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Research Note

Morphological architecture of foliar stomata in M_2 Carnation (*Dianthus caryophyllus* L.) genotypes using Scanning Electron Microscopy (SEM)

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Abstract:
Dianthus caryophyllus is an important floricultural crop in temperate climates and worldwide popular as cut-flowers for its variegated petal's colour. The development of this cultivar with more desirable floral characteristics and higher productivity are also very much important. Their identifications as well as taxonomy had been studied in the literatures using different laboratory methods. Both morphological and/or genetical characteristics were considered in the reported studies. However, to the best of our knowledge, there does not exist any study involving an image analysis based approach. For this, we undertook the mutation breeding programme with selected chemical mutagens, viz. Colchicine (COL), Ethyl Methane Sulphonate (EMS) and Maleic Hydrazide (MH) with different concentrations. These mutagens were applied on the young leaves of M_2 plants of *Dianthus* cultivar. The results of the present study on peculiar morphological architectures of leaf stomata in *Dianthus* at different concentrations of three potent chemical mutagens were analyzed on the basis of their Scanning Electron Microscopy (SEM) images which is more informative than the classical approach. Number of stomata and its shape, aperture length and its dimension, characteristics of guard cells in both dorsal and ventral surfaces of leaf also varied from treatment to treatment.

Key words: *Dianthus caryophyllus,* chemical mutagen, mutation breeding, stomata, Scanning Electron Microscopy.

Dianthus caryophyllus L., commonly known as Carnation, belongs to the angiospermic family: Caryophyllaceae, is an important floricultural crop which grows very well in temperate climate all over the world and ranks next to Rose in popularity (Laurie *et al*., 1968; Staby *et al*., 1978). This crop is very profitable and has a good market demand worldwide due to its vase quality and variegated colour of petals. This genus is important by having pharmacological and aromatic properties and is polymorphic in morphology, genetics and hybridization (Facciola, 1990; Hughes, 1993; McGeorge and Hammett, 2002; Su Yeons, 2002; Lee *et al*., 2005). In this modern era, an agronomic demand of high yielding cultivar of this crop was noticed. One way of creating variability in such a self pollinated crop is attempting crosses between two genotypes complementing the characters of each other but, due to autogamous nature of this crop, hybridization at appropriate time is a difficult process. The only alternative left with breeders to create variability is mutation breeding (Roychowdhury and Tah, 2011a). This method can be used as a potential source of creating variability (Novak and Brunner, 1992). Inducible mutation by chemical or physical agents can accelerate the *Dianthus* cultivars with more desirable floral characteristics and higher productivity (Roychowdhury and Tah, 2011b). It is possible to induce gene-mutations artificially with the help of potent chemical mutagens like Colchicine (Col), Ethyl Methane Sulphonate (EMS), Maleic hydrazide (MH) and other mutagenic chemicals to create new variations (Roychowdhury, 2011). From economical point of view, we need better quality and yields from commercial floricultural crop like *Dianthus*. Such components, yield and quality are obtained when plant's physiology and biochemical metabolic pathways are perfect; for this, leaf plays a major vital role. Leaf surfaces of plant species contain a large variety of biological and non-biological structures of various sizes and shapes (Davis *et al.*, 1976). Stomata are one of the important biological structures that are responsible for gaseous exchange in plants (Majada *et al*, 2001). Stomata having small pores found in epidermis of the leaves, which may be open or close under the control of a pair of kidney-shaped cells called guard cells and stomata occupy a central position in the pathway for the transport of water vapor, CO_2 and O_2 (Pospisilova, 2003; Taiz and Zeiger, 2006). To study the effect of different chemical mutagen on the stomata of *Dianthus*

cultivar, we have to perform the image analysis based approach, like scanning electron microscopy (SEM), which directly produces an image of the three-dimensional structure of the specimen's surface (stomata in leaves). The SEM is a cheaper device than a transmission electron microscope (TEM), whereas the TEM uses the electrons that have passed through the specimen to form an image, the SEM uses electrons that are scattered or emitted from the surface of the specimen. The specimen to be examined is fixed, dried, and coated with a thin layer of heavy metal. The specimen, thus prepared in any of these ways, is then scanned with a very narrow beam of electrons. The SEM technique provides great depth of field; moreover, since the amount of electron scattering depends on the angle of the surface relative to the beam, the image has highlights and shadows that give it a three-dimensional appearance. Only surface feature can be examined. However, in most forms of SEM, the resolution attainable is not very high; about 10 nm with an effective magnification of up to 20,000 times (Bozzola and Russell, 1992). As a result, this technique is usually used to study whole cells or tissues rather than sub-cellular organelles. The SEM photograph of foliar stomata has a good demand in modern plant taxonomy for identification purposes. Biasiolo *et al.* (2004) and Singhal *et al.* (2009) found micro morphological features of 10 mulberry cultivars as an important tool for mulberry description. The aim of this study is to use scanning electron microscopy (SEM) to compare the stomatal morphology of chemical mutagen treated *Dianthus* cultivar with untreated control plants under the same environmental condition.

The field trial of *Dianthus caryophyllus* cultivar for the mutation breeding programme was conducted in the Crop Research Farm (latitude 23.53^o N, 22.56^o S and longitude 83.25^o E, 86^o W) under the Botany Department at The University of Burdwan, West Bengal, India. This experiment was started in the winter season (2009-2010). The study on M_2 generation (second mutant generation) of *Dianthus* cultivar was obtained from previously harvested M_1 seeds of chemical mutagenic treatment by Colchicine (COL), Ethyl methane sulphonate (EMS) and maleic hydrazide (MH). Foliar treatment was conducted on the leaves, after 21 days of control seed germination, by using different concentrations (0.1%, 0.4% and 0.7% as w/v for each) of COL, EMS and MH. Then, tap water was sprayed on those treated leaves to remove the surface sticking chemicals. The seeds of M_1 plants were dried in hot air oven at 40^{o}C for 3 days and were sown in the well prepared field separately, depending upon the concentration of chemicals with proper marking. This sowing process followed randomized block design (RBD) having three replications for each treatment, where spacing between the plants in rows and columns was 2 ft x 2 ft. New codes were given to each mutagenic treatment such as: Control = $FT_{1,}$ 0.4% Colchicine = $FT_{3,}$ 0.4% Ethyl Methane Sulphonate = $FT_{6,}$ 0.4% Maleic Hydrazide = FT_9.

First, the fresh healthy treatment wise leaves were picked up with the help of autoclaved scissors and forceps from the plants in experimental field without any injury. The sampled leaves were prepared for electron microscopical analysis as described by Robinson *et al.* (1987), with slight modifications. The tenth leaf from the first fully opened top leaf of the longest branch of each genotype was cut into 3 mm^2 pieces and subsequently fixed for 16 hours in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and washed three times with the same buffer and post-fixed in 2% Osmium tetroxide for 1 hour. Then again washed in the same buffer. The leaf pieces were then dipped into 50 % ethanol for 5 minutes, then shifted in 70% alcohol for 30 minutes in two changes, then in 90% alcohol for 30 minutes in two changes and then in absolute alcohol for 30 minutes in two changes at the room temperature of 25 ± 2 °C. The leaves were immersed in absolute alcohol : amilo iso-acetate in the ratio of 3:1, 2:2 & 1:3 for 30 minutes in each case and then leaves were preserved in amilo iso-acetate for 30 minutes and the preserved leaves were kept for 2 days. After that, preserved leaves were cut into small pieces for Critical point dryer (CPD). The leaf segments of *Dianthus* first put in the chambers of cassettes set at 10 °C into the following manner: control in chamber 1, 0.4% Col. in chamber 2, 0.4% EMS in chamber 3 and 0.4% MH in chamber 4. Then the inlet valve was opened to pass the liquid CO_2 in the chambers of cassette and pressure was raised to 62 kgf /cm^2 or gauge and the inlet valve was closed. When temperature arose to 20^{o}C, and the experimental set in this condition was maintained for 25 minutes and then set the critical temperature for $CO_{2,}$ i.e., 31^{o}C for 5 minutes. Then opened the leakage valve and pressure slowly let down at zero to release the specimen from the chambers of cassette. Thus critical point drying was made (Blazers CDP030), the samples were very quickly handled to avoid rehydration and they were fixed with Dotite CX-12 (Jeol/S-C). After cutting the dried leaf segments into small pieces, they were mounted on the cupper stubs, keeping the abaxial leaf surface up by using double-sided sticky tape, and then coated with gold (20 nm thickness) in an argon atmosphere 22 mA for 90 seconds using a sputter coater (EMS-550, Electron Microscopy Sciences, USA). At last, the coated

samples were examined under the scanning electron microscope (JEOL 100 CX II-ASID 4D, Japan) at 15 kV. The dorsal and ventral surfaces of leaf samples were examined under the Electron Microscope, after gold coating the stubs, to determine stomatal number and its structure related parameters.

The data are presented in Table 1 which indicates the stomatal characteristic such as aperture length and dimension, number of stomata and major features of guard cells.

Control leaf (FT_1) showing the number of stomata was 4 (×800) in the ventral side of the leaf. Under ×3000, stomata looked like oval- shaped with a thin aperture of 2.2±1.32 µm in length and guard cells were surrounded by thick waxy layers in the ventral side of the leaf. In the case of dorsal side, oval-shaped stomata were seen with large aperture of 4.0±2.35 µm in length and guard cells were surrounded by heavy waxy layers when observed under ×3000. The more thick guard cells with waxy layers indicate more stress resistance in any season which is also triggering the rigidity and strong metabolic capability of the crop plant. 0.4% Colchicine (FT_3) treated leaf showed 3 numbers of stomata under ×800 in the ventral side of the leaf. The number of stomata decreased from that of the Control set. The tendency of increasing number of stomata results in smaller size. When it decreased in number, it results in the bigger size of the stomata. This treatment showing more or less oval-shaped stomata (×3000) with large aperture of 3.9±2.18 µm in length and the dimension of the aperture increased from that of the Control; guard cells with less thick wax deposition in the ventral side of the leaf. Whereas in dorsal side, oval–shaped stomata (×3000) with large aperture 2.6±2.86 µm in length and the dimension of the aperture is decreased from that of Control; guard cells were surrounded by heavy waxy layers.

0.4% EMS (FT_6) treatment showed 3 stomata (×800) in the ventral side of the leaf. This number decreased from that of the Control. Under ×3000, this treatment showing more or less rounded stomata with the aperture length of 3.2±2.69 µm in ventral side and 3.6±3.12 µm in dorsal side. The dimension of the aperture was increased from that of Control; the guard cells were surrounded by thin waxy layers in the ventral side of the leaf. On the other hand, dorsal side showed that the dimension of the aperture was decreased from that of the Control; the guard cells were surrounded by heavy waxy layers.

0.4% MH treatment or FT_9 showed 3 stomata (×800) in the ventral side of the leaf, i.e., lower value than that of the Control. Under ×3000, it showed oval-shaped stomata with the aperture of 4±3.25 µm (in the ventral side) and 4.6±2.71 µm (in the dorsal side) in length. For both sides of the leaf, the dimension of the aperture was increased from the corresponding control value and the guard cells were surrounded by thick waxy layers of the leaf. Figure 1 showed the scanning electron micrographs of the stomatal outlooks that fluctuates from the Control set to different mutagenic treatments.

On the contrary to mature stomata, immature ones were characterized structurally by crescent-shaped guard cells and closed pores. Plugged stomata have the same shape but an amorphous material occurs between their guard cells. In Control or FT_1, the number of stomata was 4 under x800. In all the mutated plant, stomata number was decreased, i.e., 0.4% (COL) or FT_3, 0.4% (EMS) or FT_6 and 0.4% (MH) or FT_9 showed 3 stomata under the same view. The shape of the stomata in both ventral and dorsal sides of the leaf varied in different mutagenic treatments. An earlier study of Werker and Leshem (1987) showed that the density of stomata in plants varied greatly from one plant to another and abnormal size and shape of one or both of the guard cells has been observed. These abnormalities include the following: stomatal pore permanently closed; cuticular ledges from the guard cells much thicker; stomata reduced to only one guard cell; guard cell unusually narrow; and adjacent stomata with common subsidiary cells (Wilkinson 1979). Oval-shaped stomata was observed in control (FT_1), more or less oval shaped in 0.4% COL, more or less rounded in 0.4% EMS and oval-shaped in in 0.4% MH. So in the leaf of treated plants, the stomatal structure changes from that of the control. The length of the aperture in ventral side of the leaf in Control set was 2.2±1.32 µm, 3.9±2.18 µm for 0.4% COL, 3.2±2.69 µm for 0.4% EMS and 4±3.25 µm for 0.4% MH, i.e., the aperture length was increased in treated leaf compared to the control, maximum increase was noticed in 0.4% MH (FT_9) and minimum increase in 0.4% EMS (FT_6). The dimension of the stomata in ventral side of the treated leaf increased when compared to the control. The guard cells in ventral side of the leaf in control showed thick waxy layers. On the other hand, less thick waxy layers in 0.4% COL, thin waxy layers in 0.4% EMS and thick waxy layers in 0.4% MH were observed. The length of the aperture in dorsal side of the leaf is 4±2.35 µm in Control, 2.6±2.86 µm for 0.4% COL, 3.6±3.12 µm for 0.4% EMS and 4.6±2.71 µm for 0.4% MH, i.e., the aperture length decreased in the treated leaf when

compared to the control except in FT_9 (0.4% MH), where the aperture length was increased. Maximum decreases of aperture length was found in FT_6 (0.4% EMS) and minimum decreases in FT_3 (0.4% COL). The dimension of the stomata in dorsal side of the treated leaf decreases when compared to the control except in MH treatment (FT_9), where the dimension of the stomata increased. In Control, the guard cells in dorsal side of the leaf showed the presence of thick waxy layers; whereas, thick waxy layers in FT_3, heavy waxy layers in FT_6 and thick waxy layers in FT_9 were observed. Scanning electron microscopy of stomata and their replicas showed that when there are protruding edges on the guard cells forming a vestibule over the stomatal pore, replicas may fail to show the pore dimensions.

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Table 1. Details of morphological characteristics of stomata in *Dianthus* species after SEM photograph analysis.

Treatments	Number of stomata (×800) and its shape	Structure of stomata (×3000)					
		Ventral side of the leaf			Dorsal side of the leaf		
		Mean length of aperture (µm)±S.E	Dimension of aperture	Guard cells	Mean length of aperture (µm)±S.E	Dimension of aperture	Guard cells
Control (FT_1)	4 and oval shaped in both ventral and dorsal side.	2.2±1.32 µm	_	Guard cells with thick waxy layers	4.0±2.35 µm	_	Guard cells with heavy waxy layers
0.4 % Col. (FT_3)	3 and more or less oval shaped stomata in both ventral and dorsal side.	3.9±2.18 µm	Increases from that of control	Guard cells with less thick waxy deposition	2.6±2.86 µm	decreases from that of control	Guard cells with heavy waxy layers
0.4 %EMS (FT_6)	3 and more or less rounded in both ventral and dorsal side.	3.2±2.69 µm	increases from that of control	Guard cells with thin waxy layers	3.6±3.12 µm	decreases from that of control	Guard cells with heavy waxy layers
0.4 % MH (FT_9)	3 and oval shaped in both ventral and dorsal side.	4.0±3.25 µm	increases from that of control	Guard cells with thick waxy layers	4.6±2.71 µm	increases from that of control	Guard cells with heavy waxy layers

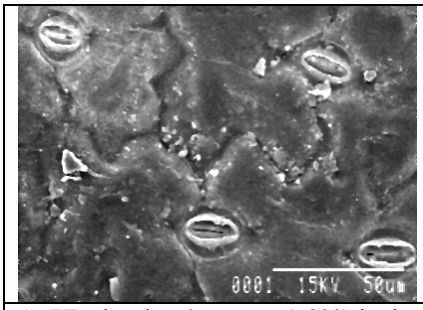
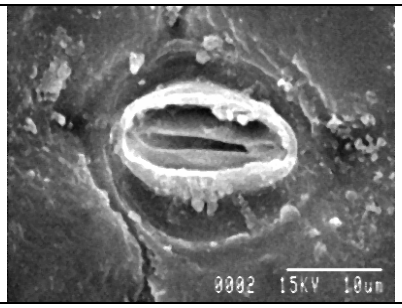
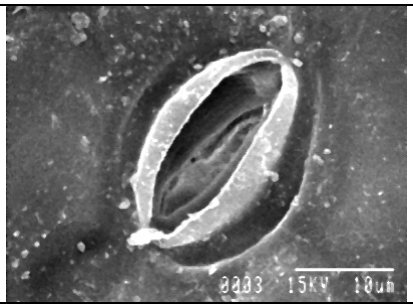
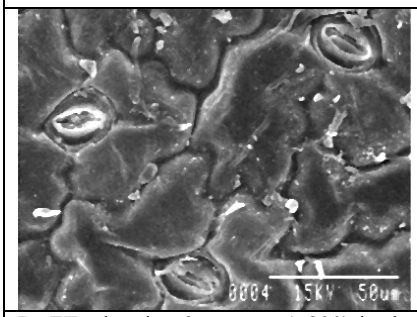
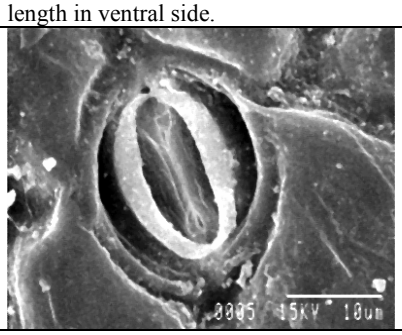
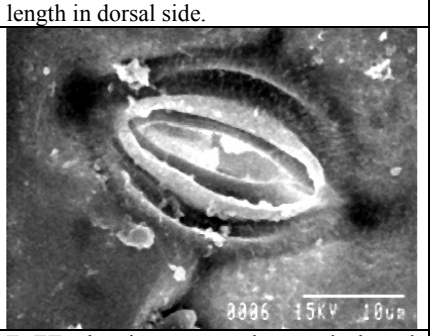
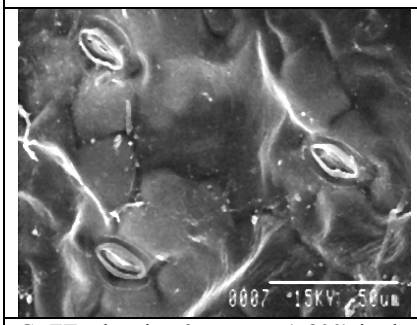
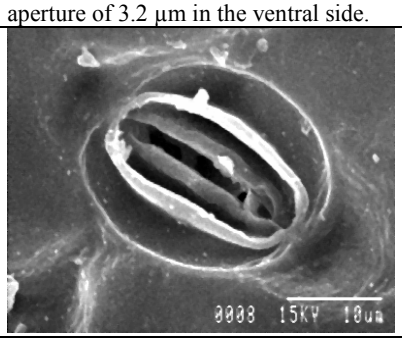
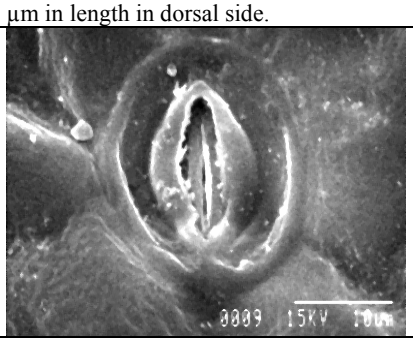
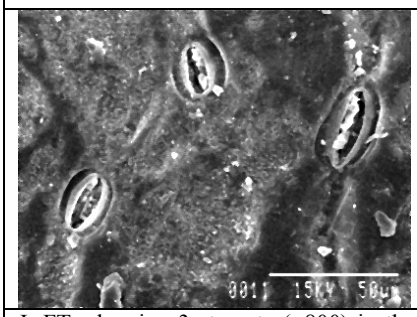
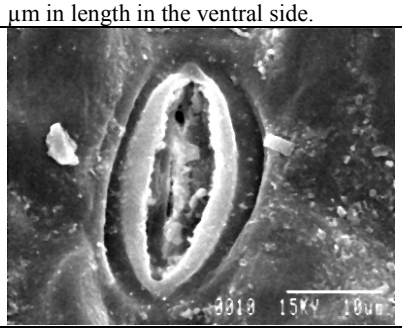
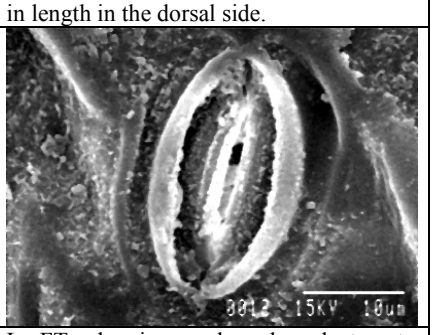
		
A: FT_1 showing 4 stomata (×800) in the ventral side of the leaf.	B: FT_1 showing oval- shaped stomata (×3000) with thin aperture of 2.2 µm in length in ventral side.	C: FT_1 showing oval – shaped stomata (×3000) with large aperture of 4 µm in length in dorsal side.
		
D: FT_3 showing 3 stomata (×800) in the ventral side of the leaf.	E: FT_3 showing more or less oval-shaped stomata (×3000) with large aperture of 3.2 µm in the ventral side.	F: FT_3 showing more or less oval–shaped stomata (×3000) with large aperture of 3.6 µm in length in dorsal side.
		
G: FT_6 showing 3 stomata (×800) in the ventral side of the leaf.	H: FT_6 showing more or less rounded stomata (×3000) with aperture of 3.9 µm in length in the ventral side.	I: FT_6 showing more or less rounded stomata (×3000) with aperture of 2.6 µm in length in the dorsal side.
		
J: FT_9 showing 3 stomata (×800) in the ventral side of the leaf.	K: FT_9 showing oval- shaped stomata (×3000) with aperture of 4 µm in length in the ventral side.	L: FT_9 showing oval – shaped stomata (×3000) with large aperture of 4.6 µm in length in the dorsal side.

Figure 1. Scanning electron micrographs showing morphological outlooks of leaf stomata (A-L) in control and different mutagenic concentrations in *Dianthus caryophyllus*