J. Appl. Genet. 36(1), 1995, pp. 1-10

Transient expression assay for the optimization of direct gene transfer into cucumber meristem protoplasts by electroporation

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Abstract. The paper presents a new way of obtaining viable and very homogeneous cucumber protoplasts. Protoplasts from cells formed in the shoot tip meristem culture were isolated from suspension. Plasmid pBI121 was introduced using impulse electric field. Effectiveness of transformation process was determined by the transient expression of β-glucuronidase (GUS) gene, controlled by promotor 35S. The activity of β-glucuronidase enzyme as a product of GUS reporter gene was estimated by fluorimetric method (JEFFERSON 1987). Parameters of electroporation process were optimized. The transient expression of GUS gene was measured 24 h after electroporation. The highest effectiveness of transformation process was achieved using three electric impulses at the initial voltage of 250-350 V at 10-sec. intervals as a result of discharging a 140 μF capacitor and 50-70 $\mu g \times cm^{-3}$ plasmid DNA in the presence of 50 $\mu g \times cm^{-3}$ carrier DNA. The system presented is an effective method of exogenic DNA transfer, which is indicated by a high transient expression of the reporter gene. In comparison to *Agrobacterium tumefaciens* and *A. rhizogenes*, this alternative method of gene transfer can be used for obtaining transgenic cucumber plants.

Key words: *Cucumis sativus*, electroporation, transient expression.

Transfer of new genes into the cells of living organisms can be performed in different ways. Beside *Agrobacterium tumefaciens* and *A. rhizogenes* mediated transformation (effective application of the both systems in cucumber was described by TRULSON et al. 1986 and CHEE 1990), techniques of direct DNA transfer are being developed. These techniques called "direct gene transfer" consist in placing recipient's cells, most frequently protoplasts, into a medium

Received: April 1994. This work was performed within the framework of CPBP 01.12.
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with isolated DNA. They have been used for the first time in the protoplast cultures of petunia (KRENS et al. 1982) and tobacco (PASZKOWSKI et al. 1984).

A tentative tester of effectiveness of "direct gene transfer" is a value defined as "transient gene expression". It is established on the basis of a measured amount of the reporter gene product present in a transferred DNA fragment. It is generally accepted that transformation conditions providing the highest transient expression are the most suitable for obtaining stable transformation. In the case of electroporation, conditions milder than those providing a maximum transient expression are frequently used. Owing to the protoplast survival increased in this way it is possible to increase the number of transformants (RIGGS, BATES 1986, GUERCHE et al. 1987a).

The basic condition, which should be fulfilled in electroporation, especially at the moment of parameter optimization, is the availability of a repeated method providing stable protoplasts of equal size, which are not easily subjected to destruction during functioning of electric field. Isolation and culture of cucumber protoplasts are developed quite well (MALEPSZY 1988). It, however, has appeared that none of the known procedures meet the above requirements to a satisfactory degree.

In this paper we present a system, the source of which is a cell suspension obtained from the shoot tip meristems. It makes possible to obtain very stable protoplasts of equal size, which most probably will be also suitable for studies on somatic hybridization. This source has not yet been described in the literature on the family *Cucurbitaceae*. Electric parameters of electroporation process as well as plasmid DNA concentration were subjected to optimization, whereas other values were not tested, accepting that data obtained for other species are adequate.

Material and methods

In vitro plant culture

Cucumber seeds were sterilized in 3% solution of sodium hypochlorite for 15 minutes and rinsed several times with sterile distilled water. Then, they were transferred into glass jars with a medium containing 1/2 macroelements as well as MS microelements (MURASHIGE, SKOOG 1962), 7g dm^{-3} Difco agar, 15 g dm^{-4} sucrose. The culture was kept at 23°C and 8000 Lx lighting with a 16 h photoperiod. After three weeks of culture, 1-mm shoot tip meristems were picked out and transferred into 10-ml Erlenmeyer's flasks with a liquid MS medium containing 1/2 macroelements, microelements and vitamins,

30 g dm^{-3} sucrose and 1 mg dm^{-3} 2,4-D. Flasks with meristems (0.5-1.0 meristem × cm^{-3}) were placed in darkness on a shaker at 120 rpm. at 23°C. Cell-releasing cultures were transferred onto a fresh medium each seven days.

Isolation of protoplasts

For isolation of protoplasts a homogeneous suspension consisting of single, homogeneous cells from a liquid culture of shoot tip meristems were used (WRÓBLEWSKI, MALEPSZY 1992).

Protoplasts were digested according to modified methods of ORCZYK and MALEPSZY (1985). They were centrifuged twice at 600 rpm for 10 minutes and finally placed for electroporation into a solution of 0.4M mannitol, 4 mM $MgCl_2$ and 0.5% MES, pH 5.6 (KOH). The suspension density was established by counting protoplasts in 8 fields of a Fuchs-Rosethal chamber. The degree of the cell wall digestion was estimated by Calcofluor White (NAGATA, TAKEBE 1971).

Electroporation

The obtained protoplasts after estimating their viability, were exposed to a heat shock on a water bath at 45°C for 5 min. and then, the suspension was transferred to a temperature of 0°C. Ten minutes after, electroporation was started.

A spherical form of plasmid pBI 121 containing two marker genes, beta-glucuronidase (GUS) and neomycin phosphotransferase (NPT2), was transferred to the protoplasts; pBI 121 is a modified plasmid pBI 101 with incorporated 800 bp fragment containing strong promotor 35S from the cauliflower mosaic virus (CaMV) (JEFFERSON 1987). The plasmid concentrations 10, 30, 50 and 70 µg cm^{-3} were applied in the presence of 50 µg cm^{-3} carrier DNA from salmon sperm (Sigma D-3159) previously subjected to sonication. DNA was sterilized in 70% ethyl alcohol.

A set of equipment for electroporation included:
- a generator of exponentially decreasing impulses (a laboratory feeder, stabilized type IZS-S/71),
- a changing-over set of 16 µF, 32 µF and 140 µF capacitors,
- a resistance measuring RLC bridge Automatic RLC meter type E-318,
- an oscilloscope DT 5200 for analysis of a capacitor discharging,
- plexiglass cylindrical chambers (ø 10 mm) with two parallel electrodes made of platinum rods (ø 0.5 mm) placed in a stainless steel block adapted to a water bath.

To the suspension of protoplasts at the density of 1.6×10^6 cm^{-3}, the both DNA forms were added. After 10 minutes, 1 cm^{-3} of the suspension prepared in this way was transferred into the chambers. The solution resistance was established per 1.0-1.1 kΩ using 300 mM $MgCl_2$. Electroporation was carried out at 0°C. After cooling down and stirring the chamber contents, electric pulses, decreasing exponentially at 10 sec. intervals, were generated. The influence of the following factors was tested: the number of pulses (1, 3, 5, 7), volume of the applied capacitor (16, 32, 140 μF), electric field strength (50, 150, 250, 350 V cm^{-1}) on the expression of GUS reporter gene.

The activity of beta-glucuronidase enzyme as a product of GUS reporter gene was determined by fluorimetric method according to the procedure described by JEFFERSON (1987). Ten minutes after electroporation, the solution with protoplasts was expelled into Petri-dishes, adding a liquid medium to the protoplast culture (ORCZYK, MALEPSZY 1985) in the 1:1 ratio.

The protoplast culture after electroporation was transferred to darkness at 24°C for 24 h. The GUS activity was measured in a way making it possible to transfer 250 μl of the protoplast extract into a measuring vessel containing 0.5 cm^{-3} of 4-Metyl-umbelliferyl-beta-D-glucuronide (MUG) solution, further incubated at 37°C. The incubation was interrupted after 0, 1, 3, 5 h, by adding 100 μl solution to 0.9 ml buffer (0.2M Na_2CO_3). The dynamics of the enzymatic activity of beta-glucuronidase was measured on the spectrofluorimeter ELS-5B Perkin Elmer (the excitation wave 365 nm, excitation gap 5 nm, emission wave 455 nm, emission gap 2.5 nm). For individual parameters under study, the average level of GUS expression noted in two independent replications was compared. The experiments were conducted on populations of protoplasts from one isolation. This prerequisition was made on account of the known fact – a significant differentiation in biophysical properties of protoplasts observed sometimes despite identical conditions applied for their isolation. The experiment was repeated in each case, when no linear increase was observed in the level of GUS expression while incubating protoplast suspension in MUG solution.

Results and discussion

The paper presents a new, not described in the literature, method of obtaining viable and homogeneous protoplasts isolated from single cells formed in shoot tip meristem culture (WRÓBLEWSKI, MALEPSZY 1992). They appeared to be exceptionally resistant to a destructive action of electric field, injuring first of all cytoplasmic membranes. Protoplasts of the best quality were obtained from

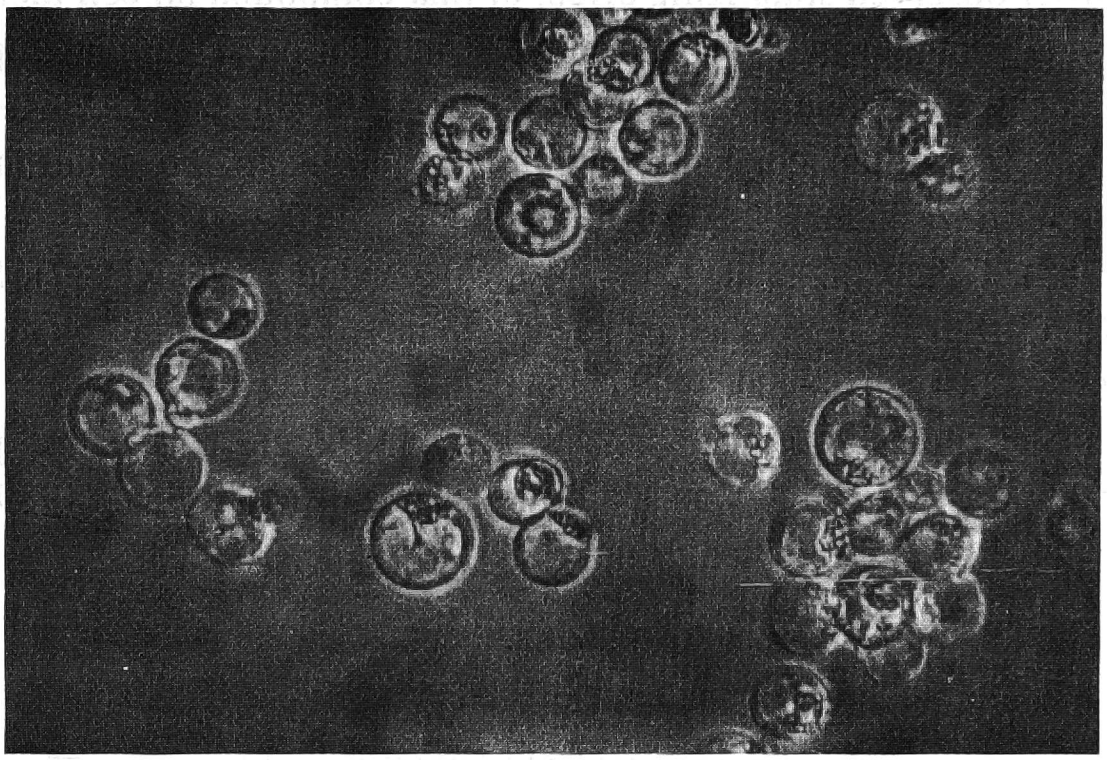

Fig. 1. The cucumber protoplasts isolated from cells produced in liquid culture of shoot meristems

the cells originating from the suspension of meristems between the 4th and 7th subcultures (Fig. 1). The then viability determined by phenosafranine exceeded 80%. To isolate protoplasts, the cells were taken at five days after the culture transfer to a fresh medium. The obtained production rate of protoplasts from the cells released from a single tip meristem was $1.5\text{-}2 \times 10^5$, i.e. about 8×10^6 protoplasts per 1 g of tissue. On account of a more safe structure of the cell wall it appeared necessary to modify the original isolation procedure of cucumber mesophyll protoplasts (ORCZYK, MALEPSZY 1985). In the enzymatic solution, mannitol concentration was increased to 0.3M and 1.2% cellulase, 1.2% macerizyme, 0.3% driselase were used. The optimal incubation time was established for 16 h and the temperature – for 27°C. A preliminary filtration of the suspension through a 30 μM-sieve permitted to decrease the necessary amount of enzyme to 1 cm^3 per 10^6 cells.

When choosing liquid for electroporation, the solution used by SAUL et al. (1988) in tobacco was taken as a model. However, $MgCl_2$ content was reduced from 6 mM to 4 mM, which ensured the desired resistance of the solution on the level of 1.0-1.1 kΩ.

A stimulating effect of Mg^{2+} ions on the level of transient expression was shown by LARKIN et al. (1990), though many authors used other salts, for instance, KCl (GUERCHE et al. 1987b, JONES et al. 1989), $CaCl_2$ (ŁYŻNIK et

al. 1989), NaCl (FROMM et al. 1985). However, $MgCl_2$ was chosen on account of the earlier observed stabilizing effect of that compound on cell membranes of cucumber protoplasts. A short protoplast incubation at a high temperature before DNA was applied acc. to SHILLITO et al. (1985) and GUERCHE et al. (1987a, b). The initial concentration of protoplasts in the solution before electroporation was established on the level of 1.6×10^6 on the basis of FROMM et al. (1985), GUERCHE et al. (1987a), LINDESY and JONES (1987), SAUL et al. (1988). Carrier DNA was added in the amount of 50 μM cm^{-3} as in FROMM et al. (1985), GUERCHE et al. (1987b) and TAUTORUS et al. (1989).

In the experiments optimizing parameters of the electroporation process, 10 μg cm^{-3} plasmid DNA was used like in CHUPEAU et al. (1989). Only SHILLITO et al. (1985) applied 4 μg cm^{-3}.

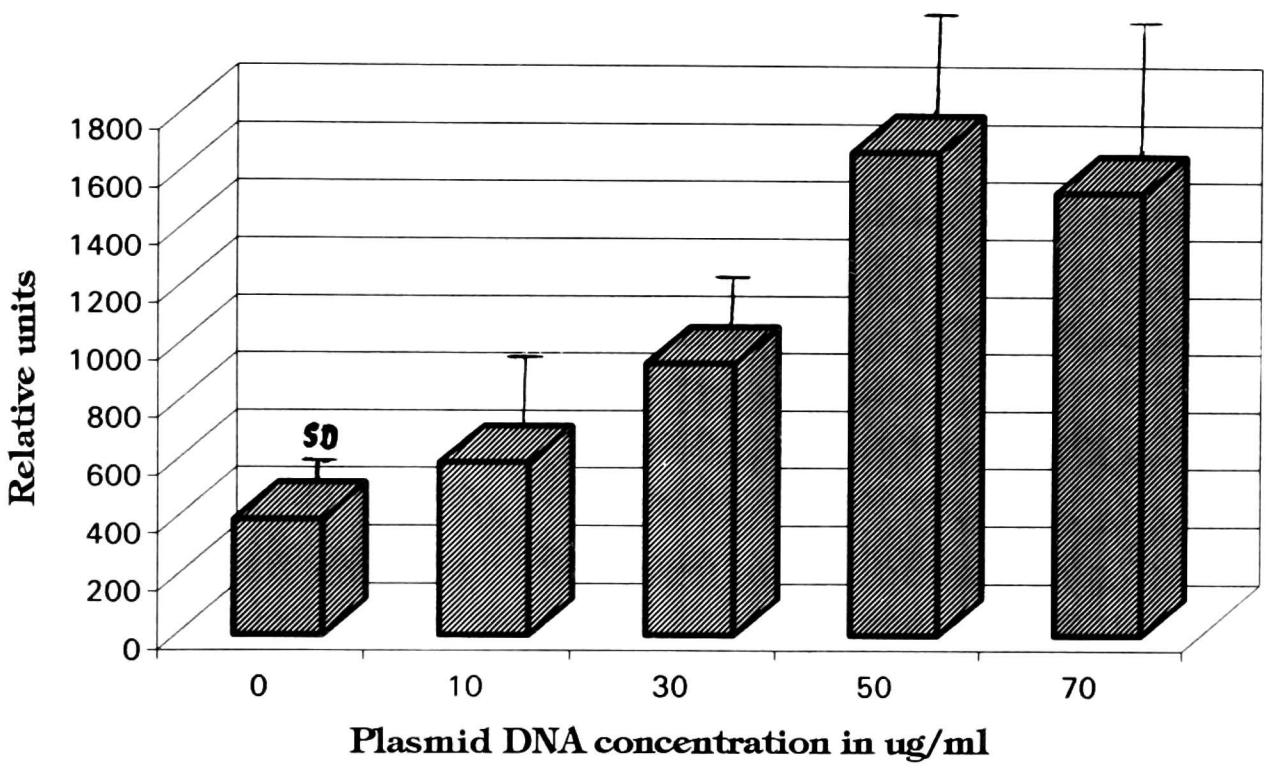

Fig. 2. The influence of the number of electric pulses on the transient expression of GUS reporter gene in cucumber protoplasts

In the present study, the transient expression of GUS gene was found to increase with an increase in plasmid DNA concentration up to 50 μg cm^{-3} (Fig. 2), which agrees with results of FROMM et al. (1985).

The highest expression of GUS reporter gene counted in relative units was obtained for three electric impulses occurring as a result of 140 μF capacitor discharge at the intensity of electric field of 300 V cm^{-1} (Fig. 3). Observations concerning a relationship between the number of used impulses and transient

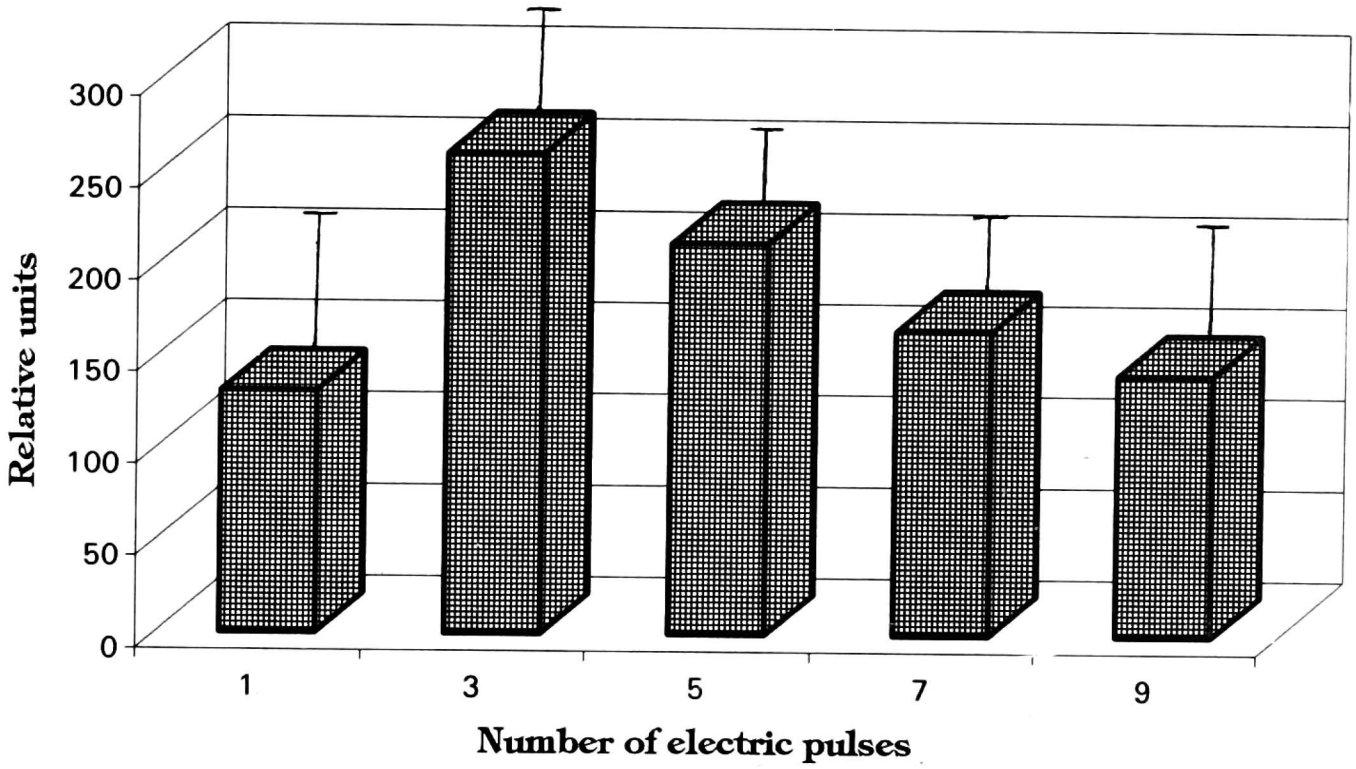

Fig. 3. The influence of capacitor characteristics on the transient expression of GUS reporter gene in cucumber protoplasts

expression in the case of cucumber protoplasts are similar to the results obtained by FROMM et al. (1985), SHILLITO et al. (1985) and LARKIN et al. (1990). Generally it has been found that the impulse number larger than 10 has a negative influence on the survival and dividing ability of protoplasts exposed to electroporation. In the papers of FROMM et al. (1986), LINDSEY and JONES

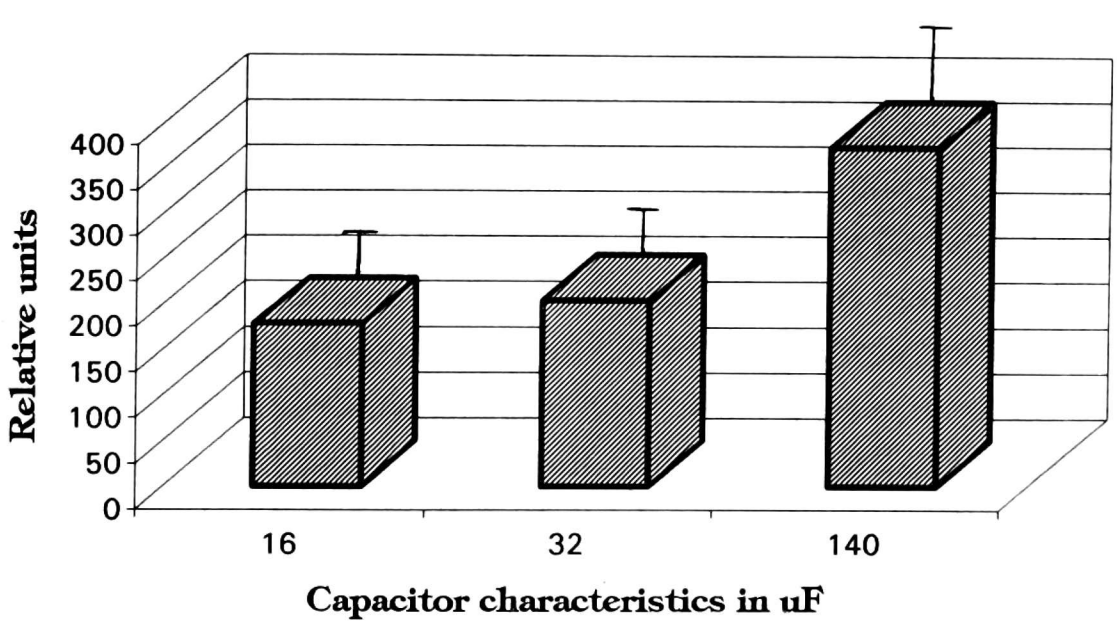

Fig. 4. The influence of electric field strength on the transient expression of GUS reporter gene in cucumber protoplasts

(1987), the use of a single impulse was recognized optimal on account of the obtained higher viability of protoplasts.

Using three electric impulses and electric field intensity of 300 V cm^{-1} for 16 and 32 μF capacitors, a similar level of GUS expression was obtained. The application of 140 μF capacitor and thereby a considerable increase of the electric impulse length increased twofold the level of reporter gene expression (Fig. 4).

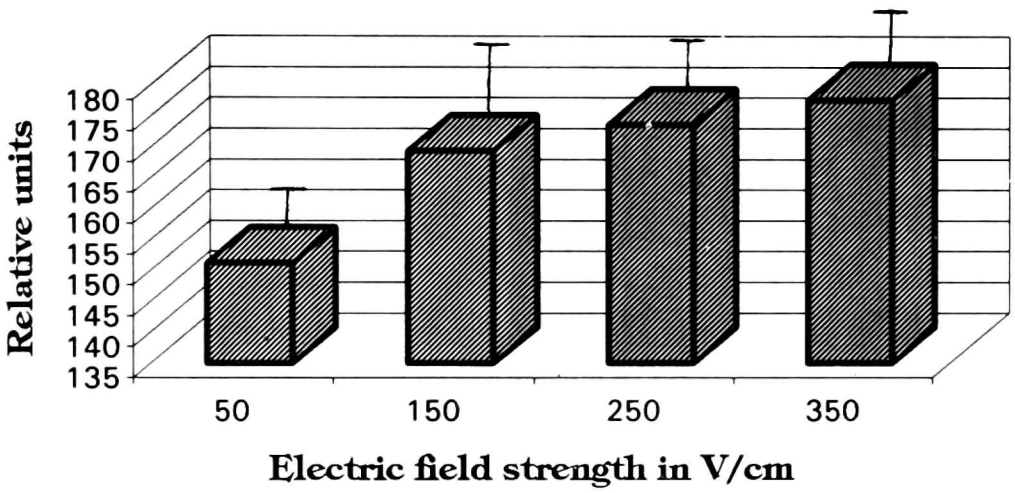

Fig. 5. The influence of plasmid DNA concentration on the transient expression of GUS reporter gene in cucumber protoplasts

The values 250-350 V cm^{-1} of the electric field strength experimentally established using three electric impulses occurring as a result of 140 μF capacitor discharge as the most effective in transformation of cucumber protoplasts are supported, among others, in the works of GUERCHE et al. (1987a, b), CHUPEAU et al. (1989), JONES et al. (1989), LARKIN et al. (1990). In these works there appeared a tendency to use larger capacitors from several to several hundred μF at a simultaneous decrease of the voltage of generated impulses below 400 V cm^{-1}. According to the mentioned authors such parameters ensure a high effectiveness of transformation and a simultaneous preservation of a good survival of protoplasts. In the case of cucumber protoplasts a stronger influence on the transient expression increase was exerted not by an increase of the electric field intensity, like, for instance, in GUERCHE et al. (1987a), but by the length increase of expotentially falling impulse due to multiplication of the applied capacitors volume (16, 32, 140 F).

The system presented in this paper is characterized by a relatively high transient expression of GUS reporter gene, and further studies on regeneration

should determine possibilities of its efficient application for obtaining transgenic plants.

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