Journal of Fruit and Ornamental Plant Research Vol. 13, 2005: 109-122

SOMACLONAL VARIATION IN MICROPROPAGATED TULIPS BASED ON PHENOTYPE OBSERVATION

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(Received November 28, 2005/Accepted December 8, 2005)

ABSTRACT

A new protocol for tulip micropropagation was developed to produce virus-free stock plants, speed up breeding, and to introduce new cultivars on the market. This method is based on cyclic multiplication of adventitious shoots in the presence of thidiazuron. Before this method can be recommended for general use, it had to be verified that it could be used to produce high quality, true-to-type plants. Tulips of the cultivars 'Blue Parrot' and 'Prominence' were cultured *in vitro* from 1.5 to 6 years before being cultured *ex vitro*. Plants were observed for morphological changes when they began to flower. Juvenile plants were also examined for leaf variegation and other abnormalities. The first plants to flower did so in their third or fourth growing season. Reliable information on somaclonal variation introduced during *in vitro* propagation can be obtained when more than 30% of the plants have flowered. This can occur as early as the fourth or fifth growing season. The frequency of variation was less than 3.3%, in progeny lines derived from cultures maintained *in vitro* for less than three years. But in the progeny lines derived from four-year-old cultures, all plants of 'Blue Parrot' and the half of plants of 'Prominence' were changed. In 'Blue Parrot', all of the off-type plants had flowers which were red-purple instead of purple-violet. In 'Prominence', most of the off-type plants had minor changes, such as lily-like flowers, although some plants did have strongly malformed flowers. Variegation was observed only in juvenile plants of progeny lines derived from shoot cultures which had been propagated *in vitro* for four years or longer. Micropropagation of tulips with this new method can increase the risk of mutation, especially when the *in vitro* cultures are maintained for longer period. By limiting this period to three years or less, this risk can be significantly reduced. Systematic monitoring of the plant material with DNA markers would further reduce the risk.

Key words: *Tulipa gesneriana*, somaclonal variation, *in vitro* propagation, flowering, variegated plants

INTRODUCTION

A new protocol for tulip micropropagation was developed to produce virus-free stock plants, speed up breeding, and to introduce new genotypes on the market. The method is based on the cyclic multiplication of adventitious shoots in the presence of thidiazuron (Podwyszyńska, 2001; Podwyszyńska and Marasek, 2003). In the tulip, the natural propagation rate is very low. Because each bulb produces only two or three new bulbs a year, twenty or more years can pass between the initial crossing and commercial release of a new cultivar. The new method can shorten this process by ten years.

Before this method can be recommended for general use, it had to be checked to see if it could be used to produce high quality, true-to-type plants. It also had to be determined whether *in vitro* culture could induce genetic or epigenetic changes detectable in the plant phenotype.

Genetic and epigenetic variation which occurs during vegetative propagation, including *in vitro* multiplication, is called somaclonal variation (Larkin and Scowcroft, 1981). The nature and causes of somaclonal variation have been elucidated, and the types of changes observed and their usefulness in plant breeding have been reviewed by several researchers (Sabała and Orlikowska, 1993; Jerzy et al., 1994; Borkowska, 1995; Karp, 1995; Pląder, 1997; Jain and De Klerk, 1998).

Changes can be heritable or transient, depending on whether the changes occur in the germ line. Somaclonal variants can be detected using morphological, cytological, biochemical and molecular methods (Chen et al., 1998; Al-Zahim et al., 1999; Zhao et al., 2005).

Spontaneous mutations in a particular gene occur once every 10^4 to 10^7 cell divisions, depending on the gene in question. The mutation rate is considerable higher in tissue cultures, and, in extreme cases, can be as high as several percent per locus (Nadolska-Orczyk, 1991).

The mutation rate depends on the genotype, the explant source, the regeneration system, the concentration of growth regulators, the number of multiplication cycles, and the duration of the culture period.

Until now, little was known about the occurrence of somaclonal variation during micropropagation of tulips because this method has only been used on a very limited scale.

In one study, no off-types were found among flowering tulips which had been propagated *in vitro*, even if they had been produced with the help of 2,4-D, an auxin with a relatively high mutagenic activity (Langens, 2001). In fact, flower quality in these plants was higher than flower quality in plants which had been propagated by conventional methods.

When testing a new micropropagation method, the tulips produced should be morphologically evaluated for true-to-typeness when they are in the flowering phase. This can be done as early as four years after the bulblets were produced *in vitro* (Le Nard et al., 1987). Some

modifications which permit earlier testing have been investigated (Podwyszyńska and Nowak, 2004). For example, plants which had been produced *in vitro* were maintained in a growth chamber or greenhouse in order to let them go through two growing cycles a year with minimum loss of bulbs due to winter freezing. However, the reproduction rate was very low. Better results were obtained by growing the tulips outdoors under optimized conditions. In this way, two to five daughter bulbs per mother plant as well as a high rate of bulb weight increase could be obtained every year.

The aim of this study was to evaluate the new method in terms of true-to-typeness and the rate of morphologically detectable somaclonal variation.

MATERIAL AND METHODS

The tulip cultivars used in this experiment were 'Blue Parrot' and 'Prominence'. Shoots were multiplied *in vitro* by the means of adventitious regeneration for 1.5 to 6 years before bulblets were generated and cultivated *ex vitro* for two to six years (Podwyszyńska and Marasek, 2003; Podwyszyńska, 2001; Podwyszyńska and Nowak, 2004). Two progeny lines were produced for 'Blue Parrot', BP-A and BP-B. Five progeny lines were produced for 'Prominence', Pr-A to Pr-E. The conditions under which they were cultured and propagated are presented in Table 1. Additionally, two progeny lines of 'Blue Parrot' derived from the four- and six-year-old cultures were planted in an insect-proof tunnel in 2001 and 2003, respectively. These plants did not flower in 2005 (juvenile plants) and were observed for leaf changes.

BP-A, Pr-A, Pr-B, Pr-D and Pr-E were grown in a growth chamber or greenhouse for the first or second growing cycle (GC) before being transferred outside to an insect-proof tunnel. BP-B and Pr-C were planted directly outdoors (Podwyszyńska and Nowak, 2004).

Bulbs were transferred to the insect-proof tunnels in mid-October, planted in plastic boxes filled with growth substrate, and mulched with bark to protect them from the frost. For each progeny line, bulbs were planted in order from largest to smallest. In the spring, the plants were fertilized, watered as needed, and treated to protect them from pests and diseases. At the beginning of July, the bulbs were lifted, dried, counted, weighed and stored at 20°C until they were planted again in the fall. In 2000, the first year plants were transferred outdoors, no insect-proof tunnel was used and the plants were accidentally exposed to aphids.

Bulbs of the progeny line Pr-E were grown in a greenhouse during the first GC. The next year, they were rooted in a growth chamber at 9°C from February until May, when they were transferred outdoors to an insect-proof tunnel. In August, the bulbs were lifted and stored until they were replanted in October.

Morphology was evaluated when the plants were in full bloom, which

occurred at the beginning of May for ‘Prominence’, and in mid-May for ‘Blue Parrot’.

The ratio of flowering plants to the number of bulbs planted in the first GC was calculated. Morphological evaluation was performed in accordance with UPOV guidelines with the help of published cultivar descriptions (Anonymous, 1988; Krause, 1986; Holitscher, 1968; 1972; Van Scheepen, 1996).

The morphological traits recorded were: plant height, leaf number per plant, leaf color, flower shape, flower length, flower color, base shape, base color, stamen color, and pollen color. Flower color was determined with the help of the color chart developed by the Royal Horticultural Society of London (Anonymous, 1996). Data were also collected on juvenile plants with altered leaf shape or with variegated leaves.

Data on plant height and flower length were statistically elaborated by analysis of variance, followed by Student's t-test at $P \leq 0.05$.

The main characteristics of ‘Blue Parrot’ are: plant 55 to 60 cm in height; flower purple-violet (CC 80C); tepal goffering weaker than in other parrot tulips; flower 7.5 cm long; base greenish blue edged with yellow; pollen grayish purple.

The main characteristics of ‘Prominence’ are: plant 40 to 45 cm in height; flower dark red (46B); flower 7.0 cm long; base ivy green edged with yellow; pollen bluish black.

Plants of the original cultivars which had been propagated by conventional means served as the reference.

In order to rule out morphological changes due to viral infection, all off-type plants and randomly selected true-to-type plants were tested for viruses by ELISA in 2004 and 2005. The viruses tested for were: *Tulip breaking virus* (TBV), *Lily symptomless virus* (LSV), *Cucumber mosaic cucumovirus* (CMV), *Tobacco rattle tobavirus* (TRV), *Tobacco necrosis virus* (TNV), and the potyvirus group. Leaf samples for testing were collected from mid-April to mid-May. Testing was carried out as previously described (Podwyszyńska et al., 2005).

RESULTS

In 2003, the first micropropagated plants flowered after 2.5 to 3 years of cultivation (Tab. 1). The progeny line BP-A was in its fourth GC and had a flowering rate of 20.8%. Flower color was typical for ‘Blue Parrot’, although the tepals did not have any of the traits typical for parrot tulips. Pr-A was also in its fourth GC and had flowers which were true-to-type, except for one plant which had red and white tepals. This plant was infected by TBV and removed from further study.

In 2004, BP-A, Pr-A and Pr-B were in their fifth GC. BP-A had a flowering rate of 37.5%, Pr-A had a flowering rate of 25.0%, and Pr-B had a flowering rate of 22.5%. Pr-C was in its fourth GC after a 0.5 years in the growth chamber and 2.5 years outdoors, and had a flowering rate of 23.5%. BP-B and Pr-D were in their

Table 1. Flowering and somaclonal variation in tulips derived from micropropagation

Cultivar, progeny line, number of growing cycles (GCs) until 2005, place of cultivation c – growth chamber g – greenhouse t – insect-proof tunnel	*In vitro* multiplication period [years]	Year of planting *ex vitro*	Number of bulbs planted in first GC	Flowering plants [%]			Number of off-types in 2005		Somaclonal variation in 2005 [%]
				2003	2004	2005	minor changes	major changes	
'Blue Parrot'									
BP-A: 6 GCs: 1c, 5t	2	1999	48	20.8	37.5	72.9	0	0	0.0
BP-B: 4 GCs: 4t	4	2001	91	0	3.3	5.8	0	6	100.0
'Prominence'									
Pr-A: 6 GCs: 1c, 5t	1.5	1999	32	3.1	25.0	78.1	0	0	0.0
Pr-B: 6 GCs: 2c, 4t	2	1999	45	-	22.2	133.3	1	1	3.3
Pr-C: 5 GCs: 5t	3	2000	17	-	23.5	58.5	0	0	0.0
Pr-D: 5 GCs: 1c, 4t	4	2001	120	-	1.5	30.8	17	2	51.4
Pr-E: 5 GCs: 1g, 4t	4	2001	68	-	-	32.4	10	1	50.0

third GC and flowered sporadically. BP-B had a flowering rate of 3.3%, and Pr-D had a flowering rate of 1.5%. Some of the plants which had been grown outdoors in 2000 without the protection of an insect-proof tunnel showed symptoms of viral infection. In 'Blue Parrot', the tepals had dark purple and green streaks, and in 'Prominence', they had dark streaks. In both cultivars, the leaves had light green stripes and streaks. ELISA revealed the presence of *Tulip breaking virus* (TBV). All symptomatic plants were removed.

No distinct off-types were observed among the healthy plants in 2004. 'Blue Parrot' plants grown from bulbs over 10 grams generally were true-to-type, whereas plants grown from bulbs under 10 grams often did not have flowers of the characteristic parrot shape. Plant height ranged from 34 to 50 cm, depending on bulb weight. 'Prominence' plants were phenotypically true-to-type. Flowers developed on plants grown from bulbs weighing at least 6 g. Plant height ranged from 37 to 46 cm.

In 2005, BP-A, Pr-A and Pr-B were in their sixth GC. BP-A had a flowering rate of 72.9%, Pr-A had a flowering rate of 78.1%, and Pr-B had a flowering rate of 133.3%. The reason why the percentage was higher than 100% for Pr-B was that flowers developed not only from the primary bulbs, but from many of the daughter bulbs as well. For 'Prominence' progeny lines in their fifth season, those which had been grown outdoors in all growing seasons had flowering rates higher than lines which had been grown in a greenhouse or growth chamber before being transfered outdoors. All plants of BP-A and Pr-A were true-to-type. These were the progeny lines which

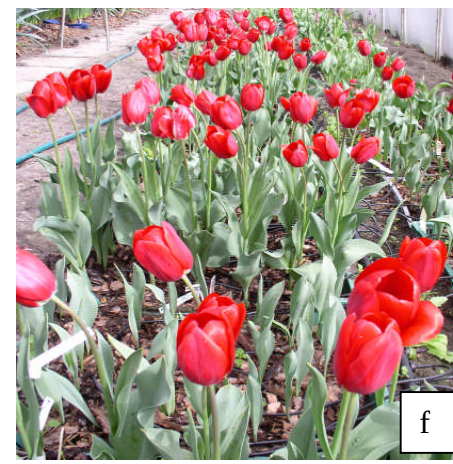

a


b


c


d


e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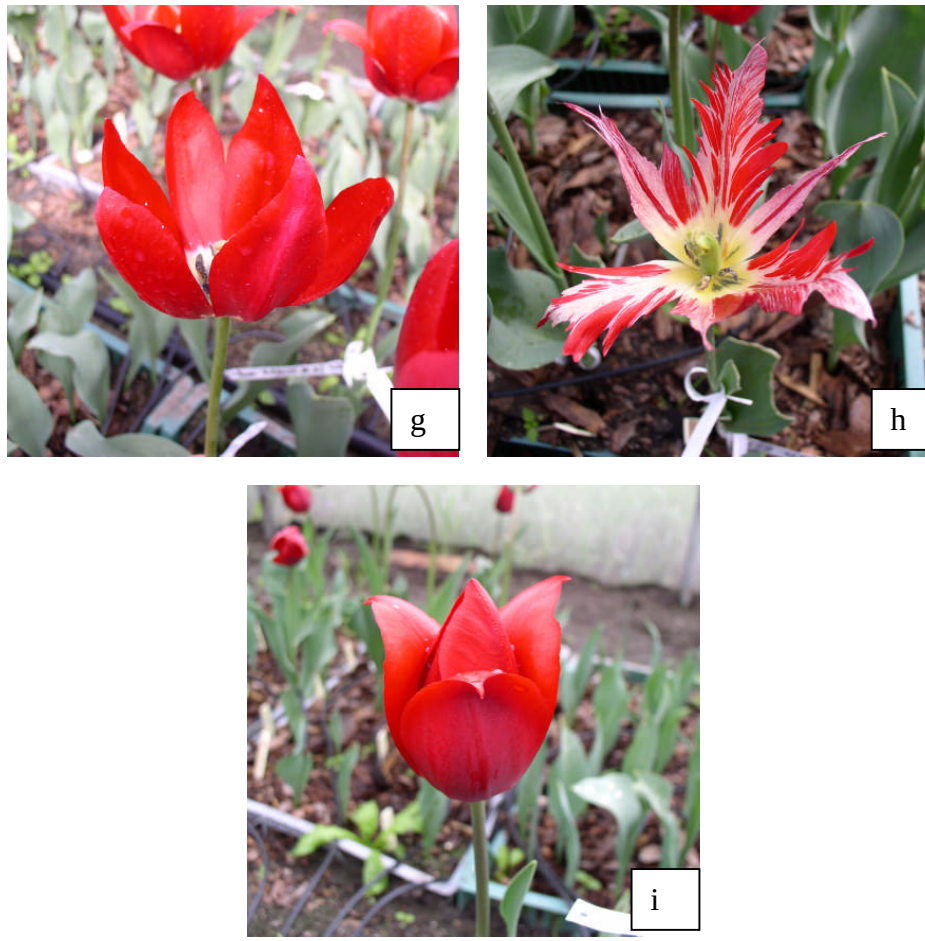

Figure 1. *In vitro*-produced tulip plants observed at full flowering in 2005
a) True-to-type plants of progeny line BP-A.
b) Off-type plant of BP-B with red purple flower.
c, d, e) Variegated plants of 'Blue Parrot' derived from six-year-old *in vitro* culture.
f) True-to-type plants of Pr-A.
g) Off-type plant of Pr-D with narrow tepals.
h) Off-type plant of Pr-D with abnormal flower.
i) Off-type plant of Pr-D with lily-like flower.

had been cultivated *ex vitro* for the longest time (Fig. 1a,f). In these progeny lines, flower length was the same as in the conventionally propagated reference plants, but plant height and bulb weight were lower (Tab. 2). In Pr-D and Pr-E, both flower length and plant height were significantly lower than in the reference plants. Plant height was generally positively correlated with bulb weight.

Table 2. Quality parameters of true-to-type tulips derived from micropropagation. Bulb weights from 2004. Plant height and flower length from 2005

Cultivar, progeny	Number of plants observed	Mean bulb weight [g]	Weight of largest bulb [g]	Plant height [cm]	Flower length [cm]
'Blue Parrot'					
BP-A	21	7.3	15.5	57.6 a	6.4 a
Reference	**20**	**18.3**	**40.1**	**60.9 b**	**6.3 a**
F_{emp}				*5.98***	*< 1*
'Prominence'					
Pr-A	21	12.0	26.1	42.2 b	7.8 a
Pr-B	53	11.9	28.7	42.2 b	7.7 a
Pr-C	9	14.5	26.5	40.1 bc	7.6 a
Pr-D	19	8.3	15.8	40.9 b	7.2 b
Pr-E	12	7.3	13.3	36.6 c	7.0 b
Reference	**20**	**21.0**	**38.6**	**45.1 a**	**7.7 a**
F_{emp}				*7.02***	*6.85***

Means marked with the same letter do not differ significantly at $P \leq 0.05$ according to Student t-test
** level of significance ≤ 0.01 according to F-test

In 2005, some off-types were observed, especially in those progeny lines, which had been cultured *in vitro* for four years (Tab. 1). In BP-B, all of the flowers were red-purple (CC 66A) instead of purple-violet. Tepal goffering was also atypical for this cultivar (Fig.1b). In Pr-D and Pr-E, about half of the plants were off-types, although only three plants were extremely atypical. One of these plants had very narrow tepals (Fig.1g). The other two plants were only 25 cm high and had abnormal flowers with yellow bases, mal-formed stamens and anthers, and fringed, narrow tepals with white streaks (Fig. 1h). The other off-types had normal colored flowers and only minor changes: tepals with acute tips or tepals which were curved back as in lily-flowered tulips (Fig.1i).

In Pr-B, which had been cultured *in vitro* for two years, two off-types were found. One had lily-like flowers. The other was strongly changed. It was only 30 cm high and had abnormal flower like those observed in Pr-D.

Variegation was observed only in juvenile plants of 'Blue Parrot' progeny lines derived from shoot cultures which had been propagated *in vitro* for long periods. For example, the percentage of variegated plants was 1.0% in one progeny line which was planted *ex vitro* after four years of *in vitro culture*, and 1.7% in another line which was planted *ex vitro* after six years of *in vitro* culture (Tab. 3, Fig. 1c,d,e). These juvenile plants always had only one leaf with various patterns of white or yellow stripes consisting of cells which did not contain chlorophyll. These patters could be either symmetrical or asym-

metrical, regular or irregular, wide or narrow, marginal or central.

Table 3. Number of variegated plants in 'Blue Parrot' progeny from long-term *in vitro* cultures in 2005

In vitro culture period [years]	First season of *ex vitro* culture	Total plants	Variegated plants
4	2001	1545	15 (1.0%)
6	2003	469	8 (1.7%)

Sporadic juvenile plants with very narrow leaves or longitudinally folded leaves were also observed. ELISA did not reveal the presence of any of the viruses tested for in these plants. We are currently studying variegation and other morphological changes observed in these juvenile plants.

DISCUSSION

Plants which had been micropropagated *in vitro* began flowering in the fourth growing season. The only atypical trait observed at this stage was the lack of parrot flowers in the 'Blue Parrot' progeny line BP-A. These flowers were very similar to those of 'Bleu Aimable', the non-parrot cultivar from which 'Blue Parrot' originated as a mutant. It has been supposed that 'Blue Parrot' is a chimera with elements of two genotypes, one with parrot flowers and the other without. The lack of parrot flowers in some of the micropropagated plants might be due to segregation of the genotypes during adventitious shoot regeneration. The plants which failed to produce typical parrot flowers in 2003 did produce them in 2004, however. The ability to produce parrot flowers was thus related to bulb size. Bulbs over a critical weight produced parrot flowers, whereas smaller bulbs did not. Further study is needed to more accurately determine the critical bulb weight, but it is about 10 grams.

In Pr-D, which had been cultured *in vitro* for four years, 1.5% of the plants flowered in fourth season. None of them were phenotypically abnormal. The next season, 30.8% of the plants flowered, and about half of them were phenotypically abnormal. Based on the results of this study, we can conclude that reliable information on somaclonal variation introduced during *in vitro* propagation can be obtained when more than 30% of the plants have flowered. This can occur as early as in the fourth or fifth growing season.

Very few off-types if any were observed in progeny lines derived from shoot cultures which had been propagated *in vitro* for three years or less. Micropropagated plants were shorter than the reference plants, but that is because of smaller bulb size. In outdoor cultivation, the annual rate of weight increase in micropropagated bulbs was between two and five (Podwyszyńska and Nowak, 2004). Thus most of the bulbs should reach the size of the reference bulbs in the next season.

Somaclonal variation was observed far more often in progeny lines derived from shoot cultures which had been propagated *in vitro* for four

years or more. Because these changes were widespread, distinct and repeated, the source of the variation may be a mutation.

There have been several reports of somaclonal changes in various plants which had been propagated *in vitro*, such as bushy growth in rhubarb, and malformed flowers in *Phalenopsis* and the oil palm (Zhao et al., 2005; Chen et al., 1998; Rival et al., 2000).

Whether the changes induced by *in vitro* propagation are stable can be confirmed by growing the resulting progeny line from seed. This has been done with *Capsicum annuum* (Anu et al., 2004). However, this is not possible with plants which take a long time to mature or in plants with highly heterogenous genomes, as in fruit trees and ornamental perennials, including tulips. Therefore, it has been suggested that somaclonal variation in tulips would be studied with the help of DNA markers, as has been done in the date palm (Gurevich et al., 2005). Work is currently underway to develop methods of detecting somaclonal changes using molecular techniques such as randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR).

Some of the changes observed in this study may be due to epigenetic changes resulting from gene expression or repression. One of the best known mechanisms of regulating gene expression is DNA methylation, which can be strongly influenced by various factors of *in vitro* culture, such as growth regulators. In the oil palm trees produced *in vitro*, DNA hypomethylation was found to cause flower malformation in 50% of the plants (Rival et al., 2000). This phenomenon vanished after nine years of cultivation. Variation due to the changes in the rate of DNA methylation has been also detected in micropropagated plants of cucumber, potato and almond (Pląder et al., 1998; Cassells et al., 1999; Channuntapipat et al., 2003).

Our results show that the occurrence of somaclonal changes and variegation increases with the time the progeny lines are maintained *in vitro*. Marked changes could be observed after four, five or six years. This confirms earlier reports that somaclonal variation increases with the duration of *in vitro* culture, and especially with the number of multiplication cycles (Gavidia et al., 1996; Yang et al., 1999; Al-Zahim et al., 1999; Devarumath et al., 2002). A statistical model to predict the theoretical mutation rate in *in vitro* plant culture has been developed using the number of multiplication cycles as the primary parameter (Côte et al., 2001).

The frequency of somaclonal variation also depends on the regeneration system and growth regulators used. Variation is more often observed in multiplication by adventitious shoot regeneration than in micropropagation by lateral bud development (Karp, 1995; Pląder et al., 1998). The new method employed in this study is based on adventitious shoot regeneration stimulated by thidiazuron, a growth regulator with a high

cytokinin activity. In this method, thidiazuron was the main factor which made successful cyclic shoot multiplication possible. So far, no methods based on micropropagation of lateral shoots have been developed for use in tulip. This is not surprising, considering how difficult it is to induce lateral bud formation in monocotyledonous plants like the tulip.

Micropropagation of tulips with our new method can therefore increase the risk of mutation, especially when the progeny lines are maintained *in vitro* for four years or more. By limiting the period of *in vitro* culture to three years or less, this risk can be significantly reduced. Systematic monitoring of the plant material with DNA markers would further reduce the risk. Molecular techniques have been used to assess the genotype stability during *in vitro* culture, and have been recommended for routine use (Rani et al., 1995; Al-Zahim et al., 1999; Cassells et al.,1999; Devarumath et al., 2002; Martins et al., 2004; Gurevich et al., 2005).

The aim of micropropagation is to rapidly produce a large number of true-to-type plants. Somaclonal variation is therefore undesirable. However, in plant breeding, somaclonal variation can be a valuable source of new genetic material. In this study, several interesting variants were seen, including 'Blue Parrot' progeny with red-purple flowers or variegated leaves, and 'Prominence' progeny with lily-like flowers. These variants may provide interesting material for breeding.

This study was supported by Ministry of Science and Information Society Technologies, Grant No. PBZ-MIN-007/P04/2003.

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ZMIENNOŚĆ SOMAKLONALNA TULIPANÓW ROZMNOŻONYCH *IN VITRO* NA PODSTAWIE OCENY FENOTYPU

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S T R E S Z C Z E N I E

Celem badań była weryfikacja nowej technologii mikrorozmnażania tulipana przez ocenę tożsamości odmianowej rozmnożonych *in vitro* roślin. Metoda ta opiera się na cyklicznym namnażaniu pędów w obecności tidiazuronu. W badaniach wykorzystano rośliny tulipanów 'Blue Parrot' i 'Prominence' uzyskane z kultur pędów rozmnażanych *in vitro* przez okres od 1,5 do 6 lat. Obserwacje fenotypu dotyczyły roślin uprawianych w tunelu owadoszczelnym przez 2-5 lat. Wykonywano je w okresie pełni kwitnienia. W przypadku roślin juwenilnych obserwowano pojawianie się chimer typu *variegata*. Wykazano, że u tulipana rozmnażanego *in vitro* pierwsze rośliny zakwitły w 3 lub 4 cyklu uprawy (1,5-23,1%). W kolejnym sezonie zakwitło od **58,5 do 133,3%** roślin. Wiarygodne obserwacje można było przeprowadzić po 4 latach uprawy, gdy zakwitło ponad 30% roślin. U tulipanów uzyskanych z kultur pędów *in vitro* rozmnażanych przez 1,5-3 lata zmienność nie występowała lub była sporadyczna. Wysoki poziom zmienności – 100% u 'Blue Parrot' i około 50% u 'Prominence', stwierdzono wśród roślin pochodzących z 4-letnich kultur *in vitro.* U 'Blue Parrot' zmany dotyczyły barwy kwiatów, a u 'Prominence' ich zmienionego kształtu, np. na „liliokształtny" (większość somaklonów), czy kwiatów silnie zdeformowanych (kilka somaklonów). Wśród roślin 'Blue Parrot' otrzymanych z długoterminowych kultur *in vitro* pojawiły się chimery typu *variegata* (1,0-1,7%).

Uzyskane wyniki wskazują, że nowoopracowana metoda może być stosowana przy zachowaniu zasady, iż cykliczne namnażanie pędów nie powinno trwać dłużej niż 2-3 lata, a materiał roślinny powinien być systematycznie kontrolowany z użyciem markerów molekularnych (obecnie prowadzone są takie badania).

Słowa kluczowe: *Tulipa gesneriana*, zmienność somaklonalna, rozmnażanie *in vitro,* rośliny typu *variegata*