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Comparative LC-ESI/MS Chemical Profile, HPLC Analysis of Isoflavonoids and Genetic Diversity of Five Soybean Genotypes

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Research Article

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ABSTRACT

Soybean (*Glycine max* L. Merr.) is one of the most important and widely consumed seed legumes worldwide. The aim of this study was to investigate the metabolic and genetic profile of four Egyptian soybean genotypes ($Giza_{21}$, $Giza_{22}$, $Giza_{35}$ and $Giza_{111}$) versus Crawford, a USA imported genotype. DNA fingerprinting was tested using Inter Simple Sequence Repeats (ISSR). Twenty primers were used, five of which showed 12.5-42.8% of polymorphism clustering the tested genotypes into two major clusters of which Crawford was the most divergent genotype. Applying LC-ESI/MS analysis, twenty-seven to forty three metabolites were recognized from the defatted ethanol extract of all genotypes. Crawford, G_{35} and G_{111} were dominated by amino acids as a major constituent (83.84-74.95-42.22%) respectively. While genotype G_{21} contained the highest percentage of total phenolics (51.42%) and vitamins (26.44%). Organic acids represented the major constituent (54.76%) in G_{22}. HPLC analysis of isoflavonoid content against authentic compounds showed that G35 contained the highest percentage of both total isoflavonoids and their glycosides (7.911 and 7.375 mg% respectively) while genotype Crawford showed the highest isoflavone aglycone percentage (0.875 mg%) with genistein being the major component.

INTRODUCTION

Soybean (*Glycine max* L. Merrill) is an important crop following cotton, in Egypt. It is widely used as a cheap source of food for humans and animals; it is extensively researched for its content of many chemical metabolites such as vitamins, amino acids, lipids, soysaponins, flavonoids, phenolics and tannins [1]. Acting as a factory for the production of several

classes of compounds reflects its medicinal importance and its nutritional value which have been known for decades. There are more than twenty five genotypes of G. max grown in Egypt. Several attempts for crop improvement programs were done throughout the world which is justified by its vast uses in different fields both medical and economic. Concentration and composition of phytochemical compounds in legumes vary significantly based on several intrinsic and extrinsic factors [2]. Furthermore, several prepared soy products such as tofu, soy milk, soy sauce, miso and soya meal (oil extraction by-product) have been developed for both human and animal consumption [3]. The present study aimed to compare between five of the commonly used genotypes in Egypt of G. max genotypes (G_{21}, G_{22}, G_{35} and G_{111}) which are locally grown in Egypt and genotype (Crawford) which is imported from USA. All the four local genotypes are a pedigree of genotype Crawford.

Soybean isoflavones are polyphenolic compounds which exist in twelve different chemical forms. Daidzein, glycitein and genistein are the aglycone forms of soy isoflavones. In conjunction with sugars, they build the β-glycoside form (daidzin, glycitin and genistin respectively). This class of compounds exhibits diverse pharmacological effects that have been studied extensively. They are known for their role as phytoestrogen which affects women health in addition to several other human health effects [4-6].

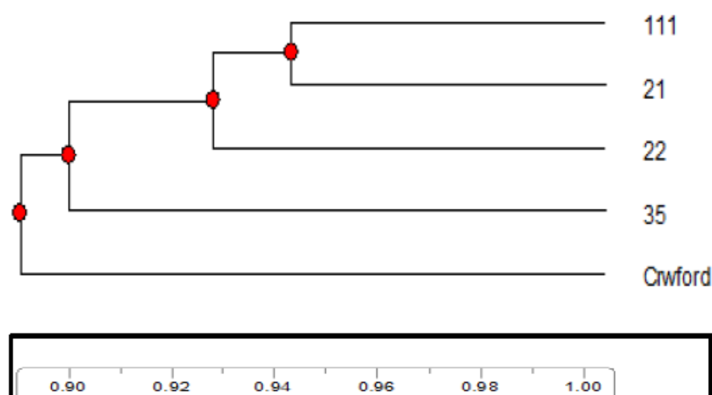
The great external similarity in the appearance of the five tested genotypes seeds encouraged us to apply an ISSR analysis for DNA finger printing studying patterns associated with genetic markers for the five genotypes. LC-ESI mass profiling of the defatted ethanol extract and an HPLC analysis of three isoflavones markers and their derivatives were assessed. Combing DNA and chemical fingerprinting will be an effective tool in the authentication of the five genotypes under investigation.

MATERIALS AND METHODS

Plant material

Dried seeds and fresh leaves of the five genotypes (G_{21}, G_{22}, G_{35}, G_{111}, and Crawford) of soybeans (*Glycine max* L. Merrill) were collected from Agriculture Research Centre, Giza, Egypt in March 2014. All used seeds were botanically identified and authenticated by Dr. Mona Marzouk, consultant of plant taxonomy, at National Research Centre, Department of Phytochemistry and Plant Systematic. A voucher specimen of the five seeds number (9*Gma*9, 10, 11, 12, 13/2018) respectively was deposited in Pharmacognosy Department Herbarium, Faculty of Pharmacy, Helwan University (Figure 1).

Figure 1. Dendrogram of the genotypes under investigation



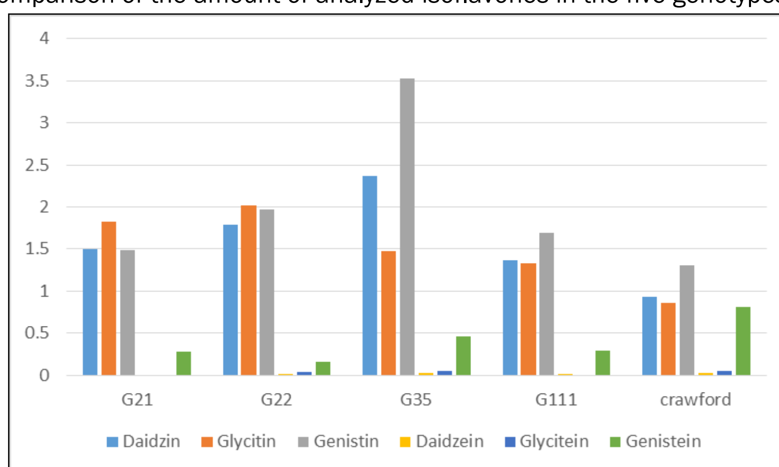
*111= genotype 111, 21= genotype 21, 22= genotype 22, 35= genotype 35, Crwford= genotype crowford

Methods for DNA fingerprint

The DNA was extracted from young leaves, using QIAGEN DNeasy kit (Cat No. /ID: 69106). DNA quality was determined visually on 0.8% agarose gel. The DNA concentration was quantitatively measured on Bio-photometer (Eppendorph, Germany) and adjusted to 50 ng /µL. ISSR bands were recorded as present (1) or absent (0) for all soybean genotypes

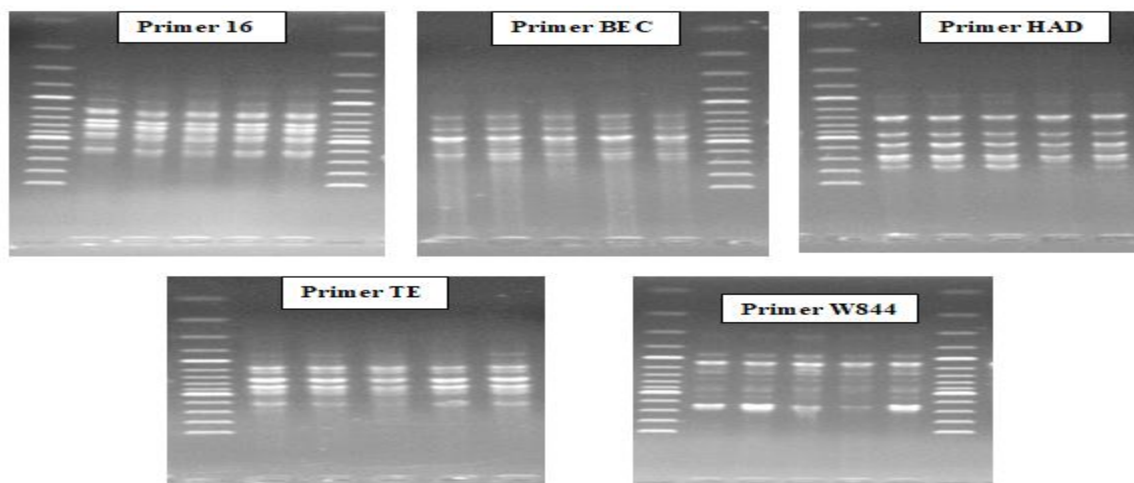
and were examined to estimate the relationships among the investigated genotypes. The difference matrix was estimated by pairwise comparisons of the genotypes based on the percentage of total fragments. Results were analyzed visually with Phoretix ID Pro-software from nonlinear dynamics. A dendrogram (Figure 2) was generated using the Un-weighted Pair-Group Method using Arithmetic Averages (UPGMA). PCR was performed in 25 µl reaction volume containing 2X ready mix (Emerald Amp Max PCR master mix) 20 pm oligonucleotide primer and 50 ng genomic DNA. This reaction was performed on Eppendorph Master Cycler programmed to 35 cycles as follows: an initial denaturation step at 95˚C for 5 minutes, followed by 35 cycles of denaturation step at 94˚C for 1 minute, annealing Temperature (Ta) for 1 minute and an extension step at 72˚C for 1 minutes, and final extension step at 72˚C for 10 minutes. The obtained ISSR-PCR products using the twenty primers (Table 1), was detected by gel electrophoresis for *G. max* genotypes, only five primers gave scorable bands (Figure 3), they are reported in Table 2, these primers were synthesized by HVD Corporation, Germany. Ladder DNA used 100 bp plus (Thermo Scientific™ SM1153).

Figure 2. Comparison of the amount of analyzed isoflavones in the five genotypes of soybean.



*G= genotype 21, 22, 35, 111 and Crawford

Figure 3. TLC plate of the five promising primers with the five genotypes of soybean G_{21}, G_{22}, G_{35}, Crawford and G_{111} respectively.



HPLC analysis of isoflavones

One gram of each sample after defatting with petroleum ether was shaken with 10 ml of acetonitrile and 6 ml water for 60 min. the final volume was adjusted to 25 ml with distilled water and centrifuged. A standard stock solution in DMSO was prepared to contain 2 mg/ml of daidzin, 0.5 mg/ml of glycitin, 2 mg/ml of genistin, 0.2 g/ml of daidzein, 0.2 mg/ml of glycitein and 0.2 mg/ml of genistein. Different dilutions of stock solution (0.5 - 2.5 ml) were prepared and diluted with a solvent mixture of acetonitrile and water (2:3) to 25 ml. Standard solutions and soy samples were separately filtered through a 0.45 µm filter. 20 µl injected into HPLC Agilent 1200 series chromatograph coupled to a quaternary pump (DE 62975591), C_{18} column (300×4 mm) at 32°C. UV detector (DE 82800737) and equipped with thermo-scientific (ODS HYPERSIL) set at λ_{max} 254 nm and temperature 40°C.eluting solvent mixture, 0.05% v/v phosphoric acid (solvent A) and acetonitrile (solvent B),flow rate 0.65 ml/min. Gradient elution: A from 90% to 70% (min 0-60), A from 70% to 10% (min 60-60.5), A 10% (min 60.5-63.5).Compounds identification was based on comparison of their retention times with those of the authentic compounds (Figure 1). A calibration curve of each analyte obtained from the standard solutions was plotted to determine the regression line by least squares analysis (Figure 2) [7]. The concentration (mg/ml) of the relevant analyte in the sample solution was determined and the percentages of daidzin, glycitin, genistin, daidzein, glycitein and genistein were calculated from the following equation:

% Analyt=(C/W) x V x 100

C: Is the concentration of each isoflavone as determined above for the sample solution (mg/ml)

W: Weight of powdered soy sample taken to prepare the sample solution (mg)

V: Final solution volume of the sample solution (ml)

LC-ESI-MS analysis

One gram of each dried sample after defatting with petroleum ether, they were separately macerated in (15 ml x 3) 70% ethanol and left to stand 72 hours with shaking. After filtration the alcoholic extracts were concentrated under reduced pressure at 45˚C. 100 µg/ml of tested samples were prepared using Methanol (analytical grade), filtered on membrane disc filter (0.2 µm). Injection volume: 10 µl. Samples were injected into the UPLC instrument equipped with reverse phase C-18 column (ACQUITY UPLC-BEH C18 1.7 µm (2.1 × 50 mm). Mobile phase was filtered using 0.2 µm filter membrane disc and degassed by sonication before injection. Elution flow rate of 0.2 ml/min using gradient mobile phase comprising eluent A (H_2O acidified with 0.1% formic acid), eluent B (MeOH acidified with 0.1% formic acid). Elution was performed using the gradient (90% A: 10% B to 100%B). Both positive and negative ion mode were applied (Figure 3A-12A): source temperature 150˚C, cone voltage 30 eV, capillary voltage 3 kV, dsolvation temperature 440˚C, cone gas flow 50 l/hr., and desolvation gas flow 900 L/hr. Mass spectra were detected in the ESI negative ion mode between m/z 100–1000. The

peaks and spectra were processed using the Maslyn x 4.1 software and the identification of the major components in the analyzed samples were based on computer matching of their molecular weights with those stored in NIST/EPA/NIH mass spectral library version 2.0/2011, in addition to comparison to literature data [8].

RESULTS AND DISCUSSION

DNA fingerprint results

The ISSRs technique have shown rapid, simple, reproducible and inexpensive means in molecular taxonomy, conservation breeding and genetic diversity analysis and it was previously successfully used to identify genetic diversity and relationships among soybean genotypes within a population [9,10]. In the present study, twenty ISSR primers were used in the ISSR analysis; it is worth mentioning that previous attempt to identify the genetic diversity of G_{22}, G_{35} and G_{11} genotypes was done using a different technique RAPD [11]. Only five primers gave scorable bands illustrated in Table 1, these five primers generated a total of 39 fragments, twenty eight fragments were monomorphic and eleven were polymorphic, with an average of 7.8 per primer. Primer (HAD) and primer (W844) gave 42.8 and 40.0% of polymorphism respectively which renders them promising primers for the identification of the five genotypes of soybean under investigation. Although genotype Crawford is a member of the pedigree of the four tested local genotypes, two main clusters were seen as shown in dendrogram. The first includes genotype Crawford which represents the most divergent genotype. While the second cluster include all the remaining four genotypes (G_{21}, G_{22}, G_{35}, G_{111}). And G_{35} was the most different genotype among the 4 locally grown genotypes. While genotypes G_{21}, G_{111} were the most similar (94 % similarity) (Table 2) [12].

Table 1. The ISSR of the five promising primers for the five genotypes fingerprint.

NO	name	Primer sequence	(Ta)	T. no of amp	Amp. size range (bp)	NO of mono amp.	NO of poly amp	% Poly
1	16	CGTC (AC) 6	50˚C	8	521-1255	7	1	12.5
2	W844	(CT) 8TG	50˚C	10	477-1401	6	4	40
3	TE	GT (GGT) 3GAC	42˚C	7	572-1246	6	1	14.2
4	BEC	(CA) 7TC	42˚C	7	646-1372	5	2	28.5
5	HAD	CT (CCT) 3CAC	42˚C	7	691-1823	4	3	42.8
T*				39		28	11	28
Ave*				7.8		5.6	2.2	

T*=Total, Ave*=Average, Annealing Temp=Ta, % poly= percent polymorphism, T. NO of amp= total number of amplicons, NO of mono amp=number of monomorphic amplicons, NO of poly amp= number of polynorphic amplicons, Amp. = amplicons.

Table 2. The percentages of different classes of tentatively identified compounds of each genotype.

Class of compounds	G_{21}	G_{22}	G_{35}	G_{111}	Crawford
Total phenolics %	51.42	29.78	15.33	29.29	16.29
Total amino acid %	39.75	37.25	74.95	42.22	83.84

Total organic acid %	32.57	54.76	13.6	16.08	16.70
Total flavonoid %	3.82	3.41	3.02	17.59	9.39
Total isoflavonoid %	5.30	4.15	3.82	7.89	5.73
Total vitamins %	26.44	7.20	-	1.16	-
Total carbohydrate %	8.26	11.84	35.89	0.75	3.03
Total saponin %	6.4	5.52	17.04	6.56	23.99
Total lignan %	0.46	19.72	25.49	-	1.49
Total anthocyanin %	0.55	-	-	-	1.91

Isoflavonoids analysis

The total isoflavone content in the five genotypes of *G. max* seeds ranges from 3.971 mg% to 7.911 mg% of the dry material. Although distribution of individual isoflavones within these values can vary significantly, daidzein, genistein and glycitein and their conjugates are the three major isoflavones found in soybean. In our finding, the total glycosidic form percentage ranging between (3.096-7.375 mg%) with the highest content found in genotype G_{35}, surpassed the aglycone percentage in all tested genotypes ranging between (0.205- 0.875 mg %) with the highest percentage detected in genotype Crawford (Figure 2). Genotype G_{35} represented the richest genotype with the total isoflavonoid content (7.911 mg%) thus it may be recommended as a healthy dietary supplement in postmenopausal females [13] . In the present study genistein aglycone represented the major aglycone in all tested genotypes. It is worth mentioning that although most obtainable products contain isoflavones in the glycoside form, the isoflavones absorption only happen in the aglycone form. Hydrolysis of isoflavones to their aglycones has been supposed to be mainly catalyzed by microflora in the colon and in some case it can start in the mouth mediated by saliva [14,15].

LC-ESI-MS analysis

Extracts of the five genotypes under investigation were analyzed by both positive and negative Electro Spray Ionization (ESI) modes, for all genotypes ionization showed more abundant and sharp peaks in the positive mode showing 31 up to 46 peaks in different genotypes as seen in Tables (3A to 7A). Eight compounds were common compounds detected in all tested genotypes at different concentrations including four amino acids (valine, proline, tyramine and glycine derivatives) two phenolic acids (quinic acid and Succinic acid derivative), one flavonoid (chrysoeriol) and one isoflavonoid (Daidzein). Amino acids presented the major constituent in three genotypes (G_{35}, G_{111} and Crawford), in the range of (37.25-83.84%). Also phenolic acids recorded the highest percentage in genotype G_{21} (51.42%) and organic acids showed the highest percentage in genotype G_{22} (54.76%). It was observed that the most different genotype from the phytochemical point of view was genotype Crawford which corresponds with the results obtained in the DNA analysis. The highest similarity was established between both genotypes G_{21} and G_{22} as they showed 23 common compounds. Genotype Crawford is recommended as a very good source of protein and amino acids (reaching up to 83% of its constituents) (Table 2), with

highly nutritive value, as well as a good source of soya-saponins (soyasaponin Bd 14.79%) well known for their anticancer activity [16]. Genotype G_{21} is recommended as a good source of phenolic compounds (51.42%) and vitamins (26.44%) with vitamin E representing a major component (25.92%) in contradiction to previous reports stating that genotype G_{22} is the richest with phenolic content. This variation in phytochemical composition between different genotypes was previously reported by different studies [17,18].

CONCLUSION

ISSR technique proved to be a successful tool for the study of the genetic diversity of soybean genotypes and their differentiation. Combining DNA and chemical fingerprinting is an effective tool in the authentication of the five genotypes under investigation. Genotype Crawford is recommended as a very good source of protein and amino acids with highly nutritive value, while Genotype G_{21} is recommended as a good source of phenolic compounds and vitamins and genotypes G_{35} proves to be a good source of carbohydrates. The present study confirms that different phyto-constituents percentages may vary greatly according to the environmental conditions & area of cultivation.

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