# THERAPY AND CITRUS IMPROVEMENT

## Improving Therapy Methods for Citrus Germplasm Exchange

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ABSTRACT. Movement of citrus germplasm between citrus-growing countries carries the risk of inadvertent spread of serious virus and viruslike pathogens. Current procedures have not been well tested for all pathogens, or are not completely efficient. The exclusion of the two pathovars of *Xanthomonas campestris* causing citrus canker and citrus bacterial spot, the greening pathogen, severe isolates of citrus tristeza virus (CTV) and citrus tatterleaf virus (TLV) was studied using a tissue culture quarantine methodology. Infected budsticks were cultured *in vitro* at 32 C. Shoot tips excised from sprouting buds were grafted *in vitro* to axenically grown seedlings of Troyer citrange. The citrus canker and the greening pathogens were consistently eliminated, even by grafting large shoot tips of 0.5-0.7 mm. Severe strains of CTV were consistently eliminated only by the use of small shoot tips of 0.15-0.2 mm. TLV was difficult to eliminate, but 42% of the plants propagated from shoot tips with three-leaf primordia were TLV-free. Shoot-tip grafting eliminated greening and CTV from chronically infected plants from Africa and Asia. Severe CTV isolates from Africa, Asia, South America, and North America were also eliminated easily. One isolate of naturally-spreading psorosis from Argentina was also eliminated.

Movement of citrus germplasm between different citrus areas is often desirable for commercial and scientific purposes, including establishment of germplasm repositories. Uncontrolled movement of whole or propagative plant tissue carries the risk of introducing new pests and pathogens. This risk can be overcome by the introduction of new materials through quarantine stations (9). However, the classical methods of quarantine are slow and require greenhouse facilities remote from citrus production areas. Such facilities are often not available in many countries or are situated where expertise and/or financial support for citriculture are not available.

An alternative citrus tissue culture system was developed for safe introduction of citrus germplasm (13). It consists of culturing imported budsticks *in vitro* at 32 C to induce the sprouting of lateral buds and formation of flushes from which shoot tips are excised and micrografted *in vitro* (shoot-tip grafting) (11). The method is being used successfully in Spain to import citrus germplasm from different growing areas (14), but it has not been experimentally tested for elimination of pathogens causing severe diseases not present in Spain. In addition, shoot-tip grafting (STG) requires a high manual dexterity to isolate the very small shoot tips needed. Grafting success increases as shoot tip size increases, but success in eliminatin pathogens declines rapidly.

Discovery of two bacterial diseases of citrus in Florida (citrus canker and citrus bacterial spot diseases) has also increased concern about the hazard of introducing these pathogens via imported budwood. The efficacy of STG to retrieve disease-free plants from budwood contaminated or infected with these diseases has not been tested.

Results are presented in this paper on the application of STG for elimination of the citrus canker and bacterial

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spot pathogens, greening agent, tatterleaf virus, severe citus tristeza virus isolates and naturally-spreading psorosis, and on the influence of shoot tip size, and addition of viricides and antibiotics in the budstick culture medium on elimination of bacterial and viral pathogens.

## MATERIALS AND METHODS

### Sources of infected materials

**Citrus canker and citrus bacterial spot.** Two strains of *Xanthomonas campestris* were used (6). Strain XC62 of *X. campestris* pv. *citri* causes the "A" type of citrus canker. Strain F1 of *X. campestris* pv. *citrumelo* causes citrus bacterial spot disease in Florida nurseries. Tissue sources for experimentation were: a) glasshouse-grown plants of Duncan grapefruit experimentally inoculated with bacterial suspensions and expressing lesions on leaves and twigs typical of the respective pathovars, and b) budsticks from glasshouse-grown healthy plants of Duncan grapefruit that were contaminated by immersion for 30 or 120 sec in bacterial suspensions of XC62 or F1 ($10^8$ cells/ml), dried at room temperature for 30 min, placed in plastic bags and incubated at 30 C in continuous darkness for 20 hr.

**Greening.** Sources of greening-infected tissue were glasshouse-grown Valencia sweet orange plants infected with the greening pathogen prior to the experiment by graft-inoculation with a source of likubin from Taiwan (coded as B121). This likubin isolate was free from CTV based on ELISA and by indexing to citrus indicators. At the time of budwood selection, the source plants had moderate greening symptoms and infection was verified by graft inoculation to Cleopatra mandarin seedlings. Limited experiments were done with glasshouse-grown plants of several hosts chronically infected with greening isolates from Reunion (B1), South Africa (B49), Taiwan (B121), Philippines (B140 and B142), and China (B144). These plants had very severe symptoms of greening. All sources, except B121, were coinfected with CTV.

**Citrus tristeza virus (CTV).** Sources of CTV-infected budwood were glasshouse-grown plants of Valencia and Washington navel sweet orange plants graft-inoculated several months prior to the experiments with a seedling yellows (SY) source of CTV (B28). Limited experiments were done with glasshouse-grown plants of several hosts chronically infected with CTV isolates from Brazil (B77), California (B6), China (B81, B82, B144), Colombia (B128, B131), Japan (B31), Peru (B133), Philippines (B140, B142), Reunion (B1), and South Africa (B49). These all cause stem pitting in grapefruit and/or sweet orange. The source plants for B1, B140, B142, B144 and B149 were coinfected with greening. All chronically infected plants were showing CTV and/or greening symptoms.

**Tatterleaf virus (TLV).** The sources of TLV-infected tissue were glasshouse-grown plants of Valencia and Washington navel sweet oranges graft-inoculated with an isolate of TLV which had been passaged through *Nicotiana clevelandii* by mechanical inoculation (4). The TLV-infected plants were coinfected by graft-inoculation with the CTV isolate B28 (see above).

**Naturally-spreading psorosis.** The source was Duncan grapefruit plants graft-inoculated with isolate B84 (free from CTV) from Concordia, Argentina.

### Tissue culture techniques

**Budstick culture in vitro.** Pencil-sized budsticks of triangular to circular cross section which contained four to eight buds were collected from glasshouse-grown source plants. They were washed with detergent and tap water and then disinfested by immersion for 10 min in a 1.5% solution of sodium hypochlorite which contained 0.1% Tween 20 wetting agent. The sticks were rinsed three times with sterile,

deionized water, and cultured individually in 38 x 200-mm test tubes containing 50 ml of the plant cell culture salt solution of Murashige and Skoog (10) solidified with 1.2% Bacto agar. To study the influence of antibiotics on greening in newly formed side shoots, 0.5 g/l penicillin and 1.25 g/l tetracycline were added to the culture medium. In one test, CTV- and TLV-infected budwood was cultured in medium amended with 50 mg/l Virazole [1-Ă-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide (ribavirin)] (5) to study the influence of this viricide on CTV and TLV elimination. Cultures were placed in an incubator at a constant 32 C and exposed to 45 $\mu E\ s^{-1}m^{-2}$ light for 16 hr a day.

**Shoot-tip grafting (STG) in vitro.** The standard technique of STG (10, 14) was used in all experiments. Grafting was done on Troyer citrange seedlings 4 to 6 cm tall and 1.6 to 1.8 mm in diameter at the point of decapitation. This size was reached 9-10 days after seed germination at 30 C in continuous darkness.

Young flushes from sprouting buds on the budsticks cultured *in vitro* were surface sterilized by immersion for 5 min in a 0.25% sodium hypochlorite solution which contained 0.1% Tween 20 wetting agent. They were rinsed three times with sterile, deionized water and used as the source of shoot tips.

Shoot tips composed of the apical meristem plus three-leaf primordia (0.15-0.2 mm) were used in most experiments. Shoot tips composed of the apical meristem plus six-leaf primordia, (0.5-0.7 mm) were also used in the experiments on the influence of shoot tip size on pathogen elimination. Shoot tips with three-leaf primordia were grafted inside an inverted-T incision made at the point of decapitation of the rootstock, whereas the shoot tips with six-leaf primordia were grafted inside a "window" made by excising a 1-$mm^2$ piece of bark of the rootstock 3-4 mm below the decapitation point. Shoot-tip-grafted cultures were placed in a culture room at 25 C and exposed 16 hr daily to 40 $\mu E\ s^{-1}m^{-2}$ light.

**Transplanting to soil.** Successfully grafted plants were transferred from the test tubes to pots containing a commercial potting mix (Metro Mix 500, W. R. Grace Co.). The pots were covered with a transparent plastic cup to maintain humidity and incubated under a bank of artificial lights at approximately 25 C. Ventilation in the transparent cover was gradually increased over a several-week acclimatization period, and the cover was removed when the plants did not show stress when exposed to normal room humidity conditions. The plants were transferred to a glasshouse and evaluated for growth.

**Indexing procedures**

**Citrus canker.** Plants recovered by STG were examined for symptoms periodically for 9 months. At the termination of the experiment, washes from each plant were plated on two general and one selective media to detect any *X. campestris* present (17). The same procedures were used to test budwood sources following sterilization and *in vitro* culture. Pathogenicity was tested by detached-leaf assay (17).

**CTV.** Presence of CTV in STG progeny plants was detected by ELISA using a standard double-antibody sandwich procedure and polyclonal antiserum 1052 made to isolate T-36 (2, 16). Extracts were prepared from young stem or leaf midrib tissue at a ratio of 1 part tissue to 20 parts of 0.05 M Tris Buffer., pH 7.8. Plants were considered positive if the $OD_{405}$ was twice the value for the extracts of healthy tissue (or the reading of healthy extract plus 0.05 if the healthy extract reading was $< 0.05$).

**TLV.** Presence of TLV in STG progeny was evaluated by several procedures. STG plants which were stunted and chlorotic when propagated on Troyer citrange were considered suspicious for TLV. Biological assays were made by graft inoculation to rough lemon seedlings which were sub-

sequently top worked to Rusk citrange, and by mechanical inoculation to *Chenopodium quinoa* using extracts of young leaf tissue macerated at a 1 to 10 ratio in cold, 0.05 M Tris buffer, pH 7.8. Plants that were negative by bioassay were further indexed by ELISA using a polyclonal antiserum to the lily isolate of TLV (7) generously donated by M. Koizumi. A standard double-antibody procedure similar to that used for CTV was used, except that the conjugate was mixed with an extract of healthy citrus just prior to use to reduce nonspecific reaction to healthy plant antigens. The threshold for a positive reaction was twice the value for extracts of healthy tissue (usually in the range of 0.05 to 0.12).

**Greening.** The presence of greening was evaluated by periodic visual evaluation of STG progeny plants. Duncan grapefruit source plants were considered self-indicating. STG plants from sour orange and rough lemon were indexed on two Duncan grapefruit and two Orlando tangelo seedlings each. STG plants from sweet orange (also considered a self-indicator) were indexed by graft-inoculation to a single Duncan grapefruit. Where sources of greening were coinfected with CTV, STG progeny or graft-inoculated indicators with possible symptoms were also indexed for CTV by ELISA as described above.

**Naturally-spreading psorosis.** STG plants were observed periodically for symptoms. The host, Duncan grapefruit, was considered to be a self-indicator of the disease.

## RESUITS

**Tissue culture.** Overall, 90% of the budsticks of good quality sprouted when placed in culture and 57% of the total buds produced flushes. Each budstick produced an average of 2.9 flushes.

Budsticks from plants chronically infected with greening or severe isolates of CTV were of poor quality, sprouting was erratic, and most of the flushes produced were weak.

Flushes produced by budsticks cultured in media containing Virazole or antibiotics were somewhat yellowish and grew more slowly than the controls, apparently due to phytotoxic effects of these compounds.

Sprouting of the Washington navel budsticks used in the experiments for elimination of CTV and TLV was abnormally low even though the budsticks were in excellent condition at the time of collection. Many budsticks died and several flushes abscised at an early stage for unknown reasons.

The average incidence of successful grafts was 68% for shoot tips composed of the apical meristem and three-leaf primordia, and 80% for shoot tips composed of the apical meristem plus six-leaf primordia.

The average rate of survival of micrografted plants after transplanting to potting mix was 85%.

**Elimination of citrus canker.** *X. campestris* was not isolated from any of the 103 plants obtained by grafting *in vitro* shoot tips from budwood of Duncan grapefruit which was infected or contaminated with strains XC62 or F1 (table 1). Moreover, none of the STG plants developed lesions characteristic of citrus canker or bacterial spot diseases. This included plants regenerated from budwood with typical and severe lesions of the two dieases and from budwood heavily contaminated with bacterial suspensions made from pure cultures. Strain XC62 was easily eliminated, even by grafting very large shoot tips (up to 0.7 mm).

Indexing of budsticks from infected plants after 10-12 days in culture showed that the strain XC62 could be isolated from the original lesions, but not from asymptomatic tissue, whereas the strain F1 could not be isolated even from the original lesions. Both strains were readily isolated from contaminated budsticks which had been cultured for 10-12 days.

**Elimination of greening.** All 74 plants recovered by STG from plants infected with the B121 isolate of green-

TABLE 1
ELIMINATION OF PATHOGENIC XANTHOMONAS FROM INFECTED OR CONTAMINATED BUDWOOD OF DUNCAN GRAPEFRUIT BY GRAFTING *IN VITRO* SHOOT TIPS EXCISED FROM FLUSHES PRODUCED BY BUDWOOD CULTURED *IN VITRO*

| Type of material | Strain | Treatment | No. healthy plants/ No. indexed plants |
|---|---|---|---|
| Infected budwood | XC62 | 3-leaf primordia[z] | 24/24 |
| | XC62 | 6-leaf primordia | 19/19 |
| | F1 | 3-leaf primordia | 20/20 |
| Contaminated budwood | XC62 | Immersion for 30 sec[y] | 15/15 |
| | XC62 | Immersion for 120 sec | 24/24 |
| | F1 | Immersion for 30 sec | 11/11 |
| | F1 | Immersion for 120 sec | 15/15 |

[z]Shoot tip composed of the apical meristem plus 3-leaf primordia.
[y]Budwood immersed in suspension of $10^8$ bacterial cells per ml.

ing were free from greening, regardless of shoot tip size or use of antibiotics (table 2). Seventy plants of different hosts were generated by STG using shoot tips with three-leaf primordia from plants chronically infected with greening sources from Reunion, South Africa, Taiwan, the Philippines and the People's Republic of China. All of these plants were also free from greening (table 3).

**Elimination of CTV.** All plants of Valencia and Washington navel produced by grafting shoot tips with three leaf primordia were free from CTV (table 4). A number of CTV-free Washington navel plants were obtained using shoot tips with six-leaf primordia, but all Valencia plants generated from six-leaf primordia were infected (table 4). The inclusion of Virazole in the budstick culture medium did not improve recovery of CTV-free plants. Virus-free plants of four different varieties were recovered from plants chronically infected with 13 CTV sources which originated in citrus-growing areas of Africa, South and North America, and Asia (table 5). Seven of the 88 plants generated were still infected with CTV.

TABLE 2
ELIMINATION OF THE GREENING AGENT (B121) FROM INFECTED PLANTS OF VALENCIA SWEET ORANGE BY GRAFTING *IN VITRO* SHOOT TIPS EXCISED FROM FLUSHES PRODUCED BY BUDWOOD CULTURED *IN VITRO*

| Medium | Shoot tip size | No. healthy plants/ no. plants tested |
|---|---|---|
| Basal | 3-leaf primordia | 28/28 |
| Basal | 6-leaf primordia | 18/18 |
| Basal + anti-biotics[z] | 6-leaf primordia | 28/28 |

[z]Medium containing 0.5 g/liter penicillin and 1.25 g/liter tetracycline.

**Elimination of TLV.** TLV was more difficult to eliminate than CTV (table 4). Forty-two per cent of the micrografted plants were free of TLV when shoot tips with three-leaf primordia were used, and nearly all micrografted plants derived from shoot tips with six-leaf primordia were infected. The presence of Virazole in the budstick culture medium did not increase recovery of TLV-free plants.

**Elimination of naturally-spreading psorosis.** In a single experiment, using shoot tips with three-leaf primordia, six micrografted plants were obtained from infected Duncan grapefruit plants. All plants were free from this disease agent.

## DISCUSSION

The results described in this paper show that the technique of grafting *in vitro* shoot tips excised from flushes produced by budsticks cultured *in vitro* is very effective for exclusion of pathogens that cause the most severe citrus disases. Direct *in vitro* culture of imported budwood for STG without propagation on rootstocks greatly reduces

TABLE 3
ELIMINATION OF THE GREENING AGENT FROM CHRONICALLY INFECTED PLANTS BY GRAFTING *IN VITRO* SHOOT TIPS[z] EXCISED FROM FLUSHES PRODUCED BY BUDSTICKS CULTURED *IN VITRO*

| Strain | Origin | Host | No. healthy plants/ no. plants tested |
|---|---|---|---|
| B1 | Reunion | Duncan grapefruit | 8/8 |
| B1 | Reunion | Rough lemon | 12/12 |
| B1 | Reunion | Sour orange | 7/7 |
| B49 | South Africa | Madame Vinous | 3/3 |
| B121 | Taiwan | Duncan grapefruit | 6/6 |
| B121 | Taiwan | Orlando tangelo | 9/9 |
| B140 | Philippines | Duncan grapefruit | 3/3 |
| B140 | Philippines | Valencia | 6/6 |
| B142 | Philippines | Duncan grapefruit | 8/8 |
| B144 | China | Valencia | 7/7 |
| B144 | China | Duncan grapefruit | 1/1 |

[z]Shoot tips consisted of apical meristem plus three-leaf primordia.

potential hazards to the receiving country during an exchange of citrus germplasm.

Results of budstick culture obtained were typical for this type of work. The problems found with budsticks from plants chronically infected with greening, and to lesser extent with CTV, were apparently associated with the severe physiological damage induced by these pathogens. The budwood source plants were weak and chlorotic and yielded very low-quality budwood. The poor results obtained with budstick cultures of Washington navel remain unexplained and could be due to some unrecognized damage that occurred during transportation, or to some physiological problem in the source plant. The budwood was cut from vigorous plants grown in Orlando, Florida. Budwood was collected, brought by plane to Beltsville, Maryland, and cultured within 2 days. The problem was apparently not produced by the disinfesting treatment or culture conditions, since Valencia budwood collected from the same location and handled in the same manner grew normally.

Grafting success was higher than normal for routine work and demonstrates that, with some advance planning, large numbers of STG plants can be produced in a new location in a very short period of time. The success in

TABLE 4
ELIMINATION OF CITRUS TRISTEZA VIRUS (CTV) AND CITRUS TATTERLEAF VIRUS (TLV) BY GRAFTING *IN VITRO* SHOOT TIPS EXCISED FROM FLUSHES PRODUCED BY BUDSTICKS CULTURED *IN VITRO*

| Host | Treatment | No. healthy plants/ no. plants tested[z] | |
|---|---|---|---|
| | | CTV | TLV |
| Valencia | 3 LP[y] | 15/15 | 8/16 |
| Valencia | 6 LP | 0/8 | 0/8 |
| Washington navel | 3 LP | 15/15 | 6/17 |
| Washington navel | 3 LP + Virazole[x] | 9/9 | 2/12 |
| Washington navel | 6 LP | 6/8 | 1/8 |
| Washington navel | 6 LP + Virazole | 2/8 | 0/8 |

[z]Indexing for CTV by ELISA, indexing for TLV based on ELISA and biological assay on *Chenopodium quinoa*.
[y]LP = leaf primordia.
[x]Medium for budstick culture contained 50 mg/l Virazole.

TABLE 5
ELIMINATION OF CITRUS TRISTEZA VIRUS (CTV) FROM CHRONICALLY INFECTED PLANTS BY GRAFTING *IN VITRO* SHOOT TIPS[z] EXCISED FROM FLUSHES PRODUCED BY BUDSTICKS CULTURED *IN VITRO*

| Strain | Origin | Host | No. healthy plants/ no. plants tested |
|---|---|---|---|
| B1 | Reunion | Duncan grapefruit | 8/8 |
| B1 | Reunion | Rough lemon | 10/12 |
| B1 | Reunion | Sour orange | 7/7 |
| B49 | South Africa | Madam Vinous | 2/3 |
| B77 | Brazil | Madam Vinous | 2/3 |
| B128 | Colombia | Madam Vinous | 5/5 |
| B131 | Colombia | Madam Vinous | 1/1 |
| B133 | Peru | Madam Vinous | 8/8 |
| B6 | California | Madam Vinous | 1/2 |
| B140 | Philippines | Duncan grapefruit | 3/3 |
| B140 | Philippines | Valencia | 5/6 |
| B142 | Philippines | Duncan grapefruit | 8/8 |
| B81 | China | Madam Vinous | 8/8 |
| B82 | China | Madam Vinous | 4/5 |
| B144 | China | Valencia | 7/7 |
| B31 | Japan | Madam Vinous | 2/2 |
| | | Total | 81/88 |

[z]Shoot tips consisted of apical meristem plus three-leaf primordia.
[y]Indexing for CTV was by ELISA.

transplanting STG plants to potting mix was somewhat lower than normal (12), and part of the plant loss was attributable to the stunted and weak condition of TLV-infected STG scions on Troyer citrange. The combination of sweet orange on Troyer is apparently self-indexing for TLV, and many infected propagations were very weak and hard to propagate. Grafting successful young STG progeny to large, healthy rootstocks would have probably increased survival.

The tissue culture quarantine system effectively excluded citrus canker, a result which was expected but which needed documentation. The experiments mimicked the most adverse possible conditions, using budwood with canker lesions and budwood heavily contaminated with bacterial suspensions. The disinfestation of the budsticks prior to culturing eliminated strain F1 from infected budwood, but it was ineffective for eliminating the same strain in contaminated budwood. Disinfestation did not eliminate strain XC62 in infected or contaminated budwood. However, all plants recovered by grafting *in vitro* shoot tips excised from the flushes produced by the infected or contaminated budwood cultured *in vitro* were free from citrus canker. Elimination of the two strains used in the experiments was equally effective, and strain XC62 was readily eliminated from infested budwood, even by the use of large shoot tips with six-leaf primordia. Based on these results, it is unlikely that citrus canker and bacterial spot pathogens would ever be introduced via STG of source plants which had only low levels of contamination.

The greening organism was also easily eliminated. Even by grafting large shoot tips with six-leaf primordia, all of the plants recovered were disease-free. Plants recovered from budsticks cultured in media with penicillin and tetracycline were free from greening. These antibiotics are effective against the greening organism (3), but a positive influence on recovery of greening-free plants by the tissue culture method here could not be evaluated because the untreated budwood also yielded 100% greening-free plants.

The South African and the Asian types of greening were easily eliminated, even from chronically infected

source plants with severe symptoms; however, some plants were still infected with CTV. Use of indicators for greening which are reactive to severe isolates of CTV, such as Duncan grapefruit, requires additional testing to identify the specific pathogen.

CTV was easily eliminatd from infected budwood only when small shoot tips with three leaf-primordia were used. The use of shoot tips with six-leaf primordia was not equally effective. Even severe CTV sources were eliminated from chronically infected plants using shoot tips with three-leaf primordia. The 92% success rate was better than previously reported for greenhouse-grown plants (18, 19).

The presence of Virazole in the budstick culture medium did not improve recovery of CTV-free plants as expected. Virazole may not be effective for CTV, or the slower growth of flushes on the treated budsticks may increase shoot tip invasion by CTV. Additional experimentation is needed to determine the usefulness of viricides in combination with STG.

Tatterleaf virus was the most difficult pathogen to eliminate, but some TLV-free plants were recovered by STG. In two previous experiments TLV was not eliminated by grafting *in vitro* shoot tips with three-leaf primordia excised from flushes produced in glasshouse-grown plants (18, 19). Koizumi (8) recovered TLV-free plants by grafting *in vitro* 0.2-mm-long shoot tips excised from flushes produced by plants subject to a heat treatment of 32 days at continuous 35 C, or at 40/30 C for 9 days followed by 35/30 C for a minimum of 13 days.

The use of Troyer citrange as the rootstock provided a direct primary index for TLV. Nearly all propagations with poor vigor subsequently indexed positively for TLV, while vigorous propagations indexed negatively.

The naturally-spreading psorosis from Argentina was easily eliminated in the small experiment carried out. In previous work, a few plants free from this serious disease were recovered at relatively low efficiency by grafting *in vitro* shoot tips excised from field- or glasshouse-grown plants (1).

TLV remains the most difficult pathogen to eliminate by shoot-tip grafting. Additional experiments should be done using increased temperature for budstick culture and/or even smaller shoot tips to try to increase the efficiency. Because TLV is difficult to eliminate and a variety of detection procedures are available, it makes a good "marker virus" to evaluate STG efficiency.

Although it is possible to use relatively large shoot tips safely to exclude some severe pathogens, small shoot tips with a maximum of three-leaf primordia should be used where possible to increase efficiency and safety.

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