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RESEARCH ARTICLE

GENETIC ENHANCEMENT OF WHITE ONION FOR DEHYDRATION

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SUMMARY White onions are in great demand for processing. One of the requirements of processing white onions is high total soluble solids (TSS). Sixtyfive white onion genotypes were assessed for TSS and 5 populations were subjected to selection and hybridization. There was a slow progress for TSS on selection due to low heritability and genetic advance. None of the parameters served as selection indices for high TSS. The character was controlled by both additive and dominant genes. Therefore, appropriate breeding methods are suggested.

Keywords: White onion, *Allium cepa*, selection, genetics, breeding, dehydration.

INTRODUCTION

White onions are mainly grown for processing purpose although being eaten as fresh are known to have medicinal properties due to quarcetin compound. One reason why white onions preferred for processing is that they do not impart brown colour to the processed product unlike red onions (Singh & Kumar 1984). But other quality parameters that they need to possess by processors are high total soluble solids (TSS) (>15°Brix), > 6 cm diameter, high pungency (>7 µmol pyruvic acid per g of fresh weight) (Sharma & Nath 1991), globular in shape (LD ratio 1:1) apart from being high yielding variety (>25–30t/ha) (Mahajan & Pathak 2014). At present, there are no varieties of onion fulfilling the above characters in India (Kulkarni 2011) although many processing companies exist using available white onion varieties as there is great demand for processed products of onion in European and western countries. There is a need to breed one such variety or hybrid to meet out country's demand. For this purpose, breeder has to understand genetics of these traits and their association to apply suitable breeding method. Therefore, TSS as a character, this study has been taken up.

MATERIAL AND METHODS

Sixtyfive white onion germplasms at I & B were constantly tested for their TSS and processing qualities from 2016 to 2020 of which 5 genotypes (populations) viz., 16/2A, 16/6B, 16/21, 16/47 and 16/50 were used to evaluate response to selection towards TSS from S_0 to S_3 cycles and genetic control of TSS was worked out . The correlated response of TSS with other traits which contribute to processing qualities like bulb weight, bulb diameter and L:D ratio (that indicates globose shape) along with regression was estimated as selection index. Since 16/2A and 16/6B were male sterile and maintainers respectively they were utilized to develop some of

the white hybrids to illustrate gene action and possible use of hybrids in commerce.

OBSERVATIONS

Response of 5 populations to selection cycles for TSS is given in Fig. 1. The base population of

Correlation and regression of TSS with other characters suggest that TSS is negatively correlated with bulb weight and so with diameter though nonsignificant. There was a weak correlation with L:D and it was independent of diameter (r = 0). The regression coefficient was

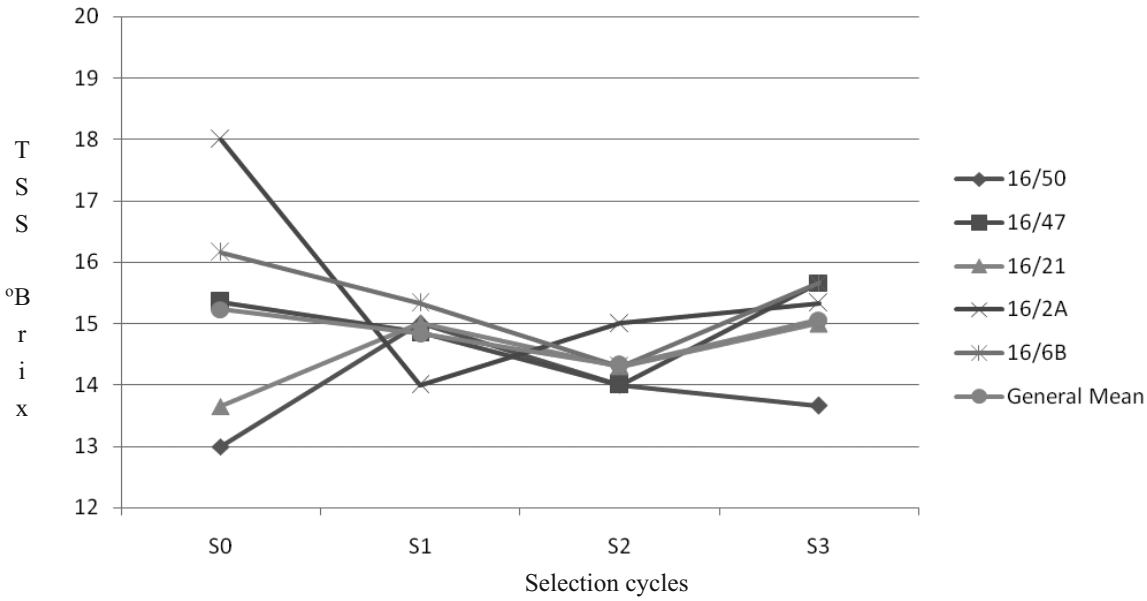

Fig. 1: Graphical representation of TSS on selection in white onion genotypes.

each genotype varies from 13°Brix to 18°Brix. On selection for high TSS (>15° Brix) at each cycle exhibited different pathway. In general, TSS went on decreasing up to S_2 and increased at S_3. However, 16/21 and 16/50 genotypes improved from S_0 to S_1 and thereon 16/50 continues to decrease. Most of the genotypes stabilized at around 15° Brix at S_3 cycle. Genetic parameters (Table 1) indicated that heritability of TSS for almost all the genotypes was less than unity (1) and the genetic advance varied from 1.21 to 7.08, while the means were around 15 except of 16/50.

also strongly negative with bulb weight but positive with diameter and L: D (Table 2).

The hybrids on male sterile line revealed that 4 out of 6 hybrids had additive genes while WH4 and WH5 showed overdominance and dominance genes respectively. However, the mean over hybrids indicated additivity (Table 3). Inheritance of colour to hybrids was varying. Although all the parents were of white skin colour , hybrid WH5 bulbs were of light red colour (all other hybrids were white).

TABLE 1: Genetic parameters for white onion lines.

Genetic parameter	16/2A	16/2B	16/21	16/47	16/50
Mean	15.58	15.36	14.48	14.94	13.91
Genotypic variance (Vg)	2.91	0.61	0.42	0.53	0.69
Phenotypic variance (Vp)	3.77	1.01	0.74	0.89	1.11
Heritability	0.87	0.78	0.75	0.76	0.79
Genetic advance	7.08	1.7	1.20	1.45	1.89

TABLE 2: Correlation and regression analysis for white onion lines.

Variable	TSS	Yield/bulb (g)	Diameter (cm)	LD ratio
TSS	1	0.498*	0.291NS	0.065NS
Yield/bulb		1(-0.038*)	0.816*	0.188NS
Diameter			1(1.082NS)	0
LD ratio				1(1.671*)

Note: Regression values in parenthesis indicate direct effect. Intercept (a): 11.120.

TABLE 3 : *Per se* values for TSS in parents and hybrids.

				TSS *per se* values				
P_1	P_2	Hybrid	Hybrid bulb colour	P_1	P_2	F_1	%Heterosis over check (Dhawal)	Probable gene action
W	W	WH1	W	14.5	14	14.2	7.6	Additive
W	W	WH3	W	14.5	11.2	14.3	8.85	Additive
W	W	WH4	W	14.5	13.7	14.8	12.61	Overdominant
W	W	WH5	LR	14.5	14	14.5	11.89	Dominant
W	W	WH7	W	14.5	12.3	13.8	5.35	Additive
W	W	WH8	W	14.5	13.8	14.2	7.63	Additive
						SEm ±	3.66	
						CV%	57.65	
						CD@5%	NS	

P_1, Parent1; P_2, Parent 2; W, White; LR, Light red.

TABLE 4: Stability analysis in white onion hybrids for TSS.

Hybrid	KB mean	WB mean	SB mean	SH mean	OverSall mean	Regression (bi)	Standard deviation (s^2d)
WH1	14.2	14.5	12.8	13.8	13.8	1.1	-0.187
WH3	14.3	12.5	11.8	13.3	13.0	1.7	-0.128
WH4	14.8	13.7	13.3	12.7	13.6	1.0	0.256
WH5	14.7	13.7	13.5	14.9	14.2	0.8	-0.017
WH7	13.9	13.9	11.5	14.1	13.3	1.8	0.181
WH8	14.2	14.7	14.7	13.5	14.3	-0.3	-0.064
Over all mean	14.33	13.8	12.9	13.7	13.7		

P, Parent 1; P2, Parent 2; KB, Kharif Bangalore; WB,Winter Bangalore; SB, Summer Bangalore; SH, Summer Haveri.

Extending the hybrid performance on TSS for stability, only 3 hybrids, WH1, WH5 and WH8 had means better than general mean (13.70° Brix) and highest being WH8 (14.25° Brix). Though WH3 had unit regression for stability, it could not be considered as its mean TSS was less than general mean. WH5 is next highest with 14.20° Brix but has a light red skin colour (Table 4).

DISCUSSION

Dry matter content (DMC) of the processed white onion is directly correlated with refractometer reading (TSS) of the expressed juice (Mc Collum 1968, Mann & Hoyle 1945) and served as selection index. Selection for quantitative character, over a period of generation, takes a longer time to fix the character due to factors like low heritability and polygenic gene actions. In the population we handled, there was a slow progress on selection due to low heritability (less than unity) and expected genetic advance. Most of the populations reached the minimum threshold (15° Brix) at 3rd generation of selection, which is advantageous to use them in the breeding programme. Since dry matter content is the product of TSS and bulb weight and these two are negatively correlated, the breeder has to work out a balancing media to select such a type that has good weight of bulb that can give an estimated yield of around 25–30t/ha with desired TSS (> 15° Brix). The character, TSS appears to be controlled by additive and dominant genes, and hence an appropriate breeding methods like recurrent selection or heterosis breeding can be employed to develop a suitable processing onion (Madalageri & Bojappa 1986). Although none of the present hybrids meets out the standard TSS, yet WH8 has higher TSS of 14.3° Brix with globular shape which can be further improved by improving its parents through selection for higher levels of TSS. Although WH5 is next best candidate, it cannot be considered due to its red bulb colour that is expected to have been inherited from R line that might be having a colour inhibiting gene, *'I'* in heterozygous condition.

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RESEARCH ARTICLE

ETHYL METHANESULPHONATE INDUCED INVERSION HETEROZYGOTE IN *CHLOROPHYTUM BORIVILIANUM*

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SUMMARY Tubers of *Chlorophytum borivilianum* were treated with 0.2% ethyl methanesulphonate (EMS) for various durations of 6, 12, 18 and 24 h. Inversion heterozygote was isolated in a population raised from 0.2% EMS treatment for 18 h. The inversion heterozygote displayed various types of chromosomal configurations such as bridge, fragment, bridge/fragment and micronuclei at anaphase/telophase I at meiosis. It was characterized by the commonly presence of bridge/fragment. Pollen fertility was found to be very low in inversion heterozygote.

Keywords: *Chlorophytum borivilianum,* ethyl methanesulphonate, inversion heterozygote.

INTRODUCTION

Chlorophytum borivilianum belonging to the family Asperagaceae is commonly called Safed Musli. It is a small perennial herb with a full crown of radical leaves appearing over the ground with the advent of summer rain. Roots (tubers) are fleshy, fascicled and directly originate from the stem disc. The flowers are small, white, bracteate, pedicellate, usually arranged in alternate clusters, each cluster consisting of 3 flowers (Nikam & Chavan 2009). *C. borivilianum* is a traditional rare Indian medicinal herb which has many therapeutic applications in ayurvedic, unani, homeopathic and allopathic systems of medicines. Its roots (tubers) are very famous for various therapeutic applications viz., adaptogenic, aphrodisiac, immunomodulatory and antidiabetic (Thakur et al. 2009). It is a rich source of over 25 alkaloids, vitamins, minerals, proteins, steroids and carbohydrates (Jakkulwar & Wadhai 2012).

C. borivilianum is an autotetraploid with chromosome number of 2n = 4x = 28 (Basu & Jha 2011, Ahirwar & Verma 2014). For development of a new species, induced mutations improved varieties at a swifter rate (Ahirwar & Verma 2016). In the present study, *C. borivilianum* has been selected for induction of mutation by ethyl methanesulphonate (EMS) to produce new and useful mutants and carried out cytological analysis.

MATERIALAND METHODS

EMS treatment was carried out in the laboratory

using 0.2% concentration for 4 durations. 400 healthy tubers of *C. borivilianum* were pricked with the help of needle so that solution penetrates the shoot apex and then immersed in 0.2% EMS for 6, 12, 18 and 24 h. Materials were washed for 3 h with tap water to completely remove EMS and then sown in earthen pots. For meiotic studies, young flower buds of appropriate size were fixed in Carnoy's fluid, anthers teased in a drop of 2% iron acetocarmine on a clean slide and squashed under a cover slip. PMCs were analyzed for various stages of meiosis. Slides with well spread cells and clear chromosomes were selected for scoring and photomicrographs were taken from temporary preparations under oil immersion.

OBSERVATIONS

Meiosis in normal plant

Meiosis in the normal plant was regular with 14 bivalents at diakinesis/metaphase I (Figs 1, 2). Chromosomes were equally distributed (14:14) at each pole at anaphase I (Fig. 3). Pollen fertility was 93.2%.

EMS induced inversion heterozygote

One hundred tubers of *C. borivilianum* were treated with 0.2% EMS for various durations of 6, 12, 18 and 24 h. Out of 100 tubers , 83 in 6 h, 69 in 12 h, 56 in 18 h and 54 in 24 h were germinated in which 24, 19, 14 and 11 survived respectively (Table 1). During meiotic analysis, one inversion heterozygote was isolated and raised from 18 h EMS treated population. The inversion heterozygote showed bridge/fragment configuration at anaphase/telophase I. At anaphase/telophase I, 174 PMCs were analyzed in which 110 cells had various configurations of chromosomes that confirmed the presence of inversion heterozygosity. At anaphase/telophase I, bridge, fragment, bridge/fragment and micronucleus were observed in 63.2% of PMCs (Table 2, Figs 4–12). Pollen fertility was relatively low (48.6%) in the abnormal plant (Table 2).

TABLE 1: Percentage of germination in EMS treated fingers of *C. borivilianum.*

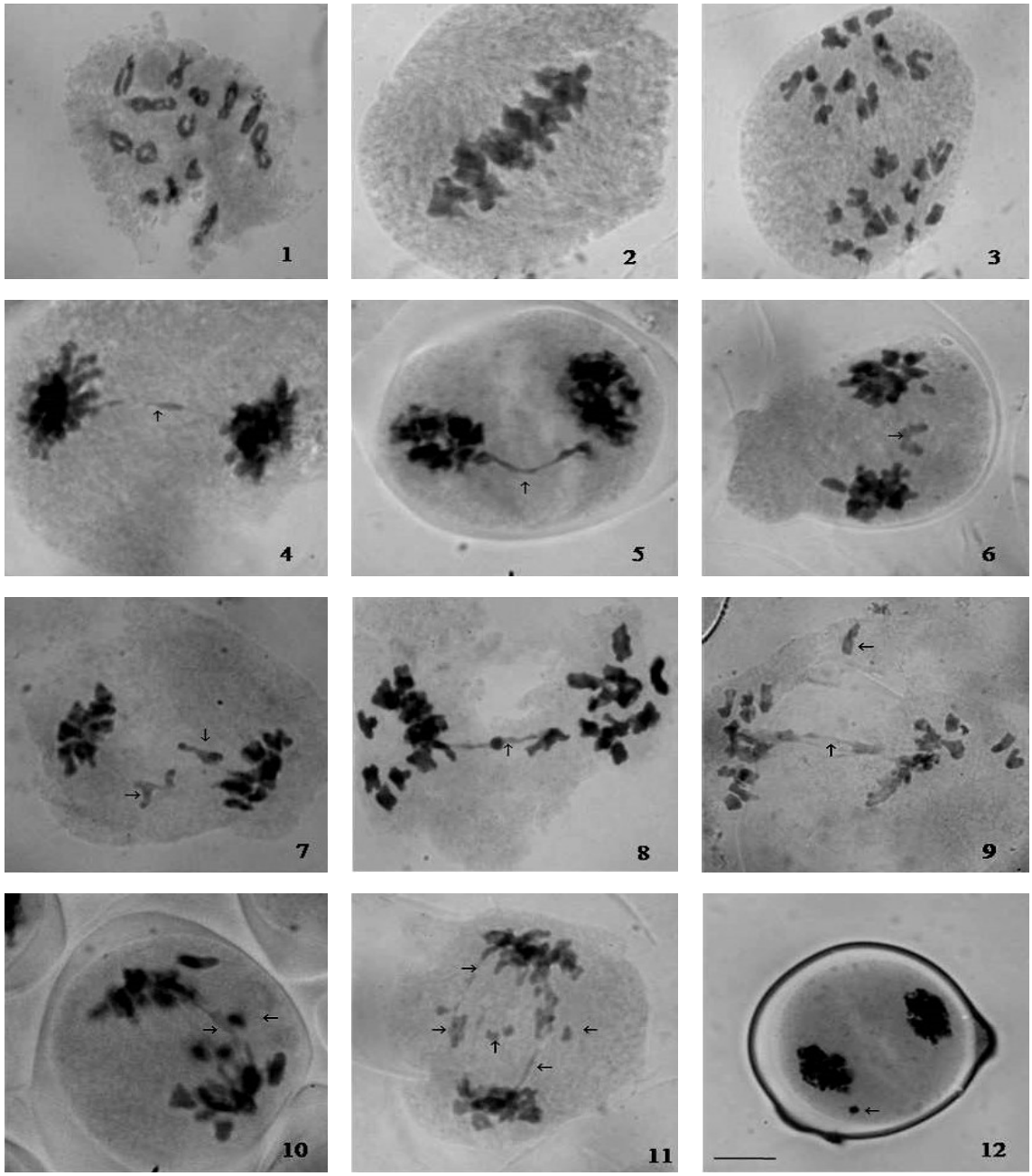
100 fingers treated with 0.2% EMS	Germination (%)	Survived plants	Flowering plants	Mutant
Control	96	92	56	
6 h	83	24	7	
12 h	69	19	5	
18 h	56	14	4	1
24 h	54	11	5	

TABLE 2: Various types of chromosomal configurations at anaphase/telophase I in meiosis of inversion heterozygote in *C. borivilianum.*

Abnormality	No. of cells	%
Anaphas/Telophase I		
Normal cells	64	36.7
Bridge	26	14.9
Fragment	10	5.74
Bridge + fragment	40	22.9
2 bridges + 2 fragments	16	9.19
Micronucleus	18	10.3
Total abnormal cells	**110**	**63.2**
Pollen grains		
Fertile	148	48.6
Sterile	156	41.4
Total	304	

DISCUSSION

Induced mutations serve as a complimentary approach in genetic improvement of crops.



Figs 1–12: *C. borivilianum.* 1–3. Meiosis in control. 1. Diakinesis. 2. Metaphase I. 3. Anaphase I (8:8). 4–12. Meiosis in inversion heterozygote. 4,5. Bridge at telophase I (arrows). 6,7. Laggard at telophase I (arrows). 8–10. Bridge/fragment at anaphase I (arrows). 11. Bridges/fragments at telophase I (arrows). 12. Macronucleus at telophase I (arrow). (Scale bar = 4 μm).

Various physical and chemical mutagenic agents are used to induce favourable mutations at high frequency in plants. For improvement of crops by mutation breeding, it is very important to determine a suitable mutagen dose (Ahirwar & Verma 2016). Several workers have attempted to induce mutations to generate genetic variability in various plants such as in *Pisum sativum* (Verma & Goyal 2012), *Lens culinaris* (Goyal &Verma 2015), *Triticum aestivum* (Verma & Khah 2016), *Pennisetum glaucum* (Khah & Verma 2017) and *Commelina benghalensis* (Shaikh et al. 2020).

In the present investigation, inversion heterozygote was isolated in *C. borivilianum* treated with 0.2% EMS for 18 h which was confirmed on the their chromosomal configurations at anaphase I. In inversion heterozygote, bridges may originate from chiasma formation in heterozygous inversion. When cell division occurs, a broken chromosome with two centromeres is pulled to the opposite poles of the cell, forming a long chromosome bridge, the chromatid bridge. Bridge and fragments are the result of spontaneous breakage and fusion of the chromosomes (Ahirwar et al. 2015). Bridge and fragment are moderately constant; it is most likely due to a paracentric inversion.

Formation of these bridge/loop fragments greatly depend upon the position of crossover in the pachytene loop. Four possible types of configurations of paracentric inversion resulting due to the involvement of chromatids in crossover within the inverted region which may or may not be accompanied by the crossover (outside the loop) between centromere and inverted region. A single crossover inside the inversion loop results in a bridge and a fragment at anaphase I, which gives a single fragment without bridge at anaphase II. A four stand crossover inside the inversion loop yields two bridges and two fragments at anaphase I and simply two fragments at anaphase II. A single crossover inside the inversion loop and a single crossover outside the inversion loop give a loop and fragment at anaphase I and a bridge and a fragment at anaphase II. Two crossovers inside the inversion loop and a single crossover outside the inversion loop gives two loops and two fragments at anaphase I and two bridges and two fragments at anaphase II.

In the present investigation, most common configurations, bridge/fragment observed at anaphase/telophase I in the inversion heterozygous *C. borivilianum* are the result of a crossover inside the loop. As is expected, the loss of acentric fragment as a result of paracentric inversion leads to deficient gamete and high sterility (Ahirwar & Verma 2013).

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RESEARCH ARTICLE

A STUDY ON MUTAGENICITY AND GENOTOXICITY IN *ALLIUM CEPA* L.

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SUMMARY Pollution of water is a serious and growing problem. Despite the existence of relevant legislation, the pollution of the aquatic environment by toxic chemical pollutants continues to occur, with domestic and industrial effluents being the main sources responsible for the contamination of aquatic environments. Here, we study the effect of untreated and treated effluent from a dye industry on somatic cells of the test system, *Allium cepa*. The bioassay indicated that the mitotic index of the root meristematic cells of *A. cepa* can be affected by the untreated textile effluents and the occurrence of chromosomal stickiness, clumping, star metaphase, bridges, disturbed anaphase, micronuclei, giant cells, bi- and trinucleate cells, chromosomal breaks, nuclear vacuolation, and elongated chromatin indicating a dangerous mutagenic and carcinogenic effects.

Keywords: *Allium cepa,* chromosomal aberrations, dye effluent, mutagenicity.

INTRODUCTION

There is a growing sense of global urgency regarding environmental pollution by a variety of pollutants. Contamination of the environment results in enormous damage both at the physiological and genetic levels of all living organisms. Textile industries have shown a significant increase in the use of synthetic complex organic dyes as colouring material such as direct dye, azoic dye, basic dye, vat dye, azo dye, nitro, nitrosodye and anthrax-quinone dye etc. Among them, azo dyes are of major concern for human health (Prakash & Chaurasia 2020 a, b). In textile production, opportunities exist for the release of potentially hazardous compounds into the ecosystem. Studies of mutagenicity in plants are essential because of their importance as food sources and plants cannot escape from their site and may, therefore, be directly attacked by these mutagenic agents. Response of the plant genetic material to the presence of potential cytotoxic and genotoxic substances in aquatic environments was routinely used to evaluate the presence of toxic substances (El Shahaby 2003). The *Allium* test provides a rapid screening procedure for chemicals, pollutants, contaminants, etc. which may represent environmental hazards (Pathirathne et al. 2015). Levan (1938) showed that colchicine could cause spindle disturbances and polyploidy in *Allium* root meristem cells. Observations of the root system of *A. cepa*, has shown that this plant is particularly sensitive to the harmful effects of such environmental contaminants. The present investigation was under-

taken to study the effect of untreated effluent on chromosomes in the root meristem of *A. cepa*.

MATERIAL AND METHODS

Fresh bulbs of uniform size of onion var. LCM-85 were used in the present investigation. The dye industry effluent was collected from the discharge outlet of a medium to large-scale industry located near Tirupur town of Coimbatore district, Tamil Nadu, India. Healthy and uniform bulbs of *A. cepa* were used as a test system. Outer scales were removed and apices of the root primordia exposed (Grant 1982). Bulbs were germinated in a sand-saw dust mixture for 72 h at 25 ± 1° C in dark. When the bulbs started rooting (1–1.5 mm length), they were transferred to glass bottles containing distilled water (for control) and to glass bottles containing effluents. The treatment was carried out for 12, 24, and 36 h of duration. The experiment was conducted at room temperature (25 ± 1° C). Five bulbs were used for each treatment. Following treatments, the bulbs were transferred to distilled water up to 72 h for recovery. Following recovery, bulbs were transferred to the sand-filled tray and allowed for rooting which was followed by cytological studies. 2–4 root tips from each bulb (control as well as treated) were collected and transferred to 3:1 fixative (absolute alcohol : glacial acetic acid) for 24 h. Root tips were hydrolyzed in 1N HCL at 60° C for 5–10 min and squashes were made in 2% acetocarmine. About 500 dividing cells were scored for each dose for observing various kinds of aberrations and for studying mitotic index. Mitotic index (MI), Relative division rate (RDR), and Relative abnormality rate (RAR) were calculated using the following formulae:

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

$$RDR = \frac{\text{\% of dividing cells in treatments} - \text{\% of dividing cells in control}}{100 - \text{\% of dividing cells in control}} \times 100$$

$$RAR = \frac{\text{\% of abnormal cells in treatments} - \text{\% of dividing cells in control}}{100 - \text{\% of dividing cells in control}} \times 100$$

OBSERVATIONS

The data on MI, RDR and RAR were noticed up to 72 h of recovery period treatments and presented in Table1.The cells showing chromosomal abnormalities at different stages of cell division such as chromosomal breaks, clumping of chromosomes, the stickiness of chromosomes, gaps in the chromosomes, lagging chromosomes,

TABLE 1 : Mitotic index, Relative division rate and Relative abnormality rate in dye effluent treated root tip cells of *A. cepa.*

Treatment / Control	Recovery period	MI (%)	RDR (%)	Total (%)	RAR (%)
Control		13.44	-	-	-
12 h effluent treatment	0 h	4.09	-10.8	62.12	56.24
	24 h	4.24	-10.63	58.56	52.12
	48 h	4.38	-10.46	52.63	45.27
	72 h	**4.4**	**-10.44**	**48.92**	**40.99**
24 h effluent treatment	0 h	3.96	-10.95	72.1	67.77
	24 h	4.12	-10.77	68.21	63.27
	48 h	4.2	-10.67	64.84	59.38
	72 h	**4.34**	**-10.51**	**61.56**	**55.59**
36 h effluent treatment	0 h	3.84	-11.09	85.31	83.03
	24 h	4.0	-10.9	81.81	78.98
	48 h	4.09	-10.8	78.08	74.68
	72 h	**4.23**	**-10.64**	**74.8**	**70.89**

TABLE 2 : Chromosomal abnormalities (%) induced at metaphase stage in *A.cepa* due to dye effluent.

Treatment/ Control	Recovery period	Non-orientation	Star metaphase	Stickiness	Clumping	Rings	Univalents	Breaks/ gaps
Control	-	-	-	-	-	-	-	-
12 h effluent treatment	0 h	3.1	4.1	9.1	10.56	2	2.96	5.52
	24 h	2.84	3.81	8.86	10.42	1.82	2.74	5.2
	48 h	2.22	3.41	8.44	9.76	1.7	2.56	4.96
	72 h	**1.86**	**3.16**	**8.1**	**9.58**	**1.35**	**2.41**	**4.63**
24 h effluent treatment	0 h	2.87	5.21	9.66	11.68	2.43	3.34	6.52
	24 h	2.58	5	9.51	11.56	2.31	3.26	6.22
	48 h	2.5	4.74	9.38	11.44	2.08	3.16	5.97
	72 h	**2.21**	**4.23**	**9.24**	**11.26**	**1.93**	**3**	**5.76**
36 h effluent treatment	0 h	4.11	5.56	9.8	11.86	3.26	4.38	8.4
	24 h	4.07	5.42	9.74	11.7	2.98	3.97	8.2
	48 h	3.72	5.36	9.62	11.66	2.95	3.63	7.64
	72 h	**3.58**	**5.28**	**9.58**	**11.64**	**2.12**	**3.46**	**7.48**

bridges, ring chromosomes, abnormal cells, giant cells, chromatic breaks, micronuclei, disturbed anaphase, polyploid cells, multinucleated cells binucleate cells, multipolar cells, elongated cells, vacuoles in the cells and fragments were recorded at appropriate stages of mitosis and they were presented in Tables 2 and 3 and depicted in Figs 1–9.

DISCUSSION

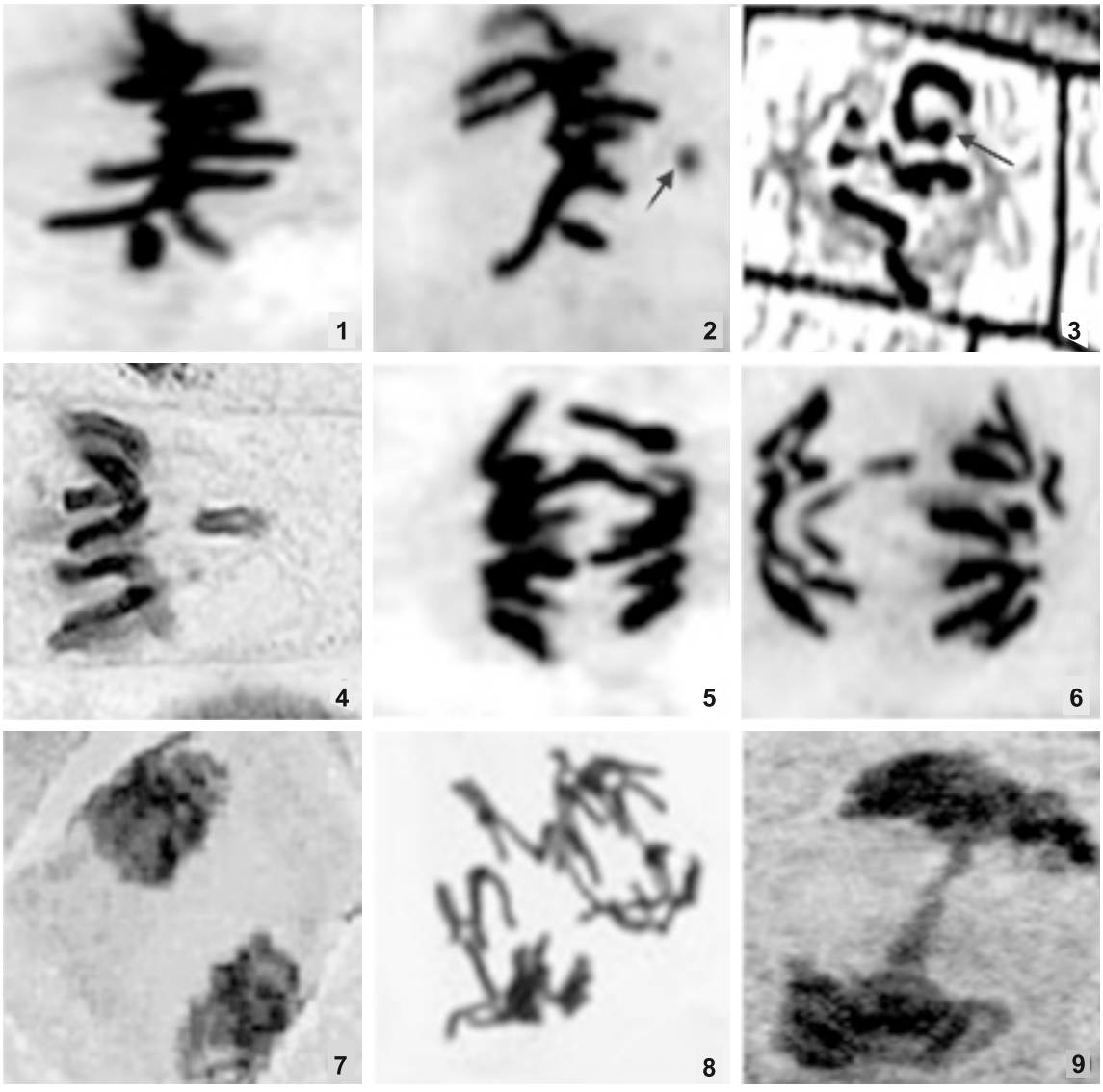
Mitosis in the control root tips was perfectly normal at all the stages. Irrespective of treatment, the division rate was considerably reduced in all the treatments where the recovery period was not given. Mitotic index reduction with an increase in the duration of effluent treatment indicates the total disappearance of the mitotic cycle. Effluents probably cause increased formation of base analogue to act as a mutagen that induces chromosomal abnormalities (Prakash & Chaurasia 2020 a, b). Mitotic inhibition might be due to toxic effect which may cause metabolic imbalance and thus interfere with the synthesis and structure of DNA resulting in physiological effects and structural change in chromosomes during division. Poon (2016) reported that the reduction in MI is due to the disturbance caused by the agent in the internal milieu of the cell during interphase. An increase in mitotic depression and per cent abnormal mitosis is the direct result of various metabolic disturbances which generally lead to inhibition of cell division and produce various types of chromosomal abnormalities. Aleem Yoosuf et al. (2020) reported most of the chromosomal abnormalities such as lesions in prophase,

TABLE 3: Chromosomal abnormalities (%) induced at anaphase, telophase and interphase stages in *A cepa* due to dye effluent.

Treatment/ Control	Recovery period	Laggards/ bridges	Frag-ments	Multi-polar cells	Non-disjun-ction	Micro-nuclei	Giant cells	Binuc-leate cells	Trinuc-leate cells	Nuclear vacula-tion	Elongated chroma-toin
Control	-	-	-	-	-	-	-	-	-	-	-
12 h effluent treatment	0 h	1.9	1	0.21	12.03	0.21	0.25	1.18	0.03	7.22	0.75
	24 h	1.72	0.96	0.16	11.82	0.17	0.18	1	-	6.43	0.43
	48 h	1.46	0.82	0.12	10.03	0.12	0.09	0.86	-	5.76	0.32
	72 h	**1**	**0.64**	**0.08**	**9.83**	**0.08**	**0.04**	**0.61**	**-**	**5.34**	**0.21**
24 h effluent treatment	0 h	2.96	1.55	0.34	12.73	0.41	0.64	2.26	0.04	8.36	1.1
	24 h	2.71	1.4	0.25	12.18	0.37	0.41	1.98	0.01	7.76	0.7
	48 h	2.42	1.21	0.17	11.74	0.27	0.21	1.64	-	7.3	0.61
	72 h	**2.05**	**1.13**	**0.12**	**11.56**	**0.2**	**0.13**	**1.26**	**-**	**6.97**	**0.51**
36 h effluent treatment	0 h	4.44	2.1	0.56	13.69	0.67	1.12	3.26	0.1	9.26	2.74
	24 h	4.1	1.98	0.46	13.19	0.59	1.06	3.03	0.07	8.96	2.29
	48 h	3.7	1.76	0.3	12.83	0.54	0.9	2.76	0.04	8.7	1.97
	72 h	**3.57**	**1.6**	**0.2**	**12.59**	**0.46**	**0.76**	**2.16**	**0.02**	**8.58**	**1.82**

ring chromosome, clumped/sticky chromosome, C-metaphase, diagonal delayed, multipolar in metaphase, bridge, diagonal, multipolar in anaphase and clumped, multipolar, chromosomal breaks in telophase.

Meristematic cells of the untreated control roots showed normal mitotic figures with normal prophase, metaphase, anaphase and telophase. Chromosomal aberrations have been considered as reliable indicators of mutagenic activity (Akyil et al. 2017). Presence of fragments and bridges in the chromosomes observed in the present study is a clear indication of the occurrence of chromosomal breakages. Breakage followed by rejoining may repair the chromosome or may lead to various anomalies such as laggards, micronuclei, etc. The stickiness of chromosomes both at metaphase and anaphase was recorded in the present investigation which reveals the polymerization effect of dye on the nucleic acid of the chromosomes. Similar observations were made with insecticide food additives (Sreela Raj & Omanakumari 2007) and food preservatives (Gomurgen 2005). Scattered chromosomes at metaphase and multipolar observed in some cells, also indicate that mercuric chloride is acting on spindle apparatus, which may ultimately lead to accumulation of polyploid cells. Few cells possessed one to many micro - and meganuclei. The number of micronuclei is either low or higher than that of



Figs 1–9: Cell showing 1. Clumping of chromosomes. 2. Arrow points out breakage of chromosome. 3. Arrow points out ring chromosome at metaphase. 4. Sticky chromosomes. 5. Non-disjunction at anaphase. 6. Lagging chromosome. 7. Non-oriented telophase. 8. Irregular separation of chromosomes at anaphase. 9. Chromatin bridge at telophase.

fragments, indicating that both laggards and/or fragments are involved in micro-nuclei formation (Mojidra et al. 2009, Nusaifa Beevi & Stephen 2010). Further, the occurrence of these genotoxic and mutations can surely cause serious environmental pollution, sometimes to levels that can threaten human health, livestock, wildlife, aquatic lives and indeed the entire ecosystem.

From the results obtained in this study it is concluded that effluent from dye industry induces chromosomal aberrations in root tips of *A. cepa* in a significant way as compared to control. Further, since there was much improvement either in mitotic index and reduction in cytological aberrations in recovery period treatments, it is suggested that 100% purification of effluent discharge has to be followed to reduce the risk for the life of all living organisms.

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RESEARCH ARTICLE

A COMPARATIVE EFFECTS OF BIOSYNTHESIZED AND SYNTHETIC NANOPARTICLES ON MITOTIC CELLS OF *ALLIUM CEPA*

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SUMMARY The extensive use of silver nanoparticles (AgNPs) in various fields has increased significantly in the recent years and expected to enter the natural environment ultimately affecting biotic factors of ecosystem. In the present study, we examined the comparative effects of biosynthesized and synthetic 20 nm AgNPs on chromosomes of *Allium cepa*. For green synthesis, *Ficus geniculata* a nobel plant of Jharkhand has been used. As compared to the control, the results clearly revealed that the percentage of mitotic index decreased in the synthetic nanoparticles treated root tips whereas root tips treated with biosynthesized AgNPs showed similar results as shown in the control. At concentration of 20 ppm of 20 nm of nanoparticles (NPs), the chromosomal abnormalities like disturbed and sticky metaphase, laggards and bridge at anaphase and telophase, micronuclei and stathmoanaphase were observed whereas at lower concentration of 5 ppm of 20 nm, similar abnormalities were observed except micronuclei but frequency of abnormalities was low. However, no chromosomal aberration was observed in green AgNPs treated root tips cells. Our findings suggest that test plant cells are susceptible to both size and duration of treatment of NPs.

Keywords: *Allium cepa*, green/biosynthesised silver nanoparticles, *Ficus geniculata,* dynamic light scattering, stathmoanaphase.

INTRODUCTION

Nanoparticles (NPs) are the most popular revolutionary materials for modern industrial product especially used in day-to-day activity. Among various NPs, silver nanoparticles (AgNPs) are used as one of the key components of cosmetics, toys, packaging material, preservatives, water purifiers and textile materials. Due to its size, it also became very impressive material for modern medical scientists to facilitate target therapy in several diseases. Wide usage of AgNPs in wound dressing, impregnated catheters in therapeutic applications can cause easy entry of AgNPs into the cells but few reports on the toxicity of AgNPs are also available (Patolla et al. 2012). Silver based compounds arouse significant interest in food packaging field for they are most promising inorganic antibacterial agents against several bacteria and fungi (Becardo et al. 2016, Siqueria et al. 2014, Derbalah et al. 2011). Through

inhalation exposure, AgNPs are deposited in olfactory mucosa and subsequently translocated to olfactory nerves which may impair the functioning of brain cells (Rahman et al. 2009). In *Lemna gibba* growth inhibition was observed with decrease in frond number with increase in concentration of AgNPs (Yin et al. 2011). Therefore, directly or indirectly its entrance into the environment and ecosystem are also increasing day-by-day. In many recently published papers, it is reported that despite many promises of AgNPs, there are some unknown risks which have not been adequately assessed before its customary use in various fields. It is also reported that barring mercury, silver is more toxic in aquatic plants and animals as compared to other metals. Hence, it is classified as an environmental hazard by US Environmental Protection Agency (EPA). AgNP itself is not toxic but increases the effectiveness of delivering toxic silver ions to locations where they can cause toxicity (Rani et al. 2009). Most of the publications practically identify the release of silver ions and reactive oxygen species (ROS) production, which cause cellular damage. Some of them describe the size dependent toxicity, as size of the NP is inversely proportional to the toxicity observed (Akter el al. 2018, Antony et al. 2015, Liu et al. 2010, Mili et al. 2015). The interaction of NPs with plant cells has not been fully elucidated. There have been conflicting reports on the translocation, absorption, accumulation and toxicity of NPs in various plants. Many researchers have synthesized NPs using microorganisms, animals such as fish and plant sources. In case of plant sources, different plant parts such as leaf, stem, root, seed and fruit have been used for the synthesis of biosynthesized/green AgNPs. In the present study, leaf extract of novel plant of Jharkhand, *F. geniculata* has been used for the synthesis of eco-friendly gAgNPs. There has not been any report on the green synthesis of AgNPs using either leaf extract or any other part of *F. geniculata*. *F. geniculata* is known as putkal in Jharkhand and an indigenous plant of this state. This plant is not much exploited in the field of research till now. Leaves are consumed by tribals for making pickle, saag and its dried form used in pulses and for making *chutney*. It has medicinal values as it contains polyphenol as a largest group (Kumari et al. 2019). So it is used in treatment of neuro-degenerative diseases. Since 1920, to evaluate the mutagenic potential of various mutagens, cytotoxicity and genotoxicity tests have been widely used through different types of chromosomal aberrations. Use of plant material for genetic test is recommended by various national and international environmental entities, such as WHO (World Health Organization), UNEP (United Nations Environment Programme) and EPA (Ma et al. 1995). As plants are more preferable test materials for toxicity test, among which *A. cepa* is a favoured test material to observe cytotoxicity and genotoxicity due to its easy availability, large size and less number of chromosomes. Rapid root growth helps to collect the meristematic root tips with plenty of dividing cells, It was first introduced by Levan in 1938 (Fiskesjo 1985). The present study deals with the synthesis of non-toxic gAgNPs that could be a

better substitute of synthetic AgNPs because every day human beings are being exposed to synthetic AgNPs through different sources like environment, household, textile, medical field etc. As there is limited number of well-controlled studies on the potential toxicity, these studies tend to suggest that NPs can induce cytotoxicity as well as genotoxicity in living beings. The growing public debate on toxicity of exposure to synthetic AgNPs has yet not thoroughly established. Therefore, it is imperative to determine a comparative assay for evaluation of the biological hazards of green and synthetic AgNPs. The findings of the present study will point out how and at what level synthetic AgNPs are affecting the nucleus as well as cell.

MATERIAL AND METHODS

F. geniculata is a large tree belonging to family Moraceae. It is the source of underutilized leafy vegetables. The vegetative characteristics were highly variable; these are generally organized as stipules, leaves and fruits. A pair of stipules encloses the end of each twig forming a sheath for the new leaf. Leaves are clustered, alternate, glabrous, ovate, oblong to oval, 3.5–8 in by 1.8–4 in. Fresh and healthy leaves of *F. geniculata* were collected locally and rinsed thoroughly first with tap water followed by distilled water to remove all the dust and unwanted particles. Leaves were dried at room temperature under clean environment. After 3–4 d, dried leaves were powdered by using mixer grinder. Then, 40 g of leaf powder was weighed and put into a beaker with 1000 ml of double distilled water and boiled at 100º C for 10 min. The aqueous extract was cooled at room temperature and then filtered using Whatman filter paper 1. Finally, filtrate was collected and the aqueous extract of faint yellow in colour was stored in the refrigerator at 4º C. The crystal powder of silver nitrate ($AgNO_3$) was weighed and dissolved in 100 ml of double distilled water to prepare 1 mM $AgNO_3$ stock solution. Then, extract of $AgNO_3$ at a ratio of 1:3 (v/v) was mixed in 100 ml beaker which shows reddish brown colour. The beaker was completely wrapped with an aluminium foil and kept for 24 h in the dark room at room temperature (27º C), for bioreduction process. After completion of the reaction, a visible colour change from reddish brown to colloidal brown indicates formation of AgNPs. Then, the reaction mixture was centrifuged at 11000 rpm at 25º C for 30 min and pellet was collected in Eppendorf tubes and kept in silica based vacuum dessicator (Tarson vacuum dessicator) for drying. The formation and size of AgNPs was confirmed by different characterization procedures.

Characterization of synthesized silver nanoparticles

Characterization was done for the size, shape and number of AgNPs. Several instrumental techniques are used for this purpose, such as UV-Visible spectroscopy and dynamic light scattering (DLS). The synthesized bioreduced AgNPs in colloidal brown solution and pellet were analysed by UV-Visible spectroscopy (Cecil instrument Cambridge, England and model no CE 7200, 7000 series) at the wavelength of 200–800 nm. For spectra analysis 0.1 mg of pellet and 100 µl of colloidal brown solution were diluted with 900 µl

of double distilled water separately. Both these solutions were used for observing the absorption peak. For observation of peak, double distilled water was used as a blank. For DLS analysis, synthesized AgNPs were studied using Nano ZS, Malvern Instruments Ltd, UK in a disposable cuvette at 25º C and results were analysed using zeta-sizer 7.01 software. The size of the particles was determined by zeta sizer and potential of the particle by zeta potential. All measurements were performed in triplicate with a temperature equilibration and the high multi-model resolution is set for data processing.

Treatment of *A. cepa* cells and microscopic examination

Synthetic AgNP suspension was procured from Sigma Aldrich, the manufactured characteristic of particles are of size 20 nm, purity 99.7% concentration 0.02 µg/ml. For experimentation, three different concentrations i.e. 20 ppm, 10 ppm and 5 ppm of 20 nm synthetic NPs were prepared from stock solution of 20 nm. Healthy seeds of *A. cepa* were collected from ICAR, Plandu. Seeds were soaked in double distilled water for 9 to 10 h. Soaked seeds were kept on moist filter paper for germination in petri dishes separately. After germination they were transferred to different concentrations of synthetic AgNPs and in the green AgNPs synthesized from leaf extract of *F. geniculata* for different time intervals (Table 1). For microscopic examination, root tips around 1–2 cm were cut with sterilized blade and fixed in Carnoy's fixative (1:3 glacial acetic acid: absolute alcohol) for 24 h and then transferred to 70% alcohol for preservation. For cytological study, slides were prepared by squash technique, using 1.5% acetocarmine as a stain. Slides were observed under Magnus s/n: C197050239 microscope and photographs were taken in 40 × and 100 ×**.** The mitotic index and total abnormality percentage (Abn%) were calculated using following formulae:

$$MI\% = \frac{\text{Total number of dividing cells}}{\text{Total cell count}} \times 100$$

$$Abn\% = \frac{\text{Total number of abnormal cells}}{\text{Total number of cell divisions}} \times 100$$

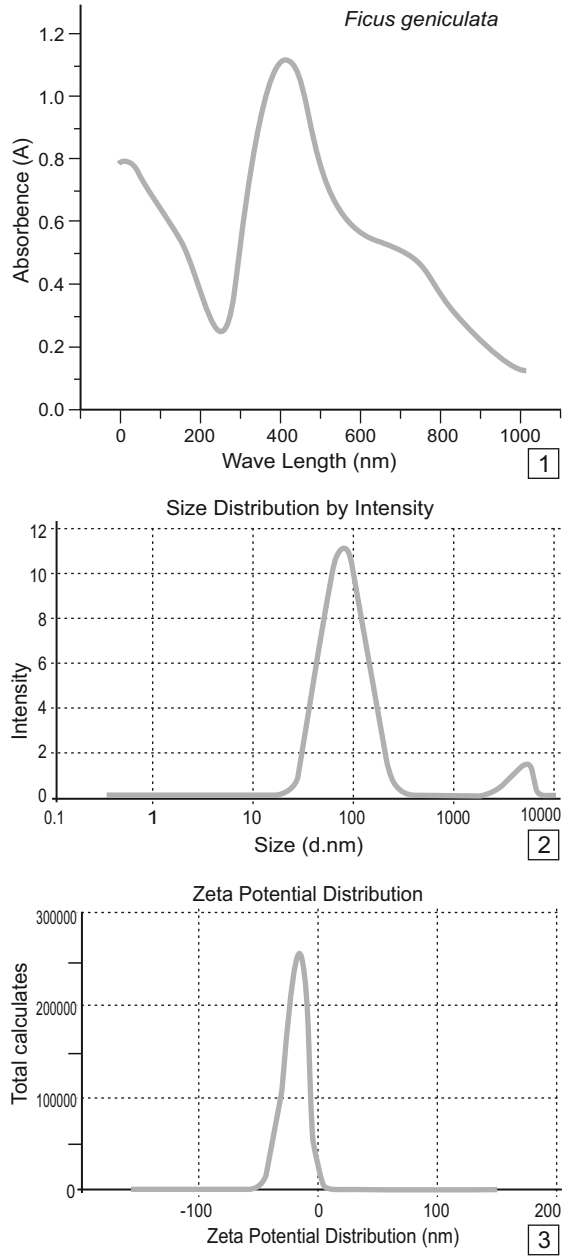
OBSERVATIONS

F. geniculata was used for biosynthesis of AgNPs. Aqueous extract of leaf powder was able to reduce Ag + into Ag^0. However, after addition of $AgNO_3$ to extract, the pH value was maintained because it was observed that any variation in the pH from 8 affects the synthesis of NPs. Then, the reaction mixture kept under incubation at 28º C for 24 h. The colour of the reaction mixture changes from pale yellow to brown indicating the formation of AgNPs. When pH of reaction mixture was above or below 8, then after completion of AgNPs synthetic process, it did not show any peak in UV-Vis spectroscopy characterization in the range of 400–420 nm, which is the characteristic limit of AgNPs.

UV-Vis-Spectroscopy analysis: UV visible spectroscopy of AgNPs formed from extract of *F. geniculata* was done by measuring the surface plasmon resonance (SPR) over the wavelength range of 200–800 nm. Usually, for confirmation of synthesis of AgNPs, directly reaction mixture

is used for characterization but in the present study reaction mixture did not show any positive sign of synthesis of AgNPs. Therefore, in successive trial, first mixture was centrifuged and then supernatant and pellet were separately used for characterization, but in UV-Vis spectroscopy supernatant did not show any peak while double distilled water diluted pellet showed presence of AgNPs in the range of 400–420 nm. Various studies have shown that spherical shaped AgNPs have absorption band at around 400–420 nm in UV-visible spectra (Stepanov 1997). In the present study, the absorbance peak at 402 nm confirms the presence of AGNPs (Fig.1). There was no significant change either in colour or in SPR peak after 24 h of incubation, even after one month by using refrigerated sample at 4º C. Therefore, through trial and error, it is established that pH should be maintained and instead, supernatant pellet could be used for biosynthesis of AgNPs.

Dynamic Light Scattering (DLS) analysis: The DLS size distribution image of bio-synthesised AgNPs is shown in Fig. 2. The size distribution of silver nanoparticles range is 4.6 nm to 111 nm. After final calculation, average hydrodynamic size (z-average diameter) of AgNPs was recorded as 42.83 nm with polydispersity index (PDI) 0.629 which indicates a slightly high polydisperse sample with multiple particle size populations present in the AgNP. The zeta potential of the biosynthesised AgNPs was equal to 20.2 mV which indicates that synthesized AgNPs are fairly stable (Fig. 3). The presence of single sharp peak both in size and zeta potential shows that the



Figs 1–3: Characterization of biosynthesized AgNPs biosynthe sized from *F. geniculata.* 1. UV-Vis spectroscopy showing the absorption peak at 402 nm. 2. Graph showing average hydrodynamic size of nanoparticle. 3. Graph showing Zeta potential of nanoparticle.

quality of AgNPs was good and also there is sufficient repulsion between the synthesized NPs and there will be no tendency of particles to assemble together and precipitate subsequently (Roy et al. 2013). The surface of the AgNPs is negatively charged and dispersed in the medium.

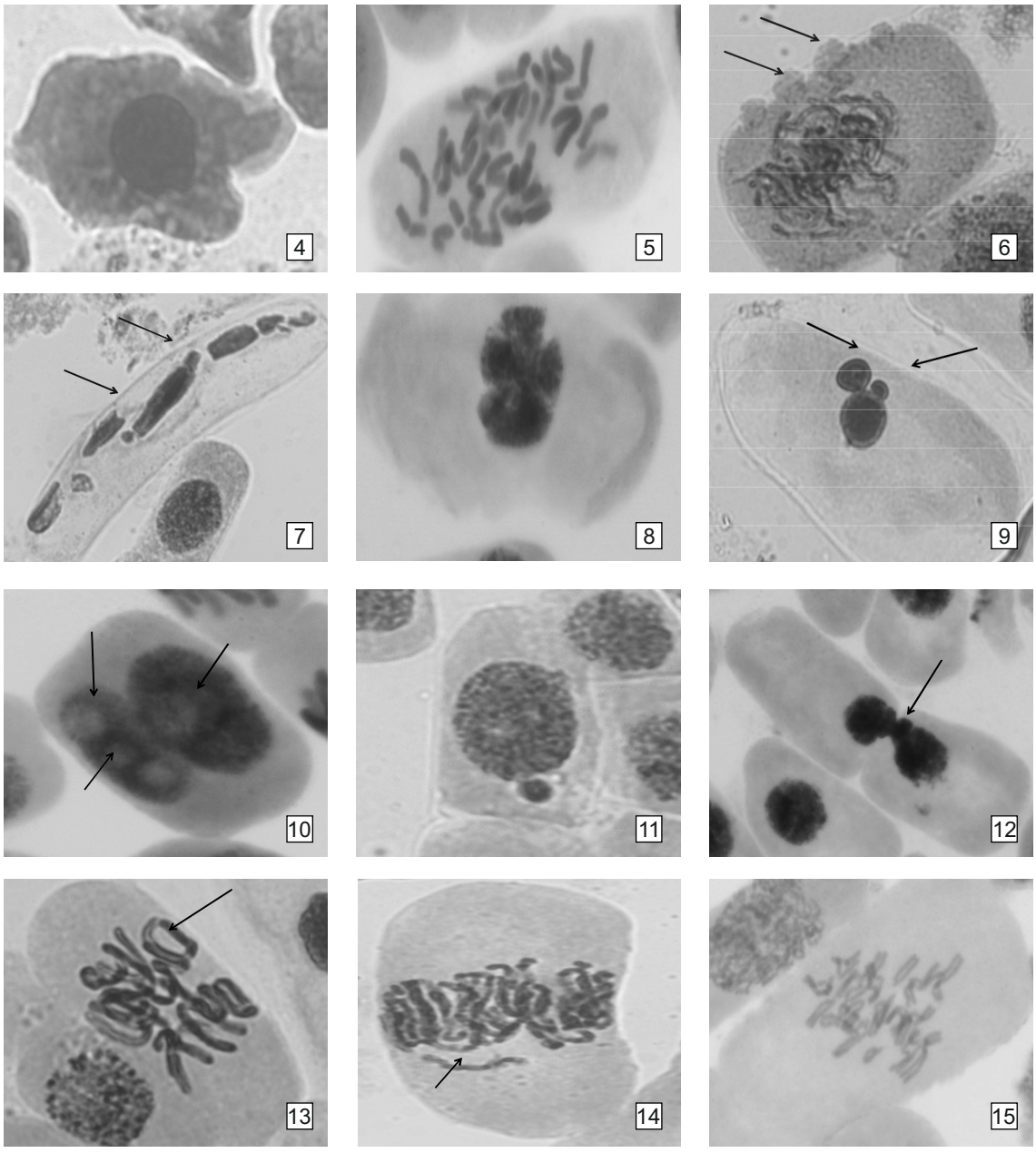
Mitotic abnormalities: A mitodepressive effect of AgNPs on the root tip cells of *A. cepa* has been observed. AgNPs show negative effect on mitotic index (MI) when compared with control. The inhibitory effects on different phases of mitotic division were duration and concentration dependent (Table 1). Decrease in the percentage of MI from 5 ppm to 20 ppm/1 h to 5 h in five replicates indicates that AgNPs interfere in the normal cell cycle. Various types of chromosomal aberrations were also observed (Table 1, Figs 4–23). The whole experiment was carried out in five replicates.

When the root tips were treated with high concentration of AgNPs such as 10 ppm for 5 h and 20 ppm for 1, 3 and 5 h, high level of chromosomal stickiness at metaphase stage was observed. The most common abnormalities observed in all concentrations of AgNPs exposed for different time periods were appearance of laggards, bridges at anaphase and telophase. The occurrence of different types of bridges like multiple bridges at anaphase, net like bridges, bridges with laggard, dicentric chromosome with

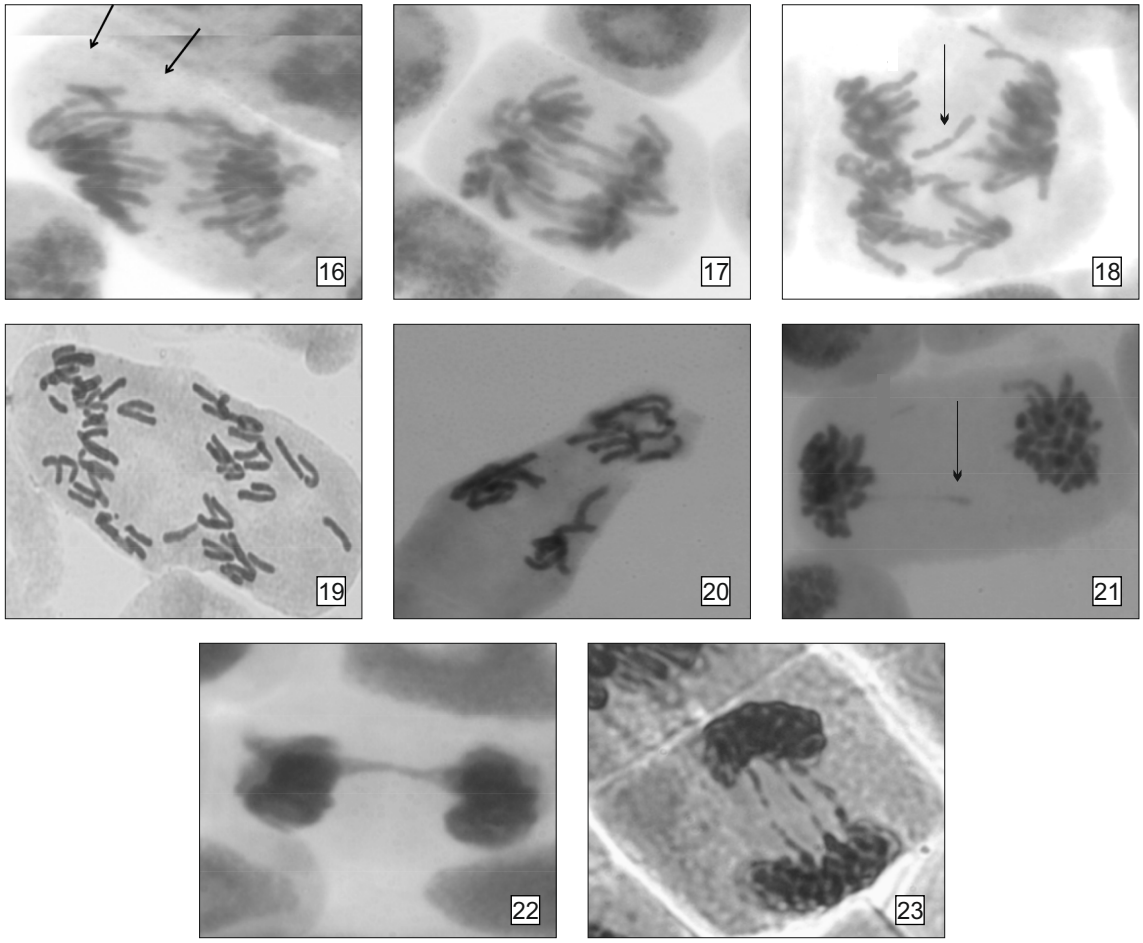
TABLE 1: Effect of gAgNPs and synthetic 20 nm AgNPs on root tip cells of *A.cepa*.

AgNPs		TCC	NCD	MI% ± SD	TAC	BB	BD	SN	LG	MN	NPB	PC	SK	SM	Abn% ± SD
	Control	3000	603	17.36 ± 1.23	-	-	-	-	-	-	-	-	-	-	-
	1 h	3000	610	17.53 ± 1.72	1	-	-	-	-	-	-	-	-	1	0.16 ± 0.15
gAgNP	3 h	3000	598	17.06 ± 1.76	-	-	-	-	-	-	-	-	-	-	-
	5 h	3000	596	16.96 ± 1.82	1	-	-	-	-	-	-	-	-	1	0.16 ± 0.15
	1 h	3000	546	18.20 ± 1.87	45	5	9	1	11	2	1	1	2	14	8.24 ± 0.64
	3 h	3000	546	18.20 ± 1.74	52	4	10	3	8	6	2	1	5	13	9.52 ± 0.52
20 ppm	5 h	3000	521	17.36 ± 2.53	63	6	15	2	10	8	1	5	2	14	12.09± 0.58
	1 h	3000	571	19.03 ± 1.79	31	1	7	1	7	2	2	1	2	8	5.42 ± 0.42
	3 h	3000	563	18.76 ± 1.14	36	2	4	3	8	4	2	3	4	6	6.39 ± 0.44
10 ppm	5 h	3000	549	18.30 ± 2.03	40	2	7	2	10	4	1	1	3	10	7.28 ± 0.42
	1 h	3000	591	19.70 ± 0.92	16	3	1	0	4	3	0	1	1	3	2.70 ± 0.37
	3 h	3000	587	19.56 ± 2.81	22	0	4	1	7	1	1	1	2	5	3.74 ± 0.51
5 ppm	5 h	3000	574	19.13 ± 1.43	23	1	3	1	3	2	2	2	4	5	4.00 ± 0.84

gAgNP- biosynthesized silver nanoparticles, TCC- Total cell count, NCD- Number of cell division, MI-Mitotic index,TAC- Total abnormal cell, BB- Bleb, BD- Bridge, SN- Star shaped nucleus, LG- Laggard, MN- Micronuclei, NPB- Nucleoplasmic bridge, PC- phagocytic cell, SK- Stathmokinesis, SM- Sticky metaphase, Abn- Abnormality, ± - Standard deviation.



Figs 4–15: Effect of 20 nm AgNPs on *A. cepa.* Abnormal cells showing 4. Phagocytosis. 5. Polyploid cell. 6. Blebs (arrows). 7. Karyorrhexis (arrows). 8. Star shaped nucleus. 9. Multiple nuclear buds (arrows). 10. Binucleate cell with abnormal vacuolation (arrows). 11. Micronucleus . 12. Nucleoplasmic bridge (arrow). 13. Ring at metaphase (arrow). 14. Abnormal metaphase with a laggard (arrow). 15. Metaphase chromosomes with separated chromatids.



Figs 16–23: Effect of 20 nm AgNPs on *A. cepa.* Abnormal cells showing 16. Anaphase bridge with one complete chromosome at one pole (arrows). 17. Multiple bridges at anaphase. 18. Multipolar anaphase with dicentric bridge (arrow). 19. Stathmo- anaphase, 20. Tripolar anaphase. 21. Telophase with laggard (arrow). 22. Telophase with a single bridge. 23. Telophase with multiple bridges.

a bridge were the characteristic features and indicate cytotoxic nature of AgNPs. AgNPs have the ability to induce polyploidy in root tip cells of *A. cepa* (Fig. 5). These results are in agreement with those obtained by treating cells with chemicals such as colchicine (Ramel 1969). Similar results were also observed in root tip cells of *Vicia faba* treated with 'Rogor' (Soheire et al. 1974). The author considered it as being a fore-step of the complete disturbance of spindle (Amer 1965). Laggard at metaphase was also observed as a result of inappropriate congression may be due to the failure of spindle kinetochore interaction. Interphase cell with micronuclei was a common feature in high concentration. Stathmoanaphase and tripolar anaphase are the

interesting abnormalities which might have caused by spindle disorientation. Non-disjunction during anaphase in which one complete chromosome moved to one pole with anaphase bridge in the same cell, and occurrence of anaphase bridge with one dicentric chromosome as laggard are two novel findings of the present study (Figs 16, 18). In addition to the above mentioned abnormalities, binucleate interphase cells with abnormal vacuolation were also observed (Fig. 10). Separation of chromatids at metaphase and stickiness in chromosome in different concentrations were observed (Fig. 15). The phosphoryalation of histone H3 is required for proper chromosome condensation and segregation (Wei et al. 1999). When treated with AgNPs, phosphorylation of H3S10 occurs which is caused by abnormalities in actin polymerization and depolymerisation leading to activation of Aurora kinases (Zhao et al. 2017a). Recent studies showed that pH3S10 induced by AgNPs can also occur via the activation of mitogen-activated protein kinase (MAPK) pathway specifically the Jun-N-terminal protein kinase (JNK) and extracellularly signal regulated kinase (ERK) pathway (Zhao et al. 2017b), thus various observations clearly manifest that H3 phosphorylation plays an important role in mitotic chromosome condensation. Hyperphosphorylation and site specific phosphorylation of core H3 protein may be the cause of premature chromatin condensation. Appropriate condensation of mitotic chromosomes is essential for the precise segregation of sister chromatids into daughter chromosomes and ultimately into two daughter cells. Recent studies reveal that SMC protein are incriminate as heavy hitter in mitotic chromosome condensation. It was also observed that mutation in SMC protein affects mitotic chromosome condensation in yeast (Saka et al. 1994). Breakage-fusion-bridge cycles might create dicentric chromosome that can cause genome instability (Fig. 18). Prolonged AgNPs exposure can increase the number of micronuclei (Fig. 11) indicating that AgNPs can promote some of the genetic changes found in cancer cells (Garcia et al. 2019). As was already reported by Garcia et al. (2019), when epithelial cells treated with higher concentration of AgNPs showed positive kinetochore MN while in the present study, when plant cells were treated with different concentrations and duration of AgNPs, cells with acentric fragments were observed very frequently; occurrence of acentric fragments which was common feature at higher concentration that may be a precursor of kinetochore negative MN. Thus, in AgNPs exposed plant cells there is prevalence of kinetochore negative MN instead of kinetochore positive MN. Treatment of plant cells with AgNPs can produce excess ROS which can increase the oxidative stress causing lipid peroxidation and damages cell membrane permeability, cell structure, directly damaging protein, DNA and consequently result in cell death (Arruda et al. 2015, Yuan et al. 2018). Thus, bleb formation (Fig. 6) observed here might be the consequence of damaged cytoskeleton. Study also reveals that nucleoplasmic bridge (Fig.12) increased significantly in dose related manner after exposure to ROS generated by activated

neutrophils, super-oxide and hydrogen peroxide (Umegaki et al. 2000). As AgNPs increase the ROS, occurrence of NPB may be the toxicity of AgNPs. Among various clastogenic and non-clastogenic aberrations caused by AgNPs, phagocytosis, star-shaped nucleus, bleb, ana-phase bridge with one dicentric chromosome as laggard, non-disjunction during anaphase in which one complete chromosome moved to one pole with anaphase bridge, were observed for the first time as far as our knowledge goes.

DISCUSSION

Silver nanoparticles of 20 nm size impede the different stages of metaphase. In comparison to the control, variation in MI and various chromosomal aberrations like ring at metaphase, dicentric chromosome bridge at anaphase and telophase were observed. The significant nuclear and morphological variations observed are, phagocytosis, nuclear budding, karyorrhexis, blebs, star-shaped nucleus. Cytological findings which were obtained from this study showed that synthetic NPs are potential danger along with chromosomal aberrations. It is also affecting shape of nucleus as well as cells. gAgNPs synthesized from leaf extract of *F. geniculata* showed non-toxic effect, unaltered cell division without any chromosomal abnormities. Therefore, this indigenous tree of Jharkhand could be potential source of gAgNPs for its production at commercial level. At the same time, it will encourage the poor tribals and farmers of Jharkhand to grow more and more trees to enhance their livelihood.

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RESEARCH ARTICLE

FLOWCYTOMETRIC ANALYSIS OF INDUCED POLYPLOIDY IN *ANDROGRAPHIS PANICULATA* (BURM. F.) NEES

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SUMMARY *Andrographis paniculata* is one of the important medicinal taxa of the family Acanthaceae and is known for the production of secondary metabolite, andrographolide. In order to enhance andrographolide content, polyploidization using colchicine was conducted in the present study. 0.1% colchicine treatment for 12 h induced tetraploids in this plant. Tetraploidy was confirmed by flowcytometric analysis which is the most effective method for detecting induced changes in ploidy level. The induced tetraploidy was associated with certain changes in leaf characters such as larger stomata, increase in cholorophyll content and decrease in stomatal density and stomatal index.

Keywords: *Andrographis paniculata*, polyploidy, colchicine, tetraploids, flowcytometry.

INTRODUCTION

Polyploidy is responsible for the heritable increase in genome copy number. It is the presence of more than 2 genomes in a cell (Lutz 1907)**.** It has been a significant and eminent process in the evolutionary history of plants (Adam & Wendle 2005). 70% of land plants and 95% of ferns have polyploidy in their evolutionary history (Otto & Whitton 2000). Polyploidy determination is an important tool for crop improvement. Polyploidy is involved in developing plants with higher amount of biologically active secondary metabolites (Audris et al. 2010). Besides naturally occurring polyploidy in plants, it can be induced artificially using agents like colchicine (Blakeslee & Avery 1937). Application of such agents on plants induces chromosome doubling and variations. Colchicine is an antimitotic agent used in the studies related to doubling of chromosomes. It is widely known as mitotic poison which blocks microtubule formation by binding to β-tubulin and prevents tubulin dimer formation (Chaikam et al. 2019). Colchicine has high affinity to tubulin subunits of microtubules resulting in the inhibition of spindle fibre formation and its function during chromosome replication and cell division (Li et al. 2018). As microtubules are involved in chromosome segregation, colchicine induces polyploidy by preventing chromosome segregation during cell division which results in half of the gametes containing doubled chromosome number than usual while the other half of

the gametes without any chromosomes and produces embryos with doubled chromosome number (Ade & Rai 2010). For the improvement of many plant species and hybrids, artificially induced polyploidy plays a very important role (Paulo et al. 2000).

Andrographis paniculata is an annual herbaceous plant belonging to Acanthaceae. The plant is commonly known as *Kalmegh, Kiriyath, King of Bitters* etc. It is mainly distributed in India, China, Sri Lanka, Taiwan and other South Asian countries (Akataky & Handique 2010). The plant has been used for the treatment of common cold, fever, diarrhoea and respiratory tract infections. For medicinal purposes, the roots and leaves of the plant are used. The plant synthesizes a wide range of bioactive compounds such as diterpenoids, lactones, flavonoids and polyphenols (Negi 2008). The prime and active secondary metabolite produced by the plant is andrographolide. Andrographolide ($C_{20}H_{30}O_5$) is commercially important for its pharmacological activities such as anticancer (Mishra et al. 2015), antiinflammatory, analgesic and antioxidant (Adedapo et al. 2015), hepatoprotective (Li et al. 2007), antihypersensitive, antiviral, anti-fungal, immune enhancement and anti-HIV activities (Abdulaziz et al. 2014). In traditionally propagated plants or in wild plants, the andrographolide content has been estimated to be 2−3% and moreover conventional propagation is extremely low to meet the increasing need of pharmaceutical industries (Pandey & Rao 2017). To overcome these issues, several studies were conducted to enrich the andrographolide content in *A. paniculata* using inducers. Artificial polyploidy using colchicine has been long developed and become an important and common method of breeding for crop improvement. Colchiploidy inhibits cell division (Eigsti 1936). Polyploidy induction expands nucleus and size of the organs resulting in the enlargement of leaves, branches and flowers of the plant (Hosseini et al. 2018). In the present study, we made an attempt to induce polyploidy in *A. paniculata* using colchicine and identify the polyploids using flowcytometry.

MATERIAL AND METHODS

Seeds of *A. paniculata* (KUBH11112) were collected from Botanical Garden of S.D. College, Alappuzha. The seeds were germinated and plants were grown under greenhouse conditions. Seedlings were collected from seed-grown plants and used in the present study.

Freshly prepared filter sterilized aqueous colchicine solution of concentrations, 0.05%, 0.1%, 0.2% were prepared and used. Seeds were pre-soaked in distilled water for 24 h and then drained using blotting paper. Both seeds and seedlings were soaked in different concentrations of colchicine (Hi Media TC030) for 6, 12, 24 and 48 h at room temperature under

dark condition. Treated seeds and seedlings were washed thoroughly with distilled water for 3 or 4 times and planted in pots filled with vermiculite peat (1:3 v/v) mixture substrate and maintained in greenhouse. Seeds and seedlings without treatment were kept as control. The survival rate was determined after one month of treatment.

For flowcytometry assays, 30 mg of fresh leaves from each test plant was cut with a scalpel and placed in the center of a plastic petri dish. 1 ml of ice-cold Otto I solution (0.1 M citric acid, 0.5% Tween 20) was added to the petri dish. The tissue was immediately chopped in the buffer with a new razor blade or a sharp (disposable) scalpel. The homogenate was mixed by pipetting up and down for several times and was passed through a nylon filter (40 µm) and centrifuged at 150 g for 5 min at 4º C. The supernatant was removed leaving approximately 100 ml of the liquid above the pellet. Resuspended the nuclei by gentle shaking and added 100 ml of fresh ice-cold Otto I solution. 1 ml of Otto II solution (0.4 M Na_2HPO_4. $12H_2O$) was added to the nuclear suspension. Then added 60 µl RNase and 60 µl propidium iodide. Incubateed the sample for 30 min at 4º C protected from light (Dolezel et al. 2007). For each sample, 20000 nuclei were analyzed on a flowcytometer (BD FACS Aria ™ II). The leaves from plant without treatment were used as control.

For the determination of polyploidy, stomatal density and size were measured. For stomatal character studies, 5 leaves of same age were sampled from both control and treated plants. The lower epidermis of the leaves was peeled off and placed on a clean glass slide, then a drop of saffranin was added on it and covered with a clean coverslip. The number of stomata and epidermal cells were determined by counting their number from random fields taken from 5 samples using Olympus U-DA microscope with a digital camera DPI 12 to take the photographs.

Stomatal Index (SI) is worked out according to Salisbury (1927) using the formula:

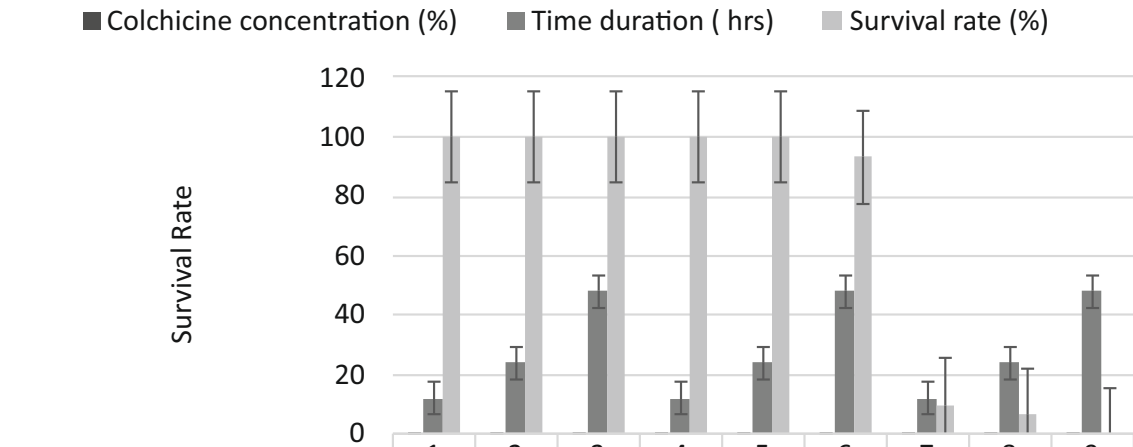
$$SI\,(\%) = (S/S + E) \times 100$$

where, S and E denote the number of stomata and epidermal cells respectively in the microscopic field**.** Length and width of 5 randomly selected stomata were measured from control and treated plants using a calibrated ocular micrometer. Then the mean was calculated for each measurement. The ANOVA results of characteristics of control and tetraploid plants were prepared.

OBSERVATIONS

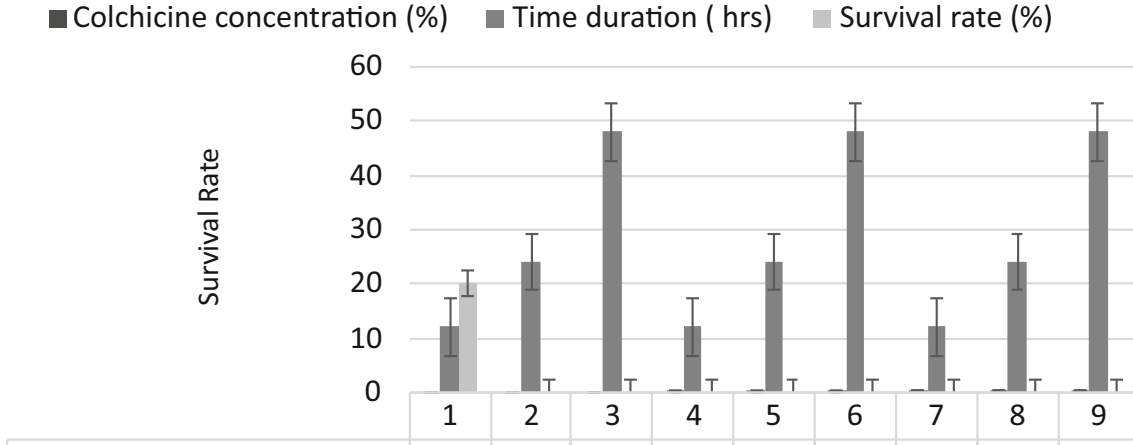
Characters such as DNA content, stomatal size and index, chlorophyll content of treated and control *A. paniculata* were compared to confirm the identity of polyploid plants.

The results on survival rate of colchicine treated seeds and seedlings show the significant effect of colchicine concentration and exposure



	1	2	3	4	5	6	7	8	9
Colchicine concentration (%)	0.05	0.05	0.05	0.1	0.1	0.1	0.2	0.2	0.2
Time duration (hrs)	12	24	48	12	24	48	12	24	48
Survival rate (%)	100	100	100	100	100	93.3	10	6.66	0

1



	1	2	3	4	5	6	7	8	9
Colchicine concentration (%)	0.05	0.05	0.05	0.1	0.1	0.1	0.2	0.2	0.2
Time duration (hrs)	12	24	48	12	24	48	12	24	48
Survival rate (%)	20	0	0	0	0	0	0	0	0

2

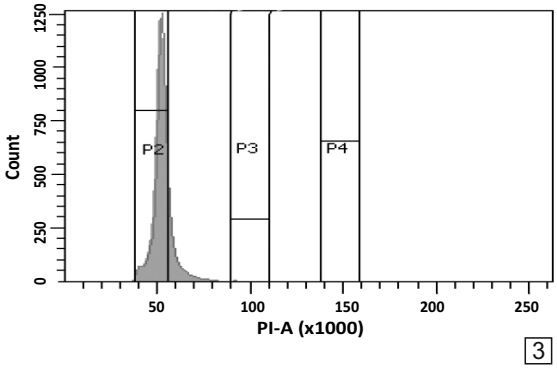
Figs 1 & 2: Effect of colchicine on *A. paniculata*. 1. Seedlings. 2. Seeds.

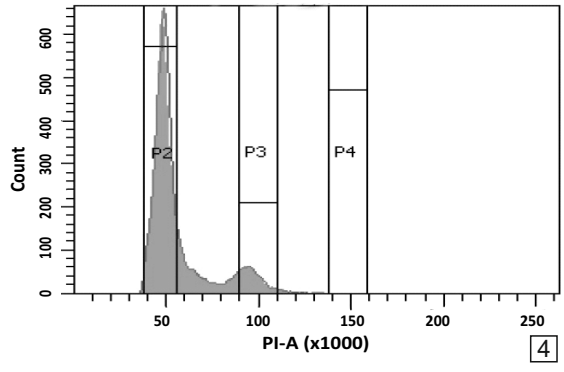
duration. With the increase in concentration and time of exposure, the viability percentage of seeds and seedlings is declined drastically. The higher percentage of survivability was observed for seeds (20%) at 0.05% colchicine for 12 h and for seedlings (100%) at 0.05% and 0.1% for 12, 24 and 48 h. 0.2% colchicine for 48 h is lethal for seedlings. The survival rate of treated seeds and seedlings was measured after 30 d (Figs 1, 2).

The ploidy levels of colchicine-treated and control were analyzed by flowcytometry (Figs 3, 4). The diploid plant (control) containing 2 C DNA showed a peak at the position of channel 50 of relative fluorescent intensity. For ploidy induced (treated) plant with both 2 C and 4 C nuclei showed a peak at channel 50 and 100 respectively.

100 stomata from the lower epidermis of leaves from control and treated plants were taken randomly. The results show that the stomatal size of colchicine treated leaves was larger (13.3 µm/9.31 µm) than that of the control (8.31 µm/4.32 µm) (Table 1).

The chlorophyll estimation values show that the chlorophyll *a* and *b* content of the treated plant is 1.34 g and 0.66 g respectively and that of the control is 0.67 g and 0.09 g respectively (Table 1, Fig. 5).





Figs 3 & 4: Flowcytometric histograms. 3. Diploid plant. 4. Tetraploid plant.

TABLE 1: Stomatal characteristics and chlorophyll contents of control and tetraploid plants.

Experiment	Stomatal length (µm ± SE)	Stomatal width (µm ± SE)	Stomatal Index (% ± SE)	Chlorophyll *a* (mg / g tissue ± SE)	Chlorophyll *b* (mg / g tissue ± SE)
Diploids	8.31 ± 0.005	4.31 ± 0.008	23.07 ± 0.005	0.67 ± 0.005	0.08 ± 0.003
Tetraploids	13.13 ± 0.088	9.31 ± 0.003	20.32 ± 0.008	1.33 ± 0.008	0.65 ± 0.005
Df (n-1) =1 F value	2978.39***	280875.13***	67732.90***	4000.00***	7140.25***

*** Significant F value at $p<0.001$ as determined by One-way Anova.

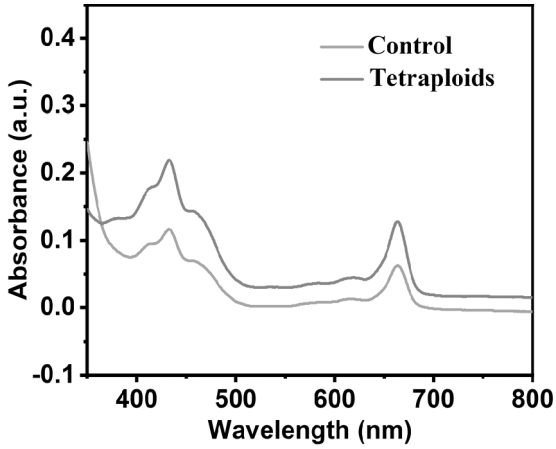


Fig. 5: The absorbance of chlorophyll pigment of control and tetraploids.

DISCUSSION

Colchicine is an antimitotic agent which has been widely used in induction of polyploidy and plant breeding (Urwin et al. 2007). Polyploidy allows plants to increase their yield and improved qualitative characteristics. In the present study, colchicine treatment changed the plants in terms of seed germination rate, growth of the plants, increase in organ size and increase in chlorophyll content which affects the photosynthetic rates of the plants. According to Lan et al. (2020), polyploids possess superior agronomic traits in agri-cultural and horti-cultural industries than diploids. Artificial polyploid induction using colchicine has been an important tool for breeding crops, flowers and economically important trees (Yue et al. 2018).

The first visible action of colchicine treatment in *A. paniculata* was delayed growth of treated seedlings. The same was reported by Omezzine et al. (2012) in *Trigonella foenum-graecum*. The delayed growth rate is due to reduced cell division rate caused by colchicine treatment (Swanson 1957). For identification of polyploids in plants, morphological, cytological, palynological characters of the plants were taken into consideration. Flow-cytometry as an advanced technique for identifying polyploid plants and has been used in the present study. Based on the flowcyto-metric analysis in the plant, the tetraploidy was achieved by applying 0.1% colchicine for 12 h. Higher colchicine concentration (0.2%) and lower concentration (0.05%) did not result in any ploidy level changes in the plant. Accor-ding to Vainola & Repo (2001), 3 *Rhodo-dendron* clones were subjected for different polyploidization procedures using colchicine solution for 24 and 48 h and tetraploids were observed in 0.05% colchicine for 48 h. Thus, it is important to optimize the correct concen-tration of colchicine and the duration of treatment for polyploid induction in different plants. According to Surochita & Debasree (2018), artificial polyploid induction in *A. paniculata* were achieved with 0.3% aqueous colchicine for 24 h. In the present study, tetra-ploids are obtained with 0.1% aqueous colchi-cine solution in 12 h treatment.

Increase in organ size which is an important characteristic feature of polyploids achieved in the present study as the stomatal size increased in treated plants than in the control. Increase in chlorophyll content was also noted in induced polyploid *A. paniculata*.

In the present study, tetraploidy was induced in *A. paniculata* at 0.1% colchicine

concentration for 12 h in seedlings. The variations seen in polyploids were delayed growth rate, increased stomatal size and chlorophyll content and decreased stomatal density and stomatal index than in the control plant.

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