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Isoflavone content of soybean [*Glycine max* (L). Merr.] cultivars with different nitrogen souces and growing season under dry land conditions

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Abstract: The objective of the research was to determine the best N nutrient management for isoflavone content in three soybean cultivars under dry land conditions. Two experiments were experiment I (June to September 2012 growing season) and Experiment II (October to December 2012 growing season). Experimental design was a randomized block design with 2 factors and 3 replications. The first factor was soybean cultivars (Anjasmoro, Wilis, Sinabung). The second factor was N source, with Urea (50 kg/ha), *Bradyrhizobium* sp., farmyard manure (10 ton/ha), a combination of *Bradyrhizobium* sp. + farmyard manure (5 ton/ha) and a control with no N. A combined analysis of variance was done to evaluate the production and the content of isoflavone in the two different growing seasons as affected by N source and cultivar. The parameters observed were the content of genistein, daidzein, glycitein and total isoflavone content. Results showed that the October to December growing season had higher genistein, daidzein, glycitein and total of isoflavones than the June to September growing season. The treatment cultivar Wilis plus *Bradyrhizobium* sp. grown at October to December growing season increased total isoflavone content more than other treatments.

Keywords: isoflavone, nitrogen, soybean.

Introduction

Soybean (*Glycine max* L. Merr.) has a strategic potential in food security as a source of protein and high quality functional food for human needs. Soybean contains secondary metabolites such as isoflavones (Sakai and Kogiso, 2008), saponins, phytic acid, oligosaccharides (Liener, 1994) and phytoestrogens (Ososki and Kennely, 2003)

which are beneficial to health. Soybean is the most common source of isoflavones in human foods, especially in many Asian countries. Soybean isoflavones have a positive impact on human health including prevention of chronic diseases such as cancer, heart disease, osteoporosis and menopausal symptoms (Messina, 1995) and beneficial effects on diabetes and renal diseases (Ranich *et al.*, 2001). Genistein, daidzein and glycitein, the known soybean isoflavones, are synthesized by a branch of the phenylpropanoid pathway (Yu and McGonigle, 2005).

Isoflavone content in soybean depends on both genetic and environmental factors. Influencing environmental factors consist of both biotic, such as wounding, nodulation and pathogen attack, and abiotic elements such as temperature, water regime, UV light, soil nutrient content and atmospheric carbon dioxide level (Dixon and Paiva, 1995, Lozovaya *et al.*, 2005, Subramanian *et al.*, 2006, Naoumkina *et al.*, 2007, Subramanian *et al.*, 2007). Planting location, crop year, planting dates within a given crop year, and storage conditions can also affect isoflavone content (Zhu *et al.*, 2005; Hoeck *et al.*, 2000; Lee *et al.*, 2003; Seguin *et al*, 2004). Previous investigations have shown that isoflavone concentrations in soybean seeds (Hoeck *et al.*, 2000; Wang and Murphy, 1994) are influenced significantly by location. Carrao-Panizzi *et al.* (1999) reported that the highest isoflavone concentrations were observed in seeds of soybean plants grown in locations with high latitudes (cooler temperatures) when compared to locations with low latitudes (warmer temperatures).

Demand for soybean in Indonesia continues to increase, along with increasing public knowledge of the benefits of soy as a functional food. Production of soybean is not keeping up with demand, so that efforts are necessary to improve national soybean production. This can be achieved through increased production approaches including expansion of soy cultivation in marginal (sub-optimal) lands. Among these are sub-optimal dry lands. Soybean cultivation on dry land has problems such as low soil fertility, low pH, higher amounts of Al, Fe and Mn, low organic matter as well as water shortages, especially in dry season because of the limited water resources. Pests, diseases and weeds along with the use of unimproved local varieties can also be contributing factors (Arsyad and Purwantoro, 2010).

Increased productivity and content of soy isoflavones on dry land can be achieved by the application of specific technologies according to the agroecology of dry land agriculture. One area of dry land in North Sumatera that was once the centre of soybean production is Sambirejo Village, District Binjei, Langkat. The dry land is classified as lowland wet climate, experiencing problems such as drought stress during the dry season (June to August), low pH, (pH 5.0), and low soil content of N, P and K. Based on these problems, the management of dry land for optimum production of soybean for yield and isoflavone characteristics can be accomplished using two basic approaches; the selection of soybean cultivars adapted to dry land and improvement of soil fertility through management of N and other nutrients.

Nitrogen is one of the essential nutrients for plants. It is a key element in proteins and nucleic acids, and is required in the synthesis of chlorophyll. Isoflavones are also one of the important secondary metabolites in soybean plants formed through the phenylpropanoid biosynthetic pathway precursor phenylalanine which is one of the essential amino acids that requires N in its synthesis.

Differences in dry seasons in dry land areas affect soybean production and content of isoflavones patterns of rainfall, humidity and temperature. It is therefore necessary to study which growing season is best for production of soybean and seasonal effect on isoflavones content and composition. Therefore, the objective of this research was to determine the effect of growing season and N management on production and soy isoflavone content in dry land.

Materials and Methods

Research was conducted in Sambirejo Village, Binjei District, Langkat, Sumatra Utara (Indonesia), a dry land area, June to December 2012. The soil texture of the experimental site was a sandy clay loam which had 11% coarse sand, 38% fine sand, 29% silt and 22% clay. Nitrogen content was low (0.14%), organic matter was 1.02%, with a pH of 5.0.

Experimental design and crop management

Two planting times (seasons) were studied. Treatments were arranged in a Randomized Block Design with two factors and three replications. The first factor was three soybean cultivars (Anjasmoro, Wilis and Sinabung). The second factor was (N) sources and consisted of urea at 50 kg/ha, innoculation of seed with *Bradyrhizobium sp*., 10 t/ha farmyard manure, the combination *Bradyrhizobium sp.* + farmyard manure at 5 t/ha and a zero N control. The research consisted of two series of experiments. The first was from June to September 2012 (dry season) and the second was from October to December 2012 (rainy season). The dry season generally goes from June to August, with the rainy season being from September to May. The climatic characteristics of the two seasons are given in Table 1.

Isoflavone extraction and HPLC analyses

Following harvest, seeds were stored at room temperature and within one month, isoflavones were extracted for determination of isoflavone composition and content. Concentration of genistein, daidzein and glycitein were determined using a high-performance liquid chromatography (HPLC) method from Vyn *et al.* (2002). Finely ground soybean seed was weighed in duplicate samples of 0.50 g each and dispersed

Table 1 - Climate characteristic factors of dry and rainy seasons at Sambirejo Village, Binjei District.

	Dry season (June –September 2012)				Rainy season (October – December 2012)		
	June	July	August	September	October	November	December
Rainfall (mm/month)	78	283.9	278.2	184	343.1	432	184.3
Mean of temperature (°C)	28.2	27.6	27.4	27.4	27.1	27.2	27.1
Maximum temperature (°C)	26.5	26.5	26.0	26.1	26.1	25.1	25.9
Maximum temperature (°C)	29	29.1	28.7	28.4	28.5	28.9	27.6
Mean of humidity (%)	79	80	80	79	85	83	84

Source: Meteorology Climatology and Geophysics Agency, Medan (2012).

in 10 mL of ethanol plus 2 mL of concentrated HCl. The resulting solutions were hydrolyzed by heating to 125 °C for 2 hours in a sand bath. After the samples were cooled, they were centrifuged at 3000 rpm for 10 minutes. The clear aliquot was filtered through a 0.45-μm PTFE filter. Individual hydrolyzed daidzein, genistein, and glycitein were separated on a HPLC equipped with a photodiode array (PDA) detector (200-300 nm). HPLC column, Waters Nove Pak C18 column (3.9 x 150 mm, 5-*m*m particle size) with C18 guard column; HPLC mobile phases, solvent A was 4% aqueous acetic acid and solvent B was 100% HPLC grade methanol; flow rate, 1.5 mL min-1; and injection volume, 5 *m*L. HPLC mobile phases were solvent A (4% aq. acetic acid) and solvent B (100% methanol), and the solvent system was as follows (%solvent A% solvent B): 0 min (70/30), 12.5 min (65/35), 13 min (50/50), 15 min (30/70), 22.5 min (25/75), and 23 min (70/30). Recovery was monitored by the addition of a recovery standard, flavone, to the sample prior to hydrolysis.

Statistical data analysis

Data were subjected to analysis of variance (ANOVA) for comparison of means. A combined analysis of variance was done to evaluate isoflavones affected by growing season. Means were separated using Duncan's Mutiple Range Test at the 0.05 probability level.

Results

Genistein

Mean comparisons for the effect of N sources in different growing seasons on genistein content of soybean cultivars are shown in Table 2. Soybean grown during the rainy season had higher genistein content (219.96 μg/g) than that grown during

Table 2 - Genistein content of soybean cultivars with different of N. sources and growing seasons under dry land conditions.

CULTIVAR	N SOURCES	GROWING SEASON		MEAN
		DRY SEASON	RAINY SEASON	
		..... µG/G SEED WEIGHT		
Anjasmoro	Without N application	220.16	208.71	214.43
(V1)	Urea (50 kg/ha)	220.23	189.32	204.77
	Bradyrhizobium sp.	206.53	246.80	226.66
	Farmyard manure (10 t/ha)	218.38	231.74	225.06
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	187.51	207.31	197.41
	Mean of V_1 x M	210.56i	216.77h	
	Mean of V_1			213.67
Wilis	Without N application	202.96	197.43	200.19
(V2)	Urea (50 kg/ha)	213.01	221.16	217.08
	Bradyrhizobium sp.	212.86	243.67	228.27
	Farmyard manure (10 t/ha)	199.18	203.12	201.15
	Bradyrhizobium sp. + Farmyard manure (5 t/ha)	165.14	232.77	198.96
	Mean of V_2 x M	198.63j	219.63gh	
	Mean of V_2			209.13
Sinabung	Without N application	188.88	210.88	199.88
(V3)	Urea (50 kg/ha)	186.14	226.26	206.20
	Bradyrhizobium sp.	179.28	234.71	206.99
	Farmyard manure (10 t/ha)	175.97	230.44	203.21
	Bradyrhizobium sp. + Farmyard manure (5 t/ha)	194.78	215.10	204.94
	Mean of V3 x M	185.01k	223.48g	
	Mean of V3			204.24
	Without N application	204.00ef	205.67def	204.84
	Urea (50 kg/ha)	206.46de	212.25cd	209.35
	Bradyrhizobium sp.	199.56ef	241.72a	220.64
	Farmyard manure (10 t/ha)	197.84f	221.77b	209.81
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	182.48g	218.39bc	200.44
	Mean of M	198.07y	219.96x	

Different letters at the same of group treatment represent significant differences at Duncan's Multiple Range Test ($p < 0.05$).

the dry season (198.07 μg/g). Cultivar Anjasmoro tended to have higher genistein content (213.67 μg/g) than Willis (209.13 μg/g) and Sinabung (204.24 μg/g). Sinabung cultivar grown during the rainy season had the highest genistein content (223.48 μg/g) than Sinabung cultivar grown during the dry season which had the lowest genistein content (185.01 μg/g). The treatment of *Bradyrhizobium sp*. during the dry season had the highest genistein content (234.71 μg/g), than the treatment of *Bradyrhizobium sp.* + farmyard manure (5 t/ha) during the dry season had the lowest genistein content (182.48 μg/g).

Daidzein

Mean comparisons for the effect of N sources at different growing seasons on daidzein content of soybean cultivars are shown in Table 3. Soybean grown during the rainy season had a higher daidzein content (686.86 μg/g) significantly than the dry season (549.65 μg/g). Cultivar of Anjasmoro tended to have a higher daidzein content (627.49 μg/g) than Willis (624.56 μg/g) and Sinabung (602.71 μg/g). Wilis cultivar grown during the dry season had the lowest content of daidzein (526.71 μg/g), while Wilis cultivar grown during the rainy season had the highest content of daidzein (722.40 μg/g). Treatment of *Bradyrhizobium sp.* had significantly higher content of daidzein (680.04 μg/g) than all other N sources treatment. The interaction among N source of *Bradyrhizobium sp*. and Wilis cultivar grown during the rainy season tended to increase daidzein content (792.17 μg/g), while the interaction among Urea and Wilis cultivar grown during the dry season tended to have the lowest content of daidzein (474.22 μg/g).

Glycitein

Mean comparisons for the effect of N sources at different of growing seasons on glycitein content of soybean cultivars are shown in Table 4. Soybean grown during the rainy season had a higher glycitein content (64.41 μg/g) significantly than the dry season (54.78 μg/g). Anjasmoro cultivar had the highest glycitein content (64.39 μg/g) than Wilis (58.39 μg/g) and Sinabung (56.02 μg/g). The treatment of *Bradyrhizobium sp.* and Anjasmoro cultivar had the highest glycitein content (71.51 μg/g) than other treatments. The treatment of Urea (50 t/ha) and Anjasmoro cultivar grown during the rainy season increased glycitein content(75.66 μg/g) significantly than other combination treatments.

Table 3 - Daidzein content of soybean cultivars with different of N sources and growing seasons under dry land conditions.

Cultivar	N. sources	Growing season		Mean
		Dry season	Rainy season	
		..... µg/g seed weight		
Anjasmoro	Without N application	570.66	673.31	621.98
(V1)	Urea (50 kg/ha)	595.94	661.43	628.68
	Bradyrhizobium sp.	598.23	727.02	662.62
	farmyard manure (10 t/ha)	610.54	688.50	649.52
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	502.67	646.65	574.66
	Mean of V1 x M	575.61D	679.38B	
	Mean of V1			627.49
Wilis	Without N application	507.44	683.00	595.22
(V2)	Urea (50 kg/ha)	474.22	709.89	592.06
	Bradyrhizobium sp.	604.36	792.17	698.27
	Farmyard manure (10 t/ha)	523.02	783.15	653.09
	Bradyrhizobium sp. + Farmyard manure (5 t/ha)	524.51	643.79	584.15
	Mean of V_2 x M	526.71e	722.40a	
	Mean of V_2			624.56
Sinabung	Without N application	542.18	560.78	551.48
(V_3)	Urea (50 kg/ha)	526.72	708.63	617.68
	Bradyrhizobium sp.	637.71	720.76	679.24
	Farmyard manure (10 t/ha)	492.61	645.20	568.91
	Bradyrhizobium sp. + Farmyard manure (5 t/ha)	533.92	658.62	596.27
	Mean of V3 x M	546.63e	658.80c	
	Mean of V3			602.71
	Without N application	540.09	639.03	589.56g
	Urea (50 kg/ha)	532.29	693.32	612.80g
	Bradyrhizobium sp.	613.43	746.65	680.04f
	Farmyard manure (10 t/ha)	542.05	705.62	623.84g
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	520.37	649.69	585.03g
	Mean of M	549.65y	686.86x	

Different letters at the same of group treatment represent significant differences at Duncan's Multiple Range Test ($p < 0.05$).

Table 4 - Glycitein content of soybean cultivars with different of N sources and growing seasons under dry land conditions.

Cultivar	N sources	Growing season		Mean
		Dry season	Rainy season	
		..... µG/G SEED WEIGHT		
Anjasmoro	Without N application	67.86a-d	62.23b-f	65.05lmn
(V1)	Urea (50 kg/ha)	58.55c-g	75.66a	67.10kl
	Bradyrhizobium sp.	69.79a-d	73.23ab	71.51k
	farmyard manure (10 t/ha)	59.85c-g	70.15a-d	65.00lmn
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	41.68ij	64.89a-f	53.28qr
	Mean of V1 x M	59.55	69.23	
	Mean of V1			64.39t
Wilis	Without N application	46.61g-j	58.12c-g	52.37qr
(V2)	Urea (50 kg/ha)	59.11c-g	61.56b-g	60.34mno
	Bradyrhizobium sp.	33.17j	78.63a	55.90op
	farmyard manure (10 t/ha)	48.58f-i	71.88abc	60.23nop
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	63.44a-f	62.79b-f	63.12l-o
	Mean of V2 x M	50.18	66.60	
	Mean of V2			58.39u
Sinabung	Without N application	61.16b-g	56.88c-h	59.02op
(V3)	Urea (50 kg/ha)	64.64a-f	67.98a-d	66.31klm
	Bradyrhizobium sp.	55.43d-i	42.14hij	48.78r
	farmyard manure (10 t/ha)	41.64ij	64.08a-f	52.86qr
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	50.24e-i	55.97d-h	53.11qr
	Mean of V3 x M	54.62	57.41	
	Mean of V3			56.02u
	Without N application	58.54	59.08	58.81
	Urea (50 kg/ha)	60.76	68.40	64.58
	Bradyrhizobium sp.	52.79	64.66	58.73
	farmyard manure (10 t/ha)	50.02	68.70	59.36
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	51.78	61.22	56.50
	Mean of M	54.78y	64.41x	

Different letters at the same of group treatment represent significant differences at Duncan's Multiple Range Test ($p < 0.05$).

Table 5 - Total isoflavones of soybean cultivars with different of N sources and growing seasons under dry land conditions.

CULTIVAR	N SOURCES	GROWING SEASON		MEAN
		DRY SEASON	RAINY SEASON	
		..... µG/G SEED WEIGHT		
Anjasmoro	Without N application	67.86a-d	62.23b-f	65.05lmn
(V1)	Urea (50 kg/ha)	58.55c-g	75.66a	67.10kl
	Bradyrhizobium sp.	69.79a-d	73.23ab	71.51k
	farmyard manure (10 t/ha)	59.85c-g	70.15a-d	65.00lmn
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	41.68ij	64.89a-f	53.28qr
	Mean of V1 x M	59.55	69.23	
	Mean of V1			64.39t
Wilis	Without N application	46.61g-j	58.12c-g	52.37qr
(V2)	Urea (50 kg/ha)	59.11c-g	61.56b-g	60.34mno
	Bradyrhizobium sp.	33.17j	78.63a	55.90op
	farmyard manure (10 t/ha)	48.58f-i	71.88abc	60.23nop
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	63.44a-f	62.79b-f	63.12l-o
	Mean of V2 x M	50.18	66.60	
	Mean of V2			58.39u
Sinabung	Without N application	61.16b-g	56.88c-h	59.02op
(V3)	Urea (50 kg/ha)	64.64a-f	67.98a-d	66.31klm
	Bradyrhizobium sp.	55.43d-i	42.14hij	48.78r
	farmyard manure (10 t/ha)	41.64ij	64.08a-f	52.86qr
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	50.24e-i	55.97d-h	53.11qr
	Mean of V3 x M	54.62	57.41	
	Mean of V3			56.02u
	Without N application	58.54	59.08	58.81
	Urea (50 kg/ha)	60.76	68.40	64.58
	Bradyrhizobium sp.	52.79	64.66	58.73
	farmyard manure (10 t/ha)	50.02	68.70	59.36
	Bradyrhizobium sp. + farmyard manure (5 t/ha)	51.78	61.22	56.50
	Mean of M	54.78y	64.41x	

Different letters at the same of group treatment represent significant differences at Duncan's Multiple Range Test ($p < 0.05$).

Total isoflavone

Mean comparisons for the effect of N sources at different of planting time on total isoflavones content of soybean cultivars are shown in Table 5. Soybean grown during the rainy season had a significantly higher isoflavones total content (971.23 μg/g) than the dry season (803.61 μg/g). Anjasmoro cultivar had higher total isoflavone content (907.21 μg/g) than Wilis or Sinabung. Wilis cultivar grown during the rainy season had higher total isoflavone content (1008.63 μg/g) that other treatments, while Wilis cultivar was grown during the dry season had the lowest total isoflavone content. The treatment of *Bradyrhizobium sp*. and Wilis cultivar grown during the rainy season tended to increase the total isoflavone than all other treatments.

Discussion and conclusion

The content all of soybean isoflavones (genistein, daidzein and glycitein) were affected by growing season. In general, soybean grown during the rainy season had higher content of genistein, daidzein, glycitein and higher total of isoflavone content compared to soybean grown during the dry season. The rainy season had higher rainfall and humidity but the temperature was lower compared to the dry season (Table 1). This suggested that the climatic conditions during rainy season were more suitable for isoflavone accumulation than in dry season. Kim and Yung (2007). Dhaubhadel *et al.* (2007) and Gonzalez *et al*. (2010) reported that accumulation of isoflavones in soybean seeds takes place during the later stages of seed maturation (R7). It suggests that their levels are greatly influenced by water availability during this period. Nevertheless, little is known about the timing and magnitude of the water deprivation required to exert a significant effect, and it is yet to be determined at which stage of seed development drought might cause more variation. In this research, the higher soil moisture due to higher of rainfall during the rainy season caused an increase in the accumulation of genistein, daidzein, glycitein and isoflavones total. This was in line with previous research by Lozovaya *et al.* (2005), who studied the effect of temperature and soil moisture status during seed development under controlled conditions and concluded that high soil moisture increased daidzein, genistein and total isoflavones. In addition, Morrison *et al.* (2010) reported that precipitation has been suggested as a potential factor influencing isoflavone concentration. Seguin *et al.* (2004) achieved the lowest total isoflavones concentration in the driest year of a two-year study.

The high accumulation of isoflavone content (genistein, daidzein, glycitein and isoflavones total) on Anjasmoro cultivar indicated that the accumulation of isoflavones contents was highly influenced by genotype. Previously research (Wang and Murphy, 1994; Hoeck *et al.,* 2000; Lee *et al*., 2003; Mebrahtu *et al*., 2004, Gonzalez

et al., 2009; 2010) reported that accumulation of soybean isoflavones compounds depend on the genetic factor (the cultivar) and the environmental factors. Influencing environmental factors consist of both biotic, such as wounding, nodulation and pathogen attack, and abiotic elements: temperature, water regime, UV light, soil nutrient content and carbon dioxide (Dixon and Paiva, 1995, Lozovaya *et al.*, 2005; Subramanian *et al.,* 2006; Naoumkina *et al.,* 2007)

The high accumulation of genistein, daidzein and total isoflavones contents on *Bradyrhizobium sp*. treatment may be related to the role of genistein and daidzein on root nodulation. Nodulation is one of the environmental factors that can influence accumulation of isoflavones (Lozovaya *et al.*, 2005; Subramanian *et al.*, 2005; Gonzalez *et al.*, 2010). In addition, it also showed the role of mutualism symbiosis between *Bradyrhizobium sp*. and soybean root in nitrogen fixation as a biochemical process that converts free N_2 into N compounds that are available to plants. N is a primary plant nutrient and a key element in proteins and nucleic acids (Wood *et al.*, 1993; Walker *et al.*, 2001), and is required in the synthesis of chlorophyll. Isoflavones are also one of the important secondary metabolites in soybean plants formed through the phenylpropanoid biosynthetic pathway precursor phenylalanine which is one of the essential amino acids that requires N in its synthesis. Genistein and daidzein are the two principal isoflavones in soybean, while glycitein is present in much lesser amounts, and is unique for soy plants. They are stored as glucosyl- and malonyl-glucosyl conjugates in vacuoles (Yu and McGonigle, 2005). Although seed isoflavone content is greatly dependent on the environment, the production is largely under genetic control (Eldridge *et al.*, 1983; Wang *et al.*, 1994; Hoeck *et al.*, 2000; Nelson *et al.*, 2002).

In this study, our results demonstrated that growing season was significantly influenced isoflavone accumulation in soybean. Soybean grown during the rainy season had a higher content of genistein, daidzein, glycitein and total isoflavones than soybean grown during the dry season. Growing season of October to December had the higher rainfall and humidity but the temperature was lower than the planting time of June to September. Wilis cultivar grown from October to December increased total isoflavone (1008.63 μg/g) more than other treatments, while Wilis cultivar was grown from June to September gave the lowest of total isoflavone content. The treatment of *Bradyrhizobium* sp. and Wilis cultivar grown during the rainy season tended to increase the total isoflavone.

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