UNIVERSITY OF PADOVA

DOCTORAL THESIS

DOCTORAL SCHOOL OF CROP SCIENCES

CURRICULUM OF ENVIRONMENTAL AGRONOMY, CYCLE XXII

Department of Environmental Agronomy and Crop Sciences

Isoflavone Accumulation in Soybean Cotyledon and Hypocotyl

Head of the School : Prof.   Andrea Battisti _____

Supervisor : Prof.   Giuliano Mosca _____

Doctoral student :   Mohammed Hewidy Mahmoud Ramadan

_____

THESIS SUBMISSION DATE

February 1st 2010

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

(_____/ Mohammed Hewidy Mahmoud Ramadan /date)

A copy of the thesis will be available at *http://paduaresearch.cab.unipd.it/*

<u>Dedication</u>

I dedicate that work for the most important person of my life ''my mother''. Although of her absence I still feel her presence with me. As well I dedicate this work for my family and my wife whom support me in all my steps.

<u>Acknowledgement</u>

Mohammed Hewidy

_____

List of Contents

List of Figures

List of Tables

Abstract

Isoflavone has demonstrated many health benefits due to its weak estrogenic effect. Different isoflavone forms of soybean have its own effect on human health or in plant system. Five different experiments were carried out in order to arrive to most effective factor on isoflavone contents. First was to study 13 varieties under organic and conventional management for 4 years (2005, 06, 07, 08). Second was irrigation and nitrogen application at (R1 in 2006 and R3 in 2007) effect on two varieties (*Ales* and *Nikir*). Third was to study the effect of irrigation level on 3 varieties. Fourth was to study the plant density. Fifth was to study the effect of soil type. Isoflavone content was evaluated in both seed organs cotyledon and embryo axis (hypocotyl).

In these studies the two major factors affecting the isoflavone contents were cultivar and environment. Conventional management showed high significant value on cotyledon isoflavone but did not influence hypocotyl. Hypocotyl has its own metabolic and physiological controls which can be one of the causes of some genotype by environment interaction that was observed. Hypocotyl is considered a poor source of total isoflavone 10-20% in compared with cotyledon 80-90%. Cotyledon isoflavone of single cultivar may vary up to 100% within years but may show up to 50% between management. However, hypocotyl may vary up to 20% within year and up to 10% between management. Cotyledon contains 30-50% daidzein and 50-70% genistein whereas hypocotyl contains 30-50% daidzein, 15-20% genistein, and 30-50% glycitein depend on the variety. Many varieties showed similarity in their profiles regardless the contents of each variety, which mean similarity of metabolic and the difference only preserved in quantity of expression or presence of gene silence. Maximum and daily range temperature were considered the most effective environment factor in our study. It was notable that varieties were maintaining their ranks among years and both management conventional and organic.

Ales and *Nikir* were different in their response for irrigation in 2007, *Ales* showed positive accumulation under water supply whereas *Nikir* showed the contrast which may reveal dependency of water effect on variety. Nitrogen application effect on cotyledon isoflavone contents varied regarding to year of application. Late N application (R3, 2006) caused negative effect on isoflavone accumulation but early (R1, 2007) showed significant

increment compared with none fertilized in both variety. Lateral shoot showed stable high significant value about 20% for both seed organs compared with principal shoot.

In 2008 level of water supply did not reveal significant effect on isoflavone contents in cotyledon and hypocotyl. Lateral shoot showed 20% greater isoflavone contents for cotyledon and 5% in hypocotyl compared with main shoot.

Plant density did not affect isoflavone contents but low density enhance ramification and consequently yield components (n° pod, pod weight and seed dry weight per plant) about 10%. Under both plant density cotyledon and hypocotyl showed higher isoflavone contents on lateral than principal shoot.

Soil type showed to be an effective factor for accumulation of isoflavone due to their properties. Cotyledon total isoflavone showed negative correlation with C:N ratio whereas hypocotyl showed slight difference without significance.

Interaction is more relevant on cotyledon due to its sensitivity and longer accumulation period of isoflavone compared with hypocotyl.

Concluding the relevance of effects on isoflavone of different factors was in order of importance, Variety, seed organs, Environment, pod position on the plant and agronomic practices.

Isoflavone contents has showed difference under certain conditions, either positive or negative regardless if that conditions were stress factor or favourable conditions for growth which mainly depend on the variety. Variety interaction with environment may result 100% difference in isoflavone contents whereas other factors may show difference up to 50%. High and low isoflavone varieties can be considered stable in their ranks and they could be recommended to certain line of production either for nutraceutical or infants food production.

Riassunto

In numerose esperienze gli isoflavoni prodotti da soia hanno dimostrato di avere un buon risultato sulla salute dell'uomo grazie al loro effetto come deboli estrogeni ed hanno manifestato di svolgere diversi ruoli nella pianta. Cinque esperimenti sono stati realizzati al fine di individuare i principali fattori che influiscono sul contenuto degli isoflavoni. Il primo era finalizzato allo studio comparativo tra 13 varietà sottoposte a due tipi di gestione agronomica (metodo biologico vs convenzionale) in 4 anni di coltivazione (2005-08). Il secondo è stato programmato per esaminare l'effetto dell'irrigazione e della concimazione azotata complementare, applicata in fase tardiva (R1-3) su due varietà di soia (*Ales* e *Nikir*). Il terzo è stato impostato per studiare l'effetto della sola irrigazione in varietà di soia di diversa precocità. Il quarto invece è stato realizzato allo scopo di studiare l'effetto della densità di piante adottando due tipi di *management* (metodo biologico vs convenzionale) in 4 varietà. L'ultimo esperimento aveva per obiettivo lo studio dell'effetto di diversi tipi di terreno sulla medesima varietà. Il contenuto degli isoflavoni è stata stimato usando separatamente la metodica HPLC sia per i cotiledoni che nel germe.

I fattori dimostratisi più efficaci sono stati la "varietà" e l' "ambiente". Il metodo di coltivazione convenzionale ha mostrato una maggiore capacità di concentrare gli isoflavoni a livello dei cotiledoni, mentre non ha influenzato il germe. Le due strutture seminali hanno dimostrato di possedere un'attività metabolica differenziata che potrà essere considerata uno dei caratteri principali di un genotipo nell'ambito dell'interazione con l' ambiente. Il germe è da considerare una struttura di peso relativo ma ricca di isoflavoni percui il risultato finale oscilla attorno al 20% della produzione totale di isoflavoni. In confronto il cotiledone rappresenta invece l' 80%. I cotiledoni di alcune varietà rispetto ad altre possono variare il loro contenuto anche del 100%, mentre la differenza tra i metodi di coltivazione può dar luogo ad una differenza in termini di isoflavoni al massimo fino del 50%. Sebbene l'ipocotile possa far variare gli isoflavoni fino al 20% tra gli anni e al 10% fra i *management*. Le varietà di soia si caratterizzano anche per il diverso profilo degli isoflavoni. Il cotiledone contiene, in forma glucosilica, il 30-50% di daidzina ed il 50-70% di genistina mentre l'ipocotile contine il 30-50% di daidzeina, il 15-20% genisteina e il 30-50% glyciteina. Varietà diverse hanno mostrato di possedere profili simili, con diverse concentrazioni. Una similarità di profilo in alcune varietà riflette la differente espressione

del gene o la presenza di geni silenti. La temperatura massima e il range giornaliero erano i fattori ambientali più correlati con il contenuto di isoflavoni totali presenti nel cotiledone, il germe invece non ha mostrato alcuna relazione con i fattori ambientali. E' stato osservato che le varietà mantengono il loro ranking di posizione negli anni e tra i metodi di coltivazione.

Per le varietà *Ales* e *Nikir* è stata accertata una diversa risposta all'irrigazione. *Ales* ha reagito positivamente all'acqua al contrario di *Nikir* che ha visto il suo incremento di isoflavoni essre assai modesto. Ciò ha rivelato che l'effetto dell'irrigazione dipende dalla varietà. L'applicazione dell'azoto in forma complementare e tardiva sul contenuto totale di isoflavoni dei cotiledoni ha mostrato diversi effetti in relazione all'epoca di applicazione mentre ancora una volta il germe non ha subito variazioni statisticamente apprezzabili.

Le ramificazioni in confronto al fusto principale hanno mostrato un capacità produttiva di isoflavoni maggiore del 20% nel cotiledone e del 5% nell'ipocotile.

La densità di semina non ha sortito alcun effetto sul contenuto di isoflavoni, però la densità bassa ha stimolato la ramificazione e di conseguenza le componenti della resa sui laterali (n° baccelli, peso del baccelli e il peso secco del semi per piante circa il 10%). Le due densità a confronto, sia nel cotiledone che nel germe hanno presentato un elevato contenuto di isoflavoni nei baccelli portati sul fusto laterale rispetto al principali.

Tipo di terreno de essere un fattore effettivo sul accumulo del isoflavone a cause di il loro caratteristica. Totali del isoflavone nel cotiledone mostrato relazione negativa con C:N rapporto mentre il ipocotile ha mostrato piccolo differenza senza significatività.

Interazione più rilevante in cotiledone a cause del lungo periodo di accumulo di isoflavone in confronto con ipocotile.

Concludendo in ordine di rilevanza dell'effetto vanno citati nell'ordine la varietà, la posizione nel seme, l'ambiente, la posizione del baccello sulla pianta, il metodo di gestione agronomica.

Il contenuto d'isoflavoni ha presentato delle differenze per effetto di qualche condizione, sia positiva sia negativa senza riguardo se queste condizioni erano state di stress o favorevoli per la crescita della soia. Comunque, questo effetto dipende sopratutto

dalla varietà. L'interazione "varietà x ambiente" può dar luogo a differenze anche del 100% mentre altri fattori hanno dimostrato di far variare gli isoflavoni solo fino al 50%. Varietà ad elevato e a basso contenuto di isoflavoni possono essere considerate stabili nel loro ranking e potranno essere utilizzate nella produzione per finalità nutraceutica o cibo per l'infanzia.

Literature Review

0 Literature Review

Soybean recently solve problem of lakeness of protein source in most poor countries or insufficiency of animal protein source. A soybean secondary metabolic component is isoflavone, considered one of the recent research objects for cancer disease.

0.1 Botanic classification, origin, and distribution

Soybean or *Glycine max* is thought to be derived from *G. ussuriensis* Regel & Maack, a slender, prostrate, twining legume, which is found wild throughout eastern Asia, possibly in hybridization with *G. tomentosa* Benth, which grows wild in southern China. *G. gracilis* Skvortzor is considered an intermediate semi-cultivated species between the wild *G. ussuriensis* and *G. max*. Soybean was cultivated in China before the times of written records. They have also been an important food crop in Manchuria, Korea and Japan since the earliest times. Soybean was introduced into the Jardin des Plants, Paris, in 1740, and the Royal Botanic Gardens, Kew, in 1790, but little interest was shown in the crop in Europe until the first shipments of soybeans were made in 1908. Soybean was first taken to the United States in 1804, but there was little commercial production until the 20th century, first mainly as a forage and pasture crop, but with increasing use of the seed since 1903 and the most rapid increase since 1942, in response to the wartime demand for edible oils and fats.

0.2 Soybean morphology

Growth of soybean varieties can be either determined (short cultivars) or undetermined (tall cultivars). Soybean growing cycle divided into main stages vegetative and reproductive stages (Fehr and Caviness, 1977).

Soybean true leaves are formed on main stem after emergence of cotyledon appears 10 days after sowing then start appear 1st trifoliate leave and that phase called V_1 and so on for the rest of leaves till V_n. Most of varieties have green leaves turned into yellow near to maturity. Generally leaves dropped before maturity of pods.

Flowers are typical to leguminous family, they are red, white or violet, small, grouped in racemes forming about 2-35 flowers posted on short peduncles. Flowers start from basal nodes going upward of apical meristem. Soybean plants produce very high number of flowers than pods. Abortion may arrive to 20-80% of total number of initiated flowers. This phenomenon is more evident with cultivars that have much flower per node. Flowers dropped after 1-7 days of formation whereas flowering period depend on seeding rate, environment and variety from 3 to more than 5 weeks.

Pods are small, (direct; slightly curved or curved), long hair and they tend to open when arrive to maturity. Colour of pods at maturity varied from yellow to grades of gray, brown till near to black. Commercial cultivars contain 2-3 seeds pod^{-1}. Each inflorescence may carry 1-2 pods to more than 20 pods. Pod length varied from less than 2 cm up to 7 cm, in some cultivars pods reach its maximum dimension in about 30 days but it takes 40 days to arrive to its maximum growth weight.

Seeds are similar to most of leguminous crops, principally composed of endosperm and consist of seed cover which surround big well developed embryo with two cotyledons. Seed shape varied from sphere to strongly plate and elongate but most of cases are sphere or oval. Colour of seed can be yellow, dark yellow, yellow-gray, green, brown or black or combination of these colours. Seed-coat marked with hilum, the place where was connected inside the pod, which is varied in shape also from linear to oval shape. Grey or yellow seed-coat stained with irregular brown or black. The dimension of seed is a fluctuate variable which the weight of 1000 seed can be 50-450 g. In general seed with big size used for human alimentation. After sowing seeds imbedded very fast depending on soil moisture but there are some genotypes (especially wild species) have hard seed-coat consequently prevent water absorption. In adequate condition of humidity and temperature the root grow-out the seed-coat in two days. Its growth in soil is rapid and arrive to 2-3 cm forming the first ramification of root the cotyledon come up of the soil in 5 -10 days because of the hypocotyl elongation.

0.3 Soybean growth

Germination of soybean needs high soil humidity more than maize and demand climate condition with minimum growth temperature of 4-0 °C. Median temperature of mid

summer 24-25 °C is the optimum temperature for all varieties. Low temperature can cause delay in flowering. In typical zone of production sum units of temperature is 3500-4500 °C in 5 month to arrive to maturity but other condition it does need lower thermal units.

Soybean is less sensitive than maize to decrease of temperature in phase of plantlet with in phase of maturity. The soybean is short season crop with sensitively to photoperiod especially with some varieties which needs 10 hour of dark per day to flower whereas artificial photoperiod of 8 hour/day, all varieties flower very fast in the same time. In normal conditions early cultivars are flowering very early than late one.

Conner *et al*. (2004) mentioned soybeans are the primary source of the world's supply of protein and vegetable oil. The demand for increased production of soybeans is forecasted to mirror the world's population growth and demand for protein and edible oil. In order to meet this demand, production acreages are increasing in key global soybean areas; moreover, technologies to increase production efficiency through transgenic trait control of yield-robbing pests and pathogens, while lowering the cost and use of insecticide and other less efficient agricultural practices, are very much a reality. In addition, the increase in more efficient and more sustainable agronomic practices will help fuel some key improvements in soybean quality and new opportunities for agricultural solutions resulting in feed improvements, health benefits through foods, and new industrial opportunities contributing to a more sustainable global environment.

0.3.1 Environment and soybean growth

Soybeans are grown in many parts of the world and are a primary source of vegetable oil and protein for use in food, feed, and industrial applications (Endres, 1992, 2001). Soybean development can be divided into two stages—vegetative and reproductive (Fehr and Caviness, 1977). Soybean plants are sensitive to photoperiod, meaning that the transition from the vegetative stage to the reproductive stage depends directly on day length. The critical photoperiod (length of the vegetative period) of soybean varieties increases progressively with higher latitude. Flowering and maturity occurs later, and plants may even fail to reach full maturity before the onset of first frosts. When grown at lower latitudes, the same varieties will flower earlier, have a smaller vegetative mass, and mature earlier, resulting in lower yield (Lima *et al.*, 2000). Photoperiod requirements,

therefore, limit the geographic distribution of a variety to a narrow belt of latitude (around 200 km, Scott and Aldrich, 1983) to which a variety has been adapted. For every soybean-growing are a, therefore, there is an optimum maturity group. Varieties that are one maturity group earlier than the optimum are too early for the area concerned, and vice versa, those that are one group later are too late.

The growth period of the crop, as well as the pod number, seed number and seed weight decreased, whereas plant height and the time to initial pod stage increased with delay in sowing. Protein and soluble sugar content increased, whereas starch content decreased with delay in sowing (Ning *et al.*, 2005).

Soybean phenology is hard to predict, because it depends on the photoperiod (Garner and Allard, 1930; Borthwick and Parker, 1939; Seddigh *et al.*, 1989) and temperature (Egli and Wardlaw, 1980; Board and Hall, 1984) as well as the amount of water available to the plant (Meckel *et al.*, 1984; Blanchet *et al.*, 1989). Even though Miladinovic *et al.* (2006) mentioned that there is single variety may have wider adaptability than previously thought possible. They concluded importance of that for at least two reasons. First, it was proved that germplasm exchange and common research programs between two institutions at distant parts of the globe make sense even for a photoperiodically very sensitive crop such as soybean. This could facilitate widening of the very narrow genetic variability of soybean which is of great importance for soybean improvement. Second, our findings also open the possibility for commercialisation of soybean varieties from geographically very distant areas such as Europe and Japan.

0.3.2 Variety of soybean and factors affect yield

In two distant locations soybean cultivars showed difference in yield due to an interaction between environment and variety also higher rainfall affect the plant height and seed weight (Toniolo and Mosca, 1981; Mosca *et al.*, 1983; Miladinovic *et al.*, 2006). Ning *et al.* (2006) mentioned increasing crop density increased plant height, but decreased stem thickness, number of nodes, number of branches, branch length, pods per plant, seeds per plant and dry matter accumulation. Crop density had no significant effect on 100 seed weight and quality of vegetable soybean.

The longer growing seasons result in higher potential crop production, defined by the insolation available when temperatures are suitable for plant growth (de Wit, 1967). Assuming there is no water limitation; biomass production is the product of the solar radiation over the duration of the crop period (Richards, 2000). So, a longer crop period means greater biomass, and according to Rao *et al.* (2002), biomass is an important determinant of seed yield.

Truong *et al.*, (2006) demonstrated days to flowering (DTF) and days to maturity (DTM) had close correlation association with agronomic traits as well as yield and yield components. Both DTF and DTM had positive correlation with the other characters except one hundred seed weight. Stepwise multiple linear regression revealed that seed and pod number were identified as being significant for plant yield. The results in this study indicated wide variation in agronomic traits including DTF and DTM, suggesting the valuable genetic resources in a soybean breeding program.

Sustainability index (SI) one of the major parameter which judged on any cultivars which can be defined on the basis of their potentiality to produce the maximum and consistent yield over the years in the zone (Majumder *et al.*, 2006).

The attachment of *rhizobium* on the root of soy bean related with culture age, inoculums size and pH (Albareda *et al.*, 2006).

Comparing between the shoot and root growth in sort S:R ratio has an pronounced indicator to root system growth in comparison to shoot growth and estimated from that the efficiency of each part for different families. Soybean S:R ratio estimated 5.2 which moderate value among the mean of S:R ratios for annual crops were typically about 5, though values ranged from 1.1 to 10.7 (Bolinder *et al.*, 2007). The efficiency of soybean on fixing CO_2 can be estimate from the result of Bolinder *et al.* (2007) that was also moderate among different examined crops (grain and cereal).

Phosphate rock (PR, 34/74) is an effective source of phosphorus in soybean production, if applied either with farmyard manure or phosphate solubilising bacteria (Kavitha and Veeraraghavaiah, 2004).Soybeans were effective in reducing soil resistance in no-till plots while corn was the opposite (Anders *et al.*, 2005).

In addition to agronomic traits, to enhance production and consistency of production of soybean grain, the quality-improvement traits to deployment potential. Conventional breeding and biotechnology-derived quality traits that may build off of enhancements in production and consistency of production for soybeans are higher oil, higher protein, modifications in essential amino acids and protein compositional changes, changes in oil composition, nutritional traits such as vitamins, and new industrial uses including biodiesel, bio-lubricants, and polymers (Conner *et al*., 2004).

0.3.3 Maturity group (MG)

Popp *et al*., (2003) choosing of MG should be related to the yield since irrigation had no effect on yield of early MG, furthermore the later maturing variety did not offer larger yield change in optimal seeding rates over time than earlier maturing varieties.

Short season cultivars aid in effectively allocation labor, equipment and irrigation water (Casey *et al*., 1998), which is MG III and IV. Also help to avoid problem of drought conditions which may happen irregularly among the years (Edwards *et al*., 2003; Edwards and Purcell 2005), but Popp *et al*. (2004) mentioned that MG IV may be under competitive of mid season drought so the selection could be depend on the yield. In order to optimize the overall return of the farm Popp *et al*. (2003) suggested growing range of relative maturity soybean.

0.4 Soybean N-fixation

N is often the most limiting nutrient in organic cropping systems where no synthetic mineral N fertilizers are applied and where total N inputs are usually lower than in conventional systems (Hansen *et al*., 2000). Therefore, N_2 fixing crops present an important option to improve N supply and to maintain soil fertility (Stockdale *et al*., 2001). Organic farms have on average more legumes in rotation (Kirchmann and Bergstrom, 2001), and estimates on N_2 fixation from the atmosphere at the farm level are greater for organic than conventional systems (Hansen *et al*., 2000). Previous researches has demonstrated that during period of drought stress N_2 fixation is the first to decrease compared to other physiological process (Durand *et al*., 1987; Sinclair *et al*., 1987; Sall and Sinclair, 1991), Which is in relation to the weakest and unreproductive part in the plant which easily to scarify rather than to affect reproductive process. Unfavourable climate

conditions have the probability to reduce the ability of germination or plant reproductively. Proponents of starter fertilizer believe that a small amount of fertilizer placed near the seed can increase soybean growth early in the season when unfavourable environmental conditions exist (Sij *et al.*, 1979). Unfavourable conditions, such as cool climates or no-till soil management, can lead to excessively cool soil temperatures thus delaying N fixation (Hardy *et al.*, 1971). Osborne and Riedell (2006) confirmed the demonstration of Scharf and Wiebold, (2003) on high yielding performance needs soil NO_3-N lower than 85 kg ha^{-1}. Non-conclusive results under different environmental conditions for soybean quality measurements and N fertilizer were obtained (Bona et al., 1991 a, b; Wesley *et al.*, 1998; Barker and Sawyer, 2005; Osborn and Riedell, 2006). High-soil N mineralization and/or low-soil P availability may have limited symbiotic N_2 fixation (Oberson *et al.*, 2007).

0.4.1 N-fertilization

Using N fertilisation targeted maximum yield since the ability of soybean to fix atmospheric N is not adequate for that (Weber, 1966; Wesley, *et al.*, 1998). Response of soybean to N fertilizer application depends mainly on soil temperature, moisture and pH (Sorensen and Penas, 1978). Hardy *et al.* (1971) determined 14 days after planting the beginning of N fixation when the plant were grown under optimum moisture and temperatures conditions, thus a small amount of N at planting could be beneficial to early growth. Bergersen (1958) concluded N applied before planting could be beneficial to soybean nodules were not present until at least 9 days from emergence. Sej *et al.* (1979) and Terman (1977) agreed on N application at planting may stimulate early vegetative growth but with out significant effect on LAI, plant height, shoot fresh weight or yield. There were theories that yield increases occurred in areas where soil and/or weather conditions limit soil moisture, reducing early vegetative growth and soil nutrient availability (Haq and Mallarino, 2000).

Additional research conducted in Missouri found that out of 48 sites a positive response to N was likely if the following conditions were present: (i) yield levels above 4000 kg ha^{-1}, (ii) fertilizer application at the beginning pod growth stage, (iii) residual soil nitrate less than 85 kg ha^{-1}, (iv) soil pH less than 7.5, and (v) irrigation (Scharf and Wiebold, 2003).

Soybean grown in high yielding environments with yield potentials greater than 3500 kg ha^{-1} had a positive response to N when applied at early flowering, compared to early pod-fill (Flannery, 1986; Wesley *et al.*, 1998). Brevedan *et al.* (1978) reported a 28 to 33% yield increase if N was applied between initial bloom (R1) to the end of bloom (R3) for soybean grown in a greenhouse. Wesley *et al.* (1999) found that application of N at the beginning pod growth stage increased yield at four irrigated sites, but had no effect on grain protein or oil concentration. Broadcast N application increased soil yield, seed weight and protein, but had no effect on seed oil concentration (Ham *et al.*, 1975).

0.4.2 Nitrogen fertilization and soybean yield and quality

Although nitrogen fertilization is not common practice there is speculation that the ability of soybean to fix atmospheric N is not always adequate for maximum yield. In most of cases full-season soybeans can be irrigated during the reproductive period and obtain the same yield response as complete-season irrigation (Grissom *et al.*, 1955; Ashley and Ethridge, 1978; Bona *et al.*, 1991 a, b). Drought in soybean decreases yield-related processes and N$_2$ fixation is more sensitive to drought than many other of these processes. Therefore, application of nitrogen (N) fertilizer may increase drought tolerance over those plants primarily dependent on N$_2$ fixation (Purcell and King, 1996). Producers desire maximum yield from irrigated plantings on soils with low residual soil nitrogen, which consider applying N at beginning pod set to ensure nitrogen deficiency does not limit yields (Heatherly, 1999; Bona et al., 1996). It is not possible to predict soybean response to N fertilizer based on soil properties. However, situations with positive responses generally have either very low residual soil nitrogen, low nitrogen mineralization capability, or soil pH so low that inhibits nodulation and nitrogen fixation (Heatherly, 2006). Brevedan *et al.* (1978) reported a 28 to 33% yield increase if N was applied between initial bloom (R1) to the end of bloom (R3) for soybean grown in a greenhouse. Combining irrigation and nitrogen application is desired to maximize yield (Bellaloui and Mengistu 2008).

0.5 Soybean products

Soybean enters in many food staff and manufactured product. Product composition of isoflavone is depending on raw material. Isoflavone levels ranged from 1 μg/g in *soy sauces* to 540 μg/g in *Tempeh*. *Soymilk* and *tofu* represented the major portion of soy foods

evaluated (Murphy *et al*., 1999). The major sources of phytoestrogens in human diets are isoflavones in soybeans and soy foods. The estrogenic isoflavones found in soy are genistein, daidzein, and glycitein predominately as their glucosides and malonylglucosides but also as acetylglucosides and aglycons (Wang and Murphy, 1994 a, b). The distribution of the isoflavone yields a picture of the processing history of a particular soy product (Wang and Murphy, 1996).

0.6   Protein and oil production

Protein quantity and quality can be increase in soybean without sacrificing agronomic performance. High protein cultivar >45% are currently available but its yield would not equal to highest yielding cultivars now in production. Development of high yielding, and high protein will require a long-term (Burton, 1984) breeding effort. Production of soybean was always connected with increment of world population.

- Protein utilization is varied due to its massive quantity in seed which may arrive 44 to 52% using near infrared spectrophotometer. Soybean bean meal uses increased occur through consumption of processed meat and eggs. A high-quality protein is used in animal feed which enhance milk and meat production. Other animal and pet foods can include soy protein to enhance its content of protein instead of using meat sources. Soy flour can be used to enhance protein content in breads and breads-like production equal to 10-13% by replacing 5% of wheat flour.

- Oil utilization for human purpose is well known since long time. About 95% of the soybean oil produced finds its way into food application. There are several industrial uses for the oil and/or the fatty acids derived from soybean. These fatty acids may arise from acidulation of the soap stock that is removed in the process that separates the natural, free fatty acids from the crude extracted oil. Other fatty acids are produced by intentional hydrolysis of triglyceride oil.

0.7   Soybean nutritional value

Soybean (*Glycine max* L. Merr.) is the world's most widely grown grain legume. It combines in one crop both the major supply of vegetable oil and protein, with a variety of uses in human food and animal feeds. Soybean also contains eight essential amino acids

that are crucial for human nutrition and are not made naturally in the body (Carpenter *et al*., 2002). Soybean seed has a unique chemical composition that makes it a valuable industrial and agricultural commodity. It contains 40% of the seed dry weight as proteins. Soybean seeds are a rich source of isoflavonoids that are associated with many health benefits (Dixon and Ferreira, 2002).

Nutritional value of soyfoods and soy dietary supplements are directly related to those found in the raw soybean that they are derived from, and can be highly variable. Messina (1999) has made overview on nutritional value of soybean and other legumes. A serving 90 g or ½ cup cooked soybean can provide 14.3-38% and 7.7-47% energy coming from protein and fat respectively. Compared to other legumes, soybean has the lowest content of riboflavin, folate and fibers but it is a good source for macro- and micro-nutrients.

Isoflavone aglycones are contained in *miso*, *natto* and *soy sauce*, Japanese traditional fermented soy foods (Wang and Murphy, 1994a).

Messina et al. (2006) indicated that

a- older Japanese adults consume approximately 6-11 g of soy protein and 25-50 mg of isoflavones (expressed as aglycone equivalents) per day;

b- Intake in Hong Kong and Singapore is lower than in Japan, whereas significant regional intake differences exist for China;

c- Evidence suggests that ≤10% of the Asian population consumes as much as 25 g of soy protein or 100 mg of isoflavones per day.

0.8   Isoflavone

0.8.1   Isoflavone importance for human health

Isoflavones are photochemical synthesized in most phenomenon vegetarian protein family member, soybean. The interest in presence of isoflavone has been growing since late 90s, as epidemiological an clinical studies have suggested that the consumption of soyfoods associated with many health benefits, such as lowered risks for beast, prostate and clone cancers (Birt *et al*., 2001; Dai *et al*. 2002; Sarkar and Li, 2003). Isoflavonoids are primarily

limited to leguminous crops. Aglyconic form is the effective form of isoflavones which is liberate from glucoside or malonyl by the microflora present in the gut (Figure 1), which may transform daidzein into equol (Akintson *et al*., 2005).
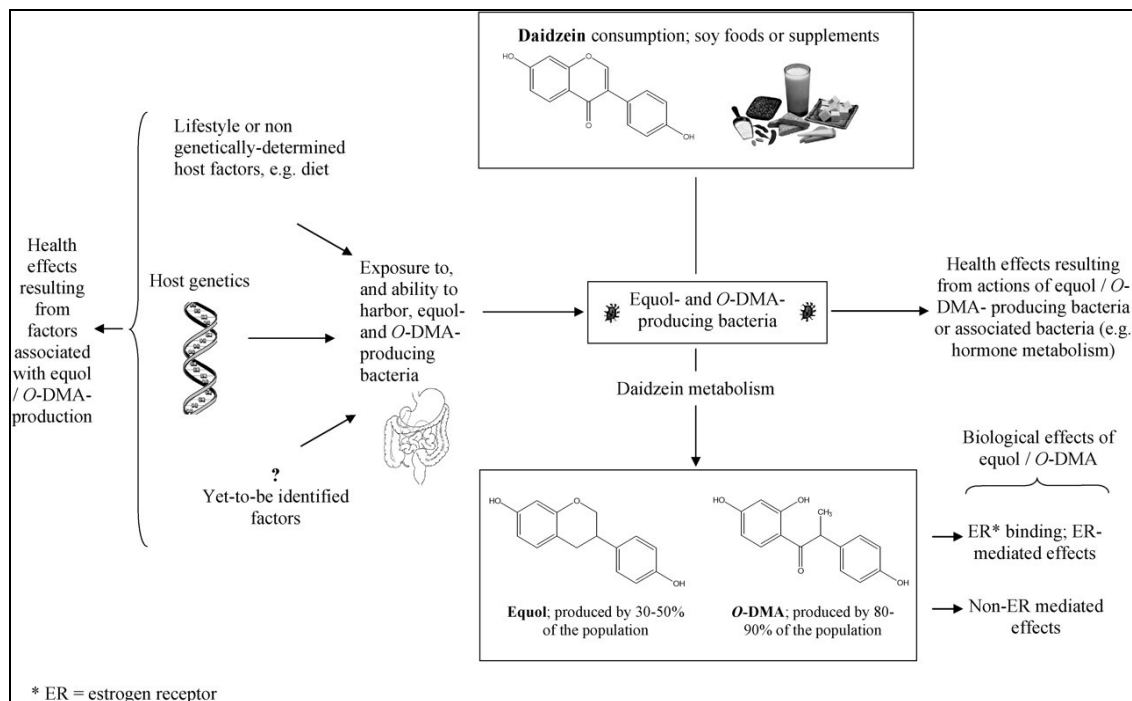
Figure 1. Gut bacterial metabolism of the soy isoflavone daidzein.
Exploring the Relevance to Human Health, (Atkinson *et al*. 2005; Yuan *et al*., 2007).

Cardiovascular Disease (Clarkson, 2002; Teede *et al*., 2001); post menopausal bone health (Setchell & Lydeking-Olsen, 2003; Dang and Lowik, 2005). Birt *et al*. (2001) demonstrated that 20% by weight of dietary soy protein significantly reduced rat intestinal mucosa levels of polyamine, a biomarker of cellular proliferation for colorectal cancer risk. Isoflavone influence on bone density was on long-term effects require continuous consumption of soybean isoflavone (Yao, *et al*., 2006).

This beneficial effect of isoflavones was due to the weak estrogenic activity which can bind estrogenic receptors (Barnes *et al*., 2000). Isoflavone can have non-estrogenic effect such as biological antioxidant (Lee *et al*., 2005).

Some other scientists were debating about that positive effect but also they expect negative effect especially hormonal effects, its safety on infants has been questioned (Chen and Rogan 2004). Regarding to that some organizations have set upper limits in daily

isoflavone intake (AFSSA and AFSSAPS, 2005). Thus, in the soyfood industry there is growing demand for soybean with low isoflavone.

In the contrast consumer interest in the health benefits of soy foods is at an unprecedented high. This is primarily the result of approval by the U.S. Food and Drug Administration (FDA) and, more recently, the Joint Health Claims Initiative (JCHI) in the United Kingdom to allow food manufacturers to make a health claim for soy protein's ability to lower the risk for coronary heart disease (FDA, 1999). There is also a large market in "over the counter" (OTC) isoflavone supplements targeting women's health (Setchell *et al.*, 2001) due to it is effect on postmenopausal problems.

0.8.2 Isoflavone structure

Plant phenylpropanoids encompass a group of phenylalanine-derived chemicals that comprise a structurally diverse group of secondary metabolites. They play vital roles in the interaction of plants with their surrounding environment. The structural diversity of phenylpropanoids is due to the action of enzymes and enzyme complexes that bring about regio-specific condensation, cyclization, aromatization, hydroxylation, glycosylation, acylation, prenylation, sulfation, and methylation reactions. Life cycle of the transcription has demonstrated great evolution in understanding transcription factors but the post-translational events need to be learned more about that control their activity, and about their subcellular localization and turnover (Broun, 2005).

Flavonoids are a large (~9000) structurally diverse class of phenolic compounds found in all higher plants (Figure 2). In addition to their physiological roles in the biochemical ecology of plants, flavonoids played a key role in the discovery of fundamental biological phenomena including *Gregor Mendel's* discovery of the laws of heredity (Ferrer *et al.*, 2008). All flavonoids are derived from the chalcone scaffold, which is biosynthesized by the ubiquitous plant enzyme chalcone synthase (CHS) (Ferrer *et al.*, 1999). CHS is pivotal for the biosynthesis of flavonoid antimicrobial phytoalexins and anthocyanin pigments in plants. It produces chalcone by condensing one p-coumaroyl- and three malonyl-coenzyme A thioesters into a polyketide reaction intermediate that cyclises (Ferrer *et al.*, 1999).

Isoflavonoids constitute a structurally distinct class of flavonoids found mainly in legumes and are defined by a dramatic C2–C3 aryl ring migration and concomitant double bond formation catalyzed by isoflavone synthase (IFS) (Ferrer *et al*., 2008). IFS play a rule in transforming naringenin into genistein and liquiritigenin into daidzein (Yu *et al*., 2003). Figure (3) shows 12 different Isoflavone forms, three main classes' daidzein, genistein and glycitein (aglycone form), glucoside conjugated with sugar only (daidzin, glycitin, and genistin), acetylglucoside derivative (6″-O-acetyldaidzin, 6″-O-acetylglycitin and 6″-O-acetylgenistin) and a malonylglucoside derivative (6″-O-malonyldaidzin, 6″-O-malonylglycitin, and 6″-O-malonylgenistin). Phenylpropanoid accumulation in cell vacuole or in defence response of soybean cells (Graham, 1995).
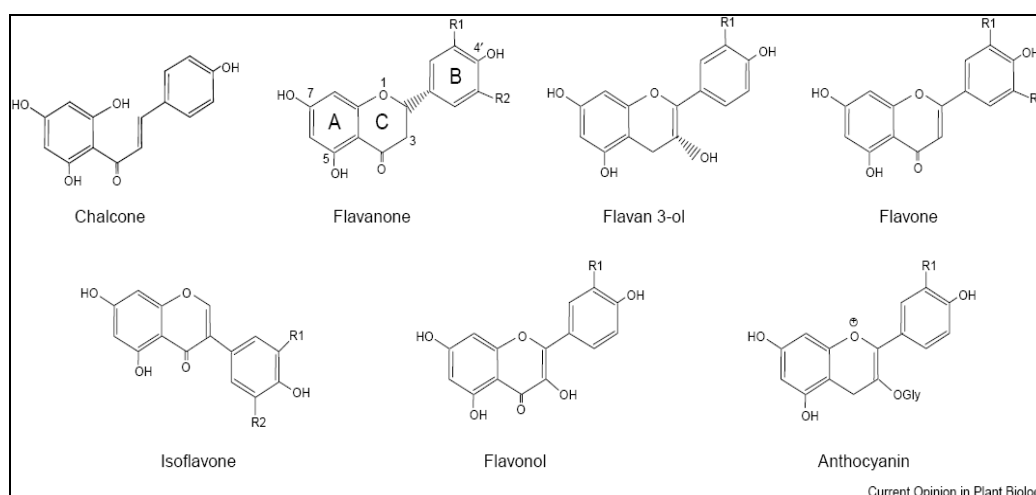
Figure 2. Structure of the main classes of flavonoids.

In which R1 and R2 indicate the sites of possible substitutions. OGly indicates a glycosidic linkage. The numbering system of the flavonoid skeleton is indicated on the flavanone structure (adapted from Taylor, and Grotewold, 2005).

0.8.3 Isoflavone pathway

In soybean, the isoflavones daidzein, genistein, and glycitein are synthesized via the phenylpropanoid pathway and stored in the vacuole as glucosyl- and malonylglucose conjugates (Graham, 1991; Kudou *et al*., 1991). Malonydaidzin and malonylglycitin constituted between 74% and 84% of total isoflavones, whereas the isoflavone levels in the four groups increased in the order malonylglucoside > glucoside > aglycon > acetylglucoside (Kim and Chung, 2007). Glycitein synthesis is not yet clearly defined but is

likely derived from isoliquiritigenin, an intermediate of daidzein synthesis (Latunde-Dada *et al*., 2001; Yu *et al*., 2003).
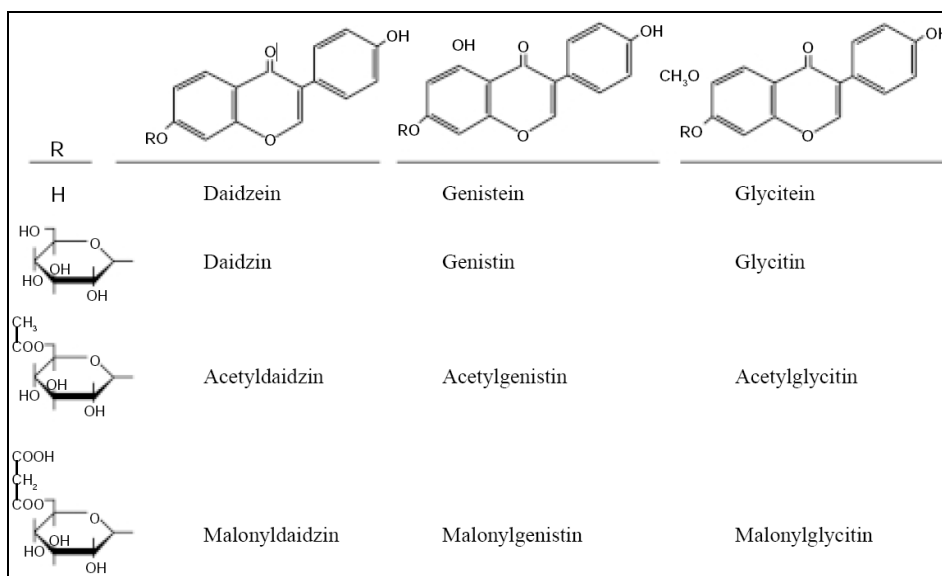
Figure 3. Structure of main isoflavones compounds.

Main skeleton in formed of three main classes daidzein, genistein and glycitein (aglycon form), glucoside conjugated with sugar only (daidzin, glycitin, and genistin), acetylglucoside derivative (6″-O-acetyldaidzin, 6″-O-acetylglycitin and 6″-O-acetylgenistin) and a malonylglucoside derivative (6″-O-malonyldaidzin, 6″-O-malonylglycitin, and 6″-O-malonylgenistin).

Genistein synthesis shares the naringenin intermediate with the flavonoid–anthocyanin branch of the phenylpropanoid pathway (Figure 4). In all cases, the unique aryl migration reaction to create the isoflavones is mediated by isoflavone synthase (IFS). The isoflavone daidzein is the precursor to the major phytoalexins including medicarpin and glyceollins, which are produced in alfalfa and soybean, respectively (Blount *et al*., 1992; Graham, 1995; Dixon and Steel, 1999), and there is hypothesis suggest that glycitein production came from glyceollin after serial of intermediates (Yu et al., 2000) hypothesized to be intermediate for glycitein formation (Kassem *et al*., 2004). Figure (5) shows plant phenylpropanoid pathway performance regarding to storing or physiological accumulation in vacuole and their release pathogen attack (Graham, 1995).

Soybean seed expresses key genes involved in isoflavonoid synthesis. However, these genes are also expressed in maternal tissues, such as seed coat or pod. Developing embryos may import isoflavonoids from a synthetic medium, and hence, the seed isoflavones can have maternal or local origin (Dhaubhadel *et al*., 2003).

Total content of isoflavone is positively correlated with genistein and daidzein contents but it can be negatively correlated with glycitein contents (Lee *et al*., 2003). Daidzein contributes 65-75% of total isoflavone while it's generally 25-35% of total isoflavones in wild-type segregating seed, while in the same altered seed genistein is close to zero but in wild-type seed it makes up about 50-60% of total isoflavones (Yu *et al*., 2003).

Wang *et al*. (2000) found that daidzein and genistein content were negatively correlated with yield, day to maturity and plant height, while total isoflavone contents was positively correlated with yield (r=0.20) among 210 soybean cultivars with wide genetic range.

In seed with altered isoflavone content, the majority of each isoflavone was about equally divided between glucose and malonyl–glucose conjugates, with very little aglycone present. These indicates that sufficient enzyme activities are present to conjugate daidzein, in genetically modified seeds, whose levels are much higher than those found in control seed, as well as genistein and glycitein. While the total isoflavone levels vary among individual seed (Yu *et al*., 2003).

Recently, a mapping study has shown that a QTL for glycitein was closely associated with three seed storage protein genes (Kassem *et al*., 2004).

Oil concentration in seed decreased very slowly, and protein concentration remained almost constant, as soybean yield increase (Yin and Vyn, 2005). Concentration and yield of individual and total isoflavones and yields of oil and protein were all positively related to seed yield. Daidzein was the most variable and glycitein was the most stable isoflavone component.

The low temperatures indeed increase the activity of key enzymes of the phenylpropanoid and flavonoid pathways such as PAL and CHS (Leyva *et al*., 1995; Janas *et al*., 2002; Posmyk *et al*., 2005). Low temperature reduces the photosynthetic capacity of the cells, leading to an excess of photon flux (Somersalo and Krause, 1989). This excess could produce an additional light stress, resulting in a further induction of the phenylpropanoid pathway (Leyva *et al*., 1995). In this way a light-inducible factor

controlling anthocyanin accumulation has recently been characterized in maize (Cone *et al.*, 1993).
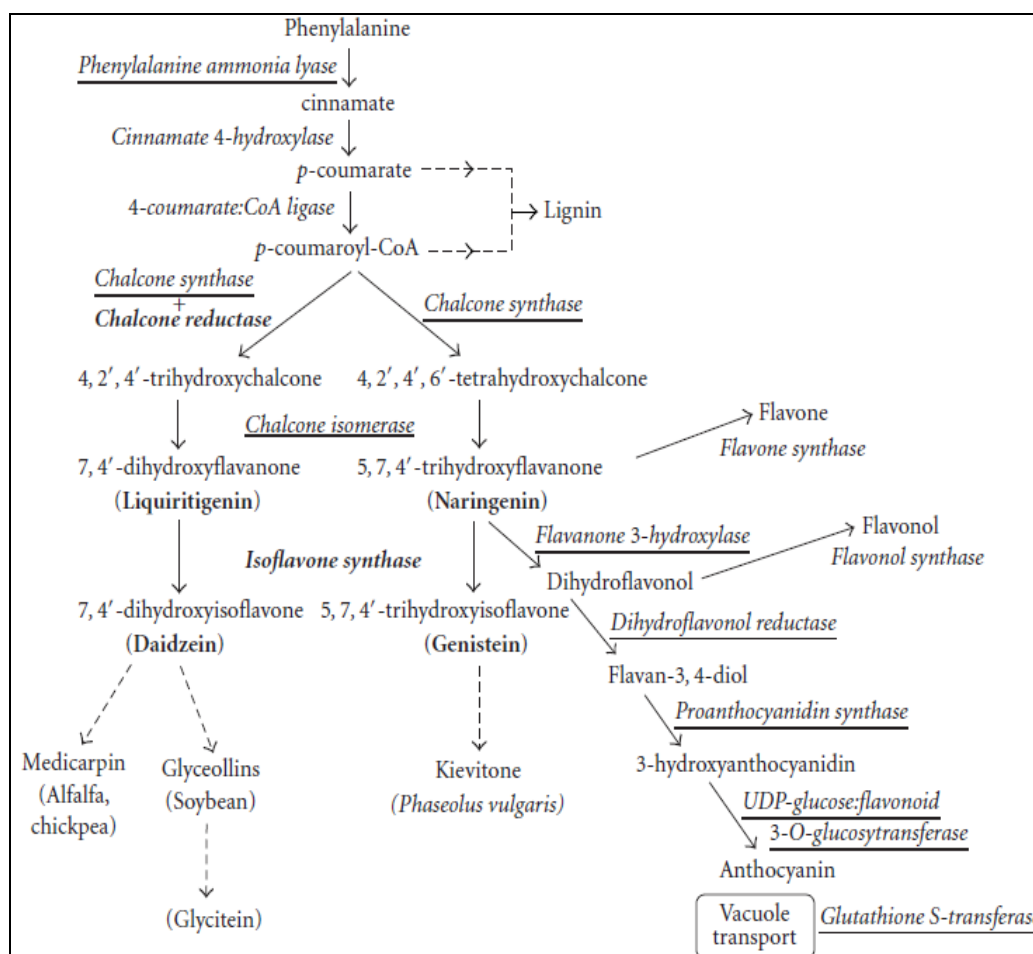
Figure 4. A partial diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis, as well as some branch pathways.

The enzymes in bold are encoded by genes expressed as transgenes in this study. Genes encoding the underlined enzymes have been shown to be activated in maize by C1 and R. Dotted arrows represent multiple steps. Enzymes are indicated in italics (adapted from (Yu *et al.*, 2000). Glycitein (a glyceollin) is hypothesized to be made from daidzein in a multistep process (hydroxylation, oxidation, and acetylation) (Kassem *et al.*, 2004).

0.8.4 Isoflavone rule in plant

To optimize growth and development, the metabolic activities of a plant are highly coordinated at the whole-plant, organ, tissue, cellular, organellar and molecular levels. Figure (5) in plants, glucosidation plays a key role in the detoxification of endogenous secondary metabolites and xenobiotics, with their glucosides often accumulating in the vacuoles (Graham, 1995; Yazaki, 2005). Phenylpropanoid compounds are very large

number and most of them have a rule in plant defence or acclimation. Phenylpropanoid defensive function is not limited to Particular compounds but found in the simple hydroxycinnamic acids and monolignols through to the more complex flavonoids, isoflavonoids and stiblenes (Dixon *et al.*, 2002). Figure (6) explore the effect of stress on different classes of phenylpropanoid (Dixon and Paiva, 1995). All classes of phenylpropanoid compounds are not present in all plant species. Natural products active in plant defense can be categorized into three broad groups: phytoalexins, phytoanticipins, and signal molecules. An obvious hypothesis is that there is a need to independently regulate the production of different phenylpropanoid products in the same or different cells and those different gene family members are somehow involved in the production of different classes of compounds. Legumes, in particular, use phenylpropanoid compounds as both phytoalexins and signal molecules for the attraction of symbiotic microbes, and the independent regulation of such pathways would clearly be necessary. In soybean, isoflavones have been shown to gene-inhibit pathogen attack by *Phytophthora sojae* Kaufmann and Gerdemann (Graham *et al.*, 1990; Morris *et al.*, 1991; Graham and Graham, 1994; Graham and Graham, 1996).

Flavonoids belongs to a group of active plant defence compounds known as phytolalexines acting as a repellent against insect feeding and pathogenic attack (Graham and Graham, 1994; Dixon *et al.*, 2002). It has ability to induce nodulation genes in *Bradyrhizobium japonicum* bacteria that form rhizobia on soybean roots (Kosslak *et al.*, 1987). The idea of a role for genistein in regulation of transcription is not new. In the soybean plant itself, genistein that is secreted through the roots into the soil is a chemo-attractant for *Bradyrhizobium sp*, in which nodulation genes are up-regulated in response to the genistein, resulting in the production of lipochitin polysaccharides. These in turn induce root nodules on the soy plant that house the bacteria. These root nodules convert atmospheric nitrogen to ammonia, which is taken up by the soybean plant, thus defining a symbiotic relation. Flavonoids and other phenylpropanoids have long been thought to play a role in protecting against UV irradiation, because they accumulate primarily in the epidermal and hypodermal layers of leaves and stem (the most illuminated layers) and strongly absorb light in the UV-6 wavelengths. (Dixon and Paiva, 1995). Importance of

flavonoids in UV protection, Auxin transport, Seed dormancy and defence against pathogens reviewed in D'Auria and Gershenzon (2005).
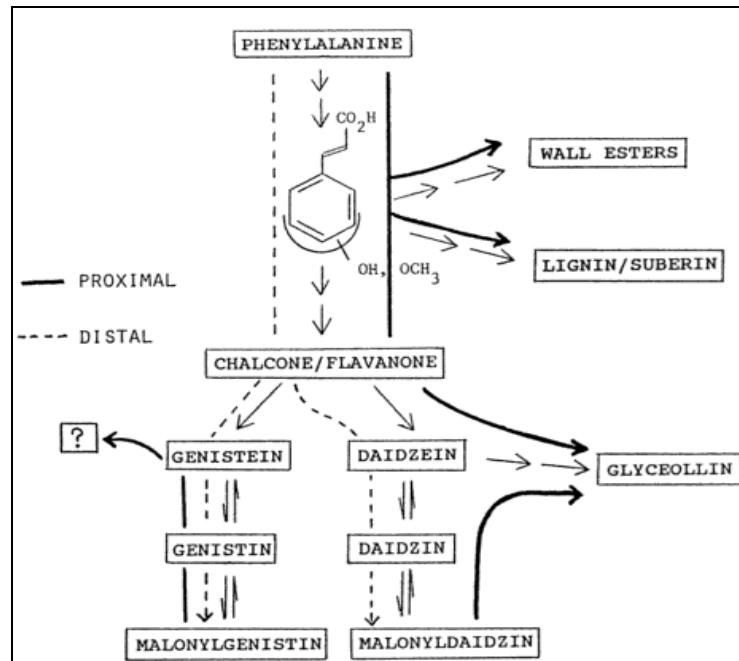
Figure 5. Phenylpropanoid accumulation in cell vacuole or in defence response of soybean cells.

Dotted line refer to distal normal accumulation in vacuole and detoxification of isoflavone in plant cell, other arrows refers to plant response to any physiological process for applying isoflavone conserved in the vacuole, adapted from (Graham, 1995).
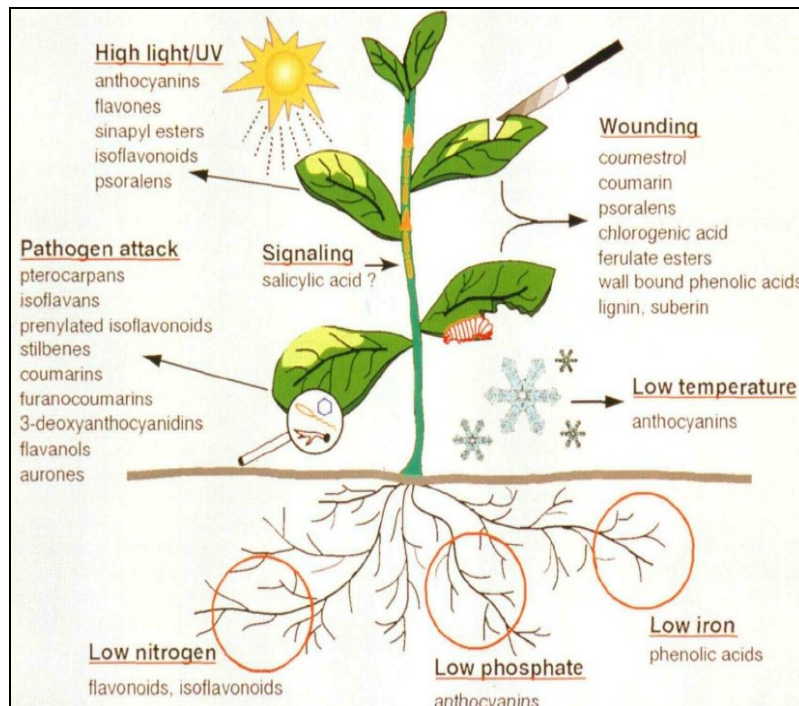
Figure 6. Examples of stress-induced phenylpropanoid (Dixon and Paiva, 1995).

0.8.5   Factors affect isoflavone accumulation

0.8.5.1   Seed hypocotyl and cotyledon

The pattern of isoflavone synthesis expression varies among tissues and development stag (Dhaubhadel *et al*., 2003; Berger *et al*., 2008), which there were studies observed that the spatial regulation of the kuntiz trypsin inhibitor gene during the soybean seed development highlighted the specificity of germ (hypocotyl) expression (Perez-Grau and Goldberg, 1989).

The profiles for a given organ are highly reproducible from plant to plant, although, as described below, the absolute amounts of any given metabolite vary in a given organ with age, developmental state, and conditions of seedling growth (Graham, 1991).

Soybean seed is virtually devoid of endosperm and comprises a well-developed embryo contains two primary organ systems, the axis or hypocotyl radical region and the cotyledons and a surrounding seed coat. During embryo development, the fertilized egg cell differentiates into a mature embryo containing cells with different roles (Figure 7). The entire embryogenesis can be divided into five stages: globular, heart, cotyledon, maturation, and dormancy (Walbot, 1978). One seed part, the hypocotyl, whose industrial name is soy germ, is often discarded during food processing because of its astringent taste resulting from a high content of secondary metabolites. Indeed, hypocotyl has 4 to 10 times more isoflavones than cotyledons and hence becomes a valuable by-product (Schryver, 2002). Berger *et al*. (2008) demonstrated that there was a clear succession of the isoflavone accumulation, which started first in hypocotyls and in the cotyledons accumulation seemed to begin when a plateau was reached in the hypocotyl. The accumulation in cotyledons that began after R6; 35-40 DAF appeared to be a late maturation event (Berger *et al*., 2008). It has been suggested that isoflavones found in the developing embryo could be simultaneously imported from the other tissues, such as seed coat, and locally synthesized from precursor molecules (Dhaubhadel *et al*., 2003).
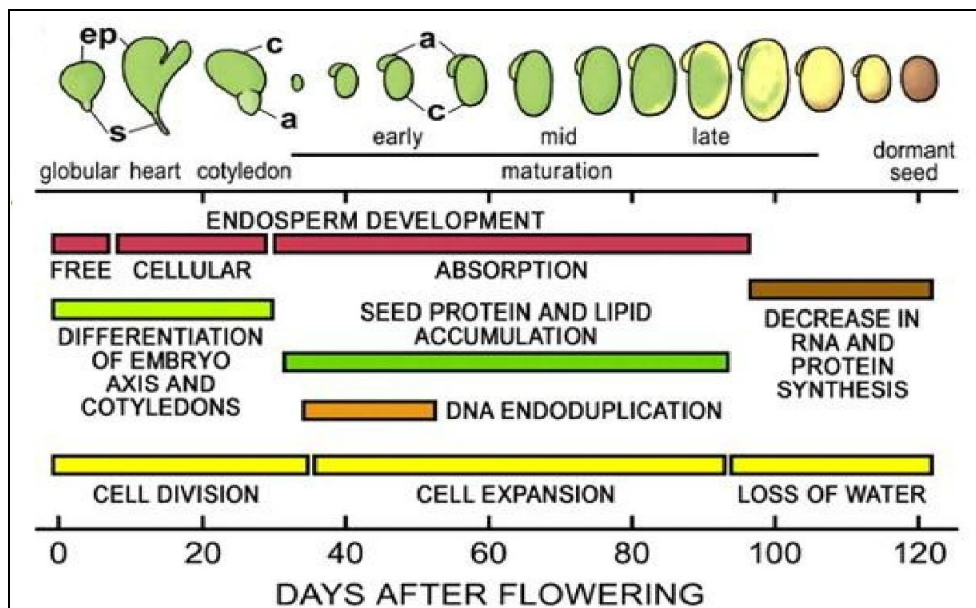
Figure 7. Seed development stages.
a, Axis; c, cotyledon; ep, embryo proper; s, suspensor; adapted from Le *et al.* (2007)

Of the total seed isoflavone, 80-90% was located in cotyledons, with the remainder in the germs (hypocotyl). The germs had a higher concentration of isoflavones on a weight basis compared with cotyledons. Isoflavone content in cotyledons exhibited a large response to temperature seed fill, but the germs isoflavone contents remained relatively constant a cross different temperature regimes (Tsukamoto *et al.*, 1995). Generally a decrease in seed weight is accompanied by an increase of the percentage weight of germ (Tsukamoto *et al.*, 1995) and germ can increase under unfavourable conditions.

Recently seeds germ is getting interest of scientists to know the effect of genotype and environment on chemical ingredient (Schryver, 2002). The soy germ represents 2-3% of total seed weight, thus it concealed 20% of the whole seed isoflavone content (Eldridge and Kwolek, 1983; Tsukamoto *et al.*, 1995). During soy food processing the germ can be separated from cotyledons, thus becoming a valuable by-product (Schryver, 2002). In hypocotyls, daidzein and genistein began their accumulation before glycitein (Berger *et al.*, 2008). The germ is particularly rich in daidzein and is the almost exclusive glycitein provider, which may lead us to conclude that cotyledons do not have a similar environment and genetic determination effect. Seed germ weight is negatively correlated with seed weight (Tsukamoto *et al.*, 1995), Nevertheless germ part weight can be increased with unfavourable conditions.

The pattern of isoflavone synthesis expression varies among tissues and development stag (Dhaubhadel *et al*., 2003); which there were studies observed that the spatial regulation of the kuntiz trypsin inhibitor gene during the soybean seed development highlighted the specificity of germ expression (Perez-Grau and Goldberg, 1989). Isoflavone daidzein, genistein and their conjugates are rapidly released from imbedding seed with small amount which is consistent with the rule in chemotaxis or nodulation gene (Graham, 1991). Seedling level of isoflavone is depleted by time. The germ (hypocotyl) is rich in daidzein and contains nearly all glycitein that is form another difference with cotyledons.

0.8.5.2   Variety and isoflavone contents

Several studies have shown that there is a large variation in isoflavone concentration and composition among soybean genotypes (Berger *et al*., 2008; Choi *et al*., 1996; Eldridge and Kwolek, 1983; Hoeck *et al*., 2000; Kitamura *et al*., 1991; Nelson *et al*., 2001; Wang and Murphy, 1994b).

Varied cultivars showed different response in correlation with year regardless the location the difference was 1335 mg g^{-1} equal 110 % (Hoeck *et al*., 2000). Dependent upon the variety Bennett *et al*. (2004) found total isoflavone content was increased as much as 1.3-fold in early-planted soybeans. High- and low-content cultivars had the same ranking above the different environmental conditions encountered, but a same cultivar could have a fourfold cotyledon isoflavone content increase in a favourable environment (Hoeck *et al*., 2000; Berger *et al*., 2008).

Wang and Murphy (1994b) reported total isoflavone contents from 1176 to 3309 mg g^{-1} across years, from 1176 to 1749 mg g^{-1} across sites within the same year for single soybean cultivars and from 2053 to 4216 mg g^{-1} a cross single cultivar.

Heritability of isoflavone has bean estimated from 64-70% (Primomo *et al*., 2005) or higher than 90% (Chiari *et al*., 2004). However, heritability for individual aglycones (daidzein, genistein or glycitein) displayed significant variation. Heritability was often higher for glycitein (Chiari *et al*., 2004) but it was some times inconsistent because of the low concentration.

Cultivars can have ability to accumulate one molecule on favour of others, which they were able to favour genistein or daidzein (Berger *et al*., 2008). The lack of predictability would make it difficult to contract before planting for production of grain with specified isoflavone contents (Hoeck *et al*., 2000).

Develop of soybean cultivars in last 60 years demonstrated increment of yield 0.43%, oil 0.24%, daidzein 1.47% and genistein 0.98% per year, while protein decreased 0.15% per year (Morrison *et al*., 2008). They mention also those recent soybean cultivars are more pore to environmental influence than older cultivars.

0.8.5.3   Environment

The main factors responsible for the variation of isoflavone content and composition are the soy genotype and the environmental conditions encountered during seed maturation (Aussenac *et al*., 1998; Lozovaya *et al*., 2005; Seguin *et al*., 2004; Tsukamoto *et al*., 1995). Total isoflavone content of a single cultivar varied among years and locations within the same year (Wang and Murphy, 1994b; Hoeck *et al*., 2000). The major environmental factor influence seed isoflavone contents is temperature during seed filling period (Kim *et al*., 2005; Lozovaya *et al*., 2005; Seguin *et al*., 2004; Tsukamoto *et al*., 1995). Probably the reason of higher isoflavone concentrations found in later maturing genotypes (Nelson *et al*., 2001; Lozovaya *et al*., 2005; Wang *et al*., 2000) or late sowing dates (Tsukamoto *et al*., 1995, Aussenac, *et al*., 1998) which encountering lower temperatures at the end of maturation. Long-term adaptation of the soybean to growth at elevated CO_2 level and high temperature might potentially increase its isoflavone content (Kim *et al*., 2005).

Since water content is very important for all plants as effective factor for plant metabolism, isoflavones compound found to be increase by irrigation (Bennett *et al*., 2004) or decreased by drought (Seguin *et al*., 2004; Caldwell *et al*., 2005; Lozovaya *et al*., 2005).

These molecules are sensitive to wound and light (Graham and Graham, 1996). Seedling growth in continuous darkness significantly affects both level and distribution of the isoflavones (Graham, 1991). The levels of daidzein and genistein in younger seedling organs (except leaves) are substantially higher.

Hoeck *et al*. (2000) tested six soybean cultivars at eight Iowa locations over 2 yr and Lee *et al*. (2003) planted 15 cultivars at three sites in Korea over 3 yr. In these two studies, genotype, genotype × year, genotype × location, and genotype × year × location interactions were significant for both total and individual isoflavone concentrations.

Carrao-Panizzi and Kitamura (1995) tested 22 soybean cultivars at one location in Brazil over 2 yr and attributed the significant differences in total isoflavone content to differences between years in temperature, precipitation and harvest date.

Berger *et al*. (2008) observed few effects of the environment on hypocotyl isoflavone contents, while nutrition has greater effect than temperature on this seed part.

0.8.5.4   Soil water content and irrigation

Since water content is very important for all plants as effective factor for plant metabolism, isoflavones compound found to be increase by irrigation (Bennett *et al*., 2004) or decreased by drought (Seguin *et al*., 2004; Caldwell *et al*., 2005; Lozovaya *et al*., 2005). Under irrigation Lozovaya *et al*., 2005 found that both genistin and daidzin concentration has the same trend as total isoflavone but glycitin concentration was much less affected. Irrigation enhanced the isoflavone and individual isoflavones content of both early- and late-planted soybeans as much as 2.5-fold (Bennett *et al*., 2004). Al-Tawaha *et al*. (2007) mentioned that irrigation response to irrigation may be non-linear in nature, with plants grown in dry soils responding to irrigation up to a certain point, after which excess irrigation could negate any potential benefits. Level of irrigation effect depends mostly on year of application due to variability of climate conditions then variety.

0.8.5.5   Soil nutrient

Soil nitrogen enrichment overall had no effect on total isoflavone concentration, where N application was not correlated with isoflavone concentration (Kim *et al*., 2005).

Vyn *et al*. (2002) demonstrated that both individual and total isoflavones were positively correlated with leaf and seed K concentrations on low-K soils. Potassium may be essential in isoflavone synthesis because K is an essential activator for more than 60 enzymes that catalyze a variety of metabolic activities (Suelter, 1985).

Negative correlation between isoflavone and glycitein content was observed in segregation populations (Primomo *et al*., 2005), after drought stress (Caldwell *et al*., 2005) or as response to atmospheric CO_2 and/or soil N enrichment (Kim *et al*., 2005).

Berger *et al*. (2008) observed few effects of the environment on hypocotyl isoflavone contents, while nutrition has greater effect than temperature on this seed part.

Objectives

1- To study the effect of management on isoflavone accumulation in cotyledon and hypocotyl of different soybean varieties;

2- To study the effect of irrigation and nitrogen fertilization synergy on isoflavone accumulation;

3- To study the effect of level of irrigation regarding to water deficit on isoflavone accumulation;

4- To study the effect of plant density in conventional and organic systems on soybean variety and their isoflavone contents;

5- Soil type effect on isoflavone contents.

Chapter 1

Organic and Conventional Management Effect on Cotyledon and Hypocotyl Isoflavone of Soybean (*Glycine max* L. Merr.)

1.1 Introduction

An increased demand for soybean production is forecast in the next few years, to reflect the world's population growth and the greater requirements for proteins and edible oils, and Conner *et al*. (2004) mentioned soybean as the primary source at world level. Soybean cultivation is very attractive in organic farms, as they generally have a larger proportion of legumes in rotation (Kirchmann and Bergstrom, 2001), suggesting the attainment of increased levels of N_2 fixation compared with conventional systems (Hansen *et al*., 2000).

Soybean seeds have also received attention due to their contents of several nutraceutical compounds, such as flavonoids, a group of phenylalanine-derived chemicals that play key roles in the interaction of plants with their surrounding environment. Flavonoids are considered secondary plant metabolites belonging to a group of active plant defence compounds known as phytolalexines, acting as repellents against insect feeding and pathogenic attack (Graham and Graham, 1994; Dixon *et al*., 2002). Since the late 1990s, interest in these compounds has focused mainly on isoflavones, a particular class of flavonoids, as epidemiological and clinical studies have suggested that the consumption of soyfoods is associated with many health benefits, such as lowered risk of breast and prostate cancer (Birt *et al*., 2001; Dai *et al*. 2002; Sarkar and Li, 2003), and cardiovascular (Teede *et al*., 2001; Clarkson, 2002) and post-menopausal bone diseases (Setchell and Lydeking-Olsen, 2003; Dang and Lowik, 2005). These beneficial effects may be due to the weak estrogenic activity of isoflavones, which can bind estrogenic receptors (Barnes *et al*., 2000), and their non estrogenic action such as a biological antioxidant effect (Lee *et al*., 2005).

Important factors responsible for variations in isoflavone contents and composition are soybean genotype (Kitamura *et al*., 1991; Choi *et al*., 1996; Nelson *et al*., 2001; Berger *et al*., 2008) and environmental conditions (Aussenac *et al*., 1998; Seguin *et al*., 2004; Lozovaya *et al*., 2005). The major portion of isoflavones (80-90%) is located in cotyledons, and the remainder in the germ, which however has higher concentrations. Isoflavone content in cotyledons exhibits a large response to temperature regime during seed filling, unlike germ isoflavones (Tsukamoto *et al*., 1995). Within seeds, isoflavone distribution

may change under unfavourable conditions, as a consequence of increased weight proportion of the germ found in small seeds (Tsukamoto *et al.*, 1995).

Various cultivars were found to show different responses in isoflavone concentration in relation to year, regardless of location of cultivation, with wide differences (Hoeck *et al.*, 2000). This result was very similar to that found by Wang and Murphy (1994 a), who identified year and variety as the two main sources of variation. Berger *et al.* (2008) found high- and low-isoflavone cultivars to have the same ranking above different environmental conditions (sites), but the same cultivar could have a four-fold increase in cotyledon isoflavone content increase in a favourable environment. These authors also found that isoflavone increases are directed to a specific class of molecules in cotyledons, genistein or daidzein, depending on variety. The studies of Hoeck *et al.* (2000) and Lee *et al.* (2003) highlighted the existence of strong "genotype × year", "genotype × location", and "genotype × year × location" interactions for both total and individual isoflavone classes. The lack of predictability makes it difficult to contract before planting for production of grain with specified isoflavone contents (Hoeck *et al.*, 2000). Among environmental variables, temperature during the seed filling period has been identified as one of the most important factors influencing seed isoflavones (Seguin *et al.*, 2004; Lozovaya *et al.*, 2005). Some authors explained the higher isoflavone concentrations found in late-maturing genotypes (Nelson *et al.*, 2001) and in late-sowing dates (Tsukamoto *et al.*, 1995, Aussenac *et al.*, 1998) as due to the lower maximum temperatures encountered at the end of maturation, although Bennett *et al.* (2004) found an opposite effect of sowing date. At biochemical level, low temperatures were found to increase the activity of key enzymes of the phenylpropanoid and flavonoid pathways, such as PAL (Phenylalanine Ammonia-Lyase) and CHS (Chalcone Synthase) (Leyva *et al.*, 1995; Janas *et al.*, 2002; Posmyk *et al.*, 2005). Low temperatures also reduce photosynthetic activity in cells, leading to an excess of photon flux (light stress) (Somersalo and Krause, 1989) responsible for further enhancement of the phenylpropanoid pathway (Leyva *et al.*, 1995).

In this framework, the present research aimed at studying variations in soybean seed isoflavone and protein accumulations in relation to: i) intraspecific variability of a wide set of Italian commercial varieties; ii) type of cultivation, comparing organic and conventional

managements. Varieties were tested within a four-year trial in North-East Italy, which also allowed the effects of climatic variables to be evaluated.

1.2 Material and methods

1.2.1 Experiment conditions

Experiments was carried out at the experimental farm of the University of Padova (45° 21' N, 11° 58' E, 12 m ASL), part of which has been following organic management since 2001. 4-years trials: 2005, 2006, 2007 and 2008, 13 soybean commercial varieties in Italy were evaluate under two different types of agricultural management, organic vs. conventional, following a 3-replicated randomised block experimental design. The soils of the two managements were quite different (Table 1.1), with organic matter content higher in the organic management (2.5% vs. 1.5%). Most of the varieties belonged to maturity group I; four had a shorter cycle (group 0). A full description of variety traits is shown in table (1.2), as regards flower, pod and seed hilum colours, and seed weight.

Following recommendations for North-East Italy, soybean was cultivated from mid-May to the beginning of October (harvest). The experimental location has a mean annual (30-year period) precipitation of 830 mm, 370 mm of which fall from May to September. Within the site, the water table fluctuates from 0.8 to 1.8 m in depth, mainly depending on precipitation. Figure (1) shows monthly temperatures during the crop cycle, evidencing a progressive reduction of precipitation from 2005, to 2006 and 2007. Minimum and maximum temperatures and their differences were registered during trials (Figure 1.1) in order to establish a possible relation with isoflavone accumulation.

Soybean was generally cultivated after wheat, and the seed bed was prepared by means of conventional tillage practices (ploughing + grubbing + harrowing). In conventional management, nutrient supply followed recommended fertilization of 100 kg ha^{-1} per year of both P_2O_5 and K_2O, without N application. In organic, before ploughing, the soil received 40 t ha^{-1} of mature cow manure once, at the beginning of the experiment only, roughly corresponding to 200 kg N ha^{-1}, and 130 and 260 kg ha^{-1} of P_2O_5 and K_2O, respectively.

Plot size was 1.8 m wide ×10 m long. Inoculated seeds (100 g inoculum per 25 kg seeds) were sown at 0.45 m inter-row distance and at 43 seeds m^{-2} density, to reach about 40 plants per m^{-2} at harvest. The crop was rainfed and kept free of weeds with herbicides (conventional) or mechanically (organic).

At harvest, yield consisted of collecting plants of the two central rows of each plot. Seed protein percentage was determined by multiplying N content (Kjeldahl method) by the coefficient 6.25.

Table 1.1. Main soil properties in two managements

	Silt	Sand	Clay	OM	N	C/N	pH	CEC (+)	Total P (P_2O_5)	Av. P (P_2O_5)	Exc. K (K_2O)
	(%)							cmol kg^{-1}	g kg^{-1}	mg kg^{-1}	
Co	65	16	19	1.8	0.12	9.7	8.0	14.8	8.5	8	59.9
Or	61	17	22	2.5	0.1	13.2	8.0	14.2	11.4	6.29	143

Co, conventional; Or, organic., CEC, cation exchange capacity; OM, organic matter,

Table 1.2. Main morphological traits and precocity of 13 tested soybean varieties.

Variety	Company	Flower colour	Pod colour	Hilum colour	Maturity group	Seed weight (mg per seed ±S.E.)	Stem length (cm)
Giulietta	Golden harvest	W	Br.	Br.	1	213.7 ±2.0	93
Dekabig	Monsanto	V	Br.	L.Br.	1+	179.7 ±2.6	85
Nikko	Monsanto	V	Br.	L.Br.	1-	165.0 ±2.6	83
B63	Pioneer	V	Be.	L.Br.	1+	150.6 ±3.5	96
Cresir	Pioneer	V	Br.	L.Br.	0+	171.0 ±2.7	86
M10	Pioneer	V	Be.	L.	0+	191.5 ±1.3	80
Nikir	Pioneer	V	Be.	L.	1	184.8 ±2.9	95
Regir	Pioneer	V	Be.	Br.	1	175.5 ±2.2	79
Sponsor	Semfor	W	Br.	Bl.	1	196.1 ±3.1	80
Aires	Sis	V	Br.	Bl.	0+	179.5 ±2.1	74
Hilario	Sis	V	Be.	Br.	1	192.3 ±2.2	77
Brillante	Syngenta	V	Be.	L.	1-	208.6 ±2.6	92
Demetra	Syngenta	W	Be.	Br.	1	178.4 ±3.1	89

W. = White; V. = Violet; Br.= Brown; Be. = Beige; L.Br. = Light Brown; D.Br. = Dark Brown; Bl. = Black; L.= Light; tendency towards longer (+) and shorter (–) cycle
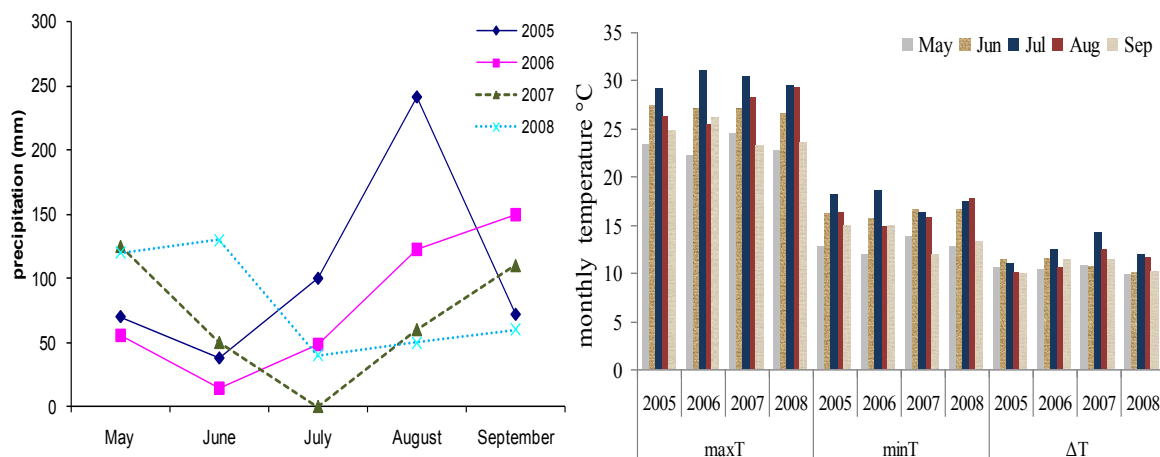
Figure 1.1. Climate data during 4 years of the trial

1.2.2 Isoflavone detection

1.2.2.1 Chemicals and standards

Genuine standards of aglycone isoflavones (daidzein, genistein, glycitein) and β-glucosyl forms (daidzin, genistin, glycitin) came from a commercial source (L.C. Laboratories, Woburn, MA, USA) so that HPLC analysis could be performed. Due to their unstable structure, the malonyl- and acetyle-conjugated forms were not quantified with external standards; their response factor (calibration curve) was calculated from the corresponding β-glucosyl forms, corrected for in molecular mass ratio (Hubert *et al*., 2005). HPLC-grade (99.95%) methanol and acetonitrile (ACN) were used as extractant and eluting chemicals, respectively. Additional elution solvent was trifluoroacetic acid (TFA).

1.2.2.2 Sample preparation and HPLC assay

After harvest, seeds were stored at room temperature until analysis. For each sample, 60 seeds were lyophilised, weighed and, after separation, weight percentages were assigned to hypocotyl and cotyledons. Cotyledons were milled mechanically whereas hypocotyl by mortar and pestle. Small flour samples (0.1 g) were treated with 7 mL of methanol solution (80% v/v) in a 10-mL tube for 2 h at room temperature (70 rpm). Clear supernatant, collected after centrifugation (5 min., 10.000 RCF), was filtered at 0.2 μm (Acrodisc Syringe Filters, GHP membranes). Samples were kept in clean tubes at -20 °C until successive processing.

HPLC analysis was carried out according to the method of Wang and Murphy (1994 b). Samples were shaken before 1 mL was extracted in vials to be set in the HPLC autosampler. The mobile phase was 0.25% v/v TFA (solvent A) and pure ACN (solvent B). A linear HPLC gradient was used: following the injection of 2 μL of sample, solvent B was kept at 16% for 2 min, and then increased gradually to 24% in 2 min, 32% in 2 min, and 100% in 3 min, a value that was maintained for 1 min more. Analysis lasted 10 min with a solvent flow rate of 1.3 mL per min. The HPLC equipment (Shimadzu®, Milan, Italy) had a UV diode array detector (SPD-M20A) and Ultratechspere C18 analytical column (CIL CLUZEAU, France) (33×4.6 mm i.d., 1.5 μm particle size), the latter kept at 35 °C.

A control sample (Figure 1.2, 1.3) was employed for each new analysis batch, to verify weather the HPLC system was operating correctly by detecting expected concentrations.
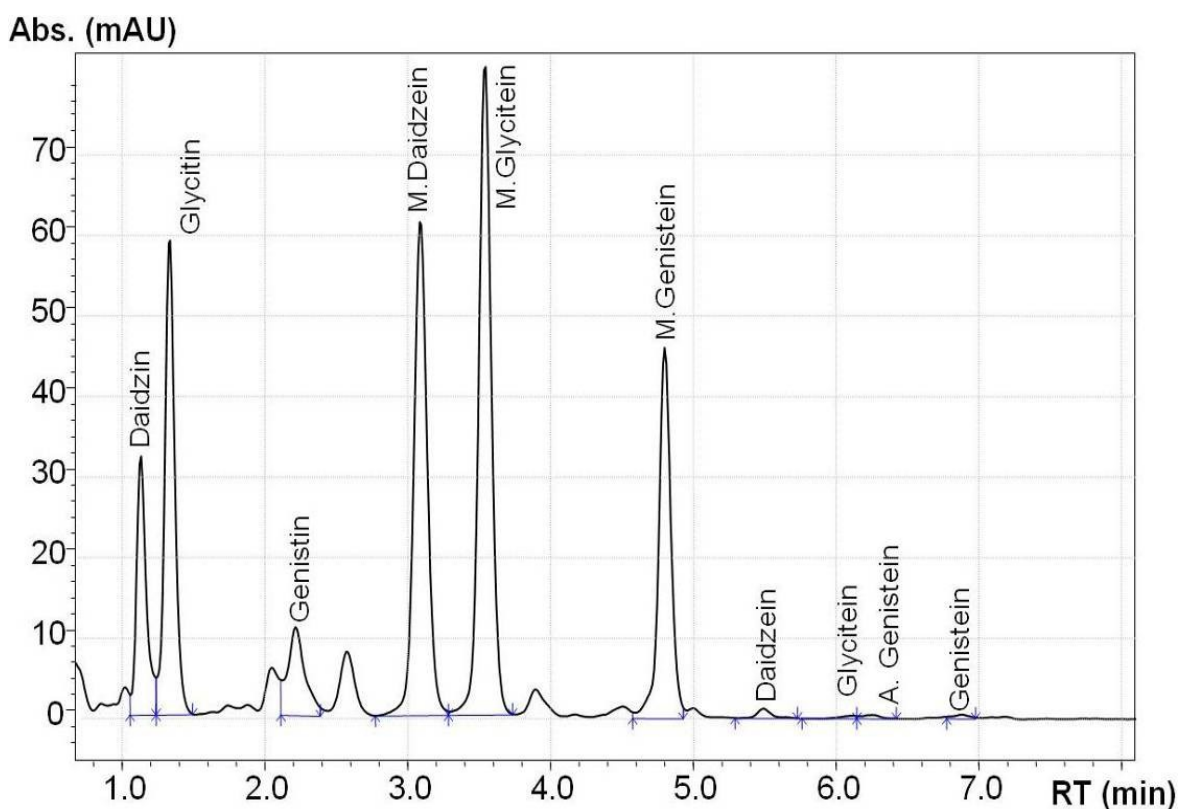
Figure 1.2. Retention time of HPLC chromatogram of soybean hypocotyl isoflavones classes.
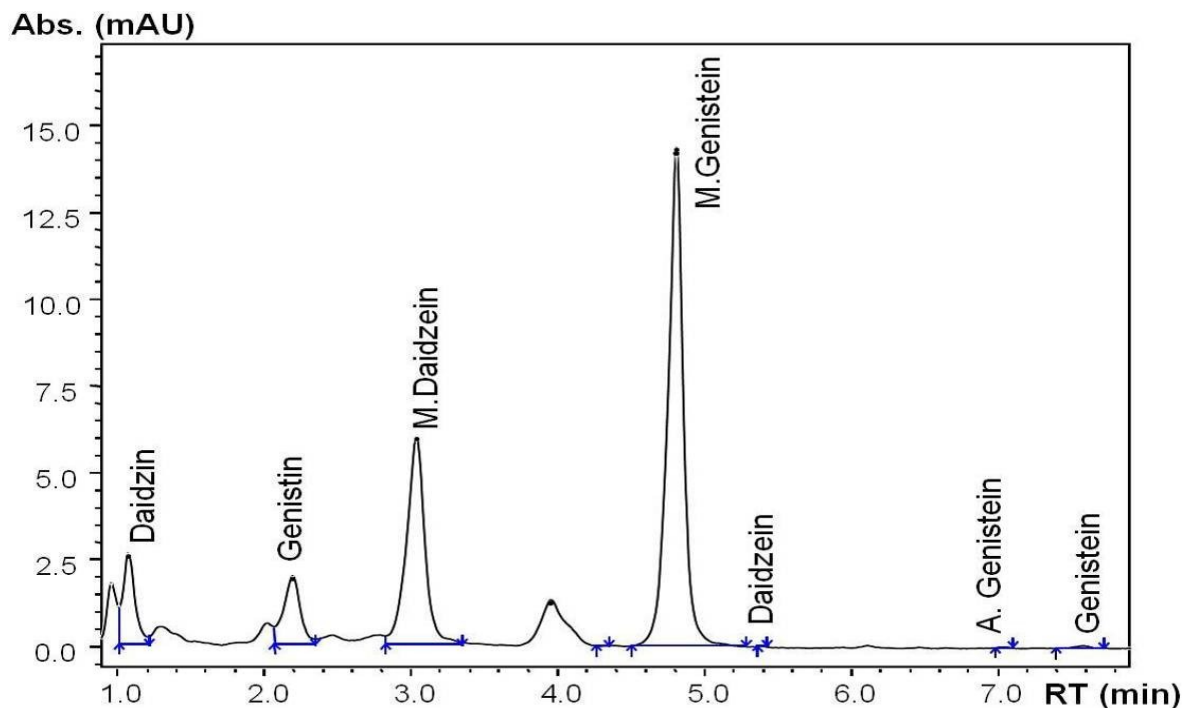
Figure 1.3. Retention time of HPLC chromatogram of soybean cotyledon isoflavones classes.

1.2.2.3   Isoflavone standards Calibration curve

Standards were prepared by adding 1 mg of each isoflavone to 0.5 mL dimethyl sulphoxide (DMSO) and bringing to 5 mL with HPLC-grade methanol, this corresponding to 0.2 mg mL-1 isoflavone concentration. This concentration was further diluted with methanol into five different powers 10, 20, 50, 80 and 100% v/v. Standard solutions were passed in HPLC to identify isoflavone molecules, their retention time (RT) and concentrations. All calibration curves had coefficients of determination >99.4% (Table 1.3).

1.2.2.4   Chromatograms analysis

Chromatograms were detected regarding to RT and there spectrophotometer view (Figure 1.4). Values were determined applying extracted equation of standard curve calibration. Peaks of daidzein, genistein, glycitein, daidzin, genistin and glycitein were determined with reference to purchased external standard. The malonyl- and acetyl-agluconic form were determined dependent upon their molecular weight.

Table 1.3. The structures of 6 isoflavone standards and their calibration curve equations.

A.

B.

structure	Isoflavones	R1	R2	calibration curve	R^2
A	Daidzein	–H	–H	y = 15.41106x - 3.211957	0.9988
	Glycitein	–H	–OCH3	y = 15.00762x - 0.5091	0.9994
	Genistein	–OH	–H	y = 10.31776x + 2.221363	0.9984
B	Daidzin	–H	–H	y = 19.36156x + 5.657396	0.9956
	Glycitin	–H	–OCH3	y = 21.65676x - 0.000413	0.9998
	Genistin	–OH	–H	y = 15.0946x + 3.8547	0.9935

A represent the aglycone (free form), B represent β-glycoside form

Figure 1.4. Following forms represent different chromatograms of spectrophotometer of different isoflavone molecules.

Daidzin

Glycitin

Genistin
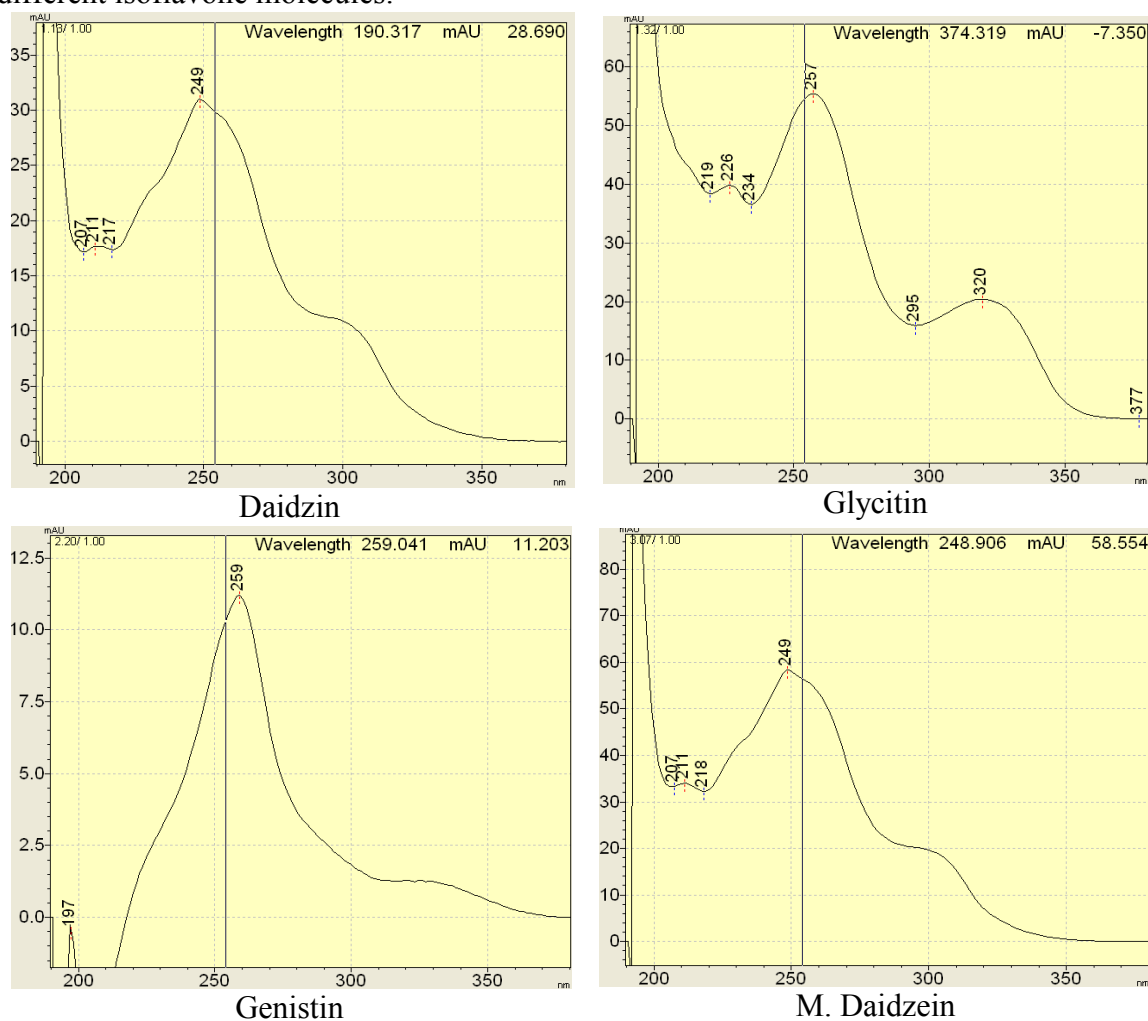
M. Daidzein

Continue Figure 1.4
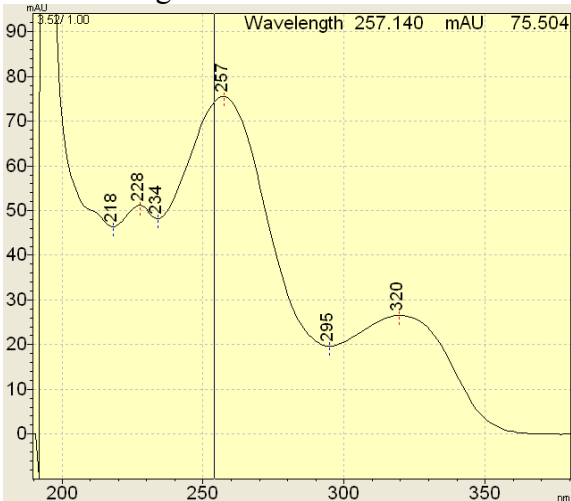
M. Glycitein
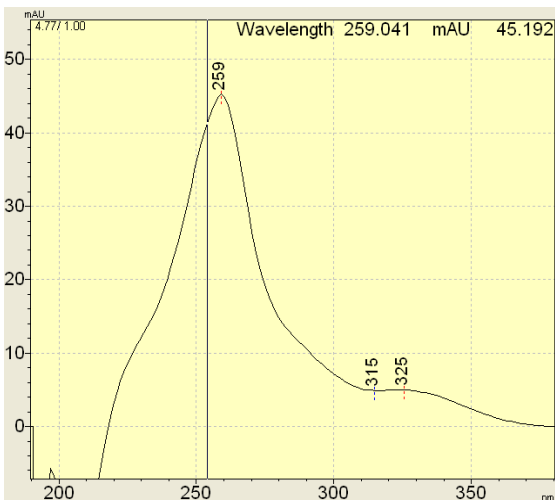
M. Genistein
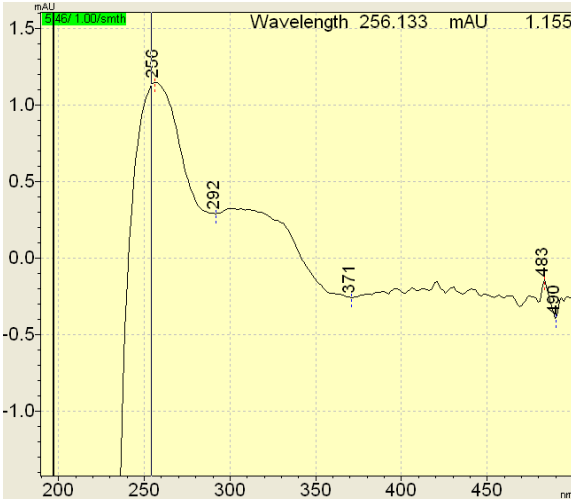
Daidzein

Glycitein
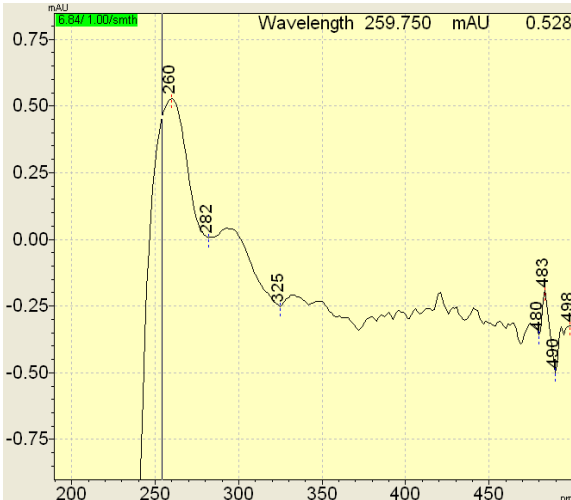
Genistein
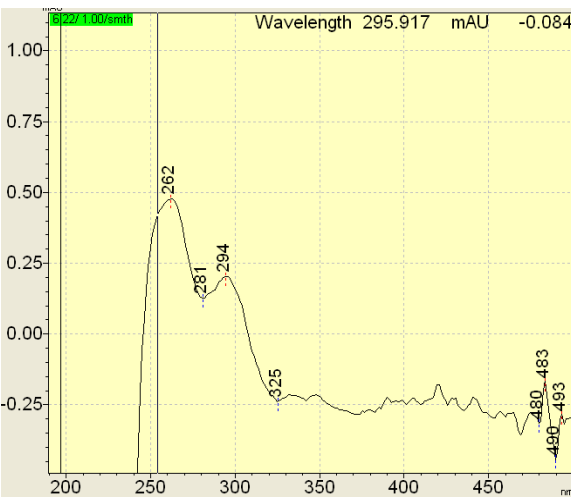
A. Glycitein

1.2.3 Statistical analysis

As a large set of data regarding various parameters was obtained, i.e., seed isoflavones and proteins, yields, and cotyledon weights in various varieties and under different managements, multivariate statistical analysis was used in order to identify common patterns in data distribution, and to reduce the number of variables by principal component analysis (PCA). These statistical methods facilitate interpretation (Castellano *et al.*, 2007) and are effective in identifying possible data adulteration (Perfetti *et al.*, 1988).

As well as analysis of variance, which was performed with Statgraphics statistical software (Statpoint, Herndon, VA, U.S.A), multivariate and cluster analysis (CA) was applied to the data, to describe characteristics of similarity among varieties concerning isoflavone production (MS Excel XLSTAT, Addinsoft, Paris, F). The behaviour of varieties was also described by CA in relation to different managements. In dendrograms, the maximum level of homogeneity within groups was calculated with the method of Calinski and Harabasz (1974) and robustness of cluster classification was tested through the stability of the cophenetic coefficient correlation (Sokal and Rohlf, 1962). Factorial discriminant analysis and PCA of data obtained by HPLC of Isoflavones were expressed as aglycone equivalents, in mg g^{-1}.

1.3 Results

The 12 isoflavones forms were analysed separately in cotyledons and hypocotyl, their contents were expressed in aglycone equivalents (AgE) as active or stored form (mg g^{-1}) and in percentage of total isoflavone in each seed fraction composition. Due to low concentration of aglycone and acetyl-conjugates forms they were not seen in all samples especially cotyledon one due to low concentration.

1.3.1 Organic and conventional management, and environment effect on isoflavone in hypocotyls and cotyledon

Year and variety illustrated significant effect on all isoflavone forms, groups and TISO in both cotyledon and hypocotyl (Table 1.4). Whereas management demonstrated significant effect on cotyledon and had no effect on hypocotyl. That reveal that cotyledon was more sensitive to management than hypocotyl. The interaction Y×V, Y×M and Y×M×V demonstrated significant effect on accumulation of isoflavone forms and groups in both seed portion. The interaction between variety and management did not show great difference great influence on cotyledon and hypocotyl except few cases that explained great influence on TISO in cotyledon. Aglycone group and TGS form in cotyledon mostly were affected by all factors and their interactions. Overall view of analysis of variance proved that hypocotyl less sensitive than cotyledon for management.

Table 1.4. Analysis of variance and significance for different isoflavones forms and groups in 4-year trial.

	Cotyledon						**Hypocotyls**						
	TISO	TDZ	TGS	GLY	AGL	MLN	TISO	TDZ	TGY	TGS	GLY	AGL	MLN
Year (Y)	**	***	***	***	**	***	*	***	ns	***	***	***	**
Var (V)	***	***	***	***	***	***	***	***	***	***	***	*	***
Man (M)	***	***	***	***	*	**	ns	ns	ns	ns	ns	ns	ns
Y×V	***	***	***	***	*	***	*	***	**	***	***	***	***
Y×M	*	**	**	**	*	**	*	***	*	***	***	*	*
V×M	ns	ns	*	ns	**	ns	ns	*	ns	ns	ns	ns	ns
Y×V×M	*	*	**	***	***	*	**	***	**	***	***	ns	***

Variety (Var); Mangement (Man); total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY); β-glycoside (GLY); Aglycone (AGL); Malonyl (MLN); *** P≤0.001; ** P≤0.01; * P≤0.05; ns= not significant. at Legnaro (n = 312; i.e., 13 soybean varieties, 2 managements, 4 years, 3 replicates).

1.3.1.1 Cotyledon

Within years cotyledon TISO and its forms showed significant variability (Figure 1.5). In 2008 cotyledon illustrated greatest accumulation of TISO and its both forms whereas 2006 and 2007 showed the lowest accumulation and 2005 showed moderate one. TDZ in 2006 and 2007 did not take the same tendency of TISO and TGS which may reveal the importance of environment on single difference within years comes from the interaction among different abiotic factors that form sort of different factors affect accumulation of isoflavone. Hoeck *et al*. (2000), Aussenac *et al*. (1998), Lozovaya *et al*. (2005), Seguin *et al*. (2004) and Tsukamoto *et al*. (1995) suggested that climatic and environmental conditions contributed to the differences among years.
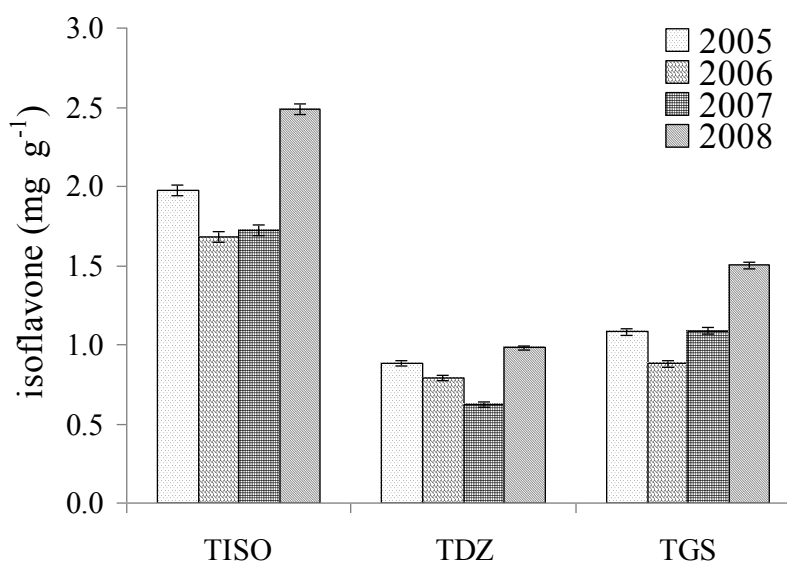
Figure 1.5. Mean of 13 variety cotyledon TISO, TDZ and TGS.

Cotyledon has showed greater accumulation of TGS compared to TDZ. Level of accumulation of both molecules varied regarding to varieties, which TGS 52-70% but TDZ 30-48%. The highest TGS% was *Nikko* and *Dekabig* but the lowest was *Regir*. Varieties can have ability to accumulate one molecule on favour of others, which they were able to favor genistein or daidzein (Berger *et al*., 2008). Interestingly in the cotyledon despite large differences in the contents, the percentages of each isoflavone were generally conserved among the growing conditions and were not correlated with total contents. The cotyledon isoflavone contents were highly genotype-dependent.

1.3.1.1.1 Cotyledon isoflavone variability under cultivation year

Ranks of varieties in different years regarding to their isoflavone contents were varied due to the difference in climate conditions and behaviours of varieties regarding to different climates. These results in consist with Hoeck *et al*. (2000) and Kitamura *et al*. (1991). However most, of varieties proved stability in their ranks above the different environmental conditions encountered, but a same variety could have a twofold cotyledon isoflavone content increase regarding to different environments (Table 1.5). That result in agree with Bergir *et al* (2008). The variation among varieties regardless the location and year 1-3 folds but single variety can vary 1-2 folds. The significant interactions were associated primarily with changes in rank and magnitude of differences among genotypes with intermediate and high isoflavone concentrations. Despite the significant interactions, the performance of the two genotypes with the highest and lowest mean total isoflavone concentration was relatively consistent among the 4 years.

Table 1.5. Mean cotyledon total isoflavone content and ranks of thirteen soybean varieties in 4 years.

Var	2005			2006			2007			2008		
	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R
Aires	2.30	ab	3	2.17	a	1	2.40	a	1	3.38	a	2
B63	2.01	b	9	2.01	a	3	2.26	a	2	3.57	a	1
Sponsor	2.37	a	2	1.92	ab	6	2.20	ab	3	2.50	bc	8
Hilario	2.38	a	1	1.95	ab	5	1.83	cd	6	2.75	b	3
Dekabig	2.12	ab	6	1.98	a	4	1.85	cd	5	2.57	bc	6
Demetra	2.14	ab	4	2.09	a	2	1.54	cde	9	2.69	b	4
Giulietta	2.10	ab	7	1.66	bc	7	1.87	bc	4	2.56	bc	7
Nikir	2.13	ab	5	1.53	cd	9	1.78	cd	7	2.58	bc	5
Cresir	2.06	b	8	1.53	cd	10	1.47	de	11	2.44	bc	9
Nikko	1.95	bc	10	1.59	cd	8	1.68	cd	8	2.30	cd	10
Regir	1.64	cd	11	1.30	de	11	1.53	cde	10	2.08	d	11
Brillante	1.36	de	12	0.98	f	13	1.16	ef	12	1.44	e	13
M10	1.09	e	13	1.11	ef	12	0.80	f	13	1.46	e	12

Variety (Var); rank (R); different small letters shows the difference between variety in each year.

Aires and B63 showed the highest content of TISO whereas *Brillante* and *M10* showed lowest content. However, the amount of isoflavone has showed only the capacity of variety in producing the isoflavone. There was no correlation between cotyledon weight and isoflavone concentration. The consistency of the ranking among genotypes for the contents of total and individual isoflavones seems to depend on the magnitude of the differences in their inherent genetic potential for the traits. These result in agree with Hoeck *et al*. (2000) and Seguin *et al*. (2004), they found out high and low TISO categories have the same tendency in different years and locations due to their magnitudes of the differences in their inherent genetic. Cotyledon TISO of single was showed variation of 1-3 folds regarding to year. Coefficient of variation was so high regarding to year and variety which may arrive to 35%.

TDZ showed good separation of three categories high, medium and low levels but TGS showed stability in low isoflavone concentration group and instability in ranking of medium and high isoflavone groups. Aires, B63 and sponsor showed mostly high ranking in different years regarding to TDZ, TGS and TISO (Table 1.6, 1.7). Moreover varieties with low TISO demonstrated stability in their ranks in different years regarding to TDZ and TGS. Varieties showed difference in their ranks regarding to their content of TDZ and TGS. However TDZ rank of varieties had almost the same ranks as TISO.

B63 showed 1-2 folds greater TISO than *Brillante* but their profile proved the same percentage of accumulation of both TDZ and TGS. Concentration of both molecules may differ but the profile could be the same due to similarity of metabolic pathway and difference in plant capacity of isoflavone production. These result in agree with Hoeck *et al*. (2000) and Seguin *et al*. (2004), they found out high and low TISO categories have the same tendency in different years and locations due to their magnitudes of the differences in their inherent genetic. TDZ can classify varieties into three categories high, medium and low TDZ. Medium and high varieties of TISO and TGS did not show big stability but mostly *Aires*, *B63* and *Sponsor* showed stability as high isoflavone category in some years. Cotyledon TDZ and TGS of single variety demonstrated 1-3 folds variation regarding to year.

Table 1.6. Mean total daidzein (TDZ) content and rank of thirteen soybean varieties in 4 years.

Var	2005			2006			2007			2008		
	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R
Aires	1.213	a	1	1.195	a	1	1.038	a	1	1.564	a	1
B63	0.964	bc	4	0.987	b	2	0.832	b	2	1.482	a	2
Hilario	1.176	a	2	1.025	b	4	0.695	bc	4	1.240	b	3
Sponsor	1.067	ab	3	0.936	b	5	0.830	b	3	1.010	cd	5
Demetra	0.981	bc	5	1.032	b	3	0.525	cde	8	1.153	bc	4
Giulietta	0.927	bcd	6	0.779	c	6	0.670	bcd	6	0.991	d	6
Cresir	0.918	cd	7	0.722	cd	7	0.553	cde	7	0.948	de	7
Nikir	0.902	cd	8	0.661	cde	9	0.532	cde	9	0.954	de	8
Dekabig	0.793	de	9	0.765	cd	8	0.666	bcd	5	0.777	f	10
Regir	0.751	ef	11	0.607	de	11	0.506	cde	10	0.822	ef	9
Nikko	0.711	ef	10	0.630	cde	10	0.490	de	11	0.697	fg	11
Brillante	0.625	f	12	0.449	f	13	0.433	ef	12	0.538	h	13
M10	0.480	fg	13	0.512	ef	12	0.264	f	13	0.554	gh	12

variety (Var); rank (R); different small letters shows the difference between variety in each year.

Table 1.7. Mean total genistein (TGS) content and rank of thirteen soybean varieties in 4 years.

Var	2005			2006			2007			2008		
	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R
B63	1.041	c	10	1.015	bc	3	1.404	a	1	2.066	a	1
Dekabig	1.320	a	1	1.214	a	1	1.174	abc	4	1.792	b	3
Aires	1.119	bc	6	0.968	bcd	4	1.349	ab	2	1.812	b	2
Sponsor	1.275	ab	4	0.976	bcd	5	1.353	ab	3	1.492	c	10
Nikko	1.224	ab	3	0.947	bcde	7	1.171	abc	7	1.602	bc	5
Giulietta	1.164	bc	9	0.882	cde	8	1.184	abc	5	1.566	c	8
Nikir	1.220	ab	2	0.867	de	9	1.084	bcd	8	1.625	bc	4
Demetra	1.153	bc	8	1.051	b	2	1.012	cd	10	1.533	c	7
Hilario	1.194	abc	5	0.922	bcde	6	1.109	abc	6	1.511	c	9
Cresir	1.129	bc	7	0.802	ef	10	0.930	cd	9	1.490	c	6
Regir	0.877	d	11	0.686	fg	11	0.988	cd	11	1.247	d	11
Brillante	0.731	de	12	0.535	h	13	0.782	de	12	0.898	e	12
M10	0.605	e	13	0.588	gh	12	0.537	e	13	0.908	e	13

variety (Var); rank (R); different small letters shows the difference between variety in each year.

1.3.1.1.2 Cotyledon isoflavone variability under cultivation year and management

Conventional management showed significant high value in all years except 2006 which organic management reveal higher value (Figure 1.6). Cultivation year has great effect on TISO that illustrated beside agronomic practices significant interaction (Table 1.8). Both TDZ and TGS showed the same tendency of TISO. However TGS ranking take the same tendency of TISO. TGS did not show great fluctuation except in 2008 which increased 25%. Wang and Murphy (1994) considered the year effects to be more important than location effects in their research. There were significant differences between managements in years (M×Y interaction)for total and individual isoflavone.
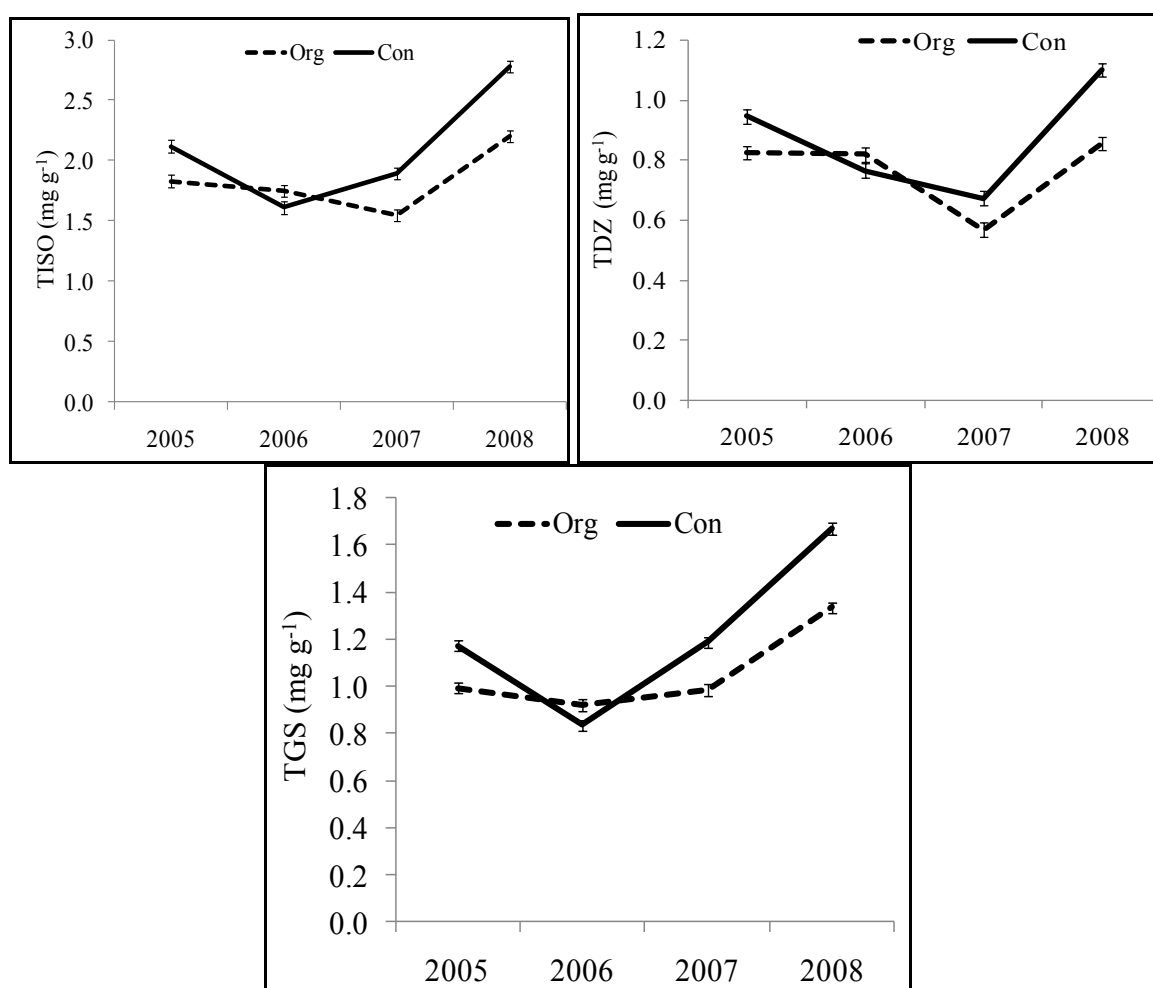
Figure 1.6. Interaction between environment and management effect on cotyledon isoflavone.

total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); organic (Org); conventional (Con); vertical bar represent standard error

Table 1.8. Mean cotyledon total isoflavone content and rank of thirteen soybean varieties under organic and conventional management in 4 years.

| | 2005 | | | | 2006 | | | | 2007 | | | | 2008 | | | |
| | Org | | Con | | Org | | Con | | Org | | Con | | Org | | Con | |
Var	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R
Air	2.21	3	2.40	4	2.27	2	2.06	2	1.96	2	2.83	1	3.26	2	3.50	2
B63	1.70	10	2.33	6	2.09	4	1.92	3	1.88	3	2.65	2	3.32	1	3.81	1
Spo	2.11	4	2.64	1	1.94	5	1.90	4	2.22	1	2.17	3	2.14	7	2.87	7
Dek	1.97	6	2.28	7	2.14	3	1.82	6	1.66	5	2.05	6	1.89	11	3.26	3
Hil	2.42	1	2.34	5	1.80	6	2.10	1	1.71	4	1.95	8	2.60	3	2.91	6
Dem	1.83	7	2.45	2	2.33	1	1.84	5	1.43	8	1.65	10	2.24	6	3.15	4
Giu	1.75	9	2.45	3	1.80	7	1.53	8	1.65	6	2.08	5	2.29	5	2.83	8
Nkr	2.08	5	2.19	8	1.61	10	1.46	9	1.46	7	2.10	4	2.11	8	3.05	5
Nko	1.79	8	2.08	9	1.63	9	1.55	7	1.38	10	1.97	7	1.97	9	2.64	9
Cre	2.27	2	1.85	10	1.65	8	1.42	10	1.39	9	1.56	11	2.41	4	2.48	10
Reg	1.49	11	1.78	11	1.25	11	1.35	11	1.28	12	1.77	9	1.92	10	2.24	11
Bri	1.23	12	1.49	12	0.98	13	0.99	12	1.30	11	1.02	12	1.34	12	1.54	13
M10	0.93	13	1.25	13	1.23	12	0.98	13	0.76	13	0.84	13	1.07	13	1.85	12
LSD	0.31		0.42		0.51		0.41		0.64		0.74		0.63		0.49	

ranks (R); organic (Org); conventional (Con), variety (Var).

Three way interaction indicates that the magnitude of differences between varieties and their ranking varied regarding to management × year. There was 50% variation in average of TISO across management and this variation arrived between 20-100%. The interaction described relative stability regarding to significant effect due to the variability of varieties behaviours under both managements. Most of varieties showed higher accumulation under conventional management. In our study cotyledon TISO ranged 0.7 to 4.0 mg/g and average 1.96 mg/g across environment and management. Ranking of varieties under different managements and years were used to estimate their division (Table 1.9). Figure (1.7) demonstrated two different cases first using absolute values or ranks form three categories of varieties respond to three group of isoflavone concentration. Second cases ranks alone showed near relation between medium and low isoflavone but absolute values showed near relation between high and medium isoflavone groups. That confirms the categories of varieties are stable and results in three different groups.

Low level isoflavone contents varieties proved greater stability in their ranks than high and medium isoflavone contents varieties. Few varieties were point up significant response for the effect of management. *Cresir* showed greater TDZ accumulation under

organic management, However Conventional management showed higher tendency for most of varieties.

Table 1.9. Mean cotyledon total isoflavone content and rank of thirteen soybean varieties under organic and conventional management in 4 years.

TDZ	2005				2006				2007				2008			
	Org		Con		Org		Con		Org		Con		Org		Con	
Var	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R
Air	1.07	2	1.34	1	1.25	1	1.14	1	0.86	1	1.21	1	1.50	1	1.63	1
B63	0.83	7	1.10	5	1.03	3	0.95	3	0.72	3	0.95	2	1.39	2	1.57	2
Hil	1.21	1	1.15	3	0.93	5	1.12	2	0.71	4	0.68	5	1.14	3	1.34	4
Spo	0.95	4	1.18	2	0.97	4	0.90	5	0.86	2	0.81	3	0.82	7	1.20	5
Dem	0.85	6	1.11	4	1.15	2	0.92	4	0.41	10	0.64	7	0.92	5	1.39	3
Giu	0.78	9	1.08	6	0.82	6	0.74	6	0.61	6	0.73	4	0.86	6	1.13	7
Nkr	0.88	5	0.92	7	0.68	9	0.64	10	0.45	9	0.58	10	0.77	8	1.14	6
Dek	0.80	8	0.79	10	0.79	7	0.72	7	0.65	5	0.68	6	0.56	11	1.00	8
Reg	0.68	10	0.83	8	0.56	12	0.65	9	0.39	11	0.63	8	0.75	9	0.89	10
Cre	1.04	3	0.80	9	0.79	8	0.66	8	0.51	8	0.57	11	0.94	4	0.96	9
Nko	0.66	11	0.75	11	0.64	10	0.62	11	0.38	12	0.59	9	0.60	10	0.79	11
Bri	0.58	12	0.67	12	0.44	13	0.46	12	0.54	7	0.36	12	0.49	12	0.58	13
M10	0.39	13	0.57	13	0.58	11	0.45	13	0.25	13	0.28	13	0.39	13	0.72	12
LSD	*0.31*		*0.42*		*0.51*		*0.41*		*0.64*		*0.74*		*0.63*		*0.49*	
TGS	2005				2006				2007				2008			
	Org		Con		Org		Con		Org		Con		Org		Con	
Var	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R
Dek	1.16	4	1.48	1	1.31	1	1.10	1	0.99	8	1.08	9	1.33	8	2.26	1
B63	0.86	10	1.22	7	1.07	3	0.96	4	1.15	2	0.65	12	1.92	1	2.21	2
Air	1.11	6	1.13	9	1.02	4	0.92	6	1.09	3	0.56	13	1.76	2	1.86	4
Nko	1.12	5	1.33	5	0.98	5	0.91	7	0.98	10	1.36	3	1.37	6	1.84	5
Spo	1.11	7	1.44	2	0.97	7	0.99	2	1.36	1	1.70	1	1.32	10	1.67	8
Giu	0.97	9	1.36	3	0.97	6	0.79	9	1.03	4	1.26	7	1.43	5	1.71	7
Nkr	1.19	3	1.25	6	0.92	8	0.81	8	1.01	6	1.36	4	1.34	7	1.91	3
Dem	0.97	8	1.33	4	1.18	2	0.93	5	1.02	5	1.13	8	1.32	9	1.75	6
Hil	1.21	2	1.18	8	0.87	9	0.97	3	0.99	9	1.33	6	1.45	4	1.57	9
Cre	1.22	1	1.04	10	0.86	10	0.74	10	0.81	12	1.01	10	1.47	3	1.51	10
Reg	0.80	11	0.95	11	0.69	11	0.68	11	0.89	11	1.61	2	1.15	11	1.34	11
Bri	0.64	12	0.82	12	0.54	13	0.53	12	0.99	7	0.97	11	0.84	12	0.95	13
M10	0.53	13	0.68	13	0.65	12	0.53	13	0.51	13	1.35	5	0.68	13	1.13	12
LSD	*0.21*		*0.26*		*0.25*		*0.20*		*0.45*		*0.45*		*0.38*		*0.30*	

total daidzein (TDZ); total genistein (TGS); ranks (R); organic (Org); conventional (Con); variety (Var).
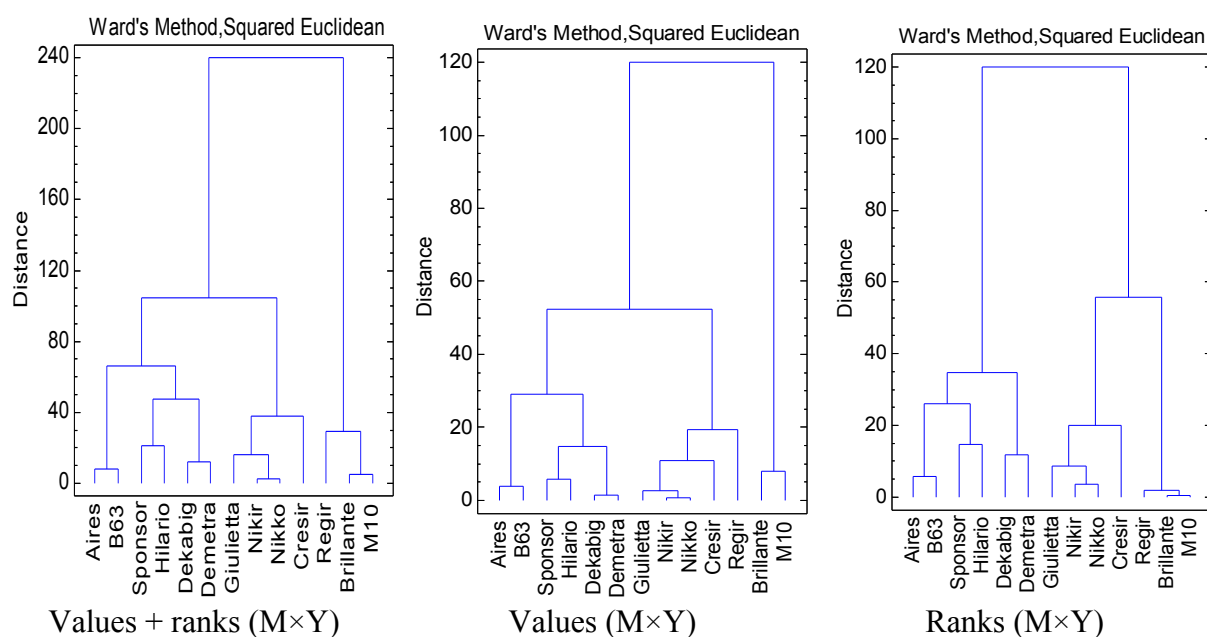
Figure 1.7. Cluster analysis of different varieties regarding to total isoflavone and ranks of varieties in 4 years.

Cluster analysis utilizing TISO cotyledon data of 4 years confirm the presence of three groups of isoflavone level varieties (Figure 1.6). Either ranks or values confirm the same hypothesis; the same result obtained applying cluster analysis on TDZ and TGS.

1.3.1.1.3 Cotyledon isoflavone conjugates variability under different varieties

Profile of cotyledon of different varieties did not great differences among varieties (Figure 1.8).

Aglycone equivalent forms (Aglycone, β-Glycone and Malonyle) in cotyledon showed the same tendency of TISO. The differences among varieties came from their content of isoflavone. Conjugated form of isoflavone, Malonyl 70-75% and β-glycone 23-28% of total aglycone form but free aglyconic form represent less than 2% (Figure 1.9). Different isoflavone group showed significant difference due to variety, management and the year but that difference was due to capacity of each variety. The percentages of different forms were varied among varieties and year and between both management. Malonyl form showed the highest portion of accumulated isoflavone in seed cells 65-73% followed by β-Glycone form 25-32% and the rest was in Aglycone form. Many varieties

showed the same percentage of different group which confirm that isoflavone pathway almost the same but the capacity of each variety depends on its genetic background. Furthermore, plant detoxification of isoflavone in seed cells has the same trend although of the difference in the concentration of isoflavone.
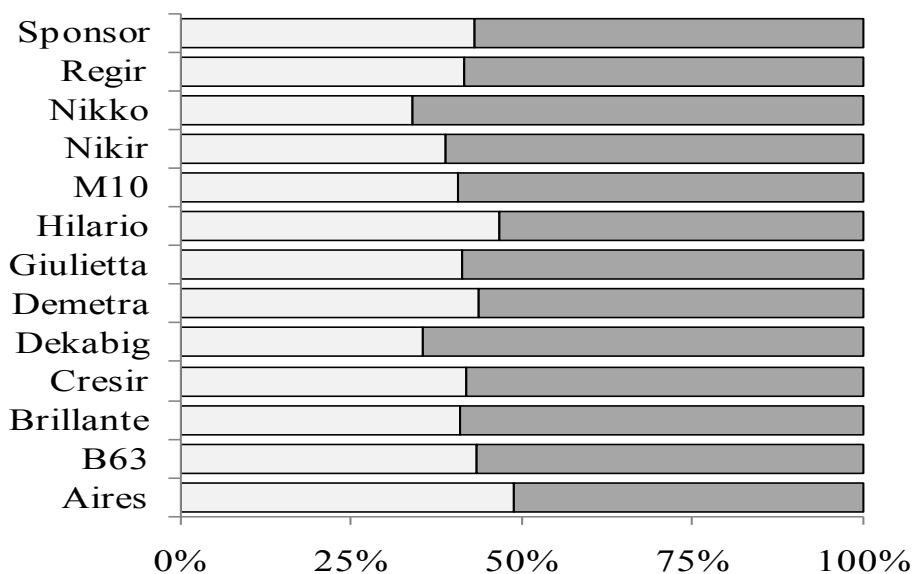
Figure 1.8. Cotyledon profile of different varieties.

■ TDZ% (total daidzein) ■ TGS% (total genistein)
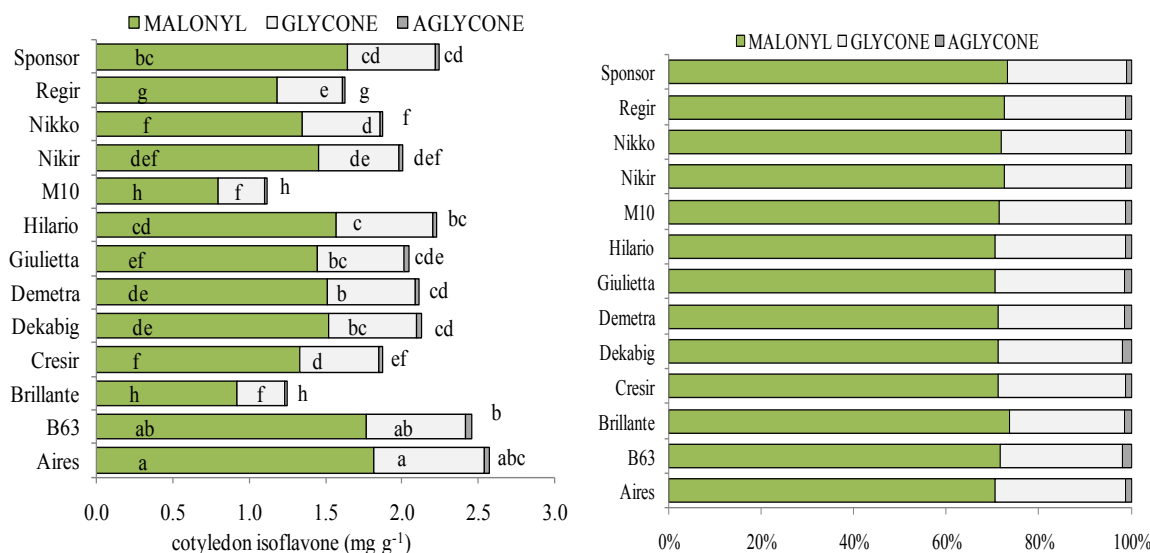
Figure 1.9. Conjugated and free isoflavone of different varieties and their profile (%).

Different letters represent the significance among the variety per each isoflavone form

1.3.1.2   Hypocotyl

TDZ varied among variety representing 35-58% and TGY represent 25-50% of but TGS accumulation in the cell take apportion of 10-12% of total isoflavone and it is positively correlated with other two fraction TDZ and TGY (Figure 1.10). Vareities illustrated preference in their accumulation of TDZ or TGY, although they are positively correlated in general but some varieties such as *Nikko* and *Cresir* showed high TGY accumulation (Bergeir *et al*., 2008). The hypocotyl isoflavone percentages are constant throughout the growing conditions and but highly taut among varieties, most of variety has more than 50% TDZ but *Cresir* and *Nikko* showed high glycitein percentage (≈55%).
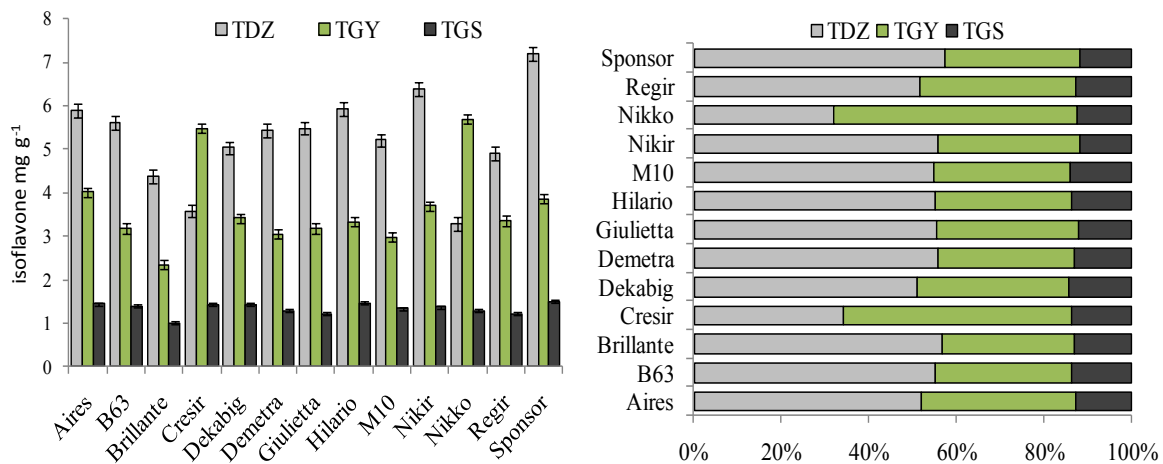
Figure 1.10. Aglycone equivalent form of different varieties and their profile (%).

total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY).
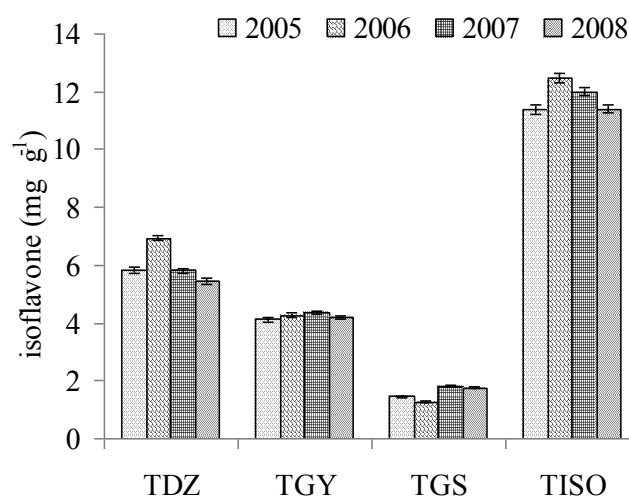
Figure 1.11. Effect of cultivation year on hypocotyl isoflavone.

Total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY), vertical bar represent standard error.

Accumulation of TISO and different isoflavone forms significantly influenced by different cultivation season due to variability of temperature and rainfall and the complex of other abiotic factors (Figure 1.11). TISO, TDZ and TGY illustrated their highest accumulation in 2006 while the lowest accumulation was in 2008. In general 2008 showed the lowest isoflavone level in hypocotyls but 2006 showed the highest accumulation of isoflavone. However, TGS showed it lower value in 2006 which may be due to excessive accumulation of TDZ.

1.3.1.2.1 Hypocotyl isoflavone variability in different years

TISO in hypocotyl was revealed the same tendency of cotyledon for overall analysis and in each year (Table 1.10). Low isoflavone concentration in hypocotyl varieties showed high stability in their ranking where as the difference among years was up to 30%. *M10*, *Regir* and *Brillante* were the lowest accumulation of TISO; they maybe selected for infants food gradients. However, high and medium TISO concentrations were demonstrated fluctuation in their ranking. Certain varieties were showed stable performance of high which, *Sponsor* and *Aires* can be accepted for high isoflavone level compared to other varieties. Fluctuation in TISO may arrive to 30% in both high and medium TISO varieties. Sponsor hypocotyl gave the highest TISO value in all years and its highest value was 16.58 mg g^{-1} in 2006. *Brillante* proved the lowest value in all years and its lowest value 8.48 mg g^{-1} in 2005. Since the ranks of different varieties more or less constant so the magnitude of TISO depends on genetic capacity and environment. The interaction between variety and environment is demonstrated in form of fluctuation of ranks of some varieties. B63 cotyledon showed high concentration of TISO but in hypocotyl level proved modest concentration. However, all low level cotyledon isoflavone concentration varieties showed low level in hypocotyl. These results confirm that low isoflavone varieties were less fluctuated regarding to genetic and environment.

Two way interactions (Y×V) was verified significant effect on accumulation of TDZ and TGS in hypocotyl (Table 1.11). Changes of varieties ranks in different years cause significant interaction. TDZ in hypocotyl proved the same tendency of TISO. *Sponsor*, *Nikir* and *Aires* can be considered high and constants source of TISO and TDZ but for low level TDZ form there is interfere of new varieties with lowest level of TDZ (*Cresir* and

Nikko) with very high stability in its rank. *Brillante* hypocotyl TDZ was in the same ranks within years which reveal its stability.

Table 1.10. Mean hypocotyl total isoflavone and rank of thirteen soybean varieties in 4 years.

Var	2005			2006			2007			2008		
	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R	mg g^{-1}		R
Spo	13.59	a	1	16.58	a	1	14.22	a	1	13.61	a	1
Nik	13.03	ab	3	13.10	bc	4	14.19	a	2	12.50	ab	2
Air	13.14	ab	2	13.66	b	2	12.11	bc	5	12.36	ab	3
Cre	11.48	cde	6	12.56	bcd	6	12.92	ab	3	12.29	ab	4
Hil	11.97	bcd	5	13.17	bc	3	11.69	bc	7	12.09	b	5
Nko	12.20	bc	4	12.27	cd	7	11.38	bc	10	11.11	bcde	9
Dek	11.45	cde	7	12.83	bc	5	11.60	bc	9	11.32	bcde	8
B63	11.28	cdef	8	12.17	cd	8	11.61	bc	8	11.54	bcd	7
Giu	11.04	def	9	11.48	d	12	12.02	bc	6	10.63	cde	10
Dem	10.27	fg	11	11.56	d	10	12.42	bc	4	10.20	def	11
M10	9.44	gh	12	11.51	d	11	11.36	bc	11	11.79	bc	6
Reg	10.79	ef	10	11.64	d	9	10.80	cd	12	10.00	ef	12
Bri	8.48	h	13	9.72	e	13	9.05	d	13	8.82	f	13

variety (Var); rank (R); different small letters shows the difference between variety in each year.

TGS verified very high fluctuation in ranking of varieties in different years. Genistein in hypocotyl genetically controlled of different varieties regarding to climatic conditions.

Glycitein considered a special characteristic for hypocotyl and it represent 25-50% of total isoflavone in hypocotyl. Glycitein pathway comes up from daidzein and it cause changes in order of varieties. *Nikko* and *Cresir* were demonstrated the highest significant value of TGY where as low TISO varieties showed the lowest value. TGY showed great stability in different years with fluctuation of 10% only. Order of varieties was invariable with very slight changes (Table 1.12). From these result, varieties showed its special profile of forming different isoflavone forms independently from the environment or management system. TGY can be considered as an indicator or special ranking indicator.

Table 1.11. Mean hypocotyl total daidzein and genistein and ranking of thirteen soybean varieties in 4 years.

TDZ	2005		2006		2007		2008	
Var	mg g^{-1}	R	mg g^{-1}	R	mg g^{-1}	R	mg g^{-1}	R
Spo	7.64 a	1	10.26 a	1	7.80 a	1	7.34 a	1
Nkr	7.27 ab	2	7.91 b	3	7.46 ab	2	6.64 ab	2
Hil	6.54 cd	4	7.93 b	2	5.98 cd	7	6.34 bc	3
Aires	6.86 bc	3	7.69 bc	4	6.35 bcd	5	5.42 de	8
B63	6.23 cde	5	7.27 cd	5	5.97 cd	8	6.04 bcd	5
Giu	6.17 cdef	6	6.87 d	8	6.39 bcd	4	5.47 de	7
Dem	5.50 fg	9	6.63 de	10	6.95 abc	3	5.49 cde	6
M10	4.96 g	10	6.88 d	7	6.04 cd	6	6.07 bcd	4
Dek	5.85 def	7	7.06 cd	6	5.79 de	9	5.33 de	9
Reg	5.76 ef	8	6.64 de	9	4.66 ef	10	5.07 e	10
Bri	4.90 g	11	6.02 e	11	4.39 fg	11	4.76 e	11
Cre	4.17 h	12	4.62 f	12	4.01 fg	12	3.74 f	12
Nko	3.96 h	13	4.42 f	13	3.37 g	13	3.10 f	13

TGS	2005		2006		2007		2008	
Var	mg g^{-1}	R	mg g^{-1}	R	mg g^{-1}	R	mg g^{-1}	R
Giu	1.29 f	12	1.06 gh	7	1.76 bcde	8	1.57 ef	2
Hil	1.59 abc	3	1.38 bc	6	1.96 abc	9	1.92 abc	6
Air	1.73 a	1	1.49 ab	1	1.59 de	7	1.84 abcd	12
B63	1.49 bcd	4	1.24 def	4	1.96 abc	10	1.81 abcd	4
Dek	1.54 bcd	6	1.38 bcd	9	1.90 abc	12	1.89 abc	10
Reg	1.31 ef	11	1.17 fg	2	1.72 cde	5	1.51 ef	7
Cre	1.52 bcd	5	1.34 cde	10	2.01 ab	1	2.04 a	13
Nkr	1.47 bcde	7	1.17 fg	5	2.15 a	13	1.69 cde	3
M10	1.38 def	10	1.20 ef	8	1.77 bcd	4	1.96 ab	1
Nko	1.42 cdef	9	1.19 fg	3	1.73 cde	3	1.73 bcde	8
Spo	1.60 ab	2	1.57 a	13	1.98 abc	6	1.90 abc	9
Dem	1.45 bcdef	8	1.30 cdef	12	1.60 de	2	1.63 de	5
Bri	1.05 g	13	0.95 h	11	1.48 e	11	1.36 f	11

total daidzein (TDZ); total genistein (TGS); variety (Var); rank (R); different small letters shows the difference between variety in each year.

Table 1.12. Hypocotyl total glycitein and ranking of thirteen soybean varieties in 4 years.

Var	2005 mg g^{-1}	R	2006 mg g^{-1}	R	2007 mg g^{-1}	R	2008 mg g^{-1}	R
Nko	6.82 a	1	6.66 a	1	6.27 a	2	6.28 a	2
Cre	5.79 b	2	6.60 a	2	6.89 a	1	6.51 a	1
Air	4.56 c	3	4.48 b	4	4.17 bcde	6	5.10 b	3
Spo	4.36 cd	4	4.75 b	3	4.45 bc	4	4.37 c	4
Nik	4.29 cd	5	4.02 cd	6	4.58 b	3	4.17 cd	5
Dek	4.05 cde	6	4.39 bc	5	3.91 bcdef	7	4.10 cd	6
Hil	3.85 def	7	3.86 de	7	3.75 cdef	10	3.84 cde	7
Reg	3.72 ef	8	3.83 de	8	4.43 bcd	5	3.43 ef	11
Giu	3.59 efg	9	3.55 ef	11	3.87 bcdef	9	3.59 def	10
B63	3.56 efg	10	3.65 def	9	3.68 def	11	3.68 de	9
Dem	3.32 fg	11	3.63 def	10	3.87 bcdef	8	3.08 fg	12
M10	3.10 g	12	3.42 f	12	3.54 ef	12	3.75 de	8
Bri	2.52 h	13	2.75 g	13	3.18 f	13	2.70 g	13

variety (Var); rank (R); different small letters shows the difference between variety in each year.

1.3.1.2.2 Hypocotyl isoflavone variability under cultivation year and management

The reason of having significant three way interaction was the differences in variety performance due to environment variation and both managements (Table 1.13). *Sponsor*, *Nikir*, *Brillante*, *Cresir* and *Nikko* showed noticed stability in their TDZ content ranks due to stability of their performance under different environments and both managements (Table 1.14). TGS concentration was illustrated separation of low level of TGS varieties, which they were constant among the year and management. However TGS fluctuated in varieties had high and medium level of TGS forming instable ranking. TGS is not influenced by management at hypocotyl level. No relation was observed between seed or hypocotyl weight with different isoflavone forms and TISO.

Low and high level TISO were demonstrated stability within the year and management in their ranks. The slight difference in ranks was responsible on the significance of three level interactions (Y×M×V). Medium level of TISO varieties were

illustrated instability on their ranks compared with high and low level TISO. *Cresir* is a modest level TISO variety showed higher accumulation of TISO under organic management compared to conventional. That characteristic was not noticed on other varieties which showed high tendency of conventional compared to organic or similarity. The effect of variety and environment was greater than type of management.

Table 1.13. Hypocotyl total isoflavone ranking of thirteen soybean varieties in 4 years and both management.

	2005				2006				2007				2008			
	Org		Con		Org		Con		Org		Con		Org		Con	
Var	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R
Spo	13.1	1	14.1	1	16.9	1	16.3	1	13.8	2	14.7	1	14.4	1	12.8	2
Nkr	12.4	2	13.6	3	12.6	7	13.6	2	15.6	1	12.6	5	12.1	5	12.9	1
Air	12.3	3	14.1	2	15.0	2	12.4	7	11.8	8	12.5	6	14.1	2	10.6	8
Hil	11.2	7	12.7	4	13.0	5	13.3	3	11.9	7	11.4	9	12.4	4	11.8	4
Dek	11.7	6	11.2	9	13.2	4	12.5	5	12.0	6	11.2	11	10.3	10	12.4	3
B63	10.5	9	12.0	6	11.8	10	12.5	4	12.5	4	10.8	12	11.8	7	11.3	7
Nko	12.1	4	12.3	5	12.2	8	12.4	6	11.5	9	11.3	10	11.7	8	10.6	10
Cre	11.8	5	11.2	8	14.3	3	10.8	11	13.1	3	12.8	3	13.2	3	11.4	6
Giu	10.4	10	11.7	7	11.6	12	11.4	9	12.3	5	11.8	7	10.7	9	10.6	9
Dem	10.4	11	10.2	11	12.1	9	11.0	10	11.3	10	13.5	2	9.9	12	10.5	11
Reg	10.8	8	10.8	10	11.6	11	11.7	8	8.9	13	12.8	4	10.2	11	9.8	12
M10	8.7	12	10.2	12	12.7	6	10.4	12	11.2	11	11.5	8	11.9	6	11.7	5
Bri	8.3	13	8.7	13	9.9	13	9.6	13	9.1	12	9.1	13	9.4	13	8.3	13
LSD	*1.91*		*1.35*		*1.81*		*1.35*		*2.68*		*2.45*		*1.45*		*2.66*	

ranks (R); variety (Var); organic (Org); conventional (Con)

Management did not show significant difference on TGY concentration in different years but the slight changes in order of varieties reveal Y×M×V significance effect on TGY (Table 1.15). *Cresir* and *Nikko* had high value significant of TGY compared to other varieties regardless the year or management. Accumulation of TGY showed greater stability than accumulation of TDZ or TGS. The difference among years of single variety was up to 25% but the difference between managements in the same year for single variety

did not exceed 10%. The major isoflavone percentage in each seed part was strongly dependent on the variety.

Table 1.14. Hypocotyl total daidzein and genistein ranking of thirteen soybean varieties in 4 years and both management.

TDZ	2005				2006				2007				2008			
	Org		Con		Org		Con		Org		Con		Org		Con	
Var	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R
Spo	7.37	1	7.91	1	10.47	1	10.05	1	7.58	2	8.01	1	7.88	1	6.80	1
Nkr	6.99	2	7.55	2	7.62	5	8.20	2	8.31	1	6.52	4	6.52	3	6.76	2
Hil	6.10	4	6.98	4	7.92	3	7.94	3	5.83	8	6.12	7	6.46	4	6.21	3
B63	5.88	6	6.58	5	7.06	8	7.48	4	6.47	4	5.48	10	6.23	5	5.86	5
Dem	5.74	9	5.27	9	7.29	6	5.98	10	5.90	7	8.00	2	5.24	9	5.74	6
Aires	6.67	3	7.09	3	8.77	2	6.62	7	5.56	9	7.14	3	6.81	2	4.03	11
Giu	5.85	7	6.49	6	6.97	9	6.77	6	6.58	3	6.20	6	5.50	7	5.45	8
Dek	6.03	5	5.68	8	7.26	7	6.87	5	6.02	5	5.55	9	4.92	11	5.74	7
Reg	5.76	8	5.77	7	6.70	10	6.59	8	2.88	13	6.45	5	5.25	8	4.89	9
M10	4.80	10	5.12	10	7.68	4	6.09	9	5.97	6	6.12	8	6.15	6	5.99	4
Bri	4.79	11	5.01	11	6.18	11	5.86	11	3.99	11	4.90	11	5.05	10	4.46	10
Cre	4.71	12	3.62	13	5.48	12	3.77	13	4.14	10	3.96	12	4.06	12	3.42	12
Nko	3.99	13	3.94	12	4.55	13	4.29	12	3.46	12	3.29	13	3.31	13	2.88	13
LSD	*1.30*		*0.72*		*1.04*		*0.80*		*2.06*		*1.27*		*0.72*		*1.55*	

TGS	2005				2006				2007				2008			
	Org		Con		Org		Con		Org		Con		Org		Con	
Var	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R	mg/g	R
Spo	1.48	3	1.71	2	1.55	1	1.58	2	1.85	7	2.10	1	2.03	5	1.78	5
Dek	1.52	2	1.57	7	1.37	3	1.39	5	1.97	5	1.83	5	1.80	8	1.99	1
Hil	1.58	1	1.59	4	1.27	5	1.50	3	2.09	3	1.82	6	2.05	4	1.78	4
Cre	1.48	4	1.55	9	1.43	2	1.24	8	2.03	4	2.00	2	2.26	1	1.81	3
Nkr	1.34	8	1.60	3	1.11	11	1.24	7	2.32	1	1.97	3	1.67	9	1.71	7
Air	1.45	5	2.02	1	1.35	4	1.62	1	1.87	6	1.32	13	2.19	2	1.49	11
B63	1.39	7	1.58	6	1.18	7	1.30	6	2.12	2	1.81	8	1.88	6	1.74	6
M10	1.21	12	1.56	8	1.27	6	1.14	11	1.73	11	1.81	7	2.09	3	1.83	2
Dem	1.32	9	1.58	5	1.16	8	1.44	4	1.79	9	1.42	12	1.67	10	1.60	9
Nko	1.42	6	1.42	10	1.14	9	1.24	9	1.74	10	1.72	10	1.82	7	1.64	8
Reg	1.28	10	1.34	12	1.11	10	1.22	10	1.49	12	1.94	4	1.60	12	1.42	12
Giu	1.22	11	1.36	11	1.08	12	1.04	12	1.80	8	1.73	9	1.61	11	1.53	10
Bri	1.00	13	1.11	13	0.91	13	0.99	13	1.42	13	1.56	11	1.47	13	1.25	13
LSD	*0.30*		*0.17*		*0.25*		*0.15*		*0.39*		*0.42*		*0.26*		*0.42*	

total daidzein (TDZ); total genistein (TGS); organic (Org), conventional (Con), variety (Var); ranks (R).

Table 1.15. Hypocotyl total glycitein ranking of thirteen soybean varieties in 4 years and both management.

VAR	2005 Org mg/g	R	2005 Con mg/g	R	2006 Org mg/g	R	2006 Con mg/g	R	2007 Org mg/g	R	2007 Con mg/g	R	2008 Org mg/g	R	2008 Con mg/g	R
Nko	6.73	1	6.90	1	6.47	2	6.86	1	6.30	2	6.25	2	6.53	2	6.03	2
Cre	5.56	2	6.03	2	7.39	1	5.81	2	6.92	1	6.86	1	6.89	1	6.12	1
Air	4.16	5	4.95	3	4.84	4	4.12	6	4.33	6	4.00	7	5.10	3	5.11	3
Spo	4.22	3	4.50	4	4.86	3	4.65	3	4.35	5	4.55	3	4.53	4	4.20	6
Nkr	4.12	6	4.46	5	3.90	6	4.13	5	5.01	3	4.15	5	3.94	5	4.39	5
Dek	4.16	4	3.95	7	4.55	5	4.25	4	3.99	8	3.84	8	3.56	9	4.63	4
Reg	3.72	7	3.72	10	3.80	8	3.86	8	4.50	4	4.36	4	3.37	11	3.48	11
Hil	3.57	8	4.12	6	3.83	7	3.89	7	4.01	7	3.48	12	3.88	6	3.80	8
Giu	3.36	9	3.82	9	3.53	12	3.56	11	3.90	9	3.83	9	3.55	10	3.63	10
B63	3.27	11	3.86	8	3.56	11	3.75	9	3.90	10	3.48	11	3.66	7	3.71	9
Dem	3.31	10	3.33	12	3.66	10	3.60	10	3.65	12	4.08	6	3.00	12	3.16	12
M10	2.70	12	3.50	11	3.71	9	3.13	12	3.48	13	3.60	10	3.66	8	3.84	7
Bri	2.48	13	2.57	13	2.79	13	2.71	13	3.74	11	2.64	13	2.84	13	2.56	13
LSD	*0.88*		*0.69*		*0.60*		*0.51*		*1.38*		*0.87*		*0.68*		*1.01*	

variety (Var); rank (R); organic (Org); conventional (Con).

1.3.1.2.3 Hypocotyl isoflavone conjugates variability under different varieties

Seed conservation of isoflavone was mainly focused on Malonyl and β-Glycoside form which are the main detoxification of this polyphenols components in soybean (Figure 1.12). The difference among year has showed the same tendency of active forms.

Figure 1.12. conjugated and free aglycone forms and profile of varieties.

1.3.2 Classification of varieties based on their isoflavone contents in cotyledon and hypocotyl

Different isoflavone forms and groups data were used to realize cluster formation (Figure 1.13). Regarding to the result obtain by Ward's cluster varieties were showed three groups of changing regarding to management and year of cultivation. Under biology and conventional management *Regir*, *M10* and *Brillante* demonstrated stability in both management and their relation with each other on the side there were many very close relation between two varieties *Demetra* and *Giulietta*; *Aires* and *B63*; *Cresir* and *Nikko*; *Sponsor* and *Nikir*. Some variety can be considered sensitive to changes in their composition regarding to management such as *Hilario* and *Dekabig*. Another type of classification based on TISO contents in hypocotyl and ranks of (Figure 1.14). Regarding to that classification two groups' can be distinguished first high and second medium to low isoflavone contents.
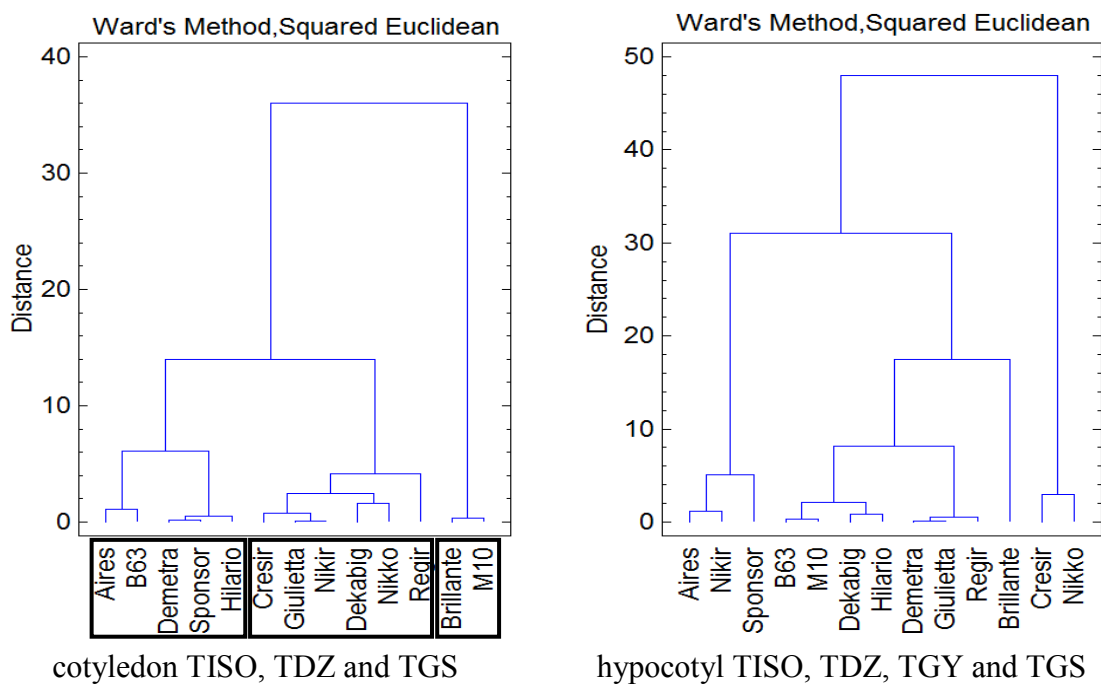
Figure 1.13. Varieties cluster analysis based on absolute values of isoflavone.

Principal components analysis demonstrated its ability to use % of different molecule and absolute TISO of both cotyledon and hypocotyl and simplify them in table (1.17). Profile component were used in principal component analysis in order to put different

variables in smaller number of factors regarding to their importance. In this case, 4 components have been extracted, since they had eigenvalues greater than 1. Together they account for 85.3% of the variability in the original data. PC1 combined TDZ% and TGY% of hypocotyl showing their relevant importance in classifying variety as demonstrated before. TISO of hypocotyl and TDZ% in cotyledon were showed relevant effect on PC2, whereas PC3 demonstrated importance of cotyledon TGS%. Cotyledon TISO and TGS% were proved a contribution to PC4.
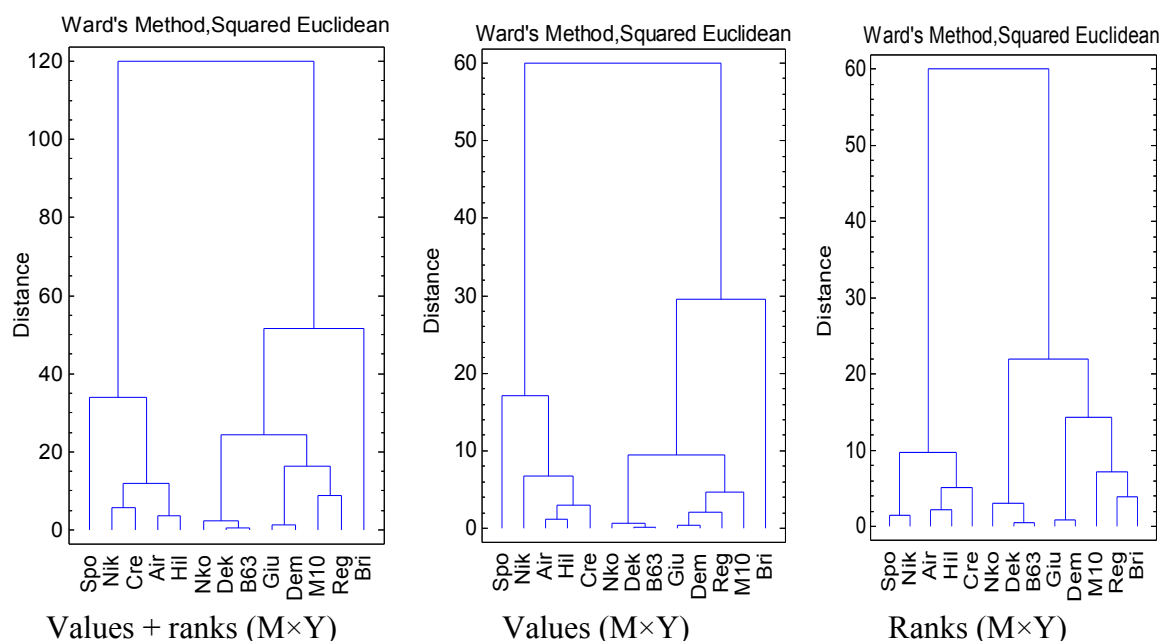
Figure 1.14. Ranks of variety based on hypocotyl total isoflavone contents and ranks of different varieties.

Table 1.16. Principal component weight of different variables of cotyledon and hypocotyl.

		PC1	PC2	PC3	PC4
Cotyledon	**TDZ%**	0.250	**0.600**	-0.357	-0.015
	TGS%	-0.253	0.383	**-0.633**	0.101
	TISO	-0.067	0.227	0.393	**0.740**
Hypocotyl	**TDZ%**	**0.624**	-0.133	-0.130	0.160
	TGY%	**-0.557**	0.217	0.226	-0.337
	TGS%	-0.392	-0.244	-0.286	**0.548**
	TISO	0.125	**0.564**	0.409	0.053

Principal components (PC), total isoflavone (TISO); total daidzein% (TDZ%); total genistein% (TGS%); total glycitein% (TGY%)

1.3.3 The relation between isoflavone composition in cotyledon and hypocotyl with environment conditions

Remarkably the cotyledon and hypocotyl did not show strong correlation. Thus yield and protein did not show significant correlation with isoflavone contents of both seed sites

Minimum temperature was connected with total isoflavone in cotyledon which showed the same tendency within years (Figure 1.15). Low temperature is the most connected climate variable with isoflavone contents. It increase accumulation of isoflavone but extremely low temperature caused reduced isoflavone accumulation in 2006 and 2007. Cotyledon considered more sensitive to low temperature since accumulation of isoflavone accumulation begins when a plateau was reached in the hypocotyl. High temperature had strong correlation with isoflavone accumulation in both seed sites. Negative correlation observed between cotyledon isoflavone and maximum daily temperature and daily range temperature (Figure 1.16).
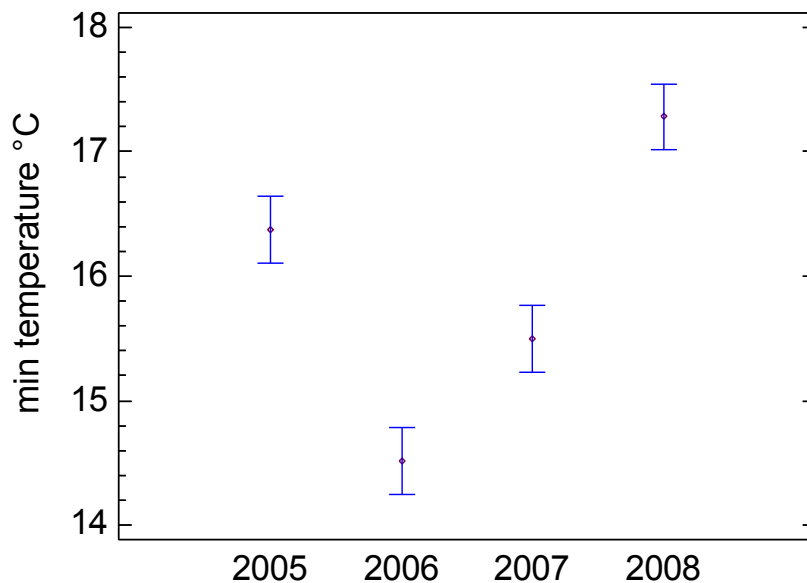
Figure 1.15. Mean of 20 days minimum temperature after 40 days of flowering.
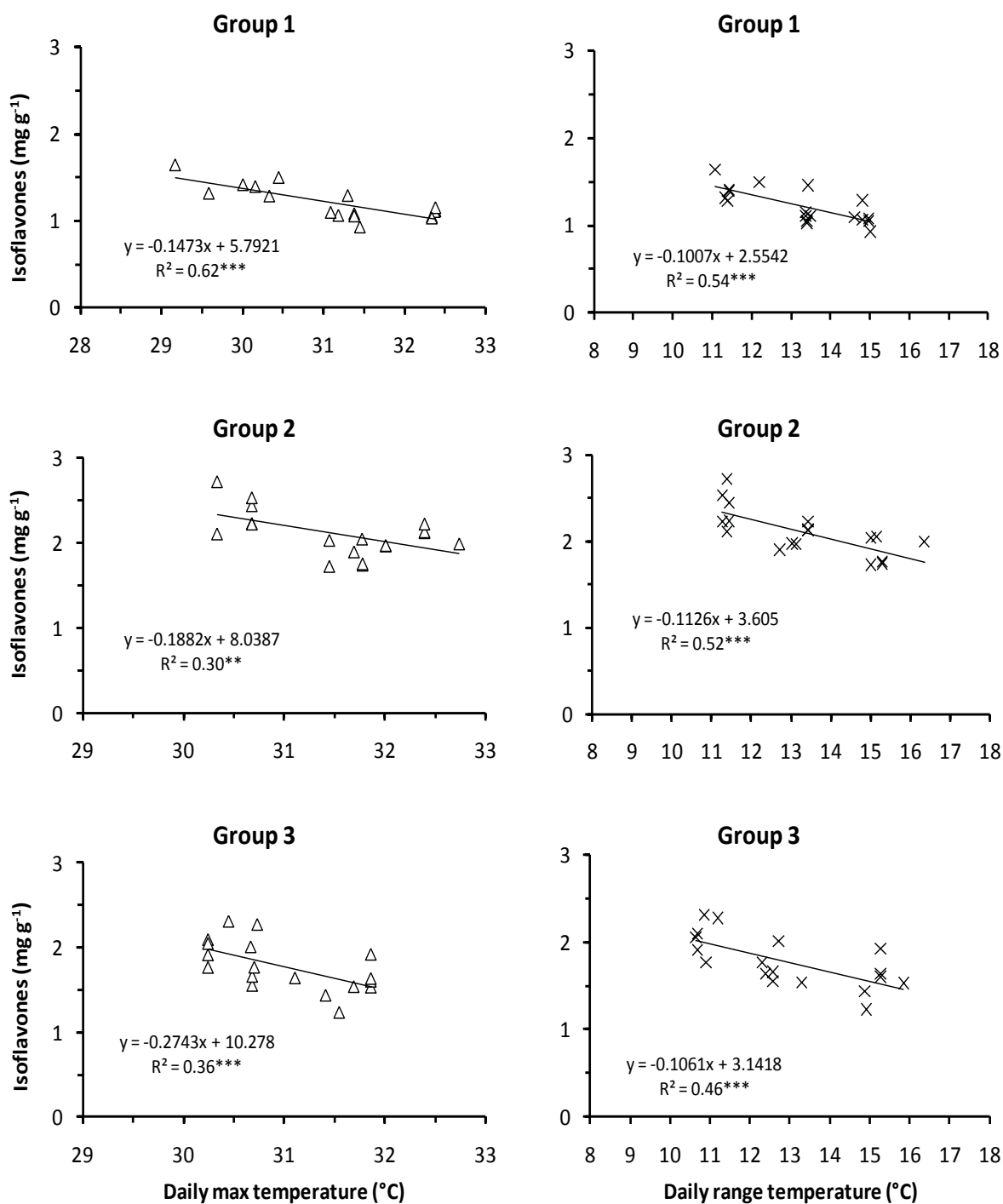
Figure 1.16. Regressions between total cotyledon isoflavone concentrations and daily maximum temperatures and daily range (maximum – minimum) temperatures (mean of first 20-day seed filling) for each variety group identified with cluster analysis.

1.4 Discussions

In this study the two major factors affecting the isoflavone contents were variety and environment. Management has showed significant effect on cotyledon but did not influence hypocotyl isoflavone. Due to sensibility of varieties the combination of different factors influences their contents. Hypocotyl has its own metabolic and physiological controls which can be one of the causes of some genotype by environment interaction that was observed (Perez-Grau and Goldberg, 1989). Due to variation on isoflavone profile, concentration and seed portion contribution maybe nowadays subjected into opposite demands concerning their isoflavone contents. Genistein is the most studied molecule due to its important (Dixon and Ferriera, 2002), hypocotyl considered a poor source of it compared with cotyledon. On the contrary glycitein was a long time considered as a minor isoflavone, remains poorly investigated (Berger *et al.*, 2008).

Cotyledon had 80-90% of total seed isoflavone, with the remainder in the hypocotyls (Tsukamoto *et al.*, 1995). The hypocotyls had a higher concentration of isoflavone on a weight basis compared with cotyledons. This variation between seed parts and organs due to distinct gene expression program was occurring in parallel in different seed compartments (embryo (cotyledon & hypocotyl), endosperm & seed coat) (Le *et al.*, 2007).

Cotyledon isoflavone of single variety may vary up to 100% within years but may show up to 50% between management. Varieties TDZ and TGS showed the same tendency of TISO due to magnitude of genetic. The aglycone equivalent form is rarly below 1% even under very drastic conditions. Cotyledon showed high TGS portion which represent ≈ 70% of TISO. However cotyledon genistein considered important source compared to hypocotyl. TGY and TDZ are present in abundant quantity in hypocotyl and it can be a good source for these molecules.

The hypocotyls are the part of soybean containing the highest concentration of isoflavones, especially daidzein aglycone equivalent form, which can be metabolized to the more potent estrogenic metabolite equol by the intestinal microflora (Yuan *et al.*, 2007).

On the other hand, percentage of each isoflavones (composition) appeared mainly under genotypic control. The key enzyme of the first common Phenylpropanoid pathway steps, phenyl ammonia lyase (PAL), chalcone syntase (CHS) and chalcone reductase (CHR) are known to be strongly over expressed when temperature is decreased (Dixon and

Paiva 1995; Leyva *et al*., 1995) and down-regulated by high temperature (Mori, 2005), leading to a general increase or decrease of the synthesis of all isoflavones.

The first branching point leads to the synthesis of either daidzein or genistein. Genotype variation in this competitive naringenin (the precursor of genistein) can be the major reason of the varieties differences in cotyledon composition (Yu *et al*., 2003; Kim *et al*., 2005). Each isoflavone may have not the same function in the seed. Daidzein, the phytoalexin glyceollin precursor, was shown to be selectively accumulated in cotyledon elicited with wall glucan preparation from *phytophthora sojae* whereas genistein could act as an internal signal triggering the local hypersensitive cell death and distally inducing the cellular competency to accumulate phytolexin (Graham and Graham, 1996; Graham and Graham, 1999).

In our study the composition in each seed fraction appeared mainly under genetic control. However, the contents of both seed fractions may fluctuate but the percentage of different molecules were constant.

Daidzein in both seed fractions proved its ability in dividing varieties into three main categories high, medium and low contents. Glycitein and daidzein in hypocotyl illustrated good ability in discriminate varieties with special characteristics. *Cresier* and *Nikko* had high glycitein and low daidzein contents. Genistein in hypocotyl did not demonstrate stability in different varieties among years and managements. Sensibility of these molecules to genotype interaction with different environment and/or management factors was observed.

Late accumulation was the main reason of sensibility of cotyledon isoflavone contents. Cotyledon showed high significant accumulation under conventional whereas hypocotyl did not demonstrate any differences. Management did not show significant effect on hypocotyl isoflavone. Thus, whatever the variety or the environmental conditions is, the accumulation in cotyledons appeared to be a late maturation event which began after R6 (35-40 DAF) (Berger *et al*., 2008).

High and low content varieties had the same ranking above the different environment and conditions but single variety can have 50-100% cotyledon isoflavone content increase. However, variability of single variety in hypocotyl did not exceed 30%. Due to late cultivation the maturity was delayed into beginning of October. At this moment night

temperature were relatively low which the responsible of increase key enzymes of Phenylpropanoid and flavonoid pathways such as PAL and CHS (Dixon and Paiva 1995; Leyva *et al.*, 1995). On the contrary high temperature during summer can have the reverse effect on these enzymes (Caldwell *et al.*, 2005; Lozovaya *et al.*, 2005). Interestingly hypocotyl did not show the same tendency that was the same observation of Lozovaya *et al.* (2005). However, hypocotyl maybe has influenced by complex of different environment variables. Berger *et al.* (2008) observed that nutrition factor could have more effect than temperature on hypocotyl.

Local variation between cotyledon and hypocotyl in the developing embryo could be explained due to the variation of isoflavone synthase enzyme IFS and chalcone syntase CHS expression. IFS2 and CHS expression come late but IFS1 already detected at 30 DAF and maintained along the development of embryo (Dhaubhadel *et al.*, 2003).

Intensive isoflavone accumulation in the cotyledon always begins when this accumulation has reached the plateau in the hypocotyl. Nevertheless, the concentration in cotyledon was very low compared to those of the hypocotyl and glycitein never observed except traces. Thus, transportation between these two parts is uncertain (Berger *et al.*, 2008). However the hypothesis of higher content and continuous accumulation in cotyledon was suggested to be translocation of formed isoflavone from maternal tissue into cotyledon which is still accumulating different nutrients till late maturity stage (Bennett *et al.*, 2004; Dhaubhadel *et al.*, 2003).

The preservation of global profiles despite very different growing conditions indicated a large part of genetic control of each single molecule. The kinetics of different molecules and their accumulation were observed to be stable due to variety and showed similar profile (Berger *et al.*, 2008). Nutrients availability for plants is more effective than temperature and that was observed by Berger *et al.* (2008), which confirm that nutrient availability of mineral fertilizer of previous crop is more relevant in general under conventional agriculture.

1.5 Conclusions

A rank of varieties was maintained throughout the years and management. Cotyledon isoflavone contents were more sensitive to climate and management compared with hypocotyl. TGY and TDZ can be a considerable mark in hypocotyl in discriminating varieties. TGS is an important molecule due to its large presence in cotyledon and its stability in hypocotyl of different varieties. Multivariate analysis showed a good comprehensive conclusion for classification of variety due to cotyledon and hypocotyl isoflavone contents. Finally these studies show that it might be possible to handle these important seed quality traits without high correlation constraints, in breeding programs as well as in crop management. However, they maybe a possibility of using hypocotyl as a raw material, more investigations are needed on the role of glycitein in the hypocotyl and on its health effect.

Chapter 2

Water and Nitrogen Supply Effect on Isoflavone Accumulation in Soybean Hypocotyls and Cotyledon

2.1 *Introduction*

Isoflavones are photochemical synthesized in most phenomenon protein family member. The interest in presence of isoflavone has been growing since late 90s, as epidemiological and clinical studies have suggested that the consumption of soyfoods associated with many health benefits (Sarkar and Li, 2003). N_2 fixing crops present an important option to improve N supply and to maintain soil fertility. Previous studies demonstrated that during drought stress period N2 fixation is the first process to decrease compared to other physiological ones (Sall and Sinclair, 1991). High N mineralization may limit symbiotic N_2 fixation (Oberson *et al*., 2007). Although N fertilization is not a common practice there is speculation that the ability of soybean to fix N_2 is not always adequate to reach the maximum yield. In most of cases full-season soybeans can be irrigated during the reproductive period and obtain the same yield as complete-season irrigation (Ashley and Ethridge, 1978). Drought stress decreases yield-related processes and N2 fixation is more sensitive to drought than many other of these processes. Therefore, application of nitrogen fertilizer may increase drought tolerance over those plants primarily dependent on N2 fixation (Purcell and King, 1996). Water content is very important for all plants as effective factor for plant metabolism, isoflavones compound found to be increase by irrigation (Bennett *et al*., 2004) or decreased by drought (Caldwell *et al*., 2005; Lozovaya *et al*., 2005; Seguin *et al*., 2004). Soil nitrogen enrichment had no effect on total isoflavone concentration, where N application was not correlated with isoflavone concentration (Kim *et al*., 2005). The objectives of this study were to examine irrigation and nitrogen applications effect on isoflavone accumulation in hypocotyl and cotyledon of two soybean varieties on main and lateral shoot seeds. Bennett *et al*. (2004) demonstrated that cotyledon isoflavone was not former in its tissue but it is translocated components from maternal tissue and other plant parts. They also did not find the mechanism of irrigation on increasing isoflavone contents.

2.2 *Material and methods*

The experiment was conducted in 2006 and 2007 at Padova University Experiment Station, Legnaro - Italy, 45°21'03" N, 11°56'54" E in loam soil. Climate data is demonstrated in table (2.1), Water deficit calculated by (water deficit= Rainfall–ETo). A pre-sowing practice were performed, weed was handly controlled twice during plant development. Factors were arranged in a split-split-plot layout. In both year included the 2 varieties (*Ales* and *Nikir*); irrigation, was irrigated vs. none irrigated; nitrogen, was applied in R1 (flower beginning) and R3 (pod set beginning) stages in 2006 and 2007 respectively. Six rows per plot were cultivated with 40 seed m^{-2} (4m length x 50cm inter-row) in 4 replicates. Irrigation was weekly based on ETm. Pods were collected on main and lateral shoot at harvest time and Seeds were collected.

Table 2.1. Medium temperature, rainfall, ETo and water deficit of Legnaro in 2006-2007.

	2006				2007			
	med T	Rainfall	ETo	Water deficit	med T	Rainfall	ETo	Water deficit
	°C		mm		°C		mm	
May	17.2	92.4	98.1	-5.7	19.15	146.6	116.4	30.2
June	21.7	14.6	123.9	-109.3	22.07	60.8	124.2	-63.4
July	25.2	47.6	131.4	-83.8	23.72	31.2	152.1	-120.9
August	19.95	122.4	94.6	27.8	22.07	48.2	110.7	-62.5
September	20.18	178.2	69.7	108.5	17.39	104.8	74.6	30.2
October	15.92	16.0	40.8	-24.8	13.28	35.8	38.4	-2.55

Data were obtained from Legnaro station belongs to ARPAV (Regional Agency for Prevention and Protection of Environment in Veneto). medT, medium temperature; ETo, evapotranspiration.

For isoflavone analysis, was estimated in the same method sited in material and methods (chapter 1). Isoflavones were expressed as aglycone equivalents, in mg g^{-1} of dry weight. Data were analyzed by ANOVA using general linear model GLM in both years considered separately because the two years contrasting in complementary N input. Correlation regression analysis used to estimate the relation between different isoflavones group. All statistical analyses were performed using statgraphics centurion XV version 15.2.06.

2.3   Results

The 12 isoflavone forms were analysed separately in cotyledons and hypocotyl, their contents were expressed in aglycone equivalents as active or stored form (mg g^{-1}) and in percentage of total isoflavone in each seed fraction composition).

In our experiments generally data set demonstrate that cotyledon had 4 times greater amount of isoflavone than hypocotyls but the relative concentration is less due to a dilution effect. In soybean hypocotyls and cotyledon isoflavone accumulation differ within years more than 30% (Table 2.2).

2.3.1   Cotyledon

Cotyledon considered as a storage unit of the seed mostly conserve all necessary product for next emergence. Cotyledon stores 5 times as much as hypocotyl isoflavone but the concentration ratio was 1:5-7. Cotyledon showed high sensitivity to agronomic practices as much as genetic and environment conditions (Table 2.4). Different isoflavone forms demonstrated increase or decrease regarding to the environment but showed stability in accumulation regarding to varieties due to absence of TGY which proved a difference in its accumulation in hypocotyl.

2.3.1.1   Variety effect on isoflavone contents in cotyledon

Ales TISO in cotyledon showed 33% greater accumulation than *Nikir* in 2006 and 44% in 2007 (Table 2.2). Variety genetic variability did influence the amount of isoflavone but did not influence its tendency. Effect of different factors on TISO confirmed the same tendency on TDZ. *Ales* gave 38% higher TDZ in cotyledon and 57% than *Nikir* in 2006 and 2007 respectively. Genistein and daidzein represents the main isoflavone forms in cotyledon. No significant effect due to variety was observed in 2006 but in 2007 *Ales* had 37% higher isoflavone than *Nikir*.

2.3.1.2   Irrigation effect on isoflavone contents in cotyledon

Water supply did not show significant effect on TISO in cotyledon but show different tendency regarding to year. Regarding to single molecules, no significant effect observed for irrigation in both year on TDZ. Drought stress showed 15% increment in TGS accumulation (Table 2.2). Protein did not influence by different factors but irrigation, that

illustrated great protein yield. The interaction did not reveal any importance on protein content.

2.3.1.3 Nitrogen effect on isoflavone contents in cotyledon

Nitrogen application date influence accumulation of TISO in cotyledon, which nitrogen application at R1 in 2007 exhibited 16% positive accumulation of TISO (Table 2.2). Nitrogen application affects TDZ accumulation negatively in 2006 and positively in 2007 and that maybe due to nitrogen application date. Nitrogen application showed diverse effect on TGS regarding to year. In 2006 nitrogen application proved negative effect on TGS whereas in 2007 it showed positive effect on TGS accumulation that maybe due to date of application.

Table 2.2. Mean data and results of ANOVA for different cotyledon AgE isoflavone in 2006 and 2007.

		2006				2007			
		TISO	TDZ	TGS	CP	TISO	TDZ	TGS	CP
		(mg g^{-1})			t ha^{-1}	(mg g^{-1})			t ha^{-1}
Var (V)	Ales	1.97**	0.99**	0.99*	1.70	2.19**	0.80**	1.37**	1.70
	Nikir	1.52	0.61	0.90	1.83**	1.24	0.35	0.87	1.85
Irig (I)	D	1.66	0.76	0.90	1.57	1.83	0.60	1.21*	1.37
	I	1.83	0.85	0.98	1.94**	1.60	0.56	1.03	2.18**
Nit (N)	-N	1.77	0.82*	0.95	1.76	1.58	0.53	1.03	1.73
	+N	1.72	0.78	0.94	1.76	1.85**	0.62**	1.21**	1.82
Stem (S)	P	1.56	0.70	0.85	ND	1.54	0.52	1.00	ND
	L	1.94**	0.90**	1.03**	ND	1.90**	0.64**	1.24**	ND
Anova									
	V×I	ns	ns	ns	ns	**	**	**	ns
	V×N	ns	*	ns	*	**	*	**	ns
	V×S	**	**	ns	ND	*	*	**	ND
	I ×N	ns	ns	ns	***	**	**	**	ns
	I ×S	*	*	ns	ND	ns	ns	*	ND
	N×S	ns	ns	ns	ND	**	*	**	ND
	V×I×N	ns	ns	ns	*	**	**	**	*
	V×I×S	ns	ns	ns	ND	ns	ns	ns	ND
	V×N×S	ns	ns	ns	ND	*	ns	**	ND
	I×N×S	**	**	ns	ND	ns	ns	ns	ND
	V×I× N×S	ns	ns	ns	ND	ns	ns	ns	ND

Total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY); drought (D); irrigation (I); without nitrogen (–N); with nitrogen (+N); crude protein (CP); not detected ND (unique sample without separation main and lateral shoot); *,** at P ≤ 0.05 and 0.01; ns= not significant.

2.3.1.4   Position of pod on stems effect on isoflavone contents in cotyledon

Previous studies did not consider shoot productions importance on isoflavone accumulation in cotyledon. High significance of lateral shoot cotyledon isoflavone contents compared with principal shoot. Lateral shoot gave 20% positive accumulation of TISO in cotyledon than main shoot in both years (Table 2.2). Lateral shoot gave about 20% significant accumulation of TDZ in both years. TGS showed the same tendency of TDZ and TISO for its accumulation in lateral shoot cotyledon. Lateral shoot cotyledon gave 20% higher TGS in both year.

2.3.1.5   Interaction among factors effect on isoflavone and protein contents

TISO accumulation influenced by the following interaction V×S, I×S and I×N×S in 2006 and most of the all the first level and second level interaction in 2007. The V×I interaction in 2007 showed positive tendency for the effect of irrigation on both variety (Table 2.3). In 2007 nitrogen application demonstrated positive effect on TISO content in *Nikir* but no effect was observed on *Ales*. TISO under I×N interaction in 2006 showed negative response for nitrogen application under both drought and irrigation application. However water supply showed higher tendency in 2006 under fertilized and none fertilized plots. In 2007 nitrogen application exhibited significant increase in accumulation of TISO, TDZ and TGS under drought plots. TISO and its components in *Nikir* cotyledon on both principal and lateral shoot in both years were higher than in *Ales*. Irrigation affected positively TDZ accumulation in cotyledon of both varieties. *Ales* TDZ in cotyledon demonstrated difference between principal and lateral shoot in 2006. In 2007 showed greater reduction in TDZ.

TGS was affected by all interaction in 2007 whereas only I×S and I×N×S interactions were significant in 2006. Irrigation showed positive accumulation of TGS in both variety cotyledons. Nitrogen application showed negative effect on TGS accumulation in cotyledon under both irrigated and non irrigated plots.

Stem showed significant effect under both varieties and conditions on cotyledon TISO and TDZ but TGS was not affected (Figure 2.1). In 2006 lateral shoot showed greater accumulation than principal shoot for both varieties. Lateral shoot showed greater difference than principal shoot in 2007 compared with 2006. Profile of both varieties did

not change under different conditions, which is genetically controlled and differ in quantity. *Ales* illustrated higher TDZ% compared with its TGS% but *Nikir* gave the contrast. Both varieties proved greater TGS than TDZ in 2007.

Table 2.3. interaction among variety, water and nitrogen supply effect on cotyledon isoflavone.

		2006			2007		
		TISO	TDZ	TGS	TISO	TDZ	TGS
		mg g^{-1}	mg g^{-1} (%)		mg g^{-1}	mg g^{-1} (%)	
Nikir	**D**	1.43	0.56 (40)	0.86 (60)	1.60	0.45 (29)	1.13 (71)
	I	1.62	0.67 (40)	0.95 (60)	0.88	0.25 (30)	0.62 (70)
Ales	**D**	1.90	0.95 (50)	0.95 (50)	2.05	0.74 (36)	1.28 (63)
	I	2.04	1.03 (50)	1.01 (50)	2.33	0.86 (37)	1.45 (62)
	LSD$_{0.05}$	*ns*	*ns (ns)*	*ns (ns)*	*0.339*	*0.109 (1.8)*	*0.232 (1.8)*
Nikir	**-N**	1.52	0.62 (40)	0.90 (60)	0.96	0.27 (28)	0.68 (71)
	+N	1.52	0.61 (40)	0.91 (60)	1.52	0.43 (30)	1.07 (69)
Ales	**-N**	2.02	1.03 (51)	0.99 (49)	2.20	0.80 (36)	1.38 (63)
	+N	1.92	0.94 (49)	0.97 (51)	2.18	0.81 (37)	1.36 (62)
	LSD$_{0.05}$	*ns*	*0.11 (0.9)*	*ns (0.9)*	*0.364*	*0.118 (1.7)*	*0.250 (1.8)*
D	**-N**	1.67	0.77 (47)	0.91 (53)	1.50	0.50 (32)	0.98 (67)
D	**+N**	1.65	0.74 (44)	0.91 (56)	2.16	0.69 (33)	1.43 (66)
I	**-N**	1.87	0.88 (45)	0.99 (55)	1.66	0.57 (33)	1.08 (67)
I	**+N**	1.79	0.82 (45)	0.98 (55)	1.55	0.54 (34)	0.99 (65)
	LSD$_{0.05}$	*ns*	*ns (ns)*	*ns (ns)*	*0.507*	*0.109 (0.9)*	*0.308 (ns)*

Total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY); drought (D); irrigation (I); without nitrogen (–N); with nitrogen (+N); crude protein (CP) (unique sample without separation main and lateral shoot); *,** at P ≤ 0.05 and 0.01; ns= not significant.

2.3.1.6   Profile and conjugates form

Isoflavone profile as shown in Figure (2.2) demonstrated that profile of cotyledons composed of daidzein and genistein form. In cotyledons, genistein and its glycoside conjugates (50-70%) were the main isoflavones, being followed by daidzein form (30-50%), and no glycitein form was found, that finding in agree with (Berger *et al*., 2008; Yuan *et al*., 2009). *Ales* showed higher TDZ% compared with *Nikir*, which showed the contrast. Both varieties demonstrated greater TGS than TDZ in 2007 but in 2006 *Ales* showed equal % from both molecules. Environment and variety illustrated higher variableness on cotyledon isoflavone profile than agronomic practices. Cotyledon isoflavone conjugates accumulation showed high sensibility to changes in agronomic

practices. *Ales* cotyledon showed the same significance in both year for all three groups aglycone, β-glycoside and malonyl-glycoside (Figure 2.3). Nitrogen and water supply proved diverse influence regarding to year of application which both treatment showed positive effect in 2006 but influenced negatively the accumulation in 2007. The effect of environment may show positive or negative impact on specific treatment due to climate scenarios in each year and way. Lateral stem showed great stability in both year on accumulation of aglycone, β-glycoside and malonyl-glycoside. Conjugated form of isoflavone showed difference in their quantity under different factors but no difference among their %. Malonyl form represent 80% ±5 followed by β-glycone 20% ±2 but free form represents less than 2%.
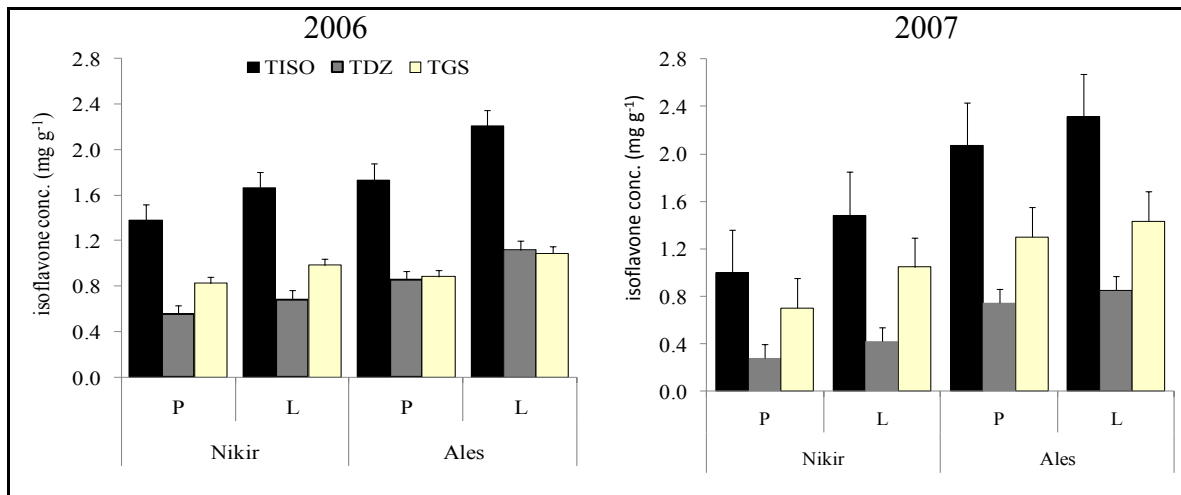
Figure 2.1. Interaction between variety × stem effect on TISO, TDZ and TGS in both years.
Principal shoot (P); lateral shoot (L); vertical bar represent $LSD_{0.05}$; Total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS)
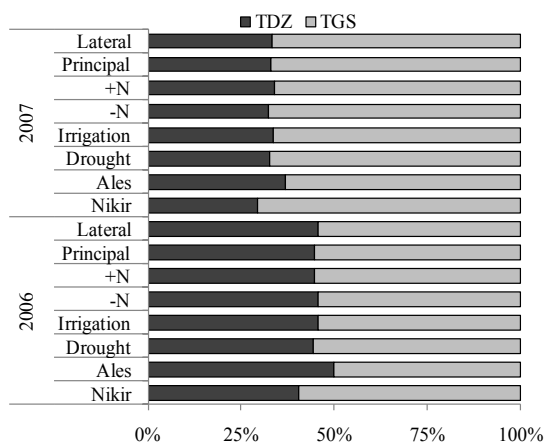
Figure 2.2. Cotyledon profile isoflavone of forms under different factors.
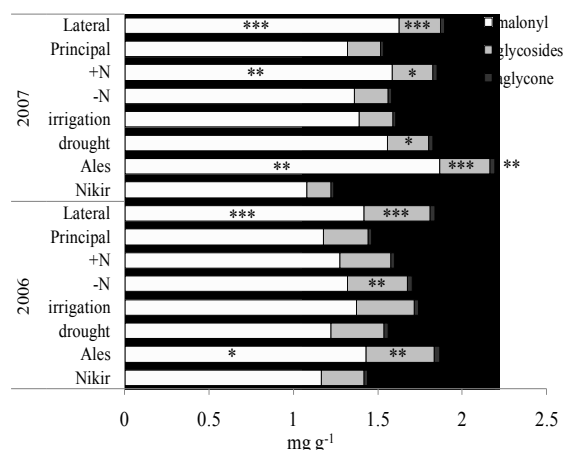
Figure 2.3. Cotyledon isoflavone values in conjugated and free aglycone forms.

Total daidzein (TDZ), total genistein (TGS); without nitrogen (−N); with nitrogen (+N).

2.3.2 Hypocotyl

Hypocotyl was more responsive to environment conditions and genetic variability than agronomic practices, which hypocotyls isoflavone accumulation expressed about 40% difference regarding to environment conditions Table (2.4). Isoflavone accumulation in hypocotyls under different irrigation, nitrogen application and stem exhibited great variability. The interaction did not reveal importance on hypocotyl.

2.3.2.1 Variety effect on isoflavone contents in hypocotyl

TISO showed in both years significant differences between both varieties. Variety performance has variation regarding to environment conditions which not affect only the quantity of specific molecule but as well change the reflectance of each variety regarding to his genetic background. *Ales* gave 5% higher TISO than *Nikir* in 2006 where as the last one verified 7% greater accumulation in 2007. Variety was the main factor, *Ales* gave about 41% greater hypocotyls TDZ than *Nikir* in both years. Variety did not show great significant effect on TGS. On the contrast of TISO and TDZ that TGY indicated greater accumulation in *Nikir* hypocotyls than *Ales* in both years (Table 2.4). This result in consist with the result obtained by Berger *et al*. (2008), which accumulation of different isoflavone molecules were variety dependent which appear clearly from the % of each molecule (Figure 2.4).

2.3.2.2 Irrigation effect on isoflavone contents in hypocotyl

Irrigation did not show significant effect on hypocotyls TISO in 2006 whereas showed 8% reduction in TISO content in 2007 compared to rain-fed plots. TDZ illustrated the same tendency as TISO under irrigation treatment (Table 2.4). Irrigation did not show stable or significant effect on TGY. However, drought demonstrated increase in hypocotyl TGS in 2007 whereas in 2006 the same tendency but no significant difference was observed molecule for water supply.

2.3.2.3 Nitrogen effect on isoflavone contents in hypocotyl

Nitrogen application demonstrated negative impact on hypocotyls TISO and all three forms in hypocotyl in 2006 but no significant effect was found in 2007 (Table 2.4). However, nitrogen supply in 2006 exhibited ≈ 3% reduction in TISO, TDZ and TGY

whereas showed ≈ 9% TGS reduction. In 2007 nitrogen supply revealed positive tendency without significance.

2.3.2.4   Position of pod on stems effect on isoflavone contents in hypocotyl

Lateral shoot verified small tendency on TISO and TDZ in 2006 and showed 7% greater accumulation in 2007 (Table 2.4). Late formation of lateral pod may be the responsible of exposure lateral shoot for lower temperature compared to main shoot (Lozovaya *et al*., 2005). Stem did not show significant effect on TGS contents in both years. Lateral shoot exhibited significant accumulation for hypocotyls TGY in 2007 but small tendency was detected in 2006.

Table 2.4. Mean data and Results of ANOVA for different hypocotyl AgE isoflavone in 2006 and 2007.

		2006				2007			
		TISO	TDZ	TGS	TGY	TISO	TDZ	TGS	TGY
					mg g^{-1}				
Var (V)	**Ales**	14.99**	5.95	1.24	7.81**	8.97	3.52	1.79	3.67**
	Nikir	14.17	8.43**	1.21	4.53	9.67*	5.50**	1.86	2.31
Irr (I)	**D**	14.57	7.20	1.24	6.13	9.70*	4.69*	1.92**	3.09*
	I	14.60	7.18	1.21	6.20	8.95	4.34	1.72	2.90
Nit (N)	**-N**	14.86**	7.30**	1.28**	6.28**	9.25	4.49	1.80	2.96
	+N	14.31	7.07	1.17	6.06	9.40	4.53	1.85	3.02
Stem (S)	**P**	14.47	7.17	1.23	6.07	9.06	4.42	1.78	2.86
	L	14.69	7.21	1.22	6.26*	9.58*	4.60*	1.87	3.12*
Anova									
V×I		**	*	ns	**	ns	ns	ns	ns
V×N		ns	ns	ns	ns	ns	ns	ns	ns
V×S		ns	ns	ns	ns	ns	ns	ns	ns
I×N		ns	ns	ns	ns	ns	ns	ns	ns
I×S		ns	ns	ns	ns	ns	ns	ns	ns
N×S		ns	ns	**	ns	ns	ns	ns	ns
V×I×N		ns	*	ns	ns	ns	*	ns	ns
V×I×S		ns	**	ns	ns	ns	ns	ns	ns
V×N×S		ns	ns	ns	ns	ns	ns	ns	ns
I×N×S		*	**	ns	ns	ns	ns	ns	ns
V×I×N×S		ns	ns	*	ns	ns	ns	ns	*

Total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY); drought (D); irrigation (I); without nitrogen (−N); with nitrogen (+N); crude protein (CP); not detected ND (unique sample without separation main and lateral shoot); *,** at P ≤ 0.05 and 0.01; ns= not significant.

2.3.2.5 Interaction among factors effect on isoflavone contents in hypocotyl

The interaction among different factors did not show great influence on TISO accumulation except V×I and I×N×S in 2006. Both varieties showed different performance under irrigation treatment in which *Nikir* gave higher content of TISO under drought where as *Ales* gave greater TISO under irrigation plots (Table 2.5). That behaviours confirmed that isoflavone accumulation is strictly connected with genetic variability and importance of practices which is variety dependence. TDZ showed the same tendency of TISO under V×I interaction. TGS exhibited significant response for N×S in 2006. The interaction V×I verified significant effect on TGY accumulation, in the same tendency of its effect on TISO and TDZ.

Table 2.5. Interaction among variety, water and nitrogen supply effect on hypocotyls isoflavone.

		2006				2007			
		TISO	TDZ	TGS	TGY	TISO	TDZ	TGS	TGY
		mg g^{-1}		mg g^{-1} (%)		mg g^{-1}		mg g^{-1} (%)	
Nikir	**D**	14.49	8.63 (59)	1.24 (9)	4.62 (32)	10.06	5.77 (57)	1.94 (19)	2.35 (23)
	I	13.86	8.23 (59)	1.19 (9)	4.44 (32)	9.29	5.23 (57)	1.78 (19)	2.28 (24)
Ales	**D**	14.65	5.76 (39)	1.24 (9)	7.65 (52)	9.34	3.60 (38)	1.91 (20)	3.83 (41)
	I	15.34	6.14 (40)	1.23 (8)	7.96 (52)	8.61	3.43 (40)	1.66 (19)	3.51 (41)
LSD$_{0.05}$		*0.505*	*0.505*	*0.277 (ns)*	*ns (ns)*	*0.224 (ns)*	*ns*	*ns (ns)*	*ns (ns)*
Nikir	**-N**	14.42	8.57 (59)	1.25 (9)	4.60 (32)	9.76	5.58 (57)	1.87 (19)	2.30 (24)
	+N	13.93	8.29 (60)	1.17 (8)	4.47 (32)	9.59	5.43 (57)	1.84 (19)	2.32(24)
Ales	**-N**	15.3	6.04 (40)	1.30 (9)	7.96 (52)	8.74	3.39 (39)	1.73 (20)	3.62 (42)
	+N	14.68	5.86 (40)	1.17 (8)	7.65 (52)	9.21	3.64 (40)	1.85 (20)	3.72 (40)
LSD$_{0.05}$		*ns*	*ns*	*ns (0.5)*	*ns (ns)*	*ns (ns)*	*ns*	*ns (ns)*	*ns (ns)*
D	**-N**	14.81	7.29 (49)	1.30 (9)	6.22 (42)	9.63	4.67 (48)	1.89 (20)	3.07 (32)
	+N	14.33	7.11 (50)	1.18 (8)	6.05 (42)	9.77	4.70 (48)	1.96 (20)	3.11 (32)
I	**-N**	14.91	7.32 (50)	1.25 (8)	6.34 (42)	8.87	4.30 (48)	1.71 (19)	2.86(33)
	+N	14.28	7.04 (50)	1.17 (8)	6.07 (42)	9.03	4.37 (49)	1.73 (19)	2.93 (32)
LSD$_{0.05}$		*ns*	*ns*	*ns (ns)*	*0.058 (0.3)*	*ns (ns)*	*ns*	*ns*	*ns*

Total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY); drought (D); irrigation (I); without nitrogen (–N); with nitrogen (+N); crude protein (CP) (unique sample without separation main and lateral shoot); *,** at P ≤ 0.05 and 0.01; ns= not significant.

2.3.2.6 Profile and conjugates form in hypocotyl

Profile of both varieties did not fluctuate within different factors (Figure 2.4). Which reveal that isoflavone formation is genetically controlled by genetic restrict, although the

difference was in quantity of isoflavone but the percentage of different molecules were the same under different factors. The difference in profile is only due to variety or year of cultivation. Due to importance of detoxification in plant cells free aglycone form represent less than 2% (Figure 2.5). Malonyl form is representing more than 70-80% and β-aglycone about 18-28%. Only variety and stem showed stable effect on different conjugates forms beside the effect of year on quantity of isoflavone.
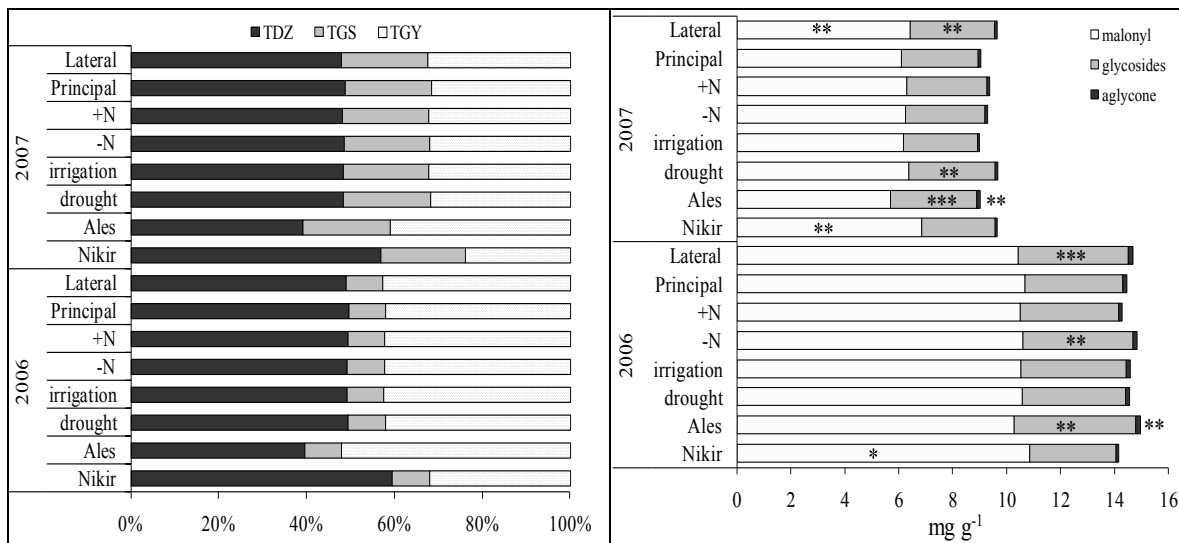
Figure 2.4. Hypocotyl profile of isoflavone accumulation regarding to variety, water, nitrogen and seed site.

Figure 2.5. The value of hypocotyls isoflavone in different conjugate forms.

total daidzein (TDZ), total genistein (TGS); without nitrogen (–N); with nitrogen (+N).

2.3.3 Metabolic of isoflavone components in soybean cotyledon and hypocotyl

Both varieties were varied in their accumulation for three main isoflavones groups. Hypocotyl contains all three groups but cotyledon contains only daidzein and genistein forms. However the accumulation of these molecules varied regarding to variety and year of accumulation (Figure 2.6). Great differences were observed between year regarding to accumulation of TDZ, TGS and TGY in hypocotyl (Figure 2.6, A, B, C). It was strictly difficult to estimate both varieties under one correlation between two molecules due to large genetic differences. In *Nikir* the correlation between TDZ and TGY hypocotyl was linear with $R^2 = 90\%$ in 2006 and 71% in 2007 but for Ales did not exceed 55% in both years (Figure 2.6, A). The correlation between TGS and TGY in 2006 was about 25% for

hypocotyl of both varieties whereas in 2007 it was 72% and 67% for *Nikir* and *Ales* respectively (Figure 2.6, B). The relation between TDZ and TGS in cotyledon was highly correlated which reflect that pathway of their synthesis is not contradictory (Figure 2.6, C). However, hypocotyl TDZ and TGS did not show the same R^2 values due to presence of TGY. Both varieties clarified difference in accumulation of TDZ and TGS in cotyledon regarding to the year, which in 2006 they produce higher TDZ but in 2007 produce more TGS (Figure 2.6, D), the effect in 2007 due to drought stress which increase accumulation of TGS. This supported the findings of Primomo *et al*. (2005), who verified that QTL for total isoflavone mapped to similar regions as daidzein and genistein but they did not mention for glycitein.
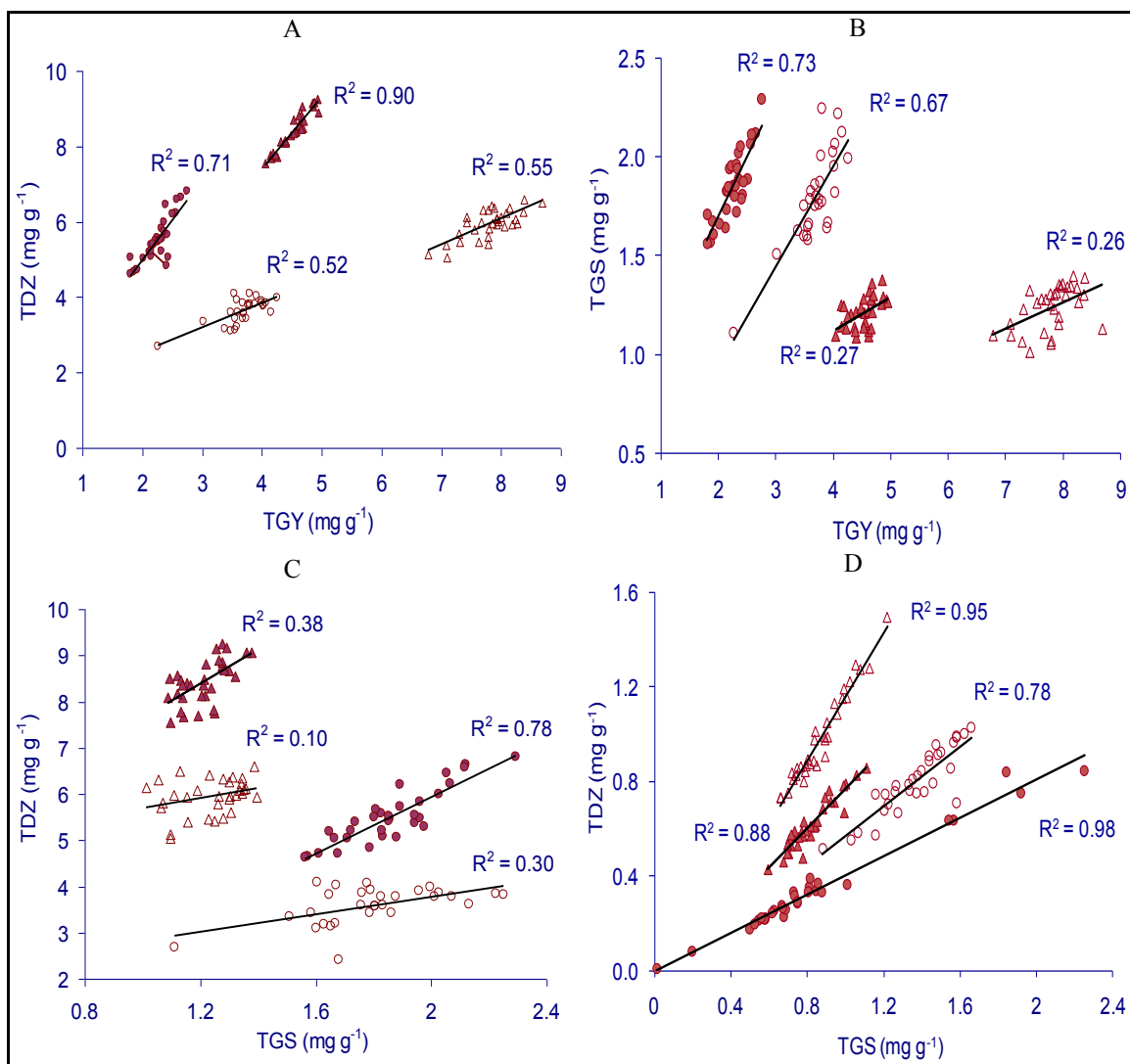
Figure 2.6. Hypocotyl and cotyledon isoflavone relationship in both varieties during 2 years under irrigation and nitrogen supply.

A, total glycitein TGY and total daidzein TDZ correlation in hypocotyls; B, hypocotyls TGY and total genistein correlation TGS; C, Hypocotyls TDZ and TGS relations. D, Cotyledon TDZ and TGS relations. ▲Nikir2006 ●Nikir2007 △Ales2006 ○Ales2007

2.3.4   The relation between different isoflavones in hypocotyls and cotyledon with yield components on main and lateral shoot

Yield components were studied in correlation with isoflavone contents in cotyledon and hypocotyl of main and lateral shoot (Table 2.6). Yield components on principal shoot have proved two different compartments, which showed positive correlation with hypocotyl isoflavone contents but were negatively correlated with cotyledon isoflavone. The contrast was detected for lateral shoot yield component which showed positive correlation with cotyledon isoflavone and negative with hypocotyl isoflavone. There was a negative correlation between principal and lateral shoot yield components with r value ≈ (-0.5). However there was no correlation between isoflavone contents on principal and lateral shoot was highly correlated r value was higher than (0.7). Cotyledon and hypocotyl did not show any correlation regarding to isoflavone contents.

Table 2.6. The correlation coefficient among different isoflavone AgE form of cotyledon and hypocotyls with yield components.

		principal x principal											
		Hypocotyl					Cotyledon				Yield comp/plant		
		TISO	TDZ	TGS	TGY	weight	TISO	TDZ	TGS	weight	nPd	wPd	SDW
Hypocotyl	TISO		0.77 ***	-0.17 ns	0.82 ***	-0.02 ns	0.15 ns	0.39 ***	-0.14 ns	-0.11 ns	**0.19 ns**	**0.36 *****	**0.44 *****
	TDZ	0.78 ***		-0.23 *	0.31 **	0.08 ns	-0.17 ns	-0.02 ns	-0.30 **	-0.13 ns	**0.33 ****	**0.31 ****	**0.36 *****
	TGS	-0.15 ns	-0.19 ns		-0.29 **	-0.60 ***	0.08 ns	-0.29 **	0.43 ***	-0.46 ***	**-0.26 ***	**-0.26 ***	**-0.20 ns**
	TGY	0.79 ***	0.26 *	-0.28 **		0.04 ns	0.35 **	0.65 ***	-0.04 ns	0.06 ns	**0.05 ns**	**0.32 ****	**0.37 *****
	weight	0.05 ns	0.06 ns	-0.61 ***	0.17 ns		0.12 ns	0.45 ***	-0.27 *	0.81 ***	**-0.09 ns**	**-0.14 ns**	**-0.13 ns**
Cotyledon	TISO	0.06 ns	-0.29 **	-0.03 ns	0.36 ***	0.01 ns		0.85 ***	0.87 ***	0.17 ns	**-0.38 *****	**-0.19 ns**	**-0.18 ns**
	TDZ	0.32 **	-0.11 Ns	-0.44 ***	0.68 ***	0.27 *	0.80 ***		0.49 ***	0.32 **	**-0.30 ****	**-0.08 ns**	**-0.06 ns**
	TGS	-0.26 *	-0.38 ***	0.40 ***	-0.12 ns	-0.26 *	0.80 ***	0.29 **		-0.06 ns	**-0.38 *****	**-0.27 ***	**-0.28 ****
	weight	-0.10 ns	-0.16 ns	-0.40 ***	0.11 ns	0.85 ***	0.26 *	0.43 ***	-0.04 ns		**-0.26 ***	**-0.23 ***	**-0.25 ***
Yield comp/plant	nPd	**-0.42 *****	**-0.43 *****	**0.26 ***	**-0.29 ****	**-0.05 ns**	**0.26 ***	**0.09 ns**	**0.36 *****	**0.09 ns**		0.85 ***	0.75 ***
	wPd	**-0.40 *****	**-0.45 *****	**0.17 ns**	**-0.21 ***	**0.04 ns**	**0.32 ****	**0.19 ns**	**0.35 ****	**0.18 ns**	0.97 ***		0.81 ***
	SDW	**-0.41 *****	**-0.44 *****	**0.13 ns**	**-0.23 ***	**0.10 ns**	**0.33 ****	**0.22 ***	**0.35 *****	**0.22 ***	0.96 ***	0.99 ***	

The upper part shows the correlation coefficient among principal shoot cotyledon, hypocotyls isoflavones and yield components and the lower part shows the correlation coefficient among lateral shoot cotyledon, hypocotyls isoflavones and yield components. $P \leq 0.05$ *, 0.01 ** and 0.001 ***; not significant (ns). Total daidzein, TDz; Total genistein (TGS), Total glycitein (TGY); Total isoflavone (TISO); n° pod per plant, (nPd); pod weight (wPd); Seed dry weight per plant (SDW).

2.4   Discussion

Cotyledon had 80-90% of total seed isoflavone, with the remainder in the hypocotyls (Tsukamoto *et al*., 1995). The hypocotyls had a higher concentration of isoflavone on a weight basis compared with cotyledons. Cotyledons exhibited large changes in response to high temperature during seed development; the isoflavone content remained high in the hypocotyls.

These results have suggested some mechanisms that permit a higher isoflavone content to be maintained in hypocotyls than in cotyledons. The observation has important practical implications because it means that isoflavone content in cotyledons and hypocotyls can to a large extent be manipulated independently of one another (Tsukamoto *et al*., 1995). This result was confirmed by Berger *et al*. (2008) but still in uncertainty if there is a relation between cotyledon and hypocotyl. Bennett *et al*. (2004) found that the relation between cotyledon and hypocotyl was not detected, changes happen in cotyledon contents of isoflavone due to long accumulation period after flowering (Berger *et al*., 2008).

The prevailing effect of environment on the isoflavone content was observed in this study. However, environment induced changes were greater than other factors (Rasolohery *et al*., 2007). Soil water supply had less effect than temperature on the isoflavone contents and compositions (Rasolohery *et al*., 2007). However, irrigation in our study was mainly depend on variety (Seguin *et al* 2004), drought stress has a role in increasing isoflavone contents in cotyledon and hypocotyl of *Nikir* but did not show effect on *Ales*. On the other hand, many studies assumes that irrigation is dramatically increased the isoflavone content of soybean seed (Bennett *et al*., 2004), or decreased by drought (Seguin *et al*., 2004; Caldwell *et al*., 2005; Lozovaya *et al*., 2005). The mechanism by which this increase was facilitated is still unknown. Effect of irrigation on cotyledon isoflavone was varied due to year and variety and this result in agree with (Seguin *et al*., 2004; Al-Tawaha *et al*., 2007).

Nitrogen effect at R3 reduced accumulation of isoflavone in both hypocotyl and cotyledon whereas N application at R1 increased accumulation of isoflavone. Late N application in 2006 showed negative effect on total isoflavone accumulation. Our result regarding to N application was not in agree with the result obtained from (Kim *et al*., 2005) due to variability of nitrogen effect due to its application date. Nitrogen application in both

years elucidated different responses due to variability of climate and nitrogen application date, which β-glycoside in 2006 was negatively influenced by nitrogen application but in 2007 no significant effect was distinguish.

Lateral shoots form their pods later which may exposure them to low temperature during pod filling consequently increase isoflavone contents of seed formed on lateral shoot (Lozovaya *et al*., 2005). Dhaubhadel *et al*. (2003) suggests that although seeds are the principal site of isoflavone synthesis, some accumulation is due to transport from other plant organs including maternal tissues. Lateral shoot demonstrate higher tendency in its accumulation for aglycone and proved significant accumulation of β-glycoside in both years, whereas malonylglycosides showed significant accumulation in hypocotyls of lateral shoot in 2007.

Hypocotyls produce all three main forms of isoflavone TDZ, TGS and TGY percentage was measured based on total isoflavone of hypocotyls. Profile of hypocotyls isoflavone under agronomic practices or site production did not show difference but variety was the only promoter for the level of isoflavone in cell. That finding is not in agree with Yuan *et al* 2009 who concluded that TDZ > TGY > TGS.

In each fraction, the isoflavones displayed synchronous accumulation kinetics. In cotyledons, however, when the environmental conditions stimulated isoflavone accumulation, the varieties may preferentially increase the accumulation of a particular isoflavone (Berger *et al*., 2008). This was the case for both *Ales* and *Nikir*, which were able to favor genistein or daidzein, respectively. Genistein, which is probably the most studied isoflavone (Dixon & Ferreira 2002), is a minor isoflavone in the hypocotyl. However, glycitein, a minor isoflavone component in the whole seed, can comprise as much as 50% of the isoflavone content of the hypocotyl and it is poorly investigated.

Malonyl-, β-glycosides and aglycone form of daidzein, genistein and glycitein were stored in hypocotyls cell. Glycosylation plays a very important role in solubilization, accumulation in vacuoles, and mobilization of isoflavonoids in legume cells (Noguchi *et al*., 2007). Most of isoflavone quantity stored in vacuoles is Malonylglycosisde derivatives form which is conjugated with both sugar and malonyl- which protects glycosides form enzymatic degradation and help in intercellular transport (Dhaubhadel *et al*., 2008). Therefore, malonylated products are the most abundant forms of isoflavonoids in soybeans.

Malonyl-, β-glycosides and aglycone form showed the tendency as functional group (daidzein, genistein, glycitein) in both cotyledon and hypocotyl.

In hypocotyl the isoflavone content (in aglycone equivalent) is rarely below 1% of the dry weight. This can probably be related to the role of isoflavones in plant–microbe interactions such as symbiosis with *Bradyrhizobium japonicum* (Kosslak *et al.*, 1987), arbuscular mycorrhizal fungi, or seedling defence against pathogens (Dixon *et al.*, 2002).

Protein content has influenced by irrigation, due to its improvement to plant metabolic (Bennett *et al* 2004) and yield increment.

Metabolism of isoflavone did not demonstrate conflict among its single molecules and all relation among them was positive in both cotyledon and hypocotyl separately. Although they have the same pathway but the plant produces all components with the same tendency with different concentrations regarding to year and varieties. The relation between molecules showed also the effect of year and variety on the difference of isoflavone level in both embryo organs. Primomo *et al.* (2005) reported that QTL for total isoflavone mapped to similar regions as daidzein and genistein but they did not mention for glycitein.

2.5 Conclusions

Isoflavone accumulation is genetically restricted by variety and seed portion (cotyledon and hypocotyl). Irrigation effect is mainly depending on the variety. Nitrogen application is important practices and its date of application may influence isoflavone contents. On single plant 30% of isoflavone contents can be found. Protein mainly influenced by water supply.

Chapter 3

Water Level Effect on Isoflavone Accumulation in Soybean Hypocotyls and Cotyledon

3.1 Introduction

Since water content is very important for all plants as effective factor for plant metabolism, isoflavones compound found to be increase by irrigation (Bennett *et al*., 2004) or decreased by drought (Seguin *et al*., 2004; Caldwell *et al*., 2005; Lozovaya *et al*., 2005). Under irrigation Lozovaya *et al*., 2005 found that both genistin and daidzin concentration had the same trend as total isoflavone but glycitin concentration was much less affected. Irrigation enhanced the isoflavone and individual isoflavones content of both early- and late-planted soybeans as much as 2.5-fold (Bennett *et al*., 2004).

That experiment was carried out to examine three different varieties under three level of irrigation and changes in isoflavone contents.

3.2 Material and Methods

The experiment was conducted in 2008 at Padova University Experiment Station, Legnaro - Italy, 45°21'03" N, 11°56'54" E in loam soil. Climate data is demonstrated in table (3.1), Water deficit calculated by (water deficit = Rainfall–ETo). Pre-sowing practices were performed; weed was handly controlled twice during plant development. Factors were arranged in a split-plot layout (Figure 3.1). There were both years included the 3 varieties (*Dekabig optimize*, *Pacific* and *Sponsor*); irrigation, was based on water deficit, water supplied in % of water deficit 25, 50 and 75%. These levels were applied for the plants to study the effect of irrigation level. Negative value of water needs reflect the efficiency of rain and irrigation to maintain water level below water needs based on accumulative value (Figure 3.2). Six rows per plot were cultivated with 40 seed m^{-2} (5m length x 50cm inter-row) in 3 replicates. Pods were collected from principal and lateral shoot at harvest time and Seeds were collected.

Table 3.1. Environment conditions and water supply under three level of irrigation in 2008.

	Temperature			**Water conditions**			**water supply**		
	med	min	max	Prec.	ETo	Wdef.	75%	50%	25%
		°C					mm		
May	17.99	12.83	22.74	95.2	nd	nd	0	0	0
Jun	21.62	16.58	26.60	80.0	87.43	-7.43	0	0	0
Jul	23.49	17.49	29.41	58.0	115.75	-57.75	45.63	30.68	16.28
Aug	23.43	17.70	29.30	77.8	118.63	-40.83	34.76	24.29	10.62
Sep	17.95	13.26	23.47	60.0	82.52	-22.52	21.58	14.62	4.18
Oct	14.73	10.08	20.66	40.6	58.35	-17.75	0	0	0

Medium (med); minimum (min); maximum (max), precipitation (Prec.), evapotranspiration (ETo), water deficit (Wdef.); Water supply is calculated % of water deficit.
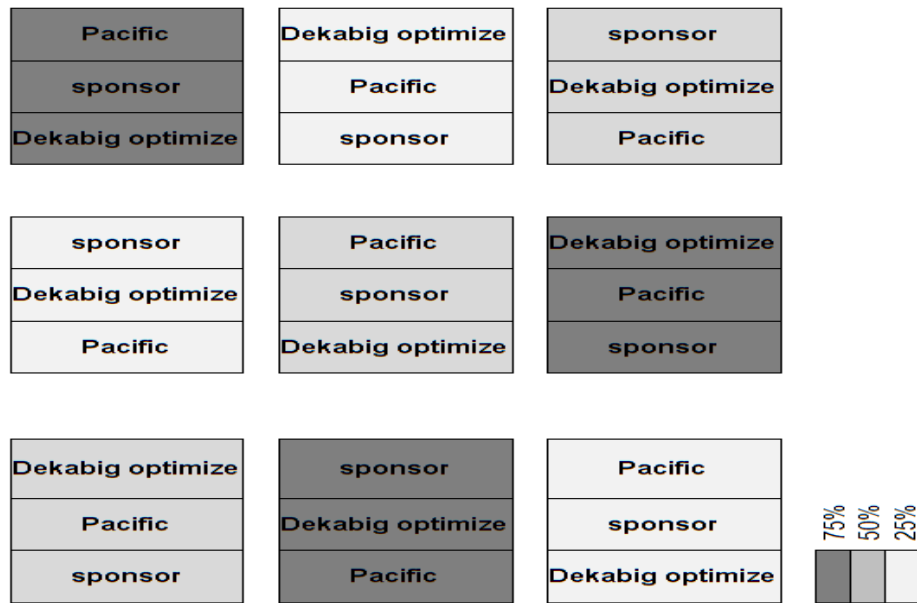
Figure 3.1. Experiment layout of soybean varieties under three level of irrigation.
75%, 50% and 25% represent the level of water supply regarding to maximum water deficiency
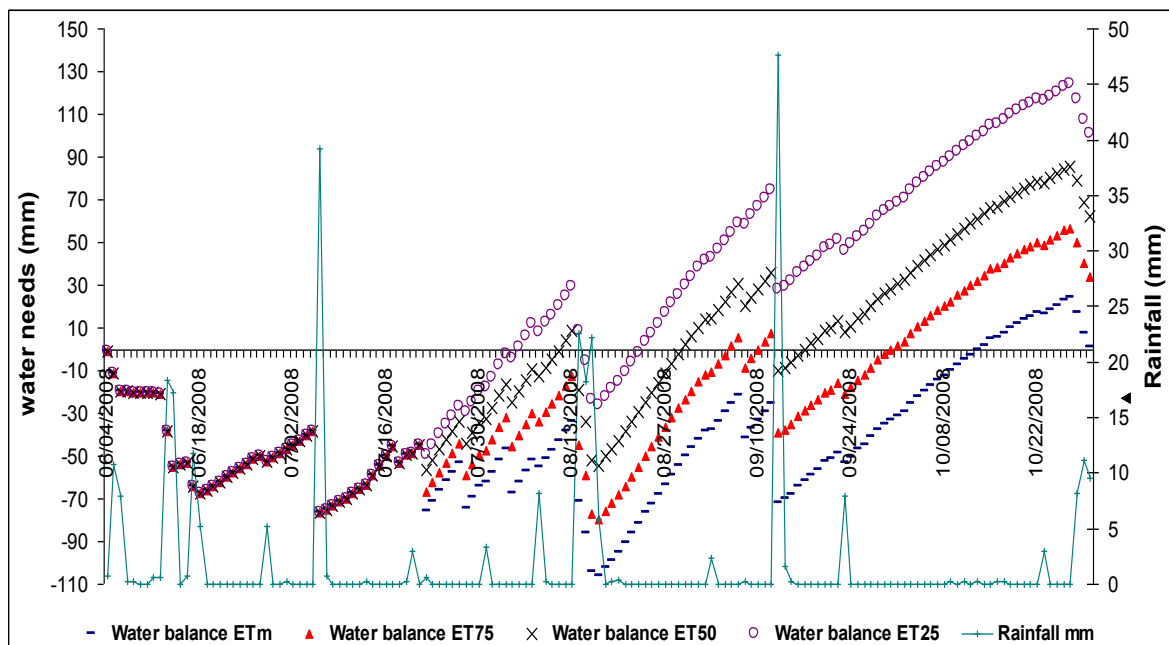
Figure 3.2. Evapotranspiration of different levels of irrigation and their water balance considering irrigation and rainfall.
ETm, ET75, ET50, ET25 represent level of water supply % from water deficiency.

For isoflavone analysis, was estimated in the same method sited in general material and methods. Data were analyzed by ANOVA using general linear model GLM. All statistical analysis was performed using statgraphics centurion XV version 15.2.06.

3.3 Results

In 2008 three varieties were selected regarding to their ability of producing moderate amount of isoflavone and settled under three level of water supply.

3.3.1 Yield components under different irrigation levels

No big difference among variety or different water supply levels on pod weight and number per plant where as the main shoot carry 80-90% of total pod carried on the plant (Table 3.2). On principal shoot varieties showed significant effect on seed yield components. *Pacific* and *Dekabig opt* main shoot demonstrated higher significant value than sponsor of yield components where as *Sponsor* had greater lateral shoot yield components than other two varieties. Medium level of irrigation showed greater number and weight of pod on principal shoot than other two levels but no effect was observed on seed dry weight. No effect was found for irrigation level on lateral shoots yield components. No correlation was noticed between isoflavone and protein content and yield component under this experiment. Although 50% and 75% irrigation treatments have illustrated higher value of number and weight of pod compared with 25% but they did not show significant difference among them regarding to seed yield weight. This result reflects the importance of plant recovery for weight instead of high reproductivity.

Table 3.2. Yield component (g plan^{-1}) under level of irrigation and varieties.

shoot		Variety			Level of irrigation		
		DEK. opt	Pacific	Sponsor	25	50	75
Principal	**nPd**	31.93 a	31.30 a	27.68 b	24.00 c	36.81 a	30.09 b
	wPd	15.72 ab	16.14 a	14.61 b	13.68 c	17.09 a	15.70 b
	SDW	11.35 a	12.14 a	9.44 b	10.69 a	10.91 a	11.33 a
Lateral	**nPd**	4.41 c	10.89 b	17.85 a	11.86 a	10.83 a	10.46 a
	wPd	2.11 c	4.45 b	9.77 a	5.86 a	5.56 a	4.91 a
	SDW	1.51 c	3.18 b	6.94 a	4.13 a	4.00 a	3.49 a

n° pod (nPd); pod weight (wPd); Seed dry weight (SDW)

3.3.2 Hypocotyl and cotyledon isoflavone and seed crude protein contents under different irrigation levels

Hypocotyl showed great sensibility compared with cotyledon regarding to variety. TGS in *Pacific* variety displayed 30% higher than *Sponsor* and 23% higher than *Dekabig Optimize* but no significant difference between last two (Table 3.2). TGY was significantly higher than both other varieties. Hypocotyl did not influence by level of irrigation or seed site production but it showed variety dependence characteristic. Lateral stem demonstrated greater accumulation for different isoflavone forms and groups except aglycone form. Greater isoflavone in cotyledon was accumulated on lateral; 14% TDZ, 17% TGS, 18% β-glycoside and 16% malonyl and TISO. Only TDZ showed significant difference among cotyledon of three varieties, sponsor verified ≈ 35% greater content than other two varieties. Lateral shoot revealed greater TISO and its forms in cotyledon than principal shoot but no effect only TGY was significant in case of hypocotyl. Other forms isoflavone forms and TISO in hypocotyl showed higher tendency on lateral shoot. Aglycone form expressed difference among varieties up to 50% but β-glycoside form showed up to 20% variation among varieties. Aglycone and β-glycoside considered as the reaction of symptoms of different varieties to biotic and abiotic stress (Dhaubhadel *et al*., 2008). Protein contents revealed significant response to variety difference but did not show any response to irrigation level. Protein content tends to be negative correlation with isoflavone contents, since they showing opposite value under verity, irrigation and stem.

Table 3.3. Isoflavone forms in hypocotyls and cotyledon and yield components under water and nitrogen supply 2008.

		Cotyledon			Hypocotyls				Weight			CP
		TDZ	TGS	TISO	TDZ	TGY	TGS	TISO	Cot	Hyp	100 seed	%
		mg g^{-1}							mg seed^{-1}		g	
Variety	**DK**	0.48	1.23	1.72	5.42	4.63	1.83	11.9	157.6	3.70	17.92	40.2
(V)	**PC**	0.47	1.23	1.72	6.05	4.81	2.37	13.3	143.0	3.32	15.97	39.4
	SP	0.67	1.18	1.86	5.94	3.91	1.70	11.6	152.6	4.06	17.42	40.4
LSD		*0.1*	*ns*	*ns*	*0.33*	*0.34*	*0.15*	*0.8*	*3.73*	*0.16*	*0.31*	*0.72*
Irrigation	**25**	0.526	1.18	1.72	5.76	4.46	1.97	12.2	151.4	3.66	17.21	40.3
(I)	**50**	0.551	1.20	1.76	5.79	4.39	1.95	12.1	151.2	3.71	16.99	40.0
	75	0.543	1.27	1.82	5.86	4.5	1.99	12.4	150.6	3.71	17.11	39.7
LSD		*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
stem (S)	**P**	0.50	1.11	1.61	5.77	4.33	1.94	12.06	150.8	3.59	17.05	40.2
	R	0.58	1.327	1.92	5.84	4.56	2.00	12.41	151.3	3.80	17.15	39.7
LSD		*0.03*	*0.123*	*0.13*	*ns*	*0.23*	*ns*	*ns*	*ns*	*0.1*	*ns*	*0.3*
V×I		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V×S		*	*	*	ns	*	*	ns	ns	ns	**	ns
I×S		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V×I×S		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Total daidzein, (TDZ); Total genistein (TGS); Total glycitein, (TGY); Total isoflavone, (TISO); crude protein (CP); cotyledon (Cot); hypocotyl (Hyp); P < (0.05 *), (0.01 **) and (0.001 ***).

3.3.3 Interaction among irrigation level, variety and seed site production on isoflavone contents

Irrigation did not show significant interaction with other factors. The interaction between variety and irrigation level did not show significant difference on different isoflavones in both seed organs. V×S interaction showed significant effect on isoflavone contents (Table 3.4). Cotyledon TISO, TDZ and TGS and hypocotyl TGY and TGS showed significant changes due to the interaction V×S and the fluctuation arrive up 20% of overall the treatment. TDZ demonstrated great significance but the coefficient of variation was about 25% due to the sensibility of isoflavone molecules to variability between blocks. The effect of interaction on TGS in both hypocotyl and cotyledon was significant

(P=0.037), in all combination contain lateral shoot showed greater isoflavone per each variety. Aglycone form did not show significant result under the interaction and CV was very high (75%) due to low concentration of aglycone form. β-glycoside and Malonyleglycoside influenced significantly by the interaction and CV was similar 21%.

Table 3.4. Isoflavone contents in hypocotyls and cotyledon of different varieties regarding to pod position on the plant.

Stem	variety	Cotyledon			Hypocotyl			
		TISO	TDZ	TGS	TISO	TDZ	TGY	TGS
					mg g^{-1}			
Principal	Dek opt	1.54	0.42	1.11	11.80	5.42	4.56	1.81
	Pacific	1.52	0.43	1.09	13.41	6.14	4.87	2.39
	Sponsor	1.77	0.65	1.12	11.81	6.11	3.94	1.75
Lateral	Dek opt	1.90	0.53	1.36	12.84	5.79	5.06	1.99
	Pacific	1.91	0.52	1.37	13.08	5.96	4.75	2.35
	Sponsor	1.81	0.64	1.16	12.63	6.44	4.33	1.85
LSD		0.27	0.08	0.19	ns	ns	0.40	0.20

Total daidzein, (TDZ); Total genistein (TGS), Total glycitein, (TGY); Total isoflavone, (TISO).

3.4 Discussion

Hypocotyl has showed difference regarding to variety but cotyledon did not demonstrate any difference among them. Hypocotyls produce all three main forms of isoflavone TDZ, TGS and TGY percentage was measured based on total isoflavone. Profile of hypocotyls isoflavone under agronomic practices or site production did not show difference but variety was the only promoter for the level of isoflavone in cell. Variability of isoflavone profile due to variety genetic capacity may create sort of classification of varieties regarding to their ability to produce specific isoflavone molecules.

This experiment confirm that irrigation may have no effect in some cases may be due to climate difference (Berger *et al*., 2008) and/or variety (as previous chapter).

Lateral shoots form their pods later which may explain the significance regarding to cotyledon compared with hypocotyl which formed in short period (Berger *et al*., 2008). Exposure to low temperature during pod filling is able to increase isoflavone contents of late formed seed or lateral shoot seeds (Lozovaya *et al*., 2005).

Yield component per plant illustrated significant difference among varieties for both principal and lateral shoots. Water supply at 50% of water deficit represents the highest significant value compared with other two levels.

Crude protein is not affected by irrigation but it show significant difference regarding to variety and pod site on the plant.

No clear relation was observed between yield components and protein with isoflavone accumulation.

Brevedan and Egli (2003) also found that water stress during soybean seed fill in greenhouse experiments results in earlier maturity. The higher isoflavone concentrations often found in later maturing genotypes (Nelson *et al*., 2001) is also consistent with our results obtained regarding to lateral shoot late formation and higher isoflavone content compared with principal shoot isoflavone contents.

Magnitude of changes in isoflavone concentration in response to changes in level of irrigation or stem is highly variety dependent that was in agree with (Lozovaya *et al*., 2005). Medium water supply at 50% of water deficit illustrated higher effect on isoflavone accumulation compared with other high and low level. This result in consist with Al-Tawaha *et al*. (2007) who showed that medium level of water supply gave higher

isoflavone value. Also they found that effect of irrigation was connected variety and year of application.

The response to irrigation observed was in accordance with previous greenhouse and field studies, which also reported that irrigation may increase isoflavone concentrations in soya bean; the response depending on varieties (Bennett *et al*., 2004; Caldwell *et al*., 2005; Lozovaya *et al*., 2005). In Missouri, Bennett *et al*. (2004), indeed, reported 130–280 % increases in total and individual isoflavone concentrations of irrigated vs. non-irrigated late-maturing soya bean varieties. Response varied depending on the seeding date and the variety, being greater in varieties of MG IV than V, and with earlier planting dates. While in our study the results were in consist with (Al-Tawaha *et al*., 2007) who reported the interference of other factor with irrigation. Al-Tawaha *et al*. (2007) mentioned that irrigation response to irrigation may be non-linear in nature, with plants grown in dry soils responding to irrigation up to a certain point, after which excess irrigation could negate any potential benefits. Level of irrigation effect depends mostly on year of application due to variability of climate conditions then variety.

Number and weight of pod per plant have showed higher value under 50% and 75% irrigation treatments compared with 25% but they did not show significant difference among them regarding to seed yield weight. This result reflects the importance of plant recovery for weight instead of high reproductivity. These large responses to irrigation most likely reflects a lower flower abortion rate in high level irrigated plots, which led to develop greater number of pod per plant (Al-Tawaha *et al*., 2007).

Protein contents revealed significant response to variety but did not show any response to irrigation level as reported by (Al-Tawaha *et al*., 2007). Protein content tends to be negatively correlated with isoflavone contents, they showing opposite tendency under variety, irrigation and stem. That was in agreeing with the result of Chiari *et al*. (2004) who mentioned the negative correlation between protein contents and seed isoflavone.

3.5 Conclusion

Increasing water supply had no effect on isoflavone of three varieties under this study. Variety was more relevant than water supply on isoflavone contents, no changes for isoflavone profile under different irrigation levels.

Chapter 4

Management and plant density effect on isoflavone contents in soybean hypocotyls and cotyledon, yield components and seed protein

4.1 Introduction

Many management practices affect soybean isoflavone contents. In 2008, 4 soybean varieties were cultivated under two managements organic and conventional. Each plot was divided into two plant density 20 and 30 plant m^2. From previous study in 2005 till now cultivating different varieties under organic and conventional, the effect of conventional management was notable on cotyledon but no effect was obtained on hypocotyl. As well as examine the plant sites of pod production main and lateral shoots reveal significant effect on isoflavone accumulation. Plant density may play a critical rule regarding to formation of lateral shoot and/or increasing isoflavone contents compared with main shoot (principal). It is expected that plant density may change the quantity of different molecules but it is not expected to demonstrate influence on plant segregation percentage of each molecules.

4.2 Material and Methods

The experiment was conducted in 2008 at Padova University Experiment Station, Legnaro - Italy, 45°21'03" N, 11°56'54" E in loam soil. Climate data is demonstrated in table (4.1), Water deficit calculated by (water deficit = Rainfall – ETo). Pre-sowing practices were performed; weed was manually controlled twice during plant development. Factors were arranged in a split-split-plot layout. Four rows per plot were cultivated with 40 seed m^{-2} (4m length x 50cm inter-row) in 3 replicates. There 4 varieties were selected for this experiment (*Brillante*, *Dekabig Optimise*, *Pedro* and *Sponsor*); at R1 plots were thinned into 20 and 30 plant/ m^2. This was followed in both management systems (organic and conventional). Pods were collected on main and lateral shoot at harvest time and Seeds were collected. Plants were faced a hail event in mid of July which affect the organic site. *Dekabig Optimise* is a different variety than *Dekabig* that was reported in chapter (1). *Dekabig Optimise* was developed by DEKALB aiming to develop their nitrogen fixation effeceincy.

For isoflavone analysis, was estimated in the same method cited in (chapter 1). Data were analyzed by ANOVA using general linear model GLM. All statistical analysis were performed using statgraphics centurion XV version 15.2.06.

Table 4.1. Environment conditions and water supply under three level of irrigation in 2008.

	Temperature			Water conditions		
	med	min	max	Prec.	ETo	Wdef.
	°C			mm		
May	17.99	12.83	22.74	95.2	nd	nd
Jun	21.62	16.58	26.60	80.0	87.43	-7.43
Jul	23.49	17.49	29.41	58.0	115.75	-57.75
Aug	23.43	17.70	29.30	77.8	118.63	-40.83
Sep	17.95	13.26	23.47	60.0	82.52	-22.52
Oct	14.73	10.08	20.66	40.6	58.35	-17.75

Medium (med); minimum (min); maximum (max), precipitation (Prec.), evapotranspiration (ETo), water deficit (Wdef.)

4.3 Results

In 2008 four varieties were selected regarding to their ability of producing different levels of isoflavone as they were cultivated under both systems showed different behaviours also under both densities.

4.3.1 Variety effect on isoflavone in cotyledon and hypocotyl

Cotyledon and hypocotyl of different varieties demonstrated the same tendency regarding to isoflavone and its molecules. The differences among varieties regarding to cotyledon isoflavone and its forms were 0-120%. *Brillante* showed the lowest cotyledon TISO and both forms, which gave 100%, 110% and 130%, lower TISO than *Dekabig Optimise*, *Pedro* and *Sponsor* respectively (Table 4.2). The two isoflavone forms in cotyledon showed the same tendency. *Dekabig Optimise* verified higher TGS than *Pedro*, in which the last demonstrated higher TDZ. Hypocotyl TISO showed the same significance among varieties but the difference was limited 30-64%. *Sponsor* showed highest TISO value among varieties which was 25%, 35% and 64% higher than *Pedro*, *Dekabig* and *Brillante* respectively.

Cotyledon and hypocotyl weight demonstrated different tendencies of isoflavone contents. *Brillante* showed 1-10% higher cotyledon weight than other varieties whereas *Dekabig Optimise* showed the higher hypocotyl weight compared with other varieties. The relation between cotyledon and hypocotyl weight with their contents of TISO was negative r^2 were 8 and 6% with tested linear model for lake of fit. Protein content did not show great difference among varieties.

4.3.2 Management effect on isoflavone in cotyledon and hypocotyl

Conventional management positively influenced the cotyledon isoflavone contents compared with organic management. Hypocotyl isoflavone was not affected by management except TDZ which exhibited 5% higher under organic management. Conventional management showed 1.4% difference in protein content compared with organic management (Table 4.2). The result of protein was in the same tendency with isoflavone contents in cotyledon. That finding reveals that isoflavone and protein are accumulated components sharing in phenylalanine the same amino acid source.

4.3.3 Density and seed site effect on isoflavone in cotyledon and hypocotyl

Density and plant ramification are connected parameters. Plant density did not demonstrate any effect either on cotyledon or hypocotyl isoflavone contents (Table 4.2). Cotyledon weight did not show react for plant density but hypocotyl showed higher weight under high plant density. Protein content under both densities did not give significant difference. Lateral stem showed positive significant difference than principal shoot on total isoflavone and its forms in both cotyledon and hypocotyl. In cotyledon the difference between lateral and principal was up to 30%, whereas in hypocotyl the difference did not exceed 10%.

Table 4.2. Analysis of variance and significance for total isoflavone and its forms in cotyledon and hypocotyl.

		Cotyledon				Hypocotyl					
		TISO	TDZ	TGS	weight	TISO	TDZ	TGS	TGY	weigh	CP
			mg g^{-1}		mg/ seed		mg g^{-1}			mg/ seed	%
Var	BR	0.63b	0.22c	0.41c	185 a	9.8d	5.0d	1.92c	2.84c	3.93b	41.3ab
(V)	DK	1.29a	0.35b	0.94a	168 c	12.0c	5.5c	2.27b	4.27b	4.21a	41.8a
	PD	1.31a	0.53a	0.78b	181 a	12.8b	6.1b	2.24b	4.41b	3.87bc	41.2b
	SP	1.43a	0.53a	0.89a	175 b	16.1a	8.2a	2.86a	4.98a	3.76c	41.0b
Mang	Bio	1.02b	0.36b	0.66b	175	12.8	6.4a	2.36	4.14	3.91	40.6b
(M)	Con	1.31a	0.46a	0.85a	179	12.5	6.1b	2.28	4.12	3.97	42.0a
Density	20	1.16	0.41	0.75	176	12.7	6.2	2.35	4.14	3.88b	41.5
(D)	30	1.17	0.41	0.76	178	12.6	6.2	2.30	4.11	4.01a	41.1
Stem	P	1.02b	0.36b	0.67b	177	12.2b	6.0b	2.24b	3.99b	3.85b	42.1a
(S)	L	1.30a	0.46a	0.84a	177	13.1a	6.4a	2.40a	4.26a	4.03a	40.5b
intera											
V×M		**	*	**	*	**	**	**	**	ns	*
V×D		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V×S		**	**	**	ns	ns	*	ns	ns	ns	ns
M×D		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
M×S		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
D×S		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V×M×D		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V×M×S		ns	*	ns	ns	ns	ns	ns	ns	ns	ns
V×D×S		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
M×D×S		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
V×M×D×S		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

1 year trial at Legnaro (n = 96; i.e., 4 soybean varieties, 2 managements, 2 plant density, 2 pod positions, 3 replicates). variety (VAR); Brillante (BR); Dekabig optimize (DK); Perdro (PD); Sponsor (SP); management (Mang); organic (Org); Conventional (Con); Density 20 or 30 plant m-2, P principal shoot, L Lateral shoot, total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS) and total glyciteln (TGY), crude protein (CP), *,** at P<0.05, 0.01; ns, not significant.

4.3.4 Interaction effect on effect on isoflavone in cotyledon and hypocotyl

The interaction confirmed some results were observed in single factor. The tendency of different varieties was similar under both managements (Table 4.3). Single variety showed greater accumulation under conventional than organic. For cotyledon and hypocotyl weight and protein contents they showed the same movement. Protein contents of *Brillante* verified 2.5% reduction under organic but other *Pedro*, *Dekabig* and *Sponsor* showed reduction of 1.8, 0.7 and 1.3% respectively.

Table 4.3. The effect of management × variety interaction on isoflavone and weight of cotyledon and hypocotyl and seed protein content.

		Cotyledon				Hypocotyl				seed	
Mang	**VAR**	**TISO**	**TDZ**	**TGS**	**weight**	**TISO**	**TDZ**	**TGS**	**TGY**	**weight**	**CP**
			mg g^{-1}		mg/ seed		mg g^{-1}			mg/ seed	%
Org	**BR**	0.61d	0.21d	0.40e	183abc	9.89f	5.08f	1.96d	2.85e	3.93	40.0d
	DK	0.95c	0.26d	0.69d	162e	11.32e	5.30ef	2.04d	3.99d	4.10	41.4bc
	PD	1.18bc	0.46c	0.71cd	184ab	13.44c	6.35c	2.55bc	4.54bc	3.85	40.8cd
	SP	1.35ab	0.50bc	0.84bc	173d	16.88a	8.76a	2.91a	5.21a	3.78	40.1d
Con	**BR**	0.64d	0.22d	0.42e	188a	9.90f	4.82f	1.91d	2.85e	3.93	42.5a
	DK	1.57a	0.42c	1.16a	174d	12.73cd	5.68de	2.49c	4.56bc	4.32	42.1ab
	PD	1.45a	0.60a	0.84bc	178bcd	12.26de	5.97cd	1.95d	4.34cd	3.89	41.5bc
	SP	1.51a	0.57ab	0.94b	176cd	15.28b	7.72b	2.80ab	4.75b	3.75	41.9ab
LSD$_{0.05}$		*0.24*	*0.09*	*0.15*	*7.51*	*1.00*	*0.50*	*0.31*	*0.36*	*ns*	*0.95*

management (Mang); organic (Org); Conventional (Con); total isoflavone (TISO); total daidzein (TDZ); total genistein (TGS); total glycitein (TGY), crude protein (CP).

Lateral shoot indicated higher TISO contents in cotyledon and its both forms compared with principal shoot. Varieties proved the same tendency under both principal and lateral shoots (Table 4.4).

Although sponsor principal shoot cotyledon showed 22% higher than *Pedro* but the last confirmed the same level of TISO in cotyledon of lateral shoot. *Brillante* lateral shoot cotyledon did not show significant difference than principal shoot cotyledon.

Table 4.4. The effect of stem × variety interaction on cotyledon isoflavone content.

Stem	Var	TISO	TDZ	TGS
			mg g^{-1}	
P	BR	0.61 c	0.21 d	0.40 e
	DK	1.00 b	0.27 d	0.73 cd
	PD	1.12 b	0.44 bc	0.67 d
	SP	1.37 a	0.51 b	0.86 bc
L	BR	0.65 c	0.22 d	0.43 e
	DK	1.55 a	0.43 c	1.13 a
	PD	1.51 a	0.62 a	0.88 bc
	SP	1.49 a	0.56 ab	0.92 b
LSD$_{0.05}$		*0.25*	*0.09*	*0.16*

principal shoot (P); Lateral shoot (L); variety (Var); Brillante (BR); Dekabig optimize (DK); Perdro (PD); Sponsor (SP); total isoflavone (TISO), total daidzein (TDZ), total genistein (TGS).

4.3.5 The profile of cotyledon and hypocotyl isoflavone in different varieties

The difference of isoflavone quantity does not reflect the profile of different varieties. Cotyledon and hypocotyl confirmed variability in the percentage of different isoflavone forms due to metabolic capability of different varieties in producing one molecule or the other (Figure 4.1). Hypocotyl showed high similarity in the percentage between *Sponsor* and *Brillante* although they have different concentrations of isoflavone and its forms (Figure 4.1). However, genistein and daidzein represent the higher portion in cotyledon and hypocotyl respectively. Genistein is an important molecule due to its stability in hypocotyl and none interfere of other two forms in its pathway. It represent great portion of its accumulation in cotyledon which reflect its importance during seed emergence. In hypocotyl was remarked that daidzein represent equivalent quantity for sum of genistein and glycitein.
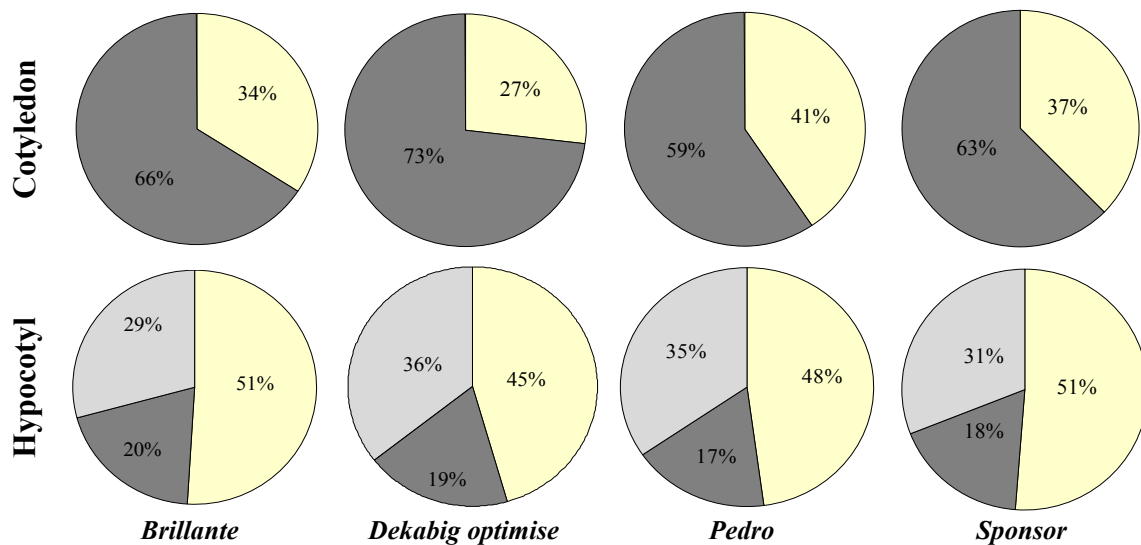
Figure 4.1. Cotyledon and hypocotyl isoflavone profile of soybean varieties ■TDZ, ■ TGS, ■ TGY.

4.3.6 Yield components under management and plant density

On principal shoot no significant between varieties yield components except *Brillante* which showed the lowest significant value (Table 4.5). Conventional and low density was significantly higher value than conventional and high density. Although low plant density gave higher yield components on main and lateral shoot per plant but it did not exceed 20% taking into consideration that the difference in plant density is more than 30%. Lateral shoot shoots revealed different behaviours due negative correlation between principal and lateral shoot on yield components. *Brillante* gave high significant value followed by *Sponsor* in nPd, wPd and SDW per plant followed by sponsor which was higher than other two varieties. Organic showed high significant yield component on lateral shoot compared with conventional management.

The interaction between variety and management illustrated significant effect on yield components except nPd and wPd on main shoot (Table 4.6). Organic management low yield on principal shoot compared to conventional which showed low yield on lateral shoot. That was attributable to the effect of hails that caused cut of apical meristem and introduce branches very early than expected. Ability of *Brillante* and *Sponsor* were high on making high yield on branches.

Table 4.5. Yield component under two plant density, two managements and varieties.

		Principal shoot			Lateral shoot		
		nPd	wPd	SDW	nPd	wPd	SDW
		g plant^{-1}					
Var	BR	27.1 b	16.3 b	10.2 b	31.9 a	19.3 a	12.5 a
(V)	DK	37.0 a	21.2 a	12.6 a	17.5 c	9.7 c	6.1 c
	PD	33.0 a	20.9 a	13.1 a	17.3 c	11.0 bc	7.1 bc
	SP	35.8 a	21.9 a	13.5 a	23.2 b	13.7 b	8.8 b
Mang	Org	26.31 b	16.6 b	10.1 b	28.2 a	17.4 a	11.1 a
(M)	Con	40.18 a	23.5 a	14.5 a	16.8 b	9.5 b	6.1 b
Density	20	35.47 a	21.2 a	13.0 a	28.0 a	16.9 a	10.7 a
(D)	30	31.02 b	19.0 b	11.7 b	17.0 b	10.0 b	6.5 b
	V×M	ns	ns	*	**	**	**
	V×D	ns	ns	ns	ns	ns	ns
	M×D	ns	*	*	ns	ns	ns
	V×M×D	ns	*	ns	ns	ns	ns

variety (Var); Brillante (BR); Dekabig optimise (DK); Perdro (PD); Sponsor (SP); management (Mang); organic (Org); Conventional (Con); Density 20 or 30 plant m^{-2}; n° pod (nPd); pod weight (wPd); Seed dry weight (SDW).

Table 4.6. The effect of management × variety interaction on yield components on principal and lateral shoots.

		principal shoot			lateral shoot		
		nPd	wPd	SDW	nPd	wPd	SDW
		g plant^{-1}					
Org	BR	22.5	14.2	9.0 b	43.2 a	27.0 a	17.4 a
	DK	27.5	16.1	9.3 b	21.4 bcd	12.3 bc	7.5 bc
	PD	26.7	18.5	11.5 b	22.3 bc	14.8 b	9.6 b
	SP	28.5	17.6	10.7 b	26.0 b	15.7 b	9.9 b
Con	BR	31.6	18.4	11.5 b	20.7 bcd	11.6 bc	7.6 bc
	DK	46.5	26.3	15.8 a	13.7 cd	7.18 c	4.7 c
	PD	38.8	23.2	14.6 a	12.3 d	7.19 c	4.6 c
	SP	43.2	26.2	16.2 a	20.4 bcd	11.81 bc	7.7 bc
LSD$_{0.05}$		ns	ns	2.52	9.61	6.17	3.8

nPd n° pod, wPd pod weight, SDW Seed dry weight, BR, Brillante, DK Dekabig optimise, PD Perdro, SP Sponsor

4.4 Discussion

Varieties can have two destination high or low isoflavone contents. Many agronomic practices are recommended to control the content of isoflavone in soybean seed.

In cotyledon cultivation conditions influence significantly total isoflavone and single molecules for all varieties due to its long accumulation period which arrived up to 60 days after flowering. However, hypocotyl did not that sensibility due to short accumulation period which arrive to its maximum peak after 40 of flowering. Hypocotyl of 4 varieties either under organic or conventional has showed high stability in their contents of isoflavone. Lateral shoot result confirm experiment in chapter 2, in which lateral shoots accumulate 10-30% isoflavone in hypocotyl and cotyledon respectively.

Plant density did not show effect on isoflavone contents but its increase ramification of the plant and consequently the yield per plant produced on lateral shoot which may represent 30-50% of plant yield. That can be a benefit for few grains with high contents of isoflavone. Lateral shoots form their pods later which may exposure them to low temperature during pod filling consequently increase isoflavone contents of seed formed on lateral shoot (Lozovaya *et al*., 2005). *Brillante* both seed portions did not show significant variation in their isoflavone contents on principal and lateral shoot, so it is better to be adapted to high plant density to reduce problem of lateral shoot with the harvesters.

Cotyledon isoflavone forms showed different percentages due to varieties but always TGS% was higher than TDZ%. Profile of hypocotyls and cotyledon isoflavone under managements, plant density and seed site on plant did not show difference but variety was the only promoter for the level of isoflavone in cell. Hypocotyls produce all three main forms of isoflavone TDZ, TGS and TGY percentage was measured based on total isoflavone of hypocotyls. In hypocotyls, daidzein and its glycoside conjugates ($\approx$50%) were the most abundant isoflavones, being followed by glycitein ($\approx$30%) and genistein series ($\approx$20%). In cotyledons, genistein and its glycoside conjugates ($\approx$60%) were the main isoflavones, being followed by daidzein series (40%), and no glycitein series was sighted. Hypocotyl profile did not show difference in % due to varieties. That reflects the stability of gene control of different varieties and importance of that balance among different isoflavone forms in hypocotyl. Cotyledon and hypocotyl weight were influenced controlled by plant genotype as well.

Variability of isoflavone profile due to variety genetic capacity may create sort of classification of varieties regarding to their ability to produce specific isoflavone molecules.

4.5 Conclusions

Soybean varieties showed wide diversity of their content of isoflavone in cotyledon and hypocotyl. Management did influence cotyledon content of isoflavone due to long accumulation period. Plant density has no effect on accumulation of isoflavone but it increased ramification under low density. Lateral shoots hypocotyl and cotyledon illustrated 10-30% higher isoflavone than principal shoot. Among three molecules in hypocotyl, genistein was showed great stability in different varieties.

Chapter 5

Soil Type Effect on Isoflavone Contents in Hypocotyl and Cotyledon

5.1 Introduction

Variety and environment represent the most effect factors that affect isoflavone contents. Management was demonstrated significant effect on accumulation of isoflavone which may introduce possibility of having effect for soil type due to their variability of different soil chemical and physical properties.

5.2 Material and Methods

The experiment was conducted in 2008 at Padova University Experiment Station, Legnaro - Italy, 45°21'03" N, 11°56'54" E. The experiment implicated four different soil types (sandy, clay-loam, silty-loam, organic) (Table 5.1), placed in 2×2 m wide lysimeters, that were cultivated with the *Brillante* variety in 2008. Each lysimeter, 2 m deep and closed at the bottom, was subdivided into 2 sectors, thus providing 2 replicates for each evaluated parameter. Lysimeters were kept at optimal water supply, based on a 10-day interval water balance. Varieties chosen were based on their contents of isoflavone from previous experiment except *Dekabig optimize,* which was recently introduced into the market.

Table 5.1. Main soil properties of different soil types.

	Sandy	Silty-loam	Clay-loam	Organic
Silt (%)	5	65	30	40
Sand (%)	80	15	35	40
Clay (%)	15	20	35	20
pH	8.11	8.15	8.09	7.91
O.M. (%)	0.64	1.77	2.42	3.6
N (%)	0.03	0.11	0.15	0.19
C/N	12.1	9.72	9.41	10.9
CEC (cmol(+) kg^{-1})	5.1	15.4	22.6	37.5
Total P (g P$_2$O$_5$ kg^{-1})	0.35	0.81	0.98	1.39
Av. P (mg P$_2$O$_5$ kg^{-1})	6.01	8.46	30.0	22.16
Exc. K (mg K$_2$O kg^{-1})	26.49	59.9	88.3	108
Exc. Mg (mg kg^{-1})	51.17	247	382	460
Exc. Ca (mg kg^{-1})	9.16	2619	3833	6659
Exc. Na (mg kg^{-1})	5.62	26.1	29.6	36.5
S (mg kg^{-1})	167	408	444	496

Water was supplied due to water needs of the crop by calculating water deficiency. That was calculated based on rain and evapotranspiration (Table 5.2).

Table 5.2. Water supply for different lysimeters during growth.

		23/07	28/07	04/08	29/08	04/09	TWS
Variety	**Lysimeter**			mm			
Brillante	**Sand**	30.00	30.25	25.25	120.75	51.50	257.75
Brillante	**Clay-loam**	33.50	32.50	29.25	83.00	36.25	214.50
Brillante	**Silty-loam**	30.75	31.25	24.50	79.25	37.00	202.75
Brillante	**Organic**	34.25	33.50	29.50	84.00	34.25	215.50
Dek opt	**Silty-loam**	34.00	36.75	28.25	75.75	35.75	210.50
Dek opt	**Organic**	34.75	37.25	28.50	78.75	38.25	217.50
Sponsor	**Silty-loam**	34.00	37.00	27.75	74.25	38.00	211.00
Sponsor	**Organic**	33.50	36.25	27.50	74.75	35.00	207.00

Quantity of water applied in different soils based water deficit level calculated by extracting rain from evapotranspiration of each period. TWS, total water supply

For isoflavone analysis, was estimated with the same method described in material and methods of chapter (1). Isoflavones were grouped as aglycone equivalent form for different active groups. Data were analyzed by ANOVA using one way ANOVA. *Brillante* was considered alone in 4 different soil types. The other 4 lysimeters were silty-loam and organic soils were cultivated by two varieties *Sponsor* and *Dekabig optimize* and they were compared with *Brillante* cultivated in the same soil type. All statistical analysis were performed using statgraphics centurion XV version 15.2.06.

5.3 RESULTS

5.3.1 Effect of soil type on isoflavone in cotyledon and hypocotyl of soybean variety (Brillante)

Variety *Brillante* (experiment in differing soils) consistently with its classification in group 1, Cotyledon isoflavone concentrations had the following order, sandy > silty-loam > clay-loam > organic (Table 5.3), with significant differences among sandy and other soil types.

The difference between sandy soil and other soil types was around 25-35%. The same tendency was observed on both isoflavone forms (daidzein and genistein) in cotyledon. Different from what observed in cotyledon that hypocotyl isoflavone concentration had the following order clay-loam > sandy = organic > silty-loam. The difference between the highest (clay-loam) and the lowest (clay-loam) was 23% and their difference with other two soil types were about ±10% compared with the highest and lowest value. The same tendency was observed on both isoflavone forms in cotyledon daidzein, glycitein and genistein. Protein concentration in seed was significant in which clay-loam soil showed the lowest significant value compared to other soil type. The tendency of protein content showed the negative tendency with total isoflavone in hypocotyl.

The preservation of global profiles despite very different growing conditions indicated a large part of genetic control of each single molecule (Figure 5.1). The kinetics of different molecules and their accumulation were observed to be stable due to variety and showed similar profile.

Table 5.3. Effect of soil type on cotyledon and hypocotyl isoflavone contents and seed protein contents.

	Cotyledon			Hypocotyl				Seed
Soil	TISO	TDZ	TGS	TISO	TDZ	TGY	TGS	protein
Sandy	0.70 a	0.25 a	0.45 a	6.34 b	3.36 b	1.93 ab	0.95 b	41.05 a
Silty-loam	0.52 b	0.18 b	0.35 b	5.57 c	2.98 c	1.75 b	0.84 a	41.01 a
Clay-loam	0.51 b	0.19 b	0.32 b	6.86 a	3.67 a	2.10 a	1.08 c	39.71 b
Organic	0.46 b	0.17 b	0.30 b	6.25 b	3.34 b	1.93 ab	0.98 b	41.25 a

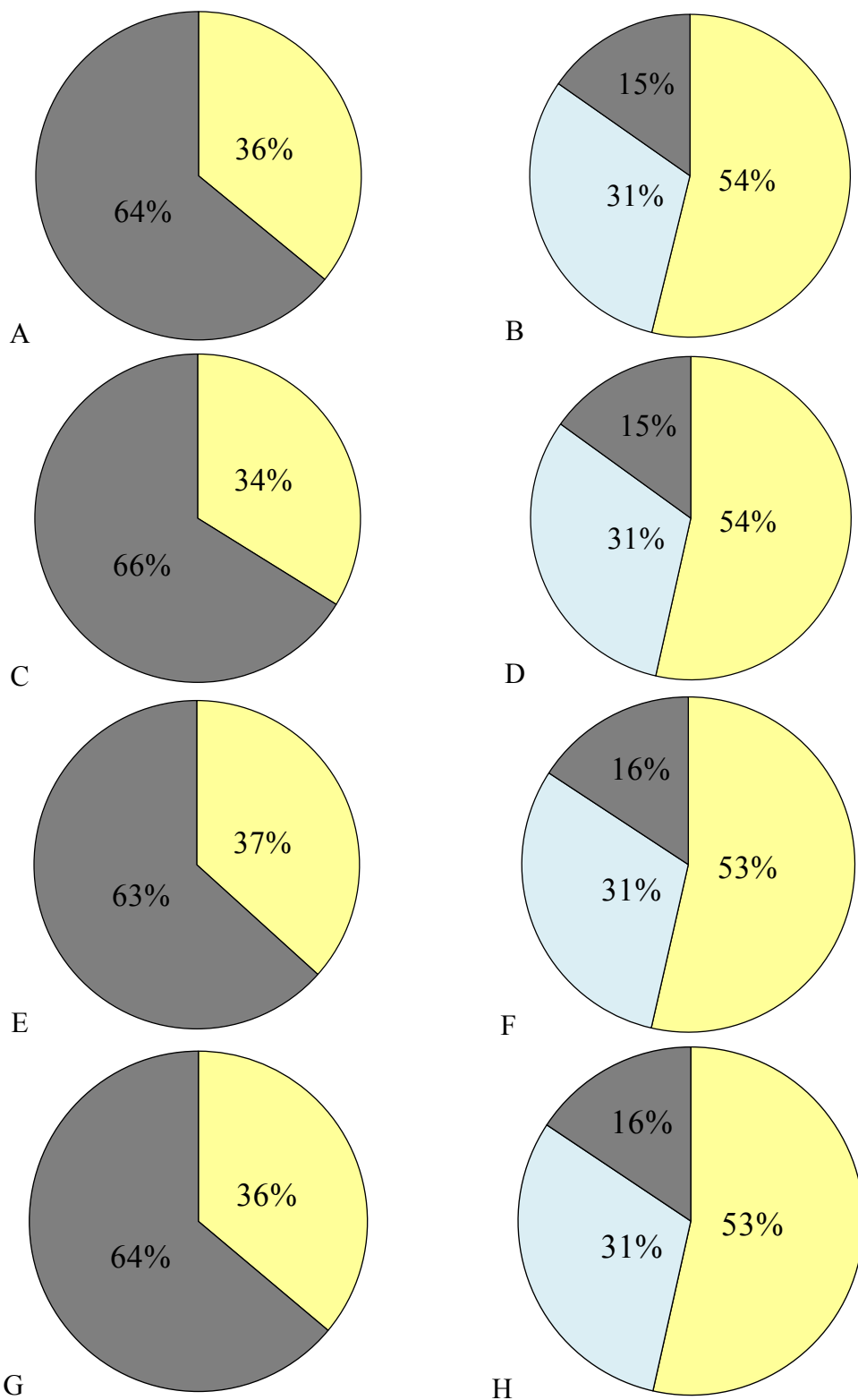Total isoflavone (TISO), total daidzein (TDZ); total genistein (TGS); total glycitein (TGY).

Figure 5.1. Cotyledon and hypocotyl profile under different soil types.

Isoflavone profile (A, C, E, G) in cotyledon and (B, D, F, G) in hypocotyl, under four soil type: (A, B) sandy, (C, D) silty-loam, (E, F) clay-loam, and (G, H) organic. ☐TDZ ☐TGS ☐TGY.

5.3.2 Effect of soil type on isoflavone in cotyledon and hypocotyl of soybean varieties

Different variety and soil type on isoflavone accumulation confirm the obtained result from organic and conventional experiment (Table 5.4). In which variability of varieties was observed in cotyledon and hypocotyl but different soil showed their effect on cotyledon isoflavone contents. The obtained results confirmed that (i) importance of genotype in accumulation of isoflavone in hypocotyl (ii) sensibility of cotyledon soil conditions.

Table 5.4. Organic and silty-loam soil effect on isoflavone content in cotyledon and hypocotyl of three varieties.

	Cotyledon			Hypocotyl			
	TISO	TDZ	TGS	TISO	TDZ	TGS	TGY
VAR							
Brillante	0.49 c	0.17 b	0.32 c	6.35 b	3.31 c	0.99 b	2.05 b
Dek opt	1.38 a	0.40 a	0.99 a	8.05 a	3.80 b	1.29 a	2.96 a
Sponsor	1.11 b	0.40 a	0.71 b	8.74 a	4.62 a	1.20 a	2.91 a
Soil type							
Organic	0.88 b	0.28 b	0.60 b	7.83 a	3.97 a	1.20 a	2.66 a
Silty-loam	1.11 a	0.36 a	0.75 a	7.59 a	3.85 a	1.12 a	2.61 a

TISO Total isoflavone, TDZ total daidzein, TGS total genistein, TGY total glycitein, VAR variety.

Profile of all three varieties did not show great changes under organic and silty-loam soil. No interaction effect was detected on different variables.

5.4 Discussion

Isoflavones are considered secondary metabolites whose biosynthetic pathway is related to protein accumulation. The crossing-point is represented by phenylalanine, an initial precursor of isoflavones and an essential amino acid in soybean proteins. It constitutes more than 5% of conglycine proteins (Zheng *et al.*, 2009), and its carbon ring represents about 34% of aglycone isoflavones as stechiometric weight. Although total mass produced during the crop cycle by secondary metabolism is very small, competition for phenylalanine would explain the generally negative (weak) correlation between isoflavones and seed proteins. As it was found that the correlation was significant in conventional but not in organic management, it is believed that available nitrogen in the plant, derived mainly from N-fixation and secondarily by uptake, was greater in our organic agriculture.

There is evidence in the literature (Rasolohery *et al.*, 2008) of great stability in isoflavone concentrations in hypocotyl, unlike cotyledons, which are affected by several environmental variables. Glycitein and its three derivatives exclusively occurred in the hypocotyl (Kudou *et al.*, 1991). Because of their polar, hydrophilic properties, the isoflavone conjugates are located in vacuoles (Barz and Welle, 1992).

Ghosh *et al.* (2004) found increased nodule mass and total chlorophyll content in soybean when organic fertilisers were used instead of inorganic. Within reasonable limits, the frequent use of mature manure in organic management may have the effect of increasing the C/N ratio over time and improving N status of the plant through N-fixation, corroborating our results showing better seed proteins and lower isoflavones.

5.5 Conclusion

Soil type showed significant effect on isoflavone contents of both seed organs cotyledon and hypocotyl. Variety confirmed its strong genetic control on isoflavone profile under different soil type.

GENERAL CONCLUSIONS

Five different experiments were carried out to reach the most effective factor on isoflavone accumulation. Within these experiments there was one conducted in 4 years, one in 2 years and 3 were conducted in one year. Cotyledon and hypocotyl have different metabolism from each other. Cotyledon showed greater sensibility to climate and cultivation conditions. However, isoflavone accumulations in both seed organs are genetically controlled but due to longer accumulation period of isoflavone in cotyledon it create great heterogeneity of its content. Low temperature in well rainy season accumulates higher isoflavone contents due to expanding accumulation period of cotyledon isoflavone under low temperature. Due to good nutrition availability of conventional farm than organic one, the first has illustrated higher accumulation of isoflavone in cotyledon. Variety proved great control in both cotyledon and hypocotyl due to stability of ranking of different varieties within four year experiment. Varieties may vary in their contents of different isoflavone forms but they showed great similarity in the profile. In all varieties genistein and daidzein were the higher percentage in cotyledon and in hypocotyl respectively. Some varieties exhibited special characteristic of presence of greater value of glycitein in hypocotyl that fact reveal that breeding program and each molecule has different rules. Maximum and daily range temperature were the most correlated climate factor with isoflavone contents in cotyledon but no relations were existing with hypocotyl isoflavone. Nutrients availability for plants is more effective than temperature and that was observed, which confirm that nutrient availability of mineral fertilizer of previous crop is more relevant in general under conventional agriculture.

Low level isoflavone and high glycitein percentage varieties considered an important source of investigation due to stability of the first and changes in metabolic pathway of the second.

Other four experiments were trials to find out most effective factor could modify the effect of environment and variety on isoflavone contents in both cotyledon and hypocotyl.

Irrigation treatment effect was mainly connect with difference in soybean varieties and environment, in which drought stress has a role in increasing isoflavone contents in cotyledon and hypocotyl. Soil water supply had less effect than environment on isoflavone contents and compositions. The mechanism by which this increase was facilitated is still unknown, but it could be connected with elongation of accumulation period of isoflavone

under low temperature. Irrigation level did not influence isoflavone contents which reflect that water supply has no importance in compare to environment conditions. However, that experiment revealed variety importance for the effect of water supply on isoflavone contents.

Surprisingly than other studies N application revealed a significant effect on isoflavone contents. Late N application at (R3) demonstrated negative effect on isoflavone contents, whereas early N application at (R1) expressed positive effect on isoflavone contents.

In all 4 small experiments, lateral shoot showed greater accumulation of isoflavone in both cotyledon and hypocotyl than principal shoot, which showed the variability within the same plant. Definitely plant density experiment confirmed that lateral shoot give higher isoflavone contents than main shoot but the only exception was for *Brillante* which showed no difference in accumulation between lateral and principal shoot.

Soil type proved difference in cotyledon and hypocotyl isoflavone contents due to their variability of C/N ratio, nutrition and water retention.

The preservation of global profiles despite very different growing conditions indicated a large part of genetic control of each single molecule. The kinetics of different molecules and their accumulation were observed to be stable due to variety and proved similar profile.

Further studies are needed to examine large maturity group range of varieties and the effect of climate variability on isoflavone contents of them in order to confirm the effect of temperature. Diversity of response of variety for water supply may restrict on trying it before taking the decision of cultivating it. N application needs further studies for examining its effect on different varieties at different growing stages. In order to stabilize the variability of soil types effect on isoflavone contents further studies are needed to examine different variable independently. Organic matter level in soil may become an interesting parameter to study its effect on isoflavone contents.

References

AFSSA and AFSSAPS (2005). Sécurité et bénéfices des phyto-estrogénes apportés par l'alimentation – Recommandations. AFSSA. p 370.

Albareda, M., Dardanelli M.S., Sousa, C., Megías, M., Temprano, F. and Rodríguez-Navarro, D.N. (2006). Factors affecting the attachment of rhizospheric bacteria to bean and soybean roots. FEMS Microbiology Letters. 259: 67–73.

Al-Tawaha, A.M., Seguin, P., Smith, D.L. and Bonnell R.B. (2007). Drought stress: irrigation level affects isoflavone concentrations of early maturing soya bean cultivars. J. Agronomy & Crop Science. 193: 238–246.

Anders, M.M., Schmid, B. and Olk, D.C. (2005). Short and long term effects of conservation tillage on soil resistance and aggregate stability in rice production systems. Proceedings of the 27th Southern Conservation Tillage Systems Conference, Florence, South Carolina, USA, 102–110.

Ashley, D.A. and Ethridge, W.J. (1978). Irrigation effect on vegetative and reprocuctive development of three soybean cultivars. Agronomy J. 70: 467–471.

Atkinson, C., Frankenfeld, C.L. and Lampe, J.W. (2005). Gut Bacterial Metabolism of the Soy Isoflavone Daidzein: Exploring the Relevance to Human Health. Experimental Biology and Medicine. 230: 155–170.

Aussenac, T., Lacombe, S. and Daydé J. (1998). Quantification of isoflavones by capillary zone electrophoresis in soybean seeds: effects of variety and environment. American Journal of Clinical Nutrition. 68: 1480S–1485S.

Barker, D.W. and Sawyer, J.E. (2005). Nitrogen application to soybean at early reproductive development. Agronomy J. 97: 615–619.

Barnes, S., Kim, H., Darley-Usmar, V., Patel, R., Xu, J., Boersma, B. and Luo, M. (2000). Beyond ERγ and ERβ: Estrogen Receptor Binding Is Only Part of the Isoflavone Story. Journal of Nutrition. 130: 656S–657S.

Bellaloui, N. and Mengistu, A. (2008). Seed composition is influenced by irrigation regimes and cultivar differences in soybean. Irrigation Science. 26: 261–268.

References

Bennett, J.O., Yu, O., Heatherly, L.G., and Krishnan, H.B. (2004). Accumulation of genistein and daidzein, soybean isoflavones implicated in promoting human health, is significantly elevated by irrigation. Journal of Agricultural and Food Chemistry. 52: 7574–7579.

Berger, M., Rasolohery, C.A., Cazalis, R., Dayde, J. (2008). Isoflavone Accumulation Kinetics in Soybean Seed Cotyledons and Hypocotyls: Distinct Pathways and Genetic Controls Crop Science. 48: 700–708.

Bergersen, F.J. (1958). The bacterial component of soybean root nodules; changes in respiratory activity, cell dry weight, and nucleic acid content with increasing nodule age. Journal of General Microbiology. 19: p 312.

Birt, D.F., Hendrich, S. and Wang, W. (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacology & Therapeutics. 90: 157– 177.

Blanchet, R., Bouniols, A., Gelfi, N. and Wallace, S.U., (1989). Response of determinate and indeterminate soybeans fertilizing irrigation in soils of different depths. In: Pascale, A.J. (Ed.), Proceedings of the World Soybean Research Conference. Buenos-Aires, Argentina, pp. 740–745.

Blount, J.W., Dixon, R.A., and Paiva, N.L. (1992). Stress response in alfalfa (Medicago sativa L.): XVI. Antifungal activity of medicarpin and its biosynthetic precursors: implications for the genetic manipulation of stress metabolites. Physiological and Molecular Plant Pathology. 41: 333–349.

Board, J.E. and Hall, W. (1984). Premature flowering in soybean yield reductions at non-optimal planting dates as influenced by temperature and photoperiod. Agronomy J. 76: 700–704.

Bolinder, M.A., Janzen, H.H., Gregorich, E.G., Angers, D.A. and Vanden Bygaart, A.J. (2007). An approach for estimating net primary productivity and annual carbon inputs to soil for common agricultural crops in Canada. Agriculture, Ecosystems and Environment. 118: 29–42.

Bona, S., Maculan, A and Mosca, G. (1991a). Behaviour of the roots nodules in soybean in the preference of nitrogen of statistical simulation model. Journal of Agronomy and Crop Science. 167: 254-258.

Bona, S., Mosca, G. and Sambo, P. (1996). Late Nitrogen fertilization in soybean (*Glycine max* (L.) Merr.) effects on some physiological and productive parameters. Eurosoya. 10: 1-8.

Bona, S., Voltan, R. and Mosca, G. (1991b). Soybean nodule development and Nitrogenase activity during the reproductive phase: statistical medeling approach. Journal of Agronomy and Crop Science. 167: 249-253.

Borthwick, H.A. and Parker, M.W. (1939). Influence of photoperiods on the differentiation of meristems and blossoming in Biloxi soybeans. Bot. Gaz. 99: 825–839.

Brevedan, R.E. and Egli, D.B. (2003). Short periods of water stress during seed filling, leaf senescence, and yield of soybean. Crop Science. 43: 2083–2088.

Brevedan, R.E., Egli, D.B. and Leggete, J.E. (1978). Influence of N nutrition on flower and pod abortion and yield of soybeans. Agronomy J. 70: 81–84.

Broun, P. (2005). Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in Arabidopsis. Current Opinion in Plant Biology. 8: 272–279.

Burton, J.W. (1984). Breeding soybean for improved protein quantity and quality. World soybean research conference III. p 361–367.

Caldwell, C.R., Britz, S.J. and Mirecki, R.M. (2005). Effect of temperature, elevated carbon dioxide and drought during seed development on the isoflavone content of dwarf soybean *Glycine max* (L.) Merrill grown in controlled environments. Journal of Agricultural and Food Chemistry. 53: 1125–1129.

Calinski, R.B. and Harabasz, J. (1974). A dendrite method for cluster analysis. Communications in Stat. 3: 1–27.

Carpenter, J., Felsot, A., Goode, T., Hamming, M., Onstad, D. and Sankula, S. (2002) Comparative Environmental Impacts of Biotechnology Derived and Traditional Soybean, Corn and Cotton Crops. Council for Agricultural Science and Technology, Ames, IA, pp 15–50.

Carrao-Panizzi, M. and Kitamura, K. (1995). Isoflavone content in Brazilian soybean cultivars. Breeding Science. 45: 295–300.

References

Casey, W.P., Dumler, T.J., Burton, R.O., Sweeney, D.W., Featherstone, A.M., Granade, G.V., 1998. A whole-farm economic analysis of early-maturing and traditional soybean. Journal of Production Agriculture. 11: 240–246.

Castellano, M., Ruiz-Filippi, G., González, W., Roca, E. and Lema, J.M. (2007). Selection of variables using factorial discrimenant analysis for the state identification of an anaerobic UASB–UAF hybrid pilot plant, fed with winery effluents. Water science and technology. 56: 139–145.

Chen, A. and Rogan, W.J. (2004). Isoflavones in soy infant formula: a review of evidence for endocrine and other activity in infants. Annual Review of Nutrition. 24: 33–54.

Chiari, L., Piovesan, N.D., Naoe, L.K., José, I.C., Viana, J.M.S., Moreira, M.A. and de Barros, E.G. (2004). Genetic parameters relating isoflavone and protein content in soybean seeds. Euphytica. 138, 55–60.

Choi, J.S., Kwon, T.W. and Kim, J.S. (1996). Isoflavone contents in some varieties of soybean. Foods and Biotechnology. 5:167–169.

Clarkson, T.B. (2002). Soy, Soy Phytoestrogens and Cardiovascular Disease. The Journal of Nutrition. 132: 566S–569S.

Cone, K.C., Cocciolone S.M., Moehlenkamp C.A., Weber T., Drummond B.J., Tagliani L.A., Bowen B.A. and Perrot G.H. (1993). Role of the regulatory gene pl in the photocontrol of maize anthocyanin pigmentation. Plant Cell. 5: 1807–1816.

Conner, T., Paschal, E.H., Barbero, A. and Johnson E. (2004). The Challenges and Potential for Future Agronomic Traits in Soybeans. AgBioForum. 7(1&2): 47–50.

D'Auria, J.C. and Gershenzon, J. (2005). The secondary metabolism of *Arabidopsis thaliana*: growing like a weed. Current Opinion in Plant Biology. 8: 308–316.

Dai, Q., Franke, A.A., Jin, F., Shu, X., Hebert, J.R., Custer, L.J., Cheng, J., Gao, Y., and Zheng, W. (2002). Urinary Excretion of Phytoestrogens and Risk of Breast Cancer among Chinese Women in Shanghai. Cancer Epidemiology Biomarkers & Prevention. 11: 815–821.

Dang, Z.C. and Lowik, C. (2005). Dose-dependent effects of phytoestrogens on bone. Trends in Endocrinology and Metabolism. 16: 207-213.

de Wit, C.T. (1967). Photosynthesis: its relationship to overpopulation. In: San Pierto, A., et al. (Eds.), Harvesting the Sun. Academic Press, New York, pp. 315–320.

References

Dhaubhadel, S., Farhangkhoee, M. and Chapman, R. (2008). Identification and characterization of isoflavonoid specific glycosyltransferase and malonyltransferase from soybean seeds. Journal of Experimental Botany. 59: 981–994.

Dhaubhadel, S., McGravey B.D., Williams, R. and Gijzen, M. (2003). Isoflavonoid biosynthesis and accumulation in developing soybean seeds. Plant Molecular Biology. 53: 733–743.

Dixon, R.A. and Ferreira, D. (2002). Genestein. Phytochemistry. 60: 205–211.

Dixon, R.A. and Paiva, N.L. (1995). Stress-lnduced Phenylpropanoid Metabolism. The Plant Cell. 7: 1085–1097.

Dixon, R.A. and Steele, C.L. (1999). Flavonoids and isoflavonoids-a gold mine for metabolic engineering. Trends in Plant Science. 4: 394–400.

Dixon, R.A., Achnine, L., Kota, P., Liu, C.J., Reddy, M.S.S. and Wang L.J. (2002). The phenylpropanoid pathway and plant defence-a genomics perspective. Molecular Plant Pathology. 5: 371–390.

Durand, J.L., Sheehy, J.E. and Minchin, F.R. (1987). Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water deprivation. Journal of Experimental Botany. 38:311–321.

Edwards, J.T. and Purcell, L.C., (2005). Soybean Yield and biomass responses to increasing plant population among diverse maturity groups: I. agronomic characteristics. Crop Science. 45: 1770–1777.

Edwards, J.T., Purcell, L.C., Vories, E.D., Shannon, J.G. and Ashlock, L.O., (2003). Short-season soybean cultivars have similar yields with less irrigation than longer-season cultivars. Crop Management Online, http://www.plantmanagementnetwork.org/pub/cm/research/2003/irrigate/. doi:10.1094/CM-2003-0922-01-RS.

Egli, D.B. and Wardlaw, I.F. (1980). Temperature response of seed growth characteristics of soybeans. Agronomy J. 72: 560–564.

Eldridge, A. and Kwolek W. (1983). Soybean isoflavones: Effect of the environment and variety on composition. Journal of Agricultural and Food Chemistry 31: 394–396.

Endres, J. (1992). Niche marketing for new oilseeds: An industrial perspective. In S.L. MacKenzie & D.C. Taylor (Eds.), Seed oils for the future. Champaign, IL: AOCS Press. pp. 1–8.

Endres, J. (2001). Soy protein products characteristics, nutritional aspects and utilization. Champaign, IL: AOCS Press.

FDA. (1999). Food and Drug Administration Talk Paper. Nov 10: T98-80.

Fehr, W.R. and Caviness, C.E. (1977). Stages of soybean development. Iowa State Univ. Agric. Exp. Stn. Spec. Rep. 80 pp.

Ferrer, J.-L., Austin, M.B., Stewart Jr., C. and Noel, J.P. (2008). Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiology and Biochemistry. 46: 356–370

Ferrer, J.-L., Jez, J.M., Bowman, M.E., Dixon, R.A. and Noel, J.P. (1999). Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nature. 6: 775–784.

Flannery, R.L. (1986). Plant food uptake in a maximum yield soybean study. Better Crops. 70: 6–7.

Garner, W.W. and Allard, H.A. (1930). Photoperiodic response of soybeans in relation to temperature and other environmental factors. J. Agric. Res. 41: 719–735.

Ghosh P.K., Bandyopadhyay K.K.A., Manna M.C., Mandal K.G., Misha A.K., Hati K.M., (2004). Comparative effectiveness of cattle manure, poultry manure, phosphocompost and fertilizer-NPK, on three cropping systems in vertisol of semiarid tropics. II. Dry matter yield, nodulation, chlorophyll content and enzyme activity. Bioresource Technology. 95: 77–83.

Gotoh, K. (1982). Limitations of yields in agricultural crops (2). Agric. Hort. 57: 737–744.

Graham, M.Y., and Graham. T.L., (1994). Wound-associated competency factors are required for the proximal cell responses of soybean to the *Phytophthora sojae* wall glucan elicitor. Plant Physiology. 105: 571–578.

Graham, T.L. (1991). Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiology. 95:594–603.

References

Graham, T.L. (1995). Cellular biochemistry of phenylpropanoid responses of soybean to infection by *Phytophthora sojae*. .In M Daniel, R.P. Purkayastha, eds, Handbook of Phytoalexin Metabolism and Action. Marcel Dekker, New York, pp 85–116.

Graham, T.L. (1999). Biosynthesis and distribution of phytoestrogen and their role in plant defense, signal transduction and cell to cell signaling. Journal of Medicinal Food. 2: 93–97.

Graham, T.L., and Graham, M.Y. (1996). Signaling in soybean phenylpropanoid responses. Plant Physiology. 110:1123–1133.

Graham, T.L., Kim, J.E. and Graham, M.Y. (1990). Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytopthora megasperma*. Molecular Plant-Microbe Interaction. 3: 157–166.

Grissom, P.; Raney, W.A.; Hogy, P. (1955). Crop response to irrigation in the Yazoo, Mississippi Delta. Mississippi State College Agric. Exp. Stn. Bull. 531.

Ham, G.E., Liener, I.E. Evans, S.D. Frazier, R.D. and Nelson, W.W. (1975). Yield and composition of soybean seed as affected by N and S fertilization. Agronomy J. 67: 293–297.

Hansen, B., Kristensen, E.S., Grant, R., Hogh-Jensen, H., Simmelsgaard, S.E., Olesen, J.E. (2000). Nitrogen leaching from conventional versus organic farming systems-a systems modelling approach. European Journal of Agronomy. 13: 65–82.

Haq, M.U. and Mallarino, A.P. (2000). Soybean Yield and Nutrient Composition as Affected by Early Season Foliar Fertilization. Agronomy. J. 92(1): 16–24.

Hardy, R.W.F., Burns, R.C., Hebert, R.R., Holsten, R.D., and Jackson, E.K. (1971). Biological nitrogen fixation: A key to world protein. p. 561–590. In T.A. Iie and E.G. Mulder (ed.) Biological nitrogen fixation in natural and agricultural habitats. Plant Soil Spec. Martinus Nijhoff, The Hague, the Netherlands.

Heatherly, L.G. (1999). Soybean irrigation. p. 119–142. In L.G. Heatherly and H.F. Hodges (ed.) Soybean production in the Mid-south. CRC Press, Boca Raton, FL.

Heatherly, L.G. (2006). Nitrogen fertility for soybeans. Penton Media, Delta Farm Press. Online link (http://deltafarmpress.com/news/060710-nitrogen-soybeans/).

Hoeck, J.A., Fehr, W.R., Murphy, P.A. and Welke G.A. (2000). Influence of genotype and environment on isoflavone contents of soybean. Crop Science. 40: 48–51.

Hubert, J., Berger, M. and Dayde. J. (2005). Use of a simplified HPLC-UV analysis for soyasaponin B determination: Study of saponin and isoflavone variability in soybean cultivars and soy-based health food products. Journal of Agriculture and Food Chemistry. 53: 3923–3930.

Janas, K.M., Cvikrova, M., Palagiewicz, A., Szafranska, K. and Posmyk. M.M. (2002). Constitutive elevated accumulation of phenylpropanoids in soybean roots at low temperature. Plant Science. 163: 369–373.

Kassem, M.A., Meksem, K., Iqbal, M.J., Njiti, V.N., Banz, W.J., Winters, T.A., Wood, A. and Lightfoot, D.A. (2004). Definition of Soybean Genomic Regions That Control Seed Phytoestrogen Amounts. Journal of Biomedicine and Biotechnology. 1: 52–60.

Kavitha, G.P. and Veeraraghavaiah, R. (2004). Agronomic evaluation of phosphate rock (34/74) in soybean production. *In* Swami, B.N., Katewa, M.K., Shaktawat, M.S., Singh, M. and Aery N.C. (2004) Phosphate rich organic manure: an alternate to phosphatic fertilizers. 93–97.

Kim J.A. and Chung I.M. (2007). Change in isoflavone concentration of soybean (*Glycine max* L.) seeds at different growth stages. Journal of the Science of Food and Agriculture. 87: 496–503.

Kim, S.H., Jung, W.S., Ahn, J.K., Kim, J.A. and Chung, I.M. (2005). Quantitative analysis of the isoflavone content and biological growth of soybean (*Glycine max* L.) at elevated temperature, CO_2 level and N application. Journal of the Science of Food and Agriculture. 85: 2557–2566.

Kirchmann, H. and Bergstrom, L. (2001). Do organic farming practices reduce nitrate leaching?. Communication in Soil Science and Plant Analysis. 32: 997–1028.

Kitamura, K., Igita, K., Kikuchi, A., Kudoi, S. and Okubo, K. (1991). Low isoflavone content in some early maturing cultivars, so-called "summer-type soybeans" (*Glycine max* (L.) Merrill). Japanese Journal of Breeding. 41: 651–654.

Kosslak, R.M., Bookland, R., Barkei, J., Paaren, H., and Appelbaum, E.R. (1987). Induction of *Bradyrhizobium japonicum* common nod genes by isoflavones isolated from *Glycine max*. Proceeding of the National Academy of Sciences. 84: 7428–7432.

References _____

Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K. and Okubo, K: (1991). Malonil isoflavone glycosides in soybeans seeds (*Glycine max* Merrill). Agriculture Biology Chemistery. 55: 2227–2233.

Latunde-Dada, A.O., Cabello-Hurtado, F., Czittrich, N., Didierjean, L., Schopfer, C., Hertkorn, N., Werck-Reichhart, D. and Ebel, J. (2001). Flavonoid 6-hydroxylase from soybean (*Glycine max* L.), a novel plant P-450 monooxygenase. The Journal of Biological Chemistry. 276: 1688–1695.

Le, B.H., Wagmaister, J.A., Kawashima, T., Bui, A.Q., Harada, J.J. and Goldberg, R.B. (2007). Using Genomics to Study Legume Seed Development. Plant Physiology. 144: 562–574.

Lee, C.H., Yang, L., Xu, Z.J., Yeung, S.Y.V., Huang, Y. and Chen, Z.Y. (2005). Relative antioxidant activity of soybean isoflavones their glycosides. Food Chemistry. 90: 735–741.

Lee, S.J., Yan, W.K., Ahn, J.K. and Chung, I.M. (2003). Effects of year, site, genotype, and their interactions on the concentration of various isoflavones in soybean. Field Crops Research. 81: 181–192.

Leyva, A., Jarillo, J.A., Salinas, J. and Martinez-Zapater J.M. (1995). Low temperature lnduces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mrnas of arabidopsis thaliana in a light-dependent manner. Plant Physiology. 108: 39–46.

Lima, W.F., De Toledo, J.F.F., Arias, C.A.A. and De Oliveira, M.F. (2000). Stability of soybean yield through different sowing periods. Pesq. Agropec. Bras. 35 (11): 2181–2189.

Lozovaya, V.V., Lygin, A.V., Ulanov, A.V., Nelson, R.L., Daydé, J. and Widholm, J.M. (2005). Effect of temperature and soil moisture status during seed development on soybean seed isoflavone concentration and composition. Crop Sci. 45: 1934–1940.

Majumder, A., Nath, R., Kundu, C.K., Chakraborty, S., Islam, S.J. and Bandopadhyay, P. (2006). Sustainability performance of soybean (*Glycine max* L. Merril) varieties under rainfed ecosystem in red and laterite zone of West Bengal. Environment and Ecology. 24S: 440-442.

Meckel, L., Egli, D.B., Phillips, R.E., Radcliffe, D. and Leggett, J.E. (1984). Effect of moisture stress on seed growth in soybean. Agron. J. 76: 647–650.

Messina, M.J. (1999). Legumes and soybeans: overview of their nutritional profiles and health effects. Am J Clin Nutr. 70: 439S–450S

Messina, M.J., Nagata., C. and Wu, A.H. (2006). Estimated Asian adult soy protein and isoflavone intakes. Nutr. Cancer. 55: 1–12.

Miladinovic, J., Kurosaki, H., Burton, J.W., Hrustic, M., and Miladinovic, D. (2006). The adaptability of short season soybean genotypes to varying longitudinal regions. *Europ. J. Agronomy*. 25: 243–249.

Morris, P.F., Savard, M.E. and Ward, E.W.B. (1991). Identification and accumulation of isoflavonoids and isoflavone glucosides in soybean leaves and hypocotyls in resistance responses to *Phytophthora megasperma* f. sp. *glycinea.* Physiological and Molecular Plant Pathology. 39: 229–234.

Morrison, M.J., Cober, E.R., Saleem, M.F., McLaughlin, N.B., Frégeau-Reid, J., Ma, B.L., Yan, W. and Woodrow, L. (2008). Changes in isoflavone concentration with 58 years of genetic improvement of short-season soybean cultivars in canada. Crop Sci. 48: 2201–2208

Mosca, G., Toniolo L., Voltan, R., Sattin, M.(1983). Soybean production in northeastern Italy. Eurosoya. 1: 43-45

Murphy, P.A., Song, T., Buseman, G., Barua, K., Beecher, G.R., Trainer, D., and Holden, J. (1999). Isoflavones in retail and institutional soy foods. J. Agric. Food Chem. 47: 2697–2704

Nelson, R.L., Lozovaya, V., Lygin, A. and Widholm. J. (2001). Variation in isoflavones in seeds of domestic and exotic soybean germplasm. *In* 2001 Agronomy Abstracts [CD-ROM]. ASA, CSSA, and SSSA, Madison, WI.

Ning, L., JianXin, Z. and Hao, M. (2005). Effect of planting date on the agronomic characters and quality of vegetable soybean. Xinjiang Agricultural Sciences. 42(5): 315–318.

Ning, L., ZhenHua, Z., JianXin, Z. and GuoFeng, Y. (2006). Effect of density on the agronomic character and yield of vegetable soybean. Xinjiang Agricultural Sciences. 43(4): 271–274

References

Noguchi, A., Saito, A., Homma, Y., Nakao, M., Sasaki, N., Nishino, T., Takahashi, S. and Nakayama, T. (2007). A UDP-Glucose: Isoflavone 7-O-Glucosyltransferase from the Roots of Soybean (*Glycine max*) Seedlings: Purification, gene cloning, phylogenetics, and an implication for an alternative strategy of enzyme catalysis. J. Biol. Chem. 282: 23581–23590.

Oberson, A., Nanzer, S. Bosshard, C., Dubois, D., Mäder, P. and Frossard, E. (2007). Symbiotic N_2 fixation by soybean in organic and conventional cropping systems estimated by ^{15}N dilution and ^{15}N natural abundance. Plant Soil. 290: 69–83.

Osborne, S.L. and Riedell, W.E. (2006). Starter nitrogen fertilizer impact on soybean yield and quality in the northern great plains. Agron. J. 98: 1569–1574.

Perez-Grau, L. and Goldberg, R.B. (1989). Soybean Seed Protein Genes Are Regulated Spatially during Embryogenesis. The Plant Cell. 1: 1095–1109.

Perfetti, G.A., Joe, F.L.Jr., Fazio, T. and Page, S.W. (1988). Liquid chromatographic methodology for the characterization of orange juice. Journal of association official analytical chemists. 71: 469–473.

Popp, M., Edwards, J., Purcell, L. and Manning, P. (2004). Early-maturity soybean in a late-maturity environment: economic considerations. Agronomy J. 96: 1711–1718.

Popp, M., Manning, P., Keisling, T., Gordon, E. and Oliver, L. (2003). Analysis of a novel bedded planting system for dry clay soil management of full-season and double-crop soybean. Communication in Soil Science and Plant Analysis. 34: 2925–2950.

Posmyk, M.M., Bailly, C., Szafranska, K., Janas, K.M. and Corbineau, F. (2005). Antioxidant enzymes and isoflavonoids in chilled soybean (*Glycine max* (L.) Merr.) seedlings. J. Plant Physiology. 162: 403–412.

Primomo, V.S., Poysa, V., Ablett, G. R., Jackson, C.J. and Rajcan, I. (2005). Agronomic performance of recombinant inbred line populations segregating for isoflavone content in soybean seeds. Crop Science. 45: 2203–2211.

Purcell, L.C. and King, C.A. (1996). Drought and nitrogen source effects on nitrogen nutrition, seed growth, and yield in soybean. Journal of Plant Nutrition. 19: 969–993.

Rao, M.S.S., Mullinix, B.G., Rangappa, M., Cebert, E., Bhagsari, A.S., Sapra, V.T., Joshi, J.M., and Dadson, R.B. (2002). Genotype × environment interactions and yield stability of food-grade soybean genotypes. Agronomy J. 94: 72–80.

References

Rasolohery, C.A., Berger, M., Lygin, A.V. Lozovaya, V.V., Nelson R.L. and Daydé J. (2008). Effect of temperature and water availability during late maturation of the soybean seed on germ and cotyledon isoflavone content and composition. Journal of the Science of Food and Agriculture. 88 (2): 218–228.

Richards, R.A. (2000). Selectable traits to increase crop photosynthesis and yield of grain crops. Journal of Experimental Botany. 51: 447–458.

Sall, K. and Sinclair, T.R. (1991). Soybean genotypic differences in sensitivity of symbiotic nitrogen fixation to soil dehydration. Plant Soil. 133: 31–37.

Sarkar, F.H. and Li, Y. (2003). Soy isoflavones and cancer prevention. Cancer Investigation. 21(5): 744–57.

Scharf, P.C. and Wiebold, W.J. (2003). Soybean yield responds minimally to nitrogen applications in Missouri. Crop Management. Available at http://www.plantmanagementnetwork.org/pub/cm/research/2003/soy/.

Schryver, T. (2002). Increasing health benefits using soy germ. Cereal Foods World. 47: 185–188.

Scott, W.O. and Aldrich, S.R. (1983). Modern Soybean Production. S & A Publications Inc., Champaign, IL, USA.

Seddigh, M., Jolliff, G.D. and Orf, J.H. (1989). Night temperature effects on soybean phenology. Crop Science. 29: 400–406.

Seguin, P., Zheng, W., Smith, D.L. and Deng, W. (2004). Isoflavone content of soybean cultivars grown in eastern Canada. Journal of the Science of Food and Agriculture. 84: 1787–1795.

Setchell, K.D.R. and Lydeking-Olsen, E. (2003). Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies. The american journal of clinical nutrition. 78: 593S–609S.

Setchell, K.D.R., Brown, N.M., Desai, P., Zimmer-Nechemias, L., Wolfe, B.E., Brashear, W.T., Kirscher, A.S., Cassidy, A. and Heubi, J. (2001). Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. Journal of Nutrition. 131: 1362S–1375S.

Sij, J.W., Turner, F.T. and Craigmiles, J.P. (1979). "Starter nitrogen" fertilization in soybean culture. Communication in Soil Science and Plant Analysis. 10: 1451–1457.

Sinclair, T.R., Muchow, R.C. Bennett, J.M. and Hammond, L.C. (1987). Relative sensitivity of nitrogen and biomass accumulation to drought in field grown soybean. Agronomy J. 79: 986–991.

Sokal, R.R. and Rohlf, F.J. (1962). The comparison of dendrograms by objective methods. Taxon. 11: 33–40.

Somersalo, S. and Krause G.H. (1989). Photoinhibition at chilling temperature. Fluorescence characteristics of unhardened and cold acclimated spinach leaves. Planta. 177: 409–416.

Sorensen, R.C., and Penas, E.J. (1978). Nitrogen fertilization of soybeans. Agronomy J. 70: 213–216.

Stockdale, E.A., Lampkin N.H., Hovi, M., Keatinge, R., Lennartsson, E.K.M., Macdonald D.W., Padel, S., Tattersall, F.H., Wolfe, M.S. and Watson, C.A. (2001). Agronomic and environmental implications of organic farming systems. Advances in Agronomy. 70: 261–327.

Suelter, C.H. (1985). Role of potassium in enzyme catalysis. In Potassium in Agriculture; Munson, R.D., Ed.; American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America: Madison, WI, pp 337–349.

Taylor, L.P. and Grotewold, E. Flavonoids as developmental regulators. (2005). Current Opinion in Plant Biology. 8: 317–323.

Teede, H.J., Dalais, F.S., Kotsopoulos, D, Liang Y.L., Davis, S. and McGrath, B.P. (2001). Dietary soy has both beneficial and potentially adverse cardiovascular effects: a placebo-controlled study in men and postmenopausal women. The Journal of Clinical Endocrinology & Metabolism. 86: 3053–3060.

Terman, G.L. (1977). Yields and nutrient accumulation by determinate soybeans, as affected by applied nutrients. Agronomy J. 69: 234–238.

Toniolo, L. and Mosca, G. (1981). Yield stability, in soybean in northeastern Italy. In E.S. Bunting (Ed). Production and utilization of protein in oilseed crops. 227-237.

Truong, N.T., KyuJung, V., Young, K.M. and SukHa, L. (2006). Genotypic variation in flowering and maturing periods and their relations with plant yield and yield components in soybean. Korean Journal of Crop Science. 51(2): 163–168. (Abstract)

References

Tsukamoto, C., Shimada, S., Igita,K. Kudou, S., Kokubun, M., Okubo, K. and K. Kitamura (1995). Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. Journal of Agricultural and Food Chemistry. 43: 1184–1192.

Vyn, T.J., Yin, X., Bruulsema, T.W., Jackson, C.C., Rajcan, I. and Brouder, S.M. (2002). Potassium fertilization effects on isoflavone concentrations in soybean (*Glycine max* (L.) Merr.). Journal of Agricultural and Food Chemistry. 50: 3501–3506.

Walbot, V. (1978). Control mechanisms for plant embryogeny. In Clutter, M. ed, Dormancy and developmental arrest. Academic Press, New York, pp 113–166.

Wang, C., Sherrard, M., Pagadala, S., Wixon, R. and Scott, R.A. (2000). Isoflavone content among maturity group 0 to II soybeans. Journal of the American Oil Chemists' Society. 77: 483-487.

Wang, H.J. and Murphy, P.A. (1994a). Isoflavone content in commercial soybean foods. Journal of Agricultural and Food Chemistry. 42: 1666–1673.

Wang, H.J. and Murphy, P.A. (1994b). Isoflavone composition of American and Japanese soybeans in Iowa: Effects of variety, crop year, and location. Journal of Agricultural and Food Chemistry. 42: 1674–1677.

Wang, H.J. and Murphy, P.A. (1996). Mass balance study of isoflavones during soybean processing. Journal of Agricultural and Food Chemistry. 44: 2377-2383.

Weber, C.R. (1966). Nodulating and nonnodulating soybean isolines: II. Response to applied nitrogen and modified soil conditions. Agronomy J. 58: 46–49.

Wesley, T.L., Lamond, R.E., Martin, V.L. and Duncan. S.R. (1998). Effects of late-season nitrogen fertilizer on irrigated soybean yield and composition. Journal of Production Agriculture. 11: 331–336.

Wesley, T.L., Lamond, R.E., Martin, V.L. and Duncan. S.R. (1999). Applied N at R3 stage bumps soybean yields. Fluid J. 25:16–19.

Yazaki, K. (2005). Transporters of secondary metabolites. Current Opinion in Plant Biology. 8: 301–307.

Yin, X. and Vyn, T.J. (2005). Relationships of isoflavone, oil, and protein in seed with yield of soybean. Agronomy J. 97: 1314–1321.

Yu, O., Jung, W., Shi, J., Croes, R.A., Fader, G.M., McGonigle, B. and Odell, J.T. (2000). Production of the isoflavones genistein and daidzein in non-legume dicot and Monocot Tissues. Plant Physiology. 124: 781–793.

Yu, O., Shi, J., Hession, A.O., Maxwell, C.A., McGonigle, B. and Odell, J.T. (2003). Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry. 63: 753–763.

Yuan, J.P., Liu, Y.B., Peng, J., Wang, J.H. and Liu, X. (2009). Changes of Isoflavone Profile in the Hypocotyls and Cotyledons of Soybeans during Dry Heating and Germination. Journal of Agricultural and Food Chemistry. 57: 9002–9010.

Yuan, J.P., Wang, J.H. and Liu, X. (2007). Metabolism of dietary soy isoflavones to equol by human intestinal microflora - Implications for health. Molecular Nutrition & Food Research. 51: 765–781.