Variable cotyledon numbers in *Mammillaria beneckei* (Cactaceae)

*Mammillaria beneckei*. Photo by Thomas Linzen

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Abstract

It was found that a complete batch of seedlings from *Mammillaria beneckei* was tricotyledonous. Further investigation shows that every available batch contains plants with only three or four cotyledons. Plants with more than 4 cotyledons were not viable. Three related species were examined: all show 2 cotyledons. The vascular system of the *M. beneckei* seedlings was examined and showed to be regular in both the tricotyledonous seedlings as in the quatrocotyledonous seedlings. The influence of these anomalous cotyledon numbers on the first growth of both groups was examined. The different groups appear to show different phyllotactic patterns. All results were compared with *Mammillaria sphacelata var. viperina*, one of the dicotyledonous related species.
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Introduction

Cotyledons

Cotyledons are the storage organs of young plants, and contain a food reserve to maintain the young plant while it is forming its first true leaves to become photosynthetic and thus self supporting. Cotyledons are found in all kinds of different shape and size, and they divide the angiosperms into two groups: the monocots and the dicots. These two groups of plants are distinct in a lot of ways, but one of the most striking differences is mentioned in the name itself: the monocots have one cotyledon, whereas the dicots have two. The gymnosperms usually have more than two. It has been questioned for a long time which is more primitive: polycotyledony, as found in gymnosperms, or dicotyledony. When looking at the vascular system of a dicot cotyledon there are two options: the whorl of cotyledons as found in gymnosperms may have fused to form two, or the two cotyledons may have split up into a whorl. According to Buchholz (1919) the dicotyledonous condition is derived from the polycotyledonous. This model has now been broadly accepted.

The gymnosperms usually produce a whorl of cotyledons, varying from 3 to 6 with an average of 4 in Pinus banksiana up to 7 to 12 with an average of 9 in Cedrus libani (Buchholz, 1919). The gymnosperms do not seem to regulate the number of cotyledons very strictly. Harrison and Von Aderkas (2004) have shown that the number of cotyledons in Larix X leptoleuropaea seems to depend on the size of the apex rather than on strict genetic regulation.

In the angiosperms the number of cotyledons seems much more fixed. Connor and Agrawal (2004) have investigated the cotyledon number maintenance in wild radish (Raphanus raphanistrum and R. sativus). Their results provide support for both a lack of genetic variation and selection as reasons for the current lack of variation in wild radish cotyledon number.

Polycotyledony

Variation in cotyledon numbers in angiosperms is however not very uncommon. In the study of Connor and Agrawal, of 1857 wild radish plants 89 (almost 5%) had cotyledon numbers less or greater than two. This phenomenon has been reported in many other species, like Sugar beet (Karuna 1959), Bean (Garnier & Cagninacci 1962), Crotolaria (8 out of 760) (Purkayastha 1940), Brassica oleracea var. capitata (Gupta & Jain 1979), Schinziophyton rautanenii (Graz 2001), and many more. Deviations of the usual number of two seem to run through the entire group of dicots.

Holthorp (1944) performed a study on the occurrence of this deviating cotyledon numbers in dicots, mostly on Brassicaceae. His main conclusions are: tricotyledony is a heritable character and one which can be easily changed by selection. Dicotyledony must then be adaptive and maintained by natural selection.

Twinning in one cotyledon

Holthorp discriminated a few types of tricotyledony: simple fission, twinning and supplementation. In fission one of the two cotyledons is separated in two, thus resulting in a
total of three cotyledons, in twinning one is being replicated, while in supplementation the
plant is at first dicotyledonous, but the first true leaf develops as a cotyledon, also resulting in
three cotyledons. In all three modes the vascular system is somewhat irregular: if for example
the normal cotyledons have four veins, the splitted ones only have two each, while the
opposite one has the normal number.

There have been reports of a fourth type of tricotyledony. In *Phaseolus vulgaris* (Harris et al,
1921) plants are reported which have three cotyledons, but not by means of separation or
supplementation. The number of veins seems to have been three or six from the start, already present in the hypocotyl, in stead of the usual two or four. These three cotyledons all
have the same size and number of veins, making it symmetrical.

While dicotyly is the rule in most dicot species, there are a few exceptions known. Species from the genera of *Syzgium* (Shavalli Khan et al. 1997), *Psittacanthus* (Kuijt
1967) and *Idiospermum* (3-4) (Wilson 1979) typically produce more than two cotyledons.

**Phyllotaxy**

Phyllotaxy is the arrangement of leaves on a stem. This can be done in basically five ways: alternate, opposite, decussate, whorled and spiral. The embryo seems to provide the initial positional references in the form of two cotyledons at opposed positions on the apex, evidenced by the fact that abnormal cotyledon positions have predictable effects on vegetative phyllotaxis. Harris et al. (1921) show that phyllotaxis in *Phaseolus vulgaris* will change if the number of cotyledons changes. While the dicot plants will have a decussate leaf arrangement, the tricotyledonous plants have a whorled arrangement, with three leaves on each node.

The effect of the cotyledon number on a spiral phyllotaxis is somewhat different. If two cotyledons are properly spaced, the observed positions of the first two rosette leaves are at maximum distance from the cotyledons and they show gradual transition to spiral phyllotaxis (Berleth & Chatfield).

When looking at the meristem, each leaf touches a certain number of other leaves, as is shown in the diagram to the right (Williams, 1974). This is an example of a so called 3:5 system, as two pairs of imaginative spirals can be drawn on the contact points where the leaves touch: in this diagram 3 (blue) and 5 (red).
Sunflower, an example of a 21:34 spiral system

There are more complex systems possible, like the 21:34 system, found in some composite flowers. One of the most outstanding properties of these spiral systems is that they are almost always composed of two adjacent numbers of the Fibonacci series. In the Fibonacci series, the next number can be calculated by taking the sum of the two preceding terms: 1+1=2, 1+2=3, 2+3=5, 3+5=8, and so on. The first terms of the Fibonacci series are: 1,1,2,3,5,8,13,21,34,55,89,144. There has been much discussion on why plants use exactly these terms for their spiral compositions, but it seems this is the most efficient way of filling a two-dimensional space. This does not depend on the exact number, but on the divergence angle where two leaves touch each other: 5 divided by 3 has about the same value as 55 divided by 34, all approaching (φ+1)/2, known as Phi, or the golden section.

As most plants have one or two cotyledons, they will use the Fibonacci series in their spiral phyllotaxy, as their starting position will always be 1 or 2. If a plant would have three cotyledons, it will simply skip the 1+2, and start with 3, going to 3+5, right into the Fibonacci series. If a plant however has four cotyledons, it is very likely that it will use the so called “first accessory series” (1,3,4,7,11,18,29), which is used in the generative part of some plant species (Williams, 1974).

Cactaceae

The Cactaceae are a highly specialised family of dicotyledons. Most of these plants have adapted to extreme drought in various ways. One of these adaptations is the reduction of cotyledon size. The typical cactus seedling has a succulent hypocotyl, and two fleshy and often very small cotyledons (Gibson & Nobel, 1986). In the most primitive genus of the Cactaceae, Pereskia, plants bear simple, entire leaves in a spiral arrangement. While this arrangement is preserved through the entire family, the leaves have shrunken dramatically, to practically invisible to the naked eye. But according to Gibson and Nobel, leaf initiation is typical of those for a dicotyledon except that leaf production is rudimentary (for the more specialised species) and accompanied by precocious and rapid development of the axillary meristem, which becomes the areolar meristem. The primordia produced by the areolar meristem develop not as leaves but as spines. This would point out that an areole can be considered as the phyllotactic equivalent of a leaf.
Mammillaria beneckei

The genus Mammillaria is likely the most species-rich and morphologically variable genus in the Cactaceae, and Mammillaria beneckei is likely the most notorious for its number of synonyms. It has even been classified in three different genera: Dolichothele (D. balsasoides), its own monophyletic genus Oehmea (O. beneckei) and Mammillaria (M. balsasensis, M. balsasoides, M. nelsonii, M. aylostera, M. barkeri, M. colonensis and M. guiengolensis). It is reported from Mexico, the state of Michoacan on cliffs at La Salada (M. nelsonii); Guerrero, near Balsas (M. balsasensis); Guerrero along the lower Rio Balsas (M. aylostera); Guerrero between Taxco and Acapulco (M. balsasoides); in the same area as the last mentioned near Colonia (M. colonensis); near the Rio Elota in Sinaloa (M. barkeri) (Pilbeam, 1981). All species were lumped into one single species, Mammillaria beneckei.

The individual heads are usually globular, clustering haphazardly at all levels. The seeds are very large for Mammillaria, 2-5 mm long, dark brown and rough. Flowers are deep yellow, up to 30 mm in diameter with dark orange stigma-lobes. The fruit is red and slender. The radial spines number is 12 to 15, spines are 6 to 8 mm long, with one or two longer and hooked, coloured brown or nearly black. The seedlings typically show 3 or 4 cotyledons. In the genus Mammillaria, and some genera of the cactaceae the areoles are mounted on a nipple-like structure, called a tubercle. This can be seen in the Diagram of a cactus shoot apex.

Mammillaria beneckei distribution map. Source: www.mammillarias.net
**The aim of this study**

A batch of seedlings of *Mammillaria beneckei* was found to show not even one specimen with the usual two cotyledons normally found in the genus *Mammillaria*, even in the entire family of *Cactaceae*. All specimens have 3 or 4 cotyledons. At first different batches of seedlings from different sources were checked to see if the first batch was anomaly, or if the polycotyledony runs through the entire species. The vascular system of the seedlings was examined to learn more about the origin of the deviating cotyledon number, if it is regular, or if it shows signs of twinning, fission or supplementation. Other related species were examined as comparison material.

Seeds of *M. sphacelata var. viperina*, *M. sphacelata var. sphacelata* and *M. zephyranthoides* were chosen as comparing material as these species are closely related to *M. beneckei* (Butterworth & Wallace 2004) and easily available.

**Phylogenetic relationship (Butterworth & Wallace)**

In a later stage of development plants will be examined to check if the different cotyledon number (3 or 4) will affect phyllotaxis.
Materials & Methods

Plant material

Seeds of *Mammillaria beneckei* were obtained from different sources:
- 24 seeds of *Dolichothele balsasoides* were obtained from nursery De Herdt, Belgium. The approximately 15 parent plants were originally collected by Alfred Lau, the field numbers are not known.
- 60 seeds of *Mammillaria balsasoides* were obtained from the nursery of Hildegard Nase, Tucson, Arizona, USA.
- 11 seeds of TL566 (probably *Mammillaria nelsonii*), near El Limon, Michoacán, Mexico, from 2 plants.
- 5 seeds of TL739 (probably *M. nelsonii*), near El Descansadero, Michoacán, Mexico, from 1 plant.
- 6 seeds of *M. beneckei* of unknown origin were donated by Wolfgang Plein.
- 60 seeds of *Mammillaria sphacelata var. viperina*, of unknown origin.
- 10 seeds of *Mammillaria zephyranthoides* from Koehres, Germany.

All seeds were sown in vermiculite.

Because germinating times are slightly different it is difficult to standardize, or to describe the exact stage of development at which the seedlings were taken. Care was taken to pick specimens which seemed about the same age, thus a fairly uniform and early stage of development was secured.

Cryo-Scanning Electron Microscopy

Seedlings were glued onto a stub with colloidal carbon adhesive and frozen in liquid nitrogen. The samples were transferred in a transfer holder under vacuum at liquid-nitrogen temperature to the cold stage at -95°C into a cryo-preparation chamber CT 1500 HF (Oxford Instruments, High Wycomb, UK). The specimens were sputter-coated with 5 nm Pt. The specimens were conveyed under a high vacuum to the cold stage of a scanning electron microscope equipped with a cold-field emission electron gun (JSM 6300F; Jeol, Tokyo, Japan), analysed and recorded at -180°C using a 5-kV accelerating voltage.

Light microscopy

**Parafin sections:** Seedlings were fixed in 87.5% EtOH (96%), 7.5% acetic acid and 5% formalin for 24 h at room temperature. Tissues were dehydrated in ethanol and xylene and embedded in Paraplast. Serial transverse sections of 15 µm were stained for light microscopy (LM) with toluidine blue (0.1% in 1% Borax).

**Spurr sections:** Seedlings were fixed in 2% glutaraldehyde in 0,1M phosphate buffer at Ph 7,2 for 4 h at room temperature. Tissues were dehydrated in ethanol and propylene oxide and embedded in Spurr’s resin. Sections of 2.5 µm were stained for light microscopy (LM) with toluidine blue (0.1% in 1% Borax).
Results

The cotyledons
The cotyledon numbers of all different seed sources of *M. beneckei*, as well as the cotyledon numbers of *M. sphacelata var. viperina* were counted (table 1). The most striking result is that none of the specimens of *M. beneckei* has two cotyledons, whereas in *M. sphacelata var. viperina* and *M. zephyranthoides*, two of the most related species, every specimen has two cotyledons. The plants of *M. beneckei* show three or four cotyledons.

Cotyledons were counted on plants which had just germinated for a few days.

Table 1. The number of cotyledons on the plants from every different seed source

<table>
<thead>
<tr>
<th>Source of seeds</th>
<th>Number of plants</th>
<th>2 Cotyledons</th>
<th>3 Cotyledons</th>
<th>4 Cotyledons</th>
<th>More</th>
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<tr>
<td>Nase</td>
<td>23</td>
<td></td>
<td>15 (65%)</td>
<td>5 (22%)</td>
<td>3 (13%)</td>
</tr>
<tr>
<td>De Herdt</td>
<td>8</td>
<td>4 (50%)</td>
<td>4 (50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wolfgang Plein</td>
<td>2</td>
<td>2 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL 566</td>
<td>8</td>
<td>6 (75%)</td>
<td>2 (25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL 739</td>
<td>2</td>
<td>2 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. sphacelata var. viperina</em></td>
<td>10</td>
<td>10 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. zephyranthoides</em></td>
<td>4</td>
<td>4 (100%)</td>
<td></td>
<td></td>
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</table>

When all the different seedlings of *M. beneckei* are put together, 29 plants have 3 cotyledons (67%), 11 plants have 4 cotyledons (26%), and 3 plants have more than 4 cotyledons (7%). The plants with more than 4 cotyledons did not grow and soon died.

The picture of *M. sphacelata var. sphacelata* was added to show that both varieties of *M. sphacelata* have the same number of cotyledons. No additional data is available on this variety.
Cryo-Scanning Electron Microscopy pictures of *M. sphacelata var. viperina*, *M. beneckei (3)* and *M. beneckei (4)*. The scale bars are 500 µM.

These pictures show again the different numbers of cotyledons. On these pictures, the cotyledons of the tricotyl are somewhat larger than those of the quatrocotyl, but overall no such trend was discovered. It is clearly visible also that the seedlings of *M. sphacelata var. viperina* are much smaller than those of *M. beneckei*.

The Vascular System

Serial transverse sections were examined to reconstruct the vascular system. In *M. sphacelata var. viperina* each cotyledons only has one vascular bundle, while in *M. beneckei* each has two bundles. The central vascular bundles shown here are coloured green and indicated with white arrows, the cotyledonary vascular bundles are coloured red and are indicated with black arrows. The photographs correspond with the region depicted in the red rectangle. Sections were taken just below the apical meristem, where the separation between the central bundles and the cotyledonary bundles is most clear. However, the right central bundle in *M. sphacelata var. viperina* was already starting to split up to provide the new shoot with a vascular system. This was also observed in both forms of *M. beneckei* when getting closer to the meristem. Seedlings just beginning to form the first areoles were used for sectioning. The scale bar of *M. sphacelata var. viperina* is 100 µM; the scale bar of the other two pictures is 200 µM.
The vascular system of *M. sphacelata var. viperina* is quite simple. In the root there is only one vascular bundle, which splits in two at the base of the hypocotyl. Each of these primary bundles provides one of the central bundles (green) and one of the cotyledonary bundles (red).

The vascular system of *M. beneckei* is somewhat more complex. In the root there is one vascular bundle, which splits up at the base of the hypocotyl in three or four primary bundles, depending on the number of cotyledons. In each plant the bundles were the same, but there was variation among the plants. Some had three (or four) primary bundles of type 1, where each primary bundle at one point splits up in three, one central (green) and two cotyledonary bundles (red). In type 2, each primary bundle splits in two, resulting in 6 or 8 central bundles. These bundles then split in two, providing one central bundle (green) and one cotyledonary bundle (red). Some of the central bundles tended to fuse again, but not in all cases, resulting in 3-6 or 4-8 central bundles after the cotyledonary bundles appeared. The third type (3) was only observed once. The bundles all split in two, resulting in eight central bundles (green), but these all fused again, resulting in four bundles, before forming the cotyledonary bundles (red). These were then formed as in type 1: a central bundle splitting up in three.

In all three types each cotyledon is connected to two primary bundles, as each cotyledonary bundle from one primary bundle leads to a different cotyledon, and each cotyledon has two bundles. These cotyledonary bundles fuse just below the tip of the cotyledon.

The Meristem

A few days after germination the meristem starts to grow and forms its first tubercles. Cryo-scanning electron images were used to study the position of the tubercles. The meristem of *M. sphacelata var. viperina* forms a normal spiral phyllotaxis, with the first two tubercles positioned at a right angle to the cotyledons. The next tubercles are formed at a slightly smaller angle to form a spiral phyllotaxy. The two cotyledons are not visible anymore, the white arrows indicate where they were originally located. The position of the tubercles *M. beneckeii* are somewhat different from those of the previous species: between every pair of cotyledons (white arrows) a tubercle is formed, like in *M. sphacelata var. viperina*, but here the result is the formation of three or four tubercles (depending on the cotyledon number) instead of the first two. The next series of tubercles will form between the pairs of already developed tubercles. The picture showing the plant with four cotyledons has one irregular tubercle (black arrow), which was absent in the rest of the quadrocotyledonous plants.

In every specimen the spines and areoles were removed for a better view at the meristem. The scale bar of *M. sphacelata var. viperina* is 500µM, the scale bar of the other two pictures is 1000µM.
Transverse sections from just above the meristem were made to determine the position of the last formed tubercles, which are not well visible in the cryo-scanning photographs, as the older tubercles cover the meristem partially.

The transverse section of *M. sphacelata var. viperina* is not very clear, as the tubercles are packed tight together. The older and larger tubercles on the outside are not distinguishable anymore. The smallest four however are clearly visible.

The section of *M. beneckei (3)* shows there are three initial tubercles, corresponding with the number of cotyledons. The newest tubercles are in a different stage of development, on two the areoles (darkest spots) are already visible, the third does not yet show up completely in this section.

The section of *M. beneckei (4)* shows there are four initial tubercles, corresponding with the number of cotyledons. The newest four tubercles are clearly in a different stage of development. This can be seen best when looking at the connection between the newest areoles and those formed earlier. As the newest areoles develop, after some time they bend somewhat away from the meristem. In the section this can be seen as two areoles which seem to be connected to each other.

The scale bar of *M. sphacelata var. viperina* is 200µM, the scale bar of the other two pictures is 500µM
Discussion

The results show that none of the plants of M. beneckei has two cotyledons, while its nearest relatives always have two (Table 1). This could mean all used plants were from a single small population. This is however unlikely, as the only two samples of known origin came from Michoacán, and are part of the main population of this species (see distribution map). In order to exclude this possibility more populations should be examined.

If this phenomenon runs through the entire species, there are two options to achieve this. One option is that having three or four cotyledons gives a great advantage to these plants, so natural selection might have eliminated all plants with two cotyledons. This seems highly unlikely however, as none of the relatives shows this mutation, and neither does any other species of the Cactaceae, which are comparable in shape and size.

A second option might be that the species went through an evolutionary bottleneck, with one small population, which showed this character. This cannot have happened recently, as this species nowadays is wide spread, and shows a lot of variation. So far, there is not sufficient information to understand how this phenomenon has originated.

One of the other interesting conclusions from table 1, is that the number of cotyledons seems to be variable between three or four. There are only a few dicot species known with variable cotyledon numbers, such as Psittacanthus schiedeanus which varies its cotyledon number between 8 and 11 (Kuijt 1967), and Idiospermum perhaps between 3 and 4 (Wilson 1979), but this conclusion was based on only 3 seeds.

In order to test if the number of cotyledons in M. beneckei is heritable, crossing experiments need to be performed.

The vascular system of the seedlings shows that the appearance of more than two cotyledons does not seem to be the same as in most cases of polycotyledony in dicots, in which the vascular system is irregular and somewhat distorted (Karuna 1959, Garnier & Cagninacci 1962, Purkayastha 1940, Gupta & Jain 1979). In M. beneckei the vascular system is already at the base of the hypocotyl split-up into three or four vascular bundles, depending on the number of cotyledons. It would be interesting to look at the early development of the embryo, to see whether the polycotyledony is caused by a split cotyledon in a very early developmental stage, or that the “heart shaped stage” may not be very heart shaped at all in this species. In other words, it might be possible that this species even at a very early stage does not have two cotyledons. To study this, very young embryos need to be examined.

In Idiospermum the phyllotaxy remains decussate, not depending on the number of cotyledons (Wilson 1979). Yet the number of cotyledons in most dicots is affecting the phyllotaxis in a predictable way: between every neighboring pair of cotyledons a leaf, and thus a tubercle, appears.

A specimen of M. beneckei with three cotyledons would give a “normal” adult plant, with a 8-13 phyllotaxy. The plants with four cotyledons however should not end up in this configuration, as 4 is not a part of the Fibonacci series. Adult plants of M. beneckei with four cotyledons however are not yet available to test this hypothesis.

The results also show that the cotyledon number may not be very stringent to the location of the new tubercles, as irregular tubercles were also observed, though only in one specimen. This however does not seem to affect the phyllotaxis in the shown specimen.

So what makes it able for this species to have more than two cotyledons, while in most other species this is so strongly selected against? In other words, what is the function of having two cotyledons?

Mammillaria beneckei, Psittacanthus schiedeanus and Idiospermum all share a quite unusual feature in dicots: they do not germinate in the soil: M. beneckei and Idiospermum germinate on
top of the soil, *P. schiedeanus* being a parasite germinates on branches of other plants. As buried seeds germinate, the hypocotyl forms an apical hook to protect the meristem as it is pulled above the ground. The cotyledons function like a glove, and two cotyledons would offer an optimal protection. *M.beneckei* is not able to form an apical hook, and does not germinate in the soil, as germination is strongly suppressed when placed in the dark, like most other cacti (Gibson & Nobel). All three lack a flexible hypocotyl to push the meristem as well as the cotyledons above the ground. If this is all true, dicotyly might have evolved as an adaptation for underground germination. Underground germination offers the advantage of protection against predation and abiotic factors like dehydration. In the three species mentioned earlier this function is no longer used anymore due to their specialized ways of life. When there is no selective pressure to dicotyledony, it may only be temporarily maintained by lack of variation and may have become rudimentary for these species.

**Main Conclusions**
*Mammillaria beneckei* has three or four cotyledons, which seem to be formed quite early in the development, as the vascular system is regular. The different cotyledon numbers have an influence on the phyllotaxy. There seems to be no advantage to this, neither a disadvantage.

**Idiospermum seedling**

**Acknowledgments**
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