ABSTRACT

Title of dissertation: THE CHEMICAL ECOLOGY OF *HYDRASTIS CANADENSIS* L. (RANUNCULACEAE):EFFECTS OF ROOT ISOQUINOLINE ALKALOIDS ON THE *HYDRASTIS* ENDOPHYTE, *FUSARIUM OXYSPORUM*

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Goldenseal (*Hydrastis canadensis* L., Ranunculaceae) is a popular medicinal plant and has been listed in Appendix II of the Convention on International Trade in Endangered Species (CITES). The herbaceous perennial is distributed in North America under deciduous forest canopies throughout much of the south and eastern seaboard north into Canada. The rhizome, rootlets and root hairs produce medicinally active alkaloids. Although berberine, one of the *Hydrastis* alkaloids, has shown anti-fungal activity, the influence of *Hydrastis* alkaloids on the plant rhizosphere fungal ecology has not been investigated. While a *Fusarium* spp. was previously isolated from *H. canadensis*, this is the first report of a *F. oxysporum* endophyte isolated from *Hydrastis* root tissue. Additionally, *F. solani* was isolated from non-rhizosphere soil surrounding *Hydrastis* root tissue. A bioassay was developed to study the effect of goldenseal isoquinoline alkaloids on three *Fusarium* isolates, including the two species isolated from *Hydrastis*. And, in order to accurately detect the alkaloids, a HPLC-MS method was developed. The whole root extract treatment stimulated macroconidia germination and chlamydospore formation, while inducing mycotoxin production in the *Hydrastis* endophyte. Chlamydospore formation, macroconidial germination and mycotoxin production of PSU isolate, *F. commune*, was reduced by the whole root extract. The second *Hydrastis* isolate, *F. solani*, responded to the whole root extract by increasing germination rates, but with no other effects. The findings suggest that the *Hydrastis* root extract appears to provide selective advantage for the endophytic isolate and influences the fungal ecology associated with its root system.

The Chemical Ecology of *Hydrastis canadensis* L. (Ranunculaceae):Effects of Root Isoquinoline Alkaloids on the *Hydrastis* Endophyte, *Fusarium oxysporum*

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Abbreviations

Concentration:conc.
DON:deoxynivalenol
Dry weight:dry wt.
High performance liquid chromatography:HPLC
High performance liquid chromatography-mass spectrometry:HPLC-MS
High performance liquid chromatography-electrospray ionization-mass spectrometry:HPLC-ESI-MS
Molecular weight:mol. wt.
Preparative thin-layer chromatography:prep. TLC
Zearalenone:ZON

CHAPTER 1:LITERATURE REVIEW

Botanical description of *Hydrastis canadensis* (goldenseal)

Goldenseal is an herbaceous perennial and a member of the buttercup family – Ranunculaceae. The plant is distributed in North American under deciduous forest canopies, in moist, loamy, well-drained soil from Alabama north through the Appalachian mountain range into New England, west through Arkansas and north again through southern Ontario (Duke & Foster, 1990). Morphologically, the flower (see Figure 1) is perfect, small not elaborate, and if fertilized, clusters of berries form. Stems are produced from a rhizome, which also produces lateral roots, and each stem produces 1-3 palmately lobed leaves. Most wild populations do not reproduce from fruit but by asexual propagation (Davis, 1999). The rhizome, rootlets and root hairs (see Figure 2), which are used as a herbal supplements, produce medicinally active alkaloids. These alkaloids are also found in the leaf tissue in different proportions, but traditionally leaf material has not been used in the preparation of herbal supplements.

The American Herbal Products Association reports that fresh weight harvest of goldenseal root for 1999 was 13,710 tons and currently retails at prices over $160 per pound. According to Robbins (2000), because of over harvesting and habitat loss in wild populations, goldenseal has been listed in Appendix II of the Convention on International Trade in Endangered Species (CITES). The CITES convention prohibits harvest before 4 years of age and since goldenseal is slow growing, commercial cultivation of goldenseal

is rapidly becoming an economically viable option to satisfy the needs of the herbal industry.

Figure 1:*Hydrastis canadensis* leaf and flower
Photo by Thomas G. Barnes @ USDA-NRCS PLANTS Database / Barnes, T.G. & S.W. Francis. 2004. *Wildflowers and ferns of Kentucky*. University Press of Kentucky

Figure 2:*Hydrastis canadensis* root mass
Unknown photographer.

Historic use of *H. canadensis*

Indigenous use of goldenseal by Native Americans was widespread as a plant dye, an eye wash, treatment for skin disorders, bitter tonic, for respiratory ailments and the variety of infectious diseases brought by European settlers (Moerman, 1986). The plant was included in *The American Eclectic Materia Medica and Therapeutics* (Ellingwood, 1919) and *King's American Dispensatory* (Felter & Lloyd, 1898), which increased its use substantially among Eclectic physicians for infections, mouth ulcers and thrush, inflamed mucous membranes, chronic gonorrhea, as a bitter tonic, a uterine tonic, for gastrointestinal complaints and jaundice. Goldenseal is now one of the most popular herbal treatments in the modern herbal repertoire for use as an antimicrobial agent to treat infections of the mucosal membrane, including mouth, upper respiratory tract, gastrointestinal tract, eyes, wounds and as a vaginal douche (Upton, 2001).

***Hydrastis* antimicrobial activity**

Experimental evidence for *H. canadensis* antimycotic activity is limited. Thus, in addition to the sparse data of direct effects from *Hydrastis* alkaloids on *Fusarium*, evidence from studies using a variety of other organism will be considered as well.

Of the major *H. canadensis* alkaloids, only berberine has been reported to have antifungal activity against *Fusarium* spp.. Mahajan et al. (1976) reported that berberine sulfate inhibited the growth of an unidentified species of *Fusarium* grown on Sabouraud's dextrose agar (SDA) at a concentration of 10 mg ml^{-1}. Mahajan (1986) also looked at

berberine disodium citrate (because of the poor solubility of berberine sulfate) and found that growth on SDA of an unnamed species of *Fusarium* was inhibited at a concentration of 10 mg ml^{-1}. Cernakova and Kostalova (2002) used 1 mg ml^{-1} of berberine chloride and reported that the growth of *F. nivale* grown on SDA was inhibited. Sarma et al. (1999) reported that berberine iodide at a concentration of 1 mg ml^{-1} significantly inhibited conidial germination of *F. udum* in sterile distilled water. Singh et al. (2001) found that conidia of an unidentified *Fusarium* species and *F. udum* were inhibited at a concentration of 0.25 – 0.5 mg ml^{-1} in sterile distilled water. The berberine salt used in the assay was also not identified. The mechanism for the inhibition in these studies is unknown.

Pasqual et al. (1993) reported that berberine inhibitory effects occurred only during cell division of various yeast strains (*Saccharomyces cervisiae*) and were concentration dependent. They suggest that berberine caused breaks in the yeast nuclear DNA and that the cytotoxic, mutagenic and recombinogenic effects caused by this were due to the activity of yeast topoisomerase enzymes. Using a battery of *in vitro* assays, Schemeller et al. (1997) reported that berberine (1.7 mg ml^{-1}) intercalated with DNA, which lead to inhibition of reverse transcriptase activity and subsequent DNA and protein synthesis. These cellular effects suggest a possible explanation for the inhibition of *Fusarium* growth or spore formation. Although human *in vitro* and *in vivo* evidence for berberine antimicrobial activity is not directly applicable, Kong et al. (2004) reports on a post-translational genomic effect of berberine. Using a small clinical trial, a hamster animal model and a human hepatoma derived cell line they reported that berberine lowered

serum cholesterol, triglycerides and LDL-c to levels similar to those activated by statins. However, the mechanism of action was different, occurring by post translation regulation of liver low-density lipoprotein receptor mRNA and proteins.

Although lacking in direct evidence for hydrastine antimycotic activity, Goel et al. (2003) isolated (±)-α-hydrastine and (±)-β-hydrastine from *Corydalis longipes* and reported that both compounds inhibited spore germination of several fungi, including *Alternaria*, *Curvularia*, *Colletotrichum*, *Helminthosporium* and *Erisyphe pisi* and that a mixture of both compounds was more effective than either compound individually, with maximum inhibition occurring at 200 ppm.

Although not directly applicable to fungi, the studies on the alkaloid anti-bacterial effects does provide information about the range of activity, the concentration of active alkaloids, and whether the activity results from multiple or single alkaloids being present. A number of authors confirmed that *H. canadensis* had antimicrobial activity against bacterial pathogens (Gentry et al., 1998; Scazzocchio et al., 2001; Hwang et al., 2003; Mahady et al., 2003; Villinski et al., 2004). Gentry et al. (1998) noted that *H. canadensis* root extract was not particularly effective at inhibiting *Mycobacterium* spp., although the MIC value for berberine was 25 µg/ml for *M. smegmatis* (vs. streptomycin sulfate MIC of 1.56 µg/ml) and 200 µg/ml against the *M. avium* complex. Scazzocchio et al. (2001), reporting on the killing time of whole root extract vs. individual alkaloids, found that a 1 mg ml^{-1} whole root extract was more effective against *Streptococcus sanguis*, as well as *E. coli* and *Pseudomonas aeruginosa* when compared with a 0.3% concentration of

individual alkaloids berberine, canadine, canadaline and β-hydrastine. The level of inhibition by *S. sanguis* whole root extract was less than for the individual alkaloids. For *Staphylococcus aureus*, canadaline was more effective than the whole root extract and the other individual alkaloids tested, but less effective than the control. Of the individual alkaloids, canadaline had the lowest MIC for the bacteria. The alkaloid content for the extract was 1% berberine, 0.5% β-hydrastine, 0.2% canadine and 0.2% canadaline, within expected range of *Hydrastis* root isoquinoline alkaloid content.

Mahady et al. (2003) reported on the *in vitro* reactions of different strains of *Helicobacter pylori* to *H. canadensis* root extract, with MIC values ranging from < 1.0 µg/ml to 50 µg/ml. They noted that the activity was associated with the alkaloid fractions of both *H. canadensis* and *Sanguinaria canadensis*, another plant with high levels of isoquinoline alkaloids, including the most active alkaloid berberine. Previously, Bae et al. (1998) reported that the extract of *C. japonica* root inhibited *H. pylori* at a MIC value of 1.0 mg ml^{-1}, whereas the MIC value for berberine was 8-200 µg/ml for strains of *H. pylori*. Villinski et al. (2004) compared the inhibitory activity of three whole root extracts from different berberine containing plant species (*Berberis thunbergii*, *B. vulgaris* and *H. canadensis*) with their alkaloid content and found their effects were equivalent. Their inhibitory effect was strongest against *S. aureus* and *Streptococcus pyogenes*, less against *S. mutans* and *P. aeruginosa*, despite the fact that the berberine content in *H. canadensis* was 5-13x greater and its total alkaloid content was approximately 3x greater. This might suggest that inhibition is based on cumulative effects, such as receptor or enzymatic binding of similar molecular species.

Hwang et al. (2003) reported that a methanol extract of *H. canadensis* was inhibitory to oral pathogens *Streptococcus mutans* and *Fusobacterium nucleatum* and that berberine was responsible for most of the activity. They did note a potential synergistic effect of berberine against *S. mutans* when combined with a C-methyl flavonoid isolated from *H. canadensis*. This supports findings by Stermitz et al. (2000a, 2000b), who showed that the flavo-lignan compounds of *Berberis* spp., including 5-methoxy-hydnocarpin, 5-methoxyhydnocarpin-D and silybin acted synergistically with berberine to inhibit *S. aureus* efflux pumps thus potentiating the inhibitory effect of berberine.

Berberine also been investigated for its *in vivo* antimicrobial activity in clinical trials for the treatment of diarrhea (Sharda, 1970; Khin et al., 1985), enterotoxigenic *E. coli* and *Vibrio cholera* (Rabbani et al., 1987), giardisis (Gupte 1975), and trachoma (Khosla et al., 1992). Only the Khin and Rabbani trials were randomized, blinded and placebo controlled. They found that berberine provided no significant effect when compared to the placebo and antibiotic treatments. Studies with guinea pig colon smooth muscle cell suspension (Cao et al., 2001), showed that although berberine did not affect resting calcium concentration, it did block KCl induced calcium increase, which may be responsible for the anti-diarrheal activity associated with berberine. Alternatively, *in vitro* cell assays developed by Kang et al. (2002) suggests that berberine antimicrobial activity was based on induction of Interleukin (IL-12), which may also be useful as an anti-cancer treatment. Kim et al. (2003) elaborated on the findings, noting that berberine altered cytokine profile of T helper cells. Although fungi do not produce immune cells, their

findings suggest that these compounds do bind to cell surfaces, which may be applicable in understanding the alkaloid effects on *Fusarium*. Since direct mechanistic evidence is lacing for the antimicrobial activity, evidence of interaction with cell surface receptors, enzymes or DNA of other organisms may indicate potential targets.

Palmery et al. (1993, 1996) reported that the root extract of *H. canadensis* showed adrenolytic activity on isolated rabbit aorta, noting that the inhibition of rabbit aorta contractions was not solely from berberine, but rather from a mixture of alkaloids. In a follow-up study, Cometa et al. (1998) reported a relaxing effect from *H. canadensis* extract on guinea pig trachea, which was later confirmed (Abdel-Haq et al., 2000), with the authors noting that the effect was due to several alkaloids, including berberine, β-hydrastine, canadine, and canadaline, but did not suggest a mechanism. Additionally, berberine induced contractions of isolated rabbit prostate smooth muscle. However, *H. canadensis* root extract did not (Baldazzi et al., 1998). This suggests that several of *Hydrastis* alkaloids provide adrenolytic activity against α-receptors in the tissue, but that inhibiting compounds may be present in the whole root extract.

Additional evidence for *Hydrastis* alkaloid receptor binding was reported from animal studies (Sun & Li, 1993). The research found that canadine increased the ventricular fibrillation (VF) threshold in electric stimulation and drug-induced VF in rabbits, rats and guinea pigs, Mechanistically, the antifibrillatory effect of canadine appears to target receptors via cell ion fluctuations with a resulting potassium, calcium and sodium current blockade. Wu and Jin (1997) used a patch-clamp perforated whole cell recording method

to show that canadine inhibited dopamine, acetylcholine, caffeine and strychnine-induced outward K+ currents, but did not affect GABA receptors. In contrast, Huang & Johnston (1990) reported that β-hydrastine was a GABA receptor antagonist, with the stereoisomer (+)-hydrastine being more potent than (-)-hydrastine. Lee et al. (1997) noted that β-(+)-hydrastine (38 μg ml^{-1}) completely inhibited bovine adrenal tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis in animals. It is unclear whether these proteins are localized on cell surface or are internal to the cell. Also, using rat dopamine synthesizing PC12 adrenal cells, Kim et al. (2001) reported that (1R,9S)-β-hydrastine decreased dopamine content of the PC12 cells, a result of tyrosine hydroxylase inhibition by β-hydrastine.

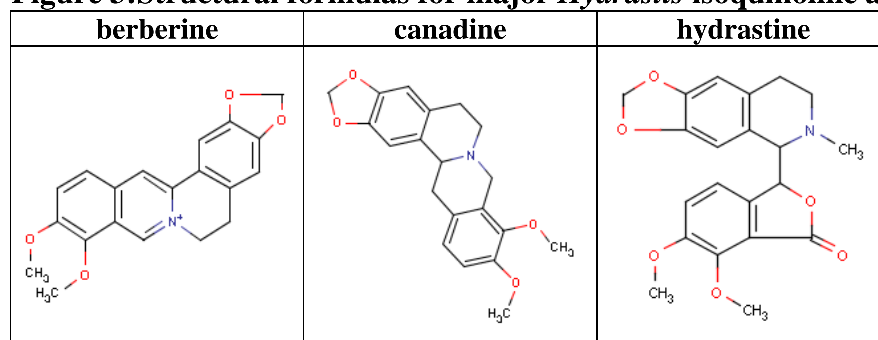
Plant/herbivore studies also found that hydrastine interacted with insect receptors. Jackson et al. (2002) found that 40 μg/ml of β-hydrastine inhibited locust (*Locusta migratoria*) nicotinic acetylcholine (ACh) receptors via a non-competitive, voltage-independent mechanism. They surmised that the inhibition at the receptor site was by a negative allosteric interaction of antagonist and binding site. The resulting effect was a deterrent to herbivory.

Important *H. canadensis* secondary metabolites

Thirteen isoquinoline alkaloids have been reported in goldenseal – berberastine, berberine, canadaline, tetrahydroberberine (canadine), canadinic acid, corypalmine, β-hydrastine, hydrastidine, hydrastinine, isocorypalmine, isohydrastidine, 8-oxotetrahydrothalifendine, and tetrahydropalmatine – plus 3 quinic feruloyl esters and a

C-methyl flavonoid (Messana et al., 1980; Galeffi et al., 1997; Gentry et al., 1998; Sturm & Stuppner, 1998; Scazzocchio et al., 2001; Hwang et al., 2003; McNamara et al., 2004). Berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium), canadine (9,10-dimethoxy-2,3-(methylenedioxy)-) and β-hydrastine (1(3H)-isobenzofuranone,6,7-dimethoxy-3-(5,6,7,8-tetrahydro-6-methyl-1,3-dioxolo[4,5-g]isoquinolin-5-yl)-, [S-(R*,S*)]-) are the primary alkaloids (see Figure 3), with total dry weight root alkaloid content range (w/w) from 2.5 – 8.0%, composed of approximately 2 – 4.0% berberine, 1% canadine and 1.5-4% hydrastine (Wagner et al., 1984; Galle et al., 1994; Gocan et al., 1996). Levels of alkaloids in leaf tissue are unknown. Both berberine and canadine are classed as protoberberine alkaloids, with the former quaternary and the later a tertiary alkaloid, lacking conjugation in ring C. Hydrastine is a phthalideisoquinoline alkaloid, possessing a tetracyclic nucleus incorporating a γ-lactone ring. Both berberine and hydrastine appear as yellow-colored pigments in root tissue, whereas canadine is colorless.

Figure 3:Structural formulas for major *Hydrastis* isoquinoline alkaloids



Isoquinoline alkaloid biosynthesis

Most research on the determination of the biosynthetic pathway for berberine and canadine focused on plant species other than goldenseal. However, using radiolabeled precursors, Barton et al. (1963) and Battersby (1963) reported that (+)-reticuline was a precursor of berberine in *H. canadensis*.

Working with cell cultures of *Berberis* sp. and *Coptis japonica*, Zenk (1985) separated vesicles of a specific gravity (1.14 g/ml) containing an enzyme system for the metabolism of the isoquinoline alkaloid berberine from the intermediate, (S)-reticuline. This was the first time that berberine synthesis was linked to enzyme containing vesicles in the cytoplasm. Funk et al. (1987) reported that fungal cell wall elicitor treated *Thalictrum rugosum* (Ranunculaceae) cell cultures accumulated berberine at higher levels in than untreated cell lines. This response was also dependent on growth stage, with berberine levels increased in actively growing cells but static during the stationary phase. In contrast, cells with high constitutive levels did not increase their berberine levels in response to elicitation. Tabata (1991) studied berberine accumulation in *Thalictrum minus* and *C. japonica* by electron microscopy and identified small vesicles in the cytoplasm along with vacuole development. He reported that in berberine producing cell lines a selective uptake mechanism operated in the tonoplast membrane that allowed the cells to accumulate exogenous cultural berberine. Yamamoto et al. (1986) suggested that specific berberine producing cell lines of *T. minus* secreted berberine into culture medium via an active transport system.

Sato et al. (1992) applied exogenous berberine hydrochloride to the media of *C. japonica* and *T. flavum* cell lines and reported that the levels of berberine accumulated in the vacuole were in excess of its solubility level. Because of the vacuole pH 5.9, they estimated that berberine remained solubilized as a dibasic malate salt and accumulating at a concentration level of 0.24 mg ml^{-1}. Kinetic studies provided evidence that the rate of uptake was based on an active transport system (Sato et al., 1993). Deliu et al. (1994) reported that electron-microscopic analysis of *Berberis parvifolia* root meristem showed that an electron dense precipitate accumulated in the vacuole and that this was the result of small vesicles fusing with the vacuole and then releasing their alkaloid contents. The TLC analysis of root apical meristem alcohol extracts confirmed the presence of protoberberine alkaloids, including berberine. Apparently synthesis occurred immediately after seed germination in *Berberis vulgaris* and the levels of alkaloid production increased as the seedling grew (Pitea & Margineanu, 1972). Suspension cell cultures appear to display the same cell structure as root apical meristems (Yeoman & Street, 1977).

Shitan et al. (2005), using *Rhizobium*-mediated transformation of *C. japonica* cells, isolated a gene (*Cjmdr1*) coding for a multidrug-resistant protein-type ABC transporter involved in berberine influx. The authors introduced the transporter in sense orientation into the transgenic cells lines and regenerated the plant. The resulting plant showed lower levels of both *Cjmdr1* mRNA and berberine. *In situ* hybridization showed that the *Cjmdr1* mRNA was expressed mainly in the xylem tissue of the rhizome. The authors suggested that the berberine transporter was responsible for translocation of berberine

from its biosynthetic origin in the roots to the rhizome, where berberine accumulates in *C. japonica*.

Deliu et al. (1994) reported that *B. parvifolia* suspension cells also contained small vacuoles of electron dense alkaloids in small vesicles in meristematic cells. Alkaloid concentrations increased as the vesicles fused with the vacuole until the culture reached stationary phase. An interesting finding was that alkaloid accumulation in *B. parvifolia* cell culture was inversely related to starch accumulation. This supported similar results by Yamamoto et al., (1986) for *C. japonica* cell culture. Bock et al. (2002) used immunocytological localization in different plant parts of *Eschscholtzia californica*, *Papaver somniferum* and *B. wilsoniae* to follow two biosynthetic enzymes, berberine-bridge enzyme (BBE) and (S)-tetrahydroprotoberberine oxidase (STOX). They reported that OsO_4 positive alkaloids, which were located exclusively in the root apex of *E. californica*, contained the isoquinoline alkaloids jatrorrhizine, palmatine and berberine. Because the vesicles contained STOX and BBE as well as the ER marker enzyme, NADH-cytochrome-*c* reductase, they hypothesized that the ER was the origin of these vesicles. The vesicles fused directly with the tonoplast or aggregated with small vacuoles before fusing. The smaller vacuoles would then fuse to the central vacuole.

Zenk 1985) was the first to describe the complete biosynthetic pathway for berberine from L-tyrosine, which uses 13 different metabolic enzymes (Sato et al., 2001). A major isoquinoline alkaloid branch-point occurs with the formation of the intermediate (S)-reticuline, at which point the first committed step is the catalytic formation of (S)-

scoulerine by the BBE (Figure 4). In Ranunculaceae, methylation of (S)-scoulerine occurs forming (S)-tetrahydrocolumbamine (Figure 5), catalyzed by scoulerine-9-O-methyltransferase followed by a cytochrome P-450 canadine synthase that forms (S)-canadine (tetrahydroberberine) the precursor to berberine. Oxidation of (S)-canadine via a (S)-canadine oxidase (CDO) or STOX, leads to the formation of berberine as reported in various cell cultures, including *E. californica* (Wing-Ming & Kutchan, 1998), *Berberis* sp. (Steffens et al., 1985), *Thalictrum* sp. (Hashimoto & Yamada, 1994) and *C. japonica* (Sato et al., 1993). The complete enzymatic pathway in *H. canadensis* has not yet been established, however, it is likely to be similar.

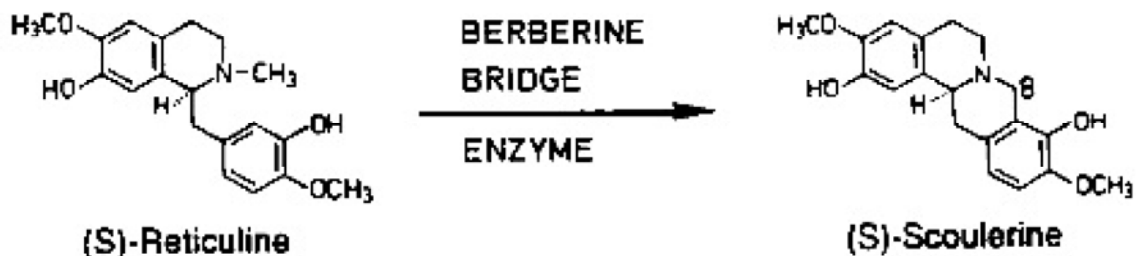


Figure 4:Berberine bridge enzyme catalyzes the conversion of (S)-reticuline to (S)-scoulerine, essential to the formation of isoquinoline alkaloids (see Dittrich and Kutchan, 1991)

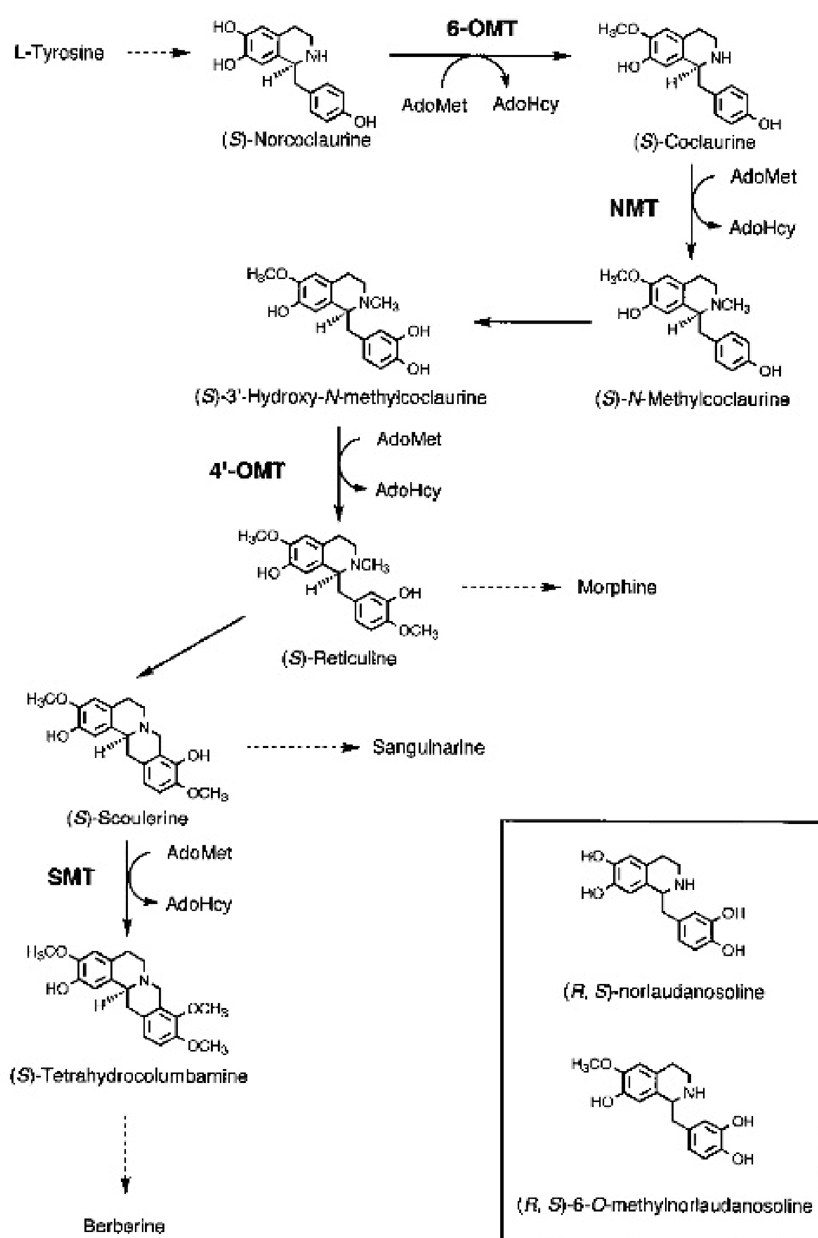


Figure 5:Schematic pathway involved in isoquinoline alkaloid biosynthesis in *Coptis japonica* as found in Morishige et al., (2000)

S-adenosyl- L-methionine:norcoclaurine 6-O-methyltransferase (6- OMT)
S- adenos yl - L- met hionine:3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'-OMT)
S-adenosyl-L-methionine:coclaurine N-methyltransferase (NMT)
S-adenosyl-L-methionine (AdoMet)
S-adenosyl-L-homocysteine (AdoHcy)

While investigating the pathway for berberine and other isoquinoline alkaloids, Dittrich & Kutchan 1991) noted that in *Eschscholtzia californica* cell culture, one of the penultimate enzymes, the BBE ([S]-reticuline:oxidoreductase) is elicitor induced, with transcripts accumulating to maximal levels in 6 hours after induction and enzyme activity also increasing for up to 20 hours. Furthermore, Kutchan & Zenk (1993) reported that methyl jasmonate and precursors also induced berberine bridge enzyme transcription. Roos et al. (1998) reported that acidification of *E. californica* suspension cell cytosol after elicitation with yeast was necessary for induction of alkaloid biosynthesis and that the vacuolar/cytosol proton gradient was the driving force. Their findings supported earlier work with tobacco, where alkaloid production depended on changes in cytosolic pH in response to elicitor induction (Mathieu & Guern, 1991).

Bernath et al. (2003) reported that phthalide-isoquinoline alkaloid biosynthesis in poppy involved a NADP-dependent 1,2-dehydroreticuline reductase acting on (S)-reticuline to produce the immediate precursor of the phthalideisoquinoline alkaloids. Depressing the enzyme activity suppressed the formation of phthalideisoquinoline alkaloids and accelerated the conversion of (S)-reticuline to (R)-reticuline, which led to isoquinoline formation. To date, a complete biosynthetic pathway for (±)-β-hydrastine has not been elucidated. Using radiolabeled precursors, early research on *H. canadensis* showed that tyrosine was a precursor to (±)-β-hydrastine (Kleinschmidt & Mothes, 1959; Gear & Spenser, 1963; Battersby et al., 1968) and that scoulerine was a precursor to both (±)-β-hydrastine and canadine (Battersby & Hirst, 1965; Battersby et al., 1967). Monkovic & Spenser (1965) reported that berberastine was a precursor to canadine in *H. canadensis*.

Galeffi et al. (1997) noted that canadinic acid was an intermediate metabolite in the biosynthesis of β-hydrastine.

<u>Detection of *H. canadensis* alkaloids</u>

Berberine and β-hydrastine have been separated and detected using thin-layer chromatography (TLC) (Genest & Hughes, 1969; Datta et al., 1971; Govindan & Govindan, 2000). Spectrophotofluorometry has been used to detect berberine, canadine, β-hydrastine and hydrastinine (Caille et al., 1970; El-Masry et al., 1980). Capillary electrophoresis-mass spectrometry has been used to detect berberastine, berberine, β-hydrastine, canadaline and canadine (Sturm and Stuppner, 1998). A pH-zone refining counter current chromatography has been used for detecting berberine, canadaline, canadine β-hydrastine and isocorypalmine (Chadwick et al., 2001). Quantitative ELISA has been used for detecting berberine (Kim et al., 2004). Shifted subtracted Raman spectroscopy (SSRS) has been used to detect berberine (Bell et al., 2002). Various high-performance liquid chromatography (HPLC) methods has been used for detecting a range of alkaloids (Leone et al., 1996; McNamara et al., 2004), including a validated HPLC methods (Wang et al., 2002; Weber et al., 2003a) and HPLC-PDA methods (Abourashed and Khan, 2001; Li and Fitzloff, 2002). HPLC-MS methods (Betz et al., 1998; Cunningham et al, 2002; Tims et al., 2000) have also been used to detect a range of alkaloids.

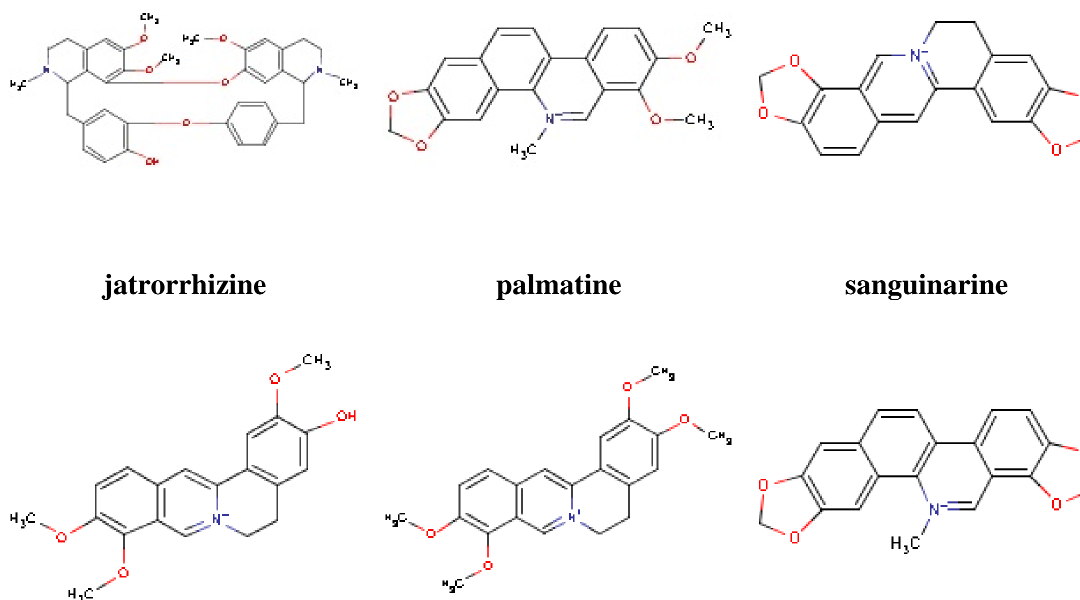
An important secondary application for any new isoquinoline alkaloid detection method involves detecting adulteration of goldenseal, which has been common for many years. In the early part of the century, at the height of the Eclectic movement in the US, the price of goldenseal had risen to the point that several plant species were being used as economic adulterants on a regular basis, including *Coptis* spp., *Xanthorrhiza simplicissima*, *Paeonia officinalis* and *Jeffersonia diphylla* (Lloyd & Lloyd, 1908; Blague & Maheu, 1926). Over the past 20 years the price has risen, based in part by erroneous use of goldenseal to negate illicit drug testing (Mikkelson and Ash, 1988). Economic adulterants have recently appeared in goldenseal products (Betz et al., 1998; Wang et al., 2002; Weber et al., 2003). Adulterant plants in use today include *Coptis japonica*, *Xanthorrhiza simplicissima*, *Mahonia aquifolium*, *Chelidonium majus*, and *Berberis* spp. (Betz et al., 1998).

Upton (2001) reported that both the width of medullary rays and absence of sclereids in *Hydrastis* but presence in common adulterants could be used to differentiate *Hydrastis* microscopically from adulterant plant species. Since most common plant adulterants contain berberine as well as at least one other unique isoquinoline alkaloid, analytical techniques can be used to detect adulterants present in various formulations. An emerging adulterant problem is the use of goldenseal leaf material, which contains both berberine and hydrastine, but in different ratios from goldenseal root (Betz et al., 1998). Of the major adulterants used, several isoquinoline alkaloids can be used as markers of adulteration, including berbamine, chelerythrine, coptisine, jatrorrhizine, palmatine, and sanguinarine (see Figure 6). In addition, when compared to the adulterants, hydrastine

and hydrastinine are unique to goldenseal. Thus adulteration can be detected by absence of goldenseal alkaloids, by berberine:hydrastine ratios, or by the presence of specific alkaloid peaks not associated with goldenseal and finally by a total alkaloid content far greater than reported values for *Hydrastis*.

Figure 6:Structural formulas for possible adulterant compounds of *Hydrastis*

berbamine **chelerythrine** **coptisine**



Ecology of *H. canadensis* and alkaloid production

According to the USDA Index of Plant Diseases in the United States (1960), fungal genera previously isolated from *H. canadensis* included *Alternaria* (leaf blight), *Botrytis* (leaf blight), *Fusarium* (vascular wilt), *Phymatrochium omnivorum*, and *Rhizoctonia solani* (root rot). There was no indication whether the plant source was commercially

farmed or wildcrafted. (Davis & McCoy, 2000) noted that the incidence of fungal disease has increased in commercially shade grown goldenseal.

Wild populations of goldenseal have been located growing at varied micro-ecologies and different elevations (Tims, personal observation). Levin (1976) provided hypothetical evidence that elevation was inversely correlated with herbivore and pathogen pressures on plant populations; the number of alkaloid containing plant populations; the toxicity of the alkaloids. Several researchers (Chandra & Purohit, 1980; Salmore & Hunter, 2001) provided follow-up data corroborating Levin's findings. Salmore and Hunter (2001) also reported *Sanguinaria canadensis* rhizome alkaloid content varied seasonally, with alkaloid levels increasing with increased seed weight and rhizome size. The results suggest that toxic compounds, such as alkaloids, help insure quality seed set and protection from pathogens and herbivores. Waller & Nowacki (1978) suggested that alkaloid rich plants are slower growing than alkaloid poor plants and that often the alkaloid rich plants grow better in low nutrient soils. The authors noted that the presence of alkaloids might prevent herbivore and pathogen effects that would limit the plants ability to maintain normal growth.

Sinclair & Catling (2003, 2000) noted that goldenseal is generally found in large populations in relatively small areas, which corroborates personal observation from field collection work. The authors noted a significant correlation between levels of disturbance from paths and edges with population sizes of goldenseal in southwestern Ontario. Cech (2001) reported that forest propagation studies showed that goldenseal is a two-phase

germinator, some seed germinating with root and bud formation but lacking aerial development until the second year. This appears to be a developmental pattern found in several native plant species growing in the same habitat as goldenseal. This pattern may allow the roots to interact with the rhizosphere ecology longer and to develop an alkaloid pool necessary for defense of aerial growth the following season. Sanders & McGraw (2002) noted that despite wide geographic distribution, seedling establishment is a constraint for wild goldenseal populations. In field studies at Bryan Nature Preserve, Indiana, they found less population loss and greater population growth along patch edges than interior habitats. Quigley & Mulhall (2002) found that vegetative growth of goldenseal proliferated under 70% shade when compared to greater or lesser shade, producing increased rhizome, rootlet and leaf biomass, as well as an increase in the number of bud primordia. These light levels may mimic those found along patch edges. Sinclair and Catling (2003) corroborated their early findings, reporting that goldenseal does benefit from disturbance simulation rather than from increased fertilization, with increased aerial growth and population density gain. Researchers using used the random amplified polymorphic DNA (RAPD) analysis generated DNA profiles from samples of cultivated and wild goldenseal populations to estimate genetic relatedness among and between these populations (Kelley et al., 2004). Their findings showed a significant increase in diversity of wild goldenseal when compared to cultivated populations.

The Fusarium Fungi

Fusarium oxysporum Schlect:Fr., is an anamorphic species that is classified taxonomically by the shape of macroconidia, the formation of chlamydospores and the

structure of the microconidiosphore (Nelson et al., 1983). The species of *Fusarium* described by Snyder & Hansen (1940) was the accepted classification scheme for more than 50 years, but the taxonomic scheme of Marasas et al. (1985) has replaced Snyder and Hansen. *Fusarium oxysporum* is cosmopolitan in distribution, with broad tolerances to climate and substrate. *Fusarium oxysporum* strains have a broad host range, but isolates that cause disease within a narrow host range are defined as the "special form" or formae specialis. Now molecular biology has grouped morphological species into a genetically heterogeneous polytypic morphospecies, or "complexes" (Waalwijk et al., 1996), since morphological differences are not enough to differentiate between saprophytic and pathogenic forms, or between two pathogenic *formae specialis* isolates. However, current molecular phylogenetic research provided evidence that the formae specialis designation is not always monophyletic, but is rather a grouping of two or more distinct and related lineages. In the past, use of formae specialis has been a loose designation for host range (Kistler, 2000) and several host specific pathogenic strains have been reported. Of note, the host-specific formae specialis of *F. oxysporum* may have been distributed by human dispersal of host plants (Backhouse, 2000).

A second important species of *Fusarium*, *F. solani* is also distributed worldwide and is considered a morphological species, comprised of a complex group of species, some that have a *Nectria* sexual state (Marasas et al., 1985). VanEtten & Kistler (1988) used DNA analysis and speciation methods to classify some of the *F. solani* species. Species associated with a Nectria sexual state normally do not produce toxins, except for those species isolated from potato that exhibit a bright blue pigment, which are known to

produce trichothecene toxic metabolites (El-Banna et al., 1984). Using molecular studies, O'Donnell (1996) suggests that the species complex contains a number of cryptic *F. solani* species each with a restricted host range.

Rhizosphere effects on *Fusarium*

Generally soils are low nutrient environments and most fungi remain dormant if not in proximity to plant root or decaying tissue. The mycostatic effect of soils can be overcome by simply adding nutrient supplements (Lockwood & Filonow, 1981). The authors noted that plant root exudates are the largest energy source for fungi in soil. The root soil complex, known as the rhizosphere, stimulates microbial growth 50-100 greater than in soil not in the rhizosphere zone. Dix & Webster (1995) defined the root surface as the rhizoplane, noting that *Fusarium* spp. are the predominant fungi in the rhizoplane. This rhizospheric effect on fungal growth is the result of root exudates of organic substances (Dix and Webster, 1995). Peterson (1958) noted that rhizosphere mycoflora populations increased with the age of the plant as greater amounts of organic material are lost from the root tissue due to damaged epidermal and cortical cells.

Early research suggested that the density of facultative fungal pathogens, such as *Fusarium*, is higher in the rhizosphere of a host plant than a non-host plant (Reyes & Mitchell, 1962). In general, the rhizosphere effect of plant roots is to increase fungal propagule number and the diversity of fungal species present. Most mycelial growth and sporulation occurs in the rhizosphere, but is inhibited outside the rhizosphere zone (Couteaudier & Alabouvette, 1990a). Humus, a complex mixture of partially

"decomposed" organic material made up of high-molecular organic compounds, is the dominant biological substance in non-rhizosphere soil and thus would be expected to play an important role in influencing growth of both plants and fungi (Kononova, 1966; Tan & Tantiwiramanond, 1983). However, Moliszewska & Pisarek (1996) reported that *F. culmorum* mycelial growth and spore germination were not very sensitive to the presence of different humic substances, whereas growth and spore germination of *Alternaria* spp., a saprophytic fungus, were inhibited by the same humic material.

Couteaudier (1989) studied nonpathogenic strains of *F. oxysporum* and *F. solani* and found that the best soil colonizers were those most able to utilize organic carbon, which resulted in an increase in their population levels. In the *Fusarium* wilt suppressive soils of Chateaurenard, France, nonpathogenic *Fusarium* propagules were present at greater levels than pathogenic *Fusarium* (Couteaudier & Alabouvette, 1990b). When comparing the effects of suppressive vs. stimulative soils on the growth of *F. oxysporum* f. sp. *niceum*, Larkin et al. (1993) found that population dynamics were no different in the two soils under constant conditions, but under fluctuating nutrient and water potential, fungal survival was less in suppressive soils. Explanations for these phenomena have been proposed. One hypothesis is that reproductive spores lyse in suppressive soils (Larkin et al., 1993), and secondly, that the fungiastatic activity was caused by high bacterial populations (Landa et al., 2001), the author reporting the suppression of *F. oxysporum* f. sp. *ciceris* by rhizosphere bacteria.

Nonpathogenic forma specialis of *Fusarium* have been reported to limit disease incidence on artificial media, in stimulatory and suppressive soils (Alabouvette, 1986; Paulitz et al., 1987; Postma, 1992; Alabouvette et al., 1993). Although the reason for this difference is not clearly understood, several hypotheses have been reported. For example, nonpathogenic isolates appear to compete more successfully for nutrients at root infection sites in both stimulatory and suppressive soils than do pathogenic strains (Couteaudier & Alabouvette, 1990b; Eparvier & Alabouvette, 1994; Turlier et al., 1994); and nonpathogenic strains are able to induce resistance in the plant (Biles, 1989; Olivain et al., 1995; Larkin et al., 1996).

Early survey data indicates that in general, *Fusarium* is more abundant in cultivated soils than forest or woodland soils (Snyder & Nash, 1968; Toussoun, 1975). In contrast, *Fusarium oxysporum* is found throughout the world in native soils that have not been cultivated (Windels & Kommedah.T, 1974; Gordon et al., 1992) and the isolates are usually associated with root surfaces and not internal to the plant. Both *F. oxysporum* and *F. solani* appear to be more widespread in forest soils than other Fusaria. Closed forests and woodlands have lower *F. oxysporum* populations compared to under story grasslands or herbaceous locations (Stoner, 1975; Stoner and Baker, 1980). Grass is more abundant in open forest settings and dicotyledonous shrubs or herbs more predominant in closed forest settings. Stoner (1981) also found that the distribution of fusaria in forest settings was greatly influenced by specific plant community associations. Edel et al. (1997) showed that plant species could influence the strains of *F. oxysporum* populations, suggesting a relationship between plant and *F. oxysporum* strains.

Host-Pathogen interaction

Hyphal growth is nonlinear, dependent on substrate variation and other factors (Rayner, 1996) and is described as a pulsed or periodic phenomenon (Lopez-Franco et al., 1994). According to Dix and Webster (1995), the vegetative body of *Fusarium*, i.e. mycelia and hyphae, is dependent on nutrient availability at the periphery of growth where hyphae excrete enzymes that solubilize the nutrients in the substrate in advance of hyphal growth. Nutrient limitations cause less branching and faster growth at the mycelial tip, and fungi can alter their hyphal organization in response to a heterogeneous environment. The area of greatest metabolic activity occurs at the hyphal tip where the apical activity includes extension and secondary wall formation, synthesis of nuclei and mitochondria, secondary metabolism and vesicle formation (Cooke, 1971).

Fusaria penetrate plant tissue with modified hyphal structure that adheres to the cuticle on the plant surface. At the site of attachment appressoria are formed that develop high turgor pressure resulting in hyphal penetration. The penetrating hyphal tip then secretes cell wall–degrading enzymes to facilitate further penetration of both cuticle and plant cell wall (Mendgen et al., 1996). Many *F. oxysporum* isolates infect the plants vascular system causing wilt disease, whereas others infect root cortical tissue, causing root rot or bulb rot (Linderman, 1981; Kroes et al., 1998; Baayen & Forch, 2000). (Katan, 1999) noted that *F. oxysporum* f. sp. *lycopersici* causes wilt disease in tomato (*Lycopersicon esculentum*), and *F. oxysporum* f. sp. *radicis-lycopersici* root rot of tomato. *Fusarium* vascular wilt occurs when the hyphal growth penetrates through the non-differentiated

endodermis to the protoxylem, and into older xylem tissue, where microconidia are produced which enter the transpiration stream (Trujillo, 1963). Systemic vessel occlusion in response to the presence of microconidia and/or degradation of xylem tissue through mycelial growth then results in vascular wilt (Baayen & Elgersma, 1983; Baayen et al., 1997).

Garrett (1970) stated that infection of young roots was most likely to occur at the immature cortex of the apical region and less likely to occur in the mature parenchyma cells of cortex, pith or xylem. Eparvier & Alabouvette (1994) used GUS transformed strains of *F. oxysporum* and, after dipping the entire seedling in a fungal suspension broth of either a pathogenic or non-pathogenic isolate, found equivalent colonization; occurring during the first 24 hours on root hairs; growing throughout the root surface after the second 24 hours hyphae; but after 4 days inoculation nutrient starvation began and stained hyphae were present only at meristematically active regions of secondary root formation, where root exudate levels could support fungal growth. Bolwerk et al. (2005) investigated soil competition at the rhizoplane of pathogenic *F. oxysporum* f. sp. *radicis-lycopersici* and a non-pathogenic *F. oxysporum* isolate Fo47. The authors found that the non-pathogen level of spore germination was greater, which provided a competitive advantage in obtaining nutrients. They also indicated that even though the non-pathogen isolate attaches to the root surface more quickly, that only when large populations of the non-pathogen were present was pathogen colonization of the root reduced and even arrested at the initial stage of attachment.

Plant defense and host signaling

Root cortices are colonized by a variety of soil borne fungi, including the toxigenic genus *Fusarium* (Sivasithamparam, 1998). Plant compounds believed to be responsible for defense against rhizosphere microorganisms have historically been designated in one of two classes of molecules, phytoalexins or phytoanticipins. According to Paxton's definition "phytoalexins are low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms" (Paxton, 1981). Phytoanticipins are defined as are" low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents" (VanEtten et al., 1994). In some instances, a compound can be considered both, depending on the nature of the pathogen-plant interaction. VanEtten et al. (1995) hypothesized that detoxification of plant compounds by fungi was the exception. Fungi that infected host plant tissue as part of a latent phase were probably spatially restricted and unlikely to encounter plant phytoanticipins or phytoalexins. Although it is possible that a smaller sub-populations of fungi do interact biochemically with these compounds and many rely on the interaction as a signal for host compatibility.

In order for fungi to respond to chemical signals from a host plant tissue fungi require expression of a gene(s) associated with spore germination, hyphal growth, attachment to plant root surfaces, appressorial development and secretion of enzymes and phytotoxins (Knogge, 1996). Such a response activates signal transduction pathways, including G proteins, cAMP signaling (Bölker, 1998), and mitogen-activated protein kinases (Lee &

Dean, 1993). Steinberg et al. (1999) concluded that the recognition mechanism between *Fusarium* and plant occurred once the fungi had penetrated the host tissue, and that non-pathogen isolates of *Fusarium* were inhibited by various plant host defenses. Di Pietro et al. (2001a) identified a mitogen-activated protein kinase encoded by the *fmk1* gene in *F. oxysporum*. Mutants lacking functional copies of *fmk1* were not pathogenic to tomato roots. Spore germination occurred near the plant root but only wild type strains attached hyphae to the root surface. In contrast, the mutant strains were unable to attach to tomato root surface. Jain et al. (2003) reported that disruption of cDNA encoding the G protein, Gβ subunit FGB1 isolated from *F. oxysporum*, changed colony morphology, conidia formation, germination frequency, pathogenicity and intracellular cAMP levels. More recently Deng et al. (2006) used analysis of *F. oxysporum* sequence tag data and gene expression profiles and found that spore germination was dependent on expression of ras-related gene and clock control gene expression; that protein levels increased during germ tube elongation, but that as hyphal growth began ubiquitin gene expression, associated with proteins degradation, increased. What remains unclear is how the gene expression profile from *in vitro* data can be correlated with profiles exhibited during *in vivo* infection of plant root tissue.

As noted in cell culture experiments, plant alkaloid production and content are dependent on the morphological stage of the plant. Root development can be clearly divided into root tip, elongation zone, maturation zone and matured zone. Additionally the degree of vacuolization decreases in older, more mature regions of the root, while increasing in younger more meristematically active areas (Gilroy & Jones, 2000). The presence of

Hydrastis alkaloids as stored metabolites and the role of the berberine-bridge enzyme as an inducible metabolic branch point (Zenk, 1995; Bock et al., 2002) suggests a dual role in defense of plant tissue against soil microbes. Although datum is lacking on the relative levels of *Hydrastis* alkaloids in younger vs. older root tissue, it is likely that berberine, canadine and hydrastine are elicited products of the vacuolar berberine-bridge enzyme in younger root tissue and constitutively stored in older root tissue. Thus *Hydrastis* alkaloids could be considered both phytoalexins and phytoanticipins depending on which tissue was involved and the timing of the fungal-plant interaction.

Host Resistance

General resistance mechanisms can be associated with the presence of plant derived enzyme accumulation at the site of host-pathogen interaction, such as hydrolases, β-1,3 – glucanase, chitinase and peroxidase (Wessels & Sietsma, 1981; Asiegbu, 2000). Interestingly, Krebs & Grummer (1993) reported that while pathogenic *F. oxysporum* f. sp. *apii* induced plant hydrolases, the non-pathogen isolate *F. oxysporum* f. sp. *cepae* did not. Okubara & Paulitz (2005) also suggested that induction of jasmonate and ethylene defense genes act on root morphology during a pathogen attack, preventing root elongation and simultaneously activating root tissue defense responses.

Suleman et al. (1996) investigated the ability of 12 *F. oxysporum* f.sp *radicis-lycopersici* isolates to infect tomato (*Lycopersicon esculentum*). The isolates all varied in the degree of disease symptoms they caused. The authors reported that that fungal sensitivity to two antifungal compounds found in tomato, rishitin and tomatine, were dependent on the

Fusarium isolate and its growth stage. Spore germination was unaffected by either compound, whereas germ tube elongation was sensitive to both compounds. Growth rate of the mycelia was associated with fungal virulence and the growth response of the isolates to both compounds varied most for this characteristic. Rishitin treatment at concentration levels of rishitin found in tomato vascular and stem tissue inhibited mycelial growth. Higher levels of rishitin exposure were less inhibitory of mycelial growth and may reflect an "inverse toxicity" response. Exposure to tomatine did not significantly inhibit mycelial growth, although, the investigators noted that isolate tolerance to both compounds was a more accurate predictor of virulence than tolerance to only one.

Structural features of tomato xylem vessel elements may provide a rational for the difference in virulence at varied fungal growth stages. They lack perforation plates, necessitating movement of conidia in the xylem rather than hyphal growth as a major virulence factor in *Fusarium* closely associated with the host plant, since movement of conidia in the xylem are prevented. Thus limits on hyphal growth would be expected to have the greatest influence on virulence. The authors also pointed out that although tomatine is constitutively present in tomato root tissue, rishitin is induced and thus is present at a different time and stage of *Fusarium* development. Tomatine may act as an initial barrier to infection for nonpathogens, whereas rishitin would be necessary to limited established infections.

Rodriquez and Mendgen (1995) noted that in order to explore other nutrient sources after rhizoplane depletion, hyphae of both pathogenic and non-pathogenic *F. oxysporum* penetrate into root tissue to access nutrients in the cytoplasm and from the plant cell wall, which requires release of degradative enzymes. In differentiating between pathogen and non-pathogen, Di Pietro et al. (2001b) noted that deficiencies in various endopolygalacturonase enzymes did not alter virulence patterns. Plants in turn produce polygalacturonase-inhibiting proteins that limit the selective advantage (Cook et al., 1999). Several authors (Skovgaard & Rosendahl, 1998; Di Pietro et al., 2001c) (investigated the role of fungal proteases necessary to break down structural plant cell wall proteins and noted that targeted inactivation of subtilisin-like *Fusarium* proteinase *PrtI* did not change pathogenicity levels.

The plant defense compounds can also have direct effect on the levels of phytotoxic compounds produced by the fungus. Desjardins et al. (1988, 1989) reported that the presence of furanocoumarin phytoalexins in parsnip (*Pastinaca sativa*), at levels below those found in plant tissue, altered trichothecene production in *F. sporotrichiodes*. Trichodiene, a precursor molecule not normally detected in liquid culture of isolates producing trichothecenes, were detected after treatment of the fungus with parsnip furanocoumarins.

The ability of plants to metabolize mycotoxins or modify their production is an additional plant resistance mechanism. Miller & Young (1985) provided evidence that resistant wheat cultivars appeared to metabolize DON in field experiments. This was confirmed in

follow up research when DON was added to head blight resistant cultivar cultures, leading to DON breakdown products and glycosides that did not appear in the susceptible cultivar cultures (Miller & Arnison, 1986a; Miller & Ewen, 1997a). The researchers were unclear by what mechanism the alteration occurred. (Miller et al., 1997b). Bakan et al. (2003) investigated resistant corn cultivars and found that rather than metabolizing mycotoxins, a corn secondary metabolite, 4-acetyl-benzoxazolin-2-one (4-ABOA) inhibited production of DON and its biosynthetic precursors without inhibiting growth of *F. culmorum* or *F. graminearum*.

Host Specificity

Research on the signal exchange between plant and root pathogenic fungi has shown that root exudate can stimulate spore germination (Curl & Truelove, 1986; Nelson, 1991), with most of the material released at the root apex (McDougall & Rovira, 1970; Griffin et al., 1976) Rarely do pathogenic or mycorrhizal fungi infect this region of the plant root; the primary site of infection is at the zone of elongation (Baluska et al., 1996). Ruan et al. (1995) discovered that flavonoids induced spore germination in *F. solani*, and that this stimulus was partially blocked by a cAMP-dependent inhibitor of protein kinase A. Hawes et al. (2003) reported that the content of border cells at the root cap was accessible only to microorganisms able to recognize and respond to specific root signals. Using the *Pisum sativa* (pea)/*Nectria haematococca* model, Gunawardena et al., (2005) reported that spore germination and mycelial growth occurred in a host specific pattern in response to root cap and border cell exudate. Spore germination appeared to be stimulated by the flavonoid pathway described by Ruan et al. (1995) and was only found

in border cell exudate. They concluded that fungi do not necrotized border cells, but rather that the cells and the secondary metabolites associated with them modulated fungal growth to establishes a stable ecological relationship without loss of root function because of infection.

Leath & Kendall (1978) used four F. *roseum* [=*semitectum*] isolates ranging in pathogenicity to alfalfa (*Medicago sativa*) roots to study *Fusarium* host specificity within forage legumes. Their study correlated the presence of internal hyphae in root tips, root elongation and root rot symptoms with virulence of the 4 isolates. Host plant roots were more severely infected by their own isolates than isolates of non-host plant roots, however root growth was also stimulated by non-pathogenic isolates. Sutherland & Pegg (1992) reported that recognition of susceptible tomato host protoplast cell lines by *F. oxysporum* f. sp. *lycopersici* was based on the presence of metabolites in the cultural filtrate and that pathogenicity and recognition of susceptible cells lines was linked to a protein fraction within the fungal filtrate.

Experimentation to determine the role of plant defense compounds in fungal plant signaling provides conflicting evidence. Pisatin, a phytoalexin of *Pisum sativum*, induced expression of a pisatin demethylase gene (*pda*) in *F. solani* f. sp. *pisi*, thus detoxifying pisatin (Pueppke & VanEtten, 1974). Straney & VanEtten (1993) noted that the pisatin demethylase was glucose suppressed, suggesting that functionally detoxification was a means for providing nutrients directly from plant defense compounds. Ruan et al. (1995) theorized a different relationship between plant defense compounds and the fungus. They

reported that two pathogenic *F. solani* strains (f. sp. *pisi*, f. sp. *phaseoli*) not only adapted to the defense mechanism of the plant, but they also found that macroconidial germination was stimulated by specific plant flavonoids, thus acting as a plant host recognition signal. Steinberg et al. (1999) suggested that nitrogen, as a nutrient or secondary plant metabolite in plant root exudates stimulates mycelial development regardless of host plant status. Of note, several authors (Sanchez et al., 1975; Roldan-Arjona et al., 1999; Namiki et al., 2001) have suggested that fungal detoxification enzymes may be repressed when nutrients are limited, thus reducing pathogenicity of the isolate.

Genetic approaches are also providing evidence of novel plant responses to fungal pathogens. Mes et al. (2000) reported that a tomato resistance protein, encoded for by the *I-2* gene, was present in young lateral root primordial and near the vascular region. The author postulated that the resistance against *F. oxysporum* was based on the protein preventing fungal growth into the plant vascular tissue. Geraats et al. (2003) noted that an *Arabidopsis* ethylene mutant, *etr-1*, was tolerant of one *F. oxysporum* isolate, but that a tobacco mutant was not. Since ethylene is a general host defense signal, the authors suggested that pathogen-host factors might also play a role.

Endophytic association or latency?

Fusarium oxysporum is commonly isolated from plant roots that do not show symptoms of infection (Katan, 1971; Gordon et al., 1989; Aloi, 1993). Both Alabouvette (1979) and Gordon et al. (1989) noted that these non-pathogenic isolates colonize root cortices, and

but do not cause disease. They are unable to reach the vascular tissue or the plant response prevents systematic infection (Gao et al., 1995). The authors suggested that *Fusarium* endophytes protect host plants by suppressing vascular wilt caused by more pathogenic *Fusarium* isolates. Thus the host plant tolerates the presence of a nonpathogenic strain resulting in an endophytic association (Chapela & Boddy, 1988; Postma, 1992; Larkin et al., 1993; Rayner, 1996). Research suggests that avirulent *Fusarium* strains can promote plant growth (Mandeel & Baker, 1991) as well as out compete virulent strains (Whipps, 1997). Gordon & Martyn (1997) noted that *F. oxysporum* is predominantly nonpathogenic in native ecologies and that endophytic associations result in a limited die back of the host plant (Sinclair & Cerkauskas, 1996). There is evidence that even at high levels in native soils *F. oxysporum* does not always invade cortical tissue, since death of the plant host would limit growth of the fungus and also stimulate saprophytic competition from other microorganisms.

Schumann et al. (1990) reported that *F. oxysporum* endophytic development in the stem and root during latency periods varied with ecological conditions, soil characteristics and plant developmental stages. The endophytic state or latency may allow *F. oxysporum* to survive in the host plant but with a concomitant reduction in its saprophytic potential (Gordon and Martyn, 1997). If endophytic status is considered a form of latency, then by definition (Sinclair and Cerkauskas, 1996), latency is a stage in parasitic fungal infection, with host and fungi coexisting for a time without damage to the host plant. Thus latency increases the chance that the fungus will survive. Gordon et al. (1989) reported that *F. oxysporum* is the most common *Fusarium* endophyte of both wild and cultivated plants.

Several researchers reported that *F. oxysporum* isolates grew among border cells during initiation of endophytic development (Turlier et al., 1994; Rodriquez-Galvez and Mendgen, 1995; Kroes et al., 1998). Using transgenic GUS-marked hyphae that allowed visualization of metabolically active mycelia, Turlier et al. (1994) reported that GUS-marked *F. oxysporum* mycelia grew extensively on root tips and young lateral roots, both of which are covered by young root caps where border cells were sloughed off and exudate levels were abundant. High levels of root exudate and sloughed root cap cells did not occur in the proximity of more mature root surfaces. The authors pointed out only metabolically active mycelia were associated with infection of the plant. Since the apical and subapical zones of root tissue are undifferentiated the authors also suggested that movement into the vascular tissue of the mature root begins with the initial infection of protoxylem tissue.

Rodriquez-Galvez and Mendgen (1995) used high pressure freezing of *Gossypium barbadense* (cotton) root tissue infected with *F. oxysporum* f.sp. *vasinfectum* and electron microscopy to study the infection process. Conidia germinated 6 hours after they were placed in contact with 2 day-old cotton seedling roots. After colonizing the root surface the fungi began penetrating the root epidermis 24 hours after inoculation. The authors found a correlation between the density of penetration and the initial number of conidia. Once *F. oxysporum* microconidia had germinated, the fungus created a dense mycelium on the plant root surface. They found that the most abundant sites of early hyphal penetration occurred at the apical meristematic zone and that the mycelial density was

correlated with root tip exudate concentration. In the lateral root zone, the antifungal terpenoid gossypol is found in high concentration in the rhizodermis, limiting hyphal penetration of this tissue. The authors were unable to find evidence of plant cell necrosis in response to *F. oxysporum* infection and described the interaction of the early period of infection as an "endophytic phase". Baayen & Forch (2000) studied bulb rot of lilies by *F. oxysporum* using electron microscopy and reported a different response to hyphal penetration - plant cell wall in-growths occurred, accompanied by transfer cell formation as the fungus accumulated host assimilates. This stage has been referred to as being biotrophic since fungal haustoria had not yet penetrated into the plant cell. Once a critical mycelial mass had formed and degradative enzymes or toxic metabolites had damaged the plant cell walls, cell death resulted.

Freeman & Rodriquez (1993a) adapted a rapid screening technique using a continuous dip inoculation method and UV mutagenesis to recover a non-pathogenic mutant (*path-1*) of *F. oxysporum* (Freeman & Rodriguez, 1993b). The mutation occurred at a single locus. When compared to the wild type, mutant spore formation, appressorial formation and infection rates were equivalent; but the pathogenic *F. oxysporum* was modified to non-pathogenic endophyte. Redman et al. (1999) corroborated the data using a cucurbit pathogen *Colletotrichum magna*. In their study, the mutant endophyte was able to protect watermelon seedlings from infection by the wild type pathogen. They found that the *path-1* mutant infected plant rapidly mobilized a defense response. The authors designated this model as "endophyte-associated resistance", and suggested that because of the mutation at a single locus and the subsequent conversion to non-pathogen

endophyte status, the mutant should be expected to have an antagonistic relationship with the wild type isolate. Given the evidence of widespread endophyte status in *F. oxysporum* such mutations may occur fairly often. Kuldau & Yates (2000) summarized the literature on the endophytic state in *Fusarium* as being a normal stage of all plant-associated *Fusarium*, and that it may reflect an evolutionary characteristic of the genus that allows it to associate with such a wide host range.

Reproductive spores

In soil with low exogenous carbon, Fusaria produce chlamydospores to aid survival in a dormant state (Cochrane et al., 1963). The most prominent chlamydospore producers in *Fusarium* spp. are *F. solani* and *F. oxysporum* (Geiser, 2002). Soil inoculated with chlamydospores was more infective for *Fusarium* wilt than soils inoculated with conidia (Alabouvette et al., 1979), germination rates of chlamydospores were higher in the rhizosphere than non-rhizosphere soil and was associated with greater saprophytic activity and disease expression (Couteaudier and Alabouvette, 1990). They also reported that in a low nutrient soil environment *F. oxysporum* f.sp. *lini* chlamydospore formation was higher than microconidia formation. They hypothesized that chlamydospores have a lower metabolic rate that allows pathogenesis to occur more rapidly once dormancy is broken. Amir & Mahdi (1992) correlated *F. oxysporum* saprophytic development in disinfected soil to rates of mycelial growth and spore production. They reported that microconidia were determinant of saprophytic development whereas chlamydospores, macroconidia and mycotoxins had a greater influence on the persistence of the fungus in soils.

Elad (1985a, 1985b) reported that *F. oxysporum* chlamydospores in *Curcumis sativus* (cucumber) rhizosphere were inhibited in a linear fashion by the level of siderophore production from fluorescent pseudomonades. But adding iron to soil reversed the effect. They also noted that *F. solani* chlamydospores in the rhizosphere of *Phaseolus vulgaris* were not affected by siderophore production. Mondal et al. (1996) noted that different aqueous root extracts of different plant species from 10 day-old seedlings caused *F. solani* to produce chlamydospores rather than macroconidia. After 3 days, micro- and macroconidia formation were stimulated and chlamydospores began to form directly from germination tubes, conidia and hyphae, up until 7 days after treatment. Of all treatments, only tomato root extract was inhibitory to chlamydospore formation. The level of chlamydospores formed was correlated with levels of mycelia produced in the latter stages of the experiment, and the authors also suggested that the number and size of the chlamydospores formed in response to root extract or exudate could affect the level of virulence of the fungus.

Hebbar et al. (1997) demonstrated that specific *F. oxysporum* isolates had different levels of chlamydospore formation, but that evidence of a baseline temporal pattern of spore formation existed:for control without treatment microconidia formed after 2 days, macroconidia formed after 4 days and chlamydospores formed after 4 days. Schroth & Snyder (1961) found that *F. solani* chlamydospores germination was confined to a 1 mm area around growing root, particularly root tips of lateral and adventitious roots and that mature roots had little effect on chlamydospore germination. Mahakul et al. (1996)

reported that chick pea (*Cicer arietinum*) root exudate stimulated chlamydospore germination in *F. oxysporum* f. sp. *ciceris*. *Fusarium solani* f. sp. *pisi* chlamydospores germinated within 24 hours after exposure within 7 mm of germinating pea seedlings (Short and Lacy, 1974). Apparently nutrient levels in the rhizosphere were maintained at below the required levels for chlamydospore germination, which suggested that chlamydospores germinated as a result of some other plant root stimulant and not the level of nutrients in the soil.

***In vitro* growth characteristics**

Monitoring media pH changes is an important variable in accessing fungal response, such as chlamydospore formation, macroconidia germination or mycelial growth, to a treatment. Several authors reported that decreasing pH of the growth media had no effect on *F. oxysporum* chlamydospore formation in either solid or liquid media (Griffith, 1964; Loffler & Schippers, 1985; Hebbar et al., 1996). Sood (1996) reported that maximum chlamydospores after 7 days, at a pH 6 to pH 7. But, in a follow up study, Hebbar et al. (1997) noted that pH effects on three strains of *F. oxysporum* chlamydospore formation were not significant up until day 10 in liquid culture, but after then, increasing pH stimulated chlamydospores formation in the culture. Beyer et al. (2004) reported that *F. graminearum* macroconidia germination rates were equivalent at pH 3-7 in liquid culture. However, research on multi-celled macroconidia of *F. culmorum* (Chitarra et al., 2005) suggested that while extracellular pH increased, intracellular pH also increased and that ungerminated macroconidia had the lowest pH levels. Their measurements were taken with a 24-hour period and thus may not reflect the adjustments to media pH changes that

may have occurred with Beyer's study. Apparently, pH levels may have a more immediate effect on mycelial growth than on either chlamydospore formation or macroconidia germination. Grigoryeva et al. (2004) noted that growth of *F. oxysporum* mycelial fragments was suppressed at pH 3.0-4.0, whereas maximum mycelial fragment growth occurred at pH 7.0-8.0. Since fungi have been shown to alter gene expression to changes in pH levels (Penalva & Arst, 2004), growth response of a fungi at a lower pH may not occur until the surrounding pH is at more optimal levels.

Media carbon to nitrogen (C/N) ratios also effects fungal sporulation and growth. Oritsejafor (1986) noted that *F. oxysporum* sporulation occurred at all C/N ratios used in their study but declined at the higher ratios up to 120:1 C/N. In the same study, the type of spore formed was also affected by the C/N ratio, with low ratios favoring macroconidial production and high ratios favoring chlamydospore formation. The most favorable C/N ratio for both chlamydospore and macroconidia production was at 30:1. In addition, increasing nitrogen concentrations increased macroconidia while decreasing chlamydospore production in *F. oxysporum* (Loffler & Schippers, 1985) and *F. solani* (Schippers, 1972). Hebbar et al. (1997) noted that irrespective of the C:N ratios, liquid media with lower "utilizable carbon content" (aqueous soya bean hull fiber) produced greater levels of *F. oxysporum* chlamydospores than media with higher "utilizable carbon content" (potato dextrose broth). This supports the observation that low carbon stress is a stimulus to chlamydospore formation.

In other studies looking at various growth media with differing carbon levels, Osman et al. (1992) reported that Czapeks agar and potato dextrose agar (PDA) increased mycelial production by *F. oxysporum* when compared to growth in lower carbon media, such as Richards solution agar and Waksman's medium. Mycelial production was equivalent for both Czapeks and PDA, suggesting it was the result of higher carbon levels in comparison with the other media. However, Kurchenko et al. (1996) compared linear growth rate of 52 strains of *F. oxysporum* on 3 high carbon level agar media (malt, potato-glucose, and meal) versus 3 media with low carbon levels (Park, soil extract, Czapeks) and found no difference. Oritsejafor (1978) suggested that since *F. oxysporum* is able to utilize the wide range of carbohydrates found in solid and liquid media, they have the ability to grow rapidly on various media.

Use of liquid culture vs. solid culture may also affect fungal response. Hebbar et al. (1997) found that the level of dissolved oxygen in liquid media influenced the type of spore by *F. oxysporum*. As dissolved oxygen was increased, chlamydospores formation decreased and microconidia levels increased. The study also showed that low levels of dissolved oxygen increased mycelial growth and chlamydospores formation. Growth phases of liquid fungal cultures are designated as lag phase, unlimited or exponential phase, stationary phase and decline or idiophase. As nutrient levels are depleted, growth decreases leading to the stationary phase, which the fungus stays in as long as nutrients are available. Secondary metabolites are not usually produced during the lag or unlimited phase (Borrow et al., 1961; Bu'Lock, 1975; Righelato, 1975; Brown, 1991). Reduction in

total mass of the fungus follows the decline phase as endogenous carbohydrates are metabolized accompanied by cell death and autolysis (Martin & Demain, 1977).

Mycotoxins

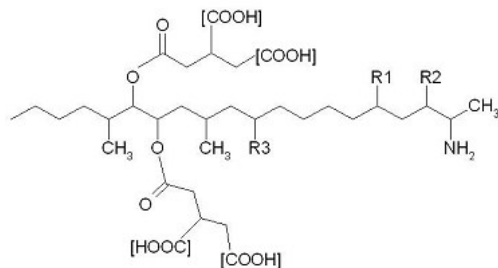
The Fusaria are prolific secondary metabolite producers (see Figure 7) and because of human and animals health risks associated with mycotoxin consumption, many strains of *Fusarium* have been designated important mycotoxigenic strains. Various *Fusarium* spp. produce mycotoxins that include several classes of compounds; the profiles of these compounds differ both within and between different species (Miller, 2002). The importance of these compounds as phytotoxins is less clear (Adams & Hart, 1989; Wakulinski, 1989; Desjardins et al., 1993; McLean, 1996). Abbas et al., (2002) noted that the toxicity of non-trichothecene mycotoxins to mammalian cell culture was considerably less compared to their phytotoxic activity towards the vascular plants *Lemna pausicosta* and *Pueraria lobata*. Phylogenetic relationships within *Fusarium* are not well characterized and a chemotaxonomic importance of mycotoxins is yet to be established. To date, eleven classes of mycotoxins have been identified which include:ennatins, fumonisins, fusaproliferin, fusaric acid, fusarins, fusarochromanone, moniliformin, naphthazarins, sambutoxin, trichothecenes and zearalenone (ZON) (Desjaardins and Proctor, 2000).

Cole et al. (2003) reported that *F. oxysporum* was capable of producing fumonisin and Marasas et al. (1984) reported that *F. oxysporum* was capable of producing the trichothecenes diacetoxyscirpenol and derivatives 7.8-dihydroxydiacetoxyscirpenol and

7-hydroxydiacetoxy-scirpenol, diacetylnivalenol, neosolaniol. *Fusarium oxysporum* was also capable of producing furanoterpenoids (dependent on host-pathogen interaction in sweet potato), fusaric acid and ZON (Marasas et al., 1984). Hermann et al. (1996) report enniatin production, and Kim et al. (1995) reported on sambutoxin producing isolates. Both studies also reported the presence of nectriafurone derivatives (fursarenone X) and moniliformin.

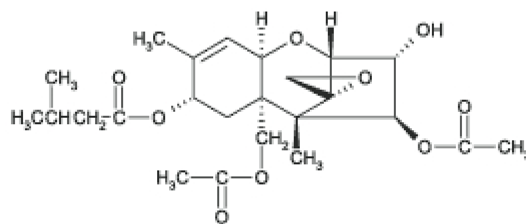
Figure 7:Structural formulas of *Fusarium* mycotoxins, fumonisin, T-2, zearalenone and deoxynivalenol

fumonisin B1-B4

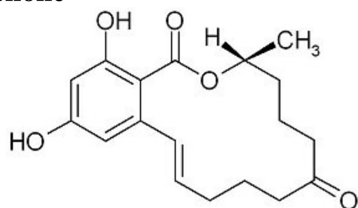


Fumonisin B_1:R1= OH; R2= OH; R3= OH
Fumonisin B_2:R1= H; R2= OH; R3= OH
Fumonisin B_3:R1= OH; R2= OH; R3= H
Fumonisin B_4:R1= H; R2= OH; R3= H

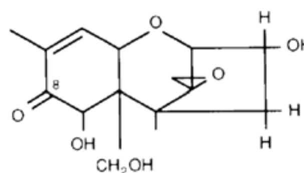
T-2 toxin (type A trichothecene)



zearalenone



deoxynivalenol (type B trichothecene)



Fusarium solani, in contrast, is reported to produce the trichothecenes T-2 toxin (3α-hydroxy-4β,14-diacetoxy-8α-[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene), neosolaniol and solaniol (Cole et al., 2003), as well as diacetoxyscirpenol, furanoterpenoids, androgens and naphthazarin-type compounds (Marasas et al., 1984). *Fusarium solani* also produces moniliformin (Rabie et al., 1982), enniatin (Hermann et al., 1996), and sambutoxin (Kim et al., 1995).

Fumonisins, as first reported by Bezuidenhout et al. (1988), produced by *F. verticillioides*, have been found in corn and rice crops. The molecule is a water-soluble polyketide with a 20-carbon backbone and an amino group at C-2. Of the various fumonisins, fumonisin B_1 occurs at the highest level (Norred et al., 1996). They also reported that fumonisin B_1 altered sphingolipid metabolism and was associated with

several animal mycotoxicoses outbreaks. Analysis of a *F. verticillioides* mutant that did not produce fumonisin had a lower level of virulence in corn compared with the wild type (Desjardins & Hohn, 1997).

The ZON compounds (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid lactone) are a complex of related estrogenic compounds and regulates sexual reproduction in *Gibberella zeae* (Mirocha & Swanson, 1982). ZON is produced by several *Fusarium* species, especially *F. graminearum* and *F. culmorum*. Maize contains more ZON than other cereal grains (Betina, 1989). Zearalenone is not strongly phytotoxic, nor highly toxic to animals, but can cause reproductive problems in animals (De Nijs et al., 1996).

Type-A trichothecene deoxynivalenol (12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-ene-8-one) and type-B trichothecene T-2 toxin (4ß,15-Diacetoxy-3α-hydroxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene) are part of a large class of tricyclic sesquiterpene toxins that are potential hazards to humans and animals. T-2 toxin is rarely isolated from intact cereal grain plants, but occurs in damaged grain and in grain dusts (De Mers, 1994). There are approximately 150 structurally related trichothecenes, mostly produced by *Fusarium*, as well as other genera, including *Myrothecium* and *Trichoderma* (Bean et al., 1984). Mechanistically, both phytotoxic and mycotoxic effects of these toxins are believed to be based on their inhibition of protein synthesis (Casale & Hart, 1988; Miller, 1989; Desjardins et al., 1993).

Cultural factors effecting mycotoxin production

Only the terminal mycelial cells of *Fusarium* produce mycotoxins while the amount of mycotoxin produced are usually a function of the total biomass of mycelia (Miller & Blackwell, 1986b). Several researchers (Borrow et al., 1961; Bu'Lock, 1975; Righelato, 1975 and Brown et al., 1991) noted that fungi growing in liquid culture produced maximum secondary metabolite at the end of the growth phase. Greenhalgh et al. (1986) reported that a number of cultural conditions influenced mycotoxin formation by *Fusarium*, included pH, osmotic tension, temperature, oxygen levels and water. Chemical composition of growth media can have a significant effect on trichothecenes production (Miller et al., 1983; Thrane, 1986). Fumonisin production by *F. verticillioides* and *F. proliferatum* was stimulated by both the addition of flavanoid antioxidants to the agar and water stress (Reynoso et al., 2002). Both T-2 toxin and ZON are produced in liquid culture at high levels of oxygen and sugar (Ueno et al., 1975; Hidy et al., 1977). Miller and Greenlalgh (1985a) reported that *F. graminearum* was very sensitive to substrate components as indicated by the levels of DON, 3-DON and ZON produced. Bukovcakova et al. (1988) found that *Fusarium* growing on ground wheat synthesized more ZON than when cultivated in liquid medium. Kim et al. (2005) reported that the production of ZON by *Gibberella zeae* requires two non-reducing polyketide synthase genes (PKS). The PKS genes are structurally similar to those involved with the biosynthesis of other fungal metabolites aflatoxin, lovastatin and fungal melanins. They also noted that when *G. zeae* grown on solid substrate such as rice it produced more ZON than when grown in liquid media. In general, they found that cultural conditions of low

carbon, nitrogen or phosphorous limited transcription of the PKS genes and that maximum transcript expression occurred at pH 4 in the cultural media.

Of note, Rosenberg et al. (1976) screened thirty fungi, including *F. moniliforme* [*F. proliferatum*], *F. nivale* and *F. semitectum*, and were able to detect primary, secondary and tertiary alkaloids in 9 of the fungal media filtrates and in 4 of the hyphal samples. No quaternary alkaloids were detected. Only *F. proliferatum* had detectable levels of alkaloids in the filtrate.

Environmental factors affecting mycotoxin production

Cuero et al. (1988) found that ZON production in *F. graminearum* decreased with decreasing temperatures. Also the presence of *Aspergillus flavus* did not affect toxin production. Ramakrishna et al. (1996) found that increased seed infection by *F. sporotrichiodes* and cfu formation was correlated with increased T-2 production in pure culture. But, there was no correlation when *F. sporotrichiodes* was grown with other fungal species (*Aspergillus flavus, Penicillium verrucosum,* and *Hyphopichia burtonii*) in the same media. *Fusarium sporotrichiodes* colonization decreased as did T-2 production. They suggested that competition for substrate was responsible. In comparison, Miedaner et al. (2004) used mixed inocula of toxigenic *Fusarium* and found that the type of mycotoxin or level of mycotoxins produced were not indicative of the competitiveness of the individual isolates. Thus it remains unclear what role mycotoxin production plays in competition for resources and pathogenicity. Also, it is unclear what benefit is gained by

producing a variety of mycotoxins. Hestbjerg et al. (2002) suggested that place of origin of the isolate also affected ZON production in five *Fusarium* spp. and that mycotoxin production should be considered the result of evolutionary selection pressure. They found significant intraspecies variation in the production of mycotoxins in chemotypes of both *F. culmorum* and *F. equiseti* isolated from different field locations. They pointed out that the greater diversity of trichothecene profiles among strains of *F. equiseti* might also be responsible for its low level of pathogenicity.

Fumonisin is produced in corn under conditions of drought stress and high insect activity, conditions that also promotes high disease levels (Schaafsma et al., 1993). Fumonisins cause both electrolyte loss in cells and interferes with formation of complex phytosphingolipids which results in reduced corn radicle elongation and seed amylase inhibition (Doehlert et al., 1994). They also found that when low and high fumonisin producing strains were inoculated simultaneously, only high fumonisin producing strains caused significant stem rot. Miller (2002) suggests that fumonisin is only produced by *F. verticillioides* in high amounts in senescent corn.

Role of mycotoxins in pathogenesis

Bean et al. (1984) suggested that trichothecenes were an important factor in pathogenesis of muskmelon by *Myrothecium roridum*. Their work was supported by findings of Kuti et al. (1989), who noted a linear relationship between tissue electrolyte leakages in muskmelon cells with macrocyclic trichothecene levels and suggested that such leakage

could be used to assay the phytotoxicity of various macrocyclic trichothecenes. Other researchers have also suggested that trichothecenes play a role in plant pathogenesis (Wang & Miller, 1988; Eudes et al., 2000). Desjardins and Plattner (2003) used *G. zeae* and transformation-mediated disruption of TR15 (trichodiene synthase gene) resulting in mutants unable to produce DON or its biosynthetic precursors. When macroconidia of these mutants and non-mutants were injected into wheat and corn the mutant isolates produced significantly less head blight in either crop.

Research on immunolocalized DON trichothecenes, and its precursors 3-ADON and 15-ADON, using infected wheat spikes (Kang & Buchenauer, 1999) found a relationship between toxin production and pathogenicity. Their results suggested that the toxin could diffuse into host tissue from the cell surface before physical invasion of the host tissue by the fungus and that during the early infection stage, toxins were within host cell cytoplasm ribosomes and the endoplasmic reticulum. Bean, in unpublished data (1999), also used immunochemical techniques to evaluate whether *Z. mays* embryo root tip growth would be stimulated or inhibited after exposure to *Fusarium* toxins. They found that ZON generally stimulated growth, whereas DON and a ZON/DON combination inhibited growth. Additionally they reported that DON-treated tissue contained abnormal cytoplasmic mitochondria and vesiculation/whorling of mitochondrial cristae, which probably resulted from uncoupled oxidative phosphorylation or changes in carbohydrate metabolism within the cell.

Vianello et al. (1978) noted that ZON interfered with pea cellular tonoplast membrane integrity as indicated by cell leakage of electrolytes, amino acids and β-cyanin. Vurro et al. (1997) found ZON and fumonisin inhibited poplar cell suspension culture growth and phenylalanine ammonium lyase (PAL) activity. Macri et al. (1978, 1996) found that ZON acted as protonophore in both animal and plant cells, eliminating the transmembrane potential associated with NADH oxidation. Corn suspension cultures metabolized ZON to less toxic metabolites, including β-D glycoside, α-zearalenol and β-zearalenol (Zill et al. 1990). Research has also indicated a possible role for ZON in regulating plant development, such as short-day induction of *Lemna* spp. growth, development and flowering (Han & Meng, 1991), initiation and development of *Nicotiana tabacum* flower buds (Fu et al., 1995) and vernalization in winter plants (Meng et al., 1992).

Mycotoxin detection methods

The 12,13 epoxy trichothecenes, type-A DON and type-B T-2 toxin are closely related structurally, but can be differentiated physiochemically by the increased polarity of T-2. Clean up procedures and analytical techniques differ for each mycotoxin. Trichothecenes as well as fumonisins require UV derivatization for detection. Methods for detecting T-2 toxin include HPLC-MS (Berger et al., 1999), GC-MS (Tanaka et al., 2000; Perkowski & Basinski, 2002), LC-MS (Razzazi-Fazeli et al., 2003) and ELISA (Usleber et al., 1992). ELISA and GC-MS T-2 toxin detection methods have been compared (Yoshizawa et al., 2004) with ELISA methods found to be highly sensitive, reproducible and accurate. Methods for detecting fumonisins include HPLC-UV (Scott & Lawrence, 1992; Ho &

Durst, 2003), HPLC-ESI/MS (Musser et al., 2002) and ELISA (Sydenham et al., 1996; Christensen et al., 2000). ZON have been detected using HPLC-UV (Joseph et al., 2001; Krska et al., 2003); HPLC using fluorescence and photodiode array detection (Mateo et al., 2002); liquid chromatography - atmospheric pressure chemical ionization LC-APCI/MS (Pallaroni et al., 2002); and LC-MS/MS (Sypecka et al., 2004)and ELISA (Park et al., 2002; Nuryono et al., 2005).

CHAPTER 2:AIM AND SCOPE

Goldenseal (*Hydrastis canadensis* L., Ranunculaceae) is an herbaceous perennial that is distributed in North American under mesic cove forest canopies throughout much of the south and eastern seaboard north into Canada. The rhizome, rootlets and root hairs produce medicinally active alkaloids berberine, canadine and hydrastine (Wagner et al., 1984; Gocan et al., 1996). Indigenous use of *Hydrastis* by Native Americans was widespread for a variety of infectious diseases (Moerman, 1986). Among Eclectic physicians at the turn of the 20th century the rhizome and rootlets was used as an anti-microbial treatment, for ulcers, skin infections, conjunctivitis, otitis media and urinary tract infections (Felter & Lloyd, 1898), which is also how the medicinal plant is used today. Research has found that the major isoquinoline alkaloids have antimicrobial activity against human bacterial pathogens (Gentry et al., 1998; Scazzocchio et al., 2001; Hwang et al., 2003; Mahady et al., 2003; Villinski et al., 2004) and interact with various cell receptors and enzymes *in vitro* (Huang & Johnston, 1990; Sun & Li, 1993; Lee et al., 1997; Wu & Jin, 1997; Baldazzi et al., 1998; Cometa et al., 1998; Abdel-Haq et al., 2000; Cao et al., 2001).

However, *Hydrastis* alkaloids collectively have not been investigated for their anti-fungal activity, although berberine alone has been investigated for activity against several fungal species (Farr et al., 1989; Vollekova et al., 2001), including *Alternaria*, *Aspergillus*, *Aureobasidium*, *Candida*, *Mucor*, *Penicillium*, *Rhizopus*, *Scopulariopsis*, *Trichoderma*, and *Verticillium*. Studies have indicated that berberine does have antimycotic activity against *Fusarium* (Mahajan, 1986; Mahakul et al., 1996; Sarma et al., 1999; Singh et al.,

2001; Cernakova & Kostalova, 2002). Although the isoquinoline alkaloid biosynthetic pathway for *Hydrastis* has not been elucidated research from other berberine producing plants with berberine as an metabolic endpoint suggests that *Hydrastis* alkaloids are synthesized and stored in the vacuole (Zenk, 1985; Tabata, 1991). Additionally, the berberine-bridge enzyme involved in the biosynthesis of berberine is inducible in several plant species (Zenk, 1985; Bock et al., 2002), which would suggest that berberine, canadine and hydrastine produced in the root tissue may be elicited in response to interactions with soil fungi.

In a similar vain, fungi that are part of the *Hydrastis* rhizosphere ecology and in proximity to root exudates may have adapted to the presence of these plant defense compounds. Isolation of fungi from and microscopic examination of *Hydrastis* rhizoplane, rhizosphere and non-rhizosphere soil, as well as from *Hydrastis* seed, rootlet, rhizome, stem and leaf tissues was performed to understand the community of fungi associated with *Hydrastis*. Increasingly more of the raw material for medicinal plant use originates from cultivated rather than from plants gathered from their native settings (wildcrafted). Our understanding of reciprocal rhizosphere influence on formation of clinically relevant plant secondary metabolites is woefully inadequate. Given a stable rhizosphere soil of a native forest setting in which *Hydrastis* is normally found, are the fungal species more or less likely to form pathogenic or endophytic associations with the root tissue? Does the plant root exudate have a selective effect on the composition of fungal community associations? And does that selective effect reciprocally alter the ratios and makeup of the clinically relevant plant metabolites? As an initial step my study

focused on understanding the nature of *Hydrastis* root alkaloids selective effect on rhizosphere fungi.

The design of the assay was influenced by an attempt to model how *Fusarium* and *Hydrastis* alkaloids might interact. As suggested by studies noted earlier, an assumption can be made that *Hydrastis* alkaloids are elicited compounds. Although stored in the cell vacuole, was there a mechanism to move elicited alkaloids from the vacuole to the rhizoplane and into the root exudates? Evidence from plant cell culture experiments suggests that the alkaloids are actively secreted. Tabata (1991) reported that a selective uptake mechanism operated in the tonoplast membrane of berberine producing cell lines of *Thalictrum minus* and *C. japonica* that allowed the cells to accumulate exogenous cultural berberine. Yamamoto et al. (1986) noted that specific berberine producing cell lines of *T. minus* secreted berberine into culture medium via an active transport system. Maddox et al. (1999) developed a mass *in vitro* nodule culture of *Hydrastis* and reported the presence of berberine in the media that originated from the nodule culture. In field experiments, J. Davis (personal communication) noted that ginseng (*Panax quinquefolius)* planted in beds that previously contained goldenseal were less likely to show disease than ginseng plants grown in locations where goldenseal had not been grown. It is uncertain if this was related to *Hydrastis* alkaloids possibly being released when goldenseal root tissue was damaged during harvest; or if the alkaloids had been released naturally in root exudate during plant growth, altering the rhizosphere ecology. Thus the assay design reflects the assumption that berberine, canadine and hydrastine

appear to be stored in the vacuole as well as elicited, and are present in *Hydrastis* root exudates.

Assuming that the alkaloids are present in the exudates, a model for the interaction between *H. canadensis* alkaloids and *Fusarium* suggests that the most likely point of contact would occur at the host plant root interface within the rhizosphere (rhizoplane). A bioassay was then developed to study the effects of *Hydrastis* isoquinoline alkaloids on three *Fusarium* species. Isoquinoline alkaloids at levels comparable to those detected in the plant (Genest & Hughes, 1969; Betz et al., 1998; Gentry et al., 1998; Tims et al., 2000) were added to liquid culture media either singly or combined in one treatment. A whole root extract was used as the treatment model for the root exudate. Mycelial growth and levels of reproductive spores produced (microconidia, macroconidia and chlamydospores) were evaluated quantitatively to determine whether *Hydrastis* isoquinoline alkaloids were stimulatory or inhibitory to mycelial growth, spore formation and macroconidial germination of the three *Fusarium* species. Production of the mycotoxins fumonisin, T-2 toxin and ZON in response to the different alkaloid treatments was also investigated to discover if the presence *Hydrastis* alkaloids in the culture media would affecting the production of *Fusarium* mycotoxins.

The study also investigated if the effects of the treatments were associated with the additive or synergistic interactions of two or more of the *Hydrastis* alkaloids. This necessitated the development of an analytical method to detect *Hydrastis* alkaloids. Since *Hydrastis* is a popular as well as an expensive medicinal plant, and there is growing

concern of the use of other adulterant plants in *Hydrastis* retail formulations, a method was developed to simultaneously detect *Hydrastis* and adulterant alkaloids, from plant species including *Coptis japonica*, *Xanthorrhiza simplicissima*, *Mahonia aquifolium*, *Chelidonium majus*, and *Berberis* spp.

Using three different *Fusarium* species, each with different levels of association with *Hydrastis* root tissue and rhizosphere, allowed the study to explore whether the level of fungal plant association can be distinguished based on measuring the range of fungal responses to the alkaloid treatments. If one isolate has adapted to the presence of alkaloids in the root exudates, it would certainly gain an advantage over competing soil flora and thus be more likely to form a closer association with *Hydrastis* root tissue. The results of the assay may then provide evidence as to whether a *Fusarium* endophyte is more responsive to compounds in the root exudates than other pathogenic isolates.

CHAPTER 3:MATERIALS AND METHODS

Fungal isolation

A. Seed isolation

Prepared media and reagents were purchased from Sigma (St. Louis, MO). Three media were prepared, Czapeks Dox medium containing antibiotics, carnation leaf agar (CLA) and water agar containing antibiotics. After Czapeks medium had cooled, penicillin G (10,000 I.U./ml) and streptomycin (10,000 μg/ml) were added to equal 0.02%. Water agar plus antibiotic medium was prepared by adding similar levels of the two antibiotics prior to pouring the media. Carnation leaf agar (CLA) was prepared by sprinkling sterile dried carnation leaf on the surface of water agar (1.5%) as it cooled.

Hydrastis canadensis seeds from native settings (05001AS, see Appendix) and cultivated (Richo Cech, Horizon Herbs, PO Box 69 Williams, OR 97544 USA) either whole or halved were treated with two sterilization protocols - 10% Chlorox for 3 minutes or 10% Chlorox for 1 minute. Ten seeds and ten half seeds from each sterilization treatment were then plated on the three media in triplicate. After 5 days at room temperature transfers were taken from seed that yielded fungal growth and plated onto potato dextrose agar (PDA) culture tubes. After 7-10 days the fungi were identified.

B. Plant tissue isolation

Hydrastis canadensis tissue, including leaf, stem, rhizome and rootlet were surface sterilized with 10% Chlorox for 1 minute and plated on water agar (1.5%). Isolates were continuously transferred by hyphal tip until pure culture was growing. Cultures that appeared to be *Fusarium* were speciated by Jean Juba of the *Fusarium* Research Center, Department of Plant Pathology, Pennsylvania State University and maintained on CLA at room temperature.

C. Soil isolation

Two soil samples were made where adult *H. canadensis* plants were collected. Approximately 10 grams of soil were collected from soil adhering to the rootlets of *H. canadensis* plants. The soil was designated rhizosphere soil (RS). The second collection was from a nearby site devoid of *H. canadensis* or any other herbaceous plant. This soil was designed non-rhizosphere soil (NRS).

To isolate fungi from the RS and NRS samples one hundred mg of soil was added to 400 ml of sterile distilled water and shaken on a rotary shaker for 30 minutes. Four dilutions of the slurry were prepared (10^{-1} – 10^{-4}) and 0.1 ml of each dilution was added to the 3 isolation media. After 3 days at room temperature, fungal growth was transferred to PDA tubes for growth and identification.

Seeds of radish and corn were also added directly to both soil samples to isolate pathogenic fungi. After 14 days corn seeds were plated on water agar containing penicillin G and streptomycin. Fungal growth was observed and transfers made. After 21 days incubation at room temperature germination rate and hypocotyls growth of radish plantlets was collected and their rootlets were plated on the same media and fungal growth observed.

D. Detection of arbuscular mycorrhizae (AM)

Hydrastis canadensis root tissue was examined for the presence of arbuscular mycorrhizae (AM) using a modified method (Koske & Gemma, 1989). Roots pieces approximately 5 mm long were soaked in 5% potassium hydroxide for 1 hour, rinsed in sterile distilled water followed by soaking in 3% H_2O_2 for 15 minutes, rinsed in sterile distilled water and placed in 50 ml of 1% hydrochloric acid for 24 hours. The sections were stained for 24 hours in 0.1% trypan blue and acidic glycerol (500 ml glycerol, 450 ml H_2O, 50 ml 1% HCl). Tissue was destained with water and stored in acidic glycerol until microscopic examination.

Using a method developed by Giovanetti and Mosse (1980) to detect spores of AM fungi in the soil, rhizosphere and non-rhizosphere soil was sieved through screens (45 um and 38 um). Material collected on the screens was examined microscopically for the presence of AM spores.

Hydrastis tissue was also examined directly for the presence of non-AM fungal growth. Root tissue was first dipped in 95% ethanol and flamed briefly. The root was cut into 5 mm sections, lactophenol blue (to detect filamentous growth) added and the sections examined microscopically at 40X. Seeds were stripped of their pericarp and directly stained and tissue examined microscopically.

E. Phylogenetic analysis of PCR amplified *Fusarium* TEF-1α

David Geiser and the Fusarium Laboratory at Pennsylvania State University performed *Fusarium* DNA extraction, PCR amplification of the translation elongation factor 1-alpha (TEF) and phylogenetic sequence analysis using reported methods (Chenna et al., 2003; Geiser et al., 2004).

Bioassay development

A. Stock *Hydrastis* extraction and standards

All *Hydrastis canadensis* tissue was air-dried and finely ground (20-mesh) using a Wiley mill. A 100 mg sample of root and rhizome were defatted with 50 ml n-heptane for 2 hours on a Labline Orbital Shaker at 100 rpm; dark brown bottles were used to prevent photo-degradation of isoquinoline alkaloids present in the plant tissue. The samples were filtered (Whatman #1) and the filtrate allowed to dry for 2 hours. Fifty ml methanol (95%) or 50% methanol:1% acetic acid (for the last liquid assay) was added to the sample and placed on a shaker (100 rpm), 25° C, 24 hours. The sample was filtered (Whatman

#1) and the mark (extracted plant material) re-extracted twice, until no alkaloids were detected with HPLC. The three filtrate samples were filter sterilized (Nalgene 0.2µm), evaporated and stored in a dark bottle at 5° C. A voucher specimen of *Hydrastis canadensis* (#05001ARZ) is stored in the Norton Brown Herbarium, University of Maryland, College Park, MD 20742. Botanical identification was performed in the field according to Gleason and Cronquist (1991).

Alkaloid standards berberine dihydrochloride (Chromadex, Santa Ana, CA), canadine (Chromadex, Santa Ana, CA), hydrastine hydrochloride (Sigma-Aldrich, St. Louis, MO), hydrastinine hydrochloride (Sigma-Aldrich, St. Louis, MO)) were stored in desiccators at 5° C. The identity of these compounds was verified by HPLC-MS, but peak purity measurements were not made. Stock solutions were prepared and sterilized with a 0.45µm filter (Nalgene). The concentration of each standard stock solution was as follows:berberine 0.12% (120 mg/100 ml), canadine 0.02% (20 mg/100 ml) and hydrastine 0.08% (80 mg/100 ml).

To construct a concentration curve for *Hydrastis* alkaloids, triplicate standards were prepared at the following concentrations for each alkaloid; berberine (0.24 mg/ml, 0.18 mg/ml, 0.12 mg/ml, 0.06 mg/ml, 0.02 mg/ml); canadine (0.04 mg/ml, 0.01 mg/ml, 0.02 mg/ml, 0.01 mg/ml, 0.004 mg/ml); hydrastine (0.16 mg/ml, 0.12 mg/ml, 0.08 mg/ml, 0.04 mg/ml, 0.20 mg/ml).

For the HPLC-MS adulterant analysis alkaloid standards were obtained; berbamine dihydrochloride, coptisine chloride, jatrorrhizine iodide (Chromadex, Santa Ana, CA): and chelerythrine chloride, palmatine chloride, sanguinarine chloride (hydrate) (Sigma-Aldrich, St. Louis, MO). The identity of these compounds was verified by HPLC-MS, but peak purity measurements were not made. Stock solutions were prepared and sterilized with 0.45μm filter (Nalgene). The concentration of each standard stock solution was 1.0% and for analysis a mixed standard solution was prepared for each standard at the concentration of 0.05 mg/ml. Powdered *Hydrastis canadensis* L. and *Berberis vulgaris* L. were purchased from Penn Herb Co. Ltd. (Philadelphia, PA). *Coptis japonica* Makino was purchased from Nuherbs Co. (Oakland, CA). Wild collection of *Hydrastis* came from Rutland OH. The powered herbs were not vouchered samples. Then 200 mg of each powdered plant material was sonicated two hours in 100 ml of 95% methanol. The liquid was decanted and filtered through a 0.45μm filter. Filtrate was diluted in mobile phase before injection. Ten mg each of powdered adulterating plant samples (*C. japonica* and *B. vulgaris*) were combined with 190 mg of *H. canadensis* and prepared as above. The fungal filtrate was re-suspended to original volume with mobile phase.

B. Solvent selection assay

The selection assay consisted of three treatments, 95% methanol, 50% aqueous methanol + 2% acetic acid, 50% methanol + 1% acetic acid and sterilize distilled water. A 0.1% water agar suspension media was prepared by melting 50 mg bacterial agar in 50 ml distilled water. Three pieces of carnation leaves with a 7 day-old *F. oxysporum*

(*Hydrastis*) isolate of CLA culture were added into the agar suspension. The mixture was then rapidly stirred to create a uniform spore suspension and the carnation leaves removed. One ml each of the fungal suspension and solvent were added to sterile 25 ml Czapeks Dox liquid media in 125 ml Erlenmeyer flasks. The inoculated flasks were wrapped in aluminum foil, placed on an orbital shaker and incubated 4, 7, 9 and14 days. Mycelial growth was filtered (Whatman #1), collected in pre-weighted vials, lyophilized and dry weight determined.

C. Preliminary fractionation method

Hydrastis canadensis root tissue isolate #900ARZ (Appendix) was dried and finely ground (20-mesh). Two 200 mg samples of *Hydrastis* were defatted with 400 ml n-heptane for two hours on an orbital shaker (100 rpm) in dark brown bottles and filtered (Whatman #1). One sample was extracted with 400 ml methanol (95%) and the other with 400 ml ethanol (95%) on a shaker (100 rpm), 25° C and 4 hours. The extracts were filtered, evaporated under nitrogen stream and stored at 5° C.

Three separations were run. The two plant samples were resuspended in aqueous methanol (40%) and aqueous ethanol (40%). Higher alcohol levels did not separate from the organic phase, creating an emulsion. A control treatment (40% methanol) was included to insure that trace contaminants were not being concentrated during the fractioning. The samples were adjusted to pH 3 with 1% glacial acetic acid.

Samples were then partitioned with anhydrous diethyl ether. The diethyl ether was first run through a Woelm column (aluminum oxide) to eliminate reactive peroxides and then filtered thru sodium sulfate to eliminate water. A glass column with a stopcock and solid filter at the base was packed with aluminum oxide and glass wool at the top. The ether was then eluted from the Woelm column on to a Buchner funnel containing a layer of sodium sulfate on Whatman #1 filter paper and collected in a Kimax screw top bottle. The lower aqueous layer was partitioned with chloroform (basified) with 5% ammonia hydroxide, pH 10. The aqueous top layer was adjusted to pH 4-5. The organic fractions (ether, chloroform) were dried under nitrogen in pre-weighted vials. The azeotropic aqueous alcohol mixture was dried using a Haaslebuchler rotoryevaporator with an iced cooled water loop. Before evaporation was complete the mixture was resuspended in 100% methanol in pre-weighted vials, dried under nitrogen, weighed and stored at 5° C.

TLC plates (Silica gel 60 F_{254} –precoated TLC plates, 10x10 cm, 200 μm thick, Merck, Darmstadt, Germany) were spotted with the ether, chloroform, and aqueous alcohol fractions from each sample. The plate was developed in n-propanol and formic acid (90:1) and, after drying, was sprayed with dragendorf reagent (Wagner et al., 1984) to detect alkaloids present in the fractions. Berberine, canadine and hydrastine were run simultaneously as standards.

E. Spore counts

Two pieces of carnation leaves from a CLA fungal culture were put into a sterile capped test tube with 25 ml of sterile distilled water. The test tube was vigorously stirred to

obtain even spore suspension. Spore concentrations were determined with a Bright line haemocytometer.

E. Growth rate study - solid media

The influence of *Hydrastis* isoquinoline alkaloids on growth rate of *Fusarium* spp. was investigated in a bioassay disc study. A dilution of 0.2% berberine, canadine, hydrastine, as well as a *Hydrastis* root extract (#07991BZ) was prepared with methanol (95%). The center of a Petri dish containing Czapeks solid media was inoculated with an unknown fungus (07991A-3) isolated from *Hydrastis* and *Fusarium oxysporum* (PSU O-1042). Bioassay disks (Shleicher and Schuell #740-E) received 80 ul each of the four-alkaloid containing solutions, methanol (95%) the control. The bioassay discs were dried for 4 hours to eliminate methanol and 4 disks were placed equidistant from each other on each plate. After ten days incubation in the dark, the radius of the inhibition zone around each disk was measured. In the final growth inhibition study on solid media the four alkaloids were added directly to the medium at a concentration of 0.28 mg/ml of media prior to inoculation. The medium used was PDA and the 3 *Fusarium* isolates were 5001AR, PSU 0-1174 and 9001ARC. After 1, 3 and 5 days incubation in the dark, colony diameters were measured. A comparison of the two solid media assays is found in Table 1.

Table 1:Comparison of two solid media bioassays

SOLID Assay	1st	Conc.	2nd	Conc.
	Disks (mg/ml)	(mg/ml)	Treatment (mg/ml)	(mg/ml)
Date	2/1/01		3/1/03	
Extract	2.000	2.0000	2.000	0.0280
Berberine	0.040	0.0400	0.120	0.0168
Canadine	0.040	0.0400	0.010	0.0001
Hydrastine	0.040	0.0400	0.080	0.1080
Application (ml)	0.080		0.280	
Inoculum (ml)	na		na	
Media	Czapeks		PDA	
Volume Media (ml)	na		20	
Hydrastis isolate	07991BZ		500ARZ	
Fungal isolate	F. *oxysporum* (Goth)		*F. oxysporum* 5001AR	
	Unknown 07991ARZ-1		*F. commune* PSU-1174	
			F. solani 9001ARC	
Duration	10 days		5 days	

F. Growth rate study - liquid media

In these studies 125 Erlenmeyer treatment flasks were wrapped in aluminum to prevent photo degradation of isoquinoline alkaloid treatments. After varying periods of time the hyphae was removed by filtration, lyophilized and dry weight determined. Filtrate was also collected, lyophilized and stored at 5° C. A comparison of all liquid media assays can be found in Table 2.

Table 2:Comparison of three liquid media bioassays

Date	7/00		7/00		7/01		7/02		7/03	
LIQUID Assay	1st	Conc.	1st	Conc.	2nd	Conc.	3rd	Conc.	4th	Conc.
	mg/ml	mg/ml media	mg/ml	mg/ml media	mg/ml	mg/ml media	mg/ml	mg/ml media	mg/ml	mg/ml media
Extract	1.0000	0.0400	2.0000	0.0800	2.0000	0.1333	2.0000	0.0800	2.0000	0.0800
Berberine	0.0500	0.0020	0.1000	0.0040	0.0800	0.0053	0.0400	0.0016	0.1200	0.0048
Canadine	0.0100	0.0005	0.0200	0.0010	0.0200	0.0013	0.0300	0.0012	0.0200	0.0008
Hydrastine	0.0500	0.0020	0.1000	0.0040	0.0200	0.0007	0.0100	0.0004	0.0800	0.0032
Application (ml)	0.6000		0.6000		1.0000		1.0000		1.0000	
Inoculum (ml)	0.2000		0.2000		0.2000		1.0000		1.0000	
Media	Czapeks Dox		Czapeks Dox		Czapeks Dox		Czapeks Dox		Czapeks Dox	
Media volume (ml)	15		15		15		25		25	
Hydrastis isolate	07791ARZ		07791ARZ		10991ARZ		5001ARZ		5001ARZ	
Fungal isolate	*F. proliferatum* Goth		*F. proliferatum* Goth		*F. proliferatum* 07991BR		*F. oxysporum* 5001AR		*F. oxysporum* 5001AR *F. oxysporum* O-1174 *F. solani* 12012R	
Duration (days)	14		14		3,7,14		7,14		1,2,3,4	

In the initial study, the following treatments were prepared in 95% methanol:0.1% and 0.2% berberine, canadine, hydrastine, a standard mix of all 3 alkaloids:0.01% and 0.02% *Hydrastis* rhizome extract (7991BZ) with methanol (95%) as control. The treatments (0.6 ml) were added to 15 ml of Czapeks media in 125 ml Erlenmeyer flasks. Each flask was inoculated with 0.2 ml spore suspensions of either *Fusarium proliferatum* or *Fusarium moniliformin* [= *F. proliferatum*] (Dr. Robert Goth, USDA). Flasks were placed on a Fisher Clinical Rotator (90 rpm) for 14 days.

In the next experiment, mycelia were collected at 3 intervals, 3, 7 and 15 days and the concentration of alkaloids reduced to 0.008% berberine, 0.002% canadine, 0.001% and 0.2% *Hydrastis* rootlets and rhizome (10991ARZ) extraction. Treatments of 1.0 ml were added to 15 ml of Czapeks media and each flask was inoculated with 0.2 ml spore suspensions of *Fusarium moniliformin*.

A third assay used increased dilutions for two *Hydrastis* alkaloids; 0.004% berberine, 0.003% canadine, *Fusarium proliferatum* (7991BRZ) and a different *Hydrastis* sample (7991BZ). The amount of Czapeks media was increased to 25 ml and incubated on at shaker (100 rpm) for 7 and 15 days.

In addition to determining the effect of *Hydrastis* alkaloids on fungal growth rates, the next study looked at changes in sporulation and hyphal morphology of *Fusarium*. Three *Fusarium* isolates 5001AR, PSU 0-1174, 9001ARC were used, 2 different species associated with *Hydrastis* and a non-*Hydrastis* isolate which was the same species (*F.*

oxysporum) as one of the *Hydrastis* isolates. The treatment solvent was changed to aqueous methanol:acetic acid (50:1). The incubation period was reduced to 1-4 days. The isoquinoline alkaloid levels for each treatment were adjusted to levels within the range reported to occur in dry weight *Hydrastis* rhizome tissue - 6% berberine, 1% canadine, 4% hydrastine. Additional treatments include a standard mix of the alkaloids and 0.2% *Hydrastis* extract (05001ARZ), a control treatment of 50% methanol (1% acetic acid) and water. A spore suspension of the 3 isolates was prepared from inoculated carnation leafs. Spore suspensions were made from pieces of carnation leaf in distilled water that was filtered thru several layers of sterile cheesecloth and re-suspended in 0.1% Tween 20. Before inoculating treatment flasks, the spore suspension of each fungus was adjusted to approximately 10^6 conidia ml^{-1}. Flasks were incubated for 0,1,2,3 and 4 days.

Lyophilized mycelia after 4 days incubation were examined with a light microscope (40X) and counts of microconidia, macroconidia, chlamydospores and germination rate of macroconidia made. Hyphae were examined for any observable morphological abnormalities.

After completion of this study, the PSU *F. oxysporum* isolate was reassigned by personnel at Penn State to a sister taxon, *F. commune*, based on phylogenetic analysis of its translation elongation factor 1-alpha, which shows 95% homology with the *F. oxysporum* (*Hydrastis*) isolate. The other *Fusarium* isolates were consistent with their special designation (Appendix, Phylogenetic Analysis, Figures 29-33).

Microscopic analysis of fungal exudates

The influence of *Hydrastis* alkaloids on the morphology of *F. oxysporum* (*Hydrastis*) was investigated using flat bottom wells (Elkay 1.7 x 1.6 cm) into which the alkaloids, plant extract, Czapeks Dox broth with 0.1% Tween 20 and *Fusarium* isolate (5001AR) were added. Controls consisted of spore suspension in Czapeks medium and Czapeks medium without spores. The wells were examined observed daily with an Olympus SZH Dissecting Scope (72X) and morphological changes recorded.

ELISA mycotoxin analysis

To determine if *Hydrastis* alkaloids altered mycotoxin production by the *Fusarium* spp., freeze dried filtrate were re-suspended as follows:DON 1:10 in water, fumonisins and ZON 1:5 in 70% methanol, T-2 1:5 in 50% methanol. Using ELISA kits (Veratox, Neogen, Lansing MI), a calibration curve was constructed for each toxin, the sample was incubated in microwell plates with toxin antibodies and antibody substrate. A reading was taken with a microwell plate reader (EL301, Neogen) and the data was analyzed using Veratox software (Neogen).

High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

HPLC/MS analysis was performed on a Hewlett Packard 1050 LC pump and autoinjector, Hewlett Packard 1100 PDA, and a Finnegan Model TSQ-7000 triple quadrapole MS with Electrospray, positive ion LC inlet. The scan range 185-685 amu at

1 scan/sec., capillary temperature at 250°C, discharge current at 4.5μA and tube lens set to 84 volts. Separation occurred using a 250x2.0 mm, 5μm phenyl column (YMC). Mobile phase consisted of gradient of 35% solvent A (80% acetonitrile, 20% methanol, 0.1% acetic acid, 0.005% heptafluorobutyric acid) and 65% solvent B (0.1% acetic acid, 0.005% heptafluorobutyric acid), changing to 100% A at 32 minutes in a linear gradient, changing back to original conditions at 50.0 minutes, and re-equilibrating for 20.0 minutes.

CHAPTER 4:RESULTS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (HPLC-MS) DEVELOPMENT

Figures 8 and 9 are LC-MS chromatograms of extracts from *H. canadensis* to which 5% (by weight) of *C. japonica* and *B. vulgaris* material respectively had been added prior to extraction. In both figures the peaks for hydrastine, canadine and berberine occur respectively at 6.45, 11.48 and 13.45 minutes. In Figure 8, MS peaks at 10.84 and 12.05, which represents jatrorrhizine and coptisine respectively are present, whereas in Fig. 9 a MS peak at 10.84 indicates the presence of the adulterant alkaloid jatrorrhizine that is present in the plant *B. vulgaris*. Figure 10 is a LC-MS chromatogram that shows retention times of the 10 alkaloids from the 5 potential plant adulterant species The alkaloid are easily separated by comparison of their respective retention times (Table 3).

Adulterant alkaloids and their plant sources are listed in Table 3 with their molecular mass. Alkaloids of *Hydrastis* are listed as well for comparison. Berberine is the only *Hydrastis* alkaloids common to all plant species in the table. *Coptis japonica* also contains the alkaloids coptisine and jatrorrhizine, whereas *B. vulgaris* contains jatrorrhizine.

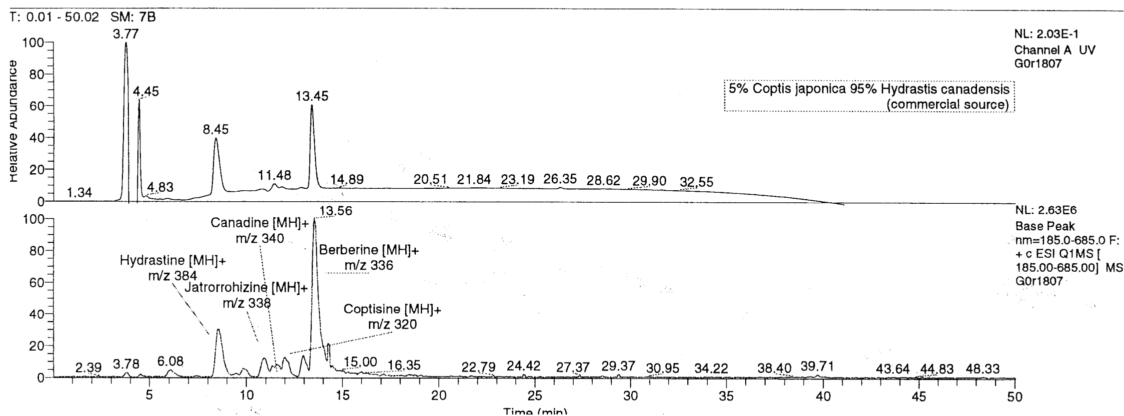


Figure 8:LC-MS chromatogram 5% *C. japonica* adulteration of *H. canadensis*.

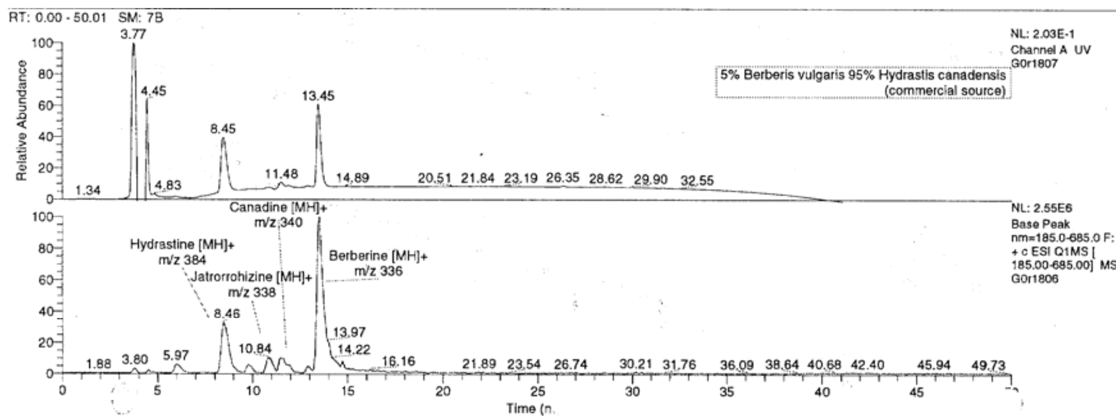


Figure 9:LC-MS chromatogram 5% *B. vulgaris* adulteration of *H. canadensis*

The chromatograms for figures 8-9 are from adulterant detection using HPLC analysis. Mobile phase - 35% solvent A (80% acetonitrile, 20% methanol, 0.1% acetic acid, 0.005% heptafluorobutyric acid) and 65% solvent B (0.1% acetic acid, 0.005% heptafluorobutyric acid), changing to 100% A at 32 minutes in a linear gradient, changing back to original conditions at 50 minutes, and re-equilibrating for 20 minutes. Separation occurred using a 250x2.0 mm, 5µm phenyl column (YMC). The spectra at the bottom are the subsequent MS analysis. The method was run in positive ion mode with a scan range 185-685 amu at 1 scan/sec., capillary temperature at 250°C, discharge current at 4.5µA and tube lens set to 84 volts.

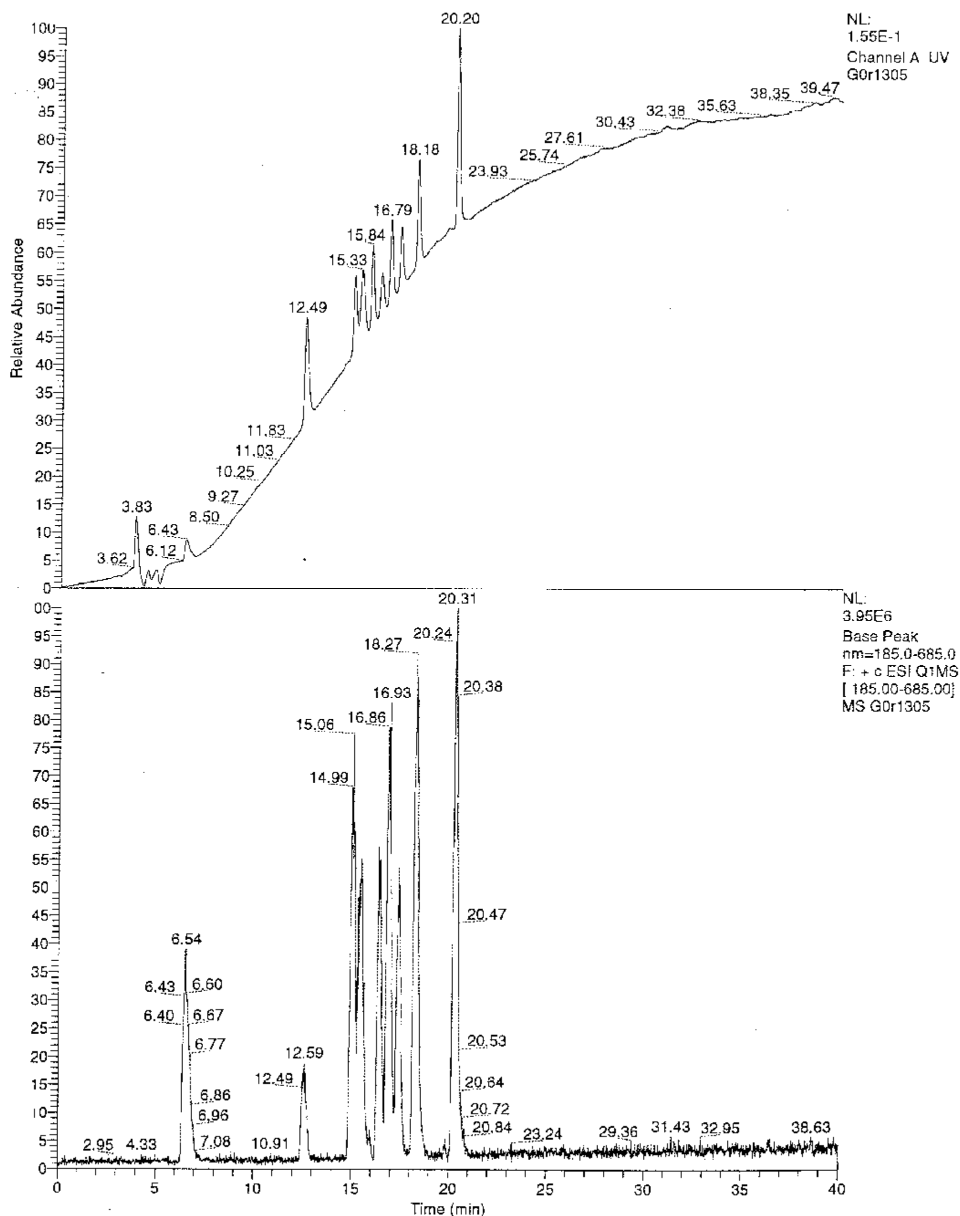


Figure 10:LC-MS chromatogram of 10 isoquinoline alkaloids present in *H. canadensis, B. vulgaris, C. japonica, C. majus, M. aquifolium, S. canadensis*

The chromatograms are from adulterant detection using HPLC analysis. Mobile phase - 35% solvent A (80% acetonitrile, 20% methanol, 0.1% acetic acid, 0.005% heptafluorobutyric acid) and 65% solvent B (0.1% acetic acid, 0.005% heptafluorobutyric acid), changing to 100% A at 32 minutes and back to original conditions at 50 minutes, and re-equilibrating for 20 minutes. Separation occurred using a 250x2.0 mm, 5µm phenyl column (YMC). The spectra at the bottom are the subsequent MS analysis. The method was run in positive ion mode with a scan range 185-685 amu at 1 scan/sec., capillary temperature at 250°C, discharge current at 4.5µA and tube lens set to 84 volts. The molecular masses and MS retention times for all of the adulterating chemical species, as well as the alkaloids found in *Hydrastis,* are listed in Table 3 that follows.

Table 3:Alkaloids molecular mass data for *Hydrastis* and adulterants.

Alkaloids	Mass[1]	Retention time	*Hydrastis*	*Coptis japonica*	*Berberis vulgaris*	*Chelidonium majus*	*Mahonia aquifolium*	*Sanguinaria canadensis*
berbamine	608.71	16.18			X		X	
berberine	336.37	18.18	X	X	X	X	X	
canadine	339.38	15.28	X					
chelerythrine	348.38	20.20				X		X
coptisine	320.33	15.84		X				
hydrastinine	207.23	6.43	X					
hydrastine	383.40	12.49	X					
jatrorrhizine	338.39	15.33		X	X		X	
palmatine	352.41	16.79				X		X
sanguinarine	332.34	17.05				X		X

[1] Merck, mol. wt.

CHAPTER 5:DISCUSSION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (HPLC-MS) DEVELOPMENT

The choice of reverse phase liquid chromatograph (RPLC) column was based on the ease with which the retention factor could be altered by manipulating the mobile phase; and the selectivity of a chromatographic system was varied by changing the solvent pH. A phenyl column was used for the LC/MS method, since at the time, end capping of the C-4 and C-18 columns was not as complete and the phenyl column with the benzene rings provided more predictable results. Now several C-18 columns have been designed with better capping and low silanol activity. Choice of the UV scanning range of 265 to 296 was based on the maximum UV absorption spectra for berberine (265nm), canadine (288 nm), hydrastine (295 nm) and hydrastinine (296 nm) (Popl et al., 1991). At the lower end of the spectrum, UV absorption picked up HFBA and unrelated plant material.

Hydrastis isoquinoline alkaloids are considered weak bases, especially berberine. The molecules are highly polar differing slightly in degree of polarity and cyclic nitrogen conjugation. Although most of these bases interact with free silanols of a silica-based alkyl bonded reverse phase HPLC column, quaternary alkaloids, such as berberine, are not retained long enough to effectively separate a series of these alkaloids. So the use of a nonpolar stationary phase in conjunction with a polar mobile phase required the addition of an ion pair and a gradient elution to improve the resolution of a mixture of tertiary and quaternary alkaloids. To provide confirmational data unavailable with HPLC-UV detection, a liquid chromatography, electrospray ionization mass spectrometric (LC-ESI-MS) detection method was developed. The ionization mechanisms in a ESI-MS system is considered a liquid-phase ionization technique where ions in solution are evaporated to

the gas phase before entering the mass spectrometer. Generally ESI-MS detection forms positive ions (M + peaks), which allows molecular mass determination directly from the LC column elution. This technique also has the advantage of creating less background interference during the ionization process. In optimizing the technique, a fine spray of droplets must be formed, thus the ion pair reagent used in this study had to be volatile. But when HPLC is coupled with electrospray ionization mass spectrometry (ESI-MS), many ion-pairing agents cause suppression of ESI analyte signals by increasing background noise (Gustavsson et al., 2001). Thus selection of an effective ion pair was vital for quantitative confirmation of analytes separated by HPLC. One study (Castro et al., 1999) examined a number of volatile ion pair reagents, heptafluorobutyric acid (HFBA), pentafluoropropionic acid (PFBA) and trifluoroacetic acid (TFA) and found that HFBA had the greatest ionization efficiency in separating quaternary ammonium pesticides using LC-MS (ESI).

Changes to acetonitrile concentration altered the selectivity of the method; increasing the concentration improved quaternary separation and decreasing concentration improved tertiary separation. Additionally, since acetonitrile is less viscous than methanol it caused less pressure change as the solvent gradient was altered. Using the ion pair HFBA to reduce polarity of berberine allowed the ion pair to increase interaction with the reverse phase column by increasing the hydrophobicity of the molecule. Subsequently, higher concentrations of organic modifiers also increased interactions of quaternary alkaloids with the column. In particular, peak broadening became an issue with methanol levels of $> 30\%$, while methanol levels above 35% increased ion suppression. Acidification of the

mobile phase ionized the bases, limiting tailing and pushing the ion pair equilibrium forward. Acetic acid was added to the mobile phase to lower the concentration of HFBA, which at higher concentrations had increased column equilibration times, caused appearances of artifactual peaks and limited column life. This change also improved reproducibility and peak shape.

An initial isocratic HPLC-MS method for the detection *Hydrastis* adulterants (Betz et al., 1998) could not satisfactorily resolve jatrorrhizine from coptisine, or sanguinarine from berberine out of 12 alkaloid molecules. Their study used 250 x 2.0 mm, 5 μm Inertsil® C4 column (MetaChem Technologies, Inc., Torrance, CA) and a 30°C temperature; a mobile phase consisting of 20% ACN/5% MeOH/75% buffer (100 mM ammonium acetate, 0.5% acetic acid) (v/v); a flow rate of 0.5 mL/min; a triple quadrapole mass spectrometry system operated in chemical ionization mode (APCI).

The gradient method used in this study was able to resolve all 10 analytes, reduce retention time and to quantitate adulterants as low as 5%. As noted above the chromatography column and solvent systems and use of electrospray ionization (ESI) were different from the Betz study. Additionally, Sato et al. (1992) reported that the maximum berberine capacity of *C. japonica* vacuoles was equivalent to 2.1 μg ml $^{-1}$. The for alkaloid content of the *Hydrastis* whole root treatment (05001AR) used in this assay was analyzed and the following results were found:berberine 1.5 μg ml^{-1}, canadine 0.9 μg ml^{-1} and hydrastine 2.9 μg ml^{-1}.

CHAPTER 6:RESULTS OF ASSAY DEVELOPMENT TO MEASURE EFFECTS OF HYDRATIS ISOQUINOLINE ALKALOIDS ON FUSARIUM SPP. GROWN IN ARTIFICIAL MEDIA

Fungal isolation

Table 4 lists fungi isolated from *Hydrastis* seed, leaf, stem, rootlet, rhizosphere soil, and non-rhizosphere soil. I was unable to test the isolates for pathogenicity. Previous reports list *Rhizoctonia solani* and *Phymatotrichum omnivorum* from *Hydrastis* roots (1960). Note that neither of these fungi is toxigenic and *Phymatotrichum* is also geographically limited to warmer areas of the U.S. (1960).

A. Hydrastis seed mycoflora

Fusarium oxysporum and *Pythium* spp. were isolated from seeds of native populations at the north facing "*Hydrastis* Heaven" site in Rutland, OH and from cultivated seed lot #1012 in Williams, OR respectively. Parent plants of the seed lot #1012 have their genetic basis in Southern Illinois goldenseal, which is an extension of the Ozark gene-center. Approximate isolation rates of *Fusarium oxysporum* from seed were <10%, whereas *Pythium* appeared 20% of the time.

B. Hydrastis plant tissue mycoflora

From native population sites in Rutland, OH *Fusarium oxysporum* was isolated only from root tissue growing at the north facing "*Hydrastis* Heaven" site, while *F. solani* was isolated from rhizosphere soil from the "*Hydrastis* Heaven" site; *F. proliferatum* was only isolated from the rootlet material from the south facing "Split Rock Hollow" site. Isolates from cultivated goldenseal tissue (lot #1012) include *F. solani* from root and leaf tissue and *F. oxysporum* isolated from seed. No fungi were isolated from rhizome or stem tissue of any goldenseal sample. Approximate isolation rates from native *Hydrastis* populations for *F. oxysporum* were << 5% from rootlets; for *F. proliferatum* 5% from rootlets; and for *F. solani* 5% from rhizosphere soil. Approximate isolation rates from cultivated *Hydrastis* populations was <10% for *F. solani* from *A. rhizogenes* transformation leaf tissue and << 5% from rootlets.

The following characteristics separate *F. oxysporum* from *F. solani* (Nelson et al., 1983): while macroconidia of both are straight, *F. oxysporum* exhibits a foot shaped basal cell and *F. solani* has blunt basal and apical cells; *F. oxysporum* microconidia are usually comma shaped and produced only in false heads, while *F. solani* microconidia are ellipsoidal (similar to *F. oxysporum*, but thicker walled); both *Fusarium* usually produce chlamydospores singly or in pairs, but *F. oxysporum* conidiophores are unbranched and branched monophialides (shorter than *F. solani*), while *F. solani* monophialides grow upward in the aerial mycelium; *F. oxysporum* culture morphology on PDA, exhibits sparse or floccose aerial mycelia, becoming felty, whitish or peach, sometimes with purple tinge; whereas *F. solani* aerial mycelia are sometimes bluish green. Although *F. proliferatum* resembles *F. oxysporum* on PDA, it can be recognized by club-shaped or

pear-shaped microconidia with a flattened base, which differentiates it from both *F. oxysporum* and *F. solani*. Additionally, chlamydospores were not detected.

C. Hydrastis soil mycoflora

Compared to non-sterilized seeds, fungal growth occurred more rapidly from seeds that had been treated with 10% Chlorox for 3 minutes. A greatest diversity of fungi and fungal growth rate occurred on Czapeks Dox, penicillin G and streptomycin medium. CLA was most selective, limiting diversity of fungi, with *Fusarium* the dominant species. Water agar, penicillin G and streptomycin medium inhibited fungal growth rate more than other media and limited the diversity of species almost as much as the CLA medium, but without the selectivity for *Fusarium*. For isolation of fungi from soil using hyphal tip methods the optimum soil dilution was 10^{-3} on water agar, penicillin G and streptomycin medium. No species predominated and compared to water agar, increased nutritional effects of Czapeks Dox and CLA made it more difficult to isolate individual species.

Radish seeds were planted in rhizosphere and non-rhizosphere soil to detect the presence of soil microbes. No visible fungal growth occurred on hypocotyl, stem or root tissue. However when seedling root tissue from the non-rhizosphere soil was plated on a water agar/antibiotic medium, *Rhizoctonia solani* growth was detected 6 days later. When corn kernels were planted in the rhizosphere and non-rhizosphere soil none of the kernels germinated, however once they were plated on a agar/antibiotic medium, *F. solani* was

detected in > 5% of the kernels from rhizosphere soil. *Pythium sp*. was also present. Species isolated from non-rhizosphere soil include *R. solani* and *Gliocladium spp.*.

Table 4:Fungi isolated from *Hydrastis* tissue and surrounding soil

Plant Tissue	ID #	Species	Collection Site
Rootlets			
	05001AR	*F. oxysporum*	Wild-crafted from the United Plant Savers sanctuary in Rutland Ohio, North slope "*Hydrastis* Heaven" stand.
	07991BR	*F. proliferatum*	Wild-crafted from the United Plant Savers sanctuary in Rutland Ohio, South slope "Split Rock Hollow" stand.
	07993BR-2	*F. proliferatum*	Wild-crafted from the United Plant Savers sanctuary in Rutland Ohio, South slope "Split Rock Hollow" stand and transplanted to Alexandria VA.
	12012R	*F. solani*	Isolated from cultivated goldenseal lot #1012 in Williams, Oregon.
Seed			
	05001AS	*F. oxysporum*	Isolated from "*Hydrastis* Heaven" plant.
	12012S-A4-2c	*F. oxysporum*	Isolated from cultivated goldenseal seed lot #1012.
	12012S-A4-1A **12012S-A4-1C**	*Pythium* spp.	Isolated from cultivated goldenseal seed lot #1012.
Leaf			
	12012L-A4-2b	*F. solani*	Isolated from cultivated goldenseal leaf lot #1012.
Soil Trapping			
	9001ARC	*F. solani*	Isolated from rhizosphere soil in "*Hydrastis* Heaven" site
		Pythium spp.	Isolated from rhizosphere soil in "*Hydrastis* Heaven" site
		Rhizoctonia solani	Isolated from non-rhizosphere soil soil in "*Hydrastis* Heaven" site
		Gliocladium spp.	Isolated from non-rhizosphere soil soil in "*Hydrastis* Heaven" site

All *Fusarium* isolates were identified at the *Fusarium* Research Center, Department of Plant Pathology, Penn State University, University Park, PA. Dr. Seonghwan Kim, Penn State University, identified Pythium isolates.

D. Detection of arbuscular mycorrhizae (AM)

Mature *Hydrastis* root tissue was examined for the presence of arbuscular mycorrhizal (AM) fungi. Microscopic examination of stained tissue showed no evidence for AM fungi, although coenocytic hyphae were found in root pericycle tissue. The *F. oxysporum*

(05001AR) used in this study was isolated from the same root tissue that was stained. Unstained root had yellow-pigmented pericycle tissue, which may indicate the presence of the berberine and hydrastine alkaloids. The rootlets appeared to be saturated with pigment throughout the tissue.

E. Phylogenetic analysis of PCR amplified *Fusarium* rDNA

Using CLUSTALW analysis (Chenna et al., 2003)**,** the phylogeny reassigned the PSU *F. oxysporum* to a sister taxon, *F. commune* (Appendix, Phylogenetic Analysis, Figures 29-33). It had 95% homology with the *F. oxysporum* (*Hydrastis*), a true *F. oxysporum*. The *F. oxysporum* (*Hydrastis*) is part of a major group/clone of *F. oxysporum* isolates that include a series of isolates from human infections, in addition to isolates from plants and other substrates. The *F. solani* isolate was placed in the clad III of the *F. solani* complex and the *F. proliferatum* (*Hydrastis*) isolate was a best match within the highly variable *F. proliferatum* species.

Preliminary Assay Development

A. Initial bioassay study

A series of experiments was designed to investigate the effects of *Hydrastis* alkaloids on growth of *Fusarium* spp. At this point, no *Fusarium* spp. had been isolated from *Hydrastis* tissue or soil. In the first study (Table 5) the 2 fungal isolates were grown on medium containing bioassay discs previously dipped in various alkaloids. In this study a non-*Hydrastis F. oxysporum* (Penn State) was compared to a fungus previously identified as a *F. oxysporum* isolated from *Hydrastis*, but later re-identified as an unknown non-*Fusarium* isolate (J. Juba, Penn State). The response of the two fungi to *Hydrastis* alkaloids was different. Whereas the PSU *Fusarium* isolate was not affected, except by hydrastine at day 7 only. Growth of the unidentified *Hydrastis* isolate was inhibited by both the whole root extract and berberine. Canadine also resulted in growth inhibition, but at a much lower level.

Table 5:Growth of unidentified fungus and *F. oxysporum* (O1042) exposed to *Hydrastis* alkaloids on solid medium

Isolate	Alkaloid Treatment	Conc. †	Inhibition Zone Measurements (mm)	
			Day 7	Day 14
7991ARZ	whole root	2.00	19.5	15.5
	berberine	0.04	16.5	10.5
	canadine	0.04	6.5	3.5
	hydrastine	0.04	0.0	0.0
	methanol (95%)	0.04	0.0	0.0
PSU-1042	whole root	2.00	0.0	0.0
	berberine	0.04	0.0	0.0
	canadine	0.04	0.0	0.0
	hydrastine	0.04	5.5	0.0
	methanol (95%)	0.04	0.0	0.0

† mg ml^{-1} of solvent
A single diameter measurement of the zone of inhibition was made for day 7 and 14**.**

B. Liquid media study

It was difficult to accurately assess the stimulus to fungal growth in the bioassay disk study since hyphal growth on the disks themselves was heterogeneous and difficult to measure, so this next study was designed to assess whether dry weight measures of *Fusarium* grown liquid culture could provide both fungal growth inhibition and stimulation data. *Fusarium proliferatum* (Goth) was also exposed to *Hydrastis* alkaloids using liquid media. The medium used was Czapeks Dox broth and two levels of alkaloid were compared, based on 0.1% and 0.2% extracts of *Hydrastis* to investigate if the fungal growth inhibition increased at higher alkaloid concentrations. After 14 days hyphal growth was separated by filtration, lyophilized and mycelial dry weight was calculated. The results of this experiment are presented in Table 6.

Table 6:Growth of *F. proliferatum* (Goth) in Czapeks Dox liquid culture containing *Hydrastis* alkaloids

Treatment	% (w/v) †	dry weight *	% (w/v) †	dry weight *
whole root	1.00	45	2.00	348
berberine	0.05	53	0.10	49
canadine	0.01	56	0.02	105
hydrastine	0.05	63	0.10	52
methanol		51		73

* mg after 14 days
† mg ml^{-1} of solvent
sampling=1x

No significant treatment effects were found at the 0.1% concentration of alkaloids with dry weights ranging from 45-63mg. However, increasing the *Hydrastis* extracts to 0.2% did elicit differential treatment effects. Whole root extract resulted in the highest amount of mycelia growth followed by canadine, methanol, hydrastine and berberine.

To verify that *Hydrastis* alkaloid could be recovered, identified and quantified from liquid Czapeks Dox medium used in the previous liquid growth studies, the filtrate from the lyophilized whole root extract treatment was resuspended in aqueous methanol and LC/MS analysis performed (Figure 11). Each of the major alkaloids, berberine, canadine and hydrastine, were separated and identified. In addition, the peaks occurring at 3.47, 3.59 and 5.96 minutes appear to be fungal metabolites present in the media filtrate. The peaks do not appear in the fungal filtrate of either the control or individual alkaloids, except canadine (data not shown). These peaks may indicate the presence of fungal metabolites produced by the fungi in response to the whole root extract. The average dry weight difference between the whole root extract treatment and individual alkaloid treatment filtrates was 300 mg, with the exception of canadine.

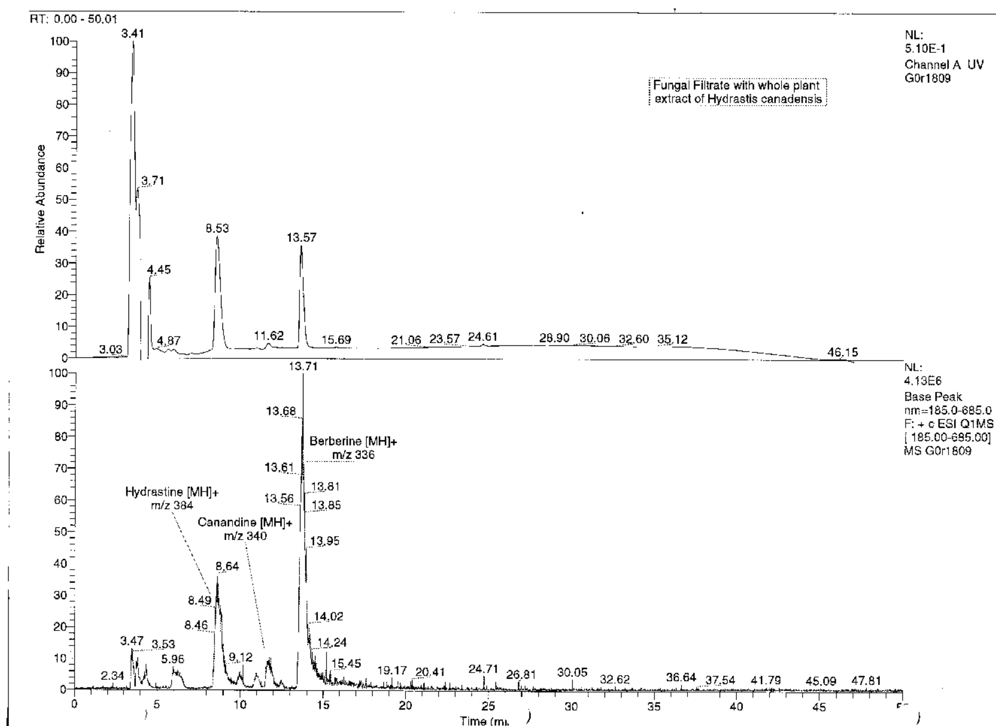


Figure 11:LC/MS chromatogram of fungal filtrate from *F. proliferatum* (Goth) treated with Hydrastis extract

This HPLC chromatogram used a mobile phase - 35% solvent A (80% acetonitrile, 20% methanol, 0.1% acetic acid, 0.005% heptafluorobutyric acid) and 65% solvent B (0.1% acetic acid, 0.005% heptafluorobutyric acid), changing to 100% A at 32 minutes in a linear gradient, changing back to original conditions at 50 minutes, and re-equilibrating for 20 minutes. Separation occurred using a 250x2.0 mm, 5µm phenyl column (YMC). The spectra at the bottom are the subsequent MS analysis. The method was run in positive ion mode with a scan range 185-685 amu at 1 scan/sec., capillary temperature at 250°C, discharge current at 4.5µA and tube lens set to 84 volts.

C. Fractionation method and preliminary liquid media assay development

Based on the results of the initial liquid assay, and published data by Stermitz et al. (2000a, 2000b) on the interactions between flavo-lignans and berberine isolated from *Berberis* spp., I wanted to investigate the effect of multiple alkaloids versus single alkaloids and a non-alkaloid fraction. Additionally evidence was lacking as to whether ethanol or methanol would be a more efficient extraction solvent. Using an isoquinoline alkaloid extraction method by Gentry (1998), the diethyl ether solvent fraction from *Hydrastis* should contain non-alkaloid compounds, the chloroform layer primary, secondary and tertiary alkaloids (including canadine, hydrastine) and the aqueous fraction quaternary alkaloids (berberine). Control blanks of ether, chloroform and methanol containing no plant material was also fractioned to be certain that artifacts that might confound treatment results would not be present. The extracts were fractioned and the fractions spotted on TLC plates. Standards of berberine, canadine and hydrastine were also spotted on the TLC plates for comparison. Visual inspection of TLC plates of ethanol and methanol extracts and comparison with the alkaloid standards indicated only that berberine was detected. Methanol appeared to extract more of the berberine than did ethanol based on the color intensity of the dragendorf stain on the TLC plate. Unexpectedly berberine appeared in the chloroform fraction as well as protonated in the aqueous alcohol fraction. In protonated form, this alkaloid base has moderate polarity and retains some solubility in chloroform. The extraction of individual alkaloids was incomplete. Also, results from the following study indicated that the effect of the

extraction control methanol blank was identical to methanol and thus solvent contaminant concentration did not occur during the fractionation process.

The dry weight of each fraction is summarized in Table 7. The methanol extract contains more material in the fractions that contain berberine; 10.0g versus 4.9g from the chloroform and 7.0g versus 2.0g from the aqueous methanol. However, the ether fraction for the ethanol extract was higher in weight than the methanol extract, 3.1g versus 0.7g. The weights of the control blank fractions were negligible. Thus it appears methanolic extraction yields more total alkaloids.

Table 7: Weight of various fractions after extraction of *Hydrastis* with methanol and ethanol.

Solvent	Fraction	Weights (mg/flask)
Methanol	1 – ether	0.7
	2 - chloroform	10.0
	3 – aq. methanol	7.0
Ethanol	1 – ether	3.1
	2 - chloroform	4.9
	3 – aq. methanol	2.0
Control	1 – ether	0.002
	2 - chloroform	.020
	3 – aq. methanol*	.016

* 50% methanol, pH=3.0

By now, *Fusarium proliferatum* had been isolated from *Hydrastis* root tissue. I incorporated the methanolic *Hydrastis* fraction components and *F. proliferatum* (*Hydrastis*) in the next study (Figure 12). Because the first liquid assay (Table 6) indicated that the methanol slightly stimulated fungal growth and the treatment alkaloids were dissolved in methanol, I used both water and the 95% methanol solvent as controls. Hyphal growth was inhibited by all treatments compared to water control, especially the fraction blank and methanol treatments through days 3 and 7. After day 12 the alkaloid fraction and canadine treatment stimulated fungal growth. By day 14 the hydrastine, fraction blank and 95% methanol treatment especially continued to inhibited fungal growth, whereas canadine, whole plant extract and the combined alkaloid fraction stimulated growth. Of note, the combined fraction treatment stimulated fungal growth more than berberine, which was unexpected since the only alkaloid detected by TLC in those fractions was berberine. In comparison with the methanol control treatment, the fungus appeared to be increasingly stimulated by all other treatments. Measurement of mycelial growth was confounded by contaminants in several treatments. For all treatments the media pH increased from pH 7 to pH 8.5-9.0 after 14 days; whereas water remained pH 8.25 after day 7 (data not shown). The pH changes did not appear to affect growth of the organism.

Figure 12:Growth of *F. proliferatum* (*Hydrastis*) in Czapeks Dox liquid media containing fractions of Hydrastis alkaloids

The treatment concentrations were as follows – berberine 0.08 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.02 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. The alkaloid fraction concentration was not known. Treatments were dissolved in 1 ml of 95% methanol solvent, which was added to 15 ml of culture medium. The Y-axis is grams of dry weight mycelia in 15 ml of the media. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 3, 7 and 14.

D. First liquid assay using *F. oxysporum* (*Hydrastis*)

A second root *Hydrastis* fungus was isolated, *F. oxysporum*, which appeared to be free of contaminants. The experimental aim was to investigate if the new isolate could also grow effectively over the same time period as the previous experiment and two time points were chosen, day 3 and 14. Note that the alkaloid concentrations used in this study were adjusted to fall more closely within the range reported as occurring naturally in *Hydrastis* root tissue, approximately 2 – 4.5% berberine, 1% canadine and 1.5-4% hydrastine (Wagner et al., 1984; Gocan et al., 1996). Also, the level of liquid media was increased to lessen the inhibitory effect of the methanol solvent control. Initially all treatments inhibited growth. By day 14 of growth the *F. oxysporum* (*Hydrastis*) treated with individual alkaloid and water showed signs of mycelial catabolism, except for the methanol and whole root extract treatments (Figure 13). Whole root extract appeared to stimulate growth significantly throughout the 14 days of the study. Water pH remained at pH 7.3 throughout, whereas the values declined from pH 6.75-7.25 to pH 6.0-6.5 over 14 days for all other treatments (data not shown). It was decided that the next experiment would incorporate a shorter time course to eliminate catabolic effects from the assay design and it appeared that the methanolic inhibitory effect for 95% methanol would continue. Using the same isolate, which at this time had been tentatively identified as an endophyte of *Hydrastis*, I attempted to alter the dilution solvent to increase or at least maintain the level of solvency of the alkaloids and minimize the inhibitory effects.

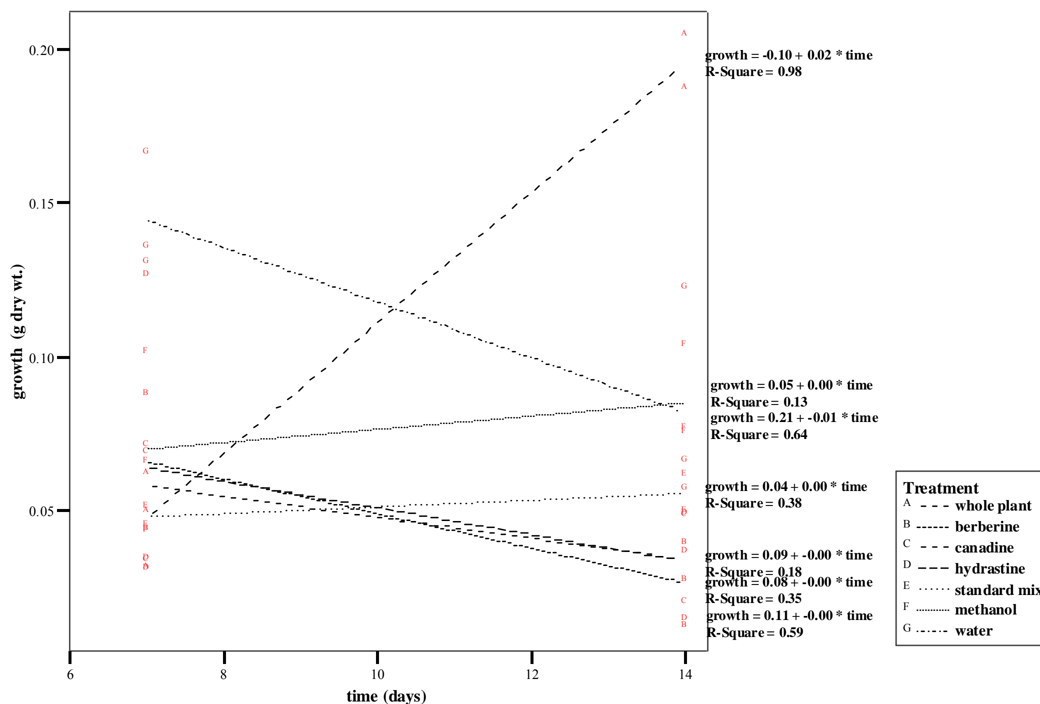


Figure 13:Growth of *F. oxysporum* (*Hydrastis*) in Czapeks Dox liquid media containing *Hydrastis* alkaloids

The treatment concentrations were as follows – berberine 0.04 mg ml^{-1}, canadine 0.03 mg ml^{-1}, hydrastine 0.01 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 95% methanol solvent, which was added to 25 ml of culture medium that contained 0.2 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}. The Y-axis is grams of dry weight mycelia in 25 ml of the media. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 7 and 14.

E. Extraction solvent selection using *F. oxysporum* (*Hydrastis*)

This additional assay was performed to determine if the extraction solvent being used might interfere with treatment effects. Figure 14 summarized the effects of 3 organic solvents plus water on *F. oxysporum* (*Hydrastis*) mycelial dry weight during 14 days of growth. With all solvents, including water, the maximum mycelia dry weight occurred at day 9 and declined thereafter. Maximum mycelial production occurred when 1 ml of two solvents, 50% methanol:acetic acid (1%) and water were added to 25 ml of medium; whereas 95% methanol caused the most inhibition of mycelial growth. Although the reduction of methanol in the 50% methanol:2% acetic acid solvent reduced the solvent hyphal growth inhibition when compared with the 95% methanol solvent, it appeared the increased level of acid contributed to inhibition of hyphal growth. It is apparent that *F. oxysporum* (*Hydrastis*) displayed a similar pattern of catabolic hyphal growth in this assay as well. For future assay design, the time component was shortened.

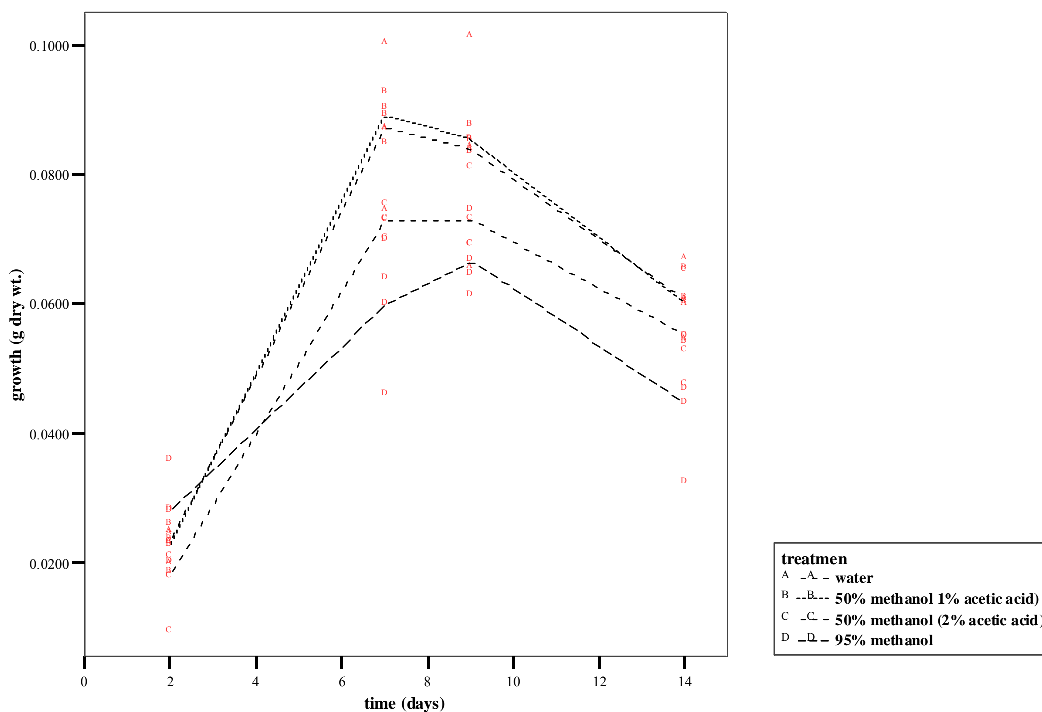


Figure 14 Effects of extraction solvent on growth of *F. oxysporum* (*Hydrastis*)
One ml of each test solvent was added to 25 ml of culture medium. The Y-axis is grams of dry weight mycelia in 25 ml of the media that contained 0.2 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}.. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 2, 7, 9 and 14.

F. Final solid media assay

A second, revised solid media method was used to determine if solid or liquid culture would provide more appropriate fungal response data. Questions remained about the availability of the alkaloid treatments saturated on the disks, and the inability to detect both inhibition and stimulation, so alkaloid treatments were incorporated directly into the medium. All *Fusarium* isolates of *Hydrastis* had been identified, including a *F. solani* isolated associated with the rhizosphere soil. A second *F. oxysporum* isolate not associated with *Hydrastis* was used to compare its response with that of the *Hydrastis* endophytic *F. oxysporum.* The PSU *F. oxysporum* isolate #1174 was chosen over the initial PSU *F. oxysporum* used (#1042) because it was known to produce mycotoxins, whereas the initial isolate was not to produce mycotoxins. Thus the 3 fungi were *F. solani* from *Hydrastis* soil and *F. oxysporum* from *Hydrastis* root and a PSU isolate, *F. commune* (host unknown).

Based on the previous experiments, a 50% methanol:acetic acid (1%) dilution solvent was used to study the effects of *Hydrastis* alkaloids on the hyphal extension of 3 *Fusarium* isolates grown on solid medium. Cernokova and Kostalova (2002) reported a minimum inhibitory concentration (MIC) of 1.0 mg ml^{-1} for berberine chloride in a growth inhibition assay using *F. nivale*. In their assay, Cernokova and Kostalova incorporated berberine in Sabouraud's agar, saturated bioassay disks with a fungal spore suspension, and placed them on the agar surface. For my experiment, Czapeks Dox media was used and treatments were incorporated into the PDA agar; 0.12 mg berberine, 0.01

mg canadine, 0.08 mg β-hydrastine and 2.0 mg whole root extract. A 2.0 mm square plug of mycelium taken from CLA was placed hyphae side down in the center of the plate.

Colony radial measurements were taken daily up to 5 days; results are summarized in Figures 15-17. The *F. solani* isolate grew at a slower rate than the *F. oxysporum* isolates, and *F. oxysporum* (*Hydrastis*) grew at a faster rate in all treatments. The growth of all 3 isolates was inhibited when *Hydrastis* alkaloids were present in the medium. The whole root extract was most inhibiting for both *Hydrastis* isolates, with the methanol control treatment almost as inhibitory to the endophyte. In comparison, the methanol treatment was most inhibiting to growth of the PSU isolate, with the whole root extract and the alkaloid standard treatment almost as inhibiting. While the hyphal growth rate of *F. oxysporum* (*Hydrastis*) remained unchanged for all treatments, the hyphal growth rate of both *F. commune* (PSU) and *F. solani* was decreased for all treatments. Hyphal growth of the two *Hydrastis* isolates appears to be more sensitive to berberine.

I was unable to measure pH changes in the media, and maximal growth occurred before day 4 for the *F. oxysporum* (*Hydrastis*) isolate. Accurate measurement of hyphal growth on solid media was confounded by vertical growth above the plane of the medium, thus liquid culture was determined to provide more accurate growth measurements. However, the three isolates provided a distinct difference in response that could be used in the final liquid assay.

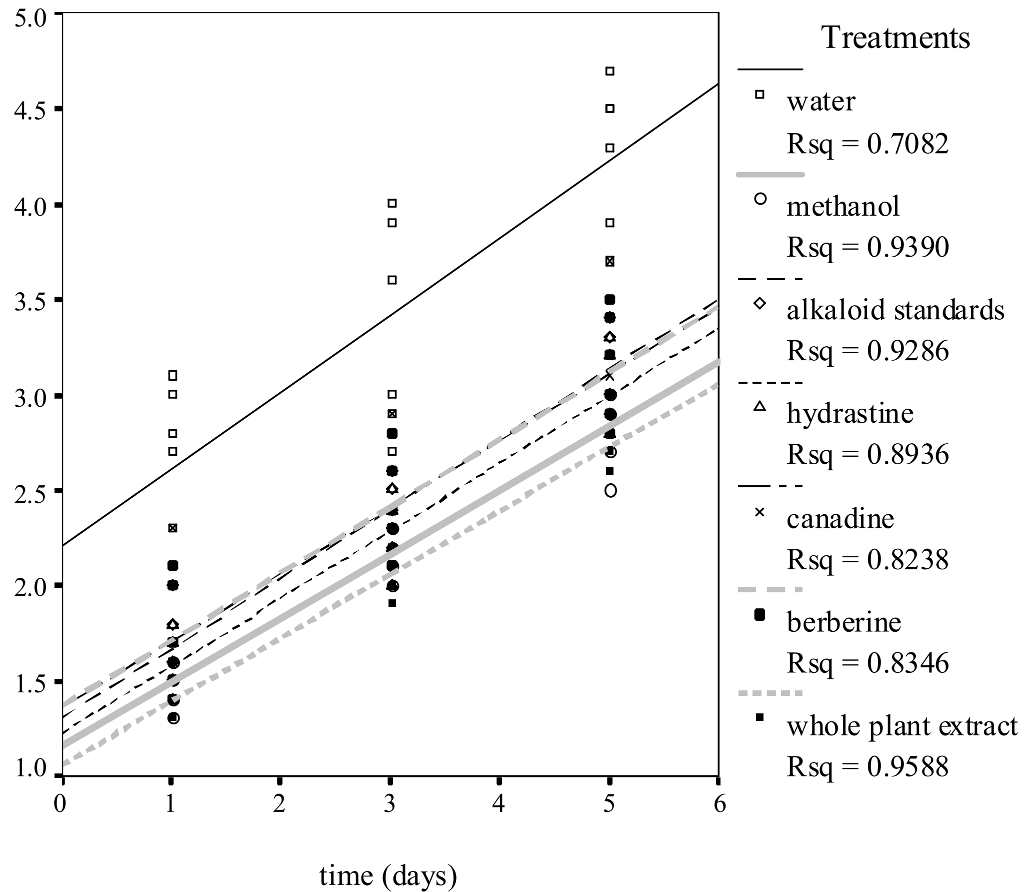


Figure 15 Growth of *F. oxysporum* (*Hydrastis*) on solid PDA medium containing isoquinoline alkaloids

The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.01 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 50% methanol:1% acetic acid solvent, and 0.28 ml of each treatment was added to 20 ml of solid culture medium/Petri dish before the media had solidified. A plug of the fungus was placed at the center of the solid media. The Y-axis is the average of hyphal growth of two radial measurements taken for each replicate time point. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-5.

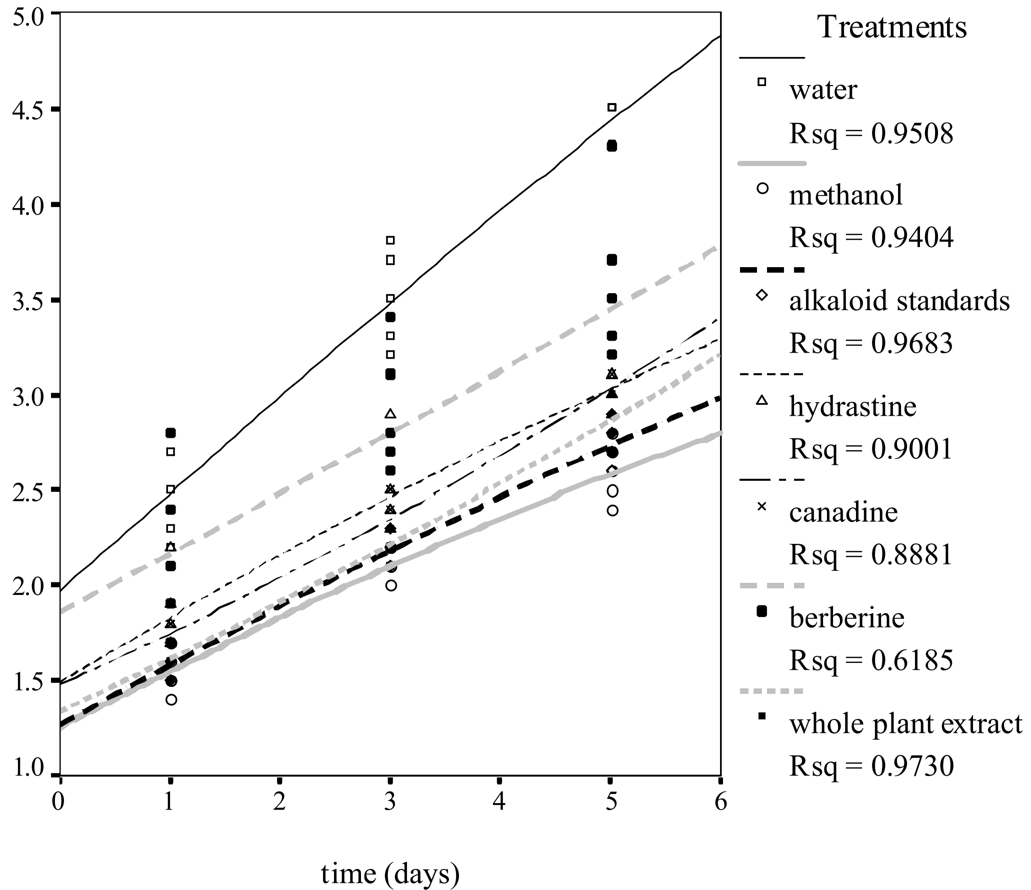


Figure 16 Growth of *F. commune* (PSU) on solid medium containing isoquinoline alkaloids

The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.01 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 50% methanol:1% acetic acid solvent, and 0.28 ml of each treatment was added to 20 ml of solid culture medium/Petri dish before the media had solidified. A plug of the fungus was placed at the center of the solid media. The Y-axis is the average of hyphal growth of two radial measurements taken for each replicate time point. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-5.

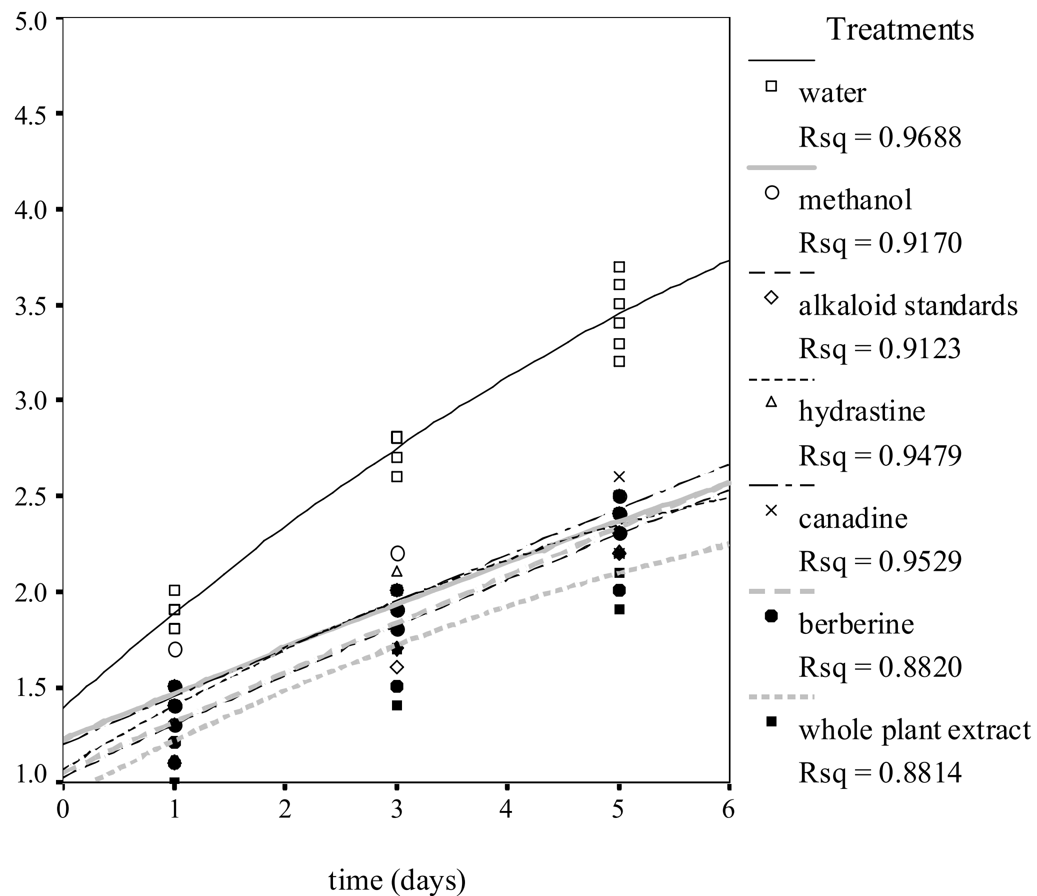


Figure 17 Growth of *F. solani* (*Hydrastis*) on solid medium containing isoquinoline alkaloids

The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.01 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 50% methanol:1% acetic acid solvent, and 0.28 ml of each treatment was added to 20 ml of solid culture medium/Petri dish before the media had solidified. A plug of the fungus was placed at the center of the solid media. The Y-axis is the average of hyphal growth of two radial measurements taken for each replicate time point. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-5.

Final Assay Development

A. Growth

Based on the preliminary experiments, I retested the isolates using a consistent timeframe (1-4 days), alkaloid concentration and methanol concentration. Figures 18-20 plot the growth of 3 *Fusarium* isolates on liquid medium containing *Hydrastis* alkaloids. In medium without alkaloids, the level of mycelia growth and the growth rate of the *F. oxysporum* (*Hydrastis*) isolate were lower than the other 2 isolates (Figure 18). The level of mycelia growth without treatment was greatest for *F. solani* (Figure 20), and this pattern remained throughout the assay. This was the opposite growth pattern noted in solid media. Treatment of *F. oxysporum* (*Hydrastis*) with whole root extract stimulated growth through day 3, but all other treatments had an inhibitory effect on growth, with the methanol control treatment least inhibitory. In comparison with the response of the isolate to the water and methanol controls, the alkaloid treatments were mildly inhibitory, while the whole root extract treatment was slightly stimulatory. After day 1, all treatments of *F. commune* (PSU) inhibited hyphal growth (Figure 19). The inhibitory effect increased throughout the study, with the methanol control treatment particularly inhibitory. Thus after day 1, all alkaloid and the whole root extract treatments were stimulatory with respect to the methanol control. The *F. commune* (PSU) isolate was the most sensitive to the methanol in comparison with the other two isolates. Initially all alkaloid treatments had a slight inhibitory effect on *F. solani* mycelial growth, except the methanol control, which had no effect. From day 2 on all treatments stimulated *F. solani*

growth except the two controls. Each treatment had a significant effect on growth of each fungus, except hydrastine for *F. commune* (Appendix, Statistics, Table 10). However, except for the relative stimulation of *F. solani* hyphal growth after day 2 by all treatments relative to the control treatments, none of the growth effects was particularly large.

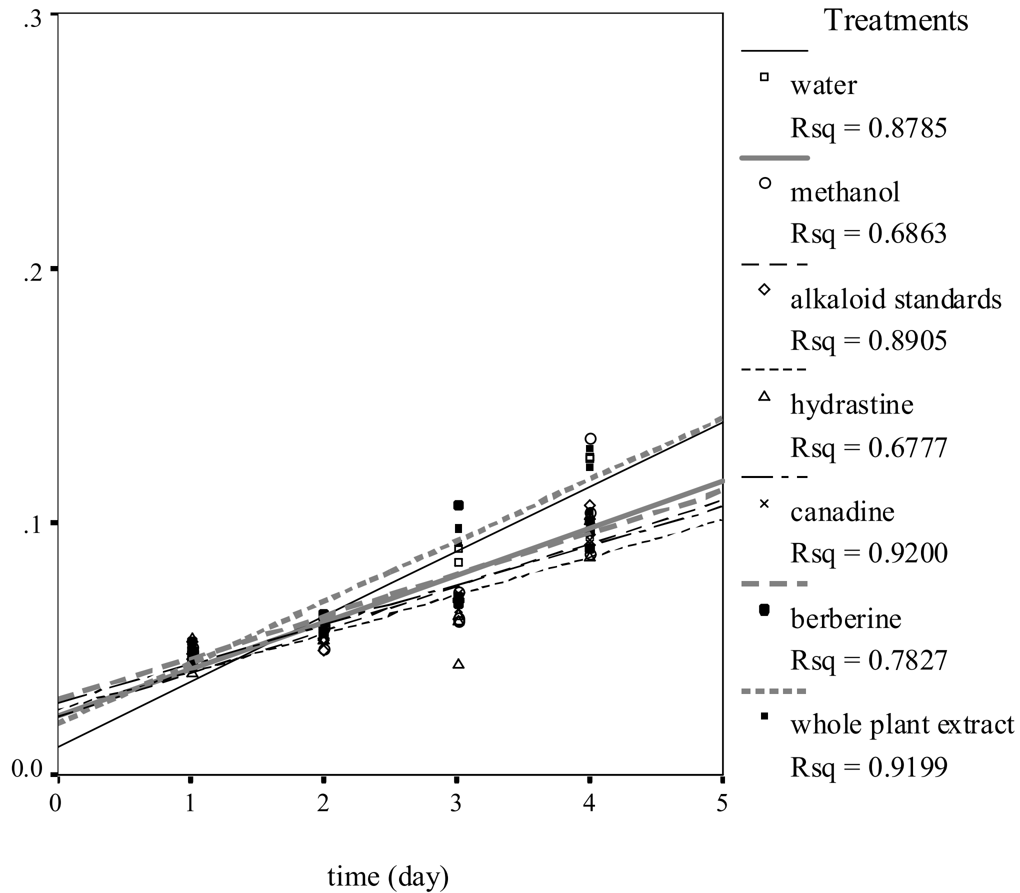


Figure 18 Growth of *F. oxysporum* (*Hydrastis*) in Czapeks Dox liquid medium containing isoquinoline alkaloids

The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 50% methanol:1% acetic acid solvent, which was added to 25 ml of culture medium that contained 1.0 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}. The Y-axis is grams of dry weight mycelia in 25 ml of the media. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-4.

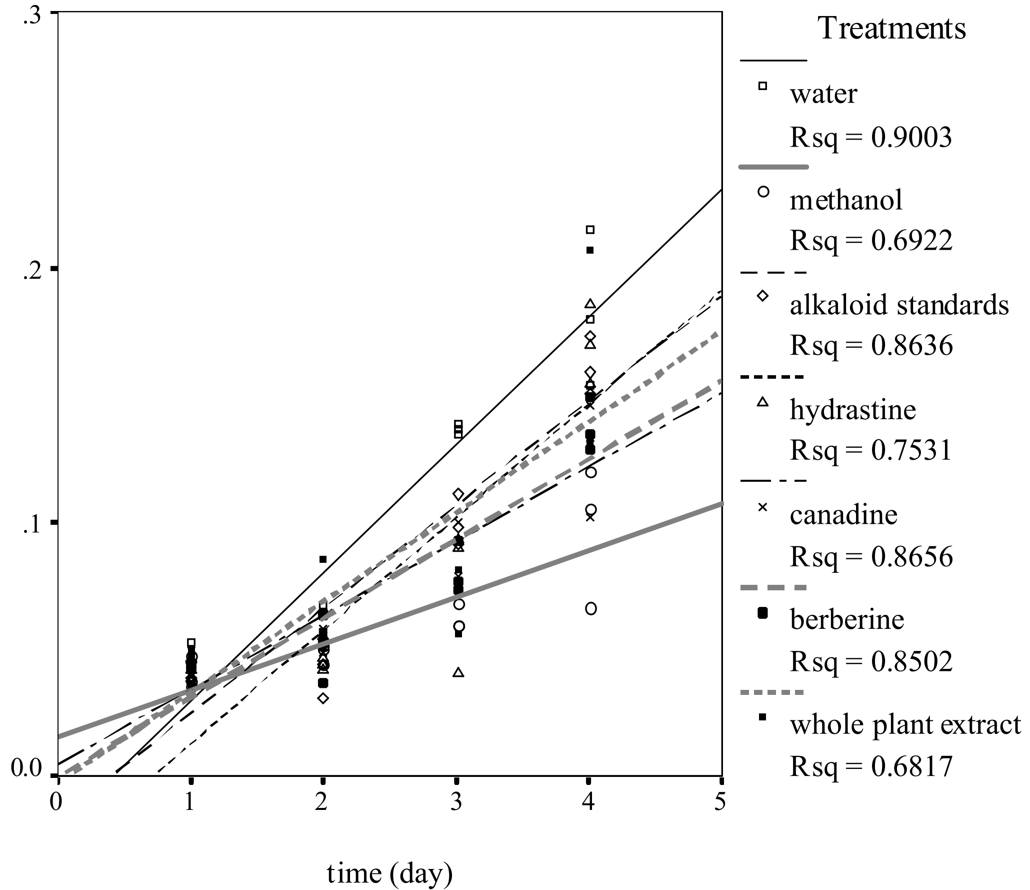


Figure 19:Growth of *F. commune* (PSU) in Czapeks Dox liquid medium containing isoquinoline alkaloids

The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 50% methanol:1% acetic acid solvent, which was added to 25 ml of culture medium that contained 1.0 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}. The Y-axis is grams of dry weight mycelia in 25 ml of the media. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-4.

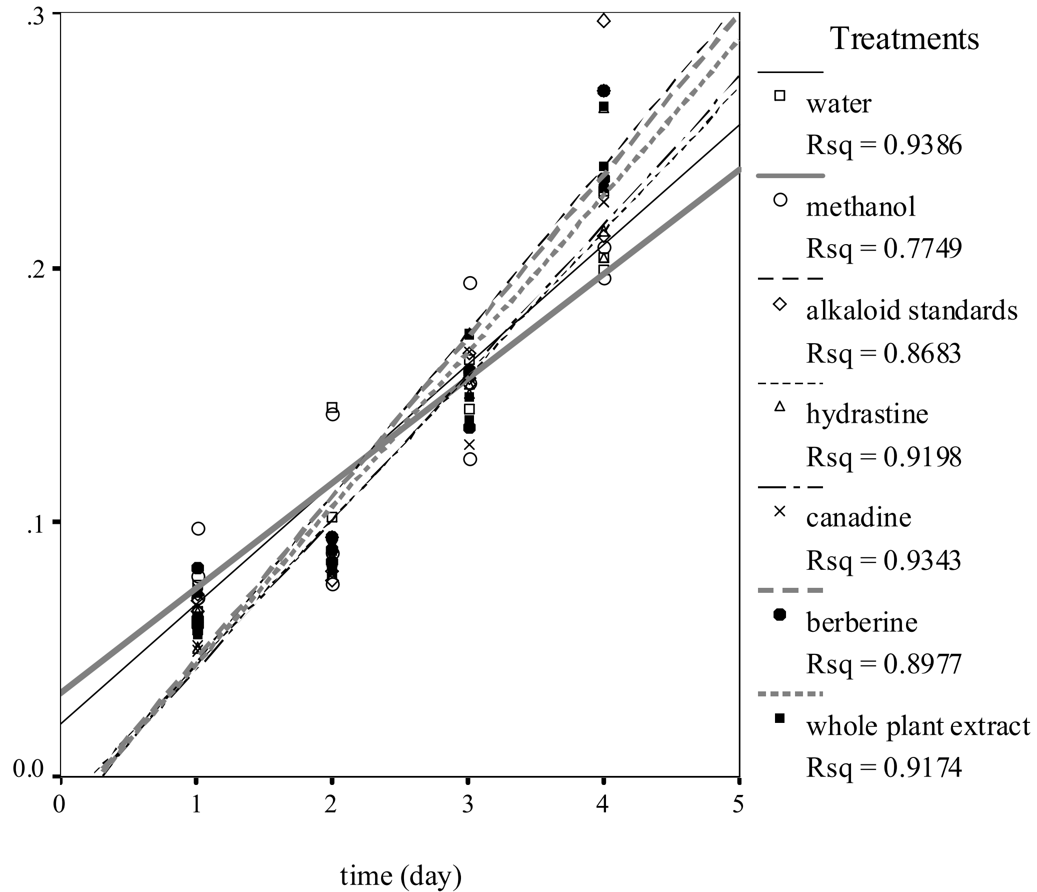


Figure 20 Growth of *F. solani* (*Hydrastis*) in Czapeks Dox liquid medium containing isoquinoline alkaloids

The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 50% methanol:1% acetic acid solvent, which was added to 25 ml of culture medium that contained 1.0 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}. The Y-axis is grams of dry weight mycelia in 25 ml of the media. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-4.

Regression analysis (Figure 21) confirmed that *F. oxysporum* (*Hydrastis*) had the lowest growth rate and that all alkaloid treatments, except whole root extract, inhibited hyphal growth rate. In the water control treatment the growth rate of *F. commune* (PSU) and *F. solani* were equivalent. However, when whole root extract, berberine, canadine and methanol were added to the medium, *F. commune* (PSU) growth rate was inhibited, especially by the methanol control. In contrast, all treatments containing berberine (whole root extract, berberine and alkaloid standards) stimulated *F. solani* growth rate.

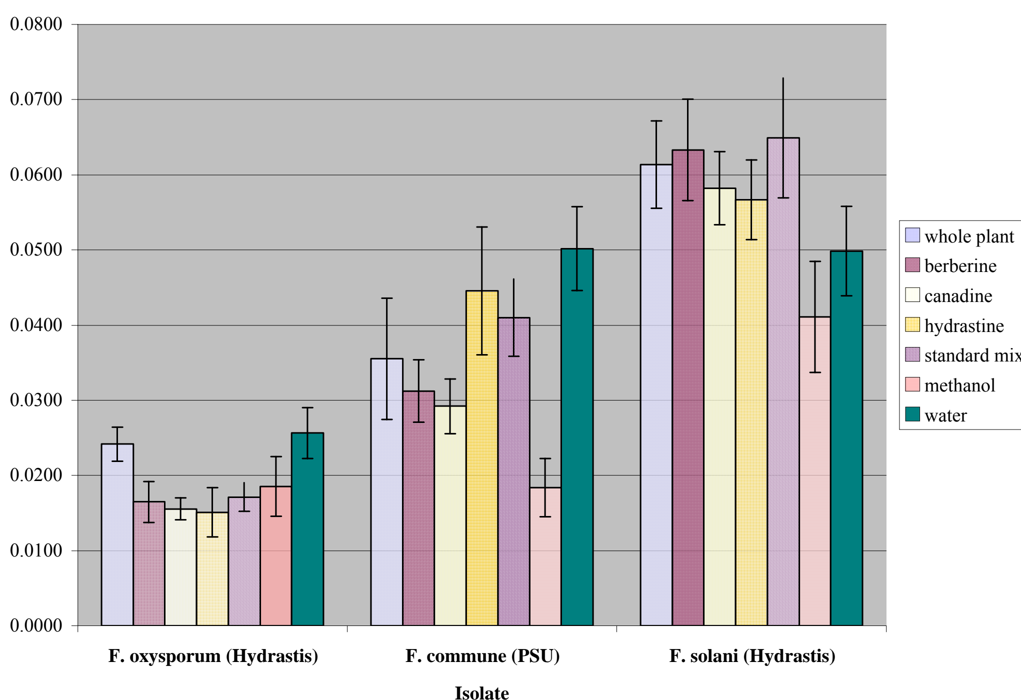


Figure 21:Growth rate (grams dry weight mycelia/day) of three *Fusarium* spp. in Czapeks Dox liquid medium containing isoquinoline alkaloids

Results are taken from Figures 18-20. The treatment concentrations were berberine 0.12 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 50% methanol:1% acetic acid, which was added to 25 ml of culture medium containing 1.0 ml fungal inoculum (10^6 conidia ml^{-1}). The Y-axis is grams of dry weight mycelia in 25 ml of the media. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-4. The error bars represent the standard deviation of error.

B. pH changes

The pH values were also plotted with respect to time in these experiments (Figures 22-24). The *F. commune* (PSU) and *F. solani* filtrate pH in water control remained steady at pH 7.5, while the pH of the water treatment for *F. oxysporum* (*Hydrastis*) decreased from pH 7.25 to pH 7.0. Both *F. commune* (PSU) and *F. solani* exhibited a more similar pattern of pH change with treatments, except that the changes in the pH values for *F. commune* (PSU) whole root extract and methanol treatments lagged until day 3, which is the time when all treatments began to stimulate growth of this isolate. There was virtually no difference in the change in pH values for all non-water treatments for the *F. solani* throughout the assay. Both the *F. commune* and *F. solani* hyphal growth patterns, with their initial inhibition, appear to reflect the fungal response to the acidic media conditions caused by the methanol solvent, with the *F. solani* altering the initial inhibition more quickly. The pH values for *F. oxysporum* (*Hydrastis*) for individual alkaloid treatments began at higher levels than the other isolates (pH 6.5 vs. pH 4.75), and remained constant during the study. It is unclear whether the basicity of alkaloid treatments was buffering the media. In comparison with the other isolates, the alkaloid treatments continued to inhibit *F. oxysporum* hyphal growth. The whole root extract and methanol treatments followed the pattern of pH change similar to the other *Fusaria*, except methanol filtrate pH lagged further behind than the other two isolates throughout the study. While the whole root extract treatment slightly stimulated hyphal growth, the methanol control treatment initially inhibited growth until day 3. Thus, when compared to the methanol control, whole root extract was stimulatory to *F. oxysporum* hyphal growth, but the

alkaloids treatments were inhibitory despite the pH lag for the whole root etract. The replicates were randomized with respect to both fungus and treatment, which suggests the pH pattern is unique to the endophytic isolate. Previous work has shown that both *F. solani* and *F. oxysporum* maximum growth/sporulation occurs in media at pH 5.5–7.0 (Tripathi 1999). Using ANOVA, variance in pH values was significantly correlated with the variance in growth except for the hydrastine treatment of the goldenseal *F. oxysporum* isolate (Appendix, Statistics, Table 12).

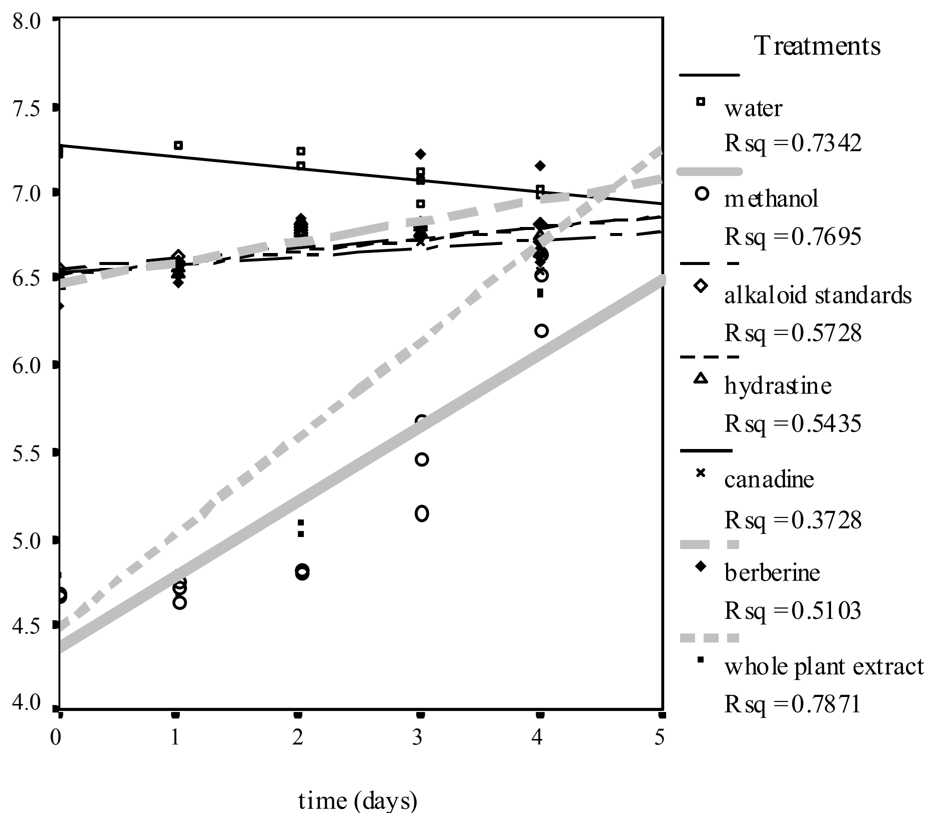


Figure 22:Change in pH with growth of *F. oxysporum* (*Hydrastis*) in Czapeks Dox liquid media treated with isoquinoline alkaloids

The data for this graph was collected from the same experiment as Figures 18-21. The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 50% methanol:1% acetic acid solvent, which was added to 25 ml of culture medium that contained 1.0 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}. The Y-axis is the pH value of the cultural media taken immediately after sample was removed from the shaker.. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-4.

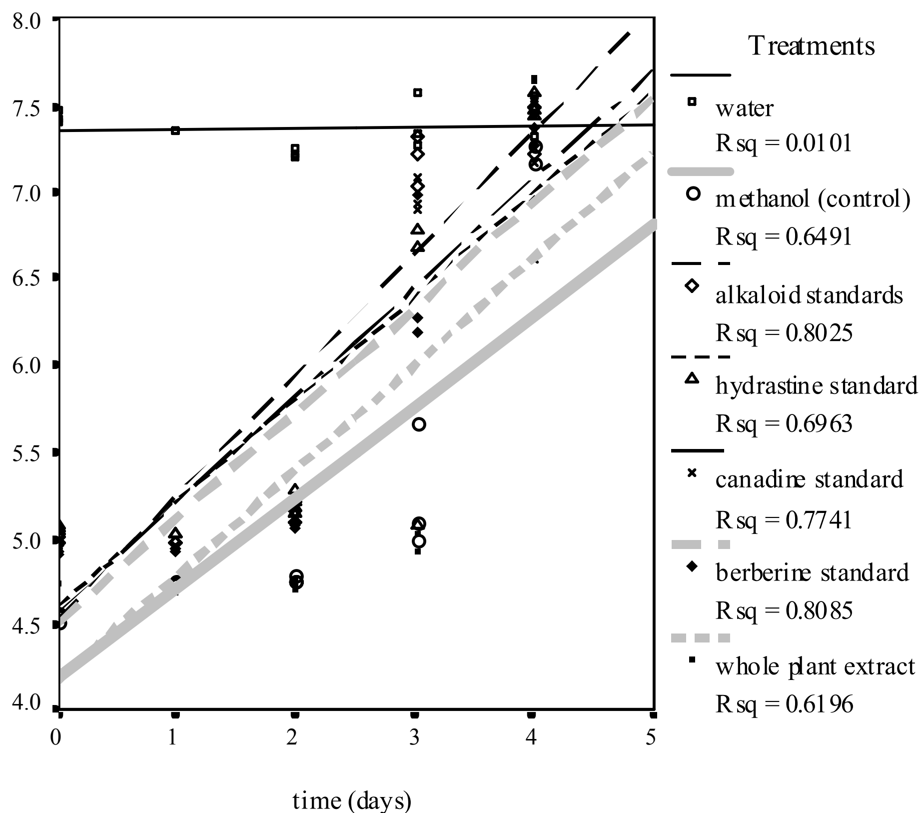


Figure 23:Change in pH with growth of *F. commune* (PSU) in Czapeks Dox liquid media treated with isoquinoline alkaloids

The data for this graph was collected from the same experiment as Figures 18-21.The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 50% methanol:1% acetic acid solvent, which was added to 25 ml of culture medium that contained 1.0 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}. The Y-axis is the pH value of the cultural media taken immediately after sample was removed from the shaker.. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-4.

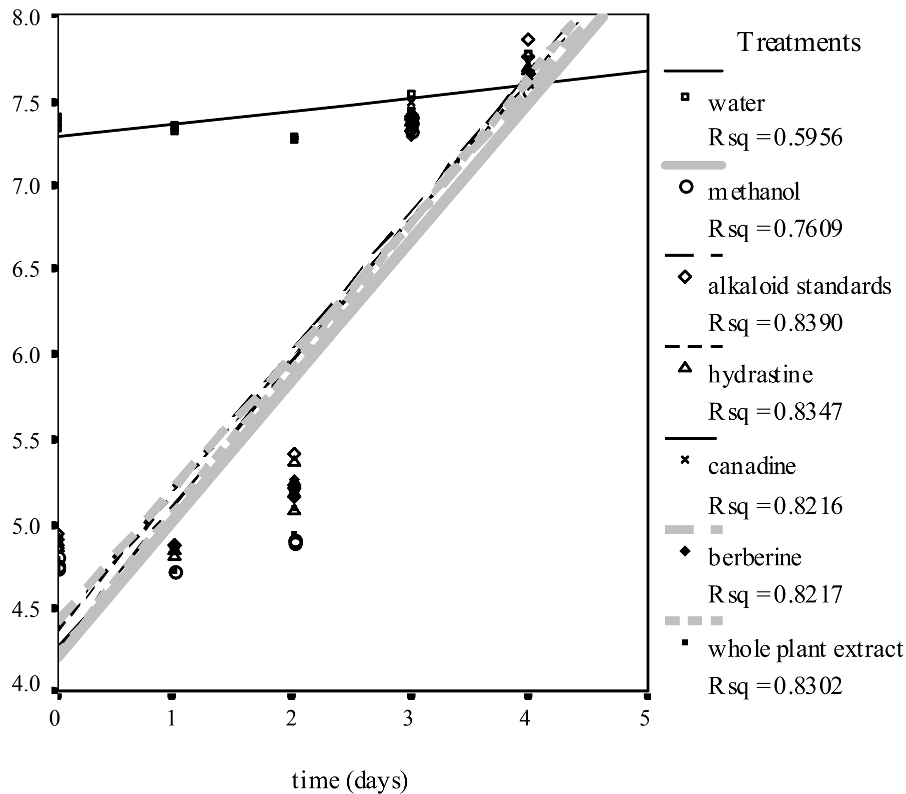


Figure 24:Change in pH with growth of *F. solani* (*Hydrastis*) in Czapeks Dox liquid media treated with isoquinoline alkaloids

The data for this graph was collected from the same experiment as Figures 18-21.The treatment concentrations were as follows – berberine 0.12 mg ml^{-1}, canadine 0.02 mg ml^{-1}, hydrastine 0.08 mg ml^{-1} and whole root extract 2.00 mg ml^{-1}. Treatments were dissolved in 1 ml of 50% methanol:1% acetic acid solvent, which was added to 25 ml of culture medium that contained 1.0 ml fungal inoculum. The fungal spore suspension was adjusted to approximately 10^6 conidia ml^{-1}. The Y-axis is the pH value of the cultural media taken immediately after sample was removed from the shaker.. The graph represents a least squares linear fit of randomized replicates (n=3) with measurements taken on days 1-4.

C. Spore formation

The influence of *Hydrastis* alkaloids on microconidia formation by *Fusarium* isolates is limited and is summarized in Figure 25. In the methanol control treatment of the *F. oxysporum* isolate had higher levels of microconidia compared to the water treatment control, whereas the 2 other isolates had lower levels. Only the reduction of *F. commune* microconidia by the methanol control treatment of approximately 30% could be considered significant. In the water control treatment the PSU *F. commune* isolate had higher levels of microconidia than either of the other 2 isolates and the *F. oxysporum* had the lowest level of production. All of the treatments except for the whole plant and methanol treatments resulted in slight lowering microconidia levels produced by the *F. oxysporum* compared with the *F. solani* isolate, for which microconidia formation was unaffected except by the methanol control, which also lowered levels. Berberine in particular reduced the levels of microconidia formed by the *F. oxysporum* isolate. All treatments of the PSU isolate produced similar levels of microconidia except in media with methanol and canadine treatments, which slightly reduced the level of microconidia. In contrast, the medium with the whole root extract stimulated microconidia production to a level higher than all other treatments, including water and methanol controls, but not to a significant level.

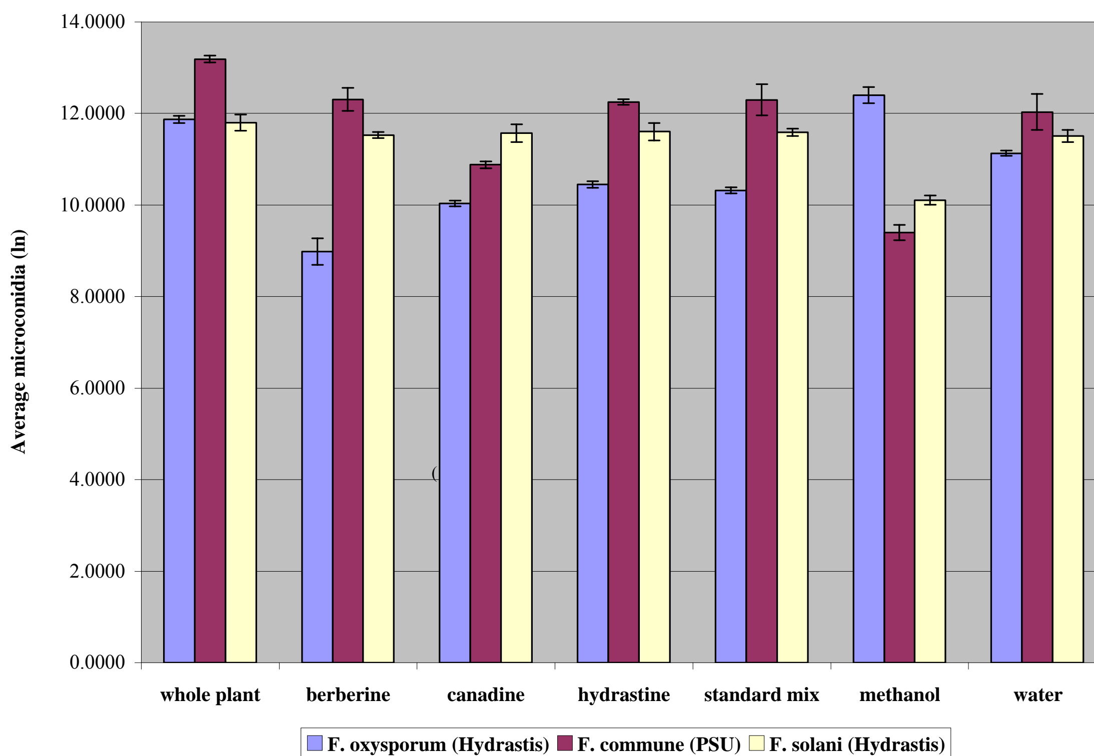


Figure 25:Isoquinoline alkaloid effect on microconidia formation by 3 *Fusarium* spp. grown in Czapeks Dox liquid media
The data for this graph was collected from the same experiment as Figures 18-21. Samples (n=12) were taken of the number of microconidia formed per mg of dry weight mycelia on the fourth day of the assay. The error bars equal the standard deviation of the error.

The influence of *Hydrastis* alkaloid treatments on macroconidia formation by *Fusarium* isolates was even less significant than the effects on microconidia, and is summarized in Figure 26. In the methanol control treatment the *F. oxysporum* isolate macroconidia were stimulated when compared to the water treatment control, whereas macroconidia formation of the other 2 isolates were inhibited. In the water control treatment the *F. solani* isolate produced lower levels of macroconidia than either of the other 2 isolates, while the PSU isolate had the highest level of production. The formation of *F. oxysporum* (*Hydrastis*) macroconidia is similar to microconidia production, with all treatments

inhibiting spore formation relative to the methanol control treatment. The inhibition pattern of *F. solani* isolate macroconidia levels was similar to the *F. oxysporum* isolate. In contrast, all treatments except canadine stimulated the *F. commune* isolate macroconidia production. The medium containing the whole root extract stimulated macroconidia production to a level higher than all other treatments, including water and methanol controls.

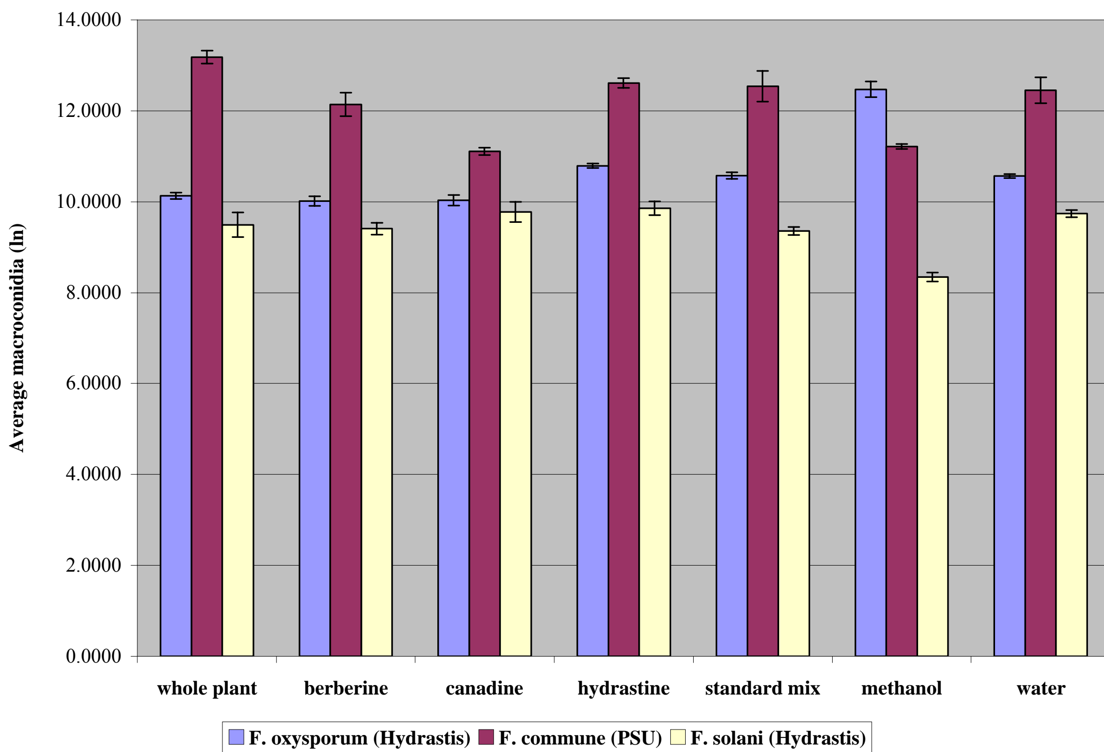


Figure 26:Isoquinoline alkaloid effect on macroconidia formation by 3 *Fusarium* spp. grown in Czapeks Dox liquid media
The data for this graph was collected from the same experiment as Figures 18-21. Samples (n=12) were taken of the number of macroconidia formed per mg of dry weight mycelia on day 4 of the assay (error bars = standard deviation of the error).

The influence of *Hydrastis* alkaloids on the germination rate of macroconidia is summarized in Figure 27. Without treatment in the water control, the greatest germination rate was found in the PSU isolate and the lowest in the *F. solani* isolate. The

methanol treatment significantly increased the germination rate in the PSU isolate compared with the water control. Whole root extract, berberine and canadine treatments reduced the production of macroconidia. Germination rate measurements were taken on day 4 and the range for the *F. commune* isolate was pH 6.5-7.5, with methanol pH at the lower end. It may be that the stimulatory affect of the methanol control treatment is related to either the differences in pH values on day 4 or the lag in pH during the course of the study. In that case, it appears that a component of the whole root extract treatment overcomes the inhibiting effects despite the lower pH values. However, the 25% increase in germination rates is not large enough to feel confident about drawing such a conclusion. Individual alkaloid treatments of *F. oxysporum* significantly lowered the germination rate compared with the control treatments; while whole root extract treatment stimulated the germination rate. The pH range was approximately 6.25-7.25, with the whole root extract and methanol control treatments at the lower end, berberine at the higher end, and water treatment in the middle. Interestingly, the whole root extract treatment increased germination rates when compared with the methanol control, despite similar pH values on day 4 and a pattern of pH change over the course of the study. This suggests that the effect is not pH related or that a component of the whole root extract is able to overcome the relatively lower pH effect to stimulate *F. oxysporum* macroconidial germination rates. Similarly the inhibition by individual alkaloid treatments might suggest that the relative minor change of pH differences over the study may be responsible; or that the alkaloids themselves and not pH is responsible for the effect. The whole root extract treatment also significantly stimulated the germination rate of the *F. solani* isolate, while the hydrastine and alkaloid standard treatments greatly reduced the

rate of macroconidial germination. The changes in pH over the study for all non-water treatments were equivalent, and on the final day cluster at pH 7.5. This strongly suggests that a component of the whole root extract is responsible for the stimulatory effect. Also hydrastine appears to be responsible for the inhibitory effect on *F. solani* germination rates for both hydrastine and alkaloid standard treatments, with the latter exhibiting signs that other alkaloids may have amplified the effect of hydrastine. Comparing the two isolate that are most similar, the whole root extract treatment stimulate *F. oxysporum* germination rates, while the same treatment inhibited the germination rates of *F. commune*. Methanol control treatment had no effect of the endophyte, while *F. commune* germination rates were greatly stimulated. Differences in pH values cannot explain the difference. They appear to be characteristics of the two isolates.

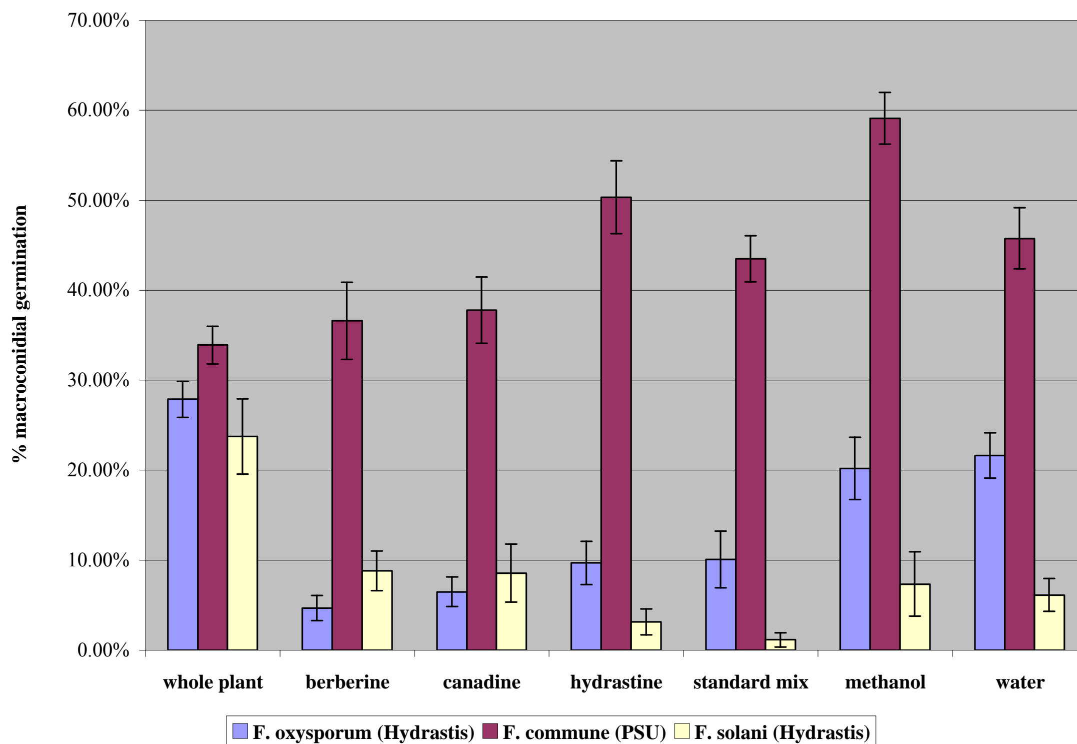


Figure 27:Isoquinoline alkaloid effect on macroconidia germination rate by 3 *Fusarium* spp. grown in Czapeks Dox liquid media
The data for this graph was collected from the same experiment as Figures 18-21. Samples (n=12) were taken of the % macroconidia germinated (Figure 26) per mg of dry weight mycelia on day 4 of the assay (error bars = the standard deviation of the error).

The influence of *Hydrastis* alkaloids on chlamydospore formation by *Fusarium* isolates is summarized in Figure 28. The methanol control treatment significantly increased levels of chlamydospore levels compared to the water control, except for the *F. solani* isolate. Whole root extract and hydrastine treatments also stimulated *F. oxysporum* chlamydospore production, but not to the degree as the methanol control. Since neither the water control nor the other alkaloid treatments had any effect on chlamydospore formation, it would appear that hydrastine may also play a role in stimulating production. Hebbar et al. (1997) noted that *F. oxysporum* chlamydospore formation was correlated with an increase in media pH from 5.5 – 7.0, but not above pH 7.0. Generally all *F.*

oxysporum (*Hydrastis*) pH values fell with that range, but not all treatments increased chlamydospore formation. Both whole root extract and the methanol control treatments started with much lower pH values and thus the change in pH could be assumed to play a role in the stimulatory effect. But because of the hydrastine effect, pH differences may not be the sole source of the effect. The response of the *F. commune* isolate was quite different. The methanol control, hydrastine and the alkaloid standard treatments significantly increased chlamydospore production, while whole extract completely inhibited formation. Hydrastine and methanol both appear to have stimulatory properties for this isolate, which components of the whole root extract is able to overcome. The individual alkaloids are also able to overcome the stimulatory effects of the methanol control alone. Only the *F. solani* isolate produced chlamydospores for every treatment, but berberine and canadine treatments reduced chlamydospore formation when compared with the water control treatment. Since the relative treatment pH values were equivalent, this appears to be a property of the two alkaloids.

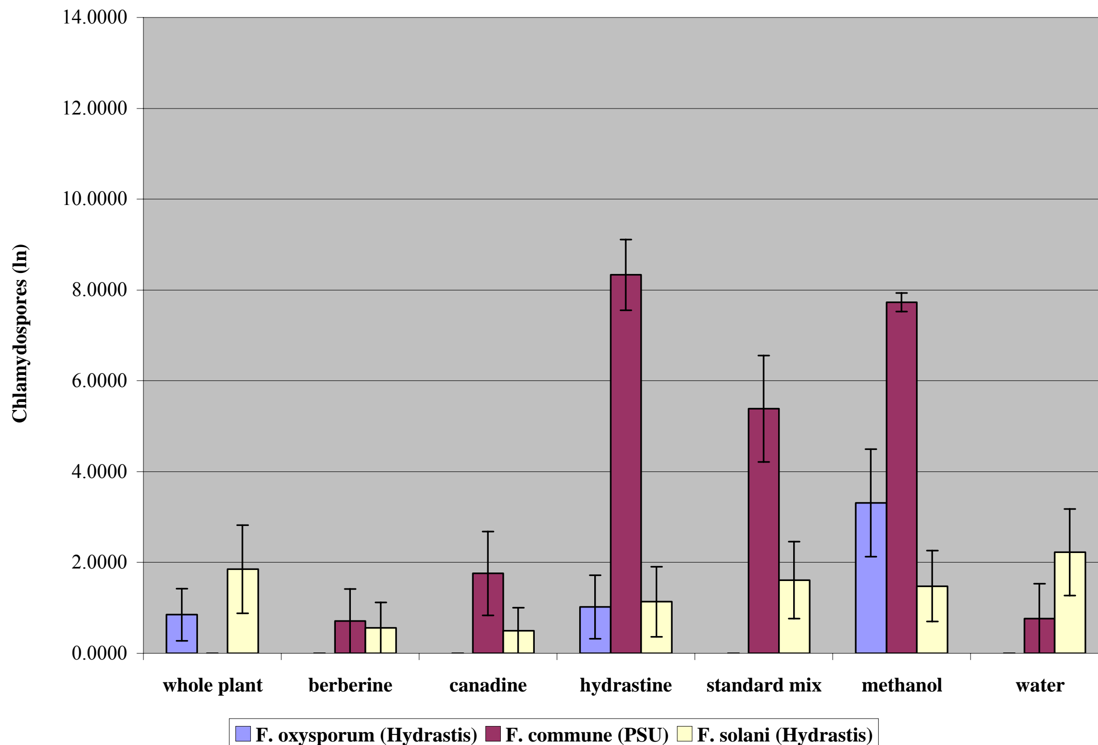


Figure 28:Isoquinoline alkaloid effect on chlamydospore formation by 3 *Fusarium* spp. grown in Czapeks Dox liquid media
The data for this graph was collected from the same experiment as Figures 18-21. Samples (n=12) were taken of the number of chlamydospores formed per mg of dry weight mycelia on day 4 of the assay (error bars = the standard deviation of the error).

Spore counts for the current study were determined after day 4. In medium without alkaloids F. oxysporum (Hydrastis) produced a maximum 4.0 x 105 microconidia/mg dry weight mycelia and 5.4 x 105 macroconidia/mg dry weight mycelia after 4 days inoculation on an orbital shaker (100 rpm) in darkness at 25º C; F. commune (PSU) produced maximum 2.7 x 105 microconidia/mg dry weight mycelia and 1.2 x 105 macroconidia/mg dry weight mycelia; and F. solani produced maximum 5.0 x 104 microconidia/mg dry weight mycelia and a maximum 7.5 x 103 macroconidia/mg dry weight mycelia. The average control germination rate for F. oxysporum (Hydrastis) was 20%, F. commune (PSU) 59% and F. solani 7%. Average chlamydospore production for

F. oxysporum (Hydrastis) was 1.3 x 10-3 per mg dry weight mycelia, F. commune (PSU) 2.8 x 10-3 per mg dry weight mycelia and F. solani 1.1 x 10-2 per mg dry weight mycelia.

D. Microscopic analysis of fungal exudates

Initially Czapeks broth control had significantly more hyphal growth from *F. oxysporum* (*Hydrastis*). The spheroid shaped objects appeared as droplets external to and at the tip of growing hyphae. The phenomenon was observed in greater proportion for all alkaloid treatments in comparison with both controls, and significantly more in whole root extract. The number of objects grew over time in all treatments with 25% more in the whole root extract treatment by the end of the study. They did not appear in the Czapeks broth control or in the Tween 20 control. After one week the droplets had darkened with a reddish tint. A sample from the whole plant treatment was removed, stained with trypan blue and viewed under a dissecting microscope. The objects were colorless and appeared lipid-like.

McPhee and Costello (1977) reported formation of similar lipid-like objects in *F. culmorum* culture shortly before macroconidia formation occurred. Pigmentation of the liquid changed as the number of the macrospores forming increased. The authors hypothesized that since the object appeared near the hyphal tip they contained a secondary metabolite pool necessary for pathogenesis of the fungus. The lack of

pigmentation within the spheroid objects in the study would suggest that no berberine or hydrastine were absorbed.

E. ELISA mycotoxin analysis

The *F. oxysporum* isolate from *Hydrastis* produced T-2 toxin, fumonisin and ZON based on ELISA analysis of mycelia (Table 8). In contrast, the *F. commune* isolate from Penn State had previously produced only ZON (Ueno et al., 1977). *Fusarium oxysporum* (*Hydrastis*) produced T-2 in all the alkaloid treatments, but none in the two controls, water and methanol. The highest level of T-2 was in media containing either canadine or hydrastine (0.24 vs. 0.11 ng metabolite mg^{-1} dry wt mycelia). In contrast, the *F. commune* (PSU) isolate did not produced T-2 or fumonisin, regardless of treatment. The *F. oxysporum* (*Hydrastis*) isolate also produced fumonisin (1.05 - 5.77 ng metabolite mg^{-1} dry wt mycelia), except when whole root extract or berberine was added to the media.

Table 8:Mycotoxin production by *Fusarium oxysporum* in media containing isoquinoline alkaloids

	Treatment	MYCOTOXIN*		
		T-2	fumonisin	ZON
F. oxysporum				
	whole root extract	0.05	0.00	1.95
	berberine	0.06	0.00	0.00
	canadine	0.24	1.05	1.87
	hydrastine	0.11	3.98	0.00
	alkaloid mix	0.04	3.17	7.61
	methanol	0.00	5.77	3.03
	water	0.00	3.19	0.00
F. commune				
	whole root extract	0.00	0.00	0.00
	berberine	0.00	0.00	0.00
	canadine	0.00	0.00	0.00
	hydrastine	0.00	0.00	1.07
	alkaloid mix	0.00	0.00	2.32
	methanol	0.00	0.00	1.30
	water	0.00	0.00	6.57
Correlation coefficient for the respective calibration curves		0.9863	0.9984	0.9996

The data for this graph was collected from the same experiment as Figures 18-21. Sample measurements (n=3) were taken of mycelia filtered from culture on day 4 using Neogen ELISA fluorescence plate reader. Results expressed as ng metabolite/mg dry wt mycelia.

The *F. commune* (PSU) isolate did however produce ZON in high quantities in the water treatment medium (6.57 ng metabolite mg^{-1} dry wt mycelia). Geiger (personal comm.) had previously reported that this isolate was capable of producing ZON. In media that contained the whole root extract, berberine or canadine, ZON production was inhibited. ZON was not produced by the *F. oxysporum* (*Hydrastis*) isolate in the water, berberine or hydrastine treatments; whereas, the alkaloid mix stimulated production of ZON more than any other treatment (7.6 ng metabolite mg^{-1} dry wt mycelia). Production of DON was not detectable. Additionally, the presence of all three mycotoxins produced by *F. oxysporum* (*Hydrastis*) occurred only in the canadine treatment.

CHAPTER 7:DISCUSSION OF ASSAY DEVELOPMENT TO MEASURE EFFECTS OF *HYDRATIS* ISOQUINOLINE ALKALOIDS ON *FUSARIUM* SPP. GROWN IN ARTIFICIAL MEDIA

Fungal isolation

Prior to plating *Hydrastis* tissue on culture media to detect microbes in or on the root, sterilization of root tissue increased the potential for isolating endophytic plant pathogens. Czapeks Dox media with antibiotics limited the diversity of fungal species isolated and appeared to select more aggressive pathogens. Carnation leaf agar with antibiotics was selective for *Fusarium* spp. and stimulated sporulation when compared to water agar medium. Antibiotics also helped to limit growth of yeasts and soil bacteria. Adding antibiotics to water agar and using a 10^{-3} soil dilution enhanced isolation of fungal species from rhizosphere soil while inhibiting bacterial growth. The nutrient limitations inherent to water agar also caused less branching of the fungal mycelia and faster growth of mycelial tips. Water agar medium also proved useful for doing hyphal tipping of germinated conidia to obtain pure cultures of the fungi used in this study.

If *Hydrastis* root exudates selectively influences the fungal rhizosphere community, what are the major pathogens in the rhizosphere of *Hydrastis* soil? Soil trapping using fresh corn kernels and radish seedlings resulted in the occurrence of isolates of *Fusarium solani*, *Pythium spp.*, and a number of unidentified soil fungi. Rhizosphere soil was from a north-facing “Hydrastis Heaven” collection site. Non-rhizosphere soil from the same collection site yielded only *Rhizoctonia solani* and *Gliocladium* spp.. *Fusarium* and *Pythium* are cosmopolitan soil fungi that occur as either saprophobes or plant pathogens,

and are often found in close proximity to plant root or seeds. In general, it does not appear that the *Hydrastis* rhizosphere soil is particularly selective except that it encourages pathogenic fungal growth rather than saprophytic fungal growth, as expected. The nutritive compounds found in the root mantle exudates, which attract soil pathogenic and saprophobic fungi, appears to have an influence on *Hydrastis* rhizosphere soil.

Non-nutritive compounds may also play a role influencing *Hydrastis* rhizosphere *Fusarium* isolates. Nagao et al. (1990) noted that the ability of *F. oxysporum* strains to colonize soil was unrelated to its ability to colonize flax root tissue (*Linum usitatissimum*), but that flax root exudates increased the population density of pathogenic *F. oxysporum* near the root. It may be that pathogenic *Fusarium* isolates predominate some distance from the root tissue but still within rhizosphere zone:whereas, as several authors noted, nonpathogenic isolates compete more successfully than pathogenic strains for soil exudates at the site of root infection (Couteaudier and Alabouvette, 1990; Eparvier and Alabouvette, 1994; Turlier et al., 1994). Not enough data is available to suggest that *Hydrastis* root selectively affects both non-pathogenic *Fusarium* isolate populations associated with plant root tissue.

Arbuscular mycorrhizae (AM) could not be detected from stained roots from either the north-facing or south-facing collection sites, as well as from wild or cultivated *Hydrastis* seed tissue. Also AM chlamydospores could not be found in the rhizosphere soil from either collection site. Such symbiotic relationships occur in poor nutrient soil conditions thus allowing the AM fungi to extend the plant root system to acquire more of limited

soil nutrients, such as phosphate. According to J, Morton, West Virginia University AM center, arbuscular mycorrhizae fungi are obligate mutualists that are reported to form symbiotic associations with the roots of more than 80% of land plants; and the mesic cove forest where *Hydrastis* was collected is not particularly conducive to high populations of arbuscular fungi, especially since the soil contains high concentrations of organic matter.

The three species of *Fusarium* isolated from *H. canadensis* were *F. oxysporum* from seeds of both native *Hydrastis* at the north-facing "*Hydrastis* Heaven" collection site (Ohio) and cultivated *Hydrastis* (Oregon), root tissue of native *Hydrastis* at the north-site; *F. solani* from the rhizosphere soil of native *Hydrastis* at the north-facing site as well as from leaf and root tissue of cultivated *Hydrastis* (Oregon); and *F. proliferatum* from root tissue of native *Hydrastis* at the south-facing "Split Rock Hollow" site (Ohio). Although the rates of *Fusarium* isolation from plant tissue at the south-facing site were almost twice the level from the north-facing site, the only *Fusarium* isolate was *F. proliferatum*; whereas north-facing site plant tissue and soil yielded both *F. oxysporum* and *F. solani*. The *F. oxysporum* isolate appears to be more closely associated with *Hydrastis* occurring in both root and seed tissue; whereas in native settings the *F. solani* isolate appears to be a soil opportunist since it occurred in both the commercially grown *Hydrastis* leaf and root samples but only in the north-facing rhizosphere soil. It is commonly known that in commercial fields cultivation disturbs populations of native soil fungi, resulting in the predominance of more aggressive isolates. In this study no fungal

isolate occurred in *Hydrastis* stem or rhizome tissue, which contains direct and continuous xylary connections from rootlet into the aerial portions of higher plants.

The coenocyte hyphae detected between *Hydrastis* root pericycle cells was probably those of *F. oxysporum*. The *F. proliferatum* (*Hydrastis*) isolate was not detected microscopically from any *Hydrastis* root tissue. It may have been present in the root cap without having penetrated the intercellular spaces of *Hydrastis* rootlets. In one study, Bacon and Hinton (1996) found that not all *Fusarium proliferatum* isolates were aggressive pathogens of maize (*Zea mays*). Some of their isolates grew intercellularly, whereas infectious isolates grew inter- and intracellularly. Intercellularly localization of hyphae in microscopic *Hydrastis* sections correlates with the isolation of *F. oxysporum* from the same root tissue. The presence of *Fusarium* in *Hydrastis* remained localized, near the tips of rootlets and could not be detected intracellularly, suggesting that the fungus was of low pathogenicity. The *Hydrastis* defense mechanism may also have prevented pathogenesis by the fungus or else a combination of low pathogenicity and plant resistance mechanisms may be responsible. Gordon and Martyn (1997) noted that *F. oxysporum* is primarily nonpathogenic in native ecologies and even when found at high levels in native soils, *F. oxysporum* does not always invade cortical tissue; since death of the plant host would limit growth of the fungus and also stimulate competition from other microorganisms. Thus being relatively non-pathogenic may be increasing the survival rate of the fungus.

According to Newsham et al. (1994), one major effect of a *Fusarium* endophytic host/pathogen relationship is to limit plant population density. They reported that the presence of *F. oxysporum* was negatively correlated with the "fecundity" of grass species, *Vulpia ciliata*, even when the fungi produced asymptomatic infections. On a cellular level, a similar relationship was reported as the inverse relationship between isoquinoline alkaloid synthesis (including berberine) and starch accumulation in cell cultures of *Berberis parvifolia* (Deliu et al., 1994) and *Coptis japonica* (Yamamoto et al., 1986). Since *Hydrastis* populations increase largely from asexual propagation, limiting population density may prevent the spread of high-density fungal leaf infections in native settings that are normally prevalent only in commercial field plots of clonal isolates. Also fungal elicitation of alkaloid production in *Hydrastis* root tissue, rather than starch accumulation, may decrease infection levels by soil pathogens.

Preliminary assay development

Although quantitative assessment of DNA is the most accurate method for measuring growth, hyphal elongation and changes in hyphal dry weight mass will be referred to as growth in this study. The initial study about *Fusarium* spp. response to isoquinoline alkaloids of *Hydrastis* used bioassay disks. One isolate was incorrectly identified as a *Fusarium* spp. and the results of that isolate will not be discussed, since the focus of the thesis is on *Fusarium*. The PSU *Fusarium oxysporum* was not inhibited by any of the treatments, except briefly by hydrastine at day 7. When the 95% methanol solvent evaporated at room temperature it left behind alkaloid residue on the bioassay disk, which

may have prevented alkaloid movement into the agar to allow contact with fungal hyphae. In a follow up study, a different approach was taken using a liquid media to measure changes in hyphal dry weight. Both of these assays were meant to provide evidence about which approach was more sensitive in measure fungal response to the alkaloid treatments and meant simply to screens for relative effects.

Using Czapeks Dox liquid media, the hyphal dry weight levels of the *Fusarium proliferatum* (Goth) isolates remained constant for the 0.1% treatments. In the 0.2% treatments, increasing the level of berberine and hydrastine in the media did not change the dry weight mycelia produced, but increasing the level of canadine and especially whole root extract did increase fungal dry weight. Unidentified LC-MS peaks were also detected in the whole root extract treatment filtrate suggesting that hyphal growth weight increase may be the result of an increase in metabolic rate of the fungi. Of the alkaloids added, berberine was expected to have caused a greater inhibitory effect on fungal growth, because of its quaternary, cationic structure would be expected to increased movement across the eukaryotic membrane of the fungus when compared to tertiary alkaloids such as canadine and hydrastine (Stermitz et al., 2000a, 2000b). For whole root extract, canadine and methanol treatments the resulting increase in dry weight may be due to a nutritive effect of the alkaloids, since canadine and methanol could be providing a carbon source to the fungus. Canadine may be influencing the metabolic process of *Fusarium* since it also occurs in the whole root extract. However, the largest hyphal dry weight increase occurred in the whole root extract treatment, which suggested that other components than just canadine may also play a role in the effect.

The fractioning method used to isolate secondary, terteriary, quaternary *Hydrastis* alkaloids from the non-alkaloid component failed to clearly separate berberine, canadine and hydrastine into individual fractions. TLC analysis showed that berberine was the only alkaloid detected in the alkaloid fraction, but response of the isolate to the alkaloid fraction treatment was very similar to that of the canadine treatment. Other major and/or minor alkaloids may have been present but could not be detected by TLC. Several authors (Mitscher et al., 1978; Gentry et al., 1998) have reported the difficulty of partitioning berberine in bulk-transfer experiments. They noted that although berberine is found largely in the more water soluble “chloroform insoluble” fraction, it also is found in smaller concentrations in more lipid soluble fractions due to the ability of berberine to add nucleophiles across the C(8)-N(7) olefinic linkage. Reversion occurs rather easily, thus the presence of berberine in several fractions. In addition, the increased level of extraction in methanol agrees with Matos et al. (1999) who reported that the methanolic extract of *Chelidonium majus*, a berberine containing plant, was more effective against several *F. culmorum* isolates than either the ethanolic or aqueous extracts.

Since the results from the initial liquid assay were more encouraging than the biodisk assay results, I continued to develop the liquid media design to study the effects of the fractions from the method above, as well as the whole root extract and individual alkaloid treatments on the hyphal growth of *Fusarium* spp.. The *F. proliferatum* (*Hydrastis*) isolate used was the first species identified from *Hydrastis* tissue. When compared to the bioassay disk study, the concentration of berberine and canadine treatments were

equivalent, hydrastine levels were lower and the whole plant extract level was higher. The *F. proliferatum* (*Hydrastis*) did not appear as sensitive to the whole root extract as the non-*Hydrastis F. proliferatum* isolate used in the previous assay, but berberine did stimulate hyphal dry weight increase by day 14. In this time course study, it is apparent the initial effect of the treatments is to inhibit growth, with only the methanol control and fraction blank inhibiting hyphal growth throughout the study. Repeated contaminant problems with the *F. proliferatum* (*Hydrastis*) isolate may have given some erroneous results as well. So the next study was begun after a second *Hydrastis* root isolate, *F. oxysporum*, had been identified. The inhibition of hyphal dry weight increase by the 95% methanol control also presented a problem, since all treatments are all diluted in this solvent. To limit the solvent effects I increase the amount of media and increased the fungal inoculum.

In this next liquid assay I also lowered the alkaloid concentrations to investigate the lower limit that could elicit a fungal response over a 14-day period. In this study, the effects of alkaloids on mycelial growth were measured by comparing the effects of whole root extract, individual alkaloid standards (berberine, canadine and hydrastine), as well as a treatment containing all three alkaloid standards combined. Without the ability to fraction the whole root extract components, comparison of the alkaloids individually and in combination with the whole root extract seemed the best alternative. By day 14 hyphal mass declined for the water control and all treatments except the whole root extract treatment, which stimulated mycelial growth and the methanol control which maintained a constant dry weight. Change in pH over time was equivalent for all treatments, with

only water maintaining a constant value. This suggests that pH change did not have a significant effect on hyphal dry weight change. The water control result suggested that by day 14 *F. oxysporum* (*Hydrastis*) growth decelerated and biomass decline occurred as endogenous carbohydrates were metabolized. In contrast, the contaminated *F. proliferatum* (*Hydrastis*) did not exhibit hyphal dry weight decrease in response to any alkaloid treatment. Either the endophytic *F. oxysporum* appears to be more sensitive to the whole root extract, or the contamination of the *F. proliferatum* culture was responsible for the increased growth in response to the whole root extract. At this point in the liquid assay design both stimulatory and inhibitory effects were discernible with respect to both the methanol and water control treatments. It is possible that the "non-alkaloid" component in the whole root extract may have provided additional nutrients not available in the other alkaloid treatments. Plant roots contain numerous proteins and carbohydrates that play a structural role. It is also possible that "non-alkaloid" component or minor alkaloids act as a chemical signal that stimulates mycelial growth.

After the all *Fusarium* isolates of *Hydrastis* were identified, the *F. oxysporum* endophyte isolate was selected as the central focus for subsequent studies because of its endophytic status. The methanol solvent had very different effects on two different *Hydrastis* root isolates. A small study was run to identify if a dilution solvent might be used that would be growth neutral and might also increase the extraction of the alkaloids from *Hydrastis* root tissue. Over the 14 days of the study, it became apparent that increased hyphal mass of the *F. oxysporum* endophyte plateaued between days 7-9, after which hyphal mass decreased. This suggested a maximum 7 day time course for any future study of the

endophyte. Also, only the 50% aqueous methanol:1% acetic acid solvent resulted in minimal growth inhibition of *F. oxysporum* comparable to the water solvent treatment. Since only two time points were used in the previous assay 14 day study, the resulting graph may not have shown hyphal mass increase and then decline for the methanol control treatment that was apparent in this solvent study.

Previous studies on the extraction and solubilization of *Hydrastis* alkaloids noted that addition of acetic acid to aqueous methanol increased the level of extraction of berberine (Li and Fitzloff, 2002), canadine and hydrastine (Cunningham, 2002). Canadine, in particular, was more soluble with the addition of acetic acid to the extraction solvent (datum not show). Obtaining maximum solubility of canadine was important since it had the lowest concentration level of the major *Hydrastis* isoquinoline alkaloids included in this study and its extraction insured that the relative ratio of alkaloids used in the assay accurately reflected the ratio of alkaloids found in *Hydrastis* plants.

Isolation of *Fusarium* isolates associated with *Hydrastis* was complete and the choice of which isolate(s) was also based on where the fungus was isolated, since *F. solani* was isolated from native *Hydrastis* rhizosphere soil but not wild *Hydrastis* tissue; whereas *F. oxysporum* was isolated from asymptomatic wild *Hydrastis* root and seed tissue, suggesting an endophytic host/pathogen relationship. Additionally, *F. proliferatum*, isolated at the south-facing site, does not produce chlamydospores, an important variable used in this study to measure the response of *Fusarium* to exogenous plant metabolites. Although phylogenetic analysis later revealed that what was originally a PSU *F.*

oxysporum isolate was reassigned to a sister taxon, *F. commune*, the 95% homology between the *F. commune* and the *F. oxysporum* (*Hydrastis*) allowed me to compare how two closely related fungal species might respond to alkaloids that ostensible only the *Hydrastis* isolate had been exposed. The *F. solani* isolate allowed me to contrast an isolate associated with the *Hydrastis* rhizosphere with one that was intimately associated root tissue. Of note, the *Hydrastis* chosen for the whole root extract treatment in the final assay was 5001ARZ, the plant root tissue with which the endophyte was associated.

Although the bioassay disk study provided little data, an attempt was made to study the growth of *Fusarium* on solid media with alkaloid treatments added to molten agar at levels found in native goldenseal root tissue. In general, the treatment effects on growth were not significant. All growth comparisons were made relative to the methanol control, to account for its inhibitory effects relative to water. The *F. commune* isolate was most inhibited by the methanol control, whereas growth of the *F. solani* isolate was least inhibited. In this solid media study, mycelial growth of the *F. oxysporum* endophyte was stimulated by all individual alkaloid treatments, the alkaloid standard treatment the most. The whole root extract treatment had little effect. This is in contrast to the previous liquid assay study where only the whole root extract stimulated growth. After the initial inhibition that occurred by day one, the level of growth inhibition of the *F. oxysporum* endophyte remained unchanged throughout the length of the study. In contrast, the mycelial growth of the *F. commune* and *F. solani* isolates was initially less inhibited by all treatments. However the rate of mycelial growth inhibition increased for the two non-

endophyte isolates over the length of the study. Interestingly, berberine had the greatest stimulatory effect on *F. commune*.

At this juncture, I was concerned about a number of issues in the using of solid media as a viable alternative to the liquid media design. Growth of *Fusarium* on solid media occurred in a central and peripheral zones. The peripheral zone, approximately 2 cm back from the edge of the colony and towards the center of the colony, is comprised largely of mycelia growing radially toward the edge of the plate. The central growth zone inside the peripheral zone includes both mycelia and spores. In studies where mycelial growth measurements are taken on solid media, they include only mycelial elongation along the media plane, whereas mycelial growth measurements in liquid culture are taken to include all the fungal tissue at the water/air interface, as well as spore formation that is occurring at the top most outer edge of the zone of mycelial growth. Another advantage of using liquid medium is that spore counts, being lower than on solid medium, are more accurately measured. Liquid media also provides a more homogeneous environment where treatments and nutrients are distributed uniformly and treatment compounds are less likely to become bound to the media matrix. Agitation of liquid media to provide sufficient oxygen also prevents pelleting of mycelia, which would inhibit sporulation.

In addition, pH values in solid media are difficult to measure, while conidia counts in the inoculum could be quantitatively estimated for the liquid culture, but not for the solid media. If the assay design is to model, as much as possible, the idea that the fungus interacts with *Hydrastis* alkaloids as root exudates components, than does one media

approximate the nutrient level found in root exudate more closely? Hestbjerg et al. (2002) suggested that the Czapeks Dox liquid media used in this assay was similar to the 35:1 ratio found in root exudates, favoring a pathogenic or non-pathogenic, as opposed to the C/N ratio of potato sucrose agar (a solid media), which was similar to that found in senescent plant tissue, favoring a saprophytic fungus.

On the other hand, the choice of media not only affects growth and sporulation, but also in the absence of plant cell wall components, normal induction of degradative fungal enzymes may not occur. *Fusarium proliferatum* produced a polygalacturonase II isoenzyme (PGII) on solid media in addition to PG I, which was produced in both solid and liquid culture (Niture & Pant, 2004). Since the treatment effect on hyphal elongation was not significant, the results on solid media probable reflect a carbohydrate utilization response unique to each strain and that the liquid assay design represents a better alternative when fungal response is measured using not only growth, but also pH change, sporulation, macroconidia germination rates, chlamydospore formation and mycotoxin production as the basis for comparing treatment effects.

Final Assay development

Hyphal dry weight growth after treatment with *Hydrastis* alkaloids

Hydrastis alkaloids had a minor affect on dry weigh hyphal mass accumulation, although the response of each *Fusarium* species could be used to differentiate the isolates based on

their level of association with *Hydrastis* root tissue and rhizosphere. Relative to the methanol control, all alkaloid treatments inhibited mycelial growth of the *F. oxysporum* endophyte throughout the study, except for the whole root extract treatment, which stimulated growth throughout. The methanol control effect on this isolate was 20% more inhibiting than the water treatment, a level greater than data from the solvent extract assay that indicated < 1% inhibition of growth of the *F. oxysporum* isolate by the 50% methanol:1% acetic acid control compared with water. Generally the individual alkaloid treatments inhibition of growth was 20% compared to the methanol control. As in previous studies, a non-alkaloid component or minor alkaloids not present in the alkaloid standard treatment may have stimulated growth of the *Hydrastis* endophyte isolate. Similar results were reported using *Senecio jacobaea*, a pyrrolizidine alkaloid containing plant (Hol & Veen, 2002). Growth of a *Fusarium* species isolated from *S. jacobaea* was stimulated by the whole root extract, whereas fungal growth was inhibited by individual *S. jacobaea* pyrrolizidine alkaloid treatments. Stimulation of growth in liquid media is often attributed to nutrients in the whole root extract. The Czapeks Dox media contains sufficient levels of nutrients for hyphal growth. The whole root extract was defatted to reduce the effects of potential nutritive components, lipids, sugars and amino acids found in the root mantle. Based on its growth response to the whole plant extract treatment, it appears that the endophytic fungus may recognize a *Hydrastis* root exudates signal.

The methanol control treatment reduced *F. commune* hyphal mass accumulation by 60% in comparison with the water control and the rate of the inhibition increased throughout the study. All treatments stimulated hyphal mass accumulation with respect to the

methanol control, ranging from 40-60%. This isolate is very sensitive to the methanol control treatment, more so than any of the other *Fusarium* isolates. The stimulatory effect of the whole root extract was based on invidual alkaloids and thus, not the same as was noted for the endophyte. In liquid media, mycelial growth of the *F. solani* isolates were initially inhibited by all treatments when compared to the endophyte isolate, but after 3 days no inhibition was detected in any of the treatments. Apparently, this isolate was not as sensitive to the methanol control, with a 15% reduction in hyphal mass accumulation. All treatments were stimulatory to growth rate when compared to both controls, ranging from 40-60%. Berberine alone and in the alkaloid standard treatment had the greatest effect. Both isolates did not respond to the whole root extract treatment as did the endophyte. Growth was stimulated by all treatments.

The findings indicate that *F. commune* is extremely sensitive to the methanol control and that the alkaloid treatments only partially overcome the effect. The alkaloid treatments were most stimulatory to the *F. solani* isolate, which was not very sensitive to the methanol control. It appears that berberine had the greatest effect on this isolate. The alkaloid standard treatment contained 3x the level of berberine than the whole root extract treatment, and was more stimulatory to hyphal growth. In comparison, the whole root extract stimulus of *F. oxysporum* growth was not related to individual alkaloids, but was probably based on some non-nutritive plant signal. Using the model of *Hydrastis* root exudate and *Fusarium* interaction at the rhizoplane, the difference isolate responds suggest that the endophyte may be unique in responding to a root exudate signal. Although the other fungus associated with the *Hydrastis* rhizosphere, *F. solani*, does not

respond to the signaling component in the whole root extract, it does respond to one of the major alkaloids that should be found in *Hydrastis* root exudates. Of note, the canadine treatment did not stimulate growth of any of the isolates, as in previous studies, possibly because of increased solubility when a 50% methanol:1% acetic acid extraction solvent was used. Although minor, apparently there is some growth adaptation on the part of *Fusarium* to extended exposure to *Hydrastis* alkaloids in the root exudates.

Some issues were not effectively optimized during the assay development. Having multiple concentrations of each treatment would have been provided additional data, but I was constrained by the number of samples (252) and the capacity of the shaker. Instead, I analysed two different concentrations used during the assay development using the endophyte. The analysis showed the inhibiting effects on hyphal growth of low concentrations of berberine (1.6 μg ml^{-1}), canadine (0.8 μg ml^{-1})and hydrastine (0.4 μg ml^{-1}) treatments in comparison with the methanol control were similar to treatments with higher alkaloid concentrations of berberine (4.8 μg ml^{-1}), canadine (1.2 μg ml^{-1}) and hydrastine (3.2 μg ml^{-1}). The concentration of the whole root extract treatment was 80μg ml^{-1} for both assays and the treatment was consistent, stimulating hyphal growth with respect to the methanol control in both studies. Interestingly, in the longer 14-day assay, the relative effects of individual alkaloids as well as the whole root extract treatment increased over time. After 14 days the inhibition of growth by the individual alkaloids was 60% versus 10% over the 4-day. The whole root extract stimulus increased from 20% over 4 days to 165% after 14 days. The solvent controls were not the same, but the relative effect of the treatments followed a similar pattern. In contending with the

difference effects the methanol control had on the 3 *Fusarium* species, optimizing the solvent for all three may have clarified the treatment affects.

And lastly, when the growth response on solid media was compared with the response of the of the 3 isolates grown on liquid media, it is interesting to note that the level of growth by the endophyte was the lowest in liquid media, but the greatest on solid media. The PDA is not as defined a media and contains more complex, less accessible carbohydrate sources. Thus an isolate would be required to degrade carbohydrate polymers to obtain nutrients. One could surmise that a non-pathogenic isolate might compete mores successfully at the site of attachment to plant roots based on that very characteristic, but that once inside the plant tissue, where nutrients are more readily available, a lower rate of growth would maintain non-pathogenic status. The opposite would then be true for more pathogenic isolates. Thus, the *F. solani* isolate, which had the lowest level of growth on the solid media, but the greatest in liquid media, would not compete as effectively at the rhizoplane and if it penetrated the plant root cell wall would not establish endophytic status.

Changes in media pH after treatment with *Hydrastis* alkaloids

At each time point where dry weight hyphal measurements were the pH of the cultural media was also recorded to detect potential effects that either the treatment or fungus had on the growing environment. The pH value for the water treatment over the course of the assay remained at pH 7.0 and was equivalent for all fungi. For two non-enophytic

isolates, the pH range (≅ pH 4.5 – 7.0) during the course of the assay was generally the same for all treatments. Over the course of the assay for all treatments where growth occured the pH values also increased. The only exception was when the pH values of the endophyte media remained flat in response to the individual alkaloid treatments and growth inhibition occurred. The pH range of the media with alkaloid treatments was 6.5 – 7.-0 while the methanol and whole root extract treatment media range was pH 4.5-7.0. If the affect of the individual alkaloid acting as bases was to buffer the acidic solvent of the endophyte culture, why only for this isolate? It is possible that the *F. oxysporum* isolate played a role in buffering the media of the individual alkaloid treatments, which then lead to a lag in growth. I cannot be absolutely certain, but randomizing the replicates and the assay design itself should have prevented experimental error as the cause for the pH trend in the *F. oxysporum* isolate data. The choice of time course for the assay may also have contributed to variability of pH values. From the preliminary studies log growth occurred through 7 days and less pH variability occurred over a 14-day time period. For the last assay a 4-day limit was chosen to be certain that hyphal growth that occurred would remain in the log phase. A longer time course, 5 to 7 days, may have flattened out some of the pH variability.

Thus if pH and growth stimulation are linked, to what degree and which is the dependent variable? Although hyphal growth of the *F. commune* was twice as sensitive to the methanol control as was either the endophyte or *F. solani*, the change in pH for the methanol control treatment was quite similar between all *Fusarium*. Using ANOVA,

changes in pH values were significantly correlated with growth for all treatments except the effect of hydrastine on the PSU isolate (Appendix, Statistics, Table 12). The pKa for hydrastine was 7.8, whereas the pKa of berberine is 2.47 (Merck). The final media pH for the hydrastine treatment for the PSU isolate was 7.5, so more than half of the solubilized hydrastine hydrochloride would presumably be occurring as a salt and not as an ion. It is unclear if the hydrastine salt is an active form of the compound and since the solvent in this study contained organic modifiers it is difficult to determine the degree to which hydrastine was ionized by comparison of pKa values. A more likely explaination is that as growth occurs, the fungus alters pH to a level that optimizes their chances of acquiring nutrients and surviving. Although the *F. oxysporum* isolate responded to the presences of individual *Hydrastis* alkaloids in a manner unlike the other isolates, it is difficult to associate the response with its endophytic status.

Spore formation after treatment with *Hydrastis* alkaloids

Spore formation and germination of spores in *Fusarium* is complex and consists of several spore types that appear to have different functions. The assay was designed to to take a snapshot of treatment effects on the fungi after four days exposure. The fungal response to treatments as measured by formation of reproductive spores, micro- and macroconidia, was minor. Relative to the methanol control all treatments inhibited *F. oxysporum* microconidia and macroconidia by 15-20%, with berberine inhibiting formation both spores by 25-30%. In contrast, all treatments stimulated *F. commune* microconidia and macroconidia formation by 20%, with the whole root extract treatment

stimulating formation of both spores by 40%. All treatments stimulated formation of both types of conidia in the *F. solani* culture by 20% relative to the methanol treatment. Thus the only significant effect was the whole root extract treatment stimulus of the *F. commune* conidia formation. However, limited advantage would be gained by the *F. commune* isolate in the rhizosphere, since these spore types are not highly correlated with fungal competitiveness at plant root surfaces or within the rhizosphere (Couteaudier 1989). Rather, once an isolate has penetrated into host root tissue, Stromberg and Corden (1980) and Suleman et al. (1996) noted that the presence of *F. oxysporum* conidia in the xylem stream was necessary for hyphal growth to occur in tomato, since hyphae would not be able to grow beyond the tomato perforation plates. *Hydrastis* metaxylem tissue contains scalariform perforation plates whereas secondary xylem contains single bar perforation plates (Carlquist, 1995) both of which are morphological structures that would prevent fungal pathogens from moving into the xylem stream by extension of hyphae. My results suggest that micro- and macroconidia spore formation is more likely to be stimulated by alkaloid components of *Hydrastis* root exudates outside the plant, including nutritive and non-alkaloid components. Formation of either spore type once the fungus has penetrated *Hydrastis* root tissue is a requirement for infection of the plant through conidia movement via the plant root vascular system. Thus the endophytic status may in part be conferred as the result of inhition of conidia formation by *Hydrastis* alkaloids. And since the *F. solani* was not isolated within *Hydrastis* root tissue, which interaction factors might favor the *F. oxysporum* over the *F. solani* isolate?

Fusaria produce chlamydospores to aid its survival in a dormant state (Cochrane et al., 1963) and pathogenesis occurs at much lower inoculum densities of chlamydospores than with micro- or macroconidia (Couteaudier and Alabouvette, 1990). Thus a fungus would be more competitive at the rhizoplane even at lower levels of mycelial mass. When the water treatment was compared for all three *Fusarium* isolates, the endophyte produced none, the *F. commune* the most, and the *F. solani* was most consistent across all treatments. The methanol control had a significant stimulatory effect on formation of *F. oxysporum* and especially *F. commune* chlamydospores. Chlamydospores of the endophyte also appeared in the hydrastine and whole root extract treatments at much lower levels compared to the methanol control. In contrast, chlamydospore formation by the *F. commune* isolate was significantly inhibited by the whole root extract. However, the alkaloid standard and the hydrastine treatment in particular, stimulated formation with respect to the methanol control. It is interesting to note that chlamydospore formation by both species is stimulated by the methanol control and less so by the hydrastine treatments. But, the presence of hydrastine in the whole plant treatment was stimulatory to *F. oxysporum* chlamydospore formation, the presence of the compound in the alkaloid standard treatment was inhibitory. The opposite was true of the *F. commune* isolate. Thus, this may reflect that a non-alkaloid component in the whole root extract is more likely to inhibit chlamydospore formation by the *F. commune* isolate, but not the endophyte. Chlamydospore formation by the *F. solani* isolated was inhibited by both berberine and canadine treatments individually, although not by either the whole root extract or the alkaloid standard treatments which were stimulatory by 10%. It may be that the hydrastine component of both those treatments was able to counter act the inhibiting

effects of the other alkaloids. More likely, the *F. solani* isolate may also be able to respond to a component of the whole root extract in forming chlamydospores. The effect might be based on non-nutritive signals, such as the C-methyl flavonoid Hwang et al. (2003) isolated from *Hydrastis* root tissue, which might play a role in altering formation of chlamydospores by the *F. commune* isolate that has no association with *Hydrastis* root system. Based on chlamydospore stimulus, the *F. solani*isolate may form a greater inoculum potential in response to the whole root extract than the *F. oxysporum* isolate. It appears to be capable of responding to components of the root exudates, and yet it does not form as intimate an association with *Hydrastis* root tissue.

Alternatively, if stimulatory or inhibitory effects of the non-alkaloid component were responses to a nutritive component, it would be expected to have similar effects on all three isolates. Several authors noted that addition of inorganic or organic nitrogen to soil or artificial media decreased levels of chlamydospore formation (Loffler and Schippers, 1985; Schippers, 1972). Even though the alkaloids and possibly non-alkaloid components of *Hydrastis* contain nitrogen, these additional amounts are not very large and do not appear to reduce chlamydospore formation in this study. And, according to Oritsejafor (1986), the C/N ratio of 40:1 (found in Czapeks Dox) should not be selectively stimulatory or inhibitory to *F. oxysporum* chlamydospore formation. Presumably, other culture conditions might have undue influence on chlamydospore formation. The increased dissolved oxygen formed because of agitation of liquid media may have inhibited *F. oxysporum* chlamydospore formation levels according to Hebbar et al. (1997). No data is available on the similar effects on the other isolates, which makes

comparison difficult. The change in isolate pH values did not appear to effect chlamydospore formation. Hebbar et al. (1997, 1996) noted that pH effects on *F. oxysporum* chlamydospore formation did not have a significant effect until day 10 in liquid media and that an increase in pH stimulated formation.

The study also measured the macroconidia germination rate of each fungus in response to the alkaloid treatments. As in chlamydospore formation, the patternof macroconidia germination in response to the alkaloid treatments for the *F. oxysporum* and the *F. solani* isolates was different than that of the *F. commune* isolate. The level of macroconidia germination by the *Hydrastis* isolates was equivalent for both controls. Whereas all individual alkaloid treatments significantly inhibited *F. oxysporum* microconidia germination by 50-75%, the whole root extract treatment stimulated germination by 40%. The inhibition by the individual alkaloids does not appear to be related to the unique pH response of the fungi to these compounds since both the levels of germination in methanol and the water control treatments are similar while their pH values were initially quite different. Similarly, germination rates of the *F. solani* isolate were also significantly stimulated by the whole root extract by 200%, but inhibited by hydrastine (50%) and the alkaloid standard treatment (75%). The findings suggest that the alkaloids are not responsible for stimulating germination, but rather another component of the whole root extract treatment. In contrast, germination rates of the *F. commune* isolate were inhibited by all treatments with respect to methanol (15%), especially berberine, canadine and the whole root extract treatments (40%). The component(s) responsible for the inhibition of macroconidia germination appears to be the alkaloids themselves. The non-*Hydrastis*

isolate is not responsive to a compound in the whole root extract, which models root exudates in our assay design. Thus it seems unlikely to be able to respond to root exudates based host/plant signalling. Again the *F. solani* responded more aggressively to the whole root extract, as it did in forming chlamydospores. Using both responses, how does the endophyte form a more intimate relationship? It is possible that the more limited response of the *F. oxysporum* isolate in comparison with the *F. solani* isolate would not overwhelm the root tissue, thus preventing disease symptoms. However, no disease symptoms were observed in sampling a large number of *Hydrastis* roots from both of the wildcrafting sites in Rutland, Ohio. An alternative experimental approach based on previous research (Bolwerk et al., 2005; Di Pietro et al., 2001a) suggests that non pathogenic *F. oxysporum* out compete pathogenic isolates at the rhizoplane based on their ability to attach to the plant root surface more quickly. Thus chlamydospore inoculum levels and rates of macroconidia germination may not be the most important characteristics to study in comparing endophytic status with.

Without gene expression data I am unable to fully discern host/pathogen signaling, nor without analysis of a wider array of compounds, including flavanoids and proteins can host defense response be fully considered. However, the data suggests that the two isolates associated with *Hydrastis* root tissue and rhizosphere soil appear to respond differentially to components in the whole root extract than the *F. commune* isolate, with the *F. solani* responding more aggressively. Despite the overt similarity in genome of *F. oxysporum* and *F. commune*, how do they differ in response to the *Hydrastis* alkaloids? The *F. commune* was isolated from river sediment (Ueno et al*.*, 1977). Microconidia and

macroconidia production by this isolate was partially stimulated by the non-alkaloid component of the whole root extract, the whole root extract treatments strongly inhibited chlamydospore formation and the presence of any alkaloid inhibited macroconidia germination rates. Couteaudier (1989) found that production of *F. oxysporum* microconidia was the most important factor in the ability of isolates to colonize non-rhizosphere soil. Depending on the extent of *Hydrastis* alkaloid diffusion, if all of the alkaloids were being excreted by *Hydrastis* into the rhizosphere the survival of non-endophytic isolates in rhizosphere soil would be limited by lower levels of chlamydospore formation and macroconida germination. However, their presence in non-rhizosphere would be substantially increased because of the increase in both micro- and macroconidia. When compared to the response of the *F. commune* isolate, the *Hydrastis* endophyte appears able to be a respond to a signaling componet found in the the whole root extract treatment that stimulates both chlamydospore formation and germination of macroconidia.

Microscopic analysis of the cultural medium provided more evidence that a component of the whole root extract treatment may also change the morphology of the fungal colony. The lipid-like globules excreted by the *Fusarium* endophyte at hyphal tips in the 96 well plate study occurred at a substantially higher level in the whole root extract treatment. The same treatment had no effect on macroconidia production in liquid media but did stimulate macroconidial germination and hyphal growth. These spheres were not observed in the liquid media assay but were observed in solid media for the whole root extract. It is not known whether the lipid spheres were fungal structures that contain

mycotoxins, or were precursors to macroconidia formation. Exudates have been known to occur at the actively growing hyphal tip in several *Fusarium* species (Cooke, 1971; Waters et al., 1975; Colotelo, 1978; Carlquist, 1995) and the formation of these droplets occurred in growing hyphae at a location where spores later form as the hyphae aged (McPhee and Colotelo 1977). The authors reported that the initial appearance of these exudates was watery and hyaline, and in bead-like groups associated with aerial mycelium of a *Fusarium culmorum* colony growing on PDA where eventually macrospores were formed. Colotelo (1978) analyzed the liquid droplets and found enzymes and/or secondary metabolites believed to be precursors to macroconidial formation, but the metabolites were never characterized. The phenomenon was not investigated any further.

Mycotoxin production after treatment with *Hydrastis* alkaloids

Mycotoxin production was the final characteristic that was studied. Since previous studies (Borrow et al., 1961; Bu'Lock, 1975; Righelato, 1975; Brown et al., 1991) reported that *Fusarium* growing in liquid culture produced maximal secondary metabolite at the end of the growth phase after 4-8 days, depending on growth media and growing conditions. This was a factor in the decision to screen the *Fusarium* isolates for mycotoxins on day four. The effect of assay treatments on mycotoxin production varied. All treatments inhibited ZON production in the *F. commune*, while the *F. solani* isolate did not produce detectable mycotoxin levels. Relative to the methanol control treatment canadine and hydrastine stimulated T-2 mycotoxin production by the *F. oxysporum*

endophyte. The alkaloid standard treatment stimulated ZON production more strongly than the whole root extract treatment, canadine or methanol alone, suggesting a interaction of the alkaloids. Fumonisin production was only stimulated in the *F. oxysporum* media containing the methanol, suggesting the alkaloid treatments were inhibitory. Interestingly, ZON, T-2 toxin and fumonisin were simultaneously produced by the endophyte in the canadine treatment. Unlike berberine, canadine is not a cationic salt and may not be extruded as easily from fungal cells by MRP or ABC transporters.

It is not clear if the absence of DON production and presence of ZON in the *F. oxysporum* endophyte isolate may have been the result of high oxygen, since DON is produced under condition of low oxygen tension (Miller and Blackwell 1986); whereas ZON requires oxygen saturation for optimal production (Hidy et al. 1977). Other cultural conditions should not have prevented production since DON appears heat stable to 120º C and does not decompose in mildly acidic conditions (Krska et al., 2001). It may be none of the isolates were capable of producing DON. Interestingly, the presence of a purple pigment in the *F. solani* cultured mycelia may have indicated the presence of naphthaquinone-type pigments, which occurred initially by day 1 in the water treatment and appeared to accumulate at higher levels on days 3-4; whereas the pigmentation of mycelia in culture occurred by days 3-4 in the canadine and methanol treatments. Without quantitative detection data it is difficult to evaluate the mycotoxin production of this isolate, but the pattern of pigmentation would suggest that alkaloid treatments, except canadine inhibited production.

The preliminary ELISA screen for mycotoxin production indicated 1.0-7.2 ng mg^{-1} of ZON, 0.04-0.24 ng mg^{-1} of T-2 and 1.0-5.8 ng mg^{-1} of fumonisin by the *Fusarium* endophyte isolate. ZON has been previously reported occurring simultaneously with type-A trichothecenes in a variety of commodity products, with an average concentration of 0.5 ng mg^{-1} using GC-MS (Tanaka et al., 2000). The limits of detection (LOD) for the Neogen ELISA kit used in this study are reported as 0.2–4.5 ng g^{-1} for T-2, 0.2-1.0 ng g^{-1} for fumonisin and 11.2-144 ng g^{-1} for ZON.

Two European laboratory intercomparison studies found a significantly higher scatter for ELISA mean values than from HPLC and GC (Josephs et al., 2001) and reported ELISA method quantitation ranges of 200 μg ml^{-1} for fumonisin, 7.5 μg ml^{-1} for T-2 toxin and 50 μg ml^{-1} for ZON. Other studies (Shelby et al., 1994; Sydenham et al., 1996) found that the ELISA method cross-reacted with structurally related fumonisins, thus overestimating fumonisin concentrations. The levels of mycotoxins detected in the liquid assay appear at the lower end of the LOD. In general, accuracy and precision of all analytical methods for mycotoxins incur sampling errors, since the metabolites are distributed unevenly (Scott, 1990). In separating and detecting multi-component *Fusarium* secondary metabolite profiles, it is difficult to get an accurate picture of all of the metabolites at any given time. The concentration of the metabolite may be below the detection level of the specific analytical method used. The isolate may not be producing the metabolite of interest because of deterioration problems associated with prolong culturing or the cultural conditions might not be conducive to the production of the metabolite (Marasas et al., 1984; Marasas et al., 1985; Thrane, 1989). But as a preliminary screen, the ELISA

method provided an indication that various mycotoxins were present in different *Fusarium* isolate cultures when highly accurate and precise methods were not required. Since the levels of various mycotoxins measured are near the LOD, accuracy of the values must be questioned. Certainly an analytical method specific to each analyte, with lower LOD and LOQ values, would provide more certainty about the presence of these compounds in the assay. But the overall trend does seem to indicate that the endophyte is metabolically more active when it comes to the production of mycotoxins. Thus, in this study under *in vivo* conditions, the ability of the *Fusarium* endophyte to produce T-2 and fumonisin as well as ZON would increase its competitive advantage in comparison with other fungi in the *Hydrastis* rhizosphere.

CHAPTER 8:CONCLUSIONS

As the use of herbal medicine continues to increase the acquisition of major medicinal plant species, such as *Hydrastis*, occurs more frequently from cultivated plants rather than from those collected in the wild. We understand little of whether such a change in rhizosphere ecology also alters levels and ratios of clinically relevant plant secondary metabolites. It is probable that *Hydrastis* major alkaloids are elicited response to interactions with soil fungi. We know even less about how these clinically relevant plant compounds reciprocally affect the rhizosphere of medicinal plants. Thus this study focused on the rhizosphere influence of *Hydrastis* isoquinoline alkaloids on the fungal community.

Extensive screening of non-rhizosphere and rhizosphere soil, *Hydrastis* seed, rootlet, rhizome, stem and leaf revealed an array of fungal species of varying levels of association with *Hydrastis* plant tissue and rhizosphere. *Hydrastis* rhizosphere influence appears to limit pathogen activity while simultaneously stimulating endophytic relationships. These findings corroborate previous research on the ability of plants to influence their rhizosphere ecology. Larkin et al. (1993) reported that a watermelon (*Citrullus lanatus*) cultivar influenced its rhizosphere soil through root exudates, creating its own "suppressive soil" by increasing non-pathogenic *F. oxysporum* isolates. The *Fusarium* spp. were more closely associated with both tissue and rhizosphere soil compared to other fungi. The isolation of a *F. oxysporum* endophyte and a *F. solani* associated with the rhizosphere soil provided an opportunity to explore how each might respond to the presence of *Hydrastis* alkaloids. To model such an interaction an assay

was designed based on the assumptions that berberine, canadine and hydrastine appear to be present in *Hydrastis* root exudates and that the most likely point of contact would occur at the host plant root interface within the rhizoplane within the rhizosphere soil. And the whole root extract treatment was chosen to model the complex of compounds present in *Hydrastis* root exudates. The rhizosphere is difficult to model and many questions remain unanswered. Are plant secreted alkaloids concentrated enough to affect soil microbes such as *Fusarium*? Do secreted alkaloids bind to various colloids in the soil? Will the soil moisture dilute the alkaloids to a level too low to affect soil microbes? Or will the alkaloids become oxidized in low oxygen surface soils? All of these variables could influence the interaction of *Hydrastis* alkaloids with *Fusarium*. Early attempts were unsuccessful at transforming *Hydrastis* with *A. rhizogenes* into a hairy root culture and/or maintaining the whole plant hydroponically. Thus, limited to growing *Hydrastis* in the greenhouse, collecting root exudates was unfeasible. Given the limitations, the *Hydrastis* whole root extract used in these studies provides the more complete estimate of *Hydrastis* root exudates. Future studies are needed for definitive data on the composition of *Hydrastis* root exudates.

Several morphological characteristics were used as fungal response indicators. For the first indicator, hyphal growth, even though both inhibition and stimulation of dry weight hyphal mass was limited the sensitivity of the assay in detecting treatment effects on hyphal growth was significantly greater than previous research. Mahajan et al. (1976, 1986) reported that 10 mg ml^{-1} of berberine added to solid Sabouraud's dextrose agar (SDA) inhibited the growth of an unidentified species of *Fusarium* species. Cernakova

and Kostalova (2002) reported even lower levels of berberine (1 mg ml^{-1} as inhibitory to the growth of *F. nivale* grown on SDA. In contrast, the current study used a final concentration in the media of 4.8 μg ml^{-1} berberine, 0.8 μg ml^{-1} canadine and 3.2 μg ml^{-1} hydrastine. All of the previous studies also reported problems with berberine solubility in aqueous solvents, which this study was able to optimize with use of an acidified 50% methanol solvent. Although the solvent was also optimized to limit its inhibitory effects on growth of the endophyte, which was a major focus of the study, the methanol solvent had a significant inhibitory effect on growth of the *F. commune* species used for comparison. Future studies would benefit not only from optimizing solvent effects on all fungal species used, but also from the use of different alkaloid concentrations to provide improved assessment of minimum inhibitory concentrations.

A more significant alkaloid effect was noted on macroconidia germination. Several authors (Singh et al., 2001; Sarma et al., 1999) reported that berberine inhibited *F. udum* spore germination while suspended in sterile distilled water, at concentrations ranging from 0.25–1.0 mg ml^{-1}. The berberine salt was not specified. Goel et al. (2003) used 0.2 mg ml^{-1} of both (±)-α-hydrastine and (±)-β-hydrastine isolated from *Corydalis longipes* to inhibit spore germination in *Alternaria*, *Curvularia*, *Colletotrichum*, *Helminthosporium* and *Erisyphe*. By comparison the current study used a final alkaloid concentration in the media of 3.2 μg ml^{-1} β-hydrastine. An increase of by an order in the sensitivity of fungal response to the same treatment indicates that development of this liquid assay significantly improved measurement of fungal spore germination. This response was an important indicator of the level of adaptation by the fungus to alkaloids

that may be present in *Hydrastis* root exudate, as germination rates for both *Fusarium* associated with *Hydrastis* increased after treatment with the whole plant extract while germination rates for the *F. commune* were greatly reduced.

Comparing fungal response to the whole root extract with the response to the alkaloid treatments indicated that a component of the whole root extract, either minor alkaloids or non-alkaloid, non-nutritive compounds, may play a role in the increased germination rates and chlamydospore formation of both *Fusarium* associated with *Hydrastis*. These factors were repressed by the same components of the whole root extract treatment in the non-*Hydrastis F. commune*. The *F. commune* responded significantly to the methanol control treatment by increasing chlamydospore formation, which appears to be a stress response by the fungus. Although all treatments inhibited ZON formation by *F. commune*, the production of mycotoxins by the endophyte in response to different treatments was more complex. Fumonisin, T-2 and ZON were produced simultaneously only in the canadine treatment. And while synergistic interactions between canadine and other alkaloids appear to play a role in stimulating ZON production by *F. oxysporum*, it does not affect T-2 or fumonisin production. When the increased macroconidia germination rate and formation of chlamydospores by the endophyte are considered in conjunction with the stimulation of ZON production, the *F. oxysporum* isolate appears more responsive to the whole root extract treatment. Although more sensitive to the whole root extract than *F. commune*, *F. solani* was not as sensitive to the whole root extract as the endophyte. Hyphal growth, microconidia and macroconidia formation by

the *F. solani* isolate was more stimulated by the berberine treatment than the whole plant extract treatment.

Using the model of interaction between *Hydrastis* root exudate and the *F. oxysporum* (*Hydrastis*), we can interpolate from the assay data to hypothesize the initial roles they each play in establishing the endophytic relationship. As previously mentioned, when *Hydrastis* seeds break dormancy two-phase germination often occurs, a characteristic that would maximize root and rhizome development during the intitial plant exposure and interaction with rhizosphere microbes. Isoquinoline alkaloid production in *Berberis vulgaris* occurs immediately after seed germination and increases with seedling age (Pitea et al. 1972). It is unknown whether the genes for these plant defense compounds are being expressed constitutively or elicited. Biesaga-Koscielniak et al. (2003) reported that ZON had auxin-like effects and work with *Arabidopsis* provides evidence that auxin promotes the development of lateral roots (Celenza et al. 1995) and influences root vascular development (Mayer et al. 1993). Bean et al. (unpublished) reported that ZON stimulated corn root tip growth. It is possible that the production of ZON by *Fusarium* may affect meristematic activity in new *Hydrastis* roots by stimulating rootlet formation, root exudation and access to plant root tissue that has not yet matured. This would also increase fungal access to root signals within *Hydrastis* root exudates, thus facilitating penetration of the host root tissue by endophytic fungi capable of recognizing such signals. Interaction with ZON may also provide stimulus to protoxylem tissue formation in undifferentiated apical and subapical root zones, which would then allow, as Turlier et al. (1994) have suggested, movement of *Fusarium* from the initial infection site into

mature vascular root tissue. Rootlet interaction with ZON during the early phase may also benefit the plantlet by increasing plant secondary metabolite formation.

Of note, Renaud and Strauss (private communication, 1999) compared seasonal variations in berberine and hydrastine content of wild goldenseal root collected from north- and south-facing collection sites in Rutland, OH during April, June, September, and November, reporting that during June the alkaloids levels at the north-facing site were 50% higher than at the south-facing site; and that the difference was equivalent for both berberine and hydrastine levels. During other collection times the total alkaloids at the north-facing site was only 10% higher than at the south-facing site. Although a larger sample size may have proven to be more statistically robust, rhizosphere competition during June would be expected to be at peak levels. The presence of the endophyte in *Hydrastis* collected from the north-facing site may explain the increased alkaloid levels. The authors also noted that the total alkaloid content was highest at both sites in November, at which point *Hydrastis* begins winter dormancy, and reallocation of alkaloid to the root may have occurred. Cech (private communication) collected organically cultivated goldenseal from Kentucky and Oregon during mid-October. The alcohol extracts were analyzed by HPTLC and although the total alkaloids were greater in the root, the relative berberine/hydrastine ratio was higher in the leaf, with berberine levels higher in the leaf and hydrastine levels higher in the root. Matos (1999) noted a similar distribution of berberine as the predominant antifungal alkaloid in the aerial portion of *C. majus*, as well as in which compounds appeared to active against *Fusarium* isolates, noting that two other isoquinoline alkaloids, chelerythrine and sanguinarine, were the

predominant alkaloids in root antifungal activity. This too would corroborate the findings of Shitan et al. (2005) that an ABC pump moves berberine from root tissue to aerial portions of the plant. Interestingly, hydrastine inhibited ZON production while the alkaloid standard was the only treatment to stimulate production. The increased expression of hydrastine in root exudate coincides with the likelihood of encountering rhizosphere fungi, such as *F. commune* and *F. solani*, neither of which produced ZON in response to the alkaloid treatments. However, the endophytic *Fusarium* was capable of producing ZON in response to the presence of all three alkaloids.

If a *Fusarium* isolate produces ZON and can recognize alkaloid or non-alkaloid signals present in root exudates, the fungus benefits from developing an early relationship with the plants roots. Additionally, ZON promoted plant root efflux of amino acids in several C-3 plants (*Medicago sativa, Medicago truncatula,* and *Triticum aestivum*) as well as a C-4 plant (*Zea mays*) (Phillips et al. 2004). The levels of amino acid efflux increased in conjunction with increased concentrations of ZON. Thus the effect of ZON production may not only increase access to root tissue, but also increase nutrient levels in the immediate vicinity of the root tissue. Of note, Hestbjerg et al. (2002) found that ZON was produced by *F. culmorum* in response to a C/N ratio that most resembles that found in root exudates. They reported that a yeast extract medium composition with a C/N ratio of approximately 35:1 is similar to root tip exudates from living plants, whereas potato sucrose media, with a higher C/N ratio, is closer to that found in senescent plant tissue. They reported the production of ZON in *F. culmorum* growing in yeast extract medium, but when the fungus was grown on either potato sucrose agar or agar containing only soil

organic matter, no ZON was produced. The ratio of the Czapeks Dox media used in this assay was 40:1 C/N. Since both the endophyte and the PSU isolates are capable of ZON production, and the nutrient and oxygen levels were appropriate to stimulate production, the endophyte response to the alkaloid standard treatment reflects a unique ability to respond to *Hydrastis* root exudates. An additional developmental question that needs to be explored is to what degree might the presence of ZON, or similar microbial compounds in the rhizosphere, favor or induce two-phase germination?

Having a sensitive method to separate, detect and quantitate the levels of *Hydrastis* alkaloids was essential for comparing the individual alkaloid treatments with the whole plant extract. It allowed me to assess whether a treatment effect was based on different concentrations of individual alkaloids or differing ratios. The MS provides improved conformational data that previously relied on PDA detection. Future studies of reciprocal influence of rhizosphere microbes on the formation of *Hydrastis* alkaloids will be able to use the method to analyze small changes in metabolite levels as a measure of plant response. Additionally, the method can be used in studies to assess the relative safety and efficacy of different *Hydrastis* formulations used in animal testing and clinical trials.

During development, the utility of the method was expanded to include detection of *Hydrastis* economic adulterants, including *Coptis japonica*, *Xanthorrhiza simplicissima*, *Mahonia aquifolium*, *Chelidonium majus*, and *Berberis* spp.. The new analytical method improved both the time required for analysis of 10 different isoquinoline alkaloids and analyte resolution, which had previously been a drawback of existing methods. Using the

method, 5% levels of *Hydrastis* contamination with *Coptis spp.* and *Berberis spp.* were successfully detected. The current market price and loss of habitat continues to provide the cost incentive for continued adulteration of *Hydrastis* raw material at levels greater than 5%. Thus, the new method will not only prove useful where good manufacturing processes (GMP) are required to detect levels of active alkaloids of raw material, but also during the manufacture of finished products. As medicinally important plants are increasingly being farmed or reintroduced into wild settings, the level and ratios of clinically relevant plant secondary metabolites need to be monitored to assess the role that different rhizosphere influences have on their formation. This information than can be used to improve safety and efficacy findings from clinical trials.

APPENDIX

Fungal media formula

Water Agar
15 g agar
1 liter dH_20

Potato Dextrose Agar (PDA)
39 g of PDA mix
1 liter dH_20

Czapeks Suspension
35 g Czapeks
1 liter dH_20

Carnation Leaf Agar (CLA)
20 g Agar
1 liter H_2O
sprinkle sterile Carnation Leaf on setting plates

Pythium
20 ml V-8 juice
200 mg $CaCO_3$
1.5 g agar
80 ml dH_20
(Long term storage, place 4 mm culture disc in 10 ml distilled sterile water with 2 hemp seeds in test tube)

Preliminary experiments

A. Germination and development of mistletoe (*Phoradendron leucarpum*) seed on artificial medium

Mistletoe lectin research has been done on the European mistletoe, *Viscum album*, because of its history of traditional use in Europe and current clinical use of mistletoe lectins as an anti-cancer agent. However, the chemical ecology of host/parasite interactions between American Mistletoe, *Phoradendron leucarpum* and its hosts has had limited study. *Phoradendron leucarpum* was successfully grown on artificial media using tissue culture protocols of other mistletoe genera. Preliminary experiments were done to determine the presence of lectins in cultured plant tissue and to use this *in-vitro* plant host/pathogen system to investigate the role of mistletoe lectins in its pathogenesis.

Methods

Lectin extraction:

Phoradendron leucarpum and *Acer saccarinum* host material were collected November 1997, from Williamsburg, Virginia. Plant material was air-dried, lyophilized and ground using a Waring blender and Wiley mill. Mistletoe haustorium, host tissue, *V. album* powder (commercial source), and mistletoe plant tissue culture were extracted using a modified protocol (Ribereau-Gayon et al., 1986). Using an Ultra Turrax homogenize, and plant material amounts and concentration levels of 4.3g at 43 mg ml-1 of haustorial tissue, 2.4g at 40 mg ml-1 of host tissue, 10g at 67 mg ml-1 of *V. album* tissue and 138mg at 6.9 mg ml-1 of culture tissue was extracted, proteins were precipitated with ammonia sulphate and resuspended in Tris buffer at 1 mg ml-1.

Lectin determination:

Gel electrophoresis was by PhastSystem (Abersham Pharmacia Biotech), SDS-PAGE buffer strips, 8-15% gradient gel and a sample buffer consisting of 4% SDS, 2% DTT, and 0.001% Bromo Phenol Blue in PBS. Sample tissue was applied at a level of 175 ng. Purified lectin from *V. album* (Sigma) was also used for comparison. One gel was stained using a Coumassie Blue for protein detection or by use of a modified Periodic acid-Schiff Glycoprotein staining kit (Sigma).

Tissue Culture:

One hundred eighty seeds were air dried, processed and plated on modified White's media (Bajaj, 1970) and incubated on slants or petri dishes under continuous diffuse light conditions (15-40 micro einsteins / m^2 s), at 20*C. In the initial medium, 300 mg of streptomycin and 100 mg of penicillin were added to prevent bacterial contamination, 250 mg of asparagine added to media because *V. album* is reported to accumulate asparagine from its host as well as lectin production (Franz). The seeds were transferred to fresh media at 10 weeks and harvested after 16 weeks.

Results

Lectin extraction:

Levels of crude protein extracted: 284mg (6.56%) from haustorial tissue, 103mg (4.29%) from host tissue, 396mg (3.96%) from *V. album*, and 107mg (74.6%) from tissue culture.

Lectin determination:

The modified Periodic acid-Schiff reaction indicated the presence of peroxidase at 44kD. The coumassie stain revealed bands at approximately the following positions. Note a standardized molecular weight gel was used as an overlay to approximate molecular weight:haustorium 24 kD, 48 kD, host 24 kD, *V. album* 24 kD, 67 kD, tissue culture 67 kD, *V. album* lectin 28 kD, 36 kD,

peroxidase 44 kD (known).

Tissue culture:

In the study to grow *P. leucarpum* under tissue culture conditions, 57% of the seed were viable after sixteen weeks. At 16 weeks, seedlings had developed suspensors and accessory embryos (see photograph). The seeds that did not grow, were contaminated with filamentous fungi.

Significance of this preliminary study

The extracts of *V. album* were reported that contained 2.11% crude protein (Eifler et al., 1994). Although micro-extraction methods increased the yield of crude proteins, the glycoprotein stain used was negative for both *P. leucarpum* haustorial and cultured tissue. This contrasts with Eifler's findings.

Sigma reports that purified *V. album* lectin was applied at 10 ug on gel electrophoresis and stained with coumassie blue resulted in two major bands. Lectin standard at 175 ng to the gel resulted in two faint bands after staining with coumassie blue. The molecular weight of *V. album* lectin (MLI) was 64kD on SDS-PAGE (Eifler, et al., 1994). The protein gel bands of 28kD and 36kD, were the MLI lectin chains described by Franz et al. (1981). Whereas the 67kD band for commercial *V. album* was likely the MLI lectin dimer. Both *P. leucarpum* haustoria and host tissue contain 24kD bands, and since the extracted haustorial tissue unavoidably contained host tissue as well, the 24kD bands were assumed to be host tissue proteins. The 48kD band for haustorial tissue did not stain for carbohydrates, and it's identity is unknown.

The only visible band on Periodic acid-Schiff stained gel was the control, peroxidase, at 44kD applied at a concentration of 1 mg ml^{-1}. Peroxidase carbohydrate content is approximately 16%, compared with the report of MLI carbohydrate content of about 10% of Eifler of et al., (1994). The absence of carbohydrate staining in plant tissue and control may be due to insufficient

material being applied to the gel.

Neither lectins nor biological activity were found in *P. leucarpum* tissue culture, mistletoe haustorium, host tree, or in *V. album* plant extract and it's standardized lectin. Since *P. leucarpum* grows at a slow future studies should concentrate on methods to stimulate growth of *P. leucarpum* on artificial media. Greater amounts of raw plant material extracted should produce larger amounts crude protein. Increased sample concentration on SDS-PAGE and agglutination assays, should also result in improved protein focusing and staining of the carbohydrate moiety of *P. leucarpum*.

B. *Hydrastis canadensis* tissue culture

As native populations of goldenseal (*Hydrastis canadensis*) come under continued pressure because of commercial demand, more herbal product manufacturers are looking at farmed and tissue cultured plant material as an economically viable alternative to wild collected plant material. However, there has been no investigation as to whether the alkaloid levels differ between wild and cultivated *Hydrastis*. A changing pattern of alkaloid levels in *H. canadensis* in the complex ecological settings native to goldenseal would suggest an influence of the environment, including the soil in which the plants are grown, on the secondary metabolites present in the plant.

The primary goldenseal alkaloids are berberine, canadine and β-hydrastine, with total alkaloid content range from 2.5 – 8.0% dry weight, composed of approximately 3% berberine, 1% canadine and 1.5-4% hydrastine (Wagner 1984, Gocan 1996). The mechanism of fungal elicitation of alkaloids from goldenseal has not been studied. In developing the biosynthetic pathway for berberine and other isoquinoline alkaloids, Dittrich and Kutchan (1991) noted that in the higher plant *Eschscholtzia californica* cell culture, a penultimate enzyme, i.e. the berberine bridge enzyme ([S]-reticuline:oxidoreductase or BBE) is elicitor induced. However the presence

of a metabolite pool of closely related compounds would indicate a more complex plant defense response to elicitation than just the formation of berberine.

Since goldenseal is a slow growing plant development of a model system using hairy root culture (*Agrobacterium rhizogenes*) was attempted to probe the elicitation response. *Agrobacterium rhizogenes* is a soil bacterium which infects and genetically transforms plant tissue producing a neoplastic culture that is fast growing and robust. In addition, hairy root cultures can be co-cultivated with plant pathogens (Flores et al., 1999) to enable studies on host-pathogen interactions. Several tissue culture methods for goldenseal have since been developed using excised embryos (Hall & Camper, 2002) and callus culture (Bedir et al., 2003; Liu et al., 2004), but no success has occurred using *A. rhizogenes* transformation.

Maddox et al. (1999) had mixed results developing a mass *in vitro* module culture of goldenseal used to propagate field ready explants. However they did report the presence of berberine in the media that originated from the nodule culture. Several medicinal plant species (*Echinacea*, *Lobelia*, *Salvia*, *Valeriana*) have been successfully transformed (Flores et al., 1999) but the frequency of transformation was < 1%. Their secondary metabolite biosynthetic capacity remained intact when compared to parent plant tissue.

Methods

Seed coat of *H. canadensis* was removed and pericarp soaked in 0.05% benomyl and 0.005% Amphotericin B for 15 min., then soaked 70% ethanol for 15 min., followed by 15 min. soaking in 10% Chlorox/0.01% Tween 20 in 100 ml of the antibiotic mix, and finally washed 3x in sterile distilled water. Standard leaf disks were prepared using a previously developed method (Horsch et al., 1985), which involved punching out disks with a paper punch from throughout non-senescent leaf tissue. Rootlets were cut into 5 mm pieces. Leaf and rootlet tissue were sterilized using the method above.

Plant material was cultivated for 6 days on ½ MS pre-cultivation media (Appendix) before co-cultivation with *Agrobacterium* using ½ MS media with an antifungal mix (Appendix). *Agrobacterium* cultures (A4) were initiated on AB culture media (Appendix) overnight at 30° C with rapid shaking then centrifuged at 10,000 Xg for 5 min., subsequently incubated in YEP rich medium (Appendix) overnight at 30° C also with rapid shaking. The cultures were then centrifuged at 10,000 Xg for 5 min. at 30° C with rapid shaking, and pre-induced overnight in *Vir* induction media (Appendix). An additional 100 mM acetosyringone was added to the bacterial suspension prior to co-cultivation. The explants were wounded with a sterile needle and incubate for 10 min. in the bacterial suspension, the inoculated explants were blotted on sterile filter paper and cultivated on ½ MS co-cultivation media (Appendix) for 48 h. The explants were transferred to a selection media, ½ MS containing antibiotics (Appendix). After 7-10 days viable explants are transferred to a ½ MS post selection media (Appendix) and sub-cultured every two weeks.

Results

Transformation rates of 0.1% occurred using leaf disk tissue and rootlets. Overgrowth of *Agrobacterium* were a consistently a problem and required addition of the antibiotic Cefatoxin to

the selection media. Because of the low transformation rate, tobacco (*Nicotiana tabacum*) leaf disks were also inoculated as a control to insure viability of the A4 culture.

Fungal contamination of the media occurred. Seeds often were contaminated with *Pythium* and *Fusarium*. Although rootlets had the least amount of fungal contaminants, their transformation rate was much lower than with leaf disk tissue.

Significance of this preliminary study

To fully elucidate the role and possible synergistic effect of goldenseal alkaloids in plant defense and ecological signaling would benefit from the development of a hairy root model system. Plant cell transformation requires cells to be in S phase of the cell cycle (Gilissen et al., 1994). Wounding and co-cultivation on a simplified cell culture media appears to stimulate S phase in plant cells of explants, which normally are not dividing cells (Bottino et al., 1989). Thus lack of competence of goldenseal plant tissue may explain their failure to undergo transformation.

Plant tissue media

½ MS Media	**AB Buffer:500 ml 20x stock solution**
1 packet (Sigma # M 5519, Murishage and Skoog Basal Medium) 1.7L dH_20 15.0g sucrose, pH 5.7 aliquot 500 ml	30g K_2HPO_4 10g Na H_2PO_4 pH 7.2 aliquot 25 ml, autoclave
½ MS Pre-cultivation 0.85L (½ MS Media) 3.0 ml kinetin stock 0.3ml NAA stock, pH 5.7 15g agar post autoclave: 5.0 ml carbinicillin stock 2.0 ml amphotericin stock	**AB salts:500 20x stock solution** 10g NH_4Cl 3g $MgSO_4 \cdot 7H_2O$ 1.5g KCl 0.1g $CaCl_2$ 25mg $FeSO_4$ aliquot 25 ml, autoclave
½ MS Co-cultivation 0.45L (½ MS Media) 7.5g agar post autoclave: 5.0 ml carbinicillin stock (400 mg/L) 1.0 ml amphotericin stock (250 mg/L)	**Glucose agar** 450 ml H_2O 7.5g agar 2.75g glucose Combine 25 ml each of AB buffer and AB salts with 450 ml glucose agar.
½ MS Selection 0.4L (½ MS Media) 7.5g agar post autoclave: 5.0 ml benolate stock (filter sterilized) 1.0 ml amphotericin stock 5.0 ml carbinicillin stock (filter sterilized)	**YEB media** 1L H_2O 5g Bacto beef extract 1g Bacto yeast extract 5g peptone 5g sucrose 0.5g $MgSO_4$ 15g agar
Vir induction media 176ml H_2O 10 ml AB salts 200µl AB buffer 4ml 25% glucose 10 ml 400 mM MES buffer, pH 5.5 Combine and filter sterilize, aliquot 5 ml Add 5µl acetosyringone stock (1mg/ml) to each 5 ml aliquot	

Mycotoxin data

Table 9:ELISA detection of Fusarium mycotoxin in liquid culture

***Fusarium oxysporum* isolate**	**Treatment**	**Hyphal mass from culture flask (mg dry wt)**	**T-2**		**Total**
			ELISA detection mg/ml	**Concentration in culture flask (mg/ml)**	**mg metabolite/mg dry wt mycelia**
Hydrastis					
	whole root extract	104.0	$2.00X10^4$	$5.00X10^3$	$4.81X10^5$
	berberine	89.5	$2.00X10^4$	$5.00X10^3$	$5.59X10^5$
	canadine	95.0	$9.00X10^4$	$2.25X10^2$	$2.37X10^4$
	hydrastine	100.5	$4.50X10^3$	$1.13X10^1$	$1.12X10^3$
	alkaloid mix	94.6	$1.70X10^3$	$4.25X10^2$	$4.49X10^4$
	methanol	103.9	0.00	0.00	0.00
	water	125.3	0.00	0.00	0.00
Penn State					
	whole root extract	131.1	0.00	0.00	0.00
	berberine	128.3	0.00	0.00	0.00
	canadine	102.0	0.00	0.00	0.00
	hydrastine	154.1	0.00	0.00	0.00
	alkaloid mix	158.7	0.00	0.00	0.00
	methanol	119.3	0.00	0.00	0.00
	water	179.2	0.00	0.00	0.00
Correlation coefficient			0.9863		
LOD			$7.50X10^3$	mg/ml	
LOQ range			$2.50X10^2$	mg/ml	
			$2.50X10^1$	mg/ml	
			Fumonisin		Total
			ELISA detection mg/ml	concentration in culture flask (mg/ml)	mg metabolite/mg dry wt mycelia
Hydrastis					
	whole root extract	104.0	0.00	0.00	0.00
	berberine	89.5	0.00	0.00	0.00
	canadine	95.0	$1.00X10^1$	2.50	$1.05X10^3$
	hydrastine	100.5	$4.00X10^1$	$1.00X10^1$	$3.98X10^3$
	alkaloid mix	94.6	$3.00X10^1$	7.50	$3.17X10^3$
	methanol	103.9	$6.00X10^1$	$1.50X10^1$	$5.77X10^3$
	water	125.3	$4.00X10^1$	$1.00X10^1$	$3.19X10^3$
Penn State					

	whole root extract	131.1	0.00	0.00	0.00
	berberine	128.3	0.00	0.00	0.00
	canadine	102.0	0.00	0.00	0.00
	hydrastine	154.1	0.00	0.00	0.00
	alkaloid mix	158.7	0.00	0.00	0.00
	methanol	119.3	0.00	0.00	0.00
	water	179.2	0.00	0.00	0.00
Correlation coefficient			0.9984		
LOD			$2.00X10^{1}$	mg/ml	
LOQ range			1.00	mg/ml	
			6.00	mg/ml	
			ZON		Total
			ELISA detection mg/ml	concentration in culture flask (mg/ml)	mg metabolite/mg dry wt mycelia
Hydrastis					
	whole root extract	104.0	$8.10X10^{3}$	$2.03X10^{1}$	$1.95X10^{3}$
	berberine	89.5	0.00	0.00	0.00
	canadine	95.0	$7.10X10^{3}$	$1.78X10^{1}$	$1.87X10^{3}$
	hydrastine	100.5	0.00	0.00	0.00
	alkaloid mix	94.6	$2.88X10^{2}$	$7.20X10^{1}$	$7.61X10^{3}$
	methanol	103.9	$1.26X10^{2}$	$3.15X10^{1}$	$3.03X10^{3}$
	water	125.3	0.00	0.00	0.00
Penn State					
	whole root extract	131.1	0.00	0.00	0.00
	berberine	128.3	0.00	0.00	0.00
	canadine	102.0	0.00	0.00	0.00
	hydrastine	154.1	$6.60X10^{3}$	$1.65X10^{1}$	$1.07X10^{3}$
	alkaloid mix	158.7	$1.47X10^{2}$	$3.68X10^{1}$	$2.32X10^{3}$
	methanol	119.3	$6.20X10^{3}$	$1.55X10^{1}$	$1.30X10^{3}$
	water	179.2	$4.71X10^{2}$	1.18	$6.57X10^{3}$
Correlation coefficient			0.9996		
LOD			$5.00X10^{2}$	mg/ml	
LOQ range			$5.00X10^{2}$	mg/ml	
			$6.00X10^{1}$	mg/ml	

The data for this graph was collected from the same experiment as Figures 18-21. Sample measurements (n=3) were taken of mycelia filtered from culture on day 4 using Neogen ELISA fluorescence plate reader. Results expressed as ng metabolite/mg dry wt mycelia.

Statistical analysis

Table 10:Regression with growth as dependent variable and time as independent variable for final solid media assay of the effects of Hydrastis alkaloids on growth of 3 Fusarium

Model=1			Unstandardized Coefficients		Standardized Coefficients	t	Sig.
Fungi	Treatment		B	Std. Error	Beta		
F. oxysporum	whole root extract	(Constant)	1.061	0.059		17.973	0.000
		TIME	0.333	0.017	0.979	19.285	0.000
	berberine	(Constant)	1.367	0.133		10.273	0.000
		TIME	0.350	0.039	0.914	8.986	0.000
	canadine	(Constant)	1.361	0.138		9.847	0.000
		TIME	0.350	0.040	0.908	8.649	0.000
	hydrastine	(Constant)	1.226	0.104		11.751	0.000
		TIME	0.354	0.031	0.945	11.592	0.000
	standards mix	(Constant)	1.306	0.087		15.039	0.000
		TIME	0.367	0.025	0.964	14.427	0.000
	methanol	(Constant)	1.167	0.073		16.077	0.000
		TIME	0.333	0.021	0.969	15.689	0.000
	water	(Constant)	2.210	0.222		9.975	0.000
		TIME	0.404	0.065	0.842	6.232	0.000
F. commune	whole root extract	(Constant)	1.296	0.045		28.883	0.000
		TIME	0.312	0.013	0.986	23.792	0.000
	berberine	(Constant)	1.843	0.215		8.564	0.000
		TIME	0.321	0.063	0.786	5.092	0.000
	canadine	(Constant)	1.415	0.098		14.464	0.000
		TIME	0.321	0.029	0.942	11.199	0.000
	hydrastine	(Constant)	1.544	0.086		18.004	0.000
		TIME	0.300	0.025	0.948	11.945	0.000
	standards mix	(Constant)	1.304	0.045		29.069	0.000
		TIME	0.288	0.013	0.984	21.888	0.000
	methanol	(Constant)	1.303	0.056		23.189	0.000
		TIME	0.258	0.016	0.969	15.706	0.000

	water	(Constant)	2.004	0.095		21.125	0.000
		TIME	0.487	0.028	0.975	17.551	0.000
F. solani	whole root extract	(Constant)	1.015	0.071		14.334	0.000
		TIME	0.221	0.021	0.936	10.649	0.000
	berberine	(Constant)	1.065	0.079		13.412	0.000
		TIME	0.254	0.023	0.939	10.930	0.000
	canadine	(Constant)	1.207	0.047		25.840	0.000
		TIME	0.246	0.014	0.976	17.977	0.000
	hydrastine	(Constant)	1.188	0.051		23.198	0.000
		TIME	0.237	0.015	0.970	15.847	0.000
	standards mix	(Constant)	1.056	0.066		15.919	0.000
		TIME	0.250	0.019	0.955	12.878	0.000
	methanol	(Constant)	1.247	0.058		21.520	0.000
		TIME	0.225	0.017	0.957	13.260	0.000
	water	(Constant)	1.519	0.064		23.898	0.000
		TIME	0.392	0.019	0.982	21.041	0.000

Table 11:ANOVA with growth the dependent variable and pH the predictor for final liquid media assay of the effects of Hydrastis alkaloids on growth of 3 Fusarium

Fungi	Treatment	Model		Sum of Squares	df	Mean Square	F	Sig.
F. oxysporum	whole root extract	1	Regression	.024	1	.024	249.353	.000
			Residual	.001	13	.000		
			Total	.025	14			
	berberine	1	Regression	.015	1	.015	89.453	.000
			Residual	.002	13	.000		
			Total	.017	14			
	canadine	1	Regression	.014	1	.014	137.068	.000
			Residual	.001	13	.000		
			Total	.015	14			
	hydrastine	1	Regression	.012	1	.012	66.045	.000
			Residual	.002	13	.000		
			Total	.015	14			
	alkaloid standards	1	Regression	.014	1	.014	156.225	.000
			Residual	.001	13	.000		
			Total	.015	14			
	50% MeOH (1% acetic acid)	1	Regression	.016	1	.016	69.790	.000
			Residual	.003	13	.000		
			Total	.019	14			
	water	1	Regression	.020	1	.020	202.799	.000
			Residual	.001	11	.000		
			Total	.021	12			
F. commune	whole root extract	1	Regression	.035	1	.035	49.008	.000
			Residual	.009	12	.001		
			Total	.044	13			
	berberine	1	Regression	.029	1	.029	147.137	.000
			Residual	.003	13	.000		
			Total	.032	14			
	canadine	1	Regression	.027	1	.027	176.284	.000
			Residual	.002	13	.000		
			Total	.029	14			
	hydrastine	1	Regression	.018	1	.018	3.380	.093
			Residual	.060	11	.005		
			Total	.078	12			

	alkaloid standards	1	Regression	.025	1	.025	63.318	.000
			Residual	.004	10	.000		
			Total	.029	11			
	50% MeOH (1% acetic acid)	1	Regression	.006	1	.006	33.890	.000
			Residual	.002	9	.000		
			Total	.007	10			
	water	1	Regression	.032	1	.032	81.240	.000
			Residual	.003	9	.000		
			Total	.035	10			
F. solani	whole root extract	1	Regression	.056	1	.056	111.076	.000
			Residual	.005	10	.001		
			Total	.062	11			
	berberine	1	Regression	.060	1	.060	87.781	.000
			Residual	.007	10	.001		
			Total	.067	11			
	canadine	1	Regression	.051	1	.051	142.120	.000
			Residual	.004	10	.000		
			Total	.054	11			
	hydrastine	1	Regression	.048	1	.048	114.647	.000
			Residual	.004	10	.000		
			Total	.052	11			
	alkaloid standards	1	Regression	.063	1	.063	65.942	.000
			Residual	.010	10	.001		
			Total	.073	11			
	50% MeOH (1% acetic acid)	1	Regression	.021	1	.021	30.976	.000
			Residual	.006	9	.001		
			Total	.027	10			
	water	1	Regression	.032	1	.032	122.264	.000
			Residual	.002	8	.000		
			Total	.034	9			

a Predictors:(Constant), TIME
b Dependent Variable:GROWTH

Table 12:ANOVA with pH the dependent variable and growth the predictor for final liquid media assay of the effects of Hydrastis alkaloids on growth of 3 Fusarium

Fungi	Treatment	Model		Sum of Squares	df	Mean Square	F	Sig.
F. oxysporum	whole root extract	1	Regression	8.737	1	8.737	38.005	.000
			Residual	2.989	13	.230		
			Total	11.726	14			
	berberine	1	Regression	.534	1	.534	22.184	.000
			Residual	.313	13	.024		
			Total	.847	14			
	canadine	1	Regression	.061	1	.061	5.924	.030
			Residual	.135	13	.010		
			Total	.196	14			
	hydrastine	1	Regression	.106	1	.106	8.933	.010
			Residual	.154	13	.012		
			Total	.260	14			
	alkaloid standards	1	Regression	.104	1	.104	15.012	.002
			Residual	.090	13	.007		
			Total	.195	14			
	50% MeOH (1% acetic acid)	1	Regression	4.942	1	4.942	29.959	.000
			Residual	2.144	13	.165		
			Total	7.086	14			
	water	1	Regression	.095	1	.095	14.925	.003
			Residual	.070	11	.006		
			Total	.165	12			
F. commune	whole root extract	1	Regression	12.986	1	12.986	34.867	.000
			Residual	4.469	12	.372		
			Total	17.455	13			
	berberine	1	Regression	11.273	1	11.273	61.491	.000
			Residual	2.383	13	.183		
			Total	13.656	14			
	canadine	1	Regression	12.094	1	12.094	51.090	.000
			Residual	3.077	13	.237		
			Total	15.171	14			
	hydrastine	1	Regression	4.152	1	4.152	4.588	.055
			Residual	9.956	11	.905		
			Total	14.108	12			
	alkaloid standards	1	Regression	12.614	1	12.614	51.867	.000

			Residual	2.432	10	.243		
			Total	15.046	11			
	50% MeOH (1% acetic acid)	1	Regression	8.951	1	8.951	170.416	.000
			Residual	.473	9	.053		
			Total	9.423	10			
	water	1	Regression	.075	1	.075	6.385	.032
			Residual	.106	9	.012		
			Total	.181	10			
F. solani	whole root extract	1	Regression	17.669	1	17.669	52.314	.000
			Residual	3.378	10	.338		
			Total	21.047	11			
	berberine	1	Regression	14.268	1	14.268	35.575	.000
			Residual	4.011	10	.401		
			Total	18.279	11			
	canadine	1	Regression	16.632	1	16.632	59.672	.000
			Residual	2.787	10	.279		
			Total	19.419	11			
	hydrastine	1	Regression	15.671	1	15.671	47.528	.000
			Residual	3.297	10	.330		
			Total	18.969	11			
	alkaloid standards	1	Regression	15.252	1	15.252	40.251	.000
			Residual	3.789	10	.379		
			Total	19.041	11			
	50% MeOH (1% acetic acid)	1	Regression	13.938	1	13.938	24.734	.001
			Residual	5.071	9	.563		
			Total	19.009	10			
	water	1	Regression	.177	1	.177	17.606	.003
			Residual	.080	8	.010		
			Total	.258	9			

a Predictors:(Constant), GROWTH
b Dependent Variable:PH

Table 13:Correlation of Fusarium growth characteristics, spore formation and mycotoxin production in response to all treatments

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	1	.494	.136	-.322	-.038	-.254	-.186	-.384	-.422	-.306
	Sig. (1-tailed)	.	.011	.278	.077	.435	.133	.210	.043	.028	.089
	N	21	21	21	21	21	21	21	21	21	21
FB	Pearson Correlation	.494	1	.305	-.121	.100	-.188	-.309	-.543	-.725	.246
	Sig. (1-tailed)	.011	.	.090	.301	.333	.207	.086	.006	.000	.141
	N	21	21	21	21	21	21	21	21	21	21
ZON	Pearson Correlation	.136	.305	1	-.181	.230	.268	-.280	-.504	-.455	-.059
	Sig. (1-tailed)	.278	.090	.	.216	.158	.120	.109	.010	.019	.400
	N	21	21	21	21	21	21	21	21	21	21
micro	Pearson Correlation	-.322	-.121	-.181	1	.501	.267	.320	.374	.371	.071
	Sig. (1-tailed)	.077	.301	.216	.	.010	.121	.078	.048	.049	.379
	N	21	21	21	21	21	21	21	21	21	21
macro	Pearson Correlation	-.038	.100	.230	.501	1	**.727**	.279	-.324	-.085	-.001
	Sig. (1-tailed)	.435	.333	.158	.010	.	.000	.111	.076	.357	.499
	N	21	21	21	21	21	21	21	21	21	21
germ rate	Pearson Correlation	-.254	-.188	.268	.267	**.727**	1	.519	-.178	.066	.066
	Sig. (1-tailed)	.133	.207	.120	.121	.000	.	.008	.220	.389	.387
	N	21	21	21	21	21	21	21	21	21	21
chlam	Pearson Correlation	-.186	-.309	-.280	.320	.279	.519	1	.308	.507	.347
	Sig. (1-tailed)	.210	.086	.109	.078	.111	.008	.	.087	.009	.062
	N	21	21	21	21	21	21	21	21	21	21
growth	Pearson Correlation	-.384	-.543	-.504	.374	-.324	-.178	.308	1	**.853**	-.072
	Sig. (1-tailed)	.043	.006	.010	.048	.076	.220	.087	.	.000	.378
	N	21	21	21	21	21	21	21	21	21	21
pH	Pearson Correlation	-.422	-.725	-.455	.371	-.085	.066	.507	**.853**	1	.024
	Sig. (1-tailed)	.028	.000	.019	.049	.357	.389	.009	.000	.	.459
	N	21	21	21	21	21	21	21	21	21	21
growth rate	Pearson Correlation	-.306	.246	-.059	.071	-.001	.066	.347	-.072	.024	1

Sig. (1-tailed)	.089	.141	.400	.379	.499	.387	.062	.378	.459	.
N	21	21	21	21	21	21	21	21	21	21

Correlation is significant at the 0.05 level (1-tailed)

Table 14:Correlation of *Fusarium* growth characteristics, spore formation and mycotoxin production in response to treatment with *Hydrastis* root extract

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	1	.	.840	-.461	-.353	-.115	.058	-.919	**-1.000**	**1.000**
	Sig. (1-tailed)	.	.	.183	.348	.385	.463	.481	.129	.006	.004
	N	3	3	3	3	3	3	3	3	3	3
FB	Pearson Correlation	.	.	.	.	.	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.	.	.	.	.	.
	N	3	3	3	3	3	3	3	3	3	3
ZON	Pearson Correlation	.840	.	1	.094	.211	.443	-.493	-.986	-.850	.834
	Sig. (1-tailed)	.183	.	.	.470	.432	.354	.336	.053	.177	.186
	N	3	3	3	3	3	3	3	3	3	3
micro	Pearson Correlation	-.461	.	.094	1	**.993**	.935	-.913	.073	.445	-.471
	Sig. (1-tailed)	.348	.	.470	.	.038	.116	.134	.477	.353	.344
	N	3	3	3	3	3	3	3	3	3	3
macro	Pearson Correlation	-.353	.	.211	**.993**	1	.970	-.955	-.045	.336	-.364
	Sig. (1-tailed)	.385	.	.432	.038	.	.078	.096	.486	.391	.381
	N	3	3	3	3	3	3	3	3	3	3
germ rate	Pearson Correlation	-.115	.	.443	.935	.970	1	-.998	-.287	.097	-.126
	Sig. (1-tailed)	.463	.	.354	.116	.078	.	.018	.407	.469	.460
	N	3	3	3	3	3	3	3	3	3	3
chlam	Pearson Correlation	.058	.	-.493	-.913	-.955	-.998	1	.340	-.040	.070
	Sig. (1-tailed)	.481	.	.336	.134	.096	.018	.	.389	.487	.478
	N	3	3	3	3	3	3	3	3	3	3
growth	Pearson Correlation	-.919	.	-.986	.073	-.045	-.287	.340	1	.926	-.914
	Sig. (1-tailed)	.129	.	.053	.477	.486	.407	.389	.	.123	.133
	N	3	3	3	3	3	3	3	3	3	3
pH	Pearson Correlation	**-1.000**	.	-.850	.445	.336	.097	-.040	.926	1	**-1.000**
	Sig. (1-tailed)	.006	.	.177	.353	.391	.469	.487	.123	.	.009
	N	3	3	3	3	3	3	3	3	3	3
growth rate	Pearson Correlation	**1.000**	.	.834	-.471	-.364	-.126	.070	-.914	**-1.000**	1

Sig. (1-tailed)	.004	.	.186	.344	.381	.460	.478	.133	.009	.
N	3	3	3	3	3	3	3	3	3	3

Correlation is significant at the 0.05 level (1-tailed)

Table 15:Correlation of Fusarium growth characteristics, spore formation and mycotoxin production in response to treatment with berberine

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	1	.	.	-.975	-.308	-.596	-.957	-.792	-.954	-.572
	Sig. (1-tailed)	.	.	.	.072	.400	.297	.093	.209	.097	.306
	N	3	3	3	3	3	3	3	3	3	3
FB	Pearson Correlation	.	.	.	.	.	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.	.	.	.	.	.
	N	3	3	3	3	3	3	3	3	3	3
ZON	Pearson Correlation	.	.	.	.	.	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.	.	.	.	.	.
	N	3	3	3	3	3	3	3	3	3	3
micro	Pearson Correlation	-.975	.	.	1	.513	.761	**.998**	.635	.862	.374
	Sig. (1-tailed)	.072	.	.	.	.329	.225	.021	.281	.169	.378
	N	3	3	3	3	3	3	3	3	3	3
macro	Pearson Correlation	-.308	.	.	.513	1	.947	.570	-.338	.007	-.604
	Sig. (1-tailed)	.400	.	.	.329	.	.104	.307	.390	.498	.293
	N	3	3	3	3	3	3	3	3	3	3
germ rate	Pearson Correlation	-.596	.	.	.761	.947	1	.803	-.019	.326	-.318
	Sig. (1-tailed)	.297	.	.	.225	.104	.	.203	.494	.394	.397
	N	3	3	3	3	3	3	3	3	3	3
chlam	Pearson Correlation	-.957	.	.	**.998**	.570	.803	1	.581	.826	.310
	Sig. (1-tailed)	.093	.	.	.021	.307	.203	.	.303	.191	.400
	N	3	3	3	3	3	3	3	3	3	3
growth	Pearson Correlation	-.792	.	.	.635	-.338	-.019	.581	1	.939	.954
	Sig. (1-tailed)	.209	.	.	.281	.390	.494	.303	.	.112	.097
	N	3	3	3	3	3	3	3	3	3	3
pH	Pearson Correlation	-.954	.	.	.862	.007	.326	.826	.939	1	.793
	Sig. (1-tailed)	.097	.	.	.169	.498	.394	.191	.112	.	.209
	N	3	3	3	3	3	3	3	3	3	3
growth rate	Pearson Correlation	-.572	.	.	.374	-.604	-.318	.310	.954	.793	1

Sig. (1-tailed)	.306	.	.	.378	.293	.397	.400	.097	.209	.
N	3	3	3	3	3	3	3	3	3	3

Correlation is significant at the 0.05 level (1-tailed)

Table 16:Correlation of Fusarium growth characteristics, spore formation and mycotoxin production in response to treatment with canadine

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	1	**1.000**	.741	-.894	-.339	-.565	-.923	-.807	**-.995**	-.829
	Sig. (1-tailed)	.	.	.234	.148	.390	.309	.126	.201	.033	.189
	N	3	3	3	3	3	3	3	3	3	3
FB	Pearson Correlation	**1.000**	1	.741	-.894	-.339	-.565	-.923	-.807	**-.995**	-.829
	Sig. (1-tailed)	.	.	.234	.148	.390	.309	.126	.201	.033	.189
	N	3	3	3	3	3	3	3	3	3	3
ZON	Pearson Correlation	.741	.741	1	-.963	.381	.135	-.424	**-.994**	-.807	-.239
	Sig. (1-tailed)	.234	.234	.	.087	.376	.457	.361	.034	.201	.423
	N	3	3	3	3	3	3	3	3	3	3
micro	Pearson Correlation	-.894	-.894	-.963	1	-.118	.137	.653	.986	.936	.492
	Sig. (1-tailed)	.148	.148	.087	.	.462	.456	.274	.053	.114	.336
	N	3	3	3	3	3	3	3	3	3	3
macro	Pearson Correlation	-.339	-.339	.381	-.118	1	.968	.676	-.281	.239	.807
	Sig. (1-tailed)	.390	.390	.376	.462	.	.081	.264	.409	.423	.201
	N	3	3	3	3	3	3	3	3	3	3
germ rate	Pearson Correlation	-.565	-.565	.135	.137	.968	1	.840	-.030	.476	.930
	Sig. (1-tailed)	.309	.309	.457	.456	.081	.	.183	.490	.342	.120
	N	3	3	3	3	3	3	3	3	3	3
chlam	Pearson Correlation	-.923	-.923	-.424	.653	.676	.840	1	.517	.877	.981
	Sig. (1-tailed)	.126	.126	.361	.274	.264	.183	.	.327	.159	.063
	N	3	3	3	3	3	3	3	3	3	3
growth	Pearson Correlation	-.807	-.807	**-.994**	.986	-.281	-.030	.517	1	.865	.340
	Sig. (1-tailed)	.201	.201	.034	.053	.409	.490	.327	.	.167	.390
	N	3	3	3	3	3	3	3	3	3	3
pH	Pearson Correlation	**-.995**	**-.995**	-.807	.936	.239	.476	.877	.865	1	.766
	Sig. (1-tailed)	.033	.033	.201	.114	.423	.342	.159	.167	.	.222
	N	3	3	3	3	3	3	3	3	3	3
growth rate	Pearson Correlation	-.829	-.829	-.239	.492	.807	.930	.981	.340	.766	1

Sig. (1-tailed)	.189	.189	.423	.336	.201	.120	.063	.390	.222	.
N	3	3	3	3	3	3	3	3	3	3

Correlation is significant at the 0.05 level (1-tailed)

Table 17:Correlation of Fusarium growth characteristics, spore formation and mycotoxin production in response to treatment with hydrastine

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	1	**1.000**	-.500	-.934	-.184	-.376	-.575	-.877	**-.999**	**-1.000**
	Sig. (1-tailed)	.	.	.333	.116	.441	.377	.305	.160	.013	.002
	N	3	3	3	3	3	3	3	3	3	3
FB	Pearson Correlation	**1.000**	1	-.500	-.934	-.184	-.376	-.575	-.877	**-.999**	**-1.000**
	Sig. (1-tailed)	.	.	.333	.116	.441	.377	.305	.160	.013	.002
	N	3	3	3	3	3	3	3	3	3	3
ZON	Pearson Correlation	-.500	-.500	1	.776	.943	**.990**	**.996**	.022	.465	.495
	Sig. (1-tailed)	.333	.333	.	.217	.108	.044	.028	.493	.346	.335
	N	3	3	3	3	3	3	3	3	3	3
micro	Pearson Correlation	-.934	-.934	.776	1	.522	.681	.829	.648	.919	.932
	Sig. (1-tailed)	.116	.116	.217	.	.325	.261	.189	.276	.129	.118
	N	3	3	3	3	3	3	3	3	3	3
macro	Pearson Correlation	-.184	-.184	.943	.522	1	.980	.910	-.312	.144	.178
	Sig. (1-tailed)	.441	.441	.108	.325	.	.064	.136	.399	.454	.443
	N	3	3	3	3	3	3	3	3	3	3
germ rate	Pearson Correlation	-.376	-.376	**.990**	.681	.980	1	.974	-.116	.339	.370
	Sig. (1-tailed)	.377	.377	.044	.261	.064	.	.072	.463	.390	.379
	N	3	3	3	3	3	3	3	3	3	3
chlam	Pearson Correlation	-.575	-.575	**.996**	.829	.910	.974	1	.110	.542	.570
	Sig. (1-tailed)	.305	.305	.028	.189	.136	.072	.	.465	.318	.307
	N	3	3	3	3	3	3	3	3	3	3
growth	Pearson Correlation	-.877	-.877	.022	.648	-.312	-.116	.110	1	.895	.880
	Sig. (1-tailed)	.160	.160	.493	.276	.399	.463	.465	.	.147	.158
	N	3	3	3	3	3	3	3	3	3	3
pH	Pearson Correlation	**-.999**	**-.999**	.465	.919	.144	.339	.542	.895	1	**.999**
	Sig. (1-tailed)	.013	.013	.346	.129	.454	.390	.318	.147	.	.011
	N	3	3	3	3	3	3	3	3	3	3
growth rate	Pearson Correlation	**-1.000**	**-1.000**	.495	.932	.178	.370	.570	.880	**.999**	1

Sig. (1-tailed)	.002	.002	.335	.118	.443	.379	.307	.158	.011	.
N	3	3	3	3	3	3	3	3	3	3

Correlation is significant at the 0.05 level (1-tailed)

Table 18:Correlation of Fusarium growth characteristics, spore formation and mycotoxin production in response to treatment with alkaloid standards mix

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	1	**1.000**	.900	-.935	-.137	-.318	-.932	-.809	-.943	-.931
	Sig. (1-tailed)	.	.	.144	.115	.456	.397	.118	.200	.108	.119
	N	3	3	3	3	3	3	3	3	3	3
FB	Pearson Correlation	**1.000**	1	.900	-.935	-.137	-.318	-.932	-.809	-.943	-.931
	Sig. (1-tailed)	.	.	.144	.115	.456	.397	.118	.200	.108	.119
	N	3	3	3	3	3	3	3	3	3	3
ZON	Pearson Correlation	.900	.900	1	-.687	.309	.127	-.680	-.985	**-.994**	**-.997**
	Sig. (1-tailed)	.144	.144	.	.259	.400	.459	.262	.056	.035	.025
	N	3	3	3	3	3	3	3	3	3	3
micro	Pearson Correlation	-.935	-.935	-.687	1	.478	.633	**1.000**	.549	.763	.741
	Sig. (1-tailed)	.115	.115	.259	.	.341	.282	.003	.315	.224	.234
	N	3	3	3	3	3	3	3	3	3	3
macro	Pearson Correlation	-.137	-.137	.309	.478	1	.983	.487	-.471	-.202	-.235
	Sig. (1-tailed)	.456	.456	.400	.341	.	.059	.338	.344	.435	.425
	N	3	3	3	3	3	3	3	3	3	3
germ rate	Pearson Correlation	-.318	-.318	.127	.633	.983	1	.641	-.299	-.017	-.050
	Sig. (1-tailed)	.397	.397	.459	.282	.059	.	.279	.403	.495	.484
	N	3	3	3	3	3	3	3	3	3	3
chlam	Pearson Correlation	-.932	-.932	-.680	**1.000**	.487	.641	1	.541	.757	.734
	Sig. (1-tailed)	.118	.118	.262	.003	.338	.279	.	.318	.227	.238
	N	3	3	3	3	3	3	3	3	3	3
growth	Pearson Correlation	-.809	-.809	-.985	.549	-.471	-.299	.541	1	.959	.968
	Sig. (1-tailed)	.200	.200	.056	.315	.344	.403	.318	.	.091	.081
	N	3	3	3	3	3	3	3	3	3	3
pH	Pearson Correlation	-.943	-.943	**-.994**	.763	-.202	-.017	.757	.959	1	**.999**
	Sig. (1-tailed)	.108	.108	.035	.224	.435	.495	.227	.091	.	.011
	N	3	3	3	3	3	3	3	3	3	3
growth rate	Pearson Correlation	-.931	-.931	**-.997**	.741	-.235	-.050	.734	.968	**.999**	1

Sig. (1-tailed)	.119	.119	.025	.234	.425	.484	.238	.081	.011	.
N	3	3	3	3	3	3	3	3	3	3

Correlation is significant at the 0.05 level (1-tailed)

Table 19:Correlation of Fusarium growth characteristics, spore formation and mycotoxin production in response to treatment with methanol

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	.	.	.	.	.	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.	.	.	.	.	.
	N	3	3	3	3	3	3	3	3	3	3
FB	Pearson Correlation	.	1	.610	-.818	-.218	-.115	-.987	-.614	-.961	**.998**
	Sig. (1-tailed)	.	.	.291	.195	.430	.463	.051	.290	.089	.019
	N	3	3	3	3	3	3	3	3	3	3
ZON	Pearson Correlation	.	.610	1	-.043	.640	.717	-.476	**-1.000**	-.804	.656
	Sig. (1-tailed)	.	.291	.	.486	.279	.245	.342	.002	.202	.272
	N	3	3	3	3	3	3	3	3	3	3
micro	Pearson Correlation	.	-.818	-.043	1	.740	.665	.899	.048	.628	-.782
	Sig. (1-tailed)	.	.195	.486	.	.235	.268	.144	.485	.284	.214
	N	3	3	3	3	3	3	3	3	3	3
macro	Pearson Correlation	.	-.218	.640	.740	1	**.994**	.371	-.636	-.059	-.160
	Sig. (1-tailed)	.	.430	.279	.235	.	.033	.379	.280	.481	.449
	N	3	3	3	3	3	3	3	3	3	3
germ rate	Pearson Correlation	.	-.115	.717	.665	**.994**	1	.272	-.714	-.163	-.055
	Sig. (1-tailed)	.	.463	.245	.268	.033	.	.412	.247	.448	.482
	N	3	3	3	3	3	3	3	3	3	3
chlam	Pearson Correlation	.	-.987	-.476	.899	.371	.272	1	.480	.905	-.976
	Sig. (1-tailed)	.	.051	.342	.144	.379	.412	.	.341	.140	.070
	N	3	3	3	3	3	3	3	3	3	3
growth	Pearson Correlation	.	-.614	**-1.000**	.048	-.636	-.714	.480	1	.807	-.660
	Sig. (1-tailed)	.	.290	.002	.485	.280	.247	.341	.	.201	.271
	N	3	3	3	3	3	3	3	3	3	3
pH	Pearson Correlation	.	-.961	-.804	.628	-.059	-.163	.905	.807	1	-.976
	Sig. (1-tailed)	.	.089	.202	.284	.481	.448	.140	.201	.	.070
	N	3	3	3	3	3	3	3	3	3	3
growth rate	Pearson Correlation	.	**.998**	.656	-.782	-.160	-.055	-.976	-.660	-.976	1

Sig. (1-tailed)	.	.019	.272	.214	.449	.482	.070	.271	.070	.
N	3	3	3	3	3	3	3	3	3	3

Correlation is significant at the 0.05 level (1-tailed)

Table 20:Correlation of Fusarium growth characteristics, spore formation and mycotoxin production in response to treatment with water

		T2	FB	ZON	micro	macro	germ rate	chlam	growth	pH	growth rate
T2	Pearson Correlation	.	.	.	.	.	.	.	.	.	.
	Sig. (1-tailed)	.	.	.	.	.	.	.	.	.	.
	N	3	3	3	3	3	3	3	3	3	3
FB	Pearson Correlation	.	1	-.500	.948	.735	-.277	.288	-.777	-.808	.876
	Sig. (1-tailed)	.	.	.333	.103	.237	.411	.407	.217	.200	.160
	N	3	3	3	3	3	3	3	3	3	3
ZON	Pearson Correlation	.	-.500	1	-.749	.220	.971	.685	-.157	-.106	-.856
	Sig. (1-tailed)	.	.333	.	.231	.430	.077	.260	.450	.466	.173
	N	3	3	3	3	3	3	3	3	3	3
micro	Pearson Correlation	.	.948	-.749	1	.482	-.567	-.030	-.538	-.580	.984
	Sig. (1-tailed)	.	.103	.231	.	.340	.308	.491	.319	.303	.058
	N	3	3	3	3	3	3	3	3	3	3
macro	Pearson Correlation	.	.735	.220	.482	1	.447	.861	**-.998**	**-.993**	.317
	Sig. (1-tailed)	.	.237	.430	.340	.	.352	.170	.020	.037	.397
	N	3	3	3	3	3	3	3	3	3	3
germ rate	Pearson Correlation	.	-.277	.971	-.567	.447	1	.840	-.389	-.342	-.706
	Sig. (1-tailed)	.	.411	.077	.308	.352	.	.183	.373	.389	.250
	N	3	3	3	3	3	3	3	3	3	3
chlam	Pearson Correlation	.	.288	.685	-.030	.861	.840	1	-.827	-.797	-.209
	Sig. (1-tailed)	.	.407	.260	.491	.170	.183	.	.190	.206	.433
	N	3	3	3	3	3	3	3	3	3	3
growth	Pearson Correlation	.	-.777	-.157	-.538	**-.998**	-.389	-.827	1	.999	-.377
	Sig. (1-tailed)	.	.217	.450	.319	.020	.373	.190	.	.016	.377
	N	3	3	3	3	3	3	3	3	3	3
pH	Pearson Correlation	.	-.808	-.106	-.580	**-.993**	-.342	-.797	.999	1	-.424
	Sig. (1-tailed)	.	.200	.466	.303	.037	.389	.206	.016	.	.361
	N	3	3	3	3	3	3	3	3	3	3
growth rate	Pearson Correlation	.	.876	-.856	.984	.317	-.706	-.209	-.377	-.424	1

Sig. (1-tailed)	.	.160	.173	.058	.397	.250	.433	.377	.361	.
N	3	3	3	3	3	3	3	3	3	3

Correlation is significant at the 0.05 level (1-tailed)

Phylogenetic analysis

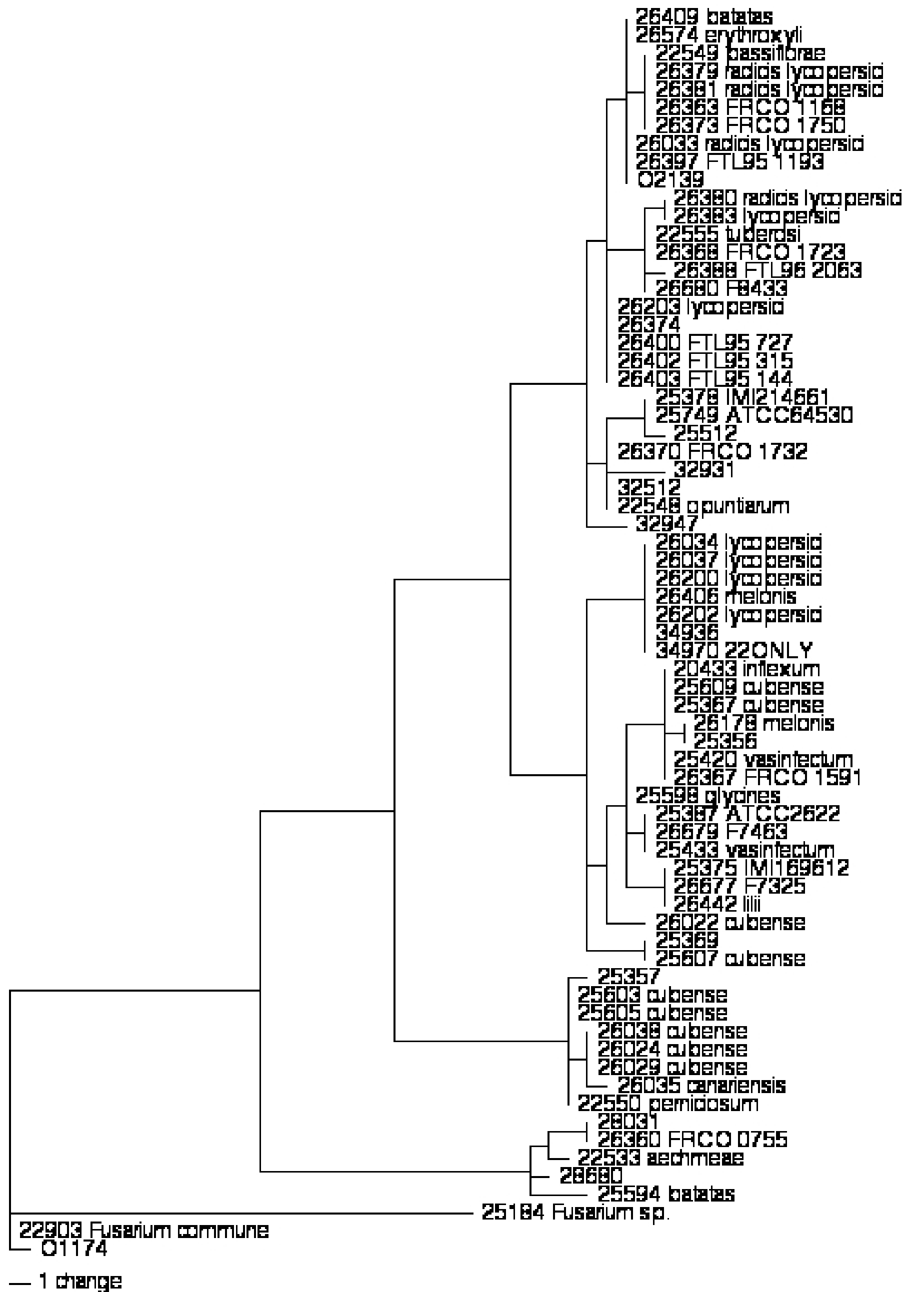


Figure 29:Phylogenetic tree analysis of *F. oxysporum* (*Hydrastis*) and *F. commune* (PSU) via ClustalW

Hydrastis isolate, *F. oxyspourm*, designated O2139 is located second from the top of the diagram. PSU isolate, *F. commune*, designated O1174, is located towards the bottom just under the *Fusarium commune.*

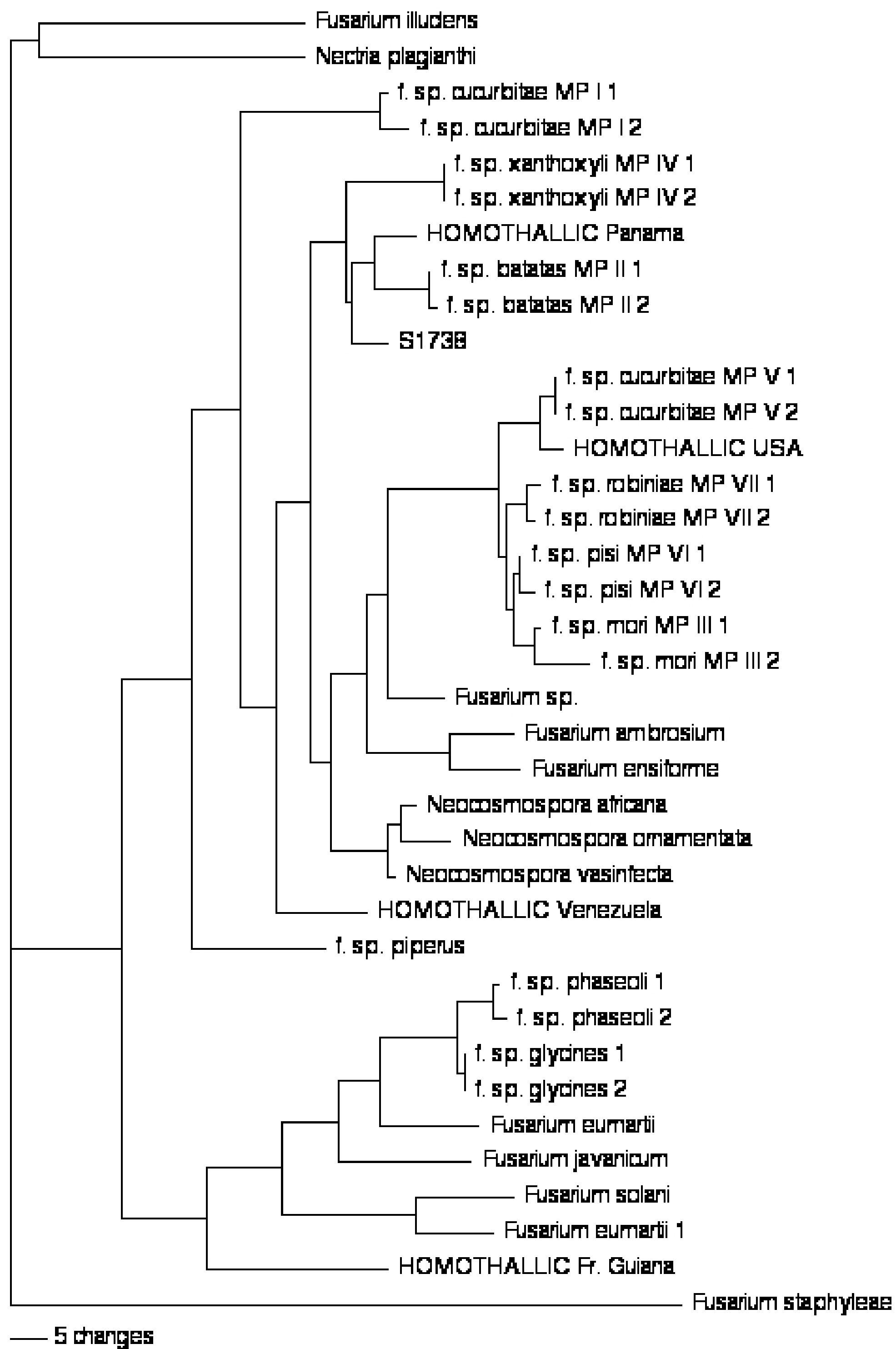


Figure 30:Phylogenetic tree analysis of F. solani (Hydrastis) via ClustalW
Hydrastis isolate, F. solani, designated S1738 is located toward the top of the diagram. All f. sp. strains represent host specific F. solani strains.

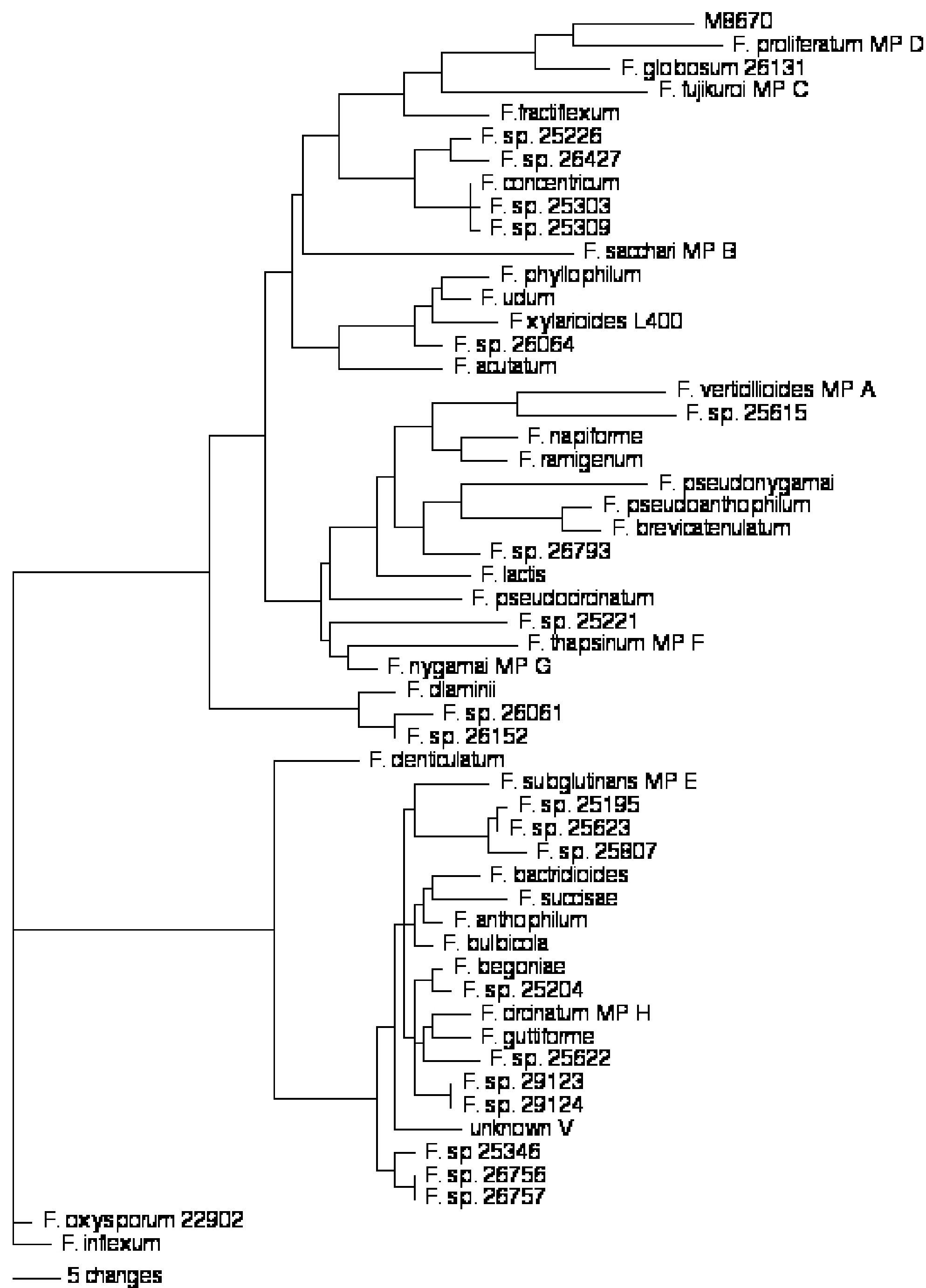


Figure 31:Phylogenetic tree analysis of F. proliferatum (Hydrastis) via ClustalW
Hydrastis isolate, F. proliferatum, designated M8670 and located at the very top of the diagram.

Figure 32:Translation Elongation Factor 1-alpha Sequence for *Fusarium* isolates of *Hydrastis*

***F. oxysporum* (02139)**
TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACTCTCCTCGA
CAATGAGCTTATCTGCCATCGTCAATCCCGACCAAGACCTGGTGGGGTATTTCTCAA
AGTCAACATACTGACATCGTTTCACAGACCGGTCACTTGATCTACCAGTGCGGTGGT
ATCGATAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCACTTTCCCTTCGATCGC
GCGTCCTTTGCCCATCGATTTCCCCTACGACTCGAAACGTGCCCGCTACCCCGCTCGA
GACCAAAAATTTTGCAATATGACTGTAATTTTTTTT-
GGTGGGGCACTTACCCCGCCACTTGAGCGACGGGA-
GCGTTTGCCCTCTTAACCATTCTCACAACCTCAATGAGTGCGTCGTCACGTGTCAAGC
AGTC-
ACTAACCATTCAACAATAGGAAGCCGCTGAGCTCGGTAAGGGTTCCTTCAAGTACGC
CTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGC
TCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCGCTC
ATGCTTCATTCTACTTCTCTTCGTACTAACAT--CACTCAGACGctCCC----------

***F. commune* (O1174)**
TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACTCCCCTTGG
ACGATGAGCTTATCTGCCATCGTTAATCCCGACCAAGACCTGGCGGGGTATTTCTCA
AAGGCAATATGCTGATATCGTTTCACAGACCGGTCACTTGATCTACCAGTGCGGTGG
TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCACTTTCCCTTCGATCG
CGCGTCCTCTGCCCATCGATTTCCCCTACGACTCGAAACCTGCCCGCTACCCCGCTCG
AGACCAAAAATTTTGCGATATGACCGTAATTTTTTTT-
GGTGGGGCATTTACCCCGCCACTCGAGCGACGGGC-GCGTTTGCCCTCCTC-
CCATTTCCACAACCTCAATGAGCGCATCGTCACGTGTCACGCAGTC-
ACTAACCATTCAATAATAGGAAGCCGCTGAGCTCGGTAAGGGTTCCTTCAAGTACGC
CTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGC
TCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCGCTC
ATGCTTCATTCTACTTCTCTTCGTACTGACATATCACTCAGACGctCCC----------

***F. solani* (S1738)**
TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACCGTAAGTCAAA--
CCCTCATCGCGATCTGCTTATCTCGGG--
TCGTGGAACCCCGCCTGGCATCTCGGGCGGGG-TATTCATCATTCACTTC-
ATGCTGACAATCATCTACAGACCGGTCACTTGATCTACCAGTGCGGTGGTATCGACA
AGCGAACCATCGAGAAGTTCGAGAAGGTTGGTGACATCTGCCCCC-GATCGCG--
CCTTGATATTCCACATCGAATTCCCCGTCGAATTCCCTCCATCGCGATACGCTCT-
GCGCCCGCTTC--TCC---CGAGTCCCAAAATTTTTGCGGTCCGACCGTAATTTTTTT-----
GGTGGGGCATTT-
ACCCCGCCACTCGGGCGACGTTGGACAAAGCCCTGATCCCTGCACACAAAA----
CACCAAACCCTCTTGGCGCGCATCATCACGTGGTTCACGACAGACGCTAACCGGTCC
AACAATAGGAAGCCGCTGAGCTCGGTAAGGGTTCCTTCAAGTACGCCTGGGTCCTTG
ACAAGCTCAAGGCCGAGCGTGAGCGTGGTATCACCATCGACATTGCCCTCTGGAAGT
TCGAGACTCCCCGCTACTATGTCACCGTCATTGGTATGTTGCTGTCACCTCTC--
TCACACATGTCTCACCACTAAC-AATCAA---CAGACGCCCCCGGCCACCGTG

***F. proliferatum* (M8670)**
TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACTACCCTGG
ACGTTGAGCTTATCTGCCATCGTGATCCTGACCAAGATCTGGCGGGGTACATCTTGG
AAGACAACATGCTGACATCGCTTCACAGACCGGTCACTTGATCTACCAGTGCGGTGG
TATCGACAAGCGAACCATCGAGAAGTTCGAGAAGGTTAGTCACTTTCCCTTCGATCG
CGCGTCCTCTGCCCACCGATTTCACTTGCGATTCGAAACGTGCCTGCTACCCCGCTCG
AGACCAAAAATTTTGCGATATGACCGTAATTTTTTTGGTGGGGCATTTACCCCGCCAC
TCGAGCGATGGGCGCGTTTTTGCCCTTTCCTGCCCACAACCTCAATGAGCGCATTGTC
ACGTGTCAAGCAGCGACTAACCATTCGACAATAGGAAGCCGCTGAGCTCGGTAAGG
GTTCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCGTGGTA
TCACCATCGATATTGCTCTCTGGAAGTTCGAGACTCCTCGCTACTATGTCACCGTCAT
TGGTATGTTGTCGCTCATACCTCATCCTACTTCCTCATACTAACACATCATTCAGACG
CTCCC

Figure 33:CLUSTAL W (1.82) multiple alignment analysis of *F. oxysporum* (*Hydrastis*), *F. commune* (PSU

22903 *F. commune* TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
O1174 F. oxysporum TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
22903 *F. commune* CCCCTTGGACGATGAGCTTATCTGCCATCGTTAATCCCGACCAAGACCTG 100
O1174 F. oxysporum CCCCTTGGACGATGAGCTTATCTGCCATCGTTAATCCCGACCAAGACCTG 100
22903 *F. commune* GCGGGGTATTTCTCAAAGGCAATATGCTGATATCGTTTCACAGACCGGTC 150
O1174 F. oxysporum GCGGGGTATTTCTCAAAGGCAATATGCTGATATCGTTTCACAGACCGGTC 150
22903 *F. commune* ACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCATCGAGAAGTTC 200
O1174 F. oxysporum ACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCATCGAGAAGTTC 200
22903 *F. commune* GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTCTGCCCATCGATT 250
O1174 F. oxysporum GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTCTGCCCATCGATT 250
22903 *F. commune* TCCCCTACGACTCGAAACCTGCCCGCTACCCCGCTCGAGACCAAAAATTT 300
O1174 F. oxysporum TCCCCTACGACTCGAAACCTGCCCGCTACCCCGCTCGAGACCAAAAATTT 300
22903 *F. commune* TGCGATATGACCGTAATTTTTTTT-GGTGGGGCATTTACCCCGCCACTCG 349
O1174 F. oxysporum TGCGATATGACCGTAATTTTTTTT-GGTGGGGCATTTACCCCGCCACTCG 349
22903 *F. commune* AGCGACGGG-CGCGTTTGCCCTCCTC-CCATTTCCACAACCTCAATGAGC 397
O1174 F. oxysporum AGCGACGGGC-GCGTTTGCCCTCCTC-CCATTTCCACAACCTCAATGAGC 397
22903 *F. commune* GCATCGTCACGTGTCACGCAGTC-ACTAACCATTCAATAATAGGAAGCCG 446
O1174 F. oxysporum GCATCGTCACGTGTCACGCAGTC-ACTAACCATTCAATAATAGGAAGCCG 446
22903 *F. commune* CTGAGCTCGGTAAGGGTTCCTTCAAGTACGCCTGGGTTCTTGACAAGCTC 496
O1174 F. oxysporum CTGAGCTCGGTAAGGGTTCCTTCAAGTACGCCTGGGTTCTTGACAAGCTC 496
22903 *F. commune* AAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGCTCTCTGGAAGTT 546
O1174 F. oxysporum AAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGCTCTCTGGAAGTT 546
22903 *F. commune* CGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCGCTCATGC 596
O1174 F. oxysporum CGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCGCTCATGC 596
22903 *F. commune* TTCATTCTACTTCTCTTCGTACTGACATATCACTCAGACGCTCCCGGTCA 646
O1174 F. oxysporum TTCATTCTACTTCTCTTCGTACTGACATATCACTCAGACGCTCCC----- 641
22903 *F. commune* CCGTG 651
O1174 F. oxysporum -----

Figure 34:CLUSTAL W (1.82) multiple alignment analysis for Cladogram for Figure 29 - *Hydrastis* isolate, *F. oxysporum* and the PSU isolate, *F. commune*, designated O1174

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28031                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26360_FRCO_0755             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
22533_aechmeae              TCGTCGTCATTGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
28680                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25594_batatas               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25357                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25603_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25605_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
22550_perniciosum           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26038_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26024_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26029_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26035_canariensis           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25369                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25607_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
20433_inflexum              TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25420_vasinfectum           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25609_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25367_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26367_FRCO_1591             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26178_melonis               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25356                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25375_IMI169612             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26677_F7325                 TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26442_lilii                 TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25598_glycines              TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25387_ATCC2622              TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26679_F7463                 TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
25433_vasinfectum           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26022_cubense               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26034_lycopersici           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26037_lycopersici           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26200_lycopersici           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26406_melonis               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26202_lycopersici           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
34936                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
34970_22ONLY                TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26380_radicis_lycopersici   TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26383_lycopersici           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
22555_tuberosi              TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26368_FRCO_1723             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26680_F8433                 TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26388_FTL96_2063            TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26403_FTL95_144             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26409_batatas               TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
O2139                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26033_radicis_lycopersici   TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26397_FTL95_1193            TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26363_FRCO_1168             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26373_FRCO_1750             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26381_radicis_lycopersici   TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26379_radicis_lycopersici   TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
22549_passiflorae           TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26402_FTL95_315             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26400_FTL95_727             TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
26374                       TCGTCGTCATCGGCCACGTCGACTCTGGCAAGTCGACCACTGTGAGTACT 50
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26680_F8433                GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
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26403_FTL95_144            GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
26409_batatas              GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
O2139                      GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
26033_radicis_lycopersici  GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
26397_FTL95_1193           GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
26363_FRCO_1168            GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
26373_FRCO_1750            GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
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26379_radicis_lycopersici  GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
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26203_lycopersici          GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
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25749_ATCC64530            GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
26370_FRCO_1732            GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
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32512                      GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
22548_opuntiarum           GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTTTGCCCATCGATT 249
22903_Fusarium_commune     GAGAAGGTTAGTCACTTTCCCTTCGATCGCGCGTCCTCTGCCCATCGATT 250
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20433_inflexum             TCCCCTACGACTCGAAACGTGCCCGCTACCCCGCTCGAGACCAAAAATTT 299
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22903_Fusarium_commune    TCCCCTACGACTCGAAACCTGCCCGCTACCCCGCTCGAGACCAAAAATTT 300
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26367_FRCO_1591            TGCAATATGACCGTAATTTTTTT--GGTGGGGCACTTACCCCGCCACTTG 347
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25184_Fusarium_sp.         TGCGTT---ACCGTAATTTTTTTT-GGTGGGGCATTTACCCCGCCACTCG 345
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28680 GGTGCCGGGC-GCGTTTGCCCTCTTA-CCATTCTCACAACCTCAATGAGC 395
25594_batatas GGTGCCGGGC-GCGTTTGCCCTTTTA-CCATTCTCACAACCTCAATGAGC 396
25357 AGCGACGGGGCGCGTTTGCCCTCTTA-CCATT---ACAACCTCAATGAGT 393
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25369 NNCGACGGGA-GCGTTTGCCCTCTTT-CCATTCTCACAACCTCGATGAGT 396
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26033_radicis_lycopersici AGCGACGGGA-GCGTTTGCCCTCTTAACCATTCTCACAACCTCAATGAGT 397
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25512 AGCGACGGGA-GCGTTTGCCCTCTTAACCATTCTCACAACCTCAATGAGT 396
25749_ATCC64530 AGCGACGGGA-GCGTTTGCCCTCTTAACCATTCTCACAACCTCAATGAGT 396
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32931 AGCGACGGGA-GCGTTTGCCCTCTTAACCATTCTCACAACCTCAATGAGT 396

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O1174                       AGCGACGGGC-GCGTTTGCCCTCCTC-CCATTTCCACAACCTCAATGAGC 397
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25607_cubense               GCGTCGTCACGTGTCAAGCAGTC-ACTAACCATTCAACAATAGGAAGCCG 445
20433_inflexum              GCGTCGTCACGTGTGAAGCAGTC-ACTAACCATTCAACAATAGGAAGCCG 444
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O2139                    CTGAGCTCGGTAAGGGTTCCTTCAAGTACGCCTGGGTTCTTGACAAGCTC 496
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26397_FTL95_1193            AAGGCCGAGCGTGAGCGTGGTATCACCATCGATATTGCTCTCTGGAAGTT 546
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34936 CGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCTCTCATGC 597
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26403_FTL95_144 CGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCGCTCATGC 595
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O2139 CGAGACTCCTCGCTACTATGTCACCGTCATTGGTATGTTGTCGCTCATGC 596
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