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Caffeine genotoxicity in the root meristem of *Anethum graveolens* L. (Dill) and *Foeniculum vulgare* Mill. (Fennel)

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Abstract
In the field of plant genetics and breeding research, mutagenesis is a stepping stone for inducing novel genetic variations in existing genotypes of crop plants. Due to the widespread consumption of tea and coffee, the majority of people are now exposed to varying levels of caffeine.The purpose of this study is to see how increasing caffeine concentrations, such as 0.5%, 1.0%, 1.5%, and 2.0%, affected chromosomal organisation in two plants: *Anethum graveolens* L. and *Foeniculum vulgare* Mill. In case of *Anethum graveolens* L., higher caffeine concentrations were found to be more chemotoxic than on *Foeniculum vulgare* Mill. In both plants, there were a significant decline in AMI % (Active mitotic index) and an increment in Total abnormality percentage (TAB%), with the increasing concentration of caffeine. Various Chromosomal aberrations like stickiness, precocious movement, laggard and unorientation were found during this experiment in which stickiness wasthe predominant abnormality in both the plants.

Keywords: active mitotic index, *Anethum graveolens* L., caffeine, *Foeniculum vulgare* mill., total abnormality percentage

Introduction

India is known around the world as the "Land of Spices". India is the best-suited country in the world for spice cultivation, production, and export from a wide range of plant species, and it dominates the global market because most countries regard it as a reliable source. Seed spices have a charismatic charm among spices due to their varietal applications [1]. Since ancient times, we have been cultivating these valuable spices to satisfy our various needs in which *Anethum graveolens* L. and *Foeniculum vulgare* Mill. are important seed spices and belong to the Apiaceae family. These seed spices were used by our forefathers to enhance the taste and flavour of food and beverages. It has been used to treat a variety of ailments, as evidenced by our old literature. *Anethum graveolens* (2n =20) is a medicinal, aromatic and spice herb commonly known as Dill and the leaves oftheplant are used as a spice and a flavouring agent in culinary preparations.Seed and herb of Dill plant contain an essential oil which isusedin folkloric medicine [2, 3]. *Anethum graveolens* seed essential oils are potent antioxidant sources, as well as antibacterial and antitumor properties [4]. Dill contains carvone, one of the most important constituents with antimicrobial activity [5].

Fennel, or *Foeniculum vulgare* Mill. (2n=22), is a well-known and important medicinal and aromatic plant that is widely used as a carminative, digestive, lactogogue, and diuretic, as well as in the treatment of respiratory and gastrointestinal disorders. Its seeds are used in baked goods, alcoholic beverages, and herb mixtures as flavourings. Fennel is used as an antispasmodic, diuretic, anti-inflammatory, and antioxidant cure [6]. It is also used as a flavouring agent in bread, cheese pharmaceuticals, and nutraceuticals. The major phytoconstituents of these spices have been identified as phenols, phenolic glycosides, and volatile aroma compounds such as trans-anethole, estragole, and fenchone.

Due to the high economic value of Fennel and Dill Plants, it is necessary to improve genotypic and phenotypic diversity, to optimise some desirable traits, and to obtain new valuable genotypes through a variety of methods, including chemical mutagenesis.In the case of chemical mutagens, certain chemical compounds induce genetic changes in DNA structure, affecting one or more genes. These chemical mutagens are present in both natural and man-made environments and products. Caffeine is one of the chemical mutagens which is found in tea and coffee both, so maximum people are exposed to different doses of caffeine in daily life. It acts as a stimulant for the central nervous, respiratory and cardiac systems [7]. Caffeine (1, 3, 7 trimethylxanthine) is a purine alkaloid from methyl xanthine chemical group, which shows low toxicity to humans as compared to another chemical mutagen.

Caffeine is a base analogue of adenine that can be incorporated into a growing DNA chain instead of adenine, causing altered base pairings and structural changes that affect DNA replication and gene transcription [8]. The mitotic study is of great significance because the root tips are often the first to be exposed to chemicals spread in nature, in the soil and in water [9]. Henceforth assessment constituting mitotic study shall assist in obtaining insight to the cytogenetic level. The present study has been conducted to reveal the comparative toxic impact of

Caffeine on the root meristems of *Anethum graveolens* L. and *Foeniculum vulgare* Mill. for examining the effect of this mutagen on the mitotic stages and their chromosomal architecture.

Materials and Methods

(a) Procurement of Seeds: The National Research Centre on Seed Spices (ICAR-NRCSS) in Ajmer, Rajasthan, provided Dill (*Anethum graveolens* L.) and Fennel (*Foeniculum vulgare* Mill.) germplasm.

(b) Seed Treatment: To make the seed coat permeable for chemical treatment, Dill and Fennel seeds were pre-soaked in distilled water for 24 hours. For three hours, the pre-soaked seeds were exposed to four different caffeine concentrations. This experiment used four different caffeine concentrations: 0.10 %, 0.25 %, 0.50 %, and 1 %. The seeds were then thoroughly rinsed under running tap water. For the mitotic study, treated seeds were kept in a seed germinator at 25^0C in a Petri plate using wet Whatman paper after a 3-hour recovery period. The germinated seeds, along with the control, were fixed in Carnoy fixative (Glacial acetic acid: Alcohol- 1:3) in their labelled bottles after seed germination. The germinated seeds were transferred into 90% alcohol after 24 hours**.**

(c) Cytological observation- Root tips were hydrolyzed in 1NHCl for cytological observation, then washed under running water to remove excess HCl. Due to hydrolysis, the root tips were softened and then hydrolyzed root tips were stained with 2% acetocarmine for 30 minutes. After that slides were prepared using the squash technique for studying mitotic frequencies. Slides were observed under Nikon Electron Microscope while photographs were clicked using the software PCTV Vision.

Data were prepared in replicates of nearly 5 microscopic field views of each slide.

Active Mitotic index and Total abnormality formula were calculated according to Edgar (1961) and Balong (1982).

Active Mitotic index (AMI) % = (Total number of dividing cells/Total number of observed cells)*100

Total abnormality percentage (TAB) % = (Total number of abnormal cells/Total number of observed cells)*100

Results

Cytological studies of treated sets provide information regarding the response of Dill as well as Fennel genotypes to a chemical mutagen and also provide greater chances for the selection of desired traits. The cytological observation of caffeine shows an impact on the cytology of root meristems in both plants (*Anethum graveolens* L. and *Foeniculum vulgare* Mill). Different concentration of caffeine causes a significant change to the chromosomal morphology and mitotic index. Table 1 represents complete comparative data of AMI% and TAB% in these two plants.

Mitosis was found to be normal in the control sets and showed regular arrangements of chromosomes at metaphase (2n=20 in *Anethum graveolens* and 2n=22 in *Foeniculum vulgare*) and having equal separation (20:20 and 22:22, respectively) at anaphase. However, various chromosomal abnormalities were recorded in root meristems of treated sets. A highly significant decrease in AMI (%) as compared to the control was evident at all the concentrations of caffeine. The reduction rate was higher in the case of *Anethum graveolens*.

Comparatively, in these two plants, the AMI% is higher in *Foeniculum vulgare* Mill. rather than the *Anethum graveolens* L. AMI% declined from 12.81 ± 0.05 (control) to 11.75± (0.1%) and 9.18 (0.5%), upto 8.97 (1.0%) in the case of *Foeniculum vulgare*, whereas in the case of *Anethum graveolens* the reduction was up to 8.4 (1.0%). Concerning the behaviour of the Active mitotic index the two examined species displayed different profiles of this cytogenetic parameter.

The rate of chromosomal aberrations was documented in the form of Total Abnormality Percentage (TAB %) in Table 1. It was increased from 2.82 to 6.38 in *Anethum graveolens* and 2.38 to 5.48 in *Foeniculum vulgare* with respect to concentration (0.10%-.1.0%). From the result, it was deduced that higher doses are more mitotoxic and impose more aberration in treated sets as compared to lower doses and control. Chromosomal Abnormality (TAB %) were recorded higher in the case of *Anethum graveolens* as compared to *Foeniculum vulgare* rather than both plants are seed spices and belonging to the Apiaceae family. In the case of lower concentration, caffeine does not have much more mito-inhibitory effect on these plants. The AMI and TAB% show an inverse relationship in a dose-dependent manner.

Some of the chromosomal abnormalities Figure 2 and 3 recorded are, scattering, stickiness, precocious movement, unorientation at metaphase and stickiness, bridge formation, laggard movement at anaphase etc. It was examined that the majority portion of chromosomal abnormalities occupied by stickness at anaphase in the case of *Anethum graveolens* L. while stickiness at metaphase was the most dominant anomaly in *Foeniculum vulgare* Mill. some other kinds of a chromosomal abnormality like scattering, precocious movement, unorientation and laggard formation at anaphase were recorded at higher in the case of *Anethum graveolens* while in *Foeniculum vulgare*, laggards, unorientation, bridge formation were recorded at higher concentration. For the above parameters, it was concluded that the more abnormalities at metaphase rather than anaphase in case of Dill and reversible result seen in Fennel.

This result also deduced that comparative metaphasic and anaphasic abnormalities were higher in *Anethum graveolens* as compared to in *Foeniculum vulgare* as mentioned in table 1.

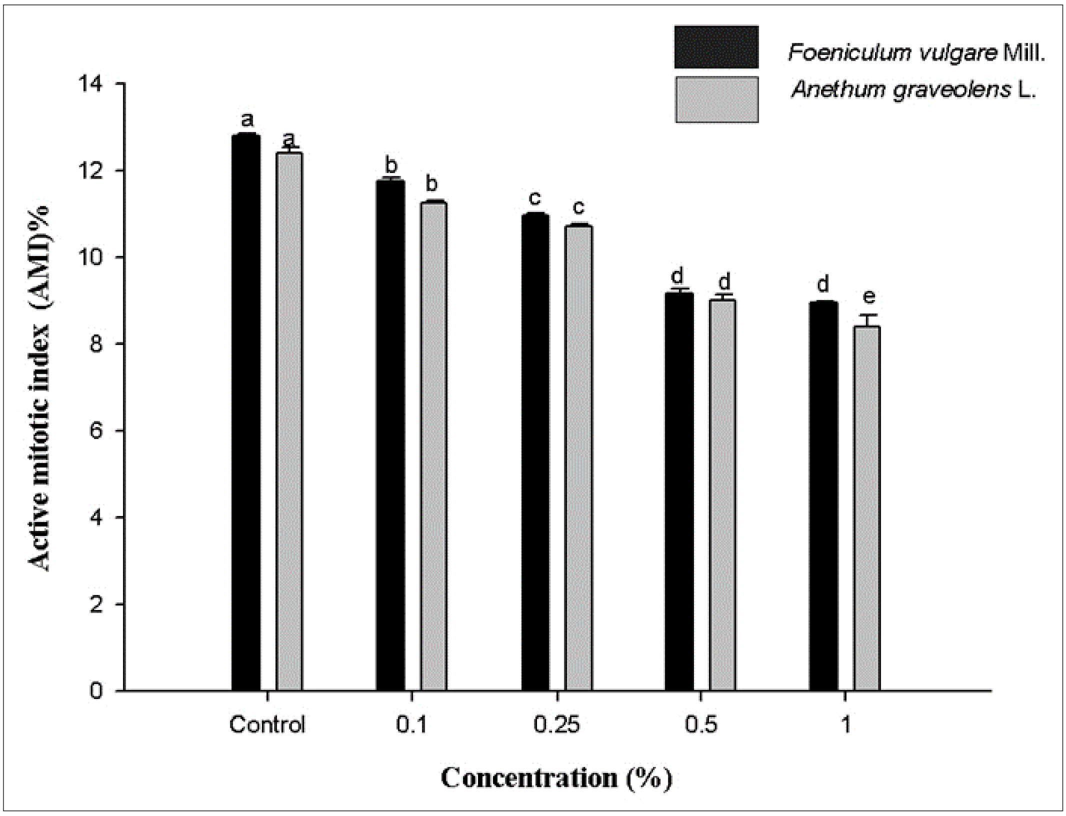

Fig 1: Comparative account of AMI % after caffeine treatment in *Foeniculum vulgare* Mill. And *Anethum graveolens* L.

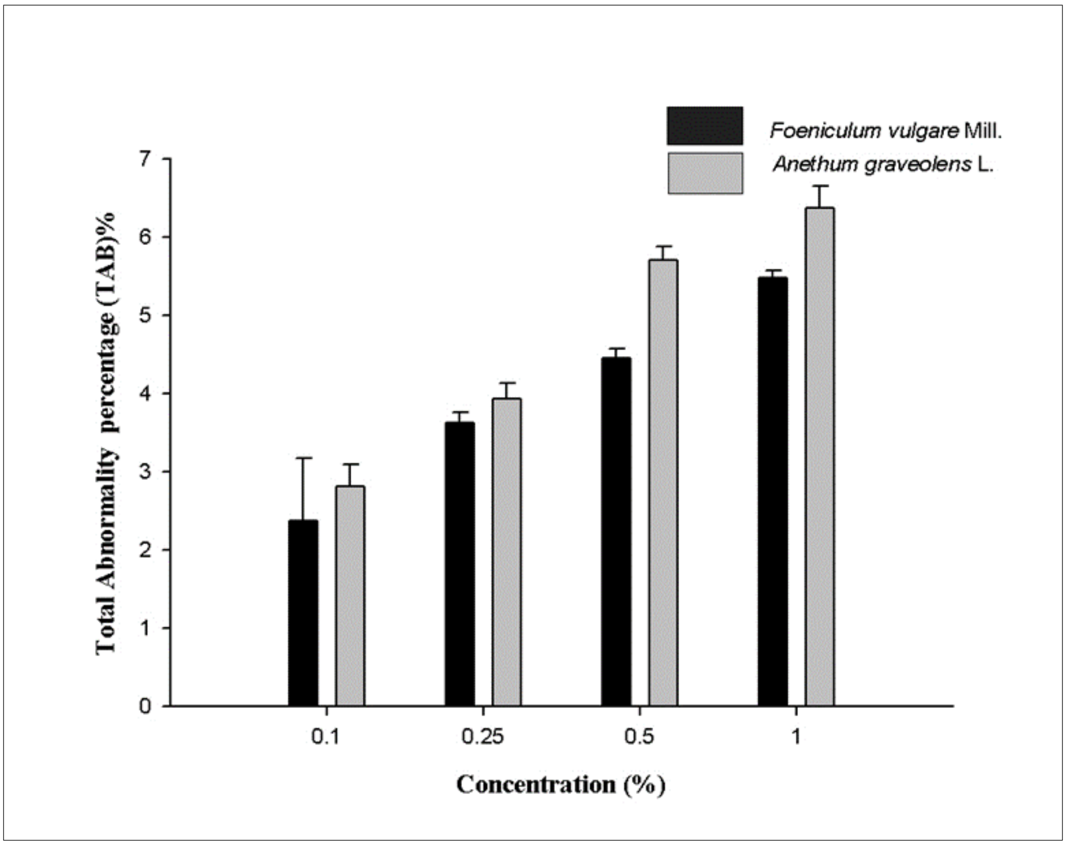

Fig 2: Comparative account of TAB % after caffeine treatment in *Foeniculum vulgare* Mill. And *Anethum graveolens* L.

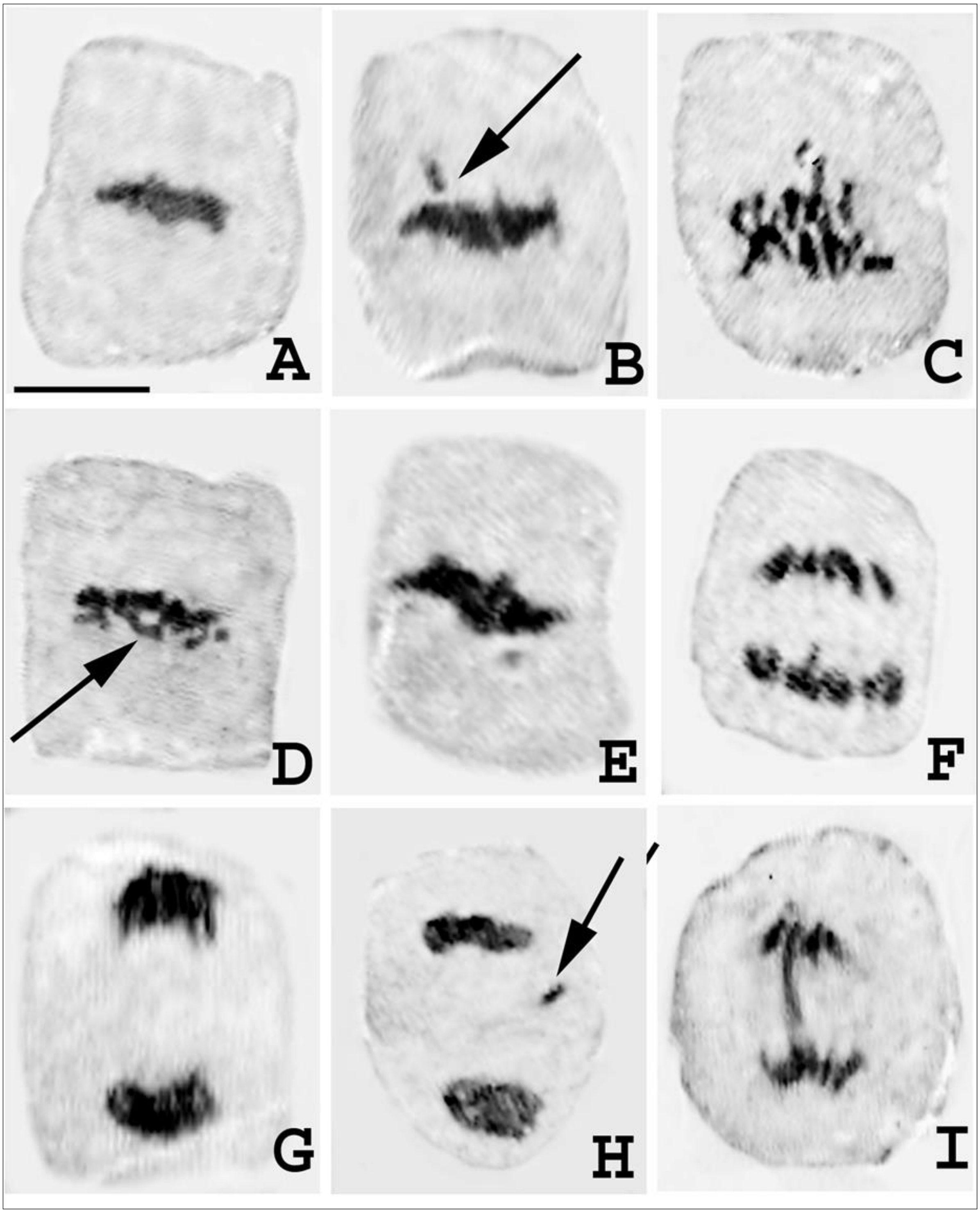

Fig 3: Mitotic Abnormalities of *Anethum graveolens* L. induced by Caffeine treatment-(A)Normal Metaphase(2n= 20), (B) Precocious movement at Metaphase, (C) Precocious with scattering at Metaphase, (D) Loop formation at Metaphase, (E) Unorientation at metaphase, (F) Normal Anaphase (20:20), (G) Stickiness at Anaphase, (H) Laggard formation at Anaphase, (I) Bridge Formation at Anaphase [Scale bar: 6.18μm]

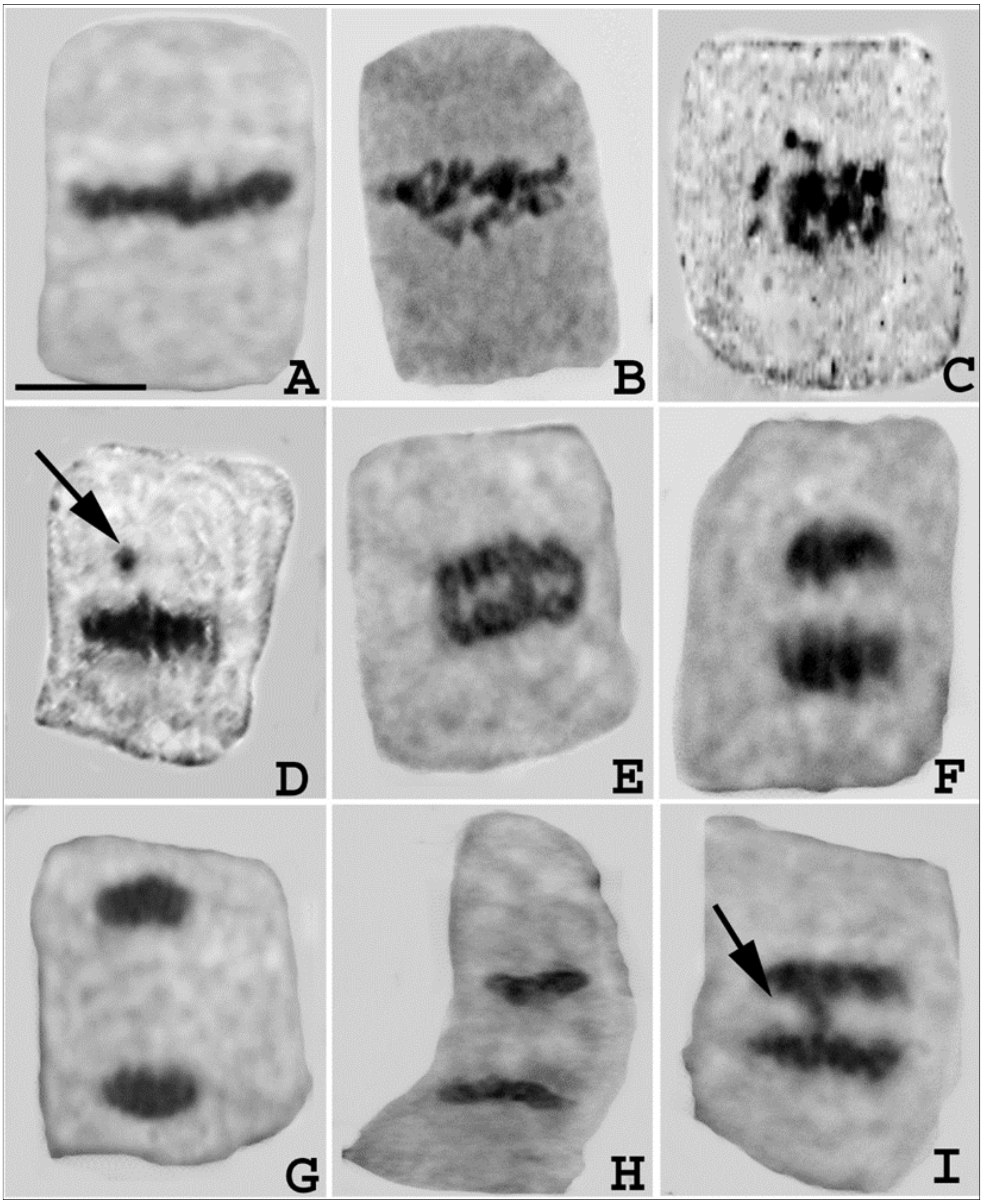

Fig 4: Mitotic Abnormalities of *Foeniculum vulgare* Mill. induced by Caffeine treatment-(A)Normal Metaphase(2n= 22), (B) Scatteringat Metaphase, (C) Precocious with scattering at Metaphase, (D) Precocious movement at Metaphase, (E) Early Anaphase, (F) Normal Anaphase (22:22), (G) Stickiness at Anaphase, (H) Unorientation at anaphase (I) Bridge Formation at anaphase [Scale bar: 6.28μm].

Table 1: Effect of caffeine on the total abnormality percentage (TAB %) in *Feoniculum vulgare* Mill. And *Anethum graveolens* L. respectively.

Treatment	**Plants**	**Cocentration**	**AMI (%) (Mean ± SE)**	**Metaphasic abnormalities % (Mean ± SE)**				**Anaphasic abnormalities % (Mean ± SE)**					**Oth % (Mean ± SE)**	**TAB (%) (Mean ± SE)**
				Sc	**St**	**Un**	**pr**	**Sc**	**St**	**Lg**	**Br**	**un**		
Caffeine	*Foeniculum vulgare* Mill.	Control	12.81 ± 0.05[a]	-	-	-	-	-	-	-	-	-	-	-
		0.10	11.75 ± 0.10[b]	0.16±0.08	0.17±0.08	0.25±0.00	0.25±0.00	0.08±0.08	0.25±0.00	0.25±0.00[a]	0.16±0.08	0.33±0.07	0.17±0.08	2.38 ± 0.8
		0.25	10.97 ± 0.05[c]	0.32±0.16	0.42±0.08	0.16±0.16	0.08±0.00	0.42±0.08	0.50±0.01	0.26±0.15[a]	0.50±0.01[a]	0.42±0.08	0.50±0.01	3.63 ±0.13
		0.50	9.18 ±	0.57±0.	0.28±0.	0.85±0.	0.28±0.	-	0.17±0.	0.85±0.	0.46±0.	-	0.85±0.	4.45 ±

			0.11^{d}	28	28	04	28		17	04^{a}	23^{a}		04	0.12
		1.00	8.97 ± 0.02^{d}	0.27±0.27	1.22±0.25	0.83±0.49	0.66±0.34	0.67±0.34	-	0.56±0.56	0.66±0.34	0.53±0.26	0.83±0.49	5.48 ±0.09
	Anethum graveolens L.	Control	12.39±0.15	-	-	-	-	-	-	-	-	-	-	-
		0.10	11.25±0.06	0.52±0.09	0.42±0.11	0.11±0.10	0.20±0.10	0.43±0.11	0.53±0.12	-	0.21±0.10	0.10±0.10	0.20±0.10	2.82±0.28
		0.25	10.71±0.07	0.55±0.05	0.44±0.06	0.35±0.05	0.25±0.13	0.44±0.06	0.55±0.05	0.35±0.05	0.35±0.05	0.45±0.06	0.20±0.10	3.93±0.20
		0.50	9.61±0.14	0.78±0.03	0.79±0.03	0.39±0.01	0.12±0.11	1.17±0.05	1.06±0.17	0.25±0.12	0.53±0.15	0.37±0.20	0.25±0.12	5.71±0.17
		1.00	8.4±0.26	1.05±0.04	1.05±0.045	0.64±0.03	0.32±0.01	1.16±0.09	1.07±0.13	0.32±0.01	0.32±0.01	0.32±0.01	0.09±0.09	6.38±0.28

Where

Sc- Scattering, Pr- Precocious movement, St- Stickiness, Un- Un-orientation, Br- Bridge formation, Lg- Laggard formation,

Oth-Others

Means followed by lowercase letter are statistically significant at $p < 0.05$ in Duncan's Multiple Range Test

Discussion

Mutagenic effects of caffeine were studied in prokaryotic as well as eukaryotic organisms, with variable results. In several cases, it was considered as a DNA repair inhibitor [10]. The researchers tested the mutagenic activity of caffeine and other purine analogues, in various biological systems, but the conclusions were not identical. Because of the lack of some identical or similar results on the mutagenic effect of caffeine in different biological systems, the mutagenic potential of caffeine and certain phenoxy-methyl-xanthine compounds is controversial and discussible. Caffeine, as well as other purine derivatives, can be incorporated into the DNA macromolecule. In the next DNA replication, the initial nitrogenous base will be changed by a base analogue, because of some incorporation errors. To be mutagens and, therefore, incorporated in DNA macromolecule, it is required to action during DNA active synthesis. Caffeine is a "base analogue" of adenine, and in fact can sometimes be incorporated into a growing DNA chain, instead of adenine. Caffeine is considered by some authors as a weak mutagen, for this reason. Itoyama & Bicudo (2000) [10] sustained that this compound has an inhibitive effect on DNA repair, stopping the repair of cytogenetic lesions,

Formation of lagging chromosomes may occur due to disfunctioning of spindle fibre, as a result spindle fibres fail to carry the respective chromosomes to the polar region [11]. It was also reported that lagging chromosomes occur because of improper movement of chromosomes during anaphase separation [12]. Precocious movement of chromosomes was also observed at each concentration of treatment but its percentage was increased with increasing the concentration. It might have been caused by the early terminalization and, stickiness of chromosomes [13]. Kumar and Rai (2007) [14] reported that unorientation at metaphase and scattering of chromosomes may be due to destruction of spindle formed.

Stickiness has been attributed to the entanglement of chromosomes which leads to inactivation of DNA replicating protein [15], but Patil and Bhat (1992) [16] explained it as a type of physical adhesion cellular matrix and chromosomal material both are involved and leads to stickiness. According to Liu. *et al* 2015 [17], the chromosome bridge might have resulted due to the treatment of caffeine, DNA breaks form, and then sides of breaks of chromosomes healed and formation of double centromere chromosome takes place, i.e. Chromosomal bridges such chromosomal abnormalities may affect adversely the vigour, fertility and yield of the exposed plant [18].

Conclusion

Concerning the influence on mitotic division, caffeine had more pronounced effects in *Anethum graveolens* as comparison to *Foeniculum vulgare* Mill. At low concentrations, it was stimulant, while at high concentrations, it shows inhibitory effect. From the above study it may concluded that caffeine have potential to causes the cytological variations in these tested biological material It induced important variations at a genetic level. This type of studies are very helpful to check the hazardous effect of chemical mutagen and also calculate its consequences on biological system. This study could be beneficial to analyzing the threshold level of chemical mutagen. Caffeine in its higher doses may be inhibitory cum toxic for plant growth and yield but further research is needed to meiotic level to confirm this fact

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Conflict of interest statement

There are no conflicts of interest declared by the authors.

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