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Leaf Disc Stress Tolerance Assay for Tobacco

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[Abstract] Stress tolerance is a multigenic trait that depends on the coordinated action of several genes. Various physiological parameters, such as plant height and weight, total yield, chlorophyll content, photosynthesis rate, level of reactive oxygen species (ROS) and anti-oxidant activity could be correlated directly with the level of stress tolerance potential of any particular genotype. To evaluate the stress tolerance potential of a plant, leaf disc stress tolerance assay is a very rapid and widely acceptable experiment with minimum instrumentation facilities.

Materials and Reagents

1. Young tobacco plants (to be tested)
2. Distilled water
3. Acetone (Sigma-Aldrich, catalog number: I10010)
4. Stress reagents (such as NaCl, Sigma-Aldrich, catalog number: S7653; for salinity stress)
5. Liquid nitrogen
6. Extraction buffer (see Recipes)

Equipment

1. Leaf puncher/ cork borer (Sigma-Aldrich, catalog number: Z165220)
2. Petri plates (60 x 15 mm)
3. Microcentrifuge tube
4. Tissue paper
5. Graduated cylinder
6. Mortar and pestle (Sigma-Aldrich, catalog number: Z112496)
7. Glass cuvette (1 ml) (Sigma-Aldrich, catalog number: C8550-1EA)
8. Electronic balance
9. Spectrophotometer
10. Benchtop refrigerated centrifuge (Eppendorf, catalog number: 5427 R)
11. Vortexer (Sigma-Aldrich, catalog number: Z755613-1EA)

Procedure

1. Grow tobacco seedlings in earthern pots filled with normal well-drained soil and keep them in the green house for 6 weeks at optimum condition (26 ± 2 °C, 75% relative humidity, and a photoperiod of 16/8 h light and dark).
2. Select healthy plants of equal height and age for all the lines to be tested for leaf disc stress tolerance assay.
3. Collect leaves (third to fifth leaf from the top) from each plant and gently wash with distilled water to remove any extraneous material associated with the tissues.
4. Cut leaves using a leaf puncher or cork borer to obtain leaf discs of similar size (1 cm diameter) and surface area. Avoid taking discs from the major veins of leaf.
5. Float equal number of leaf discs (7-8) from each line (with abaxial surface down) on sterile distilled water in triplicates as experimental control (Figure 1, 0 day).
6. Float the same number of leaf discs from each line (with abaxial surface down) on various stress containing solution such 200 mM NaCl for salinity stress (Figure 1, 0 day, lower panel).
7. The leaf discs floated on particular solutions as well as experimental controls should be kept in the same environment and visualized after every 12 h until a visual difference in their “greenness” is observed.
8. After observing a clear difference between the leaf discs floated in control solution and stress solution, photographs should be taken (Figure 1, after 3-4 days). Leaf discs can be tested for other stresses, such as oxidative, osmotic and heavy metal stresses using same protocol (Kumar *et al.*, 2012; Singh e*t al.*, 2012).
9. Quantitate this visual observation by extracting the total chlorophyll of the leaf discs as described (Arnon, 1949) and quantify according equation as described (Porra, 2002).
10. Measure fresh weight (in grams, gm) of the leaf discs from each conditions and lines.
    Note: Remove surface moisture from the discs with tissue paper before measuring weight.
11. Grind leaf discs to fine powder in a mortar and pestle using liquid nitrogen.
    Add 1 ml of extraction buffer (80% acetone), mix throughly and immediately transfer the extract into a microcentrifuge tube (MCT) in a fume hood. Alternatively, add the tissue powder to a tube containing 1 ml of extraction buffer. Mix properly by vortexing for 1 min and adjust the volume with extraction buffer in all the tubes after mixing.
    Note: Adjust volume of the mixture to 1 ml (if neccessary) with extraction buffer before centrifugation as acetone is volatile.
12. Centrifuge the suspension at 3,000 *x g* for 5 min under cold condition (4 °C).
13. Transfer the supernatant from each tube to new MCT tube and use this extract for measuring absorbance.
14. Dilute the supernatant five times by adding 200 µl of supernatant in 800 µl of extraction buffer. Absorbance of this mixture is measured in triplicates at 664 and 647 nm wavelength by taking extraction buffer (80% acetone) as blank using a spectrophotometer and 1 ml glass cuvette.

15. Calculate the content of Chlorophyll A, Chlorophyll B and total Chlorophyll using the following equations as described by Porra (2002).
    Chlorophyll A= [{(12.25xA664)-(2.55xA647)}xDF]/TW µg/ml/gm fresh weight
    Chlorophyll B= [{(20.31xA647)-(4.91xA664)}xDF]/TW µg/ml/gm fresh weight
    Total Chlorophyll= (Chlorophyll A + Chlorophyll B) µg/ml/gm fresh weight
    Here, DF (Dilution factor)= (Total volume of solution for absorbance (1 ml)/volume of supernatant taken for dilution (200 µl)
    TW (Tissue Weight) = Weight of the tissue taken (in grams)
16. Leaf discs from tolerant cultivars would be able to maintain their total chlorophyll level with minimum loss over time as compared to their control (0 day) value. However, sensitive leaves will show drastic reduction of their chlorophyll level (more stress induced chlorophyll bleaching) in response to stress.

Representative data

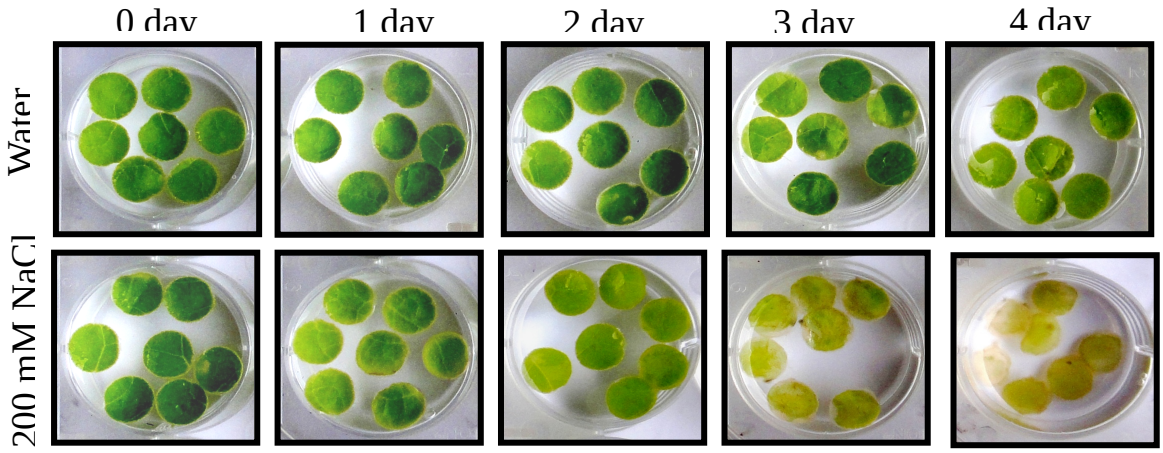

Figure 1. Response of tobacco leaf discs in presence of 200 mM NaCl for 4 days. Leaf discs are floated onto water to serve as experimental control and 200 mM NaCl for salinity stress. There is no change in the 'greenness' of discs under control condition, while discs are bleached and turned into yellow in the presence of 200 mM NaCl.

Notes

1. The size and age of the leaf is an important factor for the assay. So make sure that the leaves are collected from the same position (third to fifth leaf from the top) and almost similar in size to avoid initial variance among different lines.
2. All solutions should be made in sterile water and work under clean bench to avoid any type of bacterial or fungal infection.
3. As the response could vary based on the plant species, age of plant, type and degree of imposed stress, experiment should be monitored regularly for at least seven days (not strictly

4 days). If there is no change within seven days time period, fresh experiment should be set up with increased degree of stress.

4. As acetone could degrade disposable plastic cuvettes, glass cuvette should be used.

Recipes

1. Extraction buffer (100 ml)
   Acetone: 80 ml
   H_2O: 20 ml
   Prepare in a tightly sealed glass brown bottle and prepare fresh every time

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