Protocol

TD-P Revision 3.0

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Transformed Plant Morphology Examination after Antibiotic Selection

Introduction

Plant transformation is comprised of selection, regeneration and the elimination of the transformation vector. Usually, the elimination of the transformation vector is accomplished through the use of antibiotics. Here, we describe a protocol to determine the efficacy of aminoglycoside based antibiotics on their ability to remove *Agrobacterium* transformation vectors from a plant culture following a transformation procedure. This procedure can be modified to use whichever antibiotics are deemed favorable by the researcher provided that they are resistant to the aminoglycoside degrading genes *nptII* or *hptII*, which may be present in *Agrobacterium tumefaciens*.

Materials

- Thin cell layers (TCL) of plant to test
- Antibiotics:
  Cefotaxime (GoldBio Catalog # C-104)
  Carbenicillin (GoldBio Catalog # C-103)
  Vancomycin (GoldBio Catalog # V-200)
- Hyponex medium (20 g/L sucrose)

Optimized regeneration medium

- Murashige and Skoog (MS) medium
- 2 mg/L benzyladenine (BA)
- 0.5 mg/L α-naphthalene acetic acid (NAA)
- 40 g/L sucrose

Method

1. Place transverse TCLs from stem internode tissue of in vitro 'Lineker' and 'Shuhou-no-chikara' chrysanthemum, as well as tobacco (light and dark) at 25°C for a 16-hour photoperiod on an optimized shoot regeneration medium containing different combinations of antibiotics.
    a. The shoot regeneration medium should contain 1, 25, 50, 100, 250, 500, 1000 or 2000 µg/mL of cefotaxime (CF), vancomycin (VA), carbenicillin (CA) or 6 CA:CF:VA combinations at either 50, 100, 200 or 400 µg/mL, with (10 or 25 µg/mL

kanamycin for chrysanthemum and TOB, respectively) or without (control) selection.

2. Make new medium every two weeks.

3. Harvest the shoots derived from each medium and place on Hyponex medium containing 20 g/L sucrose.

4. Subculture the plantlets three times and maintain them under 16-hour photoperiods at 25°C.

5. Acclimatize the chrysanthemum plantlets and maintain in a greenhouse under LD conditions and place in SD condition for flower induction.

6. Perform morphological (plant) and plaque (*Agrobacterium*) scoring of all explants. Score as follows:
    a. Amount of shoots (shoot regeneration capacity or SRC)
    b. Callus
    c. Explant survival (ES)
    d. Explant fresh weight after 60 days in culture
    e. Plant threshold survival level (TSL, physiological state of the TCL in which no morphogenic development occurs)

7. Perform morphological (plant) and plaque (*Agrobacterium*) scoring of greenhouse-acclimatized chrysanthemum as follows.
    a. Vegetative and flowering normality
    b. Plantlet height
    c. Number of leaves
    d. Total fresh weight
    e. Number of disk and ray florets per flower head

8. Calculate the number of colony forming units (CFUs) and bacterial TSLs after day 4 and 14 in culture (selective 10 or 50 µg/ml kanamycin LB and MS).
    a. Standardize 1 cm^2 of non-distinguishable (solid) plaque bacterial growth at 100 CFUs/cm^2 (or 1 PFU/mm^2), with an overgrown petri dish being πr^2 x 100 CFUs = 5600 CFUs (the maximum) where r = 4.25. Percent bacterial growth can be recorded with 1% = 56 CFUs.

Associated Products

- Cefotaxime (GoldBio Catalog # C-104)

- Carbenicillin (GoldBio Catalog # C-103)
- Vancomycin (GoldBio Catalog # V-200)

References

Hammerschlag, F. A., Zimmerman, R. H., Yadava, U. L., Hunsucker, S., and Gercheva, P. (1997). Effect of antibiotics and exposure to an acidified medium on the elimination of agrobacterium tumefaciens from apple leaf explants and on shoot regeneration. *Journal of the American Society for Horticultural Science, 122*(6), 758-763.

Silva, J. D. and Fukai, S. (2001). The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and Agrobacterium growth. *J. Appl. Hort*, *3*(1), 3-12.