

Expression of the antibody 14D9 in *Nicotiana tabacum* hairy roots

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Abbreviations:
DMSO: dimethylsulfoxide;
ER: endoplasmic reticulum;
FW: fresh weight;
GI: growth index = (Final FW- Inicial FW)/ Initial FW;
PVP: polyvinylpyrrolidone;
TSP: total soluble protein.

Nicotiana tabacum hairy roots that express the antibody 14D9 were established. The 14D9 antibody yield obtained after 20 days of culture was 5.95 µg 14D9ml⁻¹. The addition of the reticulum endoplasmic retention sequence KDEL demonstrated a positive effect over the

intracellular 14D9 amounts with a yield increase up to 20.82 µg ml⁻¹. DMSO increased the antibody amount in the biomass from 20.00 to 64.03 µg ml⁻¹ while PVP (at 1.5 g l⁻¹) and gelatine (at 5.0 g l⁻¹) increased total 14D9 amounts in the culture medium to 25 µg and 14 µg respectively.

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Transgenic plants are an economical alternative for large-scale production of recombinant proteins for industrial and pharmaceutical uses. Most of the work has been performed in whole plant production methods; however, not many proteins can be produced in plants at high levels. Anyway a 0.1-1.0% TSP yield, the typical level obtained for the production of pharmaceutical proteins, is competitive enough in order to make plants economically viable. Besides, protein production in plant cells is safer than traditional techniques because of the lack of contamination with strange viral or bacterial materials, mammalian pathogens and other animal cell-culture contaminants. *In vitro* plant cell cultures appeared as an interesting alternative when a foreign protein has to be synthesized under controlled environmental conditions or when the product is required in a quicker way than that obtained by agriculture procedure (Hellwig et al. 2004). In addition, another important advantage of *in vitro* culture is the possibility to recover the foreign protein from the culture medium which reduces the cost of down stream processing. Taking into account all the aspects cited before, protein production costs could be reduced by as much as 1000-fold compared with traditional production practices (Peterson and Arntzen, 2004). On the other hand, as the economic feasibility of plant-based systems depends on the concentration or titer of foreign protein accumulated, preventing the degradation or loss of product after its assembly and/or secretion therefore it is of vital importance. The fusion of a protein to the C-terminal KDEL sequence for retention in and/or retrieve to the ER has been found to increase protein stability and to avoid addition of complex glycans in plant cells (Ko et al. 2003; Gomord et al. 2004). Also, the use of protein-stabilizing agents such as PVP and gelatine is one of the most promising methods for retaining foreign proteins secreted into plant culture medium. If the protein remains attached to biomass, permeabilising agents (such as DMSO) could be used (Magnuson, 1996; Wongsamuth and Doran, 1997).

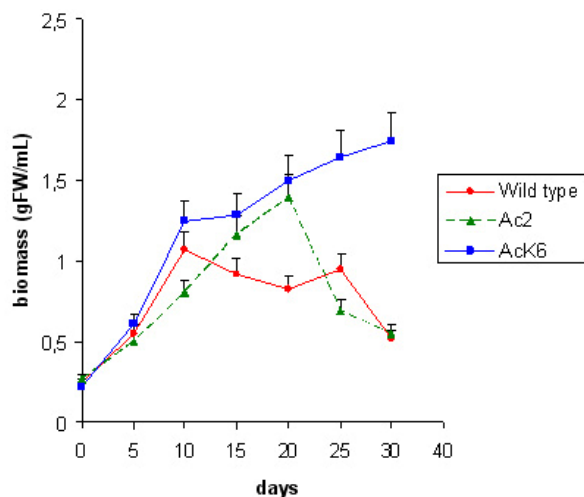


Figure 1. Time course of growth of *N. tabacum* hairy root lines. Each point represents the mean of three replicates \pm SD. Time of culture: 30 days.

At present, *in vitro* cultures and particularly hairy roots have been recognized to be an alternative for foreign protein production. Hairy roots are a stable reproducible system which grows faster than suspension cell cultures and also they are able to grow in plant growth regulator-free culture medium. Considering all the proteins that have been produced in plants, perhaps the most promising ones are monoclonal antibodies and antibody fragments. However, there have been few reports on antibody production by hairy root cultures (Wongsamuth and Doran, 1997; Sharp and Doran, 2001). The aim of this work is to study the expression of the 14D9 antibody in *Nicotiana tabacum* hairy root cultures. 14D9 is a murine antibody IgG1 type which catalyzes the stereoselective transformation of achiral enol ethers having a practical appeal for organic synthesis. The kinetic constant for the reaction ($k_{cat} = 0.4 \text{ s}^{-1}$) makes this antibody one of the faster and most useful ones (Reymond et al. 1993; Zheng et al. 2003). Moreover, the effect of the KDEL retention signal and PVP, DMSO and gelatine on antibody production was also studied.

MATERIALS AND METHODS

Plant material and culture conditions

Seeds of wild type and two transgenic lines of *N. tabacum* expressing 14D9 murine monoclonal antibody were used. The difference between the two transgenic lines, Ac and AcK, was the presence of the ER retention sequence KDEL in the last one.

Initiation and maintenance of hairy root cultures

Hairy roots of *N. tabacum* wild type and from the two transgenic lines (Ac₂ and AcK₆) were induced by direct infection of leaves from 3 to 4 week old seedlings with a 48 hrs culture of *Agrobacterium rhizogenes* LBA 9402 according Alvarez et al. (1994). The 4-5 cm hairy root tips that appeared at the inoculation sites were excised and transferred to 150-ml Erlenmeyer flasks containing 40 ml MSRT liquid medium (Alvarez et al. 1993). Cultures were maintained according to Alvarez et al (1994) and transformation confirmed according to Hamill et al. (1987). Cultures were carried on an orbital shaker operated at 100 rpm, $24 \pm 2^\circ\text{C}$ under a 16 hrs photoperiod provided by white fluorescent lamps, with an irradiance of $1.8 \text{ w m}^{-2} \text{ seg}^{-1}$.

Time-course of growth and antibody production

With the purpose of studying the time-course of growth and antibody production, 200 mg FW of 15 days-old hairy roots of wild type and transgenic clones were inoculated into 250 Erlenmeyer flasks containing 40 ml MSRT medium. Samples were taken every 5 days.

Effect of additives

DMSO was added to 14 days-old hairy root cultures of clones Ac₂ and AcK₆. The concentrations of DMSO used

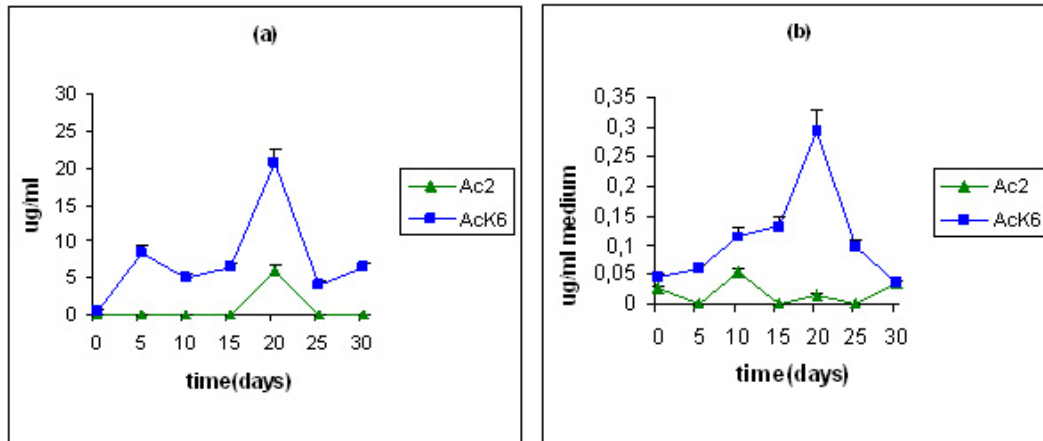


Figure 2. Time course of 14D9 expression by clones AcK₆ and Ac₂ in:

(a) biomass and,

(b) culture medium during 30 days of culture.

Each point represents the mean of three replicates \pm SD.

were 2.8% and 5% w/v. Samples were taken at the beginning and on the 6, 13, 14, 17, 26 and 33 day of culture. Gelatine (1.0, 5.0 or 9.0 g l⁻¹) and PVP (1.0, 1.5 or 2.0 g l⁻¹) were added to 2 days-old AcK₆ hairy root clone cultures. Samples were taken on the 22 day of culture. Measurements of biomass, total protein content and antibody levels in the roots and culture media were carried out in all the experiments.

Analytical methods

Samples were harvested and dried under vacuum. Biomass was estimated as FW. For antibody extraction from the plant tissues the samples were grinded using a cold mortar and pestle in cold PBS (0.24 g KH₂PO₄ l⁻¹, 1.44 g Na₂HPO₄ l⁻¹, 0.2 g KCl l⁻¹, 8 g NaCl l⁻¹, pH: 7.0-7.2) containing 10 μg leupeptin ml⁻¹, then centrifuged at 14,000g for 20 min in a cold chamber. Concentrations of antibodies possessing both heavy (γ) and light (κ) chains were measured in the biomass extracts and in the culture medium by sandwich ELISA using goat anti-mouse antibodies specific for γ and κ chains. Only antibodies assembled into γ - κ chain complexes were measured. The ability of the antibodies to recognize the hapten was evaluated by direct ELISA and their integrity by Western blot. Western blot analysis was carried out with goat anti-gamma mouse chain-conjugated peroxidase used at 1:1000. The immune complexes were detected after incubation with Supersignal West Pico Chemiluminiscent Substrate (Pierce Chem.). Proteins were evaluated according to Bradford (1976) and proteases as described by Vázquez and MacCormack (2002). Transformation with *A. rhizogenes* LBA 9402 was confirmed according to Alvarez et al. (1994). Three replicates were used in all determinations and variance analysis was conducted in each test.

Chemicals

All the culture medium components and chemicals were purchased from Sigma Chemical Co.. PVP molecular weight: 10,000. Kappa-chain specific goat anti-mouse IgG and gamma-chain specific goat anti-mouse IgG-HRP were purchased from Southern Biotechnology (Birmingham, AL).

RESULTS AND DISCUSSION

Establishment and selection of hairy root clones

The frequency of root initiation was above 85%. Several clones which present differences regarding growth as well as in antibody expression levels were obtained. Among the clones obtained we selected clones Ac₂ and AcK₆ for their characteristics of growth (high rate) and their capacity to express 14D9 antibody in an stable way. Hairy roots from line AcK₆ grew faster and stronger than the wild type and line Ac₂. That phenomenon is also observed in the whole plants and suspension cell cultures derived from AcK₆ line. We could attribute it to the site of T-DNA integration in that transgenic line.

Time-course of growth and antibody production

Figure 1 shows clone AcK₆ grew faster ($\mu = 0.190 \text{ days}^{-1}$) than both the wild type ($\mu = 0.105 \text{ days}^{-1}$) and clone Ac₂ ($\mu = 0.060 \text{ days}^{-1}$). The 14D9 antibody yield obtained after 20 days of culture was 4.26 $\mu\text{g g}^{-1}\text{FW}$ (5.95 μgml^{-1}) for clone Ac₂ and 13.70 $\mu\text{g g}^{-1}\text{FW}$ (20.82 μgml^{-1}) for clone AcK₆. On the other hand, 14D9 antibody concentration expressed as %TSP were 0.386 for clone Ac₂ and 9.34 for clone AcK₆. The antibody yield (expressed as $\mu\text{g g}^{-1}\text{FW}$) in line AcK₆ was 3.21-fold higher than in line Ac₂. Preventing the

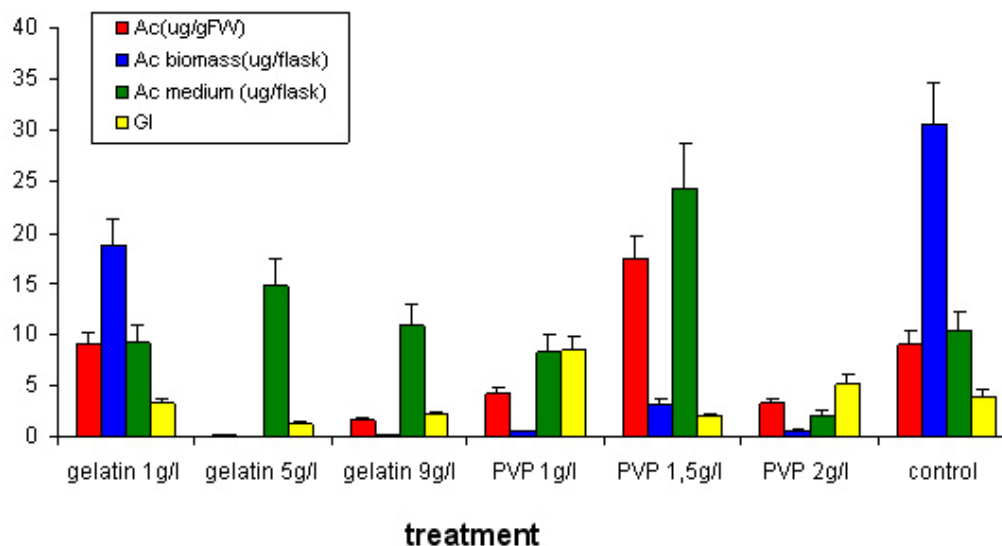


Figure 3. Growth index (GI), antibody yield in the biomass ($\mu\text{g FW}^{-1}$), total amount of 14D9 in the culture medium ($\mu\text{g 14D9 per flask}$) and in the biomass ($\mu\text{g 14Dp per flask}$) in 22 days-old *N. tabacum* AcK₆ hairy root clone with the addition of gelatine (1.0, 5.0 and 9.0 g l⁻¹), and PVP (1.0, 1.5 and 2.0 g l⁻¹). Gelatine and PVP were added to 2 days-old cultures. As control AcK₆ hairy root without any addition was used. Each point represents the mean of three replicates \pm SD.

secretion of protein by using the KDEL signal peptide for retention in the ER, has been found to minimize antibody fragmentation and improve antibody accumulation in plant cells (Tsoi and Doran, 2002). Also proteases in the secretory pathway and in the apoplast are avoided (Sharp and Doran, 2001). In the culture medium the highest values of antibodies were about 1% of the amounts reached in the biomass for both clones (Figure 2). Although at very low levels, 14D9 produced by AcK₆ hairy root clones appeared into the culture medium probably due to cellular lysis, in contrast to the results obtained in transgenic plants. It was not detected the presence of proteases in the culture media of transgenic or wild type hairy roots. In addition, Western blots showed a band that corresponds to the complete antibody, no fragments were detected in any case. Functionality of the antibody was confirmed by direct ELISA.

Culture treatments for increasing antibody accumulation and release

In order to increase antibody level in the culture medium, gelatine, PVP and DMSO were added as it was described in Materials and Methods.

Gelatine. Figure 3 shows that gelatine at 5.0 g l⁻¹ had a positive effect on antibody level in the culture medium. The maximum antibody concentration in the medium correspond to 14.0 $\mu\text{g 14D9 per flask}$ which is a 1.44-fold the corresponding level in the control culture (Figure 3). On the other hand at gelatine concentrations higher than 1.0 g l⁻¹ antibody level in the biomass fell down. Probably the intracellular environment changed unfavourably making the antibody concentration in the biomass decrease significantly. These results are in agreement with those

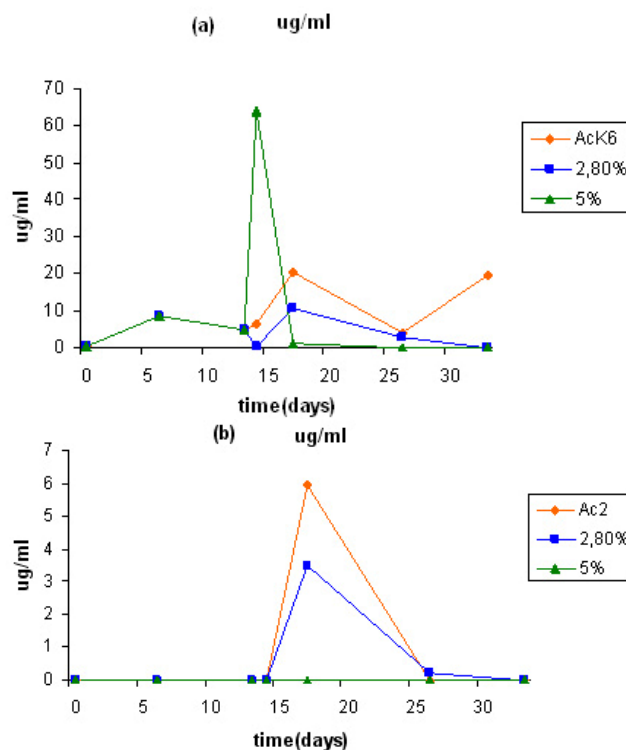


Figure 4. Time course of 14D9 expression in:

(a) AcK₆ hairy root clone cultures incubated with 2.8% or 5% v/v DMSO and, (b) Ac₂ hairy root clone cultures incubated with 2.8% or 5% v/v DMSO. DMSO was added into 14 days-old cultures. As a control AcK₆ hairy root culture without the addition of DMSO was used. Each point represents the mean of three replicates \pm SD.

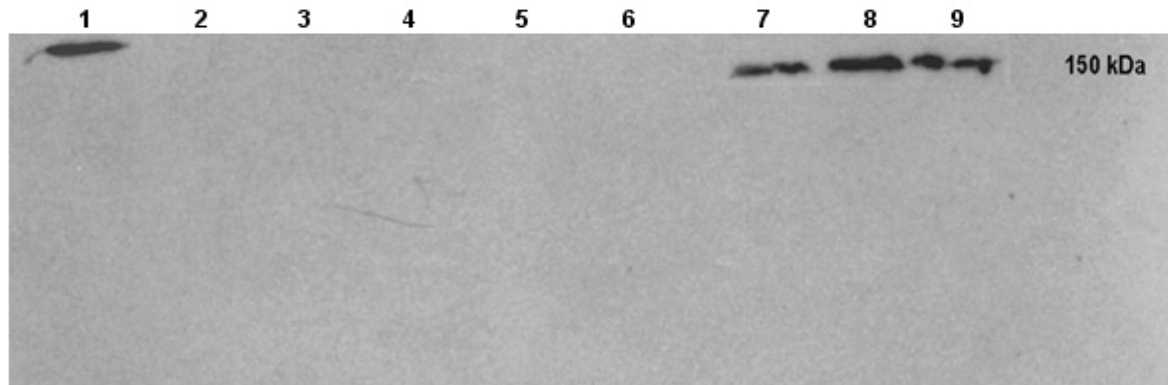


Figure 5. Western-blot of hairy root biomass. The lines were loaded with equal volumes of samples. Line 1: Standard IgG Antibody; line 2: *N. tabacum* wild type; line 3: *N. tabacum* wild type + 5% DMSO; line 4: Ac₂ + 2.8% DMSO; line 5: Ac₂ + 5% DMSO; line 6: Ac₂ control; line 7: AcK₆ + 2.8% DMSO; line 8: AcK₆+5%; line 9: AcK₆ control. Coating: goat anti gamma mouse chain conjugated with peroxidase (1:1000) in 1% (P/V) non fat milk. Antibody binding was detected after incubation with Supersignal West Pico Chemiluminiscent Substrate (Pierce Chem.) after 25 min of exposure.

reported by Lee et al. (2002) working with *N. tabacum* cell suspension cultures expressing the human granulocyte-macrophage colony stimulating factor (hGMCSF).

PVP. PVP is a versatile polymer which is water soluble, exhibiting excellent complexing, stabilizing and colloidal properties while at the same time being metabolically and physiologically inert (Magnuson et al. 1996). In our work, with the addition of 1.5 g PVP l⁻¹, the antibody concentration in culture media was significantly enhanced by 2.38-fold (Figure 3). The increased recovery cannot be attributed to antibody artifactual increases because of cell death or breakage. Therefore, the results suggest that PVP possibly functions preventing denaturation and adherence to surfaces in the culture vessel of the secreted protein. Similar results have been reported previously for tobacco cell suspension cultures (Magnuson et al. 1996; Wongsamuth and Doran, 1997).

DMSO. 14D9 antibody level in clone AcK₆ increased in about three times at the 20 day of culture using DMSO at a concentration of 5% v/v (Figure 4a). The increase of the antibody level into the biomass could be attributed to DMSO producing small channels which do not allow the antibody release. These channels would favour the entrance of small molecules of nutrients which may increase the synthesis of the antibody in the biomass. The increased metabolic activity should increase not only the 14D9 level but also other proteins. In our experiment total soluble protein content in the biomass also increased from 13.6 µg ml⁻¹ to 20.87 µg ml⁻¹. DMSO could also stabilize proteins against denaturation by forming hydrogen bonds with proton-donor groups on protein molecules (Hendersen et al. 1975). However, DMSO was not effective for increasing the release of the antibody in the culture medium, the levels remained approximately the same than those of the control (Figure 4b). The size of 14D9, about 150kDa, may be too big to pass through the pores created by the permeabilizing

action of DMSO. It has been shown that molecules with a molecular weight greater than 5,000 have difficulty passing through the cell wall even after permeabilization with DMSO concentrations up to 7.5% (Carpita et al. 1979; Wahl et al. 1995). Western blots did not show any antibody fragment Neither in the biomass nor in the culture medium (Figure 5).

CONCLUDING REMARKS

We demonstrated the ability of *N. tabacum* hairy roots to express the antibody 14D9 in a stable way in time. As far as we know, this is the first report about the use of the KDEL sequence to improve antibody expression in hairy root cultures. In our work the KDEL sequence showed a beneficial effect on antibody yields. From our results we could establish that both the addition of PVP (1.5 g l⁻¹) and gelatine (5.0 g l⁻¹) enhanced antibody accumulation in hairy root culture media probably by protecting the antibody to some extent. No antibody fragment was detected into the medium. DMSO at 5.0 % (V/V) concentration had a positive effect on antibody accumulation in the biomass of AcK₆ hairy root cultures. As Wahl et al. (1995) reported before, we found that the effect of the increasing of the DMSO concentration on antibody production was more drastic for intracellular production than for extracellular. The hairy roots accumulated by 13-15% with respect to the antibody titers typically obtained in batch hybridoma cultures (Bibila and Filckinger, 1991; Miller et al. 1988). In contrast with hybridoma cells, however, most of the antibody remained associated with the root tissue rather than being secreted extracellularly. The controversial results informed by different authors lead us to the conclusion that the influence of additives such as permeabilizant and stabilizers must be studied and optimized according to the plant cell tissue culture for each expressed antibody in order to establish a competitive production system.

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