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Induction of polyploidy to check the effect of polyploidization over the diploid wild ones in Berseem (*Trifolium alexandrinum* L.)

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Abstract
Berseem is exclusively cultivated as a winter season fodder crop in about 2mha of land in India. In addition of being a Multicut nature crop, it is high yielding with high nutritional value crop which is why it is widely cultivated. A plausible approach towards increasing forage yield could be through enhancing the vegetative growth. The present experiment deals with inducing auto-polyploidy along with evaluation of obtained polyploids to check the polyploidy effect over the diploid counterparts. Induction of polyploidy was conducted in Berseem clover (*Trifolium alexandrinum* L.) genotype, Mescavi and BB 3; the effect of polyploidy was checked over their diploid counterparts. 3 different colchicine concentrations (control, 0.01, 0.02 and 0.05%) were applied to the seeds by soaking them in 3 replicates for each treatment. Stomatal observations, nucleus size along with morphological parameters were examined for polyploidy. Polyploids were evident in populations of all the 3 treatments of Mescavi. BB 3 did not show any interaction with any of the colchicine treatment. Interactions between Mescavi genotype and colchicine treatments showed significant differences on morpho-physiological parameters. Karyotypic study evident the same. Leaflet shape, size of leaf area and leaf number showed higher variation. Multifoliate leaves were obtained from resultant autopolyploids. Increased variations are seen in floral as well as seed dimensions. Seed no. and floret no. showed significant differences. Induction of polyploidy resulted in taller plants, and more branching plant^{-1}, showed an increase in 10 seed weight. This methodology can be further used in plant breeding programs to increase efficiency of fodder production and in selection of more superior adaptive genotypes.

Keywords: Berseem clover, colchicine, polyploidy

Introduction
Berseem is a most important winter fodder of Indian region, has a high nutritional value for animal feed due to its higher contents of protein and mineral when compared to other fodder crops (Laghari *et al*., 2000 and Roy *et al*., 2019) [14, 26]. It is widely accepted because of its multi-cut nature (4–8 cuts). It is the most potent milk multiplier in the lactating cattle, provides fodder for a long duration (November to April), high digestibility (up to 65%) and palatability (Pathak *et al*., 2015) [24]. In repeated cuttings, Berseem provided highly nutritious fodder for livestock in winter season. It contains 20% of crude protein, 25.9% of crude fiber and 60-65% of Total Digestible Nutrients (TDN) content which is maximum among all the forage crops. Berseem is superior to grasses in minerals and protein content, mainly in calcium (1.5% - 1.6%), phosphorus (0.1% - 3%), magnesium, copper, and cobalt, whereas non legume forages contain 5-10% crude protein, 0.3-0.5% calcium and 0.2-0.3% phosphorus. (Frame, 2005, Egyptian Clover (*Trifolium alexandrinum* L). Berseem can also be used for phytoremediation of heavy metals, namely, Cd, Pb, Cu and Zn due to its multi-cut nature, short life span and high production of considerable biomass (Ali *et al*., 2012) [1]. It is also a valuable source for honey. Crossing in berseem is difficult because of its small sized flowers, which creates problem for new variety development. Kehr, 1996 [12] stated, induced autopolyploidy is proven to be most successful as the obtained polyploids have larger flower size, longer blooming period, and relatively longer lasting flowers. Also, variety development through traditional breeding requires a long period of time around 6-8 years. Polyploidy fulfills the aim of higher yield in Berseem.
Berseem, evidently with low chromosome number (2n=14) offers efficient results because the chromosome most beneficial of the species could not be surpassed in autopolyploid.

There is a higher chance of bringing approximately a powerful recombination of genetic material in allogamous plant species than in autogamous ones. In addition, Berseem is a cross-pollination species and is a leguminous crop which is cultivated for its vegetative yield (Levan, 1942) [17]. Induced polyploid leads to crop's better vigour, higher adaptability to environmental conditions, increased cell size which is the prerequisite to fodder crops. Other benefits of being polyploid include better salinity, drought, or extreme temperature stress tolerance (Sattler *et al*., 2016) [28]. Induced autopolyploidy has been most successful as they have bigger flower size, longer blooming period, and relatively longer lasting flowers (Kehr, 1996) [12]. Potential evolutionary/adaptive advantages of being polyploid include increased heterosis, gene redundancy and mutational robustness, and phenotypic plasticity (Comai, 2005; Sattler *et al*., 2016) [5, 28]. Doubling a cell's genome is anticipated to double the extent of area occupied by using the chromosomes in the nucleus, but it causes most effective a 1.6-fold growth in the surface area of the nuclear envelope (Melaragno *et al*., 1993) [22]. This can disrupt the balance of factors that usually mediate interactions among the chromosomes and nuclear components, such as envelope-bound proteins. The disturbed peripheral positioning of telomeric and centromeric heterochromatin as there might be less relative surface area on the nuclear envelope to deal with this positioning (Fransz *et al*., 2002) [11].

Polyploidy is a problem for the normal completion of mitosis and meiosis due to the complex chromosome pairing that can occur when more than two copies of each chromosome are present (Comai, 2005) [5]. Working on polyploidy is a tedious job & it adds cost to the experiment. Requires technical knowledge along with sophisticated laboratories for successful results with min. error. Survival rate of plants is less, use of colchicine along with being effective is toxic to humans. With the advancement in science and technology and efficient breeding methodologies, improvement in fodder crops is getting done (Smyth *et al*., 2014) [31]. Molecular techniques like RFLP, AFLP, RAPD, isozymes are also being used for cultivar identification, detection of hybrids and genetic mapping and QTL identification and gene tagging (Smyth *et al*., 2014) [31].

The study aimed to induce polyploid, evaluate & to assess the effect of polyploids over their diploid counterparts of Berseem derived from genetically homogenous plant material treated with colchicine.

Materials and Methods

Materials

The present study comprised of 2 genotypes of Berseem (*Trifolium alexandrinum* L.) *viz*., Mescavi & BB 3. A total of 450 seeds were taken from each genotype and 150 seeds of the same as their control. Seeds with bright yellow colour were chosen in Berseem as these kinds of seeds are good for germination as stated by Luthra (1932). Seeds were obtained from Forage Breeding department of Indira Gandhi Krishi Vishwavidyalaya, Raipur in Rabi 2020-21 season.

Table 1: Salient features of varieties used in the experiment.

Features	Mescavi	BB 3
Releasing centre	Chaudhary Charan Singh Haryana Agricultural University, Hissar, Haryana	ICAR-IGFRI, Jhansi
Year of release	1975	2001
Genome/ Chromosome No.	2n=2X=16	2n=4X=32
Features	Possesses basal branching and is multi-cut in nature. Fast regrowth and wide adaptability. Gives green fodder yield of 70 t/ha.	An autotetraploid variety which yields more fodder production and good regeneration capacity, uniform and higher yield throughout the season than diploid varieties Gives green fodder yield of 65-80t/ha.
Reason for taking	Good performance in C.G in terms of fodder yield.	Used as a check and to develop auto-octaploid.

Method:

For the induction of polyploidy, seeds were treated using the chemical, colchicine. 3 treatments were used *viz*., 0.01%, 0.02% and 0.05% for 2.5 hours. A total of 150 seeds were used for each treatment in 3 replications (50 seeds each replication) for both the varieties. Seeds were made to germinate in protrays with cocopeat used as a germinating substratum. Root tips of size 0.5 cm from each treatment were cut, fixed using Acetic acid: Alcohol (1:3) and stored at 4 ^{0}C. To screen the polyploids, firstly, the induced polyploids were isolated on the basis of morphological differences when compared to control, secondly, they were confirmed on the basis of stomatal observations and then were confirmed later on from cytological studies i.e., by comparing the nucleus size of the suspected polyploids with the controlled ones. Height of the plant, leaf size & leaf area were the sole parameters for primary screening. The obtained auto-tetraploids were further made to check the effect of polyploids over their diploid counterparts. On the ground of different morphological traits.

Screening via stomatal observations

The phenotypically selected plants were screened to confirm the induced polyploidy on the basis of stomatal study which included stomatal length (µm), stomatal width (µm) and stomatal frequency for each treatment for both the varieties *viz*. Mescavi and BB 3. Leaf impression using nail polish and sticky tape on the microscope slide method was used to take stomatal observations. Zeiss Primostar microscope with the camera type- US300 and camera name US300-0 was used. A software, namely, VImage 2013 was used. Images were taken at 40X magnification.

Screening via nucleus size

For nucleus study, squash-method was followed. The pre-stored root tips of size 0.5 cm were taken which were already fixed using Acetic acid: Alcohol (1:3) for 24 hrs. Feuschin solution was used as a nuclear stain on the microscopic slide. The prepared microscopic slide was viewed under the microscope at 100X magnification. Zeiss Primostar microscope with the camera type- US300 and camera name US300-0 was used. Observations, including images and measurements (area, (µm^2)) of the nucleus was done with using the software, VImage 2013.

Statistical analysis to check the effect of autopolyploids over the diploids (control): Atleast, ten individual plants from each of the three (0.01%, 0.02% and 0.05%) treatments were evaluated for 14 characteristics (plant height cm, no. of branches plant^{-}, no. of leaves plant$^{-1,}$ leaf dimensions including length (cm), width (cm) and leaf area (m^2), flower length (cm) and width (cm), days to first flowering, no. of flowers plant$^{-1,}$ no. of seeds plant$^{-1,}$ seed length (cm), seed width (cm) and 10 seed wt. mg). Tetraploid plants were checked for significant variations from their diploid counterparts statistically (t-test: Two sample Assuming Unequal Variances) for size of the nucleus (area, μm^2) and stomatal observations including its length (µm), width (µm) and count (no.).

Statistical analysis of nucleus size and stomatal size and count: Confirmed seed-yielding tetraploids were compared with the diploids (controls). Stomatal and nucleus size measurements were taken. Results with $p< 0.05$ and $p< 0.01$ were considered to be statistically significant.

Results

Post-treatment results of colchicine application and transplanting

It is evident from Table 2 that the germination percentage of seeds of Mescavi which were treated with colchicine was very low ranging from 82.66% to 89.34% while in control the percentage was high i.e., 94%. In the variety BB 3 germination percentage ranged from 92.66% to 93% while in its control, 95% germination was recorded. There was a decrease in germination percentage with increase in colchicine concentration which was recorded in Mescavi. The same was not true for BB 3.

Table 2: Effect of colchicine treatment on germination & survival percentage

	Duration in hr.	Conc.%	No. of seeds treated	No. of seeds germinated	Germination %	No. of seedlings survived upto flowering	Survival %
Mescavi	2.5	0.01	150	134	89.34	128	95.52
	2.5	0.02	150	131	87.34	121	92.36
	2.5	0.05	150	124	82.66	111	89.51
Control	-	-	150	144	94	141	97.91
BB 3	2.5	0.01	150	138	92	136	98.55
	2.5	0.02	150	140	93.33	139	99.2
	2.5	0.05	150	139	92.66	136	97.8
Control	-	-	150	143	95	142	99.3

Results obtained on morphological screening

Periodical observations were taken for morphological parameters (plant height, leaf length, width and leaf area) at intervals of 45, 60, 75 and 95 days after sowing for 10 suspected plants. Mescavi reported an initial stunted growth and then an increase in plant height at subsequent intervals, whereas, BB 3 did not respond with any such abnormal growth. Few plants from each treatment showed an abnormal increase in leaf dimensions and leaf area of Mescavi. So, the suspected plants were further made for confirmations using stoma and nucleus observations. BB 3 did not respond in the similar way, hence the suspected populations were screened for stomata and nucleus.

Results obtained on stomatal screening

Mescavi showed greater positive variations in stomatal size. Frequency of stomata showed negative results as the ploidy increased against of diploids and hence, using the recorded data, number of confirmed plants were recorded from individual treatments of Mescavi. BB3 did not show any variations for polyploidy. Maximum polyploids were obtained from highest dose (4 confirmed plants from 0.05%) and minimum from lowest (2 confirmed plants from 0.01%). Second dose which was 0.02% gave 3 confirmed plants when screened for stomata. BB 3, an autotetraploid was used as a check variety to confirm the tetraploids of Mescavi. Length of tetraploid stomata varied from 44.13 µm to 59.24 µm against 40.16 µm to 49.64 µm of diploids (controlled ones) and width of tetraploids ranged from 23.02 µm to 32.81 µm against 19.69 µm to 26.02µm of wild diploid. The average number of tetraploid stomata per microscopic field ranged from 12-14 in number against 15-17 of diploids. The same result was concluded by Leal-Bertioli *et al*. (2012) []. Red clover experiment which was conducted by Levan (1940) [16] and study of white clover and lucerne by Frandsen (1945) [10] showed that an increase in ploidy of a species was accompanied by an increase in the length of stomata. The confirmed plants were screened for variations at nuclear stage i.e., differences at size (area, μm^2) of nucleus were checked.

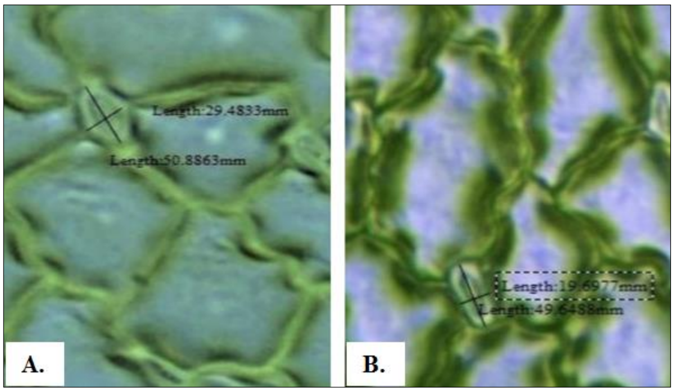

Fig 1: A. Length & width of tetraploid stomata. B. Length & width of diploid stomata at 40X magnification at 2.22mm microscopic field of view.

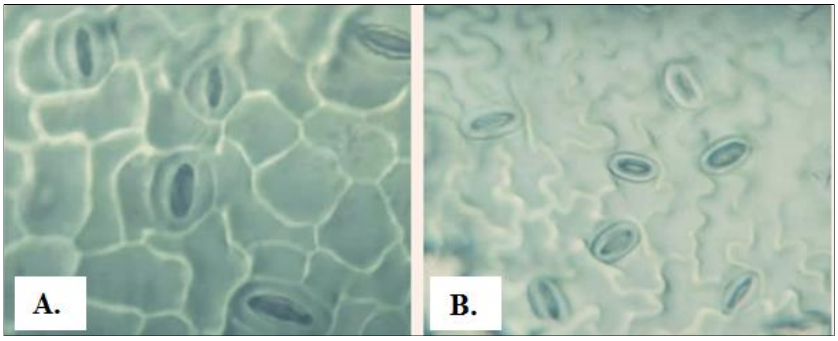

Fig 2: A. Frequency of tetraploid stomata (5 in no.) & B. frequency of diploid stomata (7 in no.) at 100X magnification at 2.22mm microscopic field of view.

Results obtained on nucleus screening

Evans (1955) [8] stated that an increase in size of the individual cells of the plant is perhaps the most widespread effect of polyploidy. An increment in the size of nucleus was obtained in tetraploids of Mescavi. Increased mean area value was obtained for tetraploids when compared to the diploid ones. A significant increase was reported. Out of 10 suspected plants, 9 were get confirmed for autopolyploids which was the end result of screening.

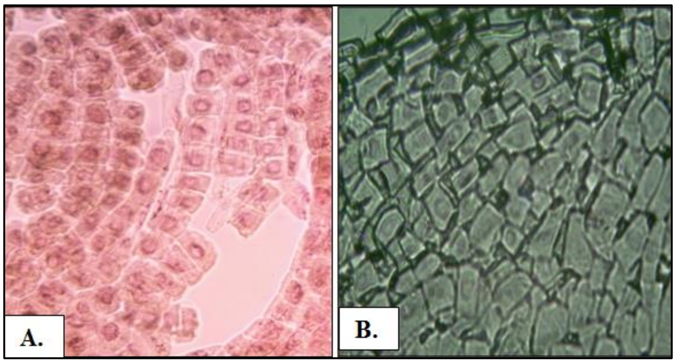

Fig 3: Variations between and nucleus size of tetraploids and diploids. A. Microscopic view of a diploid cell with nucleus at 100x magnification. b. microscopic view of a tetraploid cell with nucleus at 100x magnification.

Effect of polyploids over their diploid counterparts

Rauf *et al*. (2021) [25] stated "There are numerous positive effects of induced polyploidy when compared with diploidy in various forage species; i.e., larger leaves, herbage yield and increased plant height". Out of 9 autotetraploids obtained, only 6 yielded viable seeds. Hence, those were tested for a significant variation for all the morphological, physiological and cytological traits. Atleast ten individual plants from each of the three (0.01%, 0.02% and 0.05%) treatments were evaluated for 14 characteristics (plant height cm, no. of branches plant$^{-1,}$ no. of leaves plant$^{-1,}$ leaf dimensions including length (cm), width (cm) and leaf area (m^2), flower length (cm) and width (cm), days to first flowering, no. of flowers plant$^{-1,}$ no. of seeds plant$^{-1,}$ seed length (cm), seed width (cm) and 10 seed wt. mg).

Increment in plant height, no. of branches and no. of leaves per plant was observed. Number of seeds per plant showed negative significant difference at p value of less than 0.05. Rest of the above-mentioned characteristics showed positive significant difference at p value of less than 0.01.

20 stomata for control and tetraploid plants were taken for stomatal measurements. Similar observations were taken for nucleus size analysis. Data analysis was performed using MS-Excel, results with $p< 0.05$ and $p< 0.01$ were considered to be statistically significant. Stomatal length, width and count of tetraploids were significantly different from diploids. Stomatal length (t-stat=6.33**) and width (t-stat= 5.5**) showed positive significant difference while no. of stomata showed negative significant difference (t-stat= -9.28**) when compared to untreated control plants.

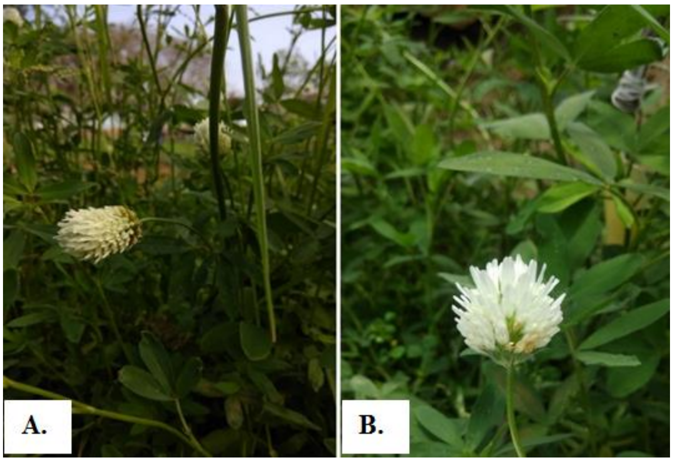

Fig 4: Flower size variation in tetraploid and diploid. A-Tetraploid flower. B- Diploid flower.

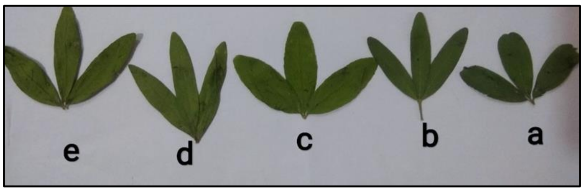

Fig 5: Variation in leaf length and width of tetraploids and diploids. a & b-leaves of diploid (control). c, d & e- leaves of tetraploid (of conc. 0.05%, 0.01% & 0.02% respectively).

Multifoliation in auto tetraploids

Most commonly cultivars of Egyptian clover (*Trifolium alexandrinum* L.) possess trifoliate leaves. However, occasional occurrence of multifoliate plants in natural population is reported (Shukla and Malaviya, 1984) [20]. Pathak *et al.* (2015) [24] had shown multifoliate leaf formation in induced tetraploids in *Trifolium alexandrinum* L. A pentafoliate line of *Trifolium alexandrinum* L. Penta-1 was registered by IGFRI as a novel genetic stock (Malaviya *et al.*, 2009) [19]. Tetraploids from all of the three (0.01%, 0.02% and

0.05%) were showed trifoliate as well as tetrafoliate leaves. Penta-foliate leaves were obtained with concentration of 0.01% and 0.05% whereas, with treatment 0.02%, only tetrafoliate leaves were obtained. Frequency of these multifoliate leaves per plant was ranging from 6-13, amongst which tetrafoliate leaves appeared more than the pentafoliate leaves. Tetraploids leaves were dark in colour in comparison to green colour of diploid leaves. Khushk and Abidi (1970) [13] reported that polyploid Egyptian clover plants with 4, 5 and 6 leaflets displayed increased vegetative growth. Similar results were drawn by Mehta *et al*. (1964) that tetraploid Egyptian clover showed better performance for green weight when compared to diploids.

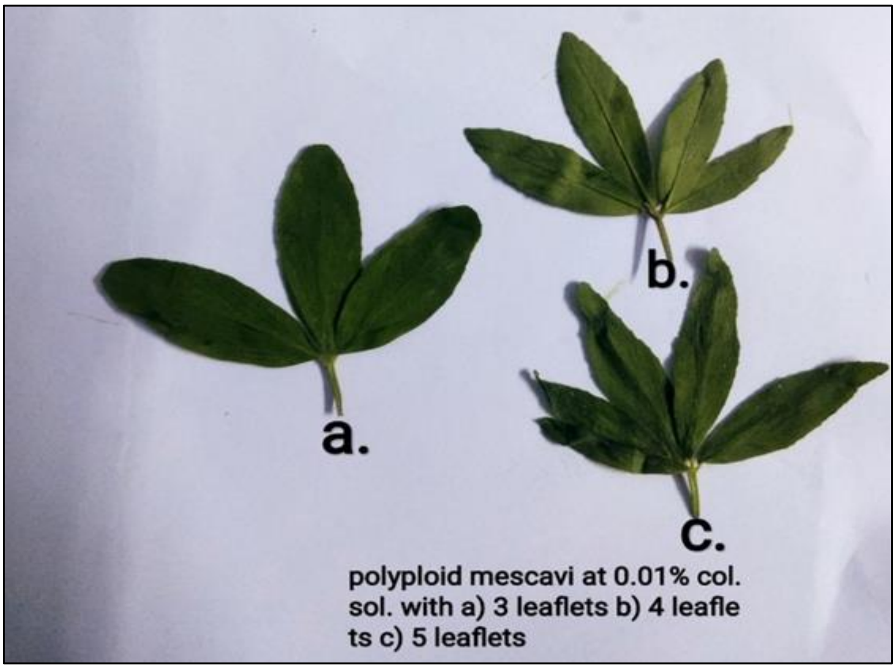

Fig 6: Multifoliate leaves of tetraploids at 0.01% conc.

Fig 7: Multifoliate leaves of tetraploids at 0.02% conc. A- Leaves of tetraploids. B- Leaves of diploids

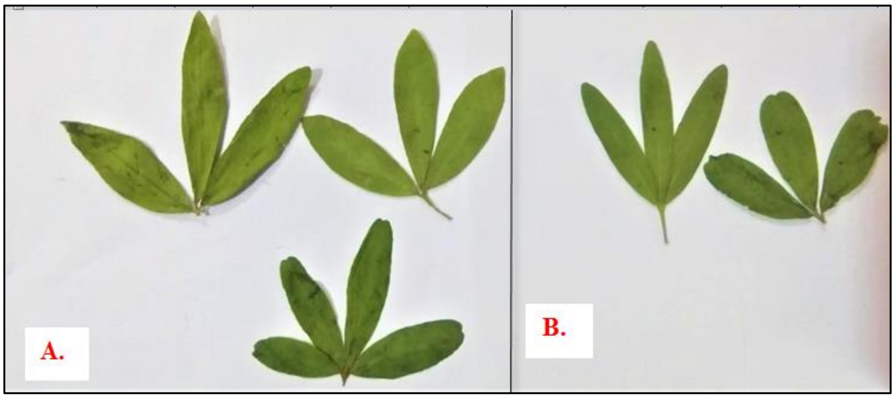

Fig 8: Multifoliate leaves of tetraploids at 0.05% conc. A- Leaves of tetraploids. B- Leaves of diploids.

Fig 9: Penta-foliate leaf of tetraploid plant at 0.05% concentration of colchicine.

Table 3: Significance among morpho-physio & cytological traits

S.no.	Parameter	t-test value		
		0.01% col. Conc.	0.02% col. Conc.	0.05% col. Conc.
1.	Plant height (cm) (n=10)	0.20	0.33	0.52
2.	No. of branches (n=10)	1.79	2.12	2.30
3.	No. of leaves per plant (n=10)	0.64	0.99	1.16
4.	Leaf length (cm) (n=10)	5.19**	5.57**	8.02**
5.	Leaf width (cm) (n=10)	5.05**	6.57**	8.01**
6.	Leaf area (m^2) (n=10)	4.92**	7.12**	5.31**
7.	Flower length (cm) (n=10)	2.73**	2.73**	4.18**
8.	Flower width (cm) (n=10)	5.96**	6.95**	7.81**
9.	Number of seeds per plant (n=10)	-9.67*	-9.67*	-1.41*
		t-test value		
		Tetraploids over diploids		
10.	Days to first flowering (n=10)	12.09**		
11.	Number of flowers per plant (n=10)	4.19**		
12.	Seed length (cm) (n=10)	2.71**		
13.	Seed width (cm) (n=10)	2.97**		
14.	10 seed weight (mg) (n=10)	58.04**		
15.	Stomatal length (µm) (n=20)	6.33**		
16.	Stomatal width (µm) (n=20)	5.5**		
17.	Stomatal count (no.) (n=20)	-9.28**		
18.	Nucleus size (μm^2) (n=20)	7.62**		

*Significant at 0.05 probability level. ** Significant at 0.01 probability level.

Discussion

El-Naby *et al.* (2012) [7] outlined that polyploidization in Berseem (*Trifolium alexandrinum* L.) using colchicine treatment can significantly increase the variability in plant morphology, fertility and yield potential. Pathak *et al.* (2015) [24] reported distinct characteristic features among induced penta-foliate tetraploid plants. The better expression of multifoliate trait in tetraploids than in diploid plants has been explained by induction of auto-tetraploids. Polyploidy resulted in gigantism effect in morphology of the plant including more robust habit, and increase in plant height, thicker stem, broader and greener but fewer leaves with larger stomata, and fewer but bigger plant and pollen and fewer but bigger seeds which was outlined by Blischak (2016) [3] Similar findings have been reported by Sikka *et al.* (1954) [27], Mehta *et al.* (1964) [21] and Dabkeviciene *et al*. (2016) [6]. A strong evidence that increasing ploidy is coupled with, and contributes directly to, an increase in cell size was concluded by Breuer *et al*. (2007) [4] in their study of isogenic diploid and tetraploid Arabidopsis.

In the present study, increased height of the plants, increased tillering and branching per plant, significant increase in leaf dimensions and leaf area, flower dimensions, no. of flowers per plant, delayed flowering, negative significant differences for number of seeds per plant and increased 10 seed weight can also be explained by induction of autotetraploids. A better expression of tetra and penta-foliate traits observed in autotetraploids than in diploid ones, in the present study can be elucidated with polyploidization. A positive significant increment was observed in nucleus size which could be explained via an increase of ploidy level. Observations inferred a positive increment in stomatal size and a decrease in stomatal number that can be explained with an increase in ploidy level of the plant. The similar results were validated by Ardabili *et al.* (2015) [2], Shreshtha *et al*. (2016) and Nagat *et al*. (2020) [23] in *Vicia faba* L. This methodology is a foremost approach for enhancing the vegetative yield, in turn upsurge the fodder production.

Summary

Polyploidization is a widespread phenomenon among forages. There are number of chemicals through which polyploidy can be induced namely, Colchicine, trifluralin or oryzalin. Physical methods are also there for the same purpose such as temperature shock and protoplast fusion. Chemical method using colchicine was used to induce polyploidy in leguminous fodder crop i.e., Berseem (*Trifolium alexandrinum* L.).

Two of the Berseem varieties, Mescavi and BB 3 were used for the polyploidization purpose. The seed samples of the above-mentioned varieties were taken and treated using 3 (0.01%, 0.02% and 0.05%) different treatments of colchicine solution. Abnormal morphological growth is a characteristic feature of polyploids. Stomatal measurements have been proven to be an efficient method to distinguish plants at their ploidy level. Morphological and stomatal parameters were used to screen the polyploids and hence were used to screen the induced plants of both the varieties. However, plants were also screened on cytological basis through microscopy. Out of the two varieties taken, the positive result for polyploidization is observed only in Mescavi. None of the treated plants from BB 3 population showed positive results for polyploidization.

All the confirmed autopolyploids of Mescavi were analyzed on different morphological parameters to check the effect of induced polyploidy over their diploid counterparts. Significant variations from morpho-physio and cytological parameters were marked for the tetraploids obtained from the genotype, Mescavi via statistical analysis, t-test: Two sample Assuming Unequal Variances.

Conclusions

- Among all the 3 treatments used to induce polyploidy *viz*., 0.01%, 0.02% and 0.05%, third treatment (0.05%) gave better results in terms of number of autopolyploids obtained. Also, confirmed tetraploids at 0.05% treatment, showed slightly more variations than rest of the two treatments.
- Induction of polyploidy in *Trifolium alexandrinum* L. using colchicine treatment can bring more variability in plant morphology.
- Along with abnormal morphological growth, microscopy method via, stomatal measurements and nucleus size study proved to be an efficient way to distinguish plant population at their ploidy level. However, other methods of screening such as molecular markers, use of flow cytometry method should be employed for tetraploid selection.
- Autotetraploids have greater forage yield in terms of plant height, larger leaf area as compared to the diploid ones.
- Induced tetraploids showed increased branching, number of leaves per plant, number of flowers per plant, delay in flowering, leaf size, flower size, seed size and increase in 10 seed weight as compared to their diploid counterparts. A decrease in number of seeds per plant was observed.
- Number of seeds per plant showed negative significant difference at p value of less than 0.05. Rest of the characteristics (leaf dimensions including length (cm), width (cm) and leaf area (m^2), flower length (cm) and width (cm), days to first flowering, no. of flowers $plant^{-1}$, no. of seeds $plant^{-1,}$ seed length (cm), seed width (cm) and 10 seed wt. mg)) showed positive significant difference at p value of less than 0.01.
- Significant variations were observed for stomatal dimensions and its count along with the size of nucleus. Stomatal length (t-stat=6.33**) and width (t-stat= 5.5**) showed positive significant difference while no. of stomata showed negative significant difference (t-stat= -9.28**) when compared to untreated control plants. Size of the nucleus (μm^2) of tetraploids showed positive significant variations (t-stat= 7.62**).
- This methodology can be used for selection of more superior adaptive genotypes in plant breeding programs.
- Research results suggested that this polyploidy approach may be pursued in forage breeding.
- Genetically polyploid plants carried multiple alleles which may be helpful to increase allelic diversity and may provide several evolutionary and adaptability advantages.

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