Stress Tolerance Assay at the Seed Germination Stage for Tobacco

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[Abstract] Stress tolerance of plants is a complex phenomenon that depends on the inter-related action of several morphological, physiological and biochemical parameters. Although stress affects normal physiological growth of a plant irrespective of its developmental stage, seed germination and seed setting are considered to be the most sensitive two. Therefore, to evaluate the stress tolerance potential of a particular plant species or variety, rate of seed germination in presence of stress is an important agronomic trait. This will provide a clear indication about the stress tolerance potential with minimum instrumentation facilities. The method is very simple, effective and highly reproducible that would provide quick and reliable results to the researchers.

Materials and Reagents

1. Tobacco seeds (*Nicotiana tabacum* L. cv. Petit Havana, wild type and transgenic)
2. Sterile distilled H_2O
3. Ethanol
4. Para film
5. Murashige and Skoog medium salt (Caisson Laboratories, catalog number: MSP09-1lt)
6. Sucrose (Sigma-Aldrich, catalog number: S0389)
7. Agar (Plant tissue culture grade, Sigma-Aldrich, catalog number: A7921)
8. Stress reagents (such as NaCl for salinity stress, and H_2O_2 for oxidative stress)
9. Germination media (see Recipes)

Equipment

1. Microcentrifuge tubes
2. Growth chamber
3. Laminar flow cabinet
4. Petri plates (100 x 20 mm)
5. Whatman filter paper
6. Autoclaved forceps

7. Electronic balance

Procedure

1. Preparation of media

   Prepare half strength MS medium as described in the recipe. There is no need to add anything externally to the medium for control plates, and autoclave directly. To mimic stress condition during germination, add stress inducing agents such as 200 mM NaCl for salinity stress to the medium before autoclaving. But for oxidative stress, add 5 mM H_2O_2 to the medium after autoclaving. After autoclaving, allow the medium to cool down to around 40 to 50 °C temperature and then pour into sterilized petridishes (100 x 20 mm). After that, let the medium to solidify and seal the petridishes properly with para film if not to be used immediately.
2. Seeds sterilization

   As the seeds could be contaminated by fungi or bacteria during maturation or harvesting or storage, they need to be surface sterilized properly before germinating in a nutrient rich medium. The steps of seed sterilization are described below:

   a. Put certain amount of seeds (around 200) into a 1.5 ml Eppendorf tube.
   b. Add 1 ml of 70% ethanol.
   c. Briefly shake for less than 1 min (strictly not more than that).
   d. Pour off ethanol.
   e. Rinse with sterile distilled water for 3 to 5 times.
3. Germination of seeds

   Keep the plates with media open in a laminar flow cabinet for some time to remove the surface moisture (if any). Then place the sterile seeds (around 20) on the media in three separate plates (for biological replicates) with autoclaved sharp end forceps. After putting seeds, seal the plates properly with para film and keep in the growth chamber under dark (Figure 1b). The plates should be kept in dark for 3 to 4 days and then keep under normal long day condition (16/8 h light/dark cycle) at 26 ± 2 °C for 15 days. Monitor the growth of seedlings every day (Figure 1c-d).
4. Rate of seed germination calculation

   Allow the seedlings to germinate and grow for certain period (10 to 15 days) under both control and stress condition. Count the number of germinated seedlings for each line in all the three biological replicates of both control and stress condition. Rate of seed germination for each line was calculated using the following equation.

   Germination rate (%) = (Average number of seeds germinated under stress condition/ Average number of seeds germinated under control condition) x 100%
5. Stress tolerance index (STI) calculation

Measure the fresh weight of 10 seedlings in triplicates for each line under both control and stress conditions and calculate the stress tolerance index (STI) using the following equation (Mustafiz *et al.*, 2014).

STI (%) = (Average fresh weight of 10 stressed seedlings/Average fresh weight of 10 control seedlings) x 100%

Representative data

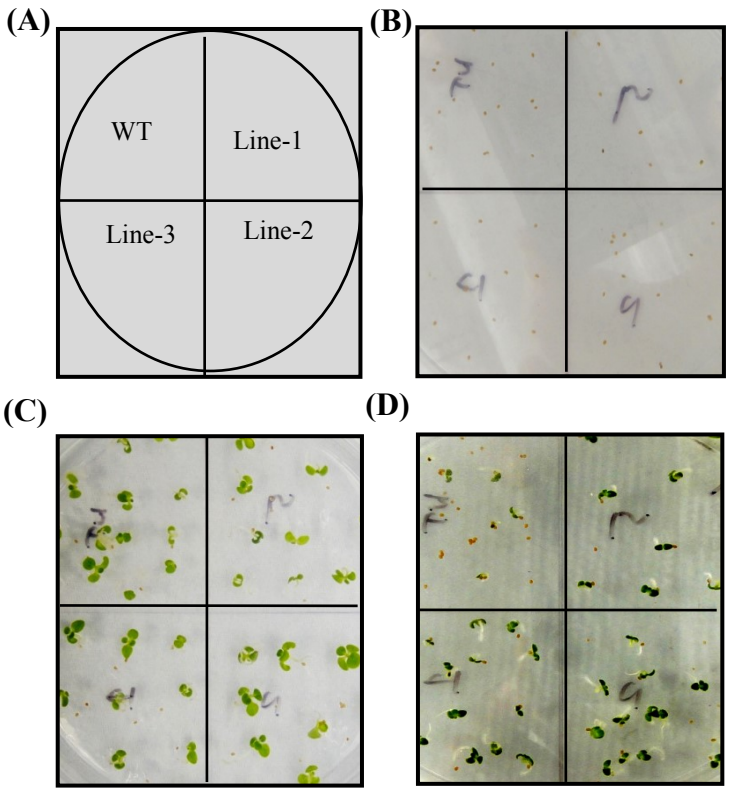

Figure 1. Seed germination assay for stress tolerance. (A) Pictorial depiction of the position of various type of tobacco seeds WT (wild type; non-transgenic) and three different transgenic lines; line-1, line-2 and line-3) used in the study. (B) Inoculation of seeds in the MS medium alone (experimental control) or MS medium supplemented with 200 mM NaCl for salinity stress. Germination of seeds after 15 days under control condition (C) and stress condition (D). Transgenic seeds showed higher germination rate and better seedling growth as compared to the WT under stress condition.

Notes

1. Seed sterilization with 70% ethanol for more than one minute will lead to complete inhibition of germination. So do not exceed the time.

2. As germination of seeds may vary based on plant species, seed storage conditions, type and degree of imposed stress, experiment should be monitored regularly (not strictly 15 days) for distinguishable results.

Recipes

1. Germination media (1 L)
   1/2 Murashige and Skoog medium salt (2.215 gm)
   30 g sucrose
   Adjust pH to 5.7 with 1 M NaOH
   Add 0.8% agar
   Then autoclave for 15 min

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References

1. Mustafiz, A., Ghosh, A., Tripathi, A. K., Kaur, C., Ganguly, A. K., Bhavesh, N. S., Tripathi, J. K., Pareek, A., Sopory, S. K. and Singla-Pareek, S. L. (2014). A unique Ni^{2+} -dependent and methylglyoxal-inducible rice glyoxalase I possesses a single active site and functions in abiotic stress response. *Plant J* 78(6): 951-963.