National Science Foundation Plant Genome

Cereal Plant Transformation Workshop

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Maize Transformation

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Agrobacterium-mediated Maize Transformation From Immature Embryos

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Agrobacterium-mediated Maize Transformation

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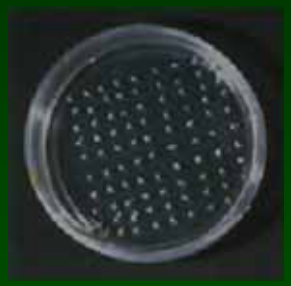
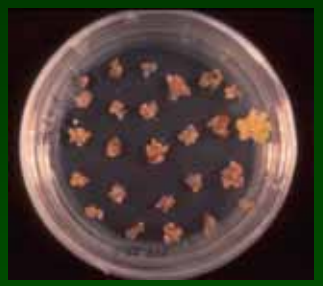
University of Rhode Island

Monocot


maize

Maize Transformation:
Autoclave all materials (beakers, tools, dd water, etc.) ahead of time

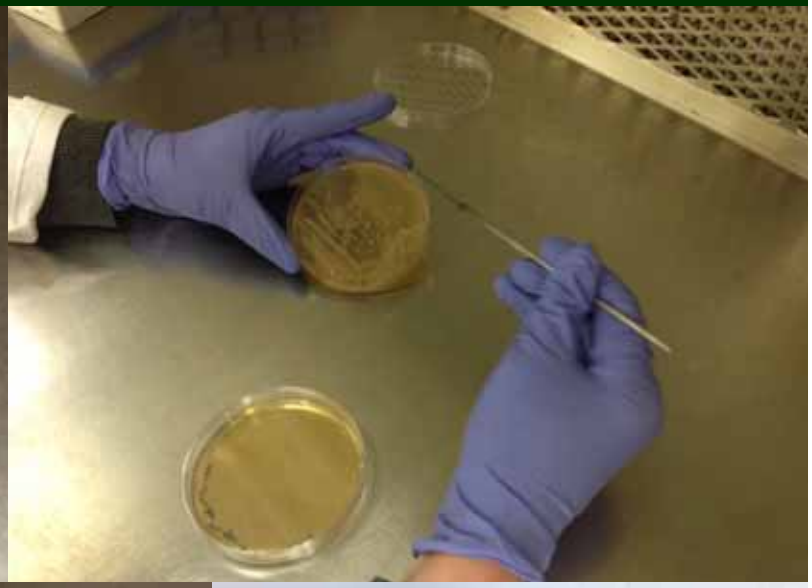
Preparation of *Agrobacterium*

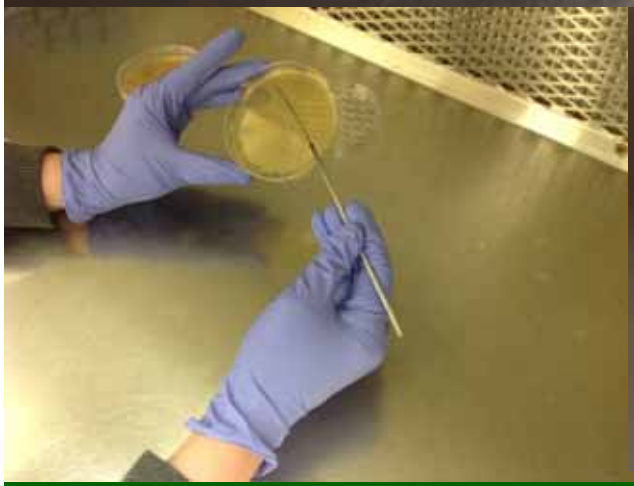
Initiation of *Agrobacterium tumefaciens* from stock culture:

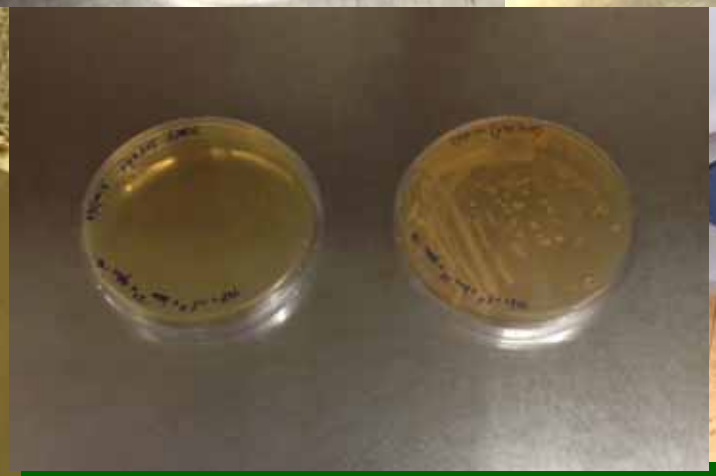
Streak the *Agrobacterium* culture for colony isolation from an -80°C glycerol stock onto a YEP medium plate containing appropriate antibiotics. Incubate the plate at 28°C for 3 days. This plate is the "master" plate, and should be stored at 4°C and can be reused for 30 days. A new master plate needs to be initiated after this period.

To begin a maize transformation experiment, streak a single colony onto a fresh YEP plate containing the appropriate antibiotics. Incubate this plate for 3 days at 20°C or 2 days at 25°C.

Preparation of *Agrobacterium*

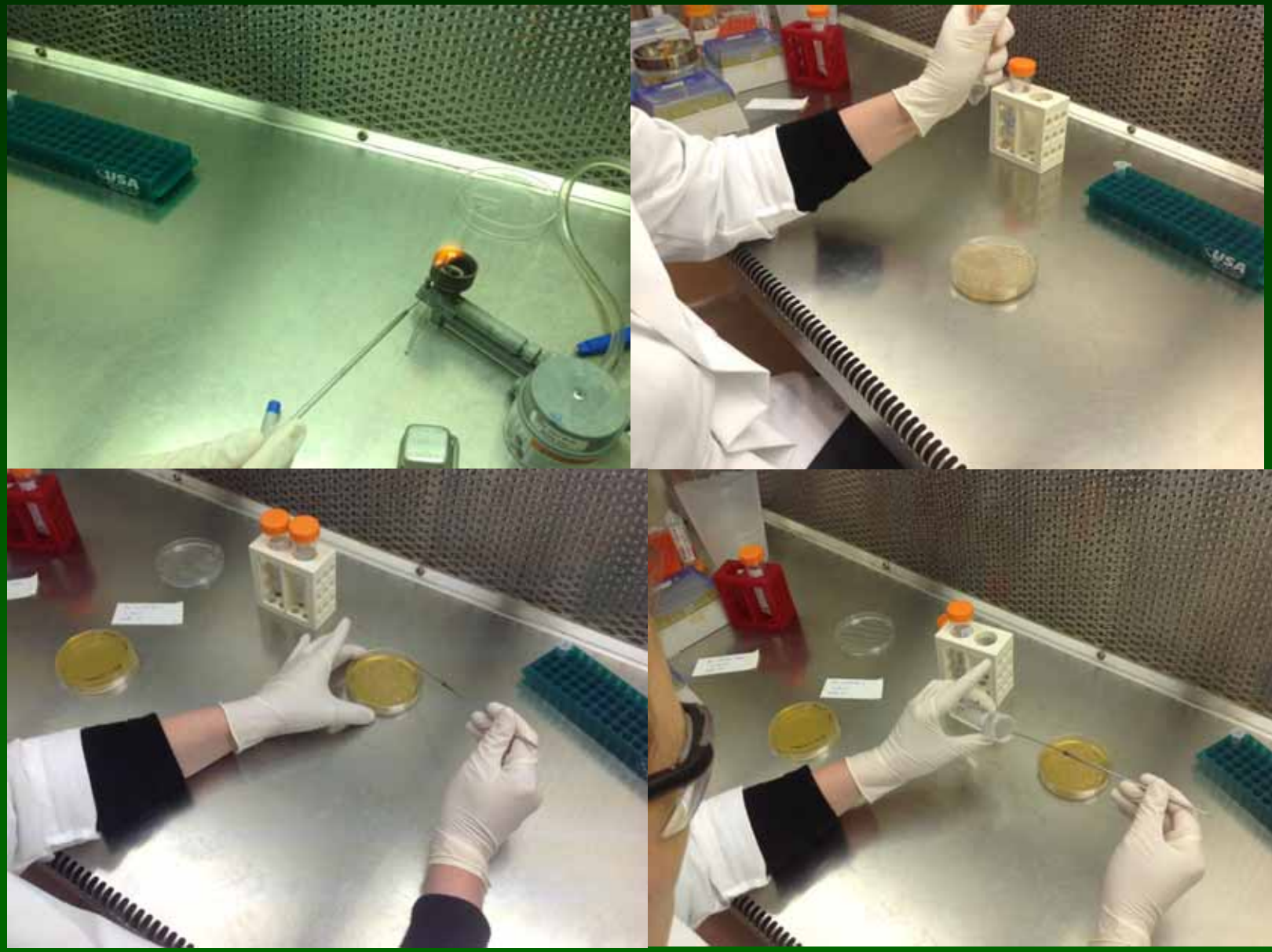






Initiation of *Agrobacterium tumefaciens* from stock culture: To begin a maize transformation experiment, streak a single colony onto a fresh YEP plate containing the appropriate antibiotics. Incubate this plate for 3 days at 20°C , 2 days at 25°C, or 28°C Overnight (caution Agro plasmids may be cured over 28°C)

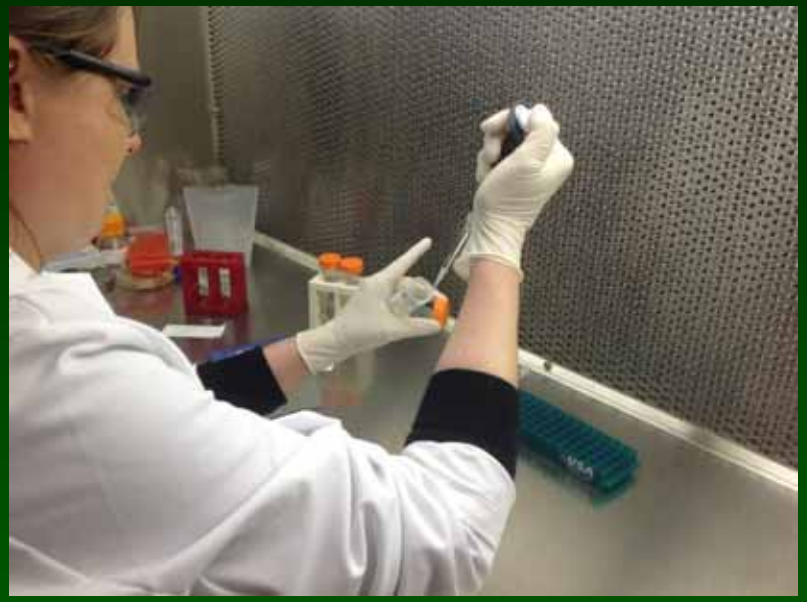
Preparation of *Agrobacterium* Infection Media



Maize Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
N_6 salt	2.0 g	2.0 g	4.0 g	4.0 g	4.0 g	-	-
MS salt	-	-	-	-	-	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	-	-
L-proline	0.7 g	0.7 g	0.7 g	0.7 g	0.7 g	-	-
Sucrose	68.5 g	30 g	30 g	30 g	30 g	60 g	30 g
Glucose	36 g	-	-	-	-	-	-
2,4-D (1 mg/mL stock)	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	-	-
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Agar (washed)*	-	8.0	-	-	-	-	-
Agar, Plant cell culture tested**	-	-	8.0 g	8.0 g	8.0 g	-	-
Gelrite	-	-	-	-	-	3.0 g	3.0 g
Autoclave	filtered	20min	20min	20min	20min	20min	20min
N_6 vitamin stock, 1000×	1 mL	1 mL	1 mL	1 mL	1 mL		
MS vitamin, 1000×						1 mL	1 mL
Silver Nitrate	0.85 mg	0.85 mg	0.85 mg	0.85 mg	0.85 mg	-	-
L-cysteine	-	0.4 g	-	-	-	-	-
DTT	-	0.15 g	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Acetosyringone (100 mM/mL)	100 µL	100 µL	-	-	-	-	-
Vancomycin (100 mg/mL)	-	-	1 mL	-	-	-	-
Carbenicillin (200 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Timentin (150 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Bialaphos (3 mg/mL stock)	-	-	-	0.5 mL	1 mL	1 mL	-

Preparation of *Agrobacterium* Infection Medium



Add 15-25 mL (depending on the size of the experiment) each of sterile co-cultivation infection medium into two sterile 50 mL Falcon tubes. One tube will be used to make the co-cultivation infection with the *Agrobacterium*, the other, will be used to adjust the concentration of bacteria after the OD is read and make tubes used in the embryo isolation prior to infection.

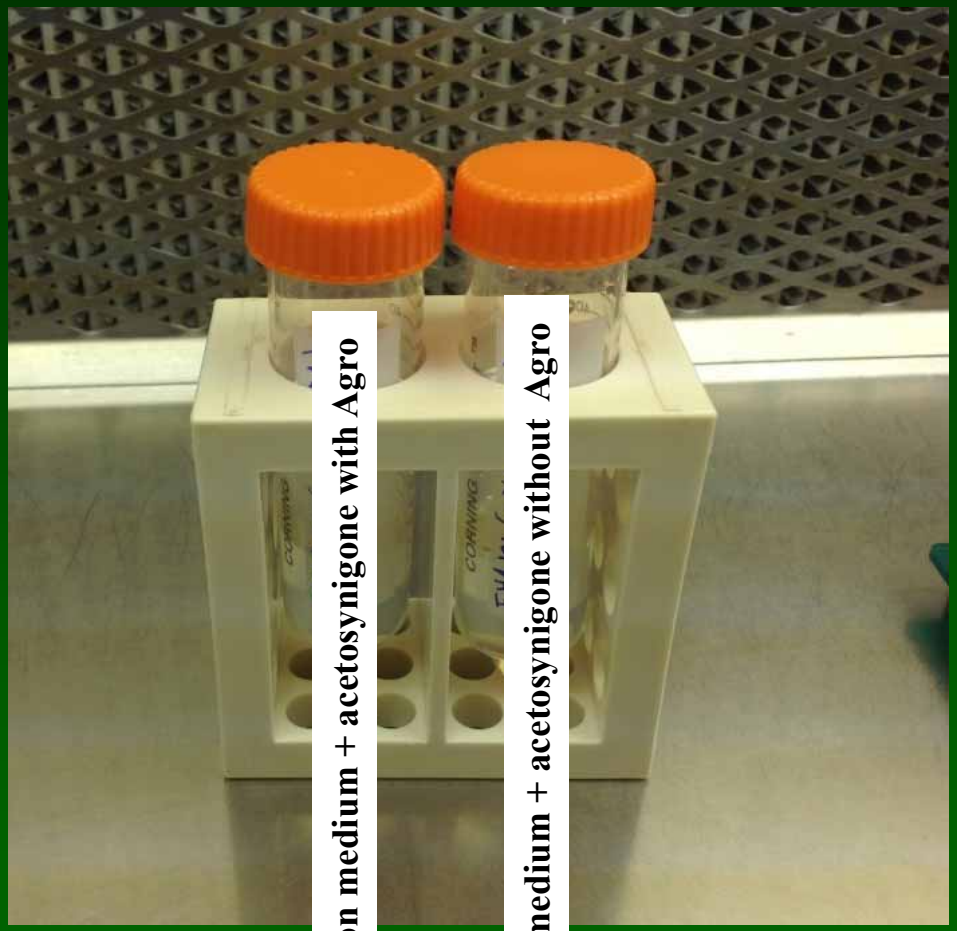
Add acetosyringone (100 mM/mL stock solution) to the each of the tubes containing infection media for a final concentration of 100 μM/mL acetosyringone.

Preparation of *Agrobacterium* Infection Medium

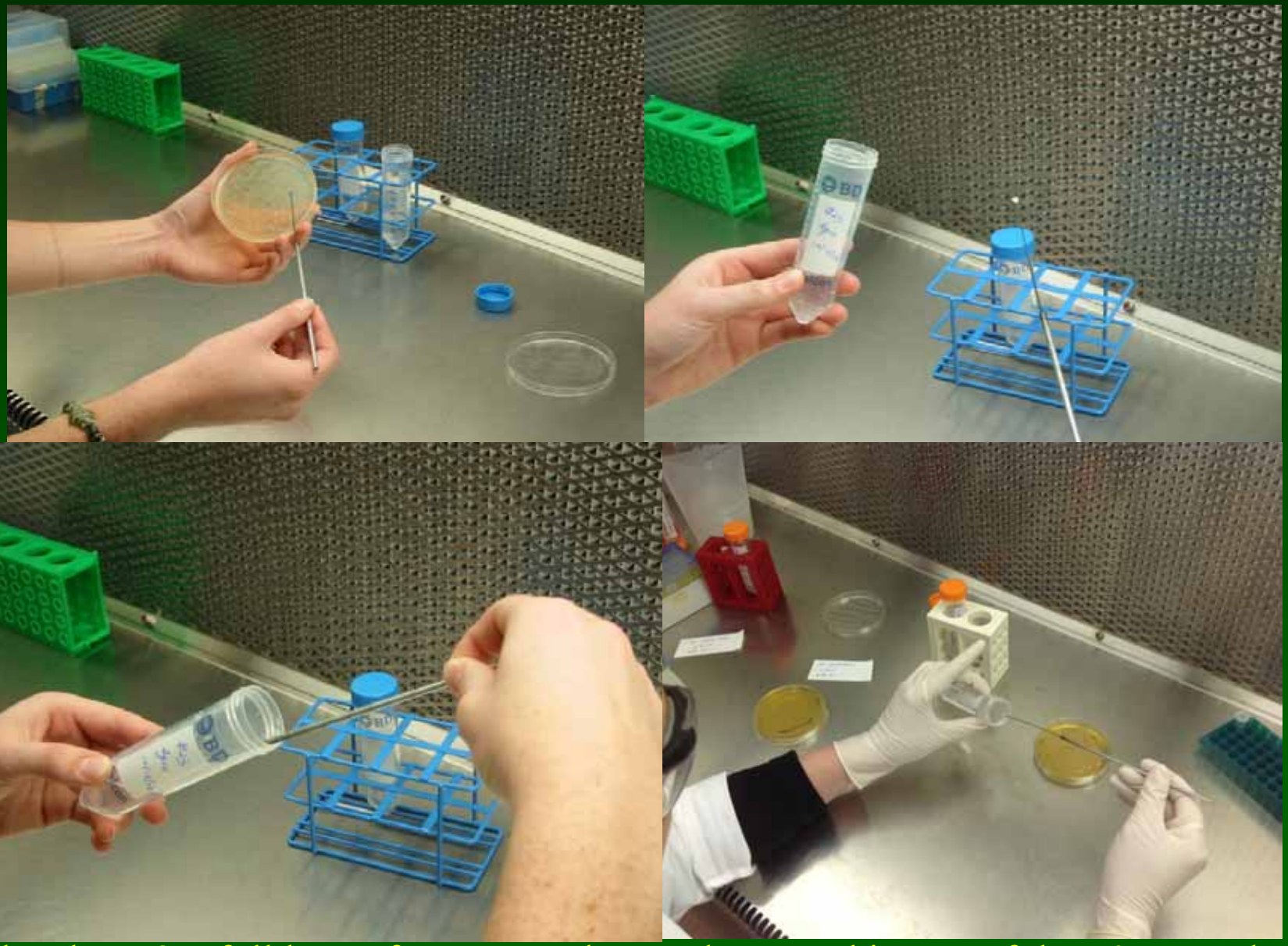
Take about 2.5 full loops from YEP plate and suspend in one of the 50 mL Falcon tubes (about 1 full loopful/10 mL of co-cultivation infection medium.

Using the other 50 ml Falcon tube containing the infection media + acteosyringone, using a sterile tip transfer ~ 1.8 mL of the infection medium + acetosyringone (without *Agrobacterium*) to 10 sterile 2 mL microcentrifuge tubes. These will be used to transfer the isolated embryos prior to co-cultivation. This can be done while the *Agrobacterium* suspension is shaking .

Preparation of *Agrobacterium* Infection Medium



Preparation of *Agrobacterium* Infection Medium



Take about 2.5 full loops from YEP plate and suspend in one of the 50 mL Falcon tubes (about 1 full loopful/10 mL of infection medium + 100 μM acetosynigone

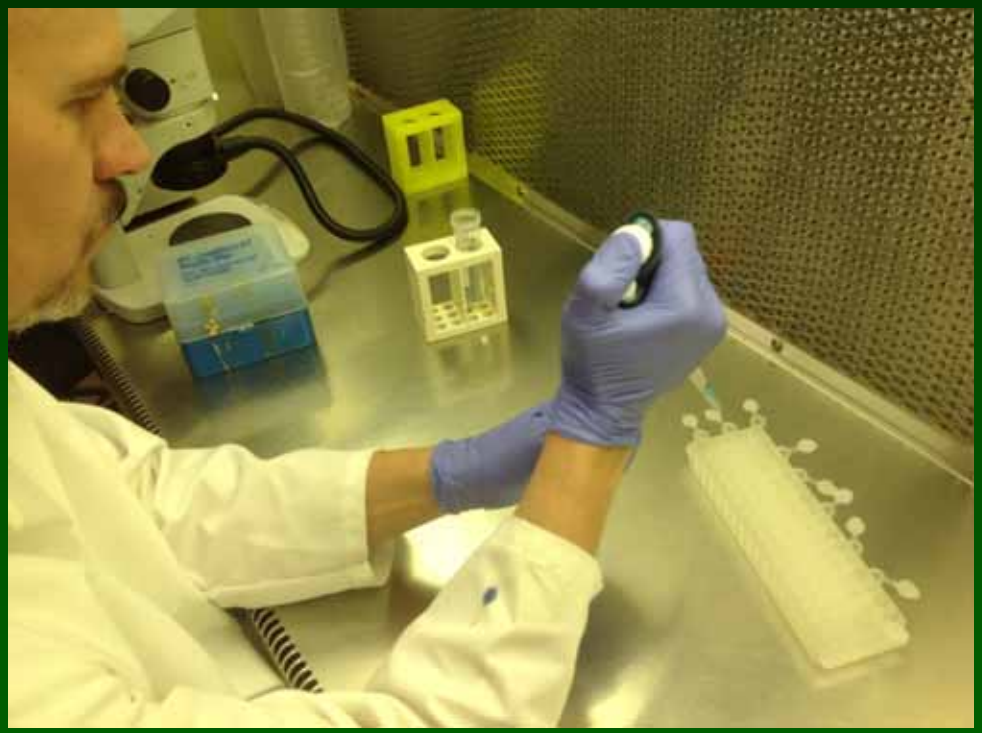
Preparation of Infection Medium Containing the *Agrobacterium*

Shake the *Agrobacterium* suspension at 25-27°C at 150-175 rpm for 1 hour to re-suspend fully.

Re-suspension of *Agrobacterium* in Infection Medium

Shake the *Agrobacterium* suspension at 25°C at 150-175 rpm for 1 hour to re-suspend fully.

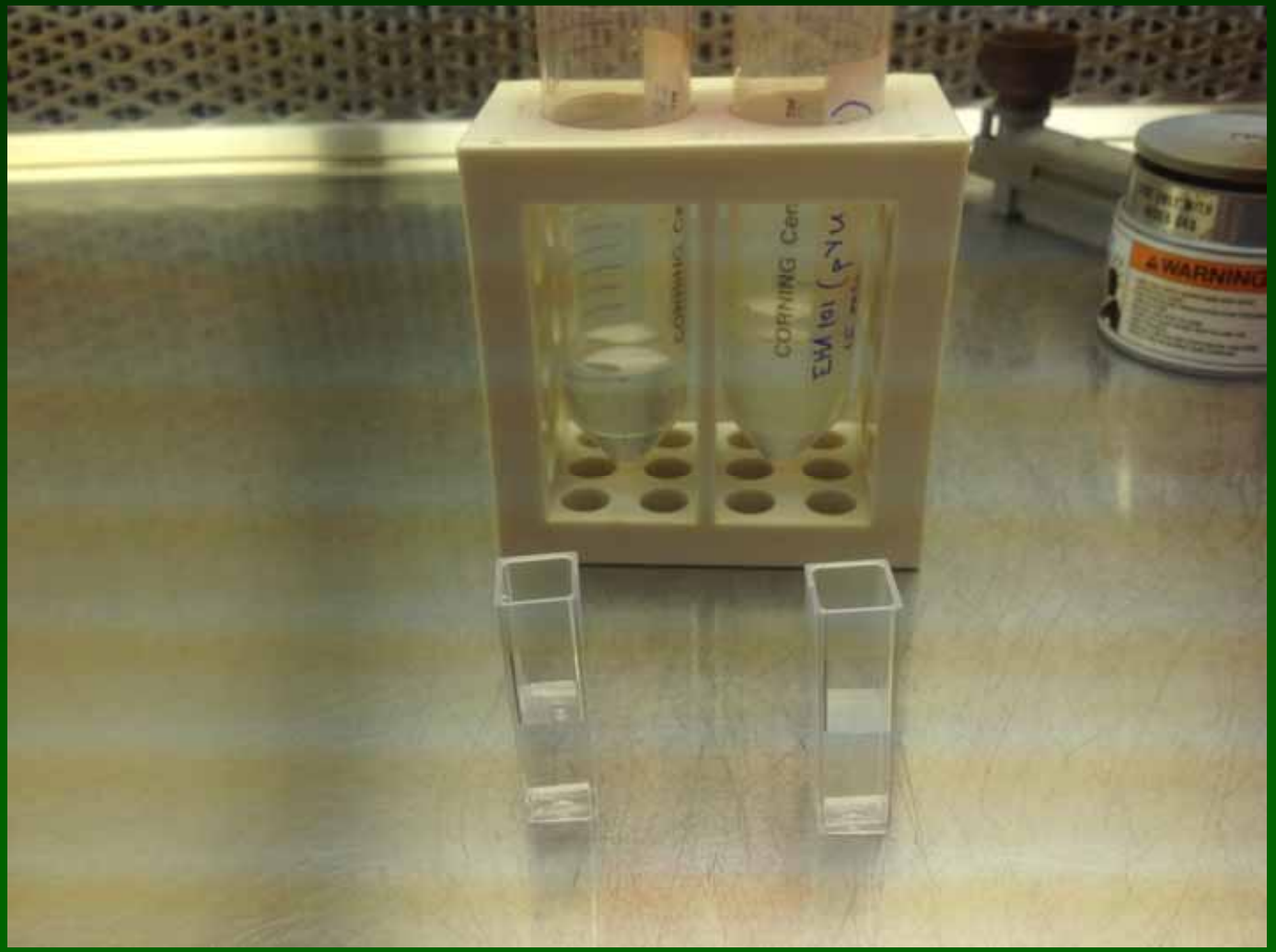
Preparation of microfuge tubes containing infection medium without *Agrobacterium* for embryo isolation



While the *Agrobacterium* suspension is shaking using the other 50 ml Falcon tube containing the infection media + 100 µM acteosyringone, and using a sterile tip transfer ~ 1.8 mL of the infection medium + 100 µM acetosyringone (without the *Agrobacterium*) to 10 sterile 2 mL microcentrifuge tubes. These will be used to transfer the isolated embryos prior to the infection step

Determination of Agro Concentration at OD_{600}

Determination of Agro Concentration at OD_{600}



Use Infection medium + 100 µM acetosyngone to blank the spectrophotometer

The final suspension should be adjusted to OD_{600}. = 0.30-0.45.

After re-suspension for 1 hr, transfer an appropriate portion (2 ml) of the *Agrobacterium* suspension to a spectrophotometer curette and measure the OD_{600}. The final suspension should be adjusted to = 0.30-0.45.


BECKMAN
FIXED WAVELENGTH
Group 0853 Sample BLNK
0.000 ABS
READ SAMPLE
TIMER 00:00
Measurement control:
READ
BLANK
VIEW: ABS
OPTIONS
START TIMER


FIXED WAVELENGTH
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06-MAY-16 14:38
Group 0853 Sample 0004
0.415 ABS
READ SAMPLE
TIMER 00:00
Measurement control:
READ
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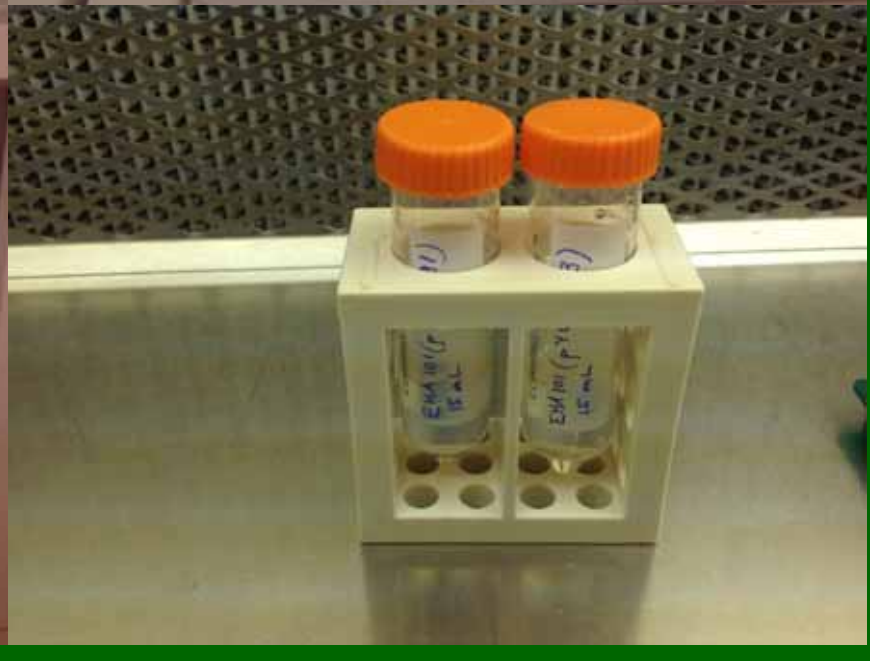
VIEW: ABS
OPTIONS
START TIMER



Donor Plant Material

Plant Material

Immature embryos are used as the preferred explant for maize transformation. Maize cv. Hi II (A188X B73) is widely considered as the model system. F_1 seeds are used for the production of donor plants supplying Hi II ears used as the source for the immature embryos. The plants can be grown under appropriate greenhouse conditions. Proper lighting, soil, containers and watering regimes are essential to plant donor health. Plants can also be grown in the field but is, of course, seasonally dependent. Upon flowering and initiation of pollen shedding, the plants are sib-pollinated. Ears should then be harvested between 9 to 12 d post-pollination, depending upon the size of the embryos found on each ear. Embryo size is more significant than days post pollination. Ears are harvested for transformation experiments when the immature embryos are between 0.9 and 1.8 mm in length. Embryo size can be sampled easily prior to harvest as a check.

Donor Plant Material

Plant Material Genotype Specificity

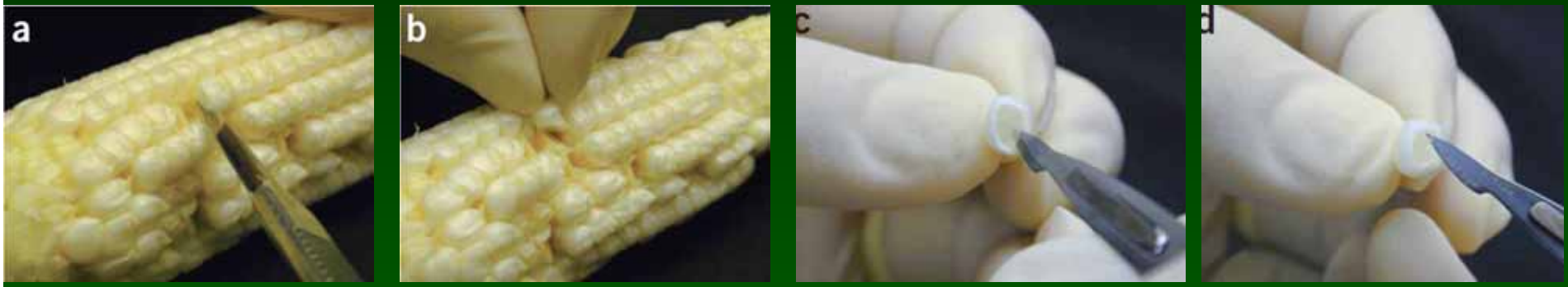
Maize cv. Hi II (A188X B73) is widely considered as the model system.

FBLL (see Monsanto paper)
Recent work by Pioneer (in press)

Recalcitrant Varieties with marginal success

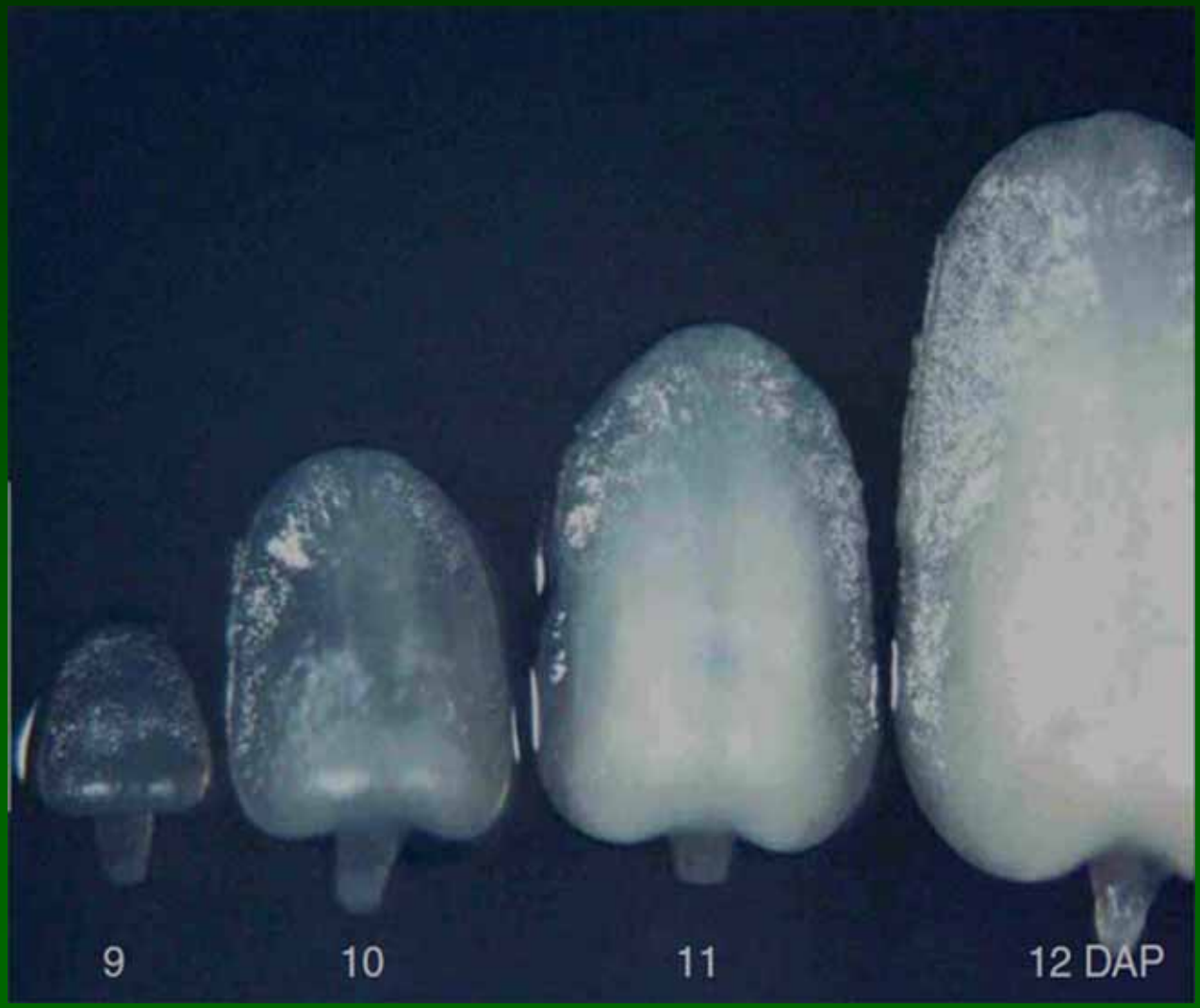
B73

Sizing the maize embryo



0.9-1.8 mm

Sizing the maize embryo DAP



Ears with the Proper Sized embryo are husked

Ideally embryos should be 0.9-1.8 mm

Note: ears with the proper size may be stored at 4C for up to 3-4 days or shipped on wet ice and will maintain their size and still work for transformation. Harvested ears which have embryos which are two small can be stored at room temp for 1-2 days until the embryos mature to their proper size.

Embryo isolation and infection

Note: This is best conducted by a 2 person team, with one person doing the embryo isolation and another person rinsing, infecting and plating the embryos

Surface sterilization of the cob (overview)

Prepare a 50% bleach solution by mixing 2 L of bleach (Chlorox) with 2 L of sterile ddH_2O in an autoclaved 5 L pitcher. Insert a sterilized stainless steel spatula into the cob of each husked ear to act as a handle, and then place the ears into the bleach solution for 30 min.

After 30 min., rinse the ears with sterile ddH_2O 3 times, leaving the final rinse water in the pitcher. It is important to maintain aseptic technique throughout. Embryos can then be isolated for infection.

Embryo isolation and infection

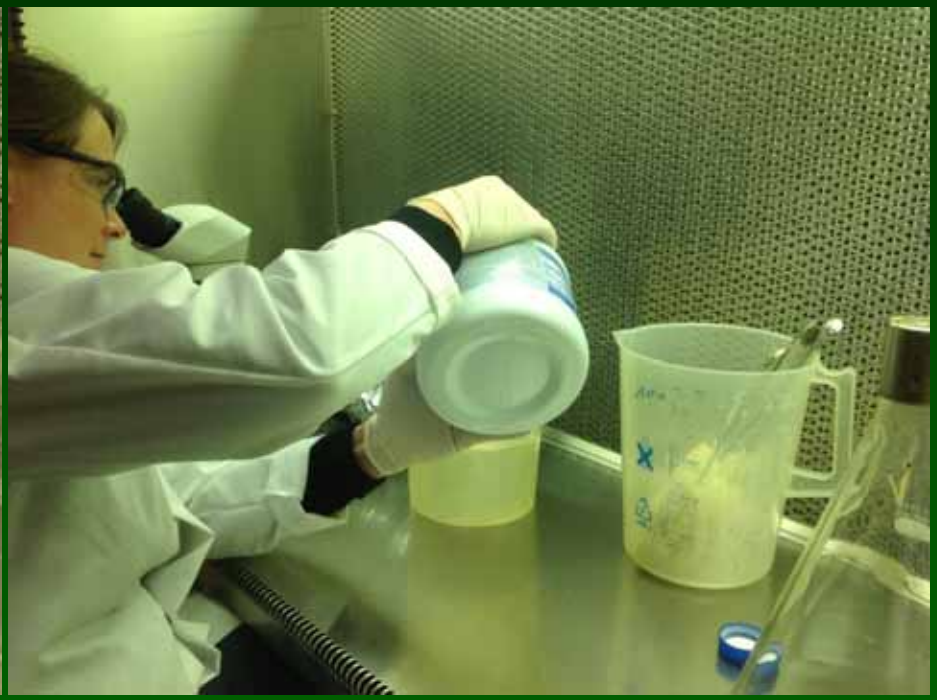
Note: This is best conducted by a 2 person team, with one person doing the embryo isolations and another person rinsing, infecting and plating the embryos

Insert a sterilized stainless steel spatula into the cob of each husked ear to act as a handle. Place each cob into an autoclaved 5 L Nalgene pitcher, beaker (or similar container).

Surface Sterilization of the Cobs

Place each cob to be used in the experiment into an autoclaved 5 L Nalgene pitcher, beaker (or similar container).

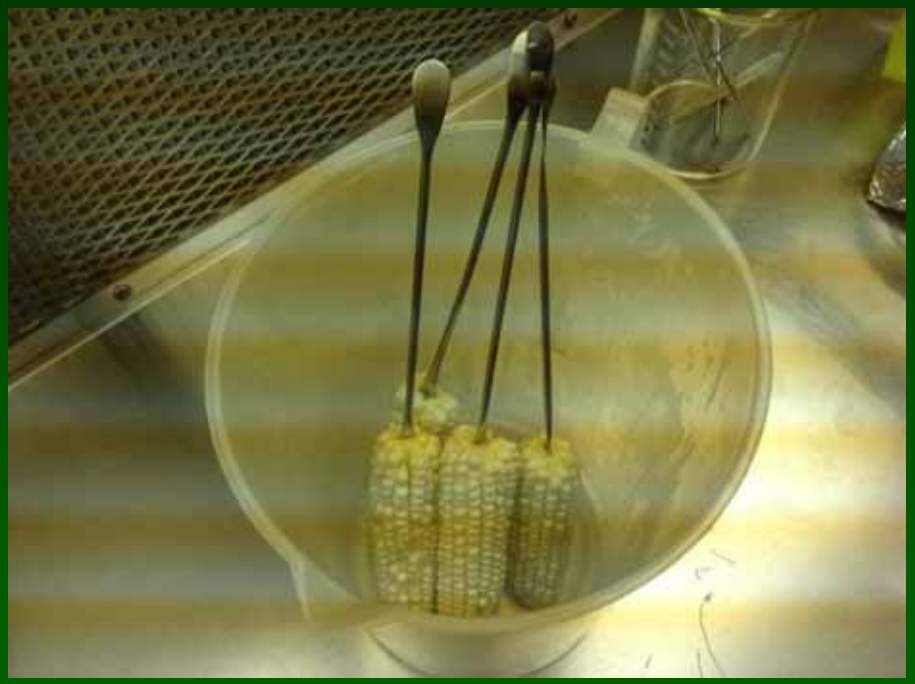
Surface Sterilization of the Cobs

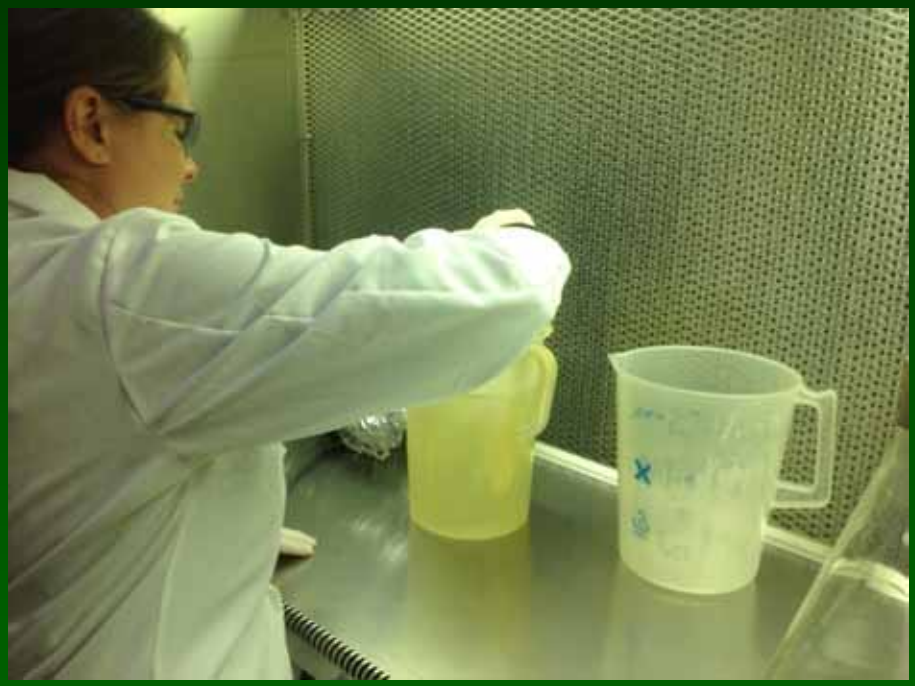


Surface Sterilization Solution

Prepare a 50% bleach solution by mixing 2 L of bleach (Chlorox) with 2 L of sterile ddH_2O in an autoclaved 5 L pitcher.

Surface Sterilization of the Cobs





Place the ears into the bleach solution. Submerge the cobs in the solution until all air escapes and stir them gently to ensure the entire surface is wetted.

Surface Sterilization of the Cobs

Submerge the cobs in the solution until all air escapes and stir them gently to ensure the entire surface is wetted.

Surface Sterilization of the Cobs

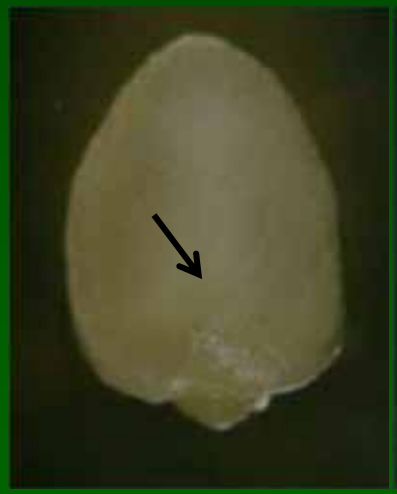
Set timer for 30 min.

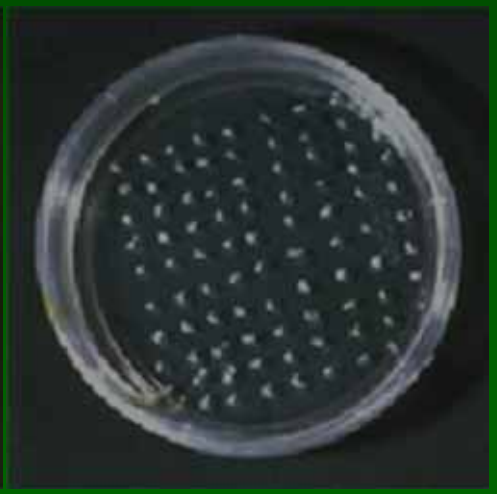
Surface Sterilization of the Cobs

After 30 min., rinse the ears with sterile ddH_2O (3) times, leaving the final rinse water in the pitcher. It is important to maintain aseptic technique throughout. Embryos can now be isolated for infection.

Embryo isolation

Avoid breaking the embryos





Recipient Cell Biology

Epidermal cells of abaxial side of immature embryo contribute to embryogenesis callus formation

Embryo isolation

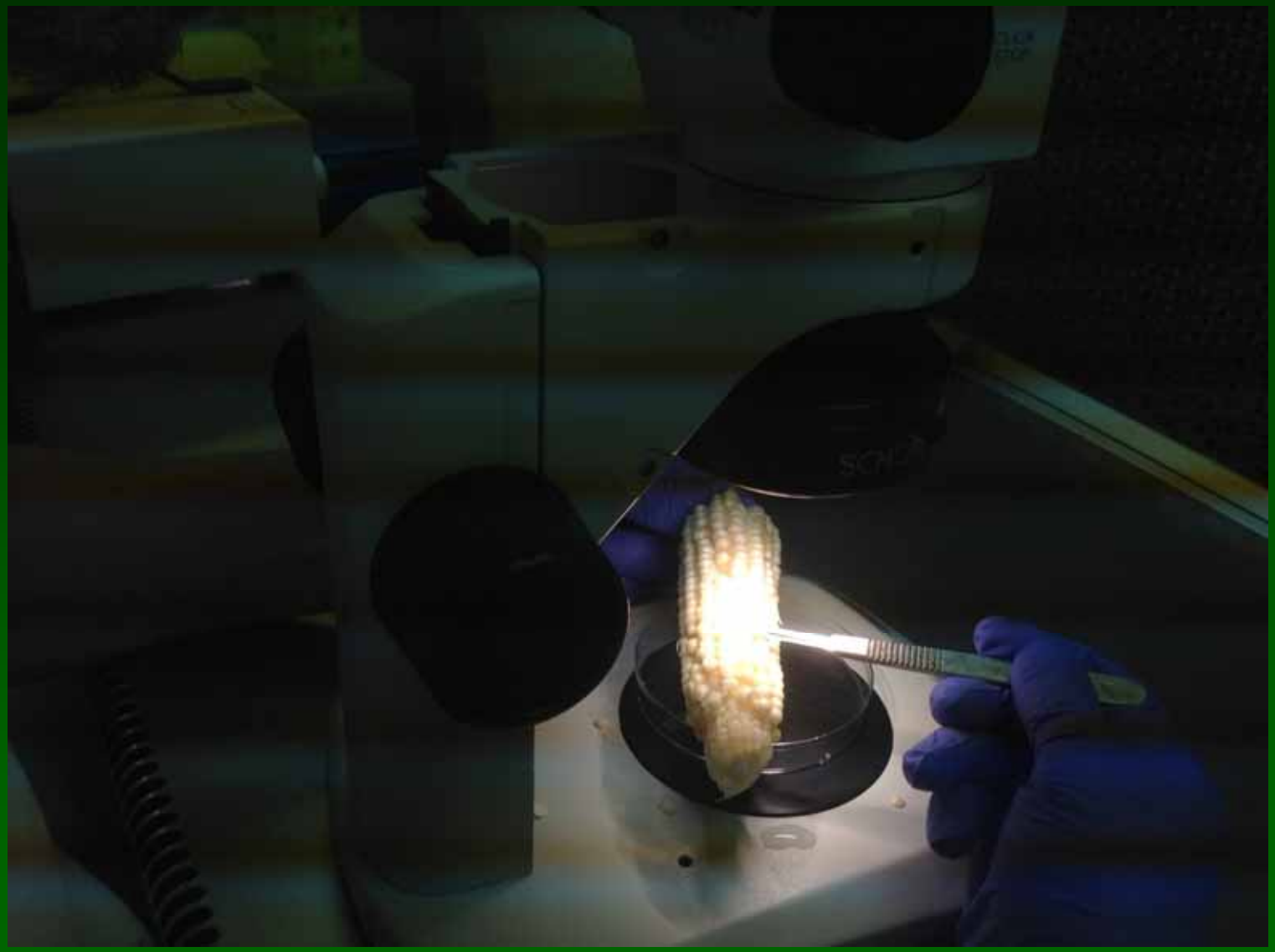
To isolate the embryos, place a sterile Petri dish on the stage of a dissecting scope. Use a sterile scalpel to shave away about 1/3 off the top of each kernel for 3 – 5 rows.

Embryo isolation.

The immature embryos are carefully removed by a twisting motion using the tapered end of a stainless steel spatula. The immature embryo is located on the side of the caryopsis that faces the tip of the ear. The embryo size should be 0.9 - 1.8 mm.

Embryo isolation.

A dissecting light microscope is useful

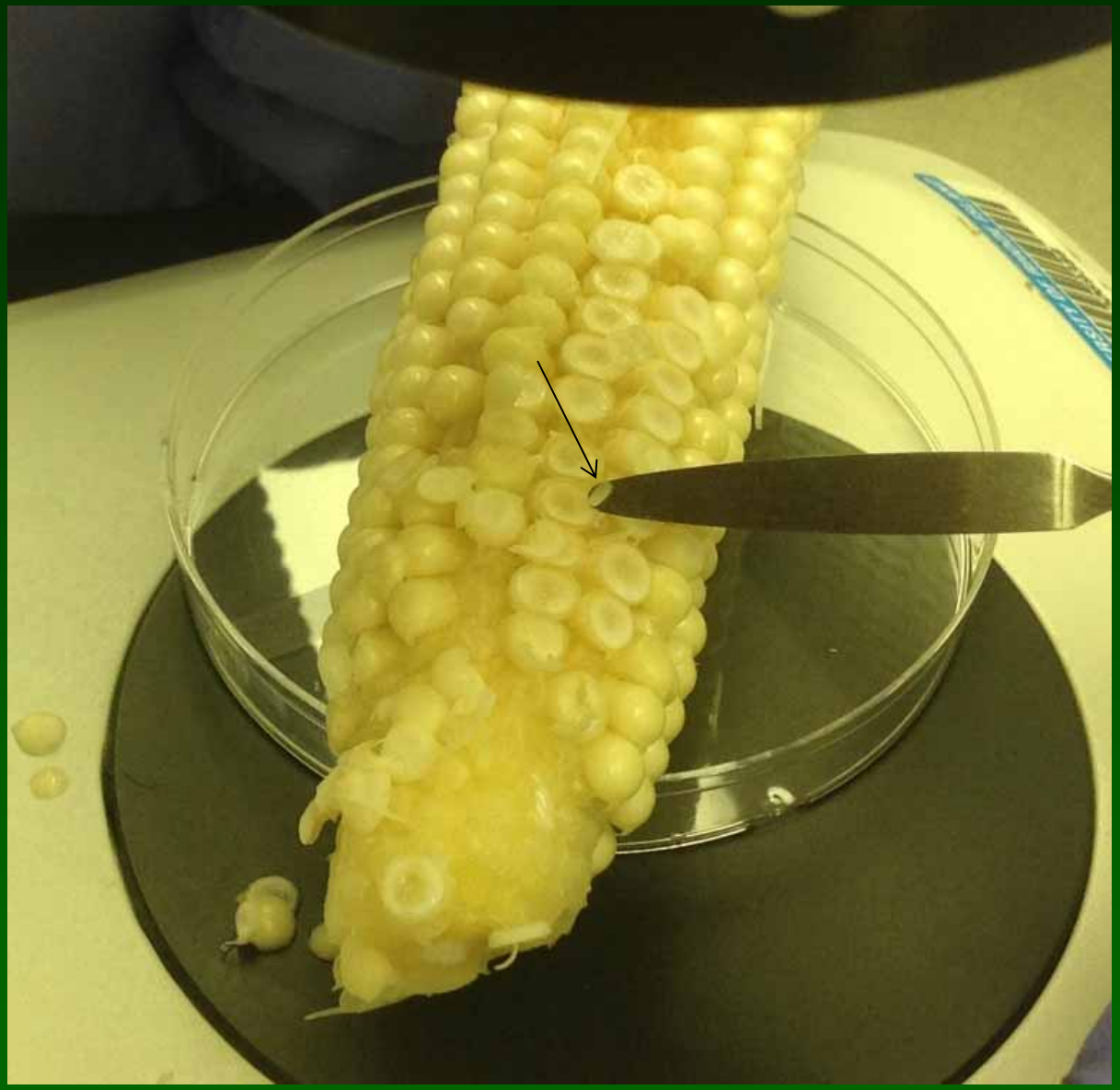


A dissecting light microscope is useful.
Using a sterile scalpel to remove the top of the embryo

Embryo isolation.

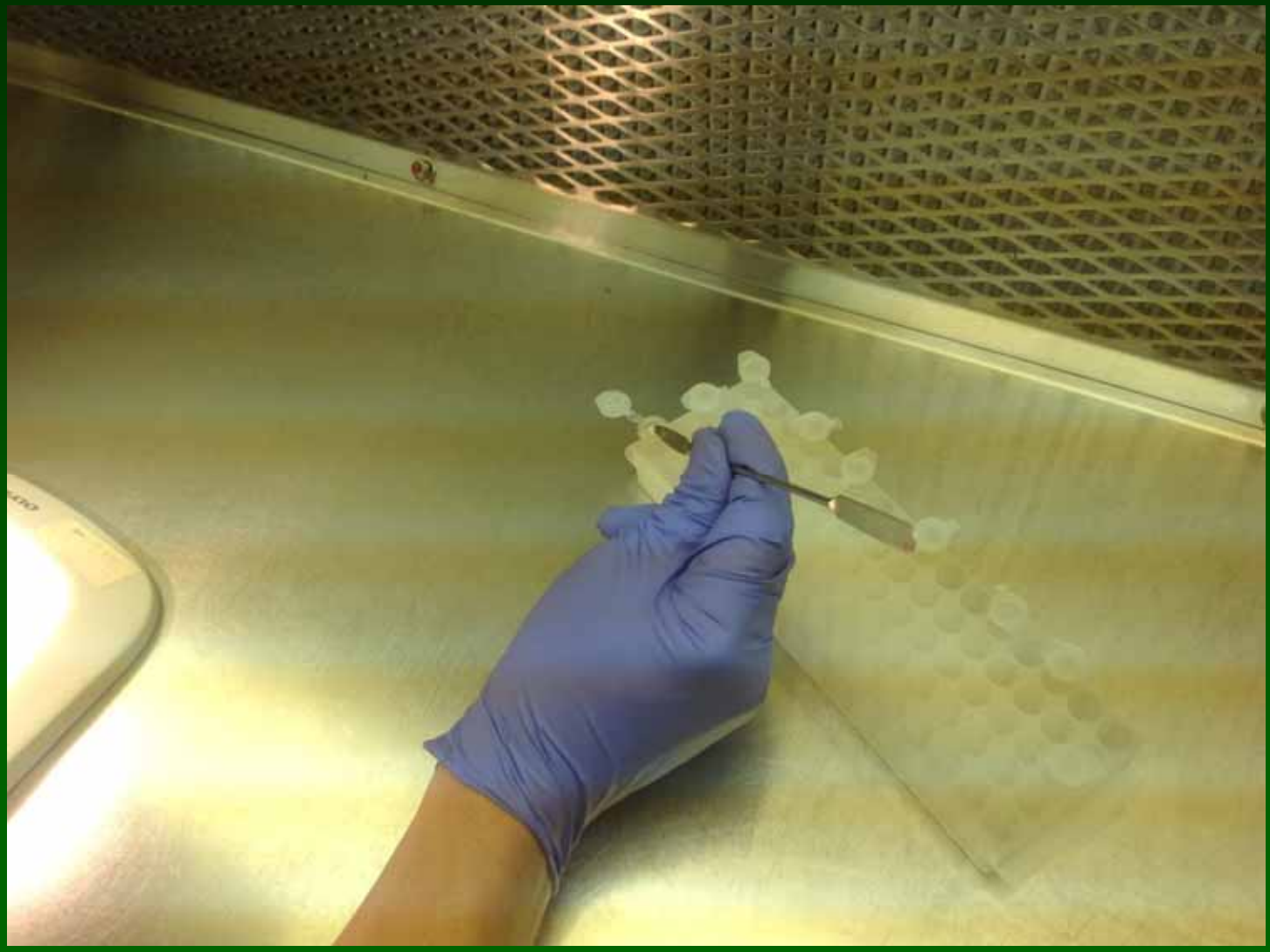
A dissecting light microscope is useful.
Using a sterile spatula to remove the embryo

Embryo isolation.



Note the embryo on the tip of the spatula

Embryo isolation.



The embryo is transferred to the prepared 2.0 mL microfuge tube containing 100um acetosyringone (without *Agro*)

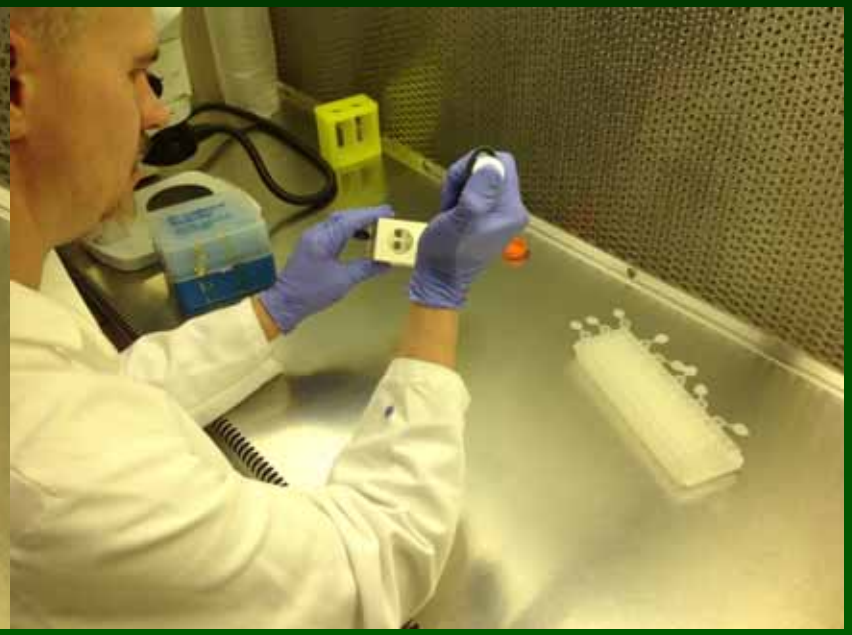
Embryo isolation.

Continue to collect embryos into micro-centrifuge tube for no longer than 20 minutes to prevent hypoxia of the embryos.

Embryo isolation.

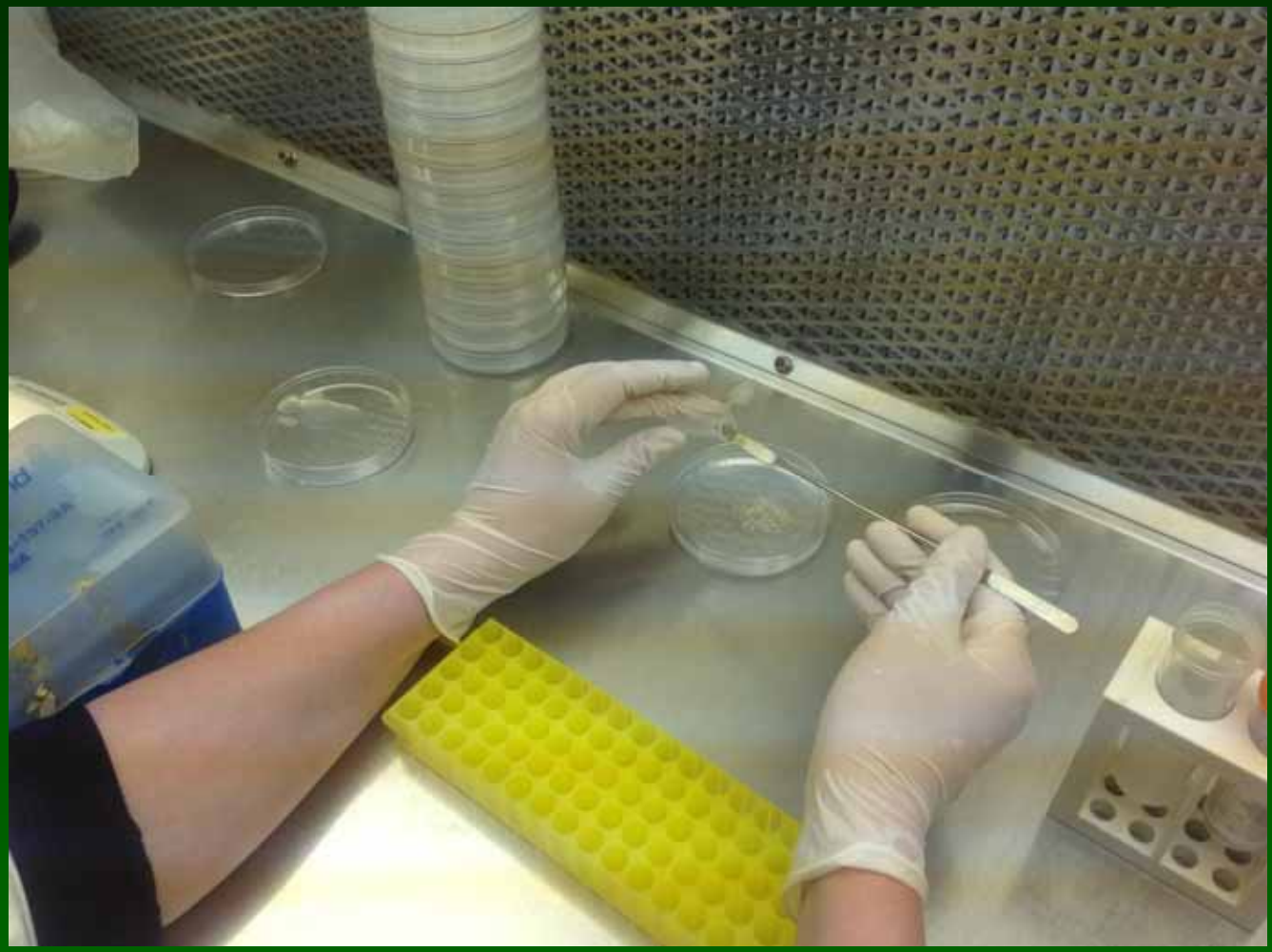
Transfer capped tube containing the embryos to your partner in a adjacent hood prior to proceeding with embryo excision.

Embryo infection.



Remove the infection media using a sterile a 1 mL micropipet and replace with infection medium containing the *Agrobacterium* OD_{600}. = 0.20-0.45). *Note: some protocols use one rinse of infection media prior to this step, or one rinse of the Agro medium to remove endosperm cells which may inhibit tissue culture response. Incubation in Agro also varies from 2-10 min. Addition modifications at this step may include a 45 C heat treatment for 1-3 min, centrifugation (500rpm for 5 min. or dehydration on Whatman filter paper in the hood for 10 min.*

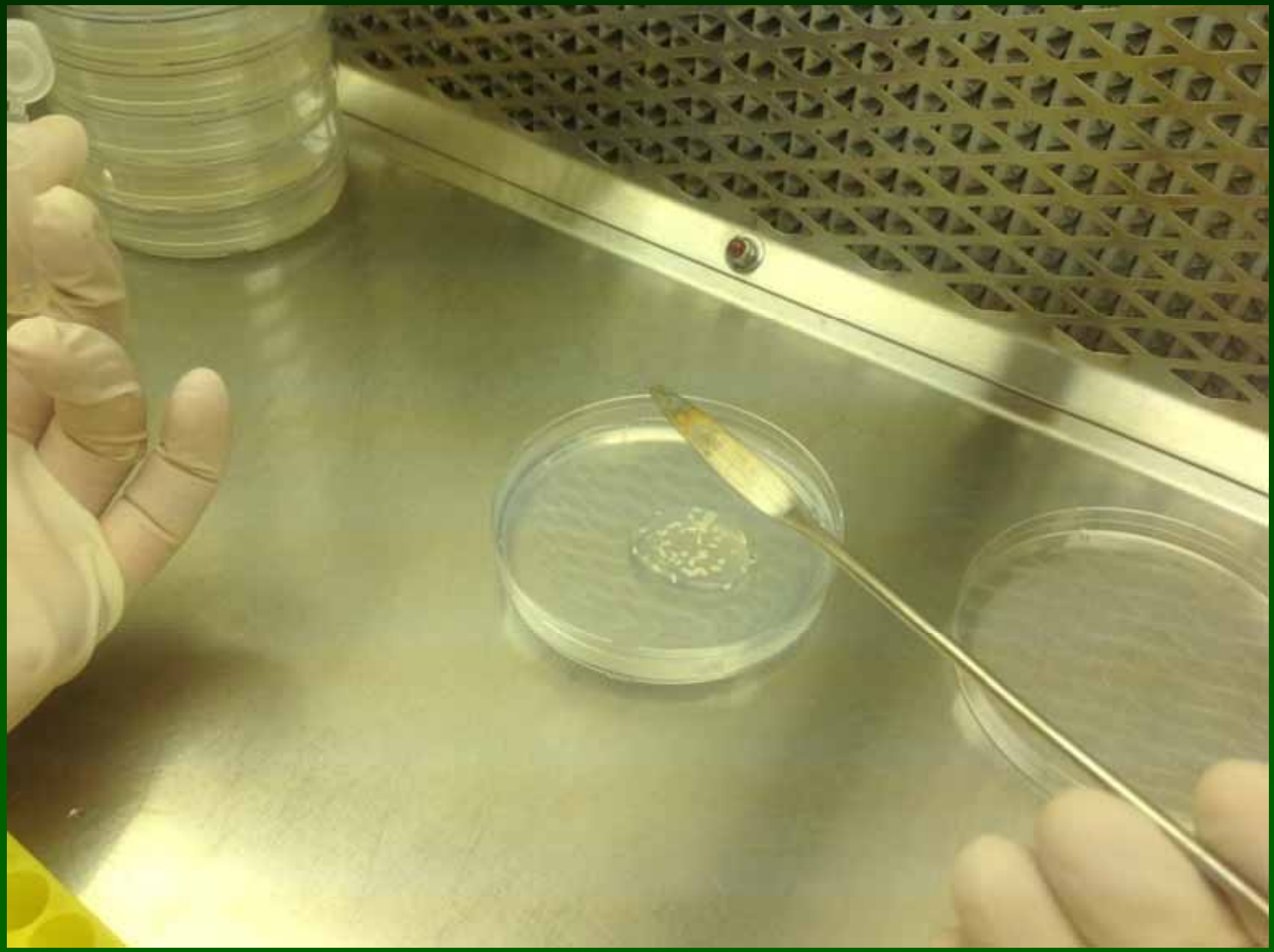
Embryo infection.



Transfer embryos from capped 2mL tube onto Co-Cultivation media. Invert the capped tube gently to suspend the embryos open the cap and dump the contents onto the plate. Alternatively a 1 mL micropipet can be used to pipette the solution up and down to suspend the embryos A spatula may be used, but care must be taken to prevent damage to the embryos

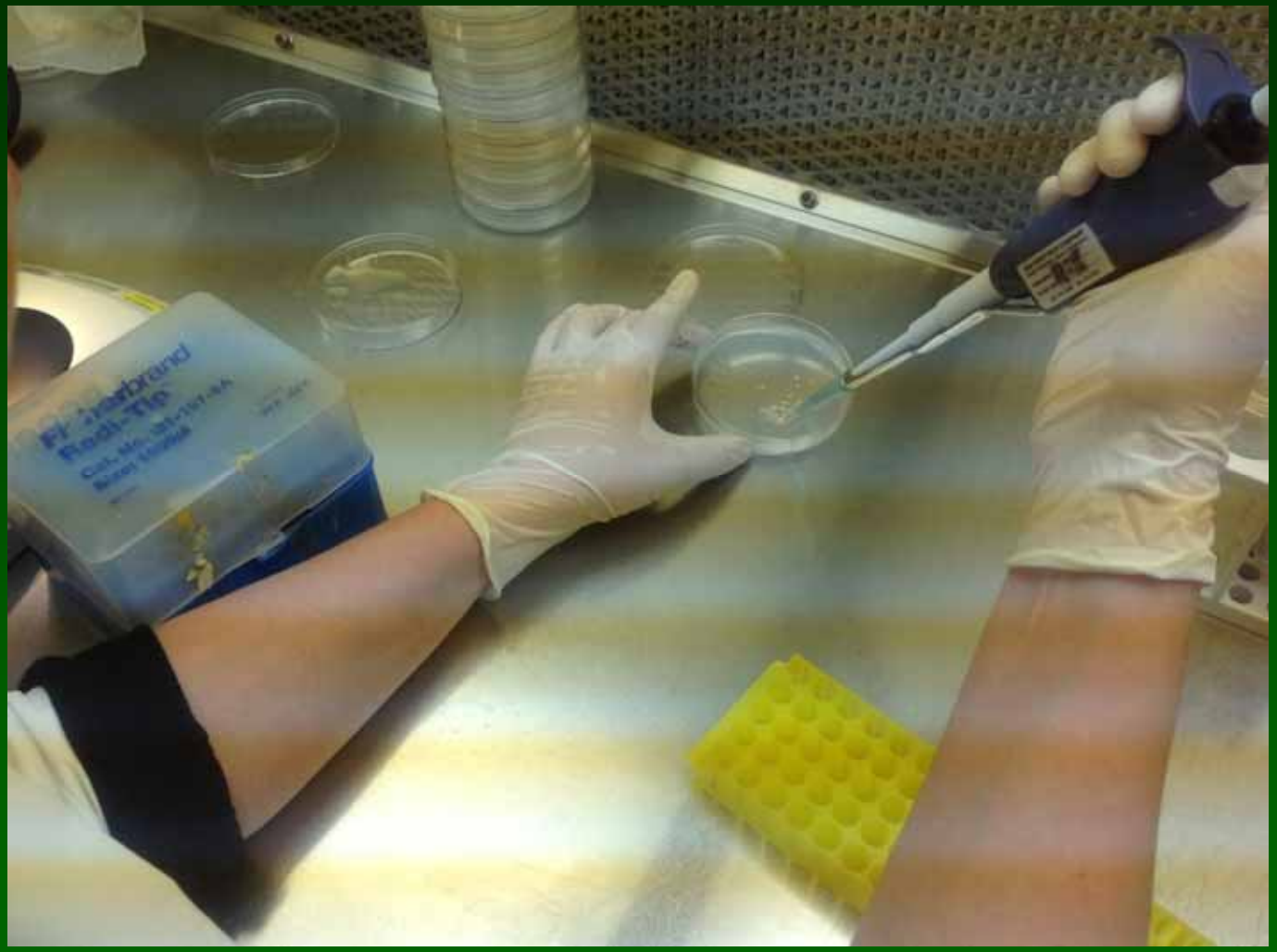
Maize Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
N_6 salt	2.0 g	2.0 g	4.0 g	4.0 g	4.0 g	-	-
MS salt	-	-	-	-	-	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	-	-
L-proline	0.7 g	0.7 g	0.7 g	0.7 g	0.7 g	-	-
Sucrose	68.5 g	30 g	30 g	30 g	30 g	60 g	30 g
Glucose	36 g	-	-	-	-	-	-
2,4-D (1 mg/mL stock)	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	-	-
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Agar (washed)*	-	8.0	-	-	-	-	-
Agar, Plant cell culture tested**	-	-	8.0 g	8.0 g	8.0 g	-	-
Gelrite	-	-	-	-	-	3.0 g	3.0 g
Autoclave	filtered	20min	20min	20min	20min	20min	20min
N_6 vitamin stock, 1000×	1 mL	1 mL	1 mL	1 mL	1 mL		
MS vitamin, 1000×						1 mL	1 mL
Silver Nitrate	0.85 mg	0.85 mg	0.85 mg	0.85 mg	0.85 mg	-	-
L-cysteine	-	0.4 g	-	-	-	-	-
DTT	-	0.15 g	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Acetosyringone (100 mM/mL)	100 µL	100 µL	-	-	-	-	-
Vancomycin (100 mg/mL)	-	-	1 mL	-	-	-	-
Carbenicillin (200 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Timentin (150 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Bialaphos (3 mg/mL stock)	-	-	-	0.5 mL	1 mL	1 mL	-



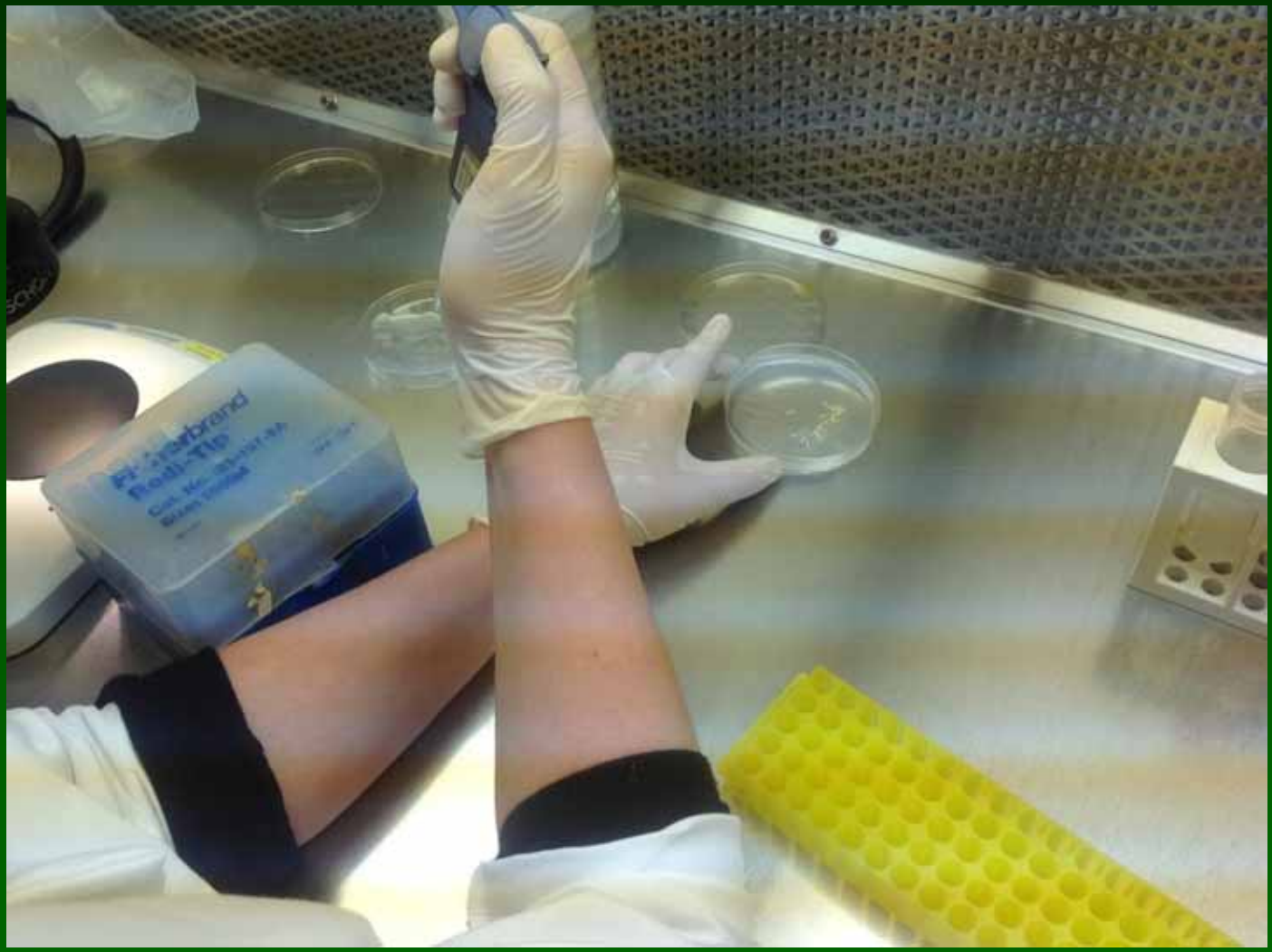
The transferred the embryos are now in the infection medium on a plate containing Co-cultivation medium . *Note: the infection is still proceeding during this step*

Embryo infection.



The infection medium is removed using a 1 mL sterile micropipet as close to dryness as possible. This is also easily accomplished using the dissecting light microscope. Avoid damaging the embryos.

Embryo infection.



Dispense the infection medium into a clean Petri dish for this purpose. It will be disposed of later into a designated Biohazard bag. Note ALL transgenic materials must be properly disposed of in designated bioharzard containers or bags

Embryo Co-Cultivation.

The Co-Cultivation process begins with the orientation of the embryos. Using a spatula and a dissecting light microscope, orient the embryos scutellum side up and spaced at least 3 to 4 mm from each other. Take care not to damage the embryos

Embryo Co-Cultivation.

Allow the plates to sit ajar with no lid for up to 30 min to dry in the laminar flow hood (not shown). Wrap the plate in micropore tape (3M 1530-1) and incubate @ 20°C in darkness for 3 days. Parafilm is not preferred at this step

Embryo Co-Cultivation.

Some investigators seem to think that a neat organization improves transformation efficiency…(just kidding!

Embryo Co-Cultivation.

Incubate @ 20°C in darkness for 3 days.

Resting Phase

After the co-cultivation period, the embryos are transferred to Resting Medium and incubated for 7 days at 28°C. The orientation of the embryos with scutellum side up must be maintained.

Maize Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
N_6 salt	2.0 g	2.0 g	4.0 g	4.0 g	4.0 g	-	-
MS salt	-	-	-	-	-	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	-	-
L-proline	0.7 g	0.7 g	0.7 g	0.7 g	0.7 g	-	-
Sucrose	68.5 g	30 g	30 g	30 g	30 g	60 g	30 g
Glucose	36 g	-	-	-	-	-	-
2,4-D (1 mg/mL stock)	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	-	-
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Agar (washed)*	-	8.0	-	-	-	-	-
Agar, Plant cell culture tested**	-	-	8.0 g	8.0 g	8.0 g	-	-
Gelrite	-	-	-	-	-	3.0 g	3.0 g
Autoclave	filtered	20min	20min	20min	20min	20min	20min
N_6 vitamin stock, 1000×	1 mL	1 mL	1 mL	1 mL	1 mL		
MS vitamin, 1000×						1 mL	1 mL
Silver Nitrate	0.85 mg	0.85 mg	0.85 mg	0.85 mg	0.85 mg	-	-
L-cysteine	-	0.4 g	-	-	-	-	-
DTT	-	0.15 g	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Acetosyringone (100 mM/mL)	100 µL	100 µL	-	-	-	-	-
Vancomycin (100 mg/mL)	-	-	1 mL	-	-	-	-
Carbenicillin (200 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Timentin (150 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Bialaphos (3 mg/mL stock)	-	-	-	0.5 mL	1 mL	1 mL	-

Selection of Transgenic Events

After the resting phase, transfer embryos to Selection I medium with half-strength (1.5 mg/mL) bialaphos selection. The orientation of the embryos with scutellum side up must be maintained. Incubate for 14 d at 28°C in darkness

Maize Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
N_6 salt	2.0 g	2.0 g	4.0 g	4.0 g	4.0 g	-	-
MS salt	-	-	-	-	-	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	-	-
L-proline	0.7 g	0.7 g	0.7 g	0.7 g	0.7 g	-	-
Sucrose	68.5 g	30 g	30 g	30 g	30 g	60 g	30 g
Glucose	36 g	-	-	-	-	-	-
2,4-D (1 mg/mL stock)	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	-	-
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Agar (washed)*	-	8.0	-	-	-	-	-
Agar, Plant cell culture tested**	-	-	8.0 g	8.0 g	8.0 g	-	-
Gelrite	-	-	-	-	-	3.0 g	3.0 g
Autoclave	filtered	20min	20min	20min	20min	20min	20min
N_6 vitamin stock, 1000×	1 mL	1 mL	1 mL	1 mL	1 mL		
MS vitamin, 1000×						1 mL	1 mL
Silver Nitrate	0.85 mg	0.85 mg	0.85 mg	0.85 mg	0.85 mg	-	-
L-cysteine	-	0.4 g	-	-	-	-	-
DTT	-	0.15 g	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Acetosyringone (100 mM/mL)	100 µL	100 µL	-	-	-	-	-
Vancomycin (100 mg/mL)	-	-	1 mL	-	-	-	-
Carbenicillin (200 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Timentin (150 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Bialaphos (3 mg/mL stock)	-	-	-	0.5 mL	1 mL	1 mL	-

Selection of Transgenic Events

After 14 d, transfer the embryos to full strength Selection II medium (3.0 mg/mL). After two 14 d rounds of selection on 3.0 mg/mL bialaphos, putatively transformed calli will be distinguishable from non-transformed tissue.

Maize Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
N_6 salt	2.0 g	2.0 g	4.0 g	4.0 g	4.0 g	-	-
MS salt	-	-	-	-	-	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	-	-
L-proline	0.7 g	0.7 g	0.7 g	0.7 g	0.7 g	-	-
Sucrose	68.5 g	30 g	30 g	30 g	30 g	60 g	30 g
Glucose	36 g	-	-	-	-	-	-
2,4-D (1 mg/mL stock)	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	-	-
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Agar (washed)*	-	8.0	-	-	-	-	-
Agar, Plant cell culture tested**	-	-	8.0 g	8.0 g	8.0 g	-	-
Gelrite	-	-	-	-	-	3.0 g	3.0 g
Autoclave	filtered	20min	20min	20min	20min	20min	20min
N_6 vitamin stock, 1000×	1 mL	1 mL	1 mL	1 mL	1 mL		
MS vitamin, 1000×						1 mL	1 mL
Silver Nitrate	0.85 mg	0.85 mg	0.85 mg	0.85 mg	0.85 mg	-	-
L-cysteine	-	0.4 g	-	-	-	-	-
DTT	-	0.15 g	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Acetosyringone (100 mM/mL)	100 µL	100 µL	-	-	-	-	-
Vancomycin (100 mg/mL)	-	-	1 mL	-	-	-	-
Carbenicillin (200 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Timentin (150 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Bialaphos (3 mg/mL stock)	-	-	-	0.5 mL	1 mL	1 mL	-

Selection of Transgenic Events

Nearly all maize transformed events using the cv Hi II genotype , be a pale yellow color and have a distinct “type II” tissue culture response with abundant somatic embryos. There are very few escapes using bialaphos as a selectable marker for the presence of the bar gene. Non-transformed material will not have a type II response, will clearly be stressed, and dying.

Selection of Transgenic Events

An additional subculture for 14 d will allow for identification of all putative transformants. Transformed material will be a pale yellow color and have a "type II" tissue culture response with abundant somatic embryos. Non-transformed material will not have a type II response, will clearly be stressed, and dying.

Selection of Transgenic Events

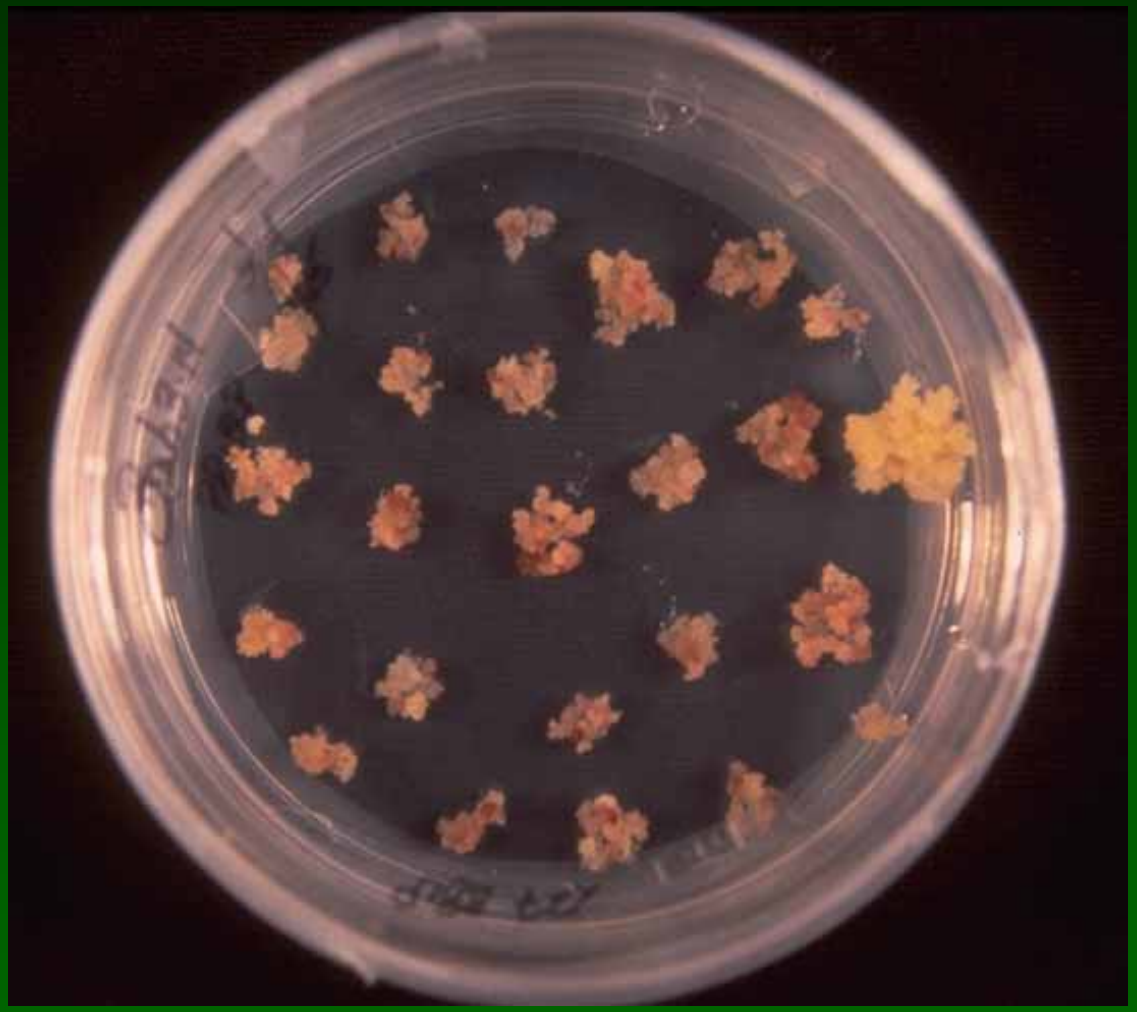


Image of the first transgenic bialaphos event which was subsequently regenerated to the first reported fertile transgenic maize plant
(The Plant Cell 1991. photo Dr. Albert Kausch)

Regeneration Step 1

Once a given callus has grown to roughly the area of a circle with a diameter of 2.5 cm that callus can be given an event ID number and ~ ¾ of the callus is then sub-cultured to Regeneration I medium. The remainder of the callus is sub-cultured to Selection II and maintained as a backup until regeneration and transplantation to soil of the putative event is successful.

Maize Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
N_6 salt	2.0 g	2.0 g	4.0 g	4.0 g	4.0 g	-	-
MS salt	-	-	-	-	-	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	-	-
L-proline	0.7 g	0.7 g	0.7 g	0.7 g	0.7 g	-	-
Sucrose	68.5 g	30 g	30 g	30 g	30 g	60 g	30 g
Glucose	36 g	-	-	-	-	-	-
2,4-D (1 mg/mL stock)	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	-	-
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Agar (washed)*	-	8.0	-	-	-	-	-
Agar, Plant cell culture tested**	-	-	8.0 g	8.0 g	8.0 g	-	-
Gelrite	-	-	-	-	-	3.0 g	3.0 g
Autoclave	filtered	20min	20min	20min	20min	20min	20min
N_6 vitamin stock, 1000×	1 mL	1 mL	1 mL	1 mL	1 mL		
MS vitamin, 1000×						1 mL	1 mL
Silver Nitrate	0.85 mg	0.85 mg	0.85 mg	0.85 mg	0.85 mg	-	-
L-cysteine	-	0.4 g	-	-	-	-	-
DTT	-	0.15 g	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Acetosyringone (100 mM/mL)	100 µL	100 µL	-	-	-	-	-
Vancomycin (100 mg/mL)	-	-	1 mL	-	-	-	-
Carbenicillin (200 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Timentin (150 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Bialaphos (3 mg/mL stock)	-	-	-	0.5 mL	1 mL	1 mL	-

Regeneration Step 1

Once a given callus has grown to roughly the area of a circle with a diameter of 2.5 cm that callus can be given an event ID number and ~ ¾ of the callus is then sub-cultured to Regeneration I medium. The remainder of the callus is sub-cultured to Selection II and maintained as a backup until regeneration and transplantation to soil of the putative event is successful.

Regeneration Step 1-2

Putative transgenic calli on Regeneration I medium are cultured for 14 - 21 d to allow the somatic embryos to mature. They will take on an ivory-white appearance, and are ready for subculture to Regeneration II medium after this period of time.

Transfer small clusters of mature somatic embryos to Regeneration II medium in 100 × 15–mm Petri dishes. It is easier to isolate well-formed individual plants if the embryos are not densely packed onto the surface of the medium. Use the bottom portion of sterile Petri dishes as lids for these dishes to form a "double tall" container for each event. Incubate at 25°C under 16-hr photoperiod for 2 to 3 weeks until a number of well formed plants are present.

Maize Medium Recipes (1L)

Component	Infection	Co-cultivation	Resting	Selection I	Selection II	Regeneration I	Regeneration II
N_6 salt	2.0 g	2.0 g	4.0 g	4.0 g	4.0 g	-	-
MS salt	-	-	-	-	-	4.3 g	4.3 g
MES	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g	-	-
L-proline	0.7 g	0.7 g	0.7 g	0.7 g	0.7 g	-	-
Sucrose	68.5 g	30 g	30 g	30 g	30 g	60 g	30 g
Glucose	36 g	-	-	-	-	-	-
2,4-D (1 mg/mL stock)	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	-	-
pH	5.2	5.8	5.8	5.8	5.8	5.6	5.6
Agar (washed)*	-	8.0	-	-	-	-	-
Agar, Plant cell culture tested**	-	-	8.0 g	8.0 g	8.0 g	-	-
Gelrite	-	-	-	-	-	3.0 g	3.0 g
Autoclave	filtered	20min	20min	20min	20min	20min	20min
N_6 vitamin stock, 1000×	1 mL	1 mL	1 mL	1 mL	1 mL		
MS vitamin, 1000×						1 mL	1 mL
Silver Nitrate	0.85 mg	0.85 mg	0.85 mg	0.85 mg	0.85 mg	-	-
L-cysteine	-	0.4 g	-	-	-	-	-
DTT	-	0.15 g	0.2 g	0.2 g	0.2 g	0.2 g	0.2 g
Acetosyringone (100 mM/mL)	100 µL	100 µL	-	-	-	-	-
Vancomycin (100 mg/mL)	-	-	1 mL	-	-	-	-
Carbenicillin (200 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Timentin (150 mg/mL)	-	-	1 mL	1 mL	1 mL*	1 mL*	-
Bialaphos (3 mg/mL stock)	-	-	-	0.5 mL	1 mL	1 mL	-

Regeneration Step 2

Transfer 4 individual well formed plants to a Plant Con® (MP Biomedicals cat. No 26-722-06) containing ~ 80 mL of Regeneration II medium and continue to incubate under 16-hr photoperiod at 25°C until the plantlets reach the top of the Plant Con®. Because individual T_0 transgenic plants will often exhibit reproductive abnormalities (e.g., no tassel formation or pollen shed), it is advisable to make 3 Plant Con® containers as described above per putative transgenic event. Once the plantlets have reached the top of the Plant Con® container, they are ready for transplantation to soil.

Transplantation to Soil

Transplantation to Soil:

Carefully remove the individual plantlets from the Plant Con. It can be helpful to remove the medium as solid block from the container and then gently break up the medium with gloved hands. It is absolutely necessary to rinse the roots free of any medium particles, and best results are obtained when plants with well developed root systems are chosen.

Transfer the plants to 6" plastic pots with moist soil mixture (e.g., MetroMix 510 potting soil). Place the individual pots into a standard greenhouse tray (8 per tray). Each tray should be covered with a "humidome" (Hummert International, cat no. 143850-1). It is absolutely critical that a very humid environment is maintained at the start of transplantation. If necessary, tape the humidomes to the trays. The plants should be maintained at 25°C with a 16 hr photoperiod. Condensation should form within the humidome for the first 48 hrs, and the humidity should only be reduced after it is apparent that the plantlets have survived transplantation (~ 72 hrs.).

At this point, gradual venting of the trays is accomplished by either punching a small hole in the humidomes or removing the tape gradually so as to reduce the humidity gradually. This should take a period of one week.

Transplantation to Soil

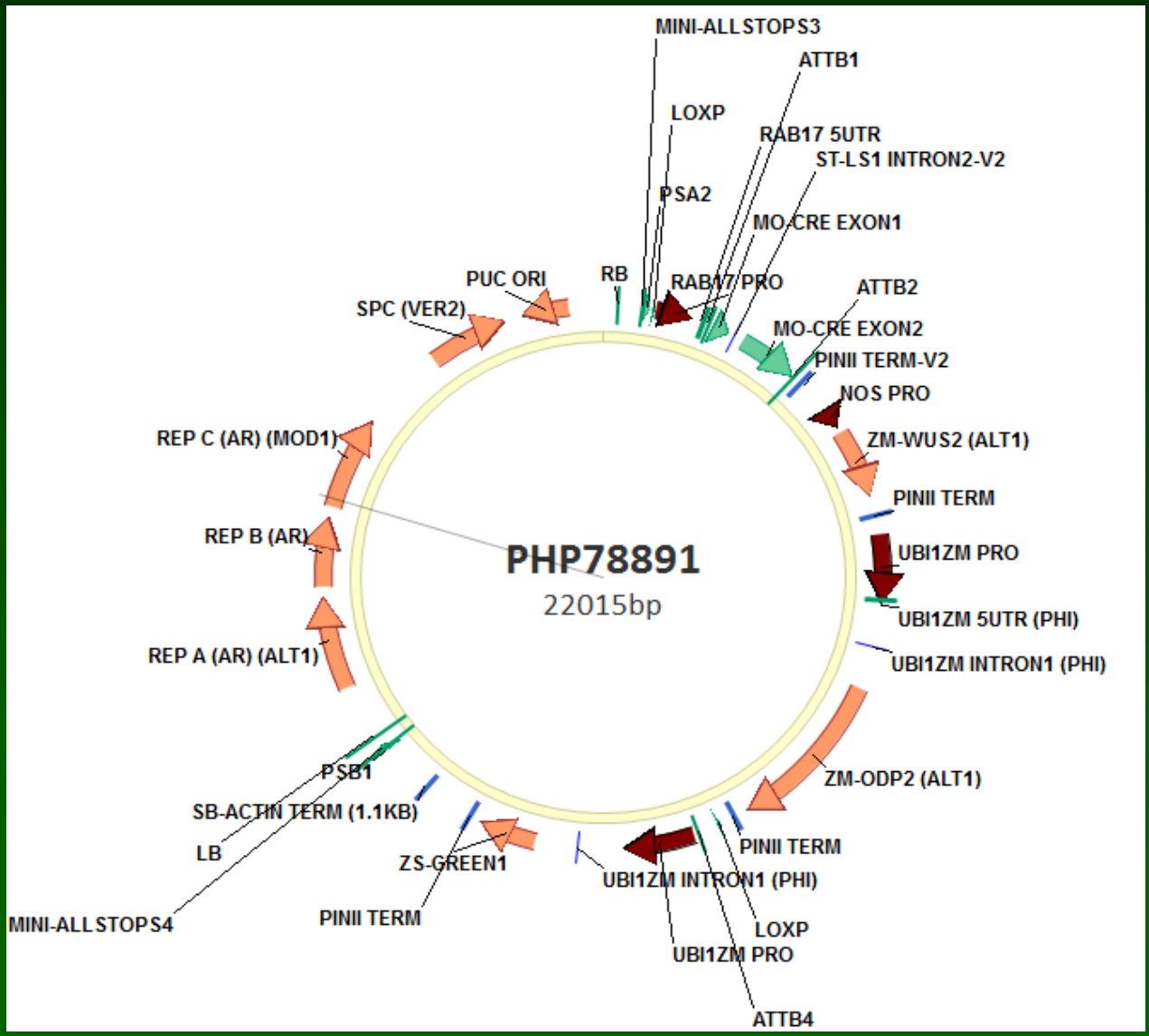
T0 plants are most typically transferred to pots and grown in the greenhouse. These plants are either, selfed, sibed, or crossed to make T1 plants, depending on the purposes of the research. All T0 plants are hemizygous for the transgene which is Mendelianly inherited. Plants can be grown in the field with proper permits.

Transgenic Seed Storage

T1 seed can be stored for further use and analysis

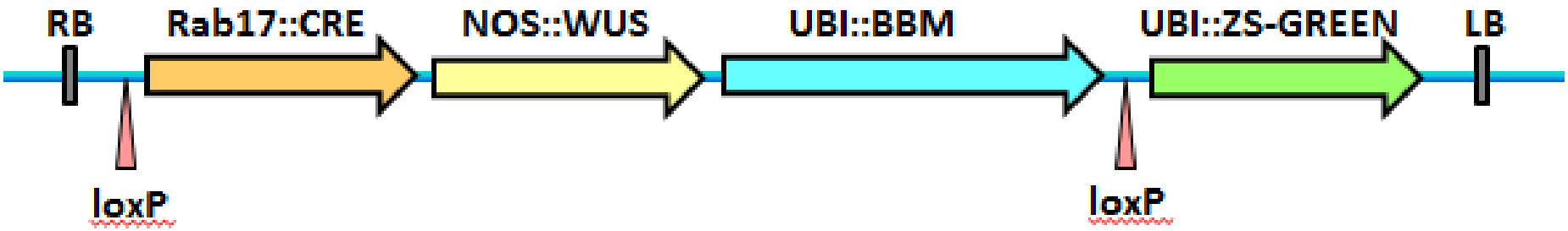
Thank You

Recalcitrant varieties



A Revolutionary New Approach -Pioneered by Pioneer

Recalcitrant varieties



A Revolutionary New Approach -Pioneered by Pioneer

The use of Transcription factors Baby Boom (BBM) and Wuschel (WUS) overexpressed causes somatic embryogenesis in many cell types. A drought inducible promoter (RAB17 driving CRE a (a site specific recombinase) is activated by placing calli on dry filter paper. Activation of CRE causes excision at recombination site (loxP) removing BBM and WUS allowing for regeneration of transformant. This approach appears to be genotype independent. A newer version reported at the SIVB meeting June 2016 is even more robust.