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RESEARCH NOTE

Induction of tetraploid poplar and black locust plants using colchicine: chloroplast number as an early marker for selecting polyploids in vitro

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Abstract Tetraploid plants were produced by inducing chromosome doubling using colchicine in in vitro shoot tips of poplar and black locust clones. Many of the plants treated with colchicine showed modified morphological characteristics like stunted growth, thicker leaves and modified leaf morphology. The counting of chloroplast number in the epidermal guard cells of stomata was used for the rapid screening of tetraploids. The differences in mean chloroplast numbers between diploid and tetraploid plants were highly significant. For all plants tested, the tetraploid genotype had almost double the number of chloroplasts per guard cell compared to the diploid origin. Some plants were further analysed by flow cytometry to verify their ploidy status that was determined by chloroplast numbers. The results of this study demonstrated for the first time that chloroplast counting in poplar and black locust could be an effective and reliable method for pre-screening large numbers of plants for their ploidy level. The protocol might be applicable in a wide scope of breeding programs.

Keywords Chloroplast number · Colchicine · In vitro · Flow cytometry · Poplar breeding · Tetraploid

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Abbreviations
2× Diploid
3× Triploid
4× Tetraploid

Since the first triploid tree with high growth performance and resistance traits was discovered (Müntzing 1936; Nilsson-Ehle 1936), breeding polyploid individuals, mainly tetraploids, as a parent for producing triploid trees has become a breeding aim. Different methods have been used to attain such trees, but mainly colchicine treatment showed suitable results. This mitotic poison inhibits microtubule polymerization by binding to tubulin. Therefore it disturbs chromosome segregation during meiosis and results in polyploidy in plants. Seedlings of unknown growth performance were induced as well as terminal buds of selected trees (Eifler 1967; Johnsson 1975). Nevertheless, final success in breeding polyploids was restricted. Although studies concerning triploids in forestry declined in the last decades in Europe, the interest in such plants in horticulture did not wane, especially when it became obvious that reduced fertility led to so-called "seedless" fruits in for example citrus or watermelon. In many other fruit bearing plants, triploids yielded superior fruit quality and size. Therefore, it was necessary to create a lot of tetraploid plants for new breeding material. Through tissue culture methods it became possible to treat large amounts of clonal shoot tips with colchicine, to regenerate new shoots and select tetraploids in vitro (Van Duren et al. 1996; Li et al. 2008). Within recently enhanced activities aimed at selecting and creating new plant material for biomass production, ploidy breeding might regain importance for woody plants too.

The conventional method of ploidy assessment via chromosome counting in root tip cells is labour intensive and time consuming, while modern flow cytometric analyses require a special laboratory equipment (Eeckhaut et al. 2005; Doležel et al. 2007). Alternative methods like e.g. measurement of stomatal length and frequency (Beck et al. 2003a) or quantification of chlorophyll content (Mathura et al. 2006) are therefore essential for pre-screening the ploidy level of large quantities of in vitro plants. Butterfass (1973) reported that for a given genotype a positive correlation between chloroplast numbers in leaf guard cells and ploidy level exists for different plants. Jacobs and Yoder (1989) could demonstrate the ploidy variation in transgenic tomato plants by counting the number of chloroplasts in stomatal cells. The usefulness of chloroplast counting for indicating ploidy levels was also shown in anther-derived calli of petunia (Mitchell et al. 1980), in maize (Ho et al. 1990), watermelon (Compton et al. 1999), in tetraploids of banana generated in vitro (Ganga et al. 2002) and in ploidy analysis of black wattle (Beck et al. 2003b).

The aim of this study was to find a fast and easy method for inducing tetraploid poplar and black locust plants through colchicine treatment of in vitro shoots followed by regeneration of plants. Furthermore, the efficiency of a simple and reliable procedure of pre-screening tetraploid plants using chloroplast numbers was proven.

Twenty micropropagated shoot tips (2 cm in length) from each of four poplar clones [Esch 5 (*Populus tremula* L. × *P. tremuloides*/Brauna11 × Tur141), L447 [*Populus* × *canescens* (Ait.)], Brauna 11 (*P. tremula* L.), T89 (*P. tremula* × *P. tremuloides* Michx.)] and from black locust clone 81/62 (*Robinia pseudoacacia*) were placed into sterile Eppendorf tubes containing 100 µl of filtersterile solution of 0.1% colchicine stained with Coomassie′s blue to trace the uptake of the solution by the explants. The cultures were kept at 23°C with a 16 h photoperiod supplied by warm-white fluorescent light at 30–40 µE m^{-2} s^{-1}. After 18 h, the shoots were transferred to propagation medium. LS_{yang} medium (a modified Linsmaier-Skoog medium described by Yang et al. 2003) was used for poplar and WTD_5 (Ahuja 1987) for black locust. After 4–8 weeks of cultivation, slightly morphologically different shoots with stunted growth, thicker leaves and modified leaf morphology became visible in some explants. Plants showing these characteristics were subcultured in vitro by transferring the respective shoot tips. This step was repeated about 8 times. In these intervals chloroplast numbers were counted for ploidy determination.

Sixty black locust seeds (*Robinia pseudoacacia*) were aseptically germinated on filter paper bridges. After 10 days, roots were cut to 1 cm in length and the plantlets were placed into 0.1% colchicine solution as described above. One week later, explants were transferred to half-concentrated modified Woody Plant Medium (WPM, according to Lloyd and McCown 1980) with 0.1% activated charcoal. One week after, shoot tips from each germinated plant were cut above the cotyledons to induce sprouting of axillary buds. Shoot development was stimulated by cultivating the explants on fully concentrated fresh WPM supplemented with 0.045 µM thidiazuron every month.

For chloroplast counting, three leaves were sampled from respective plants. Small pieces of the leaves were placed on a wet filter paper, coated with 0.5% fluorescein dibutyrate and covered with cellophane. After fifteen minutes of incubation, the leaf pieces were placed in a drop of water on a microscope slide and covered with a cover slip. Finally, the tissue was pressed carefully by knocking on the microscope slide with a pencil. The chloroplasts were counted immediately using a fluorescence microscope (Olympus BH2-RFCA) with either 250× or 500× magnification. From each clone or genotype, 3–6 single plants were used for the calculation. Chloroplasts of at least 20 guard cells from each plant were counted (in total at least 100 guard cells per clone). Grand means according to Everitt (2002) were averaged from the means of the individual plant chloroplast numbers. Standard deviations were calculated from the grand means. Data sets were normally distributed. Differences in chloroplast numbers were analysed for significance by the Welch test ($P < 0.01$). Descriptive statistics, the Shapiro–Wilk W test for testing for normality and the Welch test were performed using Statistica vers. 6.1 (StatSoft Inc.).

Flow cytometry was performed to detect the ploidy level of seven plants. Nuclei suspensions were prepared from leaf tissues as described by González Castañón and Schröder (2002). Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) and the fluorescence was analysed using a Partec Ploidy Analyser (Partec GmbH, Münster, Germany). For evaluating the ploidy level of colchicine-treated plants, untreated plants of the same origin and triploid plants of known origin were also analysed. Peaks representing the frequency of cells with certain DNA content were compared and the ploidy level was determined.

In all tested clones, relatively high amounts of plants showed modified phenotypic characteristics resulting from colchicine induction. Such poplar plants showed strongly toothed leaf margins, longer leaves and thicker veins. Polyploid black locust plants generated slightly bigger, dark green single leaves that were sometimes less oval. After subcultivating shoot tips that showed tetraploid characteristics (morphological features and corresponding numbers of chloroplasts) about 6–8 times, detailed analyses of the ploidy level were performed. About 50 plants from

each colchicine-treated clone were analysed by counting the chloroplast numbers in stomatal guard cells. In order to verify the ploidy status determined by chloroplast numbers, some plants were additionally analysed by flow cytometry.

Colchicine-treated plants which were proved to be tetraploid showed significantly higher chloroplast numbers in comparison to untreated control plants (Fig. 1). According to the results from flow cytometry, chloroplast numbers could be assigned to the different ploidy levels—diploid (2×), triploid (3×), tetraploid (4×) and mixoploid. The differences in mean chloroplast numbers between diploid and tetraploid plants were highly significant (Table 1). Doubling the ploidy level from 2× to 4× increased chloroplast density by a factor of approximately 1.8–1.9. For all plants tested, the tetraploid genotype had nearly double the chloroplast count per guard cell when compared to the diploid origin. Doubling of chromosomes seemed to cause an approximate doubling of chloroplasts in guard cells. Both methods, chloroplast counting and flow cytometry, detected doubling in the chromosome numbers. Examples of chloroplast numbers in stomatal guard cells of colchicine-treated poplar clones and black locust clones are presented in Table 1.

For the poplar clones Esch5, Brauna11, T89 and the two black locust clones, only tetraploid and mixoploid plants could be found. For the well performing grey poplar clone L447 (*P. x canescens*), triploid plants were also regenerated showing significantly different chloroplast numbers compared to both 2× and 4× plants. This is in accordance with results from Sivolapov and Blagodarova's (1997) chromosome counting experiments in two selected gigantic grey poplar clones. They described the occurrence of mixoploid individuals, which showed mainly triploid cells (>70%), but to a certain degree also di- and tetraploid ones. The accurate frequency of stable tetraploid plants regenerated in the experiments presented here was difficult to define, because confirmation of already stable tetraploids was not possible after such a short period. However, the estimated frequency for poplar clone Esch5 was about 20%.

In this study, shoot tips were used for colchicine treatment, but other types of explants were additionally tested. Root meristems were also used because of their regeneration potential (see also Haque et al. 2006). The treatment of root meristems offered a more locally restricted response to colchicine because of their smaller size. However, they consisted of a highly defined and already specialized tissue. During adventitious regeneration of shoots from root tips, induced by higher growth regulator concentrations than normally necessary for multiplication of shoots, several chloroplast-deficient plants were observed. The velocity of plant regeneration was delayed by 2–3 subcultures compared with regeneration from treated shoots. Small shoots—as used in this study—were a suitable explant type for colchicine treatment and provided sufficient amounts of modified shoots after multiple subcultures. Apical meristems as well as all meristems within shoot axils were stimulated simultaneously but differentially by colchicine. The disadvantage was the large number of subsequently appearing axillary shoots, which had to be tested later on. The most restricted reaction was noted in single node explants. In this case, there was a single meristem of restricted size, which was already determined to form shoots. The small size and the preset course of development allowed a defined treatment. All appearing axillary shoots were derived from one common origin. As a result of these observations, single leaf axils could be recommended as the explant type of choice for further experiments.

Tetraploid and triploid plants selected from the experiments were cultivated on root inducing medium and then

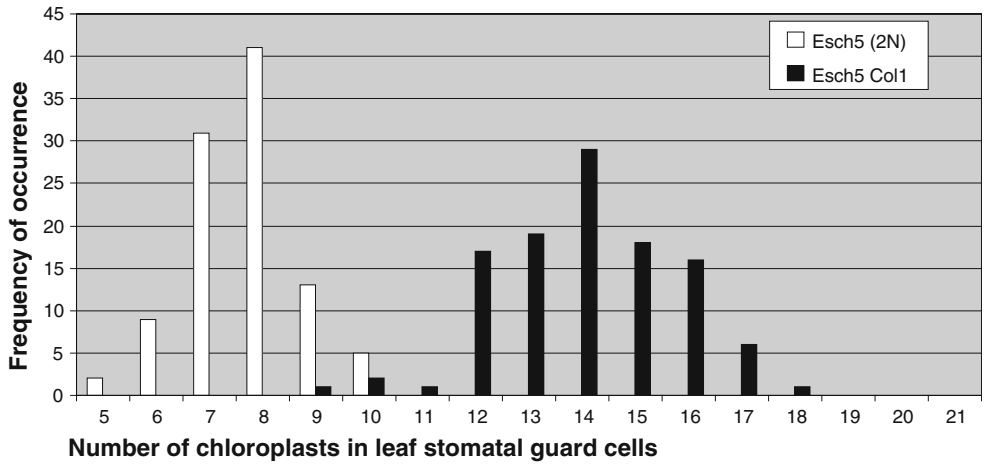

Fig. 1 Frequency scale of chloroplast number per leaf guard cell in in vitro plants of the poplar clone Esch5: Diploid original clone (*white*), tetraploid plant after colchicine treatment (*black*)

Table 1 Chloroplast numbers in leaf guard cells of different poplar and black locust clones/genotypes (clones after colchicine treatment are written in italic)

Plant clone (known ploidy level)	Mean[a] number of chloroplasts per guard cell (S)	Number of plants (guard cells) analysed	Ploidy level (ploidy determined by using flow cytometry)
Poplar			
Astria (3N)[b]	10.85 (0.38)	4 (128)	3N (3N)
L433 (3N)[b]	11.14 (0.08)	4 (104)	3N
Esch5 (2N)	7.68 (0.16)	4 (102)	2N (2N)
Esch5 Col 1	*14.01 (0.25)**	*4 (111)*	*4N (4N)*
Brauna11 (2N)	6.42 (0.15)	5 (199)	2N (2N)
Brauna11 Col 1	*12.33 (0.18)**	*4 (100)*	*4N (4N)*
Brauna11 Col 8	*11.67 (0.27)**	*4 (135)*	*4N*
T89 (2N)	7.23 (0.15)	4 (110)	2N
T89 Col 2	*13.6 (0.37)**	*4 (132)*	*4N*
L447 (2N)	8.13 (0.28)	5 (206)	2N (2N)
L447 Col 6	*12.24 (0.18)**	*6 (296)*	*3N*
L447 Col 11	*14.55 (0.18)**	*5 (118)*	*4N*
Black locust			
46(2) (2N)	4.57 (0.10)	4 (138)	2N (2N)
46(2) Col	*8.64 (0.33)**	*3 (179)*	*4N*
81(62)Col	*9.11 (0.23)**	*3 (105)*	*4N*

* Significant at $P < 0.01$ (Welch test) for comparison between colchicine treated plants and the respective untreated control plants

[a] The values in the table represent the mean values of all the chloroplast numbers per guard cell per plant averaged for the respective tree clone or genotype (after colchicine treatment -*Col*-). Standard deviation is given in brackets

[b] Clones Astria (*P. tremula* × *P. tremuloides*) and L433 (*P. tremula*) were used as triploid plants of known origin

transferred to the greenhouse. This is a precondition for subsequent field-testing for growth behaviour and stimulating early flower formation in these plants.

In conclusion, the possibility of inducing tetraploids in poplar and black locust using colchicine treatment of shoot tips could have a significant impact on tree breeding. Prescreening based on chloroplast numbers was useful for the identification of stable tetraploids and the separation from mixoploid plants to reduce population size after in vitro passage. Thus early ploidy estimation based on chloroplast counts of in vitro grown plantlets may accelerate tree-breeding programmes.

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