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Induction of tetraploidy in *Juncus effusus* by colchicine

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Abstract

Tetraploidy was induced *in vitro* in mat rush (*Juncus effusus* L.) cultivar Nonglin-4 by exposure to colchicine (0, 50, 100 and 500 mg dm^{-3}) for 6, 12 and 24 h. Flow cytometric analysis was used to confirm the ploidy level. Anatomical and ultrastructural analyses at cellular and subcellular levels in tetraploid and diploid control plants revealed differences between diploid and tetraploid plants. The leaf epidermis had larger stomata but lower stomatal density in tetraploid plants. In addition, mesophyll cells in tetraploid plants appeared more compact and showed less intercellular spaces along with increased size of vascular bundles. However, a significant reduction of chlorophyll content was observed in tetraploid plants that might be the result of structural modification in the lamellar membranes of chloroplasts.

Additional key words: chlorophyll, chloroplast ultrastructure, diploid, *in vitro* mutation, vascular bundle.

Introduction

Polyploidization of economically important crops has been in practice since long time (Lewis 1980, Zhang *et al.* 2003) and has largely contributed to the production of crops possessing beneficial traits (Gu *et al.* 2003b,c, Alishah and Bagherieh-Najjar 2008). Changes in the polyploidy level alter a number of morphological and anatomical traits like thickness of leaves, stems and roots, width-to-length ratio of leaves, stomata size, size and texture of flowers, flowering period, and pollen grain diameter (Gao *et al.* 1996). In addition, polyploids provide a wider germplasm base for breeding studies (Thao *et al.* 2003). The production of useful tetraploid plants using colchicine has been often reported (Gu *et al.* 2003a, Shao *et al.* 2003, Yang *et al.* 2006). The mutagenic effect of colchicine has been also studied (*e.g.* Gu *et al.* 2003a). The *ex vitro* colchicine treatment is conventionally employed to induce polyploidy in nodal cuttings. However, production of polyploid plants is often low while frequency of chimeras is high. *In vitro* production, on the other hand, offers a low cost, highly efficient technique (Shao *et al.* 2003). It has resulted in production of tetraploid plants in cotton (Alishah and Bagherieh-Najjar 2008), wild turmeric (Mohanty *et al.* 2008), grapevine (Yang *et al.* 2006), citrus (Wu and Mooney 2002), and oilseed *Brassica* crops (Gu *et al.* 2003c, Zhang *et al.* 2006).

Flow cytometry is commonly used to estimate the DNA quantity in cell nuclei (Bennett *et al.* 2000, Doležel and Bartoš 2005). It enables us to estimate the DNA amount directly from the leaf tissue, which is a convenient method to study ploidy levels especially in the plants where chromosome counting is unreliable due to inefficient chromosome spreading and the small size of the chromosomes (Beck *et al.* 2003c, Xu *et al.* 2007).

Mat rush (*Juncus effusus* L.) is a diploid plant. It is an ecologically important and cultivated as crop especially in China and Japan. Mat rush is mainly vegetatively propagated. The stem and leaves are economically important and tetraploid plants are reported to result in more vigorous growth than diploid plants (Gao *et al.* 1996, Gu *et al.* 2003c). The objectives of this study were *1*) to induce tetraploidy in *Juncus effusus* by colchicine treatment *in vitro*, and *2*) to investigate the anatomical, ultrastructural and physiological differences between diploid and tetraploid plants.

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Abbreviations: MS - Murashige and Skoog; BA - benzylaminopurine; FCM - flow cytometry; Cu - cuticle; Epi - epidermis; Mes - mesophyll; Vas - vascular bundle; CH - chloroplast; CW - cell wall; NU - nucleus; VAC - vacuole; PG - plastoglobuli; TH - thylakoid membrane.
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Materials and methods

Regenerated plantlets of a commercial cultivar (Nonglin-4) of mat rush (*Juncus effusus* L.) were used for *in vitro* mutagenic treatment. Basal culm segments (approximately 0.5 - 1.0 cm in length) were treated with various concentrations of colchicine (0, 50, 100 and 500 mg dm^{-3}) for 6, 12 and 24 h. The treated segments were transferred to 1/2 strength Murashige and Skoog (1962; MS) medium with 0.5 mg dm^{-3} benzyladenine (BA). Eight segments per flask were grown under 16-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1}, and temperature of 19 ± 2 °C for three weeks (for detail see Xu *et al*. 2009). Then the basal segments of fully developed plantlets were transferred to fresh MS media. This process was repeated three times and then the plants were shifted to pots with soil-*Perlite* mixture and were grown for 3 weeks in the greenhouse.

Flow-cytometer (FCM) (*BD FACSCalibur*, San Jose, USA) was used to analyze the ploidy level. The newly emerged young leaves were chopped in a detergent solution, and stained with 4',6-diamidino-2-phenylindole (DAPI; Zhou *et al.* 2002a). The suspension of cell nuclei and debris was filtered through a 50-μm nylon gauze and the filtrate was immediately analyzed with the FCM. The instrument was calibrated against normal diploid *Juncus effusus* L. which was used as a standard. The frequency curve of the sample DNA was compared to the standard peak and assigned to a ploidy level of diploid and tetraploid. The tetraploid plants were subcultured and retested after 4 weeks, as previously described. The confirmed tetraploid plants (twice tested) were used for further physiological and ultrastructural analyses.

Five leaves (samples) were randomly selected for every treatment, and every sample was repeated 3 times. The epidermis was peeled off, placed on a glass slide and measured under stereo microscope. Stomata length and width were taken by employing 5 measurements per leaf. Stomatal frequency and size were determined using software *Image-Pro plus 5.1*. The transverse sections of samples embeded in paraffin were used for observations of vascular bundles under light microscope and their size was recorded using software *JeDa 801D*.

The leaf chlorophyll content was determined according to Leul and Zhou (1999). A sample of 0.5 g of fresh leaves was homogenized in 5.0 cm^3 of 50 % acetone and 50 % ethanol mixture and kept in the dark for 8 h at room temperature. The supernatant was then used and absorbance was measured at 645 and 663 nm using *LKB Ultraspec II* (*Pharmacia LKB Biotechnology*, Uppsala, Sweden) spectrophotometer. Chlorophyll *a* and *b* contents were calculated by the equations of Joffrey and Humphrey (1975).

For transmission electron microscopy, leaf segments (approximately 2 - 3 mm in length) of randomly selected samples were fixed in 2.5 % glutaraldehyde (v/v) in 0.1 M sodium phosphate buffer (PBS; pH 7.4) at room temperature and washed three times with the same PBS. The samples were post fixed in 1 % OsO_4 for 1 h, then washed three times in 0.1 M PBS with 10-min interval between each washing. Later on, they were dehydrated in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100 %) with 15 to 20-min intervals and finally by absolute acetone for 20 min. The samples were then infiltrated and embedded in Spurr's resin overnight. After heating the specimens at 70 °C for 9 h, the ultrathin sections (80 nm) were prepared and mounted on copper grids for viewing in the transmission electron microscope (*JEOL TEM-1230EX*, Tokyo, Japan) at an accelerating voltage of 60.0 kV. Data for cell and subcellular organelles were recorded using software *JeDa 801D*.

Analysis of variance (*ANOVA*) was performed for the morphological characters, plant survival rate, induction of tetraploids, chlorophyll content and other physiological parameters. Data were analyzed using the *SAS v. 9* software. Where the *F*-test showed significant differences among means, multiple range tests were performed at the 0.05 level of probability. Before *ANOVA*, the data of survival rate and induction of tetraploids were arc sin transformed.

Results and discussion

Plant survival rate decreased significantly with the increasing concentration at the same treatment time as well as prolonged treatment time (*i.e*., from 6 to 24 h) under the same concentration of colchicine (Table 1). The highest number of lethal mutants appeared at 500 mg dm^{-3} colchicine applied for 24 h.

The colchicine at concentration 50 mg dm^{-3} did not induce any tetraploid plants when applied for 6 h. However, when the same concentration was applied for 12 or 24 h the number of tetraploid plants increased (Table 1). Also increasing colchicine concentration caused significant enhancement in the number of tetraploid plants. Under the same concentration of colchicine no significant difference was observed in the number of tetraploid plants produced after exposure 12 or 24 h. Thus the highest number of tetraploid plants (16 %) appeared at 500 mg dm^{-3} colchicine applied for 12 or 24 h (Table 1). Initial growth of tetraploid plants just after colchicine treatment was slow as compared to the diploid control plants. However, these tetraploid plants appeared thicker, stronger and longer than the controls after all subcultures on fresh media and transfer to the soil. Similar dose- and time-dependent effects of colchicine on generation of tetraploidy has been reported by Alishah and Bagherieh-Najjar (2008) in cotton. Moreover, similar effects of high doses of colchicine on growth inhibition were reported by Rauf *et al.* (2006).

Length and width of stomata in tetraploid plants (41.5 and 31.7 μm, respectively) were significantly higher compared to diploid plants, however, the stomatal density

was lower in tetraploid plants (Table 2, Fig. 1*A*). The epidermal cells of tetraploid plants were longer and wider than in diploid plants. No significant difference in the arrangement of epidermal cells was observed between tetraploid and diploid plants (Fig. 1*A*). Stomata length is considered as an accurate and a fast indicator to estimate the rate of polyploidy in many plants (Beck *et al.* 2003a). There are a number of reports showing larger stomata and lower density of in tetraploid plants over control plants in different plant species. Our results are in line with the findings of Yang *et al.* (2006) and Thao *et al.* (2003).

Transverse sections of leaves of both diploid and tetraploid plants showed significant differences in the arrangement of tissues and cell size. The bundle sheath cells were tightly arranged around the vascular bundles in both the control and tetraploid plants. However, tetraploid plants had more compact arrangement of mesophyll chlorenchyma cells with less intercellular spaces as compared to the controls (Fig. 1). The vascular bundles were relatively smaller in size and higher in number in the outer circle, and larger in size with lower frequency in the inner circle. Average size and number of vascular bundles were higher in tetraploid plants as compared to diploid plants along with significant increase in leaf length, width and dry mass (Table 2). Similar to our results, Fagerberg *et al.* (1990) reported that tetraploid chickpea plants had a greater mesophyll cell volume and less air space density than diploid plants.

Chlorophyll *a*, *b* and total chlorophyll contents were reduced significantly in tetraploid mutant plants (Table 2). A decrease in chlorophyll contents in the present study may be due to disintegration of thylakoid membrane system in tetraploid plants. However, no significant difference was observed in the chlorophyll *a*/*b* ratio which suggested that the stoichiometry of the various chlorophyll-protein complexes remained the same (Harper *et al.* 2004). In contrast, an increase in the ploidy level induced a greater number of chloroplasts and higher amount of chlorophyll in black wattle (Beck *et al.* 2003b) and pomegranate (Shao *et al.* 2003). However, Warner and Edwards (1989) described no changes in chlorophyll content in response to an increase in ploidy level.

Morphometric analysis of numerous electron

Table 1. Effect of colchicine concentration and treatment duration on survival rate and induction of tetraploids in *Juncus effusus*. Means ± SD, $n = 3$; means in each column followed by different letters are significantly different at $P < 0.05$.

Colchicine [mg dm^{-3}]	Treatment [h]	Survival rate [%]	Tetraploids [%]
50	6	85.80 ± 2.60 a	0f
	12	84.58 ± 3.15 a	4.46 ± 0.40 de
	24	78.33 ± 1.91 b	4.83 ± 0.47d
100	6	80.00 ± 2.50 b	4.38 ± 0.37 e
	12	60.63 ± 2.25 c	7.71 ± 0.51 c
	24	50.00 ± 0.00 d	7.83 ± 0.56 c
500	6	41.67 ± 1.86 e	8.96 ± 0.44 b
	12	26.79 ± 1.56 f	16.92 ± 0.19 a
	24	19.08 ± 0.38 g	16.33 ± 0.45 a

Table 2. Morphological and physiological variation in tetraploid plants compared to diploid control *Juncus effusus* plants. Means ± SD, $n = 3$; means in each column followed by different letters are significantly different at $P < 0.05$.

Ploidy level	Epidermal cell length [μm]	Epidermal cell width [μm]	Stomata length [μm]	Stomata width [μm]	Stomata length/width	Stomata density [No mm^{-2}]	Leaf fresh mass [mg]
Diploid	135.2 ± 6.78 b	18.3 ± 1.09 b	37.6 ± 2.44 b	28.9 ± 1.84 b	1.30 ± 0.06 a	99.2 ± 2.03 a	356.81 ± 13.5 b
Tetraploid	158.4 ± 8.59 a	20.4 ± 1.76 a	41.5 ± 0.79 a	31.7 ± 2.85 a	1.31 ± 0.10 a	59.5 ± 1.83 b	642.03 ± 14.2 a

Ploidy level	Number of vascular bundles		Diameter of vascular bundle [μm]		Leaf length [cm]	Leaf width [μm]	Leaf dry mass [mg]
	inner circle	outer circle	inner circle	outer circle			
Diploid	6 ± 0.34 b	9 ± 0.56 b	54.15 ± 3.26 b	15.24 ± 1.21 b	0.8 ± 0.11 b	90.6 ± 3.21 b	114.18 ± 8.7 b
Tetraploid	8 ± 0.52 a	12 ± 0.93 a	67.16 ± 4.89 a	18.21 ± 1.08 a	1.4 ± 0.15 a	121.6 ± 3.15 a	186.19 ± 6.6 a

Ploidy level	Chlorophyll *a* [mg g^{-1}(f.m.)]	Chlorophyll *b* [mg g^{-1}(f.m.)]	Chlorophyll *a*/*b*	Chlorophyll *a*+*b* [mg g^{-1}(f.m.)]	Cell circumference [μm]	Chloroplast circumference [μm]	Cell wall thickness [μm]
Diploid	1.32 ± 0.01 a	0.51 ± 0.02 a	2.58 ± 0.08 a	1.83 ± 0.03 a	54.98 ± 1.87 b	11.93 ± 1.03 a	0.31 ± 0.03 a
Tetraploid	1.16 ± 0.01 b	0.46 ± 0.02 b	2.52 ± 0.08 a	1.62 ± 0.03 b	61.63 ± 1.78 a	13.05 ± 1.07 a	0.22 ± 0.01 b

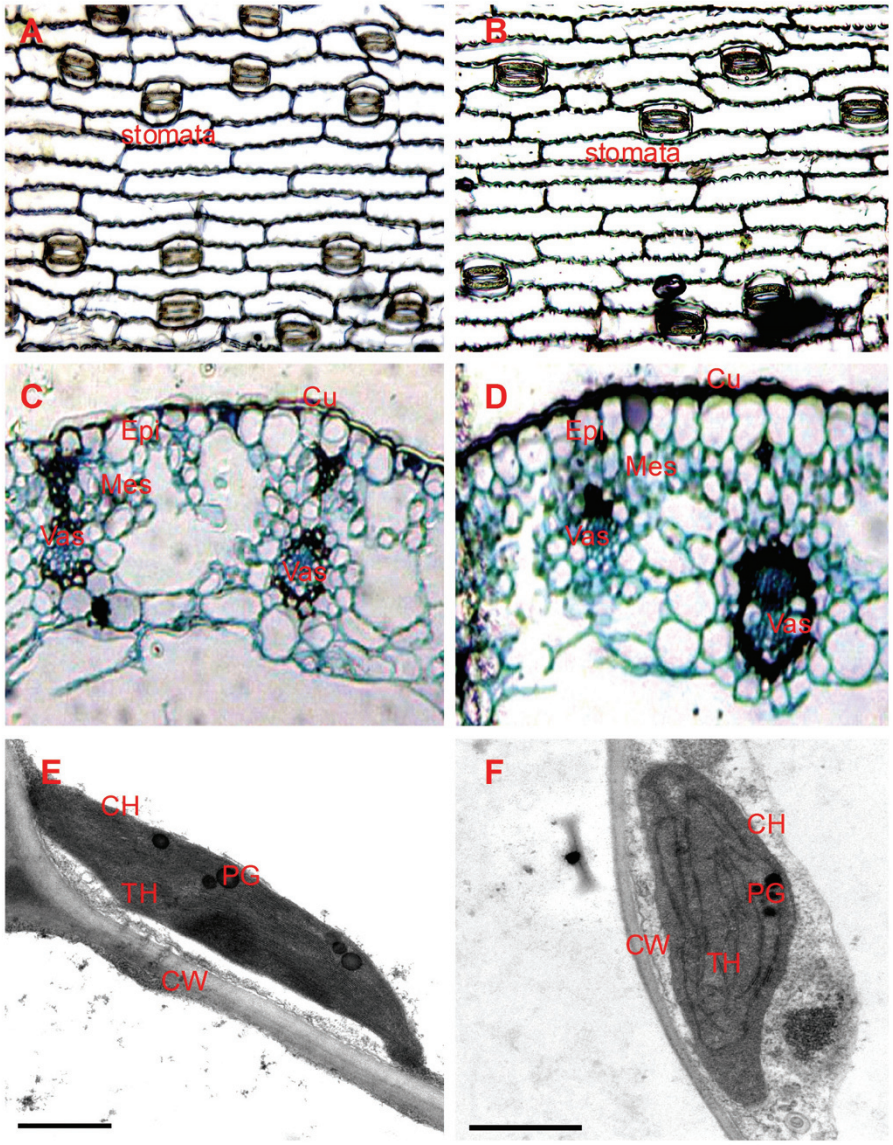

Fig. 1. Epidermal cells and stomata of diploid (*A*) and tetraploid (*B*) plants of mat rush (*Juncus effusus* L.) from *in vitro* colchicine treatments. Transverse section of a leaf, showing tissue arrangements and changes in size of vascular bundles in colchicine induced tetraploid mutant (*D*) compared to diploid control (*C*) (Cu - cuticle, Epi - epidermal cell, Mes - mesophyll cell, Vas - vascular bundle). Chloroplast ultrastructure of the tetraploid mutant cells (*F*) compared with the diploid control (*E*). (CW - cell wall, CH - chloroplast, PG - plastoglobuli (lipid droplets), TH - thylakoids membrane, *bar* = 1 μm).

microscope images of mesophyll cells revealed significant changes in the cells and subcellular organelles of these plants. Overall, tetraploid plants had larger size of cells and chloroplasts than the diploid plants (Table 2). Chloroplast swelling was recorded resulting in a significant increase in the chloroplast width over the control, however, significant reduction in cell wall thickness was noted (Fig. 1*F*). Modification in the lamellar membrane system of the chloroplast was obvious in tetraploid plants. Instead of compact thylakoid membrane system seen in diploid cells it appeared distorted into small segments and spread lengthwise in the stroma. In addition, increased number of plastoglobuli of varying electron densities was observed in the chloroplast of tetraploid compared to diploid plants (Fig. 1*F*). Our results are in agreement with the findings of Eleftheriou (1994), who observed in tetraploid plants the chloroplast containing exceptionally dense stroma, swollen vesicular structures and numerous plastoglobuli. Wise and Cook (1998) also found chloroplast swelling in maize mutant. Increase in the number of plastoglobuli is also considered to be the result of different stresses (Navarro *et al.* 2007).

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