

**EFFECT OF GREEN HOUSE CONDITION ON
DEVELOPMENT OF SOYBEAN SEED SAPONINS,
SPHINGOLIPIDS, AND PHYTOSTEROLS
CONCENTRATIONS AND COMPOSITIONS**

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ABSTRACT

This study investigated the effect of green house conditions (temperature and soil moisture) on soybean [*Glycine max* (L.) Merr.] seeds bioactive compound concentrations. The analyses included plant characters and bioactive compounds contents. There were a good relationship between maturity date and total sphingolipids. Jack and Queen, among the latest and earliest had maturing entries, respectively. Jack had the highest concentrations of total saponins and total phytosterols. Imari had the lowest total saponins, total sphingolipids, and total phytosterols concentrations but Queen had the lowest total sphingolipids among all cultivars. Each cultivar had specific characters and maintained its level along different analyses. Under controlled conditions, sphingolipids levels had changed more than saponins and phytosterols.

Key words: Soybean, Saponins, Phytosterols, Sphingolipids, Green-house plantation.

INTRODUCTION

Water scarcity and soil moisture are important limitations for agricultural production in semiarid region especially for soybean

production. Soybean is the world's primary source of protein and oil and is often called the miracle crop because of its numerous uses. Soybean seeds contain an average 40% protein, 35% carbohydrate, 20% oil, and 5% ash (Liu, 1997). Soybean is now an essential and dominant source of protein and oil with over 200 uses in feed, food and industrial applications. Recent studies indicate that consumption of soybean reduces cancer, blood serum cholesterol, osteoporosis and heart disease (Birt *et al.*, 2004). Also soybeans are a good source of minerals, vitamins, folic acid, and isoflavones which are credited with slow development of many diseases (Wilson, 2004). Scarcity of water resources and growing competition for water in many sectors reduce its availability for irrigation. Effective management of water for crop production in water scarce areas requires efficient approaches. Increasing crop water productivity (WP) and drought tolerance by genetic improvement and physiological regulation may be the means to achieve efficient and effective use of water. But only high water productivity values carry little or no interest if they are not associated with high or acceptable yields (Ali and Talukder, 2008).

Abiotic stresses are responsible for more than 50% yield losses worldwide. Soybean plantation stresses are primarily due to too little (drought) or too much water (water logging/submergence), salinity, nutrient deficiency or toxicity and low or high temperatures (Bray *et al.*, 2000). Drought and salinity are major abiotic stresses that adversely affect soybean production and quality. When plants are subjected to abiotic stress, they activate different physiological, cellular, metabolic and defense mechanisms to survive and sustain growth until maturity (Pathan *et al.* 2007).

Several studies have shown that there is a large variation in soybean contents such as phytosterols, sphingolipids and saponins. More than 100 types of phytosterols have been reported in plant species, but the more abundant are β -sitosterol, campesterol, brassicasterol and stigmasterol (Law, 2000; Berger *et al.*, 2004 and Kritchevsky and Chen, 2005). Phytosterols play major roles in several areas, mainly in

pharmaceuticals (production of therapeutic steroids), nutrition (anti-cholesterol additives in functional foods and anti-cancer properties), and cosmetics (creams, lipstick). Academia has devoted considerable efforts into identifying the role of phytosterols in health, pharmaceutical and food and feed sectors (Fernandes and Cabra, 2007).

The sphingolipids are a family of membrane lipids with important structural roles in the regulation of the fluidity and subdomain structure of the lipid bilayer, especially lipid RAFTS (Dynamic assemblies of cholesterol and sphingolipids in the plasma membrane that are important for amassing signalling complexes or specific aggregations of particular proteins). Many sphingolipid-regulated functions have significant and specific links to various aspects of cancer initiation, progression and response to anticancer treatments (Ogretmen and Hannun, 2004). Saponins from various plant sources are known to exhibit a variety of biological and pharmacological activities. Plant saponins exhibit anti-fungal, antibacterial, anti-inflammatory, anti-oxidant, and anticancer properties (Lin et al. 1996). Soybean saponins, easily accessible dietary saponins among the plant saponins, suppress the growth of colon adenocarcinoma cells *in vitro*. Recent studies suggested that soybean saponins suppress the formation of aflatoxin-DNA adduct *in vitro* (Jeon *et al.*, 1998).

In our study, HPLC was used to separate and quantify bioactive compounds in soybean seeds. The objective of our research was to evaluate the effects of air temperature and soil moisture under controlled green house conditions during soybean seed fill on seed contents of phytosterols, sphingolipids and saponins concentrations within three soybean cultivars. The relationship between different soybean bioactive compounds species and their concentrations were also studied.

MATERIALS AND METHODS

Two French soybean cultivars, Imari and Queen, and US cultivar, Jack, were selected based on similar times of maturity and preliminary

data that indicated differential responses to environmental changes (Lozovaya *et al.* 2005). All entries were grown one plant per 30-cm diameter plastic pot under intermediate night/daytime temperature of 18/28 °C (23 °C mean) with high soil moisture in the plant sciences Laboratory green houses on the campus of the University of Illinois (Urbana-IL). A sand/soil/perlite (1:1:1) mixture was used and plants were fertilized by watering with 20-20-20 (N-P-K; 250 mg Kg⁻¹ each) with micronutrients (plant Marvel laboratories Inc., Chicago Heights, IL). Plants were grown under a 14.5-h photoperiod that was maintained with use of 1000-W m⁻²) high pressure sodium Lamps for lighting (600-W m⁻²). When the plants reached the harvesting (R6) stage, they were moved into high soil moisture (approximately 70% of soil holding capacity) as measured with a Hydrosense Soil Water Measurement System device, SC620 (Spectrum Technologies, Inc., Plainfield, IL)). Soil moisture was measured and adjusted every morning. Maturity date, plant height, seed yield per plant, and 100 seed weight were measured. Phytosterols, sphingolipids and saponins concentrations were determined in mature seed samples from each plant. The experiment was repeated twice.

1. Soybean lipids Extraction and purification:

Two hundred mg soybean powder were extracted by 4 ml mixture of Chloroform –methanol (2:1) and transferred to glass centrifuge tube. 800 µl of water and 200 µl balanced salt solution were added to lipid extract, vortex and centrifuged at 5000 rpm for 5 min. Upper layer was carefully removed and this procedure was repeated twice. The purified extract was filtered through layer of anhydrous Na₂SO₄ on glass filter funnel. Chloroform was removed by using the rotary evaporator to dryness; lipids were dissolved in 400 µl of chloroform and transfer to glass tube.

2. Phytosterols Extraction and Analysis:

Phytosterols were done directly with raw purified soybean and extraction was done by Diethyl ether after saponification by 2 N KOH for 1.5 h at 80°C. Analysis of phytosterols was performed on the HPLC Separation Module 2690 with 960 PDA Detector from Waters (Milford,

MA) supplied with a Hypersil BDS (Alltech, Deerfield, IL), 150 X 4.6 mm, 3 μ m, flow rate 1 ml min⁻¹. Sterols were separated isocratically by methanol-water (1000:1.5). Monitoring of phytosterols was done by PDA detector on wave length 205 nm and authentic standard compounds purchased in the Cromadex (Santa, Anna, CA) (Phillips et al., 2002).

3. Sphingolipids Extraction and Analysis:

Sphingolipids were done directly with raw purified soybean lipids and extracted by Diethyl ether after hydrolyzed by 2 N KOH at 100°C for 1.5 h. The extracted sphingolipids were derivatized with fresh prepared O-phthalaldehyde (OPA) reagent to be analyzed by HPLC. Separation of sphingobase derivatives was carried out on the same system as shown for phytosterols but with different solvents. Solvent A was water (pH 2.8 adjusted by acetic acid) with 5% acetonitrile and solvent B was acetonitrile with 5% water with pH 2.8. Gradient elution was carried out with 85% to 90% B for 10 min. then hold it during 9 min. and increased to 95% for 1 min. Column was flushed with 95% of B during 2 min. and then returned to initial 85% of B. Detection of OPA derivatives of sphingobases with performed with fluorescent detector Waters 470 (Waters) with excitation wave length 340 nm and emission wave length 455 nm (Wang et al., 2006).

4. Saponins Extraction and Analysis:

Two hundred mg of soybean powder was extracted by 2 ml 80% methanol and 1 ml extract was hydrolyzed by 1 ml of 1N HCl in methanol under incubation 6 h at 85°C for purpose to hydrolyze sugar conjugates and release aglucones sapogenols A and B. HPLC separations of sapogenol were carried out in a Separation Module 2690 with 960 PDA Detector from Waters (Milford, MA) supplied with a Hypersil BDS (Alltech, Deerfield, IL), 150 X 4.6 mm, 3 μ m, flow rate 1 ml min⁻¹. Solvent A was acetonitrile-propnol-water-acetic acid (65:6:25:0.1). Elution was carried out with isocratically for 15 min. with solvent A and then solvent B (acetonitrile with 5% water with pH 2.8) increased to 100% in 2 min. and remaining at this level 2 min. Gradient was returned

to 100% for 3 min. the monitoring of saponins was done by UV absorbance at 205 nm. Identification saponin A and B was carried out by comparison of retention times and UV spectra (PDA detector, Waters Corp.) of the eluting peaks and authentic standard compounds purchased in the Cromadex, (Santa, Anna, CA) (Lin et al., 1996).

5. Statistical Analysis

The experiment was planted in a completely randomized design with five replications. The entire experiment was repeated twice. Analysis of variance was used to partition the total variance for all variables measured using PC SAS (SAS Institute)

RESULTS AND DISCUSSION

Seeds of three cultivars were used to investigate the effect of controlled temperature and soil moisture under green house conditions on maturity date, plant height, seed yield and weight, total saponins, total sphingolipids, and total phytosterol, all of which are expressed the degree of seed-quality (Table 1). These cultivars were chosen to make it possible to investigate the effects of temperature and watering on seed-health values. Total saponins, total sphingolipids, and total phytosterol were taken into account because its contents were associated with seed quality. For evaluation of the growth conditions, seeds were grown under normal conditions as control. There was a good relationship between maturity date and total sphingolipids. Jack and Queen, among the latest and earliest had maturing entries, respectively. Jack had the highest concentrations of total Saponins, total sphingolipids, and total phytosterols. Imari had the lowest total Saponins, total sphingolipids, and total phytosterols concentrations but Queen had the lowest total sphingolipids among all cultivars. Each cultivar had specific characters and maintained its level along different analyses. These results were in agreement with the results obtained with the effects of germination and the germination conditions (temperature, light, moisture, and germination

time) on bioactive compounds can vary greatly with the plant species, seed varieties or cultivars (Egli, et al. 2005)

Table (1) Characteristics of soybean (*Glycine max* (L.) Merr.) cultivars under conditions of temperature and soil moisture (average and standard deviation).

Cultivars	Maturity date (d)	Plant height (cm)	Seed yield (g/plant)	100 seed weight	Total saponins nmol/g DW	Total sphingolipids pmol/g DW	Total phytosterols umol/g DW
Imari	49±1.3	89±11.1	28±2.3	18.6±2.0	1619±90	102.6±21.2	5950±409
Jack	59±2.8	143±15.2	42±3.7	12.4±1.4	1967±145	148.2±13.6	6383±565
Queen	45±1.3	161±16.3	36±4.0	16.5±2.7	1784±71	94.4±18.6	6092±600

1. Saponins content of different cultivars under different conditions:

For saponins concentration in the three soybean cultivars, the most marked decrease in case of Imari cultivars comparing with others. Based on percentage change in saponins classes (saponogol A and sapogeniol B), Jack was the most consistent cultivar among other by 46.8% comparing to control but Imari and Queen were 56.9% and 73.9%, respectively (Figure 1). In contrast, no significant changes of total saponins were found in the all cultivars. Also, under different conditions except the low sever conditions; there were no significant differences among three cultivars. On basis of sapogenols, the sapogenols B of Jack were recorded the highest among both French cultivars with significant differences ($p < 0.05$) but there were no significant differences on basis of sapogenols A under all different conditions (Table 2). These results were in agreement with the results obtained in the field at 23°C in comparison to higher or lower temperatures (Wang et al., 2008). Saponins are glycosylated compounds, classified as either triterpenoids, steroids, steroidal glycoalkaloids. A biologically active triterpenoid saponins performed plant defenses secondary metabolites (Buchanan et al. 2000).

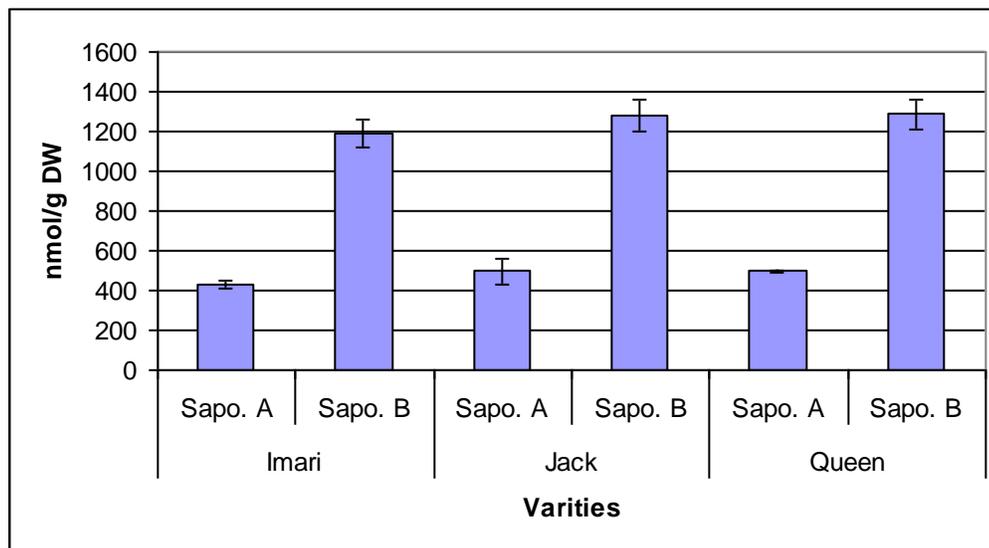


Figure (1) Total Saponins analysis in average with standard errors of three soybean cultivars under green house conditions.

Saponins are glycoside compounds composed of a fat-soluble nucleus (aglycone) that is a triterpenoid (C-30) as in soybean. One or more side-chains of water-soluble sugars (glycone) are attached through ester linkages to the aglycone nucleus at different carbon sites. Saponins have several biological effects, among them antibacterial and hemolytic activities. Not all saponins have the same biological activities, while some saponins are beneficial, others are considered harmful to animal performance (Hassan et al. 2010).

2. Phytosterols content of different cultivars under different conditions:

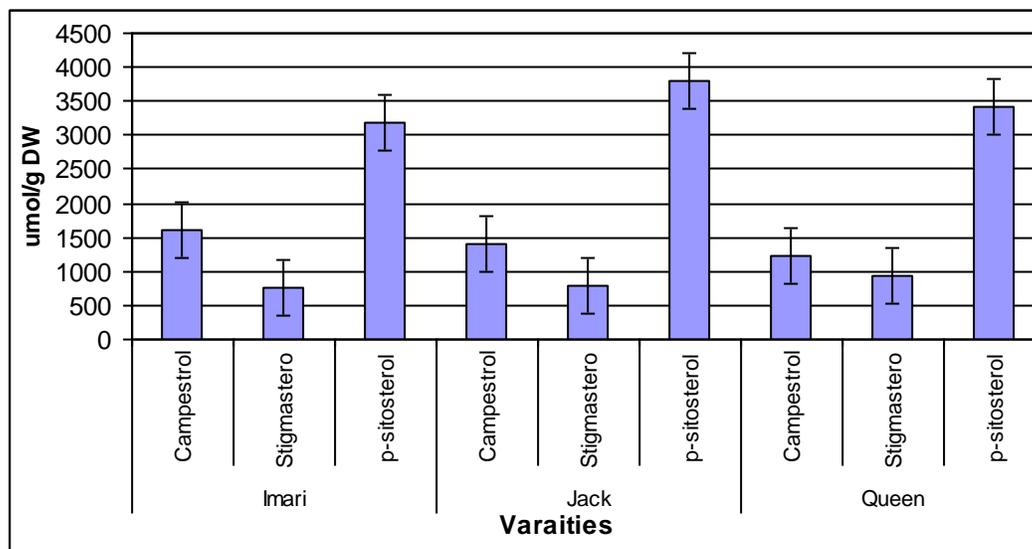
On basis of total phytosterol, the cultivars were showed no significantly differences with slight increase in case of Jack cultivar. Based on mean responses for phytosterol sub-classes (Campesterol, Stigmasterol and β -Sitosterol), Jack produced the most consistent changes to temperature and soil moisture. However, Jack showed changes from

6383 $\mu\text{mol g}^{-1}$ of total phytosterol to 7319 $\mu\text{mol g}^{-1}$ under controlled conditions. Based on percentage change, Queen and Imari were the most constant cultivars under different conditions by 130.7% and 138.9%, respectively but the most changeable cultivar was Jack by 171.1%. In case of Imari Cultivar, the only change that happened for all phytosterol subclasses was β -sitosterol and there was slight increase. Contrary, there was slight non-significant decreasing in β -sitosterol contents with normal soil moisture as shown in Figure (2).

In case of Jack Cultivar, the only change that happened for all phytosterol sub-classes was campesterol and β -sitosterol. There were significant increases under green house conditions. But there were non-significant differences for other subclasses under different conditions. In case of Queen Cultivar, the only change that happened for all phytosterol subclasses was β -sitosterol. There was slight increase under normal temperature. Contrary, there were significant decreases in β -sitosterol comparing to other phytosterols subclasses.

Phytosterols are triterpenes similar to cholesterol, both in structure, given the four-ring steroid nucleus, the 3 hydroxyl groups and often a 5,6-double bond, as in function, given their role in the stabilization of the phospholipids bilayers in cell membranes. However, cholesterol has a side-chain composed of eight carbon atoms, whereas more common phytosterols have a side-chain composed of 9 or 10 carbon atoms, out of a total of 28 or 29 carbon atoms. The alkyl side chain may also contain a double bond. Sterols in plants exist in the form of free alcohols, fatty-acid esters, steryl glycosides and acylated steryl glycosides (Moreau *et al.*, 2002; Phillips *et al.*, 2002). Free sterols control the membrane fluidity and permeability. They probably play a role in the adaptation of membranes to temperature, whereas sterylestere are located intracellularly and are mainly a storage form of sterols (Piironen *et al.*, 2000).

Figure (2) Phytosterol species analysis in average with standard errors of three soybean cultivars under green house conditions.



3. Sphingolipids content of different cultivars under different conditions:

Sphingolipids (SL) levels had changed more than what happened for other two compounds (saponins and phytosterols). On base of green house conditions, the Jack produced the highest levels of sphingolipids. The Jack levels were significantly higher than both Imari and Queen. Based on percentage change for total sphingobases , Queen was the most consistent cultivar and less sensitive to different conditions by 55.6% but Jack and Imari were recorded percent of change by 65.0% and 85.9% respectively, as shown in Figure (3). However, Imari had significant production of sphingolipids from 205 $\mu\text{mol g}^{-1}$ to 298 $\mu\text{mol g}^{-1}$. On the same trend, Jack showed changes from 296 $\mu\text{mol g}^{-1}$ of total sphingolipids to 379 $\mu\text{mol g}^{-1}$ under normal soil moisture levels.

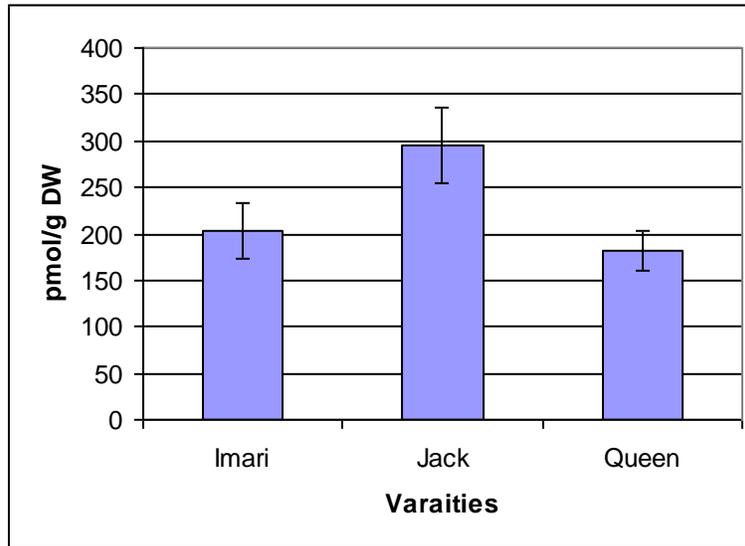


Figure (3) Sphingolipids analysis in average with standard errors of three soybean cultivars under Green house conditions.

Sphingolipids are ubiquitous components of cell membranes that play critical roles in many physiological processes including cell recognition, adhesion and signaling (Merrill and Sandhoff, 2002). Merrill et al. (1988) studied the influence of extracellular precursors on the formation of sphingoid bases (SB) and reported that a high concentration of free palmitic acid in cell culture enhanced the long-chain SB biosynthesis. On the basis of the results reported it is possible that SL content may be positively correlated with the palmitate content of soybean seeds. Wang et al. (2006) reported that the HPLC methods can be effectively used to quantify GlcCer and Cer (TA), as well as certain glycolipids in soybeans, without any chemical treatment of the extracted lipid. SL contents were significantly different among soybean lines that differed in palmitate content. No significant correlations were observed between SL and palmitate contents among the 15 soybean lines. A

significant and positive association ($r= 0.70$) was observed between GlcCer and Cer contents.

Acknowledgements

This study was supported by a grant received from Dr. Jack M. Widholm, Crop Science Department, University of Illinois at Urbana-Champaign (UIUC), USA. My sincere thanks to all members of the laboratory staffs who gave me a helping hand towards getting this work accomplished.

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الملخص العربي

دراسة أثر ظروف الصوب الزجاجية على تكوين محتويات بذور فول الصويا من مواد السابونين ودهون الأسفنجو والفيسترولس التراكيب والتركيزات

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تتضمن هذه الدراسة تأثير ظروف الصوب الزجاجية (درجة الحرارة ورطوبة التربة) في بذور فول الصويا [ماكس جليكاين (لام) Merr]. من حيث تركيزات المركبات النشطة بيولوجيا. وتضمنت الدراسة تحليل المركبات النشطة بيولوجيا وخواص النباتات. كانت هناك علاقة جيدة بين فترة النضوج ومادة دهون الأسفنجو الإجمالي. وكان الصنفان جاك وكويين لهما أحدث وأقرب فترات النضج على التوالي. وكان الصنف جاك له أعلى تركيزات مجموع السابونين الكلي ودهون الأسفنجو الكلي ، ومجموع الفيتوسترولس. وكان الصنف أمرى له أدنى تركيزات مجموع السابونين ومجموع الفيتوسترولس. لكن الصنف كويين كانت له أدنى مجموع دهون الأسفنجو بين جميع الأصناف. وكان لكل صنف ما يميزه من تركيزات خاصة به والحفاظ على مستواه على طول التحليلات المختلفة. تحت ظروف خاضعة للرقابة في الصوب الزجاجية كانت التغييرت في مستويات مادة دهون الأسفنجو أكثر من مادتي السابونين والفيسترولس.