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Homogeneous Triploid and Tetraploid Production through Crossing with Mixoploid Parents in Pointed Gourd (*Trichosanthes dioica* Roxb.)

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Abstract

This paper elucidates a procedure for isolating homogeneous triploid and tetraploid progeny from mixoploids, which are the most desirable genetic resources to develop genetically stable seedless variety in pointed gourd (*Trichosanthes dioica* Roxb.) as seeds are unpalatable. All the colchicine concentrations (0.05, 0.1, 0.5%) effectively led to the production of mixoploid for 48 and 72 h exposure time whereas 24 h did not response to induce mixoploid. These mixoploids (female and male) exhibit cross compatability with diploid (female and male) parents for F_1 seed generation. Interestingly, mixoploid parents (either female or male) produced a mixture of normal diploid size seeds and some abnormally large ones, almost twice normal size. Density plot and principal component analysis (PCA) of 603 seeds resulted in separation of diploid, mixoploid and tetraploid accessions involved in different ploidy crosses. To develop a method for the isolation of sexually derived triploid and tetraploid progeny from the induced mixoploids, we examined the ploidy level of F_1 populations by flow cytometry where 18.7 % F_1 seedlings were confirmed as triploid and tetraploid progeny when female mixoploid crossed with male mixoploid while 16.7% triploids were isolated crossed with male diploid. These findings suggest that mixoploid female parents were the best options for developing triploid and tetraploid progeny. Overall, the results of this study provide a framework to explore the genetic basis of polyploids isolated from colchicine induced mixoploids in *in vivo* conditions.

Highlights

- Mixoploid parents generated seed lot comprises of diploid and tetraploid type seeds.
- Mixoploids are reported for the first time as potential for F_1 seed generation led to the homogeneous triploid and tetraploid progeny development which confirmed by flow cytometry in pointed gourd.
- Ploidy analysis of sexually derived seedlings proposed as an efficient *in vivo* method of isolation triploid and tetraploid progeny from colchicine induced pointed gourd mixoloids.

Key words: Cucurbits, Colchicine, Flow cytometry, Mixoploid, Polyploidy breeding, *Trichosanthes dioica.*

1. Introduction

Pointed gourd (*Trichosanthes dioica* Roxb.) is one of the most economically viable and nutrition rich summer vegetables belongs to the cucurbitaceae family. It is native to Indian subcontinent and potential to cultivate in the temperate regions (Singh and Whitehead 1999; Hassan and Miyajima 2019a). It is dioecious and vegetatively propagated fruit type vegetable with 11 basic chromosome number (2n=2x=22) (Kumar and Singh, 2012). Although green fruit with soft seeds is the main edible part, matured seeds sometimes cause the unpalatability due to their hard seed coats (Hassan et al. 2020). Therefore, it becomes the most desirable for pointed gourd to be produced without seeds that can be possible by chemical application (Hassan and Miyajima, 2019b) or by natural and artificial polyploidization process. Indeed, clinical evidence suggests that pointed gourd has multiple health benefits (Pandit and Hazra 2008; Rai et al. 2008) and a tremendous effort has been made in the field of phenotypic and genetic diversity, management approaches, clone selection and ecological adaptability (Hassan and Miyajima, 2019c; Verma et al. 2017; Adhikari et al. 2014 Khan et al. 2009). But other ways to increase variability and varietal improvement through polyploidization have not been sufficiently explored. Hazra (2001) took the first initiative to induce polyploidization in pointed gourd with colchicine but unsuccessful to produce tetraploids. Besides, a successful colchicine induced polyploidization in pointed gourd was reported for the first time by Hassan et al. (2020) that creates the opportunity of subsequent studies with these advanced genetic resources for further improvement.

Naturally and artificially induced polyploids are recognized as one of the most frequent ways for introducing variability, generating new species with improved various agronomic and commercial traits of different horticultural crops such as fruit quality (Wu et al. 2013; Blasco et al. 2014), large fruit size (Rugini et al. 1996), seedless fruits (Kagan-Zur et al. 1991), disease resistance (Predieri 2001), high yield and wide adaptability (Liu et al. 2009). Within the polyploidy, triploidy and tetraploidy are the important features for pointed gourd those can lead to seedless or less seeded fruit production as observed in watermelon (Kihara 1951), citrus (Ollitrault et al. 2008), banana (Simmands and Sheperd 1955), loquat (Guo et al. 2007) and pointed gourd (Hassan et al. 2020). In our previous study, colchicine treatment of pointed gourd seeds was employed for generating tetraploids and used in crossing with diploid counterparts to produce less-seeded fruits (Hassan et al. 2020). Although the success rate of tetraploid induction was low (0.33%), a considerable amount of mixoploids (2.11%) (chimeras consisting of diploid and tetraploid cells) were also generated at that study. Usually these mixoploids are of much less important compared to homogeneous polyploidy plants due to their genetic instability (Dhooghe et al. 2011; Rose et al. 2000). Therefore, it has been hypothesized that mixoploids would become another breeding method if we could isolate homogeneous (pure) triploid and tetraploid from the colchicine induced mixoploids of pointed gourd (Figure 1).

Various *in vitro* isolation techniques have been developed in recent years, such as callus re-differentiation (Chen and Gao 2007; Roy et al. 2001), multiple subculture (Rugini et al 1996), and re-differentiation of shoot primordia in the shoot apex (Fujishige et al. 1996). However, all of these methods are inefficient due to time-consuming and difficult to apply because of high-throughput procedures (Liu et al. 2020). In contrast, Beatson et al. (2003) described an easier method for the identification of sexually derived triploids and tetraploids progeny of hop from seedling population by flow cytometry (FCM) compared to in vitro cultured derived tetraploid parents. They identified the highest number of triploids than tetraploids from the progeny analysis and screening by FCM is now considered an essential part of the New Zealand hop breeding program to develop genetically stable triploid genotypes. Another subsequent study, Koutoulis et al. (2005) reported that triploid and tetraploid progeny have been produced from the crosses of mixoploids Galena-4n (hop) with a male diploid. This may have been due to the presence of some or maximum tetraploid cells in the reproductive tissues of mixoploid Galena-4n. In the past, cytological approach of chromosome counting and stomatal conductance were classically applied to screen the ploidy level that is time consuming as well and possibility of environment factors involvement lead to confusion in results. Conversely, FCM application is revealed as the best alternative for rapid and high accuracy maintaining throughput ploidy screening visualized with clear histograms (Dhooghe et al. 2011). Most importantly, no studies have yet identified potential strategy to utilize mixoploids (chimeras) in species evolution of cucurbitaceae family as widely evident in Solanaceae and Cruciferae (Burge et al. 2002). Therefore, extensive research is required for isolation in chimera or mixed-ploidy populations to make a standard polyploidy breeding approach to produce pure triploid and tetraploid from mixoploids.

In this study, we profiled seed traits (individual seed weight and diameter) in 603 seeds across the intra and inter-ploidy crosses among mixoploids, tetraploid (as standard) and their diploid counterparts to prove the hypothesis that mixoploid comprises diploid and tetraploid cells. The main objectives of this study were to: (1) investigate the effect of colchicine treatment on pointed gourd seeds for mixoploid induction, (2) assess the cross compatibility of colchicine induced mixoploids with diploid parents for F_1 seed production, (3) establish an in vivo polyploidy breeding strategy with the isolation of homogeneous triploid and tetraploid progeny from the ploidy level assessment of the sexually derived F_1 progenies.

2. Materials and Methods

2.1. Plant materials

This study was conducted for two consecutive years (2018 and 2019) in the non-heated glasshouse at Hakozaki campus (lat. 33° 37′ N; long. 130° 25′ E), Kyushu University, Japan with the plant materials of pointed gourd (*Trichosanthes dioica*), originated in India and Bangladesh (Hassan and Miyajima, 2019a). Mature vines of female and male *T. dioica* parent were collected from different locations of Bangladesh. The vines were planted in the akadama soil: peat (2:1) mix filled plastic containers and grown in glasshouse (32/20 ºC day/night, 50-70% relative humidity) at Kyushu University to enforce flowering during the cultivation period from February 2017 to September 2017. Due to its dioecism, cross-pollination is inevitable for fruit setting and seed production. Crossing was conducted when stigmas of female *T. dioica* attained optimal receptivity during night (9~10 pm) at anthesis with fresh pollen. When the seeds were matured in the ripen fruits (60 days after pollination), fruits were harvested and seeds were prepared according to the procedure of Hassan and Miyajima (2019a). The seeds were extracted from the fruits, dried and used to conduct chromosome doubling experiment with colchicine treatment in November 2017. Since amount of seeds per fruit was not same for all harvested fruits and in some cases not sufficient to perform chromosome doubling study with more treatments, prior to conduct, the seeds were pooled and then separated into four technical replicates for each treatment. The technical replicates could minimize errors associated with data processing of seed chromosome doubling study traits.

2.2. Colchicine treatment of seeds

Seeds were soaked in the aqueous colchicine at concentrations of 0.05, 0.1, or 0.5% (w/v) for 24, 48, or 72 h and kept in dark condition. Experiments were replicated four times with 25 seeds per treatment. The similar amount of seeds was also soaked in tap water (without colchicine) for 24, 48, or 72 h as control. Following the colchicine treatment, the seeds were thoroughly rinsed three times with sterile distilled water and air dried at room temperature. The seeds were transferred for germination to the vermiculite filled plastic tray. The seedlings were cultured in the glasshouse until February 2018 and regular observation was done to assess the seed germination and seedling survival rate for each treatment. The seedlings were shifted to the small plastic pot filled with akadama soil: peat mix (2:1) and maintained in the same glasshouse for flow cytometry study.

2.3. Ploidy level confirmation by flow cytometry

The ploidy levels of colchicine treated seedlings and F_1 progenies generated from inter and intra-ploidy crosses were determined by flow cytometry. Young leaves (third leaves from the stem tip) from each treated seedling and F_1 progeny were collected and about 1 cm^2 of the middle epidermis were chopped in a petri dish with 400 µl nuclei extraction buffer HR-A (CyStain UV Precise P, Sysmex-Partec High Resolution Staining Kit, Sysmex-Partec GmbH, Germany) using a sharp razor blade. The nuclear suspension was then filtered through a 42 µm nylon mesh (Partec CellTrics filter) to remove debris. Nuclei were stained with 1.6 ml of DAPI (4,6-diamidino-2-phenylindole) (CyStain UV precise P, Partec High Resolution Staining Buffer Kit, HR-B) for 3 min and then analyzed using a Partec CyFlow Ploidy Analyzer. More than 7,000 nuclei were assessed in each sample. Nuclear DNA histograms were constructed with the help of default CyView software in Sysmex-Partec, GmbH, Germany.

At first 2-3 leaves from the parental species (without colchicine treatment) were used to determine the standard peak of diploid mother cells and considered as diploid (2x) ploidy level criteria of pointed gourd. Putative tetraploid (4x) seedlings were confirmed according to the peak positions compared to diploid parental species (Hassan et al., 2020). Meanwhile, mixoploids [hereafter denoted as (2x+4x)] were recognized with the cumulative peaks of tetraploid and diploid.

All colchicine treated seeds germinated seedlings those survived were subjected to flow cytometry to confirm their ploidy. Individual seedlings were classified as diploid, mixoploid or tetraploid according to the peaks (fluorescence profile of nuclei) obtained by flow cytometry are shown in Figure 2. The diploid parents (female or male) were used as diploid selection criteria with which colchicine treated seedlings were compared to categorize into diploid, tetraploid or mixoploid. Histograms generated by flow cytometry analysis demonstrated that diploids depicted two standard peaks at about channel 50 and 100 of relative fluorescent intensity (Figure 2A), while the peaks responsible for representing tetraploid were observed at around 100 and 200 (Figure 2B). Besides, the presence of three peaks at 50, 100 and 200 of relative fluorescent intensity (Figure 2C) confirmed the induction of mixoploid consisting of diploid as well as tetraploid cells.

2.4. Intra and inter-ploidy crosses for F_1 seed production

Intra and inter-ploidy crosses among diploids and colchicine induced mixoploids of pointed gourd were conducted from 25 April to 28 July 2019. A total of 9 from each of female and male pointed gourd plants (3 plants of each sexes belongs to the diploid and mixoploid lines were used per treatment in three replicates) were used as parents in the full-diallel crossing design for F_1 seed generation. In this case, each diploid was paired with mixoploid and crossed reciprocally where all the diploids and mixoploids were served as maternal and paternal parents. Five diploid and mixoploid female flowers were pollinated with fresh pollen of mixoploid and diploid males during night (9-10 pm) at anthesis as an interploidy cross [2x × (2x+4x); (2x+4x) × 2x]. Meanwhile, intraploidy cross was done with each ploidy member of the crossing pair pollinated with the same ploidy [2x × 2x; (2x+4x) × (2x+4x)]. Controlled pollination was ensured by covering flowers with paper bags prior to anthesis and all the pollinated flowers were re-covered to prevent them from undesirable cross pollination. Ripen fruits were harvested 60 days after pollination, and fruit setting rate, number of seeds per fruit and seed germination rate were scored.

2.5. Seed traits evaluation revealed mixoploid comprise diploid and tetraploid cytotypes

Individual seed weight and seed diameter of total 603 F_1 seeds obtained from the ripen fruits of different ploidy cross combinations was analyzed to evident the hypothesis that mixoploid comprise diploid and tetraploid cytotypes of pointed gourd. Of the 603 F_1 seeds, 162 generated from 2x × 2x; 185 from 2x × (2x+4x); 199 from (2x+4x) × 2x; 48 from (2x+4x) × (2x+4x) and 9 seeds of 4x × 4x which were used as standard of tetraploid traits. Thus, density scatter plots for seed weight and seed diameter variables were created using R where density plot determines the normal distribution of seed traits data. In addition to density plot, we performed PCA (principal component analysis) to evaluate these seeds traits that will help to distinguish different ploidy level involved in ploidy crosses according to the seed traits.

2.6. Ploidy detection of progeny

Seeds generated through different ploidy crosses were sown to establish F_1 progenies in August 2019. Experiments were replicated three times with 30 seeds per cross combination (treatment). The ploidy levels of all the survived F_1 progenies were determined by flow cytometry procedure described previously. It was done to know about the genetic stability and heredity of the ploidy level of the parents involved in inter and intra-ploidy crosses to their successive progeny.

2.7. Data processing and statistical analyses

Statistical analyses were performed using R software (version 4.0.2). Before performing analysis of variance (ANOVA), the model fitness of data was evaluated using model accuracy and a log2 transformation was applied to normalize the data as per necessary. To assess the magnitude of variation within and between the treatments of colchicine concentrations, exposure durations, interaction effect, ploidy crosses; we computed a minimum, maximum, average and range of variation for all the studied parameters. Differences in individual seed weight and seed diameter features between ploidy level counterparts in cross combinations were evaluated by paired t-tests. These variations were visualized by boxplot using R-package "ggplot2". Differences were determined to be statistically significant at $P<0.05$, and highly significant at $P<0.01$. When treatments differed significantly, an honestly significant difference (HSD) as multiple comparisons test was used for pairwise comparison. PCA (principal component analysis) was performed using the R package "FactoMiner" (Le et al., 2008) to classify the key seed traits according to ploidy groups and to evaluate the effect of ploidy profiles on seed traits among different crosses. Density plot shows the classification of ploidy groups/species according to the features of seeds generated from inter and intra-ploidy crosses.

3. Results

3.1. Colchicine treatment of pointed gourd seeds for ploidy induction

Polyploids induction was confirmed by flow cytometry of six-week old seedling survived after colchicine treatment of pointed gourd seeds for different durations. The survival rate of germinated seedling after colchicine treatment ranged from 5.0 to 34.2%, and was differed significantly by the interaction effect of the colchicine concentration and the exposure duration (Table 1). The survival frequency reached to the maximum (34.2%) following treatment with 0.05% colchicine for 72 h; however, mixoploids were induced at lower and no tetraploids induced under this condition. The mixoploid induction efficiency (%) was significantly affected by the colchicine concentrations and the treatment durations (Figure 3). The optimum condition for mixoploid induction was treatment the seeds with 0.5% colchicine for 72 h, and the highest induction frequency was 7.95%. Tetraploid induction efficiency was not significantly varied due to the colchicine concentration and soaking duration, though, maximum tetraploid was induced (3.7%) with the highest concentration of colchicine for maximum soaking duration of 72 h. Meanwhile, the highest seedlings were survived with untreated seeds and ploidy level determination showed that they were all diploids.

3.2. Inter and intra-ploidy crosses for F_1 seed generation

In order to determine which cross combinations produced fertile seeds and assess seed traits in further studies, inter and intra-ploidy crossing among colchicine induced mixoploids and diploid parental counterparts was carried out (Table 2). Reproductive success (fruit set and seed production) differed significantly among the parents involved in different cross combinations. In intra-ploidy crosses with diploid parents (female and male), 2x × 2x crosses produced higher fruit set (100%) than [(2x+4x) × (2x+4x)] crosses (33.3%) with mixoploid parents. Fruiting success was statistically identical between interploidy crosses where mixoploid female pollinated with diploid male [(2x+4x) × 2x] produced 86.6% while it's reciprocal cross of diploid female pollinated with mixoploid male [2x × (2x+4x)] produced 60.0% fruit set (Table 2). Similarly, the maximum number of seeds per fruit (26.4) was obtained from the intra-ploidy crosses with diploid parents of 2x × 2x cross than those from mixoploid parents of [(2x+4x) × (2x+4x)] cross (20.3). Meanwhile, statistically similar amount of seeds per fruit was gained from the interploidy crosses of [2x × (2x+4x)] (19.7) and [(2x+4x) × 2x] (18.5). Differences in the seed germination ability were significant according to the different types of intra and inter-ploidy crosses. Perfect seeds with 100% germination were found in 2x × 2x cross followed by [2x × (2x+4x)] cross (96.6%). Besides, relatively lower seed germination rate was observed in the seeds produced in the crosses of [(2x+4x) × 2x] (60.0%) and [(2x+4x) × (2x+4x)] (53.3%).

3.3. Phenotypic variability of seed traits

Phenotypic data for seed traits including individual seed diameter and seed weight summarized by ploidy levels involved in different inter and intra-ploidy cross combinations. The accessions evaluated here showed a

considerable variation for seed weight and diameter within and between ploidy-groups (Figure 4). These variations were compared with the seed traits derived from the intraploidy cross of tetraploid parents (4x × 4x). Individual seed weight and diameter exhibited remarkable variation within and between the accession developed from the intra and interploidy crosses of diploid, mixoploid and tetraploid genotypes and these variations were visualized in different boxplots with significance level (Figure 4). Overall, tetraploid accessions exhibited the highest value for seed traits compared to diploid. While the highest level of significance was observed in the mixod (mixoploid female crossed with mixoploid male generated seeds) for seed weight that was similar with tetraploid and diploid. For seed diameter, the variation was highly significant in all the crosses generated seeds. However, moderate variability was observed in the crosses with mixoploid as either seed parent or pollen parent. Moreover, combined analysis of variance showed significant effects of different ploidy accessions involved in the crosses on individual seed traits (weight and diameter). These findings suggesting that these seed traits may play a major role as the genetic improvement component for the phenotypic selection in further breeding program.

3.4. Density scattered plot (DSP) analysis revealed mixoploid comprises diploid and tetraploid seed traits

We found a hypothesis after seed traits phenology (Figure 4) that F_1 seeds generated from the crosses with mixoploid (female or male) parent are a mixture of diploid and tetraploid categorized seeds. To make this hypothesis statistically reliable, density scattered plot (DSP) analysis was performed (Figure 5). DSP visualize the shape information with the trends in variance distribution and central tendency of multivariate dataset of different ploidy crosses seed traits. As observed, accessions separated into five quadrants each representing the following features: (I) Diploid (F1 seeds of 2x × 2x) showed distinct normal distribution for both seed diameter and seed weight, (II) Dmixo (F1 seeds of [2x × (2x+4x)]) widely distributed covering with diploid and tetraploid generated seed traits, (III) Mixod (F1 seeds of [(2x+4x) × 2x]) also represent the distribution of seed traits data values with wide spreading including diploid and tetraploid features, (IV) Mixo (F1 seeds of [(2x+4x) × (2x+4x)]) moderate level density of seed trait distribution compared to other ploidy level accessions, (V) Tetra (F1 seeds of 4x × 4x) shows clear discrimination from diploid while overlapping with dmixo, mixod and tetra ploidy accession for both seed diameter and seed weight distribution density. As expected, a large number of seeds were in Mixod accession those comprises both diploid and tetraploid seed features followed by Dmixo and Mixo ploidy level produced seeds.

3.5. Principal component analysis (PCA) of ploidy crosses generated seed traits

Following DSP analysis, we examined the seed traits data using PCA to justify whether seed diameter and seed weight could differentiate accessions based on ploidy levels (Figure 6). PCA analysis revealed that diploid and tetraploid accessions clustered in a distinct group from each other. In contrast, no definitive separation was observed among dmixo, mixod and mixo accessions for the seed traits. Moreover, the seeds generated by the dmixo, mixod and mixo accessions showed a tendency to form cluster together with diploid and tetraploid formed clusters. PCA examination suggests that the variables individual seed diameter and seed weight significantly contributed to separate the accessions based on ploidy level and made evident as mixoploid parents produced seeds were comprising both diploid and tetraploid types seeds.

3.6. In vivo separation of triploid and tetraploid progenies confirmed by flow cytometry (FCM)

Attempt was made to isolate pure triploid and tetraploid advanced lines from F_1 progenies derived from ploidy crosses among colchicine induced mixoploids and diploid counterparts. Thus, F_1 progenies (germinating seeds) were analyzed by flow cytometry to determine their ploidy status (Figure 7, Table 3). Triploid plants were obtained from the seeds of the crosses where mixoploids females were crossed with either mixoploid or diploid parent. Therefore, no triploid progeny was identified among the tested F1 seedlings derived from the [2x × (2x+4x)] cross while the highest percentage (18.7%) of F1 progenies of the [(2x+4x) × (2x+4x)] cross confirmed as triploid followed by [(2x+4x) × 2x] cross (16.7%) (Table 3.). Interestingly, both triploid and tetraploid progenies were identified from the germinating seeds of [(2x+4x) × (2x+4x)] cross at the rate of 18.7%. Besides, the rest of the analyzed seedlings of all the crosses were recognized as diploid, while no

mixoploid (consisting of diploid and tetraploid cells) seedling was recorded. These observations indicate that in vivo progeny separation of mixoploids into pure triploid and tetraploid advanced lines will be an effective and efficient alternative of in vitro ploidy isolation technique.

4. Discussion

Pointed gourd (*T. dioica* Roxb.) is recognized as one of the most important economic summer cucurbit vegetables in Asian countries that have contributed to meet vegetable requirement for long duration (February to October) of a year (Kumar and Singh, 2012; Hassan and Miyajima, 2019c). Multiple studies have been conducted over the last four decades for the systematic management improvement and breeding strategies of pointed gourd (Hassan and Miyajima, 2020). Despite its importance, limited research has been conducted in parthenocarpy or seedless breeding approaches of pointed gourd which is the most desirable to increase consumer acceptance. Recently, successful polyploidization by colchicine treatment of pointed gourd seed has been reported and advanced genetic lines have been developed as the prerequisite of genetically stable seedless triploid variety (Hassan et al., 2020). As a number of mixoploids (consisting of 2x and 4x cells) had been identified in our previous study, we made a hypothesis that it is possible to obtain pure triploid and tetraploids from these mixoploids after crossing with the diploid counterparts. Results of the present experiments confirmed our hypothesis.

The last two decades have seen a remarkable advance in the field of polyploidization for crop improvement where *in vitro* somatic chromosome doubling using colchicine as antimitotic agent was frequently applied (Cai and Kang 2011; Shi et al. 2015; Liu et al.2018; Xu et al. 2018). In these reports, it was primarily focused on the time point of adventitious bud formation after antimitotic treatment that depends on the pre-culture duration, colchicine concentration and exposure duration for successful ploidy induction. Consequently, it was a time-consuming and high throughput cost schedule. By contrast, in this study, multiple pure triploid and tetraploid advanced lines were produced more efficiently following *in vivo* separation of mixoploid. Mixoploids could be induced easily through seed chromosome doubling, and it was not necessary to consider the pre-culture condition like *in vitro* derived mixoploids. Meanwhile, mixoploidy being described as relatively common phenomena in colchicine induced polyploidization in other species (Sun et al. 2009; Zhang et al. 2010; Harbard et al. 2012) as classified in our present study. Mixoploid induction is evident due to the asynchrony of cell division in seed (Dhooghe et al. 2011). In this study, a total of 19 mixoploids were obtained by treating seeds of pointed gourd (*T. dioica*) with colchicine and these were used as inter and intra ploidy cross materials for isolation of homogenous triploid and tetraploid progenies.

F_1 seeds have been produced by hybridizing mixoploid plants in full diallel crossing with diploid parents. Interestingly, the resultant seeds in the crosses where mixoploids were used as either male or female plant were the mixture of thin and bold type seeds compared to diploid generated seeds. At that stage, we assumed mixoploid parent generated seeds might be comprised of diploid and tetraploid seeds (Figure 1). To justify this hypothesis, we did frequency distribution analysis, visualize by boxplot, density scatter plot and PCA based on seed trait variables (individual seed weight and seed diameter) of all the produced seeds and compared with the tetraploid and diploid parents originated seeds. As revealed, ploidy was the most distinctive descriptor to differentiate the accessions involved in different ploidy crosses (Figure 4, 5, 6). Indeed, PCA analysis clearly separated into five ploidy groups; where diploid and tetraploid are separated from each other while dmixo (diploid x mixoploid), mixod (mixoploid x diploid), mixo (mixoploid x mixoploid) generated clusters were inter connected with the diploid and tetraploid groups (Figure 6). This observation was concurrent with Shomotsuma and Matsumoto (1957) where they distinguished 3x (triploid) watermelon seeds from 4x (tetraploid) based on seed weight and thickness. They reported that 3x seeds were thinner and lighter than 4x seeds, but both were thicker and heavier than diploid seeds. According to their suggestions seed weight can be considered in order to distinguish self-pollinated and crossed seeds in an open seed block.

Afterwards, F_1 seed germinated seedlings tested by flow cytometry to highlight the contribution of mixoploid parents for producing triploid and tetraploid progeny in the successive generation. Both triploid and tetraploid progeny were produced when mixoploid females were crossed with a mixoploid male, while only triploids were produced when mixoploid female crossed with diploid male (Table 3). In that case, the question becomes how a triploid and tetraploid arise from mixoploid involved inter and intraploidy crosses with a male diploid or mixoploid. There are generally two paths to form triploid like hybridization between diploid and tetraploid or

parent having 2n gamete with 1n gamete parent. Besides, for tetraploid it would be 4x × 4x or hybridization between parents having both unreduced 2n gametes of male and female reproductive tissues. This has been proven in the previous reports for Populus tomentosa (Zhu et al. 1995; Han et al. 2018; Zhou et al. 2020) and Humulus lupulus (hops) (Koutoulis et al. 2005). In the present study, as all the FCM tested progenies were derived from the crosses among mixoploids and diploid counterparts, so there is no possibility for tetraploid accession to take part in the crossing process. Thus, it can be speculated that the mixoploids (both male and female) in the present study could produce a few of viable unreduced 2n female and male gametes that might contribute in tetraploid and triploid generation. In support of this, we admitted the previous findings in other crops, as mixoploid pointed gourd (*T. dioica*) plants have not been reported before. Koutoulis et al (2005) stated that tetraploid and triploid progeny could arise from the contribution of an unreduced diploid (2n) male gamete from a diploid male or a normally reduced diploid (2n) male gamete from a tetraploid male parent. Maletskii and Maletskaya (1996) postulated that induced mixoploidy underlies gametophytic agamospermy, i.e. the presence of tetraploid cell admixtures among the bulk of diploid cells. Reduction division of admixed tetraploid cells results in the formation of a diploid embryo sac with cells capable of embryogenesis and in the majority of the cases mixoploidy was confined mostly to somatic tissues, although there are some existence reports revealed of its occurrence in germinal cells (Ranjbar et al., 2011). In addition, polyploidised shoot of mixoploid could be involved in the production of triploids that could resulted of the zygote development from the endosperm, or more likely, should be from one gamete that fails to undergo meiotic reduction lead to the production of unreduced or aneuploidy gamete by improper segregation of chromosomes during anaphase/telophase stages (Ramsey and Schemske, 1998; Dzialuk et al., 2007).

There are multiple cytological mechanisms of unreduced gamete formation have been reported in different inter and intraploidy crosses of several species of populus (Wang et al., 2017; Tian et al., 2015; Zhang and Kang, 2010; Kang, 2002; Wang and Kang, 2009). The formation of 2n-gamete can be attributed due to parthenogenesis, different abnormal meiotic aberrations including pre-meiotic doubling, anomaly chromosome pairing, misorientation of spindles and failure of cytokinesis, FDR (first division restitution), SDR (second division restitution, IMR (indeterminate meiotic restitution) and PMR (post meiotic restitution) (Zhang and Kang, 2010; Kang, 2002; Wang and Kang, 2009; Ramanna and Jacobsen, 2003).

Moreover, inter and intra-ploidy cross was found as an effective way to induce variation in the progenies with extensive ploidy level segregation, including triploids, tetraploids, pentaploids, heptaploids, octaploids, and aneuploids (Johnsson, 1940; Ozaki et al., 2004; Rao et al., 2012). In this study, interploidy hybridization between the mixoploid (2x+4x) female with the diploid and mixoploid male resulted in segregation of ploidy levels among progeny, with diploids, triploids, and tetraploids. However, the progeny ploidy level was detected by flow cytometry using leaf sample where triploid showed three peaks similar to mixoploid parent (Fig 7 B) that prompt us to further assume the evolution of such ploidy progeny. As this progeny was developed from the inter and intra-ploidy crosses so there is rare scope of mixoploid segregation. Therefore, this progeny could be considered as the putative triploid with mixed cells of diploids, tetraploids and octaploids and derived due to the compensated aneuploids. Such compensated aneuploids play important roles in polyploidy breeding with maintaining genome balance, overcome sterility and chromosome stability (Birchler et al., 2005; Birchler and Veitia, 2012) that extensively studied in wheat (Law et al., 1987). Wang et al. (2017) inferred 18 compensated triploid progenies of poplar those were unlikely to contain three integrated chromosome sets owing to unbalanced segregation of meiotic chromosomes in triploids. Similar result was also reported by Beatson et al. (2003) in hop and Varela-Alvarez et al. (2018) in the genus Porphyra where triploid produce gametes of different ploidy levels act as bridge among cytotypes. Interestingly, similar features of our present finding on triploid flow cytometry (FCM) profile (Fig. 7B) was also concomitant the tetraploid hop (Galena-4n) which is widely used as tetraploid parent in Australian hop breeding program. Even though this genotype showed a mixoploid FCM profile when using leaf material and a tetraploid FCM profile when using root materials and reproductive tissues. Koutoulis et al. (2005) claimed the possibilities of such differences include parthenogenesis or unreduced gamete with aneuploidy.

In the present study, no triploid progeny was generated in Diploid × Mixoploid [2x × (2x+4x)] cross as observed in the two other mixoploid parents mediated crosses (Table 6), indicating that 2n pollen of mixoploid male might be weakly competed with haploid (n) gamete of diploid seed parent during fertilization. It might happen due to the slower growth rate and maturity of 2n pollen than normal haploid gamete. These results support the observations of Kang and Zhu (1997) about low rate of acquired triploid from a crossing using monoploidy pollen (1n) and 2n pollen in White populars. They reported that the germinating process of 2n pollens was

delayed than that of 1n pollens, and 2n pollens had weaker competition in mixed pollination than 1n ones, responsible for developing very low rate of triploid in White populars. Accordingly, the similar trend of pollen viability reduction in tetraploid genotypes than that of the corresponding diploid genotypes were demonstrated by Aleza et al. (2012) in Citrus. As the pollen mother cells degenerate before the reduction division during meiosis in tetraploids much more frequently than those of corresponding diploids (Frost and Soost, 1968). Meanwhile, Van Breukelen (1982) claimed the existence of interploidy certation in Solanum tuberosum showed 2x pollen grew faster than x pollen, both in 4x and 2x styles that enhances the high number of 4x (tetraploid) hybrids production from S. tuberosum × S. phureja crosses. This factor might be responsible in the present study of tetraploid production when mixoploid females were crossed with a mixoploid male.

Although this study does not identify whether colchicine treatment correlated with pollen morphology, cytology of reproductive organs, exact mechanism resulting in the unreduced (2n) gametes and aneuploids involvement in mixoploid pointed gourd. However, the present findings provide a solid framework for follow up genetic and advanced functional analysis of 2n gamete formation in colchicine induced mixoploid in our future work.

5. Conclusion

Overall, the results of our study highlighted the first report of the *in vivo* separation technique of colchicine induced mixoploid into triploid and tetraploid progenies of pointed gourd (*T. dioica*). It can provide a better substitute for the sophisticated *in vitro* mixoploid isolation methods used in different breeding programs. The isolated triploid and tetraploid advanced genetic resources are the most desirable approaches for genetically stable triploid breeding program in pointed gourd (*T. dioica*) to facilitate the production of seedless fruits. This output would improve the overall quality of produce and fetches a premium price in the market. Finally, the vegetative propagation option in *T. dioica* will assist to make these advanced triploid and tetraploid resources available for the breeders and scientists for future genetic studies and improvement of pointed gourd polyploidy breeding.

6. Author Contributions:

J. Hassan, conceived the idea of the study, designed experiment, performed the research and data analysis, and wrote the manuscript. I. Miyajima and Y. Ozaki supervised the work, provided suggestions and comments on the manuscript. Y. Mizunoe and K. Sakai assisted in sample preparation and laboratory analysis. All authors have read, edited the manuscript and approved for submission.

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645

646 **Figure caption**

647 **Fig. 1** Hypothetical diagram of generating homogeneous triploid and tetraploid progeny from the colchicine
648 induced mixoploid of pointed gourd (*Trichosanthes dioica* Roxb.). (A) Mature diploid seeds (2n=2x=22), (B)
649 Colchicine treatment of seeds, (C) Flow cytometry (FCM) confirmed polyploidization representing mixoploid
650 and tetraploid induction, (D) Tetraploid females pointed gourd evident to produce less-seeded fruit production
651 crossed with diploid male (Hassan et al., 2020), (E) Mixoploids (female and male) used in inter and intra-ploidy
652 crosses with diploid counterparts, (F) Mixoploid female pollinated with diploid and mixoploid male produced
653 F1 seeds, those indicated mix of diploid and tetraploid seeds (almost twice in diploid size), (G) F1 progeny
654 establishment after germination of different ploidy crosses seeds, (H) Homogeneous triploid (3x) and tetraploid
655 (4x) progeny isolation confirmation after ploidy level analysis of the sexually derived seedlings by FCM.

656

657 **Fig. 2** Histograms of the relative nuclear DNA content obtained from the flow cytometry (FCM) analysis of the
658 colchicine treated pointed gourd (*Trichosanthes dioica* Roxb.) seeds generated seedlings. (A) Diploid plant
659 (control); (B) Tetraploid plant; (C) Mixoploid plant.

660

661 **Fig. 3** Mixoploid induction efficiency percentage (MEP) influenced by colchicine treatments of pointed gourd
662 seeds at different exposure times. (A) MEP at different colchicine concentrations indicated as C1=0.0%
663 (Control); C2=0.05%; C3=0.1%; C4=0.5%. (B) MEP at different exposure times of colchicine treatment
664 indicated as ET1=24h; ET2=48h; ET3=72h. (C) Interaction effect of colchicine concentrations and exposure
665 times on MEP. Each box plot visualizes the distribution of MEP data influenced by colchicine concentration and
666 exposure duration explaining median (middle line), first and third quartile (lower and upper edge of box),
667 minimum and maximum value (the bottom and top of the box). Boxplot itself represents the middle 50% of the
668 data and significant variability observed comparing the boxplots.

669

670 **Fig. 4** Seed characteristics of inter and intra-ploidy cross combinations. Accession indicated as Diploid=
671 Diploid×Diploid; Dmixo= Diploid×Mixoploid; Mixod= Mixoploid×Diploid; Mixo= Mixoploid×Mixoploid; [z]
672 Tetra= Tetraploid×Tetraploid, [z] Tetraploid cross generated seed traits was used as standard to compare with the
673 other inter and intraploidy crosses produced seed traits. (A) Individual seed diameter (SD); (B) Individual seed
674 weight (SW).

675

676 **Fig. 5** Density plot of individual seed diameter (SD) and seed weight (SW) of inter and intra-ploidy cross
677 combinations. Accession indicated as Diploid= Diploid×Diploid; Dmixo= Diploid×Mixoploid; Mixod=
678 Mixoploid×Diploid; Mixo= Mixoploid×Mixoploid; [z]Tetra= Tetraploid×Tetraploid, [z] Tetraploid cross generated
679 seed traits was used as standard to compare with the other inter and intraploidy crosses produced seed traits.

680

681 **Fig. 6** Principal component analysis (PCA) of seed traits of inter and intra-ploidy cross combinations. Groups
682 indicated as Diploid= Diploid×Diploid; Dmixo= Diploid×Mixoploid; Mixod= Mixoploid×Diploid; Mixo=
683 Mixoploid×Mixoploid; [z] Tetra= Tetraploid×Tetraploid, [z] Tetraploid cross generated seed traits was used as
684 standard to compare with the other inter and intraploidy crosses produced seed traits. The data obtained from
685 individual seed diameter (Dim1) and seed weight (Dim2) of 603 seeds generated from different crosses across
686 three ploidy levels (diploid, mixoploid and tetraploid).

687

688 **Fig. 7** Histograms of the flow cytometry (FCM) confirmed the homogeneous triploid and tetraploid progeny
689 development after ploidy level analysis of the sexually derived F_1 seedlings of mixoploid involved ploidy
690 crosses seeds of pointed gourd (*Trichosanthes dioica* Roxb.). (A) Diploid progeny; (B) Triploid progeny; (C)
691 Tetraploid progeny.

692

Fig. 1

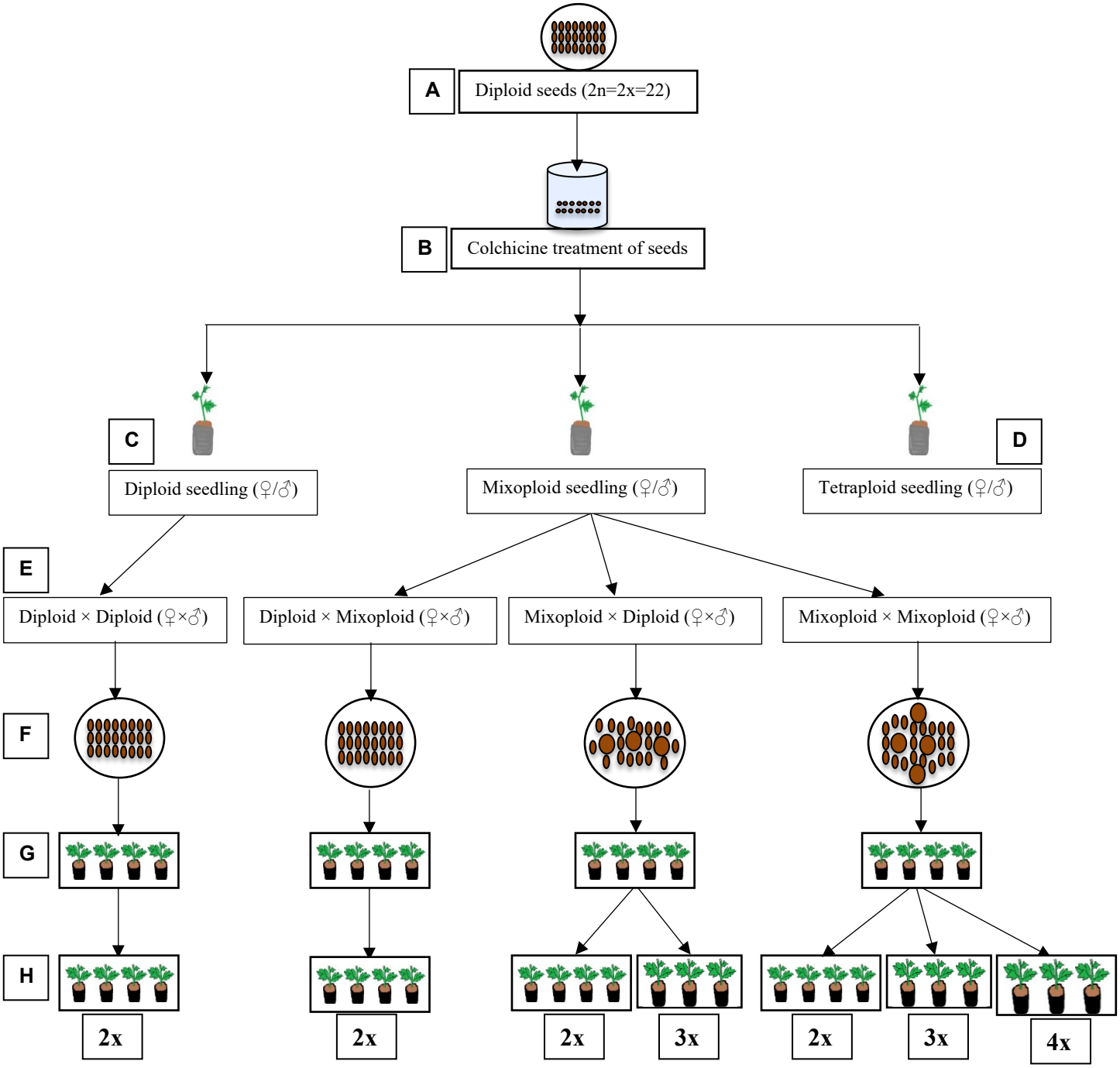
A
Diploid seeds (2n=2x=22)
B
Colchicine treatment of seeds
C
Diploid seedling (♀/♂)
Mixoploid seedling (♀/♂)
D
Tetraploid seedling (♀/♂)
E
Diploid × Diploid (♀×♂)
Diploid × Mixoploid (♀×♂)
Mixoploid × Diploid (♀×♂)
Mixoploid × Mixoploid (♀×♂)
F
G
H
2x
2x
2x
3x
2x
3x
4x

Fig. 2

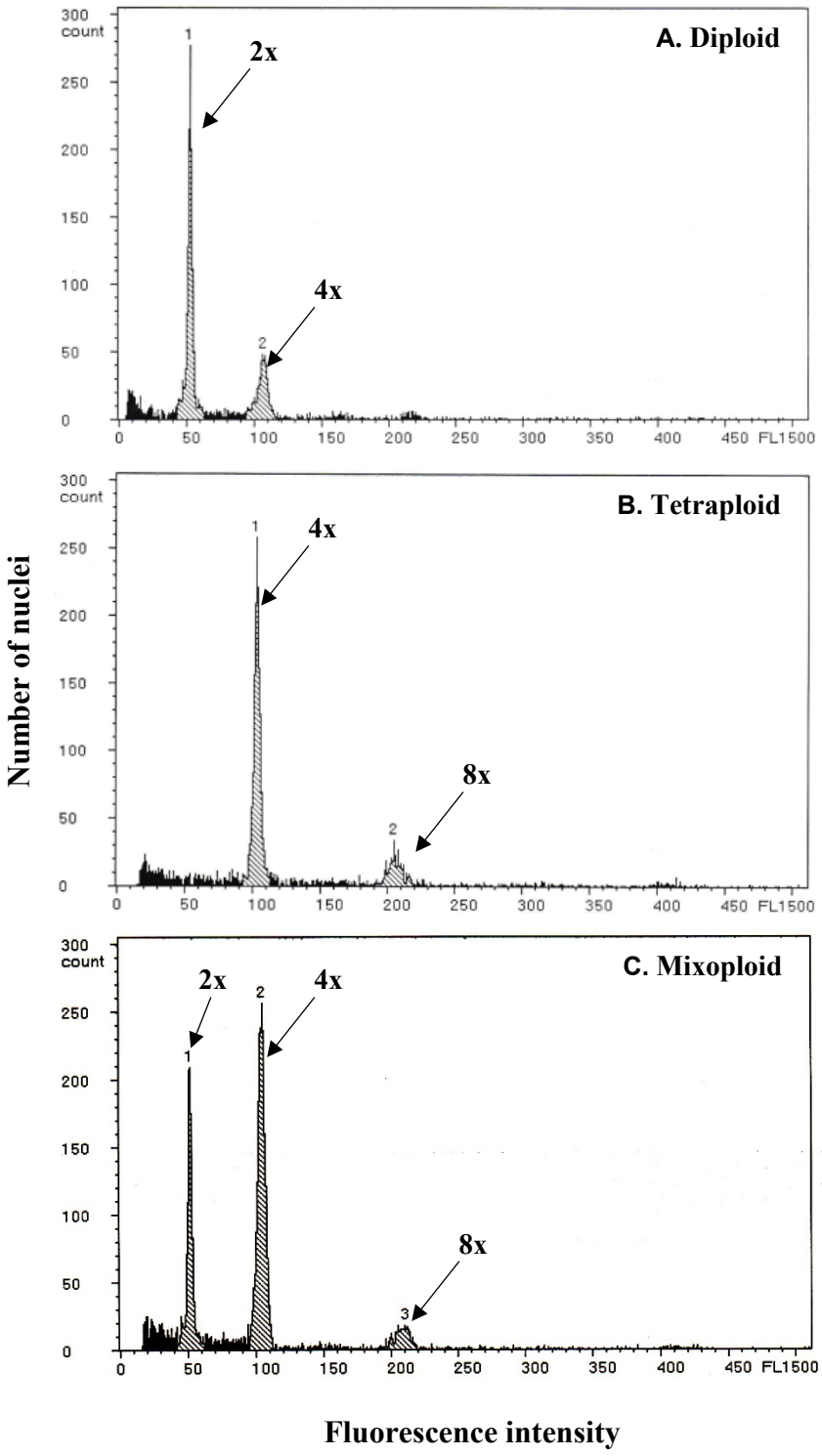
A. Diploid
2x
4x
B. Tetraploid
4x
8x
C. Mixoploid
2x
4x
8x
300
count
250
200
150
100
50
0
0
50
100
150
200
250
300
350
400
450
FL1500
Number of nuclei
Fluorescence intensity

Fig. 3

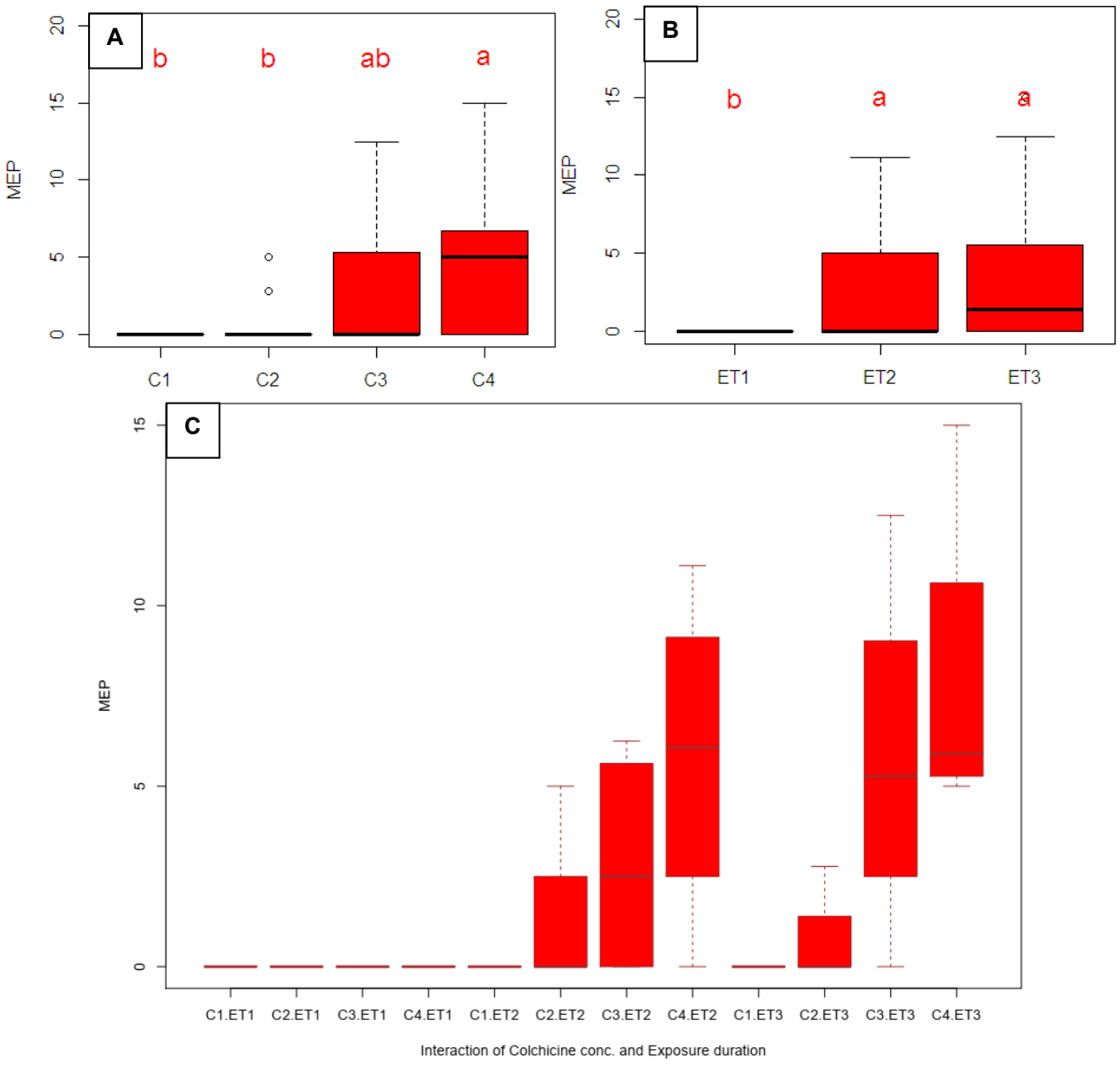
A
b
b
ab
a
MEP
0
5
10
15
20
C1
C2
C3
C4
B
b
a
a
MEP
0
5
10
15
20
ET1
ET2
ET3
C
MEP
0
5
10
15
C1.ET1
C2.ET1
C3.ET1
C4.ET1
C1.ET2
C2.ET2
C3.ET2
C4.ET2
C1.ET3
C2.ET3
C3.ET3
C4.ET3
Interaction of Colchicine conc. and Exposure duration

Fig. 4

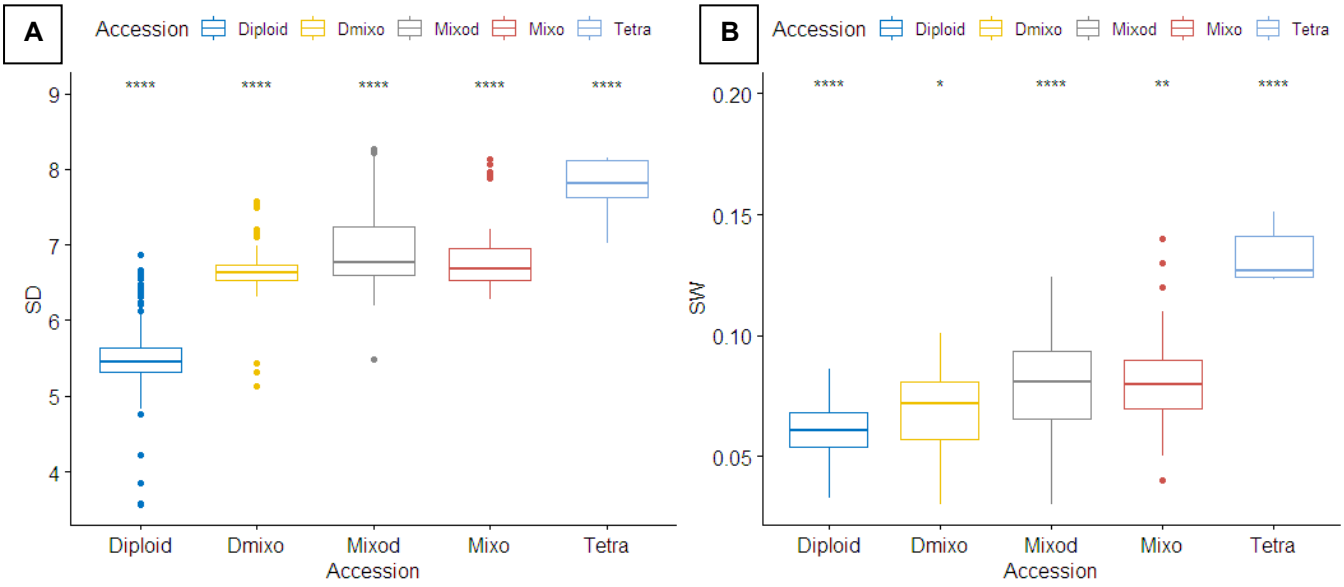
A
Accession
Diploid
Dmixo
Mixod
Mixo
Tetra
SD
Diploid
Dmixo
Mixod
Mixo
Tetra
Accession
B
SW

Fig. 5

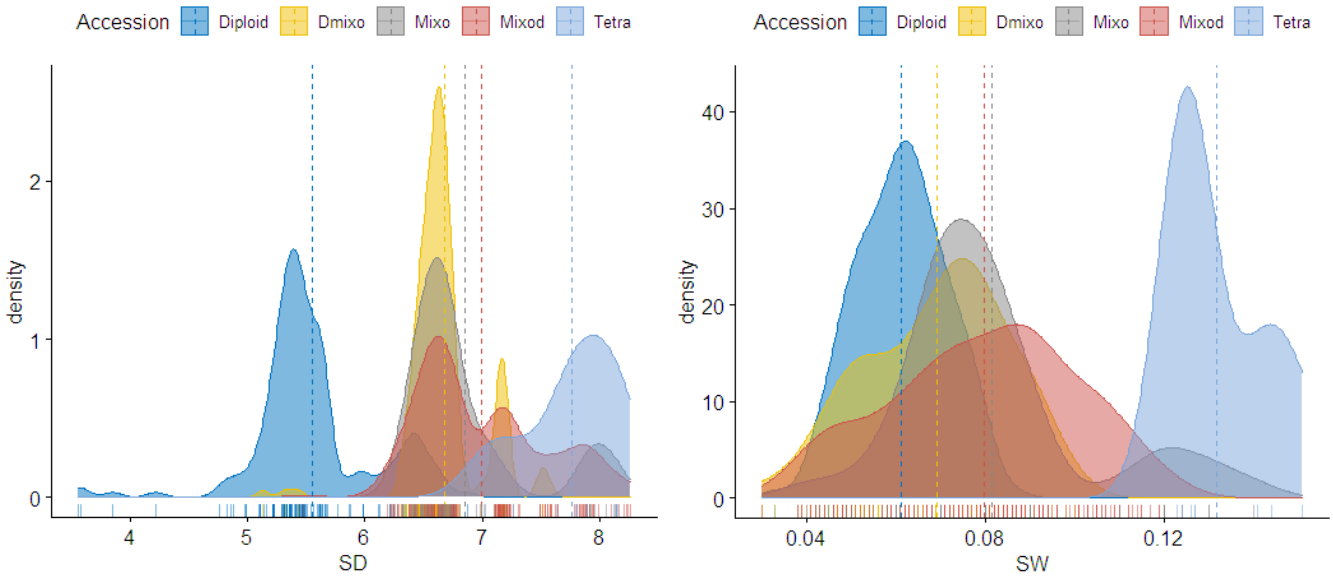
Accession
Diploid
Dmixo
Mixo
Mixod
Tetra
density
SD
SW
0
1
2
4
5
6
7
8
10
20
30
40
0.04
0.08
0.12

Fig. 6

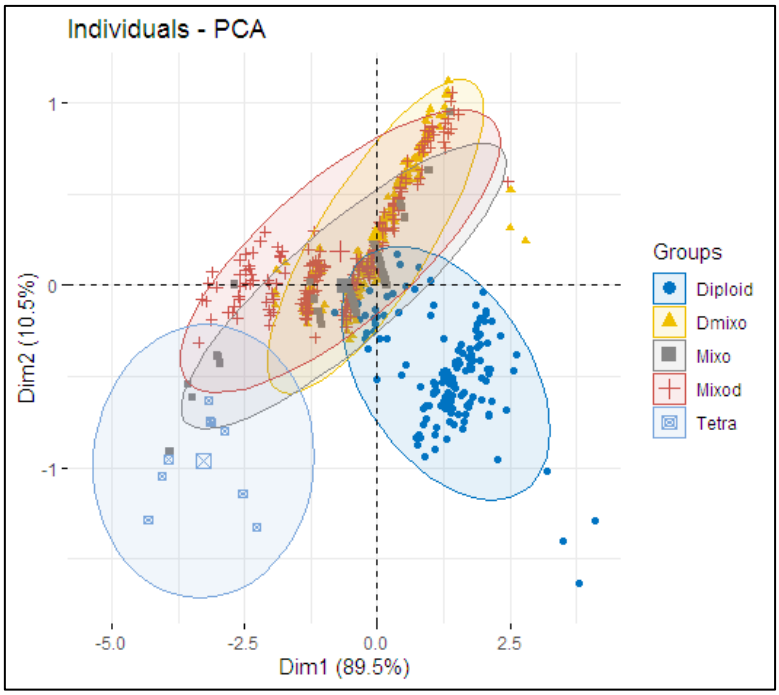
Individuals - PCA
Dim2 (10.5%)
Dim1 (89.5%)
1
0
-1
-5.0
-2.5
0.0
2.5
Groups
Diploid
Dmixo
Mixo
Mixod
Tetra

Fig. 7

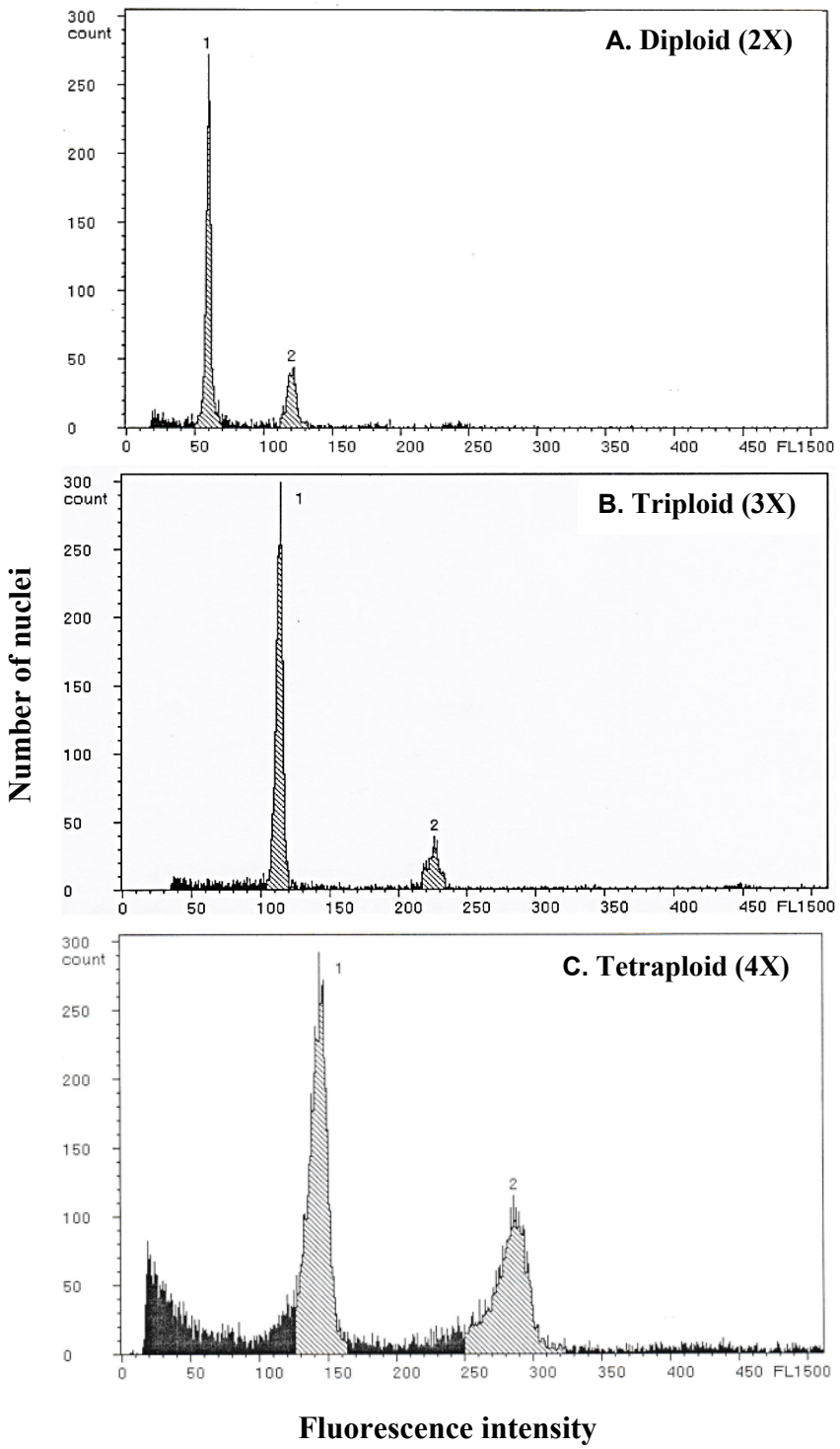
A. Diploid (2X)
B. Triploid (3X)
C. Tetraploid (4X)
300
count
250
200
150
100
50
0
1
2
0 50 100 150 200 250 300 350 400 450 FL1500
Number of nuclei
Fluorescence intensity

Table 1 Effect of colchicine concentrations and exposure durations on the seedling survival rate and tetraploid induction of pointed gourd (*Trichosanthes dioica* Roxb.).

Colchicine concentration (%)	Survival rate (%) [z]			Mixoploid number			Tetraploid number			Tetraploid induction efficiency (%) [y]		
	Exposure duration (h)			Exposure duration (h)			Exposure duration (h)			Exposure duration (h)		
	24	48	72	24	48	72	24	48	72	24	48	72
0.0	90.0±1.6a [x]	85.5±4.0a	69.7±1.5b	0.0±0.0b	0.0±0.0b	0.0±0.0b	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
0.05	6.0±2.3e	19.0±1.1d	34.2±2.0c	0.0±0.0b	0.2±0.5ab	0.2±0.5ab	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
0.1	5.0±1.1e	18.5±3.0d	21.7±3.3d	0.0±0.0b	0.5±0.5ab	1.2±1.2ab	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
0.5	6.5±1.9e	16.0±3.6d	18.5±1.9d	0.0±0.0b	1.0±0.8ab	1.5±1.0a	0.0±0.0	0.2±0.5	0.7±1.5	0.0±0.0	1.2±2.5	3.7±7.5

[z] Hundred (100) seeds per treatment were used in the germination test after colchicine treatment for four repetitions and seedling survival data recorded after six week of germination.

[y] Ploidy induction efficiency (%) = $\frac{\text{No.of induced polyploidy plant}}{\text{Survived Seedling}} \times 100$

[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 (HSD) test using the R software.

Table 2 Cross compatibility of mixoploids involved inter and intra-ploidy crosses based on fruit set and seed characteristics

Cross combinations (♀ × ♂) [z]	Fruit set (%)	Number of seeds/fruit	100-seeds weight (g)	Seed germination (%) [x]
Diploid × Diploid	100±00.0a [y]	26.4±0.9a	6.4±0.3b	100.0±0.0a
Diploid × Mixoploid	60.0±20.0ab	19.7±0.4b	7.4±0.5ab	96.6±0.5a
Mixoploid × Diploid	86.6±11.5a	18.5±0.7b	8.5±0.6a	60.0±0.0b
Mixoploid ×Mixoploid	33.3±11.5b	20.3±1.0b	7.9±0.2a	53.3±0.5b

[z] Fifteen (15) female flowers were pollinated per cross for three repetitions.

[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 (HSD) test using the R software.

[x] Ninety (90) seeds of each cross were used in the germination test for three repetitions.

Table 3 Flow cytometry confirmed pure triploid and tetraploid progeny derived from mixoploid involved ploidy crosses

F1 seedling population [z] (♀ × ♂)	Ploidy level of FCM identified progeny (%) [y]			Total number of seedlings tested
	2x (Diploid)	3x (Triploid)	4x (Tetraploid)	
Diploid × Diploid	30 (100.0) [x]	-	-	30
Diploid × Mixoploid	29 (100.0)	-	-	29
Mixoploid × Diploid	15 (83.3)	3 (16.7)	-	18
Mixoploid × Mixoploid	10 (62.5)	3 (18.7)	3 (18.7)	16
Ploidy total	84	6	3	93
Ploidy (%)	90.32	6.45	3.23	

[x] Data represent average value of three replicates (n=30 seeds of each cross were used for germination test in one replication).

[y] Flow cytometry (FCM) confirmed progeny with respective ploidy level and the progenies frequency % of total tested seedlings is in the parenthesis. Dashes indicate that not such category ploidy level identified.

[z] Sexually derived F1 seedling population of inter and intra-ploidy crosses of mixoploid and diploid counterparts; Dip, Mixo represent as Diploid and Mixoploid.

Supplement Table 1 Colchicine concentrations efficiency on survival rate and ploidy induction of pointed gourd (*Trichosanthes dioica* Roxb.)

Colchicine concentration (%)	Survival rate (%) [z]	Mixoploid number	Tetraploid number	Tetraploid induction efficiency (%) [y]
0.0	81.7±9.3a [x]	0.0±0.0b	0.0±0.0 [ns]	0.0±0.0 [ns]
0.05	19.7±12.1b	0.1±0.3b	0.0±0.0	0.0±0.0
0.1	15.1±7.9c	0.5±0.9ab	0.0±0.0	0.0±0.0
0.5	13.6±5.8c	0.8±0.9a	0.3±0.8	1.6±4.4

[z] Hundred (100) seeds per treatment were used in the germination test after colchicine treatment for four repetitions and seedling survival data recorded after six week of germination.

[y] Ploidy induction efficiency (%) = $\frac{\text{No.of induced polyploidy plant}}{\text{Survived Seedling}} \times 100$

[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 (HSD) test using the R software.

Supplement Table 2 Exposure time efficiency on survival rate and ploidy induction of pointed gourd (*Trichosanthes dioica* Roxb.)

Exposure time of colchicine treatment (h)	Survival rate (%) [z]	Mixoploid number	Tetraploid number	Tetraploid induction efficiency (%) [y]
24	26.8±37.6b [x]	0.0±0.0b	0.0±0.0 [ns]	0.0±0.0 [ns]
48	34.7±30.4a	0.4±0.6ab	0.1±0.2	0.3±1.2
72	36.0±21.0a	0.7±1.0a	0.2±0.7	0.9±3.7

[z] Hundred (100) seeds per treatment were used in the germination test after colchicine treatment for four repetitions and seedling survival data recorded after six week of germination.

[y] Ploidy induction efficiency (%) = $\frac{\text{No.of induced polyploidy plant}}{\text{Survived Seedling}} \times 100$

[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 (HSD) test using the R software.

Supplement Table 3 Sex differentiation of colchicine treated seed induced mixoploids and tetraploids pointed gourd (*Trichosanthes dioica* Roxb.)

Colchicine treated seed induced polyploids	Total number of plants	Sex differentiation of colchicine induced mixoploids and tetraploids	
		Female plants	Male Plants
Mixoploid	19	9	10
Tetraploid	4	2	2