International Journal of Biotechnology and Biochemistry
ISSN 0973-2691 Volume 6 Number 1 (2010) pp. 131–138

http://www.ripublication.com/ijbb.htm

Production of Human Epidermal Growth Factor (hEGF) by in Vitro Cultures of *Nicotiana tabacum*: Effect of Tissue Differentiation and Sodium Nitroprusside Addition

J. Parsons[1], S. Wirth[2], M. Dominguez[1], F. Bravo-Almonacid[2], A. M. Giulietti[1] and J. Rodriguez Talou[1†]

[1]Cátedra de Microbiología Industrial y Biotecnología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires Junín 956 (1113), Buenos Aires, Argentina.
[2]Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, INGEBI-CONICET, and FCEN-UBA, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina
[†]Corresponding author E-mail address: jrtalou@ffyb.uba.ar.

Abstract

The aim of this work was to study the production of human epidermal growth factor (hEGF) by *in vitro* cultures of *Nicotiana tabacum*. Suspension and hairy root cultures expressing hEGF were established and the time course of growth and hEGF production were evaluated. In hairy root cultures, hEGF content was about 2 μg g^{-1}FW, with a productivity of 15.9 μg $l^{-1}d^{-1}$ whereas in cell suspensions, a specific production of 0.48-0.53 μg g^{-1}FW and a productivity of 31 μg l^{-1} d^{-1} were obtained. From these results, it is clear that hEGF synthesis and accumulation depend on tissue differentiation. In order to increase hEGF production, cell suspension cultures were treated with casaminoacids and sodium nitroprusside (SNP, a nitric oxide donor). SNP treatment resulted in an increase of hEGF productivity of 48% compared with control cultures. In conclusion, cell suspension and hairy root cultures established from transgenic *N. tabacum* were able to produce hEGF and the concentration and yield obtained depended on the level of tissue differentiation and the treatment applied.

Keywords: Human Epidermal Growth Factor; *Nicotiana tabacum;* Suspension cultures; Hairy roots.

Introduction

Plants are a promising platform for biopharmaceutical production purposes (1,2), because they are able to carry out post translational modifications in a way similar to that of human cells. In addition, plant cell cultures, which are also used for foreign protein production, present some advantages when compared with whole plants, since they can be carried out in controlled environmental conditions independent of climatic changes, pathogen attack and with a low risk of dissemination of transgenic material. Moreover, secreted products can be easily purified from the culture media due to the low content of contaminant proteins. Besides, these cultures do not require the use of land destined to agricultural crops and the production time is shorter than that of agronomic practices.

In *in vitro* cultures, it is possible to increase the yield of foreign protein using strategies that include genetic approaches and/or enhancement of biomass. The first approach comprises promoters, translation enhancers and other regulatory sequences that affect recombinant protein levels and can direct them into a particular cellular compartment (endoplasmic reticulum or apoplastic space). The second approach involves, manipulation of medium components like sugars, plant growth regulators, nitrogen, and mineral sources are employed (2).

Human Epidermal Growth Factor (hEGF) is a valuable and useful therapeutic protein in clinical medicine but, is also used as a component in mammalian cell culture media. Wirth et al. (3) obtained *Nicotiana tabacum* transgenic plants expressing Human Epidermal Growth Factor (hEGF), with a biological activity comparable to commercial hEGF (3). More recently, a patent has described the production of hEGF in rice, which is then purified and used as a component of cell culture media (4).

The aim of this work was to establish suspension and hairy root cultures of *N. tabacum* transgenic plants expressing hEGF in order to study the production performance in both types of *in vitro* cultures. The effect of the addition of sodium nitroprusside (a nitric oxide donor) and casaminoacids on EGF production in plant cell cultures was also evaluated.

Materials and Methods

Plant material and *in vitro* cultures

The suspensions and hairy root cultures assayed were obtained from *N. tabacum* plants expressing the hEGF gene under the control of the long CaMV 35S promoter and the TMV Ω translational enhancer sequence. A signal sequence from AP24 osmotin was fused to hEGF to lead the synthesis of the protein to the apoplastic compartment (3). For callus proliferation, shoot internodes from transgenic and wild type plants were transferred to MSRT (5) solid medium supplemented with 2,4-D (0.2 mg l^{-1}). Calli were grown at 25±2 °C with a 16 h photoperiod by using cool white fluorescent lamps at a light intensity of 1.8W/m^2. Cell suspension cultures were obtained from friable calli grown in MS medium with 2,4-D (0.2 mg l^{-1}) and maintained in the same liquid medium. Subculturing was performed every 7 days by a four-fold dilution of the cells into fresh medium.

Hairy roots were obtained from transgenic and wild type plants by infection with *Agrobacterium rhizogenes* LB9402 as described by Massera et al (6). Hairy root cultures were subcultured on MS medium every 10 days.

Suspensions and hairy root cultures were grown at 25±2 °C on a gyratory shaker at 100 rpm with a 16 h photoperiod by using cool white fluorescent lamps at a light intensity of 1.8W/ m^2.

Time course experiments

Experiments were carried out in 125 ml flasks containing 25 ml of culture medium inoculated with 0.15 g of fresh weight (FW) for hairy roots and 2.5 g for cell suspension cultures. Samples were harvested every 3-4 days in triplicate, weighed and frozen at -80ºC. Casaminoacids were added to MSRT culture medium at concentrations of 0.5 %. Sodium nitroprusside (SNP, 100 μM) was added to the cells 24 h before inoculation.

Protein extraction and hEGF quantification

Plant tissue was harvested and frozen at –80 ºC. Cells were homogenized on ice with pestle and mortar in PBS buffer containing leupeptin 10 μM (1 ml per gram of fresh tissue). Protein concentration was determined according to Bradford (7) using bovine serum albumin as standard. EGF was quantified by ELISA according to Wirth et al. (3).

Chemicals

All chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St Louis MO, USA). Anti-hEGF antibodies for ELISA determination were from R&D Systems Inc., USA. hEGF standard and streptavidin-peroxidase were from Sigma.

Statistical analysis

The significance of treatment effects was determined by using variance analysis. Variations between treatment means were analyzed using Tukey´s procedure ($p= 0.05$)

Results

Hairy root cultures

Hairy roots were obtained from leaves of transgenic and wild type *N. tabacum* (3) infected with *A.rhizogenes* LB 9402. The axenic hairy root suspension cultures were obtained by growing single root tips on MSRT medium containing ampicillin. The growth time course and hEGF production by transgenic *N. tabacum* hairy root cultures are shown in Figure 1. The maximum biomass obtained was about 280 g FW l^{-1} at 30 days of culture. The growth rates of the cultures were 0.14 and 0.19 d^{-1} for transgenic and wild type cultures respectively (data not shown). hEGF production was associated with growth until 24^{th} days of culture. From 7 to 24 days of culture, the specific production in roots was about 2 μg g^{-1} FW. The amount of hEGF in

culture medium increased during the culture period; reaching a maximum value of 11% on day 21st. Considering the first 24 days of culture, the maximum productivity in hairy root cultures was 15.9 μg l^{-1} d^{-1}. Non proteolytic activities were detected in the culture medium.

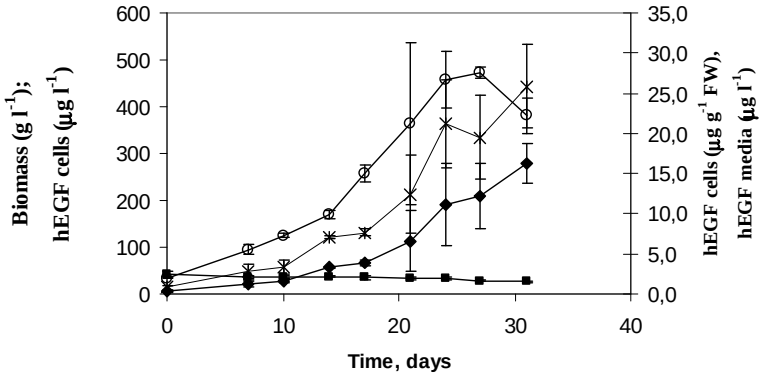

Figure 1: Time course of biomass (♦); hEGF specific production (■); hEGF volumetric production (∗) and hEGF in media (○) of transgenic *N. tabacum* hairy root cultures.

Suspension Cultures

Fast-growing cell suspensions were achieved using disaggregated calli from 2, 4-D 0.2 mg l^{-1} medium after culture on the same liquid medium. Figure 2 shows the time course of growth and hEGF production by transgenic *N. tabacum* suspension cultures.

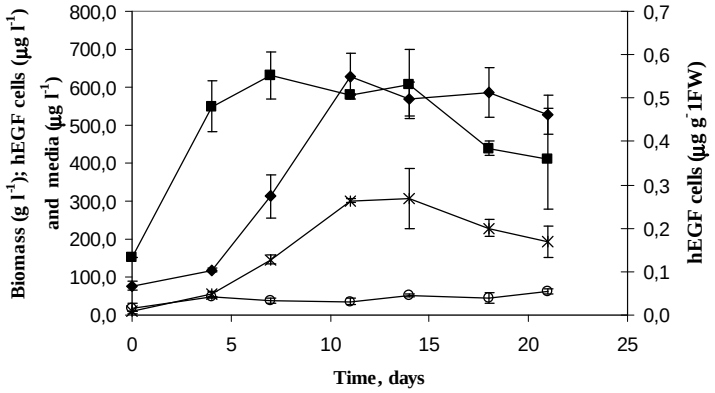

Figure 2: Time course of biomass (♦); hEGF specific production (■); hEGF volumetric production (∗) and hEGF in media (○) of transgenic *N. tabacum* suspension cultures.

In this case, the increase in biomass was about 8-fold, with its maximum value (628 g FW l^{-1}) at 11 days of culture. The maximum growth rate (0.32 d^{-1}) occurred between 7-11days with a doubling time of 2.17 days. When the time course of biomass production of wild type suspension cultures (data not shown, growth rate = 0.26 d^{-1}, doubling time = 2.67 days) was compared with their morphological aspect, it was possible to infer that the expression of the foreign protein hEGF in *N. tabacum* did not affect the growth or morphology of the cultures. hEGF production was a growth associated phenomenon. The specific production reached 0.48-0.53 μg g^{-1}FW between the 4th and 14th day of culture and then decreased slowly, being the total volumetric production 350 μg l^{-1} at 14 days of culture. The amount of recombinant protein in the culture medium reached a maximum value at 14 days (50 μg l^{-1}), representing an 11% of total hEGF. The productivity was 31 μg l^{-1} d^{-1} at 11-14 days of culture. Non proteolytic activities were detected in the culture medium.

In order to increase hEGF accumulation, two strategies were used: the culture medium was first supplemented with casaminoacids at 2% and the cells were then pretreated with SNP (100 μM). As it is well known, SNP is used as a NO donor. In plants, NO plays an important role (direct and indirect) in transduction of environmental and hormonal stimuli (8). In addition, NO affects the plant growth and also is able to promote cell multiplication (9). In some cases, NO interacts with reactive oxygen species (ROS), showing a protective effect against oxidative stress (8).

Figure 3 shows that the lag phase is longer in the medium with casaminoacids than in controls, being the growth rate similar (0.32 d^{-1}) in both cases. The hEGF specific production at 7 days of culture increased from 0.55 μg g^{-1} FW to 0.87 μg g^{-1} FW in the medium containing casaminoacids. This effect is lower than the increase of 300% in TMV antibody production reported by Fischer et al (10), although this effect was transient and decreased after 36 h.

Cell suspensions treated with SNP showed a higher growth rate (0.36 d^{-1}) than control cultures. This increase in growth rate was previously reported in *N. tabacum* cell suspensions by Parsons et al. [11] and was also observed in *Catharanthus roseus* cultures by Xu et al (11). Figure 3 C shows an increase in biomass of 44 % respect to the control at 7 days of culture. As a result of this increase, the hEGF volumetric production reached a level of 300 μg l^{-1} with 18% of it released into the culture medium. The productivity at this time was 46 μg l^{-1} d^{-1} being 48 % higher than 31 μg l^{-1} d^{-1} (control maximum productivity at day 11).

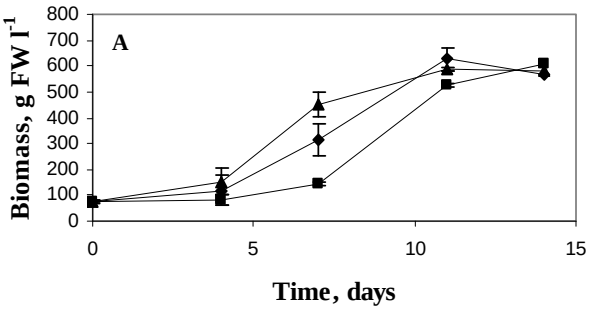

Figure 3A: Effect of casaminoacids and SNP on biomass production by transgenic *N. tabacum* suspension cultures. Control (♦); addition of casaminoacids (■) and SNP treatment (▲).

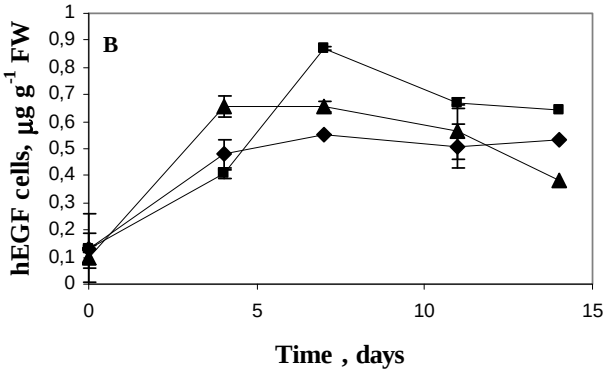

Figure 3B: Effect of casaminoacids and SNP on hEGF specific production by transgenic *N. tabacum* suspension cultures. Control (♦); addition of casaminoacids (■) and SNP treatment (▲).

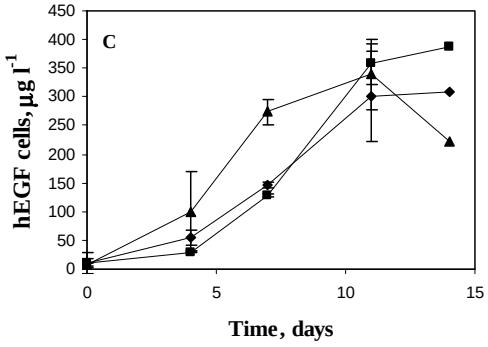

Figure 3C: Effect of casaminoacids and SNP on hEGF volumetric production by transgenic *N. tabacum* suspension cultures. Control (♦); addition of casaminoacids (■) and SNP treatment (▲).

Discussion

The establishment of both differentiated and dedifferentiated *in vitro* cultures of *N. tabacum* is very well known. This plant has been widely used as a platform for foreign proteins expression (1,2). Our results show that the expression of recombinant EGF in *N. tabacum* does not affect the growth or morphology in both suspension and hairy root cultures.

Although the concentration of hEGF in the culture medium was two times higher in suspension cultures than in hairy roots, the specific production was four times higher in hairy root cultures. These results suggest that the synthesis and the accumulation of hEGF depend on the level of tissue differentiation. This is in accordance with the hEGF content reported by Wirth et al. (3) in fresh leaf tissue of tobacco transgenic plants (34.2 ± 2.6 μg of hEGF per g of fresh leaf tissue). The release of hEGF in both hairy roots and suspension cultures was approximately 11% of the maximum amount obtained, although proteolytic activities were not detected in culture medium. The low levels of recombinant protein in the medium may be ascribed to irreversible surface adsorption as reported by Doran (12).

By analyzing the effect of casaminoacids and SNP on suspension cultures of transgenic *N. tabacum,* it is clear that only SNP treatment produces an increase in hEGF productivity. Casaminoacids produced a transient increase in the specific production of EGF this effect was also described by Fisher et al. [10]. We found that the addition of SNP a positive effect in increasing the growth rate of suspension cultures and may thus an become important tool for increasing productivity of recombinant proteins and secondary metabolites in plant cell culture systems.

Acknowledgements

This work was supported by grant PICT 2003 14-15112, Agencia Nacional de Promoción Científica y Técnica (ANPCyT) and by grant UBACyT 093 University of Buenos Aires. JRT, FBA and AMG are researchers from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas. Argentina).

References

[1] Hellwig, S., J. Drossard, R.M. Twyman, and R. Fischer. 2004. Plant cell cultures for the production of recombinant proteins. Nat Biotechnol *22*:1415-1422.

[2] Huang, T.K., M.A. Plesha, B.W. Falk, A.M. Dandekar, and K.A. McDonald. 2009. Bioreactor strategies for improving production yield and functionality of a recombinant human protein in transgenic tobacco cell cultures. Biotechnol Bioeng *102*:508-520.

[3] Wirth, S., G. Calamante, A. Mentaberry, L. Bussmann, M. Lattanzi, L. Barañao, and F. Bravo-Almonacid. 2004. Expression of active human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative systems. Mol. Breed *13*:23-35.

[4] Deeter, S., J.E. Schmidt, K.J. Mabery, D.R. Bethell, N. Huang, and S.C. Pettit. 2007. Components of cell culture media produced from plant cells. . World Intellectual Property Organization Patent *WO/2007/002762*.

[5] Khana, P. and J. Staba. 1968. Antimicrobials from plant tissue culture. Lloydia *31*:180-189.

[6] Massera, P., J. Rodriguez Talou, and A.M. Giulietti. 1998. Thiophenes production in transformed root cultures of Tagetes filifolia. Biotechnol. Lett. *20*:573-577.

[7] Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*:248-254.

[8] Lamattina, L., C. Garcia-Mata, M. Graziano, and G. Pagnussat. 2003. Nitric oxide: the versatility of an extensive signal molecule. Annu Rev Plant Biol *54*:109-136.

[9] Otvos, K., T.P. Pasternak, P. Miskolczi, M. Domoki, D. Dorjgotov, A. Szucs, S. Bottka, D. Dudits, and A. Feher. 2005. Nitric oxide is required for, and promotes auxin-mediated activation of, cell division and embryogenic cell formation but does not influence cell cycle progression in alfalfa cell cultures. Plant J *43*:849-860.

[10] Fischer, R., Y.C. Liao, and J. Drossard. 1999. Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture. J Immunol Methods *226*:1-10.

[11] Xu, M. and J. Dong. 2005. Elicitor-induced nitric oxide burst is essential for triggering catharanthine synthesis in Catharanthus roseus suspension cells. Appl Microbiol Biotechnol *67*:40-44.

[12] Doran, P.M. 2006. Loss of secreted antibody from transgenic plant tissue cultures due to surface adsorption. J Biotechnol *122*:39-54.