various levels of Cd tolerance. Since wheat is tolerant, maize moderately tolerant and pea sensitive to Cd, different Cd concentrations were used.

To minimize the standard deviation, discs with uniform area were cut from the pea and maize leaves for the electrolyte leakage measurement. The disadvantage of discs compared to leaf segments, which can be cut from narrow leaves like those of wheat plants, is the greater damage to the membrane surface, which caused an elevation in the control values but did not influence the tendencies. Electrolyte leakage measurements proved to be a useful technique

for leaves, and their reproducibility was shown by the low standard deviations. In contrast, the roots showed a higher level of electrolyte leakage compared to the leaves, and the standard deviations were also higher. The higher values measured in the roots could have been due to traces of nutrient solution ions, or to the uniform surface of the roots, which was also shown by the higher standard deviation. Electrolyte leakage measurement is thus a suitable physical parameter for detecting the level of stress in the leaves, but not in the roots. In special cases, such as for young maize, it may be applicable, as demonstrated in the present experiments.

Since electrolyte leakage was not always the best parameter for the detection of root viability, other methods were also tested. When several viability assays were compared to determine the most sensitive and appropriate method for estimating the freezing, heat and salt tolerance of *Bromus inermis* Leyss cells, the sensitivity and reliability of the tests was found to depend on the type of stress applied and the degree of injury (Ishikawa et al., 1995). The reduction of TTC was also tested for leaves and roots. This viability test was less useful in the case of the leaves than electrolyte leakage. The difference between the control and the completely killed samples was not significant for maize leaves, probably due to the overlap of the absorption of leaf pigments with that of formazan. It gave better results for pea and wheat leaves, but was still less efficient than the electrolyte leakage method, which provided the same or better data than TTC, while the measurements were simpler and faster. It is thus expedient to use electrolyte leakage measurement for leaves. By contrast, this parameter gave more reliable results than electrolyte leakage in maize roots, but the method did not work in wheat roots.

Since TTC cannot be used in wheat roots, another reduction viability test, NBT, was applied. NBT has already been used to determine the viability of the roots in pea plants. It was found that cadmium caused a reduction in NBT staining, and hence in the viability of the outer cap cells (Fusconi et al., 2007). A modification of this method was used in the present work: the reduced form of NBT was extracted from the tissues and quantified using a spectrophotometer. A substantial difference was found between the control and the completely killed wheat roots, while a Cd concentration-dependent decrease in viability was measured using NBT reduction. Pea roots showed similar tendencies, although the Cd-induced differences were not significant. In the case of maize, the difference between the control and the completely killed roots was not sufficiently great, and the level of reduction did not depend on the Cd concentration, though the viability of these roots could be measured well using TTC or electrolyte leakage. The NBT reduction method can thus be used for wheat and pea roots, but not for maize. In the case of maize and wheat leaves no changes could be measured after Cd treatment or killing the plants in liquid nitrogen. In contrast, a Cd concentration-dependent decrease in viability was detected in pea leaves.

In conclusion, the results suggest that electrolyte leakage is the most useful method for measuring leaf viability, being simple, fast, reliable and reproducible. TTC reduction measurements proved the most useful for maize roots, while NBT reduction was the best method for detecting the viability of pea and wheat roots.

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Corresponding author: O. K. Gondor
Phone: +36-22-569-500
E-mail: gondor.kinga@agrar.mta.hu