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Variation in Phytoecdysteroid Accumulation in Seeds and Shoots of *Spinacia oleracea* L. Accessions

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***Abstract*. Spinach (*Spinacea oleracea* L.) is a valuable agricultural crop that accumulates phytoecdysteroids, polyhydroxylated triterpenoids, which may play a role in plant defense and have purported health benefits for human consumers. In this study, phytoecdysteroid accumulation was measured in seeds and shoots of 15 spinach accessions to determine whether phytoecdysteroid levels vary between spinach varieties and whether seed content could reliably predict relative levels in the edible foliage. Additionally, phytosterols, precursors to phytoecdysteroids, were examined to determine potential points of regulation of spinach phytoecdysteroid biosynthesis. Significant variations in phytoecdysteroid levels between accessions were observed ($P < 0.05$), suggesting the potential for genetic manipulation through traditional breeding or genetic engineering to increase phytoecdysteroid levels in spinach. However, results suggest that estimation of phytoecdysteroid levels in shoots may not be achieved by measuring levels in the seeds. Levels of phytoecdysteroids in spinach ranged from 19.9 to 44.1 μg per shoot, 0.7 to 1.2 μg·mg^{-1} dry mass shoot, 3.2 to 9.6 μg per seed, and 0.5 to 1.1 μg·mg^{-1} seed. Several phytosterols connected to the phytoecdysteroid biosynthetic pathway were identified by gas chromatography–mass spectroscopy, predominantly spinasterol, 5-dihydroergosterol, and 22-dihydrospinasterol, which comprised 79.8%, 6.3%, and 4.6% of the total phytosterol content, respectively. Detection of the phytosterols cycloartenol and lanosterol in spinach suggests that spinach may also have dual biosynthetic pathways to phytosterols that contribute to the production of phytoecdysteroids.**

Spinach (*Spinacea oleracea* L.) is a valuable agricultural crop that accumulates phytoecdysteroids and is a model plant for the study of phytoecdysteroid biosynthesis (Grebenok et al., 1991). The main phytoecdysteroids that accumulate in spinach are 20-hydroxyecdysone (20E) and polypodine B (Grebenok et al., 1991, 1994). Phytoecdysteroids are polyhydroxylated triterpenoids biosynthesized from phytosterols through the mevalonic acid pathway (Adler and Grebenok, 1999). Although the role of phytoecdysteroids in plants has not been established, attributable in part to their dynamic and polar characteristics, they have been hypothesized to function as a long-distance water-soluble transport form of non-polar phytosterols and as plant defense compounds against non-adapted insects (Grebenok and Adler, 1993; Schmelz et al., 1999, 2000).

In spinach, phytoecdysteroids are actively biosynthesized in older leaves and transported to newly developing apical parts of the plant, including flowers, seeds, and young leaves (Bakrim et al., 2008). Phytoecdysteroids can accumulate in spinach leaves at levels greater than 100 μg·g^{-1} fresh weight, which are physiologically capable of deterring non-adapted insect species (Adler and Grebenok, 1995; Grebenok et al., 1991; Kubo and Kloche, 1983). Ingestion of phytoecdysteroids by insects and nematodes caused premature molting and death, because the analogous structures produced by insects, ecdysteroids, are arthropod-molting hormones (Nakagawa and Henrich, 2009; Soriano et al., 2004). The levels of dietary phytoecdysteroids that cause physiological effects in insects range from 0.03 to 100 mg·kg^{-1} fresh weight and depend on the particular insect species, stage of insect development, and specific physiological response measured (Adler and Grebenok, 1999; Jones and Firn, 1978; Soriano et al., 2004). Mechanical damage or the application of the plant-defense signaling compound methyl jasmonate to spinach prompted roots to rapidly increase phytoecdysteroid production (Schmelz et al., 1998). In subsequent trials by Schmelz et al. (2002), spinach roots with increased phytoecdysteroid levels (25 to 50 μg·g^{-1} wet mass) deterred *Bradysia impatiens* (dark-winged fungus gnat) feeding and had lower levels of damage compared with untreated control roots with lower levels of phytoecdysteroids. Taken together, these and many other studies make a firm case that phytoecdysteroids play a role in plant defense. Thus, improved agricultural yields may be gained through the development or identification of high phytoecdysteroid-accumulating spinach genotypes with enhanced insect resistance.

Phytoecdysteroids have also been associated with various pharmacological properties in mammals, including enhanced physical performance and stimulation of growth (Báthori et al., 2008). Phytoecdysteroids are the purported bioactive components in perennial medicinal plants such as *Ajuga turkestanica* and *Rhaponticum carthamoides* and accumulate in high levels (up to 0.5% and 1.2% 20E of dried aerial tissue, respectively) (Gorelick-Feldman et al., 2008; Kokoska and Janovska, 2009; Syrov et al., 2008). Phytoecdysteroid concentrations in spinach, on the other hand, are generally below the purported pharmacologically active levels, which could be achieved through normal dietary consumption by mammals; the average phytoecdysteroid content in spinach foliage (40 μg·g^{-1} dry mass) was reported to be over 100-fold lower than for *A. turkestanica* (5 mg·g^{-1} dried aerial portion) (Gorelick-Feldman et al., 2008). However, health benefits from dietary consumption of phytoecdysteroids may potentially be achieved through the development of high phytoecdysteroid-accumulating spinach cultivars or hybrids.

Phytosterols, precursors to phytoecdysteroids, have been linked to health benefits such as lowering serum cholesterol levels and protection against certain cancers (Jones and AbuMweis, 2009; Piironen et al., 2003). Phytosterols in general provide membrane stability and rigidity in plants (Moreau et al., 2002). Levels of total phytosterols increased or decreased in coordination with phytoecdysteroid levels during the growth and development of spinach (Grebenok et al., 1991). In an excised leaf assay, [2-^{14}C] mevalonic acid was incorporated into the phytosterol lathosterol before incorporation into 20E and other phytoecdysteroids (Grebenok and Adler, 1993). Additionally, elevated levels of phytoecdysteroid intermediates and end products inhibited endogenous phytoecdysteroid production and prevented mevalonic acid incorporation into lathosterol (Bakrim et al., 2008; Grebenok et al., 1994, 1996). Inhibition of carbon flux into lathosterol suggests that phytoecdysteroid regulation may also occur before the final hydroxylation steps, impacting carbon allocation early in the phytoecdysteroid pathway such as during phytosterol biosynthesis. The phytosterol biosynthetic network has been well investigated and therefore affords molecular tools and specific sterol biosynthesis inhibitors to further evaluate the influence of phytosterols on phytoecdysteroid biosynthesis (Espenshade and Hughes, 2007; Palani and Lalithakumari, 1999). A proposed biosynthetic pathway for phytoecdysteroids in spinach and phytosterol intermediates is presented in Figure 1.

Phytoecdysteroid content has been evaluated among numerous species within plant genera to investigate their chemotaxonomic applications; however, to the best of our knowledge, levels of phytoecdysteroid accumulation among numerous varieties of the *S. oleracea* have not been evaluated. Production and accumulation of phytoecdysteroids differ between plant species, which allows the presence and levels of specific phytoecdysteroids to serve as chemotaxonomic markers (Dinan et al., 2001c; Zibareva et al., 2003). Within a plant species,

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the phytoecdysteroid content in seeds may provide a gauge of the foliar content for the plant (Dinan et al., 2001b). For *Chenopodium* species, if phytoecdysteroids were detected in the seed, they were consistently detected in the germinated plant foliage (Dinan, 1992). In an evaluation of 180 randomly selected plant species, phytoecdysteroids were more readily detected in leaves than in seeds; however, the highest levels of phytoecdysteroids were detected only for species that were also positive for phytoecdysteroids in the seeds (Dinan et al., 2001b). Similar relationships between secondary compound accumulation in seed and vegetative tissues have been demonstrated for aliphatic glucosinolates, which were correlated in 35 different *Arabidopsis* ecotypes (Kliebenstein et al., 2001).

The aims of this study were to measure phytoecdysteroid accumulation in seeds and shoots of various spinach accessions and evaluate whether seed content could reliably predict relative levels in the edible foliage. Establishing a correlation between levels of phytoecdysteroid in the seed and corresponding levels in spinach foliage would expedite the screening of germplasm for higher phytoecdysteroid content and subsequent breeding trials. Identification of differences in phytoecdysteroid content between genotypes could be used to determine whether greater levels of phytoecdysteroid accumulation result in enhanced resistance to herbivory and to further investigate genetic regulation of phytoecdysteroid biosynthesis. In addition, establishing a metabolic profile of phytoecdysteroid precursors, phytosterols, could help to elucidate a network of potential regulatory points of de novo phytoecdysteroid biosynthesis.

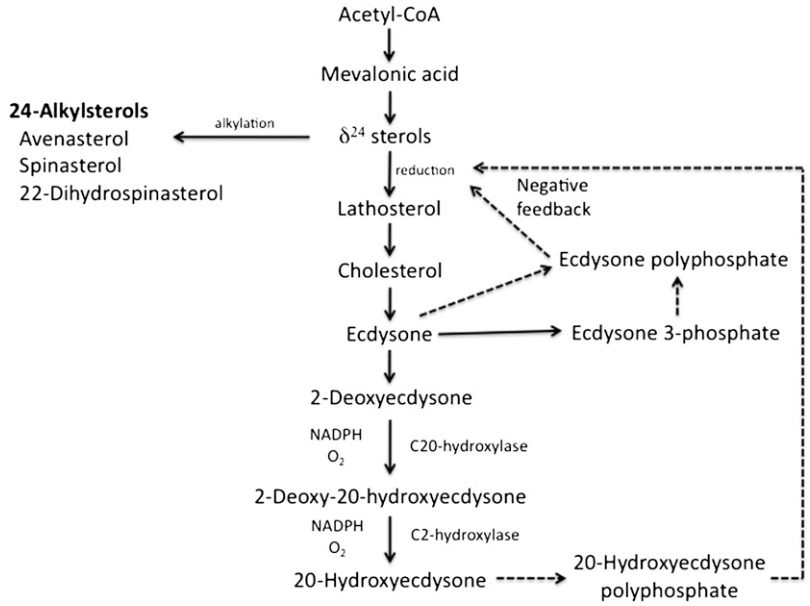

Fig. 1. Phytoecdysteroid biosynthetic pathway in spinach (from Adler and Grebenok, 1999; Bakrim et al., 2008).

Table 1. *Spinacia oleracea* accessions obtained from the U.S. Department of Agricultural Resource Service, Germplasm Resources Information Network and Mou (2008).

Accession[z]	Plant name[z]	Origin[z]	Percent flowering[zy]	Traits
Ames 20169	Hu shi yuan ye bo cai	China	73	
NSL 6084	Giant thick leaved/Nobel	California, U.S.	22	
NSL 6092	Viking	New York, U.S.	33	Very late to flower[z]
NSL 92513	Bloomsdale long standing	Oregon, U.S.	7	
PI 169673	Harlan 1788	Aydin, Turkey	22	Fewest mines[x]
PI 173130	Harlan 8557	Malatya, Turkey	93	
PI 174385	CGN 9504	Diyarbakir, Turkey	45	Fewest mines[x]
PI 175312	Palak	India	59	High sting density and number of mines[x]
PI 179589	Giant spinach	Belgium	75	
PI 274065	Wisemona	England/Germany	0	Lowest sting density[x]
PI 433208	Xiao Ye	China	47	High sting density and number of mines[x]
PI 445782	Shami	Syria	100	
PI 499372	Ispolinskij	Former Soviet Union	38	
PI 531456	Popey	Hungary	28	
PI 606707	America	The Netherlands	0	Matures later than other Bloomsdale varieties and extremely slow bolting[z]

[z]Data from USDA-ARS National Plant Germplasm System Germplasm Resources Information Network.
[y]Percentage of plants that flowered after 28 d with 16 h of light.
[x]Leafminer mines and sting density indicating level of insect resistance (Mou, 2008).

Materials and Methods

Plant material. Fifteen spinach (*Spinacia oleracea*) accessions (Table 1) were obtained through the U.S. Department of Agriculture's Agricultural Research Service–Germplasm Resources Information Network (USDA ARS GRIN; http://www.ars-grin.gov) from a range of geographic locations, including Turkey, Hungary, The Netherlands, and the United States. Seeds were surface-sterilized by immersing for 15 to 20 min in 10% sodium hypochlorite followed by a 70% ethanol rinse and immersion in a plant preservative mixture (PPM; Plant Cell Technology, Washington, DC) for 4 to 8 h. Water purified by filtering ddH_2O through a Barnstead NANOpure II ultrafiltration system (greater than 18 megohm/cm; Sybron, Boston, MA) was used for all experiments. Seeds were germinated on sterile moist filter paper in petri dishes before transferring to culture vessels (Magenta® GA; Phytotechnology Laboratories, Shawnee Mission, KS) containing 45 mL Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 0.1 g·L^{-1} myoinositol, 30 g·L^{-1} sucrose, and rose vitamins (Rogers and Smith, 1992). Plants were grown in vitro at 25 °C on a short daylight cycle (8-h light:16-h dark) with 120 μmol·m^{-2}·s^{-1} irradiance from cool-white fluorescent lights. Spinach shoots (whole aerial portion of plantlets) from each accession (five to nine individual plants per accession) were harvested after the development of the sixth true leaf, 21 to 36 d after germination (Table 2) and frozen at –80 °C before lyophilization. Dried plant tissue was ground to a powder with a glass rod in a 20-mL vial and a 25-mg subsample was extracted as described subsequently for seeds.

Extraction. Spinach seeds from each accession were sampled by measuring ≈100 mg of seed (three replicates per accession), which were ground with a mortar and pestle. A 25-mg subsample of ground seed from each pool was extracted in 1 mL of methanol for 1 h at 55 °C, and the process was repeated two more times for each sample. Methanolic extracts were pooled (total of 3 mL) and water (1.3 mL) was added followed by partitioning with 2 mL hexane. Samples were briefly centrifuged for 1 min at 1400 rpm. The upper hexane layer was removed and the extract was dried down by rotary evaporation and stored at –20 °C (Dinan et al., 2001a). All samples were redissolved in 775 μL of 70% methanol, filtered through 0.45-nm nylon filters (Fisher

Table 2. Mean seed mass ± SEM, days to harvest ± SEM, and phytoecdysteroid content (20-hydroxyecdysone equivalent) in shoots and seeds of 15 accessions of *Spinacia oleracea*.[z]

			Phytoecdysteroid content			
			Shoots		Seeds	
S. oleracea accession	Seed mass (mg)	Days to harvest	µg/shoot	µg/mg dried shoot	µg/seed	µg/mg seed
Ames 20169	9.1 ± 0.5	21 ± 0	20.9 ± 3.6 c	0.7 ± 0.2 b	5.2 ± 0.7 bcd	0.6 ± 0.0 bc
NSL 6084	6.4 ± 0.7	24 ± 3	40.3 ± 4.0 ab	0.9 ± 0.1 ab	3.2 ± 0.3 d	0.5 ± 0.0 c
NSL 6092	13.0 ± 0.7	32 ± 3	28.4 ± 3.7 bc	0.8 ± 0.1 ab	6.0 ± 0.5 b	0.5 ± 0.0 c
NSL 92513	9.2 ± 0.7	25 ± 2	33.7 ± 5.6 abc	1.2 ± 0.2 a	9.6 ± 2.3 a	1.1 ± 0.3 a
PI 169673	6.7 ± 0.1	30 ± 1	38.5 ± 5.2 ab	0.9 ± 0.0 ab	3.4 ± 0.4 d	0.5 ± 0.1 c
PI 173130	8.2 ± 0.5	30 ± 4	34.2 ± 6.0 abc	0.8 ± 0.1 b	4.3 ± 0.3 bcd	0.5 ± 0.1 bc
PI 174385	7.7 ± 0.7	27 ± 2	31.9 ± 5.0 abc	0.8 ± 0.0 b	4.2 ± 0.7 bcd	0.5 ± 0.0 bc
PI 175312	8.9 ± 1.3	27 ± 3	22.6 ± 3.6 c	0.8 ± 0.1 b	4.5 ± 0.4 bcd	0.5 ± 0.1 bc
PI 179589	7.0 ± 0.1	36 ± 4	31.3 ± 4.3 abc	0.9 ± 0.1 ab	3.7 ± 0.2 cd	0.5 ± 0.0 bc
PI 274065	8.3 ± 0.4	34 ± 4	21.8 ± 3.9 c	1.0 ± 0.2 ab	5.1 ± 0.5 bcd	0.6 ± 0.0 bc
PI 433208	6.0 ± 1.1	28 ± 2	38.7 ± 1.8 ab	0.9 ± 0.1 ab	4.2 ± 0.4 bcd	0.6 ± 0.1 bc
PI 445782	9.3 ± 0.6	26 ± 2	20.4 ± 5.5 c	0.9 ± 0.2 ab	4.6 ± 0.2 bcd	0.5 ± 0.0 bc
PI 499372	6.8 ± 0.5	24 ± 2	31.4 ± 3.2 abc	0.8 ± 0.1 b	3.7 ± 0.1 cd	0.5 ± 0.1 bc
PI 531456	9.8 ± 0.2	26 ± 2	44.1 ± 9.3 a	0.9 ± 0.1 ab	5.5 ± 0.4 bc	0.6 ± 0.0 bc
PI 606707	5.7 ± 0.4	35 ± 2	19.9 ± 1.1 c	0.7 ± 0.1 b	4.5 ± 0.5 bcd	0.8 ± 0.0 b

[z]Mean phytoecdysteroid content (n = 5–9) ± SEM followed by letters indicating least significant difference groupings ($P < 0.05$). Means for accessions with different letters within each column are statistically different.

Scientific, Pittsburgh, PA), and 30 µL was injected for high-performance liquid chromatography (HPLC) analysis.

Phytoecdysteroid analysis. A commercial standard of 20E (Bosche Scientific, New Brunswick, NJ) was dissolved in 70% methanol and used for quantification by HPLC at concentrations of 250, 125, and 62.5 µg·mL^{-1} with 5-µL injection volumes. Phytoecdysteroid content was measured as 20E equivalents resulting from coelution of 20E and polypodine B. Analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies Inc., Wilmington, DE) with autosampler, DAD (242 nm) and Kromasil 100-5C$_{18}$ reverse-phase column (250 × 5 µM × 4.6 mm; Eka Chemicals, Brewster, NY). The mobile phase solvents consisted of 0.1% trifluoroacetic acid (TFA; Acros Organics, Fair Lawn, NJ) in water (A) and 0.1% TFA in 90% acetonitrile (B). The system was eluted using a step gradient as follows: Solvent A from 0 to 30 min, then 70% Solvent B from 30 to 40 min, followed by 100% Solvent B from 40 to 50 min and 100% Solvent A from 50 to 60 min to re-equilibrate the column, all at a constant flow rate of 0.5 mL·min^{-1}.

Sterol extraction. Phytosterols were extracted from spinach as described by Piironen et al. (2002) using a method that measured total phytosterol content, which includes free phytosterols and bound phytosterol conjugates. Briefly, three groups (five plants each, ≈140 mg) of dried spinach shoots were extracted with 20 mL of hexane-diethyl ether (1:1) by moderately shaking for 10 min using a vortex (Vortex Genie 2; Scientific Industries Inc., Bohemia, NY). The organic layer was separated by centrifuging for 10 min at 2600 rpm and then transferred to a round-bottom flask and evaporated to dryness in a rotary evaporator with a less than 40 °C water bath. For saponification, 8 mL of absolute ethanol was added to the dry residue and transferred to a 50 mL Falcon tube. Next, 0.5 mL of saturated aqueous KOH solution was added and vortexed for 10 s before placing the tube in a shaking water bath (80 to 85 °C) for 30 min. After the sample was cooled, 12 mL of water and 20 mL of cyclohexane were added followed by shaking for 10 min to extract unsaponifiable lipids. An aliquot of 15 mL of the organic layer was transferred to a round-bottomed flask, rotary evaporated, then redissolved in 1 mL chloroform. A Sep-Pak® C18 cartridge (Waters Corporation, Milford, MA) was activated with 5 mL of methanol followed by 5 mL of water. The chloroform solution was eluted by gravity flow for 2 min and pressed through with a syringe to purify the unsaponifiable fraction. The bound sterol fraction was eluted with 15 mL of methanol–chloroform (5:95) and rotary-evaporated to dryness.

Sterol sample processing for gas chromatography–mass spectroscopy analyses. Extracted sterol samples were prepared and analyzed in triplicate by the Metabolomics Center in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana Champaign. They were derivatized in two steps as follows: 60 min at 50 °C with 80 µL of methoxyamine hydrochloride in pyridine (20 mg·mL^{-1}; Sigma, St. Louis, MO) followed by 60-min treatment at 50 °C with 80 µl N-methyl-N-(trimethylsilyl) trifluoroacetamide (Fisher Scientific). Sample volume of 5 µL was injected in splitless mode. The gas chromatography–mass spectroscopy system consisted of an Agilent 7890A (Agilent Technologies Inc.) gas chromatograph, an Agilent 5975C mass selective detector, and Agilent 7683B autosampler. Gas chromatography was performed on a 60 m HP-5MS column with 0.25-mm inner diameter and 0.25-µm film thickness (Agilent Technologies Inc.) with an injection temperature of 250 °C, the interface set to 250 °C, and the ion source adjusted to 230 °C. The helium carrier gas was set at a constant flow rate of 1.5 mL·min^{-1}. The temperature program was set to have 5 min of isothermal heating at 70 °C followed by an oven temperature increase of 5 °C·min^{-1} to 310 °C and a final 20 min at 310 °C. The mass spectrometer was operated in positive electron impact mode at 69.9 eV ionization energy in the m/z 50 to 800 scanning range.

The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries: NIST08 [National Institute of Standards and Technology (NIST), Gaithersburg, MD], WILEY08 (Palisade Corporation, Ithaca, NY), and the custom library. The chromatograms and mass spectra were evaluated using the MSD ChemStation (Agilent Technologies Inc.) and the Automated Mass-spectral Deconvolution and Identification System (AMDIS) (NIST) programs. The retention time and mass spectra were implemented within the AMDIS method formats. To allow comparison between samples, all data were normalized to the internal standard, lathosterol (Sigma), which was not detected in preliminary extracts of this accession. Lathosterol was added immediately before the derivatization procedure at 1 mg·mL^{-1} in each chromatogram and a 100% recovery rate was achieved. Relative concentrations of phytosterols were calculated as the ratio of the target peak area divided by the lathosterol peak area over the dry mass of each sample and reported as lathosterol equivalents per gram dry mass.

Statistical analysis. Statistically significant differences in mean phytoecdysteroid content between seeds or shoots of spinach accessions were determined by analysis of variance using Proc GLM and Fisher's least significant difference using SAS Version 9.2 for Windows (SAS Institute Inc., Cary, NC). A P value < 0.05 was considered statistically significant. Pearson's correlation coefficients between mean seed and shoot phytoecdysteroid accumulation were calculated by the Proc CORR function.

Results and Discussion

Spinach seeds were obtained from the USDA germplasm repository and are representative varieties from a range of geographic locations. Both genetic diversity and the plant's ecological environment may influence phytoecdysteroid levels (Volodin et al., 2002). There were over 300 accessions of *S. oleracea* in the germplasm repository, and 15 accessions were selected for this study based on rankings of insect resistance (Mou, 2008), seed size, and flowering and bolting times available on the ARS GRIN database (www.ars-grin.gov; Tables 1 and 2). Accumulation of phytoecdysteroids, measured as 20E equivalents in seeds and shoots of *S. oleracea*, varied significantly among accessions on a per shoot, per dry mass shoot, per seed, and per seed mass basis (Table 2). Levels of phytoecdysteroids from spinach grown in vitro ranged from 19.9 to 44.1 µg per shoot and from 0.7 to 1.2 µg·mg^{-1} dry mass shoot. In seeds, the phytoecdysteroid content ranged from 3.2 to 9.6 µg per seed and 0.5 to 1.1 µg·mg^{-1} per seed. Accessions PI 531456 ('Popey') and PI 606707 ('America') had the highest and lowest phytoecdysteroid content per shoot, respectively, and NSL 92513 ('Bloomsdale Long-Standing') and PI 606707

('America') had the highest and lowest phytoecdysteroid content per dry mass of shoots, respectively. Accession NSL 92513 also had the highest phytoecdysteroid content per gram seed and per seed. These values are within the range previously reported for spinach seeds and foliage (Dinan, 1995; Grebenok et al., 1991). The significant differences in phytoecdysteroid accumulation between varieties illustrate the potential for further manipulation of phytoecdysteroid levels. The selection of high- and low-accumulating varieties would be of use for breeding programs or studying the biological regulation of phytoecdysteroid biosynthesis.

A moderate but significant correlation was found in phytoecdysteroid accumulation per mass of seeds and per dry mass of shoots (r = 0.52, P = 0.04, n = 15) and between phytoecdysteroid accumulation per seed and phytoecdysteroid accumulation per dry mass of shoots (r = 0.58, P = 0.02, n = 15) among the 15 spinach accessions. However, the significant correlation was driven by an outlier and when this data point was removed, the Pearson's correlation coefficients were no longer significant. These results suggest that estimation of phytoecdysteroid levels in shoots may not be achieved by measuring levels in the seeds.

Among the 15 *S. oleracea* accessions selected for screening three were genotypes that demonstrated leafminer resistance and two genotypes that demonstrated high susceptibility (Mou, 2008). Leafminer (*Liriomyza* spp.) is a major agricultural pest around the world, damaging vegetable crops such as spinach (Mou, 2008; Parrella, 1987). Because phytoecdysteroids demonstrated strong antifeedant effects and disrupted insect development when ingested, higher inherent levels of 20E may be expected in genotypes that demonstrate enhanced insect resistance (Adler and Grebenok, 1999; Jones and Firn, 1978; Mele et al., 1992; Robbins et al., 1970; Singh and Russell, 1980). In cage and field tests, *S. oleracea* accessions PI 274065, PI 174385, and PI 169673 had the fewest mines or lowest sting density produced by leafminers and PI 175312 and PI 433208 had high sting density in field trials (Mou, 2008). However, the level of 20E in these genotypes, as measured in our screening (Tables 2), did not reflect the hypothesis that high levels of phytoecdysteroids may account for greater leafminer resistance. Possible explanations could be that leafminers in the Mou (2008) study were adapted to and undeterred by phytoecdysteroids or that the phytoecdysteroid content in these varieties were below leafminer antifeedant levels, and other plant secondary compounds may be responsible for conferring resistance.

The highest level of phytoecdysteroid accumulation on a $\mu g \cdot mg^{-1}$ dry mass shoot basis was found in accession NSL 92513, and therefore this accession was used to investigate phytoecdysteroid precursors to map potential pathways where phytosterols might regulate phytoecdysteroid accumulation. Phytosterols and their relative levels are presented in Table 3. Spinasterol was the predominant phytosterol, making up an average of 79.8% of total phytosterols identified, which was consistent with previous studies (Grebenok and Adler, 1993; Piironen et al., 2003). Other common phytosterols accumulated lower levels, including 5-dihydroergosterol (6.3%), 22-dihydrospinasterol (4.6%), stigmasterol (2.4%), and cholesterol (2.2%). Although the formation of lathosterol was previously identified as an intermediate in 20E biosynthesis using radiolabeled [2-^{14}C] mevalonic acid (Grebenok and Adler, 1993), lathosterol was not detected in this study. However, Grebenok and Adler (1993) also reported that lathosterol did not accumulate and instead was subsequently metabolized, which likely explains why lathosterol was not identified in this analysis.

Table 3. Spinach phytosterol content reported as lathosterol equivalents per gram dry mass spinach shoots ± SEM and the percentages of each phytosterol to total phytosterols.[z]

Phytosterol	Lathosterol equivalents/g dry mass	Percent
(24Z)-3-Hydroxystigmasta-7,24(28)-diene	116 ± 15	1.7
22-Dihydrospinasterol	313 ± 34	4.6
24-Methylenecycloartanol	12 ± 2	0.2
3-Hydroxycholest-8(14)-ene	14 ± 1	0.2
3-Hydroxyergost-8(14)-ene	26 ± 1	0.4
5-Dihydroergosterol	426 ± 44	6.3
Cholesterol	151 ± 11	2.2
Cycloartenol	57 ± 5	0.8
Lanosterol	43 ± 1	0.6
Sitosterol	43 ± 3	0.6
Spinasterol	5394 ± 220	79.8
Stigmasterol	160 ± 10	2.4
Total		100

[z]Concentrations are the average of three samples standardized relative to lathosterol.

Cycloartenol (0.8% of total phytosterols) and lanosterol (0.6% of total phytosterols) were detected in spinach. In higher plants, there are two biosynthetic pathways for phytosterol biosynthesis, through cycloartenol or through lanosterol (Benveniste, 2004). Lanosterol synthase genes were only recently identified in dicotyledonous plants (previously only found in animals and fungi). Lanosterol synthase genes have been induced by methyl jasmonate, suggesting that secondary metabolites produced through the lanosterol pathway may contribute to plant defense (Ohyama et al., 2009; Suzuki et al., 2006). The influence of lanosterol and other phytosterols on phytoecdysteroid production is an interesting avenue of investigation. Phytosterol biosynthetic pathways have been extensively researched as a result of their importance in plants and benefits to human health (Moreau et al., 2002). Thus, molecular tools such as phytosterol biosynthesis inhibitors and gene overexpression or knockout constructs are available technologies that can be used to evaluate regulatory roles of phytosterols on phytoecdysteroid biosynthesis (Fig. 1).

In this study, phytoecdysteroids were detected in all seeds and shoots of *S. oleracea* accessions selected for this study. Significant variation in phytoecdysteroid content between spinach accessions suggests potential for molecular biotechnology or conventional breeding to enhance levels of phytoecdysteroid accumulation. Additionally, manipulation of phytosterol biosynthesis may help to elucidate regulation of phytoecdysteroid biosynthesis. The ability to manipulate phytoecdysteroid levels may be used to determine their effectiveness on insect deterrence and potential to improve plant fitness and yield.

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