PHOTOSYNTHETIC CHARACTERISTICS OF TRANSFORMED TOBACCO PLANTS GROWN *IN VITRO* AFTER THEIR TRANSPLANTATION IN NATURAL CONDITIONS

Katya Georgieva*, Ivan Yordanov, Antoaneta Kroumova

Acad. M. Popov Institute of Plant Physiology, Acad. G. Bonchev Str., Bl. 21, Sofia 1113, Bulgaria

Summary. The rate of photosynthesis and changes in chlorophyll fluorescence parameters of transformed tobacco plants grown *in vitro* and after their transplantation in natural conditions were studied. It was found that *in vitro* conditions limited photosynthetic incorporation of CO_2 in the leaves of transformants probably because of restricted gas exchange in the vials. *In vitro* conditions also caused lower photochemical effectiveness (lower F_v, and F_v/F_m ratio). However, after transplanting the transformed tobacco plants and their acclimation to natural conditions the parameters investigated reached normal values. These results show that the genetic transformation had not influence the photosynthetic characteristics of tobacco line CMS/81 as well as its acclimation ability.

Key words: acclimation, chlorophyll fluorescence parameters, gas exchange, transformed tobacco plants

Abbreviations: BAP – benzylaminopurine; F_0, F_v and F_m – initial, variable and maximal chlorophyll fluorescence; Km – kanamycin; PS1, PS2 – photosystem 1, photosystem 2; q_P and q_N – photochemical and non-photochemical quenching; RC(s) – reaction centre(s); NAA – naphtalene acetic acid; PSA – photosynthetic apparatus

Introduction

In vitro cultivation of plants is a necessary step in a large amount of experiments: micropropagation, creation of virusfree material, genetic transformation, etc. The

---

* Corresponding author, e-mail Katya@obzor.bio21.acad.bg

number of plants to which these approaches are applied increases continuously. However, there are some serious problems, connected with *in vitro* development which should be solved in order to prevent significant plant loses. Among them is the difficulty of plant acclimatization from *in vitro* environment to soil conditions. The major reasons for this difficulty are considered to be desiccation through uncontroled foliar water loss (Schort et al., 1987; Roberts and Matthews, 1995) and low photosynthetic activity of the foliage, produced *in vitro* (Grout and Aston, 1978; Donnelly and Vidaver, 1984; Lee et al., 1985).

The possibility that photosynthetic factors in cultivated plants could be the reason for slow or lack of acclimatization could be investigated by analysis of the functional activity of the photosynthetic apparatus (PSA). The characteristics of chlorophyll fluorescence kinetics give an important information about its functional state. This method is a trustworthy and sensitive indicator characterizing the PS2 and O_2 evolution activities which are among the most susceptible links of the photosynthetic process to unfavourable factors. An advantage of this method is also the possibility of conducting the investigations on intact plants, leaves or leaf discs. Using this method we found, in our previous work with tobacco (Kroumova and Georgieva, 1994) that the conditions *in vitro* are quite unfavourable for photosynthesis (low light and CO_2 concentration, high humidity) which lead to decrease of initial (F_0) and variable (F_v) fluorescence and some other parameters.

The aim of this investigation was to follow the functional activity of photosynthetic apparatus in control (wild type) and genetically transformed tobacco regenerants at *in vitro* conditions and during their acclimatization to natural conditions.

Materials and Methods

Plant tissue cultures, regeneration and transformation

Tobacco plants, line CMS/81 were propagated *in vitro* from shoot internodes in half strength solid Murashige and Skoog (MS) (1962) medium without growth regulators. They were used as a source of leaf segments. The experiments with leaf segments were conducted in three variants, namely:

Variant 1. Control

Leaves were cut into pieces of $0.5\,cm^2$ and placed on solid complete, shoot induced MS medium, containing $1.0\,mg/l$ benzylaminopurine (BAP) and $0.1\,mg/l$ naphthalene acetic acid (NAA). Newly formed shoots were transferred to MS hormone-free solid medium for elongation. The regenerants after reaching $10\,cm$ were micropropagated from shoot's internodes for about one month. Half of the plants were left after micropropagation in culture and the other half were transferred directly to soil or, at first,

to water culture and then to soil. The photosynthetic parameters of both groups of plants (*in vitro* and *in vivo* growing) were measured.

Variant 2. Plants transformed with kanamycin (Km) selection

Leaf petioles of *in vitro* grown tobacco plants were transformed with *Agrobacterium* strain C58C1Rif(pMP90)(pGSR780A) (DeBlock et al., 1989), containing chimaeric *neo* and *bar* genes. This strain was kindly provided by Dr. M. DeBlock (Plants Genetic System, Belgium). The *neo* gene codes for the enzyme neomycin phosphotransferase which determines Km resistance. Transformation was performed using the method of Deblaere et al. (1977). Leaves were cut in pieces of $0.5\,cm^2$ and placed (about 15 per Petri dish) into $10\,ml$ liquid complete MS medium containing $100\,\mu l$ of bacterial culture (0.1–0.2 OD). The plates were incubated for 48 h at $25\,^{\circ}C$. The segments were washed two times with liquid complete MS medium and transferred to a plate with shoot-inducing medium. The antibiotic Cefotaxime $500\,\mu g/l$ (Calbiochem, La Jolla, CA) was added to the medium to eliminate *Agrobacterium*, as well as Km (50 mg/l, Sigma) to kill non-transformed tobacco shoots. The plates were incubated at $25\,^{\circ}C$, light intensity about $50\,\mu mol.m^{-2}.s^{-1}$ photons and 16/8 h day/night photoperiod. *De novo* formed shoots were transformed to a hormone-free solid MS medium, supplemented with $500\,\mu g/l$ Cefotaxime and 50 mg/l Km. After having reached about 10 cm and having developed foliage, regenerants were tested again for phenotypic expression of the chimaeric *neo* gene (Deblaere et al., 1977). Although indirect, this test is promising and cheap. Leaf segments of *de novo* formed plants were placed on the shoot-induced medium containing 50 mg/l Km. Only regenerants which leaf segments intensively proliferated and formed plantlets in Km containing medium were considered as transformed (Fig.1). These transformed regenerants were micropropagated from shoot internodes for one month. Then half of the plants grown after micropropagation were transferred to water culture for 15 days and later to soil. Then as in the control the photosynthesis of both groups of plants was measured.

Variant 3. Plants transformation without initial Km selection

The procedure was essentially the same as in the former variant.

Photosynthetic rate was measured by radiometric method – $^{14}CO_2$ incorporation (Yordanov et al., 1969).

Fluorescence was measured using a pulse modulation chlorophyll fluorometer (PAM 101, H. Walz, Effelrich, Germany) as described by Schreiber et al. (1986). Maximal variable fluorescence (F_v) of 3 min dark adapted sample was determined by application of a saturating white light. Photochemical (q_P) and non-photochemical (q_N) quenching ($3500\,\mu mol.m^{-2}.s^{-1}$) with 20 s intervals were calculated according to van Kooten and Snel (1990). Experimental data were processed statistically after Student.

Results and Discussion

Plant regeneration and transformation

Nutritional MS medium containing 1.0 mg/l BAP and 0.1 mg/l NAA is a well established one for regeneration of tobacco tissue. Line CMS/81 was no exception. A 100% of the leaf explants formed *de novo* plantlets (Table 1). A total of 92 plantlets were recovered. In the second variant leaf explants were cocultivated with *Agrobacterium* and Km selection of 50 mg/l was applied. Preliminary experiments with our line showed that this concentration was enough to inhibit any proliferaton of the wild type plant tissue and as a result neither callus formation nor regeneration was observed (Fig. 1A, Table 1). On this medium 30% of leaf explants produced regenerants. From them 46 primary regenerants were totally recovered. Leaf segments of these plants were transformed to Km containing medium. Segments from only 36 regenerants developed callus and superficial meristem in two weeks and two weeks later we had well formed plantlets (Fig. 1B). This means that 36 transformed plants were obtained from the second experiment. Leaf segments from the remaining 10 plants did not show any development. They turned yellow and died on Km. Regeneration of non-transformed plants in the presence of selective antibiotics has still no satisfactory explanation (Horsch et al., 1985; Deroles and Gardner, 1988). It is supposed that the regeneration sites are not in contact with the medium (respectively with the antibiotic) or that cells with the potential for regeneration are surrounded and protected from the antibiotic by the cell transiently expressing antibiotic resistance.

In the third variant leaf explants were cocultivated with *Agrobacterium*. However, Km selection was not applied immediately after that. All of the explants formed callus and plantlets. Totally 92 regenerants were recovered. Leaf segments of these

Table 1. Frequency of tobacco leaf discs line CMS/81 transformation in the presence of *Agrobacterium*, strain C58C1 Rif (pMP90)(pGSR780A)

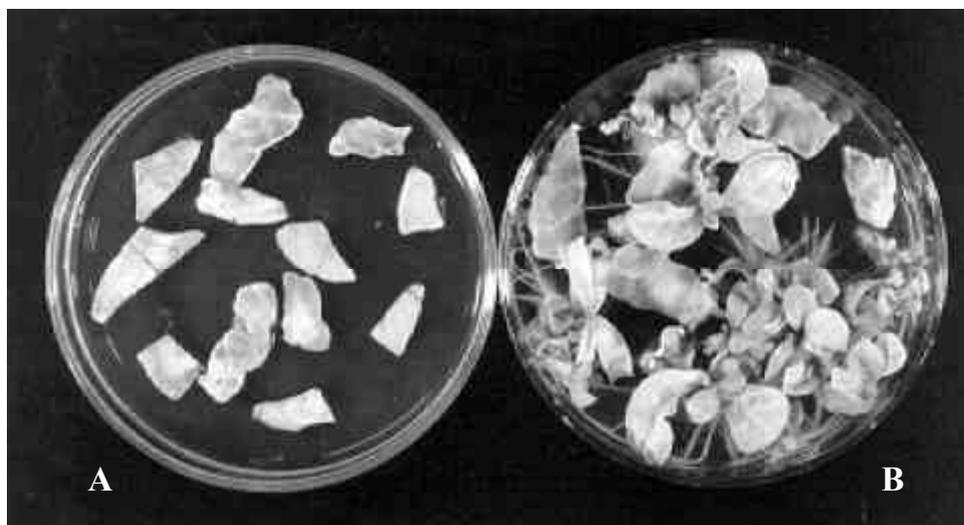| Variants	Treatment	Regenerated leaf segments, %	Number regenerants	Number regenerants with Km resistance
1. Control	- *Agrobacterium* - Km in regeneration medium	100	92	0
2.	+ *Agrobacterium* + Km in regeneration medium	30	46	36
3.	+ *Agrobacterium* - Km in regeneration medium	100	94	0

Fig. 1. Indirect test proving the transgenic nature of regenerants. A – Leaf segments from var. 1 (control) regenerants were transferred on shoot-forming MS medium, containing 50 mg/l Km. Each leaf segment was taken from a separate plant. There were not any callus- or shoot-formation. B – Leaf segments from var. 2 (cocultivated with *Agrobacterium* and direct regeneration in the presence of Km, 50 mg/l) regenerants were transferred to shoot-forming MS medium, containing 50 mg/l Km. Each leaf segment was taken from a separate plant. It can be seen that in a part of the leaf segments there was not proliferation. These segments belong to non-transformed plants. The other part of segments gave rise to small plants with well developed roots. These segments belonged to transformed plants.

regenerants were tested for Km resistance. Not one of the leaf explants survived Km selection and no proliferation was observed. The results showed that there was no transformation in variant 3. Similar results of non-transformation in the absence of selective antibiotics after *Agrobacterium* cultivation were observed in petunia (Janssen and Gardner, 1989; deJong et al., 1993) and poplar (Jowe et al., 1994). According to Janssen and Gardner (1989) stable integration of foreign DNA into the plant genome is probable only if antibiotic pressure is applied at least for 10–14 days, starting immediately after cocultivation with *Agrobacterium*.

Transfer of plants in natural conditions

The transfer of plants from *in vitro* to *in vivo* conditions is a serious stress factor, that leads to high death rate, if appropriate conditions are not ensured. Our earlier investigations showed, for example, that substratum "perlite" was not suitable for the line CMS/81, because more than 50% of transferred regenerants died (Kroumova and Georgieva, 1994).

In this paper the control tobacco regenerants from variant 1 were either transplanted directly in soil or they were at first cultivated two weeks as water culture,

on 1/2 strength Knop's nutrient solution and then planted in soil. About 70–80% of the plants transplanted directly in soil survived. But their adaptation was too long and their development – very weak and retarded. Transplantation of regenerants from *in vitro* conditions to water culture and than to soil proved more favourable for their acclimation to, and development in various environmental conditions (Georgieva et al., 1995). A month after planting in the soil the plants having passed two weeks in water culture were more vital and considerably better developed than those transplanted directly in the soil. On the basis of these results the authors suppose that transplantation of all regenerants must begin with their transfer on water culture.

Photosynthetic characteristics of regenerants

A photosynthetic characteristic was made of control and transformed regenerants, cultivated *in vitro* and then transfered to natural (soil) conditions. The rate of photosynthesis was measured radiometrically by $^{14}CO_2$ incorporation in leaf discs. Results show that there are no differences in photosynthetic rate between control and transformed plants both *in vitro* and *in vivo* conditions (Table 2). During the acclimation process, however, the intensity of photosynthesis increased in all regenerants. The higher rate of photosynthesis in natural conditions was the result of higher CO_2 concentration and light intensity as compared to *in vitro* conditions. According to Solarova (1989) the intensity of photosynthesis *in vitro* is limited generally by low CO_2 concentration, which on its part retards autotrophic growth.

The photochemical activity of PS2 and the functional state of PSA were evaluated by chlorophyll fluorescence parameters (Table 3). At physiological temperature fluorescence was emitted generally by chlorophyll *a* of PS2 (Papageorgiou, 1975) and characterized the primary processes of photosynthesis (Krause and Weis, 1984; 1991; Lichtenthaler and Rinderle, 1988). In view of the close functional connection of PS2 with other photosynthetic components fluorescence yield can serve as an indirect indicator of the functional state of integral photosynthetic process (Schreiber and Bilger, 1985). The results show that there are no essential differences in F_0 values between control and transformed plants both *in vitro* condition and during their acclimatization to soil conditions. In native photosynthetic objects F_0 fluorescence describes excitation loses during the transfer of excitation from the pigment bed to RC

Table 2. Photosynthetic rate of control and transformed tobacco plants cultivated *in vitro* and 15 and 30 days after their planting in soil

Variants	mg $CO_2.dm^{-2}.h^{-1}$		
	in vitro	15 d in soil	30 d in soil
Control plants	11.5 ± 0.8	14.0 ± 1.6	13.3 ± 0.8
Transformed plants	12.6 ± 0.7	14.3 ± 0.5	13.8 ± 0.3

(Baker and Horton, 1987) and is a measure for the efficiency of P_{680} capture. The variable (F_v) fluorescence is sensitive to the rate of electron transport through PS2-RC and to the changes in thylakoid membrane ultrastructure. We observed that F_v of control and of transformants did not differed *in vitro*, but its value in all plants increased significantly after their transfer to soil. These results are supported by the data received for the F_v/F_0 and F_v/F_m ratios which are an indicator for PS2 photochemical effectiveness in primary photosynthetic reactions.

Photochemical and non-photochemical chlorophyll fluorescence quenching of control and transformed plants did not differ after a month acclimation to soil conditions (Table 4). q_P quenching reflects the capacity of RCs to compete for chlorophyll excitation states and is connected with the redox state of Q_A. The q_P values in our experiments showed a normal functional activity of RCs. We did not observe noticeable differences between control and transformed plants in q_N quenching which is greatly dependent on proton gradient formation and is a protective mechanism for PS2 against photoinhibition.

In addition, all regenerants were additionally tested for their resistance to short (3 min) high temperature stress. Our preliminary investigations (Georgieva and Yordanov, 1994) showed as suitable testing the temperatures 40 and 45°C. As criteria for the PSA state were used the rate of CO_2 assimilation, the changes in the values of prompt chlorophyll fluorescence parameters and the temperature dependence of induction kinetics of prompt fluorescence. The data showed that 3 min treatment with 45°C influenced noticeably CO_2 uptake, i.e. the integral photosynthetic process. This temperature inhibited over 2-fold its rate – with 59% in control plants and with 55% in transformants (Table 5). In the same conditions the values of F_0 increased more than two fold in both groups of plants (Table 6), i.e. the effectiveness of excitation transfer from pigment bed to RCs was reduced. Therefore, the data obtained show that the genetic transformation of tobacco line CMS/81 investigated did not influence the state of PSA, as well as its acclimation capacity. There were no noticeable differences between control and transformed plants in respect to the thermostability of photosynthesis.

Similar data were also obtained in other objects. Contrary to the expectations that hybrid poplar plants transformed with mutant *aro* gene for EPSP could ultimately decrease their photosynthetic capacity Donahue et al. (1994) did not observe any differences in biomass accumulation and photosynthetic capacity. Tobacco plants were genetically transformed with antisence Rubisco gene in order to decrease Rubisco content. Under enriched CO_2 conditions plants with lower Rubisco levels acclimated photosynthetically just as plants with normal Rubisco level.

It looks most probable that genetic transformation itself doesn't influence PSA and its acclimation to different factors. *In vitro* conditions were much more unfavourable for photosynthesis. Some plants species were especially sensitive to these conditions. For example, cultured cauliflower plantlets had lower chlorophyll levels, Hill

Table 3. Chlorophyll fluorescence parameters of control and transformed tobacco plants cultivated *in vitro* and 15 and 30 days after their planting in soil

Variants	Control plants			Transformed plants		
	in vitro	15 d in soil	30 d in soil	*in vitro*	15 d in soil	30 d in soil
F_0	623 ±33	621 ± 28	603 ± 37	601 ±16	674 ± 17	648 ±16
F_v	3349 ±41	4212 ± 95	4426 ±110	3452 ±56	4275 ± 50	4466 ±49
F_m	3972 ±74	4833 ±123	5029 ±147	4053 ±72	4949 ± 67	5114 ±65
F_v/F_0	5.50 ± 0.27	6.86 ± 0.35	7.47 ± 0.28	5.77 ± 0.18	6.49 ± 0.17	7.03 ± 0.14
F_v/F_m	0.844 ± 0.007	0.871 ± 0.005	0.881 ± 0.005	0.848 ± 0.007	0.864 ± 0.003	0.874 ± 0.002

Table 4. Photochemical (q_P) and non-photochemical (q_N) quenching of chlorophyll fluorescence in control and transformed tobacco plants cultivated 30 days in soil

Variants	q_P	q_N
Control plants	0.88±0.00	0.40±0.02
Transformed plants	0.86±0.01	0.42±0.01

Table 5. CO_2 fixation rate of control and transformed tobacco leaf discs after 3 min treatment at 25 and 45°C

Variants	$mg\ CO_2.dm^{-2}.h^{-1}$			
	25°C		45°C	
Control plants	12.3±1.0	100%	7.3±0.4	59%
Transformed plants	10.5±0.5	100%	5.8±0.3	55%

Table 6. Temperature sensitivity of chlorophyll fluorescence parameters of control and transformed tobacco leaf discs

Variants	Control plants			Transformed plants		
	25°C	40°C	45°C	25°C	40°C	45°C
F_0	569 ± 72	854 ±113	1183 ± 131	677 ±38	1056 ± 51	1419 ±50
F_v	3049 ±142	2221 ± 65	1576 ± 55	3314 ±90	2418 ± 58	1720 ±42
F_v/F_0	5.58 ± 0.48	2.82 ± 0.23	1.42 ± 0.12	5.43 ± 0.39	2.45 ± 0.14	1.24 ± 0.05
F_v/F_m	0.831 ± 0.01	0.742 ± 0.01	0.578 ± 0.02	0.834 ± 0.007	0.697 ± 0.01	0.549 ± 0.02

activity and CO_2 fixation than the respective control. The poor development of the photosynthetic system in tissue cultures was a major factor which caused newly transplanted plants to be very vulnerable to any sort of environmental stress (Grout and Aston, 1978). After transplanting, the existing foliage rapidly degenerated and the survival of the transplants was dependent upon new growth. A similar pattern of development was also found with strawberry (Grout and Millan, 1985). Other plants, like *Diefenbachia* (Grout and Aston, 1978) demonstrated a significant positive carbon balance *in vitro* and the foliage did not degenerate following transplanting. *Liquidambar* cultured plants also showed good photosynthetic capacity which was not a limiting characteristic for plantlet acclimatization and transplant growth. However, plantlets displayed comparatively lower growth vigour in culture than non-cultured plants and had not full photosynthetic capacity (Lee et al., 1985).

The data presented indicate that *in vitro* conditions cause lower incorporation of CO_2 in the leaves probably because of the restricted gas exchange in the vials. *In vitro* conditions also cause lower photosynthetic effectiveness of PS2 in primary photosynthetic reactions (lower F_v, F_v/F_0 and F_v/F_m ratios). However, after transplantation these parameters reach their normal values. The improved acclimatization of regenerants by transfering them to water culture for two weeks indicate that probably in line CMS/81 the problems of transplanting are reduced predominantly to the control of water loss.

References

Baker, N. R., P. Horton, 1987. Chlorophyll fluorescence quenching during photoinhibition. In: Photoinhibition. Eds. D. J. Kyle et al., Elsevier Sci. Publs B. V. (Biomed. Division).

Deblaere, R., A. Reynaerts, H. Hofte, J.-P. Hernalsteenns, J. Leemans, M. Van Montagu, 1977. Vector for cloning in plant cells. Methods in Enzymology, 153 , 277–292.

DeBlock, M., D. Brouwer, P. Tenning, 1989. Transformation of *Brassica napus* and *Brassica oleracea Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol., 91, 694–701.

De Jong, J.,W. Rademacher, M. van Wordragen, 1993. Restoring adventitious shoot formation on chrysanthemum leaf explants following cocultivation with *Agrobacterium tumefaciens*. Plant Cell Tiss. Organ Cult., 32, 263–270.

Deroles, S. G., R. C. Gardner, 1988. Analysis of tDNA structure in a large number of transgenic perunias genetated by *Agrobacterium*-mediated transformation. Plant Mol. Biol., 11, 365–377.

Donahue, R. A., T. D. Davis, C. H. Michel, D. E. Riemenschneider, D. R. Carter, 1994. Growth, photosynthesis, and herbicide tolerance of genetically modified hybrid poplar. Can. J. For. Res., 24, 2377–83.

Donnelly, D. J., W. E. Vidaver, 1984. Pigment content and gas exchange of red raspberry *in vitro* and *ex vitro*. J. Amer. Soc. Hort. Sci., 109, 177–181.

Georgieva, K., I. Yordanov, 1994. Temperature dependence of photochemical and non-photochemical fluorescence quenching in intact pea leaves. J. Plant Physiol., 144, 754–759.

Georgieva, K., I. Yordanov, A. Kroumova, 1995. Optimisation of the tobacco plantlets transplantation to *in vivo* conditions. Compt. rend. Acad. bulg. Sci., 48(2), 101–104.

Grout, B. W., M. J. Aston, 1978. Transplanting of cauliflower plants regenerated from meristem culture. II. Carbon dioxide fixation and development of photosynthetic ability. Hort. Res., 17, 65–71.

Grout, B. W., S. Millam, 1985. Photosynthetic development of micropropagated strawberry plantlets following transplanting. Annals of Botany, 55, 129–131.

Horsch, R. B., J. E. Fry, N. L. Hoffmann, D. Eichholz, S.G. Rogers, R.T. Fraley, 1985. An simple and general method for transferring gene into plants. Science, 277, 1229–1231.

Janssen, B.-J., R. Gardner, 1989. Localized transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Molec. Biol., 14, 61–72.

Jowe, G. T., B. Golsfarb, S. H. Strauss, 1994. *Agrobacterium*-mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants. Plant Cell Tiss. Org. Cult., 36, 59–71.

Krause,G., E. Weis, 1984. Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. Photosynth. Res., 5, 139–157.

Krause, G., E. Weis, 1991. Chlorophyll fluorescence and photosynthsis. The basics. Annu. Rev. Plant Physiol. Plant Mol. Biol., 42, 313–349.

Kroumova, A., K. Georgieva, 1993. Functioning of photosynthetic apparatus of tobacco regenerants *in vitro* and *in vivo*. Compt. rend. Acad. bulg. Sci., 46, 97–98.

Lee, Ni, H. Y. Wetzstein, H. Sommer, 1985. Effect of quantum flux density on photosynthesis and chloroplast ultrastructure in tissue cultured plantlets and seedlings of *Liquidambar styraciflua* L. towards improved acclimation and field survival. Plant Physiol.,78, 637–641.

Lichtenthaler, H. K., U. Rinderle, 1988. The role of chlorophyll fluorescence in the detection of stress conditions in plants. CRC Critical Rev. in Analyt. Chem., 19, 529–585.

Murashige, T., F. Skoog, 1962. A reversed medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15, 473–497.

Papageorgiou, G., 1975. Chlorophyll fluorescence. An intrinsic probe of photosynthesis. In: Bioenergetics of Photosynthesis, Ed. Govingjee, Acad. Press, New York, 320–366.

Pospisilova, J., J. Solarova, J. Catsky, M. Ondrej, Z. Opatrny, 1988. The photosynthetic characteristics during the micropropagation of tobacco and potato plants. Photosynthetica, 22, 205–213.

Roberts, A. V., D. Mattews, 1995. The preparation *in vitro* of chrysanthemum for transplantation to soil. Plant Cell Tiss. Org. Cult., 40, 191–193.

Schreiber, U., W. Bilger, 1985. Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. NATO Advanced Res. Workshop , Portugal, Sesembra.

Schreiber, U., U. Schliwa, W. Bilger, 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorimeter. Photosynth. Res.,10, 51–62.

Short, K. C., J. Warburton, A.V. Roberts, 1987. *In vitro* hardening of cultured cauliflower and chrisanthemum plantlets to humidity. Acta Horticult., 212, 329–334.

Sicher, R. C., D. F. Kremer, S. R. Rodermal, 1994. Photosynthetic acclimation to elevated CO_2 occurs in transformed tobacco with decreased ribulose 1,5 bisphosphate carboxilase/oxygenase content. Plant Physiol., 104, 409–415.

Solarova, J., 1989. Photosynthesis in plant regenerants. Diurnal variations in CO_2 concentration in cultivation vessels resulting from plantlets photosynthetic activity. Photosynthetica, 23, 100–107.

Sutter, W. C., K. Shackel, C. Diaz, 1992. Acclimatization of tissue cultures plants. Acta Agricult., 314, 115–119.

Van Kooten, O., J. F. H. Snel, 1990. The use of chlorophyll fluorescence nomenclature in plant stress physiology. Photosynth. Res., 25, 147–150.

Yordanov, I., K. Popov, P. Chichev, 1969. Using ^{14}C in investigation the intensity and products of photosynthesis. In: Using of Ionizing Irradiation and Isotopes in Biology and Agriculture. Bulg. Acad. Publ. House, Sofia, 59–72 (In Bulg.).