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# Improved Survival of in Vitro-stored *Rubus* Germplasm

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*Additional index words.* raspberry, blackberry, shoot culture, cold storage, photoperiod, storage container, genotype

**Abstract.** Medium-term in vitro cold storage of *Rubus* germplasm was investigated using various temperatures, photoperiods, and storage containers. Shoot cultures of several *Rubus* taxa were grown either in tissue-culture bags or 20 × 150-mm glass tubes. Cultures stored at 10C in darkness were in poor condition after 6 months. Overall survival and condition ratings were significantly better for bags than tubes when cultures were kept at 4C. Contamination was present in 14% of the tubes, but only 3% of the bags. Addition of a 12-hour photoperiod to 4C storage significantly improved both condition ratings and survival percentages of many individual genotypes. Evaluation of the 250-accession germplasm collection after 12 months at 4C (dark) showed 92% of accessions in bags and 85% in tubes in suitable condition to remain in storage. Storage of cold-sensitive genotypes in tissue-culture bags at 25C with a 16-hour daylength was extended to 9 months when the MS-medium nitrogen level was reduced to 25% of standard concentration. Survival of 'Mandarin' raspberry stored for 9 months improved from 40% at 4C (100% N) to 90% at 25C (25% N). Results of these studies suggest that most *Rubus* germplasm can be stored safely at 4C with 12 hours of light. Plastic tissue-culture bags are preferred over tubes due to higher survival and lower contamination rates. Storage at 25C on reduced-nitrogen medium is an alternative method for cold-sensitive genotypes.

Genetic resources of some crop plants are stored as in vitro cultures; however, there are many genera for which in vitro culture and storage conditions have not been determined. Medium-term (3 months to 3 years) storage conditions for in vitro cultures of temperate genera are typically 4 or 5C in darkness (Druart, 1985; Marino et al., 1985; Reed, 1992; Wilkins et al., 1989), although some are stored with a photoperiod (Wanas et al., 1986). There is often genotypic variation in response to cold storage temperatures (Reed, 1991). Tropical crops such as banana (*Musa* spp. *cus*), ginger (*Zingiber officinale* Roscoe & spp.), and kiwi [*Actinidia deliciosa* (A. Chev.) C. F. Liang and A. R. Ferguson cv. *deliciosa*] are often stored at 8 to 15C with a photoperiod (Banerjee and de Langhe, 1985; Dekkers et al., 1991; Monette, 1986). The low tolerance of many genera to cold conditions has led to a search for alternative storage systems. Methods have been tested that use the addition of mannitol (Wanas et al., 1986), growth inhibitors (Gunning and Lagerstedt, 1985), oil overlays (Dekkers et al., 1991), or changes in nutrient levels (Moriguchi and Yamaki, 1989).

Little information is available on the in vitro storage of *Rubus* germplasm. Storage of the full range of *Rubus* variability at the USDA/ARS National Clonal Germplasm Repository (NCGR), Corvallis, Ore., including, at present, several hundred accessions of cultivar and species materials, is needed as a back-up to greenhouse collections and for worldwide germplasm exchange. While some *Rubus* genotypes in the NCGR collection store well at 4C in darkness, others survive storage for only 3 to 6 months (my unpublished data).

This study explored the effects of light, storage container, and genotype on the condition and survival of cold-stored *Rubus* cultures. Storage for non-cold-hardy accessions under growth room conditions was also investigated.

## Materials and Methods

**General conditions.** Micropropagated plants were multiplied on pH 5.7 medium containing the salts and vitamins of Murashige

and Skoog (1962) and, per liter: 30 g sucrose, 1 mg N<sup>6</sup>benzyladenine (BA), 0.1 mg indole-3-butyric acid (IBA), 0.1 mg gibberellic acid (GA<sub>3</sub>), 3.5 g agar (Bitec, Difco, Detroit), and 1.45 g Gelrite (Kelco, San Diego). Plantlets were transferred to either 20 × 150-mm glass tubes (Corning Glass Works, Corning, N.Y.) with 10 ml of medium, capped with plastic caps, and wrapped with parafilm (American Can Co., Greenwich, Conn.) or to five-chamber heat-sealed plastic tissue-culture bags (CultuSAK, Becton Dickinson, Lincoln Park, N.J.) with 10 ml of medium per chamber. The same medium without growth regulators was used for storage with Gelrite concentrations increased to 1.75 g-liter<sup>-1</sup> in the medium for bags (Reed, 1991). One plant was placed in each tube or chamber and cultures were grown at 25C with 16-h days (25 μmol·m<sup>-2</sup>·s<sup>-1</sup>) for 1 week, then cold-hardened for 1 week in an incubator with 8-h days at 22C and 16-h nights at -1C before being placed in the experimental conditions (Reed, 1991). Plant materials are referred to by both name and accession code. Experiments are summarized in Table 1.

**Comparison of storage containers under three storage conditions.** Micropropagated plants of *Rubus leucodermis* Douglass ex. Torrey and A. Gray (R 599), *Rubus* hybrid raspberry cv. Malling Promise (R 444), *R. caesius* L. (R 485), and *Rubus* hybrid blackberry cv. Kotata (R 992) were used for comparison of bags and tubes for storage under three conditions. Twenty tubes and four bags (20 chambers) of each accession were placed in one of three environments: 1) a dark refrigerator at 10C; 2) a dark cold room (D) at 4C; or 3) an incubator at 4C with a 12-h dark/light (D/L) cycle (12 μmol·m<sup>-2</sup>·s<sup>-1</sup>). Twenty replicate cultures were tested for each treatment.

**Cold storage in bags in dark or D/L conditions.** Six additional *Rubus* accessions [*R. idaeus* L. (R 239), *R. caesius* L. (R 819), *R. illecebrosus* Focke (R 838), *Rubus* hybrid blackberry cv. Anderson (R 393), *Rubus* hybrid blackberry selections ORUS 1620 (R 348), and ORUS 1362 (R 459)] were stored in bags in a 4C dark cold room (D) or a 4C incubator with a 12-h dark/light (D/L) cycle (12 μmol·m<sup>-2</sup>·s<sup>-1</sup>). *R. idaeus*, *R. illecebrosus*, Anderson, and ORUS 1620 were chosen because they performed poorly in the general cold-storage collection. Two five-chamber bags were stored under each of the two storage conditions for a total of 10 replicates per treatment.

**Nitrogen concentrations at 25C with 10 ml of medium.** Storage at 25C under 16 h of light conditions (25 μmol·m<sup>-2</sup>·s<sup>-1</sup>) at four N concentrations was tested for five accessions [raspberry cv. Malling

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Table 1. Summary of storage experiments performed with *Rubus* germplasm listing treatment, genotype tested, type of container, and conditions of storage.

Comparisons	Genotypes	Conditions
Storage containers, light and darkness (D/L)	Kotata	
	Malling Promise	
	<i>R. caesius</i>	
Cold storage in bags in dark or D/L conditions	<i>R. leucodermis</i>	
	Anderson	
	ORUS 1362	
	ORUS 1620	
	<i>R. caesius</i>	
Nitrogen concentration, storage at 25C, 10 ml of medium	<i>R. idaeus</i>	
	<i>R. illecebrosus</i>	
	Kotata	25C, 16-h light
	Malling Promise	N = 0%, 6%, 25%, 100% MS
	Mandarin	10 ml of medium/section
Nitrogen concentration, storage at 25C, 20 ml of medium	<i>R. caesius</i>	10 ml of medium/section
	<i>R. leucodermis</i>	25C, 16-h light
	Malling Promise	N = 6%, 25%, 50%, 100% MS
	Mandarin	20 ml of medium/section
		Dark, 4C
Germplasm, container	250 different genotypes (28 listed in Table 5)	Bag or tube storage

Promise (R 444), *R. leucodermis* (R 599), *Rubus* hybrid raspberry cv. Mandarin (R 743), *R. caesius* (R 814), and blackberry cv. Kotata (R 992)]. Twenty plants of each accession were planted on medium without growth regulators and with 0%, 6%, 25%, and 100% total MS nitrogen levels. Bags contained 10 ml of medium and one plant per section of five-section bags, for 20 replicates per accession.

**Nitrogen concentration at 25C with 20 ml of medium.** A storage test at 25C using a larger volume of medium was conducted with two accessions, raspberry cv. Mandarin (R 743) and raspberry cv. Malling Promise (R 444). Four five-section bags containing 20 ml, rather than 10 ml, of medium per section were used per treatment (20 replicates). Nitrogen was at 6%, 25%, 50%, and 100% of the normal MS nitrogen concentrations in the medium. An additional set of four bags of each accession with 100% of MS nitrogen level medium was placed into D/L storage at 4C.

**General germplasm storage.** Survival and condition ratings were taken at 3-month intervals on the general germplasm collection (250 accessions) kept in bags and tubes at 4C in darkness to provide information about the broad spectrum of *Rubus* germplasm. Five tubes and one five-section bag were used for each accession.

**Evaluation and analysis.** The number of living, dead, and contaminated plants and the condition rating of the cultures were recorded at 3-month intervals. Analysis of variance, factorial analysis, and mean separation were performed using MSTAT-C software (Michigan State Univ., East Lansing). Condition ratings were based on plant appearance (0, dead, brown; 1, etiolated, pale tan, no green color; 2, etiolated, pale green color; 3, etiolated, retaining medium-green color; 4, not etiolated, medium-green color; 5, not etiolated, dark green). Cultures with a rating of 2 or 1 are considered to be at the end of their storage life and are removed for repropagation.

## Results

The survival and condition of *Rubus* germplasm in vitro were compared for storage container, photoperiod, temperature, and genotype to determine the best conditions for storage of a large, genetically diverse collection.

**Comparison of storage containers under three conditions.** Storage at 10C in darkness was unsuccessful for most of the accessions tested. At 6 months, most plants were in a state of decline and were rated fair or poor (2 or 1), ratings that signaled the need for repropagation. 'Malling Promise' produced the highest mean condition rating (3). No significant differences occurred between the mean ratings for tubes and bags for condition or survival at 10C (data not shown). This section of the experiment was not followed past 6 months because of the poor condition of the plants. Cultures stored at 4C were in good condition at 6 months.

After 18 months at 4C in either darkness or D/L storage, condition ratings were affected significantly by interactions between accession and container ( $P \leq 0.01$ ) and between container and photoperiod ( $P \leq 0.05$ ) (Tables 2 and 3). 'Malling Promise' and 'Kotata' had significantly higher mean condition ratings (2.7 and 2.6) than the other genotypes, and the rating of *R. leucodermis* (1.8) was significantly higher ( $P \leq 0.001$ ) than *R. caesius* (0.3). Plants stored in bags were in significantly ( $P \leq 0.05$ ) better condition (2.4) than those in tubes (1.4), and plants in darkness were better (2.2) than those with D/L (1.5). Differences due to photoperiod were significant only for *R. leucodermis* stored in bags (Table 3).

Mean survival percentages at 18 months were significantly

Table 2. Mean squares and degrees of freedom from factorial analysis of condition and survival ratings for four *Rubus* accessions stored in an incubator at 4C with a 12-h photoperiod (D/L) ( $12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) or in darkness in tubes or tissue culture bags.

Factor	df	Mean squares	
		Condition	Survival
Accession stored (A)		17.98***	
Container (C)		14.52***	
Photoperiod (P)		6.52**	
A × C		2.98**	
A × P		1.42	
C × P		2.74*	
A × C × P		1.60	
Error		0.62	

\*\*\*\*Significant at  $P \leq 0.05$ , 0.01, or 0.001, respectively.

Table 3. Mean condition ratings of *Rubus* cultures stored in tubes or bags at 4C in darkness or in an incubator with a 12-h photoperiod (D/L) ( $12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 18 months. Ratings were: 5 = excellent, 0 = dead.

Accession	Tubes		Bags	
	Dark	D/L	Dark	D/L

<sup>a</sup>Means separation by Duncan's multiple range test. Mean s in rows followed by the same letter are not significantly different ( $P \leq 0.05$ ). N = 20. Storage of 20 plants per treatment: Four bags with five plants per bag and 20 tubes with single plants.

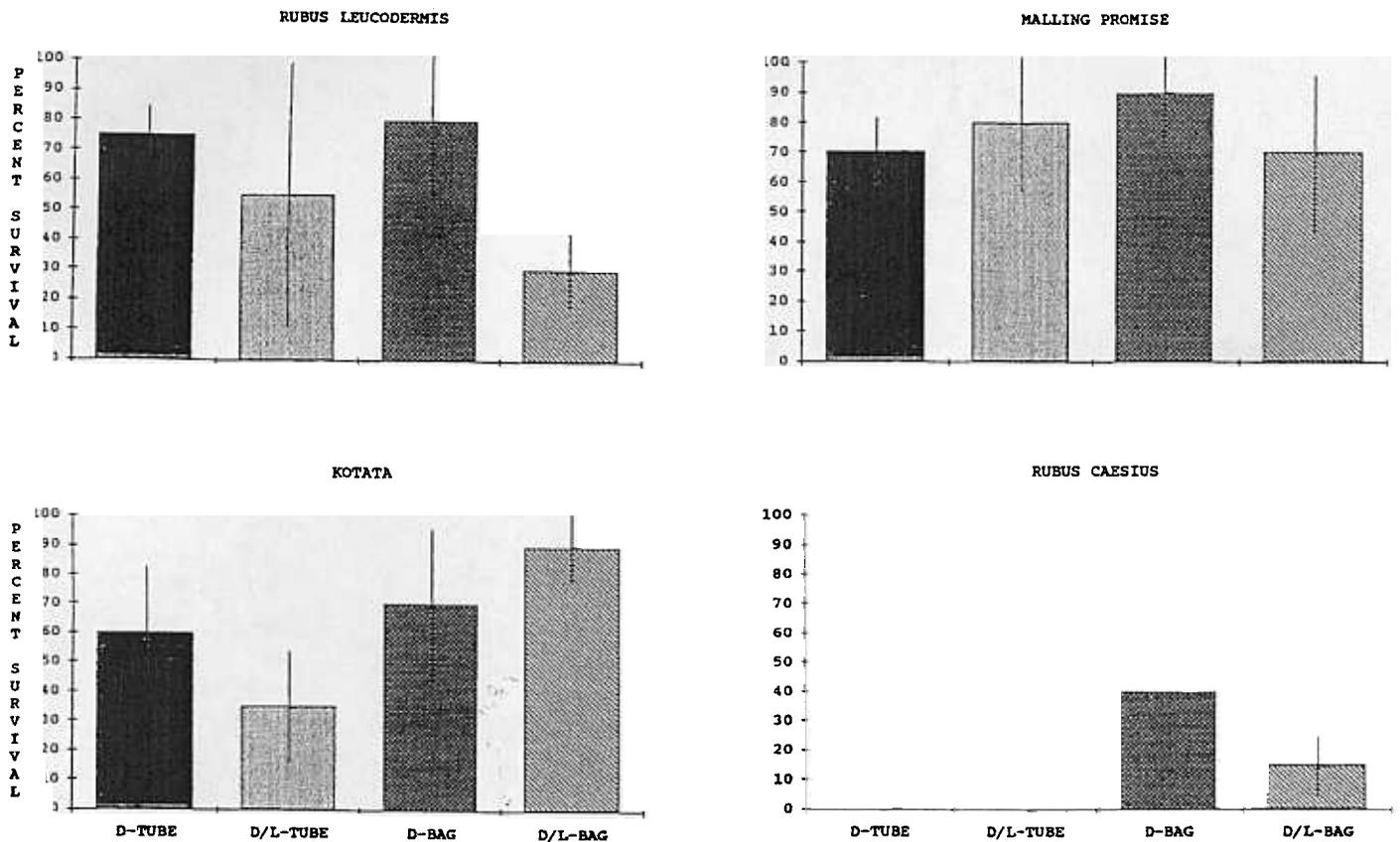


Fig. 1. Survival rates of four *Rubus* genotypes following 18 months of storage at 4C in either 20 × 150-mm tubes or plastic tissue culture bags in darkness or with a 12-h photoperiod. There was an interaction between container type (tube or bag) and photoperiod [dark (D) or 12-h photoperiod (D/L)].

affected by the interaction of accession, container, and photoperiod ( $P \leq 0.05$ ) (Table 2). Most of the interaction was due to variation among accessions due to genotype, with smaller contributions by container and photoperiod (Fig. 1). 'Malling Promise' had consistently high survival in all conditions tested, and *R. caesius* survival was always low. Survival percentages were not significantly different for container or photoperiod.

**Cold storage in bags in darkness or D/L conditions of six additional genotypes.** After 15 months of storage at 4C, both mean survival and condition ratings for *R. illecebrosus* and *R. idaeus* were lower ( $P \leq 0.001$ ) than those for the other four accessions (Table 4). Mean condition ratings of D/L-stored cultures (2.7) were higher ( $P \leq 0.001$ ) than those in darkness (1.3), and survival rates were also significantly better (D/L 3.4, D 2.3) ( $P \leq 0.01$ ). For individual genotypes, D/L conditions were the same as or significantly better than darkness for survival percentages and condition ratings (Fig. 2). There were no interactions between accession and photoperiod for either condition or survival ratings.

**Effect of nitrogen levels on storage at 25C.** The five accessions stored in the growth room on 10 ml of medium with four N levels were rated at 6 months. Mean survival and condition ratings were best at the 25% N level ( $P \leq 0.001$ ). No plants survived without N, and few or none survived at 6% or 100% of MS levels (data not shown). Genotype effects and the interaction between genotype and N level were not significant. Very little medium remained in most of the bag sections at 6 months, so the experiment was terminated.

**Nitrogen concentration at 25C with 20 ml of medium.** 'Malling Promise' and 'Mandarin' were stored at 25C under growth room conditions as in the previous 25C experiment, but with 20 ml of medium rather than 10. At 9 months, the quadratic component of

Table 4. Means of condition and survival ratings from factorial analysis of six *Rubus* accessions stored in bags at 4C in darkness or in incubator with a 12-h photoperiod (D/L) ( $12 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 15 months. Ratings were: 5 = excellent, 0 = dead.

Accession	Means	
	Condition (rating)	Survival (%) <sup>2</sup>
<i>R. illecebrosus</i>	0.3 b <sup>3</sup>	10 b
<i>R. idaeus</i>	0.4 b	15 b
<i>R. caesius</i>	2.8 a	80 a
Anderson	3.1 a	80 a
ORUS 1362	3.1 a	85 a
ORUS 1620	2.7 a	70 a

<sup>2</sup>Percentage alive of 20 plants.

<sup>3</sup>Mean separation by Duncan's multiple range test,  $P \leq 0.05$ . N = 20. Storage of 10 plants per treatment with five plants per bag.

the sum of squares showed that the mean survival rate was best on 25% of MS nitrogen, but mean condition ratings were similar for 25% and 50% N levels (Fig. 3). A comparison of the data at 6 months for these two genotypes on the two volumes of media showed lower condition and survival rates for 'Mandarin' ( $P \leq 0.05$ ) on 10 ml of agar, but no difference in response for 'Malling Promise' (Fig. 4), although the comparison is not direct, as the data are from separate experiments.

At 9 months, bags of 'Malling Promise' stored at 4C with D/L conditions on 100% of the MS nitrogen concentrations had high mean condition (3.75) and survival ratings (100%), but 'Mandarin' had low condition (2) and survival (40%) ratings. By comparison, after 9 months at 25C on 25% N, 'Malling Promise' had the same condition rating (3.75) with 65% survival and 'Mandarin'

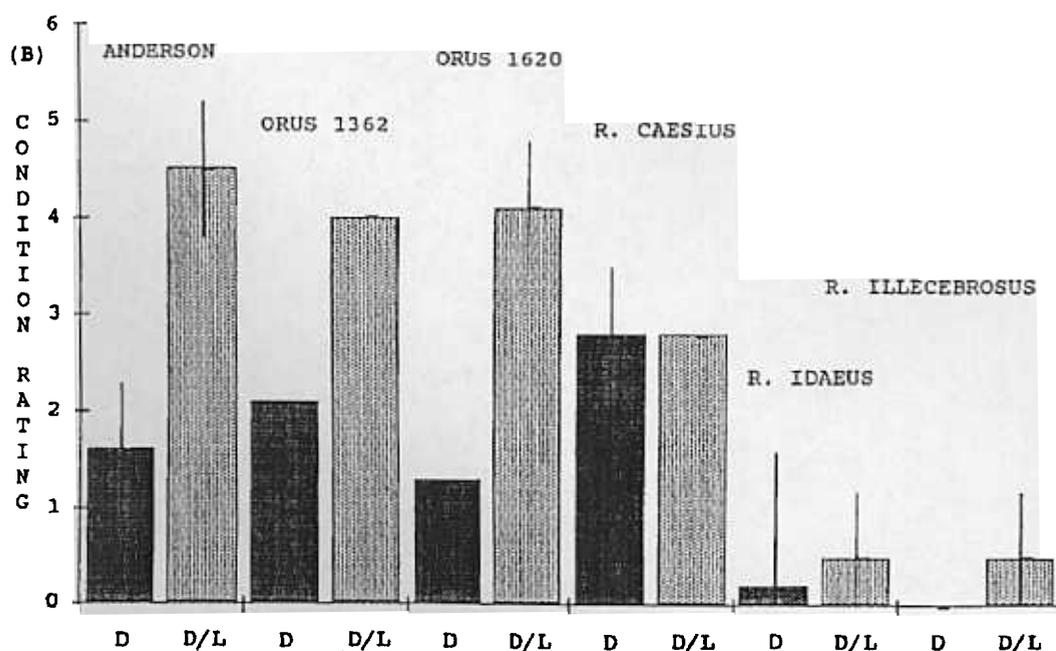
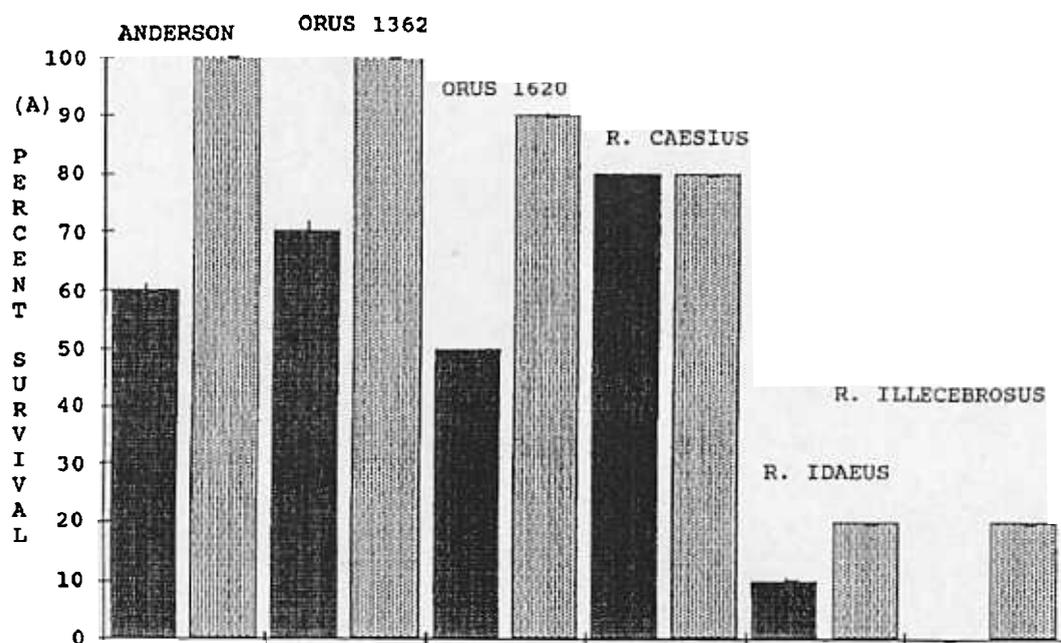


Fig. 2. Mean survival (A) and condition ratings (B) for six *Rubus* taxa stored at 4C in bags for 15 months in either darkness (D) or with a 12-h photoperiod (D/L). Storage of two bags (10 plants) of each genotype per treatment.

had an improved condition rating of 3.5 with 90% survival.

**Germplasm storage.** For the 250-accession germplasm collection, 92% of bags and 85% of tubes had viable plantlets after 1 year of storage at 4C in darkness. Fungal contamination was evident in 14% of tubes and 3% of bags, and the remainder required repropagation before 12 months (a rating of 2 or less). For this collection, the average condition ratings for surviving plants in bags ( $2.8 \pm 1$ ) and tubes ( $3.3 \pm 1$ ) remaining in storage at 12 months were not significantly different, and there were no differences in the overall survival means (both  $0.94 \pm 0.2$ ); however, for individual genotypes there was considerable variation in response to the storage container (Table 5). If condition and survival means include those removed from storage before 12 months (8% of bags and 15% of tubes), then both ratings are higher for bags than for tubes.

## Discussion

Cold storage of the four *Rubus* accessions was better in bags than in tubes at 4C in either photoperiod, but storage at 10C was not successful. Because less-hardy species such as *Actinidia* tend to grow better at 8C (Monette, 1986), it was expected that some of the less-hardy *Rubus* would store best at temperatures >4C. Wilkins et al. (1989) found 4C to be a better storage temperature than 10C for hardy species such as *Prunus* and *Malus* species and cultivars. In my study, the importance of photoperiod appeared to be dependent on genotype. In the initial tests, dark storage was better for the accessions tested than D/L, while in the second group tested, D/L storage was best. I did not determine the optimum photoperiod. Marino et al. (1985) found that a 16-h photoperiod was beneficial for 6 months of storage at 4C of *Prunus* rootstocks, but, at -3C,

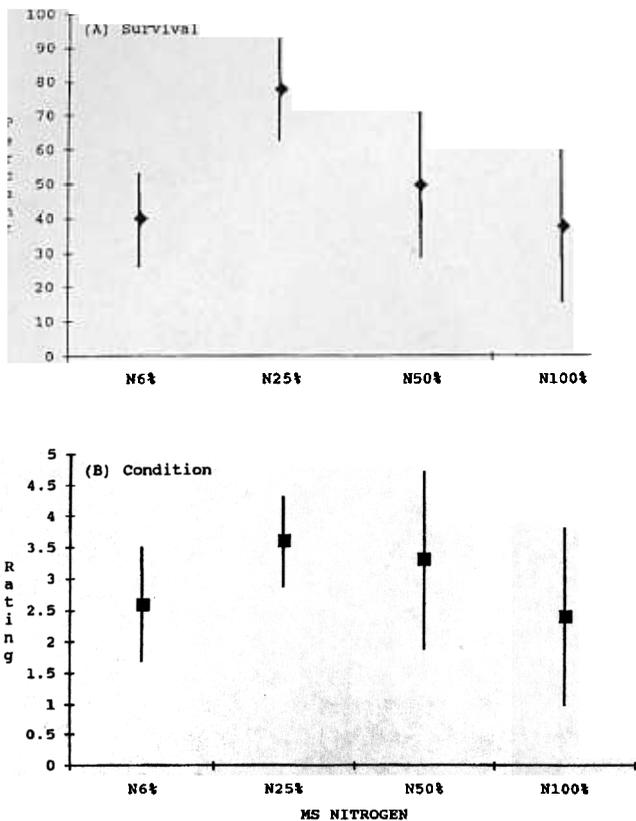


Fig. 3. Mean survival (A) and condition ratings (B) for *Rubus* cultivars grown in bags at 25C with a 16-h photoperiod for 9 months on 20 ml of MS medium with regular or reduced N concentrations relative to standard MS medium. Storage of 10 plants of each cultivar per treatment with five plants per bag. N = 40.

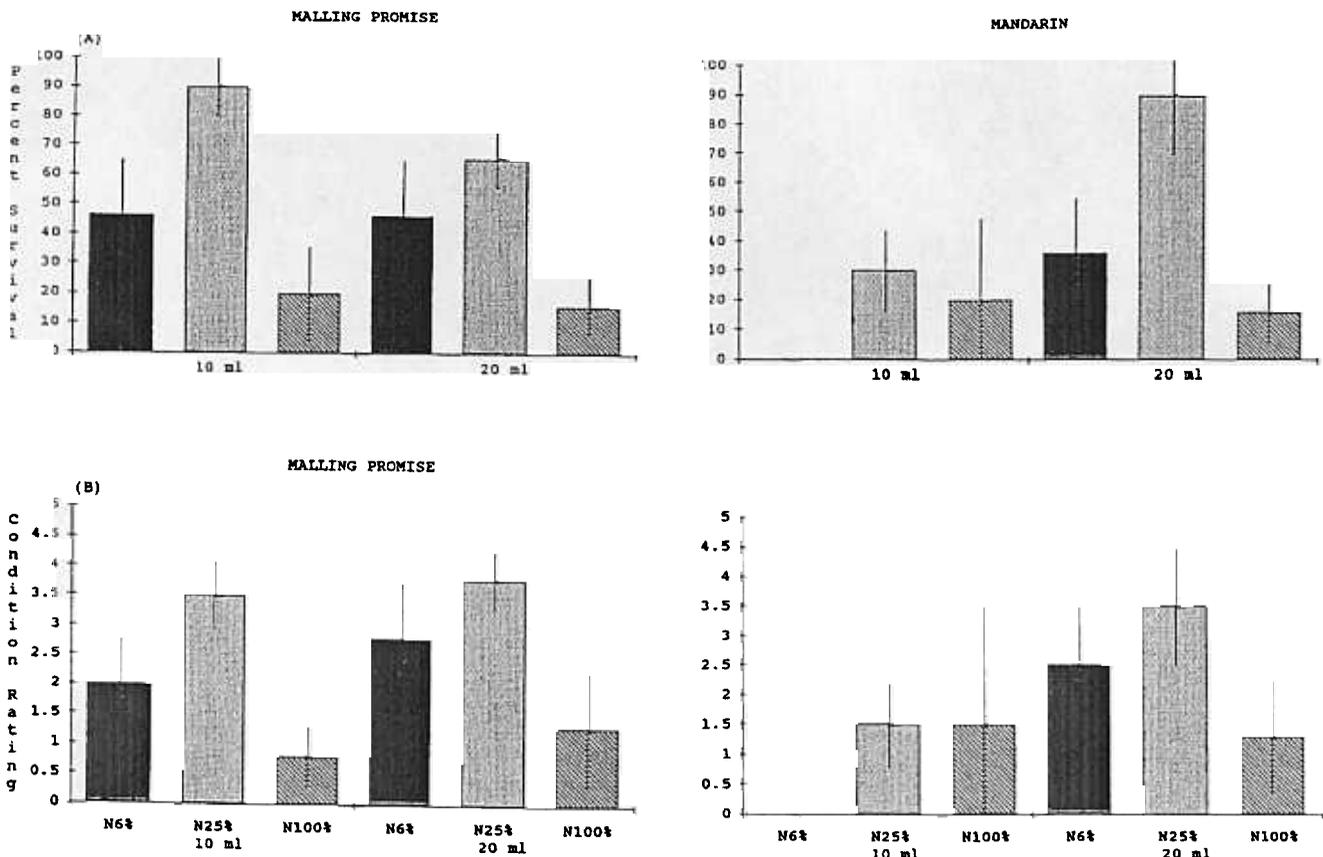


Fig. 4. Survival percentages (A) and condition ratings (B) for 'Malling Promise' and 'Mandarin' raspberry following 6 months of storage at 25C with a 16-h photoperiod in tissue-culture bags. Bags contained either 10 or 20 ml of medium and 6%, 25%, or 100% of the N concentration of standard MS medium. Means for 10 and 20 ml of medium can not be compared directly because they were evaluated in separate experiments.

light was detrimental for 6 and 10 months of storage. Storage in bags for condition rating and survival percentage was superior to storage in tubes for the genotypes tested in this study—results similar to those observed for *Fragaria* (Reed, 1992).

The genotypic differences evident in all the experiments are consistent with observations of cold-stored *Rubus* germplasm by Gunning and Lagerstedt (1985), as well as with studies of other genera (Marino et al., 1985; Wilkins et al., 1989). There were significant differences in survival rates between cultures in bags and tubes for some individual genotypes in the experimental conditions tested and the larger germplasm collection (Fig. 1, Table 5). Overall, survival of accessions from the general germplasm collection following 1 year of dark storage at 4C was higher for bags than for tubes. Occasionally, an accession was a total loss due to fungal contamination in tubes, but never in bags. Most of the variation within treatments was due to the wide genetic variability present in the genus *Rubus*. This finding points out the difficulty of applying one standard cold storage procedure for all taxa, and the need for further investigation of storage techniques. Variability in the length of viable storage among taxa of germplasm collections also mandates regular inventory and repropagation of materials (Mix-Wagner and Schittenhelm, 1991; Reed, 1991).

Cold storage is not always appropriate for all genotypes within a collection. Mix-Wagner and Schittenhelm (1991) found that 20C was better than 10C for 6 months of storage of *Helianthus tuberosus* L., and Moriguchi and Yamaki (1989) determined that 28C for 8 months at reduced ammonium nitrate concentrations was best for *Vitis* genotypes, which did not tolerate 5 or 10C. In my study, storage of several *Rubus* genotypes was possible for 6 months at 25C on low-N (25% of MS concentration) storage medium. Survival and length of storage were increased to 9 months with a

Table 5. Survival percentages of a representative sample (28 accessions) of the 250-accession general *Rubus* germplasm collection stored at 4C in darkness for 1 year as single plantlets in either five 20-mm tubes or one five-chamber plastic bag.

Accession		Alive after 12 months (%)	
No.	Name	Bag	Tube
357	Austin Thornless	100	80 <sup>z</sup>
250	Burbank Thornless	100	80 <sup>z</sup>
956	Carolina	100	100
203	Chehalem	100	80 <sup>z</sup>
252	Hillemeier	80	100
137	Jenner	100	100
1003	Malling Enterprise	40	60 <sup>z</sup>
444	Malling Promise	100	100
347	ORUS 992	100	100
970	ORUS 1308	100	100
350	ORUS 1465	100	80 <sup>z</sup>
345	ORUS 1467	100	100
6	<i>R. crataegifolius</i> Bunge	80	100
1	<i>R. hirsutus</i> Thunb.	100	60 <sup>z</sup>
56	<i>R. hirtus</i> Waldst. & Kit.	80	80 <sup>z</sup>
794	<i>R. hispidus</i> L.	100	100
261	<i>R. lasiococcus</i> A. Gray	100	100
599	<i>R. leucodermis</i> Douglass	100	80 <sup>z</sup>
199	<i>R. parviflorus</i> Nutt.	80	100
29	<i>R. parvifolius</i> L.	100	100
140	<i>R. plicatus</i> Weihe & Nees	100	100
24	<i>R. sanctus</i> Schreber	100	0 <sup>z</sup>
7	<i>R. sumatranus</i> Miq.	100	80 <sup>z</sup>
611	<i>R. ursinus</i> Cham. & Schldl.	80	100
42	<i>R. wahlbergii</i> J. Arrh.	100	100
77	Raven	100	100
633	Silvan	100	80
210	Snyder	60	80 <sup>z</sup>
991	Thornless Oregon Evergreen	100	100

<sup>z</sup>Percent surviving, but contaminated by fungi.

larger volume of medium (20 rather than 10 ml) and with N levels at 25% of normal MS medium. This result is similar to the 6% ammonium nitrate concentration (35% of total MS nitrogen) in the growth medium that Moriguchi and Yamaki (1989) used for 290 days of storage of chilling-sensitive grape cultures. The reduction in total N used for *Rubus* appears to have similar effects to reduction in ammonium nitrate only in *Vitis*. The use of a low-N medium to extend storage at room temperature to 9 months

provides a useful alternative for genotypes such as 'Mandarin', certain accessions of *R. idaeus* and *R. illecebrosus* and tropical genotypes that typically survive only a short time in cold storage.

Storage facilities for germplasm collections vary; however, 4C storage in darkness or with a photoperiod appears to be acceptable for most *Rubus* germplasm if quarterly inventories are used to evaluate materials and remove those at risk. For optimum lengths of storage, I recommend that most in vitro *Rubus* germplasm be stored at 4C with a photoperiod and that tissue-culture bags be used to increase survival and decrease contamination losses. Cold-sensitive genotypes can be stored in a growth room at 25C on a medium that contains 25% of the N concentration found in MS medium.

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