



Figure 1. *Cypripedium kentuckiense* orchid showing the distinctive lady's slipper features of its flowers and leaves. Photo courtesy of Converse Griffith, Kistachie National Forest

Restoring the rare Kentucky lady's slipper orchid to the Kisatchie National Forest

James P Barnett, Kevin Allen, and David Moore

ABSTRACT

The Kentucky lady's slipper (*Cypripedium kentuckiense* C.F. Reed [Orchidaceae]) is a spectacular orchid native to the southeastern US. Although its range includes much of the Southeast, it is rare due to loss of appropriate edaphic and climatic habitats. Efforts to restore this species to the Kisatchie National Forest in Louisiana were initiated by a high school student who located a flowering orchid, pollinated it, and collected a fertile seedpod as a collaborative effort to understand the reproductive potential of the species. Results from these evaluations are inconclusive and must be considered preliminary. Additional studies are planned to develop protocols that will facilitate the production of Kentucky lady's slipper orchids on a scale that will allow significant reintroductions of the rare orchid to the Kisatchie National Forest.

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KEY WORDS

Cypripedium kentuckiense, Orchidaceae, *in vitro* propagation, outplanting, cultural guidelines

NOMENCLATURE

USDA NRCS (2012)

CONVERSIONS

1 ha = 2.47 ac
1 cm = 0.4 in

The Kentucky lady's slipper orchid (*Cypripedium kentuckiense* C.F. Reed [Orchidaceae]) is a tall, stately, perennial plant with the largest flowers of any known native *Cypripedium* (Figure 1). Its range includes much of the southern US (USDA NRCS 2012), but the species is now endangered throughout its range, and populations continue to decline. In Louisiana, only a few plants are located on 4 sites within the 242,811-ha Kisatchie National Forest (KNF).

The efforts we describe here to restore one of the most spectacular orchids native to the region began with the initiative of a 16-y-old high school student. As a student and amateur botanist, Kevin Allen located a flowering orchid on the KNF, caused it to be pollinated, and later collected a seed capsule. He sent the seedpod to Spangle Creek Laboratory in Bovey, Minnesota, and these specialists in producing lady's slipper orchids determined the seeds were fertile.

Allen approached KNF botanists with the idea of producing plants for reintroduction efforts on the KNF. Not having expertise in growing orchid seedlings to a size suitable for outplanting, KNF botanists approached the Forest Service Southern Research Station and Central Louisiana Orchid Society (CLOS) for help in evaluating the potential for producing stock suitable for restoration efforts (Barnett 2008).

Thus began a collaborative effort to develop protocols for producing Kentucky lady's slipper orchid seedlings with the goal of reintroducing them into the KNF. Here we describe our efforts, successes, and failures and provide some suggestions for future research work in establishing this plant.

KENTUCKY LADY'S SLIPPER

The Kentucky lady's slipper is a terrestrial orchid, in contrast to the epiphytic nature of the plants most orchid hobbyists grow in their greenhouses. Underground rhizomes allow the plant to undergo periods of dormancy. Stems are erect, ranging from 35 to 80 cm tall with alternate, broadly ovate leaves about 15 to 20 cm long that have conspicuous parallel veins (Berkshire 2011). The large flowers, usually one or two occurring on the stem terminals, have cream-colored lips and yellow-green with spotted or mottled maroon sepals and petals. The lips are large and have a dorsal opening characteristic of lady's slippers (Figure 1). Flowering occurs in the late spring, typically in late April to June. The seed capsule is about 3 to 5 cm long and can produce thousands of dust-sized seeds (Reed 1981).

The habitat of Kentucky lady's slippers is usually mesic to dry-mesic forests on stream floodplains, ravine slopes, and acid steep forests. They may occur as well on forested springhead seeps in sandy soils (Berkshire 2011). Indicator species growing on sites typical of Kentucky lady's slippers include American beech (*Fagus grandifolia* Ehrh. [Fagaceae]), eastern hophornbeam (*Ostrya virginiana* (Mill.) K. Koch [Betulaceae]), horse-

sugar (*Symplocos tinctoria* (L.) L'Hér [Symplocaceae]), and witch hazel (*Hamamelis virginiana* L. [Hamamelidaceae]) in the overstory and an abundance of eastern poison ivy (*Toxicodendron radicans* (L.) Kuntze [Anacardiaceae]) and broad beechfern (*Phegopteris hexagonoptera* (Michx.) Fée [Thelypteridaceae]) in the understory (Allen and others 2004; Megyeri and Allen 2011).

SEED GERMINATION AND SEEDLING PROPAGATION

Information on seed germination, seedling propagation, and reintroduction of this terrestrial orchid species is sparse. Huber (2002) had some success in establishing mountain lady's slippers (*Cypripedium montanum* Douglas ex Lindl.) in the western US by mixing seeds with carriers such as forest soil, sugar, and cracked corn prior to sowing in forest openings. A significant outplanting effort with a *Cypripedium* species has been reported by Ramsey and Stewart (1998) in the United Kingdom with yellow lady's slipper orchid (*Cypripedium calceolus* L.). Good survival was obtained after planting, but further monitoring will be needed to determine the long-term success of these outplantings.

Seed Collection and Germination

One of Kevin Allen's self-pollinated orchids produced a capsule with viable seeds (Figure 2). In nature, orchid seeds have essentially no stored food resources and require external sources of nutrition for germination (Curtis 1943; Zettler 2005). Seeds obtain nutrients and energy by absorption from a symbiotic fungus that invades the seeds (Ramsey and Stewart 1998). This fungal associate is required for further growth and development of the plant (Zettler 2005; Dutra and others 2008). Orchid mycorrhizae are unique in that the fungus is actually consumed by the orchid host as an energy host, a process called *mycothrophy* (Zettler 1997).

To facilitate *in vitro* germination and efficient seedling production, Steele (1995, 2007) developed asymbiotic germination



Figure 2. A green seed capsule (typically 3 to 5 cm long) of a Kentucky lady's slipper orchid. Photo courtesy of Converse Griffith, Kisatchie National Forest

protocols and seedling culture procedures for native *Cypripedium* orchids. Essentially, the seeds have their nutrient requirements supplied by an agar media within a flask; Steele (2007) found that different species of orchids, even within the same genus, may require media of varying nutritional composition. The agar media and the seeds require sterilization, otherwise the cultures would quickly be overcome by fungi and bacteria (Steele 2007).

After 3 to 4 mo on specialized media within flasks, the embryos swelled, ruptured the seedcoats, and bodies known as *protocorms* were formed. Flasks were kept in darkness to encourage root rather than shoot growth. After germination and initial development, protocorms were transferred from the germination flasks to fresh media in another flask for further development.

Many species of *Cypripediums* require chilling to hasten germination and shoot development (Curtis 1943). Because *C. kentuckiense* has a more southern range than most *Cypripedium* species, extent of seed or protocorm dormancy of this species is not fully understood. After removal from flasks, the protocorms were thoroughly rinsed with a small amount of water, and because of uncertainty about the need for lengthy chilling, containers with the seedlings were then placed in a refrigerator for several months to assure the seedlings were properly vernalized (Steele 2007).

Transfer of Protocorms to Trays for Acclimation and Development

Protocorms must be transplanted into a greenhouse or nursery environment for growth to suitable size for outplanting. This transition from *in vitro* conditions requires a significant period of acclimation; unfortunately, no guidelines yet exist.

In our study, protocorms received from Spangle Creek Laboratory had buds nearly 1 cm long and roots 4 cm or more in length (Figure 3). These were planted into trays filled with media consisting of compost, commercial potting mix (Promix®; Premier Tech Ltd, Quebec, Canada), and sand (approximately 1:1:1 by volume). The 200 plantlets received in late May 2006 were distributed to several growers within the CLOS in an attempt to determine guidelines for consistent production. Although initial shoot growth was excellent (Figure 4), survival of plantlets during the next several months was poor.

Most growers, accustomed to growing epiphytic orchids rather than terrestrial orchids such as *C. kentuckiense*, started plantlets in greenhouse environments, but it soon became apparent that summer greenhouse temperatures of up to 45 °C (113 °F) were excessive for the succulent young seedlings. *Cypripedium* roots are known to prefer cool temperatures (Martin 2010), so high greenhouse temperature resulted in mortality or seedling dormancy. Subsequently, plants were found to perform better when transferred to partially shaded outdoor environments.



Figure 3. Size of the bud (1 cm) and roots (3 to 4 cm) of plantlets when received from the Spangle Creek Laboratory. Photo by James P Barnett



Figure 4. Growth and development of Kentucky lady's slipper orchids 4 wk after transplanting protocorms into pots. Average height was about 5 cm. Photo by James P Barnett

In 2007, grants from the KNF and the Southern Research Station allowed the purchase of 700 additional plants. A commercial potting mix (Promix®) and sand (2:1 ratio by volume) was used by growers, and plants transplanted into trays in mid-April were grown in shaded outdoor conditions. Survival percentage in the trays at the end of the growing season greatly improved—to about 60%.

OUTPLANTING TRIALS

We outplanted seedlings 3 times: 18 December 2007, 10 March 2008, and 30 December 2008. Seedlings were outplanted in the Catahoula Ranger District of the KNF (Figure 5) on a site chosen along a stream near a small group of native Kentucky lady's slipper orchids (Barnett and others forthcoming). This site had the requisite indicator species described earlier.



Figure 5. This outplanting site on the Catahoula District of the Kisatchie National Forest met the site criteria established by Allen and others (2004) and had a small native population of Kentucky lady's slipper orchids. Photo by James P Barnett

Before outplanting, the number of roots and shoots of each seedling were measured and recorded (Figure 6). Procedures for outplanting followed recommendations developed by Culina (2011) for planting divisions of *Cypripedium* plants. The procedure involved working compost or litter from the seedling location into the planting site to a depth of 10 to 12 cm. A cavity was then made into this soil, the root system placed so that the bud was level with the groundline, and the soil was replaced around the root system. This resulted, how-

ever, in the plant essentially being in a shallow basin; although the apical bud was at groundline, it was about 1.2 cm below the surrounding, undisturbed soil surface (Figure 7). The site was then covered with mulch to protect the plant and to conserve moisture.

December 2007 Trial

At the time of outplanting, the 2006 crop averaged 6.5 roots and 1.0 shoot, whereas the 2007 crop averaged 8.3 roots and 1.3 shoots. We planted 4 replicates of 5 seedlings for each age group (2006 and 2007). We measured survival after 4 (April 2008), 7 (July 2008), and 17 (May 2009) mo. Although 4-mo survival was relatively good, seedling mortality was high after 7 mo in the field (Figure 8). Rapid increases in seedling mortality were likely related to droughty conditions at the planting site. Too, at planting, the seedlings were small with poorly developed root systems. Steele (2007) reports lack of success in growing ram's head lady's slipper (*Cypripedium arietinum* W.T. Aiton) seedlings held in any sort of peat-based mix for longer than one season. Although the original shoot and root numbers suggested that holding the 2006 seedlings through 2007 in the same trays without additional nutrients reduced seedling vitality, that was not particularly evident in the survival data.

Figure 6. This dormant seedling with 2 buds and a dead shoot is typical of the better seedlings outplanted in the study. Photo by James P Barnett



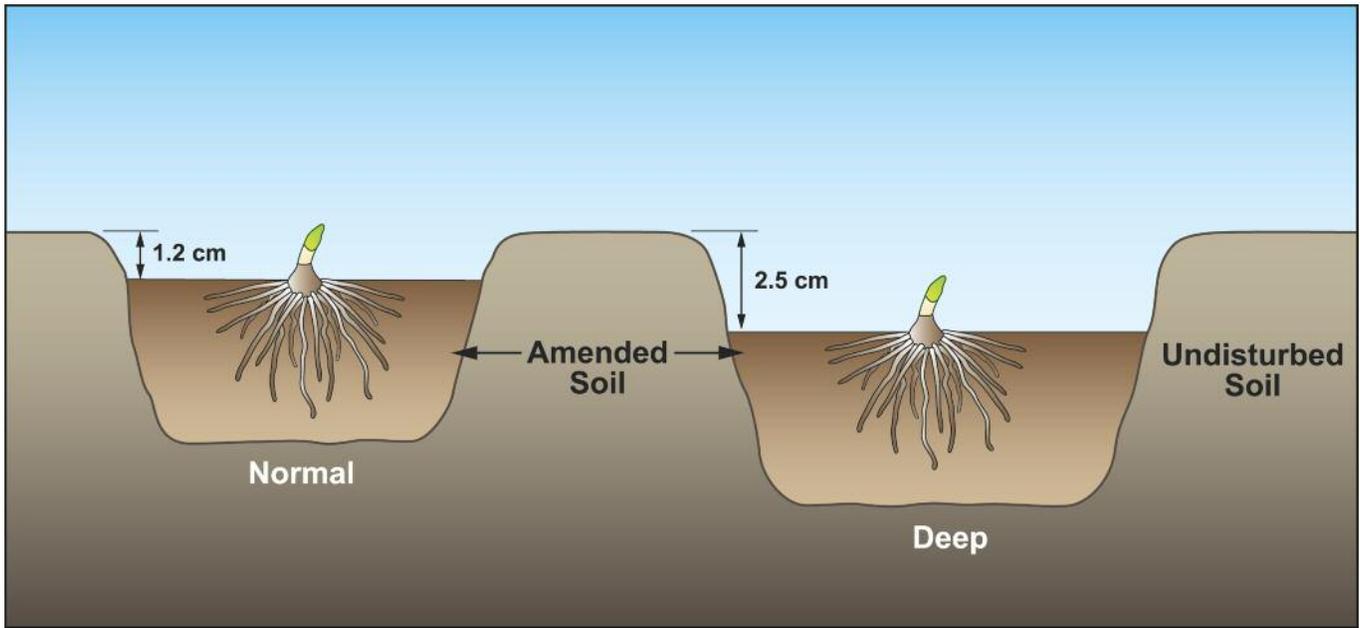


Figure 7. Seedlings outplanted at “normal” and “deeper” depths. Note the bud was always positioned at groundline. Illustration by Jim Marin Graphics

March 2008 Trial

We had 4 replications of 5 seedlings in each of 2 treatments: control (no fertilizer) or fertilization accomplished by scattering 5 g (1 tsp) of Osmocote® 19N:6P₂O₅:12K₂O (The Scotts Company, Marysville, Ohio) around each seedling after outplanting. At the time of outplanting, seedlings averaged 8.25 roots and 1.25 shoots. We measured survival after 1 (April 2008), 4 (July 2008), and 14 (May 2009) mo. Seedling mortality was high after just 4 mo in the field regardless of treatment (Figure 8), probably

for the same reasons suggested above. We assumed that some level of fertilization would help seedling growth and potential flowering but had no data as a base for application rate. The lower survival noted for fertilized seedlings at each observation date would suggest our rates were too high.

December 2008 Trial

A portion of the seedlings from the 2007 crop, held in trays until 30 December 2008, were used for the depth of planting

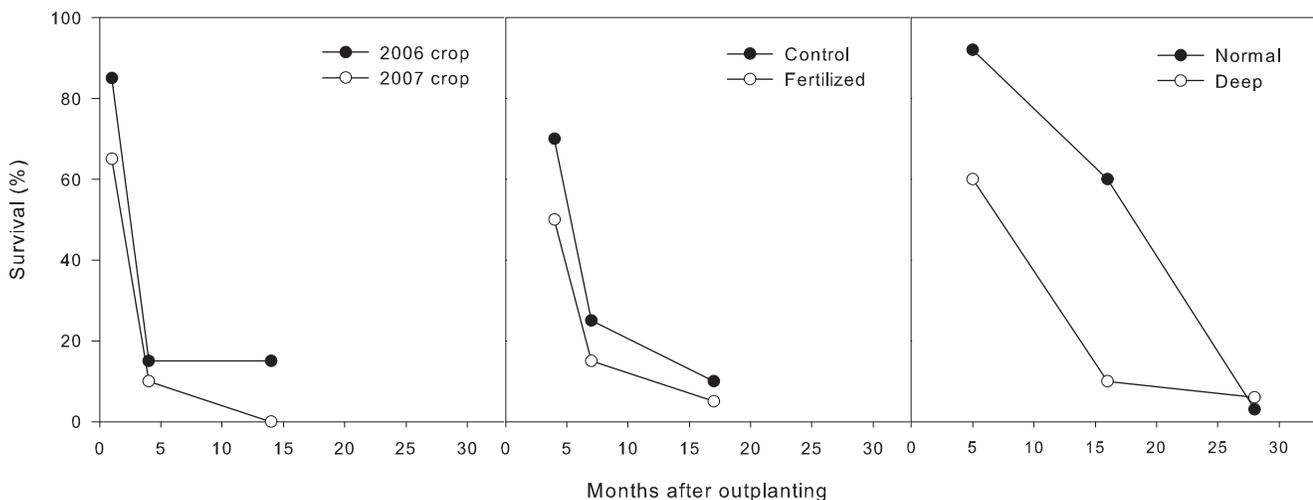


Figure 8. Field survival of *Cypripedium kentuckiense* seedlings in studies evaluating age of planting stock (2006 compared with 2007 crops), fertilization at planting (none compared with some), and depth of planting (1.25 compared with 2.50 cm below the surrounding soil surface).

treatments. At the time of outplanting, seedlings averaged 10.6 roots and 1.0 shoot. Eight replications of 5 seedlings per planting depth (normal and deep) were evaluated. As described earlier, a basin was made into amended soil and the root system placed so that the bud was level with the groundline. With normal planting, even though the bud was at groundline, the groundline was about 1.2 cm below the surrounding, undisturbed soil surface. Deep-planted seedlings had their buds at groundline but about 2.5 cm lower than the undisturbed soil (Figure 7). We measured survival after 5 (May 2009), 16 (April 2010), and 28 (April 2011) mo. After 5 mo, survival averaged 92% and 62% for normal and deep planting, respectively, but after 2 y, survival was poor regardless of treatment (Figure 8). These results indicate the superiority of normal planting of these small seedlings. Apparently, they lack the resources to overcome deeper planting. In nature, the orchid root system is shallow, growing only in the top 2.5 to 5.0 cm of soil (Stoutmire 1991); thus, optimum planting depth may depend on the size of the root system. Results when using larger planting stock may vary from these. The overall poor survival after 1 and 2 y is likely influenced by the extreme droughty conditions at the planting site.

Mycorrhizal Fungi Trial

It has been long established that orchid seeds require associated mycorrhizal fungi for germination under natural conditions (Curtis 1939). It seems logical, and others have observed, that these plants have long-term mycotrophic needs (Zettler and Piskin 2011). Therefore, inoculating these orchids with the appropriate fungal association to successfully reintroduce them into native habitat is important. Unfortunately, the specific fungi related to the germination, establishment, and growth of *Cypripedium* species are yet to be determined, but the development of seed-baiting technology may provide an approach to identifying fungi specific for *C. kentuckiense* (Brundrett and others 2003). Until the symbiotic organisms are identified, a practical approach would be to inoculate seedlings with soil collected from sites where native populations exist. This soil can be distributed throughout the potting soil used for orchid production.

In our evaluations, soil and humus were collected near native populations and applied to *C. kentuckiense* seedlings from the 2007 crop repotted 30 December 2008. One hundred potted plants were available for the evaluation—50 non-treated and 50 inoculated. Although this test was not properly replicated, our results showed that survival of seedlings held in a greenhouse for 6 mo improved from 35 to 52% due to inoculation treatment. Seedling inoculation with the appropriate fungi will clearly play an important role in the successful reintroductions of these orchids (Zettler and others 2005).

Our results indicate that collection of mature capsules will provide highly viable seeds, and that *in vitro* germination and development of protocorms on an appropriate nutrient-based agar are feasible.

The acclimatization of plantlets to southern *in situ* conditions remains a significant challenge. From our seedling acclimation work, we make the following conclusions.

- Summer temperatures inside greenhouses used by hobby orchid growers in Louisiana are excessive for growing these terrestrial orchids.
- Seedlings develop well, however, in dappled-shade outdoor environments.
- A peat-sand mix that maintains a moist but well-aerated medium works well for initial plant development.
- Seedling growth during the first growing season seldom exceeds that obtained in the first few weeks after transplanting, although limited nutrients may contribute to this lack of continued growth.
- Seedlings should not be held in the same tray or pot for more than one y before outplanting, and nutrients should be added to the potting media to stimulate plant development.

Our outplanting results, albeit discouraging over the long term, suggest the following.

- The outplanting site is important. Although presence of Allen and others' (2004) indicator plants is a good starting place, other considerations are important, particularly availability of soil moisture. The site used in this study was droughty and the entire region experienced a severe drought during the study, which is likely one reason for the poor results.
- Season of planting affects performance. The comparison of non-fertilized seedlings planted in December 2007 with those planted in March 2008 indicated that outplanting late in the seedlings' dormant season resulted in higher initial survival than when outplanted earlier in the dormant period (85% as compared with 50%).
- Plant age is unimportant. Extent of seedling development is more important than the age of the plant. Seedlings held 2 y in trays did no better than those held 1 y, but they were only slightly larger. Better cultural practices may improve size and performance.
- Fertilization at outplanting is detrimental at the rates we used. Applying nutrients at outplanting was not successful in overcoming the same size of the planting stock, and apparently the rate of application was much too high. Nutrients applied to the plants during the acclimation period in pots should result in more successful seedling establishment.

- Depth of planting is important. Seedlings outplanted with roots only slightly deeper than another treatment survived less well. At both outplanting depths, the bud was only slightly covered, but the total root system was deeper with deep-planted seedlings. It may be that debris and soil washed into the outplanting holes and covered the seedlings, thereby reducing performance.
- Inoculation with mycorrhizae is important, especially for these specialized orchids. Results of research with other mycorrhizae-dependent orchids show that establishment is significantly enhanced when seedlings have mycorrhizae-infected root systems (McCormick and others 2012).

FUTURE NEEDS AND DIRECTION

In order for these preliminary research efforts to proceed and produce meaningful guidelines, a source for large quantities of plantlets is needed. In an effort to meet this need, Kevin Allen, now a science teacher at Captain Shreve High School in Shreveport, Louisiana, is developing a laboratory equipped to germinate seeds and to produce large numbers of developing protocorms. To equip the Captain Shreve High School laboratory with the appropriate equipment and supplies, grants were provided by the Forest Service through the KNF and the Southern Research Station. Allen will use his honor's level chemistry students to develop procedures that result in consistent production of quality seedlings.

Plantlets will be grown from protocorms in garden situations and on sites near native populations where active orchid regeneration is occurring. Seedlings of various stages of development will be produced to use in outplanting trials. With availability of additional protocorms, tests will continue on seedling size at planting, depth of planting, fungal inoculation, and selection of improved planting sites.

In addition, a grant from Southwest Regional Orchid Growers Association supported Allen's travel to Lawrence Zettler's laboratory at Illinois College for training on seed germination and identification of mycorrhizal associations. Zettler is a leading authority on orchid seed baiting and on identification of fungal genera associated with specific orchids.

Until seed-baiting techniques can be used to identify fungicidal associates, mycorrhizal inoculation will be accomplished by mixing soil collected near native populations with the potting mix. Once appropriate fungal associations are identified, studies will be conducted to determine the implications of fungal inoculation to seed germination, seedling development, and outplanting establishment and development.

Coordinating the direction of this program with the program at the Atlanta Botanical Garden, which is studying techniques for propagating native orchids, including *C. kentuckiense* (Richards and Cruze-Sanders 2010), will be important, too.



Figure 9. Development of protocols to produce these spectacular lady's slipper orchids in quantities that will allow reintroductions into the Kisatchie National Forest is the goal of this effort. Photo courtesy of Jeff McMillian

Our overall objective remains the same: develop protocols that will facilitate the production of Kentucky lady's slipper orchids on a scale that will allow significant reintroductions of this rare orchid to the Kisatchie National Forest (Figure 9).

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