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# CLONING CROPS IN A CELSS VIA TISSUE CULTURE: PROSPECTS AND PROBLEMS

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## ABSTRACT

Micropropagation is currently used to clone fruits, nuts and vegetables and involves controlling the outgrowth *in vitro* of basal, axillary or adventitious buds. Following clonal multiplication shoots are divided and rooted. This process has greatly reduced space and energy requirements in greenhouses and field nurseries and has increased multiplication rates by greater than 20-fold for some vegetatively-propagated crops and breeding lines. Cereal and legume crops also can be cloned by tissue culture through somatic embryogenesis. Somatic embryos can be used to produce "synthetic seed", which can tolerate desiccation and storage and germinate upon rehydration.

Synthetic seed of hybrid wheat, rice, soybean and other crops could be produced in a controlled environment life support system (CELSS). Thus yield advantages of hybrids over inbreds (10% to 20%) would be exploited without having to provide additional facilities and energy for parental-line and hybrid seed nurseries. In our laboratory media costs for producing 1000 viable somatic embryos of wheat are about $ 0.12. This compares to $ 0.02 per 1000 for hybrid seed produced commercially and $ 0.40 per 1000 when seeds are produced in controlled environments with artificial lighting. Mass and energy requirements for seed and propagule production in a lunar or martian CELSS will be substantially reduced by innovations in micropropagation and synthetic seed technology.

## INTRODUCTION

The list of agricultural crops cloned *in vitro* for research, breeding or commercial purposes has expanded rapidly in recent years (Table 1). The rationale for using tissue culture for terrestrial applications includes rapid multiplication rates, low energy and space requirements, and maintenance of specific genotypes. This rationale will likely be of even greater importance in an extraterrestrial controlled environment life support system

Table 1. Partial listing of crops that are cloned *in vitro* for either research, breeding, hybrid seed production, commercial production, or multiplication of virus-free nuclear stock.

| Crop | Explant of choice | *In vitro* system[1] | Purpose[2] | References |
|---|---|---|---|---|
| Cereals | | | | |
| Wheat | Immature embryo & inflorescence | SE | GE, SV, SS | 1, 2 |
| Barley | Immature embryo | SE | GE, SV | 3 |
| Maize | Immature embryo | SE | GE, SV | 4, 5 |
| Rice | Immature embryo & inflorescence | SE | GE, SV | 6 |
| Sorghum | Embryos, shoot tip | SE | GE, SV | 7 |
| Legumes | | | | |
| Soybean | Immature embryo | SE | GE | 8, 9, 10 |
| Vegetables | | | | |
| Carrot | Hypocotyl, meristem | SE | BR, SS | 11, 12, 13 |
| Cassava | meristem tip, mature embryo | AvB, SE | VE, MNS, SS | 14, 15 |
| Cocoyam | shoot tip | AvB | VE, MNS | 14 |
| Hausa potato | leaf | AvB | MNS | 14 |
| Taro | shoot tip | AvB | VE, MNS | 14 |
| Potato | Axillary bud, petiole, tuber disc, meristem | ABB, AvB | VE, MNS, SV | 16, 20 |
| Sweet potato | Meristem, tuber disc, petiole | SE, AdB | SS, MNS | 14, 17, 18 |
| Sweet yam | corm segment | AdB | MNS | 14 |

Table 1 (cont.).

| Crop | Explant of choice | *In vitro* system[1] | Purpose[2] | References |
|---|---|---|---|---|
| Vegetable crops cont. | | | | |
| White yam | Mature embryo, nodal cutting | SE, AdB | VE, CM, SS | 14, 19 |
| Papaya | Axillary bud | AdB | CM | 20 |
| Artichoke | Apical bud | ABB, AdB | CM | 21 |
| Asparagus | Basal bud | ABB, AdB | CM, MNS | 20, 22 |
| Celery | Immature petiole | SE | SV, SS | 23 |
| Lettuce | Leaf | SE | SV | 24 |
| Mustard | Immature embryo | SE | SS | 25 |
| Cucumber | Leaf | SE | SS | 26 |
| Sugar and oil crops | | | | |
| Sugarcane | Meristem, leaf | SE | SV, SS | 27, 28 |
| Sugarbeet | Lateral bud, petiole | ABB, AdB | MB | 29 |
| Oil palm | Embryo, leaf | SE, AdB | MB | 30 |
| Fruit and nut crops | | | | |
| Tomato | Embryo, leaf, hypocotyl | SE, AvB | GE, SV, MB, SS | 31, 32 |
| Strawberry | Immature embryo, meristem | SE, ABB, AvB | VE, SS, CM SV, MPN | 16, 22, 33, 34, 35 |
| Raspberry | Apical and axillary bud | ABB, AvB | VE, CM | 22, 35 |
| Blackberry | Root, Apical & axillary bud | ABB, AvB | VE, CM | 22, 35, 36 |
| Blueberry | Axillary bud | ABB, AvB | CM | 22, 35 |

Table 1 (cont.).

| Crop | Explant of choice | *In vitro* system[1] | Purpose[2] | References |
|---|---|---|---|---|
| **Fruit and nut crops cont.** | | | | |
| Peach & nectarine | Shoot tip | AvB | CM | 37 |
| Apricot | Shoot tip | AvB | CM | 37 |
| Apple and crabapple | Bud | AvB | CM | 37, 38 |
| Cherry | Root | AvB | CM | 37, 39 |
| Plum, prune | Shoot tip | AvB | CM | 37 |
| Pineapple | Shoot tip & axillary bud | AvB | CM, MNS | 14, 16, 40 |
| Banana | Axillary bud, corm | AvB | CM | 14, 16, 41 |
| Grape | Anther, ovary, node | SE, ABB, AvB | SS, CM | 35, 42, 43 |
| Date palm | Lateral bud | SE, AvB | SS, CM | 44 |
| Pecan walnut chestnut filbert | Shoot tip | AvB | CM | 37 |
| Spice and fiber crops | | | | |
| Caraway | hypocotyl | SE | SS | 45 |
| Cacao | Immature embryo | SE | SS | 46 |
| Coffee | Leaf | SE, AvB, ABB | SS, CM | 47 |
| Cotton | Cotyledon | SE | BR, GE | 48 |

[1]ABB, Axillary or basis buds; AvB, adventitious buds; SE, somatic embryogenesis

[2]BR, basic research; CM, commercial micropropagation; GE, genetic engineering; MB, micropropagation for breeding purposes; MNS, micropropagation for nuclear stock; SS, synthetic seed research; SV, somaclonal variation; VE, virus eradication

(CELSS). The purpose of this article is to summarize the commercial use of *in vitro* cloning with todays crops and to describe areas of research important to the application of *in vitro* cloning for food production in a CELSS.

## TISSUE CULTURE AND CROP PRODUCTION

Plant regeneration *in vitro* occurs through one of three developmentally-distinct processes: branching from normally-formed basal or axillary buds, branching from adventitiously-formed buds, or somatic embryogenesis. Cloning from basal, axillary or adventitious buds involves cuttings from vegetative tissues and is thus a form of vegetative propagation. The term "micropropagation" is reserved for these processes. In contrast multiplication by somatic embryogenesis is not a vegetative process but involves the formation of entirely new plants, usually of single or near single cell origin, without the cutting and rooting procedures associated with micropropagation. Procedures for cloning horticultural, agricultural or forestry crops by somatic embryogenesis are still largely in a developmental stage.

Shoot formation (or branching) from preexistent basal or axillary buds occurs when dormancy or quiescence of buds is released *in vitro* by hormone treatments. Tissues generally do not dedifferentiate and there is no callus intermediate. Hence this process is considered to be genetically stable. Shoots produced are cut into pieces that contain axillary or basal nodes and the process is repeated. The multiplication potential is calculated

by the formula $x=n^c$, where x = the total number of plants produced, n = the average number of propagules produced per explant during each cycle of multiplication, and c = the number of multiplication cycles. Generally values of n range from 5 to 10 and multiplication cycles range from 3 to 6 weeks in duration.

In the second process adventitious buds form directly from dedifferentiated cells of the explant or from cells of a callus intermediate. Genetic and epigenetic stability are more readily compromised in this process, which may cause somaclonal variation (49), especially when a callus intermediate is involved. The multiplication procedure is similar to that described above. While multiplication cycles are typically of a long duration (4 to 10 weeks), the n value from the formula listed above can be much higher (50 to 100), which results in a higher overall multiplication rate. Most micropropagation systems involve shoot formation from a mixture of both preexistent and adventitious buds. Rates of multiplication by tissue culture are often far superior to those obtained by conventional procedures (Table 2).

Micropropagation is important not only for commercial production but for cloning male sterile, gynoecious or polyploid parental lines used to produce hybrid seed. For example male sterility is a homozygous recessive trait in tomatoes. Multiplication of these parental lines by seed requires use of heterozygotes as pollinators. Segregation from the required crosses results in an undesirable 1:1 ratio of male sterile to male fertile plants. The latter plants must be manually removed from hybrid seed production nurseries upon their identification at flowering. Micropropagation

Table 2. Multiplication rates of selected crops by micropropagation and conventional propagation.

| Crop | Propagules produced[1] | *In vitro* | Conventional | References |
|---|---|---|---|---|
| | | ----------years---------- | | |
| Potato | 125 | 0.2 | 2 | 16 |
| Pineapple | 40-380 | 1.0 | 5-9 | 40 |
| Stone fruit | 9-35 | 0.1 | 1-3 | 37 |
| Strawberry | 20 | 0.1 | 1 | 22 |

[1]Number of propagules produced is squared or cubed when time invested (*in vitro* or conventional) is doubled or tripled.

of these male sterile lines offers an attractive alternative. Similar situations exist for asparagus, cucumber, broccoli and triploid hybrids such as watermelon and sugar beets (see 50 for a review). *In vitro* procedures are being developed not only for clonal propagation but for somaclonal variation, genetic engineering or other research or breeding purposes (Table 1).

## SEED AND PROPAGULE PRODUCTION IN A CELSS

The operation of a CELSS will be limited by availability of human resources. Because of this limitation crops selected for a CELSS will be restricted to those crops where sowing, crop growth, harvesting, and seed or propagule processing can be automated. The CELSS Initial Reference Configuration (Nov. 1988) identifies a plant growth facility, for production of crops, seeds and propa-

gules, and a storage facility, where the cleaning and storage of seeds and propagules will occur. Most procedures associated with these facilities lend themselves to current automation technology.

*Micropropagation.* Any system proposed to improve or streamline conventional procedures of crop production in a CELSS will need to be automated. On earth, where human resources are plentiful, micropropagation is beginning to replace conventional procedures for seed and propagule production (Table 1). Labor, energy and/or space considerations are driving private-sector decisions in this direction. Costs of skilled labor for micropropagation are becoming cheaper than the greenhouse, nursery and labor costs of conventional propagation. In contrast the human labor variable in a CELSS will be heavily weighted, and micropropagation will need to meet a higher level of automation than is currently employed on earth.

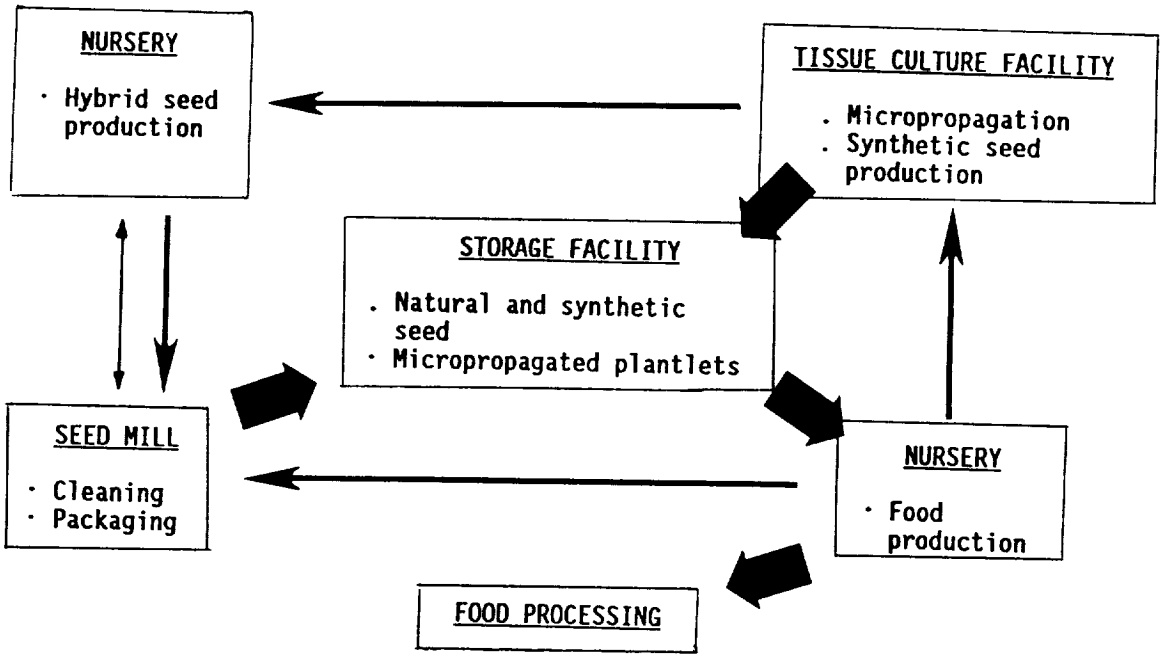
Automating micropropagation will be complicated. Micropropagation usually involves at least one mechanical cutting for every propagule produced. Cuttings often need to be made in precise locations to assure proliferation of additional shoots. Furthermore micropropagated tissues are sensitive to desiccation and mechanical injury. Thus machinery designed to handle these operations must "visualize", to a greater or lesser extent, the tissue to be propagated, make decisions based on "visual" images, and manipulate and slice tissues of varying sizes in a delicate manner. Finally nearly all of these operations must be conducted aseptically. Progress in tissue culture automation is being made (51)

but equipment designs appropriate for a CELSS probably will not be available for some time. An automated micropropagation system could be used in a large-scale CELSS to clone potato, asparagus, cucumber, sugar beet, yam, plantain, papaya, pineapple, banana, raspberry, strawberry, grape, filbert, coffee and others (Table 1).

In addition to being automated, innovations for increasing yields in a CELSS must also be energy efficient. For example nurseries for parental-line and hybrid seed production could be incorporated into the plant growth facility. This would permit the exploitation of hybrid vigor, which often means a 10% to 20% increase in yield. However, even with automation, yield advantages simply might not justify the added mass and energy required to maintain parental and hybrid seed nurseries. In this respect a combined approach where an automated micropropagation system is used to eliminate some of the parental-line nurseries may justify production of hybrid seed (Fig. 1). Currently crops for which this strategy may be of value include broccoli, cauliflower, cucumber, tomato, watermelon and sugar beets (Table 1).

*Synthetic seed production.* Somatic embryogenesis is the highest expression of cellular totipotency in plant tissue cultures and offers the greatest potential in terms of mass cloning and automation. With carrot as many as 5000 uniform somatic embryos can be obtained within 14 days from 1 ml of packed cells of a cell suspension (52). Unfortunately rates of production of somatic embryos of other crops are generally much lower and, even in the carrot system, most somatic embryos are abnormal and fail to germinate.

Figure 1. Seed and propagule production in a CELSS, current capabilities . Currently tissue culture could reduce space and energy requirements for production of propagules of many crops (Table 1). Most seed and propagules would be produced conventionally by the lower loop, nursery to seed mill to storage facility to nursery.




Nevertheless procedures for selectively multiplying highly totipotent "embryogenic" cells and inducing these to form somatic embryos are being improved for numerous crops (Table 1).

Research efforts are now focusing not only on inducing somatic embryogenesis but on defining conditions that cause normal embryo development. Recently procedures were developed for producing somatic embryos of carrot without use of an exogenous auxin. Embryos so produced are more normal and can be encapsulated in calcium alginate for "synthetic seed" production. Germination

rates are as high as 50 % (13). Synthetic seed technology and its future application have recently been reviewed and discussed by Redenbaugh et al. (53, 54).

A goal in our laboratory is to understand and increase the production of embryogenic cells in wheat tissue cultures. We have observed increases by specifically altering the type of media and auxin used (55, 56, 57), by reducing oxygen availability to tissues (1), and by pretreatments that alter endogenous hormone levels prior to tissue culture (58, 59). We are also exposing embryogenic cells to environments that simulate *in ovulo* conditions. Partial simulation of *in ovulo* oxygen, hormone, and desiccation environments has increased numbers of somatic embryos produced by six-fold (3600 per gm of callus) and have increased germinability of somatic embryos from 10 % to 40 % (1, 60).

Synthetic seed technology may be perfected by the time a lunar CELSS is constructed (approx. 2015). This technology will probably involve 3 to 4 stages. The first stage will occur in suspension culture where embryogenic cells will be mass produced. By definition such cells are capable of immediately beginning to form somatic embryos if exposed to appropriate conditions. However, during the first stage, conditions will remain inappropriate for both embryo formation and for multiplication of nonembryogenic cells.

Proliferation of embryogenic cells will be instantaneously terminated in the second stage. This will be followed by a synchronous initiation of embryogenesis. Conditions appropriate for embryo initiation and early formation may not be satisfactory for

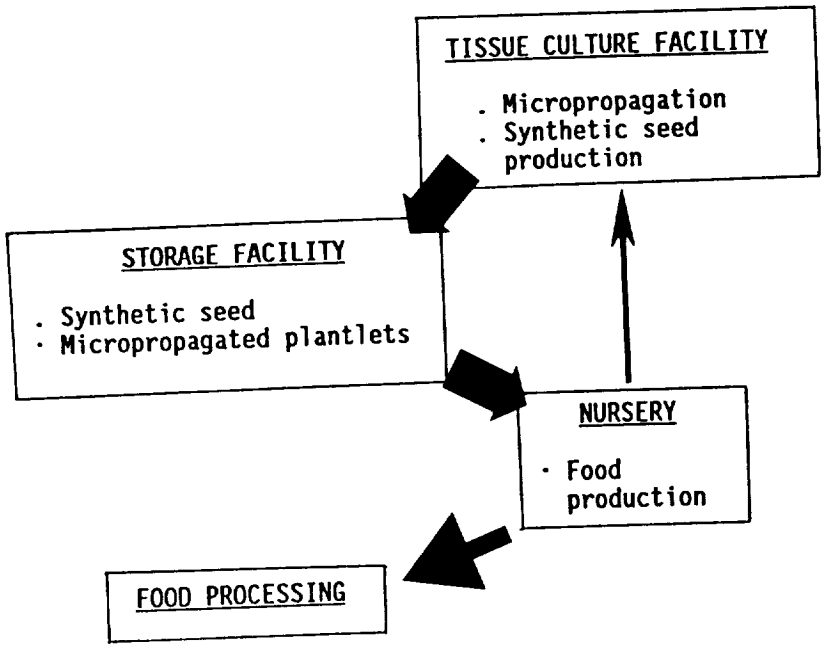
embryo maturation (1, 60, 61, 62). Thus a third stage, for embryo maturation and desiccation, will probably be required. Somatic embryos of albuminous species (food reserves of seeds associated with endosperm) will require encapsulation with an artificial endosperm (53, 54). Encapsulation might not be required for somatic embryos of legume and various other dicotyledonous crops, where food reserves are primarily stored in the cotyledons (63).

*Costs of synthetic seed: what to expect.* Replacement of true seed with synthetic seed in a lunar CELSS (Fig. 2) could be cost effective. Yields would be higher because harvested material would not be used as seed (3 % to 5 % savings in yield) and hybrids (10 % to 20 % yield advantage) could be used without the mass and energy drains of conventional parental-line and hybrid seed nurseries. Furthermore this technology could be used with nearly all crops.

It is difficult to predict what the costs of synthetic seed will be 25 years from now. However the economics of synthetic seed will certainly be more attractive in a lunar CELSS than on earth. This is because production of true seed in a lunar CELSS will require supplemental lighting for from 50 % to 100 % of the entire production cycle. In contrast somatic embryogenesis requires little or no light.

On earth pure line seed of wheat is purchased for about $ 0.01 per 1000 while hybrid seed is about twice this much. The energy cost of producing 1000 pure line wheat seed in a controlled environment with 100 % supplemental lighting is 40-fold higher, approx.

Figure 2. Seed and propagule production in a CELSS, future capabilities. By 2015 tissue culture may nearly eliminate space and energy requirements for conventional production of seeds and propagules. Vegetative propagules and synthetic hybrid seed (of numerous crops) would be produced through the upright loop, nursery to tissue culture facility to storage facility to nursery.




$ 0.40 (calculations assume a $ 0.05 per KWH energy cost and current production levels at Utah State University, 64). In our laboratory somatic embryos of wheat are produced in the dark at ambient temperatures with a media cost per 1000 viable embryos of $ 0.12 (calculated from production and germination data in 1). Energy costs are negligible.

If the costs of media and energy rise proportionately when produced in a CELSS, then true seed will remain approx. 4x more expensive. Furthermore production of 1000 viable somatic embryos requires about 75 $cm^3$. The area required to produce 1000 true seed

in a controlled environment is about 200-fold greater, or 15,000 $cm^3$. Clearly if somatic embryogenesis can be perfected and automated, then substantial savings in energy and space should be achievable. Automated systems of micropropagating potato, yam, sweet potato, asparagus and others (Table 1) may also be cost effective in terms of the mass and energy constraints of a lunar or martian CELSS.

Our cost analysis of wheat synthetic seed assumes use of current somatic embryogenesis technology, which is far from optimal. In our system callus is produced on semi-solid medium and nearly 50 % is nonembryogenic. Another problem is a structural interconnection of embryos that reduces germination frequencies and requires that plantlets be separated manually. Our goal is to produce fine suspensions of uniformly-embryogenic cells that will synchronously form singular somatic embryos. Such a system is being approached with carrot (11) where the cost of media per 1000 somatic embryos is approx. $ 0.01 (based on 5000 somatic embryos per ml packed cells, a 5:1 ratio of embryogenic suspension to packed cells, and a 40:1 ratio of embryo induction medium to packed cells). Development of such a system for wheat could reduce media costs per 1000 somatic embryos to about $ 0.02.

## *CONCLUSIONS AND RECOMMENDATIONS*

Micropropagation systems are becoming more cost effective than conventional propagation systems, particularly for certain vegetable and fruit crops and for male-sterile, gynoecious or polyploid parental lines used to produce hybrid seed (Table 1). In a lunar

CELSS conventional propagation will require high light intensities from artificial lights. In contrast micropropagation requires low intensities and can be accomplished in a much smaller area. Such variables could make automated systems of micropropagation attractive for numerous crops.

By the time a lunar CELSS is constructed (approx. 2015), private industry may have replaced many micropropagation and true seed systems with synthetic seed, particularly for high cash-value crops. It is doubtful that private industry will apply these innovations to major field crops, where the cost of natural seed is extremely low. However energy and mass limitations in a lunar CELSS may present a very different scenario. Advantages of producing synthetic seed of wheat over true seed in a CELSS could include a reduction in cost of as high as a 95 %, a reduction in required space of as high as 99.5 %, yield increases of 3 % to 5 % due to harvested seed not being used in the sowing of subsequent crops, and yield increases of 10 % to 20 % due to the use of hybrids. Both micropropagation and synthetic seed technology should receive further investigation in terms of providing mass and energy savings in a future CELSS.

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