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ORCID

IS: 0000-0001-7882-0090
DB: 0000-0002-3449-8543
BG: 0000-0002-4396-2088

Chromosomal characterization mediated by karyomorphological analysis and differential banding pattern in fenugreek (*Trigonella foenum-graecum* L.): a neglected legume

Indranil Santra, Diptesh Biswas, Biswajit Ghosh*

Plant Cytogenetics Laboratory, Post Graduate Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata -700118, India
*Corresponding author. E-mail: ghosh_b2000@yahoo.co.in

Abstract. Fenugreek or *Trigonella foenum-graecum* L. is a commercially important yet neglected crop of the family Fabaceae, with potent medicinal applications, and can treat several diseases as well. Conventional breeding studies for higher yields of commercial crops largely depend on chromosomal information of the particular species. Despite a number of cytological research being conducted on *T. foenum-graecum*, a complete characterization of its chromosomes has not been achieved due to the limitations of traditional karyotype analysis methods. A range of chromosomal markers are advantageous to characterize at full extent and identify individual chromosomes rather than relying on only physical metrics. Thus, in this study, in addition to giemsa staining, other approaches like fluorochrome and silver staining were used for the precise karyomorphological analysis of this species. Enzyme maceration and air drying (EMA) based fluorochrome banding with GC-specific stain Chromomycin A3 (CMA), and AT-specific stain 4',6-diamidino-2-phenylindole (DAPI) applied for the first time for chromosome characterization. The results showed $2n = 16$ chromosomes in metaphase cells, with karyotype formula of 2m+6sm. The unique banding pattern observed in the CMA/DAPI and AgNOR staining highlights the AT and GC-rich regions as well as the nucleolar organizer regions (NORs). All this crucial information can further assist in conducting breeding studies of more precision with simultaneously encouraging similar studies that need to be done in other unexploited species of importance.

Keywords: *Trigonella foenum-graecum,* Karyotype, CMA-DAPI, AgNOR, Fenugreek.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum* L.) belongs to the family Fabaceae and has been consumed by the human race as food, spices and medicine since ancient times; nevertheless, it is still neglected from a global perspective (Mikić 2015). The term "fenugreek" is derived from the Greek language, which translates to "Greek hay," offering a glimpse into the plant's historical usage as a forage crop. The plant is cultivated in various regions, including India, Pakistan, Mediterranean Europe, Australia, and North America

(Acharya et al. 2008). India is a preeminent producer of fenugreek, claiming a staggering 80% of the global production (Rasheed et al. 2015). In addition to its culinary applications, the seeds and leaves of fenugreek have been utilized in traditional medicine to treat a plethora of conditions such as hyperglycemia, cardiovascular disease, neurological disorders, pulmonary fibrosis, obesity, asthma, and inflammation.

Fenugreek also possesses a large variety of nutritional compounds that are important for basic maintenance of biological systems. In general the fenugreek seeds contains 58% carbohydrates, 23-26% proteins, 0.9% fats and 25% fibers (Wani et al. 2018; Syed et al. 2020). Different kinds of minerals for example potassium (603 mg/100 g), magnesium (42 mg/100 g), calcium (75 mg/100 g), zinc (2.4 mg/100 g), manganese (0.9 mg/100 g), copper (0.9 mg/100 g) and iron (25.8 mg/100 g) can be found in *T. foenum-graecum*. Vitamin C (220 mg/100 g) and β carotene (19 mg/100 g) are also present in higher amounts in fenugreek (Al-Jasass and Al-Jasser 2012; Wani et al. 2018). In addition, fenugreek contains several nutritionally valuable flavonoids such as quercetin, luteolin, vitexin, 7, 4-dimethoxy flavanones, kaempferol, tricin, and naringenin (Petropoulos 2002). Important amino acids including aspartic acid, glutamic acid, leucine, tyrosine, phenylalanine and free amino acid (2S, 3 R, 4S)-4-hydroxyisoleusine are abundantly present in fenugreek (Syed et al. 2020). In a study fenugreek seeds have been found to contain greater amounts of protein with better amino acid profile than soybean protein isolate (Feyzi et al. 2002).

Karyotyping is the process of classifying the chromosomal makeup of a cell by examining the number, size, and structure of each chromosome, which can provide insights into the relationship between different species (Levin 2002). It is a commonly used technique in crop plant research for various purposes, such as characterizing cultivars, linking genetic and physical maps, and studying the evolutionary relationships among different species (de Moraes et al. 2007). Despite its utility, karyotyping is often hindered by the scarcity of chromosome markers, which makes it challenging to identify individual chromosomes (She and Jiang 2015). The utilization of traditional staining techniques can assist in examining the shape, size, and number of chromosomes, but it falls short of being able to differentiate between chromosomes that have similar physical characteristics (Shabir et al. 2017). In order to address the difficulty in distinguishing morphologically similar chromosomes, a number of chromosome banding techniques have been developed which offer a significant advantage for the identification of chromosomes and karyotyping (Andras et al. 2000). Chromosome staining with the combination of both chromomycin A3 (CMA) and 4',6-diamidino-2-phenylindole (DAPI) fluorochromes has been widely used as a method to distinguish chromosome bands (Guerra 2000). CMA and DAPI, due to their proclivity for binding to GC- and AT-rich sequences, respectively, allow for the discernment of various forms of heterochromatin as GC-abundant ($DAPI^{-ve}/CMA^{+ve}$), AT-abundant ($DAPI^{+ve}/CMA^{-ve}$), or AT/GC-balanced (DAPI neutral/CMA neutral) bands (Barros e Silva and Guerra 2010). Nucleolar organizer regions (NORs) are another excellent chromosome landmark effective in chromosomal characterization. The localization of NORs serves as a valuable marker for identifying chromosomes, offering a precise and dependable method of characterizing them (Maragheh et al. 2019). The presence and number of NORs in a cell can help distinguish between different types of chromosomes and provide important information for karyotyping.

Cytological studies using karyotype analysis have been conducted for an extended period in different species and cultivars of fenugreek, having somatic chromosome number $2n = 16$ (Table 1). The karyotype reports concludes that any of the available species of the Foenum-graecum section cannot be considered as the wild progenitor of fenugreek (Ladizinsky and Vosa 1986). The previous studies have been primarily limited to conventional karyotype analysis, with little emphasis placed on documenting and disseminating the findings (Table 1) (Agarwal and Gupta 1983; Bairiganjan and Patnaik 1989; Martin et al. 2011; Najafi et al. 2013). As far as our knowledge extends, the application of advanced differential chromosome banding techniques such as CMA and DAPI has not been previously employed in the study of *T. foenum-graecum*. In light of this deficiency, the present study aims to fill this gap by utilizing these advanced techniques, in conjunction with silver staining (AgNOR), to perform a comprehensive characterization of the chromosomal structure of this species. The comprehensive characterization of chromosomes plays a crucial role in breeding programs. This process provides important information that enables breeders to make informed mating decisions, leading to the production of offspring that possess both desirable traits and optimal health.

MATERIAL AND METHODS

Somatic chromosome preparation

Seeds of *T. foenum-graecum* have been collected from the cultivated fields of Sainthia, Birbhum (24°00'55.9"N 87°44'09.4"E) West Bengal. The growing roots from germinated seeds of *T. foenum-graecum* were taken for

Table 1. Previous chromosome reports in *Trigonella foenum-graecum.*

Sl. No.	Chromosome counts		Karyotype	Symmetry/Asymmetry	Reference
	Gametophytic (*n*) cells	Sporophytic (2*n*) cells			
1.	8	16	$1scA^{sm}+5A^{sm}+1B^{m}+1C^{sm}$	Asymmetrical	Agarwal and Gupta (1983)
2.	8	16	–	–	Laxmi et al. (1983)
3.	–	16	–	Asymmetrical	Ladizinsky and Vosa (1986)
4.	8	16	–	–	Arya et al. (1988)
5.	–	16	1m+5sm+2st	Asymmetrical	Bairiganjan and Patnaik (1989)
6.	–	16	$A_2B_{12}D_2$	–	Kar and Sen (1991)
7.	–	16	–	–	Jahan et al. (1994)
8.	–	16	–	–	Ahmed et al. (1999)
9.	–	16	–	–	Das et al. (2000)
10.	–	16	–	Symmetrical	Das et al. (2001)
11.	–	16	–	Symmetrical	Das et al. (2002)
12.	–	16	2m+6sm	–	Martin et al. (2011)
13.	–	16	$10sm + 4sm^{sat} + 2m$	–	Najafi et al. (2013)
14.	–	16	–	–	Ranjbar and Zahra (2016)

chromosome preparation. Chromosomes were prepared following Santra et al. (2020) with minor modifications. Roots were pretreated with 0.5 g L^{-1} 8-hydroxyquinoline solution at 16 °C for 6 h and then fixed in acetic acid:methanol solution (1:3) overnight. Digestion of the cell wall was performed with an enzyme mixture containing 1% cellulase (Onozuka-RS, Sigma, USA), 0.5% pectolyase (Sigma, USA), and 0.75% macerozyme (Serva, Germany) in a sodium citrate buffer (pH 4.6) at 37 °C for 90 mins. After washing with the same buffer twice, the root tip was broken down into small pieces on a clean slide with the addition of freshly prepared fixative. The slide was air-dried for at least 24 h before staining.

Giemsa staining

The chromosomes on the air-dried slide were firstly stained with 2% giemsa solution in phosphate buffer, with a ratio of 1:15 (pH 6.8), followed by rinsing with distilled water and analyzed under a microscope. Photomicrographs were taken with an AxioCam ICc 5 camera and ZEN application suite. Individual chromosomes were measured with AxioVision 4.9.1 and categorized based on the arm ratio following Levan et al. (1964).

CMA and DAPI double staining

Prior to simultaneous fluorochrome staining, with CMA and DAPI, the giemsa stained slides were destained with 70% methanol for 15 mins and air-dried. After preincubation of the slides in McIlvaine buffer (pH 7.0) for 10 mins, chromosomes were stained with 0.2 µg mL^{-1} DAPI solution for another 10 mins in the dark. After DAPI staining, slides were preincubated in McIlvaine buffer (pH 7.0) supplemented with 5 mM $MgCl_2$ and air dried. CMA staining was done with 0.25 mg mL^{-1} CMA solution for 60 min in the dark. After a short rinse in the same buffer, slides were mounted with 50% glycerol containing 5 mM $MgCl_2$ and kept at 4 °C for 72 hrs before further analysis. Chromosomes were analyzed under the fluorescent microscope Zeiss Axio Scope A1 equipped with CMA and DAPI-specific filter cassettes. AxioCam ICc 5 and ZEN application suite were used to take the suitable photomicrographs. The karyogram has been carried out using Adobe Photoshop CS6.

Silver staining

In this study, the AgNOR staining was performed using the Ag-I procedure by Bloom and Goodpasture (1976), with a modification introduced by Kodama et al. (1980) of using nylon cloth instead of coverslips. Silver nitrate solution was added to slides, placed in moisture-proof plastic containers and covered with nylon mesh. To keep the environment moist, distilled deionized water is placed at the bottom of the containers, away from the slides. The slides are left to incubate in the water bath for 48 h at 45 °C. The NOR region appeared as dark brown color bands over light brown chromosome arms.

RESULTS

In this analysis with *Trigonella foenum-graecum*, more than 40 root tips were initially studied through giemsa staining, which confirmed that the somatic cells of the present cultivar contain $2n$ =16 chromosomes (Fig. 1a). Additionally, differential chromosome banding with CMA, DAPI and AgNOR have also been performed in metaphase as well as in prometaphase chromosomes (Fig. 1b-f). The somatic chromosomes are small to medium in size and range between 4.70 to 5.92 µm. Individual chromosome sizes, arm ratio, and the centromeric index has been mentioned in Table 2. Analysis through detailed karyomorphological studies revealed two pairs with median (m) to nearly median primary constriction and six pairs of chromosomes having submedian (sm) primary constriction (Fig. 2a-d). Thus, the karyotype formula is 2m+6sm (Fig. 2d). Secondary constrictions are also present in the long arm of one pair of metacentric chromosomes (pair 1) and in the short arm of one pair of submetacentric chromosomes (pair 4) (Fig. 2b,c). The secondary constrictions are intercalary in position. The karyotype is symmetric and falls into 3A category of Stebbins's (1971) classification. Later, fluorochrome staining with CMA and DAPI, revealed all eight pairs of chromosomes with bright, distinct and scorable CMA^{+ve} bands, in their primary constriction (Fig. 2c). DAPI mostly stained the somatic metaphase chromosomes uniformly, however a single $DAPI^{+ve}$ band has been found in the chromosome pair 4 (Fig. 2b), in the intercalary position of short arm, colocalized with a CMA^{-ve} band. $DAPI^{-ve}$ bands have been detected to be colocalized with the CMA^{+ve} bands (Fig. 2b,c). Besides the single $DAPI^{+ve}$ band found in chromosome 4, several DAPI-brilliant regions were found in the prometaphase chromosomes (Fig. 1f), which also showed corresponding CMA^{-ve} bands (Fig. 1e). However, in condensed meta-

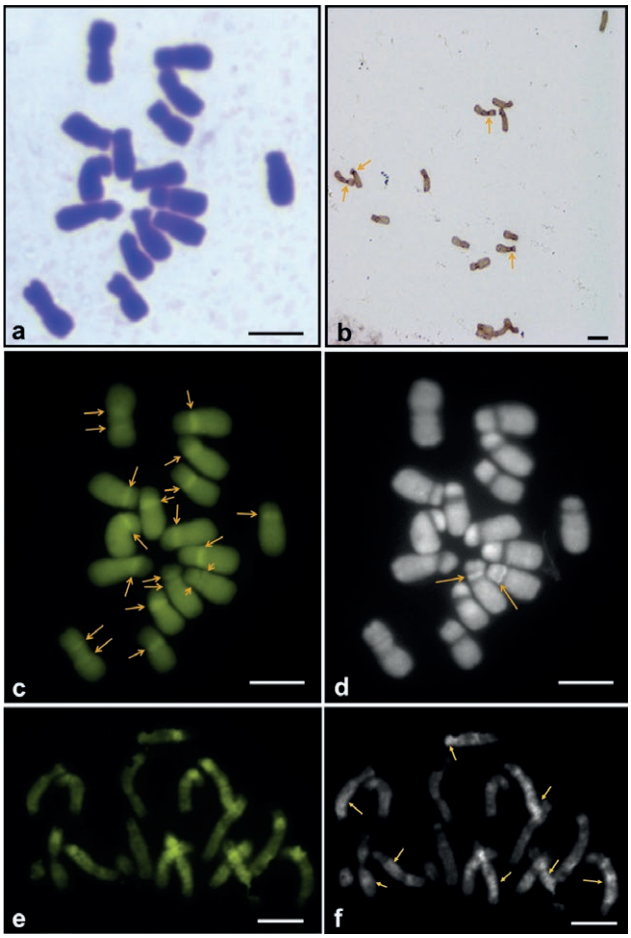

Figure 1. Differential chromosome banding in the somatic cells of *Trigonella foenum-graecum*. (a) giemsa stained metaphase plate; (b) silver staining (arrows indicate AgNOR bands); (c) CMA stained metaphase plate (arrows indicate CMA bands); (d) DAPI stained metaphase plate (arrow indicates DAPI bands); (e-f) CMA and DAPI stained prometaphase chromosomes. Scale bars of 5 µm.

Table 2. Chromosome parameters and banding patterns in *Trigonella foenum-graceum*.

Chromosome number	S (µm)	L (µm)	Total (µm)	Arm ratio	Centromeric index	Chromosome type*	CMA bands (+/-)	DAPI bands (+/-)	AgNOR bands (+/-)
1	2.575 ± 0.019	3.350 ± 0.043	5.925 ± 0.053	1.301	0.434599	m	+	-	+
2	1.789 ± 0.005	4.041 ± 0.005	5.830 ± 0.007	2.259	0.306872	sm	+	-	-
3	1.527 ± 0.016	3.961 ± 0.010	5.487 ± 0.008	2.595	0.278186	sm	+	-	-
4	1.886 ± 0.024	3.587 ± 0.007	5.473 ± 0.018	1.902	0.344541	sm	+/-	+/-	+
5	1.388 ± 0.014	3.526 ± 0.012	4.914 ± 0.003	2.542	0.282366	sm	+	-	-
6	1.231 ± 0.015	3.552 ± 0.018	4.783 ± 0.032	2.885	0.315542	sm	+	-	-
7	1.5 ± 0.002	3.253 ± 0.004	4.753 ± 0.005	2.169	0.406730	sm	+	-	-
8	1.912 ± 0.004	2.789 ± 0.002	4.701 ± 0.003	1.459	0.257422	m	+	-	-

*m = metacentric, sm = submetacentric. Total Chromatin Length (TCL) = 41.866 µm.

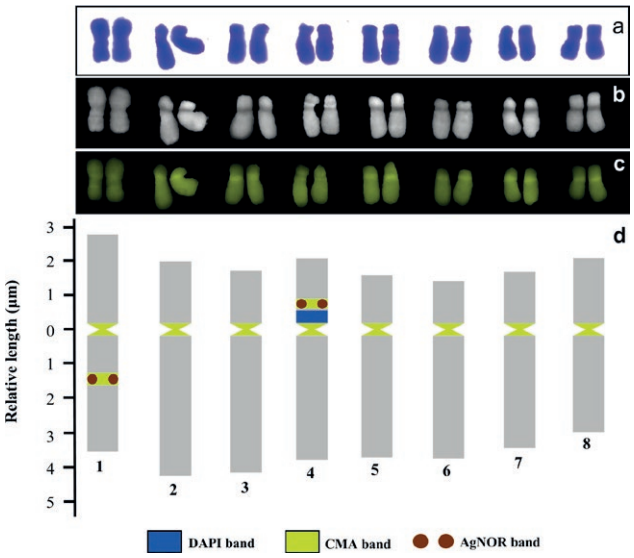

Figure 2. Karyogram and Idiogram representation of the somatic chromosomes of *Trigonella foenum-graecum.* (a) Stained with giemsa; (b) Stained with DAPI; (c) Stained with CMA; (d) Idiogram of the chromosomes along with the localization of different bands.

phase chromosomes, these regions are found to be either dispersed or not clearly visible. Lastly, AgNOR staining specifically stained intercalary positions of chromosomes 1 and 4 (Fig. 1b). Thus, *T. foenum-graecum* chromosomes can be identified and characterized based on the number and position of the CMA^{+ve}/ $DAPI^{-ve}$/ AgNOR bands (Fig. 2d).

DISCUSSION

According to Hutchinson (1964), the genus *Trigonella* is one of the six genera of the tribe Trifoliae and subtribe Trigonellinae. The genus *Trigonella* consists of approximately 134 species, which are found all over the world. These species can be diploid or polyploid, and there is evidence to suggest that their basic chromosome number could be x = 7, 8, or 9, as reported by different studies over the years. (Biddak 1996; Martin et al. 2011; Sharghi et al. 2020). The species *T. foenum-graecum* L. with basic chromosome number 8 ($2n$ = 16) comes under the section Foenum-graecum along with eight other species (Basu 2023). Karyotype studies, chromosome banding and Fluorescent In Situ Hybridization techniques have depicted finer variation in species and cultivars of *T. foenum-graecum* L. (Agarwal and Gupta 1983; Ahmed et al. 1999; Das et al. 2000). *T. foenum-graecum,* in the present study shows $2n$ = 16 chromosomes in the somatic cell with the basic chromosome number x = 8 (Fig. 2d). The present study revealed the size of the somatic chromosomes was within a moderate range, ranging from 4.70-5.92 µm (Table 2). The karyotype formula, which is used to describe the number and appearance of chromosomes in a cell, was determined to be 2m+6sm. These findings were consistent with previous studies, indicating a similarity in the chromosome size and formula between the present investigation and prior research (Martin et al. 2011). The process of enzymatic maceration of plant cells helps to prepare the chromosomes in a way that enables clear and unobstructed visualization during cytological analysis. The use of fluorescent banding techniques with CMA and DAPI, has significantly advanced the field of plant cytogenetics by identifying GC- and AT-rich constitutive heterochromatin regions on chromosomes, leading to increased knowledge and advancements in plant chromosome research (Schweizer 1976; Yamamoto 2012). The current study represents the first documented use of a double staining approach combining CMA and DAPI on chromosomes in *T. foenum-graecum* to date, producing a clear and easily distinguishable banding pattern, marking the first recorded instance of fluorochrome banding in this species based on our current knowledge. The centromeres, along with secondary constrictions, were reliably designated as CMA^{+ve} and were also correlated with $DAPI^{-ve}$ bands. This establishes that the centromere region has a high concentration of GC nucleotides. A thorough examination of several species unveiled that the DNA found in centromeres can possess a substantial richness of GC nucleotides. While some animal species exhibit, a predilection for AT-rich tandem repeats, no such tendency was apparent in the plant kingdom (Melters et al. 2013). The detection of CMA^{+ve} centromeric heterochromatin in *Crotalaria*, a member of the Fabaceae family, implies the existence of GC-rich DNA repeat units at the centromere (Mondin and Aguiar-Perecin 2011). In most species, the rDNA sites exhibit a positive stain, when subjected to CMA staining and a negative stain when treated with DAPI. These sites are frequently the sole regions displaying positive CMA staining (de Melo and Guerra 2003). A common characteristic of plants is the association of GC-rich regions with 35S rDNA sites, resulting in the generation of CMA^{+ve} bands in the NOR (Marcon et al. 2005; Dydak et al. 2009; Kolano et al. 2013). The rDNA sites are generally positively stained with CMA and negatively stained with DAPI. In many species, the rDNA sites are the only regions that are positively stained with CMA. In one pair of chromosomes, positive bands detected through DAPI staining have been identified in the region between the primary and secondary constrictions (Fig. 2b). During prometaphase, when the chromatins are less compact,

distinct signals were observed through DAPI staining. This has been documented in several plant species, and the observation that the DAPI signal is only present during prometaphase and disappears during metaphase suggests that it is not a manifestation of heterochromatin, but instead an early stage of chromatin condensation (Berjano et al. 2009; Santra et al. 2021). The use of silver nitrate staining enables the recognition of ribosomal DNA (rDNA) sites that were transcribing during the preceding interphase of the cell cycle, as visualized in the metaphase stage (Jiménez et al. 1988). In *T. foenum-graecum*, two pairs of chromosomes have been observed with AgNOR bands at secondary constrictions corresponding to the CMA^{+ve} bands. In this species, previous studies have documented information about the count and placement of the AgNOR bands, which are in agreement with the results of the current research (Ahmad et al. 1999). The authors also hypothesized that the origin of the two satellite chromosome pairs in fenugreek remains unclear, but it may stem from the hybridization of two distinct species or cytotypes. The localization of AgNOR, CMA, and DAPI bands appear to be valuable cytological markers, which have enabled us to distinguish and identify the chromosomes in *T. foenum-graecum*. The standardized techniques of EMA, Giemsa staining, silver staining and fluorochrome banding are considered to be reliable and reproducible. The results of this study hold great significance in understanding the genetic makeup of fenugreek. The study offers critical knowledge on the characterization and preservation of this neglected crop and its diversity, leading to an enrichment of its improvement program. This is vital for maintaining the sustainability of food production and the environment's well-being.

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