Variation in Phytoecdysteroid Accumulation in Seeds and Shoots of Spinacea 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, phytoesterols, 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 phytoecdysteroids connected to the phytoecdysteroid biosynthetic pathway were identified by gas chromatography–mass spectroscopy, predominantly spirostanes, 5-dihydroergostanol, and 22-dihydrospirostane, which comprised 79.8%, 6.3%, and 4.6% of the total phytoesteroid content, respectively. Detection of the phytoestrols cycloartenol and lanosterol in spinach suggests that spinach may also have dual biosynthetic pathways to phytoesterols 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 polypropylene B (Grebenok et al., 1991, 1994). Phytoecdysteroids are polyhydroxylated triterpenoids biosynthesized from phytoesterols 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 phytoesterols 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. Phytoesterols, 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). Phytoesterols in general provide membrane stability and rigidity in plants (Moreau et al., 2002). Levels of total phytoesterols increased or decreased in coordination with phytoecdysteroid levels during the growth and development of spinach (Grebenok et al., 1991). In an excised leaf assay, [2-¹⁴C] 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 phytoesterols 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,
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, phytosteroles, could help to elucidate a network of potential regulatory points of de novo phytoecdysteroid biosynthesis.

**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, States) for 4 to 8 h. Water purified by filtering ddH₂O 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 approximately 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 1. Spinacia oleracea** accessions obtained from the U.S. Department of Agricultural Research Service, Germplasm Resources Information Network and Mou (2008).

| Accession | Plant name | Origin | Percent flowering | Traits |
|-----------|------------|--------|------------------|--------|
| Ames 20169 | Hu shi yuan | China | 73 | 
| NSL 6084 | Giant thick leaved/Nobel | California, U.S. | 22 | 
| NSL 6092 | Viking | New York, U.S. | 33 | Very late to flower
| NSL 92513 | Bloomsdale long standing | Oregon, U.S. | 7 | 
| PI 169673 | Harlan 1788 | Aydin, Turkey | 22 | Fewest mines
| PI 173310 | Harlan 8557 | Malatya, Turkey | 93 | 
| PI 174385 | CGN 9504 | Diyarbakir, Turkey | 45 | Fewest mines
| PI 175312 | Palak | India | 59 | High sting density and number of mines
| PI 179589 | Giant spinach | Belgium | 75 | 
| PI 274065 | Wisemona | England/Germany | 0 | Lowest sting density
| PI 433208 | Xiao Ye | China | 47 | High sting density and number of mines
| 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

*Data from USDA-ARS National Plant Germplasm System Germplasm Resources Information Network.

*Percent of plants that flowered after 28 d with 16 h of light.

*Leafminer mines and sting density indicating level of insect resistance (Mou, 2008).
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 polypropylene B. Analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies Inc., Wilmington, DE) with autosampler, DAD (242 nm) and Kromasil 100-5C₁₈ 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: 60 min at 50% Solvent A from 50 to 30 min, followed by 100% Solvent B from 30 to 20 min, and then 70% Solvent B from 20 to 10 min 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-bottom 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 μg·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 an 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/ε 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 DMS) 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 basis (Table 2). Levels of phytoecdysteroids from spinach grown in vitro ranged from 19.9 to 44.1 μg·g⁻¹ dry mass and from 0.7 to 1.2 μg·mg⁻¹ dry mass shoot. Levels of phytoecdysteroid content ranged from 3.2 to 9.6 μg·mg⁻¹ dry mass and 0.5 to 1.1 μg·g⁻¹ dry mass. Accessions PI 531456 (‘Popey’) and PI 606707 (‘America’) had the highest and lowest phytoecdysteroid content per shoot, respectively, and NSL 92513 (‘Bloomdale Long-Standing’) and PI 606707

| S. oleracea accession | Seed mass (mg) | Days to harvest | Phytoecdysteroid content | Shoots | Seeds |
|-----------------------|---------------|----------------|--------------------------|--------|-------|
|                       | µg/shoot      | µg/mg dried shoot | µg/seed | µg/mg seed |
| Ames 20169             | 9.1 ± 0.5     | 21 ± 0           | 20.9 ± 3.6 c              | 5.2 ± 0.7 bcd | 0.6 ± 0.0 bc |
| NSL 6084              | 6.4 ± 0.7     | 24 ± 3           | 40.3 ± 4.0 ab             | 3.2 ± 0.3 d  | 0.5 ± 0.0 c |
| 169692                | 13.0 ± 0.7    | 32 ± 3           | 28.4 ± 3.7 bc             | 6.0 ± 0.5 b  | 0.5 ± 0.0 c |
| NSL 92513             | 9.2 ± 0.7     | 25 ± 2           | 33.7 ± 5.6 ab             | 9.6 ± 2.3 a  | 1.1 ± 0.3 a |
| PI 169673             | 6.7 ± 0.1     | 30 ± 1           | 38.5 ± 5.2 ab             | 3.4 ± 0.4 d  | 0.5 ± 0.1 c |
| PI 187310             | 8.2 ± 0.5     | 20 ± 4           | 34.2 ± 6.0 ab             | 4.3 ± 0.3 bcd| 0.5 ± 0.1 bc |
| PI 174385             | 7.7 ± 0.7     | 27 ± 2           | 31.9 ± 5.0 ab             | 4.2 ± 0.7 bcd| 0.5 ± 0.0 bc |
| PI 175312             | 8.9 ± 1.3     | 23 ± 3           | 22.6 ± 3.6 c              | 4.5 ± 0.4 bcd| 0.5 ± 0.1 bc |
| PI 179589             | 7.0 ± 0.1     | 36 ± 4           | 31.3 ± 4.3 ab             | 3.7 ± 0.2 cd | 0.5 ± 0.0 bc |
| PI 274065             | 8.3 ± 0.4     | 34 ± 2           | 21.8 ± 3.9 ab             | 5.1 ± 0.5 bcd| 0.6 ± 0.0 bc |
| PI 4332008            | 6.0 ± 1.1     | 28 ± 2           | 38.7 ± 1.8 ab             | 4.2 ± 0.4 bcd| 0.6 ± 0.0 bc |
| PI 457452             | 9.3 ± 0.6     | 26 ± 2           | 20.4 ± 5.5 c              | 6.0 ± 0.1 bcd| 0.5 ± 0.0 bc |
| PI 499372             | 6.8 ± 0.5     | 24 ± 2           | 31.4 ± 3.2 ab             | 3.7 ± 0.1 cd | 0.5 ± 0.1 bc |
| PI 531456             | 9.8 ± 0.2     | 26 ± 2           | 44.1 ± 9.3 a              | 5.5 ± 0.4 bc | 0.6 ± 0.0 bc |
| PI 606707             | 5.7 ± 0.4     | 35 ± 2           | 19.9 ± 1.1 c              | 4.5 ± 0.5 bcd| 0.8 ± 0.0 b |

*Mean phytoecdysteroid content (n = 5–9) ± SEM followed by letters indicating least significant difference (P < 0.05). Means for accessions with different letters within each column are statistically different.*
Spinasterol was the predominant 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 Firm, 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 \(\mu\)-g \(^{-1}\) 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 phytoecdysterols might regulate phytoecdysteroid accumulation. Phytoecdysterols and their relative levels are presented in Table 3. Spinasterol was the predominant phytoecdysterol, making up an average of 79.8% of total phytoecdysterols identified, which was consistent with previous studies (Grebenok and Adler, 1993; Pironen et al., 2003). Other common phytoecdysteroids accumulated lower levels, including 5-dihydroergosterol (6.3%), 22-dihydroxyergosterol (4.6%), stigmasterol (2.4%), and cholesterol (2.2%). Although the formation of lathosterol was previously identified as an intermediate in 20E biosynthesis using radioisotopic methods (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.

Cycloartenol (0.8% of total phytoecdysterols) and lanosterol (0.6% of total phytoecdysterols) were detected in spinach. In higher plants, there are two biosynthetic pathways for phytoecdysteroid 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. Phytoecdysteroid 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 phytoecdysteroid biosynthesis inhibitors and gene overexpression or knockout constructs are available technologies that can be used to evaluate regulatory roles of phytoecdysterols 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 phytoecdysterol 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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### Table 3. Spinach phytosterol content reported as lathosterol equivalents per gram dry mass spinach shoots ± SEM and the percentages of each phytoecdysterol to total phytoecdysterols.

| Phytosterol | Lathosterol equivalents/g dry mass | Percent |
|-------------|-----------------------------------|---------|
| (24Z)-3-Hydroxystigmasta-7,24(28)-diene | 116 ± 15 | 1.7 |
| 22-Dihydrospinosasterol | 313 ± 34 | 4.6 |
| 24-Methylencycloartenol | 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 | 3594 ± 220 | 79.8 |
| Stigmasterol | 160 ± 10 | 2.4 |
| **Total** | 100 |

*Concentrations are the average of three samples standardized relative to lathosterol.
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