Seed Propagation of *Trillium grandiflorum*

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Trillium is a predominantly North American genus of shade-tolerant herbs from deciduous forests. Many species are useful garden plants because of their large, showy flowers or attractively mottled leaves (Case and Case, 1997; Jacobs and Jacobs, 1997). Nearly all trillium plants in commerce are wild dug, often in large quantities, such as the 600,000 plants sold from Tennessee in 1989 (Sawkins, and McGough, 1993). Clearly a procedure for the practical horticultural production of trillium from seed would provide gardeners access to these plants without damage to native populations.

Often trillium seed takes 2 years to produce seedlings. It is said to be "doubly dormant" (Barton, 1944). However, there are reports of seedlings emerging outdoors after one winter (Jacobs and Jacobs, 1997; Gyer, 1997) or no germination after several cold cycles (Deno, 1993). The objective of my research is the development of a procedure that reliably produces significant germination the spring after the seed ripens. This paper reviews aspects of trillium seed physiology and development that relate to the proposed germination procedure. The research was possible because the Winterthur Garden Department graciously permitted me to study the large naturalized population of *T. grandiflorum* in the Azalea Woods area of Winterthur Museum, Garden and Library in Delaware.

SEED CHARACTERISTICS

At maturity trillium seed consists of a minute embryo, copious storage endosperm, a chalazal cap of two easily identified layers, and a complex funiculus and aril. The development of the endosperm and chalazal cap are relevant to trillium seed production, collection, and germination. Trillium endosperm is helobial (Berg, 1958), that is, the first division of the triploid endosperm nucleus forms a large and a small cell. The large cell produces storage endosperm after a series of divisions that do not immediately form cell walls. The small cell migrates to the chalazal cap area and forms a thin cellular layer. This layer is positioned to restrict the entry of photosynthate at ripening and help seal the endosperm after the seed is shed. The complex funiculus and aril are attractive to some ant species that distribute the seed throughout its environment (Berg, 1958).

The seed size variation between *Trillium* species is an adaptation to specific environments (Ohara, 1989). However, the embryo volumes I have observed are nearly constant across species. The typically *T. grandiflorum* embryo volume is 0.013 mm^3 and the typical endosperm/embryo volume ratio is 770.

POLLINATION

Trilliums are protandrous, that is, anthers shed pollen at anthesis before the stigma becomes receptive. The protandrous lag time between pollen production and stigma fertility depends on the species. *Trillium grandiflorum* has a protandrous lag of about a week.

Single plants in gardens where insect vectors are absent often fail to set seed because the protandrous lag time prevents automatic self pollination. These seed set

failures have led some to the idea that *Trillium* species are self-incompatible. If protandry is considered in pollination experiments, the *Trillium* species I have examined are self-compatible and most can produce a heavy annual seed yield. An average *T. grandiflorum* berry contains about 40 seeds, but vigorous plants produce 90 or more seeds when fully pollinated.

SEED DEVELOPMENT

Trillium seed forms in an indehiscent berry that falls from the plant at ripening. Because the seed is quickly dispersed by insects, sometimes even before the berry falls, seed collection is best done before the berry is fully ripe. Because no literature related berry age to seed development and germination, I measured berry volume and seed germination as a function of time from pollination.

Berry volume was calculated as that of a prolate ellipsoid from the caliper measurement of the distance between the receptacle and the base of the style as the major axis and the width across the berry between the wings as the minor axis. In all species measured the berry volume followed the *T. grandiflorum* growth pattern in Fig. 1. The length of the initial slow growth induction time varied with species. During the induction time the plant grows new rhizome and bud tissue before seed production makes demands on its supply of photosynthate. In most cases the induction phase is followed by an essentially constant rate of volume growth until the berry falls.

The induction time reflects the very slow rate of pollen tube growth. Blain (1945) found that pollen tubes grew for about 2 weeks before they fertilized the ovules of *T. grandiflorum*. This slow growth rate and the asynchronous ovule development she observed suggests that not all ovules in a berry are fertilized at the same time and implies that a berry may contain a range of individual seed ages.

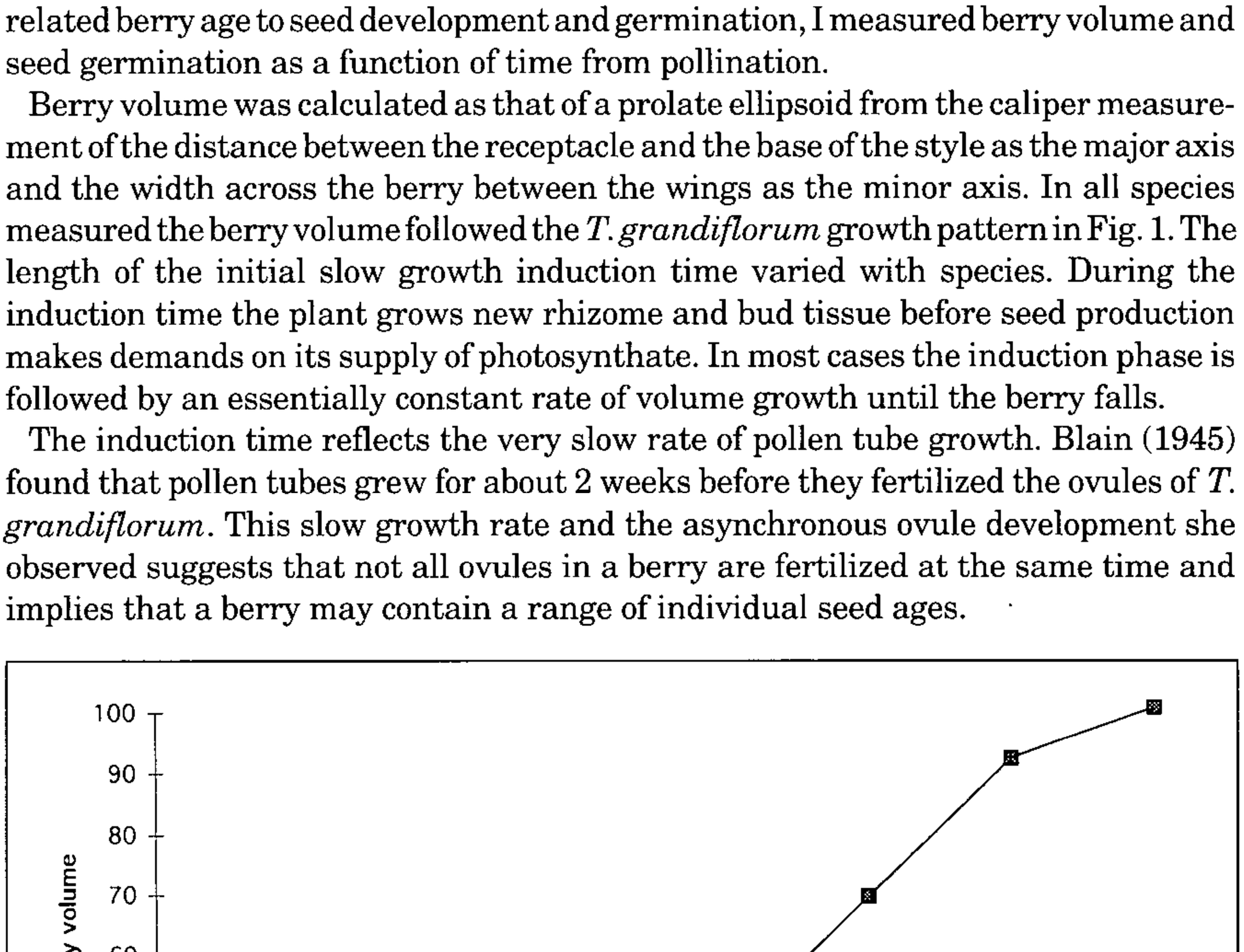

Figure 1. Berry growth rate, *T. grandiflorum*.

In Fig. 1 the slight growth rate increase at about 35 days probably reflects the end of free nuclear endosperm cell division, the onset of cell wall formation, and the initiation of food storage.

Table 1 shows the effect of age on aril and seed development. The aril or enlarged funiculus is relatively small until about 55 days post-pollination when the seed ceases to add food to its endosperm. At that time the photosynthate that has gone into endosperm tissue is stored in the aril. Because there is no change in the rate of photosynthate storage, only a change in the storage site, the berry volume continues to grow as observed in Fig. 1.

At 30× magnification the part of the chalazal cap derived from the first small endosperm cell becomes apparent about the time of this storage site shift. Because it lies between the storage endosperm and end of the vascular strand that delivers photosynthate to the seed, its position suggests that changes in this tissue have a role in seed ripening.

At about 40 days post-pollination seeds can germinate at 20 to 25C. The proportion of dormant seeds increases with age and reaches about 70% in the 66 day sample.

Table 1. Age effects on *Trillium grandiflorum* seed and germination.

Seed age[1]	Water %	Weight (mg) Seed	Aril	Embryo length (mm)	Germination at 21C (%)
32	---	4.0[2]	---	---	0
39	---	5.8[2]	---	---	0
46	67	8.2	0.9	0.15	>80
53	57	9.7	2.1	0.20	67[3]
60	---	10.7	2.9	0.25	48
66	51	10.6	5.9	0.25	35

[1] Days after hand pollination.
[2] Weight of seed + aril, seeds at these ages all decomposed.
[3] Calculated from a 30 seed dissection of a wild population between 50 and 55 days old.

EMBRYO GROWTH AND GERMINATION

Early germination stages are seen only by sectioning the seed and staining the cut surfaces with I_2/KI solution (Lugol, Sigma Chemical Co.); this turns starch in the embryonic rhizome blue-black. Trillium endosperm contains little or no starch. Embryo growth and germination occurs in warmth, 20 to 25C. The earliest evidence of embryo growth is an unstained halo around the embryo that suggests endosperm digestion. A suctorial cotyledon develops from the embryo apex, absorbs the digested endosperm, and deposits it as starch in the embryonic rhizome. After 40 to 60 days the cotyledon/rhizome axis grows to about 2 mm and the rhizome emerges from a collar of enlarged, starch-filled cells at the micropyle.

At soil temperatures above about 18C, endosperm digestion continues to drive root growth and starch storage in the young rhizome. Generally a good root system is produced by about 60 days growth after the rhizome emerges from the seed. A period of low soil temperature (5 to 12C) reduces root growth rate. The cold prepares the seedling for cotyledon elongation and emergence as a photosynthetic organ when soil temperature rises above about 15C. This cotyledon growth is driven by starch stored in the new rhizome and what little residual endosperm remains in the seed.

Table 2. *Trillium grandiflorum* embryo growth in culture[1].

Percent of embryos in each length group								
Embryo length group (mm)	Time in culture	13 days		27 days		48 days		63 days	
	GA_3 conc. (ppm)	0	1000	0	1000	0	1000	0	1000
0 - 0.49		83	96	65	45	66	37	62	35
0.5 - 0.99		17	4	35	52	10	12	7	3
1.0 - 1.49		0	0	0	0	2	21	4	0
1.5 - 1.99		0	0	0	3	0	3	0	9
>= 2.0 Germination		0	0	0	0	22	27	27	53

[1]Agar medium as in Pence and Soukup (1986) maintained at 24C.

TRIGGERING EMBRYO GROWTH

A substantial fraction of most seed collections will be dormant because growers are averse to using "green" seed. In outdoor beds dormant seeds germinate the 2nd year after collection. Plants from these seeds will be a year behind those that grow from the 20% to 30% of the collection that germinates during the fall of the year they are produced. The dormant seeds produce a propagation bed of uneven age and increase by a year the time needed to go from seed to flowering rhizome.

To determine if the embryo is dormant in mature trillium seed, I excised embryos from seed in two berries collected at Winterthur 29 June 1993, and plated them onto half-strength MS agar medium supplemented with 3% sucrose (Pence and Soukup, 1986). Table 2 shows that about 30% "germinated" in 63 days at 24C. The similarity of this germination and that of the mature 66-day seeds in Table 1 suggests that the cultured embryo population responds like intact seeds. It also supports the idea that seeds in a single berry have different physiological ages. When 1 ppm GA_3 was included in the medium, the "germination" doubled indicating that GA_3 acts directly to trigger the growth of dormant embryos. The GA_3-treated excisions that did not grow represent errors in technique; endosperm explants or embryos injured by disinfectant or mechanical damage.

After 155 days in culture some embryos formed rhizomes from outgrowths of the cotyledons. Dr. Sherry Kitto at the University of Delaware, in whose laboratory this work was done, has grown the progeny of these proliferative embryo cultures into viable plants.

Moist storage at low temperature for about 90 days induces embryo growth after seed is moved to 25C. Germination follows in about 60 days (Stolt, 1996). I have seen no embryo growth at low temperature. In fact I have kept some seeds in moist storage for 15 months at 5C. The endosperms softened somewhat, but the seeds germinated in about 60 days at 25C and produced normal plants in outdoor beds.

Although Donovan (1995) has shown that low temperature can trigger the germination of sterile seeds, most of the germinating seeds that I have exhumed from outdoor beds or sectioned during indoor germination tests show signs of disease invasion at the chalazal end. Dormant seeds without embryo growth usually do not have chalazal infection. There are at least two explanations. One suggests that infections increase the permeability of the chalazal cap and expose the seed contents to growth stimulants either from the environment or the metabolism of the infecting agent. The other proposes that endosperm softens as it is digested by the growing embryo. Softening may cause some endosperm cell contents to leak out through the chalazal cap. Bacteria and fungus attracted to the exudate then grow to produce a pathological endosperm infection.

SEED DECOMPOSITION AND ROT

Soon after harvest the seed coat and chalazal cap darken. These changes, often accompanied by the formation of a brown exudate, appear to prepare the seed to resist attack by pathogens. During this time aril decomposition feeds the development of pathogens that can attack the seed before these protective changes are complete. Because of this, arils should be promptly removed, particularly when large numbers of seeds are germinated by the procedure in Note 1.

The endosperm cell walls of immature seed are permeable and can leak cell contents into the germination medium through the chalazal cap. This begins a

process of decomposition that will kill the seed and contaminate the surrounding medium. Seeds that are undergoing decomposition should be periodically removed from the germinating mass by the sieve/wash process in Note 2.

SEEDLING PRODUCTION

The following protocol produces a significant stand of seedlings the spring of the year after the seed is collected. It primes the seed by inducing embryo growth before planting and improves the seedling stand by removing damaged or diseased seed.

- **Harvest the berries**. Berries can be harvested from about 50 days after pollination to just before they fall from the plant. Remove the seed from the berry and free it from placental tissue by the sieve/wash procedure of Note 2.
- **Remove the arils**. The bubbling action of the final peroxide rinse of Note 2 disrupts the aril tissue. If the seed is immature, use a shorter peroxide rinse because peroxide can more easily diffuse into and damage the endosperm of immature seed than that of mature seed. Rinse the seed and store it at room temperature in a moist environment, see Note 1. After 4 to 6 days, sieve and wash the seed to remove the decomposing aril tissue. Return it to moist 20C to 25C storage and repeat the process, if necessary, in another 4 to 6 days.
- **Controlled germination triggers embryo growth** in time for fall root establishment in outdoor beds. Good fall root growth assures early spring cotyledon emergence. There are three controlled germination options.

1) ***Immature seed*** (50 to 55 days post-pollination) contains metabolically active embryos that will have 40% to 60% germination in about 60 days at 25C without further treatment. However, decomposing seed must be removed and the storage medium changed about every 2 weeks.

2) ***Moist storage at 5C*** prepares mature seeds for embryo growth at 25C. In the Delaware Valley the combined times for stratification (about 90 days at 5C) and embryo growth (about 60 days at 25C) delay outdoor planting until the soil is too cold for fall root growth and early spring cotyledon emergence.

3) ***Hormones such as GA_3*** trigger embryo growth. After the aril is removed, soak the seed for 24 h in 1000 ppm GA_3 (0.2 g GA_3 dissolved in 2 ml dimethyl sulfoxide, DMSO, and diluted to 200 ml with distilled water). There is measurable embryo growth about 14 days after treatment. Micropyle rupture is apparent after 60 to 80 days of moist storage at about 25C. This allows time for root growth in outdoor beds before soil temperature falls below 12C. Expect some seed loss during embryo growth because GA_3 softens endosperm and may increase cell leakage or endosperm abnormalities. Every 2 weeks after the GA_3 treatment, remove decomposed seed with the sieve/wash procedure of Note 2, but omit the peroxide rinse.

Although GA_3-induced embryo growth appears normal in most

cases, experience with this hormone in trillium is limited. The effect of soak times and concentrations, for instance, remain to be investigated.

- **Plant the Seed** when micropyle rupture is apparent. Broadcast seed onto moist, but well drained compost/sand in outdoor propagation beds. For immature or GA_3 treated seed this will be early September in the Delaware Valley. Cover the bed with 1/2 to 3/4 inches of a mineral aggregate such as Turface or Axis. This reduces disturbance from rain, provides a disease-free substrate for foliage, and seems to reduce frost heaving damage. For me frost heaving and subsequent seedling desiccation is a major source of loss. The beds should be kept moist in the fall to promote good root growth.

  Although developed for *T. grandiflorum*, this protocol with the GA_3 embryo growth trigger has germinated species related to *T. erectum*, all the sessile species tested, some Asiatic species, and some species in the related genus *Paris*.

NOTE 1. All germinations mentioned in this paper were done with the technique used by Deno (1993). When modified to accommodate large numbers of seeds, his technique provides optimum conditions for embryo growth within the seed prior to planting. Paper towels or other inert, absorbent, smooth material surrounds the seed and is a reservoir of moisture that maintains 100% humidity in a closed plastic bag or other container. Sterile, boiled water is used, but no free water contacts the seed because it will increase rot. A single gallon-sized plastic bag can easily germinate 2000 seeds.

NOTE 2. Remove dead and decomposing seed about every 2 weeks. Wash the seeds into a sieve and gently rub under cool running water to remove decomposing aril tissue and break up decomposing seeds. Put the washed seeds into a beaker, stir, and pour off floating debris. Before aril removal, add some detergent to the wash water because fresh seed is hydrophobic and some will float. After aril removal most seed becomes hydrophilic and sound seed will sink. Disinfect by a brief rinse (about 1 to 3 min) in 3% hydrogen peroxide followed by washing with sterile (boiled and cooled) water and packaging into a clean germination bag moistened with sterile water. Do not use the peroxide rinse if there is any sign of micropyle rupture. It will damage the growing embryo.

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