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EFFECT OF DIFFERENT MUTAGENSIS ON MICROSTRUCTURE AND ULTRASTRUCTURE OF ALFALFA

FENG PENG[1,2&*], SHEN XIAOHUI[1,2&], LI RULAI[1], SUN LI[1], WU HONGDA[4], SHI CHEN[5], LI ZENGJIE[1], LI ZHIMIN[1], ZHENG HAIYAN[1], LI DAOMING[3] AND LIU JUNGANG[1]

[1]Jiamusi Branch of Heilongjiang Academy of Agricultural Sciences, 154007 Jiamusi, China
[2]Postdoctoral Workstation of Heilongjiang Academy of Agricultural Sciences, Harbin 150086, China
[3]Institute of Pratacultural Sciences of Heilongjiang Academy of Agricultural Sciences, 150086 Harbin, China
[4]Institute of Animal Husbandry of Heilongjiang Academy of Agricultural Sciences, 150086 Harbin, China
[5]Daqing Branch of Heilongjiang Academy of Agricultural Sciences, 163000 Daqing, China
[&]Means both authors contributed equally to this work and should be considered co-first authors
[*]Corresponding author's email: fenggrass@163.com

Abstract

This study aims to check the effect of mutagenesis on microstructure and ultrastructure of alfalfa (*M. sativa* L.) leaf. Four alfalfa cultivars were treated with four kinds of mutagenesis methods, and then the leaf microstructure and ultrastructure of the tested alfalfa were determined respectively. The alfalfa cultivars namely i.e. Gongnong 1, Wega7F, WL319HQ and Aohan were used as the test materials. The mutagenesis methods including $^{60}CO\gamma$-ray irradiation (150, 300, 450 Gy), ultraviolet (30, 60, 90 min), EMS (ethyl methane sulfonate 0.1%, 0.2%, 0.4% (v/v)) and magnetic field-free space (180 d) were used for the leaf treatment. The results showed that the leaf thickness was increased under mutagenesis treatment, among them Gongnong 1 alfalfa under EMS with 0.4% (v/v) was the highest, its leaf thickness was as much as 446.16μm with an increase of 25.86μm. Wega7F's cell become closer under all mutagenic treatments. The ultra microstructure of the chloroplast in the alfalfa leaves was observed under different mutagenic treatments. When making the dose or the concentration of mutagenic treatments lower, the chloroplast of the cell showed distortion, ambiguity in membrane, loose in grana lamella, disintegration in thylakoid, increased in lipid ball. Under the highest dose or concentration mutagenic treatment and magnetic field-free space, the chloroplast of the cell displayed swelling and distortion, fracture in grana lamella and disintegration in membrane system. Membrane systems of the chloroplast were disintegrated and its chloroplast structures were damaged more seriously, both plastids and starch grain were increased. $^{60}CO\gamma$-ray irradiation and EMS had higher mutagenic efficiency, their mutant materials were more abundant, their operations were simple, which was considered as being the reasonable mutation methods. Being more conducive for screening mutants, $^{60}CO\gamma$-ray irradiation with150Gy, ultraviolet for 60mins and EMS with 0.4% were the better mutagenic treatments.

Key words: Mutagenesis, Alfalfa, Microstructure, Ultrastructure, Mutants.

Introduction

In higher plants, leaf is the vegetative organ which along with other functions perform photosynthesis, and it is also considered as one of the most sensitive organ responding to environmental changes. Hence, the morphological structure of leaves can be accounted perfectly as a result of the effects of the environmental factors, which in general is termed as adaptive feature of plants to the environment (Mei *et al.,* 2014). Plant's structure is the foundation of its function. The changes in physiological functions of plants are caused by the changes in plant leaf structures (Liu *et al.,* 2015; Wang *et al.,* 2017). Therefore, the morphological and anatomical structure of plant leaves demonstrate their responses and adaptability to the prevailing environment. The study on the response mechanism of the leaf morphology and anatomical structure sometime forms the basis for exploring the adaptive capability of plants to their immediate environment.

Mutation breeding is a breeding method in which physical, chemical and biological mutagenic agents are used in order to induce hereditary changes in plants. If the mutation breeding program becomes successful, then the desired ones are selected from the mutants and further bred into new varieties or new germplasm (Jiang *et al.,* 2007). In higher plants, the structure of the cell changes under different degrees of environmental and mutagenic stresses and is closely related to the degree of stresses implied and the inherent stress resistance capacity of the plant. The mutants provide materials to be selected for breeding. Zhang *et al.,* (2010) reported that after the space mutagenesis, the leaf thicknesses of four alfalfa varieties namely, Telford, Derby, Algonquin and Suntory were increased, the protuberant degrees of leaf vein were decreased, and the palisade tissues were significantly thickened. There was no significant difference in the compactness and looseness of cell structure between the Telford variety of alfalfa and the control. The same experiment also revealed no significant difference in respect to the looseness of cell structure between the variety Algonquin and the control. Whereas, the compactness and looseness of cell structure among the two other varieties namely, Derby and Suntory, as compared with the control, showed significant differences. Feng *et al.,* (2009) studied the effects of ultrastructural changes of alfalfa leaves under the environmental condition of a satellite. The results showed that after the space flight, the leaf ultrastructure of the plants showed different degrees of changes. Strong effects were evident in respect to the chloroplast and mitochondria where clear pore and overflow were present. Xia *et al.,* (2014) treated the alfalfa seeds with different concentrations of ethyl methane sulfonate (EMS) and found that the EMS treatment inhibited the final germination percentage of M1 seeds, germination

index and radicle length. The treatment also affected the leaf color, which might be due to the changes in chloroplast structure. Zhang *et al.,* (2004) discovered that the Sainfoin mutant, by space mutagenesis, exhibited irregularly thickened and distorted cell walls, thinner cytoplasm, larger vacuoles, and smaller and irregular shaped chloroplasts. The chloroplast showed smaller but more in number starch granules, less grana, and a decreased diameter of the lamellar structure.

Based on above mentioned observations in the present study, we evaluated the effect of ^{60}COγ-ray irradiation, ultraviolet, EMS and magnetic field-free space on microstructure and ultrastructure of alfalfa, by which we hope to provide technical supports for screening the optimal induction mutation method and further breeding new alfalfa varieties for the alpine region.

Materials and Methods

Site and soil details: An experimental field was established in the Jiamusi City, which was located in the east part of the Heilongjiang Province, P. R. of China. It is situated at the region with the climate type of cold humid monsoon, with annual average temperature of 3℃, annual average precipitation of 468.4 mm, annual rainfall of 510 mm, annual sunshine of 2046.2 hrs., annual active accumulative temperature of 2500℃, and the annual average frost free period of 128.9 days. The organic matter was 2.49% in the tested meadow black soil with available nitrogen 86.3 mg·kg^{-1}, available phosphorus 64.6 mg·kg^{-1}, available potassium 79.9 mg·kg^{-1}. Its total nitrogen, phosphorus, potassium was 0.14%, 0.14%, and 3.12%, respectively. Its soil pH was 6.5. The soil Pb background value was 0.24 mg·kg^{-1}, Cd was 0.031 mg·kg^{-1}.

Materials: Four alfalfa (*M. sativa* L.) cultivars were used as the tested materials, three of them including Gongnong1, Wega7F and Aohan were provided by the Institute of Animal Husbandry of the Heilongjiang Province of P. R. of China, the rest of WL319HQ was provided by the Beijing Zhengdao Ecological Technology Co., Ltd.

Experimental design

Magnetic field-free space: By using coil compensation method, a magnetic-free field was developed with a large 26-sided magnetic shielding device and a double magnetic shielding structure. With diameter of 2.3 m, this device could control the magnetic flux density less than 20 nT, which is 4×10^{-4} times as more as the Earth's magnetic flux density. In this experiment, 3 replications were carried out at each treatment level and for each variety of alfalfa. Before sowing, the dried seeds (10 g) taken from each of the aforementioned alfalfa was treated at room temperature for 180 days in the magnetic-free field.

^{60}COγ-ray irradiation: For ^{60}CO-γ irradiation treatment, 300 seeds taken from each of the aforementioned alfalfa were treated with ^{60}CO-γ irradiation at a dose of 0, 150, 300, 450 Gy, the dose of 0 was set as control. Each treatment lasted for 7 days.

Ultraviolet: 300 seeds taken from each of the aforementioned alfalfa were wrapped with a gauze, immersed in distilled water at 45°C for 1 hrs., and dried by soaking those with a piece of filter paper. Further the seeds were disinfected by immersing those in 0.1% mercuric chloride solution for 10 min and then taken out and rinsed with sterile distilled water for 3-5 times. Finally the seeds were placed 40 cm away from the source of UV light and exposed for 30, 60 and 90 mins, respectively.

Ethyl methyl sulfonate (EMS): 900 seeds taken from each of the aforementioned alfalfa were soaked with concentrated sulfuric acid for 5 mins, and then rinsed with distilled water several times. The treated seeds were completely water-swelled by soaking in the phosphate buffer (100 mmol·L^{-1}, pH 7.0) at 4°C for 12 hrs. The seeds thus treated with phosphate buffer, were subjected to 0.1, 0.2 and 0.4% (v/v) EMS and incubated in the dark at room temperature for 15 hrs. During the procedure, the seeds were gently shaken. Finally, the seeds were rinsed with distilled water repeatedly for removing the residual EMS from the seed surface.

Microstructure: The functional leaves of the M0 generation plants were selected and cut along the middle veins in an inverted trapezoidal shape having a size of 5 × 5 mm. The samples were fixed with FAA (formalin-acetic acid-alcohol) solution and underwent conventional paraffin sections with a thickness of 5 μm and then stained with Safranin'O/Fast Green. The slides were observed under a Nikon-DIAPHOT microscope.

Ultrastructure: The central functional leaves of the mutant and the untreated alfalfa were cut into pieces with a size of 10 × 5 mm, avoiding the veins. Then the samples were quickly transferred into the glutaraldehyde fixative with a concentration of 2.5% and a pH of 6.8 under vacuum and fixed at 4 °C for 2 hrs. Afterwards, the samples were washed twice with 0.1 mol·L^{-1} phosphate buffer (pH = 6.8) at 4°C for 2 hrs. Then they were dehydrated in 50%, 70%, 80%, 95% and 100% ethanol sequentially, transferred into 100% acetone, and finally embedded with Epon 812. The resin embedded materials were sliced using an ultracute slicer into 50-70 nm sections. The sections were then double stained with uranyl acetate-lead citrate and observed under a transmission electron microscope (Hitachi HT7700, Japan), and photographed at 30000× magnification.

Statistical analysis: The mean of the 4 values of leaf thickness, vein thickness, palisade thickness and spongy thickness of the tested alfalfa was determined by Motic Image 2000 1.3. Cell tense ratio, Spongy ratio, Vein protuberant degree were calculated as follows:

$$\text{Cell tense ratio (CTR \%)} = \frac{\text{Palisade tissue thickness}}{\text{Leaf thickness}} \times 100$$

$$\text{Spongy Ratio (SR \%)} = \frac{\text{Spongy tissue thickness}}{\text{Leaf thickness}} \times 100$$

$$\text{Vein protuberant degree (VPD)} = \frac{\text{Vein thickness}}{\text{Leaf thickness}} \times 100$$

Results

Microstructure

Leaf thickness and vein protuberant degree: In the experiment, different mutagenic treatments showed different effects on the microstructure of the alfalfa leaves from the M0 generation. The mutagenic direction and degrees varied with the mutagenic methods and doses (Loutou *et al.,* 2016; Zhu *et al.,* 2015). The changes in the microstructure, particularly in the leaf thicknesses of four alfalfa cultivars differed after mutagenesis, but mainly with an increased thickness (p<0.05). In all the experiments, the leaf thickness of Gongnong No.1 alfalfa were significantly higher than their corresponding controls except for the treatments with 30 mins of UV and 0.1% EMS (Table 1). At this treatment, the leaves were thinner than their corresponding controls (p<0.05). For Wega7F alfalfa, its leaf thickness with 0.1% EMS treatment was thinner than its control. For rest treatments, the leaf thicknesses were significantly higher than their corresponding controls (p<0.05). The leaf thicknesses of WL319HQ and Aohan alfalfa in all the treatments were significantly higher than their corresponding controls (p<0.05). After mutagenic treatment, the vein protuberant degrees of all the treatments were significantly less than their corresponding controls (p<0.05) (Tables 1-4).

Palisade tissue and spongy tissue thickness: The leaf thickness of plants primarily depends upon the thickness of the palisade tissue and the spongy tissue (Peguero *et al.,* 2008). It means that thicker leaf develops with a corresponding increase in both categories of leaf tissues in plants. When the alfalfa cultivars were treated with different mutagenic agents, a change in the thickness of the palisade tissue did correspond with a change in the leaf thickness (Wang *et al.,* 2010). For Gongnong No.1 alfalfa, the thicknesses of the palisade tissues for the treatments with ^{60}CO-γ radiation, UV and high concentrations of EMS were higher than their corresponding controls. This proves that a thicker palisade tissue could be the result of a higher dose of treatment. On the contrary, the thicknesses of the palisade tissues for all the other treatments were lower than their corresponding controls. The thicknesses of the spongy tissues in the anatomical structure of the alfalfa leaves under the treatments with UV, low concentrations of EMS and the magnetic-free field were significantly lower than their corresponding controls (p<0.05). Whereas, the thicknesses of leaves for all the other treatments were higher than their corresponding controls. In case of Wega7F cultivar, the thicknesses of the palisade tissues were significantly higher than their corresponding controls in all the treatments; its thicknesses of the spongy tissues were significantly higher than their corresponding controls excepting the treatments carried out by EMS. In the same treatments, its spongy tissues of leaves were thinner (Table 2). The thicknesses of the palisade tissues in the leaves of WL319HQ alfalfa were all significantly higher than their corresponding controls (p<0.05) (Table 3).

Cell tense ratio and spongy ratio: In the present experiment, the changes occurred in the compactness and looseness of cell structure of the four tested varieties of alfalfa were different. The Gongnong No.1 alfalfa, when treated with UV, only the compactness of cell structure was significantly lower than that of the control (p<0.05). In the same experiment, the compactness of cell structure for all other treatments was not significantly different from their corresponding controls. The looseness of cell structure for the ^{60}CO-γ and UV treatment was higher than their corresponding controls but lower in all other treatments. For Wega7F, in different treatments, the compactness of cell structure increased compared to their corresponding controls. But the looseness of cell structure for the treatments at 150 Gy ^{60}CO-γ, UV and 0.4% EMS the results showed higher values than their corresponding controls. At the treatment of 150 Gy ^{60}CO-γ, Aohan alfalfa showed higher compactness of cell structure than the controls (Table 4). Lower compactness of cell structure was shown in all other treatments compared to their controls. But at 0.1% EMS treatment, only the looseness of cell structure was higher than that of the control. All these evidences prove that a looser cell structure of the Aohan alfalfa occurred after mutagenesis (Tables 1-4).

Ultrastructure

The leaf ultrastructure after treatment is shown in Figure 1. The chloroplasts in the mesophyll cells of the experimental controls are routinely spindle shaped and neatly arranged, which is closer to the cell walls. The chloroplast membranes are intact and the lamellar structures are neatly, uniformly and compactly arranged. The starch granules are small sized and numbered. For the treatments with UV and low doses of EMS, the chloroplasts in the mesophyll cells still retained their regular shape and size, the lamellar structures and stacking structures were most neatly arranged, but some of the stacking structures began to blur. From which it showed that part of the cristae ridges of mitochondria disappeared, the number of plastids decreased, and a small amount of lamellar structure was also dissolved. However, for the treatment with 150 Gy radiation, in addition to the ultrastructure characteristics of the mesophyll cells with low-dose treatments, all the stacking structures were swollen. For the treatments with high-doses, most cell walls remained intact, but a few were completely dissolved and the cell contents extravagated into the intercellular spaces. In addition, the chloroplast membranes were dissolved and a few exhibited outward protrusions. The amount of plastids was significantly increased, the lamellar structures were gradually dissolved, a few stacking structures were thickened and some were dissolved and blurred. Inside the intact cells, the cytoplasm did spread to the surroundings. The number of the starch granules was increased and most of the cristae ridges of mitochondria disappeared. Interestingly, treatments with the magnetic-free field, the changes in cells were similar to those of the high-dose treatments. Nevertheless, besides the phenomenon of completely dissolved cell walls, there were secretory substances on the cell walls, the number of plastids decreased, and the cells were filled with many and larger starch granules.

Table 1. Comparison of main structural features (Mean±SE) of Gongnong 1 leaves under different mutagenic treatments (μm).

Treatments		Leaf thickness	Vein thickness	Palisade thickness	Spongy thickness	VPD	CTR	SR
CK		354.50±9.78e	933.03±1.63f	152.12±4.50c	123.17±7.78c	263.20±3.70a	42.91±0.75a	34.75±0.08d
^{60}CO-γ (Gy)	150	425.76±2.71b	727.63±9.01h	184.02±1.90b	191.27±8.04a	170.90±9.51e	43.22±0.47a	44.92±0.21a
	300	379.86±2.69d	957.35±0.15d	164.84±8.57bc	131.59±2.92c	252.02±2.32b	43.40±0.89a	34.64±0.41d
	450	432.19±7.60b	990.55±2.76b	173.37±2.33b	185.26±3.02a	229.19±6.35c	40.11±0.45ab	42.87±0.08b
UV (min)	30	324.83±4.52f	604.15±1.53i	126.65±7.16d	117.52±3.23d	185.99±0.60d	38.99±0.79b	39.30±0.33c
	90	396.14±3.38c	970.47±8.22c	154.31±6.35c	155.67±4.55b	244.98±1.96b	38.95±0.61b	39.30±0.33c
EMS (%)	0.1	319.31±6.86f	933.31±3.51e	134.37±5.16d	102.11±8.17d	260.97±5.88a	42.08±0.17a	31.98±0.36de
	0.4	446.16±6.92a	999.34±4.06a	198.66±7.11a	158.40±7.12b	223.99±0.37c	44.53±0.69a	35.50±0.43d
MFFS		357.26±3.55e	786.60±0.63g	149.94±4.19cd	100.44±6.62e	220.17±4.39c	41.97±0.66a	28.11±0.75e

Note: Data in the table is the mean ± SE; In the same column, being the same small letters (a, b, c…) means that the difference is not significant, conversely it means significant (p<0.05). Similarly hereinafter

Table 2. Comparison of main structural features (Mean±SE) of Wega 7F leaves under different mutagenic treatments (μm).

Treatments		Leaf thickness	Vein thickness	Palisade thickness	Spongy thickness	VPD	CTR	SR
CK		333.74±6.06h	971.62±2.41b	119.28±6.41e	97.10±3.12f	291.13±6.73a	35.74±0.11e	29.09±0.24bc
^{60}CO-γ (Gy)	150	412.63±4.94e	856.02±3.31e	152.12±8.31d	133.78±5.15d	207.46±9.13d	36.87±0.54d	32.42±0.99b
	300	533.97±2.82a	913.34±4.19c	218.25±1.2b	151.08±0.8c	171.05±9.25f	40.87±0.26c	28.29±0.51bc
	450	394.99±6.9f	911.67±7.09c	195.6±3.14c	104.86±1.99ef	230.81±2.90c	49.52±0.73b	26.55±0.37c
UV (min)	30	442.33±1.79d	686.66±0.03g	219.79±0.58b	198.79±8.78a	155.24±1.63g	49.69±0.64b	44.94±0.72a
	90	511.66±4.34b	962.91±9.14b	258.36±6.69a	176.78±0.42b	188.19±7.41e	50.49±0.92b	34.55±0.52b
EMS (%)	0.1	290.52±5.31i	763.99±0.42f	152.12±5.08d	71.69±4.13g	262.97±1.55b	52.36±0.38a	24.68±0.29c
	0.4	481.94±1.43c	1069.83±5.95a	248.68±9.82a	89.89±8.05f	221.98±5.72c	51.60±0.58a	39.40±0.46ab
MFFS		383.9±7.05g	873.03±6.7d	144.84±8.66d	113.70±1.01e	227.41±4.46c	37.73±0.70d	29.62±0.04bc

Table 3. Comparison of main structural features (Mean±SE) of WL319HQ leaves under different mutagenic treatments (μm).

Treatments		Leaf thickness	Vein thickness	Palisade thickness	Spongy thickness	VPD	CTR	SR
CK		294.28±7.61g	960.34±6.38b	130.00±3.65f	98.08±1.99g	326.34±5.24a	44.18±0.53c	33.33±0.17c
^{60}CO-γ (Gy)	150	421.70±6.49c	786.94±8.01f	149.35±2.28e	122.02±3.05e	186.61±2.14e	42.02±0.37cd	28.93±0.46d
	300	355.45±2.09e	520.79±9.96h	192.01±8.05c	151.14±1.03d	174.65±3.72f	45.53±1.55c	42.50±0.98a
	450	375.16±4.51d	826.18±9.13d	177.91±6.96cd	109.08±3.20f	220.22±3.87c	47.42±0.92c	29.09±0.09d
UV (min)	30	449.75±1.09b	1065.21±9.13a	230.90±1.92b	148.65±4.74d	236.84±6.84b	51.34±0.2b	33.05±1.00c
	90	623.16±9.35a	820.37±7.60d	278.01±8.47a	241.01±1.32a	147.69±5.61g	44.61±0.02c	38.68±0.05b
EMS (%)	0.1	432.42±8.65c	861.63±0.28c	190.69±1.01 c	158.15±3.66c	199.26±9.91d	62.29±0.97a	36.57±0.55b
	0.4	306.11±9.31f	660.69±4.61g	169.45±3.98d	113.70±0.58e	215.83±5.94c	39.19±0.29d	37.14±0.91b
MFFS		432.41±6.92c	964.21±5.04b	186.47±9.67c	195.02±4.88b	222.98±9.40c	45.10±0.92c	45.10±0.82a

Table 4. Comparison of main structural features (Mean±SE) of Aohan leaves under different mutagenic treatments (μm).

Treatments		Leaf thickness	Vein thickness	Palisade thickness	Spongy thickness	VPD	CTR	SR
CK		284.29±4.59g	717.93±7.74g	137.20±3.19f	100.42±5.48e	252.76±3.79a	44.28±0.73b	35.64±0.81b
^{60}CO-γ (Gy)	150	371.45±7.75f	808.63±5.97e	172.05±7.51d	130.00±2.10c	217.70±5.35b	46.32±0.59a	35.00±0.77bc
	300	566.35±0.88a	1061.94±5.87b	249.82±6.00a	193.04±7.14a	187.50±0.74c	44.11±0.44b	34.08±0.41c
	450	564.15±5.68a	1093.09±7.52a	215.13±3.89b	150.57±5.27b	193.76±2.57c	38.13±0.27d	26.69±0.68e
UV (min)	30	389.51±2.42e	835.43±3.86d	147.32±1.76f	114.63±4.48d	214.48±6.05b	37.82±0.54de	29.43±0.22e
	90	366.66±4.48f	714.84±4.67g	190.42±6.75c	153.08±5.96b	194.96±5.54c	37.42±0.72de	35.32±0.42b
EMS (%)	0.1	417.17±3.34d	731.71±5.67f	163.77±5.04e	197.41±4.36a	175.40±8.07de	39.26±0.06c	47.24±0.55a
	0.4	521.00±0.97b	933.87±8.58c	221.05±5.58b	157.20±7.59b	179.25±5.07d	42.41±0.59b	30.17±0.44d
MFFS		429.53±5.07c	843.65±7.17d	125.87±8.91g	153.38±5.90b	196.41±3.89c	44.33±0.77b	31.47±0.38c

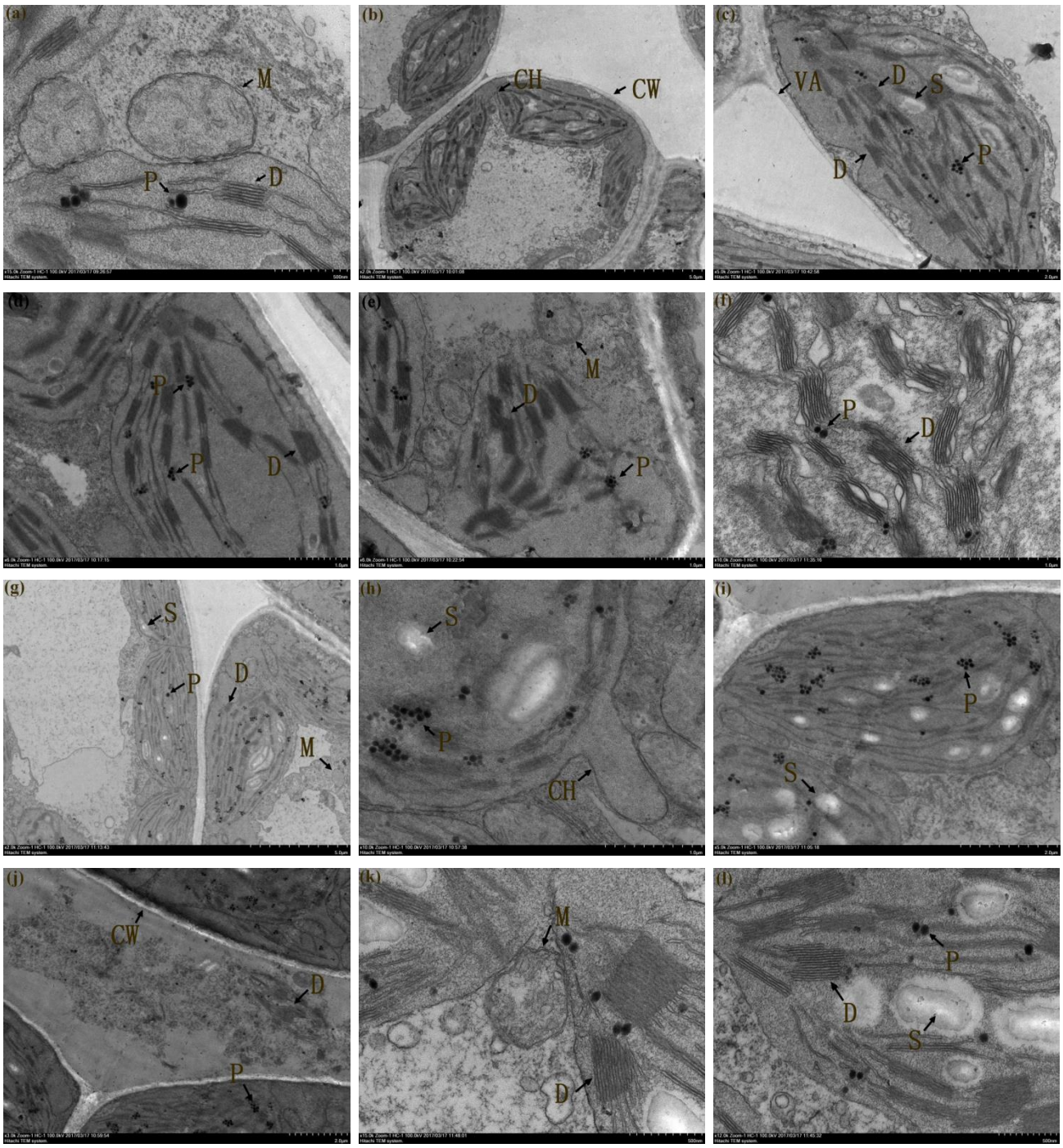

Fig. 1. Ultrastructure of WL319HQ ×30000
a: CK; b: 0.1% EMS treatment; c: 30min UV treatment; d-e: 90min UV treatment; f:150Gy radiation treatment; g: 300Gy radiation treatment; h-i: 450Gy radiation treatment; j-k: 0.4% EMS treatment; l: magnetic field free space. CW: cell wall; VA: vacuole; CH: chloroplast; M: mitochondrion; S: starch grain; D: crib fold structure; P: plasmid

Discussions

Under stress, the morphological, physiological and biochemical characteristics and cell structure of plants undergo various changes. The changes in cell structure depends upon the responses of both the genetic variation and the manifestation of their adaptability to the environment (Li *et al.*, 2015; Suriyan *et al.*, 2009; He *et al.*, 2013). Therefore, the analysis of microstructural changes can contribute knowledge to the exploration of the mechanism of mutagenesis. This knowledge regarding the microstructural characteristics can, therefore, be used to screen for the high cold-resistant mutagenic methods as well as the desired mutants. In nature, the morphological and anatomical changes in leaves e.g., the thickness of palisade tissue, spongy tissue and veins, mostly depend upon the external environment (Tian *et al.*, 2014). The structural characteristics of cells, including the thicknesses of the palisade tissue and the spongy tissue and the compactness and the looseness of the leaf tissue structures all contribute to the resistance mechanism of

plants. Because the compactness of mesophyll cells is primarily related to the palisade tissue. On the other hand, the compactness of cell structure is represented by the relative ratio of the palisade tissue thickness to the leaf thickness. The palisade tissue can also be used as a cold resistant index (Suarez *et al.,* 2011; He *et al.,* 2017). According to Pavel *et al.,* (2016) more compact the cell structure, the stronger the cold resistance of the species. Based on their analysis on the cell structure of tea plants, Yong *et al.,* suggested that the thickness, the layers and the compactness of the palisade tissue as well as the ratio of the palisade tissue thickness to the spongy tissue thickness were all positively related to the cold resistance of the tea plants (Fang *et al.,* 2014). Feng *et al.,* (2008) demonstrated that after completing a space flight, leaves of alfalfa sowed increased thickness with high relative water content. In the same experiment, the thickness and compactness of spongy tissue was also increased. Zhang *et al.,* 2005) showed that with respect to the microstructure of the alfalfa leaves, the spongy tissue and the palisade tissue reacted differently to the space mutagenesis, which had a bigger effect on the spongy tissue than on the palisade tissue. After a space flight, in alfalfa, structure of the spongy tissue differed significantly with that of control. But palisade tissue did not show any such difference. Results obtained in the present investigation showed that through mutagenesis, changes in leaf thickness, vein protuberant degrees, palisade tissue thickness, spongy tissue thickness and the compactness of cell structure all underwent changes. The degree of changes varied with respect to the different mutagenic methods and alfalfa cultivars. Compared to the control, the leaf thicknesses of the tested alfalfa cultivars mostly demonstrated an increasing trend. The compactness of the cell structure was also increased, which seems to be beneficial in increasing the adversity resistance and cold resistance capacity of alfalfa. The thickness of palisade tissue of alfalfa cultivars Gongnong No.1 increased at 450 Gy radiation and 90 min UV treatment. The thickness of palisade tissue of Wega7F, WL319HQ and Aohan showed an increasing trend compared with the control. However, the thickness of spongy tissue of the alfalfa cultivars varied in respect to different mutagenic treatments, in which the changes in the compactness structure of cells and looseness were similar to that of the sponge tissue. This indicates the uncertainty of the mutagenic directions and degrees. In this study, the half sibling seeds of the four alfalfa cultivars were selected depending on the mutagenic materials used and this had greatly reduced the differences in genetic background. Furthermore, the leaves were selected for the purpose of microstructure study as they could demonstrate the plant adaptation to mutagenic conditions more accurately.

Plant leaves are highly sensitive to all kinds of mutagenic reactions (Sun *et al.,* 2017). The microstructure and ultrastructure of leaf cells change significantly after mutagenesis. The changes are manifested as ununiformed cell walls(Ma *et al.,* 2007), degenerated and disappeared organelles inside the mesophyll cells(Bonaventura *et al.,* 2005), enlarged cell vacuoles (Guo *et al.,* 2011), organelles pushed aside cell walls (Nechitailo *et al.,* 2005) and chloroplast deformation or membrane disintegration (Zhao *et al.,* 2010). Yang *et al.,* (2001) also observed disintegration of the chloroplast membranes and several abnormal chloroplasts stacked together under low temperature stress. Most of the stroma lamellae were disordered, and some lamellae were fused together and blurred or even completely disappeared. Wang *et al.,* (2004) showed that the chloroplasts of the tomato leaves were swollen and rounded under high temperature stress. In addition, the chloroplast envelopes were broken and disintegrated in different degrees, and the packing of thylakoids was loosen and disordered, which were due to the stress response caused by the cell injury [28]. In this study, compared to the control, especially in the cases of high-dose mutagenic reagents and magnetic-free field, the chloroplast membranes were partially blurred or disintegrated. The granum lamellae were swollen and dissolved and the grana stacking were decreased or only a few were increased. Some of the cell walls were completely ruptured and the contents of the cell were extravagated. The cristae ridges of mitochondria were blurred and the plastids were increased significantly and aggregated. Since plastids are a kind of organelles associated with carbohydrate synthesis and storage, an increase of plastids can increase the viability of cells and have a beneficial effects on the improvement of the cold resistance of alfalfa (Stupnikova *et al.,* 2001; Gao *et al.,* 2009). The results also showed that after mutagenesis, the starch granules in chlorophyll were increased both in size and in number. This indicated an accumulation of photosynthetic starch in the chloroplasts of mesophyll cells, which could greatly enhance the cold resistance of alfalfa. Because the increased starch could release energy when broken, which may ascertain a higher and better cell viability. At the same time the breaking of starch granules could also improve cytoplasmic concentration, enhance osmotic pressure and water absorption, and provide adversity resistance to the plant.

Conclusions

According to the test, it showed that Leaf thickness was increased, vein protuberant degree was decreased, and starch grain were increased after treated by mutagenesis methods, it can lead to be helpful to improve cold resistance of alfalfa. ^{60}COγ-ray irradiation and EMS had higher mutagenic efficiency, their mutant materials were more abundant, their operations were simple, which was considered as being the reasonable mutation methods; Being more conducive for screening mutants, ^{60}COγ-ray irradiation with150Gy, ultraviolet for 60mins and EMS with 0.4% were the better mutagenic treatments.

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