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Influence of Rhizome Propagule Size on Yields and Triterpene Glycoside Concentrations of Black Cohosh [*Actaea racemosa* L. syn *Cimicifuga racemosa* (L.) Nuttal]

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Abstract. **Black cohosh [*Actaea racemosa* L. syn. *Cimicifuga racemosa* (L.) Nuttal] is a native North American medicinal plant traditionally harvested for its rhizomes and roots. Black cohosh products were listed in the top 10 selling herbal supplements from 2002 to 2005. As a result of increasing commercial demand, there is a need to develop propagation protocols suitable for production purposes to replace current methods of harvesting from wild populations. The objectives of this study were to 1) determine optimal rhizome propagule division size for successful regeneration, 2) analyze triterpene glycoside concentrations, 3) quantify survival rates after 3 years of production, and 4) evaluate net yield results. Experimental sites included a shade cloth structure in an agricultural research field, a shaded forest interior, and a shaded, disturbed forest edge. Plant emergence, growth, and survival were assessed at each site over a 3-year period. Optimal rhizome division size for propagation was a 10 to 30-g section originating from terminal rhizome portions. Rhizome survival averaged 97% among all treatments tested by year 3 at three sites. No differences in mean triterpene glycoside concentrations were detected between rhizome size classes or sites tested. Mean cimiracemoside concentrations ranged from 0.80 to 1.39 mg·g^{-1} d/w tissue, deoxyactein 0.47 to 0.92 mg·g^{-1}, and actein 10.41 to 13.69 mg·g^{-1}. No differences in triterpene levels were detected between flowering and nonflowering plants, nor were yields reduced. Net yields from a shade cloth production site were 9 and 17 times higher than a disturbed forest edge and forest site respectively. Black cohosh is a strong candidate for commercial propagation under adequate site selection.**

Actaea racemosa L. (black cohosh), formerly *Cimicifuga racemosa* (L.) Nutt., is a native North American plant of the Ranunculaceae family recently reorganized based on DNA sequence analyses (Compton and Culham, 1998; Hasegawa, 1993). With a well-established history of medicinal use, beginning with Native Americans, demand has continued to increase in international markets. Black cohosh products have consistently ranked in the top 10 selling herbal supplements from 2002 to 2005 (Blumenthal, 2003). The German government's safety regulatory board, the Commission E, has approved the efficacy and safety of black cohosh for specific symptoms associated with menopause, which is the primary focus of current demand (Blumenthal et al., 1998).

Rhizomes and roots are traditionally harvested in the fall and are standardized to various concentrations of three triterpene glycosides, actein, 23-epi-26-deoxyactein, and cimiracemoside A (Ganzera et al., 2000; Upton, 2002). The specific mode of action associated with their bioactivity has not been identified conclusively, although there have been numerous studies testing both triterpene glycosides and flavonoids (Kennelly et al., 2002; Upton, 2002). High intrapopulation variability of triterpene glycoside concentrations has been reported (Lata et al., 2002) as well as interpopulation variability (Al-Amier et al., 2005; Fabricant et al., 2001). Large variations in triterpene glycoside constituents among commercial products have also been reported along with adulterants from closely related species (Ganzera et al., 2000).

The majority of commercial black cohosh material available is "wild harvested" or collected from native eastern North American hardwood forests where it grows as an understory, shade-tolerant, hardy perennial. Because of increasing harvest pressures, black cohosh is listed among the top species of concern by both The Nature Conservancy and The United Plant Saver's lists of medicinal species at risk due to "wild collection" or "wild harvesting" (United Plant Savers, 1997; Nielsen, 2002, unpublished report). As a result of increasing commercial demand and high variability in triterpene glycosides reported, the overall objective of this study was to determine experimentally whether a successful rhizome propagation protocol could be established for future field or forest production purposes.

The study was conducted in the southeastern Appalachian Mountains within the native range of black cohosh. Specific objectives were to 1) determine optimal rhizome propagule division size for successful regeneration, 2) quantify survival rates after 3 years of production, 3) compare triterpene glycoside concentrations between sites and rhizome propagule size classes tested, and 4) evaluate net yield results.

Materials and Methods

All rhizome propagule material for the experiment was collected from a native population on U.S. Forest Service property in Graham County, N.C. A voucher specimen, along with site details and global positioning satellite coordinates are stored in the Clemson University herbarium. Two hundred twenty-five mature, nonflowering rhizomes were randomly collected and divided into three groups to be used for rhizome division experiments at three sites. Rhizomes with attached roots were washed thoroughly then excised into four treatment size classes: control, entire rhizome with roots; treatment 1, excised rhizome/root portions originating from terminal, actively growing rhizomes (10–30 g); treatment 2, excised portions with one to two buds originating from rhizome midsection (10–20 g), and treatment 3, excised

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portions with two to four buds originating from the rhizome midsection (20–30 g). Initial weights were recorded before planting. A total of 384 rhizome propagules were planted in 1999 within 1 week of harvesting. Field plots at each site consisted of raised beds prepared with a tractor mounded bed shaper using site soil on 152-cm centers with plants separated alternately 45 cm apart in rows. Beds were 15 to 20 cm high × 79 cm wide × 200 cm long per experimental unit. There were 16 experimental units per site with a 45-cm space between units. Experimental design was a randomized complete block with four replicates and four treatments; each experimental unit contained eight rhizome divisions or entire rhizomes.

Rhizome sections were planted 2.5 cm deep and were covered with a layer of mixed hardwood leaf mulch in both forested sites. A thick layer of partially decomposed hardwood bark/wood chips was used to mulch the shade cloth site. No soil amendments, fertilizer, or chemical controls were added to any test sites.

Site information. The original native population where propagule material was collected for all experiments is located on a north-facing steep slope in a mesic cove hardwood forest with a 90% shaded canopy (elevation, 1255 m; soil pH, 6.0). Site 1 was under a 78% shade, lath weave, polypropylene shade cloth structure located at the North Carolina State University Mountain Horticultural Crops Research Sta. in Fletcher, N.C. (elevation, 600 m; soil pH, 6.9). Shade cloth dimensions were 24.5 × 33 × 2.75 m. Overhead irrigation was used periodically.

Site 2 was a disturbed forest edge habitat in Graham County, N.C. (elevation, 665 m; soil pH, 5.2) located on a northwest-facing moderate-to-steep slope under a 90% shaded canopy with a history of disturbance.

Site 3 was located within 1.6 km of the original native population in a forested habitat with a history of timber management similar to the original population (elevation, 1037 m; soil pH, 4.2) on a southeast-facing moderate-to-steep slope under a 92% shaded canopy.

Data collected. Data collected annually for each individual plant included survival (presence/absence), foliage width (in centimeters), foliage height (in centimeters), stem number, three largest stem caliper measurements (stem diameter in centimeters), stem measurement to first node (in centimeters), and total number of seed capsules produced per plant. The stem measurement was measured from soil level to the first branching stem node. Data were recorded annually for the study duration. After three growing seasons, all rhizomes and roots were harvested from the three sites in October 2002. Individuals were washed with a pressure hose then dried to a constant weight in a forced air drier at 40 °C. Both fresh and dry weights were recorded with percent moisture calculated for 384 individuals from three sites.

After harvesting and recording data in year 3, 192 individuals (four individuals per replicate) were prepared for high-performance liquid chromatography with evaporative light scattering detector (HPLC/ELSD) analysis of triterpene glycosides. Individual dried rhizomes were ground by Wiley mill (1-mm mesh; Thomas Scientific, Swedesboro, N.J.) with 10-g samples prepared for extraction and HPLC analysis. An HPLC/ELSD method developed at the University of Mississippi (Ganzera et al., 2000) was used for analysis and quantification of the three primary triterpene glycosides: actein, 23-epi-26-deoxyactein, and cimiracemoside. These are the three marker compounds that phytopharmaceutical companies use for standardization of black cohosh products. Standard compounds used for HPLC analysis and quantification were isolated at the National Center for Natural Products Research (University of Mississippi, Oxford, Miss.). Triterpene glycosides were separated by reverse-phase HPLC (Waters; Milford, Mass.) using a Discovery C-18 column (150 × 4.6 mm, 5 µm; Supelco, Bellefonte, Pa.) and an ELSD (Sedex, France). The mobile phase consisted of water (A), acetonitrile (B), and reagent alcohol (C) using a gradient elution (58A/21B/21C to 52A/14B/34C) with flow rate adjusted to 1.0 $\text{mL}\cdot\text{min}^{-1}$. Deoxyactein, actein, and cimiracemoside were separated and quantified within 35 min. Specifics of extraction and analytical methodology including calibration of standards and peak purity have been published by Ganzera et al. (2000).

Three soil samples were collected per test site during the study duration measuring pH, cation exchange capacity (CEC), base saturation (%Ca, %Mg, %K), organic matter, and elemental analysis (P, K, Ca, Mg, Zn, Mn, Cu, B, Na). A spherical densiometer was used to calculate forest overstory density per site as a measure of percent cover by taking the mean of four recordings in late summer before leaf abscission in 2002.

All statistical analysis was performed using SAS software (version 8e; SAS Institute, Cary, N.C.). Overall differences in measured means among locations, years, and treatments used analysis of variance (ANOVA), which included a comparison of location, years, treatment, and replicate effects (using pairwise t tests, $\alpha = 0.05$). Analysis of triterpene glycosides consisted of testing site and treatment effects on mean cimiracemoside, actein, and deoxyactein concentrations using ANOVA followed by Fishers LSD, pairwise t test ($\alpha = 0.05$).

Results and Discussion

Vigorous emergence was observed at all sites in May 2000 after planting in October 1999, regardless of treatment. By year 3, survival rates averaged 97% across all treatments tested when rhizomes were harvested for analysis. Cech (2002) and Thomas et al. (2006) have also reported high emergence rates with black cohosh propagation by rhizome division, although Thomas et al. (2006) also reported high disease incidence. Thomas et al. (2006) tested the hypothesis that overwinter cold storage of fall-harvested rhizomes followed by spring planting would reduce incidence of root rot as opposed to fall planting methods. Their study reported more than 90% emergence after spring planting followed by more than 90% mortality resulting from *Phytophthora* root rot. Conversely, this study tested fall planting rhizomes in raised beds, which produced high survival rates after 3 years of growth.

A primary objective of the study was to determine optimal rhizome division size for developing a successful propagation protocol. Treatments tested were control, entire mature rhizome; treatment 1, terminal, actively growing portions of rhizomes (10–30 g); treatment 2, rhizome midsection with one to two buds (10–20 g); and treatment 3, rhizome midsection with two to four buds (20–30 g). All propagule treatments shared high survival rates, but when mean fresh harvest weights were compared for combined sites, the results were control > treatment 1 > treatment 3 > treatment 2 (Table 1). In addition, when net yields were calculated across all sites, trends indicated treatment 1 had higher overall net yields than treatments 2 and 3 (Table 2). Higher yields from treatment 1, originating from terminal sections of rhizomes, may be attributed to their apical regions of meristematic cell division. Rhizomes decay on distal portions while continuously regenerating meristematic tissue on terminal ends. Lateral meristems are secondarily produced by the apical meristems and produce lateral buds, nodes, and secondary shoots, from which treatments 2 and 3 originated. Midsections with two to four buds (20–30 g) produced higher net yields than smaller sections with one to two buds (10–20 g).

Table 1. Comparison of mean individual aboveground measurements combined across three sites at the end of the 3-year study.

Treatment	No. of stems	No. of seed caps	Plant height (cm)	Plant width (cm)	Mean stem diameter (cm)	Stem height (cm)	Fresh wt. harvested (g)
Control	2.4^{z}	103.0^{z}	65.5^{x}	96.6^{z}	0.73^{z}	29.8^{z}	213.8^{z}
1	1.7^{y}	66.0^{y}	51.3^{y}	80.1^{y}	0.67^{yz}	23.9^{y}	131.2^{y}
2	1.6^{y}	37.4^{x}	44.0^{x}	62.6^{x}	0.51^{x}	19.6^{x}	85.9^{w}
3	1.5^{y}	43.1^{x}	47.2^{x}	71.6^{y}	0.60^{y}	22.9^{y}	112.0^{x}

^{z,y,x,w}Vertical means within columns with different letters indicate significant differences based on t-test ($P \leq 0.05$).

Table 2. Net yield: total rhizome yield defined as initial fresh weight planted minus final fresh weight harvested in grams per treatment per plot (32 plants per treatment, 128 plants per site).

	Net yield (g)				
Site	Control	Treatment 1	Treatment 2	Treatment 3	Total wt.
Shade cloth	9268	7760	5575	6631	29234
Forest	–93	833	297	725	1762
Disturbed forest	–67	1489	871	879	3172

Treatments: control, entire rhizome; treatment 1, terminal section; treatment 2, midsection with one to two buds; treatment 3, midsection with three to four buds.

All rhizome sections tested will be suitable for production purposes, although the terminal sections (treatment 1) produced both highest net yields and mean fresh weights. In addition, when individual rhizome treatments were analyzed across all sites, treatment 1 produced more seed capsules, taller plants, larger stem diameter, and higher weight per plant than treatments 2 and 3 (Table 1).

The third objective was to analyze triterpene glycoside concentrations between treatments and sites. For all samples analyzed, mean cimiracemoside concentrations ranged from 0.80 to 1.39 mg·g^{-1}, deoxyactein from 0.47 to 0.92 mg·g^{-1}, and actein from 10.41 to 13.69 mg·g^{-1} dry weight (Table 3). There were no concentration differences between sites or treatments tested after three growing seasons, but because of net yields 9 to 17 times higher at the shade cloth site, triterpene glycoside concentrations were higher by weight. Mature control rhizomes did not bioaccumulate higher concentrations of triterpene glycosides in comparison with division treatments after 3 years of growth (Table 3).

Another objective of the study was to evaluate net yield results. Final fresh weights harvested were compared with initial weights planted to determine net yields after 3 years of growth. When summed across all treatments, trends indicated that rhizomes from the shade cloth site had highest net yields (29,234 g) when compared with the forest (1762 g) and disturbed forest (3172 g) sites (Table 2). When yields were analyzed across treatments to determine the fold increase of rhizomes harvested to rhizomes planted per site, the shade cloth site had an overall 9.1-fold increase, whereas the forest site had a 0.85-fold increase, and the disturbed forest a 1.6-fold increase. Mature control plants at both forested sites exhibited a decrease in both growth and net yield after 3 years, although no mortality was observed. Growth rates and net yield trends for mature control individuals at the shade cloth site were higher than both forested sites. A combination of factors may be responsible for the large differences in variables tested between the forest sites and the shade cloth site. One may be attributed to higher light conditions at the shade cloth site (78% shade) as opposed to the forest sites (90%, 92% shade). Marino et al. (1997) found another shade-tolerant understory herb, bloodroot (*Sanguinaria canadensis* L.*)*, had increased growth rates under higher light conditions than lower light. In addition, the shade cloth site had optimal soil pH (6.9), CEC 11.4, and less weed pressure resulting from heavy hardwood bark mulch. In contrast, the forest site soil pH was 5.2 and CEC 10.8 with moderate weed pressure, while the disturbed forest edge site pH 5.2 and CEC 7.7 with severe weed pressure recorded (Table 4). Both forest sites had acidic soil properties with low Ca levels, characteristic of typical woodland soils (Beyfus, 2000), whereas the shade cloth site soil was neutral, with Ca levels six to eight times higher than both forest sites (Table 4). Both soil pH and cation exchange capacity at the shade cloth site were more ideally suited for optimal growth as opposed to comparable parameters from the forest site soils (Whipker, 2000). Magnesium to potassium ratios were 3:1 at the shadecloth site and 2:1 at both forest sites. Optimal ratios are reported to be greater than 2:1, thus neither forested site provided optimal conditions (Table 4). High K levels are associated with reduced uptake of Mg; thus, the addition of Mg via dolomite lime may be beneficial in forest settings.

Flowering trends were higher at the agricultural shade cloth site (73%) when compared with both forest sites (7%, 4%) after 3 years (Fig. 1). In a phenology and pollination biology study of five other Ranunculaceae species, Steinbach and Gottsberger (1995) found pollinator visitation "habitat specific" as opposed to "species specific," with higher pollinator visitation observed in agricultural plots as opposed to forested sites. Steinbach and Gottsberger (1995) also found higher

Table 3. Mean concentrations of cimiracemoside, actein, and deoxyactein from rhizomes of *A. racemosa* (black cohosh).

		Concentrations (mg·g^{-1} dry wt)		
Site	Treatment	Cimiracemoside	Deoxyactein	Actein
Disturbed Forest	Control	1.39	0.89	13.69
	1	0.96	0.67	11.39
	2	1.12	0.87	13.58
	3	1.10	0.77	12.63
		NS	NS	NS
Shade cloth	Control	0.93	0.74	11.96
	1	1.14	0.92	14.10
	2	0.89	0.78	13.34
	3	0.74	0.73	11.99
		NS	NS	NS
Forest	Control	1.33	0.76	12.99
	1	1.07	0.52	11.33
	2	0.80	0.48	10.41
	3	0.84	0.47	11.52
		NS	NS	NS

Within individual sites and treatments (n = 16), means were significantly different at $P \leq 0.05$.
^{NS}Nonsignificant.

Table 4. Soil sample data for three field sites.

			Base saturations (%)				
	pH	CEC (Meq/100 g)	Ca	Mg	K	Total base saturation (%)	Organic matter (%)
Part 1: Site							
Shade cloth	6.9	11.4	74.7	7.3	2.3	84.3	8.6
Forest	4.2	10.8	14	2.3	1.3	17.6	12.7
Disturbed forest	5.2	7.7	36.0	6.7	3.0	45.7	5.7

	P	K	Ca	Mg	Zn	Mn	Cu	B	Na
Part 2: Elemental Analysis (kg·ha^{-1})									
Shade cloth	28	231.0	3818	229	10.9	92.3	0.45	1.3	19.1
Forest	18.7	123	686.4	67.6	5.8	83.3	1.0	0.8	12.4
Disturbed forest	8.6	186	454.8	133.4	2.0	32.2	0.9	0.7	9.3

Data presented as mean of three samples per site (Clemson Agricultural Service Laboratory, Clemson, S.C.).

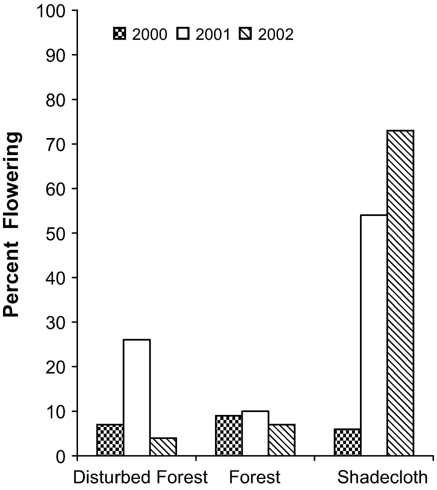

Fig. 1. Annual *A. racemosa* (black cohosh) flowering trends observed at three sites (shade cloth, disturbed forest and forest). Data are presented as total percent flowering individuals per site from 2000 to 2002.

Table 5. Comparison of mean individual seed capsule production at three sites for 3 years (1999–2001).

Year	Treatment	Disturbed forest	Shade cloth	Forest
1	Control	2.5^z	43.2^z	2.2^z
1	1	0^z	0^y	0^z
1	2	0^z	0^y	0^z
1	3	0^z	0^y	0^z
2	Control	92.9^z	401.8^z	15.8^z
2	1	2.4^y	260.9^y	0^y
2	2	2.8^y	117^x	0^y
2	3	2.1^y	100^x	0^y
3	Control	5.6^z	317.4^z	2.3^z
3	1	14.5^z	309.2^z	0^z
3	2	0^z	245.4^z	0^z
3	3	0^z	312.4^z	0^z

^{z,y,x}Separation within columns between years at $P \leq 0.05$.

flower and fruit production in open agricultural sites as a result of longer flowering periods. In our study, flowering trends in the disturbed forest site were inconsistent between years, producing more flowers in year 2 than years 1 and 3 (Fig. 1). Similar results were observed by Cook (1993) in a forest demographic study with *Cimicifuga rubifolia* (syn. *Actaea rubifolia*), in which large fluctuations in annual flowering between individuals were observed. In year 1 (2000) only mature control rhizomes produced flowers and seed at all sites (Table 5) and regenerants from rhizome divisions did not produce seed until year 2. Seed production rates at the shade cloth site were higher with mature control plants in the first 2 years of the study when compared with rhizome division treatments; but, by year 3, there were no differences in seed production between treatments, including the control. Individuals from the shade cloth site averaged 296 capsules per plant with each capsule containing 8 to 10 seeds. In contrast, both forest sites averaged less than five capsules per plant when capsule production was averaged across treatments in year 3 (Table 5). When treatment means were combined across all three sites tested, treatment 1 produced more seed capsules per plant (66.0) than treatments 2 and 3 respectively (37 seed capsules per plant and 43 seed capsules per plant) (Table 1).

Available light may be a contributing factor to higher seed production at the shade cloth site as plants were grown under 78% shade whereas the forested sites had a 90% to 92% shaded canopy. In a study comparing the influence of sunlight on growth in another understory perennial, bloodroot (*Sanguinaria canadensis*), Marino et al. (1997) found greater seed production in a high-light test site when compared with a low-light site with no sexual reproduction observed under low-light conditions. In the current study, the shade cloth site produced seed yields 53 times higher than the forest sites and rhizome yields 9 to 17 times higher.

A valid concern with rhizome production strategy is whether flowering and capsule production have negative impacts on rhizome yields or chemical concentrations. No differences in net yields or triterpene glycoside accumulation were found between flowering and nonflowering individuals after 3 years. If seed production does not affect rhizome productivity, growers could potentially harvest a seed crop in year 2 followed by a rhizome harvest in year 3 under shade cloth production.

In conclusion, robust rhizome propagation of black cohosh was observed without soil amendments or fertilizer addition. All rhizome size classes tested produced good survival rates, but propagules originating from terminal meristematic portions of the rhizome produced higher net yields, higher seed numbers, and both higher height and width. Of three sites tested, an agricultural shade cloth site produced net rhizome yields 9 to 17 times higher and net seed yields 53 times higher than two forest sites. When rhizomes were analyzed for triterpene glycoside concentrations, there were no differences between sites or treatments tested, but because net yields were 9 to 17 times higher by weight, so were associated triterpene glycosides. Future spacing and soil amendment studies are necessary to optimize yields. Future selection studies are also necessary to establish superior genotypes. In conclusion, *Actaea racemosa* is a strong candidate for commercial propagation under adequate site selection.

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