REGENERATION OF STRAWBERRY PLANTS FROM TISSUE CULTURES

EDWARD C.M. LEE and R. A. DE FOSSARD

Department of Botany,
University of New England,
Armidale, N.S.W. 2351, Australia

Abstract. Aseptic strawberry plants were obtained by culturing axillary buds from stolon scale leaves on medium-MLLM (3). When the basal stem region of these aseptic plants was cultured on medium-MMMM (3) in the light, numerous buds developed, each of which formed into a complete plant; repeated culture of the basal region of each plant thus formed constitutes a rapid method of clonal propagation, with a potential in one year of millions of plants from each original plant.

When the basal region of aseptic plants was cultured on medium-MMMM in the dark, callus was formed along with several etiolated shoots. Apical meristems from these etiolated shoots were much easier to excise than from field-grown plants. A programme for using a combination of meristem culture and heat treatment of buds and plants in culture tubes, is described for the production of virus-free material from virus-infected strains of strawberry.

Other methods for obtaining aseptic strawberry plants using tissue culture techniques are also described, including the regeneration of plants from callus derived from anthers. This is thought to be the first report of organogenesis from unorganized strawberry callus.

REVIEW OF LITERATURE

Our research with strawberries is aimed at ridding virus-infected strains of strawberries of their virus content by a combination of tissue culture, meristem culture and heat treatment. And, having done this and having obtained virus-free certification, to use rapid clonal propagation of this certified material through tissue culture techniques; our calculations indicate a potential of millions of clonal strawberry plants in one year from each certified virus-free plant.

One method of eliminating viruses from infected cultivars is by heat treatment of the mother plant but, at East Malling, some cultivars treated in this way remained infected (6). The use of single-bud cuttings following heat treatment removed crinkle virus from some clones but not from others, and few cuttings of some cultivars survived (7). Meristem culture holds more promise than stem tip culture for ridding virus-infected strains of their viruses. In general, the smaller the stem tip excised and cultured the greater the likelihood of obtaining virus-free cultures; the apical meristem, about 0.1 mm in length, is the ultimate in smallness in this respect and thus has the greatest potential for achieving the stated objective. Unfortunately, the smaller the stem tip the greater the difficulty of inducing it to grow in culture; meristems are thus the most difficult to grow in culture. Parenthetically, much that is described as meristem cuture, e.g. for *Cymbidium*, is in reality stem tip culture, often 5 or even 10 mm in length; such techniques are aimed at clonal propaga-

tion - not at virus elimination. The culture of strawberry apical meristems has been described by Belkengren and Miller (1) as a method of freeing *Fragaria vesca* strawberries of latent A virus. These authors (5) later reported freeing *F. vesca* from vein-banding and crinkle, and the cultivars Northwest and Rockhill from yellow virus complex. McGrew (4) eliminated latent C virus from plants of the Suwannee variety. Vine (9) reported freeing five cultivars from crinkle and vein chlorosis, and the elimination of yellow edge virus from 90 percent of cultured apical meristems of another cultivar. A generalized account of meristem culture for elimination of strawberry viruses has been given by Smith, Hilton and Frazier (8). Boxus (2) has reported a stem tip culture method for the rapid multiplication of strawberry clones.

MATERIALS AND METHODS

Strawberry cultivars: 'Kendall,' 'Red Gauntlet' and 'Torrey' (*Fragaria chiloensis* Duchesne var. *ananassa* Bailey) have been used at various stages of this research but most work has been done with the first two.

Sites of explants: Sites of explants are illustrated in Figure 1. Site A is the bud in the axil of the stolon scale leaf. Site B is the young plant at the end of the stolon prior to the development of roots. Site C is the immature flower.

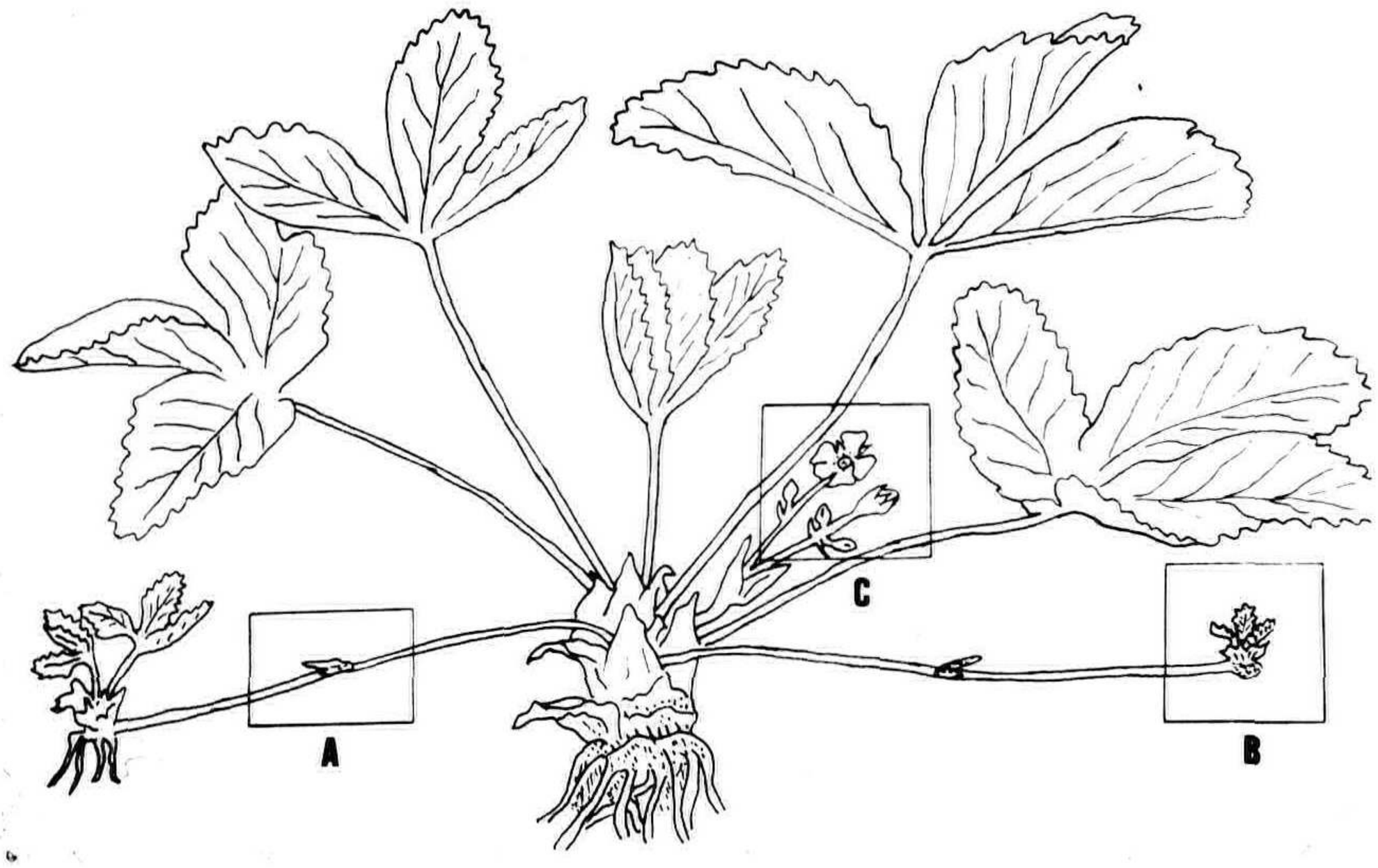

Figure 1. Diagrammatic representation of a strawberry plant with runners showing explant sites A, B and C, which are, respectively, the bud from the axil of a stolon scale leaf, the young plant without roots at the tip of the stolon, and the anthers and receptacle of immature flowers.

Disinfestation of explant sites: Site A: stolons with tightly closed scale leaves were selected. The scale leaf part of the stolon

was cut off, washed in detergent (in lots of ten nodes at a time), disinfested with chlorinated lime (saturated solution, freshly prepared and filtered before use) for 20 min., and then rinsed in two changes of sterile water. The axillary buds were then excised aseptically and individually disinfested with chlorinated lime (10 min.), rinsed twice in sterile water, and placed on the surface of autoclaved culture medium. *Site B:* the young plants were cut off from the stolon and the apex, leaves and basal region removed, followed by paring off the surface layers of the remaining piece; these pieces were then dipped in alcohol and flamed briefly, disinfested in chlorinated lime (20 min.), followed by a second paring off of the surface layers, and further disinfestation in chlorinated lime (20 min.) and three washes in sterile water; the explants were then placed on culture medium. *Site C:* Immature flower buds were dipped in alcohol and flamed briefly and disinfested in chlorinated lime (20 min.) followed by three washes in sterile water; anthers and receptacles were then aseptically excised from the flower buds and placed on the surface of culture medium.

Culture media: A broad spectrum experiment (3) was used to find media suitable for the culture of explanted materials (sites A, B, C), for the initiation of callus, for callus growth and for the regeneration of plants from the callus. The broad spectrum experiment consisted of combinations of four broad categories of constituents, namely: (1) minerals, (2) auxins, (3) cytokinins and (4) sucrose plus growth factors plus amino acids, each at three concentrations, low, medium and high. This gives an experiment with 81 treatments (media). When necessary, other substances such as coconut milk, yeast extract and casein hydrolysate, are also included in the experiment. These experiments resulted in the selection of two broad spectrum media, codes MLLM and MMMM, for bud culture, callus induction and regeneration of plants from calluses. These two media are defined in Table 1. It may later be found that several of the listed constituents are not essential, and that others are not at their optimal concentrations. (A booklet describing the logistical aspects of preparation of these broad spectrum media will be sent on request). The main differences between medium-MMMM and -MLLM are that the former has ten times more auxins and cytokinins than the latter.

Incubation conditions: Site A cultures were incubated in 12/12 (h light/dark regimen) at 25°C, and illuminated during the photoperiod with Gro-Lux fluorescent lights (2,000-16,000 lux). Sites B and C and callus cultures were incubated in the dark at 25°C. Only limited experimentation with incubation treatments has been done so far; other regimens might be as good or better than those described.

Table 1. Constituents and their concentrations of two broad spectrum media (MLLM and MMMM) selected for strawberry tissue culture.

Macronutrient elements (mmol.1^{-1})
NH_4NO_3 (10), KNO_3 (10), NaH_2PO_4 (1), $CaCl_2$ (2), $MgSO_4$ (1.5)

Micronutrient elements (μmol.1^{-1})
H_3BO_3 (50), $MnSO_4$ (50), $ZnSO_4$ (20), $CuSO_4$ (0.1), Na_2MoO_4 (0.1), $CoCl_2$ (0.5), KI (2.5), $FeSO_4$ (50), Na_2EDTA (50), Na_2SO_4 (450)

Main carbon source (mmol.1^{-1})
Sucrose (60)

Growth factors (μmol.1^{-1})
Inositol (300), Nicotinic Acid (20), Pyridoxine. HCl (3), Thiamine. HCl (2), Biotin (0.2), Folic Acid (1), D-Ca-Pantothenate (1), Riboflavin (1), Ascorbic Acid (1), Choline Chloride (1).

Amino acids (μmol.1^{-1})
L-Cysteine. HCl (60), Glycine (5)

Auxins (μmol.1^{-1})
MLLM-medium 1 μmol.1^{-1} of each of the following auxins, in contrast to MMMM-medium with 10 μmol.1^{-1} of each auxin: IAA (indole acetic acid), IBA (indole butyric acid), NAA (α-naphthalene acetic acid), NOA (2-naphthoxy acetic acid), 2,4-D (2,4-dichlorophenoxy acetic acid), pCPA (*para* chlorophenoxy acetic acid).

Cytokinins (μmol.1^{-1})
MLLM-medium 1 μmol. 1^{-1} of each of the following cytokinins, in contrast to MMMM-medium with 10 μmol. 1^{-1}: Kinetin, BAP (benzyl amino purine).

RESULTS

Bud Culture: The first experiment was with axillary buds from site A. There were 2^5 (=32) treatments (media) using low and medium concentrations of the four broad spectrum categories of constituents; half of the experimental media included 150 ml coconut milk per litre. There were five replicates and a total of 160 cultures. The experiment was examined after two weeks incubation in 8/16 (*h light/dark*) at 25°C. *More cultures on coconut* milk media had grown and were healthy looking than on plain media, and particularly good cultures were associated with the medium concentrations of minerals and auxins. On plain media, the best treatment was MLLM, that is, the medium concentrations of minerals and sucrose plus growth factors plus amino acids, and the low concentrations of auxins and cytokinins. The experiment was prematurely terminated due to a fault in an incubator.

A second experiment using coconut milk media only was planned but, because of the non-availability of coconuts at that time, an experiment was done with the plain medium-MLLM using various additives which were possible substitutes for *coconut milk. The additives were: glutamine (1 mmol.*1^{-1}), *yeast* extract (1 g.1^{-1}), casein hydrolysate (1 g.1^{-1}) and a fourth treatment included all three additives. One half of this experiment was done with agar (8 g.1^{-1}) and the other half was made up as liquid media using a filter-paper "thimble" to support the cultures. There were

10 treatments and 20 replicates, and the cultures were incubated at 12/12 (h light/dark) at 25°C. The experiment was examined after 3 weeks incubation and medium-MLLM without additives supported the best and healthiest looking cultures; none of the additives improved the growth of the cultures. The cultures on agar media suffered an average 63% loss due to microbial contamination, whereas cultures on liquid media had a 35% loss. This was a reflection of the greater growth of contaminating organisms on agar media and, in consequence, their earlier detection. Non-contaminated cultures on agar medium-MLLM were as healthy as those on liquid medium-MLLM. So, for two reasons, namely ease of detection of contaminated cultures and ease of preparation and handling, it was decided to standardize bud culture on agar medium-MLLM. A later planting with nearly 100 buds resulted in only 15 percent contaminated cultures using the disinfestation methods described above.

Induction of callus from anthers and receptacles: Anthers were excised from immature flower buds (4-5 mm in length), and from buds with petals exposed but only slightly opened. Ten anthers per culture tube were used in the case of the smaller buds, whereas 4 to 8 anthers per tube were planted from the more mature buds; one receptacle per tube was also planted from each mature bud. There were 81 broad spectrum treatments.

Callus formation on anthers from the smaller buds was associated mainly with the low concentration of sucrose plus growth factors plus amino acids and, in the case of anthers from older buds, it was also associated with the high concentration of cytokinins. These calluses were subcultured on medium-MMMM (judged on qualitative criteria as supporting the best callus growth of both 'Kendall' and 'Red Gauntlet') and, after two passages (sub-cultures) some calluses produced roots and one (derived from anthers from the more mature buds cultured initially on medium-MMHM) produced shoots. These examples of organogenesis are evidence of the totipotency of anther tissues. In addition, some calluses from receptacle explants produced roots when subcultured on medium-MMMM.

The culture which produced shoots continued to produce several buds when subcultured again on medium-MMMM in the dark (Fig. 2). When pieces of these calluses plus shoots were subcultured on the same medium but incubated in 12/12 (h light/dark) at 25°C, the shoots developed into whole plants, with healthy fibrous root systems and green leaves; numerous buds developed from the basal part of each plant (Fig. 3). Buds and plants from this source have, at the time of writing, been subcultured six times on medium-MMMM always with the same result: whole plants with 12/12 incubation and callus plus shoots (but no roots) on dark incubation. However regeneration of shoots from calluses

in the dark were less vigorous on each of the later subcultures.

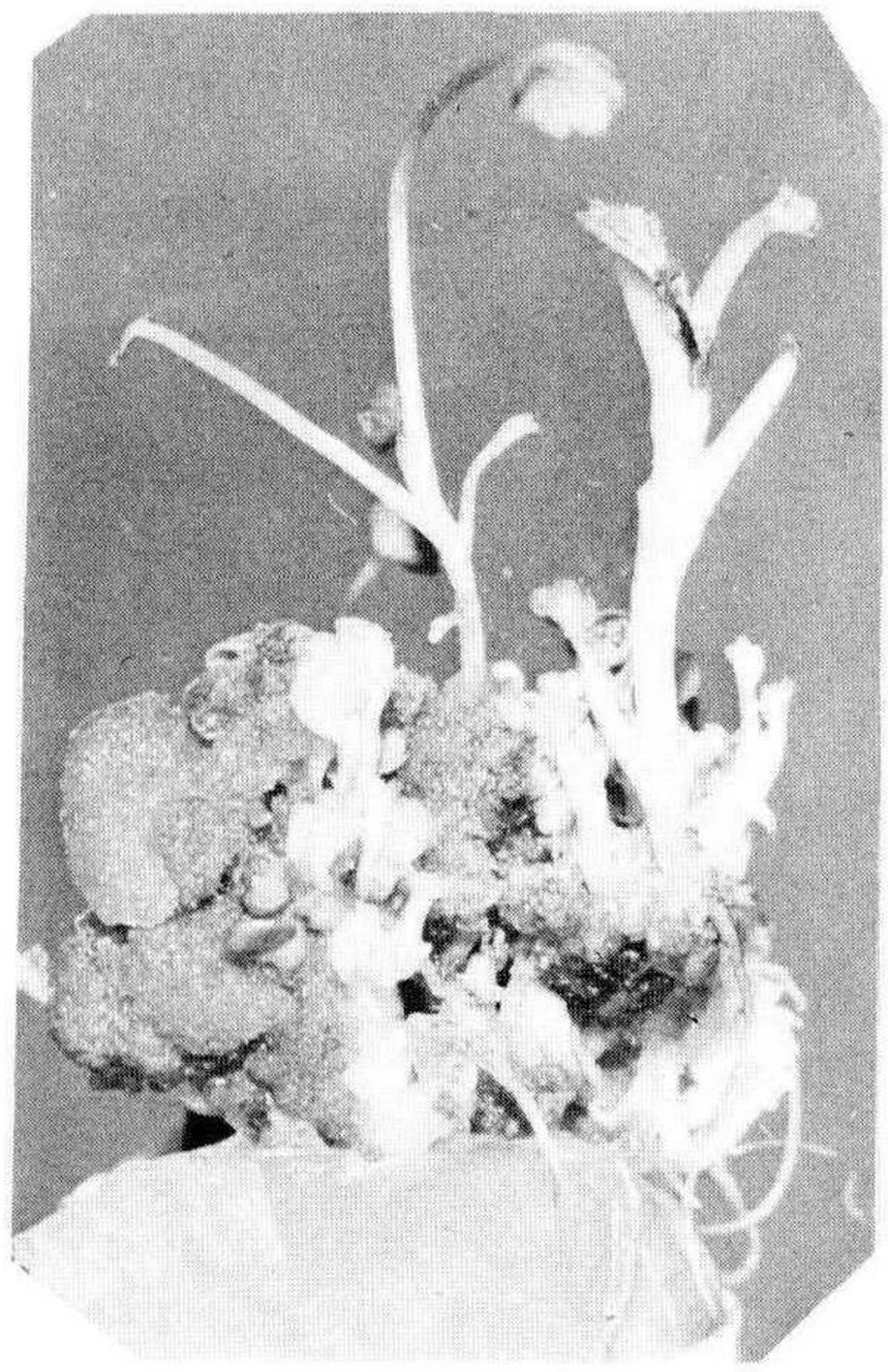

Figure 2. Etiolated shoots from callus of anthers cultured on medium-MMMM in the dark at 25°C (magnification x 7).

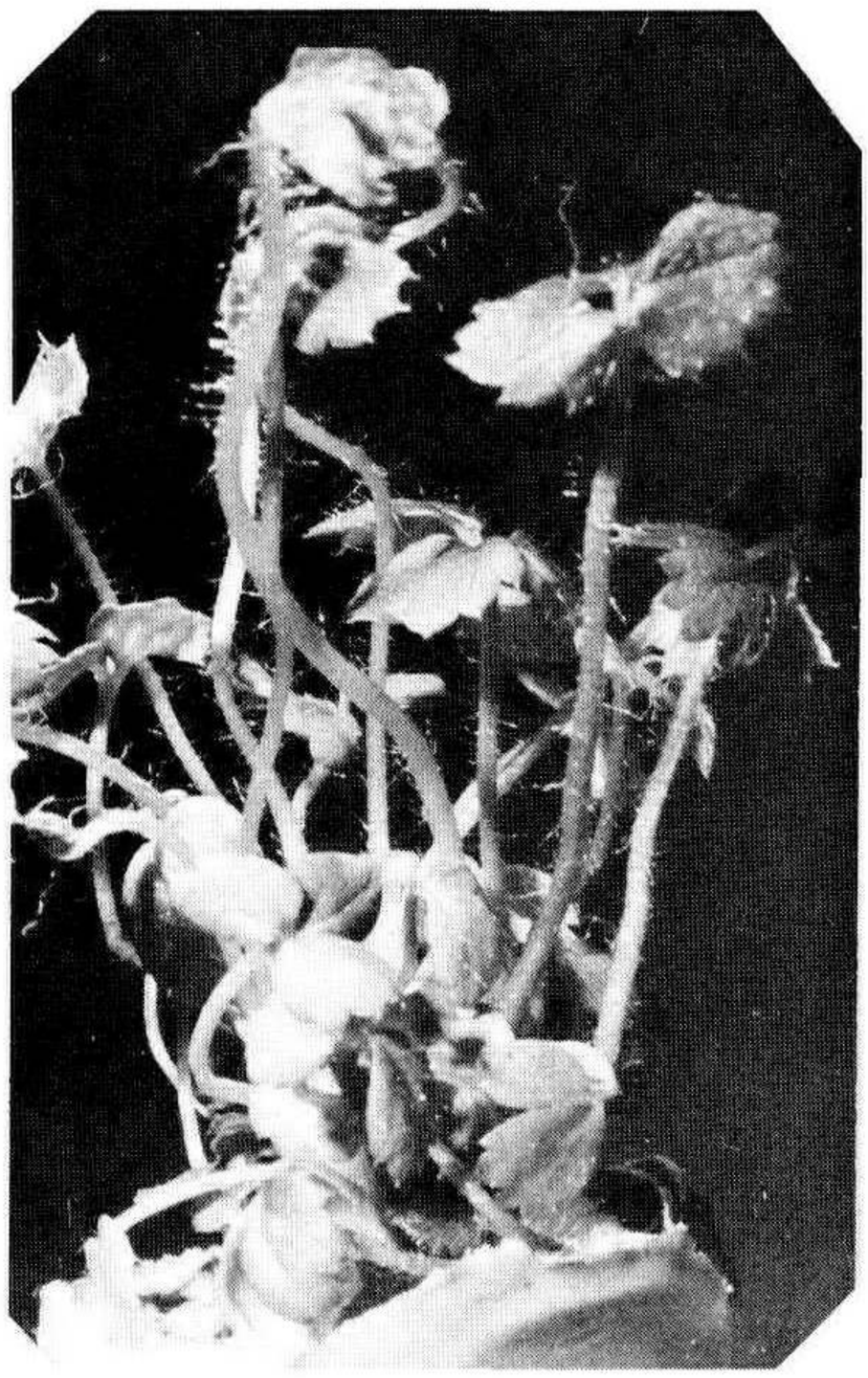

Figure 3. Complete plants with numerous buds at the base; cultured on medium-MMMM in 12/12 (h light/dark regimen) at 25°C (magnification x 7).

Callus and shoot induction from aseptic plants: Site A buds were cultured on medium-MLLM and then transplanted on medium-MMMM (both with 12/12 incubation). Pieces of stems containing buds, excised from vigorous plants, were then cultured in the dark. These developed callus and a few etiolated shoots and, when transplanted to fresh medium-MMMM and incubated in 12/12, these etiolated shoots developed roots and formed complete, healthy plants. Again, numerous buds developed from the basal part of each plant. Each of these buds formed a complete plant when subcultured on fresh medium-MMMM and exposed to 12/12 incubation, yet again with the formation of numerous buds at their base.

Site B explants planted directly on medium-MMMM, avoiding the initial medium-MLLM step, developed callus and buds on dark incubation in the first passage, and later transfer to fresh medium and 12/12 incubation again led to the formation of complete plants with numerous buds at the base of each plant.

DISCUSSION

The results described above have led to the following strategy for our forthcoming research with virus-infected strains of strawberry:

1. Culture the buds from the axils of stolon scale leaves of virus-infected strains on medium-MLLM and incubate in 12/12 (h light/dark regimen) at 25°C. If stolons are not produced, culture buds from the axils of leaves on the plant. Transfer of young plants to medium-MMMM accelerates the development of these plants.
2. Pieces of stem-containing buds are then cultured on medium-MMMM in the dark for induction of etiolated shoots.
3. Excise apical meristems from the etiolated shoots of the cultures prepared in (2); apical meristems are very much easier to dissect from aseptic etiolated shoots than from field-grown plants; culture the meristems on experimentally-determined suitable media.
4. Transfer shoots that develop from (3) to medium-MMMM and incubate in 12/12 (h light/dark) at 25°C.
5. Excise the numerous buds that develop on the complete plants produced in (4) and culture on fresh medium; repeat this process to obtain a stock of putative virus-free material.
6. Rear some of these plants under normal conditions for virus-indexation and virus-free certification by appropriate authorities.
7. Virus-free certified material can then be rapidly multiplied

by repeated subculture on medium-MMMM in 12/12 incubation, prior to release to specialist runner-producers and growers; virus-free material would be maintained in culture tubes rather than in insect-proof houses, the present practice.

Meristem culture alone may be sufficient to rid virus-infected strains of their viruses. This assumption will be tested as an integral part of the above programme concurrent with the testing of a new way of heat-treatment in combination with meristem culture. The usual practice is to heat-treat whole plants, and this is followed by application of disinfestation treatments, excision and culture of either stem tips or meristems. However the disinfestation is rarely complete and more often substantial loss of cultures is incurred due to microbial contaminants. Moreover, poor growth of excised meristems in culture could be, at least partially, attributable to tissue injury caused by either the heat treatment, the disinfestation treatment, or to both. We plan to reduce, and in the case of disinfestation treatment to avoid, tissue injury by heat-treatment of *aseptic* buds, plants, calluses and/or meristems in culture tubes. Heat treatment of plant tissue in culture tubes would avoid dessication injury to the tissue because the tissue is in contact with an aqueous-based medium. This way of heat-treatment, if successful in the elimination of viruses, would have the additional advantage of being more controllable than heat treatment of whole plants.

Finally, we wish to emphasize two results: one is the induction of organogenesis, both roots and shoots, in calluses derived from tissues (anthers, receptacles) which normally would not develop either of these organs. The second result relates to the culture of twice-pared explants of young stolon-plants (site B explants) in which callus was initiated and which simultaneously initiated buds. Theoretically, the paring off of superficial layers should have removed all buds since these have a superficial (exogenous) origin. We are currently investigating whether such explants (site B explants) have deeper-seated buds or whether *de novo* organogenesis, as with anthers and receptacles, has occurred.

Acknowledgments. A Commonwealth Post-Graduate Scholarship to E.C.M. Lee is gratefully acknowledged. We thank Mr. R. A. Bourne for his excellent technical assistance, and Dr. R. C. Smith (Department of Agronomy, University of New England) for his comments on this paper.

LITERATURE CITED

1. Belkengren, R. O., and P. W. Miller. 1962. Culture of apical meristems of *Fragaria vesca* strawberry plants as a method of excluding latent virus. *Plant Disease Reporter* 46:119-121.

2. Boxus, P. H. 1974. The production of strawberry plants by *in vitro* mi-

cropropagation *J. Hort. Sci.* 49:209-210.

3. De Fossard, R. A., Aung Myint and E. C. M. Lee. 1974. A broad spectrum tissue culture experiment with tobacco (*Nicotiana tabacum*L.) pith tissue callus. *Physiol. Plantarum* 31:125-130.
4. McGrew, J. R. 1965. Eradication of latent C virus in the Suwannee variety of strawberry by heat plus excised runner tip culture. *Phytopathology* 55:480-481.
5. Miller, P. W., and R. O. Belkengren. 1963. Elimination of yellow edge, crinkle and vein banding viruses and certain other virus complexes from strawberries by excision and culturing of apical meristems. *Plant Disease Reporter* 47:298-300.
6. Posnette, A. F., and R. Cropley. 1958. Heat treatment for the inactivation of strawberry viruses. *J. Hort. Sci.* 33:282-288.
7. Posnette, A. F., and A. Jha. 1960. The use of cuttings and heat treatment to obtain virus-free strawberry plants. *Report. East Malling Res. Stn.* 1959: 98.
8. Smith, S. H., Hilton, R. E., and N. W. Frazier. 1970. Meristem culture for elimination of strawberry viruses. *California Agriculture* 24:9-10.
9. Vine, S. J. 1968. Improved culture of apical tissues for production of virus-free strawberries. *J. Hort. Sci.* 43:293-297.

QUESTIONS

In reply to questions it was stated that neither the cutting of such small meristems nor the heat treatment had induced observable mutants but that the progeny must be watched closely for any such development.