POLYPLOIDY INDUCTION OF OIL PALM THROUGH COLCHICINE AND ORYZALIN TREATMENTS

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ABSTRACT

Germinated oil palm seeds were treated with two antimitotic chemicals, colchicine and oryzalin, to induce polyploidy. The colchicine concentrations used varied from 2.5 to 10.0 mM, and the oryzalin concentrations from 15 μM to 120 μM. The incubation period vary inversely with the chemical concentration from 48 to 6 hr. Colchicine treatments produced nine tetraploids, two triploids and a number of mixoploids. The oryzalin treatments produced four triploids and many mixoploids of 2n+3n and 3n+4n. However, one-way ANOVA showed that none of the treatments were able to induce polyploidy efficiently. Flow cytometry was found to be the most efficient method for detecting induced changes in the genome size or polyploidy level compared to stomata and chromosome counts. One-way ANOVA showed no significant difference in the stomata density between polyploids and controls (2n) while for plant morphology, polyploids seemed to be significantly shorter than their respective controls.

Keywords: tetraploids, triploids, mixoploids, flow cytometry, stomata counts.

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INTRODUCTION

The commercial oil palm *Elaeis guineensis* Jacq. (2n=32) planted in Malaysia is the *tenera* (DxP) fruit type with thick mesocarp and thin-shell. It is a hybrid obtained by crossing the thick-shelled *dura* (D) with the shell-less female sterile *pisifera* (P). This perennial monocot grows to a height of 12 m. It starts to bear fruit as early as three years after field planting and continues to do so for up to 25 years. The oil palm yields 4-6 t ha^{-1} yr^{-1} when at full yield. Currently, major DxP seed programmes are based on very restricted breeding populations derived from a few specific breeding materials, *i.e.* Deli *dura* and AVROS *pisifera* (Kushairi and Rajanaidu, 2000). Realizing this, MPOB (Malaysian Palm Oil Board) initiated and established a sizeable oil palm gene bank with materials collected from Africa and South America since 1975 for conservation as well as to broaden the genetic base of the breeding populations (Rajanaidu, 1994). All these palms are presumably diploid (2n). Rival *et al.* (1997), using flow cytometry, estimated the DNA content for *E. guineensis* as 2C = 3.786 ± 0.125 pg.

Ploidy manipulation has been found to be a valuable tool in the genetic improvement of many plants such as *Solanum* (Chauvin *et al.*, 2003); citrus (Wu and Mooney, 2002); *Scutellaria baicalensis* (Gao *et al.*, 2002); pomegranate (Shao *et al.*, 2003); *Miscanthus sinensis* (Petersen *et al.*, 2003); *Artemisia annua* (De Jesus-Gonzalez and Weathers, 2003); *Allium cepa* (Jakse *et al.*, 2003); *Alocasia* (Thao *et al.*, 2003) and azaleas (De Schepper *et al.*, 2004). Ploidy induction has been carried out for a variety of reasons. In citrus, tetraploid (4n) parents were produced to create seedless triploids by crossing 4n and 2n parents (Wu and Mooney, 2002). In the medicinal plants, *Scutellaria* (Gao *et al.*, 2002) and *Artemisia* (De Jesus-Gonzalez and Weathers, 2003), tetraploidy increases the amounts of the secondary metabolites, baicalin and artemisinin. In azalea (De Schepper *et al.*, 2004) and pomegranate (Shao *et al.*,

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2003), chromosome doubling has been used to obtain new ornamental characteristics. Besides, polyploidy often generates variants that may possess useful characteristics and, by doubling the gene products, polyploids also provide a wider germplasm base for breeding studies (Thao *et al.*, 2003).

Colchicine has been traditionally used to induce polyploids (Blakeslee and Avery, 1937). Other anti-mitotic agents such as amiprophos-methyl or the dinitroaniline herbicides, oryzalin and trifluralin, have also been used on many plants. All these chemicals act by binding to the tubulin dimers preventing the formation of microtubules, and, consequently, spindle fibres during cell division (Petersen *et al.*, 2003). Seeds exposed to these mutagens may give rise to polyploid plants that can be exploited for their improved characters or used in breeding programs. This paper describes the first work on polyploidy induction in oil palm using colchicine and oryzalin as a means to increase the genetic variability in oil palm.

MATERIALS AND METHODS

Plant Materials

The MPOB Research Station in Johor, Malaysia supplied three thousand 30-day old germinated DxP seeds. The seeds were examined to ensure only one embryo in each. They were rinsed several times with distilled water prior to chemical treatment.

Treatment with anti-mitotic agents and planting. Germinated seeds were immersed in 2.5, 5.0, 7.5, 10.0 or 12.5 mM colchicine or 15, 30, 60, 90 or 120 µM oryzalin dissolved in either distilled water only or in distilled water plus 2% DMSO (dimethylsulfoxide), a plant cryopreservant (Chauvin *et al.*, 2003). For the controls, the germinated seeds were immersed in distilled water only or in distilled water plus 2% DMSO. The incubation periods (6, 8, 18, 24 and 48 hr) were varied inversely with the concentrations of colchicine or oryzalin, *i.e.*, the seeds exposed to the lowest concentration were subjected to the longest incubation. Fifteen seeds were used for every control and treatment with five replicates. The incubations were carried out in fume cupboards due to the hazardous nature of anti-mitotic chemicals involved. Upon completion of their incubation, the seeds were rinsed thoroughly with distilled water and planted carefully in polybags filled with a mixture of two parts sand and one part soil. These polybags were then placed in a nursery under 60% sunlight. After one and a half years, the seedlings were examined morphologically and also screened for any ploidy changes using flow cytometry, stomata counts and chromosome counts.

Flow cytometric analysis. Flow cytometric (FCM) screenings were done when the seedlings were approximately one and a half years old. All the surviving seedlings were screened for changes in their ploidy level. At this age, the seedlings were more robust and better able to survive in areas with more sunlight. For FCM analysis *Glycine max* cv. Polanka and untreated *Elaeis guineensis* (tenera) leaves were used as the standard and control, respectively. The first leaf of each oil palm seedling was used to screen for nuclear DNA content, with *Glycine max* cv. Polanka leaves as the external standard (Dolezel *et al.*, 1989). *Glycine max* was chosen because its DNA content (2C=2.5 pg) is quite close to that of the oil palm. The nuclei were extracted by placing 50 mg sliced tissue from mid-leaf in a 50 µm Medicon (Becton Dickinson) container containing 1 ml LBO1 lysis buffer (Dolezel *et al.*, 1989). The buffer contained 15 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1% Triton X-100, pH 7.5, 50 µg propidium iodide and 50 µg RNaseA. The samples were blended for 3 to 5 min, and the suspension nuclei sucked out using a 1 ml syringe and filtered through a 50-µm filcon (Becton Dickinson) into a 10 ml falcon tube. The filtrates were analysed by a FACSCalibur flow cytometer (Becton Dickinson) equipped with an argon ion laser (15 mW) at 488 nm. Histograms were collected over 1024 channels; and, for each sample, 10 000 events were captured.

Stomata counts. Random seedlings identified from flow cytometric analysis as putative triploids, tetraploids or mixoploids together with their respective controls, were further screened by stomata counting. The first leaf was also used for the stomata counts. Small areas at the base, middle and end of the leaf (abaxial side) were covered with a thin layer of clear nail polish and left to dry. Next, a piece of clear adhesive tape was placed on the treated area and lifted swiftly. The taped pieces with the epidermal layer of the leaf were then placed on a glass slide and observed through a 40x phase contrast objective (LeicaQF550W-image analysis workstation). The stomata count was taken three times for each region on an area of 1 mm^2 (field of view), and one-way ANOVA carried out to determine the differences in stomata density between their polyploids and their respective controls.

Chromosome counts. The root tips of identified polyploids and their controls were sampled and pre-treated to increase their metaphase indices. This was done to verify the chromosome numbers of the polyploids and controls. However, many of the seedlings did not have suitable roots and the

chromosome counting could not be done on all of them. The root tips of the controls and polyploids were treated in 2 mM 8-hydroxyquinoline for 5-6 hr at 18ºC. They were subsequently fixed in freshly prepared absolute ethanol-glacial acetic acid (3:1) at 4ºC for 24 hr, followed by another fixation for 18 hr and, finally, stored in 70% ethanol at 4ºC. Metaphase spreads were obtained by a squash and protoplast technique derived from the methods of Ambros *et al.* (1986), Schwarzacher *et al.* (1989) and Maria *et al.* (1995). The root tips were rinsed several times with enzyme buffer (0.01M citric acid-sodium citrate, pH 4.6) to remove the fixative, and a section of approximately 1 mm cut from each root tip meristem as identified by its opaque colour. These materials were incubated in an enzyme solution containing 2% cellulase (Onozuka R10, Yakult Honsha Co., Tokyo) and 20% pectinase (from *Aspergillus niger*, Sigma Chemicals Co., St. Louis, Mo.) in a citric-citrate buffer at 37ºC for 3-5 hr depending on the size of the root tip. The softened tissues were rinsed in enzyme buffer and placed on a glass slide with a drop of 60% acetic acid. A pair of fine forceps was used to squeeze the root tip to expel the protoplasts of the meristematic cells. The protoplast suspension was then covered with a cover slip. A few layers of filter paper were placed over the cover slip and the suspension squashed using firm thumb pressure. The edges of the cover slips were sealed with nail polish to prevent drying out. Slides were screened with a LeicaQF550W (image analysis workstation) using a 40x objective.

RESULTS AND DISCUSSION

Flow Cytometric (FCM) Analysis

Figures 1a to *d* show the histograms of fluorescent intensity peaks for the control *E. guineensis* (DxP, 2n), tetraploid (4n), mixoploid (2n + 4n) and *Glycine max* cv. Polanka (2n). *Tables 1a* and *b* and *Figures 2a* and *b* show the percentages of 2n, 3n, 4n, mixoploids and dead seedlings obtained from the treatments with colchicine and oryzalin, respectively. *Figure 2a* shows the presence of the 4n ploidy state, or tetraploid seedlings, in the colchicine-treated samples. These were not found in the seedlings exposed to oryzalin, showing that colchicine was more successful in inducing chromosome doubling than oryzalin. Awoleye *et al.* (1994) and Petersen *et al.* (2002; 2003) also found that colchicine effectively induced chromosome doubling in cassava and *Miscanthus sinensis*, respectively. In our study, the result may also be due to the higher colchicine concentration used compared to that for oryzalin. *Figure 2a* also shows that colchicine caused a higher mortality than oryzalin treatment. This may be due to chromosome losses or rearrangements and gene mutation (Luckett, 1989; Wan *et al.*, 1989). *Figure 2b* shows several oryzalin-treated seedlings with triploidy. However, most of the seedlings were diploids or mixoploids, similar as in the colchicine treatments.

Effects of Dimethylsulfoxide (DMSO)

DMSO is used as a plant cryopreservant, but it can affect tubulin proteins in microtubules resulting in chromosomal damage (Vannini and Poli, 1983). In this study, one-way ANOVA showed that addition of DMSO had no significant effect in inducing polyploidy in all the treatments. However, one replicate of the treatment with 0.015 mM oryzalin had all the surviving treated seedlings as mixoploids (*Table 1b*). Similarly, in other studies with such as onion gynogenic embryos, addition of 2% DMSO had no effect in all the treatments (Jakse *et al.*, 2003).

Stomata Counts

In banana, van Duren *et al.* (1996) and Azhar *et al.* (2000) demonstrated that stomata density was significantly higher in mixoploid than diploid plants. Whether or not this difference also occurs in oil palm seedlings was investigated. *Figure 3* shows the stomata distribution on the oil palm seedling abaxial epidermis. One-way ANOVA (Minitab 12.1), however, showed no significant difference in the stomata density between the control, diploids and polyploids (*Table 2*). Hence, stomata counting is not an effective method to assess the ploidy level in oil palm. This was not surprising as it is known that various physiological factors, for example, light intensity, leaf development and water content of the plant can influence the stomata density (van Duren *et al.*, 1996). In *Scutellaria baicalensis*, the stomata of autotetraploids were larger but fewer compared to the controls (Gao *et al.*, 2002) and for ornamental Alocasia, the stomatal length increased with the ploidy level (Thao *et al.*, 2003).

Chromosome Counts

The seedlings identified as triploids (*Figure 4a*) and tetraploids (*Figure 4b*), had 48 and 64 chromosomes, respectively, while the diploid controls exhibited 2n=32 chromosomes (*Figure 4c*). On the whole, chromosome count is not a practical way to assess the ploidy level in oil palm due to the difficulty in obtaining suitable root tips and good metaphase spreads.

Morphology of Polyploids

Morphological observations of the seedlings were made at 18, 19 and 20 months and their heights recorded (*Table 2*). One-way ANOVA (Minitab 12.1) showed a significant difference in height between

the polyploids and controls. There have been reports that polyploids are bigger than diploids, for example, banana (Azhar *et al.*, 2000), cassava (Awoleye *et al.*, 1994) and *Scutellaria baicalensis* (Gao *et al.*, 2002). However, this is not so far in oil palm as the polyploids were actually shorter and their leaves thicker and greener than those of the controls (*Figures 5a* and *b*). Shao *et al.* (2003) also observed this on tetraploid *vs.* diploid of pomegranate.

CONCLUSION

This study has demonstrated that in oil palm, the anti-mitotic chemicals, oryzalin and colchicine can increase the ploidy level. However, the stability of the changes needs to be monitored along with that of the other morphological traits. The triploid, tetraploid, mixoploid and control palms will be planted in the field for further study. Other ways of inducing genome doubling should be explored, particularly using *in vitro* embryogenic calli and plantlets. Higher concentrations of oryzalin for different periods of time should also be investigated for a more reliable method of doubling the genome size of oil palm. As colchicine is very toxic, oryzalin being an herbicide can be considered as a preferable alternative (Ramulu *et al.*, 1991; van Tuyl *et al.*, 1992). Besides, in many species, colchicine causes side effects such as sterility, abnormal growth, chromosome losses or rearrangements and gene mutation (Luckett, 1989; Wan *et al.*, 1989). In this study, the most efficient method to analyse for the ploidy level was flow cytometry as it was more accurate and convenient than the conventional methods of chromosome counting and measuring stomatal length or density. This is supported by the findings of several researchers such as Pinherro *et al.* (2000), Thao *et al.* (2003) and Roux *et al.* (2003). However, even though not routinely done, chromosomal counts will still be required for confirmation.

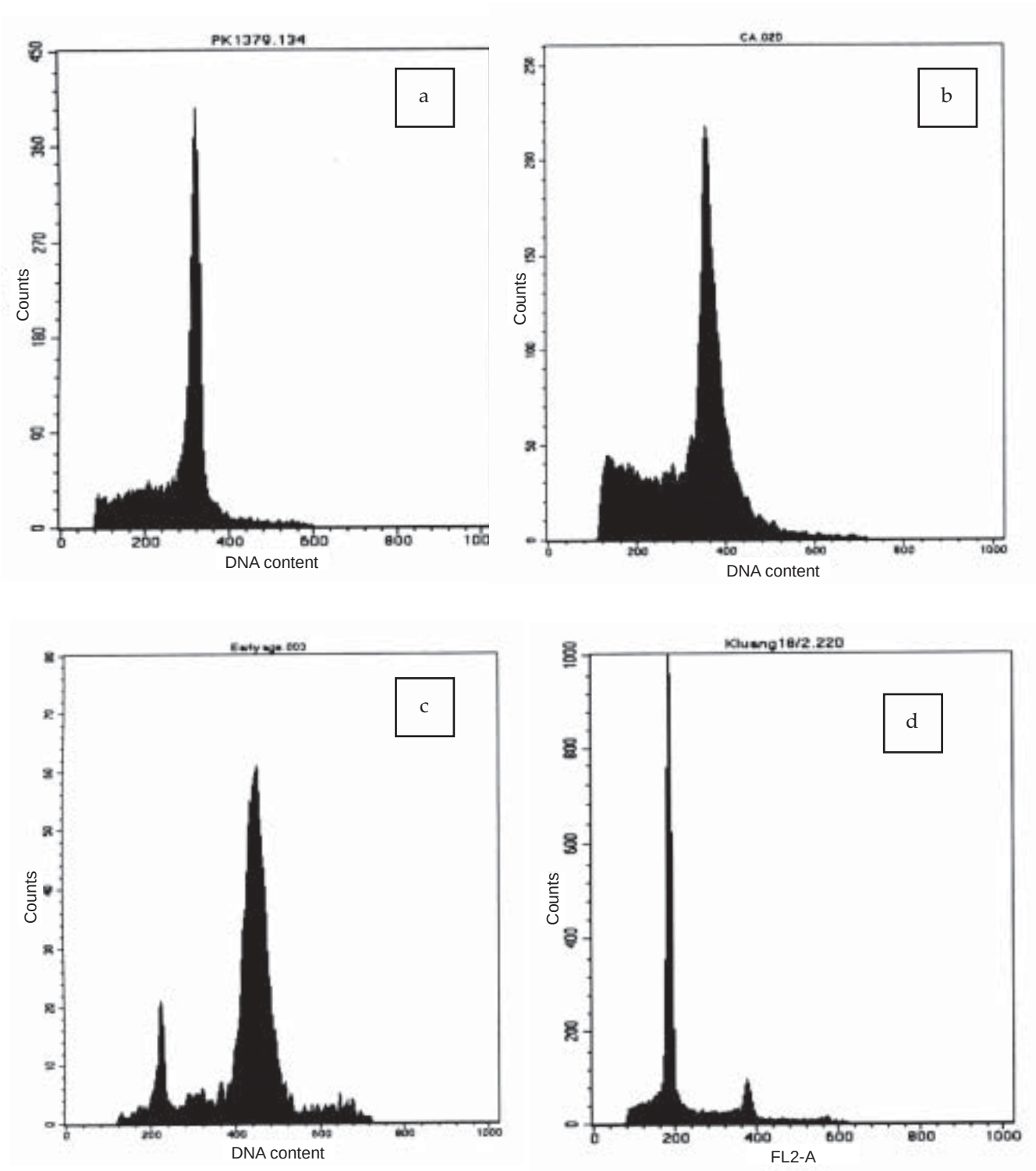

Figure 1. Examples of fluorescent intensity peaks for (a) E. guineensis *(control, 2n) (b) tetraploid, 4n (c) mixoploid, 2n + 4n and (d) standard* Glycine max *cv. Polanka (2n).*

TABLE 1a. PERCENTAGES OF DIPLOID (2n), TRIPLOID (3n), TETRAPLOID (4n), MIXOPLOID AND DEAD SEEDLINGS AFTER TREATMENTS WITH DIFFERENT CONCENTRATIONS AND VARIOUS TIMES OF EXPOSURES TO COLCHICINE (five replicates, r1-r5)

6 hr exposure

12.5 mM (%)					12.5 mM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
6,0, 88,68,0	0,0, 0,0,0	14,0, 0,0,0	20,20, 0,12,6	60,80, 12,20,94	0,0, 59,6,0	0,0, 0,0,0	6,0, 0,0,0	27,6, 19,6,0	67,94, 32,88,100	67,80, 100,94,68	-	-	-	33,20, 0,6,32	73,88, 94,88,60	-	-	-	27,12, 6,12,40

8 hr exposure

10.0 mM(%)					10.0 mM + 2% DMSO(%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
20,0, 49,0,12	0,0, 0,0,0	0,0, 0,6,0	13,25, 51,25,0	67,75, 0,69,88	14,6, 40,0,0	0,0, 0,0,0	6,0, 0,6,6	67,6, 20,38,0	13,88, 40,56,94	73,94, 100,88,94	-	-	-	27,6, 0,12,6	80,88, 100,94,80	-	-	20,12, 0,6,20	

18 hr exposure

7.5 mM (%)					7.5 mM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
0,12, 24,0,6	0,0, 0,0,0	0,0, 0,0,0	20,12, 0,12,0	80,76, 76,88,94	0,6, 12,0,0	0,0, 0,0,0	0,0, 0,0,0	6,6, 48,12,0	94,88, 40,88,100	80,88, 88,100,60	-	-	-	20,12, 12,0,40	60,80, 76,94,60	-	-	-	40,20, 24,6,40

24 hr exposure

5.0 mM (%)					5.0 mM + 2% DMSO(%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
6,0, 35,6,6	0,0, 0,0,0	6,0, 0,6,0	34,6, 45,6,0	54,94, 20,82,94	6,6, 6,6,6	0,0, 0,0,0	0,0, 0,0,0	40,0, 45,0,0	54,94, 49,94,94	80,68, 94,94,80	-	-	-	20,32, 6,6,20	53,68, 94,100,88	-	-	-	47,32, 6,0,12

48 hr exposure

2.5 mM (%)					2.5 mM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
6,0, 35,0,0	0,6, 0,0,0	0,0, 0,0,0	0,0, 20,32,12	94,94, 45,68,88	6,12, 49,0,0	6,0, 0,0,0	0,0, 0,0,0	55,32, 39,32,20	33,56, 12,68,80	46,88, 88,75,94	-	-	-	54,12, 12,25,6	46,88, 68,80,80	-	-	-	54,12, 32,20,20

TABLE 1b. PERCENTAGES OF DIPLOID (2n), TRIPLOID (3n), TETRAPLOID (4n), MIXOPLOID AND DEAD SEEDLINGS AFTER TREATMENTS WITH DIFFERENT CONCENTRATIONS AND VARIOUS TIMES OF EXPOSURES TO ORYZALIN (five replicates, r1-r5)

6 hr exposure

120 μM (%)					120 μM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
86,27, 72,60,7	0,0, 0,0,0	0,0, 0,0,0	0,53, 14,0,0	14,20, 14,40,93	86,27, 33,80,86	0,0, 0,6,0	0,0, 0,0,0	0,0, 0,14,7	14,73, 67,0,7	86,80, 88,100,67	-	-	-	14,20, 14,0,33	80,80, 80,100,73	-	-	-	20,20, 20,0,27

8 hr exposure

90 μM (%)					90 μM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
47,13, 66,73,53	0,0, 0,0,0	0,0, 0,0,0	33,0, 20,0,27	20,87, 14,27,20	47,13, 40,60,27	0,0, 0,0,0	0,0, 0,0,0	39,0, 40,40,59	14,87, 20,0,14	68,66, 66,86,80	-	-	-	32,34, 34,14,20	87,80, 46,93,100	-	-	-	13,20, 54,7,0

18 hr exposure

60 μM (%)					60 μM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
68,13, 40,73,60	0,0, 0,0,0	0,0, 0,0,0	12,0, 0,7,0	20,87, 60,20,40	47,6, 53,60,80	7,0, 0,0,0	0,0, 0,0,0	40,0, 13,0,0	6,94, 34,40,20	80,80, 80,93,86	-	-	-	20,20, 20,7,14	80,47, 93,86,93	-	-	-	20,53, 7,14,7

24 hr exposure

30 μM (%)					30 μM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
53,27, 40,67,88	0,0, 0,0,0	0,0, 0,0,0	40,0, 0,33,0	7,73, 60,0,14	74,93, 60,93,73	6,0, 0,0,7	0,0, 0,0,0,	0,0, 0,7,13	20,7, 40,0,7	87,100, 53,86,73	-	-	-	13,0, 47,14,27	87,93, 6,100,66	-	-	-	13,7, 94,0,34

48 hr exposure

15 μM (%)					15 μM + 2% DMSO (%)					Control									
										Water (%)					Water + 2% DMSO (%)				
2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead	2n	3n	4n	Mix	Dead
40,6, 73,80,73	0,0, 0,0,0	0,0, 0,0,0	26,0, 0,6,7	34,94, 27,14,20	0,80, 93,93,73	0,0, 0,0,0	0,0, 0,0,0	73,0, 0,0,0	27,20, 7,7,27	66,60, 80,86,55	-	-	-	34,40, 20,14,45	73,47, 86,80,93	-	-	-	27,53, 14,20,7

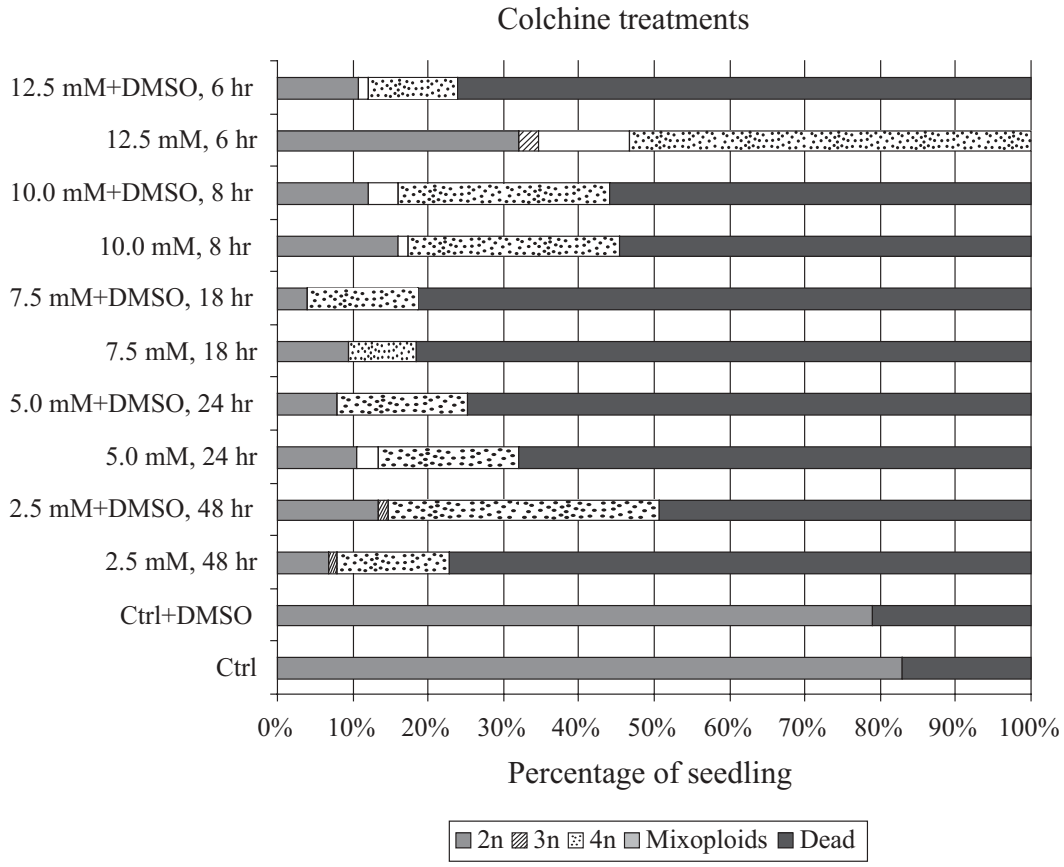

Figure 2a. Percentages of colchicine-treated seedlings with different ploidy levels.

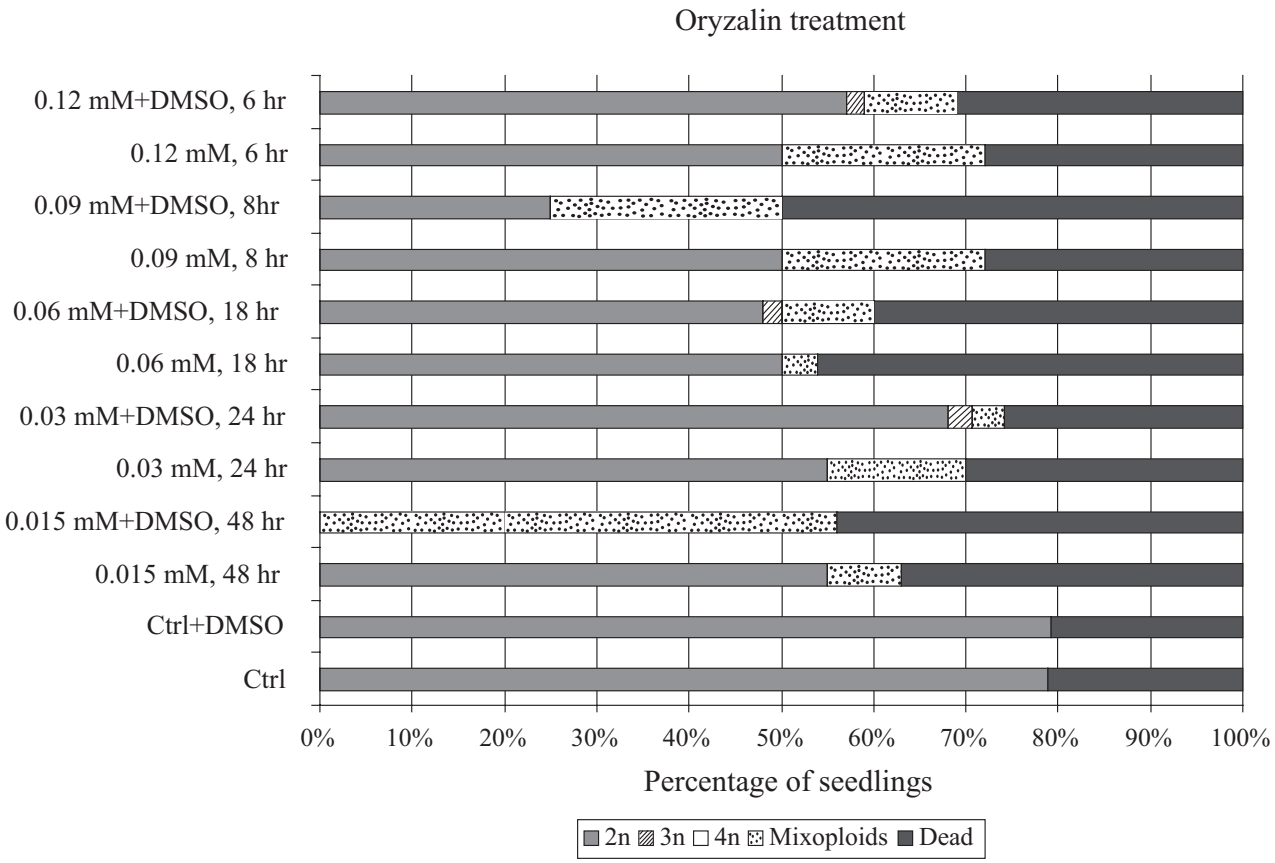

Figure 2b. Percentages of oryzalin-treated seedlings with different ploidy levels.

TABLE 2. STOMATA COUNTS AT THE BASE (B), MIDDLE (M) AND END (E) OF LEAVES AND PLANT HEIGHTS AT 18, 19 AND 20 MONTHS AND OF SELECTED POLYPLOIDS AND THEIR CONTROLS

Ploidy level	Treatments	Stomata count				Plant heights (cm)			
		Base	Mid	End	Average	18mo.	19mo.	20mo.	Average
2n + 4n	2.5 mM colchicine + 2% DMSO, 48 hr	**50**	**62**	**42**	**51**	**24.4**	**25.1**	**26.0**	**25.2**
3n	-As above-	**57**	**54**	**65**	**59**	**38.4**	**39.3**	**41.2**	**39.6**
2n	Controls	56	70	54	60	42.5	44.8	47.6	45
2n		72	38	42	51	51.4	52.6	53.4	52.5
2n		24	62	54	47	52.1	53.0	54.5	53.2
2n		58	52	38	49	43.1	43.7	44.5	43.8
2n		32	64	40	45	51.4	52.1	53.8	52.4
2n		46	52	74	57	52.1	54.6	55.7	54.1
3n	2.5 mM colchicine, 48 hr	**41**	**40**	**40**	**40**	**34.7**	**40.7**	**45.6**	**40.3**
2n	Controls	31	54	30	38	44.4	45.9	46.2	45.5
2n		26	55	58	46	43.2	44.8	45.4	44.5
2n		60	54	42	52	45.9	46.5	47.0	46.5
2n		50	45	36	44	47.0	48.3	49.6	48.3
2n		37	60	43	47	41.3	43.8	45.9	43.7
2n		46	50	42	46	54.6	55.0	55.5	55
3n + 4n + 5n + 6n	2.5 mM colchicine + 2% DMSO, 48 hr	**43**	**59**	**66**	**56**	**29.1**	**29.7**	**30.2**	**29.7**
2n	Controls	76	75	62	71	49.3	50.7	51.4	50.5
2n		50	41	42	44	50.1	51.3	52.6	51.3
2n		69	64	36	56	48.4	48.9	49.4	48.9
2n		44	54	40	46	46.0	46.8	47.3	46.7
2n		55	60	55	57	48.6	49.4	50.2	49.4
2n		54	56	48	53	45.1	45.7	46.4	45.7
4n	5.0 mM colchicine, 24 hr	**56**	**54**	**42**	**51**	**26.3**	**26.7**	**27.0**	**26.7**
2n + 4n	-As above-	**36**	**58**	**66**	**53**	**18.4**	**19.0**	**19.9**	**19.1**
2n	Controls	38	58	41	34	63.8	64.7	65.9	64.8
2n		44	63	42	50	54.3	55.0	55.7	55
2n		58	51	39	50	46.6	47.5	48.8	47.6
2n		30	50	56	45	43.0	44.5	45.6	44.4
2n		53	54	49	52	65.5	66.4	67.6	66.5
2n		36	48	38	41	38.4	40.8	42.6	40.6
4n	5.0 mM colchicine, 24 hr	**21**	**24**	**23**	**23**	**16.6**	**21.5**	**28.4**	**22.2**
2n	Controls	55	54	46	52	29.4	32.7	36.9	33
2n		44	62	44	50	35.9	36.5	37.5	36.6
2n		56	57	32	48	47.0	47.8	48.1	47.6
2n		60	60	62	61	46.1	46.7	47.0	46.6
2n		48	60	48	52	39.1	40.2	40.8	40
2n		59	54	54	56	46.3	47.3	48.5	47.4
4n	10.0 mM colchicine + 2% DMSO, 8 hr	**55**	**33**	**58**	**49**	**22.2**	**22.8**	**23.4**	**22.8**
2n	Controls	76	56	41	58	60.4	61.3	63.5	61.7
2n		45	50	48	48	43.8	44.6	45.1	44.5
2n		32	51	42	42	48.2	48.9	49.3	48.8
2n		48	63	49	53	51.7	52.3	52.9	52.3
2n		60	58	59	59	50.7	51.8	52.0	51.5
2n		38	53	46	46	64.3	65.0	65.4	64.9
4n	10.0 mM colchicine + 2% DMSO, 8 hr	**34**	**45**	**46**	**42**	**19.5**	**24.9**	**28.4**	**24.3**
2n	Controls	64	48	54	55	47.8	48.7	49.6	48.7
2n		66	75	40	60	61.5	62.0	63.4	62.3
2n		48	51	42	47	40.5	42.5	45.7	42.9
2n		55	73	45	58	62.2	62.6	63.5	62.8
2n		47	45	54	49	49.0	49.8	50.4	49.7
2n		54	60	56	57	45.4	46.1	47.3	46.3
4n	12.5 mM colchicine, 6 hr	**40**	**52**	**53**	**48**	**31.3**	**39.8**	**47.6**	**39.6**
4n	-As above-	**28**	**48**	**40**	**39**	**18.7**	**19.7**	**20.4**	**19.6**
4n	12.5 mM colchicine + 2% DMSO, 6 hr	**47**	**55**	**48**	**50**	**30.1**	**34.6**	**39.5**	**34.7**

TABLE 2. Continued

2n	Controls	35	90	45	57	40.5	41.2	42.8	41.5
2n		45	57	41	48	46.6	47.8	48.3	47.6
2n		37	54	48	46	41.4	42.3	43.0	42.2
2n		56	55	40	50	47.6	48.4	49.1	48.4
2n		48	43	50	47	46.8	47.5	48.9	47.7
2n		49	55	36	47	56.2	57.1	58.7	57.3
2n+4n	15 µM oryzalin, 48 hr	**37**	**36**	**41**	**38.0**	**17**	**21**	**24**	**20.7**
2n	Controls	44	44	49	45.7	43	47	49	46.3
2n		47	47	43	45.7	47	56	58	53.7
2n		50	48	47	48.3	46.5	48	53	49.2
2n		45	38	46	43.0	41	44	47.5	44.2
2n		43	51	47	47.0	42.5	44	47	44.5
2n		46	43	53	47.3	43	47	49	46.3
3n	30 µM oryzalin + 2% DMSO, 24 hr	**53**	**61**	**44**	**52.7**	**9**	**11**	**16**	**12**
2n	Controls	50	58	39	49.0	40	44	48	44
2n		60	57	45	54.0	45	50	56	50.3
2n		40	44	40	41.3	47	51	57	51.7
2n		38	43	45	42.0	35	37	41	37.7
2n		41	41	42	41.3	40	45	53	46
2n		35	43	37	38.3	45	47	53	48.3
3n	30 µM oryzalin + 2% DMSO, 24 hr	**33**	**37**	**38**	**36.0**	**25**	**31**	**37**	**31**
2n	Controls	53	48	51	50.7	43	45	51	46.3
2n		42	46	50	46.0	67	69	76.6	70.9
2n		44	56	45	48.3	50	55	64	56.3
2n		49	38	36	41.0	32	36	39	35.7
2n		51	53	47	50.3	45	48	57	50
2n		44	55	50	49.7	36	46	53	45
3n	60 µM oryzalin +2% DMSO,18 hr	**43**	**38**	**58**	**46.3**	**35**	**37**	**39**	**37**
2n		37	37	41	38.3	53	54	59	55.3
2n		40	34	36	36.7	42	46	57	51.7
2n		37	42	38	39.0	56	59	68	61
2n		42	40	36	39.3	49	56	64	56.3
2n		44	50	44	46.0	50.5	57	64	57.2
2n		40	60	51	50.3	52	58	68	59.3
2n+3n+4n	90 µM oryzalin, 8 hr	**46**	**48**	**50**	**48.0**	**15**	**18**	**21**	**18**
2n+3n+4n	-As above-	**33**	**41**	**34**	**36.0**	**17**	**19**	**23**	**19.7**
2n	Controls	52	58	60	56.7	60	65	68	64.3
2n		49	53	55	52.3	61	64	67.8	64.3
2n		38	45	37	40.0	64	67	71	67.3
2n		37	41	56	44.7	20	34	38	30.7
2n		40	36	42	39.3	22.5	28	33.5	28
2n		34	42	40	38.7	27	29	31	29
2n +4n	90 µM oryzalin + 2% DMSO,8 hr	**38**	**34**	**35**	**35.7**	**7**	**11**	**12.5**	**10.2**
2n	Controls	47	52	47	48.7	32	35	39	35.3
2n		37	35	37	36.3	30	33	39	34
2n		39	43	36	39.3	38	41	47	42
2n		40	45	43	42.7	39	43	47.5	43.2
2n		50	46	45	47.0	34	37	43	38
2n		45	50	47	47.3	37	39	45	40.3
3n	120 µM oryzalin + 2% DMSO,6 hr	**37**	**40**	**39**	**38.7**	**15**	**19**	**23**	**13.9**
2n	Controls	46	41	51	46.0	21	27	29	25.7
2n		43	48	37	42.7	28	34	38	33.3
2n		45	47	46	46.0	31.5	37	41	36.5
2n		42	49	46	45.7	42	56	59	52.3
2n		40	58	50	49.3	40	45	52	45.6
2n		44	46	49	46.3	60	67	74	67

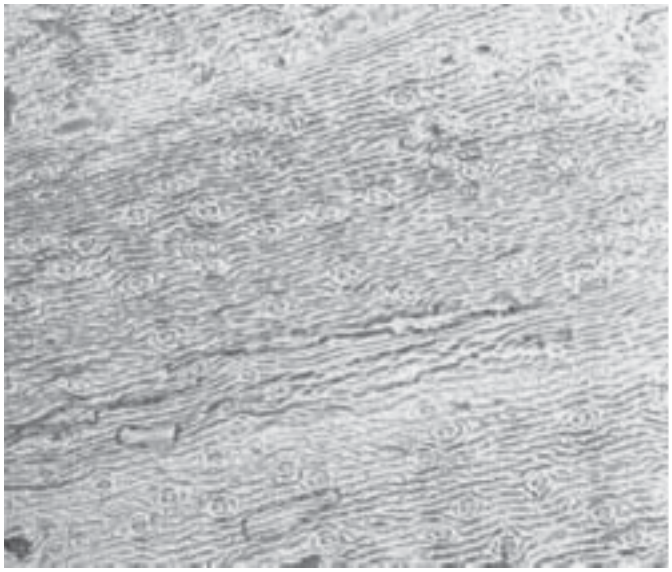

Figure 3. Stomata distribution on leaf abaxial epidermis (400x).

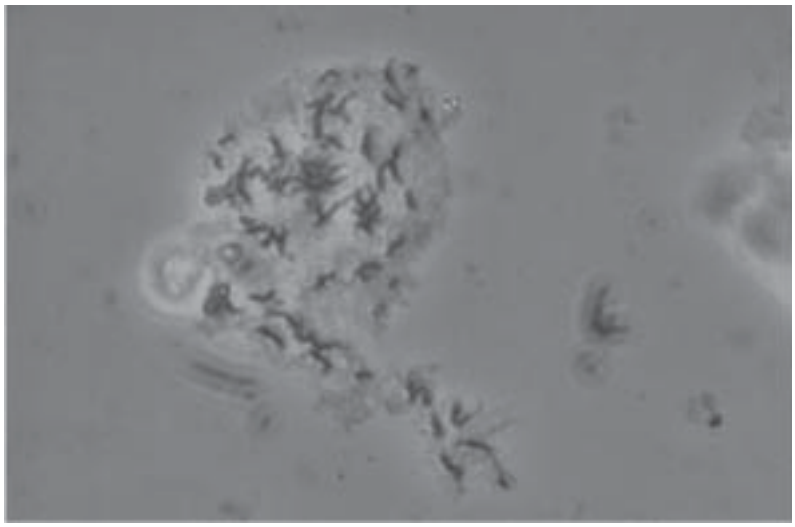

Figure 4a. A triploid from colchicine treatment showing 3n=48 chromosomes at 640x magnification.

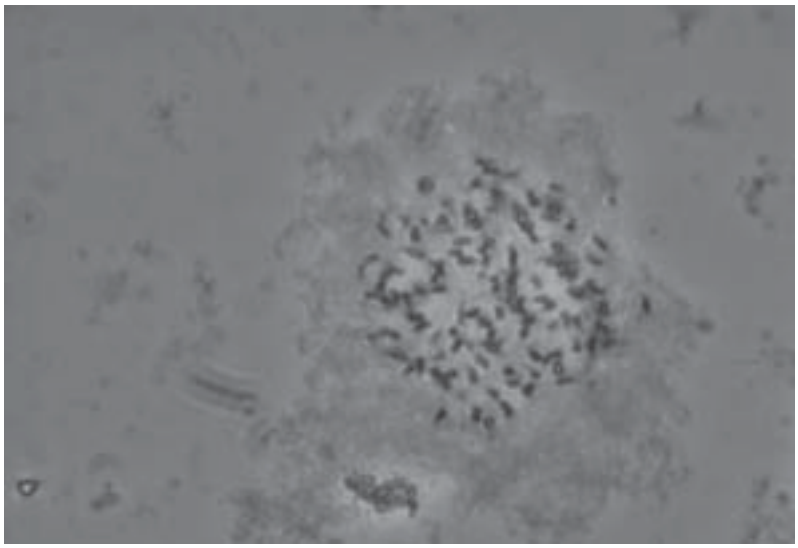

Figure 4b. A tetraploid that resulted from colchicine treatment showing 4n=64 chromosomes at 640x magnification.

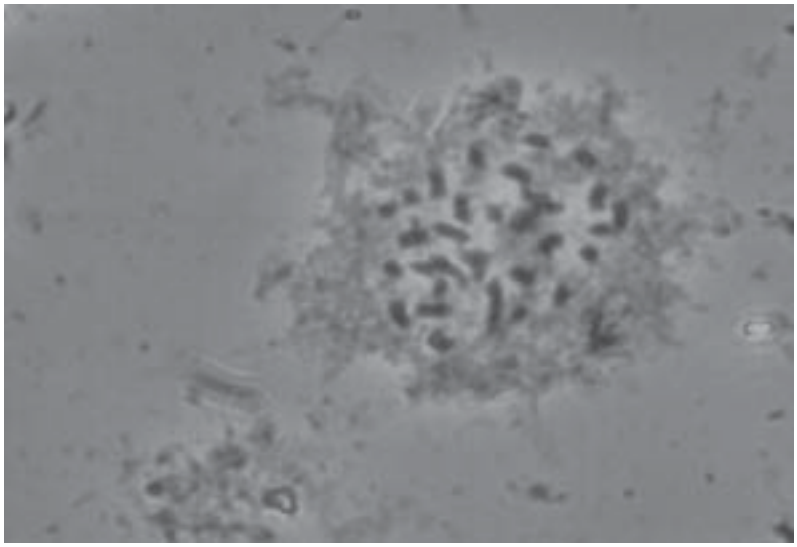

Figure 4c. A diploid (control) showing 2n=32 chromosomes at 640x magnification.

Figure 5a. A colchicine-treated triploid seedling (left) and controls.

Figure 5b. A colchicine-treated tetraploid seedling (left) and controls.

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