Phytochemistry Letters 42 (2021) 61–67

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Benzylisoquinoline alkaloid content in goldenseal (*Hydrastis canadensis* L.) is influenced by phenological stage, reproductive status, and time-of-day

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ARTICLE INFO

Keywords:
Alkaloids
Benzylisoquinoline alkaloids
Berberine
Canadine
Harvest timing
Hydrastine

ABSTRACT

Goldenseal (*Hydrastis canadensis*, Ranunculaceae/Hydrastidaceae) is a popular Appalachian medicinal forest plant whose roots and rhizomes are mostly collected from the wild for commerce. The benzylisoquinoline alkaloids (BIA) berberine, canadine, and hydrastine are believed to be primarily responsible for the bioactivity of goldenseal. To provide a consistent, quality-focused product to consumers, a better understanding of factors that influence major BIA levels is needed. We examined BIA content in relation to phenological stage, reproductive status (i.e., reproductive versus vegetative morphology), and time-of-day in wild and forest-farmed goldenseal collected in Pennsylvania, U.S.A. High performance liquid chromatography (HPLC) analysis revealed that phenological stage and reproductive status influences the BIA concentration in both dried belowground (roots, rhizomes) and aerial portions (leaves, stems). BIA levels were found to be higher in the belowground parts compared to aerial. Moreover, BIA concentrations peaked in both plant aerial and belowground portions at the flowering stage and in the belowground parts at dormancy, suggesting that an early season harvest of aerial tops could be explored in farmed populations in addition to traditional late season "root" harvests. Additionally, hydrastine and canadine levels were found to be greatest in aerial portions at 1600 h over a 24 -h range, which suggests late day as the best time for aerial harvests. Overall, these results provide guidance for optimizing alkaloid content in goldenseal harvests and contributes to the broader understanding of secondary metabolite in relation to plant phenological stage.

1. Introduction

Goldenseal (*Hydrastis canadensis* L., Ranunculaceae/Hydrastidaceae, Fig. 1) is a well-known perennial, herbaceous, medicinal forest plant indigenous to eastern North American forests. The roots and rhizome of this species are used in herbal medicine to treat inflammation and digestive disorders and have documented antimicrobial properties (Braun and Cohen, 2010; Scazzocchio et al., 2001).

The medicinal properties of goldenseal are believed to be primarily attributable to the presence of the three benzylisoquinoline alkaloids (BIA, Fig. 1) berberine, hydrastine, and canadine (Brown et al., 2008; Mahady and Chadwick, 2001; Weber et al., 2001; Scazzocchio et al., 2001), although Leyte-Lugo et al. (2017) also identified additional bioactive secondary metabolites in the leaves. The United States Pharmacopeia, an industry advisory group, has established minimum quality control standards for goldenseal roots and rhizomes, which state that berberine content and hydrastine content should be no less than 2.5% and 2.0% (w/w calculated on a dry weight basis), respectively (United States Pharmacopeia, 2021). However, these minimums are often not met, with reports on BIA content (w/w) ranging from 0.5 to 6.0% berberine and 1.5–4.0% hydrastine, and total alkaloid content of 2.5–6.0% (Burkhart and Zuiderveen, 2019; Upton, 2001). In a study of goldenseal root powder, hydrastine content was found to vary from 1.4% to 2.7% depending on the supplier (Weber et al., 2003).

Little is known about production factors that may influence BIA levels in goldenseal. This is important because available industry data suggests most of the goldenseal in commerce in the U.S. is sourced from the wild (AHPA, 2012). Kruger et al. (2020a; 2020b) found that

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https://doi.org/10.1016/j.phytol.2021.02.006
Received 1 December 2020; Received in revised form 7 February 2021; Accepted 11 February 2021

goldenseal was the third most wild-harvested Appalachian medicinal forest plant in the eastern U.S. and comprised 21% of buyer "off-root" trade volume (totaling 48,230 kg in 2014–2015). In a dealer survey within the native range of goldenseal, over 70% purchased only wild harvested goldenseal, and 25% reported that they purchased wild goldenseal whenever it was available (Zuiderveen, 2019).

Harvest timing is known to influence secondary metabolites in other plant species and has been identified as an area of goldenseal research requiring further investigation (Upton, 2001). Historic recommendations advise a summer harvest, with a spring harvest considered inferior due to high moisture content (Lloyd and Lloyd, 1884). Some buyers still avoid purchasing roots and rhizomes in the spring or early summer months (Zuiderveen, 2019). Current harvest guidance suggests rhizomes should be harvested during autumn through early winter, after the plants have senesced (AHPA, 2017; Burkhart and Zuiderveen, 2019; Davis and Persons, 2014; Upton, 2001); however, limited research suggests that alkaloids may be at their lowest concentrations during autumn (Gillis and Langenhan, 1931). In a study of goldenseal grown under artificial shade outside of its native range, researchers found seasonal variation in hydrastine, but not berberine (Douglas et al., 2010).

Additionally, beyond stage-based variation in BIA content, there is no information available on the variation in BIAs that may occur in response to time-of-day. One hypothesis is that secondary compounds, such as alkaloids, are in a state of dynamic equilibrium (Seigler and Price, 1976), and in other plant species (e.g. *Conium maculatum* and *Papaver somniferum*), secondary compounds have been found to fluctuate in a cyclic daily pattern (Fairbairn and Suwal, 1961; Fairbairn and Wassel, 1964; Figueiredo et al., 2008).

The present study examined the berberine, hydrastine, and canadine content in goldenseal roots/rhizomes (i.e., belowground portions) and shoots/leaves (i.e., aerial portions), in 10 geographically distant wild and forest farmed goldenseal populations within Pennsylvania (PA), U.S.A. Additionally, we examined the influence of reproductive versus vegetative material at a single central PA site and also collected samples every 4 h over a three-day period at this site to examine any influence of time of day on alkaloid content. Identification of how these harvest timing factors may influence BIA concentrations can be used to inform phytochemical quality in wild and farmed goldenseal along with our general understanding of secondary metabolite production in plants.

2. Results

2.1. HPLC analysis of BIA

Hydrastine, canadine, and berberine had retention times of 9 min, 14.5 min, and 16 min, respectively, in our HPLC method (Fig. 2). Hydrastine and canadine had similar HPLC response, while the response of berberine was approximately five times larger (Fig. 2A). The BIA peaks in goldenseal belowground (Fig. 2A) and aerial samples were clearly resolved by our gradient conditions.

2.2. Changes in BIA and dry weight with phenological stage

Results from ANOVA across 10 populations sampled at six harvest stages showed that BIA were highest ($p < 0.01$) at flowering and then fluctuated at lower levels with a slight upward trend appearing between senescence and dormant samples (Fig. 2B). In the belowground samples, BIA were 50% higher at flowering than at senescence. Berberine and hydrastine were highest at flowering and exhibited a general decline in concentration from flowering to senescence, with a slight increase again at dormancy. The trend for canadine in the belowground samples was

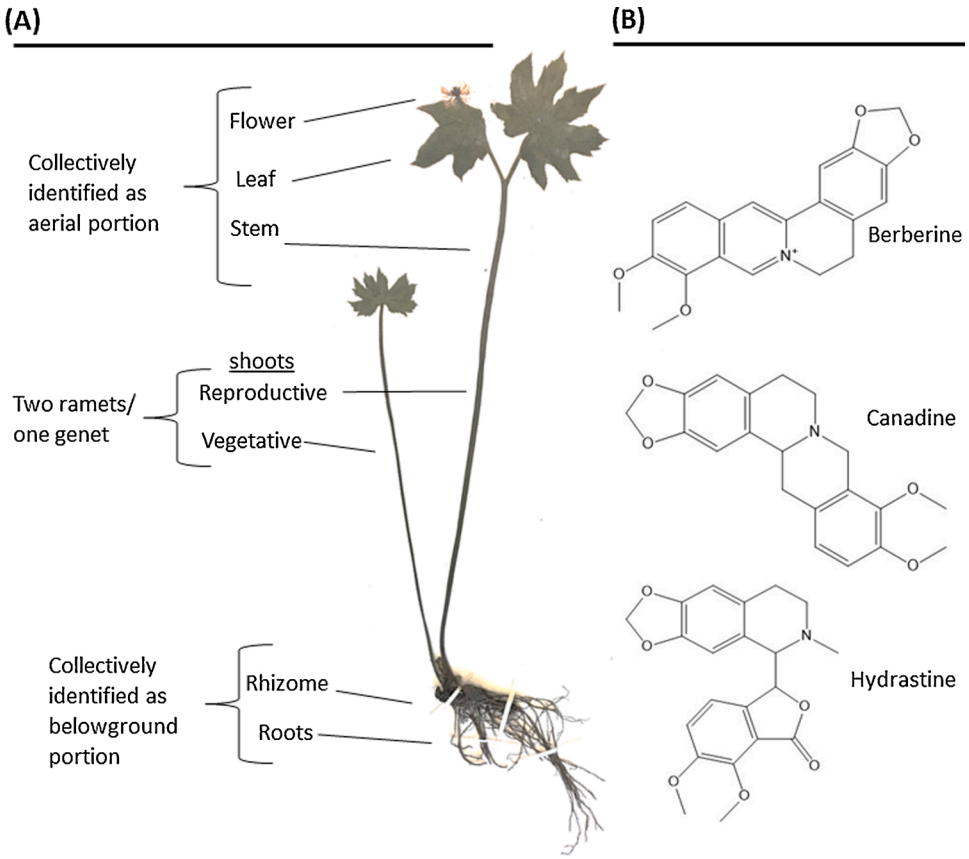


Fig. 1. Goldenseal (*Hydrastis canadensis* L.) and its major benzylisoquinoline alkaloids (BIA). (A) A dried goldenseal specimen with features identified in the text labeled. (B) Structures of the major goldenseal BIA: berberine, canadine, and hydrastine.

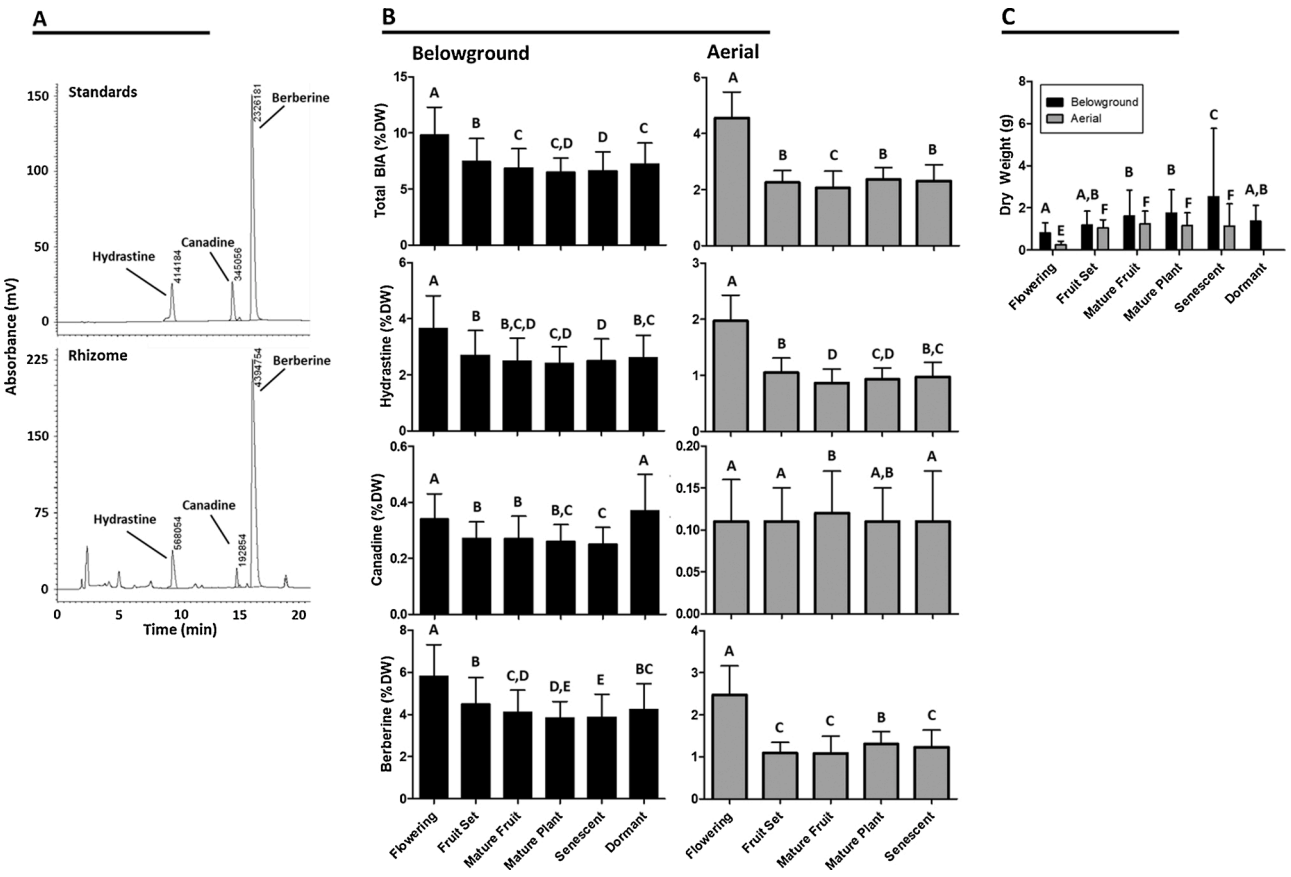


Fig. 2. The effect of phenological stage on the BIA content and dry weight of belowground portions of goldenseal. (A) Representative of HPLC−DAD chromatograms of BIA standards, belowground portion extract. (B) Changes in total BIA, berberine, canadine, and hydrastine were determined at different phenological stages in the belowground portion (left column) and the aerial portion (right column) across 10 sites in PA (n=111). (C) The effect of phenological stage on the dry weight of the belowground and aerial portions were examined at 10 sites in PA (n=111). Bars with different superscript letters are significantly different ($p < 0.05$).

similar but the levels at flowering and dormancy were not significantly different.

In the aerial samples, the concentration of each BIA was roughly half that of what was found in the belowground (Fig. 2B). The concentration of total BIA, berberine, and hydrastine were highest at flowering, and lowest at mature fruit, with more than a 50% difference in BIA levels between the two harvest stages. By contrast, the concentration of canadine was 30% higher at the mature fruit stage than at flowering.

Results from ANOVA across 10 populations sampled at six harvest stages showed that in belowground portions, dry weight increased by over 100% from flowering to the mature plant stage. Belowground dry weight did not significantly change from mature plant stage to senescence but decreased after plant senescence stage. Aerial portion dry weight increased by 360% from between flowering and the mature fruit stage, where it reached its greatest weight. After the mature fruit stage, the weight decreased by 24% by senescence (Fig. 2C).

2.3. Changes in BIA with time of day

Within the population sampled at six diurnal harvest stages, no statistically significant effect of time-of-day was observed on any of the BIA measured in the belowground portions. In the aerial portions, total BIA, hydrastine, and canadine concentrations increased with time of day: maximal levels were observed at 1600 h and minimal levels were observed at 0000 h. No time-dependent changes were observed for berberine concentration in the aerial portions (Fig. 3).

2.4. Changes in BIA content and dry weight with plant reproductive status

In four colonies, the effect of plant reproductive status (i.e., reproductive vs. vegetative morphology)) on BIA content was determined (Fig. 4A). In the belowground portion, the concentration of all three BIA examined were significantly greater in reproductive samples compared to the vegetative samples, with the sum of the three BIA being 12.1% higher on average in reproductive samples compared to vegetative samples. In the aerial portion, total BIA was 6.3% higher in the reproductive samples compared to the vegetative samples. The dry weights of both the belowground and aerial portions were nearly 50% higher on average for reproductive samples compared to vegetative samples (Fig. 4B).

3. Discussion

3.1. Implications for goldenseal phytochemical quality

The objective of our study was to determine the relative influences of phenological stage, reproductive status (i.e., reproductive vs. vegetative morphology), and time of day on BIA concentrations in belowground and aerial portions of goldenseal. Historic references such as Lloyd and Lloyd (1884) suggested that harvesting in spring resulted in more shrinkage during drying due to a higher moisture content and regarded these as inferior roots. Modern industry guidance is that goldenseal rhizomes should be harvested in the fall at senescence (AHPA, 2017; Upton, 2001). Wild harvesters, however, gather goldenseal throughout the summer months in response to buyer solicitation and opportunistic encounters with the plant in the wild (Zuiderveen, 2019). In cultivated

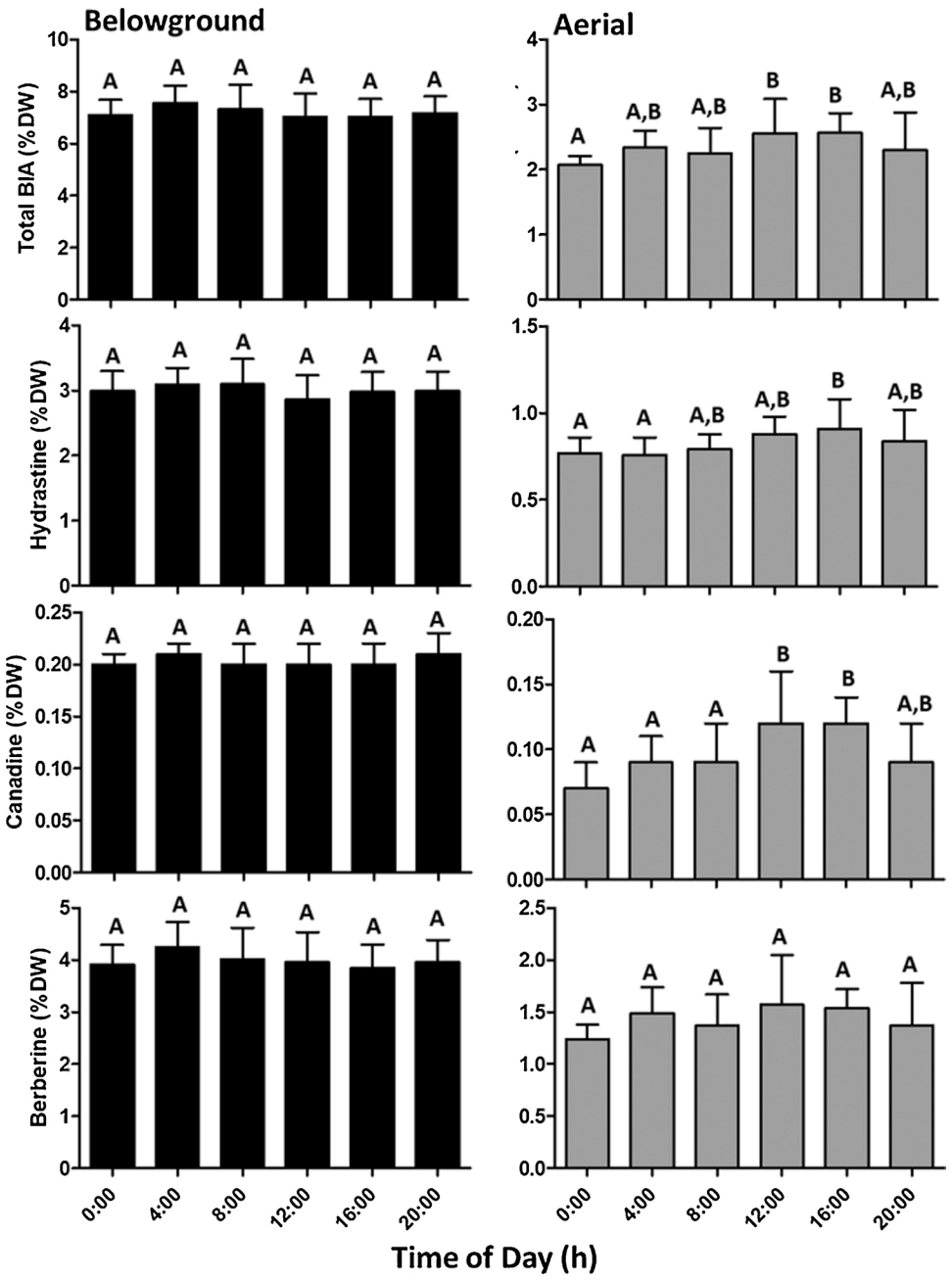
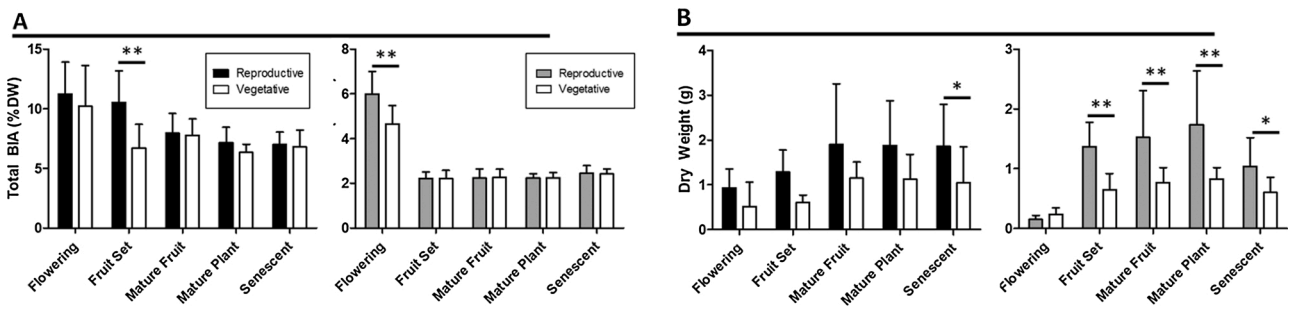


Fig. 3. The diurnal influence on the total BIA, berberine, canadine, and hydrastine in the belowground (left column) and aerial portion (right column) of goldenseal (n=15). Bars with different superscript letters are significantly different ($p < 0.05$).

plants, Douglas et al. (2010) found that concentrations of hydrastine in the belowground portions were higher during early summer when compared to autumn, but no difference when compared with late summer or winter. Further, they found no statistical difference in berberine concentrations during the different seasons.

In this study, we found that reproductive specimens, collected at flowering had the greatest BIA content in belowground as well as aerial portions, with the belowground portions having significantly higher BIA levels. These results build upon our previously published findings from a single site, using only reproductive samples collected during late season stages (mature fruit through senescent), where we found BIA content peaked at mature fruit and senescent stages (Burkhart and Zuiderveen, 2019). While early season peaks were surprising, our results are consistent with others examining the influence of key phenological stage on alkaloid levels in other taxa. In bloodroot (*Sanguinaria canadensis* L., Papaveraceae), for example, Bennett et al. (1990) found alkaloid concentration peaked during early fruit development and gradually declined through fruit maturation. Campbell et al. (2007) similarly found that the BIA sanguinarine in bloodroot rhizome was greatest at flowering. And finally, a study of the Chinese wild-harvested medicinal herb chuan bei mu (*Fritillaria cirrhosa* D. Don, Liliaceae) found that alkaloid content was greatest during early fruit maturation, providing support for harvesting during early stages of senescence (Konchar et al., 2011). Overall, our results provide support for the idea that medicinal plant harvests should be timed to coincide with key phenological stages to maximize constituent levels, and not necessarily with a season *per se*. However, this needs to be balanced by both yield and conservation considerations.

In production scenarios where reproduction is not a primary concern (e.g., forest farmed or cultivated production), our results suggest that



Factors affecting BIA (Two-Way ANOVA)	Belowground	Aerial
Morphology	p < 0.01	p < 0.01
Stage	p < 0.01	p < 0.01
Interaction	p < 0.01	p < 0.01

Factors affecting Dry Weight (Two-Way ANOVA)	Belowground	Aerial
Morphology	p < 0.01	p < 0.01
Stage	p < 0.01	p < 0.01
Interaction	p > 0.05	p < 0.01

Fig. 4. The effect of plant reproductive status (i.e., reproductive vs. vegetative morphology) on the (A) BIA content and (B) biomass yield of belowground and aerial portions of goldenseal (n=60). Bars with different superscript letters are significantly different as determined by two−way ANOVA with Bonferroni post−test.

harvesting the rhizome in the spring during flowering would result in the highest alkaloid content. However, in wild harvested populations, other factors must be considered, such as waiting until flowering and fruiting has occurred, to promote conservation of the species and populations. In wild harvested populations, a late season harvest (i.e., dormant stage) would be most appropriate as it would allow for sexual reproduction, and is associated perhaps with more rapid recovery (Albrecht and McCarthy, 2006). The harvest of goldenseal aerial parts should be further explored as a new or novel product since shoots contain all three major BIAs, albeit at a lower concentration than in the rhizomes, and there is already an established trade in wild "tops" in U.S. goldenseal supply chains (Burkhart, pers. obs.). Additionally, Leyte--Lugo et al. (2017) have identified additional bioactive secondary metabolites in the leaves worthy of further research. However, research is needed to evaluate the impact of aerial "top" (i.e., ramet) removals on the regrowth and recovery of the genet.

It has been suspected or documented that in addition to optimal seasonal/phenological stages, there are optimal times of day, for plant harvest. Many examples exist, including in *Papaver somniferum* and *Conium maculatum*, where changes in plant alkaloid levels over the course of the day/night have been observed (Fairbairn and Suwal, 1961; Fairbairn and Wassel, 1964; Itenov et al., 1999). The results of our study indicate that levels of total BIA, canadine, and hydrastine fluctuate in goldenseal aerial but not belowground portions over the course of the day. While the underlying mechanism for these changes remains unclear, these results support an optimal time of day to harvest goldenseal aerial portions; hydrastine and canadine levels were found to be greatest in aerial portions at 1600 h, which suggests late day as the best time for aerial harvests.

4. Experimental

4.1. General experimental procedures

Berberine hydrochloride [purity > 98%] and canadine (tetrahydroberberine) [purity > 98%] were purchased from Quality Phytochemicals LLC (East Brunswick, NJ, USA). Hydrastine [purity > 99%] was purchased from ChromaDex (Irvine, CA, USA). All other solvents and chemicals used in this study were of the highest grade commercially available.

4.2. Plant material

Goldenseal is a clonal, colonial plant that forms often inextricable subterranean networks over time. These networks, when spatially discernable, are commonly referred to as colonies or "patches." A colony or patch can consist of hundreds to thousands of ramets, and the number of genets is difficult to discern with time. In this study, a plot was placed in a spatially defined colony that appeared to be from a single genet. At each of the 10 locations used for the phenology study, plots were placed in three or four colonies per site, based on the overall number of colonies within a population. Harvested ramets were either reproductive (bearing fruit, possessing 2 leaves), or vegetative (no fruit, possessing a single leaf).

A total of 10 field sites were included in this study. All habitats were characterized as rich, mesic, woodland sites. The most common overstory tree associates across sampled sites were tulip-poplar (*Liriodendron tulipifera* L.) and sugar maple (*Acer saccharum* Marshall), while common understory associates included spicebush (*Lindera benzoin* L.), Jack-in-the-pulpit (*Arisaema triphyllum* (L.) Schott), mayapple (*Podophyllum peltatum* L.), and rattlesnake fern (*Botrypus virginianus* (L.) Michx.). Soils were most commonly loams (average sand, silt, and clay ratio of 50:30:20) with an average soil chemistry of pH 6.2.

Goldenseal samples at each phenological stage were harvested from 10 locations across southern PA (Fig. 5). Each location was sampled monthly between April and October, corresponding with six distinct observable, phenological stages of the plant: flowering, fruit set, mature fruit, mature plant (post-fruit), senescence, dormant (Fig. 5). Three reproductive samples were harvested from each plot at each phenological stage at 9 of the locations. At the remaining location in central PA, 3 reproductive and 3 vegetative samples were collected from each plot at each phenological stage for a comparison of reproductive and vegetative status (i.e., morphology). At the same location, five samples were collected every 4 h beginning at midnight (for a total of six times during a 24 h period), on three 24 h cycles during the mature (i.e., post-fruit, non-senescent) plant stage for examination of diurnal effects. These samples were used to investigate the influence of reproductive status and diurnal variability and were not included in the analysis of the influence of phenological stage.

The belowground portion of goldenseal consists of a horizontal rhizome (i.e., a modified stem used for carbohydrate storage) with multiple fibrous rootlets extending from the rhizome. In this study, roots and rhizomes were not separated but instead were processed and analyzed collectively and referred to collectively as belowground portions in this manuscript. We did this because industry does not differentiate between roots and rhizomes, and this study sought to apply commercial methods. Similarly, the aboveground aerial portions—stems, leaves, and fruit—were not separated when prepared and analyzed (Fig. 1). Voucher specimens were collected, digitized, and deposited at the Pennsylvania State University Herbarium (PAC), the

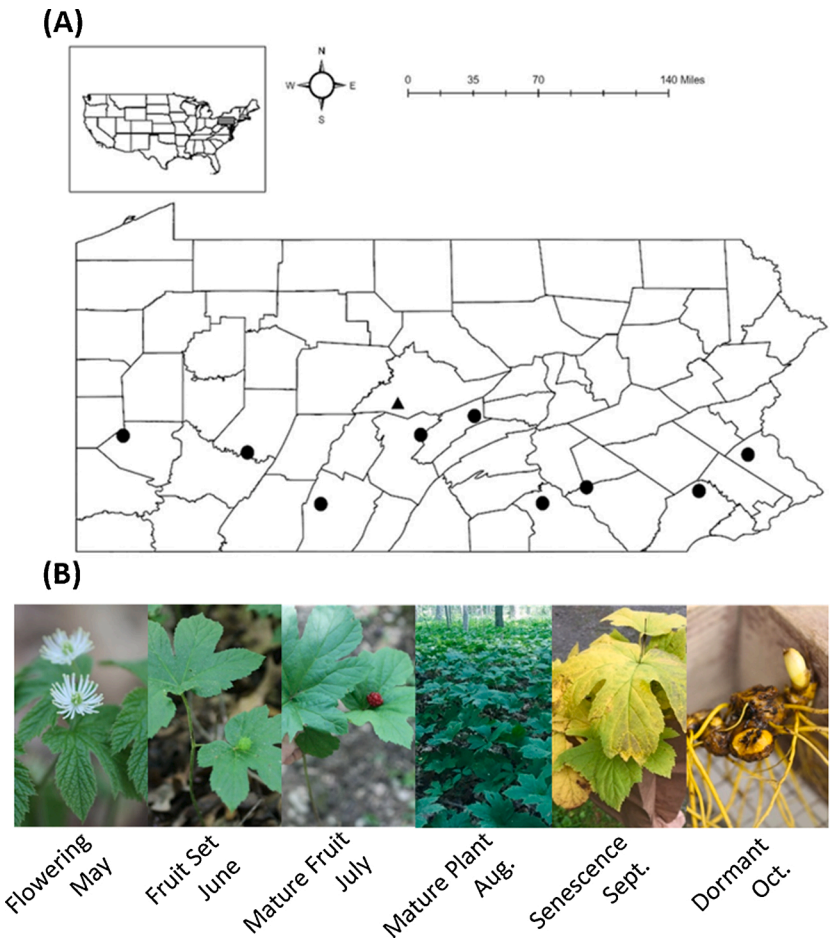


Fig. 5. Collection sites and phenological stages of goldenseal. (A) Collection sites in Pennsylvania for goldenseal samples used in the present study. All sites were included in the phenological stage study, and the triangle represents the site that was also used to examine diurnal influence and plant reproductive status (i.e., reproductive vs. vegetative morphology). All locations points are approximate to not reveal the exact location of the goldenseal populations out of conservation concerns. (B) Representative images show the six phenological stages and the approximate corresponding months when samples were collected.

Carnegie Museum of Natural History Herbarium (CM), and the Morris Arboretum of the University of Pennsylvania Herbarium (MOAR).

4.3. Post-harvest processing

All belowground samples were cleaned by hand on a screen under running water to remove any soil. The belowground and aerial portions were dried at 37.8 °C (100 °F) in a Lindberg/Blue M 260 Mechanical Oven (Model number: MO1490C-1; Thermo Scientific, Asheville, NC, USA) with an air flow rate of 25.5 L/min and specific humidity of 8.9 g H_2O/kg air beginning the day of harvest as described by Zuiderveen et al., 2021. Samples were dried until rhizome mass was 30% of fresh mass, and the rhizomes could be cleanly broken (approximately 40 h). Both fresh and dry weight of belowground and aerial portions were measured and recorded to investigate the influence of phenological stage on plant weight. Samples were then stored at 4 °C in the dark until chemical analysis.

4.4. Sample extraction

Samples were ground using a Thomas Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) with a 2 mm screen. Ground samples were combined with 30% aqueous acetonitrile containing 0.1% phosphoric acid at a ratio of 1:80, w/v. Tubes were vortexed to mix, sonicated for 10 min at room temperature, and centrifuged for 8 min at 3220 x *g*. The supernatant from rhizome samples, but not shoot samples, was diluted 1:4 with 10% aqueous acetonitrile. All samples were filtered through a 0.2 μm PTFE syringe filter prior to HPLC analysis.

4.5. HPLC analysis

HPLC analysis was based on the method developed by Weber et al. (2001) and performed using a Shimadzu HPLC system (Shimadzu Co., Columbia, MD, USA) consisting of 2 LC-20AD pumps, a SIL-20AC HT refrigerated auto injector, a column oven maintained at 24 °C, and an SPD-20AV UV/Vis detector. A binary gradient of water containing 0.1% formic acid (solvent A) and methanol containing 0.1% formic acid (solvent B) with a total flow rate of 0.75 mL/min was used. The initial mobile phase was 20% B. The concentration of B increased linearly for 15 min to 45%, and was held at this concentration for 5 min. At 21 min, the mobile phase was returned to 20% B and the HPLC was re-equilibrated for 7 min. Analytes were separated using an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 3.5 μm particle size, 80 Å pore size, Santa Clara, CA, USA). Eluent was monitored at 280 nm.

4.6. Statistical analysis

All statistical analyses were completed in R-studio (RStudio Team, 2015). Analyses of variance were conducted for both belowground and aerial samples between phenological stages, time-of-day, and plant type. Differences between individual phenological stages and time of day were analyzed using Tukey's honest significant difference (HSD) at $\alpha = 0.05$. Differences between plant type were examined using Bonferroni post-test with plant type and phenological stage considered independent factors. Both main effects and interactions between factors were analyzed at $\alpha = 0.05$.

5. Conclusion

Our results indicate that belowground and aerial portions of goldenseal contain the greatest concentrations of BIAs at the flowering phenological stage, and in reproductive (versus vegetative) samples. Additionally, aerial portions contain greater amounts of BIAs when harvested in the late afternoon (e.g., 1600 h). Therefore, phytochemical quality in goldenseal might be better controlled or improved by considering these related influences in wild harvesting, forest farming, and in cultivation. Additionally, aerial portions of goldenseal could provide an alternative or supplemental product option to the rhizomes in forest farmed and cultivated goldenseal; however, additional research is needed to determine the effects of aerial harvests on the recovery and productivity of the wild population or farmed crop.

Funding

This research was supported by Wild Resources Conservation Program research grant 16558 (to EPB), administered by the Pennsylvania DCNR, and by USDA Hatch Project PEN 4565 and the Azzara Family Food Bioactives Research Endowment (to JDL). The funding sources had no involvement in the design, analysis, or writing of this manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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