

HISTOLOGICAL DOCUMENTATION AND ECOLOGICAL IMPLICATIONS OF
BULBILS IN ACONITUM NOVEBORACENSE (RANUNCULACEAE), A
FEDERALLY PROTECTED, THREATENED PLANT SPECIES

by

BONNIE A. FARRELL

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ABSTRACT OF THE THESIS

Histological documentation and ecological implications of bulbils in *Aconitum noveboracense* (Ranunculaceae), a federally protected, threatened plant species

by BONNIE A. FARRELL

Thesis Director:

Dr. Jean Marie Hartman

Aconitum noveboracense (northern monkshood) is a federally listed, threatened plant species endemic to the states of Iowa, Ohio, New York, and Wisconsin. The presence of bulbils in some populations of this species has been noted, but previously not investigated in detail. The anatomy of vegetative propagules produced in the leaf axils and bracts of *A. noveboracense* is presented. Microscopic observations confirm that the axillary propagules are bulbils. The bulbils' outer leaves are papery and the innermost leaves are slightly fleshy at their bases and more membranous at their apices. The minute and imbricate leaves join at the base of a compressed stem and adventitious roots can develop while still attached to the parent. Vegetative propagation via bulbils occurs sporadically in *A. noveboracense*, and may have positive implications for its persistence by supplementing the production of both seeds and perennating tubers.

Keywords: *Aconitum noveboracense*, asexual reproduction, endangered species, North America, propagules, vegetative propagation.

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Chapter 1. The Status, Ecology, and Taxonomic History of *Aconitum noveboracense* (Ranunculaceae)

Aconitum noveboracense Gray ex Coville, (northern monkshood, Ranunculaceae), is protected under the aegis of the 1973 Federal Endangered Species Act (ESA). This globally rare plant species was added to the United States Department of Fish and Wildlife Service's (USFWS) Endangered Species List in 1978 (Federal Register, 43: 81: 17910-17916) with a status of threatened, meaning that it is at risk for becoming endangered in the foreseeable future throughout all or part of its range (ESA, 1973 as amended through the 108th Congress, 3: 4).

Aconitum noveboracense is a glacial relic that grows in microhabitats providing cool temperatures, low light, and excellent drainage (Fig. 1-1). It has a limited population size and a disjunct distribution. Endemic to the United States, *A. noveboracense* is found only in Iowa, Ohio, New York, and Wisconsin. At the state level, *A. noveboracense* is listed as threatened in New York, Wisconsin, and Iowa and as endangered in Ohio. There are approximately 106 populations; Iowa has the greatest number (83) followed by Wisconsin (18), New York (3), and Ohio (2: USFWS, 2006; C. Carnes & M. Moser, USFWS, personal communication, 2008). Extant populations are closely monitored in all these states yet their recovery is insecure. For example, in repeated surveys of New York populations, the Olive Natural Heritage Society and I were unable to find *A. noveboracense* growing along the lower portions of two streams in the Catskill Mountains, where they historically had been documented.

The federal recovery plan for *Aconitum noveboracense* included a comprehensive report of its distribution, ecology, and status. Populations of *A. noveboracense* were

found to be threatened primarily by human actions: construction and maintenance of dams, reservoirs, roads, and power lines, trail development, suburban and urban development, logging, quarrying, livestock grazing, and scientific over-collecting and over-visitation. The primary natural threat was flooding. The plan made detailed recommendations for addressing the threats and included the goals of searching for new populations and researching propagation (Read & Hale, 1983).

Aconitum noveboracense has biennial tubers (Kadota, 1987). In the spring, a tuber breaks dormancy, surviving only until the end of that growing season. Meanwhile, one or more ‘daughter’ tubers develop, enter dormancy when there is a killing frost, and send forth new growth the following spring. The herbaceous shoot produces flowers and seeds each year and may reach a height of 2.0 meters. The leaves are alternate, palmately lobed, simple and often highly dissected to within a few millimeters of the leaf base. The inflorescences are terminal racemes, sometimes with additional axillary racemes. Individual flowers are showy, zygomorphic and helmet-shaped (Fig. 1-1). Sepals and petals are purple or mixed shades of purple and white; petals are much reduced and may hold nectar (Figs. 1-1A and 1-1B). The flowers are protandrous, which may decrease the likelihood of selfing. Older plants are larger and produce more flowers. Pollination is commonly by bumblebees. Seed follicles are usually three per flower, but the terminal flower in a raceme may produce four follicles. Not all flowers set seed. The seeds average eight per follicle and disperse during late summer to fall. Earlier studies on germination reported very low (less than 1%) germination rates (Dixon & Cook, 1990). Fortunately, the Olive Natural Heritage Society (New York) determined that seeds collected in October then immediately sown had a 50% germination rate the following

spring (Adams & Groeters, 2001). The species can produce subterranean and stem bulbils, as well as adventitious root buds (Kuchenreuther, 1991). *Aconitum noveboracense*, similarly to other members of *Aconitum* is toxic due to the presence of aconitine alkaloids (Read & Hale, 1983).

In North America (including Alaska), there are 6 *Aconitum* species: *A. columbianum* Nutt., *A. delphiniifolium* DC., *A. maximum* Pall. ex DC., *A. noveboracense* Gray ex Coville, *A. reclinatum* Gray, and *A. uncinatum* L. (Kuchenreuther, 1996; Brink & Woods, 1997). The distribution of *A. delphiniifolium* and *A. maximum* includes Asia whereas the remaining 4 species are native to North America (Hultén, 1960; Kadota, 1987; Brink & Woods, 1997). *Aconitum columbianum* has 2 subspecies: *columbianum* and *viviparum*. Brink & Woods did not recognize Hultén's division of *A. delphiniifolium* into 3 subspecies or Hardin's division of *A. uncinatum* into 3 subspecies (Hultén, 1960; Hardin, 1964; Brink & Woods, 1997). *Aconitum maximum* has 2 subspecies: *maximum* and *kurilense*, although *kurilense* does not occur in North America. A variety of *A. noveboracense* with different pubescence was recorded (var. *quasiciliatum* Fassett or var. *pseudociliatum* Fassett ex Cratty); (Fassett, 1929; Cratty, 1933). In their treatment of *Aconitum* in *Flora of North America*, Brink and Woods did not recognize the species status or any varieties of *A. noveboracense* (1997). Rather, they subsumed *A. noveboracense* under *A. columbianum* subsp. *columbianum*, a subspecies that never produces bulbils (Brink & Woods, 1997). However, in this paper, I conserve the species status of *A. noveboracense* based on observed bulbils growing on some members of this species which conflicts with Brink's and Woods' previous determination.

Aconitum noveboracense has had an interesting, if confusing, taxonomic history. Discovered in Chenango County, New York in 1857, it was originally ascribed by Greene to *A. uncinatum* L. (southern monkshood), the first *Aconitum* named in the U.S. In 1886, after discoveries of new populations prompted a re-examination of the taxonomy, Coville published Gray's description and the new species name of *A. noveboracense* Gray ex Coville (Coville, 1886). Hardin changed the name and rank to *A. uncinatum* subsp. *noveboracense* in his revision of the eastern U. S. *Aconitum* (1964). In this treatment, *A. noveboracense* was distinguished from two other *uncinatum* subspecies by leaf morphology, helmet shape, pedicel pubescence, and geographic distribution. This change was ignored as the literature continued to use *A. noveboracense* Gray ex Coville. Reports occasionally stated that *A. noveboracense* may be more closely aligned with the *A. columbianum* complex from the western U.S. based on tuber type, pubescence, erectness, and helmet type (Mitchell & Dean, 1982; Read & Hale, 1983). The *A. columbianum* complex refers to *Aconitum* that historically grew from Alaska to California, eastward through southern British Columbia to western Montana, southward to Colorado and New Mexico, and in the Black Hills, South Dakota (Hitchcock et al., 1964). Upwards of fifteen species and varieties were reduced to three varieties of *A. columbianum* in the *Flora of the Pacific Northwest* based on the assertion that the species characters were intergradient (Hitchcock et al., 1964). Brink's revision of the U.S. *Aconitum* (except Alaska) resulted in the reduction of *A. noveboracense* and almost twenty species, subspecies and varieties to *A. columbianum* subsp. *columbianum* (Brink et al., 1994). Although some of these names are synonyms, the relevancy is that most western species and *A. noveboracense* were lumped together because their morphological

characters were considered to be a very variable complex and because they supposedly do not produce bulbils. The western species that are bulbiferous were reduced to *A. columbianum* subsp. *viviparum* based on the report that all members of those populations are consistently bulbiferous and that bulbifery is not an intergradient trait (Brink et al., 1994; Brink & Woods, 1997). Results of allozyme and RAPD studies on *A. noveboracense*, *A. columbianum*, and *A. uncinatum* were interpreted as upholding the subspecies status of *A. noveboracense* (as *A. columbianum* subsp. *columbianum*); (Cole & Kuchenreuther, 2001). However, the results only indicated that *A. noveboracense* and *A. columbianum* were more closely related to each other than either was to *A. uncinatum*. The data did not clearly resolve the relationship between *A. noveboracense* and *A. columbianum* subsp. *columbianum*. *Aconitum noveboracense* may still be a distinct genetic entity.

Approximately 300 species of *Aconitum* L. inhabit temperate to sub-arctic regions of the Northern Hemisphere with a circumboreal distribution that ranges southward to northern Mexico and northern Africa (Kadota, 1987; Brink & Woods, 1997). North America has 6 species (if *A. noveboracense* is conserved), most with disjunct distributions (Read & Hale, 1983; Brink & Woods, 1997). Europe has fewer than 20 species (Utelli et al., 2000) with its center of diversity in the Carpathian Mountains (Mucher, 1993). Asia has more than 200 species with the center of diversity the Hengduan Mountains, SW China (Luo et al., 2005). There are four *Aconitum* subgenera: *Aconitum*, *Lycoctonum* DC., *Gymnaconitum* (Stapf) Rapaics, and *Tangutica* (Wang) Kadota (Kadota, 1987, 2001). In North America, *A. columbianum*, *A. delphiniifolium*, *A. maximum*, *A. noveboracense*, and *A. uncinatum* belong to subgenus *Aconitum*,

characterized by biennial tubers. *Aconitum reclinatum* belongs to subgenus *Lycocotnum* characterized by fascicled roots.

Throughout its global range, *Aconitum* species taxonomy is complicated by many factors, e.g., conserved genetics, plasticity, hybridization including introgressive hybridization, and regional studies. Molecular markers may have too little variation (are too highly conserved) to be informative for phylogenetic purposes. For example, neither *trnL* nor *trnF* provided resolution of the tetraploid Japanese *Aconitum* (Kita et al., 1995) and I found *trnL* uninformative for North American *Aconitum* (unpublished data). *Aconitum*'s plasticity makes it challenging to determine morphological characters that are constant and thereby useful for distinguishing among species. For example, the large morphological variation in stem leaves of *A. lycocotnum* (a European species) means that this character is not helpful in determining how one species differs from another (Utelli et al., 2000). One subspecies of *A. japonicum* exhibits such a level of plasticity that in sunnier habitats, the stems are erect, the leaves deeply divided, and the inflorescences compact whereas in shadier habitats, the stems are weak, the leaves shallowly divided, and the inflorescences loose (Kadota, 1987). Introgressive hybridization has also confounded taxonomy, more so in Europe and Asia than North America, resulting in fertile hybrids that have backcrossed with the original species (Kadota, 1987). As a result, regionalized studies may have missed identifying populations that were intergradient between two supposed *Aconitum* species (Kita et al., 1995).

Aconitum is a member of Ranunculaceae (the buttercup family). Ranunculaceae contains 47 Genera and 2000 species and has a cosmopolitan distribution with a high concentration in temperate and boreal habitats (Judd et al., 2002). This herbaceous

family is characterized by tuberous or rhizomatous roots; stems with ringed or scattered vascular bundles; very dissected or compound leaves often with sheathing around the base of the petioles; showy petal-like sepals, reduced petals; many stamens and carpels; and aggregate fruits that form follicles, achenes, or berries. Plants in Ranunculaceae may contain alkaloids, glycosides, and saponins of varying degrees of toxicity (Judd et al., 2002; Zomlefer, 1994).

The monophyly of the family is supported by the molecular phylogeny based on a combined analysis of the chloroplast genes *atpB* and *rbcL* and nuclear ribosomal 18s DNA. However, the results did not support all of the traditional morphological phylogenies (Hoot, 1995). For example, although the molecular phylogeny corresponded with traditional phylogeny based on T- and R- type chromosomes it conflicted with some morphological phylogenies based on staminodia/petal morphology and fruit type. The results suggested that these character states evolved independently in several taxa (Hoot, 1995). The nuclear ribosomal 18s analysis also supported the *Aconitum* and *Delphinium* clade (Hoot, 1995), taxa that have been traditionally placed in tribe Delphinieae with *Consolida* and *Aconitella* based on floral morphology and an insect (usually bumblebee) pollination syndrome that includes high food rewards (Bosch et al., 2001).

The histological study of bulbils in *Aconitum noveboracense* was undertaken to anatomically confirm that the structures I discovered growing in the leaf axils of *A. noveboracense* during the autumn, were bulbils. This is an important finding because it conflicts with Brink's determination that *A. noveboracense* is synonymous with *A. columbianum* subsp. *columbianum*. According to his treatment, populations of *A. columbianum* subsp. *columbianum* never produce bulbils and *A. columbianum* subsp.

viviparum always produce bulbils, even in transplant experiments (Brink et al., 1994).

Yet, in *A. noveboracense*, the production of bulbils is sporadic; very few individuals appear to have them. This study was part of a larger effort to understand *A.*

noveboracense's reproductive biology, including the impact of deer herbivory on flower and seed production. In addition, the research included studying the phylogenetic relationship between *A. noveboracense* and the other *Aconitum* in North America including the Alaskan species.

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Figures



FIG. 1-1. *Aconitum noveboracense*. **A.** Shoot. Scale bar = 10 cm. **B.** Inflorescence. Upper, helmet-shaped sepal of calyx hides two nectiferous petals. Scale bar = 2 cm. Abbreviations: ls, lower sepal; us, upper sepal. **C.** Habit. (Photographed in New York State.)

Chapter 2. Histological Documentation of Bulbils in *Aconitum noveboracense* (Northern Monkshood, Ranunculaceae)

Introduction

Aconitum noveboracense is a federally protected herbaceous plant species that has a rank of threatened on the United States Fish and Wildlife Service's Endangered Species List (43 FR 17916). The previous chapter summarized the status of *A. noveboracense*, reviewed its biology, and included an overview of North American *Aconitum* taxonomy. This chapter focuses on the anatomy and morphology of bulbils in *A. noveboracense*. Microscopic documentations of *A. noveboracense* bulbils are presented for the first time and are supplemented with photographs that work together to illustrate a clearer picture of bulbifery in the species. The physiology and ecology of bulbifery among other Genera are presented that suggest mechanisms influencing bulbil production and the role bulbils play in the persistence of *A. noveboracense*.

Bulbils are asexually produced, miniature bulbs. According to Bell, a bulb has a short stem axis with fleshy scale leaves and may have membranous scale leaves on the outer side (1993). The inner leaves of the bulbil may be modified for energy storage, the outer leaves for protection, and adventitious roots may arise from the stem base. Bulbils may germinate while connected to the parent plant or separate before germination occurs. Above ground bulbils may be found in bract axils, e.g., *Globba cernua* Bak. (Zingiberaceae; Box & Rudall, 2006) and in apical and axillary shoots, e.g., *A. columbianum* ssp. *viviparum* (Greene) Brink (Brink et al., 1994).

A literature survey reveals that bulbifery occurs in over twenty families and includes species that range from invasive, e.g., *Dioscorea oppositifolia* L.

(Dioscoreaceae; Thomas et al., 2005; Main et al., 2006), to rare, e.g., *Curculigo orchioides* Gaertn. (Hypoxidaceae; Suri et al., 1999). Bulbiferous species may be found in various habitats, i.e., subtropical, e.g., *Titanotrichum oldhamii* (Hemsl.) Soler. (Gesneriaceae; Wang & Cronk, 2003), arctic, e.g., *Polygonum viviparum* L. (Polygonaceae; Wookey et al., 1994), and desert, *Agave macroacantha* Zucc. (Agavaceae; Arizaga & Ezcurra, 1995).

Throughout the plant kingdom, there is wide variation in both the initiation site and morphology of asexual reproductive structures, including bulbils. Examples of variation in initiation sites include *Remusatia vivipara* Schott (Araceae), which develops bulbils that originate at the plant base, *Dracontium asperum* Koch (Araceae), which forms tubercles that originate from accessory buds on leaves, and *Cocos nucifer* L. (Arecaceae) which rarely produces bulbil-like axillary shoots rather than spadices (Raju, 1993; Mayo et al., 1997).

Bulbifery may be influenced by a variety of endogenous and environmental factors, including herbivory, plant phenology, microclimate, genetic variation, and plant hormones. Studies from in vitro propagation experiments illustrate other potential roles that hormones may play in bulbifery. In the medicinal vine *Dioscorea bulbifera* L., indoleacetic acid (an auxin) and kinetin (a cytokinin) induced bulbil production from nodal explants (Narula et al., 2003). In *Curculigo orchioides*, another medicinal plant, benzyladenine (BAP, cytokinin) and sucrose or exogenous applications of cytokinin formed bulbils then plantlets from leaf and stem explants (Suri et al., 1999). Furthermore, in *Cycas revoluta* Thunb. (Cycadaceae), BAP induced shoot primordia formation in the middle part of a bulb scale (Chaplot & Jasrai, 2000). Conversely, in *Dioscorea*

oppositifolia, endogenously produced gibberellins promoted dormancy in bulbils as well as other effects (Kim et al., 2005).

Bulbils in North American *Aconitum* have been recorded from *A. columbianum* ssp. *viviparum* (a subspecies of western monkshood) and sporadically in populations of *A. noveboracense* (northern monkshood) and *A. uncinatum* (southern monkshood); (Brink, 1982; Mitchell & Dean, 1982; Brink et al., 1994; Brink & Woods, 1997). In Asia, bulbils have been documented from *A. bhedingense* Lauener (Nepal), *A. bulleyanum* Diels (China), and *A. tsaii* Wang (China); (Stainton, 1988; Yang, 1999).

The first specimen (and type) of *Aconitum noveboracense* was collected in New York State in 1857 (Coville, 1886; NYBG specimen ID: 353155) and early morphological comparisons among *A. noveboracense*, *A. uncinatum*, and *A. reclinatum* did not include the presence of bulbifery (Hardin, 1964). The type specimen and subsequent collections were not acquired in the autumn when bulbils are present, but during the summer at peak flowering time. This is not surprising because inflorescences help locate these otherwise obscure plants that grow in cold ravines, gravelly streambeds, algific talus slopes and cliff seeps (Fig. 1-1C). One of the first references to bulbifery in this species (as ‘bulblets’) is from a local flora covering Ranunculaceae (Mitchell & Dean, 1982). Later, bulbils from plants in Iowa and Wisconsin were described as ‘white, waxy, bipolar axillary buds’ and were illustrated in Kuchenreuther (1991, 1996).

The presence of bulbils has had traditional use as a taxonomic character to differentiate among *Aconitum* species and subspecies, but its usefulness depends on the plasticity of this trait and its frequency among members. For example, all individuals in populations of *A. columbianum* ssp. *viviparum* are bulbiferous whereas no individuals in

populations of *A. columbianum* ssp. *columbianum* produce bulbils even when grown in the same environment (Brink, 1981; Brink et al., 1994). By comparison, only some individuals exhibit bulbifery in populations of *A. uncinatum* (Brink, 1982). The method of bulbil dispersal may also be useful for taxonomic purposes, e.g., *A. columbianum* ssp. *viviparum* bulbils are deciduous, i.e., they detach from the parent whereas *A. uncinatum* bulbils are persistent, i.e., they continue to grow on the parent until the end of the growing season (Brink, 1982). To date there has been no study of the anatomical structure of *Aconitum* bulbils. In this paper, I focus on stem bulbils in *A. noveboracense* to gain a better understanding of the reproductive biology of this rare plant species, to find reliable taxonomic characters to distinguish among the North American *Aconitum*, and present the first histological study of *Aconitum* bulbils.

Materials and Methods

The populations where the two bulbils were collected were monitored over a growing season and the percent bulbifery was recorded (locality information withheld due to endangered species classification). Bulbils from one vouchered (CHRB) individual of *Aconitum noveboracense* were collected (with permission) in September and were fixed in FPA (50% ethanol: 5% formaldehyde: 5% propionic acid). The fixed bulbils underwent a dehydration series to prepare for embedding in paraffin and sectioning. The dehydration series was 30 minutes in a 50% ethanol solution, then 30 minutes each at 70%, 95%, 100% (twice) ethanol, and then each of the following percentages of 100% ethanol and CitriSolv (Fisher Scientific, Fairlawn, NJ): 90%/10%, 70%/30%, 50%/50%, 30%/70%; 10%/90%. The bulbils then were immersed in 100% CitriSolv for 12 hours (twice). Paraffin chips (Paraplast Plus, Fisher) were added at room temperature until saturated. After 12 hours of saturation, the samples were placed on a warming tray (40°C) and paraffin was again added until saturation. After 12 hours, the samples were put in a 60°C oven, with lids off. Half of the liquid was replaced with 100% melted paraffin and kept in the oven for 12 hours (twice). Finally, all the liquid was replaced with 100% melted paraffin. To create paraffin blocks, the sample was transferred to a plastic mold, half filled with melted paraffin, which was floated in ice water. The preserved bulbil was quickly and carefully placed in the paraffin and more melted paraffin was poured on top. After the paraffin solidified, excess paraffin was trimmed off by hand using a single-edge razor blade. The samples were sectioned using a HM335E rotary microtome (MICROM International GmbH, Walldorf) at a thickness of 5 µm. Sections were mounted on glass slides using Haupt's adhesive (Haupt, 1930) using one

drop per glass slide, and the ‘flooding 4% formaldehyde’ method described in Sheridan et al. (2007) on a 40°C warming plate.

Slides with sections were cleared from paraffin in a xylene bath for at least 15 min. (twice). The slides were then processed through a staining series modified after a protocol by Peter Endress (unpublished) using the stains 0.5% aqueous Astra Blue (Sigma) and 1.0% Safranin. The Astra Blue solution was prepared from 1 g Astra Blue powder, 4 g tartaric acid, 200 ml distilled water, and 2-3 crystals phenol. The Safranin solution was prepared from 2 g Safranin, 100 ml distilled water, and 100 ml 95% ethanol (EtOH). The staining series were as follows: 100% EtOH (15 min), 95% EtOH (15 min), 70% EtOH (15 min), 1% Safranin (overnight), water (quick rinse), Astra Blue (30 min-1 hour), water (dip slowly 3-5 times), 50% EtOH (dip slowly 3-5 times), 70% EtOH (dip slowly 3-5 times), 95% EtOH (5 s), 100% EtOH (10 s), 50% CitriSolv/50% EtOH (10 s), 100% CitriSolv (10 min, twice). Coverslips were mounted using Permount (Fisher, Somerville, NJ).

The slides were viewed with an Olympus Magnifier 2.0 compound light microscope (4X, 10X and 40X) equipped with an Olympus digital camera and MagnaFire 2.0 software. Microscope slides and field-collected bulbils were also examined under a Zeiss Stemi 2000-C dissecting microscope and photographed with a Nikon Coolpix8700 digital camera. Digital images were managed and enhanced using Photoshop (Adobe Systems, Inc., San Jose, CA). An overall measure of the bulbil length on the slide was made initially and then finer measurements were made using a ruler in the ocular of the microscope.

Results

Field Monitoring over one growing season at the site where the bulbils were collected showed that 4% of the plants were bulbiferous. The photographs in Fig. 2-1 suggest that the origin of bulbils in *Aconitum noveboracense* is the axillary bud. As shown in a field photograph from a NY site in September 2006 (Fig. 2-1A), two *A. noveboracense* bulbils were growing in the leaf axils. Here, the bulbils (each approximately 0.3 cm.) produced adventitious roots while attached to the parent. Both the adventitious roots and the bulbils were whitish in color. Other *A. noveboracense* bulbils had brown, papery leaves. The bulbil documented in Fig. 2-2B emerged from wrinkled, papery, scale leaves. It had a greenish white adventitious root with a brown tip and smooth, brownish white, inner bulbil leaves that were positioned with leaf tips in the same direction as the root. This bulbil was attached at the axil of a petiole and the primary stem. As shown in Fig. 2-1C, *A. noveboracense* also produced bulbils that showed leaf development but no adventitious root development. The younger bulbil had one green leaf at the apex that unfurled over the rest of the bulbil. Fig. 2-1C also shows that two bulbils grew adjacent to each other. The compressed growth pattern observed here was different from the overall symmetrical growth pattern observed in nonbulbiferous axillary shoots of *A. noveboracense* (Figs. 1-1A & 1-1C). The photographs in Fig. 2-1 also show variation in the way that the bulbils were positioned relative to the parents. The bulbils in Figs. 2-1A and 2-1B were somewhat perpendicular to the parents' upright growth axis, whereas the bulbils in Fig. 2-1C were more upright.

Microscopic sections of an immature bulbil and its adjacent petiole are illustrated in Fig. 2-2. This bulbil was 2 mm from pointed apex to rounded base and did not have

adventitious roots or leaves. The bulbil was covered with dry, dark brown, papery leaves that detached from the bulbil during the dehydration and embedding process. The bulbil and a small piece of petiole were sectioned together because it seemed possible that they may have been connected. Fig. 2-2A shows the petiole and the most distal section of the bulbil. Although the petiole epidermis is predominantly single celled, it appears to be two celled in places. Three primary leaf traces and four smaller leaf traces are embedded in the fundamental tissue. Fibers can be seen at the periphery of the larger bundles. Figure 2-2B shows a section of the bulbil base. It appears that the arrangement and relative sizes of the cells are analogous to immature, dicot root anatomy (Raven et al., 1992). (Referring back to Figs. 2-1A and 2-1B may aid the reader in visualizing the relative position of the bulbil root.) In Fig. 2-2B, the larger, more irregularly shaped cells suggest parenchymal cortex cells and the inner ring composed of smaller and more uniform cells suggest endodermis cells. The pericycle is not resolved in the immature stele. The developing vasculature appears to be forming protoxylem poles in an irregular 'x' shape. The metaxylem that would be located closer to the center, is not distinguishable. The different structure and staining of the cells between the developing xylem suggest immature phloem. Since this was not a developmental study, this region will be referred to as the vascular plexus.

Fig. 2-2C shows a longitudinal section of an immature bulbil's center (without the papery leaves). Here, the epidermis surrounds preformed leaves that fold over each other. The outer leaves are two to four cells thick at their apices and become thicker toward the base where they become indistinguishable from other leaves. At the bulbil apex, a thicker leaf (8 – 10 cells) covers leaf primordia that have darkly stained, dense

cells. Fig. 2-1C shows that the most proximal leaf primordia may develop into foliage leaves that unfurl over the rest of the bulbil. In the axil of two immature leaves is an axillary bud. A compressed stem subtends the meristematic region and is composed of darkly stained, irregularly shaped and arranged cells. The leaves and the compressed stem would be homologous to the plant's shoot system. The vascular plexus, in the lower center of the darkly stained irregularly shaped cells, does not suggest root anatomy as does the distal section (Fig. 2-2B) rather, this may be the point of attachment to the parent.

Discussion

The investigation of bulbifery in New York populations of *Aconitum noveboracense* was part of a larger effort to understand the reproductive biology of *A. noveboracense* and the phylogeny of North American *Aconitum*. In the genus *Aconitum*, the production of bulbils appears to have three character states: consistent production, inconsistent production, and consistent non-production. *Aconitum noveboracense* and two species from Yunnan Province, China, *A. bulleyanum* and *A. tsaii* (Yang, 1999), exhibit the second character state, i.e., inconsistent bulbil production. *Aconitum columbianum* subsp. *viviparum* exhibits the first character state, i.e., consistent bulbil production and *A. columbianum* subsp. *columbianum* exhibits the third character state, i.e., consistent non-production. Clearly the inconsistent production of bulbils in *A. noveboracense* is different from the consistent production of bulbils in *A. columbianum* subsp. *viviparum*, and the consistent non-production of bulbils in *A. columbianum* subsp. *columbianum*. These dissimilarities are important to the species' descriptions. The intra-population variation of bulbifery observed in *A. noveboracense* means that this characteristic would be unreliable as part of a taxonomic key for identifying an unknown specimen. In contrast, the presence or absence of bulbils in conjunction with other identifying characteristics would be useful for distinguishing *A. columbianum* subsp. *viviparum* and *A. columbianum* subsp. *columbianum* because bulbifery does not exhibit intra-population variation in these subspecies.

Aconitum noveboracense bulbils are miniature bulbs that originate in axillary meristems and bracts (Troll, 1937). The outer scale leaves are papery and the innermost leaves are very slightly fleshy at their bases, grading into more membranous apices. This

anatomical structure is similar to a membranous variation of the more common fleshy bulbil type, both described by Bell (1993). The most proximal leaves develop into foliage leaves as shown in Fig. 2-1C. The bulbils' cellular organization is typical of dicot bulbs, i.e. less organized than monocots, in particular the compressed stem area (Bell, 1993).

Macroscopic views show that the bulbils form in the leaf axils. The axillary shoot is absent when a bulbil germinates, suggesting that bulbil development originates at the axillary bud and replaces the normal shoot development at that particular location. Originating at the axillary bud would also suggest that the bulbil vasculature would be continuous with stem vascular system. This interpretation is supported by the absence of any connection between the bulbil and the petiole in any section examined. The bulbil longitudinal sections suggest that the point of attachment is at the proximal position of the bulbil base and that the basal region distal to the attachment site may differentiate into root tissue.

The versatile reproductive strategy (seeds, sporadic bulbils, and over-wintering tubers) in *Aconitum noveboracense* may increase the species' survival and fitness, especially since *A. noveboracense* seedlings have poor survival rates (Read & Hale, 1983). According to one theory of life history variation, available resources are finite; therefore, an increase in the resources invested in bulbils would be at the expense of resources allocated to seeds (Ricklefs, 1993). The reproductive strategy of *Titanotrichum oldhamii* (Hemsl.) Soler. (Gesneriaceae) supports this theory; seed failure occurred as resources were redirected to rhizome and bulbil growth (Wang et al., 2004b). Similarly, the reproductive strategy of the dioecious, bulbiferous *Dioscorea japonica* Thunb.

(Dioscoreaceae) also supports the theory; females (versus males) put more resources into sexual than asexual reproduction yet the total investment in reproductive strategies did not differ between the sexes (Mizuki et al., 2005). *Agave macroacantha* Zucc.

(Agavaceae), a desert species that grows for many years, reproduces once then dies, also produces bulbils in an adaptive response to failed seed production. Specifically, the bud removal treatment resulted in more bulbiferous plants versus the pollinator exclusion treatment and the control (Arizaga & Ezcurra, 1995). However, the aquatic *Butomus umbellatus* L. (Butomaceae), exhibited nonlinear tradeoffs between seed and bulbil formation in experiments that tested various nutrient and pollinator treatments (Thompson & Eckert, 2004).

The larger size of a bulbil relative to a seed may increase recruitment, i.e. three seasons after sowing *Dentaria bulbifera* L. (Brassicaceae) bulbils and other forest herbaceous seeds, overall survival was positively correlated to bulbil or seed size (Ehrlén & Eriksson, 2000). However, seed or bulbil size was negatively correlated with patch occupancy (small seeds are more easily dispersed). *Aconitum noveboracense* seeds and bulbils are approximately the same size (2 to 3 mm versus 1 to 3 mm, respectively) and neither disperses far unless the parent grows streamside. Perhaps the sporadic production of bulbils with proven genotypes may increase recruitment versus seeds with untested genetic variation. In highly bulbiferous species, successful initial recruitment may lead to exponential rates of dispersal. For example, both *Butomus umbellatus* and *Dioscorea oppositifolia* L. are colonizing North America primarily via bulbils (Kliber & Eckert, 2005), with the latter negatively impacting understory vegetation and biodiversity (Main et al., 2006).

The seasonality of bulbil production may also play a role in survival and fitness. Whereas populations of *Aconitum columbianum* ssp. *viviparum* form bulbils in all plants for the duration of the flowering season, *A. noveboracense* populations develop bulbils only in some plants at the end of the growing season, when a killing frost is imminent and time is running out for flower and seed development. *Butomus umbellatus* and *Titanotrichum oldhamii* also produce bulbils near the end of the growing season (Eckert et al., 2000; Wang et al., 2004a). Furthermore, increased bulbifery in *Polygonum viviparum* (Polygonaceae) may be an adaptive response to failed floral induction at colder, higher altitudes (Bauert, 1993).

Bulbils form only in some members within a population of *Aconitum noveboracense* and bulbil bearing and nonbulbil bearing plants grow next to each other indicating that in addition to environmental influences there is also genetic variation in the ability to form bulbils. Wang and Cronk (2003) noted that the phenotype of the *Antirrhinum majus* (Schrophulariaceae) double floral mutant (*squamosa* and *floricaula*) was similar to a transitional stage of bracteose inflorescences in *Titanotrichum oldhamii* that occurred after normal floral development but before bulbils replaced the inflorescences. They theorized that the ability to form bulbils may involve floral genes that had been shown to have analogous functions among different plant groups, including the ability to form bulbils.

The process of bulbil formation may be at least partially under hormonal control. For example, the growth of bulbils only in the mid to lower part of the plant may be due to the weakening of auxin's apical dominance. However this would only partly explain why many of the *Aconitum noveboracense* plants that were browsed by deer late in the

growing season were bulbil producing. Plants that were browsed by deer early in the growing season were able to sufficiently recover, i.e. produce flowers and seeds (unpublished data). Brink (1982) noted that broken or blown over *A. uncinatum* plants were the ones most likely to form bulbils.

Environmental cues such as light may also be a stimulus for hormonal activity. In the same way that low light conditions contribute to leaf senescence, *Aconitum noveboracense* produces bulbils in the autumn, when days are becoming shorter. Similarly, first year plants of *Titanotrichum oldhamii* growing in deep shade formed only bulbils (Wang & Cronk, 2003).

It is evident from the present study that a small percentage of *Aconitum noveboracense* produce aerial bulbils in the autumn. The bulbils may form in petiole and bract axils and do not replace inflorescences. Immature bulbils have scale leaves and compressed stems. The bulbils' vascular system appears to be contiguous with the parent and the bulbils often produce adventitious roots and photosynthesizing leaves while maintaining this connection.

Studying the details of the bulbil's role in *Aconitum noveboracense*'s life cycle would increase our understanding of the species' reproductive biology. For example, it is not known if the bulbil continues to grow when detached from the parent in the autumn or if it enters a dormancy period, either before or after separation. In addition, identifying and understanding the dynamic relationships among the genetic components, environmental influences, and stochastic factors (e.g., deer herbivory) involved in *A. noveboracense* bulbifery are areas for future research. At a wider focus, questions remain unanswered regarding the autumnal production of bulbils in other North American

Aconitum. Documenting the presence or absence of bulbils in *A. delphiniifolium*, *A. reclinatum*, and *A. maximum* would improve our knowledge and appreciation of *Aconitum*'s reproductive plasticity and persistence.

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Figures



FIG. 2-1. *Aconitum noveboracense* bulbils. **A.** Bulbils attached to parent. The main stem is in the background. Scale bar = 1 cm. (Photographed in New York State.) **B.** Bulbil with adventitious root, shoot, and empty scale leaf. Scale bar = 1 mm. **C.** Bulbils with leaves. White tail deer browsed the primary stem, the original axillary shoot, and one petiole. The upper bulbil has two long petioles (leaf blades not shown) and the lower bulbil has a leaf unfurling at the apex. Scale bar = 2 mm. Abbreviations: b, bulbil; p, petiole; r, adventitious root; s, stem; sl, scale leaf.



FIG. 2-2. *Aconitum noveboracense*, LM. **A.** Petiole. transverse view. Here, the base of the bulbil appears as a dark circle of cells in the upper left quadrant and the petiole is ventrally crescent shaped. Scale bar = 1 mm. **B.** Bulbil. Transverse view. The vascular plexus is the irregularly shaped structure in center. Scale bar = 1 mm. **C.** Bulbil. Longitudinal view. The bulbil is in the center and the petiole is just visible in the lower left of the image. Leaf primordia subtend the leaves at the apex and an axillary bud is on the right. The compressed stem subtends the meristematic region and the vascular plexus is interior to the compressed stem. Scale bar = 1 mm. Abbreviations: b, bulbil; cs, compressed stem; d, dorsal side; lp, leaf primordia; sl, scale leaf; v, ventral side; vp, vascular plexus.