CHAPTER 3. SEED COLLECTION MANAGEMENT

Introduction

Management of the seed collection entails eight major functions, each composed of a varying number of subroutines. This chapter describes these functions, hopefully in sufficient detail to allow someone unfamiliar with the seed collection to effectively manage it. The eight major functions are:

1. Receipt of new fruit and seed
2. Germination
3. Seedling management
4. Maintenance of the seed inventory
5. Responding to seed requests
6. Monitoring
7. Seed increases
8. Backup of seed at NCGRP

As a repository for a collection of global small fruit germplasm, new material is always arriving. New seeds must be germinated for an accession to be represented in the field or screenhouse collections and to verify identity.. Because the repository houses both cultivars and species material, and because of a history of clonal propagation, generated seedlings must be carefully labeled and guided to their ultimate destination in field, screenhouse, or species collections. A major function of the repository is providing small fruit germplasm to researchers and breeders around the world, and management of the seed collection includes responding to requests for seed. Of course, the seed collection manager should monitor storage conditions and seed viability, and when seed quantities drop, seed should be regenerated.

Receipt of new Fruit and Seed

The procedure for handling incoming fruit/seed includes six steps:

1. Completion of Acquisition Information Sheets
2. Seed Extraction and Initial Cleaning
3. Final Cleaning
4. Seed Drying
5. Packaging, and
6. Storage.Each of these steps is described in the following sections.

Acquisition (Login) Sheets

Immediately upon receipt, a login sheet should be completed for the incoming material. Passport/pedigree information for each incoming accession is presented on the login sheet. When multiple accessions in the same genus are received simultaneously, they may all be documented on the same login sheet, space permitting. A different login sheet is used for each different genus received. Completed login sheets are given to the crop manager or curator who assigns the material a local inventory number and adds the information to the inventory and accession files. Inaccurate

information on the login sheet means inaccurate information in these files. As the inventory and accession files are the foundation upon which all other operations rest, inaccuracies must be avoided.

When material has been collected by NCGR personnel, completing login sheets is a cooperative exercise. To avoid confusion, only one login sheet for each new accession should be given to the information manager. That is, a single complete login sheet should be submitted, rather than multiple incomplete sheets. If the collector needs time to review his or her collection notes before the login sheet can be completed, it is best to wait until all the information can be submitted at once. However, if the information cannot be compiled within two weeks, incomplete login sheets should be submitted to the information manager as inventory numbers need to be assigned in a timely manner. Material that lacks an inventory number cannot be incorporated into the system and remains in limbo until such numbers are assigned.

Seed Extraction/Initial Cleaning

Occasionally a clean seed lot will be received, but more often than not seed must be extracted from fruit or otherwise cleaned before it is suitable for storage. When clean seed is received, it can immediately be dried; otherwise the material must be processed first.

Freshly collected fruit is usually received packaged in plastic bags. If it is not possible to begin processing this material promptly, it may be stored for up to a month in a refrigerator. Allowing the fruit to soften and begin decomposing, often results in easier extraction. However, excessive decomposition will result in the formation of fungal sclerotia and seed cleaning will be complicated.
Pectinase at a ratio of 0.01 g pectinase per 5 mL DI water may be used to speed up the breakdown of fruit tissue.. Once fruit is placed in a zip-top bag, water is added (enough to just cover the fruit) and a few drops of the pectinase solution is added to the mixture. The contents are left to rest for approximately 48 hours. It is usually ready for seed extraction at this point.

Seed is usually extracted from small fruit using the method of Morrow et al. (1954). Fruit is placed in a blender, water is added, and the fruit macerated. Blender blades should be dulled using plastic tubing or duct tape, or by dipping them in liquid plastic (Fig.1); the liquid plastic tends to remain on the blades longer than does plastic tubing. The goal is to beat the fruit to a viscous liquid without cutting or nicking seeds. The lowest setting of the blender for a duration of 20 to 60 seconds, varying with fruit condition, is generally sufficient

Figure 1 Liquid plastic covered blades and blender.

The blended liquid is next poured into a large (1L) glass beaker. Water is added to fill the beaker and dilute the pulp solution. Sound, filled seeds should settle to the bottom, while unfilled seeds and pulp remain in suspension. Stirring the solution once or twice during settling may allow seeds to settle which would otherwise remain suspended by floating pulp or attached air bubbles. After allowing the solution to remain undisturbed for about 60 seconds, the suspension is poured off leaving the settled seeds. The pitcher of the blender is then repeatedly rinsed, with the rinse water poured into the beaker, until all seeds have been washed from the pitcher. The liquid is again poured off, leaving filled seeds. Repeatedly adding water, stirring, and pouring off the suspension will give cleaner seeds. How long settling is allowed during this cleaning is best determined by observing the behavior of seeds and debris in the beaker. However, some debris must inevitably be removed using forceps.

The blender should be disassembled and thoroughly rinsed between seed lots to avoid mixing seeds from one lot with another.

After cleaning, seeds are poured/scooped from the beaker onto a labeled brown (industrial weight) paper towel (or waxed paper) and allowed to air dry for at least three days. The paper towel is labeled with the number assigned by the collector and the species name in order to identify the accession. It is also recommended to keep the zip-top bag that the fruit came in with the seed lot while it is drying. The information on the towel must also appear on the login sheet, so that once a local inventory number is assigned by the information manager it can be linked to the correct seed lot. With reference to the sample login sheet above, the paper towels could be labeled with the genus and species of the material and the number assigned by the collector. After air drying, seeds are rubbed/scraped from the paper towel.

When very small quantities of fruit are received, simply plucking seeds from the fruit (e.g. strawberries) or crushing and smearing the fruit on a paper towel and subsequently picking seeds from the streak may prove easier.

When dried fruit is received, the seed will be particularly difficult to extract and clean. In most cases, separation of seeds and fruit is extremely difficult and tedious. Best results are usually

obtained using some method of abrasion, such as a rub-board. Rubbing the material between your hands may also be effective.

Final Cleaning

If wet cleaning has not yielded a sufficiently clean seed lot, dry seed can be further cleaned using a blower (Fig. 2). After air drying, the seeds should be gently rubbed, using either hands or a rub-board, to break up clumps of seeds which have dried together and abrade remaining debris from the seeds. The seeds are next placed in the chamber of the blower (Fig. 2). All vents and apertures should be fully closed when power is turned on and the blowing process is started. The debris pan should also be emptied before each new lot is processed. In the event of an error, the material in the debris pan can be returned to the chamber if and only if it contains only material from that lot. If the debris pan is not emptied between seed lots and seed is accidentally blown into the debris pan, it cannot be reliably separated from other light seeds already in the pan. In this case, the seed lot has been contaminated and is no longer pure. It is strongly recommended that the debris pan be emptied after each seed lot is cleaned.

The operator's manual should be consulted regarding operation of the seed blower. The gauged port, which is opened using the crank, should be opened slowly after the blower has been started. It should be set to the same level for each batch if a single seed lot is too large to be processed at one time. However, a different setting, which must be determined visually by observing seed and debris behavior during blowing and by examining contents of the debris pan after blowing, will likely be required for each different seed lot. A duration of one minute is generally adequate.

After a seed lot has been processed and the chamber emptied, the two upper ports on the side of the blower should be opened, the blower reset, and started once again. This practice will remove any remaining debris or seed from the previously processed lot from the blower as a great deal of air is moved through the column when the upper ports are open. This prevents contamination of one seed lot by another.

Figure 2: Seed Blower

Seed Drying

Before seeds can be safely stored at subfreezing temperatures, the moisture content of the seeds must be reduced. The moisture content of a seed is dependent upon the environment in which the seed has been, and seed freshly extracted from fruit will have a relatively high (too high for frozen storage) moisture content. All seeds which are received at NCGR, whether in fresh fruit, as a clean lot, or as dried fruit, should be dried before storage to maximize the longevity of stored germplasm. The procedure for drying seeds is simple though time consuming.

Material to be dried is placed in a labeled paper envelope and placed inside a moisture-proof cabinet containing a desiccant (e.g. Drierite). An indicating desiccant and an analog or digital hygrometer are recommended. Using an indicating desiccant allows one to easily ascertain whether new desiccant is required, and a hygrometer allows one to monitor relative humidity within the cabinet. The most recent research indicates that the best (least injurious to the seeds) method of seed drying is to simply allow the seeds to equilibrate with a 20% relative humidity environment (Vertucci and Roos 1990). An environment of this sort can be achieved in a moisture-proof cabinet using a standard desiccant (Fig. 3). Two weeks should be allowed for equilibration. Once the cabinet has been loaded and drying started, new seed should not be added until the two week period has expired and the dried seed removed.

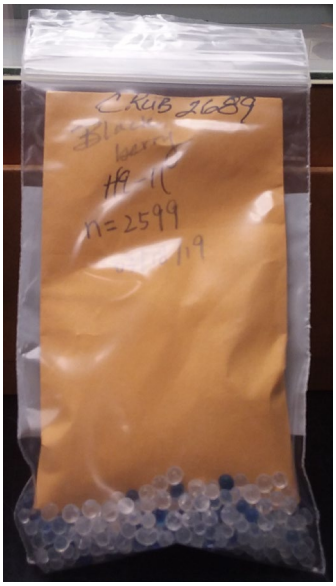

Figure 3: drying seeds with desiccant beads.

Counting and Packaging

After drying, seed quantity is determined and seeds are packaged for storage. Using the Seedburo 801 Count-A-Pak to count the total quantity of seed is the most efficient. The speed and sensitivity settings have been determined and are listed on the top of the counter machine (Fig. 4). The seed bowl is to be cleaned out using a simple water color paint brush. There is a chance that seed may become trapped in the threading where the seed bowl meets the stationary rod and to prevent contamination of one seed type or accession with another this area should be thoroughly checked before the next accession is added to the bowl. Another method to determine the quantity of seed, three replications of 100 seed are counted out and weighed (grams). Samples may be counted out by hand or using the Count-A-Pak seed counter; the seed counter is recommended. Consult the owner's manual for instructions for operating the counter. The mean of the three samples is taken as the mass of 100 seeds for that accession. The mass of the entire seed lot is then multiplied by 100 and divided by the mass of 100 seeds to calculate seed quantity. The mass of 100 seed and total number of seed are noted in both the inventory file and on the exterior of the seed packet.


Figure 4: Seedburo 801 Count-A-Pak set up for seed count.

Next, the seed lot is divided into two portions: the working sample and the reserve sample. The reserve consists of 100 seed (one of the samples used in the initial weighing is suggested) and functions as the foundation for seed multiplication or regeneration. The reserve sample is placed in a small paper (coin) envelope, which has been labeled with the accession number, genus and species, and the word "reserve". This envelope is then sealed in plastic. The remainder of the seed lot constitutes the working sample. The working sample is packaged in heavy duty Spear envelope(s) (1 oz. size). The envelope is again labeled with the accession number, genus and species, date of harvest, the collector's original number, and any other vital information. Both the reserve and working samples are then placed in a laminated envelope (paper-foil-plastic), giving a seed packet (Fig. 5 and 5a).

The coin envelope containing the seeds is labeled with a printed clear Avery 15667 Easy Peel Return Address label containing the Local number, PI number and species. The laminated envelope is labeled with a white Avery 5196 3.5" Diskette label (these are moisture resistant) with the Local number, PI number, genus and species, date of collection, date of receipt at NCGR, mass of 100 seeds (if applicable), number of seeds, and the date on which the quantity was determined or adjusted is noted. Please see a seed packet currently in use (i.e. in a freezer) for a concrete example.

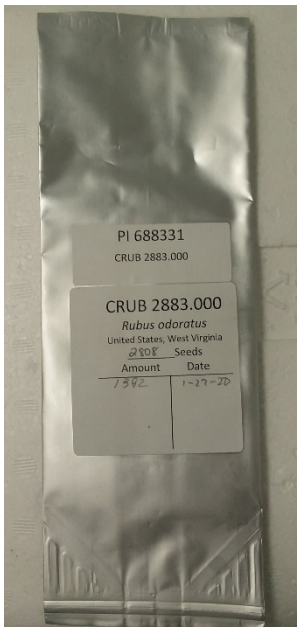

Figure 5: sealed laminated envelope.

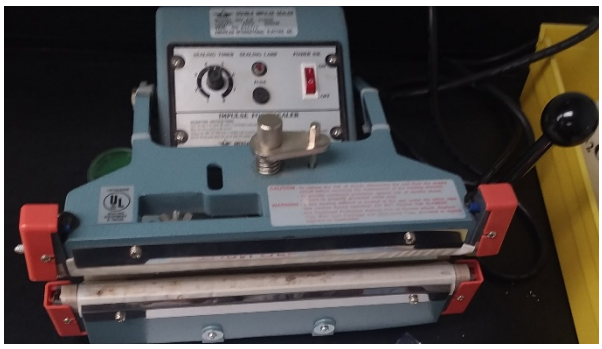
Figure 5a: sealer for laminated envelopes.

Storage

The final step in the process initiated by the arrival of new fruit or seed is storing the seed packets in freezers. Freezers are labeled regarding which genera are stored in each. Each freezer contains bins labeled with the first three letters of the genus stored in the bin (e.g. RUB indicates a bin for Rubus seed), and the new packet is placed in the appropriate bin in correct sequence by inventory number.

Germination

Germinating seeds is often a slow process, which requires both patience and planning, and includes:

1. Consultation of references
2. Pretreatments
3. Germination, and
4. Transplanting of seedlings.

Consulting References

When seeds are to be germinated, the first action taken should always be to review the literature for recommended pretreatment and germination procedures. Even when a particular protocol is known to work well for a particular genus, the available references should be consulted to determine whether the species in question requires a specific treatment. For example, within *Pyrus*, different species require prechilling for different durations (Young and Young 1992). Failure to note these differences has resulted in very poor germination and wasted seed.

There are four publications which serve as a primary source of germination protocols. These are:

> International Rules for Seed Testing (ISTA 1990),
> Rules for Testing Seeds (AOSA 1990),
> Seeds of Woody Plants in the United States (Schopmeyer 1974), and
> Seeds of Woody Plants in North America (Young and Young 1992).

Another book, Collecting, Processing, and Germinating Seeds of Wildland Plants (Young and Young 1986), may also prove helpful. Copies of all five of these references are kept in the seed manager's office. Additionally, reference files have been compiled for each of the major genera stored at the repository, some more complete and informative than others, and recommendations for minor genera have been pooled in a single file. Scientists and researchers who are obtaining seeds from or collaborating with the repository are another very valuable source of recommendations. These references will provide, in most cases, general recommendations for germinating seeds of each genus stored at the repository, and may also provide species specific recommendations. However, species-specific protocols, particularly for species which are not in cultivation in the U.S. or Europe, are often unavailable. In such cases, the protocol employed will be either the general procedure recommended for the genus, or a modification thereof designed by the seed tester.

Lastly, the repository has recently begun to actively solicit information regarding germination procedures and results from those requesting seed. This information is incorporated into the above mentioned reference files, and can prove informative in several ways, including the development of germination protocols.

Pretreatments

Once a germination procedure has been selected, any germination-enhancing pretreatments are made. These pretreatments include surface sterilization, prechilling, scarifying, presoaking,

heating, and perhaps others. Surface sterilization is recommended for all seed which are to be germinated to reduce losses to fungi and bacteria. A 1% sodium hypochlorite solution may be prepared by mixing 1 part commercial bleach with 4 parts water. Seeds should be immersed in a 1-2% solution for 10-15 minutes.

Prechilling is often called for when germinating seeds of the genera stored at the repository. Scarification and presoaking are also employed relatively often. The duration of prechilling varies with the genus and species, though 4, 8, and 12 weeks of prechilling are common, and the exterior of containers used to prechill seed must be labeled with the date on which the pretreatment was initiated and the date on which it is to end. Seeds may be prechilled in or on the germination medium, but if seeds are prechilled on the surface of the medium ("naked stratification") they are much more easily recovered and examined. Seeds which have been mixed into a germination medium are very difficult to recover. Scarification may be mechanically or chemically applied. Seeds may be mechanically scarified using a file, sandpaper, knife (nicking/cracking), or a mechanical scarifier. Seeds are chemically scarified using acids, and proper safety measures are required. Great care is required to avoid damage to seeds during scarification, and scarification often results in a higher proportion of dead or damaged seeds and abnormal seedlings (Bilsland et al. 1984). In some cases, a longer period of prechilling can be substituted for scarification. See the general recommendations by genus (below) for recommended pretreatments.

Seed Germination

Once any pretreatment is completed, seeds are transferred to conditions which favor germination. If seeds are pretreated in the same media and container in which they will be germinated, this transfer is much simplified. The Hoffman Germinator provides a chamber in which temperature fluctuations, light, and humidity may all be regulated. The germinator can be programmed to provide alternating or constant temperatures, light and dark periods, and regulated relative humidity. A continuous record of temperature can be obtained using the recording thermograph. See the owner's manual for programming and operating instructions. Use of a heated bench in a greenhouse with full spectrum lighting, heat and humidity control can also be used for germinating (Fig. 6).

Transparent, plastic germination boxes are recommended for germinating seeds. The boxes inhibit drying of seed and media, and allow the seeds to be inspected without exposing the seeds to airborne pathogens. Enclosing the germination box in a sealable plastic bag will greatly inhibit the loss of moisture from the box, thereby maintaining the seeds in a high humidity environment, without reducing the ease with which the seeds can be inspected. Germination boxes should be labeled with the accession number, genus and species, number of seeds, replication number, type of pretreatment and dates on which the pretreatment started and ended, and dates on which the germination test started and ended, and the environmental conditions of the test (e.g. 10/25 °C, 12 hrs light). This information can be written on an adhesive label and affixed to the germination box; if the label is attached to the bottom of the box, transmission of light to seeds and seed inspection is not impaired. However, care is required when reading the label (if so affixed) to avoid dislodging and clumping seeds.

There are many substrates which may be used in germination tests, including sand, paper, potting mix, and soil. Autoclaved sand provides a sterile medium and tends to inhibit fungal development.

Paper substrates are more prone to fungal contamination, but much easier to work with compared to sand. For most germination tests, paper is adequate. For very long prechilling or long germinations, sand may give better results.

Figure 6: germination boxes with transplants in greenhouse

Germination counts may be made as recommended in the rules for seed testing, or more frequently if desired. Data should be recorded on the seed germination data collection form, which should be prepared when seeds are pretreated. At the completion of the test, the data should be entered into the spreadsheet prepared for such information. Once entered, the data can be statistically analyzed and graphically described. Both computer and original paper records should be kept and filed. The paper records should be placed in the appropriate germination data file (e.g. Ribes Germination Data).

Transplanting Seedlings

Once germination commences, germinated seeds should be transplanted to pots or flats at least weekly, except when plant material is not required (as in a replicated test designed to determine germination percentage). When relatively few seedlings must be transplanted, individual two inch pots filled with fine potting mix are suitable. A coarse mix is to be avoided as it will give poor root-soil contact and may result in a desiccated seedling. When many seedlings are transplanted, a plug sheet works very well. Seedlings must be labeled with accession number, genus and species, date, and the name of the propagator. A single label for the entire group of seedlings should identify the purposes the seedlings are to serve (such as identity verification or plant representatives of a new accession). Shock to the seedlings may be reduced and fewer seedlings lost if the transplanted seedlings can be left in the germinator. Seedlings transplanted to small pots may be placed inside a transparent plastic bag (creating a terrarium) and left in the germinator until vigorous growth is

achieved. When a plug sheet is used, it should be labeled and placed on a mist bed. When large and hardy enough, seedlings are transplanted from plug sheets or small pots to four inch pots, and when ready to one gallon pots. Once in one gallon pots, the plants are ready to be sent to screenhouse, tubehouse, or field.

General Recommendations by Genus

The following protocols are recommended as general procedure for each particular genus. The protocol may work very well with some species in the genus and very poorly with others. These protocols are not substitutes for recommendations found in the literature.

***Corylus*:** The seeds of *Corylus* are recalcitrant and should be germinated promptly upon receipt; they do not store well. When *Corylus* seed is being entered and processed as a new accession, replacement seed or a seed increase, **one half of the seed sample should be given to the Cryopreservation Lab for cryogenic storage.** Pretreating the seeds with gibberellic acid (GA_3) is recommended. Also, the kernels must be removed from the shell to allow germination. Smith and Brenner (pers. comm.) recommend the following procedure:

1. Soak whole nuts in tap water for 24 to 48 hours to soften the shell and ease cracking.
2. Crack the shells, being careful to avoid damaging the kernel. Discard those kernels which were badly damaged in cracking. Damage to the seed, though it may not appear extensive, usually kills the embryo.
3. Soak the kernels in a 25 ppm GA_3 solution for 12 to 24 hours. The solution may be prepared by mixing 25 mg of GA_3 in one liter of ultrafiltered water.
4. Rinse the kernels in ultrafiltered water and treat with a fungicide solution. To prepare the solution, mix one-half teaspoon Captan 50WP in one liter of ultrafiltered water, and immerse the kernels for 30 seconds.
5. Plant the kernels either in a flat filled with a mix of equal parts peat, vermiculite, and perlite, or place the kernels on paper in a germination box. The flat is more useful when a large quantity of kernels is to be planted; germination boxes are adequate for a small quantity. If a flat is used, kernels must be planted shallowly (part of the kernel projecting above the soil line) and the flat should be placed on a mist bed. Germination paper should be moistened with the fungicide solution. Flats or boxes must be adequately labeled.
6. Use a program of 20 °C for 14 hours alternating with 30 °C for 10 hours with lights on during the warm period. If a flat is used, germinate the seeds in a warm greenhouse.
7. Allow 45-60 days for germination.

***Fragaria*:** Though the genus is quite variable regarding germination requirements, germination is often improved by after-ripening, prechilling, and light (Adams and Wilson 1967).

1. Surface sterilize the seed.
2. Prechill for 8-12 weeks at 5 °C on paper or sand moistened with ultrafiltered water.

3. Germinate the seeds with a program of alternating temperatures (20 °C for 14 hours alternating with 10 hours at 30 °C) with light during the warm period.
4. Allow 30 days for germination.

References:

Bringhurst, R. S. and Victor Voth. 1957. Effect of stratification on strawberry seed germination. Proc. Amer. Soc. Hort Sci. 70:144-149.

Henry. E. H. 1934. The germination of strawberry seeds and the technique of handling the seedlings. Proc. Amer. Soc. Hort Sci. 32:431.433.

More, J. N. and D. H. Scott. 1964. Longevity of strawberry seed in cold storage. Proc. Amer. Soc. For Hort. Sci. 85:341-343.

Scott, D. H. and A. D. Draper. 1970. A further note on the longevity of strawberry seed in cold storage. HortScience. 5(5):439.

Scott, D.H. and A. D. Draper. 1967. Light in relation to seed germination of blueberries, strawberries, and Rubus. HortScience 2:107-108.

***Humulus*:** Hop seed germination generally requires a 4-week period of moist-prechilling (5 °C) followed by a regime of alternating warm temperatures (15 °C /25 °C or 20 °C /30 °C). Germination occurs within 2 to 4 weeks. Operculum removal can increase germination rate and percent but is not a feasible procedure for large quantities of seed. Both physiological and physical factors affect germination in hops.

References:

Paine, J. 1950. The treatment of hop seed to improve germination. E. Malling Res. Sta. Ann. Rpt. A34: 139-140.

***Mentha*:** Many mints do not have viable seeds. Mint seeds generally require after-ripening before a good germination response can be achieved and prechilling may also increase the percentage of seeds that germinate (Ikeda 1961).

1. Surface sterilize the seed.
2. Prechill at 5 °C for 4 to 8 weeks on paper moistened with ultrafiltered water.
3. Germinate the seeds using the same program of light and temperature as is used for *Fragaria*.
4. Allow 30 days for germination.

***Pyrus*:** The best germination is obtained from *Pyrus* seeds after inhibitors have been leached away and seeds prechilled. Prechilling requirements vary tremendously in this genus and species-specific protocols are found in the literature.

1. Leach inhibitors from the seeds by soaking in tap water for 24 hours, changing the water roughly every 8 hours.
2. Surface sterilize the seeds.
3. Prechill at 5 °C for at least 45 days on sand or paper (see the literature for chilling requirements of different species).
4. Germinate seeds under alternating temperatures (15 °C for 14 hours, 25 °C for 10 hours) with light during the warm period.

5. Allow 30 days for germination.

***Ribes*:** Seeds of this genus show great variability in germination requirements (Young and Young 1992). Good germination may be obtained from *Ribes* seeds using the following protocol, though the literature should be consulted for species specific recommendations.

1. Surface sterilize the seeds.
2. Prechill at 1 °C for 8 to12 weeks on sand or paper moistened with ultrafiltered water.
3. Germinate the seeds using a program of alternating temperatures (10 °C for 14 hours alternating with 25 °C for 10 hours) with light during the warm period.
4. Allow 4 to 8 weeks for germination.
5. Repeat the prechilling and germination period to obtain more complete germination.

References:

Goodwin, J.R. and K.E. Hummer. 1993. Seed germination of *Ribes* hybrids. Fruit Var. J. 47(4):229-233.

Rubus: Dense, impermeable seed coats inhibit seed germination in this diverse genus. Of the genera stored at NCGR, *Rubus* is the most difficult to germinate. Chemical scarification or warm stratification followed by cold stratification is recommended, and the entire process may require in excess of six months. For each species, seed size, pigmentation, texture and, especially seed coat thickness are more important than crop type when determining optimal scarification treatments (Wada, 2009). Table 1 gives suggested scarification times for some *Rubus* species (Wada and Reed, 2011). Others should be measured for thickness of the seedcoat and hardness and given an appropriate scarification.

Seed coat thickness and hardness: Seed coat thickness was measured for ten seeds of each species. A Nikon SMZ 1000 stereomicroscopic Zoom Microscope (Nikon Instruments, Tokyo, Japan) was employed and measured with Infinity image capture and analysis software (Luminera Corporation, Ottawa, Canada). Measurements were taken in the center of the cut seed equidistant from the micropylar region and the hilar end. Hardness ratings of 1-5 were assigned after seed samples were soaked in deionized (DI) water for 2 days and hand sectioned with a scalpel. Subjective hardness grading was 1 soft, 2 slightly hard, 3 hard, 4 very hard, and 5 extremely hard.

Seed scarification: Seeds are soaked in concentrated H_2SO_4 (98% +) in an ice bath for 30 minutes for thin seeded (<0.1 mm) or 1-3+ hours for thick seeded species (>0.1 mm), then rinsed in running water for 1 hour. Then seeds are soaked for 5 min in calcium hypochlorite [$Ca(ClO)_2$; 3 g/L] completely dissolved in water with an excess of calcium hydroxide [$\underline{Ca(OH)}_2$; 3 g/L] in each treatment beaker, and rinsed for 5 min in running water. Seeds are rubbed against a strainer before stratification to remove the carbonized portions of testa.

Germination treatments: After scarification treatments, germination blotters are fully soaked and 5 mL of one of the solutions is added. The most effective treatments were: 1) GA_3 + KNO_3 (34 mg/L); and 2) Smoke gas solution (Super Smoke Plus, Claremont, South Africa) made from 1 piece of smoke infused paper and 250 mL DI water for 24 h (Wada, 2009).

Seed germination: Seeds are held in warm storage (4 weeks) in light at 18 °C, and then cold storage (12 weeks) in dark at -4 °C, then moved into a germinator (4 weeks) with 8 h of dark at 15 °C, 16 h of light at 30 °C. Plates are treated with fungicide (Cleary 3336, Cleary Chemical Corporation, Dayton, NJ; 0.13 ml/L) as needed to control fungi.

References:

Peacock, D. and K. E. Hummer. 1996. Pregermination Studies with Liquid Nitrogen and Sulfuric Acid on Several *Rubus* Species. HortScience. 31(2):238-239.

Scott, D.H. and A. D. Draper. 1967. Light in relation to seed germination of blueberries, strawberries, and *Rubus*. HortScience 2:107-108.

Wada, S. 2009. Evaluation of *Rubus* Seed Characteristics: Seed Coat Morphology, anatomy, Germination Requirements and Dormancy Breaking, Oregon State University, Corvallis.

Wada, S. and B.M. Reed. 2008. Morphological analysis of *Rubus* seed. Acta Hort. 782: 67-74.

Wada, S., H. Nonogaki, and B.M. Reed. 2010. Identification of blackberry cultivars by seed structure, Oregon State University Extension Publications Online Catalog, Oregon State University Extension Service Corvallis, OR.

Wada S. and B.M. Reed. 2011. Germination of defining germination requirements for diverse *Rubus* species. In preparation.

Wada S. and B.M. Reed. 2011. Germination of cold-stored *Rubus* seed. In preparation.

Table 1. Actual tested and suggested scarification times based on germination data, seedcoat thickness and hardness of 16 *Rubus* species. From Wada and Reed 2011.

***Rubus* species**					**H_2SO_4 (h)**	
	Thickness (mm)	**Hardness (1-5)**	**Control Germ**	**Overall Germ**	**Tested**	**Suggested**
R. arcticus	0.23	5	0	0.7-2.7%	0.5	4-5
R. chamaemorus	0.24	5	2.7	2.7-14%	1	4-5
R. glaucus	0.19	4	0	0-2.7%	3	3-4
R. hawaiensis	0.25	5	0	0-2%	1	4-5
R. hayata-koidzumii	0.12	2	0	0-16.7%	0.5	1
R. hirsutus	0.09	2	0	0-22%	0.5	1
R. leucodermis	0.07	3	0	0-49%	0.66	1
R. microphyllus	0.13	2	0	0-2.7%	0.5	1
R. niveus	0.09	3	0	5-43%	0.66	1
R. odoratus	0.13	1	72	53-87%	0.5	0.5
R. parviflorus	0.13	1	42.7	39-69%	0.66	0.5
R. sachalinensis	0.21	4	1.3	0-9.3%	1	4-5
R. sanctus	0.13	3	50.7	37-48%	3	4
R. spectabilis	0.09	4	0	0-1.3%	1	3-4
R. strigosus	0.22	5	0	0-0.7%	1	4-5
R. urticifolius	0.15	3	0	32-97%	1	1

Seed Production and Germination Procedures for *Rubus*

These procedures were developed by Dr. Chad Finn's USDA ARS Small Fruit Genetics program. The NCGR-Corvallis staff have adapted these procedures for use.

Revised from "Seed scarification and germination of *Rubus*"
Kirsten Wennstrom; November 24, 1998
Revised by Mary Peterson November 8, 2006
Adapted for NCGR use by Jill Bushakra, January 2020

1. Seed Production

Production of seed for spring planting begins about one year in advance. The breeder generates a list of proposed crosses, determined by the goals of the breeding program and the available parents. A Microsoft Access database is used to compile the list, which provides information about the parentage of each of the genotypes to be used as a parent, as well as the purpose(s) for each proposed cross.

My first step is to take inventory of the proposed plants in the field to make sure all are available for use—that they are alive and have enough floricane material to accommodate the typical number of bags. I also determine which parents I am planning to use as females and which crosses might require or benefit from also doing the reciprocal cross. I export the Cross Plan from Access to Excel, where I can manipulate it to show all possible crosses and reciprocals, and sort it by field and location to make the operation more efficient. In April and early May, when new leaves have emerged, I collect leaf samples and have all proposed plants virus tested. If any are infected with RBDV or TSV, which are pollen vectored, I designate those to be used as females only, so that pollen from the infected plants is not spread to others.

As most of the caneberries begin to bloom, I collect 10 or more buds from all genotypes that will be used as males. If the buds are collected when they are expanded to show some petal, but have not yet opened to allow contamination by pollinators, the pollen can be induced to mature and shed in the lab under clean conditions. Buds from each genotype are placed in a sterile Petri dish, cut in half, and dried overnight under a 25-watt bulb placed about nine inches above the bench. This is adequate heat to gently mature the pollen and dry it for storage. The half-buds are then stored in a small tin in a refrigerated desiccator until needed. The pollen is expected to remain viable in these storage conditions for 1-2 weeks, but if a longer period is needed between the first collection of pollen and the time of its use, additional collections are done and stored in the same way. If pollen must be stored for later in the season or the following year, it is dried and then frozen in a desiccator. Some loss of viability is expected, but even a small viable percentage of the original quantity is usually enough for a successful cross. If dried pollen becomes wet during use it is likely to germinate and/or mold, so it is typically discarded and recollected.

Emasculation of the female genotypes is most efficiently done when inflorescences have reached a stage when the primary bud has bloomed but the secondary buds are just beginning to swell to show a bit of petal. Neither the pollen nor the stigmas are mature at this stage, so the emasculation can be done without fear of self-pollination. Typically, four inflorescences (about 16 buds) are

emasculated for each cross. More are done if plant health is a problem, or the cross is interspecific and the chances of incompatibility are greater.[1]

The spent primary is removed from all targeted inflorescences, as well as any underdeveloped buds and leaves in the area to be bagged. Hands and single-edged razor blades are sterilized with 95% ethanol. Buds are then emasculated by slicing from the underside through the sepal, petal, and stamen whorls simultaneously, leaving only the receptacle and gynoecium. All stray anthers must be cleared away before a bag is secured over the inflorescence. A jeweler's tag is attached to the stem and marked with the female parent, the date, and the number of buds emasculated on that stem. The waxed paper or glassine bag (available from Lawson Pollinating Bags, Northfield, IL) is inscribed with only the name of the female parent.

Approximately three days later for blackberries (1-2 for blackcaps and raspberries) stigmas mature and become receptive to pollen. This is indicated by expansion of the cluster of styles and a change in stigma color from bright green to a pale yellowish, as well as an expansion and flattening of the stigma surfaces. Bags are removed and buds are pollinated with pollen from the intended male parent. Pollen from the storage tin is brushed onto the emasculated buds using small paint brushes which are stored inside the tins. Hands must be sterilized with ethanol again between each cross. The tags are marked with the male parent. The name of the male parent is then added to the bag, along with the date pollinated. As time permits, and depending on the length of time the stigmas are receptive, bags are removed for repollination in 1-3 days. When possible, a third pollination is also done 1-3 days later. Repollination dates are written on the bags as they are completed.

When fruit matures on the majority of the plant, bags are harvested. Data is recorded for each bag: number of buds, all dates for emasculation and pollination, number of fruit and/or drupelets (or seeds, if dry), and date harvested. We have been using a system for a number of years in which we open each bag, and record all data as we drop the fruit into Magenta boxes which we label with the parents. We then fill the boxes about halfway with tap water, add 2-4 drops of pectinase, mash with a fork and let sit in the lab, covered, overnight. (We have sometimes left the fruit in the pectinase solution longer—up to two days—and seem to have seen no loss in seed viability.)

After treatment with the pectinase, the solution is poured through a small strainer and rinsed, as the seeds are rubbed against the mesh of the strainer under running tap water. It is important to remove all the fruit tissue. This reduces future contamination and makes handling much easier. For very large seed lots, or those that contain large amounts of debris, rinsing through the strainer can be difficult. We have had success with returning the seeds and debris to the Magenta box and adding water, floating the debris or undigested fruit tissue to the surface, and decanting it off. The seeds sink safely to the bottom. A few repetitions of this step and a final rinse through the strainer is sufficient for even very large or moldy seed lots.

The cleaned seed is tapped or scraped out of the strainer onto paper toweling labeled with the cross, and allowed to dry over night. When dry, seed lots are then poured into paper envelopes, labeled,

[1]This standard protocol was developed over time to result in the final desired seedling quantity of 50-100 for field planting—it attempts to compensate for a multitude of potential losses, including: damaged or lost bags, broken stems, poor set, scarification damage, and the expected germination percentage of 25-30%.

and stored dry for a few to several weeks at room temperature until scarification and stratification begins. Seed from each lot is counted and data is entered into the database. Seed is counted by hand; seed lots with greater than 100 seeds are counted with a scanning seed counter, or count is estimated by weight. For long-term storage, envelopes are kept in a refrigerated desiccator. In 2006 we tested seed from long-term storage (1992-2000) using TTC (triphenyl tetrazolium chloride) and observed a range of 0-95% viability (average 29%).

2. Seed Pre-treatment and Germination

The size of the seed lot and the rarity of the cross determine the pre-germination and germination protocol to be used. Our target is 50-100 viable seedlings per cross. Typical crosses, and those with >50 seeds are treated with the standard protocol. Rare crosses (interspecific, expected sterility, lost parents, etc.) and those with <50 seeds are germinated *in vitro*.

Standard Germination: Basic protocol—acid scarification/water, sodium bicarbonate rinse/5-6 day calcium hypochlorite soak/water rinse/overnight warm stratification/6-10 week cold stratification/1-4 week germination and transplanting/6 weeks as greenhouse plugs/1 week acclimation/field planting.

The target date for planting determines when pre-germination treatments should begin. Counting backward from the planting date using the basic protocol shows that 18-22 weeks is the typical timeframe.

In preparation for scarification with concentrated sulfuric acid (H_2SO_4; 98%+), seeds are removed from envelopes into labeled 100 mL test tubes. We limit the number of seeds per tube to <300 so that the acid will be evenly distributed and the seeds will not clump together. Larger seed lots are divided. Because we have such a wide variety of *Rubus* germplasm, seed lots vary tremendously in seed size, and hence in thickness of seed coat. To efficiently scarify the typical number of crosses (>100 per year), we group seed lots by type (raspberry, black raspberry, blackberry, erect blackberry) and size (smallest to largest). Raspberry seeds require the least scarification (20-30 min); black raspberries 30 min-1 hour; trailing blackberries 1-4 hours, depending on size; erect blackberries might require 3-4 hours.

Seed must be dry prior to scarification. Any water present can result in splattering of the acid and death of the embryo due to the intense heat reaction where the acid and water interface.

One person can handle about ten tubes at a time. A rack of tubes is set into an ice bath and placed in a fume hood. Safety apparel, including eye protection and acid resistant gloves should be worn. Approximately 10 mL H_2SO_4 is poured into each tube, and then stirred using a vortex mixer to coat the seeds. Tubes are stirred every 30 minutes or so. For short treatments (20-30 min), tubes are stirred midway through.

Before or during the acid treatment, a super-saturated solution of sodium bicarbonate is prepared in a beaker to neutralize the acid during rinsing (2-3 heaping teaspoons of baking soda in 500 mL beaker). If electing to use the calcium hypochlorite solution, mix 3g/L calcium hypochlorite (70% active chlorine) until dissolved completely, then add excess calcium hydroxide a teaspoonful per

liter. It should be prepared at the same time and ~25 mL poured into the vessels (we use baby food jars) that will hold the seeds during the 5-6 day soak. Because the excess lime settles, it is useful to pour just after mixing. Cap the vessels until use to keep the solution from volatilizing. Do not prepare the solution before the day of treatment, as it loses potency over time through volatilization of the chlorine.

When the designated scarification time has elapsed, ice water is poured quickly into the tubes to within 1 inch of the rim to dilute the acid and slow the reaction. Rapid stirring with a glass rod or glass thermometer is necessary to keep the seed away from the heat generating interface and speed the dilution. The solution and seeds are then poured through a strainer under cold running tap water, and rinsed thoroughly while rubbing against the mesh of the strainer to remove some of the charring on the seed coat. The strainer is then set into the sodium bicarb solution, where some gentle fizzing will occur as the acid is neutralized. If the scarification time was too long, white embryo and endosperm material might be present. If most of the seeds are intact, germination will still be fine. The exposed material does not need to be separated from the intact seed. The tap water rinse and sodium bicarb drench are repeated, then a final water rinse is given to remove the bicarb before placing seeds into the waiting jar of calcium hypochlorite solution. Jars are stirred, then kept in storage at 4 °C for 5-6 days.

Germination flats have varied over time, but currently we are using 10/20 flats lined with sheets of 12 bedding plant pots. These are filled with medium textured vermiculite (fine has also been used, but required lining the pots with newspaper to keep it from draining through the holes), watered, and topped with ¼" No-Damp-Off sphagnum peat moss. These are left overnight under mist to moisten.

When the calcium hypochlorite soak is complete, seeds have usually taken on a golden color and most of the black charring has been removed from the seed coat. Each jar is stirred again, before the seed lot is rinsed under running tap water and rubbed against the mesh of the strainer to remove the residue. Seeds are then scraped onto the surface of the germination flats and pressed into the peat, but not covered, as they germinate best in light. Flats are placed into the mist bench overnight with bottom heat; they are then wrapped with clear plastic bags and stored at 4 °C with 16 hours of light for 6-10 weeks.

Stratification time varies by cross. Typically, blackberries require the least amount of chilling and raspberries require the most. Some seed might start to germinate while being stratified, so flats should be checked every couple of weeks to make sure the seed bed is still moist and to discover germinating seedlings. We have found little difference in the germination percentage of seeds from the same cross held for 6 weeks vs. 10 weeks for either raspberry or blackberry. Some crosses from parents with low chilling requirements can be removed earlier than 6 weeks if they begin germinating during stratification. *Botrytis* colonization sometimes develops during stratification, but typically decreases after removal from the cold treatment. A fungicide drench is sometimes needed if it does not subside before seedlings emerge.

After stratification, the plastic wrap is removed. Flats are moved to the mist bench and given intermittent mist and bottom heat. Seedlings generally begin to emerge in less than a week, and germination is mostly complete within four weeks. Germination of seed treated with calcium

hypochlorite is typically faster and more even than untreated, but overall germination percentage is about the same for both.

When seedlings have developed two true leaves they are pricked out using blunt ended sticks (6" long, 1/8" diameter) and transplanted into 50-72 cell plug trays filled with a bedding plant mix. Deeper cells are preferred for better root development. Plugs are watered in and grown in the greenhouse at 72-75 °F under 16-hr daylength; they are initially fertilized with a balanced fertilizer at 1-2x per week with 100ppm N for 2-3 weeks, then 200ppm N for 3-4 weeks. When roots fill the plugs and outdoor temperatures allow, the flats are moved outdoors under shade cloth for 1 week, then moved to full sun to await field planting after the last frost date.

In vitro germination: Basic protocol—surface sterilization with ethanol, then bleach/6-10 week cold stratification/repeat surface sterilization/dissection/1-2 week germination on agar medium/transplanting/6 weeks as greenhouse plugs/1 week acclimation/field planting.

In the past in our lab, seed intended for *in vitro* germination has been kept moist after extraction using moist paper towels in plastic bags in the refrigerator until needed. This was thought to improve germination. Successful germination, however, has currently been obtained *in vitro* using seed that has been dried and held at room temperature for several months. Because a cold stratification period will be applied in either case, it seems that either practice will work.

Because the *in vitro* protocol is time consuming and the chance for contamination is great, we typically reserve this procedure for very small seed lots (<50 seeds), very wide crosses in which we expect a substantial number of "empty" seed, or for a seed lot that we've tried to germinate using the basic protocol which did not produce the desired number of seedlings, but still has intact seed in the flat that we think might be simply inhibited by an unbreached seed coat.

A Petri dish with moistened filter paper is prepared for each seed lot for stratification. These are autoclaved for sterility. Have plastic bags and parafilm on hand to seal the dishes after seed is added.

Seed is surface sterilized prior to stratification using 1 minute in 70% ethanol while swirling by hand, then into 20-25 mL solution of 10% bleach + 1-2 drops Tween or other surfactant with agitation on a shaker table at 300+ RPM for 60 minutes. Rinsing is not necessary after treatment. Seed and bleach are poured through a strainer, and seeds are then placed into the sterilized Petri dishes, sealed with parafilm and labeled. Dishes are placed into sealed plastic bags and stratified at 4 °C for 6-10 weeks. They should be checked every few weeks to make sure the filter paper is still moist. If the paper dries out, which often happens due to condensation of the available water inside the lid, the dish can be unsealed, a few drops of sterile water added, and then resealed and returned to stratification. The shortest time we've tried for stratification has been 7 days; the longest time has been 15 months; 6-10 weeks is typical.

When the stratification period has been satisfied, seed is ready for seed coat removal and germination. Two test tubes of water are sterilized by autoclave for each seed lot, and cooled.

Germination vessels should be prepared ahead of time. We are currently using 48-well (0.4 mL) sterile culture plates and the following medium:

½ strength MS media with 100 mg/L myoinositol, 10 g/L sucrose, and 7 g/L agar.[2] The medium should be autoclaved prior to pipetting into the culture plates. Wells should be about ¾ filled with the medium under sterile hood conditions, recapped, cooled to room temperature in the hood, and stored in the hood until ready for seed transfer.

Seed is removed from its Petri dish and surface sterilized as previously, using the 70% ethanol for 1 min. and then bleach + Tween for 1 hr. Seed is removed from the bleach and placed into a tube of sterile water to await dissection. It is best to take out and resterilize only as many seed lots as can be handled in the time available.

We use a seed cutting method that is easier than typical embryo extraction. Using a dissection microscope in open lab conditions (no hood required) we can easily view the seeds at a magnification strong enough to easily identify the radicle end and to check seed viability. The sterile water containing sterilized seed is poured through a small strainer. Seed can then be scraped onto a large Petri dish or clear cutting surface which will allow backlighting on the microscope stage.

Non-viable seeds can be identified and discarded at this point, usually without even cutting into them. Viable seed will be uniformly yellowish or tan, with no blotchiness or variability among seeds. Underdeveloped seed might be dark, grayish, reddish or black. “Empty” seed, without a viable embryo, or that has been overprocessed in surface sterilization or destroyed by pathogens will look whitish, or pale grey, or translucent, sometimes with a dark testa (inner seed coat) visible under magnification. Discard all such seed before beginning to cut into the good seeds. Size of the seed is not a good indicator of viability, but shape can be. If the sides are flat and parallel, rather than continuously curving, it is probably immature.

Using forceps and scalpel cleaned in 70% ethanol, grasp the radicle end of each seed (identified by its more pointed shape in contrast to the more rounded edge of the cotyledon end; also, the micropyle is often visible at the radicle end and there may be tiny bracts), then sever and remove the half of the seed containing the tips of the cotyledons. Healthy, viable seed has a nearly white testa at least three cells thick, and white, firm cotyledons. Any of the following are clear indications that the seed is immature or dead and will not germinate: air spaces, brown inner seed coat, brown or red blotches on the embryo or cotyledons, oozing from within, or mushy cotyledons that fall apart at scalpel touch. An immature testa is usually brown or red, only one cell thick, and is not easily peeled away from the cotyledons.

[2]Previously we have also used: 1) Petri dishes, but contamination is easily spread from seed to seed, whereas the culture plates allow isolation of individual seeds; 2) test tubes, but this requires a relatively large amount of medium per vessel per seed, and the seedlings do not thrive if the volume of media is too large, or if multiple seeds are placed in the same tube, shared contamination is a problem; 3) 96-well ELISA plates, but the volume is rather too small for the seedling to expand much and it must be removed to a test tube for cotyledon, leaf and root expansion before it can be transplanted to a plug tray. The 48-well plate allows room for the germinated seedling to develop to about the same size as those germinated in flats using the standard protocol, and seedlings can then be removed from the culture environment into plug trays sooner.

Retain the portion containing the radicle, embryo, and a portion of the cotyledons and move it into a new tube of sterile water to await placement on the germination medium. If unsure about which half to keep, keep both halves. Make sure to remove at least half of the seed. If more remains, the embryo will have difficulty emerging from the seed coat.

Embryos will show signs of germination as quickly as 2-4 hours after initial cutting. Imbibition begins, and they begin to expand. The seed halves can be transferred to the medium under the flow hood at this point by dumping the water and its contents through a fine mesh strainer and picking them out of the strainer with a forceps cleansed in 70% ethanol, then laying them onto the surface of the medium, one seed per well. HOWEVER: If left in the sterile water for 4 or more hours before transfer to media, most of the embryos will expand enough to expel themselves from the seed coat. (This expulsion appears to be a mechanical response to the water uptake, and does not necessarily mean that germination will follow.) These can then be transferred to the medium as naked embryos without the seed coat. This is recommended, as much of the contamination remaining on the outer seed coat can be avoided. If all of the embryos are not expelled, it is worth leaving them in the water overnight to get as many naked embryos as possible. Those that are not expelled can be transferred with the seed coat intact, but expect a greater chance of contamination.

Once the embryos are loaded into the culture plates in the flow hood, the lids are replaced and the plates are sealed with parafilm. Germination will occur at room temperature on the benchtop, but better results have been obtained with 16 hour day-length and maintaining temperature around 25 °C.

Within 5-10 days the initially white embryos will have begun to take on a green color and root growth will be observed. Roots will typically orient themselves downward, but a few that were transferred with the seed coat still attached might begin with root tips pointing up; these should be repositioned to make contact with the medium. Cotyledons will expand and turn bright green.

If contamination is visible it will typically stay confined to individual cells; contaminated seedlings are seldom successfully rescued, so it is better to keep the lid on to maintain the confinement than to open the lid and let fungal spores become airborne, possibly contaminating previously clean seedlings. If desired, the contamination in individual cells can be suppressed by melting a hole in the lid and pipetting into the cell a 10% bleach solution, but this practice does not seem to be worth the effort, as the healthy seedlings will be removed within a few days before the contamination has the chance to outgrow the confinement of its cell.

Once germinated, the seedlings can follow either of two paths: 1) transfer to a test tube to allow further development before establishing in soil, or 2) remain in the culture plate for a few extra days until the first true leaves begin to show, then transfer to small plug trays in germination mix for further development before establishing in final-size plug trays.

Because the size of both roots and shoots of the seedlings in the 48-well trays is similar to those that are germinated using the standard protocol in the same timeframe, and because removing seedlings from the agar medium is easier and causes less damage before there is much root branching, we are currently using the second option. We have found that seedling survival seems

at least equal to what had been observed with the additional test tube development step. Seedlings seem to acclimate faster and the incidence of culture contamination is mostly eliminated.

It is important to use smaller size plugs first (162-200 per flat) and a lighter germination mix, as seedlings are easily overwatered in the 50-72 cell plugs with the heavier soil. When transplanting from the *in vitro* environment it is critical that seedlings be protected from drying out. Soil should be moist prior to transplanting. New transplants must either be misted with a spray bottle periodically while the rest of the flat is filled, or given mist and plastic film laid over them to prevent exposure to drying air. Once complete, the flat is placed in the mist bench with supplemental light delivering a 16-hour day-length for a day or two, then brought out for successively longer exposures to open air. Bottom heat is recommended to prevent cool conditions at the soil surface conducive to establishment of damping off pathogens.

After about a week when plants are hardened enough they can be moved to the greenhouse, grown at 16 hour days and temperatures ranging from 70-80 °F. When roots fill the cells they are potted on into 50 or 72-cell flats in the same soil medium as for the standard protocol.

Vaccinium: This genus also shows diverse germination requirements and is poorly represented in the literature. For some species, seed is not dormant and will germinate without pretreatment; other species require prechilling (Crossley 1974). Light is required for germination.

1. Surface sterilize the seeds.
2. Sow on paper moistened with ultrafiltered water.
3. Prechill at 1 °C for 8 weeks.
4. Germinate the seeds using the same program of alternating temperatures and light as is used for *Fragaria*.
5. Allow 4-8 weeks for germination.

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Minor Genera Consult the references listed at the beginning of this section for recommendations for germinating the seeds of the 14 minor genera stored at NCGR.

Seedling Management

Seedling management basically consists of two processes:

1. Labeling seedlings, and
2. Overseeing the incorporation of seedlings into the plant collections.

Because the repository holds both clonal and non-clonal plants in its collections, all seedlings must be labeled so they can be distinguished from clonally propagated individuals of the same species. Thus, the major element in seedling management is to adequately label all seedlings. Seedlings usually receive two labels: a standard label for each seedling and a group label for a group of seedlings from the same seed lot. These labels are discussed in detail below. Seedling management also entails care for young seedlings and moving seedlings through the greenhouses to their final field and screenhouse destinations.

Labeling Seedlings

There are three primary elements used to identify plant material at NCGR, and these appear on plant labels and in inventory files. The three elements are: the local inventory number, the taxonomy of the plant, and the material's storage code (which identifies the form or type of material). The alpha-numeric inventory number consists of two parts: the first three letters of the genus of the material and the inventory number assigned by the information manager (e.g. RIB 625 for the *Ribes* accession assigned an inventory number of 625). The genus and species, and variety if needed, of the material also appear on the label. Three storage codes are used at NCGR: PL for clones, SL for seedlings, and SD for seeds. The SL storage code must be written on all labels for seedlings.

The inventory number given to seedlings is also affected by two more factors. The first is that all inventory numbers assigned at NCGR include a three digit suffix, so our example above might actually appear as RIB 625.000. The suffix ".000" is reserved for seed, so our example identifies an accession represented by seed. Seedlings generated from this seedlot are assigned the same inventory number (RIB 625) but are also given sequential numeric suffixes. Thus, 10 seedlings grown out from this seedlot would be given the inventory numbers RIB 625.001, RIB 625.002, RIB 625.003,...RIB 625.010 (see the flow chart below). Numbering seedlings in this way accomplishes two things: it reveals the relatedness of the seedlot and the seedlings (all are RIB 625) and allows for individual seedlings to be tracked using the numeric suffix. Thus, evaluation data can be collected for each individual seedling. If additional seedlings are generated in future, they are assigned the next available numeric suffix (e.g. RIB 625.011 in this example).

Once they have been moved into a greenhouse, each group of seedlings is labeled with a group label. The group label serves primarily to identify the purposes the seedlings are to serve (such as plant material of a new accession received as seed only, for verification of taxonomy, or for seed production) and the person who is responsible for the seedlings. This information allows other NCGR personnel to know who to contact regarding the seedlings (as might be required in controlling greenhouse pests), and also achieves continuity through personnel changes in seeing that seedlings are put to the use for which they were intended. The group label should include the date, the responsible person, the purpose the seedlings are to serve, any special cultural practices (e.g. Seed Production, Do Not Prune Flowers), and any known sensitivity to pesticides.

Incorporation into Collections

Prior to being dispersed to their ultimate field and screenhouse destinations, seedlings should be kept on greenhouse benches designated for seedlings. Very young seedlings (e.g. first leaf stage) should first go to the mist beds after removal from the germinator. Once hardy enough, they should

be moved to a bench for seedlings. Lastly, the seed manager should closely cooperate with the screenhouse and field managers to assure that seedlings are incorporated into the screenhouse, field, and species collections in an efficient and timely manner. Seedlings not needed for the field or screenhouse collections should be retained until it is certain that plants incorporated into these collections survive transplanting. These backup plants should not be discarded until the field and screenhouse managers have been consulted. Extra seedlings and unneeded backup plants for field plantings must be disposed of eventually; they may be given away or discarded. Inventory files should be updated every time the group of seedlings is moved to a new location or when the number of seedlings in the group changes.

Inventory Maintenance

The inventory files are the foundation for the operations carried out at the repository. Inventory files must therefore be accurate. Maintaining accurate files is one of the seed manager's primary duties. Presently, maintaining an accurate seed inventory involves two processes:

1. Determination of seed quantity upon receipt of new seed, and
2. Periodic inspection of seedlots already in storage.

The underlying reason for an accurate seed inventory is the service NCGR provides to the horticultural community in providing small fruit germplasm. Seed is available for distribution only if the quantity of seed exceeds the threshold value separating reserve and working seed. So long as the inventory files are correct, seed will not be advertised as available, and hence will not be requested, if only reserve seed remains in the particular seedlot. Accurate inventory files mean fewer clients are frustrated when seed that is supposedly available is not, and saves the seed manager the labor involved in filling a seed request only to find, after preparing the paperwork and shipping containers, that the quantity of seed is too low to allow distribution.

Determining Seed Quantity

The basic procedure involved in an inventory of seed is the determination of the quantity of seeds in a particular lot. Generally, this is most easily accomplished by weighing the seed and calculating seed quantity. Standard procedure on receipt of new seed calls for determining the mass of 100 seed (see above). Given a clean seedlot and the mean mass of 100 seed from this lot, quantity can be determined quickly and accurately. Simply tare the scale and weighing boat, weigh then repackage the seeds, and calculate quantity. Occasionally, counting seeds in a lot, either by hand or using the electronic counter, is faster (e.g. very small lots). Also, when seed is in the form of dried fruit, the mean number of seeds per fruit and the mean mass of some quantity of fruit must be determined before the quantity of seed in the lot can be calculated; this generally means that dried fruit must be dissected.

However, when the seedlot is dirty or the mass of 100 seed is not known, the inventory is more difficult. In these cases, the operations that should have occurred upon receipt of the seed must now be performed -- the seed must be cleaned and the mean mass of 100 seed determined, then quantity can be calculated. Refer to the section on receipt of fruit and seed, above, for detailed discussion of this process.

Periodic Inspections

Stored seeds are inspected periodically to verify that inventory records are accurate. The procedure is the same as described above, and inventory files are adjusted as needed. Because seed management was neglected in the past at NCGR, inspection of seedlots already on hand is still a major component of the seed inventory. At NCGR, one major genus and one minor genus are inspected every year. Once an accurate count has been made for a particular seedlot, it is not difficult to maintain an accurate inventory file so long as each and every withdrawal of seed is noted and the file adjusted to reflect the changed quantity. If this practice is rigidly followed, periodic inspections are not needed. However, if seeds can be withdrawn without the knowledge of the seed manager, the periodic inspection will be necessary. Institution policy should state that all seed withdraws are made by seed management staff, or at least with their knowledge and consent.

During the periodic inspection, or any time a seedlot is handled, the packaging in which the lot is stored should be visually inspected for excessive wear or damage. Once ruptured or punctured, a moisture-proof envelope is no longer moisture proof. Damaged packaging should be replaced.

Seed Requests

The repository fills a service-oriented mission, and one of the seed manager's major tasks is responding to requests for seed. These requests primarily come from persons outside of NCGR, but repository staff may also request seed for various research programs. Responding to seed requests entails:

1. Completion of required paperwork
2. Preparation of shipping containers
3. Transfer of seed from the original seedlot to shipping containers
4. Storage of packaged seed until inspected and actually shipped, and
5. Updating of inventory files.

The seed manager prepares a labeled shipping container for each of the available seedlots requested, removes these seedlots from the freezer and transfers seeds (usually 25) to the shipping container, repackages the original seedlot, and returns the seedlot to its correct location in the freezer.

Seed Request Paperwork The distribution manager has the responsibilities of managing seeds. Requests for seed are handled in the same manner as other plant requests. See: Distribution. Paperwork for Requests and Shipping.

Shipping Containers Seeds may be shipped in paper coin envelopes or in plastic vials. If paper is used, the envelopes will need to be well sealed before shipment, but not before inspection by Animal and Plant Health Inspection Service (APHIS) personnel. The bottom of the coin envelope may be taped with scotch tape when first preparing the labeled shipping containers; however the top must not be taped until after inspection. Plastic vials are simply closed after filling. Both envelopes and vials must be labeled with the local inventory number (e.g. RIB 625) and the genus and species of the seed.

Quantity of Seed Distributed Seed requests are filled with 25 seeds from the accession requested. Occasionally, more than 25 seeds will be sent to a requestor, as when the request specifically asks

for a larger quantity and sufficient seed is available to comply with such a request or when so directed by the curator. Seeds may be counted out by hand, using the electronic counter, or determined by mass, but the number of seeds removed from the original seedlot must be known so that the quantity of seed remaining in storage can be updated.

Storage Prior to Shipment Seed packaged for shipment must be stored until it is actually shipped, and should be stored at the same low temperature as the seed collection. The filled and labeled storage containers are placed in a zip-top bag on which the last name of the requestor has been written. The labeled zip-top is placed in the bin labeled "seed requests" in the freezer; this is where the propagator will look for the seeds which will be sent to the requestor.

Adjustment of Inventory Files Because a known quantity of seed was removed from the seedlot, fewer seeds remain in storage. The change in quantity must be reflected in the inventory files. Changes in seed quantity are noted both on the exterior of the seed packet (on the laminated envelope) and in appropriate inventory files. Changes to the inventory file reflecting the distribution of seed should be made promptly, as many operations depend on an accurate inventory.

Occasionally, seed packaged for shipment will not be allowed to be distributed by the APHIS inspector. In this event, the seed must be returned to the original seedlot, and seed quantity updated on both the laminated envelope and in the inventory file.

Monitoring

The seed manager must routinely monitor three things:

1. Seed storage conditions
2. Seed packet integrity, and
3. Seed viability.

Seed is stored in standard commercial freezers, and these must be monitored to assure that they are functioning as desired. The condition of the containers in which seeds are stored must also be monitored to attain the maximum possible longevity of stored seeds. Lastly, the viability of stored seeds should be monitored so that seed can be increased in a timely manner.

Storage Conditions Once every two weeks, the seed manager should check the temperatures inside the upright and chest freezers. Additionally, the operating temperatures of the cold room and small fridge should be monitored at the same frequency as these are used to stratify seeds. Temperatures are easily checked using a digital thermometer. To measure temperature in a freezer or fridge, place the sensor (thermocouple) inside the cabinet but leave the hand-held readout unit outside, and allow 5-10 minutes for temperatures to return to a stable state after the cabinet has been opened. The entire unit, sensor and readout, may be placed in the cold room. Temperatures of the two freezers should average −20 °C, the cold room should be held at 5 °C, and the fridge should be at 1 °C. Operating temperature data should be entered into the prepared spreadsheet and long-term moving averages calculated and plotted. Operating temperatures fluctuate considerably, particularly in the freezers and fridge. If the temperature data indicates that operating temperatures are not as desired, the unit must be adjusted.

Seed Packet Integrity Seed longevity is greatest when seed temperature and moisture content are low (Harrington 1972, Justice and Bass 1978). To maintain a low moisture content in stored seeds, they must be packaged in moisture-proof containers because relative humidity inside the storage freezers is 50%. So long as they are not ruptured or punctured, laminated envelopes are adequate moisture-proof containers. Any time a seed packet is handled, the packaging should be inspected for damage. Damaged envelopes should be immediately replaced. All information (inventory number, genus and species, date collected, date received at NCGR, mass per 100 seeds, and quantity of seeds) must be transcribed from the damaged envelope to the new envelope. The printed label from the damaged laminated envelope should be cut off and attached with packing tape to the new laminated envelope. The distribution record should go inside the new laminated envelope.

The viability of stored seed should be monitored through periodic germination tests. Ideally, the frequency with which a seedlot is tested is based on the physiology of the stored seed. However, this knowledge is generally lacking in small fruit germplasm. A good rule of thumb is for viability to be tested once every five years. However, as the number of seedlots in storage increases this becomes increasing difficult to accomplish. Additionally, every germination test reduces the quantity of seed available for distribution. If requestors return the seed request response form included in their shipment, the germination percentages they obtain may be used as a proxy for the viability test.

Seed Increase

In most cases, the quantity of seed in any one seedlot is relatively small. Generally, only a small amount of fruit or seed is received when a new accession is sent to NCGR. Because this seed is distributed to requestors and because seed remains viable for a limited time, even given optimal storage, seedlots must be increased or regenerated if NCGR is to retain viable seed in the long-term. The reserved quantity of a seed accession is intended to allow for seed increase, but seed regeneration is not a simple matter. Theoretical considerations of population genetics and conservation of genetic diversity and practical limitations of land, labor, capital, and time often make seed production in small fruit difficult. In addition, seed regeneration has not been a standard practice at NCGR to date.

Population genetics theory suggests that, in the absence of detailed genetic information for a population, 500 seedlings should be used in seed regeneration to minimize genetic erosion (Hedrick and Miller 1992). The U.S. National Germplasm System, recognizing real world limitations, suggests 200 and a minimum of 100 seedlings in seed increase efforts (National Research Council 1991). Even 100 seedlings of the genera held at the repository, most of which are shrubs, would require a large area of land. Additionally, many of the small fruits genera do not produce fruit until the second year of growth. Plants used in seed production must also be caged to prevent cross pollination. To increase seed, a sufficient number of seedlings must be generated, planted in the field, watered, fertilized and trained as needed for two or more years, caged with pollinators, and finally removed from the field to make room for the next population. NCGR lacks the resources to carry out such an operation.

Seed regeneration efforts at NCGR have been limited to very small populations (e.g. 10 seedlings). *Fragaria* seedlings isolated in the greenhouses have been used to increase seed quantity. The new

species plantings may also be used for seed regeneration, but there are several important considerations to keep in mind. In many cases, these plantings consist of seedlings grown out from seed collected from up to 10 geographically distinct populations. This is appropriate for the primary function of the species planting, which is to show to plant breeders the variability found within the particular species. However, crossing among these seedlings may result in much altered allele frequencies in the seed produced and will tend to blur any ecotype differentiation which has occurred among the different populations. From the operational perspective, the arrangement of the seedlings in linear rows is not the optimal spatial arrangement for caging. The linear row will require a very long and narrow cage, with a large surface area. The larger the ratio of surface area to caged plant, the more difficult it will be to prevent immigration of undesired pollen (e.g. larger surface area probably means more holes from rocks thrown by farm machinery which can allow entry of insects carrying pollen from other plants). Additionally, the linear arrangement may restrict pollen movement within the cage. The probability of pollen being transferred to an immediate neighbor may be greater than dispersal to the seedling at the far end of the cage, though this is a function of pollinator behavior. Seed regeneration in small fruit genera is a problematic area.

Backup of Seed to NCGRP

Ideally, a representative seed sample of each seed accession should be sent to the National Center for Genetic Resource Preservation (NCGRP), Fort Collins, Colorado, for back-up in the base collection. NCGRP has the following requirements before seed can be shipped to them for inclusion into their main collection.

- The accession must have a plant introduction (PI) number,
- The viability of the accession must be greater than 65%,
- The quantity of seed must be > 1,000,
- Regeneration procedures must be defined.

Seeds which do not meet the above criteria may be sent to NCGRP for "critical storage." This allows seed lots to be sent to NCGRP for added germplasm preservation security.

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