Morphological and anatomical characterization of colchicine induced polyploids in watermelon

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Abstract

This study aimed to elucidate the effective colchicine concentration induced polyploidization distinction based on morphological and anatomical features in watermelon. Watermelon seeds were soaked in different colchicine concentrations (0.05, 0.1, 0.5%) for several durations (24, 48, 72h) to induce polyploidization. Putative polyploids were evaluated regarding the morphological and anatomical traits compared to the diploid one (control). Colchicine @ 0.5% for 72h revealed the lowest germination percentage with high mortality in putative polyploids compared to diploids. Morphological traits revealed in vigorous growth in putative tetraploids with slower germination speed whereas the putative octoploid exerted suppressed growth compared to tetraploids and diploids. Besides, in respect of the reproductive biology, the petal number (6), pollen size and viability were remarkably higher in induced polyploids that confirmed the successful tetraploid induction by 0.5% colchicine treatment in seed for 72h. Similarly, the bigger stomatal size with lower density was also noticed in induced tetraploids compared to the diploid one using the same treatment after anatomical analyses. Meanwhile, PCA and correlation matrix illustrated that among the 20 variables polyploid induction efficiency% (PIE), leaf length (LL), guard cells distance (GCD), pollen viability% (PV) were recognized as the most effective morphological and anatomical indicators for successful polyploid induction confirmation with colchicine in watermelon. The present findings would be the basis for distinguishing colchicine induced polyploids as improved genetic resources to enhance the seedless triploid breeding in watermelon.

Introduction

Watermelon (*Citrullus lanatus*) is one of the most popular and economically viable cucurbitaceous in the tropical countries including Bangladesh. It is highly relished as a fresh fruit because of its thirst-quenching attribute in addition to many other identified characteristics like size, color, sweetness, nutritional values, etc. (Barai 2016). Global production of watermelon in 2016 was about 117 million ton into which China alone accounting for 68% of the total production while secondary producers with more than 1% of world production included Turkey, Iran, Brazil, Uzbekistan, Algeria, the United States, Russia, Egypt, Mexico and Kazakhstan (FAOSTAT 2018). However, the total watermelon production in Bangladesh was 16 lakh tonnes on 38,824 hectares of land in 2019–2020.

Watermelon is a demanding fruit but one of the main limiting traits is the presence of enormous seed. Usually, consumer prefers to have less seeded or seedless watermelon because of unpalatable taste of hard seeds. About 300–500 seeds per watermelon fruit are present depending on the size of fruits (Grant 2020). So, seed lessness is the most desirable trait in watermelon and seedless cultivars with high fruit quality are presently available to the consumers of the developed countries commanding a high price (Compton et al. 1993). Polyploid induction can potentially produce less seeded watermelon. One of the most successful methods for polyploidy induction is through seed treatment with colchicine that artificially applied for a range of plant species (Abdoli et al. 2013). Simple methods for early identification of ploidy levels have important application value in breeding program, especially when many plants are to be treated. For confirmation of polyploidy, different morphological and physiological traits, particularly pollen diameter, number of chloroplasts, stomatal size and stomatal density can be studied as indirect method and chromosome counting as direct method. However, flow cytometry (FCM) is considered as a more reliable, rapid and simple direct method to analyze a large number of samples in a very short time period (Sattler et al. 2016). But it is expensive and particularly where flow cytometry facility is not available in that case morphological, physiological, and cytological characteristics would be assayed as good indicators for the confirmation of the conversion of diploidy to tetraploidy (Sabzehzari et al. 2019).

According to the advantages of polyploid, the current study is designed to obtain colchicine induced tetraploid as a logical first step for expansion of genetic resources in further triploid breeding program of watermelon (Citrullus lanatus). In addition, morphological, physiological and cytological alterations that arise from polyploidization have been documented in many crops. The triploid watermelon was first reported in 1947 by Kihara and Nishiyama in Japan

(Kihara and Nishiyama 1947). Since then, improvement efforts have been continued. But unfortunately, limited efforts have been made to produce seedless watermelon in Bangladesh despite having favorable climatic conditions for the cultivation of watermelon, along with an increasing demand in national and international markets. Therefore, the present study will verify the hypothesis that colchicine concentration might be effective to induce polyploidization with remarkable changes in morphological and anatomical features enable to distinguish polyploids than diploids (Fig. 1). The current study has been focused on to ascertain the most effective colchicine concentration and exposure duration for polyploid induction. Furthermore, also illustrate the distinguishable morphological and anatomical traits associated with the polyploid induction confirmation.

Materials And Methods

Experimental site

The experiment was conducted at the tissue culture laboratory, experimental field, advanced genetic laboratory of Department of horticulture and Department of Genetics and Plant Breeding in Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur-1706, Bangladesh during November 2020 to July 2021. This area belongs to the AEZ 28 of Bangladesh called Madhupur tract (24°09′ N latitude and 90°26′ E longitude) having mean temperature varies from 28-32º C in summer season; but winter season shows falling below 20º C and annual rainfall lies between 1,000-1500 m. The soil was clay loam in texture and acidic in nature with a pH of around 5.8 (FAO 1988; Haider et al. 1991).

Plant material and polyploidy induction

Seeds of diploid watermelon (Citrullus lanatus) Thailand-2 variety with chromosome number of 2n=22 were used for the polyploidy induction. Fresh, healthy and mature seeds of watermelon were soaked in aqueous colchicine at the concentrations of 0(C1), 0.05(C2), 0.1(C3) or 0.5% (C4) (w/v) for 24(D1), 48(D2) and 72 h (D3) and kept in dark condition. Stock colchicine solutions was prepared by dissolving various colchicine concentrations in sterilized distilled water and 2% (v/v) dimethyl sulfoxide (DMSO), which increases cell permeability and colchicine absorption (Glowacka et al. 2009). The colchicine concentrations and soaking durations was selected on the basis of the findings outlined by Hassan et al. (2020) in pointed gourd. Experiment was replicated three times with 90 seeds per treatment. The similar amount of seeds was also soaked in tapwater (without colchicine) for 24, 48 and 72 h as control. Following the colchicine treatment, the seeds were thoroughly rinsed for three times with sterile distilled water and air dried in room temperature. The seeds were planted for germination in polybag using three seeds each with garden soil and compost mix (2:1). The polybags were cultured under a shade condition in the nursery and regular observation was carried out to assess the seed germination and seedlings mortality rate for each treatment. In three to four true leaves stage, seedlings were transferred to the small plastic bag and kept in the nursery conditions for further growth and development.

Early screening and transplanting

Early screening was done using the survived germinated seedlings of 45 days old that were compared with the controls (diploid) in order to decline the workload of further studies and also helped in rudimentary isolation of putative polyploids. For achieving this goal, polyploid plants were marked according to the visual differences of the atypical morphology which was evident in their cotyledons shape and color, hypocotyl thickness in contrast with the diploid plants. After marking deformed seedlings as putative polyploids seedlings were transplanted at the experimental bed in the main research field. The field layout was maintained as the two-factor randomized complete block design (RCBD) having 3 replications consisting of 12 treatments with 4 plants each treatment. Fertilization, pesticide and fungicide spray was done at definite time of intervals with optimum doses. Also weeding, irrigation, trellising, pheromone trap setting etc. was done at appropriate time.

Morphological characterization

Vegetative attributes

Every germinated and mortal seedling were recorded from the beginning of seed sowing up to 40 days after sowing (DAS) for getting the final germination and mortality percentage. The equation given by Ajmal et al. (1998) was applied to get final germination percentage,

Germination percentage= Ni/N×100 ……(i)

where, Ni designates as the germinated seeds and N is the number of seeds used. Similarly, mortality percentage was calculated from the following equation,

Mortality (%) = (Total mortal seedling upto a certain duration)/(Total germinated seedlings upto certain duration)×100……(ii)

In addition, Speed of germination was found by using another formula used by Bradbeer (1988),

Germination speed (GS) = N1/T1+(N1+N2)/T2+⋯+(N1+N2+..+Tk)/Tk ……(iii)

Where, N1, N2, N3, ⋯⋯Nk: Number of germinated seeds observed at time (days) T1, T2, T3, ⋯, Tk: after sowing (Not accumulated/cumulative number, but the number of seeds that germinated at the specific time) and k: is the total number of time intervals.

After transplanting of seedlings, the leaf blade (third leaf from the above) was collected for identifying the external differences on the basis of their degree of lobing, apex shape and colour according to the guideline of UPOV (2010) and Sashital (2018). At 45 days after transplanting (DAT) the average leaf blade length, width, petiole length and stem diameter were measured with the help of slide caliper. Besides, average plant height also recorded at 45 DAT from the base top portion of the plant with measuring tape.

Reproductive characteristics

Days required for first flowering in both male and female flower was recorded at proper time. Likewise, node number of first male and female flower initiation was measured by the counting. Petal numbers of male flower was recorded by counting. Petal length was measured from the base of the petal to the apex and breadth was calculated at the maximum point of petal with the help of slide calipers. Furthermore, the pollen was collected from the male flower for the in-vitro viability test. The pollen collection was done at early in the morning as with the increasing temperature most of the pollens lose their viability. Afterwards, 1-2 drops of 1% acetocarmine solution added into the previously shredded pollen on the slide observed in a digital microscope (Olympus CX43 equipped with digital camera) at 40x magnification. Pollen grain with the dark red colour was considered as the fertile pollen and the pollen with lighter colour in appearance or hyaline was recorded as the sterile pollen. The snaps of the pollens were taken and size (micrometer) of the specific pollen was calibrated by using Toupview 3.7 software. The sample pictures were calibrated in micrometer by the help of ImageJ (64bit, Java 1.8.0_172). Later, the pollen diameter was recorded with that software in micrometer.

Anatomical attributes evaluation and putative polyploids estimation

Matured leaves were collected at midday for anatomical observation as it neutralizes the effect of natural opening of stomata (Sabzehzari et al. 2019). The portion of the leaf petiole was soaked in water for water absorption for the clear observation of stomata. The upper epidermal layer of expanded leaves was collected and then separated epidermis was observed on the digital microscope (Olympus CX43 equipped with digital camera) and 40x magnification was

maintained for every observation and 3 different plots were taken in each observation for stomatal length, diameter, area, distance between two guard cells, chloroplast counting, stomatal density measurement. For collecting these data multiple snaps were taken for each sample as previously done for the pollen diameter observation and measured by the ImageJ software with calibrated snaps. Furthermore, the chloroplasts were counted from the snap and stomatal area and density were measured according to the Paul et al. (2017); the stomatal area calculated through the equation of πr^2 where r designates the radius value of the stomata. These anatomical features revealed to make the decision on polyploid induction confirmation.

Polyploid induction efficiency (PIE) %

The lower density of stomata per leaf area and higher chloroplasts in stomata indicated the ploidy induction through colchicine. The PIE% was calculated by the equation followed by Hassan et al. (2020).

PIE (%)=(Number of induced polyploids)/(seedling survival rate (%))×100……(iv)

Statistical analyses

The statistical analysis like two-way ANOVA (Analysis of variance) and mean comparison using Honestly Significance Difference (HSD) test at 5% probability level was carried out by using R (version 4.1.2) program. In addition, Correlation matrix, Cluster analysis, Principal Component Analysis (PCA) and biplot analysis were also performed by using GGally, agricolae, Factoextra, Corrplot packages of R program for further clarification of polyploid induction by colchicine treatment. Visualization of experiment output was illustrated by using Microsoft Excel 2019 version and R program.

Results

Vegetative morphology

Influence of colchicine concentrations with exposure duration showed diversified responses in various vegetative parameters. Cotyledon characteristics like shape and intensity of green colour is presented in Fig. 2. Early screening according to these attributes found that diploids (control treatment) exhibited with narrow elliptic shape and light green colour whereas the putative polyploids composed with broad elliptic and darker green appearance. Then the hypocotyl thickness was much prominent for the higher concentration (0.5%) with its three exposure durations (Fig. 3). This organ thickness was helpful to make decision about the early screening for polyploid induction.

All the quantitative characters were observed at the vegetative stage of watermelon is disclosed in Table 1. At the beginning, germination response showed the prominent colchicine inhibitory effect. It was noted that for final germination (40 DAS), the highest germination (100%) was found in control at 72h duration and the lowest in the combination of 0.5% colchicine at 48h of time exposure (77.8%). On the other hand, the number of mortal seedlings were abundant in the field. Results revealed that the highest colchicine concentration (0.5%) with the 72h of time exposure expressed the highest influence on mortality percentage (42.2%) and the lowest mortality was registered at the control with the 48h of duration (6.3%). The similar trend of findings were also noticed regarding the speed of germination where higher concentration of colchicine had the less speed of germination. More specifically, it was revealed that the combination of 0.5% colchicine and 24 h of time exposure exerted the lowest speed (466.49). Meanwhile, the highest speed was found at control combined with the 48 h of exposure duration (563.10). The germination speed was found at the range of 547.96 to 5470.24, for the rest of the other treatment combination (Table 1).

In case of leaf blade, these were with some diversified responses in concern of their phenotypic appearance and quantitative attributes (Fig. 4 and Table 1). The leaf green colour intensity was found dark in nature for diploids (control treatments) and tetraploids (control 0.05, 0.1, 05% with three durations) but some of the putative octoploid plants

belongs to the 0.5% colchicine with three durations was exhibited as lighter in colour. Interestingly, the degree of lobing like medium, weak and strong was varied according the ploidy level variation of diploid, tetraploid and octoploid (Fig. 4).

Considering the phenotypic appearance of leaf structure in the cultivated duration 0.5% colchicine exhibited the smallest third leaves production whereas the control treatment always appeared with the double sized third leaves. Consequently, the highest leaf length was found at the response of control with 48h after 45 days of transplanting (10.5cm). Statistically the highest leaf width (12.7cm) was found in 0.1% colchicine concentration and 24 h of duration. Also, at C2×D2, C3×D2 in leaf width were found higher viz. 11.9, 11.1 cm respectively. On the contrary, the higher colchicine concentration (0.5%) with three duration were appeared as the representative of lower leaf width viz. 5.3, 6.3, 6.0cm for the C4×D1, C4×D2 and C4×D3 interactions.

Other organs like leaf petiole, stem was also showed some differences. Petiole length fluctuates in between 3.6-6.2cm where the lowest petiole length (3.6 cm) represented by the effect of 0.5% colchicine at 24 h duration and the highest petiole length was found in the interaction of 0.1% colchicine at 24 h of duration. Then, most of the stem diameter showing with the range of 8.3-7.6mm where the highest interaction effect (8.3mm) was observed with the 0.1% colchicine at 24 h of duration. Whereas the lowest effect (4.6mm) was found in the 0.5% at 48h of duration. In fact, the higher concentration with three different durations ranging the stem diameter value lower in tendency (4.6-5.4mm) compared to the others. Besides, the control associated interactions were assessed as massive in growth. Although at 45 DAT, 0.05% and 0.1% colchicine concentration in combination with 24h exposure duration manifested plant height of 73.5 cm of each. While the tallest plant (74.3cm) was registered in 0.1% colchicine when combined with 48h soaking durations.

Reproductive morphology

Male flower exhibited differences after colchicine treatments. Therefore, differences were noticed for the petal number of male flowers. Generally, the petal number of diploid male flowers is 5 (Zygomorphic) in watermelon plant. But in the present study some flowers were found with 6 petals treated with 0.5% colchicine (Fig. 5).

Besides, quantitative characteristics varies with the different level of colchicine concentration at their exposure durations (Table 2). So, from the Table 2 it was distinguishable that 17.0mm petal length with the 0.1% colchicine at 24 h of duration and however 0.5% colchicine at 24 h & 48 h did not produce any flowers during the whole season of flowering. Moreover, at 0.5% of colchicine with 72 h duration also had the lowest (8.5mm) interaction effect among rest of the interaction effects. The interaction between the control with the three durations shows the 6.4-6.7mm of petal breadth that is near to the lowest (5.2mm) of 0.5% concentration with the 72h duration treatment. Rest of the treatments ranging the average petal breadth in between 9.0-10.5mm where 10.5mm for the 0.05% colchicine with 24h of duration. The highest value of average node number for male flower initiation is near 5, that is 4.8 for the control at 24h, 0.1% at 48h; on the contrary the lowest (3.0) was found with treatment of 0.5% colchicine at 72h. Early days of flowering (lowest days required) was noticed in the plants treated with 0.1% at 72h of duration (20.7) and 0.5% colchicine at 72h of duration (21.8). On the other side, rest of the treatments required 42.7 to 46.0 days to initiate first male flower at 0.5% colchicine with 72h.

Pollen features like viability, diameter gave the precise outcomes to distinguish the diploid to polyploids (Fig. 6). From the Fig. 6, it has been illustrated the pollen appearances where smaller and larger pollens was considered as the diploids and tetraploids respectively; whereas mixture of different size of pollens was considered as the mixoploid. Subsequently, significant differences were observed for pollen diameter where the highest (5.6 µm) pollen diameter was measured at 0.5% colchicine concentration with 72h of duration and rest of the interactions were found to had statistically similar pollen diameter as that of control associated treatments ranged from 4-5µm. Furthermore, the lowest viability (57.0%)

was observed for the control at 48h of duration. However, the control treatment with 72h and 0.1% concentration with 48h treatment also showed higher percentage of viability viz. 95.2% and 94.4%, respectively.

Watermelon plant always mature with the tendency of less female flowers. In this study, no female flower was observed in various treatments like control with 24 h, 0.1% colchicine with 72 h duration and 0.5% colchicine with all the durations. Besides, the other treatments also contain less flowers that's why the effects of colchicine and duration was found the less effective individually derived from data analysis. Less flower production also appeared in the field throughout the season; some treatments showed no flower production in both male and female. Therefore, four treatments effect showed higher in their average node number for the first female flowering viz. 8.3 for 0.05% at 72h duration, 5.5 for 0.05% at 48h of duration and control with 72h duration, 5.2 for the 0.1% with 24h. Rest of the interactions showed average node number of ≤ 3.0. The maximum days (61.0) requirement was found at 0.05% of colchicine with 72h of duration; whereas lesser duration for the first female flowering was found at the 0.1% with the 24h, control with 72h and 0.05% colchicine with 48h of exposure duration viz. 39.2, 38.7, 35.3 respectively. The other treatments showed the days requirement ranged between 17.5-20.3.

Anatomical attributes to identify polyploids

The diploid plants have 8-9 chloroplasts were found within the stomatal guard cells of leaf blade that is observed in control treated plant. However, putative mixoploid contained 10-12 chloroplasts within the stomata and sometimes fused together within the guard cell and were not clearly visible. Interestingly, putative polyploids (tetraploid, octoploid) generally had 14-18 chloroplasts. (Fig. 7) Usually, the 0.1% and 0.5% colchicine with their three exposure durations possessed the 14-18 chloroplasts.

Quantitative anatomical characterization related to stomatal attributes are visualized in Fig. 8. These attributes were found to be beneficial for PIE% confirmation. At first, for stomatal length the lowest (15.9 µm) response was found in 0.05% colchicine at 24h (C2×D1) and the highest for 0.5% colchicine with 48h duration (C4×D2). Most of the interaction response were above 20 µm (C1×D2, C2×D3, C3×D1, C3×D2, C3×D3, C4×D1, C1×D3). Other treatments effect was below or near 20 µm (C1×D1, C1×D3, C2×D2). Then the highest concentration 0.5% with three durations (C4×D1, C4×D2, C4×D3) were found the average diameter $\geq$13.0 µm. Rest of the interactions effect were found between 10.6-12.8 µm where the lowest 10.6 µm diameter belonged to the control with 72h (C1×D3) of duration. The 0.5% colchicine concentration showed higher in average area response viz. for 48 hour 171.2 µm2, 72h 152.8 µm2, 24 h 136.4 µm2. Rest of the area maintained the value below 130 µm2. Also, the lowest one was found in control at 72h (C1×D3) below 100 µm2 viz. 88.6 µm2. So, with higher colchicine concentrations the stomata appeared as larger in size. In case of distance between two guard cells there are some distinguishable responses were found viz. highest (4.0 µm) response for the 0.5% colchicine at 72h of duration (C4×D3); whereas the lowest (1.8 µm) was found in both control at 24h duration and 0.05% colchicine at 24h duration (C1×D1, C2×D1). Most of the response were found within the range of 1.9-2.8 µm. It also indicates the stomatal enlargement by colchicine mutagen.

Finally, PIE% was assured on the basis of all the morphological and anatomical parameters (Fig. 9). From the findings it has been revealed that 0.5% colchicine with three durations showed the higher induction efficiency viz. C4×D3, C4×D2, C4×D1 followed by 14.5, 11.3, 9.7% efficiency. The colchicine treatment at 0.1% with its three durations maintaining the order of PIE% as C3×D3>C3×D1>C3×D2 were 9.4>7.8>5.2%. Besides, the other treatment combinations like C2×D3, C2×D2 and C2×D1 showed the lowest in PIE% viz. 4.0, 1.8, 1.5% respectively. So, the putative polyploids were more detected in the higher colchicine concentration (0.5%) with the highest duration (72h).

Multivariate analyses

Pearson's correlation analysis and dendrogram cluster

Polyploid induction demands the changes in various parameters with evident interrelations. Pearson's correlation matrix showed the extent of both positive and negative correlation among the 20 studied variables. The Figure 9A are distributed from negative to positive values indicated by the red to blue-colored circles. Vacant cells were considered as nonsignificant relationship among the variables at 5% level of significance. From this analysis it has been revealed that most of the studied variables related to vegetative and reproductive morphology positively correlated to each other. But stomatal attributes like SPM, SD negatively correlated to SL. Also, reproductive variables FFM, NNM, PL and PW had the negative correlations. Besides, PIE% had the negative correlation with FFM, LL, PH, PLC, SD (at $p < 0.05$). That means, if one variable response increases, then the other one will be increased too for positive correlation and vice versa for negative correlation. Big sized circle denotes the stronger correlation between the two respective variables.

Pheatmap with dendrogram cluster prepared using the 20 studied dependent variables depicted 2 major clusters. Cluster 1 showed the characters like SL, PIE, GCD and MF closely related to each other. Less difference was discoverable among them in the concern of their corralation showing tendency with other parameters. For cluster 2 SPM, NNF, FFF, GF, GS, LL, PH, PLC, LW, PV, PD, PL, PW and SD, node number of first male flower initiation (NNM) and duration of first male flowering (FFM) characters were included. Although, the SPM and GS was found in a subcluster which was seperated from the other characters and rest of the characters also form several small clusters. These multiple clusters in single major cluster indicates the variation among the characters even they were similarly correlated to each other at some extent

Principal component analysis (PCA)

The data presented in Table 3 revealed that first two principal components (PC) contributed enough to explain maximum (about 59%) of the pattern variations. Individually, only PC1 and PC2 can explained 46.68% and 12.09% of the total variations. Therefore, from the PCA (Table 4) it is evident that FFM, LL, PH, LW, PLC, SD, NNM, PL, PW have the highest positive loadings on PC1. On the other hand, for PC2 only LL comprises the highest positive loadings. Negative loadings suggested that, SL have the highest value for PC1 and MF, PIE, GCD consists most of the negative loadings. Hence, for biplot-PCA cluster analysis using colchicine concentrations illuminated that 0.5% (C4) concentration form the distinct cluster by including those variables which had the negative loading values (Fig. 11A). Also 0.1% (C3) can influence these parameters to some extent with having rest of the parameters. So, these dependent variables definitely get the dependency by the 0.5% and 0.1% concentration. Rest of the two concentrations not influence negatively loaded parameters including PIE. Therefore, it is clearly evident that among the three studied concentrations specifically 0.5% concentration have the remarkable influence in PIE. Similarly, for the duration effect 72h (D3) have the distinct cluster than the other having most of the parameters and most importantly PIE or other variables shown negative loadings (Fig. 11B).

Discussion

The experiment was initiated with the consideration of two factors viz. colchicine concentration and duration as these are the most key factor for polyploid induction through seed treatment by colchicine (Moore and Janick 1983; Hassan et al. 2020). Colchicine is effective only in the cell division stage; that's why both colchicine concentration and duration of seed soaking period can visualize the actual impact in polyploid induction (Emsweller 1961). For the confirmation of the putative polyploids the morphological traits were recorded initially; these parameters are the good sign and primary selection criteria for the ploidy level confirmation though sometimes it is not completely reliable (Compton et al. 1996; Norman et al. 1995). So, stomatal attributes are one of the important tools that were considered for identifying the polyploids in more precisely (Sabzehzari et al. 2019).

At germination stage, there were higher germination percentage and lower mortality percentage in control treatment than the treatments related to colchicine along with its various soaking duration were noticed in the present study. It is happened due to the inhibitory effect of colchicine on living parts. Consequently, seedlings not survived enough (Zlesak et al. 2005). Likewise, definite variations were also found in morphological characteristics and reproductive biology (Fig. 5–6, Table 2). Usually, tetraploids tend to have bigger leaves size, larger plants, flowers, pollen compare to diploids. But in this experiment the leaves size, plant height, other plant organs remain stunted after three months of germination. This is due to colchicine doses that influence the mitotic cell division to slow down (Stebbins 1971). Similar kind of stunted growth were also reported in Trichosantes dioica Roxb. till four months after germination (Hassan et al., 2020). However, the hypocotyl thickness was found thicker than the controlled treated seedlings. These findings was concurrent with the evident of Hassan et al. (2020) where they explained this organ thickness might occur because of larger number of chromosomes maintain balance of cytoplasm to nuclear volume and more protein expression that are the results of higher expression of genes as they available with higher allelic number (Hassan et al. 2020).

In reproductive biology the petal numbers were remarkably higher in 0.5% colchicine treated plants then that of the untreated one. But this observation was disputed with Ning et al. (2009) where they noticed that there was no significant change in petal number in case of tetraploid compared with diploid Petunia hybrida (Ning et al. 2009). Although in current study, the petal length and breadth of male flowers were small in the colchicine treated plants; but some previous experiments reported that flowering parts were always larger in the tetraploids of Basil, Myrtle (Omidbaigi et al. 2010; Zhang et al. 2009). On the other hand, the pollen shows higher in diameter of the plants treated with colchicine than the control treatments, those were substantiated with the other, findings reported in Plantago psyllium, Allium sativum, Arachis paraguariensis, Ziziphus jujube (Calvalho et al. 2005; Cheng et al. 2012; Aina et al. 2012, Cui et al. 2017). Flowering initiation occurred in both male and female earlier in higher colchicine concentration while significant variations were recorded among the node numbers of first flower position. Although flowering time were not significant enough in colchicine induced Jatropha curcas (Niu et al. 2016). Thus, chromosome doubling might cause these kinds of variations in external morphology in several species (Zhang et al. 2019). Where control, 0.05, 0.1% colchicine treated plants were capable to produce flower (male and female). This may have been due to the less photosynthetic product production (Rekika et al. 1998).

Stomatal length, diameter and distance between two guard cells of higher colchicine treated plants were larger enough to distinguish from the control or the diploid category plants. As a result, with the density become lower the enlarged stomata at colchicine treated plants compared to the stomata in the plants treated without colchicine. These findings supported by the results reported in Plantago psyllium (Sabzenhzari et al. 2019; Niu et al. 2016) and similar features are also relevant within present finding. Chloroplast number increased in the putative polyploids than the diploids. As chlorophyll content may increase by the increasing of chloroplast numbers in stomatal guard cells; thus, most of the leaves which become polyploids show dark coloured leaves (Abdoli et al. 2013). The putative polyploids are more detected in the higher colchicine concentration (0.5%) with the highest duration (72h). This data agrees with the previous reports of Trichosantes dioica Roxb. (Hassan et al. 2020). With the separation of duration effect on 24h induction efficiency is more than that of the 48h. This result is also similar to the previous studies of Platanus acerifolia, Ocimum basilicum (Liu et al. 2007; Omidbaigi et al. 2010).

The present findings suggested that substantial morphological and anatomical changes had been occurred among the treatments and significant correlations were also found among the most important dependent variables. It can be summarized in such a way that plants with suppressed growth characters at initial stage with definite anatomical and morphological attributes were noticed due to the effect of colchicine treatment on spindle fiber formation in the cell cycle at varied soaking durations (Hassan et al. 2020). Distinguishable reproductive traits both in male and female flowers were seen, even no female flower was counted at plants treated with higher colchicine concentration (0.5%) for soaking in long duration (72h). Diploid and putative polyploids were characterized morphologically; however, the final decision

was made on the putative polyploids induction efficiency (PIE) based on the stomata structure analyses. The stomata with higher guard cells distance with less density was evident in putative polyploids than the diploid variant. Likewise, the pollen diameter was noticeably increased in tetraploids than that of the diploid. Therefore, these morphological and anatomical traits would be an alternative tool of expensive flow cytometry (FCM) for the validation of polyploid induction through colchicine in watermelon.

Conclusions

Putative polyploid induction was found the highest for 0.5% colchicine concentration at 72h soaking duration. Significant morphological differences were expressed between diploid and putative polyploids regarding thick hypocotyl, stunted growth with thin stem, less leaf area. In reproductive biology male flowers having 6 petals at 0.5% colchicine with higher pollen diameter and viability compared to diploid one, that confirm the polyploid induction. Larger stomatal length, diameter, area and distance between two guard cells were correlated with each other that profoundly evident in putative polyploids. Further research should be carried with various colchicine concentrations for successful polyploid induction and cytology analysis for validation. Colchicine induced polyploid resources would be used as improved genetic resources for triploid evolution with seedless fruit in watermelon.

Declarations

Acknowledgement

The authors are highly indebting to the Research Management Wing (RMW), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh for the technical and logistic supports to conduct this study.

Ethical statement

Watermelon (*Citrullus lanatus*) was used as plant material in the present study. It has been declared that the watermelon seeds were collected from the department of Horticulture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh. The watermelon seeds are producing and preserving in the Horticulture department for using in the research purposes. The study was conducted in the research field of the same department with following standard procedures of cultivation and without violating the relevant guidelines and regulations of the plant handling process.

Data availability statement

The tables (1-2) and figures (6-11) were generated using the recorded raw data of the present study. All the used data are publicly available in the following link:

https://docs.google.com/spreadsheets/d/1_pRouoO1VU_xv7Z8MfIzru0_lkAbUu8e/edit?usp=sharing&ouid=113582438860975202973&rtpof=true&sd=true

However, the tables 3-4 were prepared based on the output of the PCA analyses that was relevant with the raw data as well. In addition, the figures 1-5 were prepared as per the hypothetical and visual appearance of the results.

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Tables

Table 1. Quantitative parameters at vegetative stage

Colchicine Treatments		Final Germination (%)	Final Mortality (%)	Germination speed	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Stem diameter (cm)	Plant height (cm)
Conc. (%)	Duration (h)								
C1(0.0)	D1(24)	88.9 ± 8.4ab[x]	23.4 ± 18.9ab	494.9 ± 4.3a	8.0 ± 0.2ab	10.3 ± 0.5ab	5.7 ± 0.4abc	7.2 ± 0.2abc	60.3 ± 10.0ab
	D2(48)	88.9 ± 1.9ab	6.3 ± 4.2b	563.1 ± 17.3a	10.5 ± 0.9a	9.8 ± 0.6abc	5.3 ± 0.1abc	6.5 ± 0.1bcd	70.2 ± 11.0a
	D3(72)	100.0 ± 0.0a	17.8 ± 12.6ab	548.0 ± 67.1a	9.1 ± 0.8a	9.5 ± 1.4bc	5.2 ± 0.0abc	7.4 ± 0.3abc	59.6 ± 15.0ab
C2(0.05)	D1(24)	92.2 ± 6.9ab	26.1 ± 9.4ab	520.0 ± 33.0a	8.6 ± 1.5a	7.3 ± 0.3cde	4.9 ± 0.4bcd	7.6 ± 1.0ab	73.5 ± 5.7a
	D2(48)	86.7 ± 11.5ab	10.2 ± 0.9b	473.3 ± 95.0a	8.1 ± 0.3ab	9.2 ± 1.6bcd	5.3 ± 0.6abc	6.0 ± 0.3cd	56.3 ± 17.3ab
	D3(72)	96.7 ± 5.8ab	28.5 ± 12.9ab	470.2 ± 81.3a	7.9 ± 1.0abc	11.9 ± 1.2ab	6.1 ± 0.5ab	7.8 ± 0.2ab	64.9 ± 9.0ab
C3(0.1)	D1(24)	98.9 ± 1.9a	22.5 ± 6.7ab	466.5 ± 71.5a	9.3 ± 0.7a	12.7 ± 1.1a	6.2 ± 0.2a	8.3 ± 0.4a	73.5 ± 21.4a
	D2(48)	85.6 ± 10.2ab	12.5 ± 7.1b	491.2 ± 92.1a	8.0 ± 1.3ab	11.1 ± 1.1ab	5.4 ± 0.3abc	7.3 ± 0.3abc	74.3 ± 14.4a
	D3(72)	94.4 ± 5.1ab	22.3 ± 3.4ab	515.4 ± 161.6a	5.9 ± 0.9bcd	9.8 ± 0.5abc	4.7 ± 0.4cde	7.2 ± 0.6abc	35.8 ± 3.2bc
C4(0.5)	D1(24)	82.2 ± 7.7ab	23.2 ± 4.0ab	481.2 ± 75.6a	5.3 ± 0.9cd	5.3 ± 0.6e	3.6 ± 0.2e	5.4 ± 0.4de	7.7 ± 0.8c
	D2(48)	77.8 ± 3.9b	28.3 ± 8.7ab	479.0 ± 110.2a	5.6 ± 1.0bcd	6.3 ± 1.4de	4.6 ± 0.4cde	4.6 ± 0.2e	9.7 ± 3.5c
	D3(72)	86.7 ± 3.3ab	42.2 ± 5.4a	539.6 ± 86.4a	4.5 ± 0.6d	6.0 ± 0.6e	4.0 ± 0.8de	5.2 ± 0.8de	8.5 ± 2.7c

[x] The values are the means ± standard error; the column means under each parameter with the same letter (s) are not significantly different at $P < 0.05$, as determined by Honestly significant difference test using the R software.

Table 2. Quantitative parameters at reproductive stage

Colchicine treatments		Male flower						Female flower	
Conc. (%)	Duration (h)	NNM[x]	FFM	PL (cm)	PW (cm)	PD (μm)	PV (%)	FFF	NNF
C1(0.0)	D1(24)	4.8 ± 0.8a[y]	43.7 ± 0.6ab	10.1 ± 0.6e	6.7 ± 0.1b	4.1 ± 0.3b	60.0 ± 20.3ab	0.0 ± 0.0a	0.0 ± 0.0a
	D2(48)	4.7 ± 1.3a	42.8 ± 1.2abc	9.9 ± 0.3e	6.6 ± 0.5b	4.2 ± 0.1b	57.0 ± 25.0ab	17.5 ± 30.3a	2.5 ± 4.3a
	D3(72)	3.5 ± 0.5ab	46.0 ± 2.6a	9.8 ± 0.3e	6.4 ± 0.5b	4.4 ± 0.3b	95.2 ± 8.2a	38.7 ± 33.6a	5.5 ± 4.8a
C2(0.05)	D1(24)	4.2 ± 0.8a	43.7 ± 1.1ab	13.8 ± 0.2cd	10.5 ± 1.4a	4.5 ± 0.2b	76.9 ± 14.8a	18.7 ± 32.3a	3.0 ± 5.2a
	D2(48)	4.3 ± 0.9a	44.8 ± 3.8a	12.9 ± 0.1d	9.0 ± 1.2a	4.4 ± 0.4b	77.2 ± 32.5a	35.3 ± 30.6a	5.5 ± 4.8a
	D3(72)	4.1 ± 1.1a	44.2 ± 1.6ab	13.1 ± 0.4cd	10.3 ± 0.4a	4.1 ± 0.3b	58.8 ± 50.9ab	61.0 ± 0.0a	8.3 ± 0.6a
C3(0.1)	D1(24)	4.2 ± 0.3a	42.7 ± 0.6abc	17.0 ± 0.1a	9.4 ± 0.6a	4.3 ± 0.0b	87.1 ± 11.3a	39.2 ± 34.0a	5.2 ± 4.5a
	D2(48)	4.8 ± 0.2a	42.9 ± 0.7abc	15.8 ± 0.2b	9.5 ± 0.3a	4.8 ± 0.6ab	94.4 ± 9.6a	20.3 ± 35.2a	3.0 ± 5.2a
	D3(72)	3.3 ± 2.9ab	20.7 ± 18.1cd	14.1 ± 0.7c	9.8 ± 1.2a	4.6 ± 0.2b	73.1 ± 23.8a	0.0 ± 0.0a	0.0 ± 0.0a
C4(0.5)	D1(24)	0.0 ± 0.0b	0.0 ± 0.0d	0.0 ± 0.0g	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0b	0.0 ± 0.0a	0.0 ± 0.0a
	D2(48)	0.0 ± 0.0b	0.0 ± 0.0d	0.0 ± 0.0g	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0b	0.0 ± 0.0a	0.0 ± 0.0a
	D3(72)	3.0 ± 2.6ab	21.8 ± 18.9bcd	8.5 ± 0.3f	5.2 ± 0.2b	5.6 ± 0.1a	100.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a

[y]The values are the means ± standard error; the column means under each parameter with the same letter (s) are not significantly different at $P < 0.05$, as determined by Honestly significant difference test using the R software.

[x]NNM=node number of first male flowering, FFM= days required for first male flowering, PL=Petal length (cm), PW=Petal width (cm), PD=pollen diameter (μm), PV=pollen viability (%), FFF= days required for first female flowering, NNF= node number of first female flowering.

Table 3. Total variance explained (two components selected) for dependent variables

Component	Standard deviation	Proportion variance	Cumulative proportion
PC1	3.06	0.47	0.47
PC2	1.56	0.12	0.59
PC3	1.38	0.09	0.68
PC4	1.08	0.06	0.74
PC5	1.01	0.05	0.79
PC6	0.91	0.04	0.83
PC7	0.87	0.04	0.87
PC8	0.73	0.03	0.90
PC9	0.71	0.03	0.92
PC10	0.64	0.02	0.94
PC11	0.54	0.01	0.96
PC12	0.46	0.01	0.97
PC13	0.45	0.01	0.98
PC14	0.39	0.01	0.99
PC15	0.26	0.00	0.99
PC16	0.25	0.00	0.99
PC17	0.22	0.00	1.00
PC18	0.20	0.00	1.00
PC19	0.15	0.00	1.00
PC20	0.04	0.00	1.00

Table 4. Component matrixes

Variables	PC1	PC2
GF	0.20	-0.19
MF	-0.11	-0.35
LL	0.25	0.24
PH	0.29	0.09
LW	0.26	-0.05
PLC	0.25	0.09
SD	0.27	-0.06
NNM	0.27	0.01
FFM	0.30	0.08
PL	0.28	-0.23
PW	0.28	-0.22
SL	-0.23	-0.18
GCD	-0.12	-0.46
PD	0.25	-0.24
PV	0.22	-0.27
PIE	-0.14	-0.43
FFF	0.18	-0.13
NNF	0.18	-0.13
SPM	0.14	0.20
GS	0.03	0.17

Figures

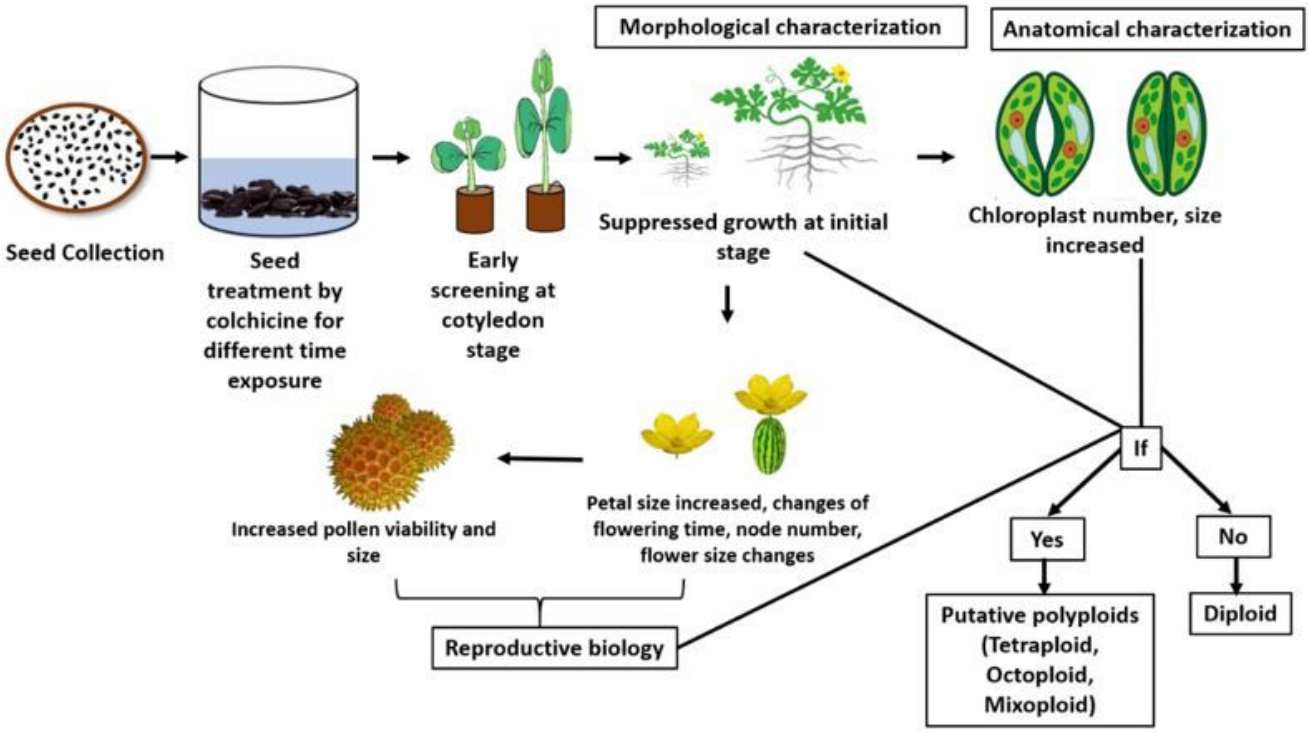

Figure 1

Schematic hypothetical diagram of polyploid induction and confirmation with colchicine treatment in watermelon seed

Figure 2

Cotyledon for diploid and putative polyploid

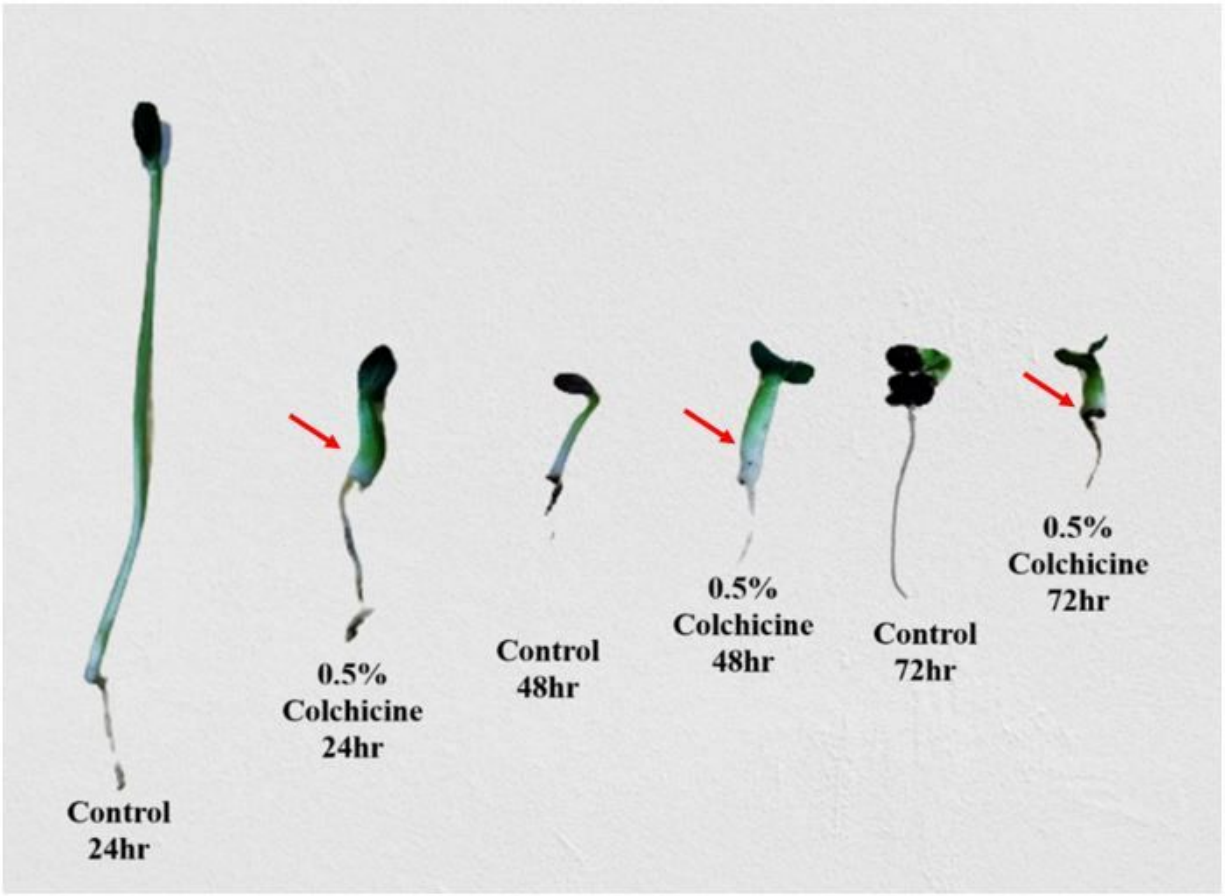

Figure 3

Hypocotyl thickness as the marker for early screening. (Arrow indicates the thick hypocotyl)

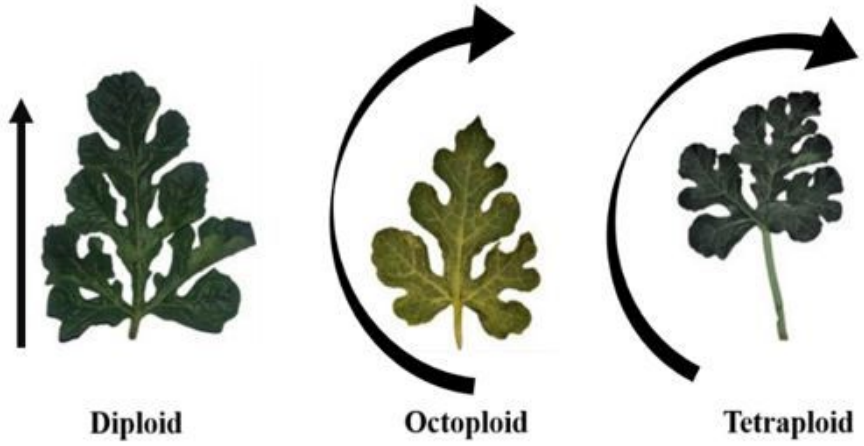

Figure 4

Leaf colour intensity and degree of lobing as the indicator of ploidy level at early vegetative stage. (Arrow indicates the degree of lobing)

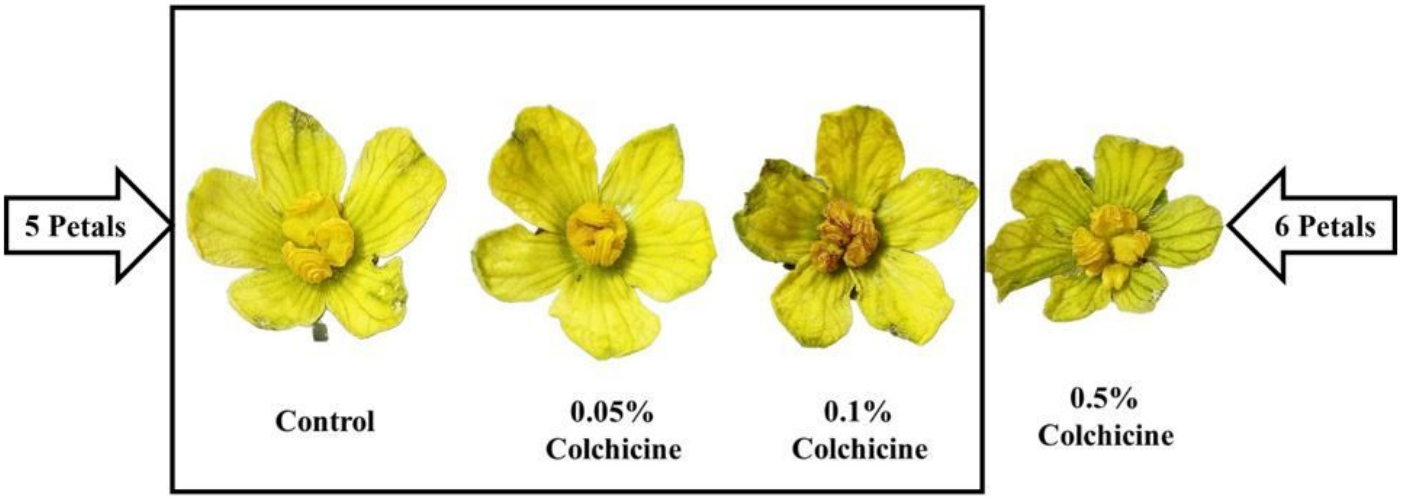

Figure 5

Difference in petal number of male flowers

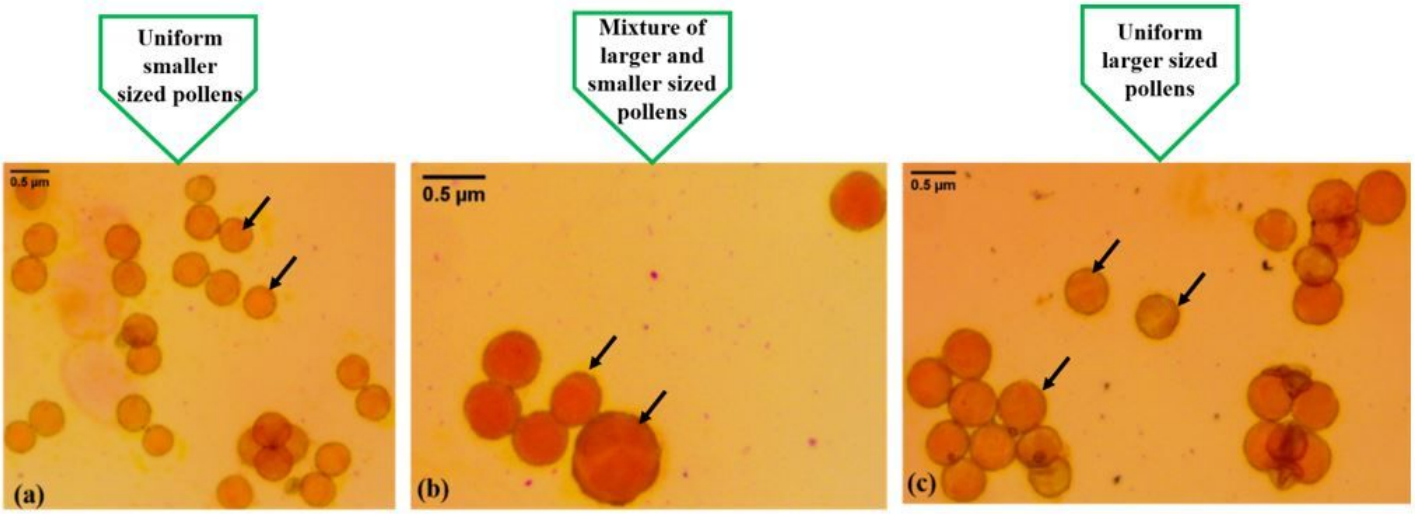

Figure 6

Pollen size of colchicine induced polyploids. (a) diploid (uniform small in size), (b) mixoploid (mixture of diploid and tetraploid) and (c) Tetraploids (larger size)

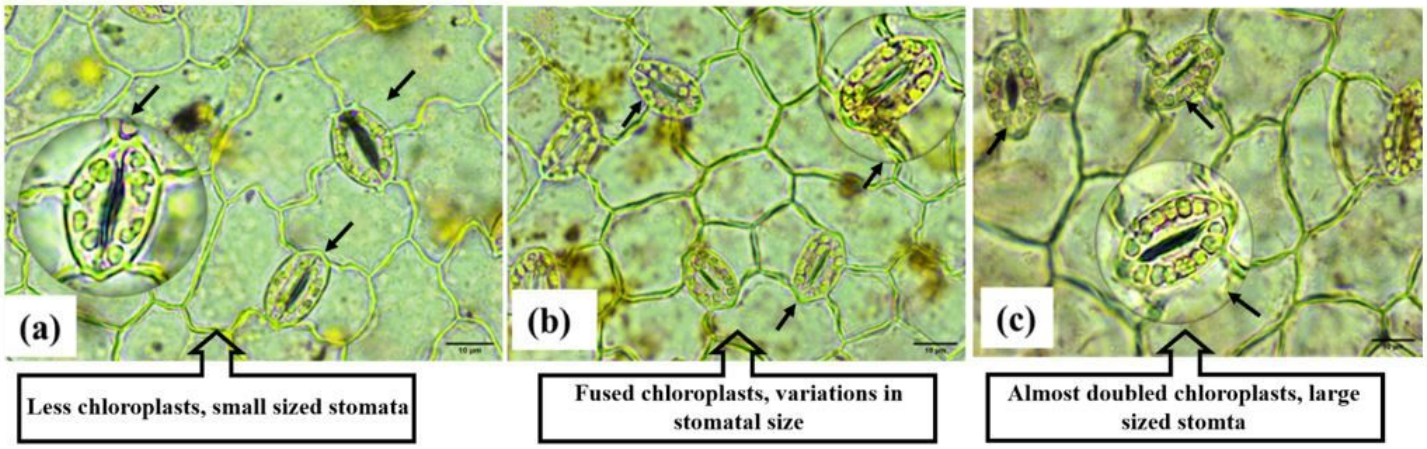

Figure 7

Stomatal observation for confirmation of ploidy induction. (a) diploid for control treatments, (b) mixoploid in various higher concentrations (0.1 and 0.5% at three durations) and (c) tetraploid or octoploid induction for most of the colchicine treated plants

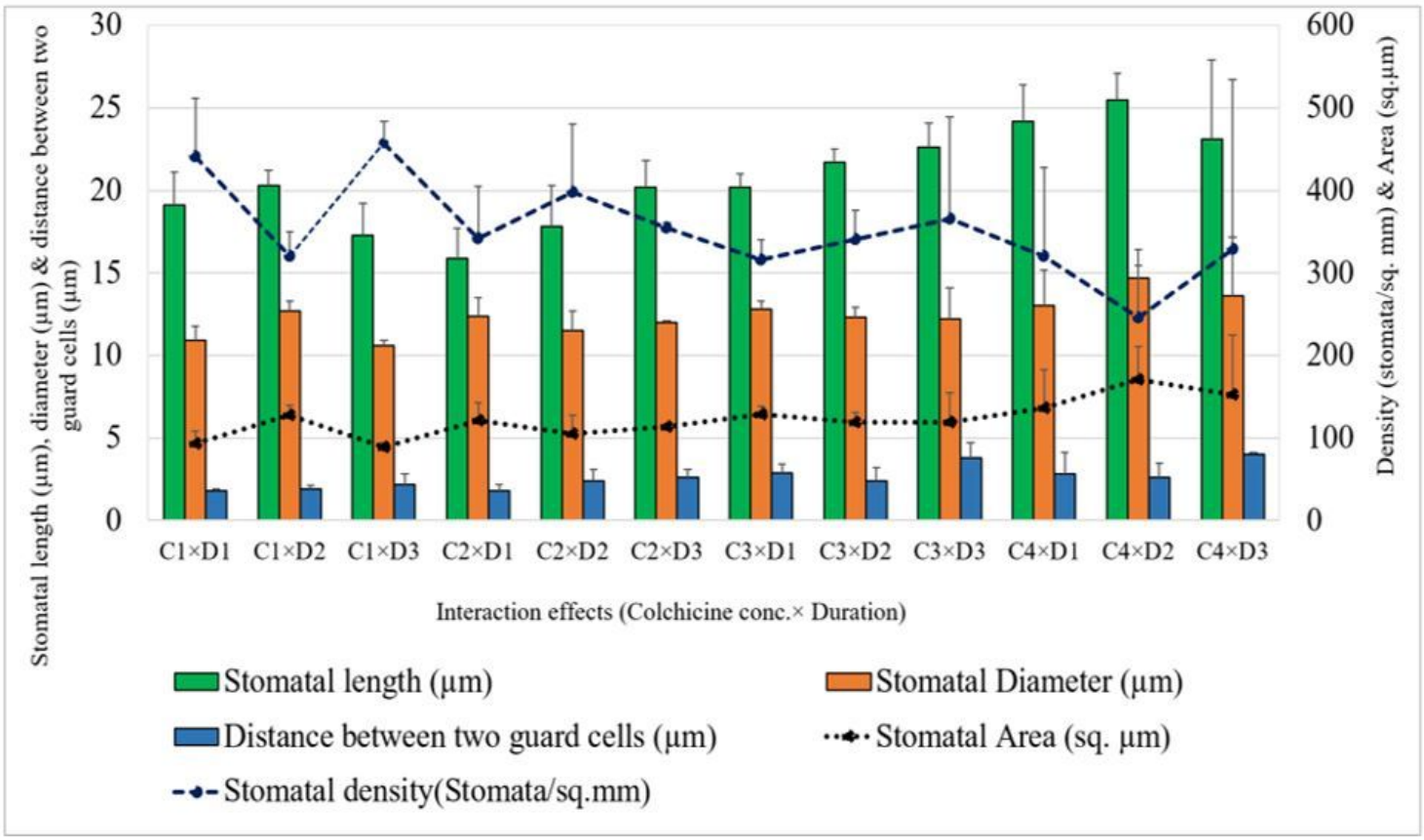

Figure 8

Quantitative stomatal attributes

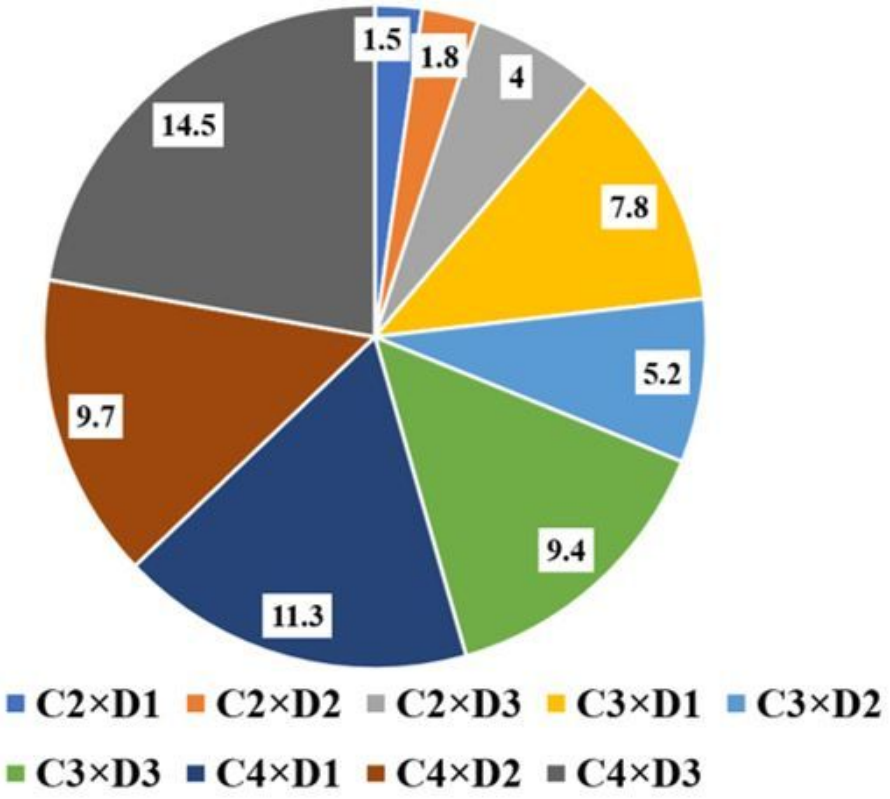

Figure 9

Polyploid induction efficiency (PIE) % by colchicine

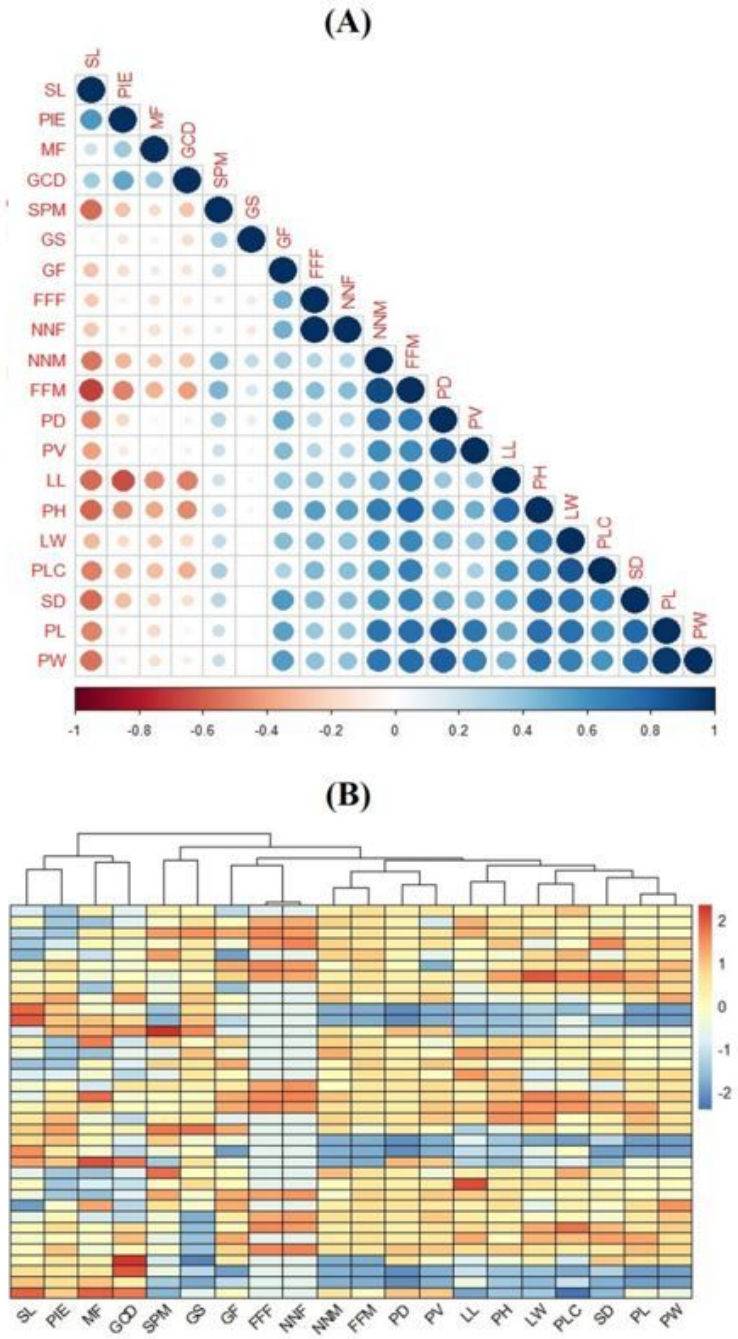

Figure 10

Multivariate analysis. (A) Correlation coefficient for variables related to polyploid induction in watermelon, (B) Distribution of 20 variables into two major clusters with Pheatmap. (SL= stomatal length, PIE=polyploid induction efficiency, MF= final mortality at 40DAS, GCD= distance between two guard cells, SPM= stomata per micrometer, GS= germination speed, GF= Final germination at 40DAS, FFF= days required for first female flowering, NNF= node number for first female flower initiation, NNM= node number for first male flower initiation, FFM= days required for first male flowering, PD= pollen diameter, PV= pollen viability, LL= leaf length, PH= plant height, LW= leaf width, PLC=petiole length, SD= stomatal diameter, PL= petal length, PW=petal width)

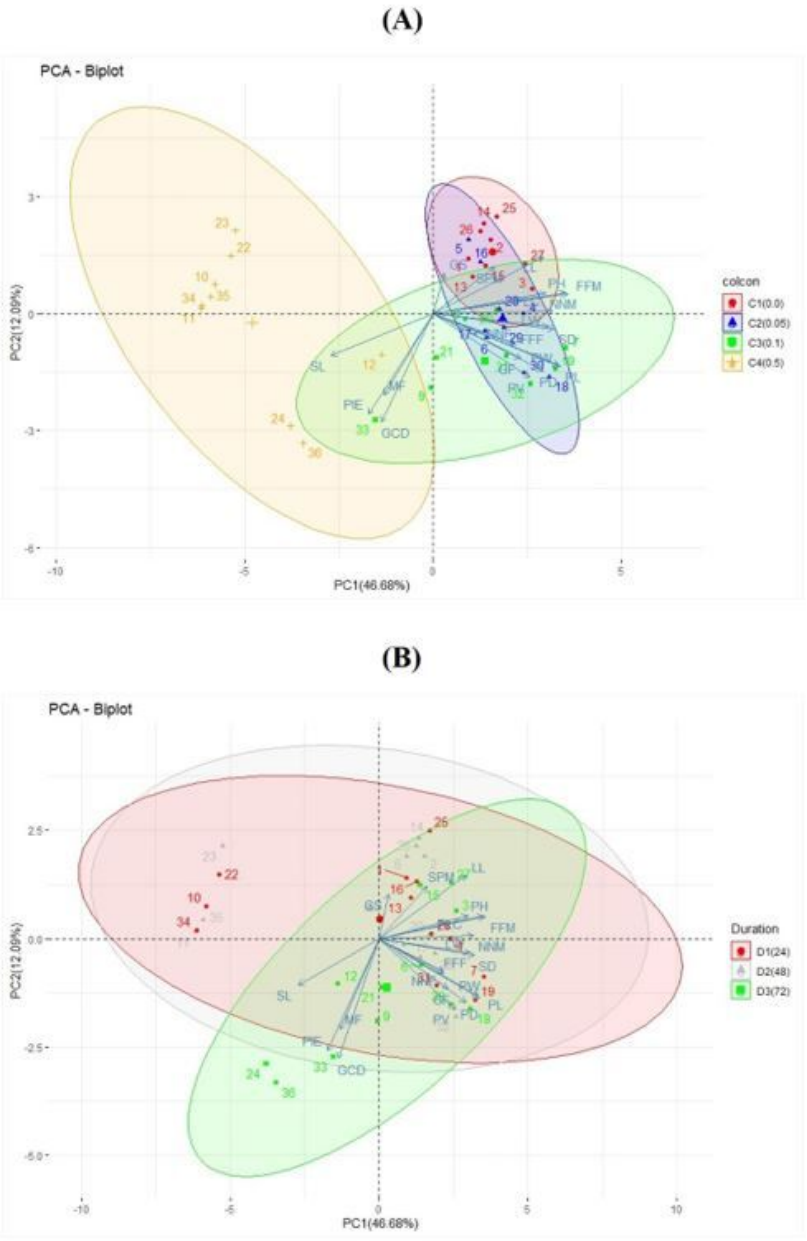

Figure 11

Principal component analysis (PCA). (A) Colchicine concentration and (B) Exposure duration