

Greenhouse care for transgenic maize plants

Materials

⌘ Containers

1. Greenhouse flat with drainage holes (Hummert Cat# 11-3000-1)
2. Plastic 4-packs (Hummert Cat. # 11-0300-1)
3. Plastic, transparent, Humi-dome (Hummert Cat. # 14-3850-1)
4. 2-gallon nursery pot with 4 drainage holes (Hummert Cat. #14-9606-1)
5. 3-gallon nursery pot with 4 drainage holes (Hummert Cat: #14-9612-1)

⌘ Soils

1. Redi-Earth (Hummert Cat. # 10-2030-1)
2. Sunshine Universal Mix SB300 (Fosters Inc.)

⌘ Fertilizers and Chemicals

1. Miracle Gro Excel 15-5-15 water-soluble fertilizer supplemented with calcium and magnesium (Hummert Cat. # 07-5660-1)
2. Osmocote Plus 16-8-12 Controlled Release Fertilizer 7.5 g tablets with trace elements (Hummert Cat. # 07-6455-1).
3. Calcium nitrate (4H₂O) for treatment of calcium deficiency (Fisher Cat. # C109-3)
4. Magnesium sulfate (7H₂O) or Epsom salts for treatment of calcium deficiency (Fisher Cat. # M65-3)
5. Marathon, general use pesticide, for aphid control (Hummert Cat. # 01-1118-1)
6. Whitemire yellow monitoring cards, for fungus gnat control (Hummert Cat. # 01-2700-1)

⌘ Bags

1. Shoot bags (Lawson Cat. #217)
2. Striped bags (Lawson Cat. # 404 tassel bags – red stripe or green stripe)
3. Plain brown bags (Lawson Cat. # 404 tassel bags – plain)

Plant Hardening

⌘ Moving plants from germination medium in petri dishes to soil

1. Plantlets in petri dishes (100 x 25 mm) are germinated on MSS media (MS major and minor salts and vitamins, no hormones, 3% sucrose and 0.3% gelrite, pH 5.8), incubated at 25°C in 80-100 $\mu\text{E m}^{-2} \text{ s}^{-1}$ intensity light (16:8 photoperiod), and wrapped in vent tape.
2. When the plantlet leaf is 4-6 cm long and has developed a good-sized root, transfer under sterile conditions from the petri dish to soil following the procedure for plant hardening in the growth chamber (below). Be sure to select strong green plants. This transfer should be done on an individual plant basis. When one plantlet reaches the appropriate size, remove it from the petri dish regardless of the stage of the other plants. Replace the lid and reseal the dish to allow the remaining plants to grow. It is important not to make this transfer before the plantlets are mature enough or they will not survive the transplant.

⌘ Plant hardening in the growth chamber and greenhouse

1. Fill the necessary number of plastic 4-packs with Redi-Earth and place into a greenhouse flat with drainage holes. Water the soil until it is reasonably wet (not completely wet, Redi-earth is wetted to make it sticky but not soaked).
2. In a laminar flow bench, to maintain sterility, take the regenerating plantlet from the petri dish and remove as much media as possible from the roots (favor those plantlets with high root/leaf ratio). Plants should always be manipulated by the callus ball at the base of the stem to avoid leaf-breakage. Avoid manipulation of fragile stem parts.
3. Place the plantlet into the middle of the soil and cover the roots completely; do not press the soil too tightly. Water each plantlet again using a squirt bottle until the soil in each small pot is thoroughly wetted but not water-logged
4. Cover plants with a plastic Humi-dome with one ventilation hole opened; place the flat in a growth chamber. The PTF growth chamber (Conviro, CMP3244) operates on a 16:8 photoperiod with a temperature setting of 26°C/22°C (day/night) and a light intensity of 350 $\mu\text{E m}^{-2} \text{ s}^{-1}$ at plant height (12 inches). Lighting is provided by a mixture of fluorescent and incandescent lights.
5. The next morning, check the soil moisture of each plantlet. Do not water them if the soil has not changed color from dark brown to light brown. If the soil is wetted properly at transplanting, nothing should need water for 48 hours. From here on, each plantlet is watered individually according to need, that is, do not simply drench the whole flat each time you check these plantlets. It is very important not to over water plants at any stage (especially at early stages before internode elongation) to ensure the development of a good root system. Excessive watering or poor drainage here promotes rot/fungal growth at the stem/root junction so drainage holes in the bottom of the 4-packs and in the flat are important. It is also necessary for the plants to have high (but not 100%) humidity the first few days after transplanting. As such, one ventilation hole needs to be cut open in the Humi-dome.
6. Check soil moisture daily. After 1 week, or just before plants reach the top of the dome, remove the plastic dome, and a few days to one week later move the flat to the greenhouse (at this point, plants are 6-8 inches tall). During the winter, the flat needs to be covered by the plastic dome wrapped over with a large plastic bag and transported to the greenhouse in a preheated vehicle. Exposure to freezing temperatures will kill these

young plants. At PTF, fewer than 3% of Hi II transplants are lost during this hardening stage.

7. When the plants can be easily lifted out of the small pot with roots holding the soil, transplant each plantlet to a big pot in the greenhouse as described in transplant section below – this should be done within a week of transfer to the greenhouse. During this week, check moisture daily and water only when dry (young plants will develop a better root system if not overwatered – a good root system will prevent a daily wilting cycle in older plants).
8. If the plantlets need to be left in small pots for longer than usual due to limited transplant space, be sure they are placed as close to the lights as feasible. At first drying, water the entire flat of plantlets once with Miracle Gro Excel 15-5-15 with Cal/Mag (50ppm). Thereafter water without fertilizer on a plant by plant basis, and get them transplanted to big pots as soon as possible.

Greenhouse Care

⌘ Greenhouse Conditions

1. The PTF greenhouse operates on a 16:8 photoperiod. The daytime temperature is set at 28°C while the night-time temperature is set at 21°C. However, with the current cooling system in the PTF greenhouse, daytime temperatures in summer can range as high as 37°C regardless of thermostat settings. Light intensity is 230 $\mu\text{E m}^{-2} \text{s}^{-1}$ at a height of 3.5 feet from the floor. This intensity was measured in February on a slightly overcast day, therefore, does not factor in any additional sunlight.
2. Any transgenic ears, seed, or tassels that are to be discarded are thrown into a red biohazard bag in the greenhouse, then autoclaved before being thrown away.
3. The greenhouse is cooled with swamp coolers.

⌘ Non-transgenic pollen donor plants

1. Non-transgenic donor plants are needed to provide pollen to cross with transgenic ears (in general, PTF does not self R₀ transgenic plants). Donor seeds should be started about the time the regenerating tissue cultures go to the light cabinet for germination. Most non-transgenic plants will require about 2 months for pollen to shed, while B73 and B104 may take up to 1 week longer. Transgenic plants will take 60 to 80 days for silks to emerge after plantlets are removed from the germination medium. For example, on 2/10/03, B73 plants seeded between 11/22/02 and 12/03/02 were shedding donor pollen.
2. Additional seeds should be planted every 3 to 4 days to ensure that pollen is consistently available. One non-transgenic donor plant can be depended on to pollinate about 3 transgenic plants, although this varies considerably with genotype, plant vigor and season.

⌘ Transgenic pollen donor plants

1. Non-transgenic plants may also be required as a recipient ear for transgenic pollen depending on the availability of transgenic pollen and recipient ears. To achieve the reciprocal cross, non-transgenic seed needs to be planted 2 weeks before transgenic plants are removed from the germination medium since transgenic plants will take about 55 to 75 days for pollen to shed after they are removed from the medium.

2. One non-transgenic plant should be sown for each transgenic plant. Transgenic tassels should be bagged (on the plant) once it starts to shed to prevent cross-contamination.

⌘ Non-transgenic embryo donor plants

1. Non-transgenic plants are also required as embryo donors for genetic transformation using immature zygotic embryo explants. Assuming 100% germination, ten seeds should be sown twice a week to achieve delivery of 15 useable ears per week to the lab. It will take about 55-60 days for the silks to emerge at which time the plants are sibbed. Ears are harvested 9 to 13 days post-pollination when embryos are 1.2 to 1.5 mm in size. This duration is season dependent under our greenhouse conditions.
2. To determine immature embryo length, a narrow swath of ear husk is peeled down from the ear and, using a spatula, the crown of a kernel mid-ear is removed. The embryo is dug out, separated from the endosperm, and its size assessed. If the embryo is 1.2- 1.8 mm, the ear is ripped from the stock and taken to the lab for experimental use. If it is 1mm or less, the ear is rewrapped (with both husk and pollination bag) and checked either at the end of the day or the next morning depending on ambient greenhouse temperature. Note that the utensil used for checking embryos must be cleaned with 70% ethanol between each use to avoid spreading bacterial or fungal contamination between ears.
3. Ears that are taken to the lab for experiments are left in their pollination bags and stored in the crisper of the refrigerator (4°C) in a large, loosely wrapped black garbage bag for up to 3 days before use for transformation.

⌘ Seed germination in the greenhouse

1. Fill a plastic 4-pack with Sunshine Universal Mix SB300.
2. Water until the soil is completely wet.
3. Place the corn seed in the middle of the soil (about 1/2" from the top).
4. Place the 4-packs in a greenhouse flat with drainage holes under a plastic Humi-dome for 1 week to avoid excessive evaporation of moisture from the soil.
5. The seeds should germinate in approximately 4 days.
6. Check moisture daily; water the plants only when the soil is dry.
7. After 9 days, or just before the plants reach the top of the dome, remove the plastic dome.
8. Transplant the seedling (~2-3 leaf stage) into a larger pot (see below).

⌘ Transplant

1. Fill 50% of a 2-gallon nursery pot with 4 drainage holes (3-gallon for B73) with Sunshine Universal Mix SB300.
2. Before filling the rest of the pot, add one, 7.5 g plug of Osmotcote Plus -16-8-12 controlled release fertilizer with trace elements (per 2 gallon pot).
3. Finish filling the pot by adding Universal Mix to 80% of the pot volume (~2" from top edge of pot). Too little doesn't hold enough water between waterings, and too much doesn't allow enough water to be added at watering.

4. Move those corn plants with soil adhering to the root ball from the 4-pack to the middle of the 2-gallon pot. The soil should cover all the roots. Be sure to plant the young plants deep enough or they will tip over when they grow taller and before their brace roots have formed.
5. Drench each pot with water alone (no fertilizer) until it reaches the top edge of the pot, and let it drain completely.
6. Marathon®, for aphid control, is added at this stage (according to instructions on container) and watered in at next step.
7. Then, water with injector fertilizer (Miracle Gro Excel 15-5-15 with Cal/Mag), at a nitrogen delivery rate of 50ppm until the water again reaches to the top edge of the pot.
8. Normally, no watering is needed during the first week after transplant, but this will depend on season. About 7 days after transplant, or at the 6-7 leaf stage, each plant is thoroughly watered using injector fertilizer to deliver 50 ppm N. Allow each pot to dry before watering again at 50 ppm N using injector fertilizer. During this time, keep these young plants as close to the greenhouse lights as possible – either by keeping them on tables, or by lowering your lights to them. To ensure adequate root system development do not overwater plants at this stage.
9. At the 7-8 leaf stage, one more 7.5 g Osmocote Plus plug is buried just below the soil surface and plantlets are now watered with water alone from here to maturity (no more liquid-injected fertilizer). In the PTF greenhouse, this is also the stage at which plants are moved from the potting tables to the floor. During summer months, during which watering frequency of larger plants increases, one more 7.5 g Osmocote plus is added to each pot before elongation.

⌘ Watering after 6-7 leaf stage

1. Check the soil moisture daily by lifting the pot and feeling its weight. Water when the pot can be lifted up by one hand easily and the soil is dry in the pot. Water thoroughly when plants are dry by filling the pot to the rim and then allowing it to drain out. If your water pressure from the hose sends soil flying out of the pot, it is too high and should be turned down.
2. Pay special attention to small plants. Young plants prefer a drier root environment to develop a good strong root system. Do not be misled by dry top-soil; the only way to tell whether they need water or not is to lift the pot up and feel it. Developing a healthy root system at an early stage is most critical for a maize plant. To stimulate a good rooting system, do not over water plants. This cannot be emphasized enough.
3. Plants need more water at the 8 to 11 leaf stage as internodes elongate and the tassel/ear is formed. During the summer, fully-grown plants will almost certainly need to be watered twice a day unless adequate greenhouse cooling is provided.
4. See “Troubleshooting” if plants develop calcium deficiency symptoms. **IMPORTANT:** In the PTF greenhouse with water pH = 8.9, we consistently confront Ca⁺⁺ deficiency symptoms therefore we use apply calcium/magnesium routinely (see Trouble Shooting section below for details).

⌘ Pollination

1. Cover any emerging ears with shoot bags to prevent contamination of the silks before pollinating. Be sure shoot bags are firmly pulled over ear shoots otherwise they will fall

off and your emerging silks will be “contaminated” with unwanted pollen and the plant will have to be discarded. Be careful in your eagerness to affix the shoot bag securely that you do not destroy or remove the ear leaf to get at the emerging silks.

2. The best cross is made when the silks are 2-3 days old, but are still viable if silks are less than 10 inches long. Even if the silks are long and have been emerged for a week, you can still cut the silks back, re-cover with the shoot bag, and pollinate the fresh "stubs" the next day.
3. Cut the top of the emerging ear before the silks come out to make the silk more uniform for pollination. This only works if the ear is big enough to start with, so be patient. Make sure to clean your scissors with 70% ethanol before cutting each ear to avoid contamination with or spread of mold or bacteria between ears.
4. Use white and light yellow pollen for pollination; do not use old and crystallized yellow pollen because it is not viable. Be careful with B73 tassels...they are very easy to accidentally break off and maneuvering the tall plant to obtain pollen can be difficult. If the tassel is accidentally broken off, its pollen shedding during can be maintained by placing the broken tassel in a vase of water.
5. Note that striped red bags are used for transgenic pollinations (both directions) and plain brown bags for non-transgenic embryo donors or non-transgenic pollen donor sibs or selfs. Green striped bags are used to for male sterile plants so they can be distinguished from other transgenic plants.
6. After pollen is taken from a transgenic plant tassel, the back of the plant tag is marked with a male sign (♂) to indicate that pollen has already been used. Note that in all crosses, the female plant ID (the plant whose ear is being pollinated) is listed first, the male or pollen donor second (female x male), followed by the date of pollination. Never use blue or red pens for any greenhouse work - instead use thick black permanent marker (all other colors and tip widths will eventually fade due to sunlight).

✂ Seed Harvest

1. After pollination, plants still need to be well taken care of to insure seed quality. At 0-15 days after pollination, plants should be watered about 3 times per week in the summer and 2 times per week in the winter. At 16-25 days after pollination, plants should be watered 2 times per week in the summer and once per week in the winter.
2. Twenty to twenty-five days after pollination, watering should be stopped altogether or the kernels will 'burst' or germinate on the ear. Plants should then be moved to a dry area (no standing water on floor) and allowed to dry down.
3. At 15 days post-pollination, the ear should be exposed to air to let it dry. This is done by raising the pollination bag. At 25-30 days, expose the cob by pulling down the husks to allow the kernels to dry further. (If mold appears on the husk before this time, lift the pollination bag up over the ear earlier).
4. At about day 40, check the seed on the ear. If the seed shows no milk line and shows a black layer at the seed base (an indication that nutrition has stopped being sent to the seed from the rest of the plant), harvest the seed.
5. If seeds are contaminated by fungus, use 70% ethanol to clean the surface before storage.

Troubleshooting

⌘ Monitoring pests

1. Common pests found in the greenhouse are aphids (on the tassels or on the underside of leaves), spider mites (on the leaf back), and thrips (on the leaf whorl). If one sees any sign of them, report to the greenhouse manager immediately and let him/her take any necessary action. The greenhouse manager sprays the PTF greenhouse for mites or thrips once a month, or as needed, using Floramite®, Pylon®, Akari®, Avid® or Samite® for spider mite control and Conserve® for thrip control. At the same time, remove and discard the infected plant part to prevent further spreading. Fungus gnats are another common pest. They are present when plants are over watered. Placing yellow pest cards (Hummert Cat. #01-2700-1), around the greenhouse will help to control them. Another disease that we see although infrequently is smut. Smutted plants should immediately be discarded.
2. In order to limit disease onset, the greenhouse must be kept clean. The floor should be frequently swept or sprayed clean, the drain hole always left unclogged, and fallen and dead leaves still clinging to the plants should be removed. Garbage should be emptied at least once a week.

⌘ Calcium deficiency- symptoms and treatment

When calcium deficiency occurs, plants develop rippled edges and un-pigmented patches or lesions on the leaves. In the worst cases, the leaves in the whorl wrap tightly around each other and eventually rot. Plants can also be severely stunted. If your plants are suffering from calcium deficiency, you should implement the following steps.

1. Plants are treated with the calcium/magnesium recipe provided by Dr. Charles Block (ISU, USDA-ARS, Plant Pathologist, North Central Regional Plant Introduction Station-Ames). In the PTF greenhouse, beginning when they are moved to the floor at the 7-8 leaf stage, plants are watered once every 2 weeks with this mix- even if they have stopped showing symptoms- as a precautionary measure.
2. To make the calcium/magnesium water, first prepare two stock solutions. The stock solutions should be made separately rather than adding both salts to one bottle of water. If they are not made separately, gypsum will immediately be formed.

Stock #1: Calcium nitrate (4H₂O): 720 g/L

Stock #2: Magnesium sulfate (7H₂O) or Epsom salts: 370g/L

3. Then to 1 gallon of water add:

5 ml Stock #1

5 ml Stock #2

4. Plants are then directly watered with this solution.

Vendors

⌘ Fosters Incorporated: PO Box 2674, Waterloo, IA 50704, 1-319-235-6548

⌘ Fisher Scientific: 1-800-766-7000

- ⌘ **Hummert International: 4500 Earth City Expressway, Earth City, MO 63045, 1-800-325-3055**

- ⌘ **Lawson Bags: 318 Happ Road, P.O. Box 8577, Northfield, IL 60093, 1-800-451-1495**