Resource Book

CrGC

Crucifer Genetics Cooperative

Dept. of Plant Pathology, 1630 Linden Dr., University of Wisconsin, Madison, WI 53706

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—Paul H. Williams

Research Notes

- *Techniques*
- *Formulations*

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RC 06-22-87 WILPAU

CHROMOSOME DOUBLING WITH COLCHICINE

Doubling of chromosomes of rapid-cycling crucifers is routinely performed by using the following procedure:

Materials needed:
1. 0.1% (w/v) aqueous colchicine solution
2. 5-10 mm dia. absorbent cotton balls
3. dew chamber at 100% R.H. and 20°C
4. five-day-old crucifer seedlings

Procedure:
1. Soak the cotton balls in the colchicine solution until saturated.
2. Place a saturated cotton ball between the cotyledons of a seedling so that the growing point is completely covered or in axils of older plants.
3. Place the treated plants in the dew chamber for 12-16 hr.
4. Remove the plants from the chamber, remove the cotton balls and gently wash off any remaining colchicine solution.

This treatment can delay growth for several days. New growth may appear abnormal. Polyploid plants or branches may be identified by their thicker and darker foliage and enlarged guard cells, flower parts and pollen grains. They grow slower than normal and often have reduced fertility. Putative polyploids are confirmed by meiotic chromosome counts.

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CYTOGENETIC TECHNIQUES

PREPARING *BRASSICA* MITOTIC OR MEIOTIC CHROMOSOMES FOR VIEWING

Materials Needed:

1. Meristematic tissues (young, rapidly growing leaves, sepals or root tips) collected between 9-11 A.M. or flower buds approximately 1 mm length.
2. 0.002M 8-hydroxyquinoline.
3. Carnoy's fixative:
    a. 60 ml anhydrous ethyl alcohol.
    b. 10 ml glacial acetic acid.
    c. 30 ml chloroform.
4. 1N HCl.
5. Modified phenol-fuchsin staining solution.
    a. Solution A-mix 3 g basic fuchsin in 100 ml 70% ethanol.
    b. Solution B-put 10 ml of solution A into 90 ml of 5% phenol.
    c. Solution C-add 45 ml of solution B to 6 ml of 37% formaldehyde + 6 ml glacial acetic acid.
    d. Combine 10 ml of solution C with 90 ml 45% acetic acid and 1 g sorbitol or mannitol.

Procedure:

1. Pretreat meristematic tissues for mitotic chromosomes in 8-hydroxyquinoline at 22-24C for 2-3 hr. (Pollen mother cells need no pretreatment)
2. Fix in Carnoy's fixative for 1 hr.-3 days.
3. Hydrolyze in HCl for 6-10 min.
4. Stain in modified phenol-fuchsin for 20 min.-12 hr.
5. Squash cells using standard methods.
6. Examine chromosomes with a microscope.

PREPARING MITOTIC CHROMOSOMES FOR PHOTOGRAPHY AND KARYOTYPING

Materials Needed:

1. 0.5-1 cm long root tips.
2. 0.002M 8-hydroxyquinoline.
3. 0.075M KCl.
4. Carnoy's fixative (see procedure above).
5. Citrate buffer at pH=4.4-4.8 (9.35 ml 0.2M Na_2HPO_4 or $Na_2HPO_4-2H_2O$ + 10.65 ml 0.1M $C_6H_8O_7-H_2O$).
6. Enzyme solution:
    a. Make a 10% pectinase solution (dilute one part purified pectinase, EC 3.2.1.15, Sigma Chem. Co. stock no. P-5146, in 10 parts citrate buffer, pH 4.4-4.8).

b. Make a 1.5% cellulase solution (1.5 g purified cellulase, EC 3.2.1.4, in 100 ml citrate buffer, pH 4.4-4.8) (cellulase preparation "Onozuka R-10" from Yalkalt Pharmaceutical Industry Co., Ltd., Shingikon-Cho, Nishinomiya, Japan, works well).
c. Mix equal parts of the pectinase and cellulase solutions. The enzyme solution may be stored at -20C for up to two mo.

7. Thoroughly cleaned glass sides.
8. 60% acetic acid.
9. 2% Giemsa staining solution:
   a. Make Giemsa stock solution (dissolve 1 g Giemsa powder in 66 ml glycerine and 66 ml methanol, by heating at 60 C) (refrigerate).
   b. Make Sorensen buffer at pH 6.81 (5 ml 0.066M Na_2HPO_4 + 5 ml 0.066M KH_2PO_4).
   c. Add 2 ml Giemsa stock solution to 98 ml Sorensen buffer.

Procedure:

1. Pretreat root tips in 8-hydroxyquinoline at 22-24 C for 2-3 hr.
2. Wash the root tips in the KCl solution at least twice for 15 min. each washing.
3. Fix root tips in Carnoy's fixative at 4 C for 2 hr-one wk.
4. Wash in citrate buffer twice for 15 min. each washing.
5. Digest root tips in enzyme solution for 1-1.5 hr at 37 C or 3-4 hr at 22 C (digestion is sufficient when the root tip region of cell division separates from the region of elongation).
6. Gently wash in citrate buffer 3 times for 10 min. each washing.
7. Remove the regions of elongation with a pipette (regions of cell division are denser than regions of elongation).
8. Transfer 3-5 root tips onto a clean glass slide and carefully blot off excess buffer.
9. Add one drop of the acetic acid to the slide and soften the cells over gentle heat.
10. Suspend the cells by agitating with a pair of forceps or similar instrument.
11. Add a few drops of Carnoy's around the cell suspension and then add several drops on top to spread the cells.
12. Flame or air dry the slide (for Giemsa C-banding, air drying works better; for karyotyping, flame drying is better).
13. Stain in Giemsa solution for 15-60 min.
14. Wash with tap water.
15. Examine chromosomes with a microscope.

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REMS 05-12-85 BOSPAU

EMS MUTAGENESIS OF *BRASSICA* SEEDS

The mutagen, ethyl methanesulphonate (EMS) has been used for the production of mutations in *Brassica oleracea* CrGC-3, *B. nigra* CrGC-2 and *Raphanus sativus* by treating seeds.

Alkylating agents such as EMS are very reactive compounds, able to react with water. To be effective, solutions must be prepared just before use and never stored. Reaction products with water give rise to compounds that are no longer mutagenic, but are still toxic. Extreme caution should be taken in handling EMS as it is a potent carcinogen.

The three factors most critical in EMS mutagenesis are concentration, duration of treatment, and temperature during treatment. Presoaking the seeds in water prior to EMS treatment is beneficial. Experiments were run to determine the most effective treatment parameters for mutagenesis that would also minimize seedling injury. The following procedure produced numerous mutations.

The best results were obtained from a water presoak of two hours at room temperature. After soaking, the water is poured off and the seeds covered with a 1.5% aqueous EMS solution (V:V). The seeds are soaked, with occasional shaking, for six hours at 20 C. The EMS solution is poured off and neutralized by mixing with copious amounts of sodium bicarbonate (powder). After 48-72 hours the neutralized solution is flushed down the drain with excess water. The seeds are flushed with running water for two hours. Seeds can then be planted immediately or dried and stored for a day or two.

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RG 05-12-85 WILPAU

GIBBERELLIN RESPONDERS

CrGC-63 stock of *Brassica campestris* is a putative gibberellin deficient mutant which exhibits seed dormancy and/or a 'rosette' phenotype. 'Rosettes' lack all internode extension, have dark green leaves with short, infertile flowering shoots. The 'rosette' phenotype is controlled by the single recessive gene *ro*.

Seed dormancy is overcome by soaking seed in the GA, exposure to light or removal of the seed coat. 'Rosettes' can be induced to produce normal, fertile flowering shoots by applying GA.

GA is applied to 5-7 day old 'rosettes' by placing a droplet of 100 ppm gibberellic acid onto the meristem and emerging leaves or atomizing the GA solution onto the foliage. Three to five applications at 2-5 day intervals will insure normal plant and flower development and good seed sets. Care should be taken to keep the foliage dry so that the GA is not washed off.

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RPS 05-11-85 HILCUR

OBSERVATION OF POLLEN-STIGMA INTERACTION

The procedure developed by Kho and Baer (1) is routinely used to evaluate pollen-stigma interactions for compatibility and incompatibility.

Materials needed:

1. Formalin-acetic-alcohol (F.A.A.) fixative.

Formalin	13 ml
Glacial acetic acid	5 ml
50% ethanol	200 ml

2. 1 N NaOH.
3. 0.1% (w/v) aniline blue dissoved in 0.1 N K_3PO_4.
4. Fluorescence microscope capable of providing short-wave illumination.
5. Crucifer pistils at least 24-48 hr after pollination.

Procedure:

1. Macerate excised pistils, which may be used fresh or fixed for 24 hr in F.A.A. (material to be stored should be placed in 70% ethanol after fixation) in 1 N NaOH for 1-2 hr at 22-24 C.
2. Wash the pistils 3 times in distilled water.
3. Place an excised style and stigma in a drop of the aniline blue solution on a glass microscope slides, cover with a #2 cover slip and apply slight pressure on the cover slip to squash and spread the stylar stigmatic tissues into a mono layer.
4. View stigmas and pollen with fluorescence microscope, under short-wave illumination (350-400 nm) preferably with epillumination. Callose deposits in the stigmatic papillae will exhibit bright yellow green fluorescence as will callose in the pollen tubes.
5. Incompatible brassica pollen frequently either fails to germinate or produces short curled and often thickened tubes which may or may not penetrate stigmatic papillae. Papillar cells in incompatible reactions may produce large amounts of callose. Compatible pollen germinates to produce long lightly fluorescing tubes which grow through the style toward the ovules. Pollen tubes often contain brightly fluorescing callose plugs.
6. It is important to run compatible and incompatible pollen-stigma controls with each test.

The intensity of the fluorescence decreases over time after the aniline blue treatment so observations must not be delayed long.

References

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RPV 05-12-85 FERJOH

POLLEN VIABILITY TESTS FOR *BRASSICA*

Johan Ferreira

I. Vital stains

MTT, tetrazolium salts have been widely used to determine viability. The methods of Hecker (1963) and Norton (1966) have been modified for *Brassica* pollen. One part of 10 g/l of MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide] was mixed with five parts of 60% sucrose solution. A drop is placed on pollen deposited on a microscope slide. Viable pollen reacts with with the MTT to produce a magenta colour. Fresh *B. juncea* pollen stains with variable intensity whereas pollen killed by 95% ethanol does not stain.

Fluorescein diacetate, a fluorescent stain uses the integrity of the plasmalemma of the cell or pollen grain as an indicator of viability. A drop of 10^{-5} to 10^{-6} M fluorescein diacetate in 0.5 M sucrose is placed on pollen grains on a slide. Fluorescein diacetate (2 mg) is dissolved in 1 ml acetone which is added drop by drop to 10 ml 0.5 M sucrose until the solution remains milky (Hauser & Morrison, 1964). Pollen is incubated in a drop of the solution for 5 min before viewing with a fluorescense microscope. Viable pollen grains fluoresce bright yellow-green under near ultra-violet (350-400 nm) epiilluminance.

II. *In vitro* pollen germination

Methods to investigate pollen viability is described by Hodgkin (1983) and Roberts *et al* (1983).

III. *In vivo* pollen germination

Aniline blue stain stains the callose surrounding the pollen tubes. A modified method as described by Kho & Baer (1968) and in (CrGC-ID# RPS - - HILCUR) is used. The tissue was lightly squashed under a cover slip and observations made with a fluorescense microscope under epiilluminance. After 24 h at 24C viable pollen grains of *B. juncea* have germinated and can be counted on the surface of the stigma. If stigmas are sampled and fixed after 24 h, the pollen tubes elongate to such an extent that visualization of the pollen tubes is difficult.

IV. Seed set

The flowers of male fertile plants are emasculated 24 h before anthesis. The mature stigmas are dusted with the pollen. Use of genic or cytoplasmic male sterile plants circumvents the need for emasculation. The flowers are protected from stray pollen and the pods are allowed to mature. The seed can be counted and compared with the appropriate controls.

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RSV 05-11-85 HILCUR

SEED VERNALIZATION PROCEDURE

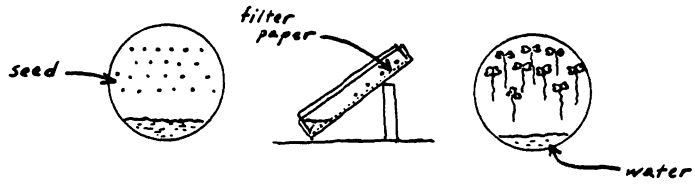
Biennial *Brassica campestris* and *B. napus* plants can be induced to flower during the seedling stage by cold treatment using the following prodcedure:

Materials needed:

1. Petri dishes
2. Whatman No. 2 filter paper (9 cm)
3. distilled water
4. Hoagland's nutrient solution (see MHO 05-07-85 WILPAU)
5. fluorescent lighting
6. cold storage set at 4 C equipped with fluorescent lighting
7. 30 x 16 x 8 cm clear plastic shoe boxes (Althor Products, Box 1236, Weston, CN 06883)
8. course vermiculite

Procedure:

1. With graphite pencil, mark the lower 1/3 of a filter paper with the seed stock # and date. Invert the petri dishes, pour a little distilled water on the inside of the lid of the petrie dish and slide the filter paper disk on to the inside of the lid so that air pockets do not form beneath the paper.
2. Place 25-30 seeds in staggered formation beginning at one edge of the paper in rows of 5 seeds. The seeds should be confined to about 2/3 of the filter paper area.



3. Place the seeded dishes in clear plastic shoe boxes on 2 cm of moist vermiculite with the seed end raised to a 30-45° angle.
4. Carefully water the dish through the opening between the inverted dish bottom and lid to create a reservoir of water in the lower 1/3 of the dish. Then close the shoe boxes.
5. Incubate the boxes at room temperature for 24 hr under low intensity fluorescent lighting. Then place them at 4 C for 2 - 6 weeks about one foot from low fluorescent lighting.
6. Replenish the water reservoir in each seed dish with 50% Hoagland's as needed.

7. When the vernalization period has ended, transplant the seedlings to rooting medium and incubate them under 250 μmol $s^{-1}m^{-2}$ at 15 C.
8. Gradually raise the temperature to 20 C over 2 - 4 weeks. Plants should flower within 6 weeks after leaving cold storage.