

# Germination In Vitro, Micropropagation, and Cryogenic Storage for Three Rare Pitcher Plants: *Sarracenia oreophila* (Kearney) Wherry (Federally Endangered), *S. leucophylla* Raf., and *S. purpurea* spp. *venosa* (Raf.) Wherry

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**Abstract.** The genus *Sarracenia* forms a group of carnivorous pitcher plants native to North America. Habitat destruction and overcollection have caused pitcher plants to become rare, including U.S. federally endangered *S. oreophila* as well as *S. leucophylla* and *S. purpurea* spp. *venosa* (Raf.) Wherry, both listed as endangered in several states. Protocols for in vitro germination, sustainable shoot micropropagation, shoot establishment in soil, and seed cryopreservation are presented. Six-min sulfuric acid scarification treatments coupled with appropriate tissue culture media resulted in germination in vitro within 3 weeks, often reaching greater than 50%. Best germination for *S. leucophylla* and *S. purpurea* occurred on one-third strength Murashige and Skoog (MS) salts, whereas *S. oreophila* germinated best on one-sixth strength MS salts. Adjustment of pH to 4.5 to simulate a bog environment further increased germination for *S. leucophylla*. Shoot multiplication occurred at optimal levels when explants were placed on media in the presence of a cytokinin without auxin with greatest multiplication on 6-benzylaminopurine (BAP) or trans-zeatin and best shoot quality on trans-zeatin. Plant establishment in soil required both an in vitro rooting treatment and use of shoot clusters resulting in greater than 80% survival in soil. Seed cryopreservation tests with all three species suggest storage in liquid N<sub>2</sub> followed by in vitro micropropagation and plant establishment can be used to preserve material long term.

The genus *Sarracenia* forms a group of carnivorous pitcher plants found mainly in North America. Pitcher plants are found in bog environments throughout the United States, usually in areas of slow-moving water where the acidic soil is poor in nutrients. Pitcher plants use deep tube-like fluid-filled leaves (pitchers) to attract and capture prey helping the plant to compete in

their nutrient-poor habitat (Wakefield et al., 2005).

With habitat destruction levels at 93% to 97%, populations have been reduced by 90% and many U.S. pitcher plants have become rare and endangered (Stiefel, 2000; Uhnak, 2003). Three species of this genus, *S. leucophylla* Raf., *S. oreophila* (Kearney) Wherry, and *S. purpurea* spp. *venosa* (Raf.) Wherry,

are considered to be endangered in the state of Georgia and vulnerable in other states (Chafin, 2007; Georgia Department of Natural Resources Wildlife Resources Division, 2011; NatureServe, 2011). *Sarracenia oreophila* is listed as federally endangered by the U.S. Fish and Wildlife Service (USFWS, 1994). The large interest in carnivorous plants has also had some adverse effects. Carnivorous plants have become popular among hobby horticulturists, fueled in part by soaring Internet sales. Although many species are endangered, plant poachers still harvest wild plants illegally to sell to collectors (Stiefel, 2000). Several *Sarracenia* species have shown medicinal and commercial value for anticancer treatment (Miles et al., 1974), betulinic acid production for regulation of hair growth (Bradbury et al., 2002), and production of insect attractants (Howse, 1996) leading to added collection pressure on natural populations.

*Sarracenia* species can be easily grown from seed or vegetatively propagated from rhizomes (Thomas, 2002). However, the process is slow and may yield small numbers of plants. Several books have been devoted to carnivorous plant ecology and cultivation (D'Amato, 1998; Rice, 2006). Reports of seed germination percentages are highly variable ranging from 5% in nature for *S. purpurea* (Ellison and Parker, 2002) to 85% germination in the light for *S. purpurea* var. *terrae-novae* de la Pylaie in a greenhouse setting after 32 d of stratification at 4 °C for 6 weeks (Gotsch and Ellison, 1998). In a study that compared taxonomic characters among species, Ellison (2001) examined germination requirements of eight *Sarracenia* species. Using a 4- to 5-week stratification treatment at 4 °C, germination ranged from 8% to 16% after 34 d for the eight species. *Sarracenia leucophylla* showed 12% germination and *S. purpurea* ssp. *purpurea* and *S. purpurea* spp. *venosa* var. *burkii* showed 10% and 18% germination, respectively.

In vitro propagation from seed, shoot tips, and even rhizomes can multiply the number of individuals of an endangered species rapidly and year round (Fay, 1992; Reed et al., 2011). Once enough plants are available, they can be used to repopulate existing populations or to establish new planting sites for conservation, research, education, or recreational purposes. In vitro-grown plants can be sold to collectors decreasing the collection pressures on wild populations. Although several plant tissue culture companies produce pitcher plants for sale, very little literature is available concerning the propagation of *Sarracenia* plants using micropropagation techniques. Several researchers have attempted to develop in vitro propagation methods for *Sarracenia* species (Arnold, 1989; Uhnak, 2003).

Cryopreservation is emerging as a reliable process for seed conservation and long-term storage of many desiccation-tolerant seeds and tissues (Englemann, 2011; Pence, 2011; Pritchard, 2007; Reed et al., 2011). Genetic preservation through long-term seed storage is indicated as a goal in the recovery plan for

endangered *Sarracenia oreophila* (USFWS, 1994). Seed cryopreservation may play an important role in the conservation of these rare and endangered plants.

The objectives of this study were to develop a reliable micropropagation protocol for endangered *Sarracenia* species using seed to start cultures. A further objective was to investigate the effect of seed cryostorage on these endangered species.

## Materials and Methods

### Plant materials and seed sterilization

*Sarracenia* seed were collected by Atlanta Botanical Garden (ABG) staff and stored as follows until experiments were performed in 2008–2011. Two lots of *S. leucophylla* seed were collected in different years from the one known wild population in Georgia in Sumter County: Lot A, collected in Oct. 2008 was stored at  $-20^{\circ}\text{C}$ . Lot B was collected in October 2006, dried for 6 weeks, and stored at  $-20^{\circ}\text{C}$ . A third lot, C, was collected in Aug. 2010 on a property of The Nature Conservancy at Splinter Hill bog in Baldwin County, AL. Seed was held at room temperature until use shortly after collection. *Sarracenia oreophila* seed were collected from a Nature Conservancy bog at Center, AL, in September 2003, dried in an airtight desiccator cabinet for 1 month over calcium sulfate desiccant at 12% relative humidity (Walls, 2009), and stored at  $-20^{\circ}\text{C}$ . *S. purpurea* spp. *venosa* (Raf.) Wherry (hereafter referred to as *S. purpurea*) seed were collected twice: Lot A was collected from an exhibit bog at ABG in Fall 2008 and stored at  $4^{\circ}\text{C}$ ; Lot B was collected in Aug. 2010 from Manassas Bog near Statesboro, GA. Seed was held at room temperature for several months until use in the experiments.

As a result of the small size and fragility of *Sarracenia* seeds, sterilization and rinsing procedures were done with metal sieves containing fine wire inserts covered with finely woven nylon fabric (fabric 06400JP-72 with  $162 \times 162$  fibers/inch and openings  $\approx 60 \mu\text{m}$  in size from Decotex Inc., Pawling, NY). The fabric tended to minimize seed damage, especially after scarification procedures that weakened the seedcoat.

### Media and culture conditions

Prior experiments by ABG staff showed excellent *Sarracenia* species growth and

vigor without signs of leaf burn on medium containing one-third MS salts (Murashige and Skoog, 1962) along with  $0.4 \text{ mg}\cdot\text{L}^{-1}$  thiamine HCL,  $100 \text{ mg}\cdot\text{L}^{-1}$  myo-inositol, 3% sucrose, and  $4.5 \text{ g}\cdot\text{L}^{-1}$  Phytagel® or Gelrite®. To induce shoot multiplication, the addition of  $4.6 \mu\text{M}$  ( $1 \text{ mg}\cdot\text{L}^{-1}$ ) kinetin was found to work well. These media provided a starting point for the following experiments.

Medium pH was adjusted with KOH or HCl after the addition of all ingredients except gelling agents. Media were autoclaved at  $121^{\circ}\text{C}$  for 20 min. Germination and shoot culture experiments were incubated at  $25\text{--}26^{\circ}\text{C}$  under a 16/8-h (day/night) photoperiod with light from cool white fluorescent lamps at an intensity of  $\approx 30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

### Experimental design and evaluation

Unless otherwise indicated, treatments were arranged in a complete randomized design. Germination responses, based on seeds that were not contaminated and indicated by emergence of the radicle, were evaluated at 3 and 6 weeks and percentages for individual replicates were transformed by arcsine  $\sqrt{(\%)}$  for analysis of variance. Significant differences by the least significant differences test at the 5% level and treatment averages are sometimes shown with SES.

### *Sarracenia* seed germination experiments

**Expt. 1: Preliminary sterilization tests with *S. purpurea*.** Preliminary tests evaluating seed germination for *S. purpurea* after sterilization treatments for 40 min with 0.6% sodium hypochlorite, 10 min with 20% hydrogen peroxide (MacKay et al., 2006; Pullman et al., 2006), or 10 min of scarification with concentrated sulfuric acid (Uhnak, 2003)

showed promising germination after scarification. In the scarification method, seeds were rinsed in tap water for 10 min, scarified by gently stirring for 10 min in 3 mL of concentrated sulfuric acid (EM Science, Gibbstown, NJ) followed by three rinses in sterilized water, each for 5 min (Uhnak, 2003). Forty scarified seeds were placed onto one-third strength modified MS without hormones and incubated under fluorescent light at room temperature.

**Expt. 2: *S. leucophylla*—Murashige and Skoog salt strength  $\pm$  kinetin.** Trial 1. A  $2 \times 2$  factorial experiment was developed with *S. leucophylla* (Lot A) to test germination on one-sixth vs. one-third strength MS salts in the presence or absence of  $4.6 \mu\text{M}$  ( $1 \text{ mg}\cdot\text{L}^{-1}$ ) kinetin (Sigma Aldrich, St. Louis, MO). Seeds were scarified in 3 mL of concentrated sulfuric acid for 10 min and rinsed aseptically in five water rinses, each for 5 min, using a sterile sieve to collect seed. Seeds were placed on four media: one-third strength MS without hormones, one-third strength MS +  $4.6 \mu\text{M}$  kinetin, one-sixth strength MS without hormones, and one-sixth strength MS +  $4.6 \mu\text{M}$  kinetin (Table 1) with five seed per plate and 11 to 12 plates per treatment.

Trial 2. Based on observations from Expt. 3, a repeat of Trial 1 was done but the sulfuric acid exposure time was reduced to 6 min.

**Expt. 3: *S. leucophylla*—sulfuric acid scarification exposure time.** Trial 1 of Expt. 2 showed abundant broken and crushed seed-coats after 10 min of scarification suggesting that reduced time in acid may damage seeds less. *S. leucophylla* (Lot A) was used to optimize scarification time by comparing germination for 4, 6, 8, or 10 min of sulfuric acid exposure. Scarified seeds were plated onto one-third MS medium without hormones, the

Table 1. Components for *Sarracenia* germination media.

Components	Media and components ( $\text{mg}\cdot\text{L}^{-1}$ )				
	One-third MS	One-third MS + kinetin	One-sixth MS	One-sixth MS + kinetin	One-third MS + NAA
$\text{NH}_4\text{NO}_3$	550	550	275	275	550
$\text{KNO}_3$	633	633	317	317	633
$\text{KH}_2\text{PO}_4$	57	57	28	28	57
$\text{CaCl}_2\cdot 2\text{H}_2\text{O}$	147	147	73	73	147
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	123	123	62	62	123
KI	0.275	0.275	0.1375	0.1375	0.275
$\text{H}_3\text{BO}_3$	2.07	2.07	1.03	1.03	2.07
$\text{MnSO}_4\cdot 4\text{H}_2\text{O}$	7.43	7.43	3.72	3.72	7.43
$\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$	2.87	2.87	1.43	1.43	2.87
$\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$	0.083	0.083	0.0415	0.0415	0.083
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	0.008	0.008	0.004	0.004	0.008
$\text{CoCl}_2\cdot 6\text{H}_2\text{O}$	0.008	0.008	0.004	0.004	0.008
$\text{FeSO}_4\cdot 7\text{H}_2\text{O}$	9.27	9.27	4.63	4.63	9.27
$\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$	12.4	12.4	6.2	6.2	12.4
Sucrose	30,000	30,000	15,000	15,000	30,000
Myo-inositol	100	100	50	50	100
Thiamine HCL	0.33	0.33	0.165	0.165	0.33
Pyridoxine HCL	0.165	0.165	0.0825	0.0825	0.165
Nicotinic acid	0.165	0.165	0.0825	0.0825	0.165
Glycine	0.66	0.66	0.33	0.33	0.66
Kinetin	—	1	—	1	—
NAA	—	—	—	—	0.1
Phytagel	4,500	4,500	4,500	4,500	4,500
TC agar	—	—	—	—	—
pH	5.7 or 4.5	5.7	5.7	5.7	5.7

MS = Murashige and Skoog; NAA = 1-naphthaleneacetic acid.

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best medium from Expt. 2. Five replicates containing eight seed per plate were tested for each treatment.

**Expt. 4:** *S. oreophila*—*Murashige and Skoog salt strength*  $\pm$  kinetin. Germination on one-sixth vs. one-third strength MS salts in the presence or absence of 4.6  $\mu\text{M}$  (1  $\text{mg}\cdot\text{L}^{-1}$ ) kinetin was compared with a different species of *Sarracenia*. *S. oreophila* seeds were scarified in concentrated sulfuric acid for 6 min and placed onto one-third and one-sixth strength MS with and without 4.6  $\mu\text{M}$  kinetin (Table 1) with 50 seeds per treatment.

**Expt. 5:** *S. leucophylla*—*medium pH*. Pitcher plants live in bog environments that are often acidic at pH 4 to 5 (Plummer, 1963). Germination was compared in media differing in pH. Eleven replications of five seed per plate of *S. leucophylla* (Lot A) were tested for germination on one-third strength MS medium without hormones and pH adjusted with KOH or HCL before autoclaving to 5.7, 5.2, 4.5, or 4.0. Based on positive results from the previous trial, a second trial was conducted at pH 5.7 and 4.5.

### ***Sarracenia* shoot multiplication experiments**

**Expt. 6:** *Cytokinin type and shoot multiplication*. When *S. purpurea* germinants were left on germination medium for 6 weeks, shoots enlarged but did not form axillary shoots. Preliminary tests on *S. purpurea* showed minimal shoot multiplication with medium containing 4.6  $\mu\text{M}$  kinetin.

Several tests were carried out to compare shoot multiplication with four cytokinin types. *S. purpurea*, *S. leucophylla*, and *S. oreophila* shoots from germination experiments were each divided randomly among multiplication medium that used one-third strength MS salts and compared no hormones with the additions of 4.6  $\mu\text{M}$  kinetin, 13.3  $\mu\text{M}$  (3  $\text{mg}\cdot\text{L}^{-1}$ ) BAP (Sigma Aldrich), or 9.1  $\mu\text{M}$  (2.0  $\text{mg}\cdot\text{L}^{-1}$ ) trans-zeatin (Sigma Aldrich). Five to eight shoots for each species were placed on 7 mL of each medium in 60  $\times$  15-mm petri plates and incubated in the light (30  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). After 2 months, resulting shoots for *S. purpurea* were counted, divided, and transferred to 20 mL fresh medium contained in Magenta boxes (Magenta, Chicago, IL) for another 2 months and then counted again. After 6 weeks, *S. leucophylla* and *S. oreophila* shoots were counted. Multiplication rates per subculture were calculated based on the number of shoots produced at the end of a subculture cycle divided by the number of shoots at the start of the cycle.

### ***Sarracenia* root induction in vitro.**

**Expt. 7:** *In vitro root induction*. Preliminary tests with shoots produced on one-third MS salts containing kinetin showed promising root induction within 4 to 6 weeks when transferred to one-third MS salts without hormones or with 0.5  $\mu\text{M}$  (0.1  $\text{mg}\cdot\text{L}^{-1}$ ) 1-naphthaleneacetic acid (NAA) (Table 1). When *S. purpurea* single shoots or shoot clusters with or without roots were transferred to pre-moistened peatmoss in the

greenhouse and maintained under high humidity with clear domes or constant mist with natural lighting (10–14-h photoperiod depending on season), only shoot clusters with roots survived. Shoot clusters established in the soil could be further divided and growth continued under greenhouse conditions.

To verify these initial observations, individual shoot clusters of *S. purpurea* or *S. leucophylla* grown on one-third MS + 4.6  $\mu\text{M}$  kinetin were transferred to magenta boxes containing 20 mL of one-third MS without hormones or one-third MS with 0.5  $\mu\text{M}$  NAA. Five shoots per species were tested in each treatment. Shoots were incubated at 25–26 °C under a 16/8-h (day/night) photoperiod with light supplied by cool white fluorescent lamps at an intensity of  $\approx 30$   $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

### **Acclimation to soil**

After 7 weeks, when roots had formed, individual shoots were rinsed to remove residual medium and planted in a mixture of five parts of ABG Carnivorous Plant Mix (five parts peatmoss:three parts milled sphagnum moss:one part builders sand) and one part ABG Cutting Mix II (four parts perlite:one part milled sphagnum moss:one part pumice) and incubated under  $\approx 40$   $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  of a 16/8-h (day/night) photoperiod with light supplied by plant and aquarium fluorescent lamps (Phillips F40-T12, 40 W HG). The previous experiment was carried out again, this time with five shoots per treatment from *S. leucophylla*, *S. oreophila* and *S. purpurea* grown on one-third strength MS containing 9.1  $\mu\text{M}$  trans-zeatin.

### ***Sarracenia* seed cryopreservation in liquid nitrogen**

The optimum moisture content for seed cryopreservation varies from 7% to 14% depending on species and seed lipid content (Pritchard, 2007). To determine seed water contents before liquid  $\text{N}_2$  exposure, one to three sets of 10 seed per species were placed in small pre-weighed glass vials covered with aluminum foil to prevent water uptake or loss from or to the air and weighed on a five-place metric balance. Seed were dried for at least 24 h at 70 °C in the uncovered vial and then covered and reweighed to obtain dry weights for water content calculations.

Seed samples were placed in Nalgene cryogenic storage vials and either rapidly immersed in liquid  $\text{N}_2$  or cooled to  $-35$  °C at a rate of 0.33 °C/min using a programmable freezer and then immersed in liquid  $\text{N}_2$ . To retrieve seed, vials were removed from liquid  $\text{N}_2$  and thawed in a 37 °C water bath for 1–2 min. Seed was retrieved, scarified, and tested for germination as described.

**Expt. 8:** *Seed cryopreservation and post-storage survival*. Seven cryopreservation and post-storage viability trials were carried out using *S. leucophylla*, *S. oreophila*, and *S. purpurea* seed (Table 4). In Trial 1, 30 seeds of Lot A of *S. leucophylla* were left untreated or placed in a vial and immersed in liquid  $\text{N}_2$  for at least 48 h and thawed. Seeds were scarified for 6 min and placed on one-third

MS without hormones at pH 5.7. Trial 2 was a repeat of Trial 1 but Lot B of *S. leucophylla* was used. Trial 3 split seeds from Lot B into three treatments: control (untreated), rapid immersion in liquid  $\text{N}_2$ , and slow cooling before immersion in liquid  $\text{N}_2$ ; germination occurred on one-third MS without hormones at pH 5.7. Trial 4 consisted of *S. leucophylla* Lot C: 30 control seeds and 30 rapid immersion seeds were germinated on one-third MS without hormones at pH 4.5. A small preliminary trial with *S. oreophila* seed placed in cryostorage showed heavy seed damage after scarification. Thus, seed in Trials 5 and 6 compared germination of *S. oreophila* on one-sixth strength MS without hormones at pH 5.7 for control and fast immersion treatments using 4 min of scarification. Trial 7 tested germination for 30 control and 30 rapid immersion seeds of *S. purpurea* Lot B on one-third strength MS without hormones at pH 4.5.

## **Results**

### ***Sarracenia* seed germination experiments**

**Expt. 1:** *Preliminary sterilization tests with S. purpurea*. Germination began after 10 to 14 d and generally reached a maximum at 3 weeks. Germination after 25 d for seeds sterilized with scarification reached 23%. Contamination was not observed.

**Expt. 2:** *S. leucophylla*—*Murashige and Skoog salt strength*  $\pm$  kinetin. Trial 1. *S. leucophylla* germination began within 10 d and approached maximum values at 3 weeks, although a few additional seed germinated later (Table 2). The highest germination (25%) occurred with 10 min scarification on one-third strength MS without hormones, although differences were not statistically significant. After 10 min of scarification, some seed had broken coats and some were crushed during handling suggesting acid treatments may be too strong (Fig. 1).

Trial 2. Based on results from Expt. 3, the 2  $\times$  2 factorial design of Expt. 2 was repeated but with scarification times reduced to 6 min. Trends similar to those seen with the 10-min scarification occurred but germination was much higher (Table 2).

When trials were combined, analysis of variance and multiple range tests for 3-week germination showed greater germination percentages with 6 vs. 10 min of scarification and differences were highly significant ( $P = 0.01$ ). Addition of kinetin to germination medium reduced germination and differences were statistically significant for one-sixth strength MS salts ( $P = 0.05$ ) but for one-third strength MS salts, differences were only statistically significant at  $P = 0.11$ . Use of one-third strength MS vs. one-sixth strength MS salts caused higher germination, but differences were not statistically significant ( $P = 0.16$ ). Between the two trials, contamination occurred on only one of 400 seed tested.

**Expt. 3:** *S. leucophylla*—*sulfuric acid scarification exposure time*. Germination averages for *S. leucophylla* at 3 weeks increased



as sulfuric acid exposure was decreased from 10 min (12.5%) to 4 min (32.5%) and differences were statistically significant between 4 and 10 min (Fig. 2). Although no contamination was observed in this experiment, 6-min scarification was selected for further experimentation to decrease contamination possibilities in more heavily contaminated seed.

*Expt. 4: S. oreophila—Murashige and Skoog salt strength ± kinetin.* *S. oreophila* germination in vitro was also high with little change after 3 weeks (Table 2). Contamination percentages were slightly higher compared with the other species because three of 40 plates showed contamination. When analyzed using the 2 × 2 factorial arrangement, germination averages were higher in one-sixth

strength MS (± kinetin treatments averaged 53%) vs. one-third strength MS treatments (± kinetin treatments averaged 39.3%); differences were close to statistical significance at  $P = 0.11$ . Germination did not change with the addition of kinetin.

*Expt. 5: S. leucophylla—medium pH.* Medium at pH 4.0 did not gel properly and some seeds sank into the medium, but high germination still occurred (Table 3). In the first trial, germination was highest at pH 4.5 (Table 3). A second trial with pH 5.7 and 4.5 treatments confirmed higher germination at pH 4.5 (Table 3). Combining trials, germination averaged 50% and 58%, respectively, at pH 5.7 and 4.5 but differences were not statistically significant ( $P = 0.26$ ).

### *Sarracenia* shoot multiplication experiments

*Expt. 6: Cytokinin type and shoot multiplication.* Average shoot multiplication per subculture (2 months) over two subcultures for *S. purpurea* was as follows (medium; rate in parentheses): one-third strength MS without hormones (2.0), 4.6  $\mu\text{M}$  kinetin (3.5), 13.3  $\mu\text{M}$  BAP (5.9), and 9.1  $\mu\text{M}$  trans-zeatin (4.8). Differences were statistically significant over the two subcultures (Fig. 3A). Shoots from hormone-free, kinetin, or BAP-containing medium were similar in size but those from trans-zeatin were larger, sometimes double that of other treatments (Fig. 3B).

After 6 weeks, *S. leucophylla* showed similar, but lower trends of multiplication by cytokinin type: one-third strength MS without hormones (1.3), 4.6  $\mu\text{M}$  kinetin (1.9), 13.3  $\mu\text{M}$  BAP (3.4), and 9.1  $\mu\text{M}$  trans-zeatin (2.9). *S. oreophila* multiplied the slowest but again with similar trends by cytokinin: one-third strength MS without hormones (1.1), 4.6  $\mu\text{M}$  kinetin (1.0), 13.3  $\mu\text{M}$  BAP (2.2), and 9.1  $\mu\text{M}$  trans-zeatin (1.6). Shoot production differences were statistically significant between hormone-free and BAP and trans-zeatin treatments and between kinetin and BAP for both species. A majority of the *S. leucophylla* and *S. oreophila* explants produced

Table 2. *Sarracenia* germination in vitro on media varying in salts and hormones.

Medium	Seed germination (%)	
	3 weeks	6 weeks
<i>Sarracenia leucophylla</i>		
Trial 1—10-min sulfuric acid sterilization		
One-third strength MS, no hormones	25.5 a	25.5 a
One-third strength MS, 1 $\text{mg}\cdot\text{L}^{-1}$ kinetin	19.3 a	22.8 a
One-sixth strength MS, no hormones	22.0 a	24.0 a
One-sixth strength MS, 1 $\text{mg}\cdot\text{L}^{-1}$ kinetin	12.0 a	12.0 a
Trial 2—6-min sulfuric acid sterilization		
One-third strength MS, no hormones	66.7 a	66.7 a
One-third strength MS, 1 $\text{mg}\cdot\text{L}^{-1}$ kinetin	50.0 ab	56.7 a
One-sixth strength MS, no hormones	65.5 a	65.5 a
One-sixth strength MS, 1 $\text{mg}\cdot\text{L}^{-1}$ kinetin	41.7 b	53.3 a
<i>Sarracenia oreophila</i>		
One-third strength MS, no hormones	40.4 a	40.4 a
One-third strength MS, 1 $\text{mg}\cdot\text{L}^{-1}$ kinetin	35.4 a	37.5 a
One-sixth strength MS, no hormones	50.0 a	52.9 a
One-sixth strength MS, 1 $\text{mg}\cdot\text{L}^{-1}$ kinetin	46.3 a	48.8 a

Values within a trial and column followed by the same letter are not statistically different by the multiple range test at  $P = 0.05$ ; analyses are based on arcsine  $\sqrt{\%}$  transformation of percentages. MS = Murashige and Skoog.

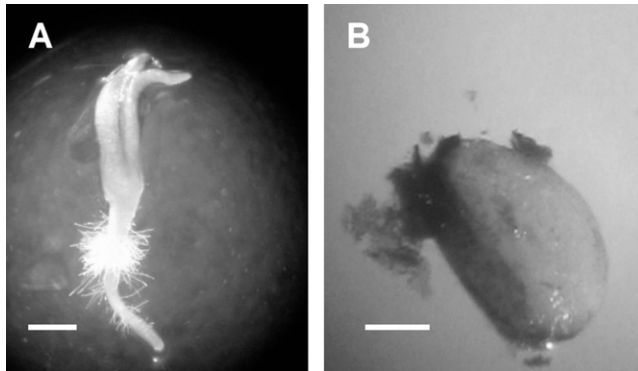


Fig. 1. (A) *Sarracenia leucophylla* seed germination. (B) Ten min of scarification has removed much of the seedcoat exposing the endosperm. Scale bars: A = 1 mm, B = 0.5 mm.

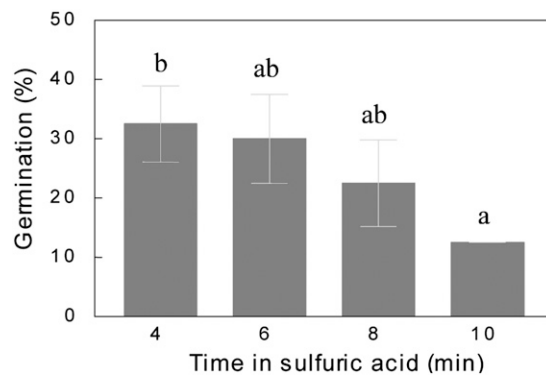


Fig. 2. *Sarracenia leucophylla* germination on one-third strength Murashige and Skoog medium without hormones after 3 weeks with varying scarification times. Values represent averages of five replications (eight seed per replication) along with SE bars. Bars with the same letter above are not statistically different by the multiple range test at  $P = 0.05$ .

Table 3. *Sarracenia leucophylla* germination on one-third strength Murashige and Skoog salts in response to medium pH.

Medium pH	Seed germination (%)	
	3 weeks	6 weeks
Trial 1		
5.7	56 ab	60 ab
5.2	42 a	44 a
4.5	66 b	66 b
4.0	62 b	62 b
Trial 2		
5.7	44 a	—
5.2	—	—
4.5	50 a	—
4.0	—	—
Averages for two trials above		
5.7	50.0 a	—
4.5	57.8 a	—

Values within a trial and column followed by the same letter are not statistically different by the multiple range test at  $P = 0.05$ ; analyses are based on arcsine  $\sqrt{\%}$  transformation of percentages.

larger shoots on trans-zeatin compared with the other cytokinins tested.

All three species, now in culture for  $\approx 2$  years, continue to show sustainable shoot multiplication when maintained in one-third strength MS medium containing  $9.1 \mu\text{M}$  trans-zeatin and transferred every 2–3 months.

#### *Sarracenia* root induction in vitro

*Expt. 7: In vitro root induction.* *S. leucophylla* roots appeared in vitro after  $\approx 3$  weeks and *S. purpurea* roots appeared after  $\approx 4$

weeks. After 7 weeks, all shoots showed slightly longer roots in medium containing NAA. Roots of *S. leucophylla* were generally longer than those of *S. purpurea*.

#### Acclimation to soil

One month after planting in soil, all shoot clusters had survived, showed new root growth, and were increasing in size (Fig. 4).

*S. leucophylla*, *S. oreophila*, and *S. purpurea* grown in one-third strength MS medium containing  $9.1 \mu\text{M}$  trans-zeatin all survived transfer to the greenhouse after 3

weeks and by 4 weeks, most shoot clusters began to show new pitchers and root growth.

#### *Sarracenia* seed cryopreservation in liquid nitrogen

*Sarracenia* seed water contents were measured as follows. *S. leucophylla*: Seed Lot A, 6.4, 8.5 (average 7.4%); seed Lot B, 11.4, 7.4, 10.6 (average 9.8%); seed Lot C, 11.0%, *S. oreophila*, 11.7, 10.6 (average 11.1%); *S. purpurea* seed Lot B 11.0%.

*Expt. 8: Seed cryopreservation and post-storage survival.* Four trials with *S. leucophylla* from three seed lots showed survival after storage in liquid  $\text{N}_2$ . Across the four trials, germination after rapid immersion cryopreservation averaged 34.3% compared with 47.5% for untreated control seed. Differences between treatments in the individual trials were not statistically significant (Table 4). *S. oreophila* seed survived cryopreservation treatments and differences between germination of control seed (18%) and cryopreserved seed (18%) were not statistically significant. *S. purpurea* survived cryopreservation with 30% germination compared with 50% in the control group. Again, differences were not statistically significant. *S. purpurea* continued to increase germination in both treatments up to 8 weeks.

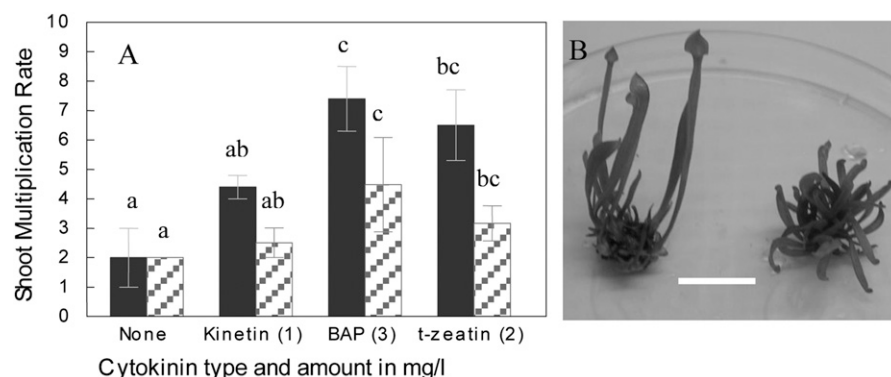


Fig. 3. (A) *Sarracenia purpurea* shoot multiplication over two subcultures on multiplication media containing one-third strength Murashige and Skoog salts and varying in cytokinin content; first subculture (solid bar), second subculture (hatched bar). Bars show means and SES. Averages with the same letters above bars from the same subculture are not statistically different by the multiple range test at  $P = 0.05$ . (B) *S. purpurea* shoot clusters grown on trans-zeatin (left) and kinetin (right). Scale bar = 1 cm.



Fig. 4. *Sarracenia* shoot clusters rooting in vitro and in greenhouse soil. (A) *S. leucophylla* rooting after 6 weeks in one-third strength Murashige and Skoog (MS) without hormones; (B) *S. oreophila* rooting after 6 weeks in one-third strength MS without hormones; (C) *S. leucophylla* rooting after 4 weeks in one-third strength MS with  $0.5 \mu\text{M}$  1-naphthaleneacetic acid; (D) *S. purpurea* with new roots visible after 1 month in greenhouse soil; (E) *S. leucophylla* with new roots visible after 1 month in greenhouse soil. Scale bars: A–C = 0.5 cm, D = 2.0 cm, E = 2.5 cm.

#### Discussion

A scarification method was developed for improved germination in vitro for several rare *Sarracenia* species. When combined with an optimized scarification time and tissue culture medium, germination percentages of 23% to 56% occurred in vitro for *S. leucophylla*, *S. oreophila*, and *S. purpurea*. The rather harsh scarification treatment both kills surface-contaminating agents and begins to dissolve and damage the seedcoat leading to removal of dormancy. Germination occurred within 3 weeks and generally exhibited less than 1% contamination, providing clean explants for shoot multiplication in vitro. Sustainable shoot micropropagation occurred in one-third strength MS medium supplemented with trans-zeatin and shoots were easily rooted in vitro and acclimated under greenhouse conditions. Protocols are presented in enough detail to enable interested parties to easily culture several *Sarracenia* species. In addition, successful seed cryopreservation protocols are reported for the first time for *Sarracenia* species.

We desired a sterilization and germination protocol that germinated seeds for in vitro studies within several weeks as opposed to several months when 4–6 weeks of stratification is used. When Gotsch and Ellison (1998) scarified *Sarracenia* seed in 1 M sulfuric acid for 60 s, germination did not occur for any of the species tested. Adding a 1M  $\text{H}_2\text{SO}_4$  scarification treatment for 30 s after stratification for 4 weeks also did not increase germination in vivo. One min in 1 M  $\text{H}_2\text{SO}_4$  or 1 or 5 min in concentrated  $\text{H}_2\text{SO}_4$  without stratification also resulted in no germination in vivo for *S. purpurea* seeds

Table 4. *S. leucophylla*, *S. oreophila* and *S. purpurea* germination after cryopreservation.

Species	Scarification time (min)	Number of seeds tested	Germination (%)			
			3 weeks	4 weeks	5 weeks	8 weeks
<i>Sarracenia leucophylla</i>						
Trial 1 (seed Lot A) <sup>z</sup>						
Control (no cryopreservation)	6	25	40	56	56 a <sup>w</sup>	
Fast immersion	6	23	22	35	35 a	
Trial 2 (seed Lot B) <sup>z</sup>						
Control (no cryopreservation)	6	26	23	31	31 a	
Fast immersion	6	24	17	17	21 a	
Trial 3 (seed Lot B) <sup>z</sup>						
Control (no cryopreservation)	6	18	50	50	50 a	
Fast immersion	6	27	37	37	41 a	
Slow immersion	6	29	34	45	45 a	
Trial 4 (seed Lot C) <sup>y</sup>						
Control (no cryopreservation)	6	30			53 a	
Fast immersion	6	30			40 a	
<i>Sarracenia oreophila</i>						
Trial 5 <sup>x</sup>						
Control (no cryopreservation)	4	27	7	15	15 a	
Fast immersion	4	30	13	13	17 a	
Trial 6 <sup>x</sup>						
Control (no cryopreservation)	4	25	7	21	21 a	
Fast immersion	4	27	4	11	19 a	
<i>Sarracenia purpurea</i>						
Trial 7 (seed Lot B) <sup>y</sup>						
Control (no cryopreservation)	6	22	23	23	36 a	50 a
Fast immersion	6	25	9	9	13 a	30 a

<sup>z</sup>Germination on one-third strength MS, no hormones, pH 5.7.<sup>y</sup>Germination on one-third strength MS, no hormones, pH 4.5.<sup>x</sup>Germination on one-sixth strength MS, no hormones, pH 5.7.<sup>w</sup>Values within a trial and column followed by the same letter are not statistically different by the multiple range test at  $P = 0.05$ ; analyses are based on arcsine  $\sqrt{\%}$  transformation of percentages.

MS = Murashige and Skoog.

(Ellison, 2001; Mandossian, 1966). Because seed usually does not germinate unless it is stratified, we speculate that our high germination in vitro may occur from immature embryo growth after scarification caused by exposure to medium sugars and minerals (Gotsch and Ellison, 1998).

The first report of *Sarracenia* germination in vitro was for *S. flava* and *S. purpurea* greenhouse-grown immature pods that were sterilized in 70% alcohol, flamed, and ovules plated onto chopped sphagnum moss agar supplemented with sucrose and potassium nitrate (Withner, 1964). When Uhnak (2003) treated *S. leucophylla* seeds in concentrated H<sub>2</sub>SO<sub>4</sub> for 4, 8, 12, or 16 min, contamination in liquid indexing medium were 52%, 40%, 15%, or 6%, respectively. However, Uhnak (2003) was unable to obtain *S. leucophylla* germination on liquid one-half strength MS medium with 20 g·L<sup>-1</sup> sucrose. Perhaps the long scarification times and use of MS medium that was too concentrated (one-half instead of one-third to one-sixth strength) contributed to the lack of *S. leucophylla* germination in vitro in Uhnak's studies. This hypothesis is supported by Uhnak's ability to achieve 11% germination in shaking vials of sterile water with pH adjusted to 5.0.

Pitcher plants usually grow in bog or fen soils that are wet or waterlogged over the growing season. Soils are mostly acidic and contain a high level of decomposing matter with low oxygen concentrations that cause a low redox potential (Adamec, 1997). These conditions further lead to low levels of macronutrients that presumably pitcher plants

have evolved carnivory to overcome. Carnivorous plants living in a wet nutrient-poor environment likely have evolved to grow under conditions of dilute nutrients. Our results show that *S. purpurea* will germinate on one-third strength MS salts, *S. leucophylla* grows better on one-third strength MS compared with one-sixth strength MS salts, and *S. oreophila* may prefer one-sixth compared with one-third strength MS salts ( $P = 0.16$ ). Additional tests with *S. leucophylla* should be conducted to confirm a preference for germination in vitro at pH 4.5, similar to that found in the bog environment. Van Winkle and Pullman (2003) found that medium pH alteration further changes availability of macronutrients and micronutrients. Arnold (1989) had some success producing *S. oreophila* shoots on half-strength MS medium with 6- $\gamma$ , $\gamma$ -dimethylallylamino]purine and other cytokinins and establishing them in a greenhouse. In two patents, Choi and Han (2004) and Xu et al. (2009) used half-strength to one-sixth strength MS salts to micropropagate several *Sarracenia* species. Other carnivorous plants have also been found to prefer dilute salt formulations for in vitro micropropagation (Adams et al., 1979; Latha and Seeni, 1994).

Because somaclonal variation, a type of genetic or epigenetic variation produced in tissue culture, is often associated with the use of auxin to induce shoot regeneration from callus (Ahmed et al., 2004), the use of cytokinins alone for shoot multiplication is preferred.

Shoots required an in vitro rooting treatment for establishment in soil along with the

use of shoot clusters rather than single shoots. When these treatments were combined, several *Sarracenia* species showed high percentages of rooted shoot clusters that established in soil and could be further divided after establishment.

Seventeen percent to 45% of the *Sarracenia* seeds placed in cryostorage germinated in vitro after retrieval from liquid N<sub>2</sub>. Rapid immersion cryopreservation treatments for *S. leucophylla*, *S. oreophila*, and *S. purpurea* showed 72%, 100%, and 60%, respectively, of the germination seen in control treatments; differences between treatments were not statistically significant. With recent evidence that seed longevity in conventional seed banks at -20 °C is not as long as hoped (Li and Pritchard, 2009), it becomes increasingly important to evaluate other options for conservation of rare and endangered species. Even during our research trials over 2 years, we saw germination decrease in the seed lots we worked with that were stored at room temperature. We would expect higher survival rates for freshly stored seed. Our results suggest seed cryopreservation may provide a highly useful long-term ex situ plant conservation approach for *Sarracenia* species.

Many plant species are becoming rare in their natural environments as a result of loss of habitat, overcollection, or disease. Tissue culture laboratories are important resources for propagation of rare and endangered plants. Methods are presented for long-term seed storage at ultralow temperatures followed by in vitro germination, micropropagation, and plant establishment in soil. The procedures developed here provide valuable protocols for preserving germplasm and genetic diversity in *Sarracenia* and will likely assist in the conservation of these beautiful and valuable carnivorous plants.

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