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Original article

Production of autotetraploid plants by *in vitro* chromosome engineering in *Allium hirtifolium*

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A B S T R A C T

Persian shallot has been propagated vegetatively through daughter bulbs and has a narrow genetic base. In the present study, to create genetic diversity in terms of ploidy level, the effects of *in vitro* polyploidization were investigated on morphological, physiological and phytochemical traits. Different colchicine treatments (0, 0.3%, 0.5% and 0.7% concentrations) for 24, 36 and 48 h and oryzalin treatments (0, 0.001%, 0.002% and 0.004% concentrations) for 4, 6 and 8 h were used. A sterile basal plate of bulbs was used for the induction of polyploidy in liquid and solid media. After obtaining plantlets, root tip chromosomes were counted for the determination of ploidy levels. Flow cytometry was performed to confirm the chromosome counting results. The regenerated diploid and tetraploid plantlets were transferred to *ex vitro* conditions. Colchicine and oryzalin were both successful in inducing polyploids and the polyploids induced were tetraploids and mixoploids. The highest induction of polyploidy was obtained in solid media using 0.5% (w/v) colchicine for 36 h (35.0% polyploidy induction) and in 0.001% (w/v) Oryzalin for 8 h (45.5% polyploidy induction). Differences in plantlet height and weight, bulblet weight, density, stomatal width and length, and chlorophyll content were observed between tetraploid and diploid plants. Chromosome duplication, as in the case of induced tetraploidy, significantly influenced the antioxidant content and enzyme activities. The results showed that total phenolic content, allicin, and antioxidant capacity were significantly higher in the tetraploid plantlets than that in the diploid plantlets. SRAP loci polymorphisms indicated that the 12 autotetraploid plants (with high allicin content) had different genotypes from the parental diploid plant. Besides, *in vitro* polyploidy induction not only duplicated chromosomes but also altered the DNA sequence in *Allium hirtifolium*. *In vitro* induction of tetraploids in *A. hirtifolium* can be a reliable way to obtain suitable plant material for breeding programs to generate new genotypic variations.

Keywords: Persian shallot; *Allium hirtifolium*; Polyploidy; Colchicine; Oryzalin; Molecular marker

1. Introduction

Biotechnological techniques offer great potential for genetic modification to improve crop yield and quality rapidly, especially in plants with narrow genetic bases or newly domesticated plants, such as medicinal plants (Oliver and Why, 2014; Gianotto et al., 2019; Dessoky et al., 2021). Chromosome engineering by *in vitro* polyploidization is one of the biotechnological methods for genetic modification of asexually propagated plants. Studies on plant evolution and speciation show that the polyploidization process has important implications for the evolutionary history of higher plants (Sanwal et al., 2010; Gao et al., 2016). Auto- or allopolyploid plants are better adapted to biotic and abiotic stresses, such as low or high temperatures, drought, salinity, nutrient deficiency, diseases, and pests (Li, 2003; Zhang et al., 2010; Sattler et al., 2016). Polyploid induction can give rise to genotypes that cannot be

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achieved by hybridization and other methods of genetic diversification (Głowacka et al., 2010). During the polyploidization process, there are changes in the DNA sequence which may lead to differential expression of homologous genes, resulting in adaptive phenotypic variation (Grant, 1999). Polyploidy is common in plants with asexual reproduction that can be easily propagated. Therefore, this makes the formation of extreme phenotypes and increased plant vigor possible, leading to the use of artificial polyploidy induction in plant breeding programs, especially in those plants used for their vegetative organs (Zhang et al., 2010).

Several antimicrotubule compounds such as alkaloids (colchicine) and phosphoric acid amides (amiprophos-methyl and butamiphos), as well as herbicides such as dinitroaniline (trifluralin and oryzalin), have been used for *in vitro* polyploidy induction with high effectiveness (Yemets and Blume, 2008; Gallone et al., 2014; Touchell et al., 2020; Tsai et al., 2021). Dixit and Chaudhary (2014) used different concentrations of colchicine to induce tetraploidy in garlic (*Allium sativum*). Oryzalin as an alternative to colchicine is more effective in inducing polyploidy because of its greater readiness to bind to plant tubulins (Kermani et al., 2003; Dhooghe et al., 2011).

The induction of polyploidy in garlic using *in vitro* treatment of stem-disc treatment with 0.5% (w/v) colchicine resulted in the production of autopolyploid plants with more biomass and higher secondary metabolite content than diploid counterparts (Shmeit et al., 2020; Touchell et al., 2020). In addition, the allicin content in autotetraploid garlic bulbs was 30.7% higher than that in the control (diploid bulbs) (Dixit and Chaudhary, 2014). According to Song et al. (1997), regeneration of tetraploid plantlets by *in vitro* colchicine treatment of callus in the interspecific hybrid *Allium fistulosum* × *Allium cepa* was effective in restoring tetraploid plants. Cheng et al. (2012) reported that trifluralin treatments successfully produced autotetraploid plants in garlic with increased plant biomass and secondary metabolites.

During *in vitro* polyploidization, changes occur in the hereditary materials, at both the epigenetic and genetic level (Gao et al., 2016). PCR-based DNA fingerprinting techniques, such as the sequence-related amplified polymorphism (SRAP) analysis, could identify molecular polymorphisms between diploid and tetraploid plants. These techniques have been successfully performed to detect genetic diversity in polyploid plants, hybridized plants (Xiang et al., 2019), and mutant plants (Harfi et al., 2021). SRAP is an effective and simple technique for producing genome-wide fragments with high flexibility and reproducibility (Robarts and Wolfe, 2014). SRAP markers are efficient tools for estimating genetic diversity in *Allium* spp. Twenty-three (11.16%) polymorphic SRAP bands were identified between autotetraploid plants of *P. grandiflorum* and diploid plants. In other words, differences in morphology and biochemistry between diploid and autopolyploid *P. grandiflorum* plants were detected by SRAP markers (Xiang et al., 2019).

One of the important bulbous and medicinal plants in Iran is Persian shallot (*Allium hirtifolium*) which is an asexually propagated plant (Ebrahimi et al., 2009; Farhadi et al., 2017). It distributed from north western to central western Iran. Persian shallot is rich in valuable secondary metabolites used in the treatment of rheumatic, inflammatory and stomach pains. The genetic resources of cultivated diploid Persian shallot are limited (Ebrahimi et al., 2014). Therefore, polyploidy induction of this plant would be an alternative method to generate suitable plant material for future breeding programs. *In vitro* cultures allow better control of the polyploidization process and increase the number of produced polyploid plants. *In vitro* plant tissue cultures therefore have the potential to standardize the polyploidy induction process to produce artificial polyploid plants (Sattler et al., 2016). There are some studies on the agronomic characterization of *A. hirtifolium*, but polyploidy induction in this plant has not been reported before. This study aimed to develop an effective system for autopolyploidization of *A. hirtifolium* using different concentrations and exposure times of colchicine ($C_{22}H_{25}NO_6$) and oryzalin ($C_{12}H_{18}N_4O_6S$) in liquid and solid medium under *in vitro* conditions. Moreover, the effects of induced polyploidy were investigated on the morphological, physiological, and some phytochemical characteristics of regenerated *A. hirtifolium* plantlets to detect the differences between diploid and tetraploid plants at the molecular level by SRAP markers.

2. Materials and methods

2.1. Plant material and explant preparation

The bulbs of *A. hirtifolium* were provided by the Department of Horticultural Sciences, Faculty of Agriculture, University of Tabriz. The bulbs were collected from the Khorramabad area (the natural habitat of Persian shallots) in 2020. The bulbs were washed under running tap water for 2 h and the outer dry scales were removed from the bulbs. Then, they were again washed in dishwashing detergent for 15 min and sterilized with 70% ethanol and 5% sodium hypochlorite for 5 min, followed by rinsing with sterile distilled water at least three times.

2.2. Polyploidy induction

2.2.1. Liquid-solid media

Liquid-solid and solid–solid media were tested for the induction of polyploids. A liquid MS basal medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose without agar and growth regulators was prepared in the soaking method. Sterilized colchicine ($C_{22}H_{25}NO_6$) (Sigma Aldrich, St. Louis, MO) at concentrations of 0%, 0.3%, 0.5%, and 0.7% and oryzalin ($C_{12}H_{18}N_4O_6S$) (Sigma Aldrich, St. Louis, MO) at concentrations of 0, 0.001%, 0.002%, and 0.004% were added to the autoclaved media (liquid). Prepared colchicine and oryzalin stock solutions, were sterilized with water containing 1.0% (v/v) dimethyl sulfoxide [$(CH_3)_2SO$ or DMSO] and filtered through 0.45 μm Millex-HV Millipore filters. After sterilizing the bulbs, the basal plates (approximately 5 mm thick) were excised and soaked in liquid medium for 24, 36, and 48 h for colchicine treatments and for 4, 6, and 8 h for oryzalin treatments. The culture media were shaken on an orbital shaker (100 r · min^{-1}) at (26 ± 2) °C during the treatment periods.

2.2.2. Solid–solid medium

In the second method, the filtered colchicine and oryzalin were added to a solid MS basal medium prepared with 0.8% (w/v) Agar–Agar and 3% (w/v) sucrose (Merck, Darmstadt, Germany) without growth regulators. *A. hirtifolium* explants with 2 mm basal plates were cultured vertically on the prepared media containing different colchicine concentrations (0, 0.3%, 0.5%, and

0.7%) for 24, 36, and 48 h and different oryzalin concentrations (0, 0.001%, 0.002%, and 0.004%) for 4, 6, and 8 h.

The treated explants of both methods (the liquid and solid methods) were removed from the medium and washed three times with distilled water to remove the residual colchicine and oryzalin. They were then transferred to an MS medium containing 3% sucrose and 0.8% Agar–Agar with 1 mg L^{-1} of 6-benzylaminopurine (BAP) (Merck, Darmstadt, Germany) and 0.5 mg L^{-1} of 1-naphthaleneacetic acid (NAA) (Merck, Darmstadt, Germany) for shoot induction in both methods (Farhadi et al., 2017). Cultures were maintained at (25 ± 2) °C under a 16 h/8 h light regime with white fluorescent tubes at 40 μmol $m^{-2} \cdot s^{-1}$. Subcultures were then performed six times on the same medium every four weeks. The survival rate, shoot regeneration percentage, and numbers of formed shoots were recorded six months after the time of culture.

2.3. Chromosome counting

Regenerated shoots were transferred to hormone-free MS medium containing 0.8% Agar–agar and 3% sucrose for bulb formation and root induction. Root tips (1–2 cm in length) were cut and pretreated with a saturated solution of 2% α-bromonaphthalene (Sigma Aldrich, St. Louis, MO) at 3 °C for 8 h. Then, the roots were rinsed for 3 min to thoroughly stop hydrolysis and placed in a fixing solution of ethanol and glacial acetic acid (Sigma Aldrich, St. Louis, MO) (3:1, v/v) for 3 days. Next, the roots were extracted from the fixative, rinsed with distilled water, and placed in 36.45 mol L^{-1} hydrochloric acid at 60 °C for 8 min. Aceto-orcein (Sigma Aldrich, St. Louis, MO), in form of a 1% solution in 45% acetic acid, was used to stain the samples for 24 h, followed by removing the stain. Then, 1–2 mm of the stained root tips were excised and transferred to a drop of 45% acetic acid, and microscopic slides were prepared by squeezing. The slides were prepared for observation with an Olympus light microscope CX22 (Zeiss Photomicroscope, Carl Zeiss, New York, USA) to count the chromosomes in the correct metaphase plates. Five cells of each of five root tips in the metaphase stage in each root tip were randomly selected for counting the number of chromosomes.

2.4. Analysis of polyploidy level by flow cytometry

Leaf samples were cut into 1 cm^2 pieces, crushed in 0.5 mL Partec nuclear extraction buffer, and mixed with 1.5 mL of 1% DAPI (4',6-diamido-2-phenylindole)-aceto-orcein staining solution (Sigma Aldrich, St. Louis, MO). After 5 min, the suspension was filtered through a 50 μm Celltrics disposable filter. Nuclear suspensions were analyzed using a Partec flow cytometer (PAI, Partec Gmbh, Germany) equipped with an HBO lamp. Leaves of the diploid full-sib family were used as controls. More than 4000 nuclei were analyzed and three independent replications were used to determine the DNA ploidy level. After the cytogenetic analysis, the diploid and tetraploid plantlets were identified and transferred to the pots filled with aseptic perlite. The pots were placed in a germinator at (25 ± 2) °C with 16 h of daylight.

2.5. Evaluation of plant characteristics of induced polyploids

Two months after acclimation of the diploid and induced polyploid plantlets under *ex vitro* conditions, the surviving autopolyploid plantlets were compared morphologically, physiologically, biochemically, and phytochemically. The measured morphological traits were plantlet height (cm) and plantlet and bulb weight (g). For stomata characteristics, small strips of lower epidermis were torn off and prepared for observation with an Olympus light microscope CX22 (Zeiss Photomicroscope, Carl Zeiss, New York, USA). The width and length of stomata (an average of 50 stomata) and density (10 randomly selected fields without overlap) were analyzed by light microscopy (Chen et al., 2009; Gao et al., 2016).

Chlorophyll and carotenoids contents were extracted with 80% acetone from five leaves of the diploid or tetraploid plantlets. The absorbance of the pigments was measured with a UV–visible spectrophotometer (Analytikjena Spekol 1500) at 470, 648, and 664 nm (Lichtenthaler, 1987). The contents of total chlorophyll and carotenoids were expressed as mg per g leaf fresh weight and each measurement was performed in triplicate.

The biochemical properties such as total soluble solids (by a refractometer), and the activity of antioxidant enzyme, including catalase, peroxidase, ascorbate peroxidase and superoxide dismutase, were evaluated in the samples. The antioxidant activity of the plant extracts against stable 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) was determined as described by Brand-Williams et al. (1995). A decrease in radical concentration at 515 nm gave a measure of the antioxidant activity of the samples. For enzyme assays, 1 g of the leaves was homogenized in 50 mmol L^{-1} of potassium phosphate buffer (pH 6.8). The homogenates were centrifuged at 12 000×*g* at 4 °C for 20 min, and the supernatant was used for the enzyme extraction. The amount of soluble protein in the extract was determined by the Bradford (1976) protein assay using bovine serum albumin (BSA) as a standard. Catalase activity was estimated by the method of Aebi (1974) and expressed as mmol $\cdot$ L^{-1} of H_2O_2 decomposed per min per mg of soluble protein (U $\cdot$ mg^{-1}) using the H_2O_2 extinction coefficient of 39.4 mmol L^{-1}. Peroxidase activity was determined using guaiacol and H_2O_2 substrates with the extinction coefficient of the tetraguaiacol product (26.6 mmol L^{-1}) (Chance and Maehly, 1955) expressed as mmol $\cdot$ L^{-1} of tetraguaiacol produced per minute per mg of soluble protein (U $\cdot$ mg^{-1}). Ascorbate peroxidase activity was measured as described by Nakano and Asada (1981) and expressed as mmol of oxidized ascorbate per min per mg of soluble protein (U $\cdot$ mg^{-1}) with 2.8 mmol $L^{-1} \cdot cm^{-1}$ as the extinction coefficient. Superoxide dismutase activity was estimated from its ability to inhibit the photochemical reduction of nitroblue tetrazolium according to Giannopolitis and Ries (1977) and expressed as units per mg of soluble protein.

The content of allicin, as a main organosulfur compound of the Persian shallot bulb was measured using high-performance liquid chromatography (Shimadzu, 150 × 4.6 mm C18 column with a Bishoff pump) and a spectrophotometer (UVD 2.1 L - KNAUER) with an internal standard, butyl peroxybenzoate (Iberl et al., 1990), expressed as mg allicin per g bulb fresh weight. Pyruvate content as a pungency indicator of the diploid and tetraploid bulbs, was determined according to Anthon and Barrett (2003). A spectrophotometer based on the reaction of 2,4-dinitrophenylhydrazine (DNPH) with pyruvic acid at 420 nm was used to measure the pyruvic acid content, expressed as μmol per bulb fresh weight.

Table 1 Primer sequences used for SRAP analysis

Forward primer		Reverse primer		Primer combinations	
Name	Sequence (5′ to 3′)	Name	Sequence (5′ to 3′)	Forward	Reverse
ME1*	TGAGTCCAAACCGGATA	EM3	GACTGCGTACGAATTGAC	ME1	EM3/EM4/EM12
ME2	TGAGTCCAAACCGGAGC	EM4	GACTGCGTACGAATTTGA	ME2	EM4/EM9/EM10/EM12
ME3	TGAGTCCAAACCGGAAT	EM6	GACTGCGTACGAATTGCA	ME3	EM1/EM3/EM9/EM8/EM11
ME4	TGAGTCCAAACCGGACC	EM8	GACTGCGTACGAATTCAC	ME4	EM2/EM6/EM9
ME6	TGAGTCCAAACCGGACA	EM9	GACTGCGTACGAATTCAG	ME6	EM3/EM4/EM6
ME8	TGAGTCCAAACCGGACT	EM10	GACTGCGTACGAATTCAT	ME8	EM6
ME9	TGAGTCCAAACCGGAGG	EM11	GACTGCGTACGAATTCTA	ME9	EM3/EM4/EM12
ME10	TGAGTCCAAACCGGAAA	EM12	GACTGCGTACGAATTCTC	ME10	EM3/EM10

Note: * ME: forward primers; EM: backward primers.

2.6. DNA extraction

Total genomic DNA was extracted from 200 mg of fresh leaf of diploid *A. hirtifolium* (control) and 12 autotetraploid variants according to Doyle and Doyle (1990) with minor modifications. The absorbance of DNA concentration was measured at 260 nm using a Smart A52 Spec™ Plus (Biorad, USA). DNA quality was examined by 0.8% agarose (Sigma–Aldrich, Germany) gel electrophoresis. The original DNA solutions were diluted to 25 ng μL^{-1} with TE buffer, confirmed by visible spectrophotometry with UV absorbance on a Smart A52 Spec™ Plus (Bio-rad, USA) and stored at −20 °C until use.

2.7. SRAP amplification and examination

SRAP primers targeting regions genome-wide (17 or 18 nucleotides long) were designed according to Li and Quiros (2001) (Table 1). A total of 24 different primer pairs were assembled, 20 pairs of which produced clear and reproducible bands. PCR amplification was performed using the following program: 5 min at 94 °C; 3 cycles of 1 min at 94 °C, 1 min at 35 °C, and 1.5 min at 72 °C; 35 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1.5 min at 72 °C, with a final elongation step of 10 min at 72 °C. SRAP-PCR Amplification was performed in 25 μL volume: 0.2 μL of *Taq* DNA polymerase 5 ($U \cdot \mu L^{-1}$), 2.5 μL of 10 × PCR buffer, 2.5 μL of 25 mmol L^{-1} $MgCl_2$, 3.0 μL of deoxynucleoside triphosphate (dNTP) mixture (2.5 mmol L^{-1}), 0.75 μL of forward (10 mmol L^{-1}) and reverse (10 mmol L^{-1}) primers, 2 μL of template DNA (20 ng), and 13.3 μL of ddH_2O. For electrophoresis, 10 μL of the PCR-amplified products were separated on a 1.5% agarose gel stained with 0.5 pg mL^{-1} of ethidium bromide. A DNA ladder was used as a size standard (DL 2000) and electrophoresis was performed at 100 V for 110–120 min. The bands were visualized and photographed under UV light using the Gel Doc 2000 image analysis system (Bio-rad, USA).

2.8. Statistical analysis

The analysis of variance (ANOVA) of morphological, biochemical, and physiological data was performed using the SPSS version 21.0 statistical analysis program (IBM Corporation; Somers, NY). Duncan's new multiple range test was used to compare the means. The ANOVA hypotheses were tested prior to ANOVA, and an arcsine transformation was used for the percentage data. The effects of colchicine (with four levels) and oryzalin concentrations (with four levels) using two separate experiments and their exposure times (with three levels) were examined as factorial experiment based on a completely randomized design with four replications and 30 explants in each experimental unit.

All clearly distinguishable SRAP fragments in PAGE (SRAP loci) between 100 bp and 950 bp were scored for the presence (1) or absence (0) using Quantity One software (BioRad, Hercules, CA, USA) to create the 0/1 matrix. Jaccard coefficients were created based on Jaccard's (1908) definition. The data matrix was created using SPSS var. 21 (Statistical Package for the Social Sciences). The unweighted pair-group method arithmetic means (UPGMA) was used to create a dendrogram.

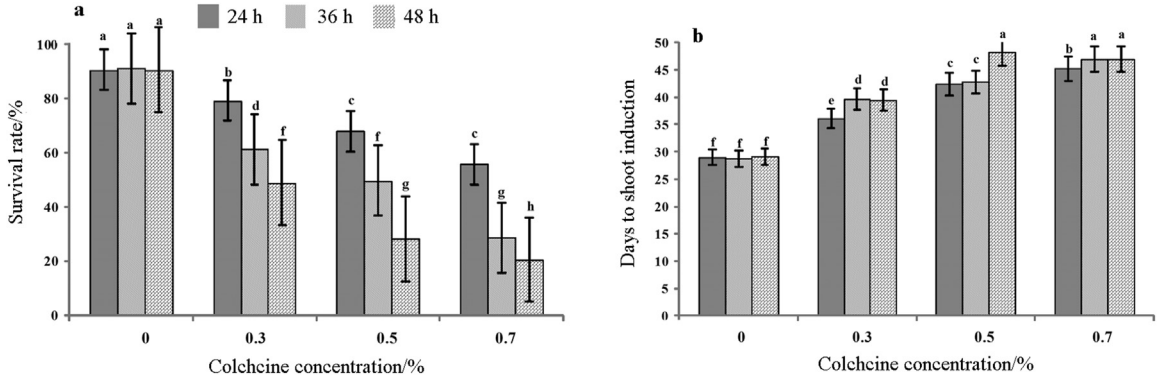

Fig. 1 Effect of different concentrations and exposure durations of colchicine treatments on survival (a) and days to regeneration (b) of basal plate explants in *Allium hirtifolium*.

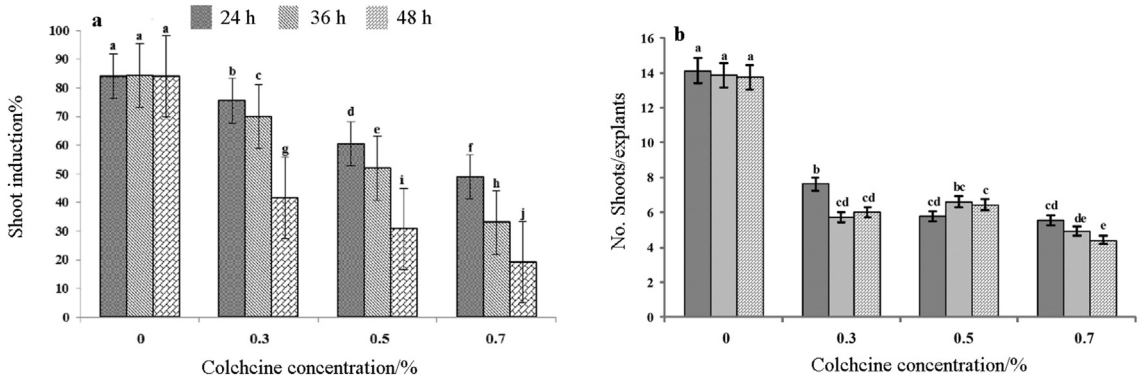

Fig. 2 Effect of different concentrations and exposure durations of colchicine treatments on shoot induction (a) and number of shoots (b) of basal plate explants in *Allium hirtifolium*.

3. Results

3.1. *Colchicine-treated explants in solid medium*

Explants that were soaked in a liquid MS medium containing various concentrations of colchicine and oryzalin died shortly after treatment. However, explants treated with colchicine in a solid medium survived successfully. The survival rate decreased significantly with an increased concentration and duration of colchicine exposure. The highest survival rate (90.67%) was observed in control explants, while explants treated with a colchicine concentration of 0.7% for 48 h had the lowest survival rate (26.98%) (Fig. 1, a). Colchicine treatment resulted in complete termination or delay of regeneration of *A. hirtifolium*-treated explants by 1–3 weeks, depending on concentration and exposure time (Fig. 1, b).

Shoot regeneration of *A. hirtifolium* explants was significantly affected by the colchicine concentration and exposure time. As shown in Fig. 2, a, the regeneration of shoots in the control treatment was 85%, which was reduced to 19.13% in explants treated with colchicine (0.7%) for 48 h. The number of regenerated shoots decreased with increasing colchicine concentrations (Fig. 2, b). Thus, the lowest number of regenerated shoots (4.43 shoots per explant) was observed at the highest colchicine concentration (0.7%) (Fig. 2, a). Reduced regeneration in colchicine-treated basal plate explants was also observed in garlic (Dixit and Chaudhary, 2014) and onion (Geoffriau et al., 1997; Grzebelus and Adamus, 2004).

Cytogenetic studies have apparently confirmed the efficiency of chromosome duplication at different concentrations of colchicine. Persian shallot contains 16 chromosomes (2n = 2x = 16) in the diploid state (Fig. 3, a), and the tetraploid plants had 32 chromosomes (2n = 4x = 32) (Fig. 3, b). The frequencies of tetraploid induction (based on both chromosome counting and flow cytometric analysis) at the colchicine concentrations of 0.3%, 0.5% and 0.7% were 10.94%, 25.41% and 19.35%, respectively. The highest number of tetraploid plantlets (35%) were observed with 0.5% colchicine for 36 h (Fig. 4).

Mixoploidy was also observed in the regenerated Persian shallot plantlets after colchicine treatment. The highest frequency of mixoploid plantlets (31.82%) was observed with 0.3% colchicine treatment for 36 h (Fig. 4). The meristematic cells are not uniformly polyploid, explaining the heterogeneous and mixoploid nature of the tissue (Zhang et al., 2020). In other words, chromosome duplication must occur in all meristematic cells to generate putative tetraploids (Touchell et al., 2020). Fig. 5, a–c shows the results of flow cytometric analysis of the

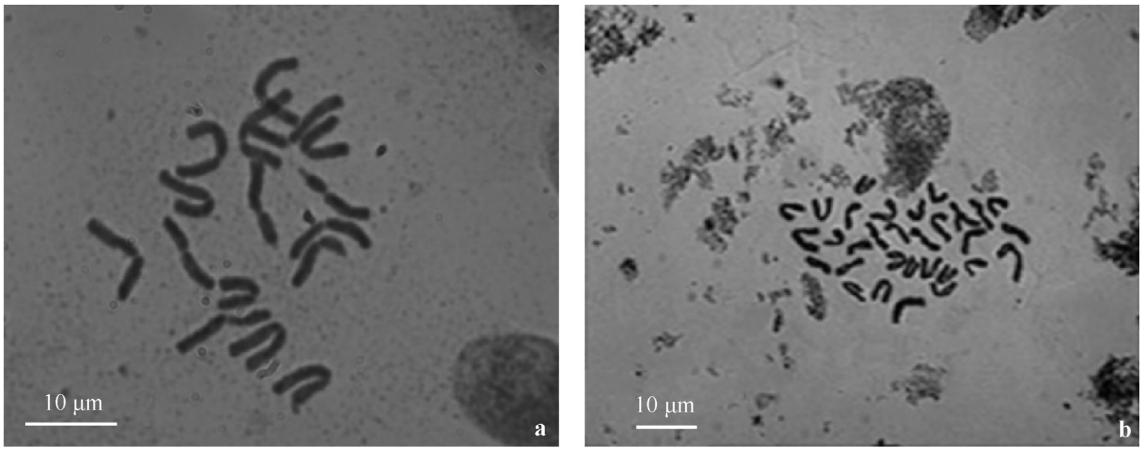

Fig. 3 Chromosome number of diploid (a) and autotetraploid (b) *Allium hirtifolium* plantlets.

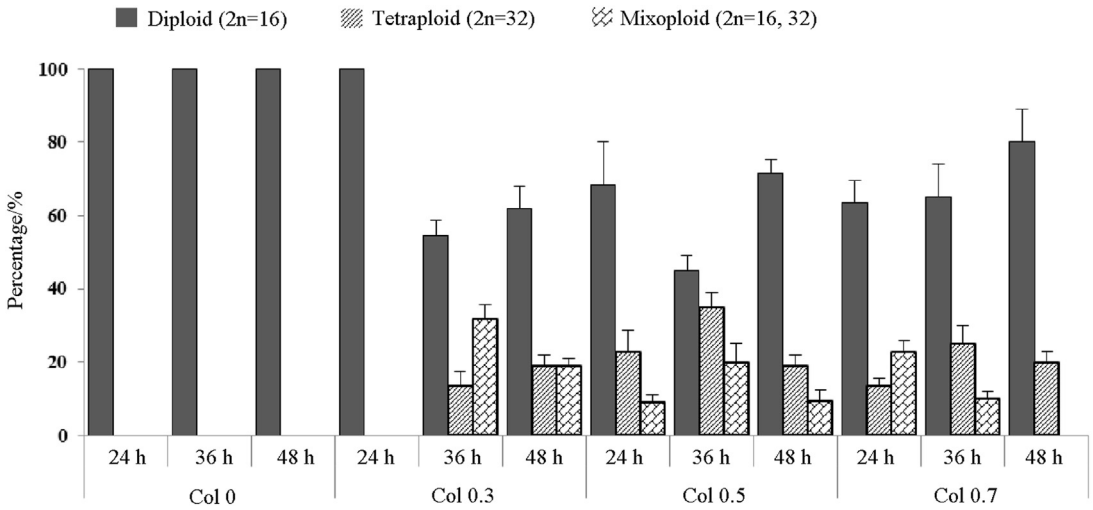

Fig. 4 Percentage of diploid, tetraploid and mixoploid of regenerated plantlets from treated basal plate explants of *Allium hirtifolium* in colchicine concentrations and exposure times

Col: Colchicine. In the absence of colchicine, all regenerated plants were diploid.

plantlets treated with colchicine. A major advantage of flow cytometry is that it permits a rapid and accurate screening of the DNA content of a large number of individuals. Furthermore, as well as being suitable for somatic cells, this technique has been used to study the relative DNA content of polyploid plants (Maceira et al., 1992).

3.2. *Oryzalin-treated explants in solid medium*

The highest survival rate and shoot regeneration of basal plate explants of Persian shallot were observed at the lowest oryzalin concentration and exposure time. The survival rate of control explants was 90.01%, which decreased to 30% in explants treated

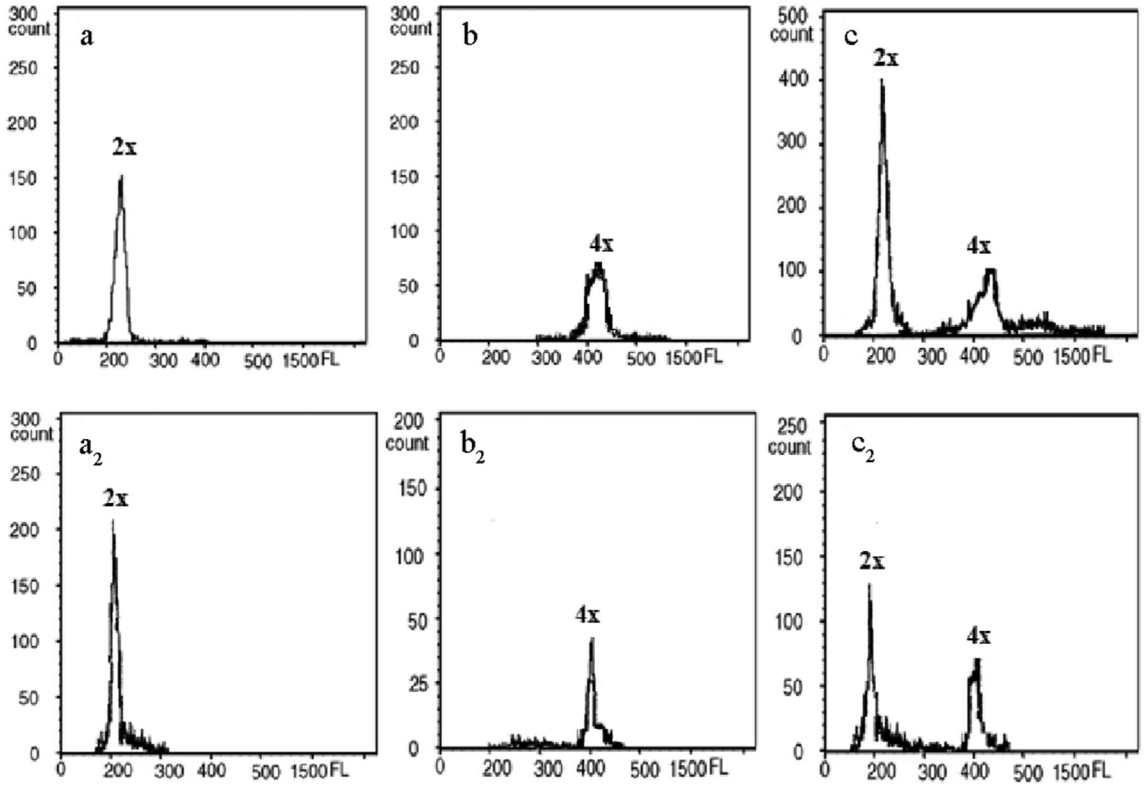

Fig. 5 Flow cytometric analysis of control, colchicine (upper graphs) and oryzalin-treated (lower graphs) *Allium hirtifolium* plantlets

a, a_2: Histogram of diploid control plantlets on Channel 200; b, b_2: histogram of colchicine- and oryzalin-treated autotetraploid plantlets on Channel 200; c, c_2: histogram of colchicine- and oryzalin-treated diploid + autotetraploid plantlets (mixoploid).

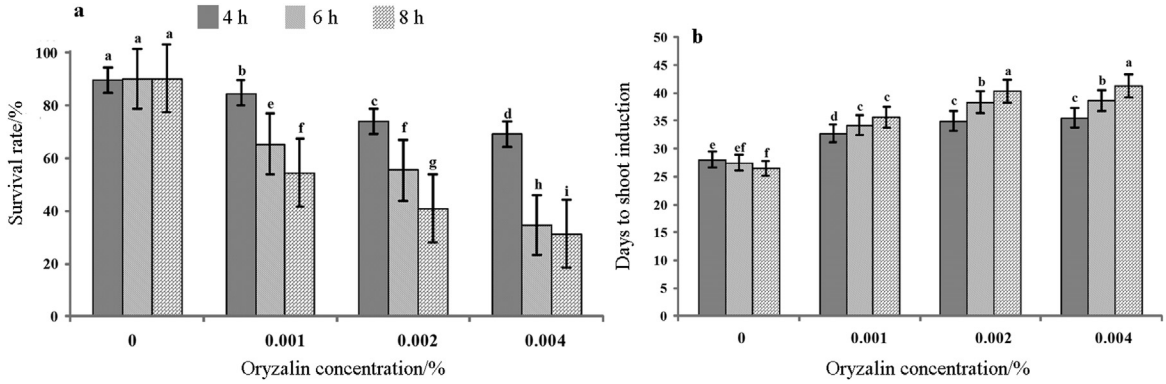

Fig. 6 Effect of different concentrations and exposure durations of oryzalin treatments on survival (a) and days to regeneration (b) of basal plate explants in *Allium hirtifolium*.

with oryzalin (0.004%) (Fig. 6, a). Moreover, oryzalin treatment significantly increased the days to shoot regeneration (up to 15 days) (Fig. 6, b). The same trend in the survival and regeneration of oryzalin-treated explants was observed in *Impatiens walleriana* (Ghanbari et al., 2019). Although oryzalin is an effective agent for chromosome duplication, a long exposure time and high concentrations of oryzalin could be toxic to the explants (Touchell et al., 2020). The factors affecting polyploidy induction under both *in vitro* and *in vivo* conditions are the concentration and exposure time of antimitotic agents. Low doses are not effective while high doses can be lethal. Therefore, the effective and non-lethal oryzalin concentration and treatment time must be determined empirically for successful production of polyploids, as plant species vary in their sensitivity to oryzalin (Dhooghe et al., 2011).

Shoot regeneration of Persian shallot explants decreased significantly with increasing concentration of oryzalin in the culture medium. Control treatment has the highest shoot induction, the 0.001% has significantly higher shoot induction than the 0.002% and 0.004%, which two had not significant difference (Fig. 7, a). On average, 14.11 shoots were formed in each control explant. The number of regenerated shoots in treated explants was affected only by the oryzalin concentration. In explants treated with 0.002% and 0.004% oryzalin, an average of 4.5 shoots were regenerated per explant (Fig. 7, b). Apparently, the *in vitro* application of oryzalin delays the regeneration time and reduces the branching of regenerated explants (Zhang et al., 2020). Colchicine and oryzalin are mitotic and spindle poisons, respectively, which act in plant cells by destroying spindle fibers and modifying the differentiation process. Therefore, the delay or inhibition of shoot regeneration was predictable after treatment with colchicine and oryzalin (Yemets and Blume, 2008).

Fig. 5 shows that the chromosome number in the regenerated plantlets was significantly influenced by the concentration and exposure time of oryzalin. Tetraploid and mixoploid plantlets were observed in all treatments. The number of these plantlets was correlated with the duration and concentration of treatment with the mitotic inhibitor (i.e. oryzalin). Fig. 5, a2–c2 shows the results of flow cytometric analysis of plantlets treated with oryzalin. The regeneration rates of autotetraploid plantlets at oryzalin concentrations of 0.001%, 0.002%, and 0.004% were about 35%, 14%, and 11%, respectively. The highest autotetraploid regeneration (45.45%) was observed when treated with 0.001% oryzalin for 8 h followed by 0.002% (28.57%) and 0.004% (8.7%) for 6 h and 4 h, respectively. Of the plantlets studied, mixoploids were detected in 10.81% (at 0.001% oryzalin), 16.13% (at 0.002% oryzalin) and 16.90% (at 0.004% oryzalin). Oryzalin acts more efficiently on microtubule polymerization and chromosome duplication in most plant cells, allowing a higher frequency of tetraploid cells. In general, it appears that low concentrations of

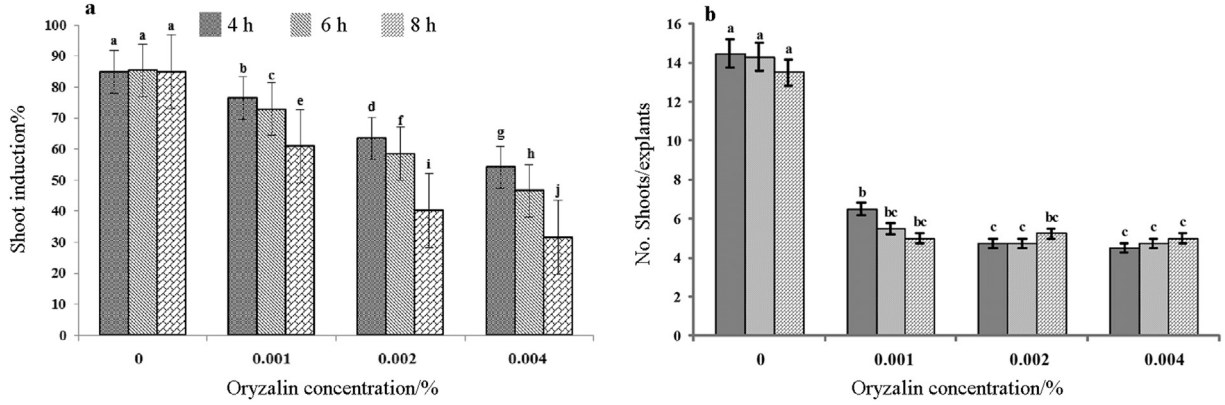

Fig. 7 Effect of different concentrations and exposure durations of oryzalin treatments on shoot induction (a) and number of shoots (b) of basal plate explants in *Allium hirtifolium*.

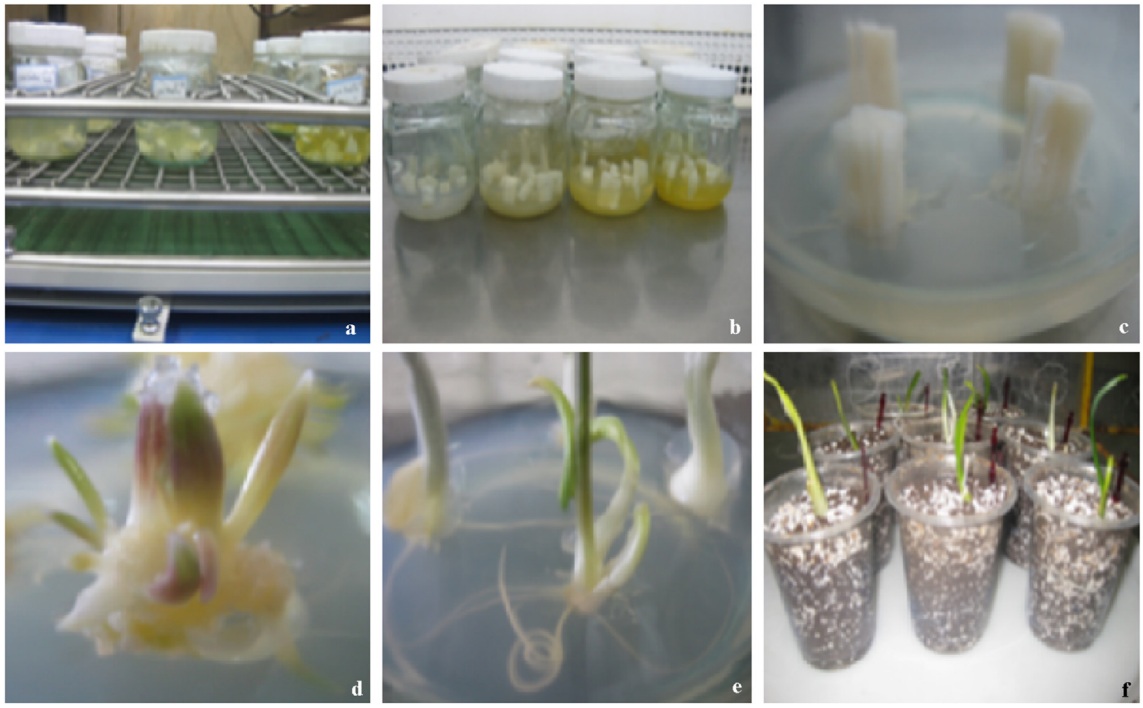

Fig. 8 Plant regeneration from basal plates of *Allium hirtifolium* treated with oryzalin

a. Soaking of explants in liquid MS medium with different concentrations of oryzalin for specific times; b. Placing of explants in solid MS medium with different concentrations of oryzalin for specific times; c. Atrophy of soaked explants; d. Shoot regeneration from treated basal plates in solid medium; e. Rooting of regenerated shoots on growth regulation free media; f. Hardening off of rooted shoots.

oryzalin are more effective for the induction of polyploidy in most plant species (Dhooghe et al., 2011).

A comparison of the results with colchicine (Figs. 1 and 2) and oryzalin (Figs. 6 and 7) shows that the basal plate explants are more sensitive to colchicine than to oryzalin as the survival rate, shoot regeneration, and growth retardation of the explants treated with colchicine were higher than those of oryzalin. According to Fig. 6, oryzalin is also more efficient in inducing polyploidy in explants of Persian shallot as the autotetraploids induced with oryzalin were almost 9% higher than the tetraploids induced with colchicine on average across all exposure durations and concentrations. Therefore, it seems that oryzalin is better for inducing autotetraploids in Persian shallot. The results found in the current study are in agreement with other studies such as that in Hebe 'Oratia Beauty', in which the highest tetraploidy rates were 28.6% and 45.7% using colchicine (1000 μmol L^{-1}) and oryzalin, respectively (Dhooghe et al., 2011; Sattler et al., 2016; Touchell et al., 2020). However, the development of an appropriate protocol for *in vitro* polyploidization for each plant species requires several tests to obtain an appropriate combination for the concentration and exposure time of the antimitotic agent (Dhooghe et al., 2011).

3.3. *Autotetraploid plantlets production and photosynthetic pigments*

Fig. 8 shows the explants treated with oryzalin in the solid MS medium, the regenerated shoots, and rooted and adapted plantlets. The ploidy level of all plantlets was verified after adaptation of the plantlets to the *ex vitro* conditions.

Autotetraploid plantlets had thicker and darker leaves (Fig. 9). According to reports by Touchell et al. (2020) and Liu et al. (2007), the growth of plantlets resulting from antimitotic treatments is slow, and these plantlets have thick cotyledons and short, swollen hypocotyls. It appears that some deviation from normal morphology in most plants was a consequence of induced polyploidy (Dhooghe et al., 2011). In the present study, the height of the diploid plant was significantly higher than that of the autotetraploid plant (Table 2). The reduction in plant height due to polyploidization stimulates plants to be more tolerant to environmental conditions (Sattler et al., 2016).

The total chlorophyll content was 1.614 mg g^{-1} fresh weight in the diploid plantlets vs. 2.433 mg g^{-1} in the autotetraploid seedlings. In this study, no significant difference was found between the carotenoid content in autotetraploid (0.35 mg g^{-1} leaf fresh weight) and diploid (0.354 mg g^{-1} leaf fresh weight) plantlets. Significant effects of polyploidy on plant photosynthetic rate have been reported elsewhere. In some cases, autotetraploid plantlets showed higher chlorophyll content and photosynthetic rate (Yemets and Blume, 2008), but the results were contradictory in other cases (Zhang et al., 2020). Photosynthetic capacity is influenced by biochemical and anatomical changes in leaves. Polyploidy often increases DNA and protein content, enzymatic activity per cell, and the volume of leaf cells such as mesophilic cells, and leaf thickness. These changes affect the biosynthesis of photosynthetic pigments, which differ according to the type of polyploidy (auto- and allopolyploids) (Sattler et al., 2016).

The density of stomata, the size of guard cells, and the number of plastid cells have often been used as morphological markers to

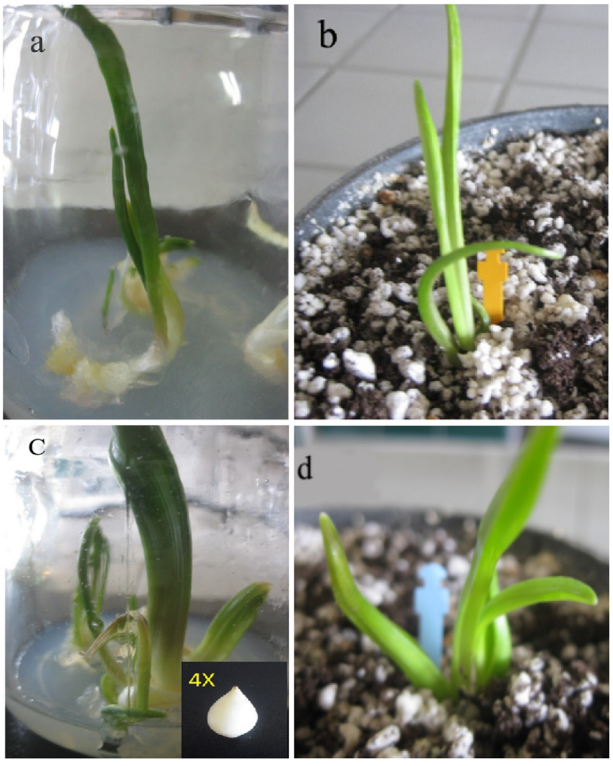

Fig. 9 Morphological characteristics of diploid and autotetraploid *Allium hirtifolium* plantlets under *in vitro* and *ex vitro* conditions
a and b: diploid; c and d: autotetraploid plantlets.

identify the degree of ploidy levels in many plant species. Fully developed leaves of treated plantlets were used for making leaf impressions and viewing the stomata (Khazaei et al., 2010; Zhang et al., 2020). Data analysis showed a significant increase in stomatal length and width in the autotetraploid plantlets of *A. hirtifolium* compared to the diploid plantlets. The mean stomatal length was 22.86 μm in the diploid plantlets, while it was 38.37 μm in the autotetraploid plantlets. The stomatal width was 10.97 μm and 19.93 μm in the diploid and autotetraploid plantlets, respectively (Table 2).

Ploidy assay based on stomata cell density offers scientists the opportunity to screen potential polyploids early without squandering the time and space to grow a large population of plants. This method is less time-consuming and permits simple and rapid screening of large groups of plants. In the present study, the mean stomatal cell density was calculated at two ploidy levels. The difference in stomatal density in one millimeter square at different parts of the leaf surface was significantly different in the diploid and autotetraploid plantlets ($P < 0.001$) (Fig. 10a and b). The mean values of stomatal density in the diploid and autotetraploid plantlets were 117.33 and 86.00 stomata per mm^2, respectively (Table 2). The decrease in stomata density with a higher ploidy level was reported in different plant species (Grzebelus and Adamus, 2004). Morphological changes induced by a higher ploidy level are the adaptation strategies of plants to different environmental conditions. Indeed, polyploid plants are more tolerant to adverse environmental conditions than their diploid ancestors. This adaptability results from reduced leaf area and increased stomata size, as well as the plants' greater ability to maintain water balance (Hollister, 2015).

Plantlet weight was higher in autotetraploids (approximately 21%, 22%) than in diploids. All diploid and autotetraploid Persian shallot plantlets produced a bulblet; however, the weight of autotetraploid bulblets (2.96 g) was significantly higher than that of diploid bulblets (2.19 g) (Table 2) (Fig. 7). Larger plant organs and higher biomass in the polyploid plants are probably related to a larger leaf area, more chloroplasts in guard cells, and better water efficiency (Kaensaksiri et al., 2011).

3.4. *Soluble solids and phytochemical compounds*

The content of soluble solids showed no significant differences in the diploid (11.20%) and autotetraploid (11.53%) plantlets (Table 3) . The autotetraploid plantlets contained higher total phenolics than diploid plantlets (0.545 mg g^{-1} and 0.475 mg g^{-1} weight of leaf fresh, respectively). Autotetraploidy caused an increase in the allicin content (the major organosulfur compound of the Persian shallot bulb). The allicin content in the autotetraploid plantlets was higher than that in the diploid plantlets (2.65 mg g^{-1} and 2.08 mg g^{-1} of bulb fresh weight, respectively). The active ingredient was higher in the polyploid genotypes of *Thymus vulgaris* than in the diploid parents (Shmeit et al., 2020). Pyruvate content has no significance between autotetraploids and diploids (Table 3).

The fact that a large number of plant species are polyploid has drawn the attention of plant breeders to the use of artificial polyploidy induction for crop improvement. Polyploidization events often appear to be associated with an increase in the vigor and adaptive potential of newly formed polyploids (Chen and Gao, 2007). These superior traits along with the higher phytochemicals result in the plants having more chances of survival under harsh environmental conditions. The higher active compounds (i.e. phytochemicals) in plant tissues probably increase

Table 2 Morphological, physiological and stomata characteristics in regenerated diploid and autotetraploid *Allium hirtifolium* plantlets

Ploidy level	Plantlet height/cm	Total plantlets weight/g	Bulblet weight/g	Total Chlorophyll/ (mg · g^{-1}FW)	Carotenoid/ (mg · g^{-1} FW)	Stomata density/ (S · mm^{-2})	Stomata width/μm	Stomata length/μm
Diploid (2n = 2×)	10.84 ± 0.63a	3.11 ± 0.02b	2.19 ± 0.09b	1.61 ± 0.03b	0.35 ± 0.01a	117.33 ± 1.45b	10.97 ± 0.57b	22.86 ± 0.72b
Autotetraploid (2n = 4×)	6.93 ± 0.72b	3.77 ± 0.14a	2.96 ± 0.15a	2.43 ± 0.06a	0.35 ± 0.01a	86.00 ± 2.08a	19.93 ± 0.15a	38.37 ± 0.63a

Note: Ddifferent letters in each column are statistically different at 5% probability.

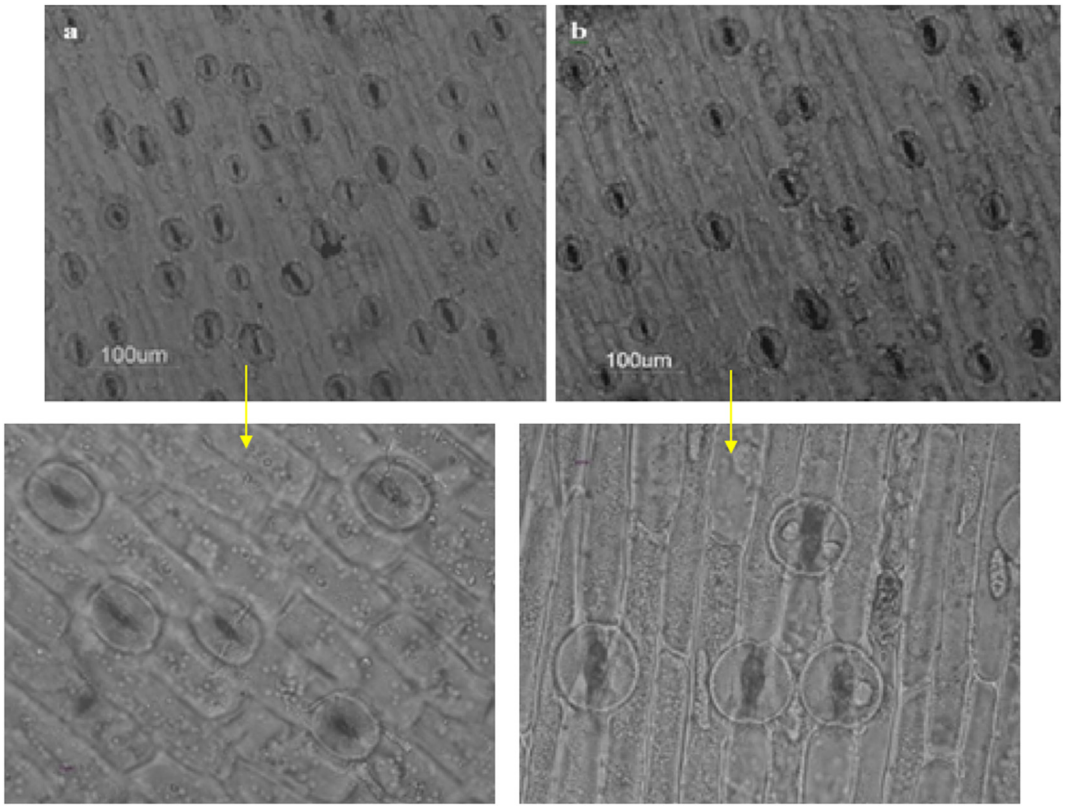

Fig. 10 Variation in stomata frequency in diploid (a) and autotetraploid (b) *Allium hirtifolium* plantlets.

the tolerance of plants to stress conditions. The higher levels of these compounds in polyploid plants could be due to the duplication of the corresponding genes and their overexpression (Zhang et al., 2020). In some species, however, polyploidy lowers the level of secondary metabolites or has an insignificant effect on this trait. These non-identical results come from complicated gene regulation pathways (Touchell et al., 2020).

The investigation of some enzymes related to antioxidant activity of diploid and autotetraploid Persian shallot plantlets revealed that it was higher in the latter than in the former (Table 4). Catalase and superoxide dismutase activities were higher in the autotetraploid plantlets than in the diploid ones. However, ascorbate peroxidase activity was higher in the diploid plantlets. Moreover, the peroxidase activity of the diploid and autotetraploid plantlets showed no significant differences (Table 4). Increased phenolic and allicin content in response to autotetraploidy could lead to the increased antioxidant activity of the studied plantlets. These results suggest that autotetraploid plantlets have a higher antioxidant capacity. The higher antioxidant potential due to enzymatic and non-enzymatic compounds in autotetraploid plants would entail a higher capacity to tolerate environmental stress (Zhang et al., 2010). The results of Lu et al. (2006) showed that ploidy-responsive genes were altered in autotetraploids of *Arabidopsis thaliana* (induction and repression, respectively). Some of these up-regulated genes are involved in various metabolic, signal transduction, transcriptional regulation, and developmental pathways. The expression of duplicated genes alters the morphological and phytochemical characteristics of tetraploid plants compared to their diploid forms.

3.5. *Cluster analysis using SRAP data*

Out of 24 SRAP primer pairs (Table 1), only 20 combinations of primer pairs were able to amplify DNA samples in 12 autotetraploid (with high allicin content) and diploid plants. These 20 primer pairs amplified 127 different amplicons (fragments) ranging in size from 100 bp to 950 bp and the number of amplified bands was 5 bands. Of these 20 primer pairs, polymorphism was observed in only eight primer pairs. In addition, 33 fragments showed polymorphisms between autotetraploid and diploid

Table 3 Bbiochemical traits in regenerated diploid and autotetraploid *Allium hirtifolium* plantlets

Ploidy level	Allicin/(mg · g^{-1} FW)	Pyruvate/(μmol · g^{-1} FW)	Total phenol/(mg · g^{-1} FW)	Soluble solids/%
Diploid (2n = 2×)	2.08 ± 0.09 b	56.52 ± 0.49 a	0.475 ± 0.03 b	11.20 ± 0.08 a
Autotetraploid (2n = 4×)	2.65 ± 0.15 a	55.31 ± 0.08 a	0.545 ± 0.04 a	11.53 ± 0.51 a

Note: Different letters in each column are statistically different at 5% probability.

Table 4 Antioxidant activity in regenerated diploid and autotetraploid *Allium hirtifolium* plantlets

Ploidy level	Antioxidant activity/%	Catalase/ (U · mg^{-1} pro)	Peroxidae/ (U · mg^{-1} pro)	Ascorbat peroxidase/ (U · mg^{-1} pro)	Super oxide dismutase/ (U · mg^{-1} pro)
Diploid (2n = 2×)	64.82 ± 0.32 b*	0.417 ± 0.01 b	0.761 ± 0.02 a	42.06 ± 1.2 a	35.32 ± 0.59 b
Autotetraploid (2n = 4×)	66.07 ± 0.05 a	0.511 ± 0.001 a	0.693 ± 0.02 a	33.89 ± 0.84 b	42.15 ± 0.54 a

Note: Different letters in each column are statistically different at 5% probability.

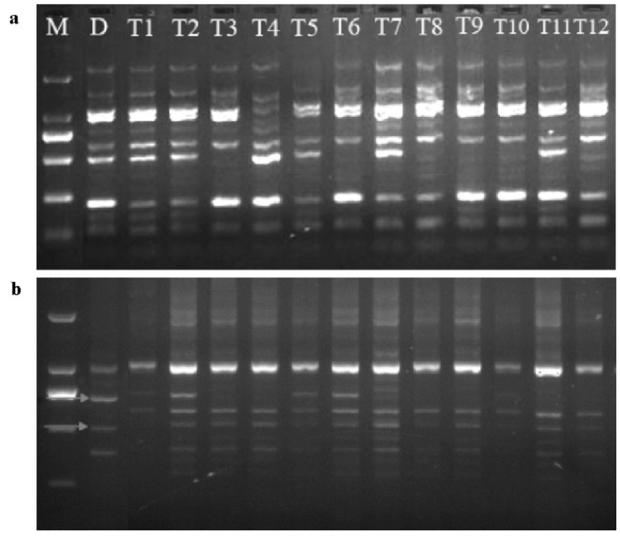

Fig. 11 SRAP of *Allium hirtifolium* using combination primers me3/em1 (A) and me4/em2 (B)
T1–T12: autotetraploid plants; D: diploid; M: DL2000 plus DNA ladder.

plants after the SRAP analysis (Fig. 11). Thus, the polymorphism observed in DNA sequences by SRAP indicates that genetic diversity at different ploidy levels can be detected during polyploidy. The SRAP polymorphic bands revealed that the 12 autotetraploid plants had different genotypes compared with the diploid plant, and that *in vitro* polyploidy induction resulted not only in duplicate chromosomes but also in altered DNA sequence in *A. hirtifolium*. The results of the SRAP analysis in *Saccharum spontaneum* L. showed the presence of genetic diversity among different genotypes with the same and different levels of ploidy (Yu et al., 2019).

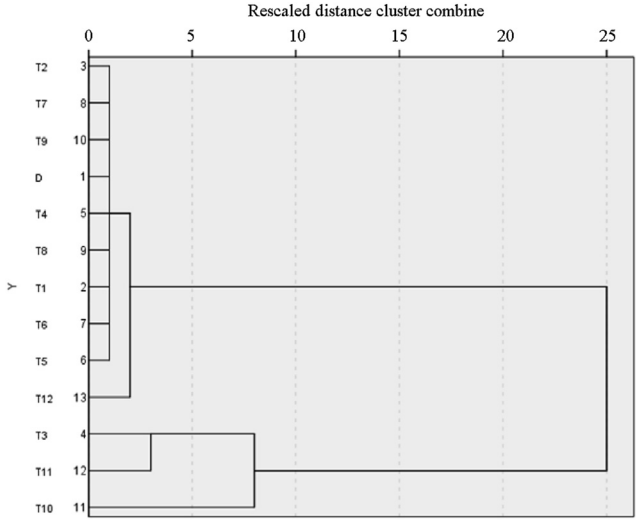

Fig. 12 Phylogenetic dendrogram of 12 autotetraploid plants (T1–T12) and diploids (D) of *Allium hirtifolium*, using 33 polymorphic SRAP markers.

The SRAP data were analyzed using the NTSYS software to create a UPGMA dendrogram (Fig. 12). The analysis of similarity coefficients between 12 autotetraploid and parental diploid plants revealed two main groups. The three autotetraploid plants (T3, T10, and T11) were in cluster I, and the other autotetraploids and the control (diploid plant) were in cluster II. The dendrogram showed a clear pattern of clustering, and the 12 autotetraploids had common differences, indicating that there were differences in their genetic relationships. In addition to the duplication of chromosomes, colchicine and oryzalin may also cause the loss or rearrangement of chromosomes and alteration of the gene sequence due to its mutagenic properties. Cytological variations, such as chromosome duplication and chromosome losses, are reported to be involved in genetic variation in caladium (*Caladium bicolor*) (Xu et al., 2009). Genetic alterations (somaclonal variation) were also observed in regenerated plants derived from somatic embryos of *Lilium distichum* and *Lilium cernuum* treated by colchicine.

4. Conclusions

Our studies have shown that oryzalin is a suitable agent for inducing autotetraploidy in *A. hirtifolium* using a solid medium. *A. hirtifolium* needs several years to produce a mature bulb. The results show that the induction of tetaraploidy in this plant improves the weight of the bulb as well as phytochemical compounds such as allicin and phenol, as well as the antioxidant activity. The SRAP loci polymorphisms indicated that the 12 autotetraploid plants (produced in chemical treatments and exposure times) had different genotypes compared to the diploid parent plant. Besides, *in vitro* polyploidy induction not only duplicated chromosomes but also altered the DNA sequence of *A. hirtifolium*. This basic information will allow researchers to further improve the protocol of *in vitro* autotetraploidy induction in *A. hirtifolium*.

Conflicts of interest

The authors declare that is no any conflict of interest.

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