PENNSTATE

College of Agricultural Sciences

INTEGRATED SYSTEM FOR VEGETATIVE PROPAGATION OF CACAO

PROTOCOL BOOK

VERSION 2.1
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Forward: Application of tissue culture to propagation of cacao

Since the early work of cacao researchers in the late 1800s, propagation systems have been an important tool, enabling the multiplication of wild or breeding genotypes for distribution, germplasm collection and for replicate performance trials. Rooted cuttings and grafting have been used throughout the world for propagation of cacao. However to date, a large percentage of production stocks are grown from seed. Due to the high heterozygosity of most cacao genotypes, this results in a high degree of yield and resistance variation, bringing the mean yield below that of individual, high yielding and/or disease resistant trees.

In the 50s and 60s plant tissue culture methods were developed for the propagation of a wide variety of species, but were not applied to cacao until the late 70s, and even then, with very limited success. Recently, research conducted at Plant DNA Technology, CIRAD, Montpellier, France, Nestles, Tours, France and at The Pennsylvania State University, has led to the development of efficient methods for somatic embryogenesis of cacao. In addition, at Penn State, we have investigated a number of related propagation systems. These systems are the topic of this Manual. Rather than relying only on published manuscripts to describe these methods, we have chosen to supplement our manuscripts with a detailed protocol book, with all of the subtle details developed by our group. These methods should be regarded as a work in progress. We will continually update this manual as new methods are proved to be more efficient. The reader is invited to contact us to obtain new versions of this manual which is also available at no charge online (http://guiltinanlab.cas.psu.edu), or to ask questions, request clarifications, or to suggest improvements.

Finally, a word of caution: while these methods are potentially very powerful, for example we have estimated that by using these methods, from a single flower, 800,000 plants could be produced in two years, it should be noted that this is new technology, that has yet to be fully tested in commercial settings. We have successfully tested SE plants in Ecuador, Brazil, St. Lucia and Puerto Rico and it appears they grow with equal or better productivity compared to seed grown or grafted plants. The production of rooted cuttings from orthotropic bentwood stock plants has not yet been fully tested. We have some indications that plants produced by this method may form developmental abnormalities after 2-3 years so for now, this protocol should not be used for commercial production. We invite our partners, the readers of this manuscript, to work together to set up additional field tests to help validate this system.

November 1, 2010
Mark Guiltinan and Siela Maximova

Alphabetical List of Necessary Materials and Equipment

Equipment

Autoclave, 121˚C, 15 lb / in^2 (1.06 kg / cm^2)
Burner for sterilizing dissecting tools
Controlled environment room or Growth chamber, light, 25˚C
Incubator, dark, set at 25˚C or a simple box placed in the 25˚C light growth chamber
Laminar airflow transfer hood, sterile
pH meter
Refrigerator with a freezer to store chemicals and stock solutions
Scale or analytical balance, preferably top loading
Stirrer and stir bars

General Supplies / Materials

Beakers or appropriate containers for mixing media
Chemicals, assorted: DMSO, 1N NaOH, 1N KOH, HCl, media reagents (listed in media recipes)
Ethanol prepared with sterile water (95% or 70% (v/v)
Forceps and scalpels with replaceable knife blades (#11)
Freezer boxes
Freezer tubes or Eppendorf tubes (1.5-1.7 ml)
Glass bottles or flasks of 1 or 2 liter volume (10 or more)
Glass or plastic petri dishes, sterile
Graduated cylinders
Milli-Q water, sterile
Paper for holding and sectioning explants, sterile
Paper towels or filter paper for blotting, sterile
Parafilm or other material to seal Petri dishes with the cultures
Pipetman set or other accurate dispenser and pipets
Spatulas
Tissue culture glass or plastic containers, sterile
Vials or test tubes (50 ml or bigger), sterile
Weigh boats

PROTOCOLS

Primary Somatic Embryogenesis From Cacao Flower Explants

Mature cacao plants used as a source of tissue culture explants are maintained at Penn State in greenhouse conditions at: 60% humidity, 30°C day, 24°C night (82-85F day and 4 degrees less for night)(+/- approx. 5C), 50% shade under full sun. Supplemental HID 400W sodium halide lights are utilized to extend photoperiod to 14 hours in short daylight periods of the year. Gentle horizontal airflow is applied continuously. Flowers can also be taken from the field (see below).

Tissue Culture Growth Chamber Conditions

Culture conditions required in the growth chamber, unless otherwise stated are: 25°-30°C, cool white fluorescent lights with an intensity of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ measured at shelf height, 16/8 hour photoperiod (light/dark).

Protocol for collection and surface-sterilization of flower buds from greenhouse or field near to the dissection site

1. One hour prior to flower collection, prepare a 1% (w/v) calcium hypochlorite solution using autoclaved, sterile Milli-Q water in a sterile plastic or glass container in a sterile hood. Shake the solution by hand or using orbital shaker at 25C, 200 rpm until powder is dissolved.

The resulting solution is cloudy and never entirely clears after mixing. Store calcium hypochlorite powder with Drierite to prevent hydration of the powder.

2. Collect immature flower buds in a clean container (screw-cap tubes) containing cold water. Use tweezers to remove the flowers from the tree.

Harvest the flower buds early in the morning, before 9 a.m. Observe and evaluate the growth and development of flower buds on individual trees and genotypes and select unopened flowers of medium-large size (relative to the size of the buds on an individual tree). Flower buds in advanced developmental stages or harvested in the afternoon often open during surface-sterilization, contributing to contamination of the tissue culture explants.

Steps 3 to 7 of this protocol are performed inside a sterile tissue culture transfer hood

3. Decant cold water from the immature flower buds and rinse 3 times with cold tap water. Transfer all the flower buds into the container with the calcium hypochlorite solution.

4. Transfer the flowers to a new tube (50 ml screw-cap tubes) with 40 ml of calcium hypochlorite solution and make sure that the flower buds are completely immersed in the solution and all buds come into contact with the hypochlorite solution. Sterilize for 20 min. with occasional gentle inversion every 5 minutes.

5. Completely remove the calcium hypochlorite solution and add 40 ml, 22-25C, autoclaved Milli-Q water to rinse the flower buds. Invert tube several times, decant water. Repeat the rinse with fresh sterile water 2 more times. Decant the water from the last rinse and remove as much water as possible. Do not let the sterile flowers remain immersed in water as they may open.

6. Using sterile forceps transfer flower buds to a sterile Petri dish inside the transfer hood.

7. Close the Petri dish to prevent desiccation of the flower buds and keep in the hood until dissection. Dissect and culture immediately. Do not store the flowers in the Petri dish for more than 3 hours.

Protocol for collection, sterilization and transportation of material from field to distant dissection sites/labs.

We have developed a protocol that allows sterilization and transportation of flowers from foreign filed sites to Penn State for dissection and culture initiation. We have observed that best results are obtained when the flowers are sterilized in a sterile hood (protocol above) prior to transportation and are introduced into tissue culture within 24-30 hours post collection. Alternatively an initial flower sterilization can be performed in open air at the collection site, transported to the lab, then resterilized as described below.

1. In the lab, pre weigh calcium hypochlorite (1% w/v or 5 g/500 ml) and add to a sterile bottle. About 5-6 hours prior to flower collection (night before) pour 500 ml of sterile water into the bottle with the calcium hypochlorite.

2. Collect the flowers from the field early in the morning before 9 am in 50 ml sterile, empty screw cap tubes with about 50 to 60 flowers per tube. Label each tube with genotype name, collection date and location.

3. At the sterilization table, add 40 ml of cold water (if possible use 4ºC water, but not iced) to each tube and rinse the flowers by gently inverting the tube. Discard the water.

4. In the same tube, add 40 ml of calcium hypochlorite solution.

5. Sterilize the flowers for 20 minutes with occasional gentle inversion.

6. Discard calcium hypochlorite solution and replace with **sterile water** (if possible use 4ºC water) and rinse with a few gentle inversions.

7. Repeat water rinse two more times and discard the last water from the final rinse.

8. Add 40 ml of liquid 4ºC DKW liquid basal, sugar free media (ED minus sugar and agar) and gently invert the tubes for a few times to wet the flowers.

9. Pour the liquid from the tube and drain for 5 seconds.

10. Hold the tube of sterile flowers horizontally and disperse the flowers across the tube so none of the flowers are bathed in any residual fluid.

11. Place the tube of flowers in a horizontal position in a plastic bag.

12. In the bottom of an insulated container (cooler) place a bag of crushed ice or ice packs and layer with a few layers of paper towels or other absorbent material.

13. Place the bag/s with the flowers on the top of the paper towels in the container and place more paper towels on the sides and the top of the flower bag.

14. Place more bags with crushed ice on the sides and the top of the flowers keeping them separated from the ice with the paper towels.

15. The temperature in the transportation container should be approximately 1ºC in the beginning and should not increase above 16-17ºC over the course of 3 days. For best results dissect the flowers within 24 hours. If not possible, flowers could be stored at 16ºC for up to 3 days post field sterilization.

16. In the lab repeat sterilization in sterile conditions prior to dissecting the flowers using the standard protocol above.

17. Make sure to have current USDA import permits (or of your country) with authorization for entry into the port you airplane will arrive at.

Packing List for Field Collection of Cacao Flowers for Tissue culture

1. Sterile liquid DKW basal, sugar-free media in sterile 250ml bottles (plastic if shipping is required). The content and the preparation of the medium are described in the Media Formulations section of this protocol book (ED medium minus sugar and agar).
2. Sterile Water in sterile 250ml bottles (plastic if shipping is required)
3. 50ml screw-cap collection tubes
4. Tweezers
5. Freezer ice packs (4) or ice
6. 1 gallon plastic Ziploc bags
7. Cooler, soft collapsible cooler with the dimensions of the overhead compartment is preferred for transportation on airplane
8. Calcium Hypochlorite powder pre weighted in 250 ml bottle/s according to sterilization protocol (1% w/v or 2.5 g for 250 ml)
9. Markers, water resistant
10. Markers or labels to identify source trees
11. Paper towels
12. Protocol
13. Parafilm for sealing tubes
14. Permit to import flowers
15. Notebook (water resistant preferred for field conditions)

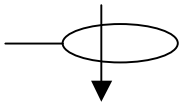
Dissection of flower buds and callus induction for Primary SE (In a sterile hood)

1. Transfer 4-10 flower buds to a sterile Petri dish.

It is important not to transfer excess moisture when moving the flower buds to the new dish. The number of buds transferred depends on a personal preference and one's tissue culture skills. Prepare in batches that will take no longer than 5 min. to avoid desiccation of the cut surfaces.

2. Slice the flower buds across at a position 1/3 of the flower length from the base using a sterile scalpel blade.

Number 11scalpel blade is recommended. Never cut/dissect opened flowers, as this may cause contamination.



3. Extract floral parts including staminodes and petal tissues together through the opening at the cut end using fine point, sterile forceps.

The two most commonly used explants for embryogenesis are staminodes and petal bases.

4. Transfer floral parts into a 100 x 15 mm Petri dish containing 30-32 ml of PCG medium. Separate individual staminodes and petal base explants on the plate with the medium by using 2 fine point forceps and distribute explants evenly on the entire surface of the medium (up to 50 explants per dish), assuring good contact of the explants with the medium without immersion.

5. Seal the Petri dishes with a double layer of parafilm and maintain cultures with the lid side up in the dark at 26 - 28^{o}C for 14 days.

6. After 14 days, transfer the explants into a 100 x 15 mm Petri dish containing 30-32 ml of SCG medium. Place 25 - 50 explants per 100 x 15 mm Petri dish. Seal the dishes and maintain cultures in the dark for another 14 days. Gently press the explants into the medium to assure good contact, but **do not** immerse them in the medium.

Callus formation should be apparent by the end of this culture period.

Somatic embryo induction and maintenance

1. Transfer explants to Petri dishes containing 30-32 ml of ED medium. Incubate cultures in the dark for 14 days. At this step the size of the individual explants has increased and calli have developed. Reduce the number of explants per plate to 20-25.

2. Subculture explants onto fresh ED medium every 14 days and maintain cultures in the dark.

During the ED culture period (6-8 weeks in culture) large numbers of somatic embryos develop. The initiation of new embryos continues for up to 10 -

12 months and embryos at different developmental stages (globular, heart, torpedo and mature) are present on individual explants at the same time.
At each transfer to fresh medium select individual mature embryos (elongated axes of at least 1-2 cm) and transfer to PEC conversion medium.

Secondary Embryogenesis
From Cotyledon Explants of Primary Somatic Embryos

1. Select recently matured, primary embryos with developed cotyledons.
The cotyledons with high embryogenic potential are light yellow or pink in color. Avoid using very young cotyledons (transparent/white) or old, thickened hairy cotyledons with visible trichomes. At PSU a maximum number of secondary embryos are produced when embryos are selected from primary cultures that are between 22 and 28 weeks of age (24 weeks being optimum).

2. Separate the cotyledons from the embryo hypocotyl and slice with a sharp scalpel blade (#10 or 11 is recommended) into 4 by 4 mm pieces.

3. Culture the explants in a Petri dish containing 30 - 32 ml of SCG medium. Press gently into the medium to assure good contact of the explants with the medium without immersion into the medium.

4. Seal the dishes and maintain cultures in the dark for 14 days. Incubate with the lid side up. Callus formation should be apparent by the end of this culture period.

5. Transfer cotyledon explants to Petri dishes containing 30-32 ml of ED medium and culture in the dark for 14 days.

6. Subculture explants onto fresh ED medium every 14 days, and maintain cultures in the dark.
Secondary embryos should form within 2 to 3 months after culture initiation with no callus or minimal callus development. The embryos produced by secondary embryogenesis appear to be of better quality and have more synchronized development than the primary embryos.

7. Maintain the individual embryos in the dark with a subculture interval of 14 days on ED medium until they reach maturity and are ready for conversion. At each transfer to fresh medium select individual mature embryos (elongated axes of at least 1-2 cm and presence of cotyledons) and transfer to PEC conversion medium.
A good indicator of maturity is a brown striations or brown spotting on the axis.

Somatic Embryo Conversion and Plant Establishment

1. Select individual mature somatic embryos (up to 1-2 cm in length) with distinctive cotyledons and an extended axis (brown striations on axis often appear). Place the embryos on PEC medium at a 90^{o} angle, root tip first, up to the base of the cotyledons at a density of 6 to 10 embryos per Petri dish. For this step use deep Petri dishes (100 x 20 mm) with 50 ml PEC medium.

2. Seal the culture dishes with parafilm and maintain cultures under light (16/8h photoperiod) at 26-28˚C.

3. Transfer embryos to fresh PEC medium every 4 weeks until shoots with leaves develop.

4. Transfer shoot-producing embryos with 2 developing leaves of at least 0.5 -1 cm in length into Magenta GA3 vessels containing 140 ml of RD medium (or glass jars). Place the embryos root tip first, at a density of 4 embryos per vessel. Close the vessels and seal the lids with Micropore surgical tape (3M Health Care, Germany) to prevent contamination but allow air exchange.

5. Maintain cultures under light with a 16/8h photoperiod. Transfer embryos to fresh RD every 4 weeks.

Before transferring and inserting plants into fresh medium, gently break up the new media with sterile forceps. Holding the plant with one pair of forceps, press the plant into the fresh media, being careful to gently work the roots into the new media so as not to break the roots.

At this stage young plants are sensitive to ethylene buildup, which can cause leaf drop. The recommendation is to use magenta GA3 vessels with vented lids (Sigma-Aldrich, St. Louis, MO Cat. #C3430) and to seal the container with Micropore surgical tape. In case vented lids cannot be purchased, alternatively puncture a circular opening in the center of the lid (approximately 1.5 cm diameter) using a cork borer and cover the opening with adhesive sterile vent patch (SSvent-10PC, Liquid lab Vent Patch pk 0.2 um membrane for gas exchange, Caisson Labs, North Logan, UT).

6. Occasionally, plantlets produce abnormal shoots, which continue to produce cotyledon like leaves with long stems/internodes.

To promote normal development of these plantlets, prune (decapitate) with a sharp scalpel by cutting the apical shoot approximately 0.5 cm above the first node. If the shoot is extraordinarily long, decapitate between any of the lower nodes, assuring the presence of at least one node for adventitious shoot proliferation.

7. Transfer plants with at least 2-5 leaves to the greenhouse (each leaf longer than 2 cm) and healthy primary and secondary roots (each root more than 2 cm long) into D40 Deepots (plastic, 62.5mm x 250mm; Hummert, Earth City, MO, USA) or bags containing medium grade commercial sand.

7.1 Add a plug of soil to the bottom of the D40 Deepots prior to the sand to prevent sand from escaping out of the drainage hole

7.2 Fill ¾ of the pot with sand.
7.3 Saturate the soil mixture (sand) with water.
7.4 Gently remove the plant from the tissue culture phytagel medium and rinse the gel from the roots in a container with cold tap water.
7.5 Gently hold the plant in the pot straight-up above the sand level and fill in with additional sand around the root system without covering the stem and the leaves with sand.
7.6 Water the additional sand.
7.7 Spray the leaves with water with fine mist and keep them moist until they are placed on misting bench.

8. Place the plants in the pots on a misting bench with misting schedule of 10 seconds every 15 minutes (100% humidity) and light level at approximately 100 µmol*m-2*s-1.

9. Observe the plants every day during the first week and water carefully or as needed.

At this stage the plants are very sensitive to overwatering (causing a lack of oxygen). Immediately after transplanting, start applications of dilute fertilizer at each watering: 1/10 Hoagland's solution.

10. After 4 weeks transfer and maintained the plants at a minimum of 60-65% humidity, with 50% shade (250-500 µmol*m-2*s-1).

By the end of the acclimatization period (2-4 weeks after transfer to 60% humidity) the plants should reach approximately 5-10 cm in height. At this point, the rate of growth should be similar to a seed grown plant.

Micropropagation of Cacao
Somatic Embryo Derived Plantlets

Once SE plantlets are established in vitro, it is possible to propagate them further in vitro through micropropagation methods.

Harvest of microcuttings and root induction

1. Select somatic embryo derived plantlets grown on RD medium with developed roots and leaves.
Plantlets ready for micropropagation should have green healthy leaves at least 3 cm long. Young purple leaves often drop after the micropropagation, reducing the rooting ability of the shoots.

2. Identify a cutting point at an internode approximately 1 to 2 cm below the apex and the first two or three leaves of the shoot. If the shoots have more than 5 or 6 nodes and leaves, nodal microcuttings can also be harvested and rooted successfully.

3. Using a sterile scalpel, trim only the green leaves above the cutting point to 1/3 of their size.

4. Holding the tip of the shoot with sterile forceps, cut the stem at the selected point/s.
See step 10 below for further instructions on culturing the remaining stock plantlet.

5. Transfer microcuttings into a Magenta box or glass jar containing 50 ml or less of RI medium containing freshly made IBA. The depth of the medium in the container needs to be 1 cm or more. Place the microcutting vertically with the basal cut end immersed 3 to 5 mm into the medium.

6. Culture for 7 days under light (16/8 h photoperiod).
A long exposure to IBA could cause formation of excessive callus and a large number of roots with stunted growth. However, some genotypes may require an extended root induction period.

Root development, maintenance and plant acclimation

7. After 7 days in root induction medium, transfer explants into a Magenta box or glass jar containing 140-150 ml of RD medium. Assure that the basal end of the microcutting is always immersed in the medium.

8. Culture explants under light (16/8 h photoperiod) and transfer as necessary every 2 months into fresh RD medium. Roots emerge and grow 2-3 cm within 30 - 40 days after root initiation.

9. Some plants fully develop within the two-month period and can be transferred to the greenhouse. Follow the embryo conversion and plant establishment protocol.

Stock plant maintenance for continuous microcutting production

10. Prepare fresh RD medium and place 140-150 ml into sterile Magenta GA3 vessels or glass jars for the decapitated stock plantlet (see step 4). Before transferring the plantlet, gently break the surface of the medium with sterile forceps.

11. Pick up the decapitated plantlet by the stem with sterile forceps and slowly pull it out of the old medium.

12. Place the plantlet on the surface of the fresh RD medium. Hold the root tip with second pair of sterile forceps and insert it into the fresh medium until the root is entirely immersed into the medium.

13. Maintain cultures under light (16/8h photoperoid) at 25 -30°C for 14 days.
New axillary shoots develop and are ready for harvest in 6-8 weeks after previous harvest.

14. When the shoots grow to approximately 3 cm in height, proceed through steps 2 to 5.
It is important to harvest the new shoots regularly to prevent the re-establishment of apical dominance and to promote axillary bud proliferation. It is not unusual that after decapitation one or more newly developed shoots grow faster and re-establish themselves as new leaders.

Rooting of Orthotropic Stem Cuttings In the Greenhouse

SE-derived plants growing in the greenhouse will grow orthotropically and could be uses as a source for rooted cuttings. Please notice the warning in the Forward of this document regarding potential developmental problems with plants coming from BENTWOOD stock plants. See the thesis of Carter Miller for full details.

Miller, C. (2009). An integrated in vitro and greenhouse orthotropic clonal propagation system for Theobroma cacao L (United States -- Pennsylvania: The Pennsylvania State University), pp. 158.
pdf download at: http://guiltinanlab.cas.psu.edu/MS_and_Ph.D._Theses.html

Semi-hardwood Stock Plant Production

1. Greenhouse stock plants for orthotropic rooted cuttings propagation are selected from juvenile somatic embryo plants at the sapling stage (at or near jorquette height).

2. Hard prune the fan branches. This promotes release of dormant axillary orthotropic meristems leading to greater orthotropic shoot production.

3. With the pots remaining upright, arch the plant over so that the main trunk is at a horizontal position and secure the shoot with a metal hook or some rope.
Many orthotropic shoots should proliferate in 1 – 2 weeks along the top side of the trunk length.

4. Two to 3 months after bending several plants, start harvesting orthotropic shoots of 0.7 – 1.0 m height for cuttings.

5. Before harvesting the cutting:
5.1 Prepare a 1 : 1 mixture of α Naphthalene acetic acid (NAA) and β Indole-3-butyric acid (IBA) at a total combined concentration of 4 g/L in 50% ethanol The solution should be stored in a dark refrigerator for no more than 2 days. Rooting mixtures should be used for only one day in the greenhouse and the remainder discarded.
5.2 Position root tutor trays (32 plug cells, 2 1/4" top cell dimension tapering to 3/4" punched hole, 4" deep) or individual pots into flat trays with drainage holes lined with water permeable synthetic fabric. Fill the cells/pots with medium grade commercial sand, saturate the sand with water and make a 4 cm deep and 1 cm wide opening in the sand.

6. Harvest hardened and dark green orthotropic shoots from the stock plants early in the morning while leaf water potential is still high. Harvest shoots at their base at the main stock plant trunk. Well-hardened and even material showing beginnings of characteristic browning of the stem works well.

7. First prune all leaves of the orthotropic shoot to 1/3 then cut the stem into single leaf cuttings with stems at least 40 mm long. Stems should be cut such that 5 –10 mm remains above the leaf axil (so that the axillary bud will not die as the stem is rooting), and 20–30 mm of the stem remains below the node. If there

are shorter internodes on some portions of the stem, remove the lower leaves excising close to the stem without damaging the stem.

8. Dip the stem in the rooting hormone mixture for 5 seconds.

9. After hormone treatment, insert the cutting into the opening in the wet sand. Gently press the sand around the stem of the cutting.

10. Place the potted cuttings with the trays under intermittent mist (10 sec. every 15 min.) or a fog for 4-6 weeks. Relative humidity should be maintained at or near 100%, keeping the leaves wet without inundating the cuttings. Light intensity should remain approximately 100 µmol * m-2 * sec. -1 PAR. During the misting period water/fertilize the cuttings every 3-4 days with at Hoagland's fertilizer at 150ppm nitrogen.

The first roots will appear at approximately 3 weeks after hormone treatment. By the end of the 6 weeks misting period all cuttings with rooting potential should form roots.

Acclimating, pot establishment, and general cultural management

11. Any rooted cutting with 2 or more roots and a live axillary bud of even the smallest size may be transplanted to polythene bags containing a potting soil or sand mix. Place the plants under 50% shade (250-500 µmol*m-2*s-1), and RH above 60-65%. Fertilizing will promote bud break. Care should be taken when watering to not damage the tender shoots.

12. After the first flush has hardened the cuttings may be treated as any other material. Light may be increased and RH may be lowered.

13. Orthotropic cuttings could jorquette prematurely. To achieve a more standard height of jorquette, remove jorquette bud as soon as it appears. Following removal, an axillary orthotropic shoot from below the cut will release, and the plant will reach a more standard height (1 – 1.5 m) prior to the appearance of the next jorquette (Bertrand & Agbodjan, 1989).

Softwood Stock Plant Production

14. Newly acclimatized SE plants may be grown for 2 – 3 months and used as stock plants for rooted cuttings. After 2 months establishment, orthotropic shoots longer than 0.15 m should be harvested by strong head cuts leaving 3 – 4 nodes with leaves of 80 mm long.

15. Successive harvests are then comprised of orthotropic shoots from 0.20 to 0.40 m length and with only 2 nodes above the previous harvests' cut. Multiple orthotropic axillary meristems swell during the first week, and bud break occurs by 7 – 10 days.

16. Individual shoots are allowed to flush mostly 2 – 3 and up to 4 times prior to harvesting orthotropic shoots depending on shoot morphological characteristics. Stem diameter less than approximately 5 mm was found to be inadequate for

rooting; therefore, shoots are allowed to flush until stem diameter 40 – 60 mm basipedal from apex is at least 5 mm.

17. Softwood cuttings of 40 – 60 mm in length and mostly 2 leaf nodes were made from the entire harvested orthotropic shoot. Node number varies from 1 to 7 and does not affect rooting success. The bottom 20 mm of each cutting is trimmed of leaves at the petiole base. A maximum of 4 leaves per cutting remain. These leaf so 1/3 of the length remains.

18. Rooting of cuttings follows the same method described above.

MEDIA FORMULATIONS

Primary Callus Growth Medium (PCG)

	1L	___L
DKW macro A (10X)	100 ml	
DKW macro B (10X)	100 ml	
DKW micro (100X)	10 ml	
DKW vitamins (1000X)	1 ml	
Glucose	20 g	
Glutamine	250 mg	
Myo-Inositol	100 mg	
2,4-D (1 mg/ml stock)	2 ml	
TDZ (0.2 mg/ml stock)	25 ul	
pH	5.8 (1 M KOH)	
Phytagel	2g	
Autoclave	18 min	

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4˚C for no longer then 4 months.
2. DKW vitamins solution is stored at - 20˚C as aliquots, made fresh every 3-4 months.
3. 2,4-D and TDZ stock solutions are stored at 4˚C and made fresh every two months.
4. Adjust autoclave time according to the specifications of the autoclave equipment used.

Secondary Callus Growth Medium (SCG)
(Also known as E5B)

	1L	___L
McCown's salts (Sigma M-6774)	2.3 g	
B5 vitamins (1000X stock)	1 ml	
Glucose	20 g	
2,4-D (1 mg/ml stock)	2 ml	
6-BA (1 mg/ml stock)	50 ul	
pH	5.7 (1 M KOH)	
Phytagel	2.2 g	
Autoclave	18 min	

Note:
1. McCown's salts (Sigma M-6774) are hygroscopic. Therefore, store at 4˚C in a desiccator.
2. B5 Vitamins solution is stored at -20˚C as aliquots and made fresh every 3-4 months.
3. 6 BA and 2,4-D stock solutions are stored at 4˚C and made fresh every two months.
4. Adjust autoclave time according to the specifications of the autoclave equipment used.

Embryo Development Medium (ED)

	1L	___L
DKW macro A (10X)	100 ml	
DKW macro B (10X)	100 ml	
DKW micro (100X)	10 ml	
DKW vitamins (1000X)	1 ml	
Sucrose	30g	
Glucose	1 g	
pH	5.7 (1 M KOH)	
Phytagel	2g	
Autoclave	18 min	

Note:
1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4˚C for no longer then 4 months.
2. DKW vitamins solution is stored at - 20˚C as aliquots, made fresh every 3-4 months.
3. Adjust autoclave time according to the specifications of the autoclave equipment used.

Primary Embryo Conversion Medium (PEC)

	1L	___L
DKW macro A (10X)	100 ml	
DKW macro B (10X)	100 ml	
DKW micro (100X)	10 ml	
DKW Vitamins (1000X)	1 ml	
Potassium Nitrate ($KN0_3$)	0.3g	
Amino Acid (1000X stock)	1 ml	
Sucrose	10g	
Glucose	20 g	
pH (1M KOH)	5.8	
Phytagel	1.75 g	
Autoclave	18 min	

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4˚C for no longer then 4 months.
2. DKW vitamins solution is stored at - 20˚C as aliquots, made fresh every 3-4 months.
3. Amino acid (AA) 1000X solution is stored at -20C as aliquots, made fresh every 3-4 months.
4. Adjust autoclave time according to the specifications of the autoclave equipment used.

Root Induction Medium (RI)

	1L	___L
DKW macro A (10X)	50 ml	
DKW macro B (10X)	50 ml	
DKW micro (100X)	5 ml	
DKW Vitamins (1000X)	0.5 ml	
Potassium Nitrate ($KN0_3$)	0.3 g	
Sucrose	5 g	
Glucose	10 g	
Indole Butyric Acid (IBA;1 mg/ml stock)	3mg (3ml)	
pH (1M KOH)	5.8	
Phytagel	1.75 g	
Autoclave	18 min	

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4˚C for no longer then 4 months.
2. DKW vitamins solution is stored at - 20˚C as aliquots, made fresh every 3-4 months.
3. IBA stock solution is stored at -20˚C as aliquots, made fresh every 3-4 months.
4. Adjust autoclave time according to the specifications of the autoclave equipment used.

Root Development and Maintenance Medium (RD)

	1L	___L
DKW macro A (10X)	50 ml	
DKW macro B (10X)	50 ml	
DKW micro (100X)	5 ml	
DKW Vitamins (1000X)	0.5 ml	
Potassium Nitrate ($KN0_3$)	0.3 g	
Sucrose	5 g	
Glucose	10 g	
pH (1M KOH)	5.8	
Phytagel	1.75 g	
Autoclave	18 min	

Note:

1. DKW macro A, DKW macro B, DKW micro stock solutions are stored at 4˚C for no longer then 4 months.
2. DKW vitamins solution is stored at - 20˚C as aliquots, made fresh every 3-4 months.
3. Adjust autoclave time according to the specifications of the autoclave equipment used.

STOCK SOLUTIONS FORMULATIONS

DKW l0X Macro Elements Solutions A & B

Solution A	**1L**
Calcium Nitrate tetrahydrate ($Ca(N0_3)_2 .4H_20$)	14.16 g
Ammonium Nitrate (NH_4N0_3)	19.69 g

Solution B	**1L**
Calcium Chloride dihydrate ($CaCl_2 .2 H_20$)	1.49 g (add first!)
Potassium Sulfate (K_2SO_4)	15.59 g
Magnesium Sulfate heptahydrate ($MgS0_4 . 7 H_20$)	7.40 g
Potassium Phosphate monobasic (KH_2PO_4)	2.65g

Solution A needs to be stirred longer than solution B. Solution A clears upon sitting in the refrigerator. DKW macro A and DKW macro B stock solutions are stored at 4˚C for no longer then 4 months. Use 100 ml of each for 1 L of medium.

DKW 100X Micro Elements Solution

	1L	___L
Zinc Nitrate hexahydrate ($Zn(N0_3)_2 .6 H_20$) _	1.700 g	
Manganese Sulfate monohydrate ($MnS0_4 . H_20$)	3.340 g	
Copper Sulfate Pentahydrate ($CuS0_4 . 5 H_20$)	0.025 g	
Boric Acid (H_3BO_3)	0.480 g	
Sodium Molybdate Dihydrate ($Na_2 MoO_4 . 2 H_20$)	0.039 g	
Iron Sulfate hyptahydrate ($FeS0_4 . 7 H_20$)	3.380 g	
Disodium Dihydrate EDTA ($C_{10}H_{14}N_2 O_8Na_2. 2H_2O$)	4.540 g	

Store solution at 4˚C for no longer then 4 months. Use 10 ml for 1 L of medium.

DKW 1000X Vitamins Stock Solution

	100 ml
Myo-Inositol	10.0 g
Thiamine-HCL	0.2 g
Nicotinic acid	0.1 g
Glycine	0.2 g

B5 1000X Vitamin Solution

	50 ml
Myo-Inositol	5.0 g
Thiamine-HCL	500 mg
Nicotinic acid	50 mg
Pyridoxine	50 mg

Store solutions at -20˚C as aliquots. Make fresh every 3-4 months. Use 1 ml per 1L of medium.

Amino Acid 1000X Stock Solution

	100 ml
Arginine	43.55 mg
Glycine	18.76 mg
Leucine	32.80 mg
Lysine	45.65 mg
Tryptophane	51.05 mg

Store solution at - 20˚C as aliquots, remake every 3-4 months. Use 1 ml per 1L of medium.

Plant Growth Regulators Stock Solutions

According to specifications for chemicals purchased from Sigma-Aldrich Co.

1. 2, 4-Dichlorophenoxyacetic Acid (1mg/ml stock solution)
Twenty-five or fifty ml batches are prepared every 2 months. 12.5 mls (25 ml batch) or 25 mls (50 ml batch) of 95% ethanol are used to dissolve the powder. Milli-Q water is used to bring the solution up to volume. The solution is kept for two months at 4^0C. (Solution is 50% ethanol).

2. 6-Benzylaminopurine (1mg/ml stock solution)
A 10 ml solution is prepared using 1 ml of 1N NaOH to dissolve the powder and then brought up to volume with 9 mls of Milli-Q water. This solution is prepared fresh every 2 months and kept at 4^0C.

3. Indole-3-Butyric Acid (1mg/ml stock solution)
Amounts of 10-25 mls are prepared each time depending on the amount needed for the experiment. The powder is dissolved in approximately one ml of 95% ethanol and a few drops (4 or 5) of 1N NaOH. The solution is brought up to volume with Milli-Q water, aliquotted and stored at -20 C.

4. Kinetin (1mg/ml stock solution)
Kinetin is dissolved in approximately 1 ml of 1N NaOH then add 9 ml of Milli-Q water for 10 ml final volume. Store at -20 C.

5. Thidiazuron {1-Phenyl-3-(1,2,3-Thiadiazol-5-YL)Urea} Thidiazuron is prepared in a 0.2 mg/ml stock concentration every 2 months and kept at 4^0 C. 1 milligram of Thidiazuron is dissolved in 1 ml of DMSO and then 4 ml of Milli-Q water is added to this to make a total volume of 5 ml. This is then aliquotted into microfuge tubes in 1 ml amounts. Store 4C.

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