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EMS Induced Desynaptic Male Sterile Lines in Buckwheat (*Fagopyrum esculentum* Moench)

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Abstract—Cytological analysis of EMS (Ethyl methane sulphonate) treated population of *Fagopyrum esculentum* revealed an abnormal behaviour of microsporogenesis that affected the meiotic events resulting in the formation of abnormal meiotic products that prevent the gamete formation and impair pollen fertility. The two desynaptic mutants were recorded, showing distinctive variations in the morphology as compared to the control plants. The seeds of *Fagopyrum esculentum* were treated with EMS solution in different concentrations such as 0.1, 0.3 and 0.5% using potassium phosphate buffer (pH 7) for 5 h. During cytological investigation, 0.5% concentration of EMS enhanced the univalent frequency per cell at diakinesis/Metaphase I, respectively along with unequal segregation at anaphase I which is pronounced to be higher in contrast to bivalents. Because of higher frequency of univalents desynapsis has been categorized as medium-strong type. EMS induced desynaptic plants showed abnormal meiotic behaviour leading to pollen sterility and no seed setting was recorded. The chemical mutagen has acted on recombination genes system which is accountable for synapsis and chiasma formation and further disrupted the complete chiasma assembly. The desynaptic mutant is a potential tool that provides genetic information on the maintenance of chiasma and the study offers the possibility for formation of aneuploids production which may be exploited successfully through chemical mutagenesis in breeding programmes.

Keywords: meiosis, EMS, univalents, medium strong type, pollen sterility, *Fagopyrum esculentum*

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INTRODUCTION

Meiosis is a highly specialized process in the life cycle of all sexually reproducing eukaryotes. During meiotic cycle, a single round of DNA replication is followed by two successive nuclear divisions characterizing meiosis I and II [1]. Segregation of chromosome homology occuring at meiosis I and meiosis II involves separation of sister chromatids [2]. Pairing of homologous chromosomes is one of the most important phenomenon of meiosis I in eukaryotic organisms that gametes from the earlier stage. Chiasma formation which occurs at diplotene is terminalized at diakinesis stage. Chiasma terminalization preserves the structure of bivalent through metaphase I division and helps appropriate disjunction of bivalent at anaphase I [3] i.e. process to be continued until the separation of chromosome homology each one moving to opposite pole at anaphase I and II [4]. Whole cytological events governed by molecular process relation to homologous recombination between DNA strands [5, 6].

Mutation in any of the genes disrupts meiosis i.e. causes failure in homologous pairing associated with indiscriminate synapsis and fold back pairing [7, 8] due to which there occurs the formation of larger number of univalents at metaphase and culminate in gametic sterility [9]. Due to changes in normal patterning of cell division, two types of mutations occur i.e. asynapsis and desynapsis. These two meiotic abnormalities disrupt the pairing of homologous chromosomes and chiasma formation.

Asynapsis can be the result of failure to pairing of homologous chromosomes or synapsis due to genetic and environmental factor. Alternatively, desynapsis is an important cytological phenomenon used to denote the normal pattern of pairing of homologous chromosomes or synapsis but the association cannot be held until anaphase I and they separate prematurely. This meiotic abnormality is referred as desynapsis [10] and in this case, most of the chromosomes arrive at the metaphase plate as univalents rather than bivalent [11].

The meiotic disruptions i.e. desynapsis and asynapsis can increase the number of univalents (unpaired chromosomes) which are usually noticed at metaphase I. Univalents either get lost or are randomly transmitted to daughter cells, resulting in the formation of unbalanced gametes [10]. Desynapsis is classified into complete type having maximum number of unpaired chromosomes at metaphase I or none of the chromosomes remain paired, another is medium strong type, some of the chromosomes remain paired

until metaphase I [11]. Desynaptic plants are significant since they offer possibility for the production of aneuploids [12, 13].

Desynapsis in *Fagopyrum esculentum* has been reported firstly in this research work. A desynaptic plant of *Fagopyrum esculentum* has been discussed with respect to cytological as well as morphological parameters. In some regions of the world, buckwheat is an important crop used as a food, as a good source of rutin which is used medicinally, and as a green manure and grown throughout a large area of Asia and Southeast Asia as a crop [14]. In India, the crop is widely grown in Jammu and Kashmir in the north and Arunachal Pradesh in the east [15]. Common buckwheat a member of family Polygonaceae is the most widely consumed pseudocereal crop with its several advantages. It is not a cereal but the seeds are classified among cereal grains because of their similar usage. It is cultivated to obtain grain for human consumption because it contains high nutritive substance such as carbohydrate (63–70%) and possess excellent quality of protein. It also contains essential amino acid in higher amount such as lysine unlike common cereals [14]. Rutin (quercetin-3-rhamnoglucoside) is very important flavonol glucoside because it was not found in cereals and pseudocereals except *Fagopyrum esculentum* [16]. It is recognized as the most health protective and anticarcinogenic as compared to other antioxidant components found in buckwheat [17]. Almost 80–90% rutin content present in the leaves and flower [18], only a small percentage is present in stems and absent in the fruits [19].

Major cytological work has not been done in *Fagopyrum esculentum*. During the present investigation, the nuclear male sterile mutant/variant has been isolated in *Fagopyrum esculentum*. Male sterility has been previously reported in the family polygonaceae by Kaul [20]. Desynapsis is one of the major abnormality firstly reported in *Fagopyrum esculentum*. The present paper deals with the genetic behaviour of sterility and its mechanism through cytological analysis of these male sterile mutant/variants. Study of desynapsis is a potentially important source of information on the chiasma maintenance [21] and offers the possibility for the production of aneuploids [12, 13].

MATERIALS AND METHODS

Procurement of Seeds

The healthy and inbred seeds of *F. esculentum*, variety VL-7 obtained from germ plasm collection of NBPGR, Phagli, Shimla which was analysed cytologically before performing experiment.

Seed Treatment

Before treatment, the fresh seeds of *F. esculentum* were presoaked in distilled water for standardization of moisture content. After 12 h of presoaking, they were treated with EMS (Ethyl methane sulphonate) at different concentrations viz. 0.1, 0.3 and 0.5%, prepared in sodium phosphate buffer at 7.0 pH with 3 h. with constant shaking (FAO/IAEA Technical Report series no. 119, 1997). Control set was maintained separately. The treated seeds were washed thoroughly in running water for the removal of residual particles of mutagen stuck to the seed coat. The treated seeds were sown immediately in the experimental pots along with control set under standard agronomic practices. Two desynaptic plants were observed during experiment.

Meiotic Analysis

For meiotic studies, the young floral buds were fixed in carnoy's fixative (GAA1: absolute alcohol 3) for 24 h. After 24 h, the young floral buds were transferred to 90% alcohol for preservation. Slides were prepared by Anther-squash technique and stained in 2% acetocarmine. About 200–300 dividing PMC's from all the treatment sets with respect to control were observed and studied. All the meiotic stages were evaluated beginning from diakinesis. Different types of meiotic configuration were observed at metaphase I and anaphase I.

Pollen Fertility

For the study of pollen fertility, pollen grains were stained with 2% acetocarmine. Viable seeds were darkly stained having a definite size whereas undersized and unstained pollen grains were considered as inviable. Slides were analyzed and photographs of PMC's were taken at 40XResolution from PCTV Software.

Statistical Analysis

For statistical analysis, three replicates for each treatment set were used. Statistical analysis was performed by SPSS 16.0 software. One way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT, $P < 0.05$) were conducted for mean separation and the graph was plotted using sigma plot 10.0 software. Actual mean and standard error were calculated.

RESULTS

The desynaptic plants from the EMS 0.5% concentration treated population were weak and showed late flowering as compared to control plants. The plants showed partial sterility whereas seeds were obtained in little amount having a smaller size as compared to control and the seed formation was almost negligible. In the normal meiotic event, diakinesis is characterized by the number of bivalents and multivalents which remained as such till metaphase I while the affected

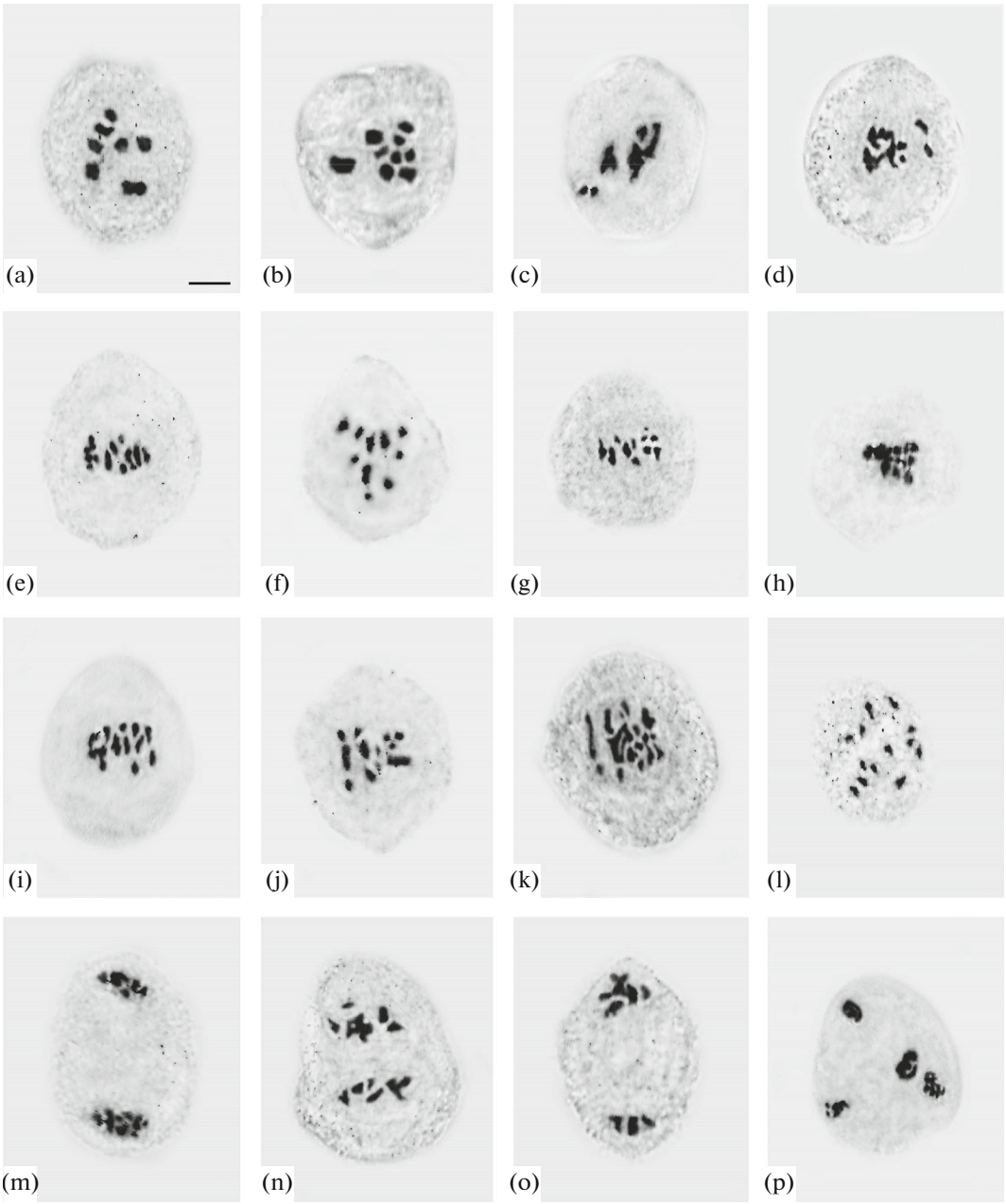

Fig. 1. Meiosis in desynaptic plants: (a) Normal 8 bivalents (scale bar: 8.62 μm), (b) $2_I + 7_{II}$ at Metaphase I, (c) $2_I + 1_{II} + 1_{III} + 1_{IV} + 1_V$ at Metaphase I, (d) $2_I + 2_{II} + 2_{III} + 1_{IV}$ at Metaphase I, (e) $4_I + 3_{II} + 1_{VI}$ at Metaphase I, (f) $5_I + 4_{II} + 1_{III}$ at diakinesis, (g) $6_I + 2_{II} + 2_{III}$ at Metaphase I, (h) $7_I + 1_{II} + 1_{III} + 1_{IV}$ at Metaphase I, (i) $7_I + 3_{II} + 1_{III}$ at Metaphase I, (j) $8_I + 4_{II}$ at Metaphase I, (k) $9_I + 2_{II} + 1_{III}$ at Metaphase I, (l) $10_I + 3_{II}$ at Metaphase I, (m) Unequal separation at Anaphase I (10 : 6), (n) Unequal separation at Anaphase I (9 : 7), (o) Unequal separation at Anaphase I (12 : 4), (p) Disturbed polarity at Anaphase II.

meiosis showed chromosomal dissociations at the end of the diakinesis, separating the chromosomes by desynapsis (Fig. 1). The two desynaptic mutants showed abnormal behaviour of chromosomes during meiotic process. Meiosis was normal with regular occurrence of 8 bivalents ($2n = 16$) at diakinesis (Fig. 1a) and 8 : 8 segregation at anaphase I in case of control. In Desynaptic plants, fewer PMC's showed complete bivalent formation, but number of univalents and unequal separation of chromosomes was found to be greater in most of the PMC's. The most important chromosomal configurations observed were $2_I + 7_{II}$, $2_I + 1_{II} + 1_{III} + 1_{IV} + 1_V$, $2_I + 2_{II} + 2_{III} + 1_{IV}$, $4_I + 3_{II} + 1_{VI}$, $5_I + 4_{II} + 1_{III}$, $6_I + 2_{II} + 2_{III}$, $7_I + 1_{II} + 1_{III} + 1_{IV}$, $7_I + 3_{II} + 1_{III}$, $8_I + 4_{II}$, $9_I + 2_{II} + 1_{III}$ and $10_I + 3_{II}$ (Figs. 1b–1l). Table 1 showed chromosomal configurations recorded at diakinesis and metaphase I in two EMS induced Desynaptic plant (d-1 and d-2) along with control.

Table 1. Meiotic configurations at diakinesis and Metaphase I in normal and two EMS (0.5%) induced desynaptics in *Fagopyrum esculentum* Moench

Plants	Stages	No. of PMC's observed	Chromosomal configurations, % (Mean ± S.E.)					
			8_{II}	$2_I + 7_{II}$	$4_I + 3_{II} + 1_{VI}$	$6_I + 2_{II} + 2_{III}$	$8_I + 4_{II}$	$10_I + 3_{II}$
Control	Diakinesis	200	70.00 ± 2.02	1.50 ± 0.04	–	–	–	–
	Metaphase I	110	34.55 ± 0.99	1.78 ± 0.04	–	–	–	–
d-1	Diakinesis	226	1.78 ± 0.05	2.21 ± 0.06	5.31 ± 0.15	11.06 ± 0.31	15.92 ± 0.45	21.23 ± 0.61
	Metaphase I	125	0.80 ± 0.02	1.60 ± 0.04	5.60 ± 0.16	8.00 ± 0.23	10.40 ± 0.30	13.60 ± 0.39
d-2	Diakinesis	247	1.21 ± 0.03	1.61 ± 0.04	6.07 ± 0.17	12.14 ± 0.34	21.05 ± 0.60	13.36 ± 0.38
	Metaphase I	132	0.76 ± 0.02	2.27 ± 0.06	6.82 ± 0.19	9.09 ± 0.26	12.12 ± 0.34	10.60 ± 0.30

The normal chromosomal configuration of 8 II in the untreated set corresponded to a percentage of 70.00 ± 0.02 and 34.55 ± 0.99 at diakinesis and metaphase I, respectively. However in the induced desynaptic plants d1 and d2, this frequency was reduced to 1.78 ± 0.05 (at diakinesis) and 0.80 ± 0.02 (at metaphase I) and 1.21 ± 0.03 (at diakinesis) and 0.76 ± 0.02 (at metaphase I), respectively. Due to some environmental factor, the control plant exhibited least frequency of univalents observed to be 1.50 ± 0.04 and 1.78 ± 0.04 at diakinesis and metaphase I. In d-1 plant, the most prevalent chromosomal configurations noticed were of 10 univalents and 3 bivalents with the frequency of 21.23 ± 0.61 at diakinesis and 13.60 ± 0.39 at metaphase I while in d-2 plant, $8_I + 4_{II}$ configuration with the frequency of 21.05 ± 0.60 was found to be the dominant configuration at diakinesis and the frequency of 12.12 ± 0.34 at metaphase I.

A huge number of univalents were recorded at diakinesis with an average of 53.99 and 58.88% in d-1 and d-2 plant, respectively. Figure 2 shows the frequencies of univalents and bivalents of two desynaptic plants.

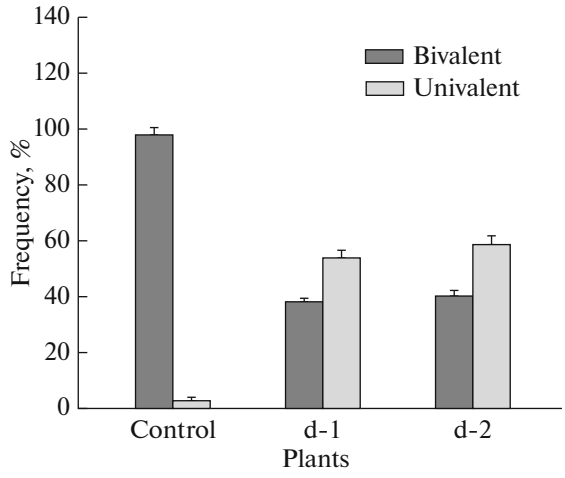

Fig. 2. The frequencies (%) of bivalent and univalent in normal and two induced desynaptic plants in *Fagopyrum esculentum* Moench.

The anaphase I had shown 8 : 8 segregations with the frequency of 2.60 ± 0.07 and 3.07 ± 0.08 in d-1 and d-2 plants, respectively (Table 2) and also showed anomalous distribution of chromosomes including 9 : 7 (Fig. 1n), 10 : 6 (Fig. 1m), 12 : 4 (Fig. 1o) and 8 : 5 : 3. The most prevalent chromosomal distribution was 9 : 7 with the frequency of 11.97 ± 0.34 and 10.25 ± 0.29 in the two desynaptic plants (Fig. 1). Different types of chromosomal abnormalities were also observed such as laggards at anaphase I and disturbed polarity was also reported at anaphase II (Fig. 1p).

The univalents and bivalents varied considerably from PMC to PMC in both the desynaptic plants and fitted in the category of medium strong type desynapsis on the basis of classification given by Prakken [22] (Fig. 2). The homosized spores of the tetrad are the characteristics of normal plant but the desynaptic mutants showed several unbalanced microspores with various size (Fig. 3). The various types of anomalies caused sterility which lead further to the reduction of seed setting. Pollen fertility was reduced in desynaptic mutants i.e. 45.10% (d-1) and 34.87% (d-2) while control plants showed 96.45% pollen fertility.

DISCUSSION

Studies of meiotic behaviour are essential for understanding the reproductive biology, fertility aspects and genomic evolution of any organisms [10]. In the present case, most of the PMC's analyzed at diakinesis and metaphase did not reveal the expected chromosome configuration of 8 bivalent. Most of the PMC's showed univalents which were quite high in number and range. Reason for it is the partial/complete absence of synapsis and chiasmata formation.

Behaviour of univalents is attributable to the actual fact that they need only one centromere, in order that they are unable to keep up the normal bipolar orientation on the metaphase I [23]. Conventionally, the occurrence of univalents at diplotene, diakinesis and metaphase I is referred as desynapsis which depends on the presence or absence of initial pairing of homologous chromosomes at pachytene stage [24]. These homologues are typically linked by both segmental

Table 2. Anaphasic abnormalities and Pollen fertility in normal and two induced desynaptic plants in *Fagopyrum esculentum* Moench

Plant no.	Normal anaphasic separation	Anaphasic abnormalities, % (Mean ± S.E.)							Pollen fertility, %		
		unequal separations				disturbed polarity	laggard	other	min.	max.	average
	8 : 8	9 : 7	10 : 6	12 : 4	8 : 5 : 3						
Control	89.69 ± 0.22	0.56 ± 0.11	–	–	–	–	–	–	93.56	99.34	96.45 ± 1.67
d-1	2.60 ± 0.07	11.97 ± 0.34	8.33 ± 0.23	6.25 ± 0.18	5.20 ± 0.15	3.12 ± 0.08	1.04 ± 0.02	1.56 ± 0.04	42.84	47.36	45.10 ± 1.30
d-2	3.07 ± 0.08	10.25 ± 0.29	9.23 ± 0.26	7.69 ± 0.22	6.15 ± 0.17	4.10 ± 0.11	2.08 ± 0.06	2.05 ± 0.05	33.13	36.61	34.87 ± 1.00

exchanges and synaptinemal complex in meiotic stage of prophase I. Variation in synaptic behaviour of homologous chromosomes during zygotene, results in total (asynapsis) or partial (desynapsis) failure of chromosome pairing. The induction of synaptic mutations using extrinsic and intrinsic agents are also widely reported.

In the present study, highest concentration of EMS (Chemical mutagen) i.e. 0.5%, is responsible for inducing desynapsis, which leads to the disruptions in meiotic process which is accountable for the creation of base pairing error. EMS is a vigorous mutagen, acted on genes which are responsible for synapsis and chiasma formation and results in early chiasmata dissociation [21]. Synaptinemal complex (SC) protein is formed via highly conserved sequence, the gene responsible for that events leads to formation of defective SC proteins during mutation which are unable to

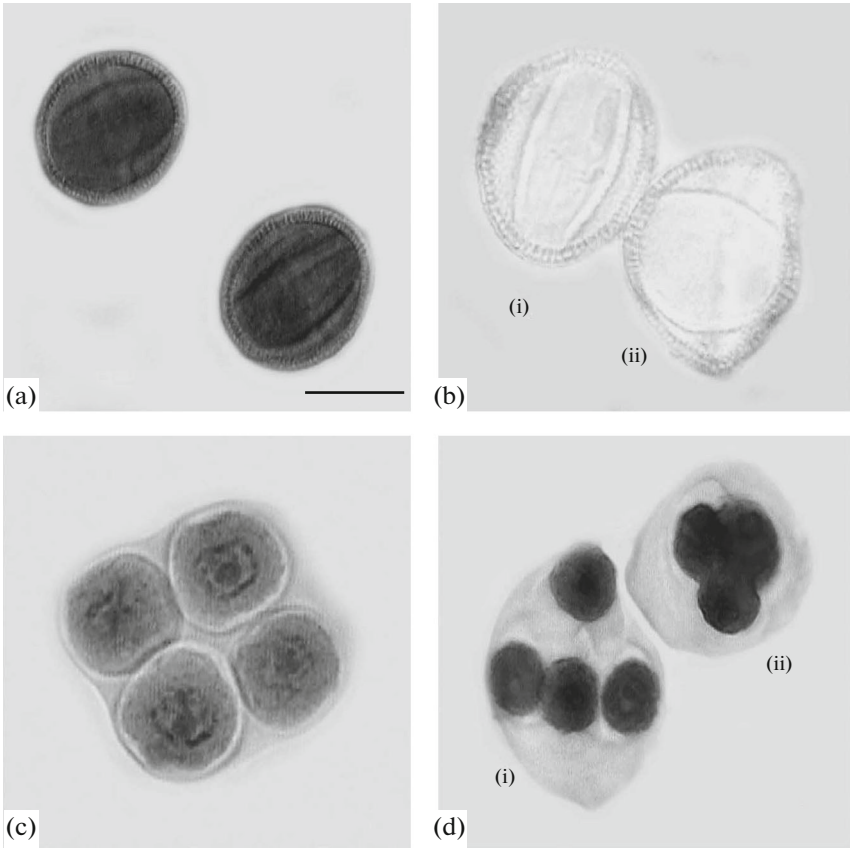

Fig. 3. (a) Fertile pollen grain (scale bar: 43.24 μm), (b) (i) Normal sterile pollen grain (ii) Abnormal sterile pollen grain (change axis), (c) Normal tetrad, (d) (i) abnormal tetrad (ii) fused microspore.

hold the chiasma in place [25–27]. Sister chromatids cohesiveness is responsible for chiasma maintenance in plants [28]. The desynaptic mutation is representative of a large group of mutations typified by formation of univalents at diakinesis [29]. Mostly, recombination defect may be caused by desynaptic mutation. In desynaptic plant, however, it is possible to determine that at least some of the univalents had undergone recombination of cytological markers: suggesting a defect in chiasma maintenance [30].

The desynapsis deliberated a tendency of close proximity of the univalents and chromosomal arrangement at metaphase I suggesting a premature dissociation. Peirson et al. [23] referred to desynaptics where univalents never align at the equator in metaphase I whereas congregation of bivalents as well as univalent is observed at metaphase plate. These desynaptic plants exhibited high prevalence of univalents as compared to bivalent. Several studies have been reported by various researchers from time to time to explain the genetics of desynapsis. The recombination modifier genes (rec gene) may scale back the recombination to a degree wherever no pairing happens [31] and results of viable cross over product. A mutation in recombination gene system may cause failure of chiasma formation and recombination [32]. The formation of a chiasma in ordinary cells presents a connection among homologues and restrains a poleward movement and purpose anxiety at the kinetochores. In the absence of chiasma, the ensuing univalent kinetochores do not feel any tension and produce a signal that causes the cell to delay anaphasic division [30] and because of this the univalents were observed scattered inside the cytoplasm and rest of the stages weren't observed, since chromosomal movement was substantially affected. Meiotic abnormalities emanated as a consequence of univalent chromosome usually results in the formation of abnormal microsporogenesis which was specified by the presence of irregular sporads with dyads, triads, tetrads (with micronuclei) and polyads [33] which could be the reason for the absence of seed formation and leads to pollen abortion and sterility.

CONCLUSIONS

From the present investigation, the desynaptic plant was morphologically affected with stunted growth and reduced flowering and the higher frequency of univalent in contrast to bivalents was observed in two desynaptic plants as compared to control. The pollen fertility was highly affected in the case of desynaptic mutants with lower chiasma frequency. Other anomalies like unequal segregation, disturbed polarity and abnormal sporads were also reported. On the basis of above observation, medium strong type of desynapsis was detected in the buckwheat plant. Hence, it can be concluded that such abnormal meiotic pattern expressed by most of the PMC's of *Fagopyrum esculentum* analyzed in the present investigation provides a better platform for the production of aneuploids in mutation breeding programme and influential mutagen i.e. EMS acting as a tool for the establishment of male sterile lines in buckwheat plant.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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