Biochemical and Molecular Criteria of Some Egyptian Species of Cassia and Senna (Subfamily: Caesalpinioideae-Leguminosae); With Reference To Their Taxonomic Significance.

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Abstract: Four species and one subspecies representing the two genera *Cassia* and *Senna* were examined for measuring some biochemical and molecular evidences; total protein content, leaf protein electrophoretic profiles, activity of some antioxidant enzymes and total nucleic acids content as well as ISSR fingerprinting using four ISSR primers. Leaf protein electrophoretic profiles and ISSR fingerprinting were found taxonomically useful among the taxa studied at both the generic and specific levels.
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Keywords: *Cassia*; *Senna*; Leaf protein electeophoresis; ISSR fingerprinting.

1. Introduction

Cassia L. *sens*. *lat*. is the largest genus in subfamily Caesalpinioideae of the Leguminosae. It comprises about 600 species **(Brenan, 1967; Singh, 2001).** This genus extends in all terrestrial habitats from the equator to the edges of dry and cold deserts but much of its diversity centred in the areas of varied topography with seasonal climates **(Laxmikanta and Pratap, 2010)**. **Irwin and Barneby (1982)** splitted *Cassia* L. *sens*. *lat*. into three genera namely; *Cassia* L., *Chamaecrista* Moench. and *Senna* Mill. They mentioned that the three genera are distinct through discrete morphological characteristics and the lack of intermediates. This segregation of *Cassia* L. is supported by several subsequent studies based on ontogenetic floral development **(Tucker, 1992)**; floral anatomy **(Hussein, 1999)**; numerical analysis of electrophoretic protein profiles, chromosome number and morphological characters of some Egyptian species of *Cassia* **(Ghareeb *et al*., 1999)**; and molecular studies **(Laxmikanta and Pratap, 2010; Tripathi and Goswami, 2011)**.

Several studies highlighted the usefulness of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate the differences and similarities between plants **(Badr, 1995; Mohamed *et al*. (2006); George *et al*., 2013)**.

Metabolic interconversions are catalyzed by enzymes. The genetic variation in metabolite levels can be explained by changes in the activities of one or several enzymes **(Sulpice *et al*., 2010)**. Enzyme activities exhibit considerable natural genetic variation which collocated with structural genes for the enzymes **(Thevenot *et al*., 2005)**.

The development of DNA markers has greatly facilitated research in a variety of biological branches such as taxonomy, phylogenetic relationships and genetics **(Halward *et al.*, 1992; Abdelsalam *et al.*, 1998)**. The most commonly used methods based on the polymerase chain reaction (PCR) are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR) and simple sequence repeats (SSR_S) or microsatellites **(Gupta and Varshney, 2000)**. ISSR is a valuable tool to analyze the genetic variability in a plant collection or wild species and are useful to identify genotypes even in closely related individuals **(Christopolous *et al*., 2010; Mariana *et al*., 2012)**. The major advantage of this method is its universality and ease of development as it does not require genome sequence information **(Jabbarazadeh *et al*., 2010)**. ISSR-PCR is a fast, reproducible and inexpensive technique **(Li *et al*., 2010)**.

The ISSR method has a wide range of uses viz., evaluating genetic diversity among plant materials, identification of closely related cultivars **(Gonzales *et al*., 2002)**, in reclassifying previously classified species and genera **(Tripathi and Goswami, 2011)**. The objective of this study is to investigate the relationships among four species and one subspecies, of *Cassia* and *Senna* based on variation of evidences derived from the total protein content, electrophoretic protein pattern, activity of some antioxidant enzymes viz., Catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO), total content of nucleic acids (RNA and DNA contents) and ISSR markers analysis.

2. Materials and Methods

This study is conducted on four species and one subspecies of the two genera *Cassia* and *Senna*, growing in parks of Zagazig University. The used taxa are: *Cassia fistula* L.; *Cassia javanica* L. subsp. *nodosa* (Roxb.) K. Larsen & S. S. Larsen (= *Cassia*

nodosa Roxb.); *Senna alexandrina* Mill. (= *Cassia senna* L.; *Cassia acutifolia* Del.); *Senna sophera* (L.) Roxb. (=*Cassia sophera* L.) and *Senna surattensis* (Burm. f.) H. S. Irwin & Barneby (= *Cassia surattensis* Burm. f.). The collected specimens were matched against authentic herbarium specimens kept at the Herbarium of Flora and Phytotaxonomy Research Department (CAIM), Horticultural Research Institute, Agricultural Research Centre, Cairo, Egypt. The scientific names and author citation of the species investigated were rechecked against the website www.ipni.org/ipni/plantnamesearchpage.do of the international plant names index and the website of the plant list: www.theplantlist.org.

Biochemical analysis:

Protein analysis:

Total protein:

According to **Bradford (1976)** total protein content of young leaves of each species was determined spectrophotometrically. The quantity of protein in samples of leaves of the studied species was calculated from standard curve by using Bovine Serum Albumin as standard protein.

Protein electrophoresis:

For SDS-protein electrophoresis, protein extracts were centrifuged at 14,000 rpm for 10 min at 4^0C and separated in 12% acrylamide-gel. Molecular weights of different bands were calibrated with mixture of standard protein markers (M.W. 7-240 KDa). The banding profile was stained in Commassie blue dye, destained then the gel was photographed and analyzed using BIO-RAD Video documentation system, Model Gel Doc 2000.

Enzyme activity:

Catalase (CAT) Assay: The activity of catalase (CAT) as well as peroxidase (POD) was assayed after the method of **Chance and Maehly (1955).**

Five milliliters of the assay mixture for the CAT activity incubated at 25^0C for 1 min., the reaction was stopped by adding 10 ml of 2% (v/v) H_2SO_4 and the residual H_2O_2 was titrated against 0.01 N $KMnO_4$ until a faint purple color persisted. One unit of (CAT) activity is defined as that amount of enzyme which breaks down 1 µmol of H_2O_2/min. (µmoles H_2O_2 destroyed/min.).

Peroxidase (POD) Assay: five milliliters of the assay mixture for the peroxidase activity was incubated for 5 min. at 25^0C after which the reaction was stopped by adding 0.5 ml (v/v) H_2SO_4. The amount of purpurogallin formed was determined by taking the absorbency at 420 nm.

Polyphenoloxidase (PPO) Assay: five milliliters assay mixture of PPO activity consisted of the same assay mixture as that the POD without H_2O_2. The absorbency of the purpurogallin formed was taken at 420 nm. Peroxidase and polyphenol oxidase activities were expressed in absorbency units.

Molecular Analysis:

Determination of Total Nucleic Acids:

For determination of nucleic acids contents the method described by **Sadasivam and Manickam (1996)** and **Devi (2000)** was adopted. RNA and DNA contents were estimated by easy colorimetric method based on the quantitative reaction of ribose with orcinol and deoxyribose with diphenylamine reagent.

ISSR analysis:

For ISSR analysis, DNA was isolated according to the method outlined by **Junghans and Metzlatt (1990)**.

PCR amplification was performed according to the protocol of **Zielkiewicz *et al.*, (1994)**. ISSR amplification reactions were carried out in 25 µl volume containing 0.75 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM), 2.5 µl PCR buffer (10 x), 1 µl Taq DNA polymerase, 0.5 µl primer (100 µM), 1 µl template DNA (10 ng/µl) and 19.5 µl ddH_2O. Eight ISSR primers were purchased from Metabion, Plonneg, Germany (Table 4). Amplified products were electrophoresed on 2% agarose in 0.5 x TBE buffer. The gels stained with ethidium bromide (1.0 µg/ ml). A marker of 1 Kb plus DNA ladder 1 µg/µl that contain a total of twelve bands ranging from 1,000 to 200 bp was used. Bands were detected on UV-transilluminator and photographed by Gel documentation system Biometra Bio Doc Analyze 2000. Primers which gave reproducible fingerprints (DNA bands) were considered for the data analysis.

For data analysis the bands produced from the banding patterns of four ISSR primers and/or leaf proteins were scored and treated by numerical analysis resulting in number of dendrograms. The presence or absence of each of all bands was treated as a binary character; coded as (1) and (0) respectively for computation using SPSS (Statistical Package for the Social Sciences) version 14. In addition, the relationships among the taxa examined have been expressed using Jaccard's coefficient of similarity.

3. Results

Biochemical analysis:

Protein analysis:

Total protein:

The percentage of the total leaf protein content of the taxa studied are given in Table (1). The total leaf protein content being the highest (46.0) in *Senna sophera* and the lowest (7.9) in *Cassia fistula*.

Protein electrophoresis:

The electrophoretic analysis of leaf protein for the five taxa examined is shown in Plate (1). A total of 28 protein bands were scored in the electrophoretic profiles of the taxa examined. The scanning of SDS-

PAGE gel of the different taxa is shown in Table (2), bands were compared with those of standard proteins with respect to their Rf values and molecular weights, and the range of M.W. of proteins has been shown in Table (2).

From both *Senna alexandrina* and *Senna surattensis* eleven polypeptides have been separated. Out of these two polypeptides with M.W. ranging between 70 and 140 are observed in leaves of *Senna surattensis* which are absent in *Senna alexandrina.* However in *Senna alexandrina* two proteins with M.W. ranging from 50 to 70 and 140 to 240 are observed but absent in *Senna surattensis*. Proteins with M.W. less than 15 KDa are commonly observed in leaves of the taxa studied. Proteins with M.W. between 15 to 20 (three bands) and 25 to 35 (two bands) are common to *Cassia fistula* as well as *Cassia javanica* subsp. *nodosa.* Eight proteins have been observed in *Senna sophera* the M.W. of which fall in the range from 7 to 50 KDa (Table 2 and Plate 1).

The dendrogram (Fig. 1B), generated on the basis of the data obtained from leaf protein electrophoretic profiles using between groups linkage clustering in the SPSS version 14, revealed that the five taxa studied were splitted into two clusters. One of them contained *Cassia fistula* and *Cassia javanica* subsp. *nodosa* while the second cluster comprised the three examined species of genus *Senna* namely; *Senna sophera*, *Senna alexandrina* and *Senna surattensis*. The two latter species presented close affinity to each other.

Enzyme activity:

In the present investigation, three antioxidant enzymes, Peroxidase (POD) Polyphenol oxidase (PPO) and Catalase(CAT) are studied as enzyme activities. The result in Table (3) (mean ± SD) and Fig. (2) illustrated that the enzymes activities differ from one species to another.

POD activity increased in *Senna alexandrina* as compared with *Cassia javanica* subsp. *nodosa.* Whereas *Senna sophera, Cassia fistula* and *Senna surattensis* possess moderate activity. PPO activity ranged from (0.63 ± 0.22) to (1.22 ± 0.25) in *Cassia fistula* and *Senna surratensis* respectively. *Senna surratensis* and *Cassia fistula* had a high CAT activity (56.05 ± 8.09) and (56.05 ± 9.38) as compared with *Senna alexandrina* (48.55 ± 7.09) which recorded the lowest activity (Table 3). Antioxidant value of the three studied enzymes (POD, PPO and CAT) in *Cassia javanica* subsp. *nodosa* is low relatively to the rest studied species (Fig. 2).

Molecular Analysis:

Total nucleic acids:

Table (1) shows the amount of total DNA and RNA contents of the studied species. The lowest and highest DNA content were found in *Senna alexandrina* and *Cassia fistula* respectively. The total RNA content being the lowest in *Cassia javanica* subsp. *nodosa* and the highest in *Senna surattensis.*

ISSR fingerprinting

In the present study, eight tested primers were used to differentiate between the studied taxa. Out of eight tested primers, four [PS4, PS5, PS7 and PS8] gave no or few amplified bands between the tested species and four [PS1, PS2, PS3 and PS6] gave rich and highly polymorphic fingerprints of which only were used, the results shown in Plate (2) and Table (4).

The size of the amplified products ranged from 200 to 2,000 bp. Four primers generated 57 bands, from which 50 were polymorphic (87.72%). The number of bands varied from 12; primer PS1 to 17; primer PS2 (Table 4). The polymorphism level, calculated as the number of polymorphic bands per primer ranged from 25% (primer PS1) to 61.54% (primer PS6) for the two species *Senna sophera* and *Cassia fistula* respectively. Maximum and minimum number of polymorphic bands was obtained with the primers PS2 (14) and PS1 (11) respectively. Details of ISSR banding patterns and bands amplified in the examined taxa with the four primers have been presented in Table (4).

The dendrogram resulted from ISSR fingerprinting (Fig. 1A) presented that the five taxa examined are segregated into two clusters. One of them involves *Cassia fistula* and *Cassia javanica* subsp. *nodosa* as well as *Senna surattensis* exhibiting high degree of dissimilarity with the two examined taxa of *Cassia*. The other cluster includes *Senna alexandrina* and *Senna sophera* representing somewhat high degree of genetic diversity. In addition, the dendrogram generated from the combination of the leaf protein electrophoretic profiles and the four ISSR primers (Fig. 1C) illustrated that the taxa examined are separated into two clusters. One of them comprised *Cassia fistula*, *Cassia javanica* subsp. *nodosa* and *Senna surattensis* which clearly appeared distantly related to them. The other cluster contained *Senna sophera* and *Senna alexandrina* exhibiting somewhat low degree of closeness or similarity.

The percentage of similarity among the taxa studied as revealed from combination of leaf protein electrophoretic patterns and ISSR primers fingerprinting is illustrated in Table (5). It ranged from 19.24% between *Senna sophera* and *Cassia javanica* subsp. *nodosa* to 48.83% between *Cassia fistula* and *Cassia javanica* subsp. *nodosa.*

Table (1): Total protein, DNA and RNA contents of the species studied.

Species	Protein(mg/ml)	DNA (µg/gm)	RNA(µg/gm)
Cassia fistula	7.9	0.421	0.251
Cassia javanica subsp. *nodosa*	16.6	0.154	0.234
Senna alexandrina	12.7	0.125	0.318
Senna sophera	46.0	0.363	0.336
Senna surattensis	36.4	0.226	0.379

Table (2): Molecular weights of leaf proteins as revealed by SDS-PAGE and number of bands scored in each species.

Species	Number of bands				
Range of M.W. protein (KDa)	*Cassia fistula*	*Cassia javanica* subsp. *nodosa*	*Senna alexandrina*	*Senna sophera*	*Senna surattensis*
7 < 15	1	1	1	1	1
15 < 20	3	3	2	3	2
20 < 25	1	4	2	1	2
25 < 35	2	2	2	1	2
35 < 50	2	1	2	2	2
50 < 70	1	2	1	_	_
70 < 100	_	2	_	_	1
100 < 140	_	1	_	_	1
140 > 240	_	_	1	_	_
Total bands	10	16	11	8	11

Table (3): Catalase, peroxidase and polyphenoloxidase activity in the species examined.

Species	Peroxidase A_{420}	Polyphenol oxidase A_{420}	Catalase µmols H_2O_2 destroyed/min.
Cassia fistula	1.5246 ±0.488	0.634 ±0.223944	56.0333±9.2795
Cassia javanica subsp. *nodosa*	0.9756±0.1579	0.910333±0.116749	49.7000±7.77
Senna alexandrina	1.639±0.4877	0.906667±0.053314	48.550±7.0897
Senna sophera	1.3067±0.581	1.186667±0.537153	51.00±2.645
Senna surattensis	1.1828 ± 0.2788	1.216667±0.246644	56.0500±8.0925

Table (4): Number and types of the amplified DNA bands produced by ISSR primers.

Primer	Primer sequence	Number of Bands					% Polymorphism per primer				
		Total bands	Monomorphic	Polymorphic bands		% Polymorphic	*Cassia fistula*	*Cassia javanica* subsp. *nodosa*	*Senna alexand.*	*Senna sophera*	*Senna surattensis*
				Unique bands	Non-Unique						
PS1	$(GA)_9T$	12	1	3	8	91.67	50.00	50.00	33.33	25.00	58.33
PS2	$C(GAG)_5$	17	3	1	13	82.35	47.05	41.17	41.17	47.05	41.17
PS3	$(AGG)_6$	15	2	4	9	86.67	46.67	60.00	40.00	33.33	20.00
PS6	$(GACA)_4$	13	1	3	9	92.31	61.54	30.77	30.77	30.77	30.77

Table (5): Jaccard's coefficient of similarity among species of *Cassia* and *Senna* as revealed from leaf protein electrophoretic pattern and ISSR primers fingerprinting.

Species	*C. fistula*	*C. javanica* subsp. *nodosa*	*S. alexandrina*	*S. sophera*	*S. surattensis*
Cassia fistula	100				
Cassia javanica subsp. *nodosa*	48.83	100			
Senna alexandrina	28.23	25.27	100		
Senna sophera	21.95	19.24	28.33	100	
Senna surattensis	23.63	29.41	30.94	29.52	100

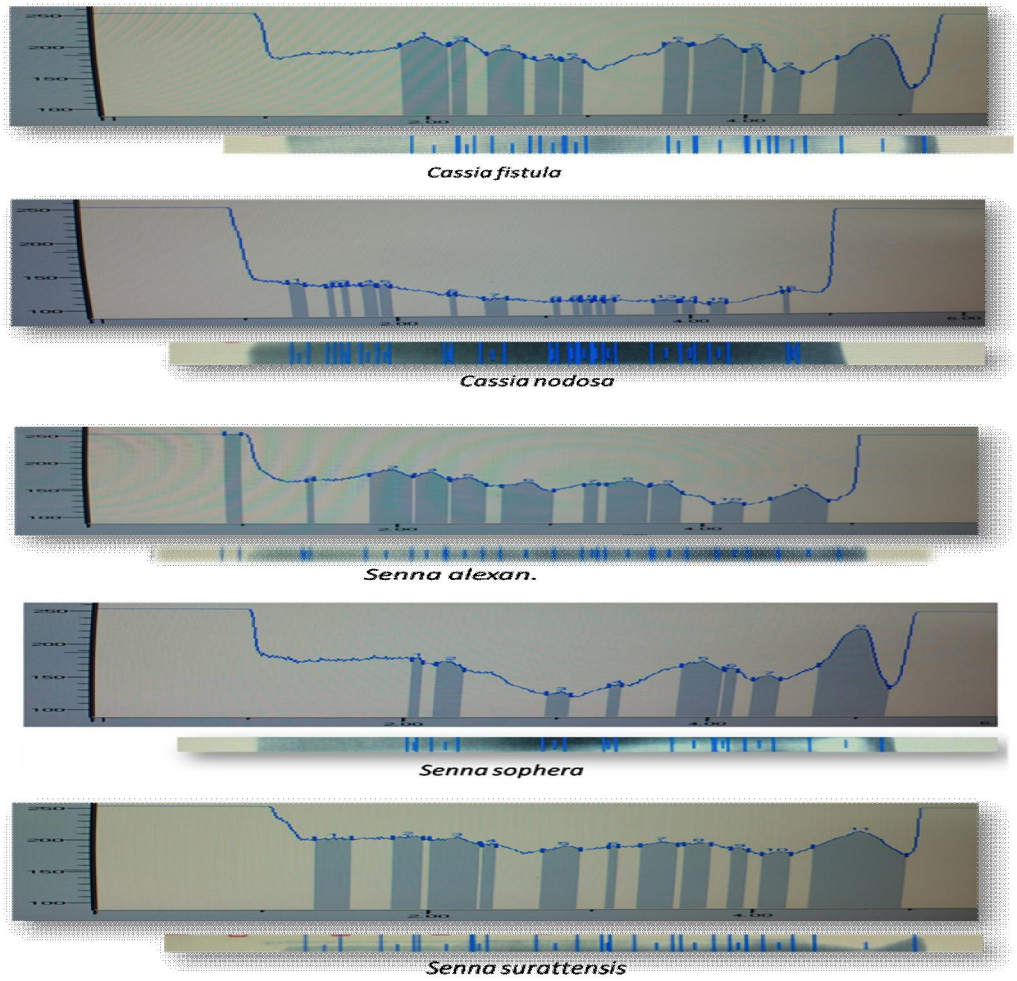


Plate (1): Leaf protein electropherograms as presented by Gel Doc 2000, BIO RAD scanner.

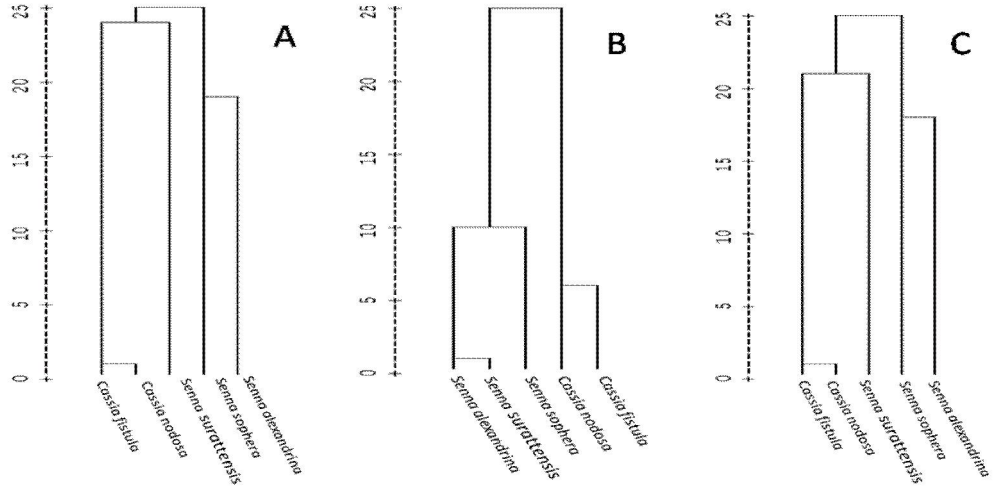
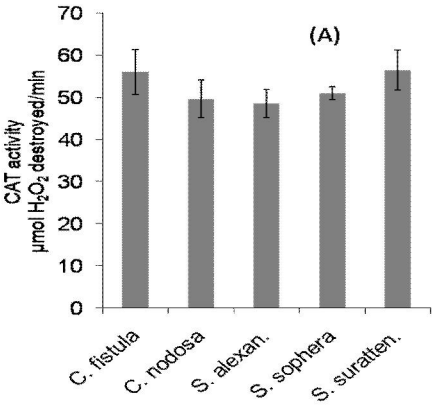
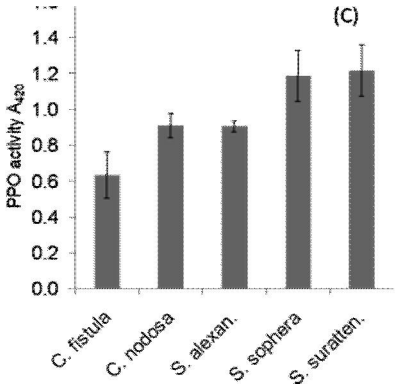


Fig. (1): Dendrograms revealing the genetic distance among the taxa examined based on ISSR data (A), leaf protein electrophoretic pattern (B) and combination of leaf protein electrophoretic pattern and ISSR data (C).





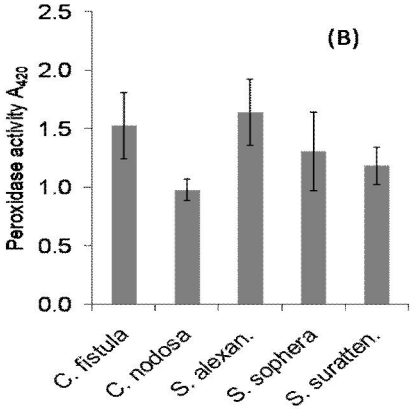


Fig. (2): Activities of three antioxidant enzymes. Enzyme activity of catalase (CAT) (A), peroxidase (POD) (B) and polyphenol oxidase (PPO) (C) in leaves of the taxa studied. Results are the mean of three replication ± SE.

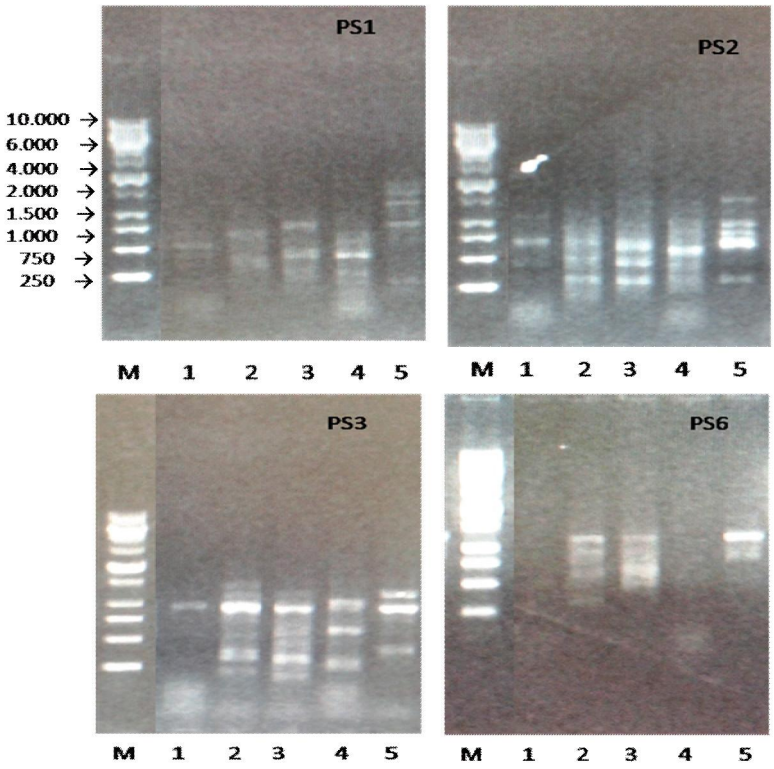


Plate (2): ISSR primers, (PS1, PS2, PS3 and PS6), fingerprinting among the taxa examined. M. Molecular marker, 1. *Senna sophera*, 2. *Cassia fistula*, 3. *Cassia javanica* subsp. *nodosa*. 4. *Senna alexandrina*, 5. *Senna surattensis*; taxa arranged, here, according to their positions in lanes during the procedure.

4. Discussion

Many taxonomic treatments have been supported the segregation of genus *Senna* from genus *Cassia* L. *sens*. *lat*. **(Tucker, 1992; Ghareeb *et al*., 1999; Hussein, 1999; Laxmikanta and Pratap, 2010; Tripathi and Goswami, 2011)**. In this study, leaf protein electrophoretic profiles and ISSR fingerprinting were proved to be effective evidences to differentiate the taxa studied of both *Cassia* and *Senna* at both the genus and species levels. The clear-cut separation of the three examined species of genus *Senna* from those of genus *Cassia*, based on leaf protein electrophoretic data, implies that such attribute can be a promising clue to evaluate the taxonomic relationships of other species of both *Cassia* and *Senna* and also in determining their generic status. Similar conclusion has been attained by **Mohamed *et al.* (2006)** when studying leaf protein electrophoretic profiles of some selected members of the Araceae. However, the intrageneric relationships among the species examined of *Senna* using ISSR primers did not fit with the results obtained from investigating the leaf protein electrophoretic pattern. This may be due to the small number of species examined from both *Cassia* and *Senna* as well as ISSR can be chiefly effective in identification of closely related varieties **(Escandon *et al*., 2007)** or cultivars **(Gonzales, 2002; Christopolous *et al*., 2010)**.

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