

Chapter 2

Biosynthesis of Natural Products in Plants by Fungal Endophytes with an Emphasis on Swainsonine

Daniel Cook, Dale R. Gardner, James A. Pfister and Daniel Grum

Abstract Plant natural products are frequently used as chemotaxonomic markers which are indicative of select members of a family, genus, and/or species. However, the erratic occurrence of some natural products raises questions about their biosynthetic origin and significance as chemotaxonomic markers. Recent research has shown that fungal endophytes associated with plants are a rich source of natural products. The objective of this review is to highlight natural products found in plants that are reported to be derived from fungal endophytes and, when appropriate, briefly comment on the plant-endophyte interaction. We will summarize current knowledge on alkaloids synthesized by Clavicipitaceae endophytes, then on other diverse secondary metabolites including taxol and camptothecin. Specifically, we will highlight the role that fungal endophytes play in the synthesis of the indolizidine alkaloid swainsonine and the interaction between host and endophyte.

Keywords Ergot alkaloids · Swainsonine · Taxol · Endophytes · Clavicipitaceae · Undifilum

2.1 Introduction

Plant natural products are frequently used as chemotaxonomic markers which are indicative of select members of a family, genus, and/or species. However, the erratic occurrence of some natural products raises questions about their biosynthetic origin and significance as chemotaxonomic markers. Four mechanisms could explain the sporadic occurrence of natural products in unrelated taxa [1]: First, plant taxa may have lost their ability to produce the natural product; second, the biosynthetic pathways of a natural product may have originated multiple times over evolutionary history; third, the genes responsible for the biosynthesis of a natural product may have

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been horizontally transferred between unrelated taxa [2]; or fourth, the natural product may be produced by a microbe associated with several, unrelated plant species.

Fungal endophytes are microbes that live within plants for part of their life cycle without causing any apparent disease symptoms [3]. Endophytes differ in their host range, tissues colonized, *in planta* biodiversity, *in planta* colonization, mode of transmission, and fitness benefit conferred to the host [4]. These criteria have been used to separate endophytes into different classes. For a more detailed review of the proposed classes of endophytes and their diagnostic criteria see Rodriguez et al. [4].

Recent research has shown that fungal endophytes associated with plants are a rich source of natural products [4, 5]. The objective of this review is to highlight natural products found in plants that are reported to be derived from fungal endophytes and, when appropriate, briefly comment on the plant-endophyte interaction. We will first summarize current knowledge on alkaloids synthesized by Clavicipitaceae endophytes (Sect. 2.2), then on diverse other secondary metabolites (Sect. 2.3). Specifically, we will highlight the role that fungal endophytes play in the synthesis of the indolizidine alkaloid swainsonine and the interaction between host and endophyte (Sect. 2.4). The review will not highlight plant mycorrhizal relationships.

2.2 Alkaloids Derived from Clavicipitaceous Endophytes

The most studied fungal endophyte system is the symbiotic association of the fungal endophytes in the genera *Epichloë* and *Neotyphodium* (Clavicipitaceae) with many cool season grasses (Poaceae). *Neotyphodium* species are asexual and grow within the intercellular spaces of their grass hosts, while *Epichloë* species represent the sexual states of several *Neotyphodium* species. *Neotyphodium* species are strictly vertically transmitted while *Epichloë* species are horizontally and vertically transmitted. *Epichloë* species are distinguished from *Neotyphodium* species because they are capable of exiting their plant hosts via the formation of sexual reproductive stroma on plant inflorescences.

Epichloë and *Neotyphodium* species may produce four classes of bioactive metabolites in their symbiotic associations with plants: ergot alkaloids, indole diterpenes, loline alkaloids, and peramine (Fig. 2.1). These four classes of alkaloids are derived from amino acid precursors, and the pathways are independent of one another. No individual fungal endophyte has been reported to produce representatives of all four classes; most produce metabolites belonging to one to three of the chemical classes [5].

The diverse suite of metabolites in the ergot alkaloid family can be grouped as clavines, simple amides of lysergic acid, or ergopeptines with the classification based on the structural complexity and position in the pathway [6, 7]. Like the ergot alkaloids, the indole-diterpenes represent a suite of products including the terpendoles, lolitrems, and janthitrems derived from oxidation and prenylation of a shared biosynthetic precursor, terpendole I [8, 9]. The lolines represent a family of aminopyrrolizidine alkaloids, derived from homoserine and proline. Lastly,

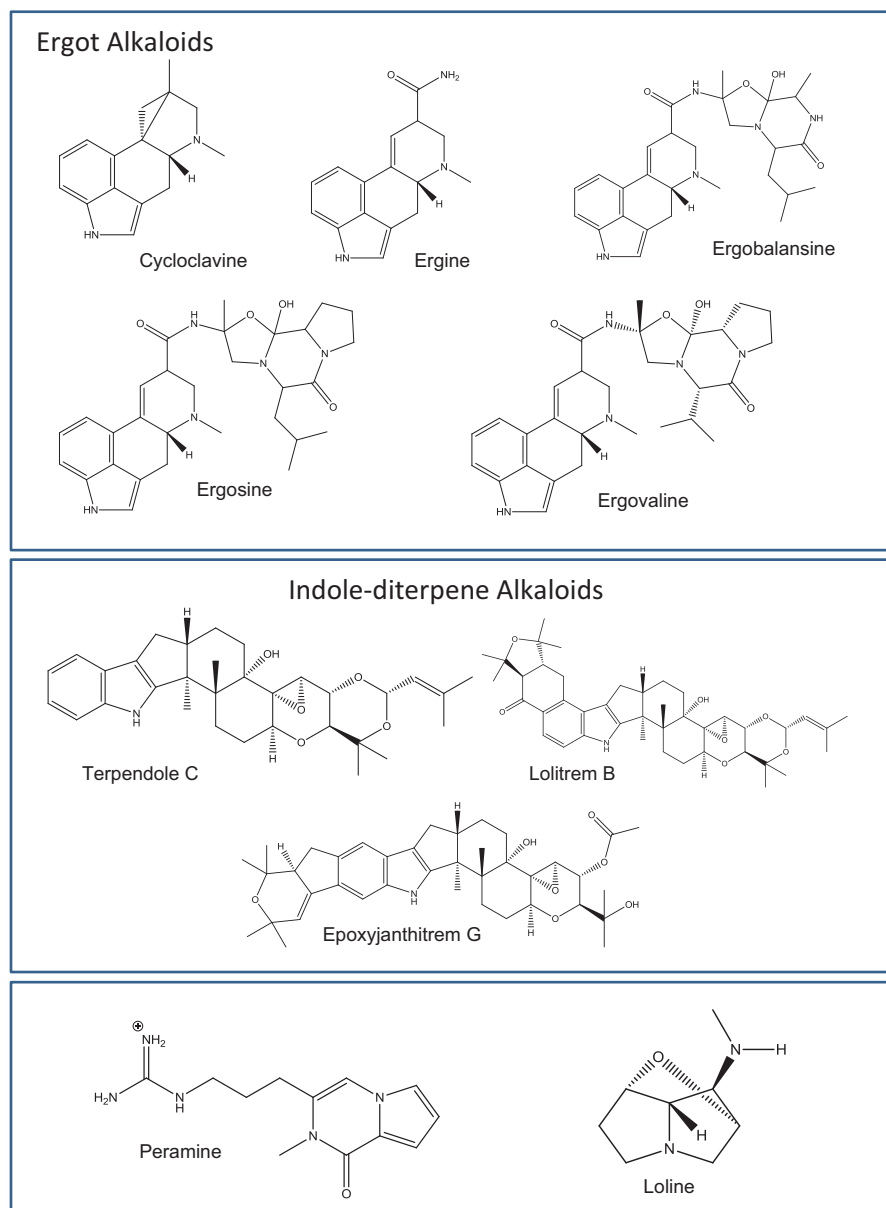


Fig. 2.1 Chemical structures of representative alkaloids produced by Clavicipitaceae endophytes associated with the grasses and morning glory families

peramine represents a single alkaloid rather than a family of alkaloids and is derived from a dipeptide possibly made up of arginine and a precursor to proline. *Epichloë* and *Neotyphodium* endophytes of grasses and the alkaloids associated with this

interaction affect herbivores and significantly impact ecological communities. For a more detailed commentary on each of these alkaloids and their biology and the effects of this symbiotic interaction, readers are referred to Schardl et al. [5, 10].

Not only are ergot alkaloids found in cool season grasses, but they are also present in select taxa of the Convolvulaceae, the morning glory family [11]. The three major types of ergot alkaloids have been detected in the Convolvulaceae [11], as well as the lolines in one taxon of the Convolvulaceae, *Argyreia mollis* [12]. In regard to the other bioactive metabolites found in grasses, there are no published reports of any Convolvulaceae taxa containing the indole diterpenes or peramine. However, there are a number of reports of livestock having a tremorgenic syndrome caused by feeding on select *Ipomoea* species including *Ipomoea muelleri* and *Ipomoea asarifolia* [13, 14]. These cases suggest that *Ipomoea* species may contain the indole diterpenes, as they are known to be tremoregenic, but this remains to be verified. Recent research has demonstrated the presence of clavicipitaceous epibiotic fungal symbionts described as *Periglandula* species in *Ipomoea* taxa [1, 15–17]. These fungi are vertically transmitted and appear to have a narrow host range, as the described species to date occur on different plant hosts. *Periglandula* species are the only clavicipitaceous fungi known to be associated with a dicot host [15]. The ecological effects of this interaction have yet to be studied; however, effects are likely to be substantial due to the bioactivities of the endophyte-derived alkaloids.

2.3 Other Natural Products

Numerous other natural products in plants are reported to be produced by fungal endophytes associated with the host. Some examples include paclitaxel (also known as taxol), podophyllotoxin, deoxypodophyllotoxin, camptothecin and structural analogues, hypericin and emodin, and azadirachtin (Fig. 2.2). In each of these cases, the compounds are thought to be produced by the plant as well as an endophyte that has been isolated from the plant host. Furthermore, in the cases of paclitaxel and camptothecin, the biosynthetic pathways *in planta* have been described, and several enzymes in the pathways have been characterized.

2.3.1 Taxol

Taxol, a taxane-type diterpene, is a tetracyclic lactam found in the genus *Taxus* [18]. All *Taxus* species are reported to contain taxanes. Taxol is an anticancer drug that acts as a microtubule inhibitor. Taxol was first reported to be produced by *Taxomyces andreanae*, an endophytic fungus associated with *Taxus brevifolia* [19]. Since then, more than 20 genera that produce taxol have been isolated, representing several species of unrelated endophytic fungi [20]. In several cases, more than one endophytic isolate from the same host has been reported to produce taxol. In one study of 109 endophytic isolates cultured from *Taxus chinensis*, 28 isolates

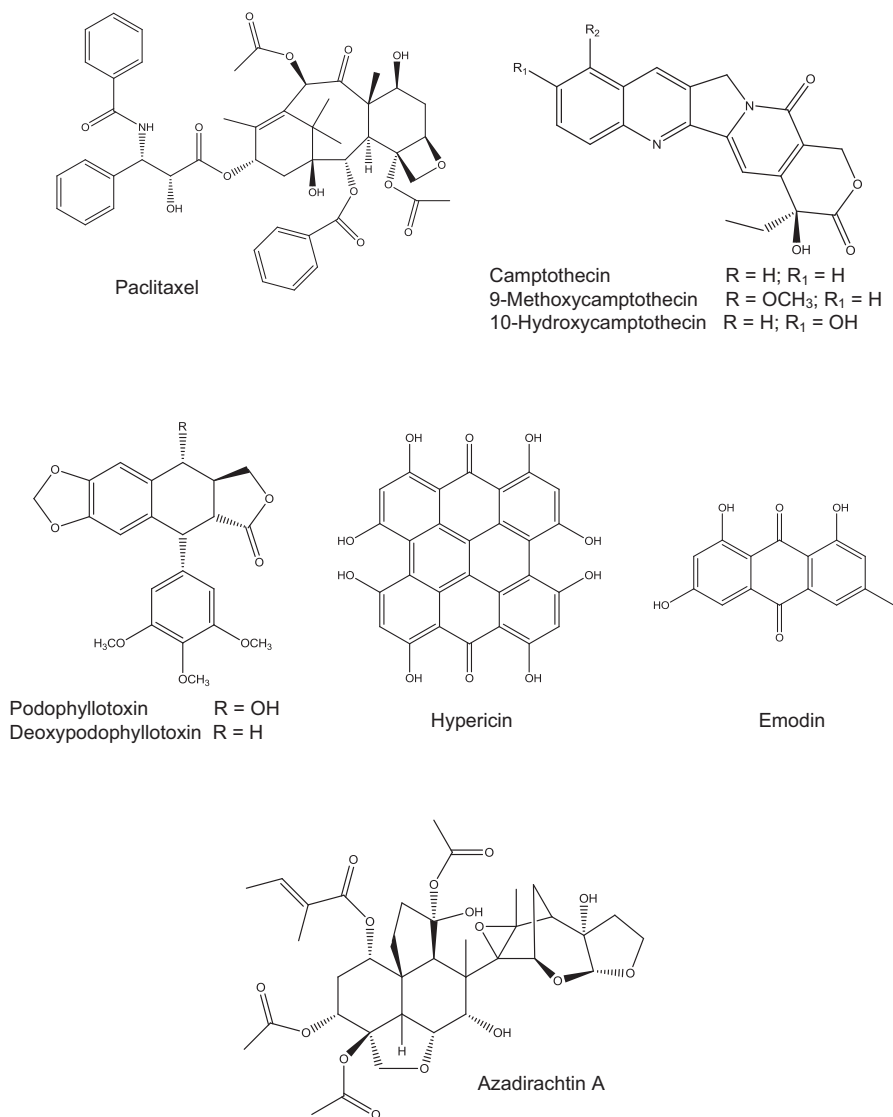


Fig. 2.2 Chemical structures of other highlighted natural products reported to be produced by endophytes

were reported to produce taxol [21]. Taxol-producing endophytes have been isolated from various hosts including *Taxus* species, cypress [22], *Wollemia* pine [23], and a variety of other non-*Taxus* species [24, 25]. These hosts include some that are reported to contain taxol and some that are not. None of the taxol-producing endophytes has been reported to contain any intermediates that are detected *in planta* for the taxol biosynthetic pathway. Furthermore, there are no reports of plant taxol biosynthetic enzymes found in fungi. In the cases where the endophytic isolates

were isolated from *Taxus* species, the authors propose that horizontal gene transfer was responsible for the biosynthetic machinery of the plant being transferred to the fungus, although no data are presented in support of this hypothesis. In cases where taxol-producing endophytic isolates were isolated from hosts that are reported not to produce taxol, the authors suggest that the endophytic isolate may have been associated with a *Taxus* species, but through time came to be associated with a different host [23].

2.3.2 Podophyllotoxin

Podophyllotoxin, podophyllotoxin glycoside, deoxypodophyllotoxin, and related analogues are present in four plant families: the Berberidaceae, Cupressaceae, Polygalaceae, and Linaceae [26]. These compounds are aryl tetralin lignans, and have various bioactivities including but not limited to antiviral [27], anticancer [28], antiproliferative [29], and insecticidal [30] properties. In fact, podophyllotoxin is in great demand as a precursor in the synthesis of topoisomerase inhibitors that are anticancer drugs. Recently, endophytes have been isolated from both *Podophyllum* and *Juniperus* species that are reported to produce these structurally related compounds [31–33]. In two *Podophyllum* species, two unrelated endophytes were identified: in *Podophyllum hexandrum* the endophyte *Trametes hirsuta* was identified that produces podophyllotoxin, podophyllotoxin glycoside, and deoxypodophyllotoxin [32], whereas in *Podophyllum peltatum* the endophyte *Phialocephala fortinii* was identified that produces podophyllotoxin [31]. In *Juniperus communis*, the endophyte *Aspergillus fumigatus* was identified and reported to produce deoxypodophyllotoxin [33]. Interestingly, in the latter two examples the authors do not mention if the endophyte produces any of the other structurally related analogues. In all three cases, the authors propose that horizontal gene transfer was responsible for the biosynthetic machinery from the plant being transferred to the fungus, although no data are presented in support of this hypothesis.

2.3.3 Hypericin

Hypericin is a naphthodianthrone derivative of significant medicinal value isolated from St. John's Wort, *Hypericum perforatum* [34]. Hypericin is reported to have a number of bioactivities including but not limited to being an antidepressant [35], anti-inflammatory, and antiviral [36]. Hypericin is a photodynamic compound activated by light, and animals consuming St. John's Wort can suffer from primary photosensitization due to hypericin. Emodin is thought to be a biosynthetic precursor of hypericin [36, 37]. Recently, an endophyte isolated from the inner stem tissues of *Hypericum perforatum* and found to produce both emodin and hypericin was identified as *Thielavia subthermophila* [37].

2.3.4 *Camptothecin*

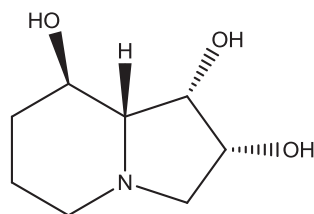
Camptothecin, a pentacyclic quinoline alkaloid, has been reported from several genera in unrelated angiosperm families [38]. For example, camptothecin has been reported in *Camptotheca* spp. (Nyssaceae) [39], *Ophiorrhiza* spp. (Rubiaceae) [40], *Nothapodytes foetida*, and *Apodytes dimidiata* (Icacinaeae) [41], and two genera of the Apocynaceae and Gelsemiaceae [42, 43]. Camptothecin and its structural analogues 9-methoxycamptothecin and 10-hydroxycamptothecin are potent neoplastic compounds that are important because they inhibit the enzyme topoisomerase I which is required for DNA replication and transcription [38]. In fact, two semisynthetic drugs targeting different cancers are derived from camptothecin [38]. Endophytes isolated from *Nothapodytes foetida*, *Apodytes dimidiata*, and *Camptotheca acuminata* have been reported to produce camptothecin [44–47]. Two unrelated endophytes have been isolated from *Nothapodytes foetida* that produce camptothecin [44, 45]. Interestingly, separate strains of *Fusarium solani* that produce camptothecin were isolated from two unrelated plants (*Apodytes dimidiata* and *Camptotheca acuminata*) [46, 47]. In two of the above examples, the isolated endophytes produce camptothecin and its structural analogues [46, 47] while in the other two examples camptothecin is produced, but the authors do not mention if the endophyte produces other structurally related compounds [44, 45].

The endophyte isolated from *Camptotheca acuminata* was particularly intriguing because it lost its ability to produce camptothecin at the third subculture and continued as such to the seventh generation [46]. To further investigate the loss of camptothecin synthesis in the endophyte isolated from *Camptotheca acuminata*, the authors took advantage of the fact that the biosynthetic pathway of camptothecin is known and several enzymes in the pathway have been described [48]. The authors identified geraniol 10-hydroxylase, secologanin synthase, and tryptophan decarboxylase in the *Fusarium solani* strain isolated from *Camptotheca acuminata* [48], which were 100% identical to the host enzymes. The authors found no evidence of the strictosidine synthase in the endophyte, the enzyme in the biosynthetic pathway of camptothecin that forms strictosidine from tryptamine and secologanin. The authors proposed a cross-species biosynthetic pathway where the endophyte uses the host strictosidine synthase. Furthermore, the authors showed that the genes involved in camptothecin biosynthesis in the endophyte had undergone random non-synonymous mutations from generation 1 to generation 7 to further explain the inability of the endophyte to produce camptothecin and its precursors [48].

2.3.5 *Azadirachtin*

Azadirachtin A and its structural analogues are highly oxygenated tetranortriterpenoids isolated from the Indian neem or Indian lilac, *Azadirachta indica* [49]. These compounds are reported to have various bioactivities, including as insecticides that act as antifeedants and have growth-regulating properties [50]. They are found in

Fig. 2.3 Chemical structure of swainsonine



Swainsonine

all parts of the plant, with the highest concentrations in the seeds [49]. Recently, an endophytic fungus identified as *Eupenicillium parvum* was isolated from the plant host, *Azadirachta indica*, that was capable of producing azadirachtin A and B but not the other structural analogues [51].

2.4 Swainsonine

Several species in the legume (Fabaceae) genera *Astragalus*, *Oxytropis*, and *Swainsona* are toxic to grazing livestock in the Americas, Asia, and Australia [52–55]. Locoism, a disease induced by prolonged grazing of some *Astragalus* and *Oxytropis* species generally called locoweeds, was first noted by the Spanish conquistadors, and again during the settlement of western North America by pioneers [52, 53, 56]. Clinical signs and pathology of locoism, a neurologic disease, are similar in animals intoxicated by locoweed species and *Swainsona* species [57, 58]. Swainsonine (Fig. 2.3), a trihydroxyindolizidine alkaloid, was first identified as the bioactive principle of the neurologic disease in *Swainsona canescens*, a legume native to Australia [59], and subsequently identified as the active principle in locoweeds [60]. Swainsonine inhibits the enzymes α -mannosidase and mannosidase II, resulting in lysosomal storage disease and altered glycoprotein synthesis [61, 62].

A fungal endophyte, *Undifilum oxytropis*, previously described as an *Embellesia* species [63], is reported to produce swainsonine in locoweeds [64, 65]. The *Undifilum* genus (Pleosporaceae) is related to the genera *Alternaria*, *Embellesia*, and *Ulocladium* [65]. In general, *Undifilum* species are associated with swainsonine-containing *Astragalus* and *Oxytropis* species in North America and China [65–67]. Other species of *Undifilum* have been found and characterized in the swainsonine-containing plant species *Astragalus lentiginosus* and *Astragalus mollissimus* [67]. *Undifilum bornmuelleri*, a pathogen of the legume *Securigera varia* [65], is reported to not produce swainsonine. *Undifilum* species associated with locoweeds are vertically transmitted and have no apparent sexual stage [68]. *Undifilum* species appear to have a narrow host range, as different plant species are associated with unique *Undifilum* species [65, 67].

Swainsonine concentrations vary greatly among plant species, varieties and populations, often ranging from not being detected to greater than 0.2%. For example, *Astragalus* locoweed species generally have greater swainsonine concentrations than *Oxytropis* locoweed species in North America, although this depends on the specific taxa being compared [69]. Swainsonine concentrations vary greatly among different varieties of some *Astragalus* and *Oxytropis* species. For example, some *Oxytropis lambertii* and *Astragalus mollissimus* varieties contain swainsonine, while others contain very little or no swainsonine [69, 70]. Additionally, swainsonine concentrations vary greatly among populations, for example some populations of *O. lambertii* var. *bigelovii* contain swainsonine while others contain very little or no swainsonine. Furthermore, within toxic populations of locoweeds, swainsonine concentrations vary greatly among individual plants.

Initial reports demonstrated that the endophyte usually could be cultured only from locoweed samples, with swainsonine concentrations greater than 0.01%, but could be detected by polymerase chain reaction (PCR) in all samples regardless of swainsonine concentration [69]. To further describe the plant–endophyte–swainsonine relationship and to investigate the differences in swainsonine concentrations among individual plants in locoweed populations, a method was developed to quantify the endophyte in plant samples. Using quantitative polymerase chain reaction (qPCR), the amount of endophyte was measured in the *Astragalus* and *Oxytropis* locoweeds with different swainsonine concentrations. Two chemotypes of plants were identified, namely chemotype 1 plants, which contain swainsonine concentrations greater than 0.01%, and chemotype 2 plants, which have concentrations below 0.01% (generally near 0.001% or not detected) (Fig. 2.4a) [71, 72]. These two chemotypes were determined to differ significantly in the amount of endophyte they contain, which may help to explain the difference in swainsonine concentrations of the respective host plants (Fig. 2.4b) [71, 72].

Swainsonine and endophyte concentrations have been shown to be influenced by the plant part [71, 72] and phenological stage of the plant [73, 74]. Swainsonine was found in all plant parts, with greater concentrations in aboveground parts than in below-ground parts (Fig. 2.4a); the endophyte *Undifilum* was also found in all plant parts, again with greater concentrations in aboveground parts than in the root (Fig. 2.4b) [71, 72]. The root crown has endophyte amounts equivalent to aboveground parts, which we believe may serve as a reservoir for the endophyte for the following year's growth, as many locoweeds are perennial plants [71, 72]. Swainsonine concentrations are greatest in floral parts and seeds [73]. This is consistent with the optimal defense theory of protecting reproductively valuable plant and endophyte tissues. Swainsonine and endophyte amounts in aboveground parts increase throughout the growing season until the plant reaches maturity [73, 74]. As plants senesce, swainsonine concentrations decrease significantly [73]. Swainsonine and endophyte amounts are highly correlated over the growing season, suggesting the endophyte amount plays a critical role in determining swainsonine concentration in the plant [73].

Three mechanisms were proposed that may explain the origin of chemotype 2 plants in *Astragalus* and *Oxytropis* locoweeds [72]. First, different endophyte

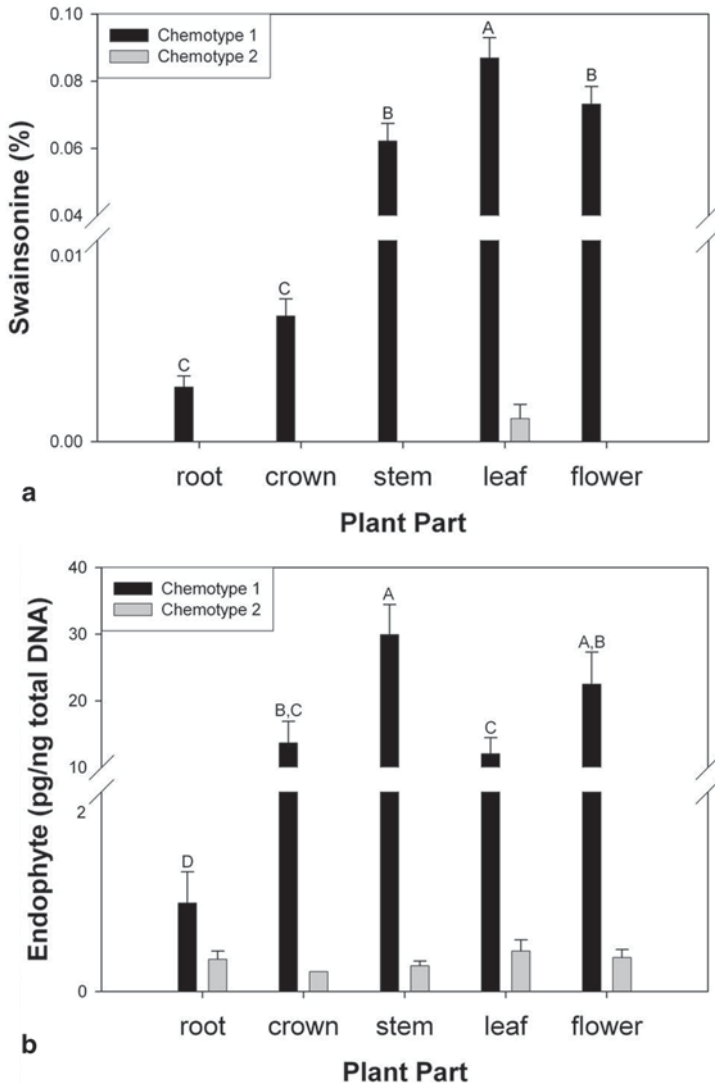


Fig. 2.4 Comparison of swainsonine and endophyte concentrations among plant parts and between two chemotypes of *Oxytropis sericea*. Mean swainsonine (%) and endophyte (pg/ng total DNA) concentrations (\pm SE) **a** from different plant parts (root, crown, scape, leaf, and flower/pod) and **b** from two chemotypes of *O. sericea* plants [71]

genotypes distributed across host plants may account for differences in swainsonine accumulation. However, recent work does not support this suggestion, as we have reported that the internal transcribed spacer (ITS) of the rDNA region of *Undifilum* spp. from three locoweed species are identical between chemotype 1 and 2 plants [72].

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