

Expression of the recombinant tE2 antigen of bovine viral diarrhea virus (BVDV) in *Nicotiana tabacum* plants and biosafety studies in mice

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RESEARCH

ABSTRACT

Antigenic glycoprotein tE2 from bovine viral diarrhea virus (BVDV) and the fluorescence marker EGFP were transiently expressed in leaves of *Nicotiana tabacum* using *Agrobacterium tumefaciens* as vector. Analyzing the *Agrobacterium* and EGFP RNA/protein persistence allowed us to estimate an environmental exposure to recombinant material during 6 days after agroinfiltration. Time course analysis for the expression of EGFP shows silencing after 6 days post-infiltration with recombinant *Agrobacterium* strain in treated leaves. The leaf extracts expressing tE2 and EGFP were clarified at 6 days after agroinfiltration. The extracts were mixed with oil or aqueous adjuvants and injected to Balb/c mice in order to establish if the plant extracts are innocuous. Four doses were applied, followed by necropsy. No significant differences between non-recombinant extracts (control treatment) or recombinant leaf extracts in clinical and biochemical analysis were observed. In all mice injected, the adjuvant caused the classical pathological lesions observed in typical inflammatory processes at the site of inoculation. Histological sections of these lesions and major organs showed no further injury.

Keywords: agroinfiltration, recombinant protein, BVDV, glycoprotein tE2, transient expression, biosafety

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RESUMEN

Expresión recombinante del antígeno tE2 del virus de la diarrea viral bovina (BVDV) en plantas de *Nicotiana tabacum* y estudio de su bioseguridad en ratones. En el presente trabajo se expresaron el antígeno vacunal tE2 del virus de la diarrea viral bovina (BVDV) y la proteína marcadora de fluorescencia EGFP en forma transitoria en hojas de *Nicotiana tabacum*, utilizando como vector *Agrobacterium tumefaciens*. Analizando la supervivencia microbiana y la persistencia del RNA/proteína de EGFP pudimos estimar un riesgo ambiental hasta 6 días después de la agroinfiltración. La cinética de tiempo y el análisis de imágenes para EGFP muestran el silenciamiento de la expresión de la proteína heteróloga 6 días después de la infiltración con la cepa de *Agrobacterium* recombinante en las hojas de tabaco tratadas. Los extractos foliares expresando tE2 fueron clarificados y mezclados con un adyuvante oleoso o acuoso. Estos candidatos a vacunas se administraron a ratones hembra Balb/c (inoculación primaria intramuscular seguida por tres dosis de refuerzo). No se observaron diferencias significativas entre los extractos no recombinantes (tratamiento control) o extractos foliares recombinantes en el análisis clínico y bioquímico. En todos los ratones inyectados, el adyuvante provocó las lesiones patológicas clásicas observadas en los procesos inflamatorios en el sitio de inoculación. Secciones histológicas de estas lesiones y de los órganos principales no mostraron lesiones adicionales.

Palabras clave: agroinfiltración, proteína recombinante, BVDV, glicoproteína tE2, expresión transitoria, bioseguridad

Introduction

The use of plants for the production of recombinant antigens is gaining interest as an alternative for the development of effective, safe and economical subunit vaccines. In particular, a transient gene expression system allows to obtain high levels of recombinant proteins in a short period of time without modifying the whole plant genome. One of the most common methods used for transient expression is agroinfiltration, where the gene of interest is cloned into a binary vector

and transformed into an *Agrobacterium* host [1]. This recombinant *Agrobacterium* is infiltrated into the apoplast space of the plant tissue, allowing the delivery of transgenes to cells at high copy number [2, 3]. Few days after agroinfiltration, significant amounts of recombinant protein can be produced and harvested.

One of those proteins, the major envelope glycoprotein E2 of Bovine Viral Diarrhoea Virus (BVDV), has been expressed as truncated version of E2 (tE2) in

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N. tabacum plants in our laboratory, and the immunogenicity of recombinant plants extracts containing it evaluated in guinea pigs. Two different formulations of plant tE2 vaccines were tested, one with aqueous (Al(OH)₃) and the other with oily (Montanide ISA 70) adjuvants [4]. The sera of vaccinated animals were tested by an indirect ELISA and by a Virus Neutralization Test. In both cases, results showed antibodies high titers in animals inoculated with the plant formulation, indicating a clear seroconversion of vaccinated animals. This suggested a good immunogenic performance of the recombinant antigen [4].

Concerning the safety of using *Agrobacterium* strains for the purpose of heterologous protein expression in plants, several studies have examined the persistence of bacterial DNA in the environment [5], but little information is available. In this work, we infiltrated *N. tabacum* leaves with *A. tumefaciens* carrying the tE2 and EGFP sequences in its T-DNA and analyzed the time course of *Agrobacterium* survival and EGFP RNA/protein persistence after infiltration. Finally, in order to establish if the plant extracts are innocuous to animals, Balb/c mice were injected with clarified leaf extracts expressing tE2 and EGFP proteins mixed with oily or aqueous adjuvants.

Materials and methods

Vector construction and agroinfiltration

The pENTR-2s2-tE2-His-KDEL entry clone [4] was recombined with the pk7WG2D destination vector using the Gateway cloning technology (Invitrogen, USA) according to the manufacturer's instructions. The resultant pK-tE2-EGFP expression clone was introduced into *A. tumefaciens* strain EHA101 (pTiBo542ΔT-DNA) by electroporation (Figure 1). A single colony from a fresh plate was inoculated in 10 mL YEB medium supplemented with rifampicin 20 mg/L, kanamycin 50 mg/L, spectinomycin 100 mg/L and 20 μM acetosyringone. Cultures were grown at 28 °C and 210 rpm until the OD₆₀₀ nm reached 0.6. The bacterial suspensions were used to agroinfiltrate the third leaf (counting from the apex) of 10 *N. tabacum* cv. Xanthi plants using a syringe without a needle. Leaves were infected in the apical, middle or basal (leaf petiole) sector. Neighboring leaves (upper and lower leaves) were also used for analysis. Control plants were tobacco leaves infected with non-recombinant *A. tumefaciens* EHA 101 following simile pattern of infection.

Microbiological assays

Samples were collected daily during 15 days after agroinfiltration from middle and basal portions of infiltrated leaves using a sterile cotton swab for each sample. Also, upper and lower neighboring leaves were sampled [6]. Each cotton swab was dipped in broth medium dispensed in agar plates (YEB medium, containing 50 μg/mL kanamycin as described [4]). The number of colonies on each plate was recorded daily during 3 days.

RT-PCR analysis

Total RNA was extracted from frozen leaf samples (100 mg) using RNeasy Plant Mini Kit (Quiagen) according to the manufacturer's instructions. The qual-

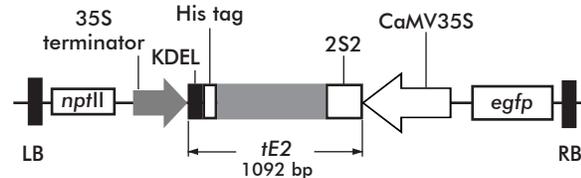


Figure 1. Schematic representation of T-DNA region of the pk7-tE2-EGFP expression clone. LB, RB: T-DNA left and right border; CaMV35S: cauliflower mosaic virus 35S promoter; 35S Terminator, cauliflower mosaic virus 35S transcription terminator; tE2: truncated E2 version lacking its transmembrane domain fused to the signal peptide of the *Arabidopsis thaliana* 2S2 seed storage protein for driving the protein to the apoplast, and to the KDEL retention signal for protein accumulation in the endoplasmic reticulum; egfp: enhanced green fluorescent protein gene. In addition, a C-terminal hexahistidine tag was introduced (His-tag). Vector used (pK7WG2D, Gateway Technology) include neomycin phosphotransferase resistance gene (nptII) and egfp marker.

ity of RNA was determined by agarose gel electrophoresis and quantified by spectrophotometric analysis. For cDNA synthesis, 2 μg of total RNA was reverse-transcribed using Superscript II First Strand Synthesis System (Invitrogen, USA) and random primers, following the manufacturer's instructions. Finally, a PCR (GenePro, BIOER) was performed using as template cDNA and EGFP specific primers (forward: 5'-catgctcctgctggagttcgtg-3' and reverse 5'-cgtcgcctc-cagctcgaccag-3'). The pK-tE2-EGFP expression clone was used as positive control and total RNA without retrotranscription was used as negative control. The PCR reaction involved a single denaturation step at 94 °C for 7 min, followed by 35 cycles at 94 °C for 60 s, 53 °C for 45 s, and 72 °C for 70 s, and a final extension at 72 °C for 5 min. The PCR products were resolved by electrophoresis in 1 % agarose gel stained with ethidium bromide in TAE 1× buffer.

Image analyzer-time course of EGFP

Leaves were peeling (squares of 5 mm² approx.) and mounted in water on glass slides with the abaxial side facing up and covered with glass slips. Images were taken under light or fluorescence by using an optical microscope (Nikon Eclipse E600) with an excitation filter of band pass of 450-490 nm and emission above 515 nm (B-2A filter). The fluorescence light source was provided by a 100 W high-pressure mercury bulb. Then image-processing techniques were applied using ImageJ® software from the acquired images, to extract useful features that are necessary for further analysis [7]. Each image was analyzed to select the areas of interest and partitioning the digital image into multiple sets of pixels. Also, images were conventional single channel RGB images (composed of red, green and blue channels), and were advantageously segmented using automated thresholding procedures for the quantification of multiple sets of pixels. Fluorescence parameter values of all pixels within each area for RGB channel were averaged.

Samples from control agroinfiltrated leaves (tobacco leaves infected with non-recombinant *A. tumefaciens*) were used as negative controls to prevent overestimation of pixels due to background due to chlorophyll and to normalize pigment emissions on inoculated leaves. Identical microscope power set-

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tings were applied for all individual images of a figure, to allow comparison of fluorescence intensities between samples.

Protein extraction and western blot analysis

Agroinfiltrated leaves (0.5 g) were disrupted with 0.5 mL PBS extraction buffer (0.24 g/L KH_2PO_4 , 1.44 g/L Na_2HPO_4 , 0.2 g/L KCl, 8 g/L NaCl, leupeptin 10 $\mu\text{g}/\text{mL}$, pH 7.0-7.2) using a tissue homogenizer (Polytron PT 10-35, Kinematica). The extract was centrifuged at 14 000 g and 4 °C for 20 min and the supernatant used for expression analysis. Total protein concentration was determined according to Bradford [8]. Proteins were separated in 10 % SDS-PAGE under non-reducing conditions and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Milipore). Western Blot was performed using a mouse monoclonal antibody anti-E2 (kindly provided by ICT Milstein, Argentina) diluted at 1:100 (titer higher than 1:4000), and a goat anti-gamma mouse chain-conjugated peroxidase antibody (Sigma) at a 1:500 dilution according to manufacturer instructions. The immune complexes were detected after incubation with Super-signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, USA).

A calibration curve was generated to estimate the tE2 accumulation in agroinfiltrated leaves, with a series of known concentration of baculovirus-derived tE2 (bE2) [9]. The amounts of tE2 protein were determined in leaf extracts by comparison with the bE2 calibration curve using the Gel-Pro analyzer software (Media Cybernetics, Rockville, MD). tE2 accumulation was estimated as tE2 μg per percentage of total soluble protein (TSP).

Balb/c Mice immunization schedule

Mice (28 day-old female, average weight 24 ± 2 g, n = 10 per treatment) were kept in cages (1144B, Tecniplast, Touch SLIM PLUS, Italy) at 22 ± 2 °C 12/12 h, 55 ± 10 % relative humidity, light/dark photoperiod (12/12 h), and fed on pellets ad libitum [10]. Animals were clinically evaluated on a daily basis, including weight control. Each mouse was immunized intramuscular with 0.2 mL of a solution containing the mineral oil base adjuvant Montanide ISA 70 SEPPIC and 20 μg tE2 in the plant extract (adjuvant:antigen ratio 60:40) followed by 3 boosters at days 14, 28 and 35. When an aqueous adjuvant ($\text{Al}(\text{OH})_3$ -Hydragel; Sigma) was used, either 20 μg of tE2 plant extract (adjuvant:antigen ratio 10:90) were injected intramuscular (0.2 mL of vaccine). Additional boosters were performed at days 14, 28 and 35. Control animals were primed and boosted with 0.2 mL of wild-type tobacco leaf extract (tobacco leaves infected with non-recombinant *A. tumefaciens* EHA 101) plus each of the two adjuvants mentioned above.

Necropsies

Animals were sacrificed by excess of ether anaesthesia, and complete necropsies were performed 42 days after the first injection. Samples were collected from heart, lungs, liver, spleen, genital tract and from skin covering the injection site with adjacent subcutaneous tissue and muscle. Tissues were fixed in Bouin's fluid, routinely embedded in paraffin, and sections

were stained with haematoxylin-eosin. This study was carried out in compliance with The Animal Welfare Regulations of the National Research Council of Argentina (CONICET).

Blood studies

Whole blood samples were collected at the time of immunization (day 0) and after 42 days post-vaccination. Sera from all animals were used to perform virus neutralization testing.

Blood hematology

Blood samples were collected from retro-orbital bleeding at the time of necropsy (day 42) in ethylenediaminetetraacetic acid (EDTA) vacuum blood collection tubes and immediately shaken to mix the blood with the EDTA-anticoagulant inside the tubes for automated haematology analyses. Blood samples were analyzed using an automated haematology analyzer (Beckman Coulter LH 750, USA) to measure total white blood cells (WBC), packed red blood cells (haematocrit), and number of platelets.

Blood biochemistry

Blood samples were drawn from mice by retro-orbital bleeding and recovered in serum vacuum blood collection tubes. Blood samples were centrifuged at 5000 rpm for 5 min and then sera were collected in Eppendorf tubes and stored at -20 °C. Liver and pancreas functionalities were tested by measuring AST (aspartate aminotransferase), ALT (alanine aminotransferase), LDH (lactic dehydrogenase), amylase enzyme activities as well as glucose concentration. Serum samples were analyzed using an automated biochemistry analyzer (Hitachi Cobas c311, Hitachi, Japan) following the manufacturer's instructions. Standard controls were run before each determination.

Statistical analyses

All assays were repeated thrice. Results of weight and serological assays were compared using analysis of variance (ANOVA). A P-value of less than 0.05 was considered significant.

Results and discussion

tE2 expression

The tE2 expression was confirmed in agroinfiltrated leaves by Western blot using a monoclonal antibody against E2 antigen (Figure 2). A band of approximately 80 kDa corresponding to the tE2 dimer was observed in protein extracts from leaves harvested 4 days post-infiltration (dpi). In addition, another band of 35 kDa was observed which corresponded to the expected molecular weight of the tE2 monomer. No immunoreactive bands were detected in samples from control plants (Figure 2A and B). The amount of tE2 accumulated in agroinfiltrated leaves was estimated by comparing the intensity of the bands corresponding to tE2 with a standard curve of baculovirus-derived tE2 (bE2)(Figure 2C). The tE2 expression level reach up 20 $\mu\text{g}/\text{g}$, representing 5.6 % respect to total soluble protein (322 $\mu\text{g}/\text{g}$ TSP) (Figure 2A). A similar pattern and concentration for the recombinant protein was reported by Nelson *et al.* [4].

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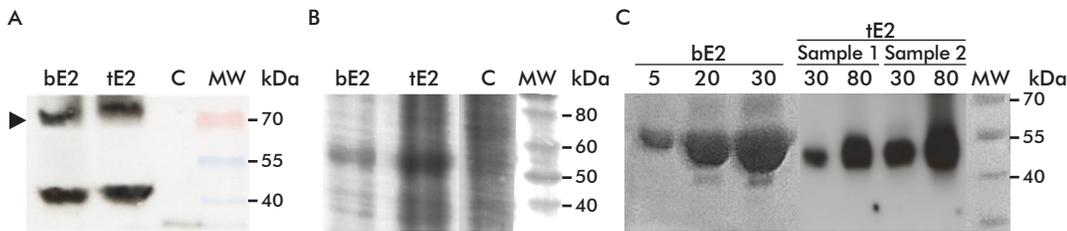


Figure 2. Expression of tE2 in *N. tabacum* leaves 4 days after agroinfiltration. A) Western blot analysis. 5 μ g of total proteins (tE2) was loaded in a 10 % SDS-PAGE gel, further transferred to a PVDF membrane and immunoblotted with an anti-tE2 monoclonal antibody. The arrowhead indicates the expected position for the dimer form. B) A Coomassie blue-stained SDS-PAGE gel was used as a loading control. C) Semi-quantification of tE2 by Western blot. A dilution series of baculovirus-derived tE2 (5, 20 and 30 ng) was used as a standard curve for tE2 quantification from two samples (30 and 80 μ g of TSP). bE2: 10 μ g baculovirus-derived tE2; C: control plants; MW: PageRuler Prestained Protein Ladder for western blot and Novex Sharp Unstained Protein Standard for Coomassie Blue (ThermoFisher).

Microbiological assays of *Agrobacterium* persistence

The transient expression is a secure methodology considering that agroinfiltration does not induce any alteration in the plant genome. However, *Agrobacterium* is a transgenic organism, covered by the currently existing legislation and risk assessment as a genetically modified organism (GMO) [5, 11]. In order to evaluate the bacterial persistence, samples were collected from leaves surface and colony-forming units were recorder during 15 dpi. Samples collected from agroinfiltrated zones during the first and second dpi produced growing colonies after 24 h in culture. After 3 dpi, no colony development was observed in samples obtained using cotton swabs from agroinfiltrated zones. These negative results were obtained from samples taken in the upper and lower neighboring leaf surfaces or control plants (tobacco leaves infected with non-recombinant *A. tumefaciens*) during 15 dpi (data not shown).

EGFP RNA persistence by RT-PCR analysis

Bacterial DNA can be released either through active secretion by living cells or passive release from dead cells or it could be present in agroinfiltrated leaves [5]. We also evaluated the persistence of EGFP RNA in agroinfiltrated leaves. RT-PCR analysis was performed in infiltrated leaves and in neighboring leaves. PCR products showed a band of approximately 700 bp in agroinfiltrated leaves (apical, basal and middle zones) at 2, 3 and 4 dpi (Figure 3). The intensity of the band decrease from day 5 and at day 7 becomes undetectable. The molecular size of these bands is coincident with the EGFP used as template (positive control). No amplification was observed in neighboring leaves (data not shown).

Time course image analysis of EGFP expression

The time course of EGFP production was determined by image analysis using a fluorescence microscope and appropriate software for 10 dpi (Figure 4). Each value corresponded to the mean of the given area for samples obtained from 5 different agroinfiltrated leaves. The conventional image could be quantified as the sum of pixels from each RGB channel. The highest EGFP expression (green channel) was obtained at

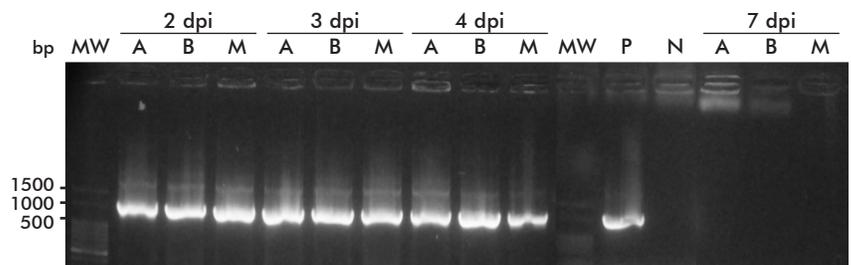


Figure 3. RT-PCR of tE2 gene visualized on 1 % agarose gel. Total RNA was isolated from agroinfiltrated *Nicotiana tabacum* leaves using RNeasy Plant Mini Kit (Qiagen, Germany). The first DNA strand was synthesized from 2 μ g RNA using random primer and Superscript II (Invitrogen) retrotranscriptase. RNAs from the tE2 gene were amplified using specific primers. dpi, days post-infiltration; A: apical; B: basal; M: middle; P and N: positive (pk7-tE2-EGFP) and negative (total RNA) controls, respectively; MW: 100 bp DNA ladder (Invitrogen, USA). Data was analyzed for up to 10 dpi, with no variation from 7 dpi on.

6 dpi at injection site (basal zone) and spread to adjacent areas of injection (middle and apical) (Figure 4A). EGFP content was higher in vascular tissue than in the lamina as previously reported [12]. After 6 dpi, the expression was reduced, which would be related to RNA silencing mechanisms [13, 14] (Figure 4A). Likewise, the fluorescence level of infiltrated leaves decayed to that of control leaves on day eight (Figure 4A). Chlorophyll fluorescence (red channel) demonstrated to be a good control as it was not sensitive to environmental light variations or EGFP expression, and provided single-channel images [15]. As shown in Figure 4B, the red channel had no significant differences among agroinfiltrated leaves as compared to controls.

Balb/c mice immunization with recombinant foliar extracts

Mice were maintained in good health conditions during all the experimental treatments. Clinical observations revealed no significant differences in the average of body weight of mice throughout the experiments. Similar weight curves with a steady weight gain were obtained in animals injected with recombinant proteins and in control animals injected with control foliar extracts without any expressions of recombinant proteins (data not shown).

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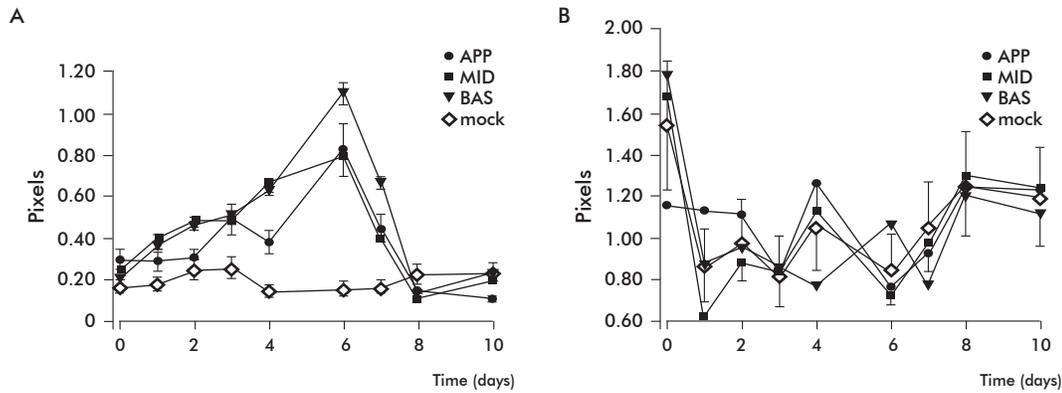


Figure 4. Time course of pixels in samples of *Nicotiana tabacum* leaves (apical, middle and basal zones; APP, MID and BAS, respectively) agroinfiltrated with *Agrobacterium tumefaciens* harboring the pK-2S2-tE2-His-KDEL vector carrying the EGFP under an independent promoter and the control leaf (mock) sample. Images were obtained by using a microscope, an image processing software and appropriate filters (see Materials and methods). A) Pixels taken in the green channel. B) Pixels taken in the red channel.

Macroscopic analysis of injection sites: in the case of the control or extracts expressing tE2 with oil adjuvant, rounded, cystic, 0.2 -0.8 cm width structures containing a milky fluid were evident (Figure 5A). After histological examination of injection sites, discrete inflammatory foci were found which coexisted with an infiltrate mainly composed of plasma cells in all the animals treated. Absence of bacterial colonies and the kind of inflammatory cells, especially surrounding injection sites, suggested a reactive cystic structure rather than a collection of infectious abscess. Macrophages with foamy cytoplasm, polymorphonuclear neutrophil leukocytes, edema, and diffuse deposits of fibrin and necrotic, and apoptotic cells were also observed as a component of inflammatory lesions (Figure 5B). These infiltrates were self-limited, with only minimal compromise of deep soft tissues.

Animals injected with tE2, containing Al(OH)₃ Hidragel® Adjuvant, showed cutaneous injuries. In all samples, the histological examination revealed a well delimited site with an inflammatory pustular injury composed of leukocytes, necrotic areas with fibrin deposition, and mild calcifications (Figure 5C).

Hematological and biochemical parameters were tested, to investigate the potential damage caused in mice by the recombinant proteins delivered in the foliar extracts. Blood hematology comprised haematocrit levels, WBC and platelet counts to evaluate anaemia, and possible pathological and inflammatory changes in connection with normal value ranges. Clinical and hematological blood values obtained from mice, either from controls or from those treated with plant foliar extracts, did not show significant differences compared to normal reference clinical and haematological values, regardless of the adjuvant used [16]. Total platelet counts showed to be lower than the reference normal range only in mice injected with Al(OH)₃ as adjuvant (Table).

The injuries observed at injection sites have been described [17-21], thus elucidating that the toxicity of the adjuvant administered is the cause of these inflammatory reactions. Moreover, DeGregorio *et al.*

[22] demonstrated in a knockout mice model that inflammation components were present at injection sites and required adjuvanticity. Furthermore, mice immunized with these extracts showed no apparent adverse effects, beyond the injection site, in contrast to the well-known and severe inflammatory lesions observed following immunization with Freund's adjuvant in rats [23].

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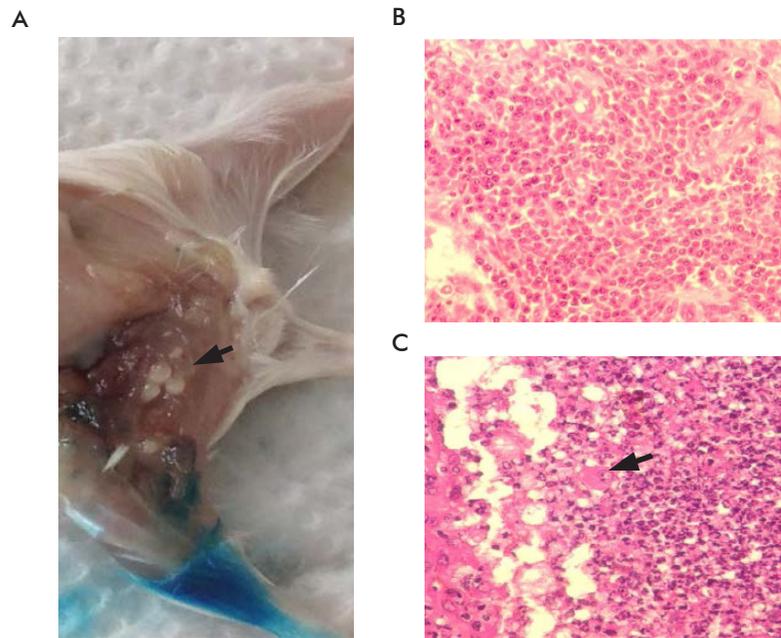


Figure 5. Macroscopic and histology sections of injected areas in female Balb/c mice immunized intramuscularly with foliar extracts of agroinfiltrated *Nicotiana tabacum* plants expressing the glycoprotein tE2. A) Macroscopic observation representative of mice inoculated with oily adjuvants; a diffuse, homogeneous infiltrate of plasma cells is shown (arrow). B) Histology section of a mouse inoculated with oily adjuvant. Inflammatory lesions at subcutaneous levels was found at low magnification. C) Histology section of a mouse inoculated with an aqueous adjuvant. Foamy macrophages surrounded by neutrophil polymorphonuclear leucocytes were detected (arrow). Magnification: 400x.

Table. Hematological chemistry values in mice injected with extracts of *Nicotiana tabacum* leaves expressing the tE2 recombinant antigen of the bovine viral diarrhoea virus or control extracts*

Adjuvant	Treatment	Hct	WBC (× mm ³)	PLT	AST (UI/L)	ALT (UI/L)	Amylase (UI/L)	LDH (UI/L)	Glu (mg/dL)
Montanide ISA 70 SEPPIC®	tE2	49.6	5400	1 327 600	0	39	657	0	147
	Wt	49.8	6260	1 072 200	0	34	798	0	137
Al(OH) ₃ Hydragel®	tE2	39.9	5563	223 125	210	55	759	1371	147
	Wt	46.6	5188	192 333	234	43	1004	1319	142
Reference value ranges		42/68	3200/14 000	470 000/1 600 000	95/132	38/52	420/589	1300/1560	82/175

* Reference values for the species as reported by Suckow MA, Danneman P & Brayton C (The laboratory mouse. Boca Raton: CRC Press; 2001). Hct: haematocrit; WBC: total white blood cell; PLT: platelets; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; Glu: glucose.

In the last years, transient gene expression technology in plants with binary or viral vectors have gained interest due to rapid and large amounts of protein production obtained even achieving industrial scale levels. Nevertheless, a commercial process requires high productivity and maximum product yield with minimum cost [24] and must follow regulations like GMP, GLP and risk assessment. Agroinfiltration, is an economic biotechnology method for transiently express a gene during a short period of time without introducing a permanent genetic modification in the plant genome [25]. Also, a variety of strategies have been develop during the last decade with the aim to increase the protein yield and to obtain an efficient transient platform production. There are several reports regarding the use of antigens produced in plants as plant extracts to immunize animals that are able to induced protection against diverse diseases [26-28]. Besides, the potential toxicity of these plant extracts

in the immunized animals was not evaluated for biosafety purposes. Previous experiments were conducted following basic concepts in biological safety and national codes of practice for the safe handling of pathogenic microorganisms in laboratories.

It is important to highlight that the testing methods used in this study could not avoid the use of animals. Hence, following the 3R principles, we designed the minimum experiments to test our hypothesis. By using the minimum animals as possible, it is considered that the results obtained herein provide enough evidence to affirm that recombinant foliar extracts containing the BVDV tE2 glycoprotein are safe to be applied in animals after 4 dpi, thus providing a new tool to deliver antigens for vaccination without protein purification.

Acknowledgements

The authors would like to thank Dr. Moises Burachik for his useful contribution.

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