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Effect of Ethyl Methanesulfonate on Induced Morphological Variation in M_3 Generation of *Chrysanthemum indicum* var. *aromaticum*

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Abstract. **Mutation breeding is considered to be economic and efficient in plant improvement, and the use of chemical mutagens such as ethyl methanesulfonate (EMS) can potentially address plant breeding challenges. The aim of this study was to induce morphological mutants in *C. indicum* var. *aromaticum* using EMS treatments with different doses, and to analyze the morphological and physiological traits of obtained mutants in expectation of finding favorable mutants. Results revealed significant effects of EMS doses on seed germination. The sample germination rate significantly decreased with increasing of EMS doses. The obtained morphological mutants were two viable types, containing leaf and stem mutants. Overall leaf size was significantly larger as a result of EMS treatments. And the height of mutant plants was significantly higher. Anatomical characteristics exhibited changes in both leaves and stems of the mutant plants. The puncture strength of the bent stem from the mutant plants was low, with weak penetration resistance. The total lignin and cellulose contents of mutant plants stem decreased significantly as a result of the EMS treatments. These results demonstrate the efficiency of EMS to induce mutations in *C. indicum* var. *aromaticum*, and this method can be useful in the future to assist breeding of this plant.**

Chrysanthemum indicum var. *aromaticum* is a perennial herb plant belonging to the family Compositae, in which all parts have a special fragrance. In China, *C. indicum* var. *aromaticum* was first cultivated as a flowering herb, and both the leaves and the flowers are mentioned in medicine to prevent colds, headaches, enteritis, diarrhea, coronary heart disease, and hypertension (Deng et al., 2006; Hussaini et al., 2018; Liu et al., 2013; Zhong et al., 2019) Furthermore, the extracts from this plant have become popular in the pharmaceutical, perfumery, daily-use chemical, and cosmetics industries, which are of crucial economic significance (Jian et al., 2014) Its consumption both as medicine and industrial raw material is increasing rapidly, and the distribution range of *C. indicum* var. *aromaticum* is limited and its resources are not so abundant. Therefore, it is necessary to expand its growth range and reduce the difficulty of its cultivation and collection. Chemical mutagens can be used to induce and select favorable mutants, thus an ideal method to improve varieties of *C. indicum* var. *aromaticum*.

Induced mutagenesis is widely used to obtain mutants with desirable plant traits (Ahloowalia et al., 2004). Improvements in mutant varieties of different crop species have been used widely in cultivation (Kharkwal and Shu, 2009; Mohan Jian and Suprasanna, 2011). EMS is a commonly used chemical mutagen in plants and has become a primary resource for development of improved varieties. EMS as a mutagen has been reported to affect the physiology, anatomy, biochemistry, and morphology of plants differently, depending on its concentration. Various studies have been conducted on using mutagens to improve morphological variation, such as herbicide tolerance in soybean (Sebastian et al., 1989), early flowering in spring rape (Thurling and Depittayanan, 1992), male sterility in wheat (Maan and Williams, 1984), and reduced fruit size in tomato (Yudhvir, 1995). Morphological characteristics are considered to be an efficient way to identify differences between mutant plants and wild species. Morphological characteristics of plants have been used to detect changes in various plants, because morphological mutants play a vital role in modifying the characteristics of cultivars and the development of new varieties of plants. EMS provides opportunities to develop the genetic variability of quantitatively inherited characteristics (Sarma et al., 1991), which has been proposed as a plausible solution to challenges encountered in plant cultivation such as *C. indicum* var. *aromaticum.*

Therefore, the objective of this study was to induce genetic variation using optimal EMS treatment and select mutants with the aim of exploiting the potential of EMS to develop improved *C. indicum* var. *aromaticum* and obtain a clear understanding of the effect of EMS solution on morphological changes of *C. indicum* var. *aromaticum* in M_3 generation. Some mutant phenotypes such as leaf size and stem shape were also identified in this study. The work described in this study will be useful in identifying interesting mutant phenotypes for further development of *C. indicum* var. *aromaticum.*

Materials and Methods

Plant materials. C. indicum var. *aromaticum* seeds were obtained from the Department of Landscape Architecture of Northeast Forestry University, Harbin, Heilongjiang, China. Seeds were treated with different concentrations of EMS (0%, 0.1%, 0.2%, and 0.5% v/v) for 8 h, then were posttreated with stop solution (0.1 M sodium thiosulfate) and water. The treated seeds were put into petri dishes, and placed in a dark incubator at 24 ± 2 C° until germinated, as described by Talebi et al. (2012). The germination rate of the EMS-treated group was collected on the day when the germination rate of the control reached 100%. When four to six true leaves had grown, M_1 seedlings were transferred into plastic pots with soil and were maintained under greenhouse conditions. M_2 consisted of individual stem-tip cuttings from M_1 that were transplanted into the field to screen for morphological traits under field conditions. To identify inheritable morphological mutants, the M_3 generation was propagated from stem-tip cuttings of M_2 and, again, planted in the field. Visible morphological traits were observed during the growing period and documented.

Leaf area. Leaf size was measured using the LI-3000A leaf area meter (LI-COR, Lincoln, NE).The scanning head scanned over a leaf and provided readouts of leaf area, leaf width and leaf length. The data were showed on the display or output through a computer (Awal et al., 2004).

Stem strength. Stem strength was measured at the top, middle, and lower segments of the stem using a stalk strength tester

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(YYD-1). The tester was set perpendicularly to the center of the stem and then pushed the basal internode back to its breaking point to measure stem bending resistance. Stem strength is expressed in Newtons (Sher et al., 2018).

Table 1. Effects of ethyl methanesulfonate (EMS) treatments on the germination rate of seeds.

Treatment	Seed no.	Germination rate of seeds (%)
CK	100	100.00 ± 0.00 a
EMS 0.1%	100	86.33 ± 2.08 b
EMS 0.2%	100	73.00 ± 7.81 c
EMS 0.5%	100	32.33 ± 6.65 d

Different lowercase letters in the same column represent significant differences at $P < 0.05$.

Paraffin sectioning. The stems and leaves were sliced into 3-mm sections, and then the samples were fixed immediately with 3:1 ethanol:acetic acid for 24 h at room temperature. Paraffin embedding was performed follow as: 50% ethanol for 2 h, 70% ethanol for 2 h, 85% ethanol for 2 h, 95% ethanol for 2 h, 100% ethanol for 1 h, xylene for 1.5 h, and paraffin wax at 60 °C for 2 h, after which the tissues were embedded in paraffin blocks. All slides were examined using a Nikon Eclipse E 600 microscope (Kamada et al., 1997).

Phloroglucinol–hydrochloric acid. Two volumes of 3% phloroglucinol (Ph) solution were mixed with one volume of concentrated hydrochloric acid (HCl; 37 N). Stems sections were transferred to a tube, and then 1 mL of the Ph-HCl solution was added. Pipette sections into the tip gently, and onto a microscope slide. Cover the sections with a coverslip and the imaging has to be completed within a period of time (Mitra and Loqué, 2014).

Stem lignin content. Stem samples were dried at 85 °C, and stem powder (500 mg) was extracted with formaldehyde solution for one night. The samples were dried at 85 °C, then cooled and weighed. The amount of lignin was determined according to the Klason method. A total of 7.5 mL 72% sulfuric acid was added to 300 mg of the sample, was placed in 30 °C water, and was bathed for 3 h. The samples were diluted to 4% sulfuric acid with about 127.5 mL water and autoclaved at 121 °C for 1 h. The precipitates were collected with a glass fiber filter using suction filtration. The filters, along with the acid-insoluble lignin, were dried at 85 °C, then cooled and weighed. The amount of lignin of each sample (measured as a percentage) was then calculated (Fagerstedt et al., 2015).

Stem cellulose content. The amount of cellulose was extracted and assessed using the method described by Updegraff (1969). A total of 0.2 g (dry weight) ground (0.5 mm) of each sample was digested using 60% sulfuric acid (H_2SO_4; 60 mL) for 30 mins. Tubes with a lid (to reduce the possibility of evaporation) were placed in a boiling water bath for 30 min. The water level of the water bath was kept at the same level as the liquid in the tubes. Samples were centrifuged at 4000 rpm for 5 min and then decanted. The samples were washed two times with water and dried, and then 67% H_2SO_4 was added. The samples were diluted from 1 to 100 mL with water. The amounts of cellulose were absorbance-measured at 620 nm using an anthrone reagent with pure cellulose as the standard (Aguiar, 2001).

Statistical analyses. Statistical analysis was carried out for the observations recorded in the experiment to determine whether there exists any significant variation for various parameters among the EMS-treated plants and control plants. All data obtained from M_3 generations were subjected to analysis of variance using SPSS 24.0 (Anderson et al., 1997). The results are presented as mean ± SE of three replicates from all measured parameters.

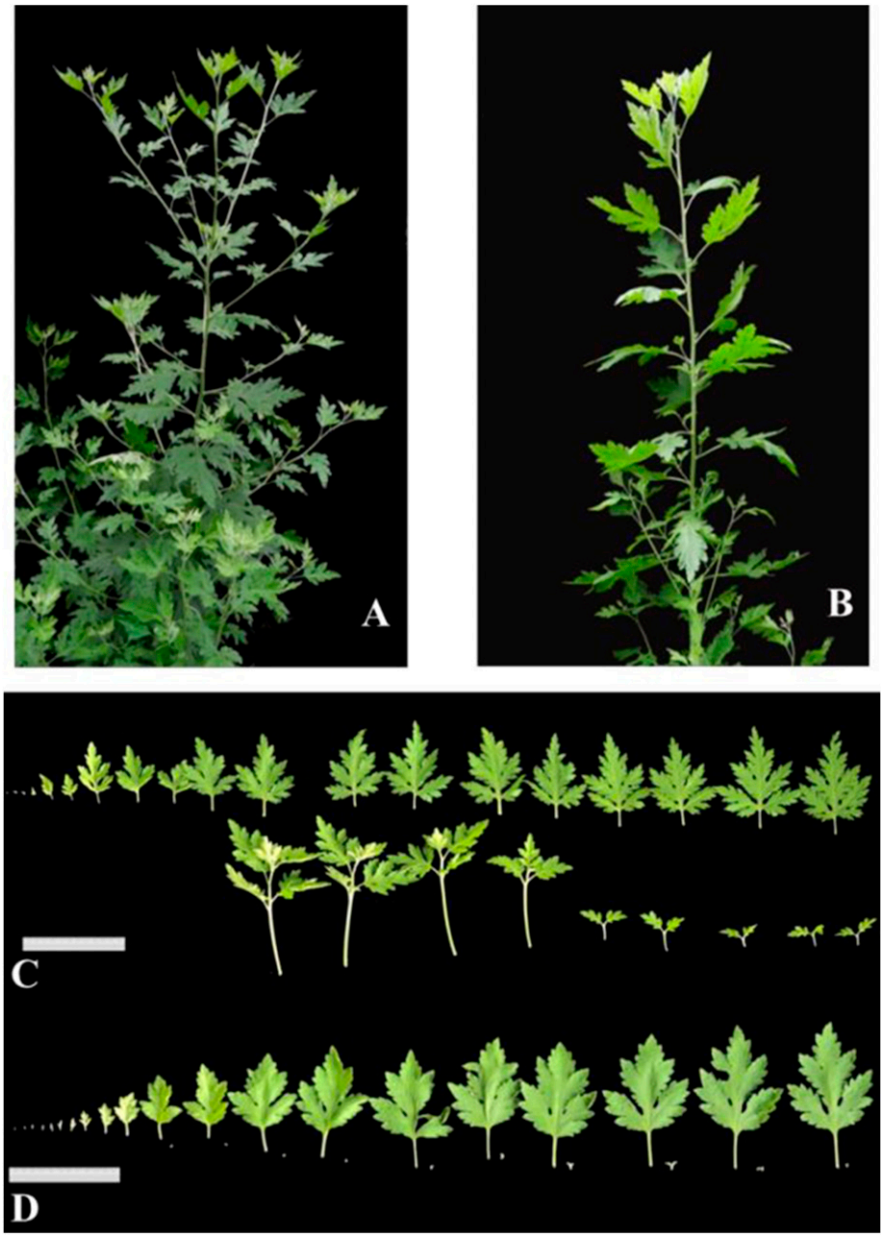

Fig. 1. Effects of ethyl methanesulfonate on M_3 leaf morphology. (**A, C**) Treatment with CK. (**B, D**) Treatment with ET2-138. Scale bar, 10 cm.

Results

Impact of EMS on seed germination. Treating seeds with EMS can result in numerous side effects on germination. The germination rate decreased with increasing doses of EMS. The highest percentage of germination (86%) was recorded at 0.1% v/v of EMS whereas the lowest percentage (32%) was recorded at 0.5% v/v of EMS (Table 1). Seeds showed that the reduction in germination might have been a result of the effect of mutagens on seed meristematic tissues.

Morphological mutants. We screened 13,500 plants in the M_3 generation, derived

Table 2. Effect of ethyl methanesulfonate on leaf morphology and plant height.

Treatment	Leaf area (cm^2)	Leaf length (cm)	Leaf width (cm)	Plant ht (cm)
CK	8.89 ± 0.40	4.22 ± 0.91	3.64 ± 0.59	26.20 ± 2.20
ET3-138	32.69 ± 0.92	8.46 ± 1.03	6.36 ± 0.45	48.10 ± 3.36
P value	0.000	0.015	0.007	0.001

Table 3. Effect of ethyl methanesulfonate on anatomical structure of leaf.

Treatment	Leaf thickness (μm)	Upper epidermis thickness (μm)	Lower epidermis thickness (μm)
CK	24.29 ± 2.91	1.53 ± 0.29	1.24 ± 0.10
ET3-138	27.80 ± 2.24	2.63 ± 0.25	1.57 ± 0.27
P value	0.007	0.000	0.003

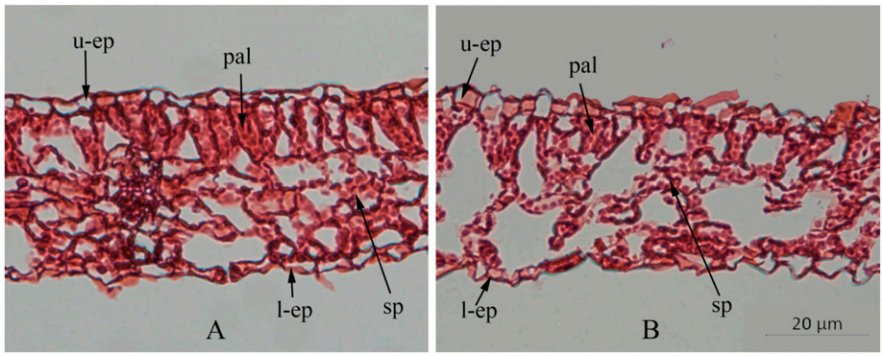

Fig. 2. Anatomical structures of leaves from mutant and CK. Scale bar, 20 μm. (**A**) CK treatment. (**B**) ET2-138 treatment. pal, palisade mesophyll; sp, spongy mesophyll; u-ep, upper epidermis; l-ep, lower epidermis.

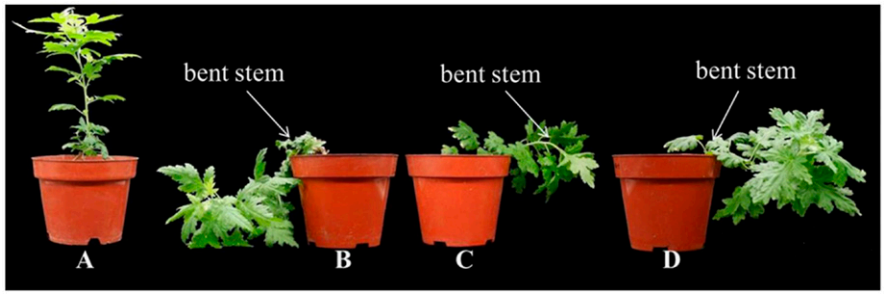

Fig. 3. Effect of ethyl methanesulfonate on M_3 bent plant stem. (**A**) CK treatment. (**B**) ET1-55 treatment. (**C**) ET1-56 treatment. (**D**) ET2-61 treatment.

from 1500 M_1 plants by two cycles of vegetative propagation through stem-tip cuttings, to identify mutants with clear visual morphological variations. Four mutants with unusual growth habits from the M_3 generation based on their morphological changes were found. These plants are predicted to represent mosaics with (heterozygous) mutant L1, L2, or L3 sectors, and it is currently unclear whether the mutant traits can be propagated by sexual reproduction. They exhibited abnormal morphological characteristics compared with CK plants. One mutant named ET2-138 (EMS 0.2% v/v) had an overall larger leaf size. Three mutants—ET1-55, ET1-56 (EMS 0.1% v/v), and ET2-61 (EMS 0.2% v/v)—had an unusually bent stem.

Morphological and anatomical leaf characteristics. The leaf mutant was ET2-138 (EMS 0.2% v/v). There was a tendency that plants were an average size in control, but mutant plants showed significant increase in leaf size and plant height (Fig. 1). Table 2 presents the effect of EMS on leaf morphology and plant height. Mutant plants (48.10 cm) were significantly taller than CK plants (26.20 cm). Leaf length and width of mutant plants (8.46 cm and 6.36 cm, respectively) were significantly longer than CK plants (4.22 cm and 3.64 cm, respectively). The leaf area of the mutant plant (32.69 cm^2) was four times larger than the CK plant (8.89 cm^2). Interestingly, these results proved the strong variation of leaf area, and plant height shows a corresponding increase as leaf area. The anatomical characteristics of mutant leaves also exhibited significant changes compared with CK plants. The thicknesses of the leaves, upper epidermis, and lower epidermis of the mutant plants were 27.80, 2.63, and 1.57 μm, respectively, whereas CK plants were 24.29, 1.53, and 1.24 μm, respectively (Table 3). Also, the leaf blade of mutant plants contained thicker, palisade, and spongy tissue cells, which were irregularly spherical and arranged with an almost large gap compared with the CK plants (Fig. 2). The results indicate that EMS affects the general processes that control cell division and leaf expansion.

Analysis of stem characteristics and puncture strength. We isolated several unusual stem mutants that developed a bent stem—namely, ET1-55, ET1-56, and ET2-61. To study the underlying reason for the bending, we carried out an analysis of stem characteristics and puncture strength on the mutant and CK plants. Their stem growth morphology was quite different. The stems of CK plants were erect and grew straight, whereas the stems of mutant plants bent transversely bend and were creeping (Fig. 3). Meanwhile, the average height of mutant plants was significantly taller than CK plants.

Stem puncture strength is a comprehensive index to evaluate the penetration resistance of stem epidermis. In m the upper to the lower segments of CK and mutant plants, puncture strength increased progressively (Table 4). In the upper to middle segments, CK and mutant plants did not show significant differences in puncture strength. However, in the middle to lower segments, three mutant plants did not show an increase in puncture strength as much as CK plants, which indicates a weaker puncture strength in the lower stem of mutant plants. Comparing puncture strength of the lower segments of CK and mutant plants, CK plants were mechanically stronger than mutant plants; mutant plants were relatively weak. Mutant plants also exhibited a stem bending morphology around lower segments. These variations could imply that mutant plants have been poorly characterized and, from a mechanical point of view, are weaker than CK plants.

Interestingly, stem anatomical structures of mutant and CK plants varied greatly (Fig. 4). The stem epidermis layer of mutant plants was a short strip with a different size and part of tissue cells arranged irregularly compared with CK plants, and the stem cortex consisted of three to five layers of

Table 4. Comparison of the puncture strength and stem length of mutant plants and CK plants.

Treatments	Puncture strength (N)			Stem length (cm)
	Upper segment	Middle segment	Lower segment	
CK	9.10 ± 0.25 ab	11.35 ± 0.34 a	15.63 ± 0.92 b	26.44 ± 0.53 a
ET1-55	9.20 ± 0.15 b	10.06 ± 0.34 a	12.70 ± 0.70 a	37.16 ± 0.44 b
ET1-56	8.23 ± 0.28 ab	10.60 ± 0.05 a	12.03 ± 0.24 a	31.93 ± 2.28 ab
ET2-61	9.16 ± 0.35 b	11.23 ± 0.20 b	12.56 ± 0.37 a	32.40 ± 1.13 ab

Different lowercase letters in the same column represent significant differences at $P < 0.05$.

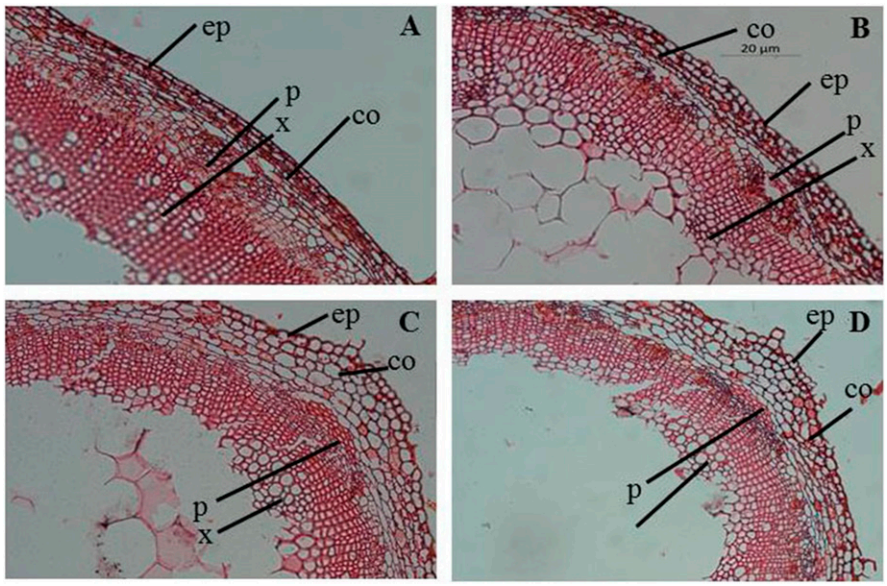

Fig. 4. Comparison of cell morphology between lower sections of stem. Scale bar, 20 µm. (**A**) CK treatment. (**B**) ET1-55 treatment. (**C**) ET1-56 treatment. (**D**) ET2-61 treatment. p, phloem; x, xylem; ep, epidermis; co, cortex.

usually oval cells that were arranged irregularly. The primary xylem was in the middle of the stem, whereas the primary phloem was pushed outward by the new cells that arose from the vascular cambium. This anatomical structure enabled the mutant plants to increase its vascular bundles. Vascular bundles consisting of secondary phloem and xylem of mutant plants were larger than CK plants. These observations indicate that EMS induced changes in the anatomical structure of bent-stem mutations, and that reduced growth is a well-documented impact of EMS treatment on M_3 generation because of its negative effect on stem cell division.

Stem lignin deposition patterns. To assess the effect these mutants had on lignin deposition patterns, cell wall material from mature stalks was analyzed with Ph-HCl staining. The Ph-HCl reaction was visible in red on the cell walls. The greater color intensity suggested greater accumulation of lignin in the cell walls. The color differences between CK plants and mutant plants in the mechanical tissues, especially in the sclerenchyma cells below and above the epidermis and around the vascular bundles, indicated an apparent decrease in lignin quantity in the mutant plants. The intricate pattern showed quite apparent changes in EMS treatments of mutants ET1-55, ET1-56, and ET2-61. Figure 5 (A-1, B-1, C-1, and D-1) shows the ground tissue structure of upper segments from mutant and CK plants. In the upper segments, the mutants' sclerenchyma cells around the vascular bundles were larger than those of CK plants. Also, gaps were observed in the ring of interfascicular parenchyma cells of mutant plants, with lignin deposited abnormally (green and yellow in Fig. 5). In contrast, the lignin of CK plants was normally deposited in the epidermis and cortex (red in Fig. 5). No color reaction in the pith in both mutant and CK plants was observed.

In the middle segments [Fig. 5 (A-2, B-2, C-2, and D-2)], mutant plants have thinner secondary cell walls than CK plants. Consistently, intense lignin staining was also observed in these sclerenchyma cells of mutant plants. The sclerenchyma cell walls were very thick as a result of the deposition of lignin. The gaps among vascular bundle of mutant plants were much smaller than in their top segments, and the Ph-HCl reaction was quite visible in them. The cortex of mutant plants was mostly composed of large thin-wall parenchyma cells, and the outer cortical cells were thicker than those in the upper segments.

Figure 5 (A-3, B-3, C-3, and D-3) presents a clear comparison of lower segments between mutant plants and CK plants. The ground tissue system from the lower segment of mutant plants was similarly thin as that in middle segments. The outer cortical cells of mutant plants were thinner than in CK plants. This pattern correlates with the position of abnormally differentiated, striped tissue in mutant plants, which indicates the mutant plants are deficient mainly in the secondary cell walls. No lignin staining was detected in the pith parenchyma cells throughout the stems of both mutant and CK plants. These results indicate that EMS induced changes in lignin deposited specifically in cell walls and stems during plant growth and development, which then affected stem shape and stem strength.

Lignin and cellulose content in C. indicum *var.* aromaticum. A comparative study of lignin and cellulose content in plant stems was performed on samples of bent-stem mutant plants (ET1-55, ET1-56, and ET2-61) and CK plants. The results of lignin content in mutant plants are shown in Fig. 6. All the mutants plants examined had a significantly reduced lignin content compared with CK plants. The lowest level was observed in ET1-56, followed by ET2-61 and ET1-55. This suggests that the mutation has influenced lignin content. The observed reduction in lignin content in these mutants is consistent with the previously described effects of EMS on cell wall lignification.

In mutant plants, cellulose content showed a decrease similar to that seen in lignin content. Accessible cellulose content in mutant plants was reduced significantly relative to the CK plants (Fig. 7), suggesting that the mutations might have directly or indirectly played an important role in cellulose content. These results indicate that the bent-stem mutations affected the organization of cellulose content.

Discussion

Induced mutations are a mechanism of creating new variability in any crop plants with a higher frequency than the occurrence of spontaneous mutations (Chopra, 2005). In our study, four EMS-induced mutants in *C. indicum* var. *aromaticum* (ET2-138, ET1-55, ET1-56, and ET2-61) were used for morphological and physiological characterization.

The results demonstrate that EMS treatments considerably influence seed germination. Seed germination rates showed a significant decrease with increasing dose of EMS. According to Laskar et al. (2018), the percentage of germination and fertility in the M_1 generation of tomato was profoundly affected by increased mutagenic treatment using EMS. A strong reduction in seed germination is an indication of useful mutagenesis (Singh and Kole, 2005). A reduction in seed germination may be the result of

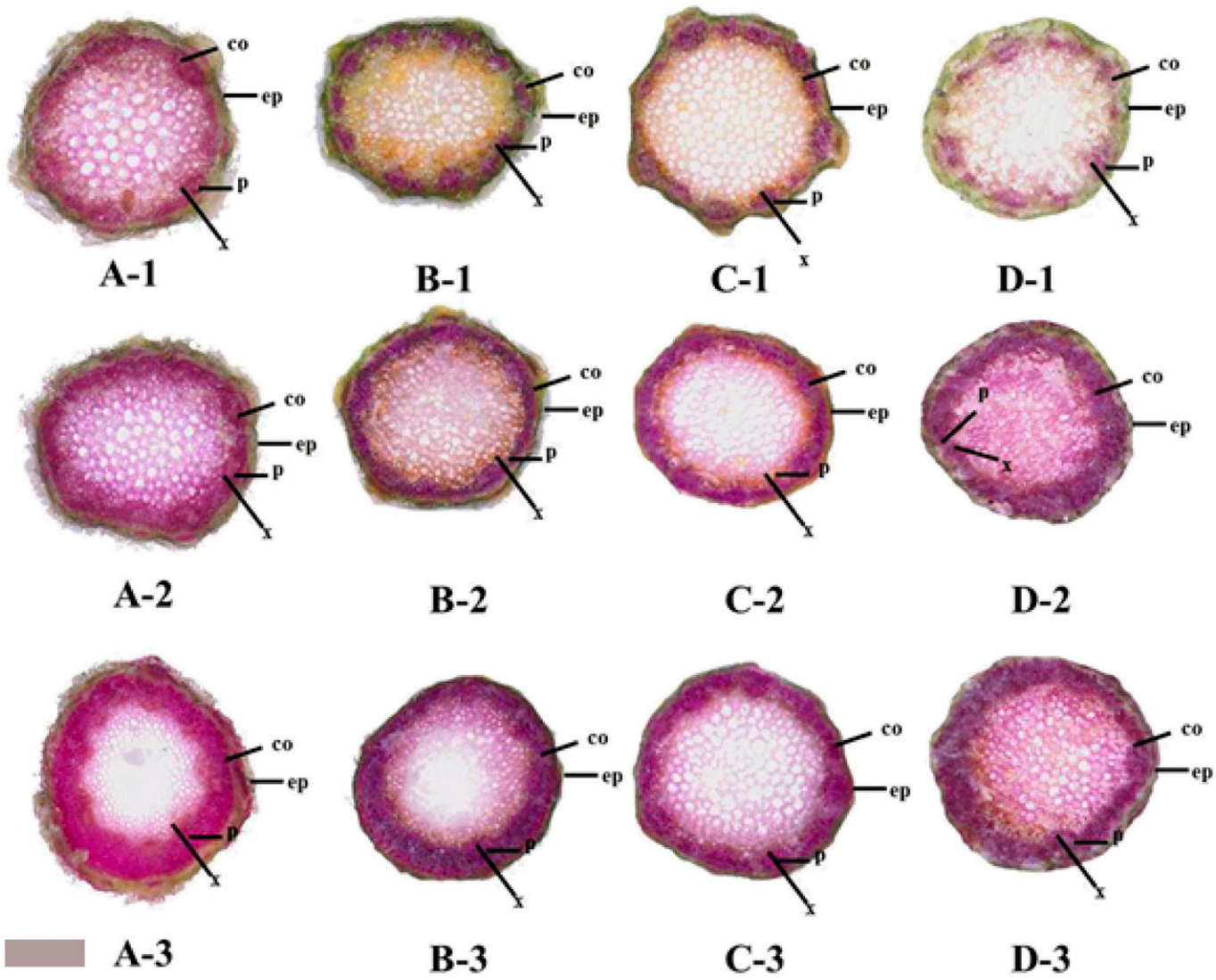

Fig. 5. Lignification pattern in the stem throughout CK (A-1, A-2, A-3), ET1-55 (B-1, B-2, B-3), ET1-56 (C-1, C-2, C-3), and ET2-61 (D-1, D-2, D-3) treatments. Scale bar, 1 mm. A stem from CK and mutant plants was divided into three equal segments. A-1, B-1, C-1, and D-1 are the sections from the top segment. A-2, B-2, C-2, and D-2 are the sections with 5 cm apart from the middle segment. A-3, B-3, C-3, and D-3 are the sections from the bottom segment. Although lignin was seen in the pith cells in all sections, lignin distribution in the pith appeared to be mosaic. p, phloem; x, xylem; ep, epidermis; co, cortex.

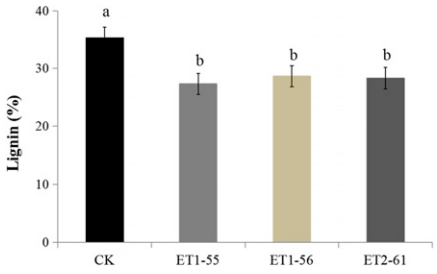

Fig. 6. Lignin contents of mutant plants and CK plants. Different lowercase letters above the error bars represent significant differences at $P < 0.05$.

disturbances in meristematic tissues or of chromosomal damage to the seed (Kumar and Yadav, 2010). EMS mutagenic treatment of seeds causes chromosomal aberrations that can adversely affect cell division. This indicated the effect of mutagenic treatment on seed germination in *C. indicum* var. *aromaticum*.

EMS mutagenesis also affected the morphological characteristics of *C. indicum* var. *aromaticum* in the M_3 generation. In our study, the growth behavior of mutant plants exhibited changed characteristics compared with CK plants. The leaves of mutant plants were thicker and longer than those of CK plants. Anatomical characteristics of mutant plants were different from those of CK plants. After considering this extreme case, it is reasonable to assume that mutations in genes related to the control of leaf ontogeny might determine functional phenotypes, characterized by changes in leaf size. The mutants obtained from this process should show disturbances in the structure, spatial arrangement, and division or expansion patterns of leaf cells. In agreement with our results, Behera et al. (2012) showed that induction of mutagenesis through EMS treatment in *Asteracantha longifolia* affected plant height, morphology, and even leaf size. Mutations in genes that control the development of leaves might have phenotypic effects that vary from lethality to the absence of visible alterations. The changes in the scape anatomy in response to treatment with EMS, including intervarietal and intertreatment differences, indicated that, similar to qualitative and quantitative characteristics, anatomical features were also as a result to alterative by mutagenic treatment (Anderson and Abbe, 1933).

In support of our results, the mutation of a bent stem was previously observed. ET1-55, ET1-56, and ET2-61 showed an abnormal stem, curved transversely and creeping, and its height was significantly taller than in CK plants. Jabeen and Mirza (2004) observed that the growth of pepper seed exposed to EMS was taller or shorter than their controls. All of our three bent-stem mutants—ET1-55, ET1-56, and ET2-61—were defective in lignin and cellulose content, and their

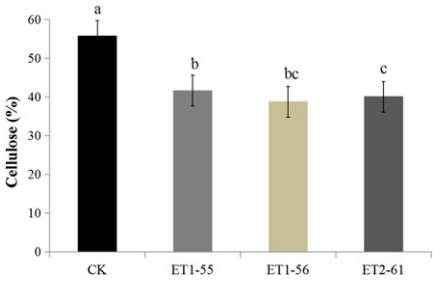

Fig. 7. Cellulose contents of mutant plants and CK plants. Different lowercase letters above the error bars represent significant differences at $P < 0.05$.

deposition in the secondary cell wall of sclerenchyma cell, so the anatomical structure of mutant plants was different compared with CK plants. The stem puncture strength, and lignin and cellulose contents were significantly less than CK plants. Our findings are in agreement with Petti et al. (2013). The lignin provides plant tissue and individual fibers with compressive strength, and strengthens the cell wall of the fibers to protect the carbohydrates from chemical and physical damage (Saheb and Jog, 1999). According to Yong and Wickneswari (2013), one of the main structural components providing stability and strength to plant cell walls is cellulose. Li et al. (2003) reported that the rice classic mutant *bc1* causes altered biosynthesis of cellulose and lignin, leading to a reduction in secondary cell wall thickness and in the mechanical strength of the rice plant. In our findings, the *C. indicum* var. *aromaticum* bent-stem mutant showed weaker mechanical strength and reduced lignin and cellulose contents, indicating a correlation between lignin and cellulose contents and mechanical strength. These results show that the mutations from EMS may impact cell wall metabolism. This also indicated that the lignin and cellulose present in the cell walls are not only a simple mixture but also chemical linkages. This further indicates that EMS could regulate the lignin and cellulose contents in the stem of *C. indicum* var. *aromaticum*.

Conclusion

Suitable doses of EMS treatments are a productive and economic method to induce mutants in *C. indicum* var. *aromaticum.* The efficiency of EMS treatment was found to depend on its concentration, and it was harmful to seed germination even at 0.1% v/v. The germination ratio decreased sharply with the increase of EMS dose. Among those mutants found in our study, improved leaf size (in mutant ET2-138) is a potential favorable and economic mutant to *C. indicum* var. *aromaticum*, because its large leaves are productive sources for Chinese traditional medicine and industrial raw materials. Consequently, this study shows that we can select variants with interesting attributes using an efficient mutant phenotype selection procedure for further development of *C. indicum* var. *aromaticum*.

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