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Research Article

Doubling of chromosomes of pearl millet napier hybrids and preliminary screening based on stomatal characteristics

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Abstract
The objective of the present work was to induce chromosome doubling in sterile triploid pearl millet napier hybrids to develop fertile hexaploids. Colchicine, an alkaloid from *Colchicum autumnale*, was used for polyploidization. F_1 seeds of the cross combination ICMV 05666 × FD 471 and setts of PMN hybrid CO (BN) 5 were used for treatment. F_1 seeds were treated with 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 1.0% of colchicine for different durations of 6hrs, 8hrs, 12hrs and 24hrs and setts treated with 0.05, 0.06, 0.07, 0.08, 0.09 0.10, 0.15, 0.20, 0.25 and 0.30% of colchicine at 3hrs intervals for 2 days. The treated seeds and setts were evaluated for germination (%), survivability (%) and stomatal measurements. In seed treatment, highest germination (78.0) and survival per cent (62.0) was recorded at 0.04% for 6hrs treatment, respectively. Pertaining to sett treatment, highest germination and survival per cent was recorded at 0.05%, followed by 0.06%, the least being at 0.25 and 0.30%. With regard to stomatal length, highest mean value (61.03 ± 7.44) was recorded from seedlings raised with seed treated at 0.06% for 24hrs with a range of 53.59 - 68.46 µm and in sett treatment raised seedlings, it was highest (56.72 ± 4.00 µm) at 0.20% with a range of 50.34 - 68.41 µm, against the control with 46.27 ± 0.57 µm. Among various treatments, seed treatment at 0.06% for 24hrs and 0.07% for 12hrs and sett treatment at 0.15 and 0. 20% were identified to be efficient in generating variants. However, these variants have to be further studied for ploidy confirmation *via.* Flow cytometry and chromosomal counts.

Key words
Pearl millet napier hybrids, colchicine, seed treatment, sett treatment and stomatal size

Introduction

India has the largest livestock population in the world and livestock production is the backbone of Indian agriculture and source of employment in rural areas for centuries. This sector has been the primary source of energy for agriculture operation and major source of animal protein for the masses. Therefore, India has been house to major draught, milch and dual-purpose breeds of cattle. Sustainable livestock production is highly dependent on the availability of quality feed and forage resources (Alemayehu *et al*., 2017). Among the several feeds available forage is the most important component in the diet of dairy cattle because of the dramatic impact it has on dry matter and nutrient consumption.

Several fodder crops are available for animal feeding of which pearl millet napier hybrid belonging to the genus *Pennisetum* is an important one. It is produced by the inter-specific cross between pearl millet (*Pennisetum glaucum* L.; 2n = 2x = 14, AA) and elephant grass *(Pennisetum purpureum* Schum; 2n = 4x = 28, A'A'BB). Pearl millet grows well on soils with low fertility, is drought tolerant and produces a high biomass quality (Serraj *et al.*, 2005). Its relative elephant grass is one of the most productive biomass plant in tropical and subtropical climates and it is an important forage and bio-energy crop. Hybrid development between these two species (2n = 3x = 21, AA'B) is a common strategy used in elephant grass breeding programs to combine the most favorable millet characteristics (resistance to drought, tolerance to diseases, and seed size) with the hardiness, aggressiveness, and high dry matter production of elephant grass (Campos *et al*., 2009). However, the sterility of the triploid hybrid is a limiting factor of this strategy in popularization and horizontal spread of hybrids. The spread of popular PMN hybrids *viz.*, CO (CN) 4 and CO (BN) 5 have a major limitation of transport of voluminous vegetative propagules to distant places which normally take longer time in transportation and quality deterioration as well during the transit. The seed multiplication ratio (SMR) is also very low (1: 20) which could be overcome through some other alternative approaches. Chromosome doubling of the triploid hybrids produces hexaploids that can restore fertility (Hanna *et al*, 1985 and Campos *et al*., 2009) to enable subsequent backcrosses with

elephant grass and may enhance biomass production and persistence in the resulting hybrids. Colchicine is the most commonly used antimitotic agent, obtained from *Colchicum autumnale* of angiosperm which binds specifically to tublins to prevent polymerization of microtubules and to induce polyploidy cells (Murali *et al*., 2013). Fertility restoration of sterile hybrids between pearl millet (*Pennisetum glaucum)* and napier grass (*Pennisetum purpureum)*, has been achieved by chromosome doubling (Falerio *et al.*, 2015).

Determination of ploidy level in plants may be performed directly by counting chromosome numbers or by flow cytometry (Ochatt *et al*., 2011). Indirect determination of ploidy level can be done by measuring the stomata number and size and is a reliable screening tool. Compos *et al*., (2009) in pearl millet napier hybrids, Quesenberry *et al*., (2010) in bahia grass, Murali *et al*.,(2013) in sorghum, Ramesh *et al.*, (2014) in mulberry and Falerio *et al.*, (2015) in pearl millet napier hybrids have successfully used stomatal size as an indirect measure of altered ploidy. Keeping this in view, the current study is aimed at standardization of the methodology and concentration of colchicine to double the chromosome number of triploid seeds (F_1) and vegetative setts of pearl millet napier hybrid using colchicine to generate colchiploids which were preliminary screened for identifying putative polyploids by stomatal size measurements.

Material and Methods

The study was conducted at Department of Forage Crops, CPBG, TNAU, Coimbatore, during 2017-18. For colchicine treatment with different concentrations and duration, the F_1seeds (triploid) obtained from interspecific cross between pearl millet (ICMV 05666) and napier grass (FD 471) and the setts of the PMN hybrid CO (BN) 5 were used. Twenty five seeds were treated for each treatment. They were surface sterilized by immersing in sodium hypochlorite for 15 mins, rinsed thrice with deionised water, carefully blotted with a paper towel and immediately sown in a petridish containing germination paper soaked in different concentrations of Colchicine (0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 1.0%) for different durations of 6, 8, 12 and 24 hrs and allowed for germination. The methodology adopted by Weiler *et al.,* (2015) for chromosomal doubling in *Paspalum notatum* was followed. The germinated seedlings were carefully transferred to the protrays. In case of setts, ten concentrations of aqueous colchicine *viz.*, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.15, 0.20, 0.25 and 0.30% were tried to treat the vegetative buds. Twenty single budded setts per treatment were prepared and planted individually in polybags filled with soil. The buds were cleaned in distilled water before the application of colchicine. Buds were covered with dry cotton swab (Fig.3a) and colchicine solution was applied (concentrations mentioned in materials and methods) from 6.00 A.M to 6.00 P.M for two consecutive days at an interval of three hour while the buds of control were treated with distilled water. The growth parameters *viz.*, germination percentage (15 days after treatment), survivability (30 days after treatment) and stomatal dimensions (30 days after transplanting) were recorded and subjected for analysis.

Longitudinal and transverse dimensions were recorded for adaxial epidermal stomata in the leaves of all survived and established plants at 30 days after transplanting in the field. Leaf surfaces were coated with clear nail polish, which were allowed to dry, peeled off, and dry mounted on a microscope slide. Five stomata from each leaf were measured using Scope Image software and the mean was arrived.

Results and Discussion

Analysis of variance for germination and survival per cent revealed the existence of high significant difference between treatments, concentration, duration and the interaction of concentration *vs* duration (Table 1). As the concentration of colchicine increased, the germination per cent decreased (Table 2 and Fig. 1). The mean germination per cent ranged from 9.0 to 48.0 against the control with 92.0 per cent. The highest germination per cent (48.0) was recorded at 0.04% concentration, followed by 0.05% (43.0), 0.06% (33.0), 0.07% (30.0), 0.08% (19.0), 0.09 % (12.0) and 0.10% (9.0). Similar scenario was observed for survival per cent and it ranged from 3.0 (0.09 and 0.1%) to 40.0 (0.04%). Considering the duration, the highest mean germination per cent (53.0) and survival per cent (35.0) were recorded at 6hrs of treatment and the least per cent (22.0) and (18.0) being at 24hrs duration, respectively.

In combination of the duration and concentration, highest germination per cent (78.0) was recorded at the treatment, 0.04% colchicine for 6hrs followed by 0.05% for 6hrs (70.0) and 0.06% for 6 hrs treatment with 54 per cent. In contrast, least germination per cent (6.0) was recorded at 0.08% for 24 hrs and 0.09% for 12 hrs treatment. The treatments, 0.9% for 24 hrs duration and 0.10% for 12 and 24 hrs recorded zero per cent germination. Pertaining to the survival per cent, highest value (62.0) was recorded at 0.04% for 6hrs, followed by 0.05% for 6 hrs (48.0) and 0.04% for 8 hrs (38.0). However, the treatments *viz.*, 0.08% for 24 hrs and 0.09% for 12 hrs resulted in complete mortality. Therefore, the germination and survival per cent

decreased as the concentration and duration of treatment increased (Fig. 1) and such similar reports were recorded by Zeinab *et al.,* (2012) in Berseem, Sourour *et al.,* (2014) in barley, Wang *et al.,* (2017) in buck wheat for germination per cent and Nair (2004) in *Lolium*; Majdi and Ghasem Karimzadeh (2010) in *Tanacetum parthenium* and Timbo *et al*., (2014) in *Bracharia ruziziensis* for survival per cent**.**

A significant difference for germination and survival per cent was observed between different concentrations of colchicine (Table 3). The highest germination per cent (92.0) was recorded at 0.05 and 0.06% against the control with 95.0%. However, these concentrations are almost similar to control in their germination per cent. Followed by 0.05 and 0.06% concentrations, 0.07 and 0.08% recorded the next highest germination per cent (87.0). In contrast, the least germination per cent (2.0) was observed at 0.25 and 0.30%. Therefore, as the concentration increased there was a decrease in germination per cent (Table 4 and Fig. 2). Pertaining to the survival per cent, highest value (92.0) was recorded at 0.05%, which was similar to control, followed by 0.06% (90.0), 0.07% (87.0) and 0.08% (82.0). The least survival per cent (2.0) was recorded at 0.25 and 0.30% treatment. Combining the germination and survival per cent, the concentrations 0.05, 0.06 and 0.07% showed similar results to the control, however their performance varied with 0.08% concentration treatment. Therefore, lower concentrations did not show much variation for germination and survival per cent in comparison to the control, but a wider variation was observed as the concentration was increased. Similar findings were reported by Liu *et al.* (2007) in *Platanus acerifolia,* Lam *et al.* (2014) in *Acacia crassicarpa* for germination percentage and for survival per cent Ramesh and Murthy (2014) in Mulberry and Timbo *et al*. (2014) in *B. ruziziensis* reported the same results.

Initial screening on the basis of stomatal size was conducted to downsize the number of putative hexaploids for further studies in the field. All the variants were studied for their adaxial stomatal length and width and a wide variation was observed among the colchiploids. In seed treatment, the control plants recorded a mean stomatal length and width of, 46.12 ± 0.26 µm and 24.04 ± 0. 81 µm, respectively (Table 5 and Fig. 3b). The highest mean value (61.03 ± 7.44 µm) for stomatal length was observed at 0.06% for 24hrs treatment with a range of 53.59 - 68.46 µm (Fig. 3c), followed by 0.07% for 12hrs treatment with mean value and range of 59.47 ± 4.83 µm and 53.15 - 68.95 µm (Fig. 3d), respectively. The range of stomatal length obtained was highest (46.97 - 66.84 µm) at 0.06% for 12 hrs treatment, followed by 0.07% for 6 hrs treatment (34.98 - 54.37 µm). Similarly, highest mean value (28.82 ± 0.45 µm) for stomatal width was recorded at 0.07% concentration for 12 hrs with wider range of 23.08 - 34.46 µm. In contrast, the least mean (45.41 ± 4.01 µm) for stomatal length was observed at 0.06% for 12 hrs treatments, however it had highest range. The least mean value (23.62 ± 0.48 µm) for stomatal width was observed at 0.05% for 8 hrs treatment. The lower concentrations *viz.*, 0.04 and 0.05%, in all the treatments showed stomatal length and width almost similar to control, indicating that these concentrations were ineffective in generating the variation (Table 5).Same variant (0.10% for 8 hrs treatment) with different sizes of stomata was seen, which might probably fall under mixoploids (Fig. 3g).

In sett treatment, highest mean value (56.72 ± 4.00 µm) for stomatal length was obtained at 0.20% with a range of 50.34 - 68.41 µm, against the control with 46.27 ± 0.57 µm (Table 6). Followed by 0.20%, the treatment 0.15% recorded high mean value of 53.31 ± 3.14 µm with highest range of 45.34 - 70.10 µm (Fig. 3e). Pertaining to the stomata width 0.15% recorded the highest mean value of 26.16 ± 1.09 µm, with widest range of 23.99 - 32.53 µm. The variation in stomatal measurements generated upon treatment with 0.05, 0.06, 0.07, 0.08 and 0.10% is very narrow and are almost similar to the control, indicating that these concentrations do not have effect on creating variation. Campos *et al*. (2009) reported that the stomatal size in induced hexaploids of pearl millet napier hybrids was approximately 1.5 times greater than their triploid counterparts.

It can be summarized that colchicine was efficient in generating variants by seed (Fig. 4a) and sett treatment (Fig. 4b), which was evident from the above results. In seed treatment, the concentration 0.06% for 24 hrs treatment and 0.07% for 12 hrs, and in sett treatment 0.15 and 0.20% are efficient in generating variants. In seed treatment, the concentrations 0.09 and 0.10, and in sett treatment, 0.20% generates considerable variants however the mortality was high. Although stomatal dimensions can be used as primary selection criteria of polyploids, further confirmation is necessary as there may be a chance of occurrence of mixoploids as reported by Zhang *et al.* (2010) in crape myrtle (*Lagerstroemia indica*). Similar reports were documented by Campos *et al.* (2009) in pearl millet napier hybrids also. It was concluded that colchicine could be used as a potential antimitotic agent for creating variability in the triploids and warrants the possibility of breaking the sterility in the pearl millet napier hybrids. The selected

variants will be subjected for flow cytometry and chromosomal counts for further confirmation.

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Table 1. Analysis of variance for germination, survival percentage and dosage effect in seed treated pearl millet napier hybrid

	Treatments (df =31)	Concentration (df= 7)	Duration (df= 3)	Concentration x Duration (df= 21)	Error (df= 32)
Germination per cent	67.59**	231.38 **	122.08 **	5.21 **	1.00
Survival per cent	126.03**	494.98 **	113.21 **	4.88 **	1.00

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Table 2. Effects of colchicine concentration and duration on the germination and survival percentage of seed treated pearl millet napier hybrid

	Germination percentage					Survival percentage				
Concentration of colchicine	**6hrs**	**8hrs**	**12hrs**	**24hrs**	**Mean of concentration**	**6hrs**	**8hrs**	**12hrs**	**24hrs**	**Mean of concentration**
Control C0	**90^{a}**	**94^{a}**	**90^{a}**	**94^{a}**	**92**	**82^{a}**	**86^{a}**	**80^{a}**	**84^{a}**	**83**
	(71.5)	**(76.4)**	**(71.5)**	**(75.8)**	**(73.8)**	**(64.9)**	**(68.0)**	**(63.4)**	**(66.4)**	**(65.7)**
0.04%	**78^{b}**	48^{cd}	42^{cdef}	26^{hijk}	**48**	**62^{b}**	38^{d}	34^{def}	26^{fg}	**40**
	(62.0)	(43.8)	(43.8)	(30.5)	**(44.1)**	(51.9)	(38.0)	(35.6)	(30.6)	**(39.0)**
0.05%	**70^{b}**	44^{cdef}	38^{defg}	20^{jk}	**43**	**48^{c}**	30^{ef}	28^{efg}	18^{hi}	**31**
	(56.8)	(41.5)	(38.0)	(26.4)	**(40.7)**	(43.8)	(33.2)	(31.9)	(25.0)	**(33.5)**
0.06%	54^{c}	34^{efgh}	28^{ghij}	18^{jkl}	**33**	36de	22^{gh}	18^{hi}	12^{jk}	**22**
	(47.2)	(35.5)	(31.8)	(24.8)	**(34.8)**	(36.8)	(27.9)	(25.0)	(20.0)	**(27.46)**
0.07%	46^{cde}	32^{fghi}	26^{hijk}	18^{jkl}	**30**	26^{fg}	18^{hi}	14^{ij}	8^{klm}	**16**
	(42.7)	(34.4)	(30.5)	(24.8)	**(33.1)**	(30.6)	(25.0)	(21.9)	(15.9)	**(23.3)**
0.08%	38^{defg}	22^{ijk}	10^{lm}	6^{m}	**19**	18^{hi}	10^{jkl}	6^{lm}	0^{n}	**8**
	(38.0)	(27.9)	(18.3)	(13.9)	**(24.5)**	(25.0)	(18.3)	(13.9)	(0.5)	**(14.4)**
0.09%	26^{hij}	18^{jkl}	6^{m}	0^{n}	**12**	6^{lm}	6^{m}	0^{n}	0^{n}	**3**
	(30.6)	(25.0)	(13.9)	(0.5)	**(17.5)**	(13.9)	(13.9)	(0.5)	(0.5)	**(07.2)**
0.10%	22^{ijk}	16^{kl}	0^{n}	0^{n}	**9**	6^{lm}	6^{lm}	0^{n}	0^{n}	**3**
	(27.9)	(23.4)	(0.5)	(0.5)	**(13.1)**	(13.9)	(13.9)	(0.5)	(0.5)	**(07.2)**
Mean of duration	53	38	30	22		35	27	22	18	
	(47.1)	(38.5)	(30.6)	(24.6)		(35.1)	(29.7)	(24.1)	(19.9)	

For comparing means of	**SEd**	**CD @ 5%**	**SEd**	**CD @ 5%**
Concentration	1.76	3.59	1.23	2.52
Duration	1.24	2.54	0.87	1.78
Concentration x Duration	3.52	7.18	2.47	5.04

Values in the parenthesis are arcsine transformed
The letters followed by the parameters indicates significance

Table 3. Analysis of variance for germination and survival percentage in sett treated pearl millet napier hybrid

	Concentration (df= 10)	Error (df= 10)
Germination per cent	71.48 **	20.31
Survival per cent	59.73 **	23.05

Table 4. Effects of colchicine concentration on the germination and survival percentage of sett treated pearl millet napier hybrid

S. No.	Concentration of Colchicine	Germination percentage	Survival percentage
1.	**Control**	**95^{a} (77.1)**	**92^{a} (74.3)**
2.	0.05%	92^{ab} (74.3)	92^{a} (74.3)
3.	0.06%	92^{ab} (74.3)	90^{ab} (71.6)
4.	0.07%	87^{ab} (69.4)	87^{ab} (69.4)
5.	0.08%	87^{ab} (69.4)	82^{ab} (65.3)
6.	0.09%	82^{b} (65.3)	77^{b} (61.7)
7.	0.10%	57^{c} (49.3)	52^{c} (46.4)
8.	0.15%	37^{d} (37.8)	35^{cd} (36.2)
9.	0.20%	22^{d} (28.3)	20^{d} (26.4)
10.	0.25%	02^{e} (06.7)	02^{e} (06.7)
11.	0.30%	02^{e} (06.7)	02^{e} (06.7)
	Mean of concentrations	**60 (50.7)**	**57 (49.1)**
	SEd	4.5	4.8
	CD @ 5%	9.9	10.5

Values in the parenthesis are arcsine transformed
The letters followed by the parameters indicates significance

Table 5. Mean performance and range of seed treated pearl millet napier colchiploids for adaxial stomata

Colchicine concentration (%)	Duration of treatment	Stomata length (µm)		Stomata width (µm)	
		Mean ± SE	Range	Mean ± SE	Range
Control	**6hrs**	**46.12 ± 0.26**	**45.66 – 46.56**	**24.04 ± 0.81**	**22.44 – 25.06**
0.04	6hrs	47.23 ± 0.63	41.97 - 53.32	24.32 ± 0.70	22.16 - 30.02
	8hrs	48.68 ± 1.12	44.66 - 53.70	25.02 ± 0.89	22.18 - 29.29
	12hrs	50.38 ± 1.33	46.06 - 55.48	25.27 ± 0.97	22.18 - 29.76
	24hrs	49.02 ± 1.52	44.84 - 53.32	24.55 ± 0.48	23.38 - 26.21
0.05	6hrs	49.48 ± 0.98	45.68 - 55.35	24.74 ± 0.48	22.71 - 28.42
	8hrs	49.48 ± 0.83	45.68 - 55.35	23.62 ± 0.48	21.72 - 28.42
	12hrs	48.28 ± 0.31	47.02 - 49.49	23.98 ± 0.45	22.16 - 25.26
	24hrs	47.56 ± 0.83	45.45 - 49.38	23.71 ± 0.63	22.79 - 25.55
0.06	6hrs	47.09 ± 1.49	42.66 - 56.09	25.43 ± 1.02	22.77 - 30.73
	8hrs	51.54 ± 1.73	46.06 - 55.46	26.24 ± 0.88	24.96 - 29.71
	12hrs	45.41 ± 4.01	**34.98 - 54.37**	24.32 ± 1.24	22.35 - 27.81
	24hrs	**61.03 ± 7.44**	53.59 - 68.46	26.22 ± 1.18	25.03 - 27.40
0.07	6hrs	52.26 ± 3.14	**46.97 - 66.84**	25.25 ± 1.50	22.18 - 32.07
	8hrs	51.06 ± 1.20	47.73 - 53.39	24.96 ± 1.12	22.71 - 27.32
	12hrs	**59.47 ± 4.83**	53.15 - 68.95	**28.82 ± 0.45**	**23.08 - 34.46**
0.08	6hrs	51.26 ± 1.60	47.17 - 53.10	25.48 ± 0.72	24.42 - 27.24
	8hrs	46.29 ± 1.50	44.79 - 47.79	24.02 ± 1.12	22.91 - 25.13

Table 6. Mean performance and range of sett treated pearl millet napier colchiploids for adaxial stomata

Colchicine concentration (%)	Stomata length (µm)		Stomata width (µm)	
	Mean ± SE	Range	Mean ± SE	Range
Control	**46.27 ± 0.57**	**45.12 – 46.87**	**24.61 ± 0.66**	**23.34 – 25.58**
0.05%	47.66 ± 0.48	44.73 - 51.88	24.69 ± 0.30	22.00 - 27.46
0.06%	47.83 ± 0.53	43.69 - 52.01	24.63 ± 0.28	22.79 - 27.60
0.07%	49.65 ± 0.65	44.83 - 53.71	24.38 ± 0.19	23.00 - 25.49
0.08%	49.84 ± 0.71	44.31 - 53.99	24.59 ± 0.29	22.77 - 27.11
0.09%	49.76 ± 1.51	35.87 - 60.45	25.83 ± 1.13	23.47 - 31.37
0.10%	49.49 ± 1.03	44.84 - 53.76	24.34 ± 0.27	23.31 - 25.55
0.15%	53.31 ± 3.14	**45.34 - 70.10**	**26.16 ± 1.09**	**23.99 - 32.53**
0.20%	**56.72 ± 4.00**	50.34 - 68.41	25.52 ± 1.69	22.61 - 30.35

Note: Data was depicted only for the treatments showing survival per cent > six

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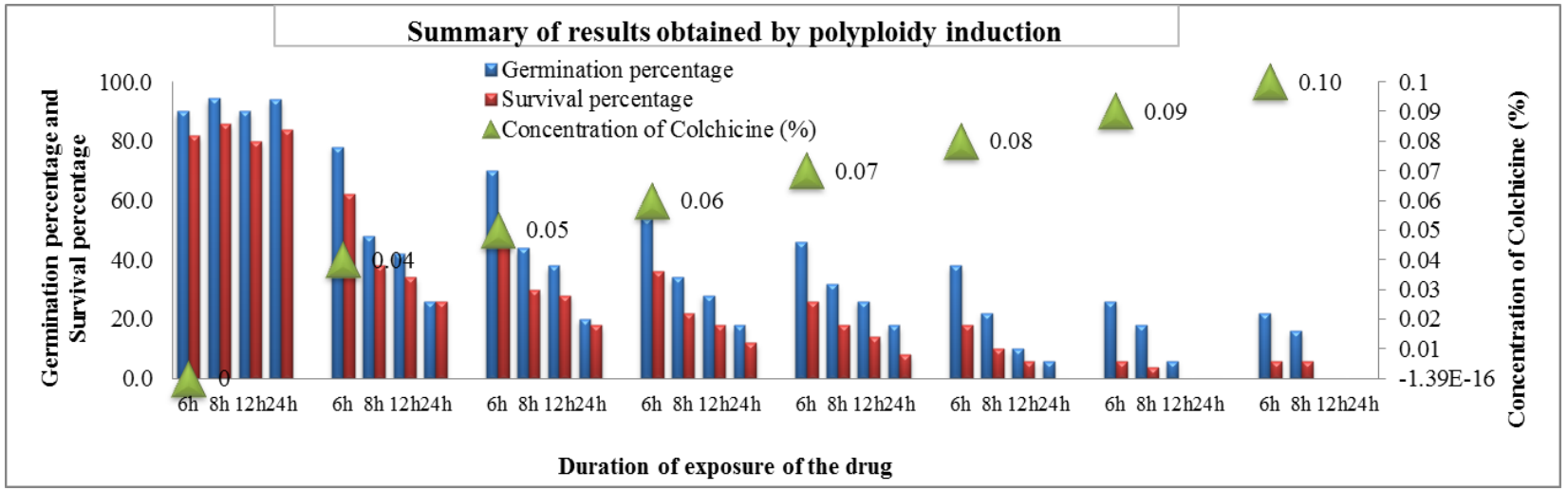


Fig. 1. Summary of germination and survival per cent of pearl millet napier hybrids obtained by seed treatment at various concentrations and durations.

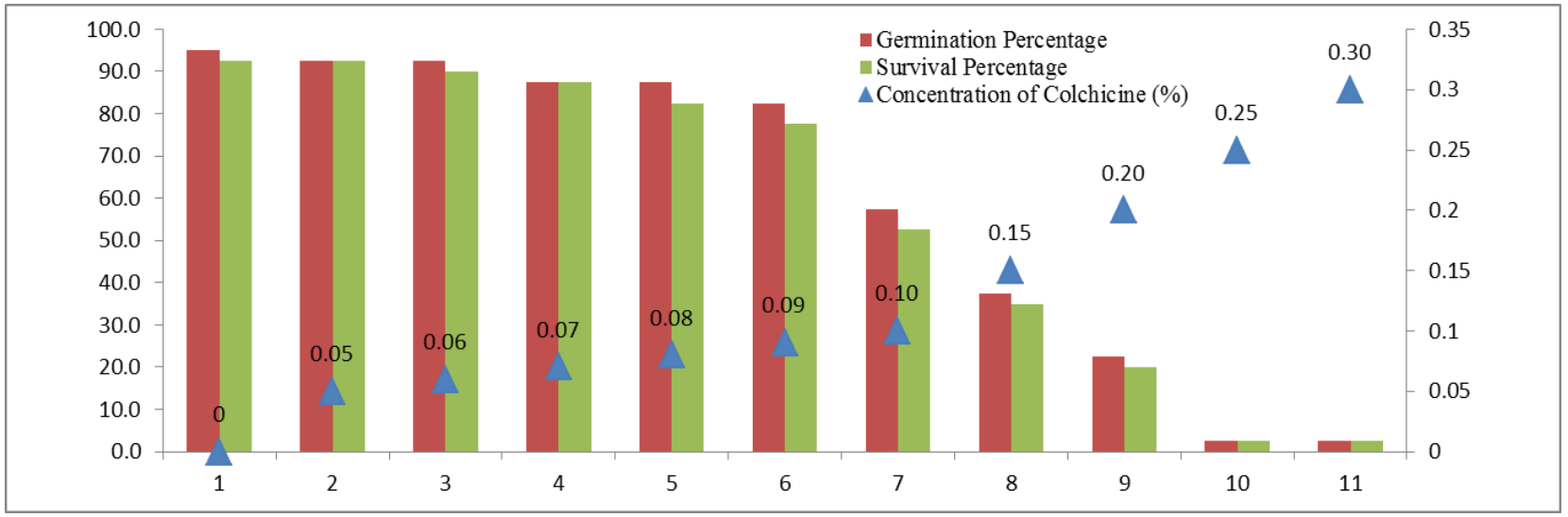
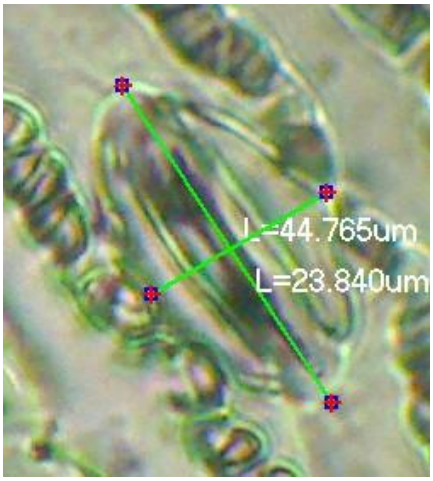
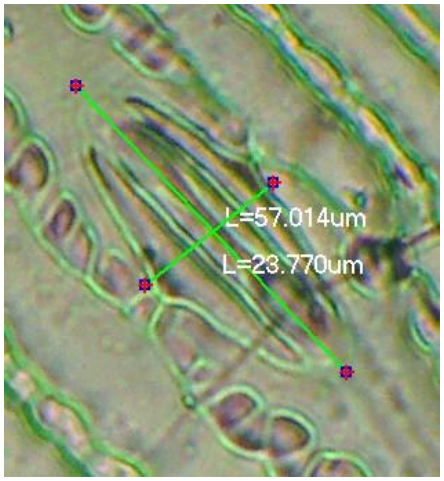


Fig. 2. Summary of germination and survival per cent of pearl millet napier hybrids obtained by sett treatment at various concentrations.

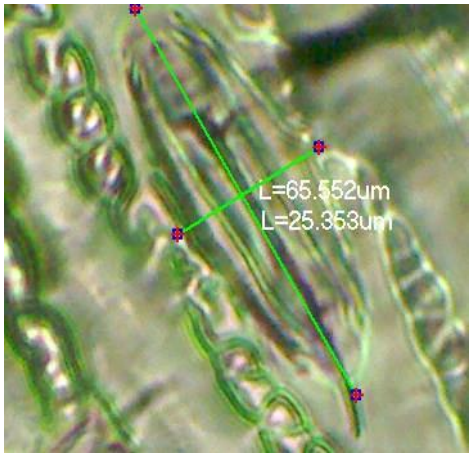
a. Set treatment



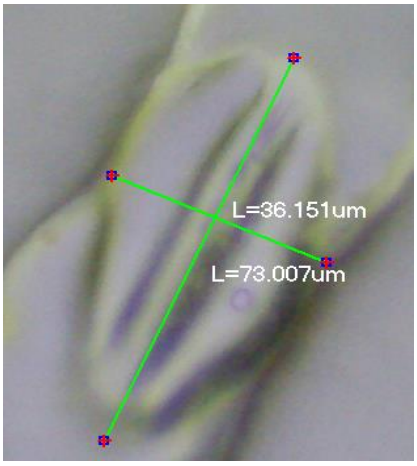
b. Control



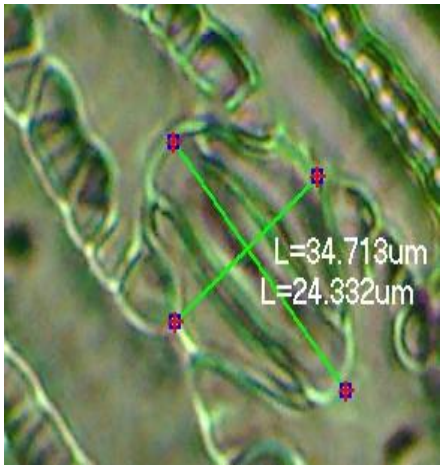
c. 0.06% for 24hrs (seed treatment)



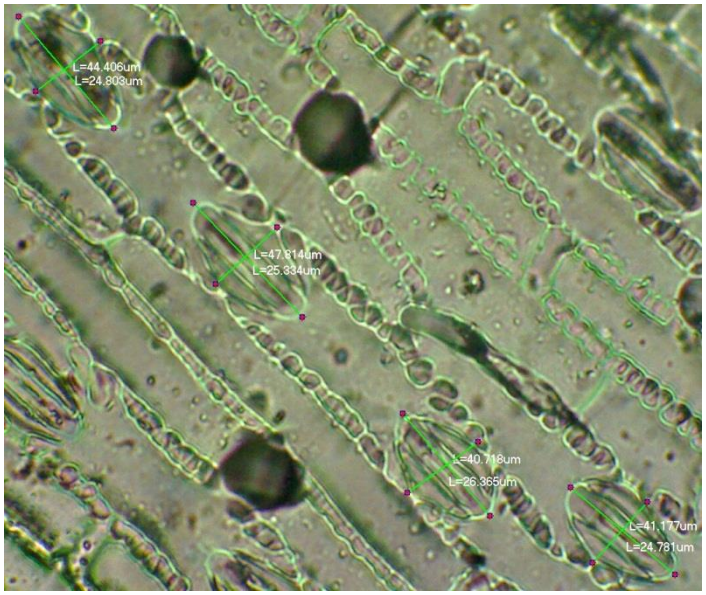
d. 0.06% for 24hrs (seed treatment)



e. 0.15% (set treatment)

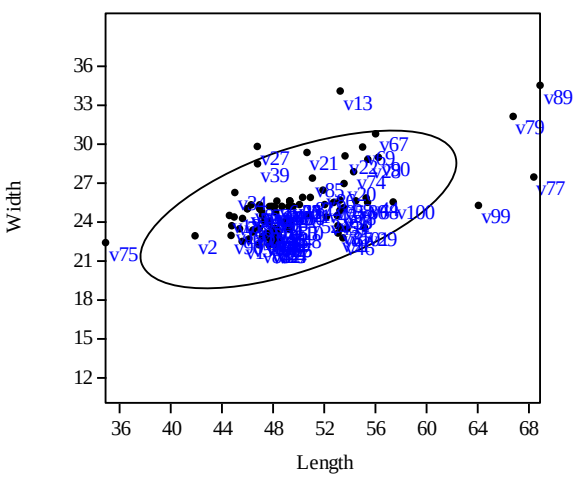


f. 0.09% set treatment

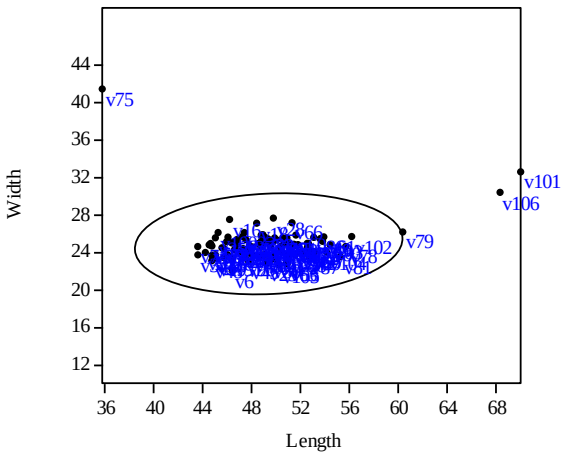


g. 0.10% for 8hrs set treatment

Fig. 3. Stomatal measurements of colchiploids

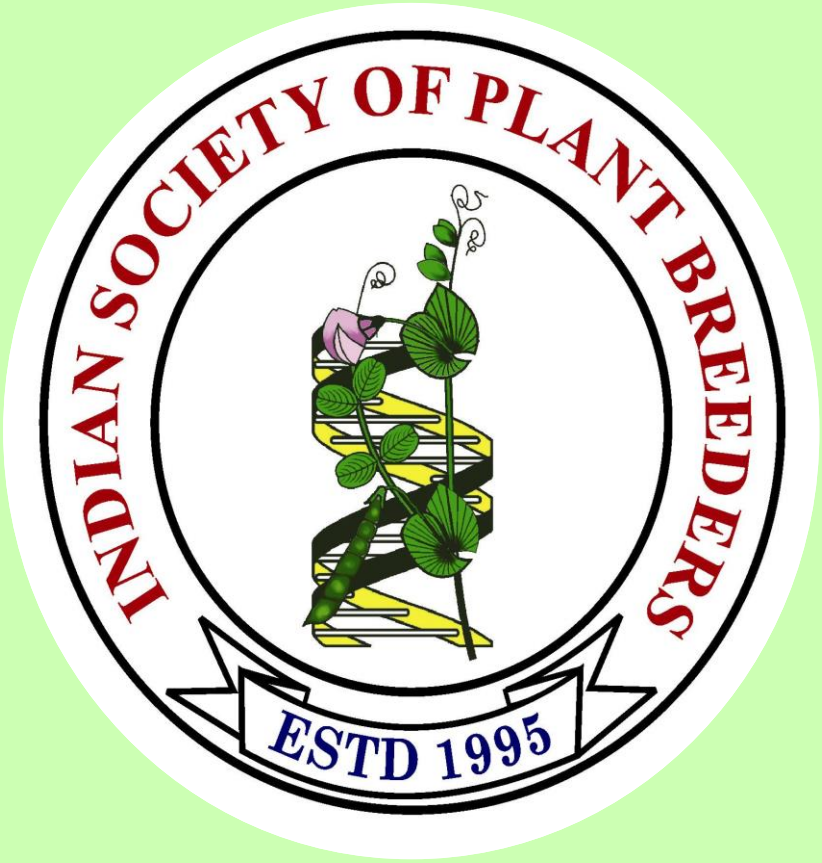


a. Seed treatment



b. Sett treatment

4. Scatter graph of stomata measurements of all the variants obtained at different treatments



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