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Use of Natural Hybridization and Karyotypic Analysis to Study the Transfer of Base Tillering from Egyptian Clover Multi-Cut Cultivar to Mono-Cut Cultivar

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ABSTRACT

This investigation was conducted at Gemmeiza Agric. Res. Station, ARC, Egypt, during the period between 2014-2016, to study the effect of natural hybridization in transferring base tillering trait from multi-cut to mono – cut cultivars of Egyptian clover. Differences between the parental types *Trifolium alexandrinum* Fahl, Miskawi and their hybrid was determined by using morphological and Karyotypic analysis. The cultivar Fahl Giza 1 was used as a female parent, Miskawi Gemmeiza 1 as a male parent and the first generation (Miskawi Gemmeiza 1 x Fahl Giza 1) were used for further cytological examination the results appeared that, the hybrid plants were multicut similar to Miskawi as will as, branching was basal similar to Miskawi and apical similar to Fahl. The blooming period in Fahl, Miskawi and hybrid was 108.00, 137.55 and 131.15 day, respectively. The differences of the blooming period among the different genotypes were highly significant. Seed number per head was 51.60 for Fahl, 44.30 for Miskawi and 68.00 for hybrid although the weight of 100 seeds in Fahl, Miskawi and hybrid was 0.31, 0.27 and 0.30 gram, respectively. Fahl and Miskawi has the same chromosome number (2n = 16) and total length of all chromosomes of each type was almost equal to the other. The length of chromosome No. 1 in Fahl was larger than of Miskawi, while chromosome No. 3 in Miskawi was larger than that of Fahl. The chromosome ratio of the long arm to the short arm in Fahl and Miskawi show difference only between the corresponding chromosomes No. 1 and 4 in both cultivars. The chiasma frequency was less in hybrid plants than in both parents. It was 12.8, 12.6 and 11.4 in Fahl, Miskawi and hypbrid, respectively. The hybrid recored the hightest percentage of cells containing chromosomal aberration (14.36%). Significant differences were obtained chromosome area, chromosome length and mitotic index among the varieties.

Keywords: Karyotype, mitotic index, chromosomal aberrations, Berseem clover.

INTRODUCTION

Egyptian clover, berseem, (*Trifolium alexandrinum*) is the most important winter forage in Egypt, india and most countries of Mediterranean region. The crop is reported to be highly self – compatible requiring normal hybridization via wind and insects. Some morphological and cytological traits of berseem were studied by (Tobgy *et al.,* 1974).

Miskawi and Fahl are two Egyptian types of berseem, based on stem branching, number. of cuttings, plant vigor and seed yield. Miskawi type is a basal or crown branching type that can be cut, from four to six times during its growing season but produces low seed yield. While, Fahl type is a stem branching and is cut only once but has more seed yield if compared with Miskawi. Out crossing between Miskawi and Fahl cultivars could be used as a tool to improve forage yield and quality Bakheit (1996) developed a new foliate multi – cut line of berseem clover by crossing a mutant of mono – cut Fahl cultivar having multi foliate leaves and a multi – cut Miskawi cultivar with trifoliate leaves. Fahl is a unicut plant while Miskawi is multicut plant and gives from 4 to 5 cuts.

Chromosomes of two subspecies diploid (2n = 2x = 16) of Miskawi, Fahl ssp and their hybrid were studied by C-banding technique Ellison *et al.,* (2006). The differences in banding patterns between these types allowed the identification of parental chromosomes in hybrid cells. (Mccoy and Bingham, 1988); (Pfeiffer and Bingham, 1983); (Ellison *et al.,* 2006).

The present investigation aimed to in traduce :

- Some light on the hybridization between Fahl and Miskawi. Through Karyotype analysis of chromosome for the both parents and their hybrid mitotic cell division, the frequency of chromosomal aberrations and develop a standard C-banded Karyotypal to identify parental chromosomes in hybrid.

MATERIALS AND METHODS

Two varieties of berseem; Miskawi Gemmiza 1 cultivar which was used as a male parent and Fahl cultivar as a female parent. These varieties were obtained from the Forage crops Res. Dept, Field Crops Research Institute, Agricultural Research Center, Egypt and the Lab procedures were carried out in Genetic Dept. Faculty of Agric., Alex Univ.

Morphological Studies, were done to distinguish between the two types (Fahl, Miskawi) and their hybrid. These included number of cuts, branching behavior, length of branches on successive nodes and length of successive internodes along the main stem. Certain reproductive characters such as : blooming date, number of florets per head, number of seeds per head, seed per floret, weight of 100 seeds.

Cytological studies

This investigation used karyotypic analysis in clouded the area of chromosome, length of long and short arm chromosome, centromeric index, position of chromosome, mitotic activity and chromosomal aberrations.

Karyotype analysis

Chromosomal studies were carried out, based on visible characteristic of the chromosomes. Karyotype analysis is a well established method based on the morphological characteristic of the chromosomes according to Fukui and Kakeda, (1994). Imaging by digital camera in the c- metaphase of dividing root tip cells, pretreated with 0.05 % colchicine and analyzed using the video test karyotype software (Ikaros Karotyping Platform). Measurement of the total length of chromosome (um), long and short arms of chromosome, area of chromosome (um), arm ration, cetnromeric position and centromeric index percentage (length of short arm/ length of chromosome) were taken for every chromosome.

From the karyotype analysis of *Trifolium* genome, the two homologues (a and b) of each chromosome pair were judged according to similarities in length of short arm, long arm, total length, arm ratio and centromeric index percentages which were calculated (a and b)/2 for each pair and the chromosome pairs were arranged in descending order and were given numbers from l to 8.

Samples preparation

Seeds were germinated on moisture filter paper in Petri dishes at 25-30°C in an incubator. Root tips were collected at 3 days after germination of about 1.5 to 2.0 cm length Schwarzacher (2016).

Colchicine treatment

The roots were placed in glass vials containing 2 ml of 0.05% colchicine for three hours at room temperature or on ice water over night Schwarzacher (2016).

Fixation and slide preparation

Fixation was done using ethanol-glacial acetic acid (3:1 V/V) fixative. Those samples were washed thoroughly with water. Samples flamed by forces and stained by the aceto orcein solution. Stained samples were used for automatic scanning experiments. Karyotype analysis was carried out using image Process Analysis System (Video Test- Karyo). The mean measurements in the c-metaphase of fifteen cells for each variety were used to construct the karyotype Schwarzacher (2016).

- **Mitotic index and chromosomal aberrations**

Seeds from the two varieties were germinated on moist filter paper on Petri dishes at room temperature in a randomized complete design with three replicates. Each replication comprised three dishes for each entry and each dish contained 30 seeds. Actively growing root-tips were cut from the seedlings and fixed in farmer solution. The aceto-carmine squash technique was used to stain the root-tip cells as described by Sayed-Ahmed (1985). Nine prepared slides were used for each variety to determine the frequencies of mitotic index and chromosomal aberrations.

Mitotic index represented the percentage of divided cells to the total cells examined. The total number of chromosomal aberrations was estimated in dividing cells. The abnormalities included cells with micronuclei, fragments, laggards, stickiness and bi-nucleate cells (Schwarzacher, 2016).

Statistical analysis

The field layout (Fig 1) consisted of 19 faddan and their first generations polycrosses a long random from the male parent variety (Miskawi Gemmiza 1).

The data were statistically analyzed according to Snedecor and Cochran (1967). The least significant difference (L.S.D), value was used to compare between means if the differences were significant.

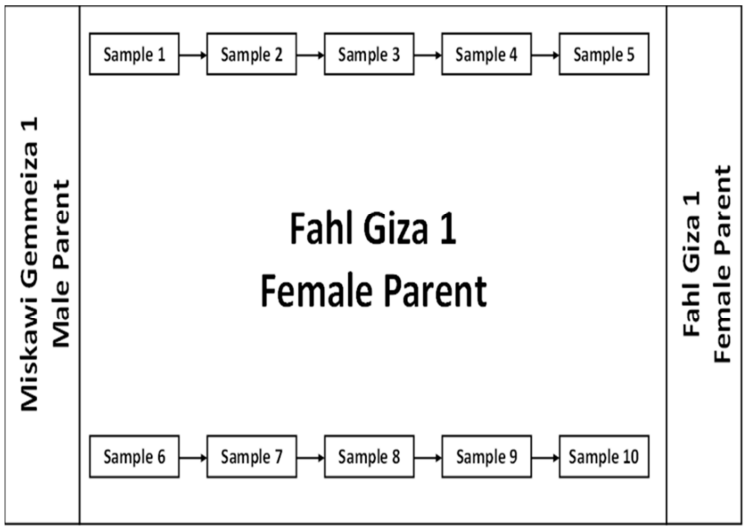

Fig. 1. Field Idiogram of Egyptian clover appeared various samples taken from the female parent.

RESULTS AND DISCUSSION

The present study includes the description of certain morphological traits to differentiate between two parental types of *Trifolum alexandrinum L*;. Fahl and Miskawi, as well as the crossability between the two types, karyological and cytological behaviour of both parents and their hybrid.

Morphological studies

Some morphological traits included number of cuts, branching behaviour, length of branches on successive nodes and length of successive internodes along the main stem, were studied.

In addition, some reproductive traits such as blooming date, number of florets per head, number of seeds per head , seed per floret, weight of 100 seeds, were studied.

Number of cuts

Fahl gives only one cut (unicut) while miskawi gives from 4 to 5 cuts (multicut). The hybrid proved to be multicut similar to miskawi.

Branching

Branching in Fahl is apical and restricted in the upper part of the main stem (Figure 2) However, in miskawi, branching is basal and profused. The basal branches are lager than those of Fahl (Figure 3). The first or second node bears the largest branch and then the length decreased gradually in the successive branches till the top of the plant (Fig 4 A-B).

Fig.2(A, B). Photograph of apical branching in the Egyptian Clover Fahl.

Although the hybrid branching was similar to the male parent Miskawi. The length of the branches of the basal zone was almost equal to that of miskawi their length was intermediate between the two parents (Figure 4).

Hybrid plants, proved to be multicut with basal and apical branching behavior similar to the parents. Consequently, number of cuts and branching behaviour were used to distinguish between the parents as well as the hybrid plants.

Zaher (1947 and 1956), Abou Sayed and Nassib (1958), Kaddah (1962) and Tanash (1970) described the branching behaviour of Fahl and Miskawi in dense growings. They found that branching in Fahl was apical and restricted to the upper part of the main stem, while in Miskawi it was basal and profuse.

Reproductive traits

Random samples of 30 plants were taken from each of Fahl, Miskawi and the hybrid to determine flowering date, number of florets per head, number of seeds per head and weight of 100 seeds.

A

Fig.3(A). Photograph of basal branching traits in the Egyptian Clover in the Miskawi.

B

Fig.3(B). Photograph of basal branching traits in the Egyptian Clover in the Miskawi.

C

Fig.3(C). Photograph of basal branching traits in the Egyptian Clover in the Miskawi.

D

Fig.3(D). Photograph of basal branching traits in the Egyptian Clover in the Miskawi.

Fig.3(D). Photograph of basal branching traits in the Egyptian Clover in the Miskawi.

Fig.4. Photogroph of apical and basal branching in the Egyptian Clover hybrid.

Flowering date

This date was determined by the number of days from sowing to flowering of the first floret in the terminal head. In this study hybrid plants were left without cutting. The blooming period was ranged from 95 to 120 days and 127 to 150 days for Fahl and Miskawi, respectively, (Table 1 and Fig. 5).

Blooming period of the hybrid was intermediate between those of the two parents, which ranged from 120 to 144 days.

Blooming date, have been determined by the number of days from sowing to blooming of the first floret in the terminal head of the main stem. Mean value of blooming period in Fahl was 108.00 days, while in Miskawi it was longer with a mean value of 137. 55 days. In hybrid plants, the blooming period was intermediate between the parental types, with an average of 131.15 days (Table 1).

Zaher (1956), found that the beginning of heading in Fahl started after 102 days from sowing, while in Miskawi was 104 days However,

Tanash (1970) demonstrated that heading in Fahl started from 95 to 140 days with a mean value of 116.2 days.

Figure 5. Flowering photograph in the Egyptian clover hybrid.

Table 1. Ranges of days from sowing to blooming date Fahl, Miskawi and their hybrid

Type	No. of Plants	Range by days	Mean of Blooming date by days
Fahl	35	95 – 120	108.00
Miskawi	35	127 – 150	137.55
Hybrid	35	120 – 144	131.15
L.S.D. 5%			**

Number of seeds per head

In Fahl, number of seeds per head was ranged from 24 to 74 and the mean value of seeds per head was equal 51.60 (Table 2).

In Miskawi, number of seeds per head was ranged from 11 to 83 and the mean value of seeds per head was equal 44.30.

In hybrid plants, number of seeds per head was ranged from 40 to 93 and the mean value of seeds per head was equal 68.00.

Table 2. Ranges of seeds per head and the mean of seeds per head for both parents and their hybrid :

Type	No. of heads	Range of seeds / head	Mean number of seeds/head
Fahl	50	24 – 74	51.60
Miskawi	50	11 – 83	44.30
Hybrid	50	40 - 93	68.00
L.S.D. 5%			*

The Same author found that the number of seeds per head was ranged from 15 to 89 seeds, with a mean value of 50.5 seeds.

Some other workers estimated number of seeds per head in Miskawi and obtained various results due to different environmental conditions (Hassanein, 1953, Said, 1954, Wafa and Ibrahim, 1960).

Weight of 100 seeds

Separate samples of 100 seeds were taken at random from samples of 10 plants of Fahl, Miskawi and their hybrid.

In Fahl, weight of 100 seeds was ranged from 0.18 to 0.42 grams with a mean value of 0.31 gram (Table 3).

In Miskawi, weight of 100 seeds was ranged from 0.21 to 0.35 grams with a mean value of 0.27 grams.

In hybrid plants, weight of 100 seeds was ranged from 0.18 to 0.40 grams with a mean value of 0.30 grams.

The data indicated that weight of 100 seeds in Fahl is higher than in Miskawi and the difference was significant.

The weight of 100 seeds in the hybrid plants was similar to that of Fahl.

Table 3. Number of Plants, Ranges of weight of 100 seeds of different plants, means value of 100 seeds of Trifolium alexandrinum (Fahl and Miskawi) and their hybrid :

Type	No. of Plants	Weight of 100 seeds by gram	Mean weight of 100 seeds by gram
Fahl	18	0.18 – 0.42	0.31
Miskawi	18	0.21 – 0.35	0.27
Hybrid	18	0.18 – 0.40	0.30
L.S.D. 5%			*

Karyotype characterization

Karyotype analysis of the parents and their hybrid chromosome number of 2n = 2x = 16 (Figures 6, 7 and 8) included area of chromosome, chromosome length, arm ratio and centromeric index, as well as, centromeric position (Tables 4, 5 and 6).

Mean of chromosome area

The data obtained from the karyotype expressed by chromosome area of the parents and their hybrid are given in Table (4). It was showed that the variation in chromosome are among the parents and their hybrid was significant. This variation was ranged from the highest value of chromosome area in hybrid (4.36 um) to lowest value in Miskawi (3.1um).

Table 4. Mean of chromosome area of the parents and their hybrid.

Type	Average chromosome area (um)									
	1	2	3	4	5	6	7	8	Total area	Mean area
Miskawi male parent	5.09	4.77	3.36	3.29	2.35	2.33	1.57	2.04	24.80	3.10
Fahl female parent	5.25	4.92	3.64	3.96	2.93	2.93	1.85	1.82	27.30	3.41
Hybrid	6.23	6.72	5.94	5.16	3.71	3.63	1.84	1.72	34.95	4.36
L.S.D. 5%										0.97

Data presented in Table (5) showed that the chromosome length is a type depended on the variety of *Trifolium alexandrinum*. These differences were ranged from the higher score of chromosome length in hybrid variety (2.57 um) to the lower value in Fahl variety (2.20 um). The maximum chromosome length was (3.59 um) which recorded in chromosome I of female variety Fahl, where as the minimum was (1.27 um) in chromosome 8 of the same female parent variety.

The high level of chromosome length indicated more crossing over and recombination Fayed *et al.,* (1990).

Table 5. Mean of chromosome total length for parents and their hybrid.

Type	Average of chromosome total length (um)									
	1	2	3	4	5	6	7	8	Total length	Mean length
Miskawi male parent	3.40	3.52	2.49	2.82	2.44	2.34	1.58	1.78	20.37	2.54
Fahl female parent	3.59	3.12	2.74	2.1	1.83	1.66	1.33	1.27	17.64	2.20
Hybrid	2.75	3.33	2.88	2.93	2.73	2.30	1.93	1.78	20.63	2.57
L.S.D. 5%										0.23

Both of them have the same chromosome number (2n = 16) and total length of all chromosomes was almost the same in both types. Chromosome number agrees with the findings of Darlington and wylie (1955) and Mehta, *et al.,* (1963) in t*rifolium alexandrinum*.

Mean of long and short arm chromosome

The mean of long and short arm chromosome was given in Tables (6) and (7).

Berseem types showed different trends between parents and their hybrid, where the highest chromosome long and short arm was exhibited by the hybrid and Miskawi, respectively while the lowest one was displayed in Fahl. The maximum mean of chromosomes long and short arm were 1.42 um and 1.02 um, respectively, which recorded in chromosome 4 of both parents, while the minimum chromosome arms were 0.62 um and 0.54 um which recorded for chromosome 8 and 7 in Fahl female parent. Similar results were recorded by Eun *et al.,* (2011) and Kurata and Omera (1978).

Table 6. Mean of chromosome long arm of the eight chromosomes in the parents and their hybrid.

Type	Average of chromosome long arm (um)									
	1	2	3	4	5	6	7	8	Total	Mean
Miskawi male parent	1.74	1.78	1.35	1.42	1.29	1.28	0.87	1.04	10.77	1.34
Fahl female parent	1.85	1.54	1.33	1.02	0.88	0.74	0.62	0.54	8.52	1.06
Hybrid	1.88	1.74	1.66	1.52	1.51	1.25	0.98	0.87	11.41	1.42
L.S.D. 5%										0.03

Table 7. Mean of chromosome short arm of the eight chromosomes in the parents and their hybrid.

Type	Average of chromosome short arm (um)									
	1	2	3	4	5	6	7	8	Total	Mean
Miskawi male parent	1.66	1.74	1.14	1.40	1.15	1.06	0.71	0.74	9.06	1.20
Fahl female parent	1.74	1.58	1.41	1.08	0.95	0.92	0.71	0.73	9.12	1.14
Hybrid	1.87	1.59	1.22	1.41	1.22	1.05	0.95	0.91	9.42	1.17
L.S.D. 5%										0.04

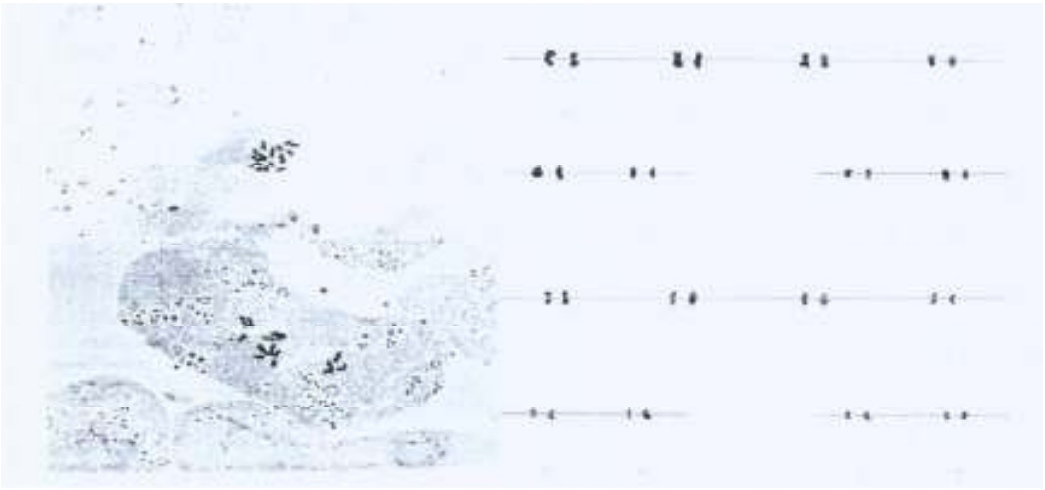

Figure 6. Metaphase and karyotype of Miskawi chromosomes.

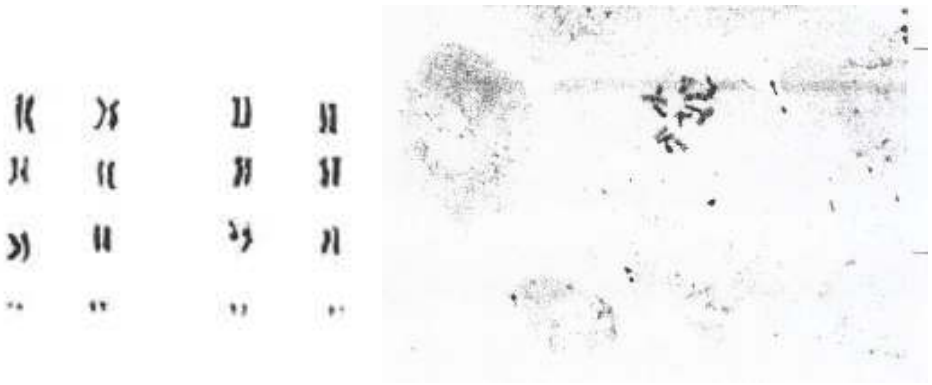

Figure 7. Metaphase and karyotype of Fahl (Giza 1) chromosomes.

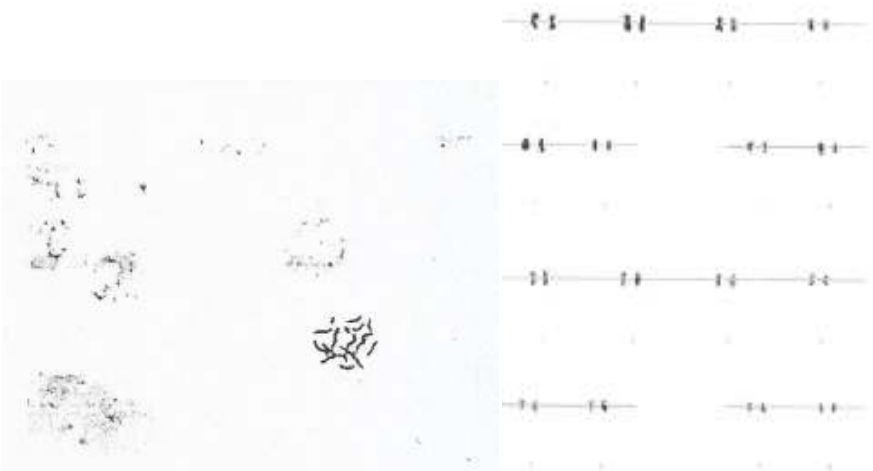

Fig. 8. Metaphase and karyotype of hybrid chromosomes.

Chromosomal aberrations

The data in Table (8) showed that the cells containing either micronuclei or chromosomal aberrations in mitotic divisions depended on the variety of parents and their hybrid These differences were ranged from the highest score of chromosomal aberrations in Fahl and hybrid. The types of chromosomal aberrations observed were fragments, stickiness, binucleate cells, laggards, micronuclei and chiasmata per cell.

The occurrence of low or high chromosomal aberrations may depend on the rate of DNA repair mechanism which differed between genotypes. In this respect, Suzanne (2008) reported that chromosomal aberrations are resulted from repaire failure of damaged DNA

Table 8. Percentage of chromosomal aberrations mitotic division of the parents and their hybrid.

Type	Total No. of cells	Total No. of Divided cells	No. of micronuclei types				Percentage of micronuclei %		Percentage of types of chromosomal aberration						
			Non-compact		Compact				Chromosomal aberrations %	Lagged %	Binculeate cells %	Stickiness	Fragment	Chiasmata frequency per cell	M.I. %
			No.	%	No.	%	No.	%							
Miskawi male parent	2170	388	-		-			-	-	-	-	-	-	12.3	28.67
Fahl female parent	2188	502	41	6.39	21	4.74	57	16.27	13.16	4.16	0.00	2.14	4.75	12.8	28.98
Hybrid	1987	423	52	5.19	22	3.64	61	17.17	14.36	4.22	0.00	2.57	3.78	11.9	21.28

M.I. % = Percentage of mitotic index.

Mean of the arm ratio

Data summarized in Table (9) shows the variation in arm ratio between the types. This variation was ranged from the highest value in Fahl (1.35um) to the lowest value in Miskawi (0.01 um). Both values are shown in chromosome number eight

Table 9. Mean of arm ratio of the eight chromosmes in the parents and their hybrid.

Type	Arm Ratio of Chromosome (um)							
	1	2	3	4	5	6	7	8
Miskawi male parent	0.95	0.97	0.84	0.98	0.89	0.82	0.81	0.01
Fahl female parent	0.94	1.02	1.06	1.05	1.07	1.24	1.14	1.35
Hybrid	0.99	0.91	0.73	0.92	0.80	0.84	0.89	1.04

CONCLUSION

Finally may be the karyotype analysis of chromosomes and morphological traits were conducted for transferring bags tillering trait from multicut variety to monocot variety of Egyptian clover.

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استخدام التهجين الطبيعي وتحليل الهيئة الكروموسومية لدراسة نقل صفة التفريع القاعدي من البرسيم المصري المتعدد الحشات إلى البرسيم المصري الفحل وحيد الحشة

عبد العزيز طلعت بندق، شيرين محمد النحراوي و عبد الكريم بدوي

قسم بحوث محاصيل العلف – معهد بحوث المحاصيل الحقلية – مركز البحوث الزراعية – الجيزة – القاهرة

أجريت تلك الدراسة في محطة البحوث الزراعية بالجميزة – مركز البحوث الزراعية – مصر حيث تم استخدام التوصيف المورفولوجي في دراسة نقل صفة التفريع القاعدي من البرسيم المصري متعدد الحشات إلى البرسيم الفحل وحيد الحشة وأيضاً استخدام توصيف المجموعة الكروموسومية Karyotypic Analysis حيث يعتمد التوصيف الكروموسومي على تقدير المساحة الكروموسومية، طول الكروموسوم، طول الذراع الطويل والقصير للكروموسوم وأيضاً دراسة نسبة السنترومير وموضع السنترومير ودراسة النشاط الميوزي والانحرافات الكروموسومية وأيضاً نسبة عدد الكيازمات في الخلية لكل من البرسيم الفحل والمسقاوي والهجين ويمكن تلخيص النتائج المتحصل عليها فيما يلي :- يتميز البرسيم الفحل بإعطاء حشة واحدة بينما المسقاوي يعطي عدة حشات وكذلك الهجين يعطي عدة حشات.- يتميز البرسيم الفحل بأن التفريع فيه علوياً أما المسقاوي فالتفريع فيه قاعدياً وكانت الفروع القاعدية هي أكبر الفروع ثم تقل تدريجياً إلى أعلا حتى نهاية النبات بينما في الهجين يكون التفريع قاعدياً وعلوياً.- ميعاد التزهير هو الفترة بين ميعاد الزراعة وتفتح أول زهرة في النورة الطرفية للساق الأصلية وكانت هذه الفترة في كل من الفحل والمسقاوي والهجين هي 108,000 يوم، 137,55 يوم، 131,15 يوم على التوالي، ويلاحظ وجود فروق في ميعاد التزهير بين البرسيم الفحل والمسقاوي والهجين وهذا يشير إلى وجود عزل موسمي نسبة إلى تباعد فترة التزهير بين الفحل والمسقاوي.- متوسط وزن 100 بذرة في كل من الفحل والمسقاوي والهجين وكانت كالآتي : 0,31 جرام، 0,27 جرام، 0,30 جرام على التوالي، وكان الفرق بين متوسط وزن 100 بذرة لكل من الفحل والمسقاوي ذو معنوية، بينما الفرق بين متوسط وزن 100 بذرة لكل من الهجين والفحل كان غير معنوي.- متوسط عدد البذور في كل نورة من الفحل والمسقاوي والهجين كان كالتالي : 51,60 بذرة في الفحل، 44,30 بذرة في المسقاوي و68,000 بذرة في الهجين.- كان عدد الكروموسومات في كل من الفحل والمسقاوي والهجين متساوية (2N = 16) حيث أن (N = 8).- قورنت أطوال الكروموسومات في الفحل بمثيلتها في المسقاوي فوجد أن الكروموسوم رقم (1) في الفحل أطول من الكروموسوم رقم (1) في المسقاوي وكان الفرق بينهما ذو معنوية عالية.- كذلك الكروموسوم رقم (4) في المسقاوي كان أطول من الكروموسوم رقم (4) في الفحل وكان الفرق بينهما معنوي.- كان عدد الكيازمات بالخلية في الفحل والمسقاوي والهجين بينهما كالآتي 12,8، 12,6، 11,9 كيازمة على التوالي وكانت الفروق بين عدد الكيازمات في الخلية الواحدة بين الفحل والمسقاوي والهجين ثم المسقاوي والهجين ذات معنوية عالية.- كانت نسبة الانحرافات الكروموسومية (Chromosomal Aberration) في الفحل والمسقاوي والهجين بينهما كالآتي: 13,16%، 11,06%، 14,36% على التوالي، حيث أن من أبرزها Fragments، Stickiness، Binucleate Cell، Laggard وكلما زاد معدل الكيازما كلما زاد معدل الاختلافات والتباينات الوراثية، والكيازما هي الأثر السيتولوجي الدال على حدوث العبور الوراثي وظهور تراكيب وراثية جديدة تختلف عن الأبوين.- بمقارنة النسبة الكروموسومية (الذراع الطويل : الذراع القصير) بين الكروموسومات المتماثلة في كل من الفحل والمسقاوي والهجين فوجد أن أعلى نسبة كانت 1,35 على الكروموسوم رقم (8) في الفحل وأقل نسبة كانت 0,73 على الكروموسوم رقم (3) في الهجين