Embryo Rescue of Rare *Camellia azalea* Hybrids

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Known only since 1986 and rediscovered in China in the late1990s by Mr. Gao Jiyin, the ever-blooming tropical *Camellia azalea* Wei. [Figure 1] is one of the rarest and most unique species of *Camellia* currently in cultivation. Pushed to near extinction by habitat loss, a wild population of only about 1,118 plants still exists in the Guangdong Province of southern China. The histories of this rare species of *Camellia* and Longwood Gardens have been intertwined since its introduction to Longwood in October,

Figure 1: Camellia azalea flowers eve ry month of the year in the Longwood Gardens research greenhouses

2000. Longwood Gardens is currently caring for the oldest specimen of *Camellia azalea* in the United States and has focused its *Camellia* breeding program on producing new hybrids with this rare and endangered species.

History is continuing to be made at Longwood Gardens by the recent production of new hybrid seedlings using embryo rescue as a technique to save the often underdeveloped seeds that result from *Camellia azalea* crosses. Embryo rescue describes a technique used to save hybrid embryos that would otherwise abort with typical in vivo germination.

The main goal of the *Camellia azalea* breeding program at Longwood Gardens is to transfer the ever-blooming trait of *Camellia azalea* to some of the most cold-hardy cultivars of *Camellia* currently available. Most *Camellia* species are not year-long bloomers because their growth is episodic and synchronous.

Episodic growth causes the average camellia to grow in phases, meaning that it flushes new growth or sets flowers only at certain times of the year. Synchronous growth causes the average *Camellia* to produce shoots or flowers that are fairly uniform in their development across the entire plant.

The zone 10 *Camellia azalea* is different from other members of the genus *Camellia* by exhibiting non-episodic, asynchronous growth typical of many tropical plants. *Camellia azalea* can bloom literally in every month of the year, and is continually covered with shoots at every stage of growth when grown in a warm environment. The branches of *Camellia azalea* even set vegetative and floral buds at the same time. With the possible exception of the *Camellia amplexicaulis*, a treasured repeat bloomer from Vietnam, the ever-blooming trait is unheard of in all other members of this genus, and offers a unique opportunity to shape the future of *Camellia* breeding.

Camellia azalea was determined by Shuang, et al. to be a primitive diploid (2n=30) after a karyological study of the species in 2001. Since then, breeding efforts at Longwood Gardens have focused on making crosses with cultivars of *Camellia japonica, Camellia pitardii, Camellia albogigas, Camellia wenshanensis,* selected hybrids made by Dr. W i l l i a m Ackerman, and non-reticulata types. However, producing viable seedlings from these crosses has proven to be extremely difficult. Prior to 2008, Longwood Gardens had only produced 8 seedlings from traditional *in vivo* seed germination, 4 from crosses with the straight species *Camellia japonica* and 4 from crosses with *Camellia pitardii.*

When using *Camellia azalea* as the female parent, most of the difficulty in obtaining viable seed originates from the rapid maturation of the fruit without complete embryo development [Figure 2]. The duration of fruit development ranges from only 2 to 2.5 months from pollination to dehiscence on the Longwood specimen of *Camellia azalea* compared to the average of about 6 to 9 months for other *Camellia* species. This extremely brief period of fruit development results in seeds that have a below average size, seeds with deformed or cracking testa (seed coat), seeds with underdeveloped embryos, and in some cases seeds that are completely empty. Most of these hybrid seeds have proven non-viable when sown in vivo, yielding extremely low germination rates for all hybrid seeds collected from *Camellia azalea*.

In an attempt to lengthen the time that these underdeveloped embryos have to mature, Longwood Gardens attempted embryo rescues of 5 seeds collected from the cross *C. azalea x C. japonica* 'Maiden of Great Promise' [Figure 3] on April 4, 2008. After collecting the immature seeds from mature fruit, the brittle testa was removed from each before undergoing a surface sterilization treatment of a 15% bleach solution for 15 minutes. Embryos were then rinsed in autoclaved distilled water under sterile conditions in a laminar flow hood and were placed (with cotyledons still attached) onto a tissue culture media consisting of 4.43g Murishige and Skoog with vitamins, 30g sucrose, and 7g BactoAgar per liter. After tissue culture, embryos were placed in a growth chamber set at 23°C (73°F) with 100 mmol.m-2.s-1 (~670 foot candles) of cool white fluorescent light. Embryos were then observed over the next several weeks, and surprisingly some of them continued to enlarge while one showed signs of germination.The cotyledons of this active embryo opened within a month of being placed on culture media, and callus began to form on the

Figure 2. Fruits shatter on Camellia azalea (left) after only 2 to 2.5 months and are significantly undersized compared to a healthy fruit of Camellia japonica (right).

Figure 3. The cold-hardy cultivar Camellia japonica 'Maiden of Great Promise' is indeed showing great promise as a compatible breeding partner for Camellia azalea.

Figure 4. An embryo of 'Maiden of Great Promise' from April 2008 has since germinated while its cotyledons are becoming embryogenic.

Figure 5. While some flowers are allowed to open for pollen collection, most buds of Camellia azalea are emasculated before breeding by the Longwood research staff.

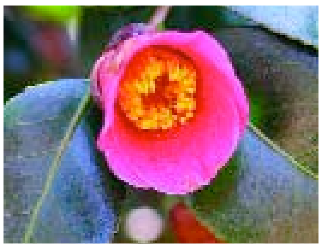

Figure 6. Bred for its cold-hardiness, Camellia japonica 'Longwood Centennial' has shown compatibility as a breeding partner for Camellia azalea.

Figure 7. Camellia japonica 'Longwood Valentine' has a more open flower than Camellia japonica 'Longwood Centennial', and has also shown compatibility with Camellia azalea.

interior of the cotyledons.

During the second month, the embryo continued to produce callus, but no shoot or radicle formation was detected. On June 6, 2008, all embryos were transferred onto new media containing 0.2 ppm cytokinin BA (6-benzylaminopurine), while all other ingredients remained the same. Cytokinin is a plant hormone that when added to tissue culture media induces the formation of callus and encourages shoot development. After being moved onto the new cytokinin-containing media, 2 of the 5 initial tissue cultures responded by increasing in size and producing callus. The most active embryo has since produced a single shoot while callus on the cotyledons has become embryogenic [Figure 4]. This type of tissue may create the opportunity for rapid multiplication by producing multiple shoots from callus, rather than a single shoot as seen with the in-vitro germination of the other rescued embryos.

Due to the level of success obtained in the first round of embryo rescues, staff at Longwood Gardens began to experiment with different sterilization and media treatments. One of the problems encountered as the number of rescues increased was contamination. Since most embryos seemed to be germinating, a new media completely eliminating sucrose and reducing Murishige and Skoog with Vitamins to 2.215 g per liter was trialed in an attempt to reduce contamination. Surface sterilization of embryos was also intensified by rinsing in a 20% Clorox solution for 20 minutes. No significant difference in contamination was observed between the new media and the old, and the stronger sterilization solution caused leaching of oxidation products from the immature embryos into the media. This leaching required an additional step of transferring the young embryos onto clean media in as little as a week after being placed in tissue culture. Therefore, the use of this new media and sterilization solution was discontinued, opting for the original procedures for all new embryo rescues.

Observations of the Longwood *Camellia azalea* specimen over the years indicate that warm temperatures following pollination are linked to successful fertilization. In the Longwood research greenhouses, *Camellia azalea* has indeed flushed more flowers and produced more fruits during the spring and summer months In 2008, Longwood's *Camellia azalea* began to increase flower production in April and yielded flowers for nearly 150 pollination attempts by October [Figure 5]. The increased number of fruits collected from these crosses has provided Longwood Gardens with the opportunity to refine its embryo rescue procedure. Consequently, as staff became more proficient with the embryo rescue technique, germination rates of embryos increased and incidences of contamination decreased across all treatments.

During most of the spring and summer of 2008, pollen from the cold-hardy cultivars *C. japonica* 'Longwood

Figure 8. A hybrid embryo of **Camellia azalea x Camellia japonica** *'Maiden of Great Promise' produces callus from its cotyledons.*

Figure 10. Mutations from in vitro culture can sometimes be beneficial as seen in this variegated mutant from the cross **Camellia azalea x Camellia japonica** *'Longwood Centennial', although seedlings usually grow out of the variegated condition.*

Figure 9. An underdeveloped **Camellia azalea x Camellia japonica 'Maiden of Great Promise'** *embryo fails to germinate, but becomes embryogenic 4 months after tissue culture*

Figure 11. **Camellia azalea x Camellia japonica** *'Longwood Centennial'*

Figure 12. *Camellia azalea x Camellia japonica* 'Longwood Centennial'

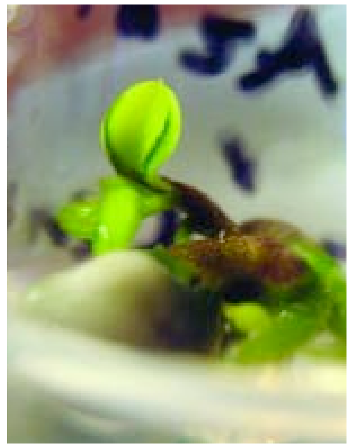

Figure 14. A germinating embryo of the cross Camellia azalea x Camellia japonica 'Longwood Valentine'

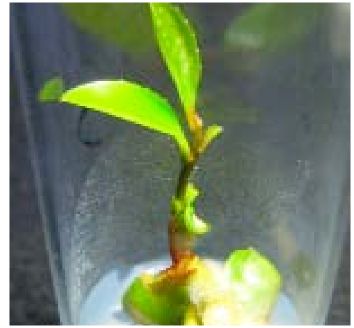

Figure 15. The same Camellia azalea x Camellia japonica 'Longwood Valentine' embryo in Figure 14 after nearly two months of growth.

Figure 16. Camellia azalea x Camellia japonica 'Maiden of Great Promise'

Centennial' [Figure 6] and *C. japonica* 'Longwood Valentine' [Figure 7] was used for fertilization. Although seeds collected from these crosses were still brittle with deformed or cracked testa, they produced larger, healthier looking embryos than seeds from the earlier crosses with *C. japonica* 'Maiden of Great Promise'. However, this could be attributable to the higher temperatures and longer days of spring and summer. All of these new embryos were cultured on BA (cytokinin)-containing media, and many of them responded within 2-3 weeks by turning green and producing a healthy radicle and shoot. While cytokinin in tissue culture media usually restricts root production, these embryos appeared to be germinating normally.

Embryo rescue of *Camellia azalea* hybrids in tissue culture has several advantages compared to traditional in vivo seed germination. One major benefit is the increase in germination rate of the underdeveloped embryos which have difficulty surviving traditional germination. By starting a seedling in tissue culture, clones of each hybrid can be produced by either callus or shoot cuttings, offering more than just one plant per seed. Also, more clones can continue to be produced while the explanted clones are being grown and evaluated. Therefore, in the event that one of the seedlings matures to have desirable leaf or flower morphology or the highly

Left: Figure 13. Camellia azalea x Camellia japonica 'Longwood Centennial'

Longwood Gardens Embryo Rescue Procedure

1) **Remove Testa**
2) **Rinse in 15% Clorox for 15 min**
3) **Rinse in Distilled Autoclaved H2O**
4) **Place on Tissue Culture Media:**

Ingredients	**Amount in 1L of Stock Solution**	**Amount in 1 L of Media**
Murishige & Skoog (w/Vitamins)		4.43g
BA (6-benzylaminopurine)	10 mg/100 ml	2.0 mL
Sucrose		30.0 g
pH (before Agar)		5.7
BactoAgar		7g

5) **Place in Growth Chamber at 670 fc and 23°C**

desirable ever-blooming trait, a stock of in vitro clones will have already been produced.

Another advantage of using embryo rescue may come from the recent embryogenesis of some of Longwood's first embryo rescues from April 2008 [Figure 8]. One embryo from a *C. azalea x C. japonica* 'Maiden of Great Promise' [Figure 9] cross has just been observed some 4 months later to be producing new embryogenic tissue after failing to germinate. Although this type of growth may be susceptible to somaclonal variation (genetic mutations induced by the tissue culture environment) [Figure 10], such a result poses an incredible opportunity to produce multiple hybrid shoots from embryos that are too immature to germinate even in the tissue culture environment.

Over the period of April to September 2008, a total of 17 successful in vitro germinations of *Camellia azalea* hybrids were accomplished from 13 crosses with *C. japonica* 'Longwood Centennial' [Figure 11-13], 2 crosses with *C. japonica* 'Longwood Valentine' [Figure 14-15], and 2 crosses with *C. japonica* 'Maiden of Great Promise' [Figure 16]. Current trials are being performed to judge the success rate of multiplying seedlings in vitro via shoot tip cuttings. Trials are also being performed to judge the success rate of producing shoots via excised embryogenic tissue in tissue culture.

Future plans for the *Camellia azalea* breeding project include trialing media with higher cytokinin and additional hormones, continuing to trial cold-hardy cultivars as breeding partners, and successfully explanting clones from in vitro culture. While embryo rescue has been used in the past for the production of more common *Camellia* cultivars, this is the first time this technique has been employed for the production of hybrids with *Camellia azalea*.

As seen in the successful results of the Longwood trials, embryo rescue promises to be a useful tool in the production of new *Camellia azalea* hybrids, and gives hope that an ever-blooming, cold-hardy *Camellia* is somewhere on the not-too-distant horizon.

References

Gao, J, 2000. A Peculiar Species of Camellia in China. *International Camellia Journal* 32: 64-65.

Harbage, J. F, 2003. Ever-blooming *Camellia azalea* is alive and well in Longwood Gardens. *American Camellia Yearbook* 2003: 8-11.

Orel, G, A.D. Marchant, and A.S. Curry, 2007. Molecular Investigation and Assessment of *C. azalea* C. F. Wei 1986 (SYN. *C. changii* YE 1985) as Potential Breeding Material. *International Camellia Journal* 39: 64-75.

Phillips, G, 2006. The Hybridizer's Corner. *Coushatta Camellia Society Yearbook* 2006-2007: 15-16.

Wang. S, et al, 2007. Karyological Study on the Endangered Species *Camellia azalea (Theaceae)*. Acta Botanica Yunnanica 29, 6: 655-658.