Millennium Seed Bank
Standard Operating Procedures

Method Code: 2.3
Subject: *Seed viability testing using the Tetrazolium Chloride stain*
Section: Conservation and Technology

---

1. Introduction & Principle

The Tetrazolium viability stain is a destructive method to determine whether an ungerminated seed is alive (viable) or dead.

In principle, the imbibed seed material to be tested is sectioned carefully and fully immersed in 1% buffered 2,3,5-triphenyl tetrazolium chloride (TTC or TZ) for a defined period, then examined microscopically. TZ solution penetrates living tissue and hydrogen ions released by enzymes (dehydrogenases), involved in the respiration process, reduce the TZ to an insoluble red compound called formazan. Therefore, respiring tissue stains various shades of red, whilst dead material remains unstained. However, the TZ test is not simply a 'colour stain test'. Other factors must be included when assessing viability.

2. Equipment & Materials

- 1% buffered 2,3,5-triphenyl tetrazolium chloride stock
- agar or suitable imbibing medium
- dissecting equipment to cut seed material
- suitably sized glass or plastic container with lid to contain seed material and excess tetrazolium stain.
- aluminium foil
- stereoscope
- seed material

3. Procedures

Preparation of 1% TZ solution. (See Health and Safety below before proceeding.)

The buffer is usually prepared in 1 litre volumes.

1) Dissolve 3.63 g of Potassium Dihydrogen Orthophosphate (KH_2PO_4) in 400 ml of distilled water.
2) Dissolve 7.13 g of Disodium Hydrogen Orthophosphate Dihydrate (Na_2HPO_4) in 600 ml of distilled water.
3) When these stocks have completely dissolved (a magnetic stirrer may aid dissolving), mix the two solutions in a l litre glass container, label and store at 4°C for not longer than 3 months.

Prepare the TZ reagent as required

4) For each 100ml of reagent required add 1g of 2,3,5-triphenyl Tetrazolium Chloride to 100ml of buffer. Allow this solution to completely dissolve with the aid of a magnetic stirrer. The pH of the solution should be between 6 and 8.

5) Wrap the bottle with foil to exclude light, label the bottle with initials, date and store in the dark at 4°C for not longer than 3 months.

Note: any reagent that develops a pink colour should be discarded.

Viability testing using TZ

It would be very beneficial for those carrying out this procedure for the first time to read Part 1 of the Tetrazolium Testing Handbook [1], which more fully explains the nature of the test, seed structures and dormancy.

1) If the TZ results are to be compared to germination results, a minimum of 25-50 seeds should be analysed in the TZ test. Seeds not yet fully imbibed, are hydrated in high humidity (over water) overnight at 20°C. Any seeds with physical dormancy must be scarified at this time. Check the Tetrazolium Testing Handbook [1], the Handbook of Tetrazolium Testing [3] and the ISTA Working Sheets on Tetrazolium Testing [6] for any special conditioning instructions. Continue general procedures below if there is no further information.

2) Seeds then go onto water agar for 2-3 days at 20°C (3 days over the weekend is easiest) to fully imbibe and initiate any metabolic processes inside the seed.

3) Check the Comparative Internal Morphology of Seeds [2] for embryo and endosperm position. Section 1-2 seeds to note positions, make sketches and notes on the TZ record sheet, and plan the sectioning of the remaining seeds, e.g. "the embryo is always at the end of the seed with a darker spot on the outside – therefore cut at the other end". Wherever possible, DO NOT cut the embryo when sectioning. The goal is to remove the seed coat entirely or from a large section of the seed to enable the TZ solution to move into the embryo. If the seeds are endospermic then some or most of the endosperm will need to be removed as well, in order to speed up movement of the solution through this tissue; however, some endosperm should be left intact as this may be important when assessing viability.

4) Put the sections containing the embryo into the TZ solution for 48 hours at 30°C in dark conditions (aluminium foil). TZ solution also reacts to light, so this step is important; however, if the solution turns red during the test this could also be due to fungal growth. Remember that after making the TZ solution, it is only properly active for 3 months, so it must be used within this time or thrown away and a new batch made (check before you imbibe your seeds).

5) Following incubation, rinse seeds with water and discard any solution and rinse water. Evaluate the sections and try to be as descriptive on the record sheet as possible. This is especially helpful when new to TZ testing or encountering a new genus, as mistakes may be better explained later with more detailed information on the sheet. All tissues need not be viable for germination to occur, e.g. the radicle root cap may be unstained but the meristematic tissue behind the root cap

must be viable in order for the cells to multiply and the radicle to emerge from the seed coat (Figure 2). Most seeds have dead endosperm, but some endosperm is live tissue and therefore needs to stain for the seed to be considered viable. There are descriptions and illustrations in [1] [2] and [6] of the amount of staining/non-staining permitted in the radicle, embryo axis, plumule, endosperm, coleoptile and scutellum seed tissues. Practice and comparing TZ results to germination results after testing will help increase accuracy.

In addition to stain evaluation, the turgidity of the seed tissue, embryo abnormalities, tissue bruising, and seed-borne fungal pathogens must be considered before a decision on viability can be made.

6) TZ results can then be compared to germination results using a chi-square statistical test.

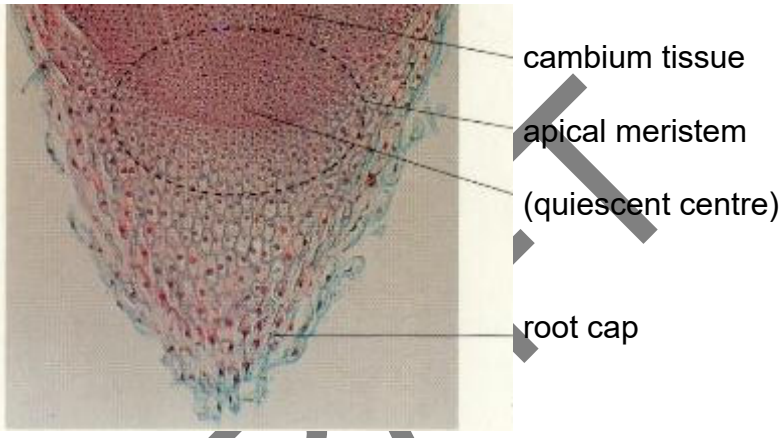

Figure 2: *Root tip showing meristematic tissue and root cap.*

Please note:

- Seeds should be cleaned prior to staining (see SOP 2.8 'Seed cleaning').
- Seeds with a high oil content (>15%) and resins or embryos with air pockets may not give accurate TZ results, as the solution may not penetrate all tissues.
- If you suspect that there has not been sufficient time for the TZ solution to penetrate the embryo completely, they may be submersed in solution for a further 24hrs.
- Seeds with a fungal infection may stain dark red, burgundy or brown.
- A false positive result is very difficult to obtain, whereas a false negative result may be due to staining method, time in the solution or deep dormancy.
- Sectioning the seed may damage tissues (necrosis). Therefore, seeds must be further sectioned after staining to evaluate.
- Discard all unwanted plant material for incineration in the yellow bins provided.

4. Health & Safety

- Only authorised and trained staff should perform this technique.
- Good laboratory practise MUST be followed.
- Laboratory coats, protective disposable gloves and safety glasses MUST be worn especially when preparing stock solution which must be carried out in the weigh station.
- Great care should be taken when sectioning plant material to avoid cutting oneself.
- The COSHH assessment indicates that the chemicals used in this technique are of low risk when used correctly. TTC (TZ) and disodium hydrogen orthophosphate dehydrate (formazan) may cause minor skin irritation but severe eye irritation. Avoid inhalation of powder, wash any contaminated skin with copious quantities of water, rinse out any eye contamination with sterile eyewash. If irritation persists seek immediate medical advice.

5. Spillages & Disposal

All spillages should be immediately mopped up with tissue and the area cleaned. Wear gloves when dealing with hazardous chemicals.
Dispose of contaminated tissue etc. in yellow bins provided.
Unless otherwise stated in the procedure, all *stock solutions* and prepared *reagents* may be discarded down the laboratory sink, rinsing away with plenty of water.
Laboratory chemicals may only be disposed of by the Laboratory Manager or the Departmental Heath & Safety Co-ordinator.

6. References

1] Tetrazolium Testing Handbook. The Tetrazolium Subcommittee of the Association of Official Seed Analysts. 1970 – revised 2008.
2] Martin, A.C. 1946. The Comparative Internal Morphology of Seeds. The American Midland Naturalist 36(3):513-661.
3] Handbook on Tetrazolium Testing, 1985. International Seed Testing Association.
4] Handbook of seed technology for genebanks - volume 1 (p125-134) R.H.Ellis, T.D.Hong & E.H.Roberts.
5] COSHH risk assessment No. 2 (24/6/98).
6] ISTA Working Sheets on Tetrazolium Testing, Vol I & II, 2003. International Seed Testing Association.

General risk assessment – pending KRM
COSHH no 105