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RESEARCH ARTICLE

ASSESSMENT OF GENETIC DIVERSITY AMONG THE THREE UNIQUE AND POTENTIAL GENOTYPES OF *PIPER NIGRUM* - 'PMM', 'PAJ' AND CV. 'KARIMUNDA' USING RAPD MARKERS

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SUMMARY RAPD marker-based genetic diversity analysis was carried out on three unique and potential genotypes of *Piper nigrum* L. (Black pepper) – 'PMM', 'PAJ' and cv. 'Karimunda'. Ten primers selected randomly for the study generated a total of 79 bands, of which 46 were polymorphic (58.75%). This points to the existence of remarkable overall genetic diversity among the three genotypes. The estimates of Jaccard's similarity coefficients between pairs of genotypes revealed their genetic interrelationships. The genotypes, 'PMM' and 'PAJ' possessed the highest Jaccard's similarity index and revealed that they are the most closely allied genotypes, whereas 'PAJ' and cv. 'Karimunda' owned the least similarity coefficient value and therefore, they are the most distantly related ones, among the three pairs of genotypes. Cluster analysis of the three genotypes based on pair-wise Jaccard's similarity coefficient of the three genotypes grouped them in two divergent clusters. The Cluster-I, a single member cluster included cv. 'Karimunda', and the Cluster-II incorporated two genotypes, 'PMM' and 'PAJ'. Hence, it may be inferred that hybridisation involving the genotypes, 'PMM' as well as the probable double haploid of 'PAJ' as one parent (since 'PAJ' is a haploid genotype, not flowered/fruited even after attaining reproductive maturity) and cv. 'Karimunda' as the other parent, aiming at exploitation of hybrid vigour for crop improvement seems worthwhile.

Keywords: *Piper nigrum*, RAPD, genetic diversity, genetic interrelationship, hybrid vigour.

INTRODUCTION

Piper nigrum L. is one of the most important spice crops of the world, and its dried fruits – Black pepper is much admired as 'King of Spices'. In addition to its wide acclaim as an indispensable culinary spice the world over, Black pepper is also well known for its therapeutic applications. It is an ingredient of a number of formulations of Indian traditional systems of medicine such as Ayurveda, Siddha and Unani. In these treatment regimens, Black pepper is used as a curative and preventive drug against diverse disease conditions. The alkaloid piperine and the essential oil, mainly present in the fruits of *P. nigrum* are the major bioactive principles of the species, which are responsible for its therapeutic properties. Piperine has been demonstrated in in vitro studies as an agent for providing protection

against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Srinivasan 2007). More importantly, piperine is found to have the property of enhancing bioavailability of bioactive molecules of therapeutic drugs (Atal 1979, Atal et al. 1985). The studies also showed that piperine is useful as a Multi Drug Resistant (MDR) reversal agent by potentiating the efficacy of chemotherapy by multiple mechanisms, suggesting that it may be a lead compound for future investigations (Li et al. 2011).

Until a few decades ago, India was the leading producer, exporter and consumer of Black pepper. But, the country lost its claim during 1990s, when Vietnam became the largest producer of Black pepper in the world. Today, in India, productivity of the crop is decreasing day by day, and the current average yield of Black pepper in the country is ca. 321 kg/ha, whereas in other countries such as Indonesia, Malaysia and Thailand the average yield is 2000–3000 kg/ha. The major causative factors for low productivity of the crop in the country are diverse, which include the prevailing practice of unorganised cultivation of the crop, cultivation of low yielding varieties and susceptibility of cultivars to drought, pest and diseases etc. Therefore, for overcoming the present day set-back of decreased yield of Black pepper in the country, breeding and popularisation of high yielding, drought and disease resistant cultivars are the key needs of the time.

The Western Ghats is considered to be the centre of origin of *P. nigrum* (Rahiman 1987) and hence, the region possesses high genetic diversity of the species. Sen et al. (2019) provided molecular evidence for the proposition that origin of *P. nigrum* is within the wet evergreen forests of the Western Ghats. The presence of remarkable genetic diversity of *P. nigrum* in the Kerala sector of the Western Ghats is well evident with the prevalence of a number of traditional cultivars (>100) in the farmers' fields in Kerala and numerous wild genotypes of the species in the forests of the region. But, today, the genetic resources of the crop are under threat of extinction due to diverse causative factors.

The fruits and roots of different species of *Piper*, especially the wild *P. nigrum* occurring in the forests of the Western Ghats have high demand in the pharmaceutical industry of the indigenous systems of medicine in the country and elsewhere. These raw drugs are collected from the forests as NWFP (Non Wood Forest Produce) items, and associated with their procurement, over exploitation and destructive harvesting of the resource are reported to occur in the forests of the Western Ghats. Degradation and destruction of habitats of the species is another causative factor resulting in depletion of populations of the species leading to gene erosion. Monocropping of high yielding/hybrid varieties and elimination of landraces and traditional cultivars from farmers' fields is yet another reason for depletion of genetic resource of the crop. The genetic entities of a species once lost is a loss forever, and therefore, extinction of genotypes is an irreversible set-back to the richness of its germplasm. In spite of the severity of the problem, in depth studies on genotypes of *P. nigrum* aiming at their conservation and effective utilisation for crop improvement are still lacking.

In this backdrop, it is interesting to note that Rameshkumar et al. (2008) discovered a unique,

lemon-scented genotype of Black pepper 'PMM' (*P. nigrum* L. 'PMM') from the forests of the Western Ghats, which may be a product of transgressive segregation as part of evolutionary advancements in the species. This genotype of *P. nigrum* possesses high percentage content of piperine in its fruits (9–10%), which is almost double the average content of piperine present in common cultivars of Black pepper. Subsequently, Mathew et al. (2016) developed a haploid genotype of 'PMM' namely, 'PAJ' (2n = 26), which is the first reported haploid Black pepper, and since then, no haploid Black pepper has been reported hitherto. The development of the haploid genotype 'PAJ' assumes significance on considering the potential of haploid breeding in Black pepper, which can lead to the development of homozygous lines of doubled haploids in the crop. This would enable the breeders to overcome the inherent heterozygous nature of the crop, which is considered to be the major bottleneck in the path of development of high yielding cultivars of Black pepper, through exploiting hybrid vigour. The third genotype, cv. 'Karimunda' is a high yielding, popular traditional cultivarof Kerala, which possesses ideal agromorphological features, enabling easy maintenance of the plants of the cultivar by farmers in their fields on relatively small-sized living standards.

DNA sequences of organisms are the fundamental units determining their genetic diversity. Hence, RAPD marker techniques which are used to evaluate DNA polymorphism are beneficial for assessing genetic diversity and interrelationships between and within species. (Chakravarthy & Naravaneni 2006, Hoshino et al. 2012).

The present investigation was aimed at RAPD-based assessment of genetic diversity among the three unique and potential genotypes of *P. nigrum*, 'PMM', 'PAJ' and cv. 'Karimunda' and elucidation of their interrelationships. The information generated through the study would be beneficial for conservation and effective utilisation of the three genotypes in general, and for planning hybridisation programmes in particular, involving them as parents/parent stock for exploiting hybrid vigour.

MATERIALS AND METHODS

Plant materials and DNA extraction

Leaf samples of the three genotypes of *P. nigrum* such as 'PMM', 'PAJ' and cv. 'Karimunda' collected from the plants maintained in the Field Gene Bank of Black pepper at the Department of Botany, University of Kerala were subjected to RAPD marker studies. For isolating pure genomic DNA from the leaf samples, Dellaporta method proposed by Dellaporta et al. (1983) was employed with minor modifications.1g leaf tissue was ground to fine powder in liquid nitrogen, followed by addition of 1 ml of 20% SDS and 15 ml of extraction buffer (0.5M EDTA - pH 8.0, 1M Tris - pH 8.0, 4M NaCl, β -Mercaptoethanol, DD H_2O). After vigorous shaking, 15 µl of RNase (20 µg/ml) was added and incubated the solution for 10 min in a waterbath. After incubation, pipetted 5 ml KOAC (5 M) solution to the samples. Mixed the solution vigorously and incubated at 0° C for 30 min and carried out spinning of the tubes at 18000 rpm at 4° C for 15 min. Poured the supernatant into 15 ml centrifuge tubes and added an equal amount of 10 mM isopropyl alcohol, mixed well and incubated at 0° C for 30 min and spun down at 18500 rpm for 25 min. Gently poured off the supernatant and dried the pellets by inversely placing the centrifuge tubes on tissue

paper towels in a laminar airflow hood. Redissolved the pellets in 0.7 ml of extraction buffer (0.5M EDTA - pH 8.0, 1M Tris - pH 8.0, DD H_2O) and transferred into 1.7 ml Eppendorf tubes and added 5 µl of Proteinase K. Spun down the tubes at maximum rpm for 15 min to remove impurities. Transferred the supernatant of the tubes to other tubes containing 75 µl of NaOAC and 500 µl of isopropyl alcohol, mixed well by inverting the centrifuge tubes containing the pellet DNA and centrifuged for 2 min. Washed the DNA pellets with 500 µl of 80% ethanol, dried thoroughly and dissolved in 100 µl of TE buffer. DNA was quantified using NanoDrop Biophotometer (Thermo Scientific, NanoDrop One). The purity of isolated DNA was confirmed by 1% agarose gel electrophoresis.

PCR amplification and estimation of percentage of polymorphism

Reaction master mix composition and temperature conditions were standardized for RAPD analysis adopted for PCR amplification. 15 µl reaction mixture (comprising 7.5 µl PCR mixture (TaKaRa), 1 µl RAPD primer, 1 µl isolated DNA and 5.5 µl DD H_2O) containing 24 ng of genomic DNA was used for carrying out the PCR reaction. A total of 12 universal RAPD primers (Operon Technologies Inc. Alameda, CA, USA) were selected randomly and tested for amplification and polymorphism. Polymerase chain reaction was carried out in a thermal cycler (BIORAD T100). An ideal amplification protocol was developed with an initial denaturation at 95° C for 3 min, and a second denaturation at 94° C for 30 sec. Annealing time given was 1 min at a standardized temperature for each primer, which ranged between 33.4–41.1^0 C. Amplification reactions were cycled 40 times at 72^{o}C for 3 min. The PCR products were then subjected to agarose gel (1.8%) electrophoresis. Out of the 12 random decamer primers tested for PCR amplification, the 10 primers which provided satisfactory results were employed for detailed analysis (Table 1). These primers were selected based on the criteria such as robustness of amplification, clarity and scorability of their banding patterns. The gel was photographed using a gel documentation system (BIORAD Molecular Imager, Gel Doc TM XR with image lab TM software). Clear and well resolved bands were scored for presence (1) and absence (0) and estimated total number of bands, total number of polymorphic bands and percentage of polymorphism.

Estimation of Jaccard's similarity coefficient

Jaccard's similarity coefficient (Jaccard 1908) between pairs of genotypes was calculated using the formula: $J = n_{xy} / (n_t - n_z)$, where n_{xy} - Number of bands common to the sample A and sample B, n_t - Total No. of bands present in all samples, n_z - Number of bands not present in sample A or B, but found in other samples.

The similarity matrix was subjected to cluster analysis using PAST (PAleontological STatistics) v.4.03 and the results were depicted in a dendrogram (Hammer et al. 2001).

OBSERVATIONS

The ten selected decamer primers generated a total of 79 bands of different sizes among the three genotypes of *P. nigrum* 'PMM', 'PAJ' and cv. 'Karimunda' (Table 1, Fig. 1). The number of bands per primer ranged from 5–11. The Primer 10 produced the highest number of bands and

TABLE 1: RAPD markers produced by the 10 selected primers as regards to the genotypes of *P. nigrum*, PMM', 'PAJ' and cv. 'Karimunda'.

Sl. No.	Primer	No. of bands generated	No. of polymorphic bands
1	OPA 19	5	5
2	OPB 17	10	6
3	OPB 20	9	3
4	OPBC 10	10	4
5	OPF 02	7	3
6	OPC 15	7	4
7	OPA 09	6	3
8	OPA 04	8	3
9	OPA 11	6	4
10	OPC 06	11	11
	Total	79	46

Primer 1 produced the lowest number of bands. The highest number of polymorphic bands (11) was also produced by the Primer 10 and the lowest number of polymorphic bands (3) was produced by a series of primers, Primers 3, 5, 7 and 8. Out of the total number of bands (79) produced, 33 were monomorphic and the rest (46) were polymorphic and the polymorphism estimated among the three genotypes was 58.75%.

Based on the RAPD data generated on the three genotypes, 'PMM', 'PAJ' and cv. 'Karimunda', Jaccard's similarity coefficient values pertaining to the pairs of genotypes were estimated (Table 2). The genotypes 'PMM' and 'PAJ' possessed the highest similarity index

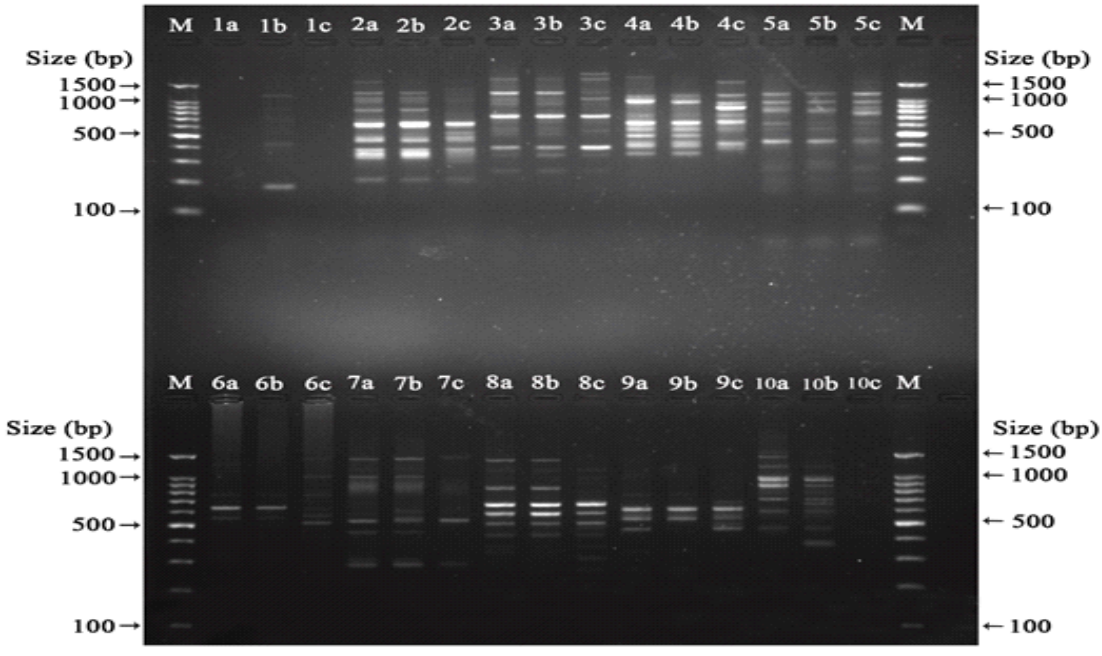

Fig. 1: RAPD profile of the genotypes of *P. nigrum* as regards to the 10 primers. M-size markers, a, b and c represent 'PMM', 'PAJ' and cv. 'Karimunda' respectively and 1–10 represent the 10 primers. e.g. 1a - bands of 'PMM' as regards to the Primer -1.

TABLE 2: Jaccard's similarity matrix of *P. nigrum* genotypes, 'PMM', 'PAJ' and cv. 'Karimunda' based on the RAPD analysis.

Genotypes	'PMM'	'PAJ'	cv. 'Karimunda'
'PMM'	1		
'PAJ'	0.77	1	
cv. 'Karimunda'	0.54	0.43	1

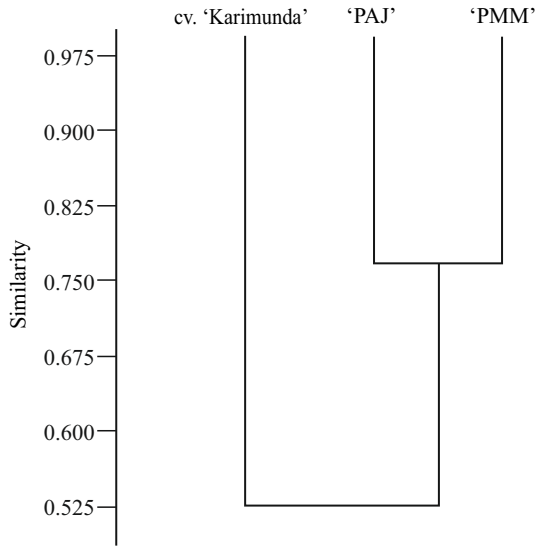

Fig. 2: Dendrogram showing the clustering based on Jaccard's similarity indices of the three genotypes of *P. nigrum*, 'PMM', 'PAJ' and cv. 'Karimunda'.

(77%) among the three pairs of genotypes and 'PAJ' and cv 'Karimunda' owned the lowest similarity index (43%). The genotype 'PMM' showed 54% similarity index with cv. 'Karimunda'.

As a result of cluster analysis using PAST v.4.03, the three genotypes were grouped into two clusters, Cluster-I and Cluster-II. The Cluster-I was a single member cluster comprising cv. 'Karimunda' and the Cluster-II included the genotypes, 'PMM' and 'PAJ'. The results of the cluster analysis were depicted in a dendrogram (Fig. 2).

DISCUSSION

DNA sequences of organisms are fundamental for determining their genetic make-up, and hence the diverse techniques used to evaluate DNA polymorphism are useful for evaluating genetic diversity of a species, and therefore, it is also used for understanding phylogenetic interrelationships among related species/genotypes (Hoshino et al. 2012). The present day growth in the field of molecular biology widens the possibility to exploit DNA polymorphism in breeding programmes, and by exploring the genomes of crop species, remarkable achievements have been made in crop improvement (Chakravarthi & Naravaneni 2006).

The present RAPD studies on three unique and potential genotypes of Black pepper for crop improvement 'PMM', 'PAJ' and cv. 'Karimunda' were aimed at (i) assessment of genetic diversity between and among the three genotypes and (ii) elucidation of their genetic interrelationship, which are useful for their conservation and effective utilization. The study estimated overall polymorphism among the three genotypes to be 58.75%, which is suggestive of remarkable genetic variability among them. In a previous study (Davis 2017) involving 30 wild accessions of *P. nigrum* occurring in the Kerala sector of the Western Ghats using 11 microsatellite markers revealed that the percentage of polymorphism among them was 24.45. Joy et al. (2007, 2011) also noted in their studies using AFLP and microsatellite based molecular techniques, remarkable genetic diversity among the genotypes/

cultivars of Black pepper occurring in the region. The origin and perpetuation of extensive genetic variation in *P. nigrum* as reported by many workers may be due to the capability of the species to reproduce both sexually and vegetatively.

The studies undertaken here showed that among the three genotypes, the highest Jaccard's similarity coefficient value (77%) was between the genotypes, 'PMM' and 'PAJ', and the lowest index (43%) was between 'PAJ' and cv. 'Karimunda'. The similarity value between 'PMM' and cv. 'Karimunda' was 54%. The similarity indices based on the RAPD data reflects the pair-wise interrelationship among the three genotypes. Thus, 'PMM' and 'PAJ' are the most closely related genotypes among the three, possessing high genetic relatedness between the two (77%) as expected since the latter is a haploid genotype of the former (Davis et al. 2013, Mathew et al. 2016). As per the results of the analysis, the most genetically distant genotypes among the three are, 'PAJ' and cv. 'Karimunda', which showed only 43% similarity value, and 'PMM' showed more genetic relatedness (54%) with cv. 'Karimunda' than 'PAJ'. In a previous RAPD analysis on 22 cultivars of *P. nigrum* using 24 primers, the Jaccard's similarity coefficient between the cultivars ranged from 0.20 to 0.66 and the mean value was 0.42 (Pradeepkumar 2003). The results of the cluster analysis revealed that cv. 'Karimunda' is remarkably divergent genetically from the genotypes, 'PMM' and 'PAJ'. The findings of the present study on interrelationship among the three genotypes corroborate the outcome of a similar study involving the three genotypes based on their morphological traits (Anchana 2020).

Hence, it may be inferred that hybridisation involving the genotypes 'PMM' as well as the probable double haploid of 'PAJ' as one parent (since 'PAJ' is a haploid genotype, not flowered/ fruited even after attaining reproductive maturity) and cv. 'Karimunda' as the other parent, aiming at exploitation of hybrid vigour for crop improvement seems worthwhile.

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RESEARCH ARTICLE

EFFECT OF BLEACHING WASTE FROM SILK INDUSTRIES ON SKIN OF MICE (*MUS MUSCULUS*)

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SUMMARY Small scale silk industries discharge toxic waste in huge amount into drains. The present study concerns the effect of bleaching waste released from the silk industries on the skin of mice. The skin of fore and hind limbs of mice was painted by different concentrations of untreated bleaching waste (1%, 2%, 4%, 8% and 16%) with different incubation periods (1^{st}, 2^{nd} , 3^{rd} and 4^{th} wk). The results on the effect of silk dye waste effluent on the skin of mice showed redness, huge number of keratin pearls, necrosis of cells and loss of melanocytes. Carcinoma-like changes were induced in the skin such as nondifferentiated cells and tumours.

Keywords: Mice, silk bleaching waste, skin.

INTRODUCTION

The silk industry is one of the productive cottage industries in India. It has developed rapidly and lucratively over the past years. Clothing is the second important need and factors like comfort, luxury, tradition, elegance, quality, design and fashion appeal play a significant role during cloth selection. Silk is a Nature's gift for humankind. India is the second largest tussar silk producing country in the world. Much of it is produced in Bhagalpur. The silk industry, as textile, provides an important economic stand to the artisans but the dye waste or spent wash arising from the manufacturing unit causes serious menace if released in open. Untreated silk dye wastes are the major source of water pollution that poses a serious threat to plants, agriculture, and human beings (Shrivastava et al. 2001). Small scale silk industries established in Bhagalpur city discharge many toxic substances in huge amounts into drains, ponds and finally into the river Ganga. These wastes contribute to a great extent to deteriorate the water quality of the surroundings (Kannan et al. 2003, Nagaraj & Boopathy 2004).

They use a variety of dyes, both natural and synthetic, for colouring the fiber/fabric. The dyes are made up of two parts chromophores and auxochromes. The colour of the dye is due to the presence of certain multiple bonded group called chromophores. The chromophores part of the dye absorbs some wavelengths from white light and reflects back the complementary colour. This compound is known as chromogen. While auxochromic group does not produce colour by themselves but intensify the colour. Auxo-chromes have acidic or basic functional groups.

The important auxochromes are, OH, SO_3H, NH_2, NR_Z etc. The dye may be classified according to the chromophore present in their structure such as direct dye, azoic dye, basic dye, vat dye, azo dye, nitro, nitrosodye and anthra- quinone dye etc. But in Bhagalpur silk industries, generally, the azo dye is used. The azo dye contains one or more azo groups (N=N–) as the primary chromophore.

The toxicity, mutagenicity and carcinogenicity of azo dyes are of major concern for human health thus posing a grave damage to life, particularly occupational persons. The workers often suffered from lung impairment and bladder cancer. Azo dyes affect the liver and kidney (Gupta & Sharma 2001) and also cause a genotoxic effect in bone marrow cells (Chaurasia et al. 2005). The azo dyes were found to be nonbiodegradable and nondecomposed by microorganisms. Humans constantly exposed to this waste. Thus it is prime concern to evaluate the toxicity caused by such waste that pollutes our water bodies. Unfortunately, the impact by such small scale silk industries located at Bhagalpur has not been assessed as yet.

The Industrial wastes released from agrochemical (Mohan et al. 2004), drugs (Kakkar & Seth 2003) cosmetic food additives (Walker et al. 1999) and various industrial effluent units (Matsumoto 2005) have been evaluated in the past. Obviously, the major routes of human and animal exposure to these toxicants are through drinking water, food and direct physical contact (Jordon & Dahl 1995). 7-12-dimethyl benzene anthracene (DMBA) developed skin tumor in mice (Koul 2006). In humans, dyeing waste causes burns and skin irritation, in severe cases, keratinization and dermatitis (NOHSC 1998).

The present work deals with the effect of bleaching waste from silk industries on the skin of mice.

MATERIAL AND METHODS

The chemical wastes were collected from the main outlets of the silk industries. The collected wastes were considered to be of 100% concentration. By dilution with distilled water, 1%, 2%, 4%, 8% and 16% concentrations of the waste were prepared.

Swiss albino mice (*Mus musculus*) were obtained from Central Drug Research Institute, Lucknow, and maintained in the laboratory. The animals were separated into 6 groups and subjected to various treatments for the histological study. The skin was cut after a suitable incubation period and treated with different concentrations of chemical waste.

Normal and treated limb's skin of mice were dehydrated by using graded alcohol such as 50%, 70%, 90% and absolute alcohol. Tissues were kept for 3 h in each concentration and 1 h in absolute alcohol and then transferred to xylene, the clearing agent, and thereafter, transferred to the mixture of melted pure paraffin and xylene. Tissues were kept for 1 h at 60º C. Paraffin block was prepared and 5–7 µm thick sections were cut with a microtome and stained with haemotoxylin and counterstained in eosin by following standard histological procedure and mounted in DPX (Kiernan 1999). The sections were examined under the Ermascope which is provided with inbuilt camera from which the photographs were taken.

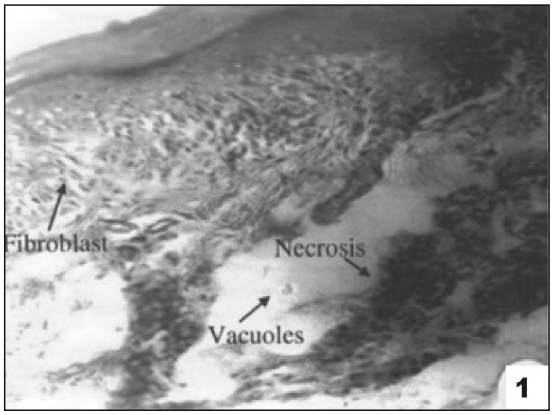
OBSERVATIONS

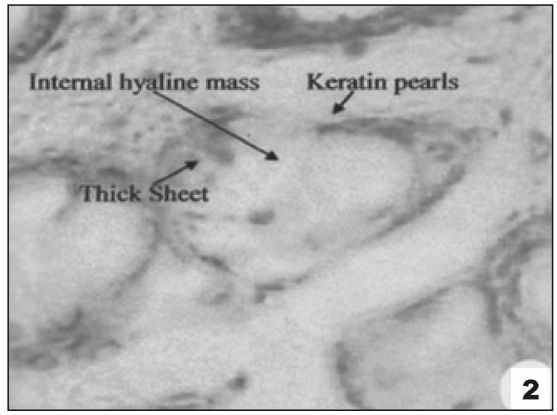
In the present study, control group showed a normal texture of skin because mice have provided only distilled water for painting the skin of fore and hind limbs.

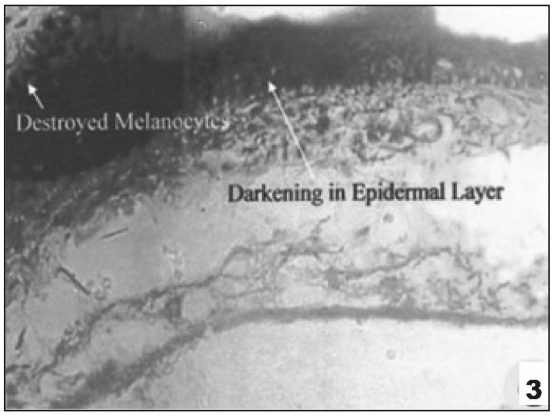
One wk after treatment with 8–16% of dyeing waste, skin showed inflammation. However, when treated with 1–4% of waste, showed only deep colouration (redness) on the skin surface.

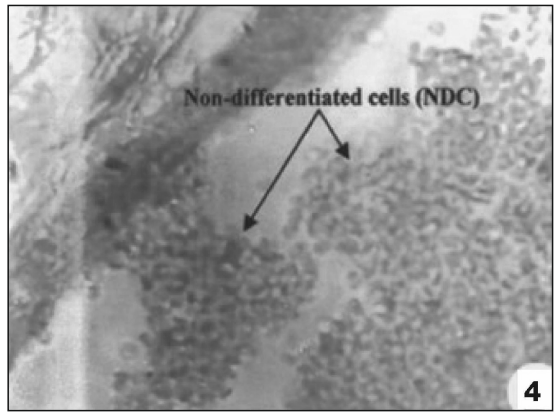
After 2 wks of incubation with different concentrations (1, 2, 4 and 8%), skin showed abnormal texture, thin epidermal layer, vacuoles and keratin layer. At 8–16% treatment, skin showed keratin layer and also necrosis (Figs 1, 2). In 16% treatment, skin showed flattened ridge.

After 3 wks of treatment with 1 and 2% waste, mice showed a dense keratin. Collagen fibers were abundant as compared to the treatment in previous wk. When treated with 16% waste, skin showed a tumour, as well as increased number of nondifferentiated cells (Fig. 4) and lumen of blood vessels were enlarged as compared to the skin treated with 8% waste.









Figs 1—4: Histological abnormalities in mice skin. 1. Arrows point out vacuoles, fibroblast and necrosis. 2. Keratin pearl (arrow). 3. Arrow points out destroyed melanocytes. 4**.** Non-differentiated cells in tumour (arrows).

In the 4th wk of incubation period, skin treated with 16% waste showed darkening in the epidermal region due to loss of melanocytes (Fig. 3) and also induced tumors in large numbers.

DISCUSSION

In the present investigation, after one wk of treatment with 1–4% of dyeing waste, skin surface of mice turned reddish; however, when treated with 8–16% waste, of the same incubation period, the skin showed inflammation. With the increase in incubation period to 2, 3 and 4 wks, different concentrations of dyeing waste caused a number of changes in the skin viz., thinning of epidermal layer, formation of vacuoles (or vacuolation), keratinization, necrosis, tumour, nondifferenciated cells, enlargement of lumen of blood vessels and finally the darkening of epidermis as reported by Roychaudhary et al. 1997.

The separation of damaged cells from their normal location in a tissue results in the formation of vacuoles (Mayer & Hendricks 1985). The concentration of glycogen is reduced in the epidermis, where keratinization takes place. It is due to carbohydrate dysmetabolism (Achtem 1959) and also is believed to be due to exfoliation of the cells due to lipid dysmetabolism (Anderson 1976). A much prominent skin lesions were found in 3rd and 4th wks of treated mice. Chemicals may interact with the genome, such that a gene involved in the normal growth and differentiation of cells is changed into oncogene resulting in loss of normal growth and differentiation (Van Bender & Ostrander 1994).

The number of abnormalities is increased with increasing concentration of dyeing waste and incubation period. Obviously, it showed the concentration (dose) and time period dependency at all concentrations. Similar observations have been made by Vainio & Gustavsson (2001) on mice skin when animals are exposed to aromatic amines.

In the view of the adverse effect of effluents seen in mice, there is a need to analyze the chemical components of the dyeing waste to identify different constituents and pinpoint the influence of specific chemical/s on the living system. Therefore, there is a need for further investigation in this direction.

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RESEARCH ARTICLE

MUTAGENICITY ASSESSMENT OF SUNSET YELLOW ON CHROMOSOMAL ABERRATIONS AND WHOLE GENOME DNA STRAND BREAKS IN *ALLIUM CEPA*

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SUMMARY Food additives are substances intentionally added to modify the visual appearance, taste, texture, flavour, processing or storage life of food. Some artificial food additives have mutagenic properties and hence have been discontinued from the market. Sunset yellow (SY) is a common food colouring agent, currently used in India and some other countries. Previous reports do indicate that SY is mutagenic. We have assessed the influence of three different concentrations [1000, 2000 and 3000 parts per millions (ppm)] of SY and four different time intervals (4, 8, 12 and 24 h) of treatment, in inducing chromosomal aberrations in *Allium cepa* L. which is routinely used as a model system to study chromosomal aberrations in higher eukaryotes. SY lead to a decrease in mitotic index and increase chromosomal aberrations, in a dose- and time-dependent manner. Clastogenic, nonclastogenic (structural) and aneugenic aberrations were observed due to SY treatment. We checked if very high concentrations of SY (2000, 5000 and 10000 ppm) could induce whole genome DNA strand breaks, if treated for short time interval (24 h). Using comet assay, we compared the SY-treated nuclei with that of malathion-treated ones as positive control. While the former treatment did not induce any DNA strand breaks, 14.71% of the latter-treated nuclei exhibited DNA strand breaks. Thus, unlike chromosomal aberrations, whole genome strand breaks did not occur when the treatment period was as short as 24 h, irrespective of the higher concentration.

Keywords: Food additives, cytotoxicity, sunset yellow, comet assay, chromosomes, aberrations, mutations, DNA strand breaks.

INTRODUCTION

Food additives are the substances added to various food products for flavouring, colouring, nutrient enrichment, texture enhancement, shelf-life extension, as well as the promotion of food safety. More than 2500 such chemicals are available in global market (Carocho et al. 2014). It is estimated that, the use of food additives started from 5000 B.C., at the time of Egyptian civilization, and usage of food dyes started from 1500 B.C. (Chequer et al. 2012, Meggos 1995). After the industrial revolution, in order to cope up with the need of food products at industrial level, various food additives were developed over the years (Jen & Chen 2017). The risks and adverse effect of food additives have been under high grade inspection for a number of years. A number of food additives were banned after confirming their carcinogenic, hepatotoxic, cardiotoxic and neurotoxic effects on body (Rangan & Barceloux

2009). Voluntary addition of over-dose of food additives beyond the allowed limit and accidental adulteration by small quantities, can cause accumulation within the body. Different strategies to examine and validate the chronic, toxic, physiological and carcinogenic risks of various food additives have developed over the years. For example, metanil yellow caused tremendous histopathological and ultrastructural changes (Sarkar & Ghosh 2012) and carmosine adversely affected the physiological function of the body by altering the biochemical markers in vital organs like kidney and liver in albino rat (Amin et al. 2010).

In the present study, mutagenic potential of sunset yellow (SY) was examined in *Allium cepa* L. using mitotic squash preparations and alkaline comet assay. SY, an azo dye, is disodium 2-hydroxy-1- (4-sulfonatophenylazo) naphthalene-6-sulfonate (Konig 2015). The dose and exposure time-related potential of SY to induce chromosomal aberrations (CA) in *A. cepa* was assessed. We also analyzed whether higher concentrations of SY could induce whole genome DNA strand breaks when treated for shorter time interval.

MATERIAL AND METHODS

Clean and healthy onions having almost similar sizes were purchased from the local market at Periya, Kasargod. Only fresh and healthy bulbs were used for the experiments. Old roots and dry scales of onion bulbs were removed and the bulbs were allowed to germinate by placing in autoclaved tap water. After 3–5 d, uniformly grown roots were selected from which, the root tips were used for further treatment and cytological studies. The bulbs with 1 or 2 cm long roots were exposed to different concentrations of SY (Manju Chemicals Pvt. Ltd., Chennai, India) or 10000 ppm of malathion (positive control; Shree Rasayan Udyog Ltd. Delhi, India) for different time intervals after diluting in autoclaved tap water (Srivastava & Singh 2020).

Mitotic squash preparation of the root tips was done as per the technique described by Sharma & Sharma (1980). The treated roots were washed in autoclaved distilled water and cut into segments having around 1 or 2 cm long from the tips and fixed in Farmer's fluid (3 parts of absolute alcohol:1 part of glacial acetic acid) for 1 h at room temperature, and stored in 70% ethanol at 4 °C until use. The root tips were put in 1N HCl for 5 min at room temperature and stained with 2% acetocarmine for 4 h and squashed in 45% acetic acid. The slides were scanned in Magnus MLX series microscope using ScopePhoto image software Ver 3.1.386, and different stages of mitosis were photographed. Different stages of mitosis were examined to score mitotic index (MI), percentage of abnormal cells (AC) and to identify the types of chromosomal aberrations. For each treatment, six preparations involving root tips from three onion bulbs (two from each bulb) were taken. About 1000 cells were scanned for each group of onions (Fiskesjo 1985).

MI and CA index were calculated using the following formula:

$$\text{MI} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

$$\text{CA} = \frac{\text{Number of abnormal cells}}{\text{Total number of dividing cells}} \times 100$$

MI and percentage of CA are denoted in percentage mean ± SE. One-way analysis of variance (ANOVA) was tested in between the treated and control groups by choosing level of significance 0.05. Treatments with significant variations ($p < 0.05$) were subjected to post-hoc analysis using Tukey-HSD in R studio.

Nuclei isolation and alkaline comet assays were performed using the protocol of Chakraborty et al. (2009) with some modifications. About 5 cm of root tips were cut and placed in a petri dish kept on an ice pack. The root tissues were sliced into small pieces using a fresh razor blade in 600 µl phosphate buffered saline buffer containing 50 mM ethylene diamine tetraacetic acid (EDTA). The nuclei suspensions were collected in buffer by just tilting the plates. This suspension was aspired using a micropipette and transferred to sterile microfuge tubes. The tubes were allowed to stand free for the debris to sediment at the bottom of the tube. Nuclei suspension without tissue debris was carefully collected in another microfuge tube.

The slides were coated with 1% normal melting point agarose (NMP) and the coverslips were placed on the slides to uniformly layer this NMP on top of the slides. The slides were kept at 4 °C for a minimum of 10 min to solidify, followed by gentle removal of the coverslips. Nuclei suspension (500 µl) was mixed with 1 ml of molten 1.5% low melting agarose at 37 °C. The mixing was done by repeatedly pipetting using a 1 ml micropipette tip. This mixture was uniformly poured on top of the slides that were pre-coated with 1% NMP. Again, coverslips were placed on the slides to uniformly layer this mixture on top of the slides, chilled and after getting solidified, gently removed. The slides were immersed into pre-chilled lysis solution, at 4 °C for 20 min and washed thrice in pre-chilled freshly prepared electrophoresis buffer for 20 min to facilitate nuclear DNA unwinding. Thereafter, the slides were immersed in an horizontal gel electrophoresis apparatus containing freshly prepared chilled electrophoresis buffer (0.03 M NaOH and 2 mM Na_2EDTA, pH > 13) and subjected to electrophoresis in order to segregate the DNA particles based on size at 0.7 V/cm (25 V/300 mA) for 20 min at 4 °C. This was followed by washing of slides with 0.4 M Tris buffer (pH 7.5) for neutralization, thrice. The slides were stained in propidium iodide (PI) for 10 min in the dark at room temperature, followed by washing in chilled autoclaved tap water to remove excess stains. All experiments were conducted under day light and the electrophoresis was done in the dark, by covering the tank with aluminum foil paper, to avoid additional DNA damage due to exposure to light. For each treatment, three slides were examined. Images of comets were viewed by epifluorescence microscopy, Leica DMI3000 B with an excitation filter of 515–560 nm and a barrier filter of 590 nm at 20–40 × magnifications.

OBSERVATIONS

Chromosomal aberrations and mitotic index

CAs induced by SY on the root tip meristem cells of *A. cepa* were analyzed first using different concentrations of SY and then at different time intervals. Normal stages of mitosis in untreated controls were compared with mitotic stages of treated roots. Various clastogenic, nonclastogenic (structural) and aneugenic CAs were observed in all treatment conditions (Figs 1–16). In prophase, lesions (single and double) were the main aberrations observed. Metaphase CAs included,

ring chromosome, clumped/sticky chromosome, C-metaphase, diagonal delayed, diagonal, multipolar, vagrant and clumped metaphases. Various anaphase aberrations included, bridge, diagonal, delayed diagonal, multipolar and misoriented stages. At telophase, clumped, multipolar and chromosome breaks were observed. Some other miscellaneous stages like interphase lesion and chromosomal loss were also observed.

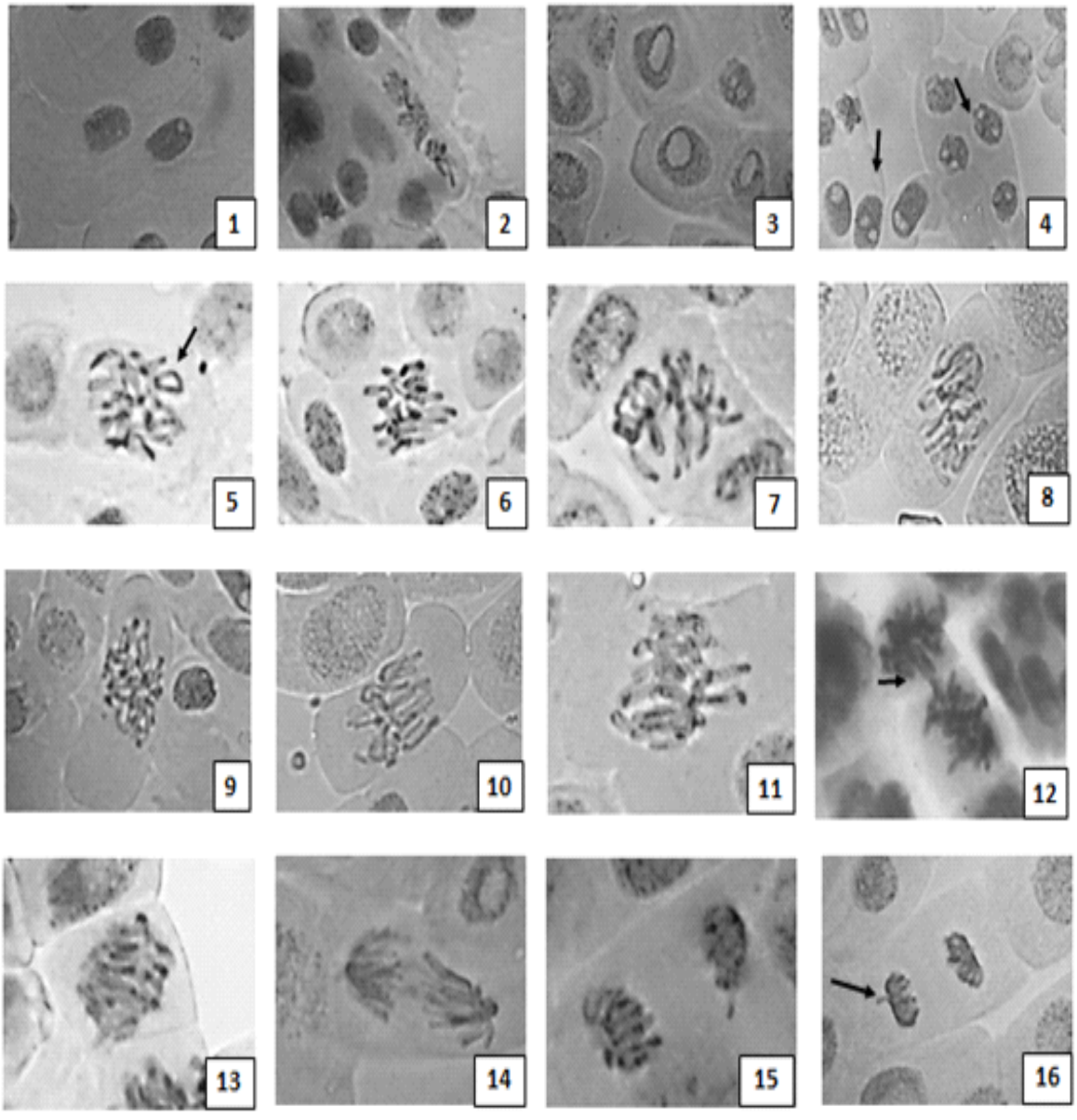
Though all the SY concentrations (1000, 2000 and 3000 ppm), subjected for 8 h, as well as untreated controls induced various categories of CA, significantly high CA and low MI were obtained in 3000 ppm-treated nuclei (Fig. 17). In order to check if prolonged exposure would influence the extent of damage induced by SY, the cells were treated with a constant concentration of 2000 ppm at four different time intervals (4, 8, 12 and 24 h). Here also like the previous case, various CAs were observed and calculated. Though 2000 ppm did not induce a significant difference in 8 h treatment, significantly high CA and low MI were obtained in 2000 ppm-treated nuclei at 24 h posttreatment. This experiment indicates that SY can enhance CA and adversely affect the MI either at higher concentration (3000 ppm) or at a lesser concentration subjected for a prolonged time interval.

Alkaline comet assay

The potential of SY to induce whole genome DNA damage in *A. cepa* root meristems was examined by comet assay. A total of 994 nuclei were isolated (treatment-758, negative control-134 and positive controls-102) and examined using alkaline comet assay. The concentrations of SY used here were the same as well as higher (2000, 5000 and 10000 ppm), compared to that used for CA study. The treatment time was fixed to 24 h as it gave the highest CAs in the above experiment. However, none of these SY concentrations induced any comets (Fig. 20). At the same time, 1% of malathion, taken as positive control, induced comets in 14.71% of the nuclei examined, 24 h exposure, suggesting whole genome DNA breakage (Fig. 21). In malathion-treated samples, about 25–30% of nuclei in comets were relatively smaller than the nuclei without comets.

DISCUSSION

Many food additives, especially dyes are known to have mutagenic property. Still they are permitted for consumption, with a recommended dose often prescribed on it. However, in India and other developing countries, due to the lack of awareness and less reports of research on their adverse effects, most of the food additives are used by the common people in amounts beyond the prescribed limit. One such food additive is the dye SY. It is sold in the market under various trade names such as Food Yellow 3 and Orange Yellow S. The recommended acceptable daily intake (ADI) concentration of SY by the Food and Agriculture Organization of World Health Organization is 4 mg/kg (EFSA 2014) and it is based on the adverse physiological impacts that SY caused in rats (Konig 2015). SY induced cellular apoptosis, physiological risks like cuts hatching rate, morphometry, eye diameter and heart rate, cardiac edema, yolk sac edema, spinal and tail distortion in zebra fish (Joshi & Pancharatna 2018). Previous research on the influence of SY on the genomic stability in



Figs 1–16: Abnormal stages of mitosis in *A. cepa*. 1. Interphase lesion. 2. Chromosomal loss. 3. Arrow indicates single lesion. 4. Arrows indicate double lesions. 5. Arrow points out a ring chromosome 6. Sticky chromosomes. 7. C-metaphases. 8. Diagonal metaphase. 9. Multipolar metaphase. 10. Vagrant metaphase. 11. Clumped metaphase. 12. Arrow points out a bridge at anaphase. 13. Diagonal delayed anaphase. 14. Misoriented anaphase. 15. Multipolar telophase. 16. Arrow points out a chromosome break at telophase.

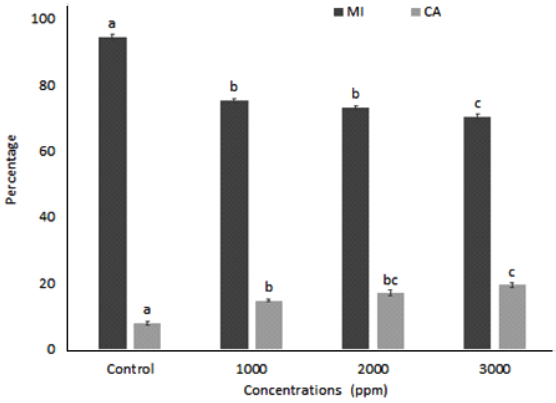


Fig. 17: Bar graph showing potential of SY to induce chromosomal aberrations and anti-mitotic effect with reduced mitotic index in *A. cepa* root meristem at different concentrations with 8 h exposure time at $p < 0.05$, as determined by ANOVA. [a, b, c] indicate significantly different groups for each treatment at 5% level, as determined by Tukey HSD. Error bar indicates ± SE of the mean values.

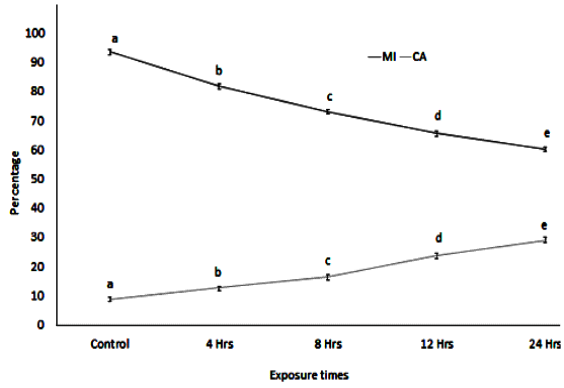
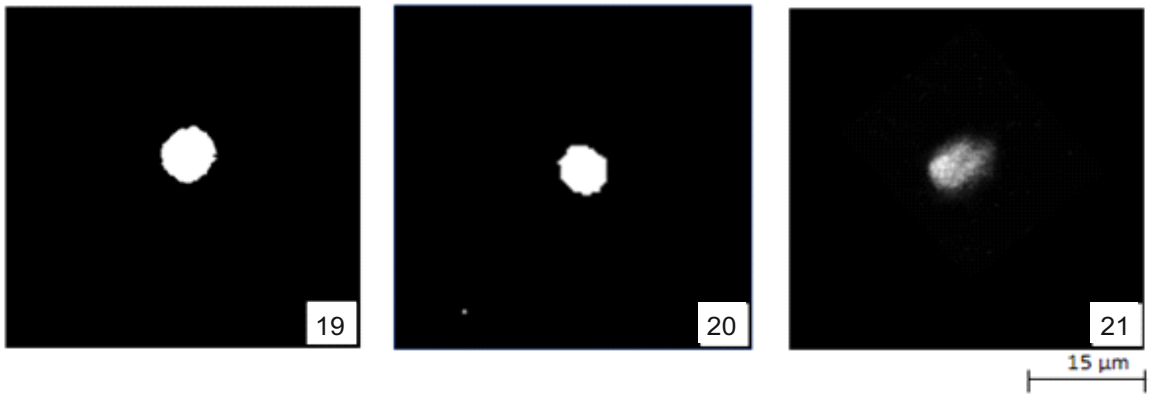


Fig. 18: Graph showing potential of SY to induce chromosomal aberrations and anti-mitotic effect with reduced mitotic index in *A. cepa* root meristem, at different exposure time intervals with 2000 ppm concentration at $p < 0.05$, as determined by ANOVA. [a,b,c,d,e] indicate significantly different groups for each treatment at 5% level, as determined by Tukey HSD. Error bar indicates ± SE of the mean values.



Figs 19–21: Nuclei isolated from root meristem of *A. cepa* after performing the comet assay by respective treatments. 19. Autoclaved tap water (negative control). 20. 10000 ppm of SY. 21. 1% malathion (positive control).

A. cepa revealed that this dye is mutagenic even at concentrations of 25 ppm at 72 h exposure, which is much lesser than the prescribed limit (Koc & Pandir 2018). In this study, we have compared the influence of various concentrations and time intervals on CAs and whole genome DNA strand breaks in *A. cepa*.

The DNA structure and chromosome organization is highly conserved across the higher eukaryotes, including plants (Dounce et al. 1973). Also, the homologs of almost all DNA repair genes in humans are present in plants too, with the same function (Gimenez & Manzano-Agugliaro 2017, Trapp et al. 2011). It is found that plants are

much adapted to tolerate chromosomal aberrations and exhibit better post-mutagenic viability than animals, by virtue of their sessile nature (Manova & Gruszka 2015). In addition, plants are easier to maintain and offer the provision to screen a large population, which is a desirable feature in any scientific study. These properties make plants appropriate to be used as model organisms to study mutations. Nevertheless, the animal systems are preferred, especially for the physiological impacts.

Many food additives induce CA which can be clastogenic or nonclastogenic. Clactogenic food additives cause disruption or breakages of chromosomes, leading to sections of the chromosomes being deleted, added, or rearranged. This type of mutagenesis can lead to carcinogenesis, as cells that are not killed by the clastogenic effect may become cancerous (Ashby 1985). Some agents cause nonclastogenic type of disruption as they affect the assembly of spindles and result in abnormalities during cell division like chromosome lagging and tropkinesis. In such cases, chromosome does not undergo any change, but results in aneuploidy and other functional anomalies.

Our study indicates that the mutagenic and antimitotic influence of SY is proportional to its concentration and treatment period. A concentration of 3000 ppm was required to induce various types of CAs and also to reduce the MI, if the treatment period was short (8 h). However, if the cells were exposed to longer period (24 h), even lesser concentration of 2000 ppm was sufficient to induce higher CAs and antimitotic effect. Previously, SY was shown to induce stickiness of chromosomes, micronuclei formation, precocious migration of chromosome, unorientation, forward movement of chromosome, laggards, and chromatin bridges in the root tip cells of *Brassica campestris* when treated with various concentrations (1%, 3%, and 5%, for 6 h) (Dwivedi & Kumar 2015). Similarly, it induced unorientation, precocious movement at metaphase, stickiness, laggard and chromosomal bridges in *Trigonella foenum-graecum* root meristems upon treatment with concentrations of 0.25%, 0.50%, 0.75% and 1% for 3 h (Kumar & Srivastava 2011). There is one report of micronuclei formation in *A. cepa* root meristems, (Gomes et al. 2013). Like SY, cytotoxicity by tartrazine food dyes on root tips of *A. cepa* has also been reported. It induces bridges at anaphase and telophase, breaks, stickiness, micronucleate and binucleate cells (Gomes et al. 2013, Lerda 2017), developmental toxicity in zebra fish (Joshi & Pancharatna 2018), sister chromatid exchange and antiproliferative effect on human peripheral blood cells (Mpountoukas et al. 2010) and severe adverse physiological risks in albino rat (Amin et al. 2010). The cherry-pink food dye (ErB) caused binucleate cell, micronuclei, nucleoplasmic bridges and nuclear buds in HepG2, HB-8065 and in hepatocellular carcinoma (Chequer et al. 2012). Apple green, another dye, induced moderate mutagenicity at higher concentration in TA98 and TA100 strains of *Salmonella typhimurium* (Kaur et al. 2010).

Present investigation revealed that SY has not caused comet formation, thus indicating lack of whole genome strand break occurrence. However, Koc & Pandir (2018) reported that even at low concentration of 25 ppm comets could be induced in *A. cepa*. However, their exposure time of 72 h was longer than the one used in the present

work. Thus, our results, along with previous reports indicate that possibly it is the prolonged exposure time to SY that has the potential to induce DNA breaks in plants, rather than concentration. In contrast to plants, dose-dependent effect of SY on comets was seen in certain animal cells. For example, significant comets were obtained due to 200 µg/mL of SY in human sperms after 1 h incubation (Pandir 2014). Also, significant comets were induced in leucocytes of male rats when 2.5 mg/kg body weight of SY was orally fed for 4 wks (Khayyat et al. 2018). In contrast, SY (2000 mg/kg), when administered orally to mice, did not induce any DNA breakage in 8 mouse organs after exposure to a lesser time interval of 3 and 24 h (Sasaki et al. 2002). SY did not induce micronucleus as well in mice (Poul et al. 2009). Other dyes, such as amaranth, allura red, new coccine, tartrazine, ErB, phloxine, and rose bengal induced DNA damage in various organs of mouse (Sasaki et al. 2002). Combinations of SY along with sodium benzoate induced an increase in the frequency of tailed nuclei (DNA damage) in liver cells of female rat (Ali et al. 2018).

Our study suggests that multiple combinations of concentration and treatment period should be checked for analyzing the adverse effect of any mutagen and calls for reconsideration on the prescribed limit of SY recommended for consumption. More of such work is needed in animal and human cells.

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