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MTT Assay for Cytotoxicity Assessment in *Oryza sativa* Root Tissue

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[Abstract] Cytotoxicity of different compounds are commonly evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay is mainly used to study cell viability in cell lines (Carmichael *et al.*, 1987). In this study, the protocol is being used to determine the cell viability of plant roots, treated with different stress inducing agents. The basis of the assay is that the dye enters the living cell's mitochondrion where it is reduced to insoluble formazan, which is solubilised by directly treating the cells with organic solvent (DMSO). Intensity of colour is directly proportional to the amount of formazan produced.

In the present study, plants were treated for 16 h, with several phytotoxic agents, then the roots were incubated in MTT solution for 4 h. To solubilise the formazan, roots were excised. 2 N potassium hydroxide (KOH) along with DMSO was used to solubilize the cell wall components and thereby liberating the formazan granules in the DMSO solution. The rate of the cell viability was measured by measuring the colour intensity of the formazan.

Keywords: Cell viability, Cytotoxicity, MTT assay, Plant tissue, Rice seedling

[Background] This protocol is designed to determine plant cell viability directly from root tissue. Till date, MTT assay has been profusely used for mammalian cell proliferation and viability assay. In case of plants usually the 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay (Kaundal *et al.*, 2012) is preferably used. Cost effective MTT assay can also be used for plant tissue viability assay instead of XTT assay. This protocol can be used to determine the cytotoxicity of different stress inducing agents and their IC_{50} doses (the concentration of phytotoxic agent at which 50% cell death is obtained). Nicotinamide adenine dinucleotide phosphate (NAD(P)H) dependent dehydrogenase enzyme present in mitochondrion of living cell is capable of reducing tetrazolium dye MTT 3-(4,5-dimethylthia zol-2-yl)-2,5-diphenyltetrazolium bromide to an insoluble purple coloured formazan, which is measured spectrophotometrically. This reduction takes place when the mitochondrial enzymes are active and hence the degree of reduction can be directly correlated with the number of viable cells. The metabolically inactive cells will not show this property.

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Materials and Reagents

1. Culture tubes (Borosil, catalog number: 9910010)
2. Tissue paper (Kim wipes) (Tarsons, catalog number: 370080)
3. 2 ml microcentrifuge tube (gem. gov. in, Genaxy, catalog number: GEN-MT-200-C)
4. Aluminium foil Freshwrapp^{R} (Hindalco)
5. Petri plates (Borosil, catalog number: 3160065)
6. Rice *Oryza sativa* cv. IR64 (6 days old) (Rice Research Station Government of West Bengal, Chinsurah R.S., Hooghly, India)
7. Stress inducing agents:
   a. Nanoscale zero-valent iron nanoparticles (nZVI) (gifted by Prof. A. Mukherjee, Vellore Institute of Technology, Tamil Nadu, India)
   b. Cadmium chloride monohydrate ($CdCl_2·H_2O$) (Merck, catalog number: 61813101001730)
   c. Sodium chloride (NaCl) (HiMedia Laboratories, catalog number: MB023-500G)
   d. Mannitol($C_6H_{14}O_6$) (Sisco Research Laboratories, catalog number: 134889)
8. Potassium hydroxide (KOH) (HiMedia Laboratories, catalog number: GRM251-500G)
9. 99.99% dimethyl sulfoxide (DMSO) (Merck, catalog number: 1.07046.0521)
10. Antifungal agent: Dithane^{R} M-45 75% WP (Dow AgroSciences)
11. Sodium dihydrogen phosphate dehydrate ($NaH_2PO_4·2H_2O$) (Merck, CAS number: 200-664-3)
12. Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) (Merck, CAS number: 231-448-7)
13. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT) (Sisco Research Laboratories, catalog number: 33611)
14. 70% alcohol (Analytical Z Reagent, Hong Young Chemicals, catalog number: 15005-51)
15. Deionised water
16. 50 mM sodium phosphate buffer (pH 7) (see Recipes)
17. 1x Hoagland's solution (see Recipes)
    a. Potassium dihydrogen phosphate (KH_2PO_4) (Sisco Research Laboratories, CAS number: 7778-77-0)
    b. Potassium nitrate (KNO_3) (Merck, CAS number: 231-818-8)
    c. Calcium nitrate tetrahydrate ($Ca(NO_3)_2·4H_2O$) (Merck, CAS number: 233-332-1)
    d. Magnesium sulphate heptahydrate ($MgSO_4·7H_2O$) (Sisco Research Laboratories, catalog number: 85611)
    e. Manganese sulphate monohydrate ($MnSO_4·H_2O$) (Sisco Research Laboratories, catalog number: 12386)
    f. Zinc suphate heptahydrate ($ZnSO_4·7H_2O$) (Sisco Research Laboratories, catalog number: 75738)
    g. Copper sulphate pentahydrate ($CuSO_4·5H_2O$) (Merck, CAS number: 231-847-6)
    h. Ammonium molybdate monohydrate ($(NH_4)_6Mo_7O_{24}·H_2O$) (Sisco Research Laboratories, catalog number: 69429)

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i. Boric acid (H_3BO_3) (Sisco Research Laboratories, MDL number: 22311)
j. Ferric chloride ($FeCl_3$) (Thermo Fisher Scientific, catalog number: MFCD00011005)

18. Stress inducing agents(see Recipes)

Equipment

1. Measuring cylinder (Tarsons, catalog numbers: 345040, 345070)
2. Weighing balance (Wensar, model: PGB 610)
3. pH meter (Global Electronics, model: DPH 500)
4. UV-VIS spectrophotometer (Techcomp, model: UH5300)
5. Centrifuge (Eppendorf, model: 5810R, catalog number: 3334)

Procedure

1. Sterilize the *Oryza sativa* seeds with 0.2% dithane-M45 and wash thoroughly with deionised water for at least 10-15 times till there is no pungent odour of dithane-M45.
2. Leave them for 24 h in deionised water for water imbibition.
3. Germinate the seeds in the dark for 72 h at 30 °C.
4. Grow seedlings in culture tubes at 30 °C with a photoperiod of 16 h light and 8 h dark for 6 days in nutrient solution (1x Hoagland's solution).
5. Add 5 ml of different stress inducing agents (see Recipes) directly to the culture tubes containing seedlings. Add 5 ml of deionised water to the control set, stir the treatments by occasional aeration through bubbling.
6. Keep the control and treated sets for 16 h.
7. Remove the seedlings from the growth solution, wipe off till dry with tissue paper.
8. Weigh ~10 mg of fresh root tissue and transfer to a fresh 2 ml microcentrifuge tube.
9. Add 1.5 ml of MTT solution (0.25 mg/L, see Recipes) and keep the tubes in the dark for 4 h.
10. Discard the MTT solution, take the root samples into clean Petriplates without any further washing, cut the roots into 1-2 mm pieces with sterile scalpel (to ensure leaching out of the formed formazan). Add 0.5 ml KOH (2 N) to this and transfer the cut pieces along with KOH solution to 2 ml microcentrifuge tubes.
11. Add 0.5 ml 99.99% DMSO solution to each tube, to make the final volume 1 ml. The colour of the solution appears as shown in Figure 1.
12. Centrifuge the tubes briefly at 500 *x g* for 5 min at room temperature so that the root pieces settle down.
13. Transfer the clear supernatant into fresh tubes.
14. Take spectrophotometric readings of the supernatants at 570 nm immediately. Keep the samples in the dark all the time due to photosensitivity of formazan formed. (see Notes 1 and 2)

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15. The O.D. values obtained are for 10 mg tissue, calculate the O.D. value for 1 mg. From the O.D. value determine the percentage of cell death using below-mentioned formula,

$$\text{Percentage of cell death/mg fresh tissue} = \frac{\text{OD value of the control set} - \text{OD value of treated set}}{\text{OD value of control set}} \times 100$$

Hence, percentage of cell viability/mg fresh tissue= 100 - percentage of cell death

Percentage of cell death is shown in Table 1 and Figure 2.

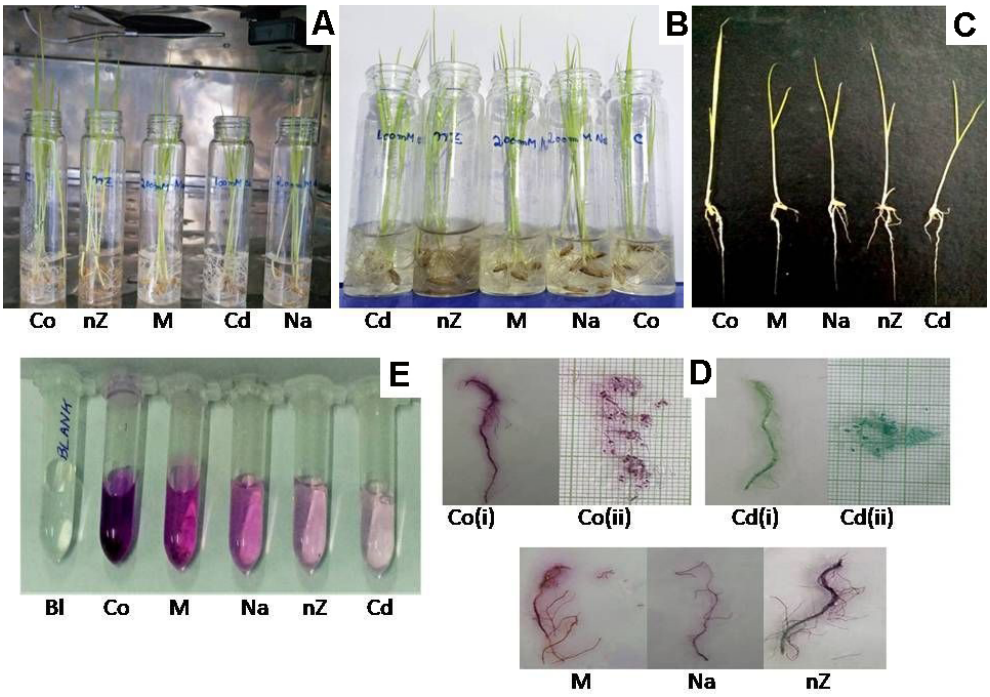

Figure 1.Pictorial representation of different stages of the MTT assay. Bl-Blank, Co-Control, M-treated with 200 mM mannitol, Na-treated with 200 mM sodium chloride, nZ-treated with 200 mg/L nZVI, Cd-treated with 100 mM cadmium chloride. A. Seedlings being grown in growth chamber; B. Seedlings after treatment with stress inducing agents for 16 h; C. Roots before MTT assay; D. Roots after MTT treatment for 4 h. Co (i), Cd (i), M, Na, nZ, Co (ii), Cd (ii) Roots chopped to 1-2 mm in length. E. Colour intensity of the formazan after solubilized in DMSO.

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Table 1. O.D. value and percentage of cell death

Sample	OD at 570 nm	Percentage of cell death: $\frac{\text{OD of control} - \text{OD of treated sample}}{\text{OD of control sample}} \times 100$
Control	1.475	0
200 mM mannitol	0.914	38.03
200 mM NaCl	0.517	64.95
200 mg/L nZVI	0.331	77.56
100 mM $CdCl_2$	0.149	89.89

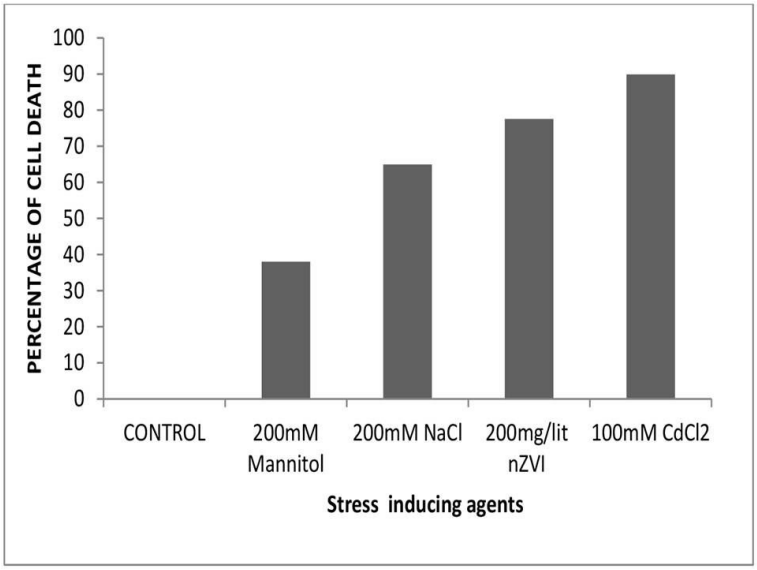

Figure 2. The percentage of cell death of the root tissue is determined from the OD value at 570 nm. The cell death percentage is maximum for 100 mM $CdCl_2$ followed by 200 mg Nzvi > 200 mM NaCl > 200 mM mannitol.

Notes

1. Avoid direct light after addition of MTT as it is photosensitive.
2. Record the absorbance immediately after solubilizing the formazan in DMSO as the colour intensity may change and give erroneous readings.

Recipes

1. 0.2% dithane solution
   Dissolve 200 mg of dithane in 100 ml autoclaved deionised water
2. 50 mM sodium phosphate buffer (pH 7)
   a. Prepare 50 mM sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) (Merck) by dissolving 0.78 g of sodium dihydrogen phosphate in 100 ml deionised water

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b. Prepare 50 mM di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) (Merck) by dissolving 0.7 g of di-sodium hydrogen phosphate in 100 ml deionised water
c. 55 ml of 50 mM sodium dihydrogen phosphate dihydrate and 45 ml of 50 mM di-sodium hydrogen phosphate anhydrous are mixed to make 50 mM sodium phosphate buffer and adjust the pH to 7

3. MTT dye
   Prepare stock solution of MTT (10 mg/ml) by dissolving the MTT in 50 mM phosphate buffer (pH 7)
4. 2 N potassium hydroxide solution
   Dissolve 11.2 g potassium hydroxide in 100 ml deionised water
5. 1x Hoagland's solution
   Macronutrients:
   1 mM potassium dihydrogen phosphate (KH_2PO_4)
   5 mM potassium nitrate (KNO_3)
   5 mM Calcium nitrate $Ca(NO_3)_2$
   Micronutrients:
   11.8 µM manganese sulphate monohydrate ($MnSO_4·H_2O$)
   0.70 µM zinc suphate heptahydrate ($ZnSO_4·7H_2O$)
   0.32 µM copper sulphatepentahydrate ($CuSO_4·5H_2O$)
   0.16 µM ammonium molybdate monohydrate ($(NH_4)_6Mo_7O_{24}·H_2O$)
   46.3 µM boric acid (H_3BO_3)
   5 µM ferric chloride ($FeCl_3$)
   Make up to 1 L with autoclaved deionised water
   Adjust the pH to 5.8
6. Stress inducing agents
   200 mM mannitol
   200 mg/L nZVI
   100 mM $CdCl_2$
   200 mM NaCl
   Dissolved in deionised water

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References

1. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. and Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semi automated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 47(4): 936-942.
2. Kaundal, A., Rojas, C. M. and Mysore, K. S. (2012). Measurement of NADPH oxidase activity in plants. *Bio Protoc* 2(20): e278.