system we have achieved improved rooting percentages and/or improved plant quality in apples, maples, blueberries, and other plants. Although optimizing plant media is not the answer to all our problems, it is a very important tool for us at Microplant Nurseries.

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LONG-TERM STORAGE TECHNIQUES FOR IN VITRO PLANT GERMPLASM[1]

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The United States Department of Agriculture has established a National system of Plant Germplasm Repositories whose goals are to collect, maintain, evaluate, and distribute plant material of economically important crops. These crops are stored as seeds or as living plants. The Corvallis Repository is a clonal repository responsible for the maintenance of pears, filberts, mint, hops, and all the small fruit crops. Plant

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material maintained includes genetically important foreign and domestic cultivars, and undeveloped species germplasm collected from around the world. At each clonal repository a tissue culture program aids in the maintenance, distribution, and health improvement of the germplasm collection.

The tissue culture lab's most important function is to maintain a back-up collection of all germplasm stored in the repository. This collection provides replacement plants for screenhouse and field collections. Eventually, the *in vitro* back-up collection could replace the labor-intensive, costly, screenhouse collection (3). To do this, ways must be found to retard *in vitro* plant growth and reduce the labor involved in long term storage. The subject of this paper is the different methods currently being investigated by the Corvallis Repository tissue culture lab to extend the storage life of *in vitro* germplasm collections.

STORAGE IN GROWTH ROOM CONDITIONS

Standard growth room conditions for cultures reported in this paper were 23°C and a light level of 25 $\mu Em^{-2}sec^{-1}$ for 16 hr/day. Cultures were grown in 13×100mm tubes, with 3.5 ml medium unless otherwise indicated, using 10 tubes per treatment.

When left on the shelf in a standard growth room, some *in vitro* shoots and plantlets maintain themselves until the culture medium is depleted. Under standard growth room conditions, four *Mentha* species survived from 6 to 13 months, depending upon their growth rate before medium depletion. *Rubus spectabilis* lasted 12 to 15 months, and 'Merton Thornless' blackberry lasted 13 to 16 months. *Vaccinium ovatum* was viable for 17 months. Moisture loss was occasionally hastened by having too thin a film of wax around the cap, or by perforation of the film seal. Use of self-sealing caps that allow gas exchange, but not water vapor exchange, eliminated this problem.

In an attempt to extend shelf life by suppressing growth, clones of six genera were grown with and without plant growth regulators (PGRs). 'Merton Thornless' blackberry cultures without PGRs were slightly stockier and darker green than those with PGRs and, after 8 months, were ranked in better overall condition. *Mentha* × *dumetorum* without PGRs had growth similar to cultures with PGRs, while mint(USDA 10467) without PGR exhibited slightly more vigorous growth than those with PGRs. All mint cultures remained viable until the medium was depleted, which occurred sometime after 8 months. Strawberry cultures of 'Bounty' and 'Dabreak' with PGRs proliferated shoots to the point of overcrowding, which

caused browning of the medium, tissue browning, and death within 3 months. Cultures without PGRs had only one or two shoots and thicker, longer petioles; cultures were viable for 5 months, at which time the medium was about depleted. Survival of strawberries 'Midway', 'Morioka 17' and 'Climax' was extended by 1.5, 2.0 and 3.5 months, respectively, where PGRs were not included in the medium.

Vaccinium ovatum cultures with PGRs were in excellent condition after 9 months, while those without had shorter shoots, more basal callus, and were slightly chlorotic after 6 months (experiment in progress). Cultures of *V. uliginosum* showed opposite results: PGR cultures began to decline after 6 months, while those lacking PGRs were in excellent health at 7 months (experiment in progress).

Cultures of *Ribes* (ORUS-11) with PGRs were crowded, and the medium brown, after 2 months. The medium was depleted during the 7th month. Cultures without PGRs died the first month. In other experiments with *Ribes* (ORUS-11), browning of the culture media containing PGRs was reduced by increasing the initial pH of the medium to pH 7.0 or pH 8.0 (initial pH of controls: pH 5.7), or by increasing agar content to 8 g/l or 10 g/l (controls: 6 g/l agar).

These results illustrate that elimination of PGRs cannot be used as a standard method for growth suppression, and that clonal response differences are apparent. In some clones, elimination of PGRs resulted in decreased vigor or death, while in other clones it resulted in improved plant vigor and/or culture longevity.

Another attempt to slow shoot growth involved increasing agar content from 6g/l (control) to 8 and 10 g/l (experiment in progress). The increased agar content resulted in healthier cultures for 'Merton Thornless' blackberry, mint (USDA 10487), *Mentha* × *dumetorum*, and *Vaccinium ovatum* when evaluated after 8 months. Control cultures of *Vaccinium uliginosum* died by the 6th month, while high agar cultures were still viable at 8 months. Cultures of *Humulus lupulus* in a treatment using 12 g/l agar were ranked in even better condition after 8 months than cultures grown in 8 or 10 g/l agar, although all were in viable condition. However, increased agar content had no observable effect on longevity of *Ribes* (ORUS-11) or 'Bounty' and 'Dabreak' strawberries. Increased agar content of the medium retards the rate of medium depletion in several genera. This may contribute to increasing culture longevity.

Another method of suppressing growth is to increase the osmotic potential of the medium by increasing the sugar content. *Mentha* cultures evaluated after 8 months (experiment in

progress), are being grown in 0.088M sucrose as the control, 0.222M sucrose and 0.222M glucose. Shoots of *Mentha aquatica* growing in 0.222M sucrose had smaller leaves and less height than controls, with heavy purpling of stems and leaf veins; their average time to death was 8 months. In the 0.222M glucose cultures, *M. aquatica* had small dense clumps of shoots and were very dark green for the first three months; thereafter, growth resulted in depletion and browning of the medium, similar to sucrose controls. In both the sucrose controls and 0.222M glucose treatments, 9 of 10 cultures were viable in the 8th month. In contrast to *M. aquatica*, *M. arvensis* showed only small differences between treatments for the first 2 months, and ultimately all treatments depleted the medium in an average of 6 months. Thus, increased sugar content did affect growth during the first few months, but did not always result in an extended shelf-life.

Decreasing the sugar content of the medium reduces the carbon source, forcing a reliance on photosynthetic products for new shoot growth. Media were prepared with 8g/l agar, glucose at 0, 0.03, 0.06, and 0.09 M concentrations, and no PGRs. Each tube contained approximately 5 ml medium per tube. *Mentha aquatica* had similar vigor at all 4 glucose levels, with leaves becoming darker and slightly smaller as the glucose level increased. Evaluated at 5 months, cultures were in good condition. Strawberry 'Dabreak' also grew very well in all 4 concentrations of glucose. Cultures in 0.09M glucose rooted, but died by the 4th month following browning of shoots and media. In a similar way, all of the 0.06M cultures and half of the 0.03M cultures were dead by the 5th month. Seven of 10 cultures grown without glucose were in good health when evaluated at 5 months. These results show that growth rate in strawberry cultures can be slowed by decreasing the glucose level.

The sugar-alcohols, mannitol and sorbitol, have been added to media to retard shoot growth. Codaccioni studied the effects of mannitol and glucose on *Mentha spicata* [syn. *M. viridis*] cultures.(1) Some of her findings were: 1) mannitol delays rooting, but ultimately results in more roots; 2) mannitol breaks apical dominance and stimulate lateral branching; and, 3) without the addition of a metabolizable sugar, mannitol is unable to support plant growth. Repository experiments with *Mentha* and mannitol supported the first two findings, but not completely the third. Experiments with *Mentha aquatica* and *M. arvensis*, using 0.028, 0.056 and 0.111M concentrations of mannitol combined with 0.111M glucose, revealed an inverse relationship between mannitol concentration and shoot height. When mannitol and glucose were increased, multiple branching and shortening of internodes occurred. Vitrification

(translucent tissue) also increased. Higher levels of 0.222M glucose + 0.222M mannitol were extremely injurious to mint, blackberry, gooseberry, strawberry, hops, and blueberry. Of two clones of *Mentha* grown in 0.028 and 0.111M mannitol without glucose, *Mentha arvensis* did not grow, while *M. aquatica* did. In 0.111M mannitol, *Mentha aquatica* was very stunted, highly branched, dark green and intensely vitrified, yet half of the cultures were viable in the 7th month. At the 0.028M level, there was no vitrification, less branching, slight chlorosis, and healthy shoots; 9 of 10 cultures were viable when evaluated in the 8th month. Only 6 of 10 control cultures in 0.111M glucose were viable. The same two mint clones were grown in 0.028M and 0.111M sorbitol, without glucose. In the lower concentration, sorbitol slowed growth without producing the great morphological changes seen with mannitol. When evaluated at 8 months, 50% of the *M. arvensis* and 100% of the *M. aquatica* cultures were viable. Growth of *M. arvensis* in the high sorbitol concentration was similar to growth in the low concentration, and viability was the same. Growth of *M. aquatica* in the high concentration was extremely branched and vitrified, yet all 10 cultures were dark green and vigorous at 8 months. In general, sorbitol appeared to effectively retard growth, while causing less severe symptoms as compared to mannitol.

Another attempt to suppress plant growth and extend storage life was by adding a growth retardant to the standard media.(2) Three concentrations of Cycocel [11.8% (2-chloroethyl) trimethylammonium chloride], 10, 20, and 30 mM, were tested on several genera. All concentrations were lethal to *Ribes* (ORUS-11) and *Vaccinium uliginosum*. With *V. ovatum*, leaves stayed green for 6 and 8 months in the 10 and 20mM treatments, respectively, but no new growth appeared. In 'Merton Thornless' blackberry, 20 and 30 mM levels led to severe stunting and death. The 10mM cultures grew well developed, dense clumps of shoots with very short internodes. After 8 months the pressure of the shoot mass against the culture tube prevented the mass from descending in the tube with the medium, resulting in a separation of shoot mass from medium. In *Humulus lupulus*, the 20 and 30 mM treatments were injurious, whereas the 10mM cultures were all viable, with growth similar to the controls, when evaluated at 7 months. Cultures of strawberries 'Dabreak' and 'Bounty' were more chlorotic as the Cycocel level increased. In all 3 treatments, shoot proliferation caused overcrowding and browning of the medium, just as in the controls. Half of the cultures in all treatments were dead by the 4th month. In contrast to the gooseberry, bluebery, blackberry, hop and strawberry cultures, mint cultures grew in all Cycocel concentrations. The higher

concentrations caused shortening of internodes, thickening of leaf blades and stems, and a deeper green color. In *Mentha dumetorum*, the 3 Cycocel treatments and the controls had a 50% death rate by the 8th month. Mint (USDA 10487) treated with 30mM Cycocel also had a 50% death rate by the 8th month, yet the control, 10 and 20mM Cycocel treatments had only a 10% death rate. Tolerance to Cycocel appears to be highly specific. Further experiments should clarify its usefulness in germplasm preservation.

STORAGE IN COLD TEMPERATURES

A commonly used cold storage temperature for *in vitro* cultures is 4°C. Mullin stored 50 *Fragaria* clones for 6 years at this temperature, in the dark. However, under these conditions regular replenishment of the liquid medium was necessary.(4) *In vitro* germplasm collections at the Corvallis Repository have been held for varying lengths of time at 5°C, in darkness. The following general response was observed for *Fragaria*, *Mentha*, *Rubus*, and *Vaccinium* cultures during a year of storage: etiolation, root growth, gradual depletion of medium, and gradual browning of medium. Contaminations occurred as endogenous bacteria or surface spore multiplied to visually observable levels. In some cases of older *Rubus*, *Fragaria* and *Vaccinium* cultures, development of new basal shoots occurred as the primary shoot-tip died. *Rubus spectabilis* cultures placed in 5°C storage were green and healthy at 4 months. At 6 months the shoots had brown leaves and stems, which appeared dead, but were actually dormant. Transfer to the growth room resulted in 39 of 40 cultures developing shoots from axillary buds. In single cultures of 27 cultivars of *Pyrus*, stored more than 8 months, the original leaves blackened and abscissed. This was followed by new, but etiolated, growth from the shoot tip. Germplasm storage at 5°C does retard growth, but is accompanied by gradual decline of tissues and browning of media.

Results of germplasm stored at −1°C have ranged from poor to excellent, depending on the genus and sometimes the clone. Three hundred clones of *Mentha*, stored for a year, exhibited no growth and no tissue breakdown; when placed in the growth room for 1 week, growth was vigorous. However, this new tender growth succumbed when cultures were returned to −1°C storage. Cultures of *Mentha spicata* [syn. *M. cordifolia*], *M. longifolia*, and *M.* × *dumetorum* survived 2½ yrs of uninterrupted −1°C storage in good condition. *Fragaria chiloensis* and 'Midway', evaluated after 17 months of storage at −1°C, were in good condition. In another experiment (in progress), the strawberries 'Climax', 'Morioka 17' and 'Midway' were grown with and without PGRs at temperatures of 1°C,

5°C and 23°C. After 11 months of storage the healthiest cultures emerged from 5°C.

Stock cultures of *Ribes* (ORUS-11) and *R. diacanthum*, as well as cultures of a dozen other *Ribes* clones, stored well for 1 yr at −1°C whereas cultures began dying after several months at 5°C. Storage of several hundred *Rubus* clones showed mixed results, suggesting broad genetic variation in tolerance to cold storage.

These experiments, and others not reported here, show that *in vitro* germplasm storage at −1°C and 5°C is highly promising. Where clones are sufficiently cold tolerant, −1°C storage is preferable, as it seems to suspend plant growth. Suspension of plant growth reduces or eliminates problems of medium depletion, etiolation, and delayed contamination during storage.

POSSIBILITIES FOR FUTURE RESEARCH

Currently under investigation at the Corvallis Repository is a preservation method suggested by Boxus, where defoliated stems are submersed in the culture medium and placed into cold storage. (Seminar, Oregon State University, 1983) *Pyrus* shoots 1.5 to 2.0cm long were submerged and placed in 23°, 5° and −1°C storage. After 4 months, the shoot tips remained green and were somewhat swollen. As yet it is too early to determine the value of this technique.

Of the methods for prolonging storage life and suppressing growth outlined in this paper, cold storage at 5°C and −1°C have been the best. Much more work needs to be done with altered and amended media. The use of various types and concentrations of growth retardants needs to be expanded. Cold-hardening treatments could be tried as pretreatments for long-term cold storage. Combinations of media amendments plus cold storage should be very promising. The research on prolonging storage life of *in vitro* cultures is in its infancy. For germplasm repositories, this research will be of long duration due to the tremendous genetic variability encountered in large, diverse plant collections.

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