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Micropropagation of ‘Awa
(Kava, *Piper methysticum*)

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Literature on micropropagation of ‘awa (*Piper methysticum* Forst. f.) is not available, partly because of proprietary restrictions and partly because of difficulty encountered in developing a protocol for the aseptic clonal increase process. The stumbling block in micropropagating ‘awa seems to lie in the very first step, eliminating bacteria and fungi from the explant. Once this problem is overcome, the subsequent steps of clonal increase under aseptic conditions are similar to methodologies for other successfully micropropagated crops.

This publication provides information on a protocol of ‘awa micropropagation and instructions for procedures to produce ‘awa in aseptic culture. This protocol was developed with an ‘awa plant of unknown origin, then successfully tested with four ‘awa clones, three of which are named cultivars.

Because the source of explants are axillary buds, a helpful botanical characteristic of ‘awa is that it has an alternate leaf arrangement; that is, it has only one leaf and one axillary bud at each node. In contrast, plants with an opposite leaf arrangement have two leaves at each node and a bud in each leaf axil. On the basal portion of ‘awa stems, the leaves have already dropped, but one can expect a bud at each leafless node.

Stages of ‘awa micropropagation

Micropropagation can usually be divided into four stages. The aim of each stage is different, and thus the nutrient medium used may differ with the stage. Recipes for these nutrient media will be given in the following section. First we provide an overview of the stages and their nutrient media.

Stage 1. Establishment of aseptic cultures

An explant of an axillary bud is disinfested and grown to a two-leaved plantlet in Medium A, then transferred to Medium B for further growth.

Medium	*Contents*
A	0.5 Murashige and Skoog medium (MS) 15% (v/v) coconut water (CW) 20 g/liter sucrose
B	0.5 MS 15% CW 20 g/liter sucrose 8 g/liter agar

Stage 2. Clonal increase by axillary shoot enhancement

When the plantlet has grown to the 5–6 node stage, basal and apical cuttings are prepared. Basal cuttings are transferred to medium C, containing benzyladenine (BA), to stimulate rapid growth of axillary shoots. Apical cuttings are placed on fresh medium B for continued apical growth.

Medium	*Contents*
C	0.5 MS 1% CW 0.2 mg/liter BA 20 g/liter sucrose 8 g/liter agar

Acknowledgment: Thanks to T. Higaki for plant materials and to J. DeFrank, T. Fujii, and D. Ogata for other assistance.

Stage 3. Rooting of cuttings

Cuttings are transferred to a medium with reduced nitrogen content (Medium D) to stimulate root emergence and retard vegetative growth.

Medium	*Contents*
D	0.5 MS (0.25 NH_4NO_3) 1% CW 20 g/liter sucrose

Stage 4. Establishment of rooted cuttings

Rooted cuttings are hardened and transplanted to pots and established under greenhouse conditions.

Nutrient media

In the previous section, nutrient media for each growth stage were mentioned. On page 3, the formula for Murashige and Skoog's inorganic salt formulation (designated "MS") is given. When MS is diluted, it is called "0.5 MS" if the salt concentration was reduced by half.

Plant growth regulators are added to the basal MS medium. Because the amount of growth regulator to be added to the medium will differ depending on the plant species and the type of growth desired, they are not listed in the formulation. The most frequently used growth regulators in tissue culture are auxins, such as 2, 4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), and indoleacetic acid (IAA), and cytokinins, such as kinetin and benzyl adenine (BA) or benzylaminopurine (BAP). Auxins frequently have been used in concentrations of 0.1–0.3 mg/liter and cytokinins at 0.01–30 mg/liter. Growth regulators must be dissolved completely before adding them to the medium. Usually they are first dissolved in a small volume of alcohol (1–2 ml), and then distilled water is added.

Stock solutions

When a nutrient medium is prepared frequently, it is convenient to prepare stock solutions first. When using stock solutions, it is not necessary to weigh each chemical every time. Following are useful stock solutions and their constituents.

Stock solution	*Constituents*	*Amount (g/liter)*
A	NH_4NO_3	82.5
B	KNO_3	95
C	H_3BO_3	0.124
	KH_2PO_4	34
	KI	0.166
	$Na_2MoO_4 \cdot 2H_2O$	0.05
	$CoCl_2 \cdot 6H_2O$	0.005
D	$CaCl_2 \cdot 2H_2O$	88
E	$MgSO_4 \cdot 7H_2O$	74
	$MnSO_4 \cdot H_2O$	3.38
	$ZnSO_4 \cdot 7H_2O$	1.72
	$CuSO_4 \cdot 5H_2O$	0.005
*F	$FeSO_4 \cdot 7H_2O$	5.57
	$Na_2 \cdot EDTA$	7.45
*G	Glycine	0.4
	Nictonic acid	0.1
	Pyridoxine·HCl	0.1
	Thiamine·HCl	0.02

*Stock solutions F and G should be stored under refrigeration.

Nutrient constituents for 1 liter of Murashige and Skoog (MS) tissue culture medium.

Mineral salt	*Chemical name*	*Amount (mg/liter)*
NH_4NO_3	Ammonium nitrate	1650
KNO_3	Potassium nitrate	1900
KH_2PO_4	Potassium phosphate monobasic	170
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate	370
$CaCl_2 \cdot 2H_2O$	Calcium chloride, dihydrate	440
$MnSO_4 \cdot H_2O$	Manganous sulfate	16.9
$FeSO_4 \cdot 7H_2O$	Ferrous sulfate	27.8
$Na_2 \cdot EDTA$	Disodium ethylene diaminetetraacetate	37.3
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate	8.6
H_3BO_3	Boric acid	6.2
KI	Potassium iodide	0.83
$CuSO_4 \cdot 5H_2O$	Cupric sulfate	0.025
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate	0.25
$CoCl_2 \cdot 6H_2O$	Cobalt chloride	0.025

Organic constituents	*Amount per liter*	
Sucrose	30	g
Glycine	2	mg
Myo-inositol	100	mg
Nicotonic acid	0.5	mg
Pyridoxine·HCl	0.5	mg
Thiamine·HCl	0.1	mg
Agar	10	g

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Growth regulators

If low concentrations of growth regulators are to be used, stock solutions should be prepared for each growth regulator and these should be stored under refrigeration to prevent rapid decrease in activity of the chemical. First, 5 mg of a growth regulator should be dissolved in 2 ml of ethyl alcohol (heat the alcohol gently if the growth regulator does not dissolve readily; *use caution:* heated alcohol may catch fire). Then, add distilled water to bring the final volume to 100 ml. The amounts of stock solution required for different concentrations are as follows:

Final concentration (mg/liter) of growth regulator in the medium	Amount (ml) of stock solution to use for 1 liter of medium
0.01	0.2
0.05	1.0
0.10	2.0
0.50	10
1.00	20

For higher concentrations of growth regulator, stock solutions need not be prepared. In these cases, the required amount should be weighed out, dissolved, and added to the medium. Because most growth regulators are not readily soluble in water, they will precipitate at high concentrations when stored in the refrigerator.

Procedure for preparing 1 liter of Murashige and Skoog's medium with the use of stock solutions

To about 500 ml of distilled water, add the following amounts of stock solution:

Stock solution	*Amount (ml)*
A	20
B	20
C	5
D	5
E	5
F	5
G	5

Weigh out 30 g of sucrose and add it to the culture medium.

Weigh out 100 mg of myo-inositol and add it to the culture medium.

Add the required amounts of plant growth regulators.

Add distilled water until 1 liter of medium is obtained.

Adjust the pH of the medium to 5.7–5.8. If the pH is higher, add drops of 1N HCl; if it is lower, add drops of 1N KOH or NaOH.

Add 10 g of agar to the medium.

Dissolve the agar in a microwave oven or on a hot plate or stove (*caution:* agar burns easily).

Dispense the medium into containers.

Autoclave for 15 minutes at 15 lb pressure.

For ‘awa micropropagation, the concentration of the MS inorganic salt mixture is reduced by half (0.5 MS). If medium is prepared using stock solutions, the required volume of each stock is decreased by half. For example, the required volume for Stock A will be 10 ml for each liter of medium. In medium D, the concentration of NH_4NO_3 is reduced to one quarter (0.25 MS); therefore, the required volume of Stock A is 5 ml for a liter of medium.

In calculating the volume of concentrated solution needed to prepare a certain volume of diluted concentration, a very useful chemistry formula is

$$V_1 \times C_1 = V_2 \times C_2$$

where V_1 = initial volume (volume of the concentrated solution)
C_1 = initial concentration
V_2 = final volume (volume of medium), and
C_2 = final concentration.

For example, to determine the volume of 95% ethanol needed to prepare 100 ml of 70% ethanol,
V_1 = volume to be determined
C_1 = 95% (or 0.95)
V_2 = 100 ml
C_2 = 70% or (0.70)
Solving for V_1, it is 73.7 ml.

For greater convenience in preparing media, commercial nutrient formulations are available.

Micropropagation procedure

Stage 1: Establishment of aseptic cultures

Supplies and equipment: (Fig. 1)

Tubes (16 x 125 mm) of medium A
Tubes (25 x 150 mm) of medium B
Petri dish (10-cm diameter)
ELIZA plate
‘Awa cuttings
10% bleach
2% bleach
Single-edge razor blade
Scalpel and no. 11 blade
1 sterile and 1 nonsterile pasteur pipets
Bulb for pipet
2 100-ml beakers
2 500-ml Erlenmeyer flasks
Stereo zoom dissecting microscope
Cell culture roller drum (Fig. 2)

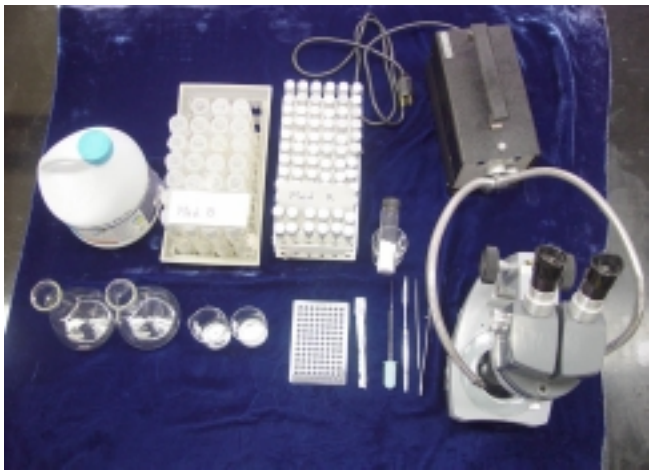

Figure 1. Supplies and equipment for Stage 1.

Figure 2. Cell culture roller drum.

Stage 1 procedure:

1. Prepare dilute bleach solutions (disinfesting solutions):
   a. 10% bleach: dilute 37.5 ml of commercial laundry bleach solution (active ingredient = 5.25% sodium hypochlorite) to 300 ml. Add 3 drops wetting agent.
   b. 2% bleach: dilute 50 ml of 10% bleach to 250 ml.
2. Add 60 ml of 10% bleach to a 100-ml beaker. Add 10–15 ml of 2% bleach to a petri dish. Add 2% bleach to row “A” wells of an ELIZA plate, filling each well to just below the brim.
3. With a single-edge razor blade, cut the leaves from the cutting.
4. Remove five clean nodes, each with an axillary bud, cutting 1 cm above and below the node (Fig. 3).
5. Soak and gently scrub the node segments in soapy solution. Soak segments in running tap water for 1 hour.
6. Soak a node segment in 10% bleach solution for 10 minutes.
7. Cut off lateral and rear portions to form a rectangular block (Fig. 4) to prevent rolling of segment during excision.
8. Starting from the bud, remove the epidermal tissue by slant-cutting away from the bud (Fig. 5). Be sure to always have the bud as the highest structure on the segment.

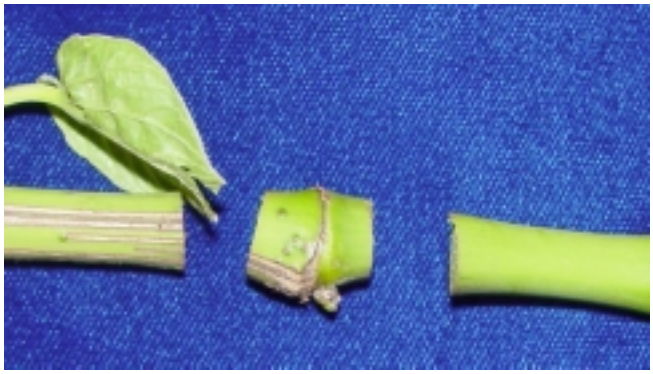

Figure 3. Cut the stem 1 cm above and below the bud.

Figure 4. Cut off lateral and rear portions.

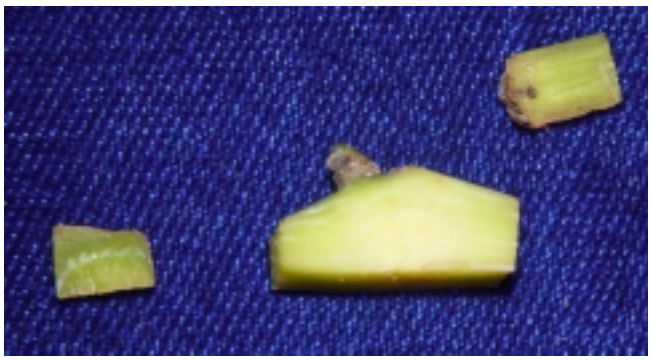

Figure 5. Remove the epidermal tissue by slant-cutting away from the bud.

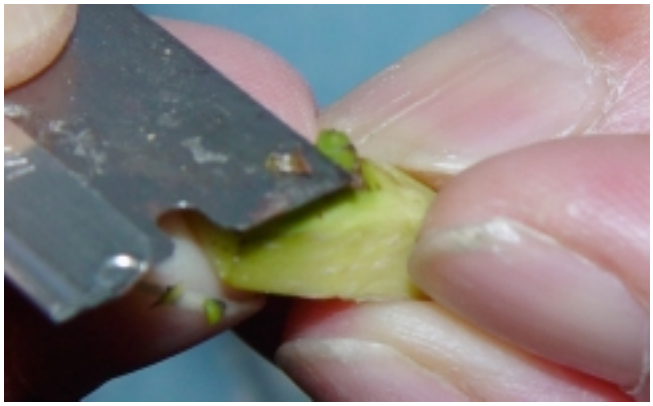

Figure 6. Lop off the tip of the axillary bud.

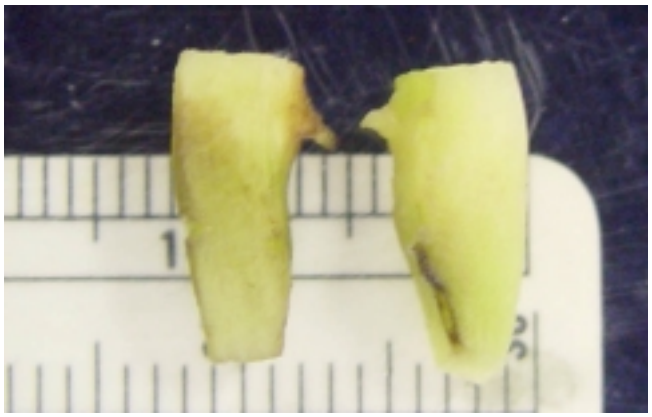

Figure 7. Bud with three to four scales removed.

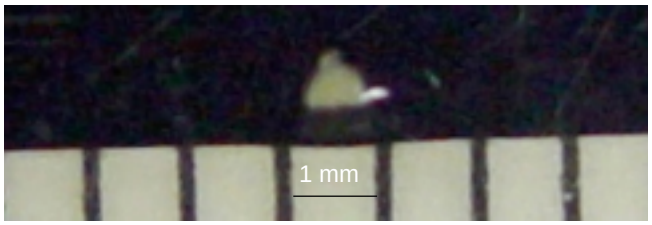

Figure 8. Carefully trim bud to 1 mm size.

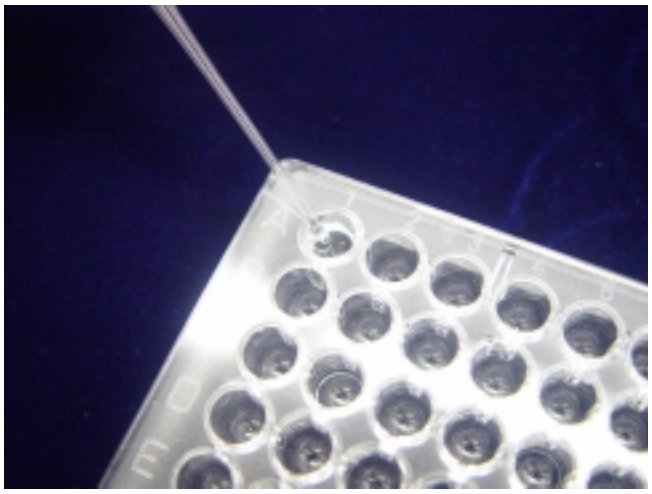

Figure 9. Transfer explant onto ELIZA plate.

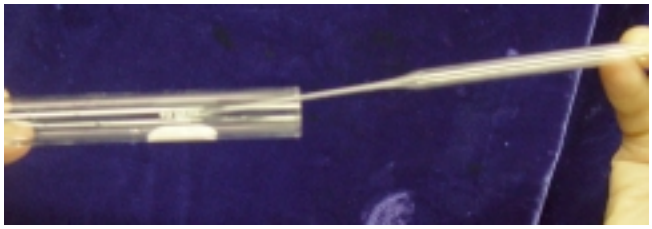

Figure 10. Transfer explant to tube containing medium A.

9. Set the segment in a petri dish with 2% bleach. Place the dish under the microscope.
10. With a razor blade, lop off the tip of axillary bud (Fig. 6), thus revealing the underlying scales. In a sequence, remove each scale completely, being sure to maintain the bud as the highest structure on the segment.
11. When three or four scales have been removed and the base of the bud is now approximately 1 mm wide (Fig. 7), remove the bud by making five incisions, four on each side of the bud and one below the bud. Carefully trim the bud so it is 1 mm in height and width (Fig. 8).
12. With a nonsterile pasteur pipet, transfer the explant to the first well on the ELIZA plate (Fig. 9). After 10 minutes, take the plate into a transfer hood.
    Note: Before this step, all work was done outside the transfer hood.
13. With the sterile pasteur pipet, transfer the explant aseptically and with care to medium A (16 x 125 mm tubes) (Fig. 10).
14. Set test tube on cell culture roller drum (Fig. 2) that can rotate at 0.2–3.5 rpm.
15. Repeat above steps for each explant.
16. Transfer the explants every two months to fresh medium A.
17. When an explant has grown into a plantlet with two leaves, transfer it to medium B; transfer again to fresh medium B every 2 months until the stem has grown to at least five nodes.

Stage 2. Clonal increase

Supplies: (Fig. 11)

Sterile petri dishes
Tubes of medium B
Tubes of medium C
Scalpel and no. 10 blade

Stage 2 procedure:

1. Select a plantlet with 4–5 leaves (Fig. 12).
2. Aseptically transfer the plantlet to a sterile petri dish.
3. Starting from the stem base, prepare two-node cuttings (Fig. 13).
4. Transfer the basal cuttings to medium C and the apical cuttings to medium B (Fig. 14).
5. After 2 months, remove axillary shoots from cuttings in medium C. Subculture excised axillary shoots in medium B.
6. Steps 1–4 are repeated until sufficient shoots are obtained.

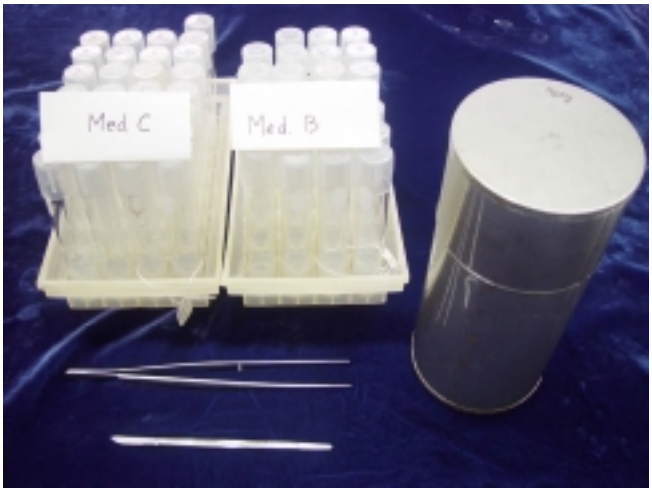

Figure 11. Supplies for Stage 2.

Figure 12. Plantlet ready for multiplication.

Figure 13. Two-node cuttings.

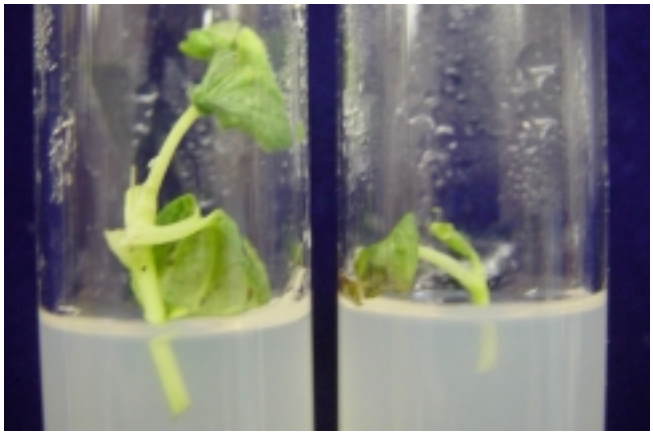

Figure 14. Basal cuttings to medium C and apical cuttings to medium B.

Stage 3. Rooting of cuttings

Supplies: (Fig. 15)

Baby food jars containing medium D
Tubes (25 x 150 mm) of sphagnum moss moistened with medium D
Scalpel and no. 10 blade
Sterile petri dishes

Stage 3 procedure:

1. Aseptically transfer four-node plantlets to sterile petri dishes.
2. Prepare two-node cuttings. Transfer cuttings to baby food jars with medium D, 5–6 cuttings per jar (Fig. 16). The stems need not touch the medium; only part of a leaf needs to touch the medium.
3. After roots have emerged on the cutting, after about 1 month, aseptically transfer the rooted cutting to a sterile petri dish. Remove half of each leaf.
4. Transfer the rooted cutting to tube containing moistened sphagnum moss (Fig. 17).

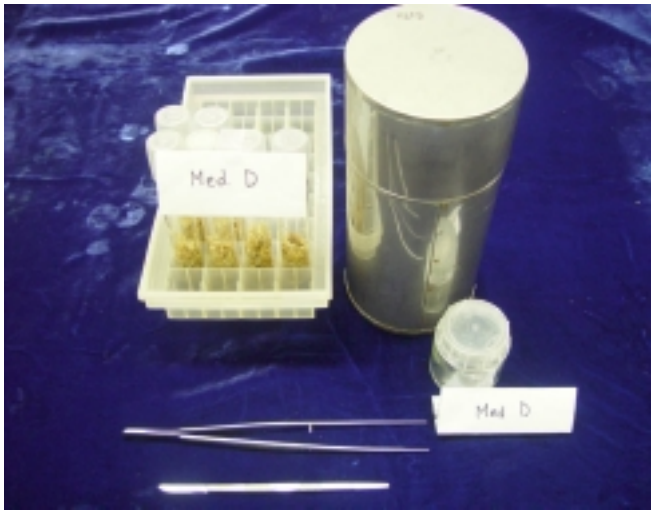

Figure 15. Supplies for Stage 3.

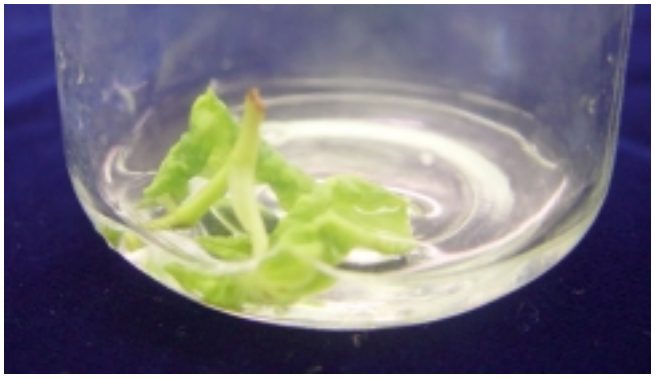

Figure 16. Apical tip in medium D.

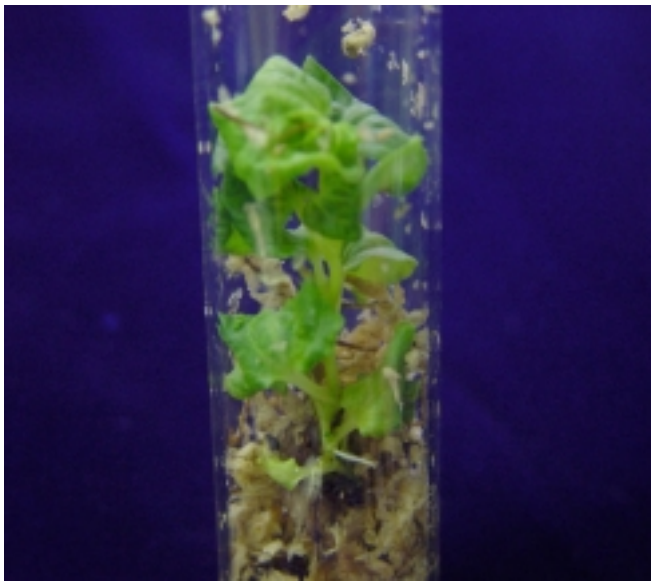

Figure 17. Rooted plantlet in sphagnum moss.

Stage 4. Establishing rooted cuttings under greenhouse conditions

Supplies: (Fig. 18)

Sphagnum moss
Plastic or clay pots (about 2 inches in diameter)

Stage 4 procedure:

1. Carefully remove rooted cutting with sphagnum moss from sterile container (Fig. 19).
2. Clip off all leaves except the terminal leaf (Figs. 20, 21).
3. Add presoaked sphagnum and plant in a plastic or clay pot (Fig. 22).
4. Shade the plants with newspaper for a few days.
5. Gradually move plants to higher light conditions.
6. When well established, repot plants into commercial plant potting mix (Figs. 23, 24).
7. Fertilize as needed.

Figure 19. Ball of sphagnum moss around explant.

Figure 20. Removal of leaves from rooted plantlet.

Figure 18. Supplies for Stage 4.

Figure 21. Terminal leaf remains as the rest of the leaves are removed.

Figure 22. Newly planted cutting.

Figure 23. Established cutting with functional leaves.

Figure 24. Established plants.

Glossary

alternate leaf arrangement: arrangement in which a node has one leaf

apical bud: a bud at the apex of a stem

aseptic: free from microorganisms; used interchangeably with *in vitro*

axil: the upper angle between a stem and a leaf

axillary bud: a bud formed in the leaf axil

bud: an undeveloped shoot, generally protected by modified leaves or scale

bud scale: a modified leaf covering a bud

disinfectant: a chemical used to disinfest explants

disinfestation: process of eliminating bacteria and fungi from explants

in vitro culture: growing of plants under aseptic conditions

lateral bud: a bud that grows out from the side of a stem

micropropagation: vegetative propagation of plant under aseptic conditions; used interchangeably with *tissue culture* or *in vitro culture*

node: the part of a stem at which leaves and buds are attached

opposite leaf arrangement: arrangement in which a node has two oppositely situated leaves

plant growth regulator: an organic compound when added in very small quantity to a nutrient medium influences growth and multiplication of explants and plantlets.

surfactant: substance added to disinfectant increases the efficiency of disinfestation; used interchangeably with *wetting agent*

terminal bud: a bud at the end of a stem

tissue culture: broadly used to indicate micropropagation, in vitro propagation, aseptic propagation

wetting agent: a chemical that increases the effectiveness of disinfectant by reducing surface tensions of contact surfaces; used interchangeably with *surfactant*