

Application of gas-permeable bags for *in vitro* cold storage of strawberry germplasm

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Summary. This study reports the first use of gas-permeable, heat-sealable polyethylene bags for cold storage of plant tissue cultures. The bags were used to develop a new cold storage system for the *in vitro* strawberry collection at the National Clonal Germplasm Repository (NCGR), Corvallis. *In vitro* *Fragaria* plantlets of 96 different accessions (species and cultivars) were transferred to bags with basal medium without growth regulators, heat-sealed, grown for one week at 25°C, cold hardened for one week, and then stored in the dark at 4°C. These *in vitro* cultures were successfully stored for up to 24 months in polyethylene bags. Evaluations at three month intervals provided information on the condition of the diverse collection. Over 75% of the accessions originally stored remained in storage for 15 months and 47% remained for over 18 months. None of the 96 accessions studied was lost due to contamination or decline in vigor. Over 300 *Fragaria* accessions are currently stored using this system.

Keywords: Gas-permeable bags-Cold storage-*Fragaria*-Micropropagation

Abbreviations: N⁶-benzyladenine (BA); indole-3-acetic acid (IAA); gibberellic acid (GA₃).

Introduction

Tissue culture conservation of germplasm has changed very little in recent years. Cold storage conservation methods for *in vitro* cultures of *Malus*, *Prunus*, *Pyrus* and other genera utilize a variety of similar methods (Lundergan and Janick 1979; Druart 1985; Wanas et al. 1986; Wilkins et al. 1988). Two storage procedures for

strawberry were developed in the 1970s. Mullin and Schlegel (1976) used a liquid medium in 13 mm tubes with filter paper bridges at 4°C and Damiano (1979) stored plantlets in jars and large tubes on solid medium at 2°C. Contamination rates were not mentioned in these reports, however, loss of stored cultures due to contamination was reported as a major problem by others (Nord and Hanniford 1989; Marino et al. 1985).

Most *in vitro* germplasm collections are stored on agar-solidified media in glass tubes, jars or plastic boxes (Wilkins et al. 1988). Tubes have the positive attributes of small size and reuse but are subject to breakage, contamination and are difficult to handle and store. Boxes and jars are easier to handle and provide larger growing spaces for the plants, but also require more storage space and are at risk for contamination. A container which provides a long storage time, requires little space and decreases or eliminates contamination risk would improve storage of *in vitro* collections.

The use of cold hardening to improve survival of cold-stored cultures was suggested by Gunning and Lagerstedt (1985). Cold-hardened apple and saskatoon berry *in vitro* cultures were 4 to 8°C hardier after 10 weeks of treatment than untreated cultures (Caswell et al. 1986). Angelo (1939) determined several points about the cold hardening of potted strawberry plants: plants harden at 0°C to resist freezing at -5°C; more than 7 days does not increase hardiness; and daily alterations of 0 and 20°C produced greater hardiness than continuous exposure at 0°C. A modification of this technique was used to harden shoot cultures of pear (Reed 1990) and blackberry (Reed 1988) for cryopreservation.

The introduction in 1988 of gas-permeable, heat-sealable, polyethylene bags for *in vitro* culture provided a potentially useful alternative to tubes, jars and boxes. We have used these bags and a cold hardening regime (Reed 1988) to develop a cold storage system for *in vitro*

germplasm collections at NCGR, Corvallis. The first use of gas-permeable, heat-sealed polyethylene bags and cold hardening for cold storage of *in vitro* germplasm is reported here.

Materials and methods

Growth conditions. *Fragaria* plantlets were aseptically initiated from runners of pot-grown greenhouse plants and multiplied on medium with Murashige and Skoog (1962) salts and (per liter): 170 mg sodium phosphate (monobasic), 80 mg adenine sulfate, 1 mg BA, 1 mg IAA, 0.01 mg GA₃, 3 g agar (Bitek agar, Difco, Detroit, MI) and 1.25 g Gelrite (Kelco, San Diego, CA). Growth room conditions were 16 h (25 mol · m⁻² · s⁻¹) days at 25°C. Cold-hardening conditions were 8 h days at 22°C and 16 h nights at -1°C for one week. Storage was at 4°C in the dark.

Preparation for storage. *In vitro* plantlets 2-3 cm in length were transferred to bags (CultuSak, Becton Dickinson, Lincoln Park, NJ) heat sealed, grown for one week in the growth room, and then placed in cold hardening conditions for one week before storage. For each accession, five plantlets were stored, each in an individual section (15 x 150 mm) of a five-section bag with 10 ml per section of basal medium without growth regulators. In preliminary experiments a firmer medium (3 g agar and 1.5 g Gelrite) was found to be necessary to compensate for the low level of water loss through the bag walls.

Accessions stored. The strawberry accessions stored included: *F. chiloensis* (L.) Duch.; *F. moschata* Duch.; *F. vesca* L.; and *F. virginiana* Duch. selections as well as hybrids; *Fragaria x ananassa* Duch. cvs.: Aliso, Blakemore, Canoga, Dana, Dover, Fairfax, Florida Belle, Francesco, Fresno, Himiko, Kurume, Lateglow, Marlata, Massey, Perle de Prague, Rubin, Sierra, Sunrise and breeders selections.

Evaluation. The condition of cold-stored *in vitro* plantlets was evaluated every three months. Each culture was rated on a scale of 0 to 5 with 0 dead, 1 very poor (plant soft, etiolated), 2 poor (plant declining but with meristems intact, would not survive until the next inventory), 3 good (solid tissue, healthy leaves and meristems), 4 very good (moderately vigorous) and 5 excellent (vigorous). Cultures were all rated five when placed into storage. At each inventory accessions rated at two or below were removed from storage for repropagation so that no plants were lost.

Results

The storage system developed here, using cold hardening and heat-sealable polyethylene bags, maintained strawberry germplasm in good condition for 9 to 24 months. Evaluations at three month intervals provided information on the condition of the collection. None of the 96 accessions studied was lost due to contamination or decline in vigor.

Length of Storage

Plants in storage were rated quarterly (Fig. 1). Accessions rated 2 or below were removed for

repropagation, so the mean ratings reflect only those remaining in storage at a particular inventory. At six months, 99% of the original 96 accessions remained in storage with a mean rating of 4.31. The largest decline occurred at 9 months with 19% removed for repropagation.

Very little change occurred at 12 months but by 15 months another large decline occurred with 28% of the original 96 removed for repropagation. At 18 months 47% of the original plants were still in storage.

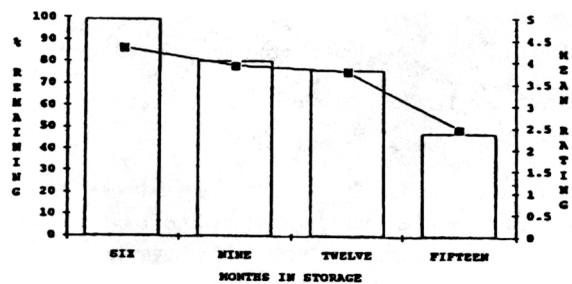


Fig. 1. The mean ratings of *Fragaria* accessions stored for 6, 9, 12 or 15 months in polyethylene bags (squares) and the percent of the 96 original accessions remaining in storage (bars).

Mean Ratings

Most *Fragaria* cultures remained in excellent to very good condition for 12 months (Table 1). All accessions were rated 5 (Excellent) when stored. Rating the cultures at three-month intervals provided consistent information on the survival in storage of the many cultivars studied.

Cultures declined no more than two units on the 0-5 rating scale within a 6 month period and most declined by one unit or less. A larger percentage of cultures was repropagated at 15 months than at earlier inventories. However, even then many cultures remained rated at 3 or above and continued in storage for many more months. *F. virginiana*, *F. chiloensis* cv. Del Norte and *Fragaria x ananassa* cvs. Lateglow, Jerseybelle and Surecrop remained in storage for 24 months.

Media Considerations

The levels of agar and Gelrite were optimized in preliminary experiments. Bags with standard tube or box concentrations of agar and Gelrite had large amounts of condensation which contributed to water-soaking of the stored plants and early decline. Firmer gels produced by increasing the Gelrite concentration by 0.25 g/l decreased the level of condensate in the bags but allowed for good growth and water availability.

Hardiness Levels

Preliminary experiments indicated that additional hardiness was produced in strawberry accessions by a cold-hardening regime used for hardening cultures for cryopreservation (Reed 1988), so it was incorporated as a part of the storage procedure.

Table 1. Ratings over time of some *Fragaria x ananassa* cultivars stored in gas-permeable polyethylene bags at 4°C in the dark. The rating scale is 0 = dead, 5 = excellent.

Cultivar	Months in storage				
	Zero	Six	Nine	Twelve	Fifteen
Aliso	5	5	4	4	2
Blakemore	5	5	4	4	2
Dana	5	5	4	4	3
Dover	5	5	5	4	2
Fairfax	5	4	4	4	2
Florida Belle	5	5	4	4	3
Francesco	5	5	3	3	2
Fresno	5	5	4	4	2
Himiko	5	5	4	4	3
Kaiser's Samling	5	5	4	3	1
Kurume	5	5	3	2	1
Lateglow	5	5	5	4	3
Mariate	5	5	4	4	3
Massey	5	5	5	4	3
Perle de Prague	5	5	4	4	2
Primella	5	4	4	4	4
Rubin	5	5	4	4	3
Sierra	5	5	3	3	1
Sunrise	5	5	4	4	3
Mean ratings	5.0	4.9	4.0	3.7	2.4

Discussion

The use of gas-permeable polyethylene bags, cold hardening and quarterly evaluations provides the basis for a new system of *in vitro* germplasm storage. Variations due to genotype and the addition of accessions at different intervals mandate a regular review of stored materials to insure high survival rates in any system. This is well illustrated by the range of survival, 10 to 100%, of 16 strawberry cultivars cold stored in tubes and jars during a 9 to 27 month period (Damiano 1979). With storage in bags and quarterly evaluations, declining cultures were identified and repropagated as needed so that none of the 96 accessions were lost due to lack of vigor.

Ratings of individual cultivars over time (Table 1) reflect the variability present in germplasm collections,

with some showing consistent ratings and others a gradual decline. Semi-annual evaluations were sufficient to identify declining *Fragaria* cultures when all were initially stored simultaneously. However, if cultures are periodically added to the cold storage collection, evaluations should be done quarterly.

The bag storage system described here requires manipulation of plant material only when it is in need of repropagation. At that point, usually after 15 months of storage, most accessions required only one passage in the growth room before being returned to 4°C. The work load of repropagation was spread out over the two year storage period. Forty-seven percent required no attention for over 18 months. Damiano (1979) observed that stored strawberries required from one to three passages to restore vigor to the culture before further storage.

Any manipulation of cultures such as adding medium also increases the risk of contamination. Contamination is minimized with storage in sealed bags and contamination in one chamber will not spread to other chambers of the bag. High contamination rates such as those observed in tubes of *Pelargonium* shoot cultures (Nord and Hanniford 1989) and jars of *Prunus* rootstock cultures (Marino et al. 1985) put stored germplasm at risk. The storage system described here virtually eliminates contamination from outside sources and requires repropagation of plants only as needed. Seventy-five percent of the plants remained in storage for 15 months and none were lost due to contamination. Although some cultivars survive for up to six years in glass tubes with the liquid medium procedure of Mullin and Schlegel (1976), the amount of time and effort involved to add medium at three-month intervals is a major drawback and could contribute to higher contamination rates.

Additional advantages of the bag storage system are small size, resistance to breakage and separate chambers for individual plants. A single bag with five sections requires about the same space as five individual test tubes and much less space than similar numbers of replicates in boxes or jars. Bags that are dropped do not break, spill or become contaminated as do tubes or boxes. In addition, as part of an active germplasm collection, single sections of a five section bag can be cut away and sent out to requestors. Plantlets shipped in the bags will not become contaminated and, if not crushed, frozen or heated, will remain viable in transit.

Over 300 *Fragaria* accessions are currently stored in bags at the Corvallis Repository. This system is also in place for *in vitro* collections of *Corylus*, *Mentha*, *Pyrus*, *Ribes*, *Rubus*, and *Vaccinium*.

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