Tissue Culture Applications

- Micropropagation
- Germplasm preservation
- Somaclonal variation & mutation selection
- Embryo Culture
- Haploid & Dihaploid Production
- *In vitro* hybridization – Protoplast Fusion

Definitions

- **Plant cell and tissue culture**: cultural techniques for regeneration of functional plants from embryonic tissues, tissue fragments, calli, isolated cells, or protoplasts
- **Totipotency**: the ability of undifferentiated plant tissues to differentiate into functional plants when cultured in vitro
- **Competency**: the endogenous potential of a given cell or tissue to develop in a particular way

Definitions

- **Organogenesis**: The process of initiation and development of a structure that shows natural organ form and/or function.
- **Embryogenesis**: The process of initiation and development of embryos or embryo-like structures from somatic cells (Somatic embryogenesis).

Basis for Plant Tissue Culture

- Two Hormones Affect Plant Differentiation:
  - Auxin: Stimulates Root Development
  - Cytokinin: Stimulates Shoot Development
- *Generally*, the ratio of these two hormones can determine plant development:
  - ↑ Auxin ↓Cytokinin = Root Development
  - ↑ Cytokinin ↓Auxin = Shoot Development
  - Auxin = Cytokinin = Callus Development

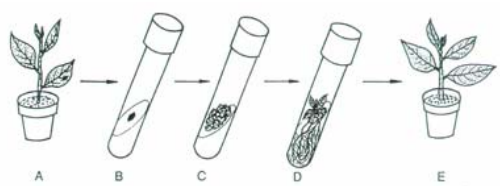


FIG. 8.1. In Vitro regeneration of plants through tissue culture. (A) Source of tissue explant. (B) Explant culture on agar medium. (C) Callus production. (D) Plantlet regenerated. (E) Plantlet transplanted into sterile soil.

Factors Affecting Plant Tissue Culture

- Growth Media
  - Minerals, Growth factors, Carbon source, Hormones
- Environmental Factors
  - Light, Temperature, Photoperiod, Sterility, Media
- Explant Source
  - Usually, the younger, less differentiated the explant, the better for tissue culture
- Genetics
  - Different species show differences in amenability to tissue culture
  - In many cases, different genotypes within a species will have variable responses to tissue culture; response to somatic embryogenesis has been transferred between melon cultivars through sexual hybridization

Micropropagation

- The art and science of plant multiplication *in vitro*
- Usually derived from meristems (or vegetative buds) without a callus stage
  - Tends to reduce or eliminate somaclonal variation, resulting in true clones
- Can be derived from other explant or callus (but these are often problematic)

Steps of Micropropagation

- Stage 0 – Selection & preparation of the mother plant
  - sterilization of the plant tissue takes place
- Stage I - Initiation of culture
  - explant placed into growth media
- Stage II - Multiplication
  - explant transferred to shoot media; shoots can be constantly divided
- Stage III - Rooting
  - explant transferred to root media
- Stage IV - Transfer to soil
  - explant returned to soil; hardened off

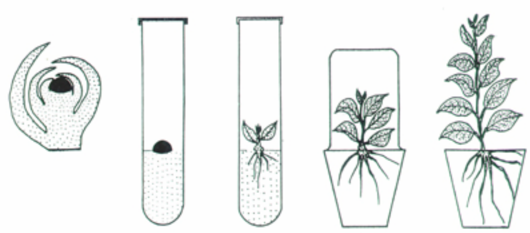


FIG. 8.3. Meristem-tip culture. (A) Apical meristem showing section to be excised. (B) Excised meristem tip cultured on agar medium. (C) Plantlet regenerated from excised meristem tip. (D) Plantlet transferred to sterile soil. (E) Virus-free plant growing in soil.

Features of Micropropagation

- Clonal reproduction
  - Way of maintaining heterozygozity
- Multiplication Stage can be recycled many times to produce an unlimited number of clones
  - Routinely used commercially for many ornamental species, some vegetatively propagated crops
- Easy to manipulate production cycles
  - Not limited by field seasons/environmental influences

Potential Uses for Micropropagation in Plant Breeding

- Eliminate virus from infected plant selection
  - Either via meristem culture or sometimes via heat treatment of cultured tissue (or combination)
- Maintain a heterozygous plant population for marker development
  - By having multiple clones, each genotype of an F_2 can be submitted for multiple evaluations
- Produce inbred plants for hybrid seed production where seed production of the inbred is limited
  - Maintenance or production of male sterile lines
  - Poor seed yielding inbred lines
  - Potential for seedless watermelon production

Germplasm Preservation

- Extension of micropropagation techniques
- Two methods:
  1. Slow growth techniques
     - *e.g.*: ↓ Temp., ↓ Light, media supplements (osmotic inhibitors, growth retardants), tissue dehydration, etc…
     - Medium-term storage (1 to 4 years)
  2. Cryopreservation
     - Ultra low temperatures
     - Stops cell division & metabolic processes
     - Very long-term (indefinite?)
     - Details to follow on next two slides →

Cryopreservation Requirements

- Preculturing
  - Usually a rapid growth rate to create cells with small vacuoles and low water content
- Cryoprotection
  - Glycerol, DMSO, PEG, etc…, to protect against ice damage and alter the form of ice crystals
- Freezing
  - The most critical phase; one of two methods:
    - Slow freezing allows for cytoplasmic dehydration
    - Quick freezing results in fast intercellular freezing with little dehydration

Cryopreservation Requirements

- Storage
  - Usually in liquid nitrogen (-196^{o}C) to avoid changes in ice crystals that occur above -100^{o}C
- Thawing
  - Usually rapid thawing to avoid damage from ice crystal growth
- Recovery (don't forget you have to get a plant)
  - Thawed cells must be washed of cryoprotectants and nursed back to normal growth
  - Avoid callus production to maintain genetic stability

Somaclonal Variation

- The source for most breeding material begins with mutations, whether the mutation occurs in a modern cultivar, a landrace, a plant accession, a wild related species, or in an unrelated organism
- Total sources of variation:
  - Mutation, Hybridization, Polyploidy

Somaclonal Variation & Mutation Breeding

- Somaclonal variation is a general phenomenon of all plant regeneration systems that involve a callus phase
- There are two general types of Somaclonal Variation:
  - Heritable, genetic changes (alter the DNA)
  - Stable, but non-heritable changes (alter gene expression, AKA epigenetic)
- Since utilizing somaclonal variation is a form of mutation breeding, we need to consider mutation breeding in more detail →

Mutation Breeding

- 1927: Muller produced mutations in fruit flies using x-rays
- 1928: Stadler produced mutations in barley
- Mutation breeding became a bandwagon for about 10 years (first claim to "replace breeders")
- Today there are three groups of breeders:
  1) Mutation breeding is useless, we can accomplish the same thing with conventional methods
  2) Mutation breeding will produce a breakthrough given enough effort
  3) Mutation breeding is a tool, useful to meet specific objectives

Inducing Mutations

- Physical Mutagens (irradiation)
  - Neutrons, Alpha rays
    - Densely ionizing ("Cannon balls"), mostly chromosome aberrations
  - Gamma, Beta, X-rays
    - Sparsely ionizing ("Bullets"), chromosome aberrations & point mutations
  - UV radiation
    - Non-ionizing, cause point mutations (if any), low penetrating
- Chemical Mutagens (carcinogens)
  - Many different chemicals
    - Most are highly toxic, usually result in point mutations
- Callus Growth in Tissue Culture
  - Somaclonal variation (can be combined with other agents)
    - Can screen large number of individual cells
    - Chromosomal aberrations, point mutations
    - Also: Uncover genetic variation in source plant

Traditional Mutation Breeding Procedures

- Treat seed with mutagen (irradiation or chemical)
- Target: 50% kill
- Grow-out M_1 plants (some call this M_0)
  - Evaluation for dominant mutations possible, but most are recessive, so →
- Grow-out M_2 plants
  - Evaluate for recessive mutations
  - Expect segregation
- Progeny test selected, putative mutants
  - Prove mutation is stable, heritable

Somaclonal Breeding Procedures

- Use plant cultures as starting material
  - Idea is to target single cells in multi-cellular culture
  - Usually suspension culture, but callus culture can work (want as much contact with selective agent as possible)
- Optional: apply physical or chemical mutagen
- Apply selection pressure to culture
  - Target: very high kill rate, you want very few cells to survive, so long as selection is effective
- Regenerate whole plants from surviving cells

Somaclonal/Mutation Breeding

- Advantages
  - Screen very high populations (cell based)
  - Can apply selection to single cells
- Disadvantages
  - Many mutations are non-heritable
  - Requires dominant mutation (or double recessive mutation); most mutations are recessive
    - Can avoid this constraint by not applying selection pressure in culture, but you loose the advantage of high through-put screening – have to grow out all regenerated plants, produce seed, and evaluate the M_2
    - How can you avoid this problem?

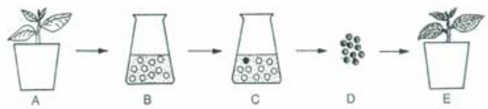


FIG. 8.8. Somaclonal variation. (A) Haploid plant from which cultured tissue was obtained. (B) Cell suspension culture from haploid plant. (C) Mutant cell in suspension culture. (D) Aggregate of mutant cells. (E) Haploid plantlet that exhibits the mutant character. Culturing cells from haploid plants permits either dominant or recessive mutations to be exhibited in the regenerated plantlet. If tissue from diploid plants is cultured, only dominant mutations will be immediately expressed but recessive mutations will be expressed in some progeny plants as a result of segregation.

Successes of Somaclonal/Mutation Breeding

Herbicide Resistance and Tolerance

- **Resistance:** able to break-down or metabolize the herbicide – introduce a new enzyme to metabolize the herbicide
- **Tolerance:** able to grow in the presence of the herbicide – either ↑ the target enzyme or altered form of enzyme
  - Most successful application of somaclonal breeding have been herbicide tolerance
  - Glyphosate *resistant* tomato, tobacco, soybean (GOX enzyme)
  - Glyphosate *tolerant* petunia, carrot, tobacco and tomato (elevated EPSP (enolpyruvyl shikimate phosphate synthase))
    - But not as effective as altered EPSP enzyme (bacterial sources)
  - Imazaquin (Sceptor) *tolerant* maize
- Theoretically possible for any enzyme-targeted herbicide – it's relatively easy to change a single enzyme by changing a single gene

Other Targets for Somaclonal Variation

- Specific amino acid accumulators
  - Screen for specific amino acid production
  - *e.g.* Lysine in cereals
- Abiotic stress tolerance
  - Add or subject cultures to selection agent
  - *e.g.*: salt tolerance, temperature stresses, etc…
- Disease resistance
  - Add toxin or culture filtrate to growth media
  - Examples shown on next slide →

Disease Resistant Success using Somaclonal Variation

Crop	Pathogen	Toxin
Alfalfa	Colletotrichum sp.	Culture filtrate
Banana	Fusarium sp.	Fusaric acid
Coffee	Colletotrichum sp.	Partially purified culture filtrate
Maize	Helminthosporium maydis	T-toxin
Oat*	Helminthosporium victoriae	Victorin
Oilseed Rape*	Phoma lingam	Culture filtrate
Peach	Xanthomonas sp.	Culture filtrate
Potato**	Phytophthora infestans	Culture filtrate
Rice*	Xanthomonas oryzae	Culture filtrate
Sugarcane**	Helminthosporium sp.	Culture filtrate
Sugarcane**	Helminthosporium sachari	Partially purified HS toxin
Tobacco*	Psedomonas tabaci	Methionine-sulfoximine
Tobacco*	Alternaria alternata	Partially purified toxin

*Shown to be heritable through sexual propagation
**Shown to be stable through vegetative propagation

Requirements for Somaclonal/Mutation Breeding

- Effective screening procedure
  - Most mutations are deleterious
    - With fruit fly, the ratio is ~800:1 deleterious to beneficial
  - Most mutations are recessive
    - Must screen M_2 or later generations
    - Consider using heterozygous plants?
    - Haploid plants seem a reasonable alternative if possible
  - Very large populations are required to identify desired mutation:
    - Can you afford to identify marginal traits with replicates & statistics? Estimate: ~10,000 plants for single gene mutant
- Clear Objective
  - Can't expect to just plant things out and see what happens; relates to having an effective screen
  - This may be why so many early experiments failed

Questions with Mutation Breeding

- Do artificial mutations differ from natural ones?
  - Most people agree that they are, since any induced mutation can be found in nature, if you look long enough & hard enough
  - If this is true, then any mutation found in nature can be induced by mutation breeding
- Is it worthwhile, given the time & expense?
- ➢ Still require conventional breeding to incorporate new variability into crop plants (will not replace plant breeders)
- ➢ Not subject to regulatory requirements (or consumer attitudes) of genetically engineered plants

Reading Assignment

•D.R. Miller, R.M. Waskom, M.A. Brick & P.L. Chapman. 1991. Transferring *in vitro* technology to the field. Bio/Technology. 9:143-146

Tissue Culture Applications

- Micropropagation
- Germplasm preservation
- Somaclonal variation & mutation selection
- ✓Embryo Culture
- Haploid & Dihaploid Production
- *In vitro* hybridization – Protoplast Fusion

Embryo Culture Uses

- Rescue F1 hybrid from a wide cross
- Overcome seed dormancy, usually with addition of hormone to media (GA)
- To overcome immaturity in seed
  - To speed generations in a breeding program
  - To rescue a cross or self (valuable genotype) from dead or dying plant

Embryo Culture as a Source of Genetic Variation

- Hybridization
  - Can transfer mutant alleles between species
  - Can introduce new genetic combinations through interspecific crosses
- Polyploidy
  - Can combine embryo culture with chromosome doubling to create new polyploid species (allopolyploidy)

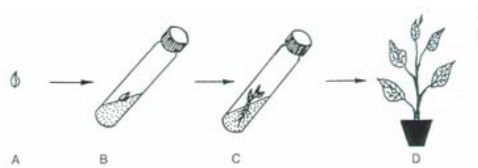


FIG. 8.4. Embryo culture. (A) Proembryo dissected 3 to 5 days after pollination. (B) Proembryo cultured on solid agar medium. (C) Plantlet developing from embryo. (D) Plantlet transplanted into soil.

Embryo Rescue Process

- Make cross between two species
- Dissect embryo (usually immature)
  - The younger the embryo, the more difficult to culture
- Grow on culture medium using basic tissue culture techniques, use for breeding if fertile
- Many times, resulting plants will be haploid because of lack of pairing between the chromosomes of the different species
  - This can be overcome by doubling the chromosomes, creating allotetraploids
  - Polyploids are another source of genetic variation →

Polyploids in Plant Breeding

Very Brief, General Overview

Definitions

- **Euploidy:** An even increase in number of genomes (entire chromosome sets)
- **Aneuploidy:** An increase in number of chromosomes within a genome
- **Autopolyploid:** Multiple structurally identical genomes with unrestricted recombination
- **Allopolyploid:** Multiple genomes so differentiated as to restrict pairing and recombination to homologous chromosomes between genomes

Euploid Polyploid Examples

Euploids	Symbol	Somatic (2n)
monoploid	x	(ABC)
diploid	2x	(ABC)(ABC)
triploid	3x	(ABC)(ABC)(ABC)
autotetraploid	4x	(ABC)(ABC)(ABC)(ABC)
allotetraploid	2x+2x'	(ABC)(ABC)(DEF)(DEF)

Aneuploid Polyploid Examples

Aneuploids	Symbol	Somatic (2n)	Description
nullisomic	2x-2	(AB)(AB)	(missing a chromosome set)
monosomic	2x-1	(ABC)(AB)	(missing a chromosome)
double monosomic	2x-1-1	(AB)(AC)	(missing 2 different chromosomes)
trisomic	2x+1	(ABC)(ABC)(A)	(additional chromosome)
double trisomic	2x+1+1	(ABC)(ABC)(A)(B)	(2 additional different chromosomes)
tetrasomic	2x+2	(ABC)(ABC)(A)(A)	(2 additional chromosomes - same)
trisomic-monosomic	2x+1-1	(ABC)(AB)(A)	(missing a chromosome + additional chromosome)

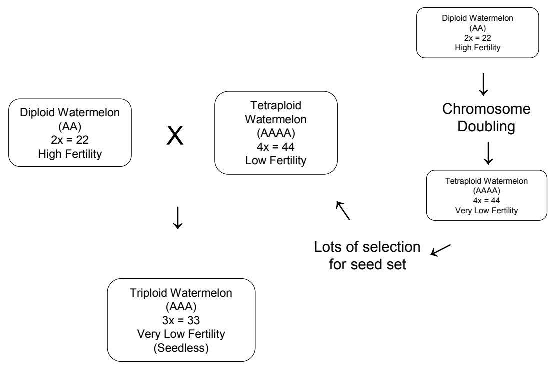
Polyploids as a Source of Genetic Variation

- Multiple genomes alter gene frequencies, induce a permanent hybridity, genetic buffering and evolutionary flexibility (esp. Allopolyploids)
- Autopolyploids typically have larger cell sizes, resulting in larger, lusher plants than the diploid version
- Chromosome doubling occurs naturally in all plants at low frequency as a result of mitotic failure
- Can be induced by chemicals (colchicine from *Colchicum autumnale*) applied to meristematic tissue
- Young zygotes respond best; vegetative tissue usually results in mixoploid chimeras

Autopolyploids

- Multiple structurally identical genomes with unrestricted recombination
- Source material is highly fertile
  - *i.e.*: diploid
- Relatively rare in crop plants:
  - Potato (4x), alfalfa (4x), banana (3x)
  - Typical feature: grown for vegetative product
  - Usually reduced seed fertility
- Limited breeding success in seed crops
  - Despite a lot of effort
  - Exception: Seedless watermelon →

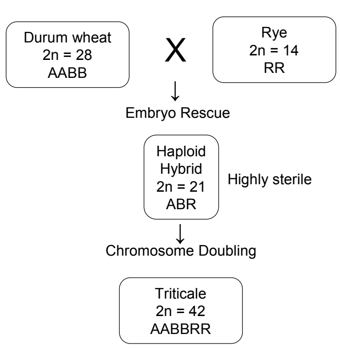
Example of Autopolyploid in Breeding



Allopolyploidy

- Multiple genomes so differentiated as to restrict pairing and recombination to homologous chromosomes between genomes
  - Functionally diploid because of preferential pairing of chromosomes
- Starting material usually an interspecific hybrid
  - F_1 usually has a high degree of sterility
  - Fertility of alloploid usually inversely correlated to sterility in source material (F_1)

Example of Man-Made Allopoloyploid



Uses for Polyploids in Breeding

- Potential for new crop development (triticale)
- Move genes between species
  - Can get recombination between genomes of alloploids, especially when combined with ionizing radiation (mutation breeding)
  - Can re-create polyploids from diploid ancestors using new genetic variation present in the diploids

Haploid Plant Production

- Embryo rescue of interspecific crosses
  - Creation of alloploids (*e.g.* triticale)
  - Bulbosum method
- Anther culture/Microspore culture
  - Culturing of Anthers or Pollen grains (microspores)
  - Derive a mature plant from a single microspore
- Ovule culture
  - Culturing of unfertilized ovules (macrospores)
  - Sometimes “trick” ovule into thinking it has been fertilized

Bulbosum Method of Haploid Production

Hordeum vulgare Barley 2n = 2X = 14 X Hordeum bulbosum Wild relative 2n = 2X = 14

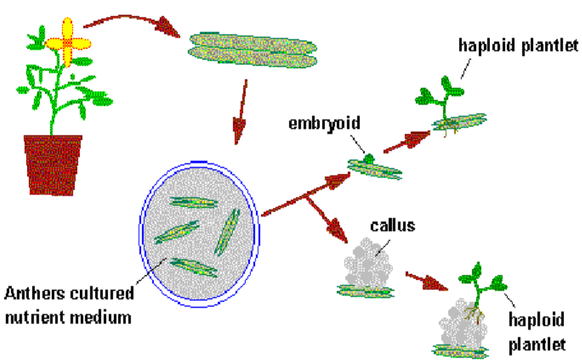
↓

Embryo Rescue

Haploid Barley 2n = X = 7 H. Bulbosum chromosomes eliminated

- This was once more efficient than microspore culture in creating haploid barley
- Now, with an improved culture media (sucrose replaced by maltose), microspore culture is much more efficient (~2000 plants per 100 anthers)

Features of Anther/Microspore Culture



Anther/Microspore Culture Factors

- Genotype
  - As with all tissue culture techniques
- Growth of mother plant
  - Usually requires optimum growing conditions
- Correct stage of pollen development
  - Need to be able to switch pollen development from gametogenesis to embryogenesis
- Pretreatment of anthers
  - Cold or heat have both been effective
- Culture media
  - Additives, Agar vs. 'Floating'

Ovule Culture for Haploid Production

- Essentially the same as embryo culture
  - Difference is an unfertilized ovule instead of a fertilized embryo
- Effective for crops that do not yet have an efficient microspore culture system
  - *e.g.*: melon, onion
    - In the case of melon, you have to "trick" the fruit into developing by using irradiated pollen, then x-ray the immature seed to find developed ovules

What do you do with the haploid?

- Weak, sterile plant
- Usually want to double the chromosomes, creating a dihaploid plant with normal growth & fertility
- Chromosomes can be doubled by
  - Colchicine treatment
  - Spontaneous doubling
    - Tends to occur in all haploids at varying levels
    - Many systems rely on it, using visual observation to detect spontaneous dihaploids
    - Can be confirmed using flow cytometry

Uses of Hapliods in Breeding

- Creation of allopolyploids
  - as previously described
- Production of homozygous diploids (dihaploids)
- Detection and selection for (or against) recessive alleles

Specific examples on next slide →

Specific Examples of DH uses

- Evaluate fixed progeny from an F_1
  - Can evaluate for recessive & quantitative traits
  - Requires very large dihaploid population, since no prior selection
  - May be effective if you can screen some qualitative traits early
- For creating permanent F2 family for molecular marker development
- For fixing inbred lines (novel use?)
  - Create a few dihaploid plants from a new inbred prior to going to Foundation Seed (allows you to uncover unseen off-types)
- For eliminating inbreeding depression (theoretical)
  - If you can select against deleterious genes in culture, and screen very large populations, you may be able to eliminate or reduce inbreeding depression
  - e.g.: inbreeding depression has been reduced to manageable level in maize through about 50+ years of breeding; this may reduce that time to a few years for a crop like onion or alfalfa

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- Micropropagation
- Germplasm preservation
- Somaclonal variation & mutation selection
- Embryo Culture
- Haploid & Dihaploid Production
- ➢ *In vitro* hybridization – Protoplast Fusion

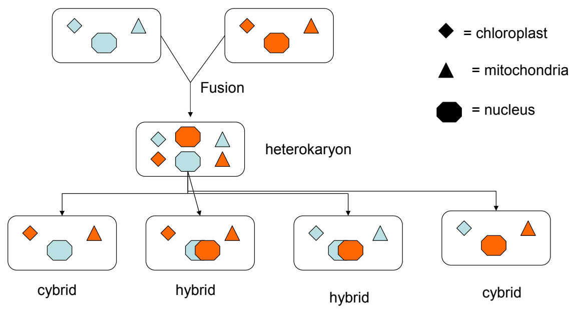
Somatic Hybridization using Protoplasts

- Created by degrading the cell wall using enzymes
- Very fragile, can't pipette
- Protoplasts can be induced to fuse with one another:
  - Electrofusion: A high frequency AC field is applied between 2 electrodes immersed in the suspension of protoplasts- this induces charges on the protoplasts and causes them to arrange themselves in lines between the electrodes. They are then subject to a high voltage discharge which causes them membranes to fuse where they are in contact.
  - Polyethylene glycol (PEG): causes agglutination of many types of small particles, including protoplasts which fuse when centrifuged in its presence
  - Addition of calcium ions at high pH values

Uses for Protoplast Fusion

- Combine two complete genomes
  - Another way to create allopolyploids
- Partial genome transfer
  - Exchange single or few traits between species
  - May or may not require ionizing radiation
- Genetic engineering
  - Micro-injection, electroporation, Agrobacterium
- Transfer of organelles
  - Unique to protoplast fusion
  - The transfer of mitochondria and/or chloroplasts between species

Possible Result of Fusion of Two Genetically Different Protoplasts



Identifying Desired Fusions

- Complementation selection
  - Can be done if each parent has a different selectable marker (e.g. antibiotic or herbicide resistance), then the fusion product should have both markers
- Fluorescence-activated cell sorters
  - First label cells with different fluorescent markers; fusion product should have both markers
- Mechanical isolation
  - Tedious, but often works when you start with different cell types
- Mass culture
  - Basically, no selection; just regenerate everything and then screen for desired traits

Reading Assignment

- Earle, E.D., and M.A. Sigareva. 1997. Direct transfer of a cold-tolerant ogura male-sterile cytoplasm into cabbage (*Brassica oleracea* ssp. *capitata*) via protoplast fusion. Theor Appl Genet. 94:213-220

Example of Protoplast Fusion

- Male sterility introduced into cabbage by making a cross with radish (as the female)
  - embryo rescue employed to recover plants
- Cabbage phenotypes were recovered that contained the radish cytoplasm and were male sterile due to radish genes in the mitochondria
- Unfortunately, the chloroplasts did not perform well in cabbage, and seedlings became chlorotic at lower temperatures (where most cabbage is grown)
- Protoplast fusion between male sterile cabbage and normal cabbage was done, and cybrids were selected that contained the radish mitochondria and the cabbage chloroplast
- Current procedure is to irradiate the cytoplasmic donor to eliminate nuclear DNA – routinely used in the industry to re-create male sterile brassica crops

One Last Role of Plant Tissue Culture

- Genetic engineering would not be possible without the development of plant tissue
  - Genetic engineering requires the regeneration of whole plants from single cells
  - Efficient regeneration systems are required for commercial success of genetically engineered products