In Vitro Tetraploidization Towards Enhancement Of Charantin Biosynthesis In *Momordica Charantia* (L.)

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Abstract
A practical and reliable method for *in vitro* tetraploidization of Bitter Gourd [*Momordica charantia* (L.)] has been established to enhance the production of charantin. Shoot tip from *in vitro*-grown culture (2n = 22) were exposed to the anti-mitotic chemical, i.e., colchicine, at various concentrations (0, 0.01, 0.05, 0.1, 0.2 and 0.3% along with 2% DMSO) for 12, 24, 36, and 48 h. The treated explants were then incubated and proliferated on Murashige and Skoog (MS) medium fortified with 1.5 mg L^{-1} benzyladenine and 0.5 mg L^{-1} naphthalene acetic acid, followed by root induction in 1.0 mg L^{-1} indole-3 acetic acid enriched 1/2MS medium. Treatment of shoot tips with 0.1% colchicine for 24 h supported the highest tetraploid induction efficiency (33.56 ±0.22%). Morphological, stomatal, and cytological characteristics along with the secondary metabolite content of the in vitro tetraploids were compared to that of diploids. The recovered tetraploid plants possessed superior plant height, stem diameter, leaf size, and increased length and width of stomata but decreased stomatal frequency. The tetraploid plants demonstrated twice the chromosome number (2n = 4x = 44) in respect to diploids as confirmed through cytology, spectrophotometry and flow cytometry. High-performance liquid chromatography showed 1.09 times enhancement of charantin content in tetraploid plants than that of diploid plants, signifying the prospective of this technique for the trade value improvement.

Keywords: Colchicine; Charantin; HPLC; Momordica charantia; Tetraploid

INTRODUCTION

Momordica charantia (L.) is a herbaceous, tendril bearing vine plant that may grown largely in tropical and subtropical climates of northern hemisphere. It is widely known for its edible fruit that is distinctly marked for its bitterness. Hereby, *Momordica charantia* (L.) belonging to the family Cucurbitaceae, considered being an old world species has long been used as a food and medicine (Rao, 2021). And is also known by many names such as *Bitter melon, Balsam pear, Bitter cucumber, or Bitter gourd, Karela, Fukwa and Ampalaya* etc. It is native to eastern India and southern China. It is found in tropical regions such as parts of the Amazon, eastern Africa, Asia and the Caribbean (Dey *et al.*, 2006) and also exhibits wide diversity. This vegetable is low in calories (17 calories/ 100 g fresh) but very rich in phyto-nutrients like, vitamin C, β-carotene, vitamin E, thiamin, riboflavin, minerals, different anti-oxidants, several other nutraceuticals and dietary fibre (Krishnendu and Nandini, 2016). It has also been found useful as antimalarials, antiseptics, bitter carminatives, antipyretic (reduces fever), hyposensitivity (reduces blood pressure), promotes milk flow, menstrual stimulator, laxative, vermifuge (increases removal of worms) and wound healing agent. It is also used to treat several other medical conditions such as dysmenorrhoea, eczema, emmenagogue, galactagogue gout, jaundice, kidney (stones), leprosy, leucorrhoea, hemorrhoids, pneumonia, rheumatism and scabies. (Grover and Yadav, 2004). There have been studies in the past where consumption of raw or juice form of *Momordica charantia* has been effective in lowering the blood glucose level. Whilst further research is required to ascertain the efficacy of tablet or capsule form of *Momordica charantia*, the usage of its concentrated extract in juice form has had a measurable improvement in lowering blood glucose levels. Bitter melon contains many pharmacologically active compounds such as momordicine and charantin (Mahmood *et al.*, 2012). It also contains a hypoglycemic agent called charantin which is a 1:1 mixture of stigmasterol glucoside (STG) and β-sitosterol glucoside (BSG) and has a hypoglycemic effect (lowering of blood sugar level) like insulin (Singh *et al.*, 2011). Charantin increases glucose uptake and glycogen synthesis in liver, muscle and adipose tissue cells and increases the number of pancreatic β-cells, such healthy body helps to produce insulin (Shetty *et al.*, 2005) thereby leading to the treatment of type 2 diabetes (Grover and Yadav, 2004). Clinical studies have also confirmed the benefits of *M. charantia* in treating diabetes. This is mainly due to the presence of charantin, a steroidal saponin that lowers blood glucose levels in both normal and diabetic rabbits (Raman and Lau, 1996). Because of its

pharmacological importance, charantin has already been isolated from chloroform extracts of dried fruits by HPTLC (Patel *et al.*, 2006), and ethanol/ water extracts of *M. charantia* from leaves and fruits by HPTLC and TLC (EI- and Al-Barak, 2011).

Compared to diploid species, polyploids have been shown to have advanced morphology and greater resilience to environmental stress (Kaensaksiri *et al.*, 2011). Furthermore, genome multiplication appears to increase the production of secondary metabolites in pharmacologically important medicinal plants, both quantitatively and qualitatively (Majdi *et al.*, 2010; Zahedi *et al.*, 2014; Javadian *et al.*, 2017), as it also results in higher gene expression. For that reason, the aim of this study was set to produce tetraploids of *M. charantia* from diploid individuals and compare the diverse morphological, cytological and phytochemical features.

MATERIALS AND METHOD

Induction of polyploidy

One centimetre long ST explants were cut off from the *in vitro* grown plantlets from seeds and considered as explants for polyploidy induction. They were immersed in liquid MS medium containing filter sterilized colchicine (Sigma-Aldrich®) (0, 0.01, 0.05, 0.1, 0.2 and 0.3%; *w/v*) along with 2% (*v/v*) DMSO (Sigma-Aldrich®) and constantly shaken at 100 rpm. After 12, 24, 36, and 48 h, the explants were washed with sterilized water to get rid of colchicine and incubated on multiplication medium containing MS nutrients, 1.5 mg L^{-1} BA, 0.5 mg L^{-1} NAA, 30 g L^{-1} sucrose and 8 g L^{-1} agar. To initiate root formation, the regenerated shoots were cultured on ½MS medium involving 1.0 mg L^{-1} IAA, 30 g L^{-1} sucrose and 8 g L^{-1} agar. Sixty explants were cultured per treatment and each treatment was repeated thrice. The records on percentage of survival, tetraploid induction and efficiency of tetraploidy induction [explant survival (%) × tetraploidy induction (%)] were taken after 30 days of culture.

Verification of polyploidy induction

To categorize the tetraploid plants, quantification of total DNA content as well as chromosome counting was carried out. Total genomic DNA was extracted from ten plantlets (taking 40 mg leaves), regenerated from each of colchicine treated and control explants and determined by spectrophotometer (Systronics®, Ahmedabad, India) at 260 nm. Extraction of genomic DNA was carried out following the CTAB method (Doyle and Doyle, 1990). DNA concentration was estimated by UV spectrophotometry at 260 nm. Subsequently, number of chromosomes in induced polyploids as well as control mother plants was verified by microscopic examination. About 0.5 cm in length, 15 root tips (RTs) were cut out from each of putative polyploid along with control diploid plants and fixed in Carnoy's solution (ethanol : glacial acetic acid, 3:1) followed by storage at 5°C for 24 h. After rinsing properly in water, RTs were macerated in 1 N HCl for 15 min at 60°C. Staining of the fixed RTs was done with 1% (*w/v*) aceto-carmine keeping for 30 min and squashed underneath a cover slip. Ten cells per sample were observed at metaphase stage under Auxioscope (Carl Zeiss, Germany).

Flow cytometry analysis

For accurate confirmation, flow cytometry was performed using leaf tissue (1.0 cm^2) obtained from both control and *in vitro* colchicine treated regenerants (taking 10 samples in three replications). A nuclei suspension was prepared by chopping the leaf tissues in a 500 mL of modified Galbraith's nuclei isolation buffer (200 mM Tris, 4 mM $MgCl_2.6H_2O$, pH 7.5, 0.5% Triton X-100) (Galbraith *et al*., 1983), followed by the addition of 500 mL 4,6-diamino-2-phenylindole for DNA staining. The staining solution also contained RNase to avoid RNA interferences. After 2 min incubation, the suspension was strained through a 30-µm nylon filter to remove cell debris. The samples were analyzed with a partec PA-I flow cytometer (Partec GmbH, Munster, Germany) and DNA histograms were made.

Phenotypic characterization of polyploid plants

After the verification of polyploid plants, employing the above mentioned direct methods, 25 plantlets each of diploids and polyploids were then categorized as per several morphological traits. The traits included diameter of the stem, length and width of petiole and length and width of lamina.

Stomatal studies

Pieces of epidermal layer were pealed from the lower leaf surface of *in vitro* tetraploid and diploid plants for stomatal analysis. With one drop of distilled water taken on a glass slide, these epidermal layers were mounted and measured the stomatal sizes microscopically under Auxioscope equipped with a linear micrometre. To estimate the stomatal length and width, 25 stomata were arbitrarily chosen from 5 leaves per plantlet and to determine the stomatal frequency (number of stomata per microscopic field) 25 microscopic field areas were considered from each of the 5 leaves per plantlet.

Extraction and estimation of charantin content from diploid and tetraploid leaves of BG1346501

For the phytochemical assessment, leaves of *in vitro* grown diploid and tetraploid plants were shade dried and pounded. It was followed by procedure as described by Pitipanapong *et al.* (2007). The charantin was analyzed using an HPLC

system (Symmetry® C18 column (75 mm × 4.6 mm, 3.5 µm) with a UV detector at 204 nm). The separation was performed on an Symmetry® C18 column with a flow rate of 0.8 mL min^{-1}. The mobile phase used was 98% MeOH and 20 µL was injected for each sample. Sample aliquots were filtered through a 0.45 µm poly (tetrafluoroethylene) filter prior to injection. All samples were run in triplicate. Identification and quantification of charantin were carried out by comparing the retention times and the peak areas, respectively, with standards. Reference standards of STG (purity 95.1% w/w) and BSG (purity 98% w/w) were purchased from Natural Remedies Pvt. Ltd. Bangalore, India. HPLC analysis of samples were performed in triplicates from the same sample.

Statistical analysis

All the conducted experiments were set in a Completely Randomized Design and the recorded data were statistically computed through one-way Analysis of Variance. The means of the data were analysed by Duncan's multiple range test (Duncan, 1955) executed at 5% level of probability via SPSS software (Version 16, SPSS Inc. Chicago, USA).

RESULTS AND DISCUSSION

Survival rate of colchicine treated explants (ST)

The different concentration and duration of colchicine treatment exhibited significant difference among the survivability of the explants (Fig. 1). The condensed regeneration rate of the explant is the primary noticeable outcome of colchicine treatment. The reduced growth rate may be because of colchicines adverse effect on cell division. However, this effect was observed to be temporary and the regeneration rate was regained after two to three sub-cultures. Shoot tips were used as an explants, bud regeneration was quicker in ST (3-4 days). The rapid response of ST may be due to the occurrence of actively dividing apical meristem. Nevertheless, the survival percentage of the explants decreased with the increase in the dosage and duration of colchicine treatment. The highest survival percentage for the ST (93.3 0.96%) was recorded with the treatment of 0.01% of colchicine for 12 h, and the lowest was found at 0.5% of colchicine treated for 36 and 48 h. The resultant slow growth and inverse correlation between the survival of explant and colchicine concentration was predictable and in harmony with other plant species, for instance, *Lagerstroemia indica* (Zhang *et al.*, 2010b); *Echinacea purpurea* (Nilanthi and Yang, 2013); *Trachyspermum ammi* (Noori *et al.*, 2017). Low survival was not a disadvantage as the recovered tetraploids were multiplied to a large scale by micropropagation.

Polyploid induction and its verification

Colchicine is a conventional antimitotic agent that has been successfully employed since long time to induce polyploidy in several medicinal plants (Salma et al., 2017). The proper combination of colchicines concentration and duration of treatment is the chief factor that significantly induced tetraploids in the current study (Fig. 2). The ploidy level of the regenerated *M. charantia* plantlets was initially confirmed by spectrophotometry (Fig. 3, a) followed by chromosome counting (Fig. 3, b) after 30 days of colchicine treatment. Estimation of total DNA content distinguished that the plants regenerated from colchicine-treated explants contained higher quantity of DNA (0.51 mg mL^{-1}) than the untreated ones (0.29 mg mL^{-1}), whereas, the mixoploid individuals recorded the DNA content of 0.38 mg mL^{-1}. Considering the spectrophotometric analyses, the highest tetraploid induction efficiency was attained at the colchicine concentration of 0.1% when treated for 24 h in case of ST (33.56 0.22%) explants. It was noted that colchicine at concentration higher than 0.1% also increased the tetraploid rates but simultaneously, the mortality rate also increased. Hence, tetraploid induction efficiency is the appropriate parameter to calculate tetraploidization for the reason that it takes into account both the survival as well as tetraploid induction rates (Abdoli *et al.*, 2013). The verification by determining the chromosome number of the tetraploid and diploid plantlets was performed following the suggestion of many researchers (Widoretno, 2016 and Noori et al., 2017). The tetraploid plantlets showed 2n = 4x = 44 chromosomes (Fig. 3, b-ii) in the RT cells whereas the control diploid plantlets showed 2n = 2x = 22 chromosomes (Fig. 3, b-i), as formerly reported by Verma et al. (2016).

Analysis by FCM further confirmed the tetraploid plants that were earlier identified by spectrophotometry and chromosome counting. Predominantly, FCM gave two kinds of peaks at different positions determining the chromosome duplication by colchicine treatment (Fig. 4). Peak position in the tetraploid plants was twice than that of the diploid plants which corresponds to 4x (Fig. 4, b) and 2x (Fig. 4.4, a), respectively. FCM is one of the quick and reliable methods used widely to verify the ploidy induction in different medicinal plants, for instance, *Thymus persicus* (Tavan *et al.*, 2015), *T. ammi* (Noori *et al.*, 2017) *etc*. STs are the majorly employed explant as it includes actively dividing apical meristem and might also more porous to colchicine. The outcome of this research are in agreement with the reports of Acanda *et al.* (2015), Yan *et al.* (2016) and Javadian *et al.* (2017) that suggested the treatments with lower colchicine concentrations for higher duration for obtaining maximum frequency of polyploidization.

Phenotypical differences between diploid and tetraploid plants

In the current research, significant variations in the phenotype between tetraploid and diploid plants were evidenced (Table 1; Fig. 5). Most of the time, the first emerged leaves of tetraploids had a deformed contour (Fig. 5, b), but subsequently with the progress of subculture the leaves come out normal. The mean diameter of stem is higher in tetraploid (1.29 0.00 cm) than diploid (0.89 0.01 cm). The average length and width of the petiole is higher in tetraploid

(3.24 0.06 cm and 0.33 0.00 cm, respectively), than in diploid (2.10 0.75 cm and 0.14 0.00 cm, respectively). The average mean of length of lamina is more in tetraploid (3.17 0.04 cm) than diploid (1.65 0.03 cm). The average mean of width of lamina is more in tetraploid (4.43 0.13 cm) than diploid (2.31 0.04 cm). The vigorous morphological features in comparison to the diploids for diameter of the stem and leaf size recorded here in tetraploid *M. charantia* corroborates to the former reports in *Pogostemon cablin* (Yan *et al.*,2016), *Trollius chinensis* (Zhang *et al.,* 2016) and *T. ammi* (Noori *et al.,* 2017). Contrary to our findings, some researchers reported lower plant height of tetraploid plants in relation to the diploids (Omidbaigi *et al.,* 2010; Yan *et al.,* 2016). Even, leaves were found smaller in length and insignificant leaf width in tetraploids in comparison to diploids (Pansuksan *et al.,* 2014). But, Shao *et al.* (2003) accounted that higher plant height, thicker stems or enlarged length-to-width leaf ratios, are essential markers for selection of putative tetraploids.

Stomatal size variation

An assessment on stomatal features showed that the size of stomata and stomatal frequencies were inversely proportional in relation to the ploidy difference (Table 2). The mean length and width of stomata in tetraploids (43.41 0.27µm; 33.62 0.09 µm, Fig. 3, c-ii) was almost double than the diploids (23.50 0.1µm; 18.45 0.09 µm,
Fig. 3, c-i), whereas the average stomatal frequency in tetraploid plants (Fig. 3, d-ii) was lower than diploids (Fig 3, d-i). However, no morphological differences in stomatal shape were noticeable between the tetraploid and diploid plantlets. These findings demarcated that doubling the genome can chiefly alter the stomata characteristics and appear to be an essential marker for polyploids. The lower frequency of stomata in tetraploids was probably due the larger epidermal and guard cells (Gantait et al., 2011) and the results of our study validates several reports (Majdi et al., 2010; Hannweg et al., 2013; Javadian et al., 2017).

Charantin content in diploid and tetraploid leaves of BG1346501

An affirmative connection between the higher ploidy level and enhanced secondary metabolite content has been established in numerous artificially induced tetraploid plants, such as, 27.5% more essential oil content in *Dracocephalum moldavica* (Omidbaigi *et al*., 2010), as high as 50% more accumulation of β-ecdysone in *P. glomerata* (Gomes *et al*., 2014), 8.66% more scopolamine content in *H. reticulatus* (Madani *et al*., 2015), 1.39- and 1.23-fold enhancement of Podophyllotoxin production respectively, in the leaves and stem (Javadian *et al*., 2017) etc. The augmentation of the charantin content in the tetraploids is probably attributable to increased metabolic activity and over expression of genes following chromosome doubling (Yun-Soo *et al*., 2004). The charantin estimates were found to be significantly higher (Table, 3; Fig. 6) in samples obtained from tetraploid leaves (1.26 mg per 100 g DW) when compared to that from diploid leaves (1.15 mg per 100 g DW). When evaluated against the control, the concentration of charantin in tetraploid (leaves) was 1.09 fold higher than that of diploid (leaves).

CONCLUSION AND FUTURE SCOPE

In vitro tetraploidy has been efficiently induced in *M. charantia*, employing low colchicine dosage. The tetraploids demonstrated noteworthy variations in their morphological traits in comparison to diploid plants; for instance, higher plant height, thicker stems, large leaves, greater size of stomata but reduced stomatal density in the leaves. Due to the doubled chromosome number, the tetraploids accumulated 1.09-fold more charantin than the diploids. The obtained results signify that the established tetraploids can be effectively used for the potential supply in pharmaceutical application. Additional research will be indispensable to resolve the mechanisms responsible for the polyploid-mediated modification in gene expression. It is also necessary to assess the effect of tetraploidy on
transcriptome and metabolome of *M. charantia*.

Table 1: Characteristics of diploid and tetraploid in BG1346501 after 30 days

	Parameter	**Diploid**	**Tetraploid**
Stem	Diameter of stem (mm)	0.89±0.01 c	1.29±0.00a
Leaf	Length of petiole (cm)	2.10±0.75c	3.24±0.06a
	width of petiole (cm)	0.14±0.00c	0.33±0.00 c
	Length of lamina (cm)	1.65±0.03 c	3.17±0.04a
	Width of lamina (cm)	2.31±0.04c	4.43±0.13a

Data in each column represents mean standard error. Different letters within columns represent significant differences according to Duncan s multiple range test (Duncan, 1955) at 5% level

Table 2: Effect of ploidy level on stomatal status after 30 days of culture

	Parameters	**Diploid**	**Tetraploid**
Stomata	Length (µm)	23.50±0.10c	43.41±0.27a
	Width (µm)	18.45±0.09c	33.62±0.09a
	No. of stomata per field	11.94±0.04c	14.04±0.06a

Data in each column represents mean standard error. Different letters within columns denote significant differences ($p<0.05$) according to Duncan s multiple range test (Duncan, 1955)

Table 3: Charantin content in polyploid tissues

Samples	Charantin content (mg per 100 g DW)
Diploid leaf	1.15
Tetraploid leaf	1.26

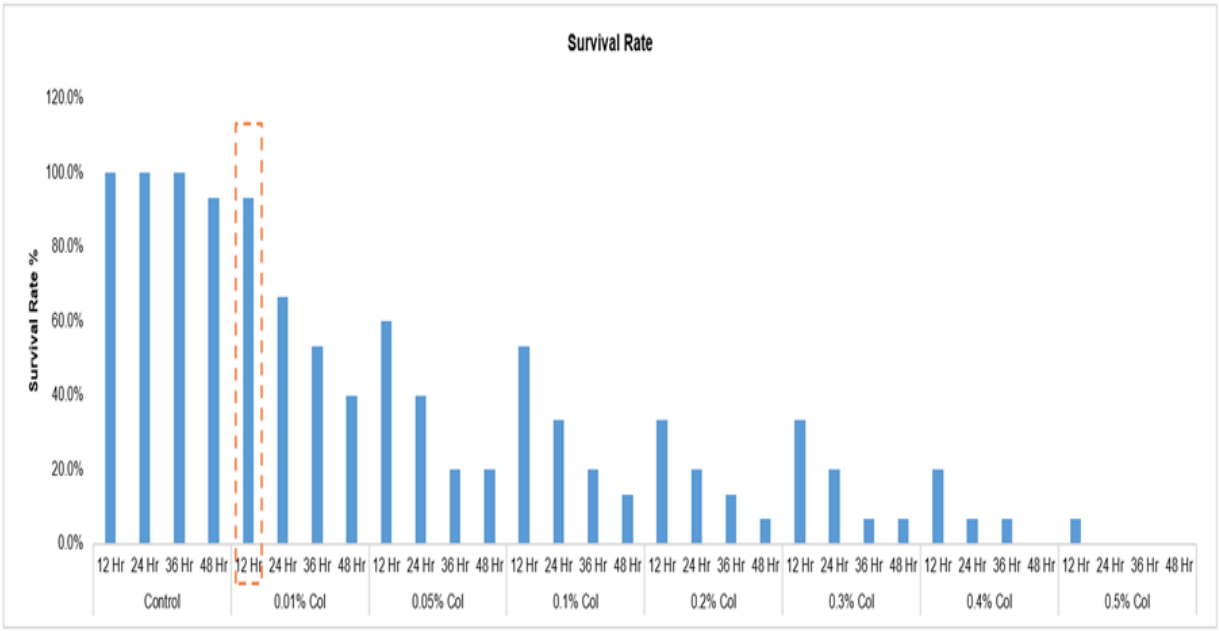

Fig. 1: Survival rate of the explants (ST) following diverse concentrations and durations of colchicine treatment on *in vitro* ST of *M. charantia*. Values in histogram represent mean standard error. Data were taken after 30 days and analyzed by Duncan s multiple range test (DMRT) at P < 0.05

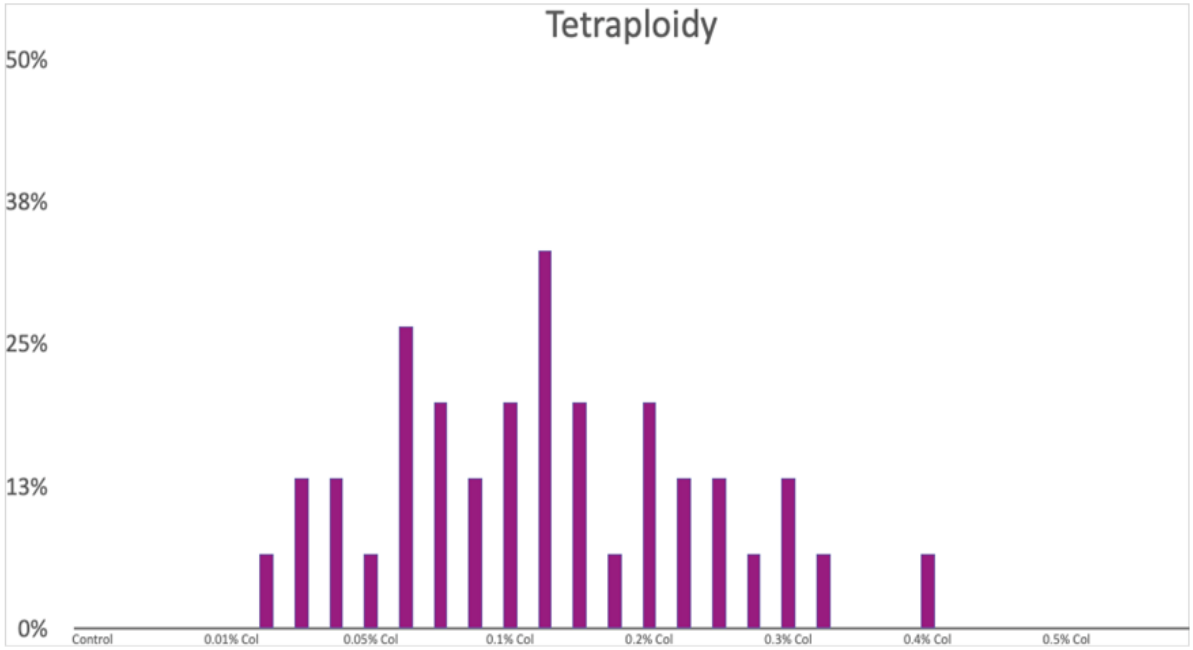

Fig. 2: Influence of diverse concentrations and durations of colchicine treatment on *in vitro* ST for tetraploid induction in *M. charantia* after 30 days of culture. Values in histogram represent mean ± standard error. Data were taken after 30 days and analyzed by Duncan's multiple range test (DMRT) at P < 0.05

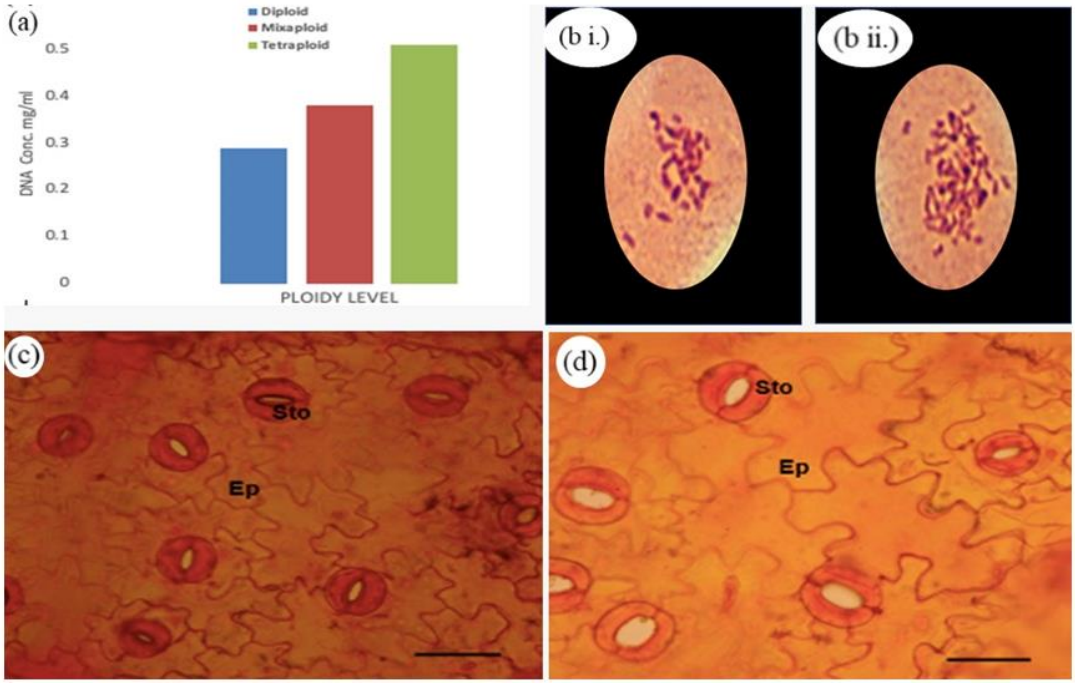

Fig. 3: Comparative cytological and stomatal characterization of *in vitro* colchicine-induced tetraploid with the diploid plantlets of *M. charantia*. (a) The effect of polyploidy on the DNA content;(b) Cytology of i. diploid and ii. tetraploid plantlets; (c) Variation in stomatal frequency of i. diploid and ii. Tetraploid plants.

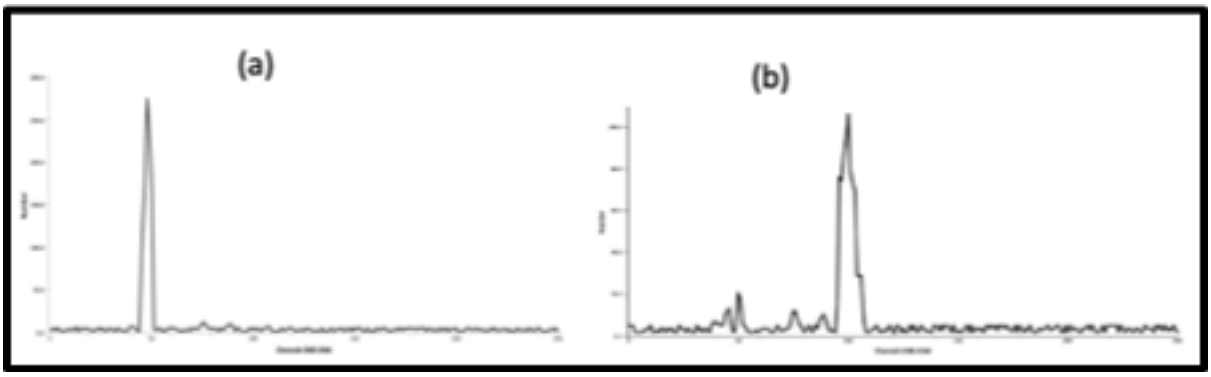

Fig. 4: Flow cytometric histograms of *M. charantia*. (a) Control plantlets and (b) colchicine treated plantlets.

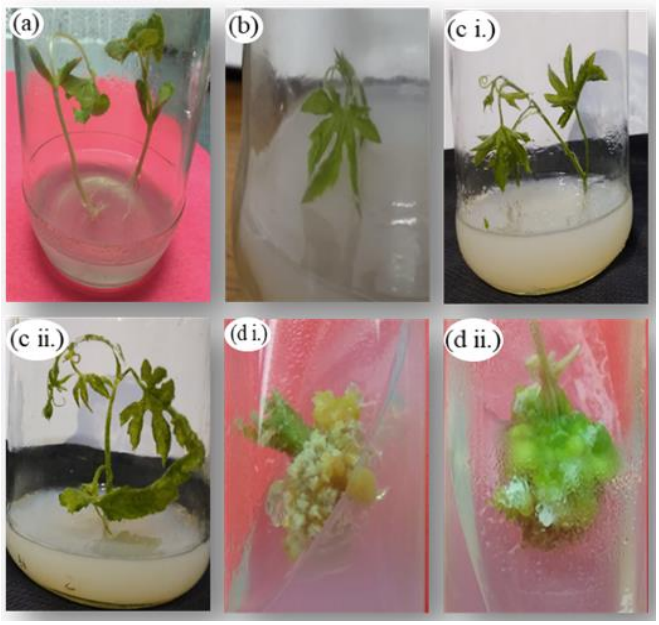

Fig. 5: Comparative morphological characterization of *in vitro* colchicine induced diploid with the tetraploid plantlets of *M.*

charantia. (a) *In vitro* diploid mother plant; (b) *In vitro* culture establishment of colchicine treated explant on MS medium fortified with 1.5 mg L^{-1} BA and 0.5 mg L^{-1} NAA; (c) Variation in size of i. *In vitro diploid* and ii. tetraploid plantlets; (d) Callus of i. Diploid and ii. tetraploid from shoot tips

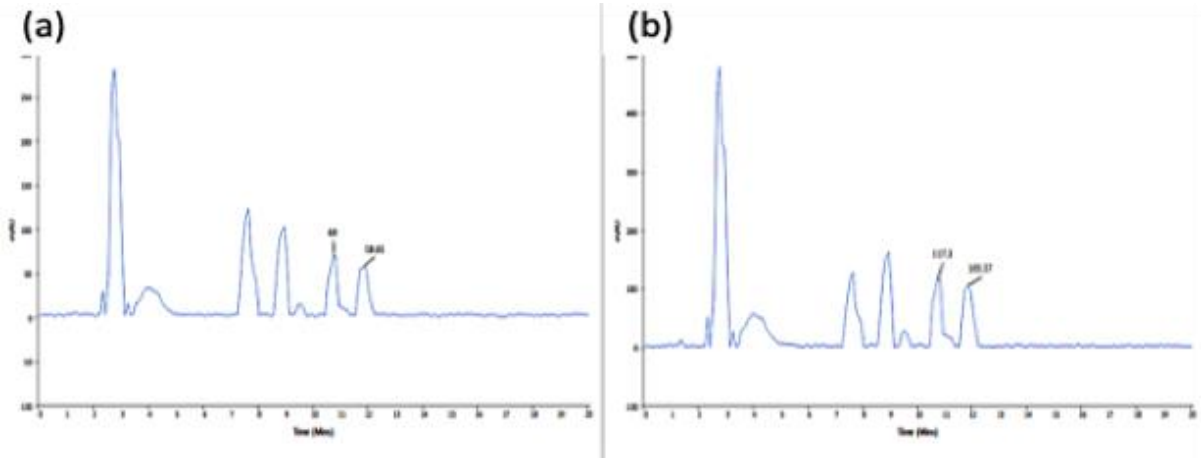

Fig. 6: HPLC graph of charantin estimation of colchicines treated *in vitro* plants of the genotype BG1346501 (a) Diploid plant leaf; (b) Tetraploid plant leaf

Conflict of Interest: The authors should declare that they do not have any conflict of interest.

Author contributions: Conceptualization and designing of the research work (MS, SK, US, NM); Execution of field/lab experiments and data collection (MS, SK); Analysis of data and interpretation (MS, SK, US); Preparation of manuscript (MS, SK, US). All the authors approved the final version of the manuscript prior to submission.

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