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ORIGINAL ARTICLE

Agrobacterium-mediated transformation of *Nicotiana tabacum* by disarmed strain At 699 resulted in considerable raising of growth and development of transgenic plants

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The question how long transgenesis invected alterations are demonstrated in a succeeding generations remains of great interest. In this study we describe the development of T_1-T_5 generations of *Nicotiana tabacum L*. transformed by *Agrobacterium tumefacience* strain 699 with disarmed plasmid. Tobacco plants were grown in the same environmental conditions. The characteristics of vegetative and generated parts had been assessed. Transgenic plants were superior to normal ones in leaf area and stem length, had earlier flowering and internodes development but not differ in a number and size of flowers. Growth activation is suggested to be a result of biotic stress induced by transformation.

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Although there are numerous investigating of the post-transgenesis effects, much less is known about ones after transformation with disarmed vector which sometimes reveals unexplainable results. It was found that in some cases plants transformed with disarmed plasmid had resistance to pests like Bt-plants (Bogomaz, 2005), the altered reactions to the selective light (Efimova et al., 2010) or had no any visible effects (Maximova et al., 1998; Cui, Ezura, 2003). Another question arises, how long will these allogenetic features continue to persist? Frequently, presented data are limited to cell cultures or to early stages of T_0-T_1 generations without detailed description of development, so the ontogenesis and physiology of mature transgenic plant in many instances are unclear till now. The objective of this work was to assess some morphochronometric characteristics of development of successive T_1-T_5 generations of tobacco plants, transformed with disarmed strain *A.tum*.699.

MATERIALS AND METHODS

Agrobacterium-mediated transformation

Transgenic lines of Nicotiana *tabacum L.* have been obtained by agrobacterium transformation of leaf disks. The vector pCNL 65 with *npt* gene in disarmed strain *Agrobacterium tumefaciense* 699 (*A.tum.*699) was used for plant transformation. The procedure of transformation, regeneration and selection of T_0 generation carried out according to method (Draper, 1991).

To make sure of transgenic status of kanamycin selected tobacco lines the PCR analysis (Fig.1) was performed. Primers to *npt* gene:

5r… ATGACTGGGCACAACAGACCATCGGCT

3r… TACTGACCCGTGTTGTCTGGTAGCCGA.

Line №1 was used for plant regenerations and following T_1-T_5 generations producing.

Tobacco plants which had no contact with A.tum. 699 were defined as normal or control plants.

Plant material

Tobacco plants were grown in greenhouse at 15°C night and 25°C day temperature and received a 16-hour natural daylight. Each plant grew in individual vessel filled with peat of the same humidity and nutrient's content.

Measurements and Statistics

Following plant characters were measured:

leaf area (weekly); number of flowers per plant (daily); stem and internodes length (after harvesting); flower parts (10 flowers from each plant were fixed in acetic acid and ethanol mixture (1:3) and then were incised into corolla, pestle and stamens, placed on glass and measured. Measurement error was 2.76%. Time scale represented a number of days after sowing.

In all figures (excluding Fig.5), data are represented by box plots with M (mediana), IQR (interquartile range) and limits. N=6. To test the statistical significance of the differences, Wilcoxon test was used ($p<0.05$) (Glantz, 1999).

RESULTS AND DISCUSSION

Transgenic plants (TP) demonstrated enhanced vegetative parameters compared with normal plants (NP). TP flowered earlier than the NP by 10-13 days (Fig.2) and produced longer stems (Fig.3). Nevertheless, period of flowering time was substantially equal (Fig.4).

TP had increased total leaf area as compared to controls (Fig 5). The diagram demonstrates the rate of development of total leaf area of TP which was more intensive then one of NP. For example, the total leaf area of T_4 plants exceeded NP more than twofold.

The flower organ's measurements showed no significant differences between all TP generations and NP (Fig.6-9). Only T_1 plants have stamen length more then normal one (Fig.8,9).

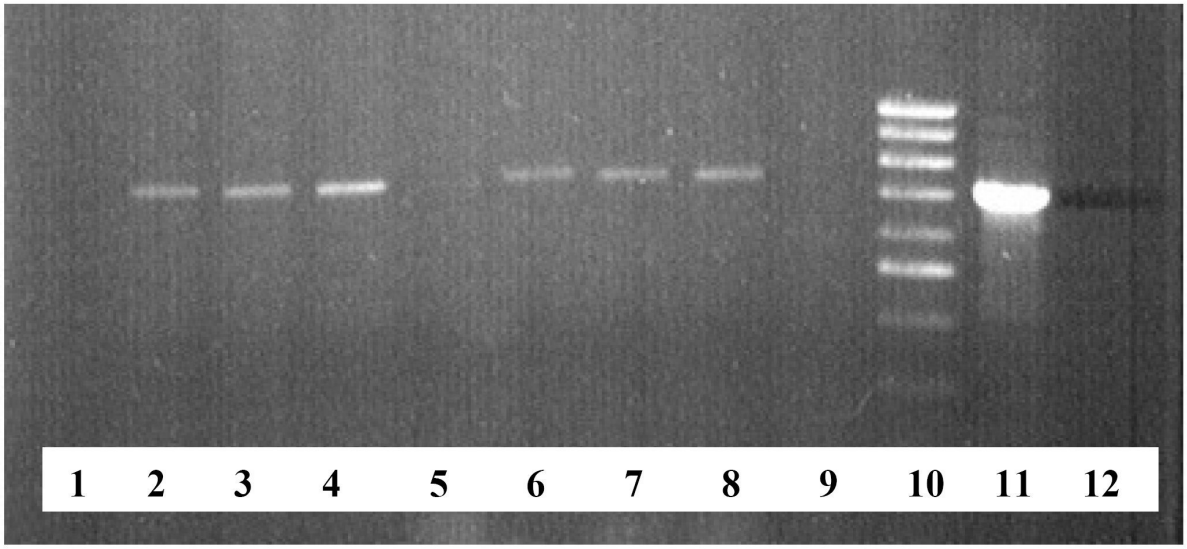

Figure 1. The amplification of *npt* gene in transgenic tobacco lines.
1,9 – control (nontransgenic plants); 2-8 – transgenic tobacco lines; 10 – marker 1000 b.p;
11 – positive control (plasmid DNA from *A. tum*.699); 12 – negative control.

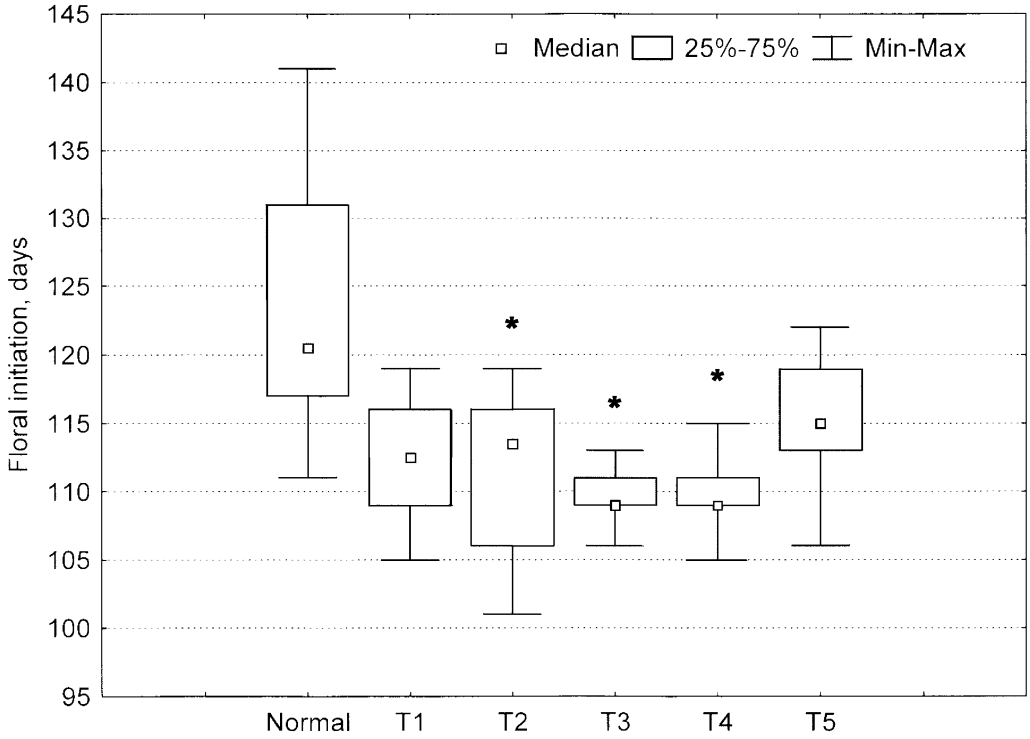

Figure 2. Effect of transformation on flower initiation of tobacco plants for succeeding generations. T_1-T_5 plant generations are compared. * - significantly different from the control ($p<0.05$)

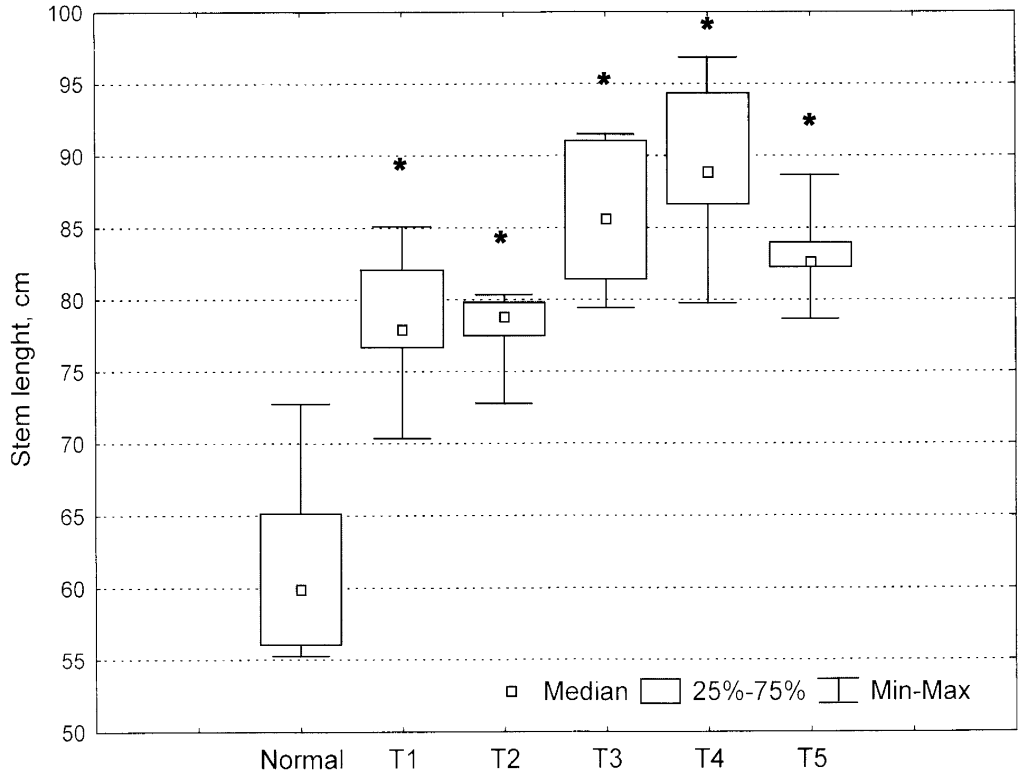

Figure 3. Effect of transformation on stem length of tobacco plants for succeeding generations. T_1-T_5 plant generations are compared. * - significantly different from the control ($p<0.05$)

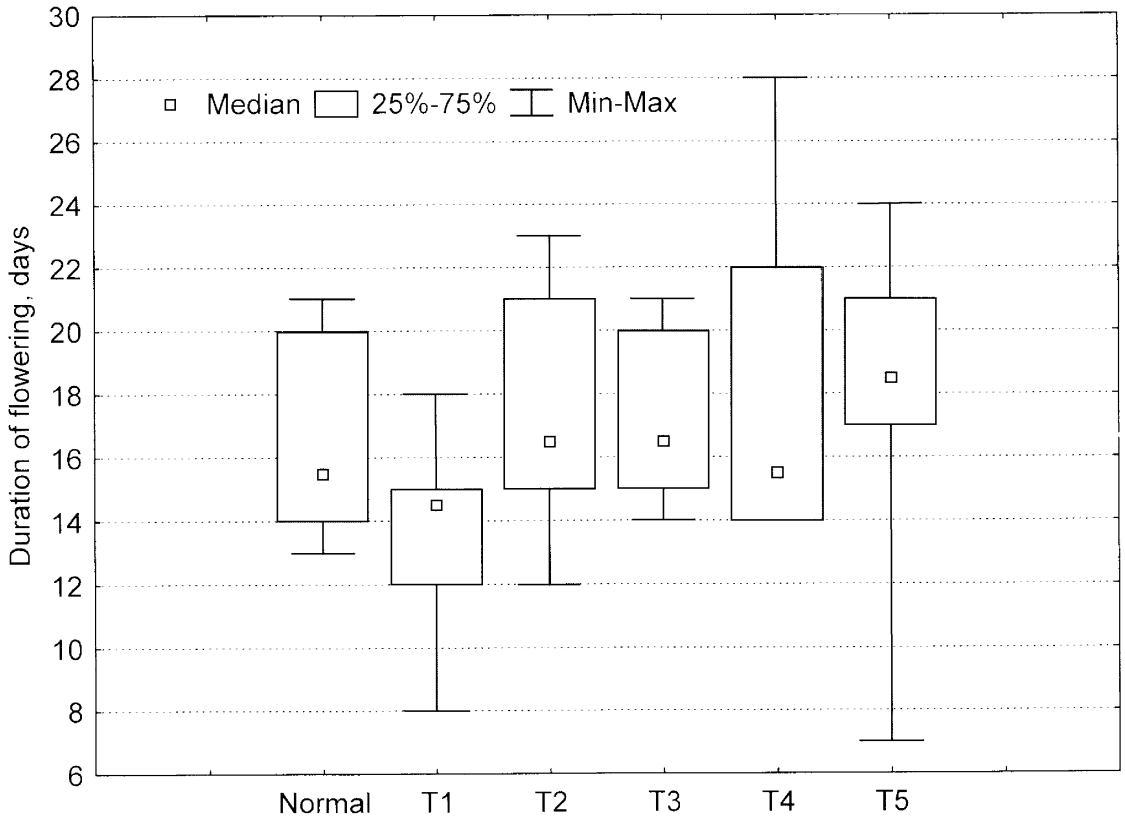

Figure 4. Effect of transformation on duration of flowering of tobacco plants for succeeding generations. T1-T5 plant generations are compared. No significant differences from the control.

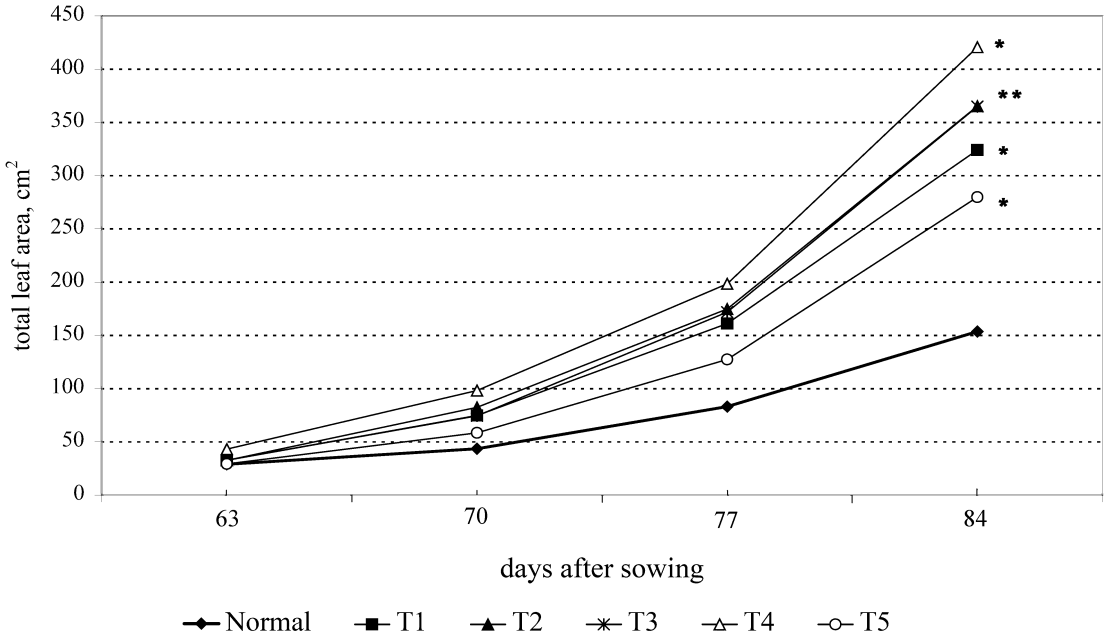

Figure 5. Effect of transformation on total leaf area's development of tobacco plants for succeeding generations. T1-T5 plant generations are compared. Values represent mean (±SE not indicated) * - significantly different from the control ($p<0.05$)

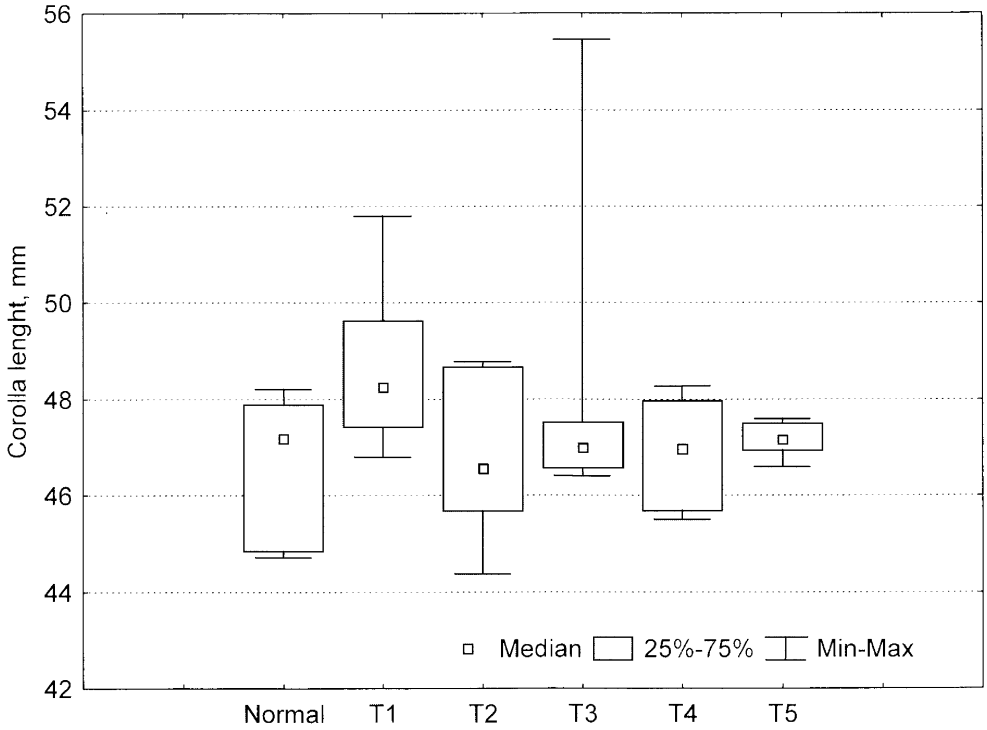

Figure 6. Effect of transformation on flower size (corolla) of tobacco plants for succeeding generations. T1-T5 plant generations are compared. No significant differences from the control.

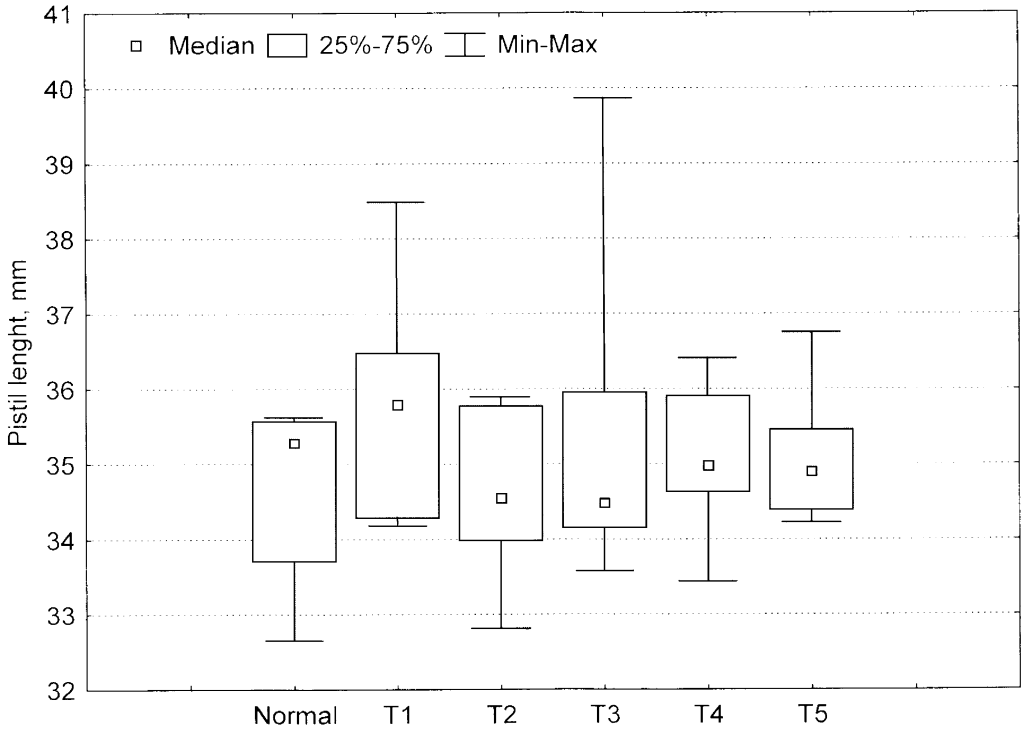

Figure 7. Effect of transformation on flower size (pistil) of tobacco plants for succeeding generations. T1-T5 plant generations are compared. * - significantly different from the control ($p<0.05$)

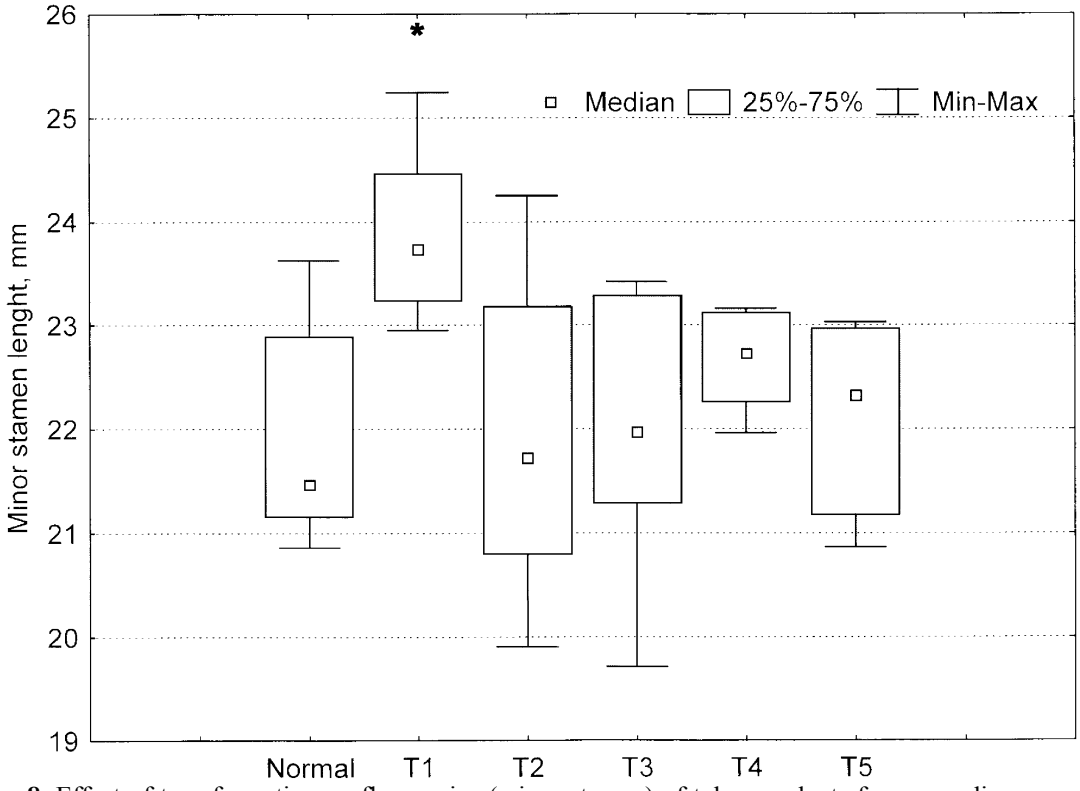

Figure 8. Effect of transformation on flower size (minor stamen) of tobacco plants for succeeding generations. T_1-T_5 plant generations are compared. * - significantly different from the control ($p<0.05$)

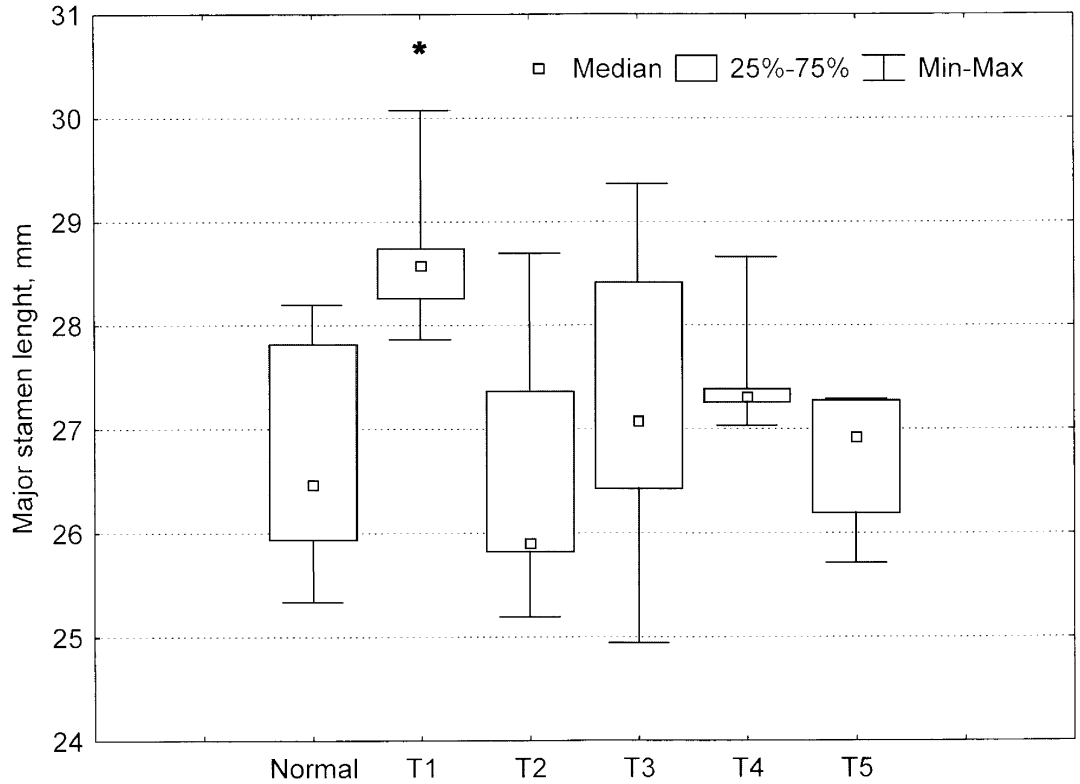

Figure 9. Effect of transformation on flower size (major stamen) of tobacco plants for succeeding generations. T_1-T_5 plant generations are compared. * - significantly different from the control ($p<0.05$)

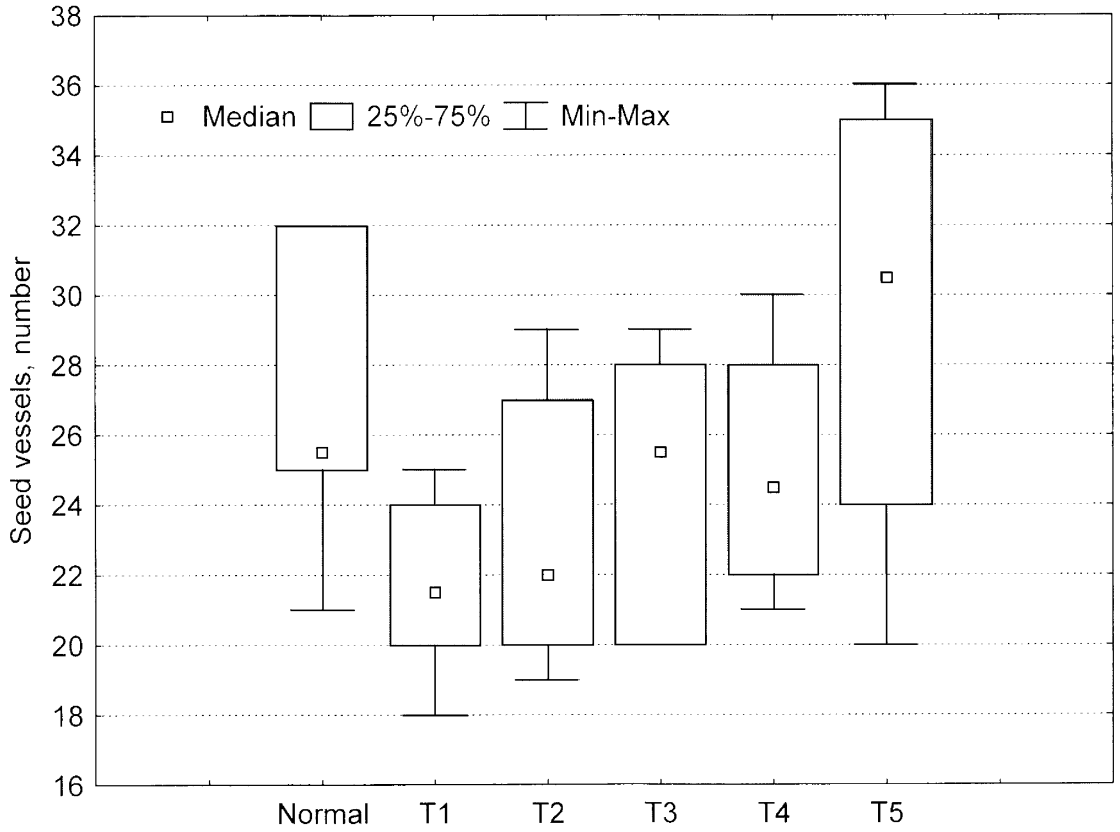

Figure 10. Effect of transformation on a number of seed vessels per plant of tobacco for succeeding generations. T_1-T_5 plant generations are compared. No significant differences from the control.

No significant alterations were found in a number of seed vessels between the transgenic plants and the untransformed control plants. Nevertheless, the number of seed vessels of TP was visibly less than one of NP (Fig.10). Thus, our analysis of morphochronometric peculiarities of normal and transformed tobacco plants showed that TP had earlier development and increased vegetative characteristics. Therefore, to provide this, they must have an enhanced biosynthesis of hormones, proteins, lipids, hydrocarbons *et cetera*, and have an additional pool of constituents; in other words, they will have enhanced metabolism. It is known that plants have a great potential for yield that is commonly unrealized because of insufficient adaptation to unfavorable environment (Boyer, 1982). But our results showed that at the same environmental conditions TP exhibited a stem length and leaf area 2–3 times greater compared to NP at maturity.

What are the sources of observed alterations in transgenic plants? Where and why plants keep metabolic reserves close? Disarmed agrobacterial strains used for transformation did not contain any sense sequences directly associated with plant metabolism like genes of biosynthesis of phytohormones, fat acids, oxidative enzymes etc (de Boer et al., 1999; Tsabary et al., 2003; Cecchetti et al., 2004; O'Hara et al., 2007). Among the numerous studies on a plant transgenesis there are the repots describing effects which can't be explained by action of inserted gene (Abdeev et al., 2005; Karnachuk et al., 2008; Zagorskaya et al., 2009; Pryadyohina et al., 2010; Puzina et al., 2010). So there should be another cause for such events. Many authors point to the elevations of antioxidant content, POL, activity of oxidative enzymes in

transgenic plants (Doubnerová et al 2007; Zhenqiang et al, 2007; Wei et al, 2007) . These alterations are like nonspecific stress response (Gaspar et al., 2002). We interpret these responses as a consequence of contact with *Agrobacterium* and a transformation procedure. Agrobacterial transformation is considered to be a complex multilevel biotic stress factor including reactions on the wounding, contact with pathogen, culturing in vitro and T-DNA insertion (Enikeev, 2008). Thus, relevant alterations in phenotypes of the transgenic plants are assumed to be more likely related to the stress-reaction after agrobacterial transformation. The majority of transgenic plants studies to date have been performed using tissue cultures or whole organisms that were sampled at an early developmental stage. Thus, considerable information on post-transformation growth and development is lost. Our results showed that first T-generations demonstrated clearly increasing plant size but then tended to decrease, returning to origin; T_5 was generally similar to control plants. It is remarkably, vegetative characters appeared more sensitive and reactive to stress. Evidently, flower as a generative organ is evolutionally more defended because of relevancy to keep its structure stabile.

Based on these results, it was suggested that effect of growth and development stimulation after agrobacterial transformation `had similarity with I st phase of the adaptation strain (Selye, 1936) and also is accompanied by releasing of hidden metabolic reserves. Causes and details of this phenomenon are appeared to be of great interest and further investigations of the plant metabolism potential need to be continued.

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