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Kinetic of Expression of a Plantibody in (*Nicotiana tabacum*) Plants Cultivated in Different Substrates (Zeolite and Soil)

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Abstract: This study reviewed the biomass production and expression of a plantibody in *Nicotiana tabacum* plants cultivated in zeolite, zeolite+soil and soil under confined conditions with the hope that results in the zeolite variant can be comparable with results in soil (best case). Production of leaves ($168.4-228.8 \text{ g plants}^{-1}$) and stems ($148.0-190.4 \text{ g plants}^{-1}$) achieved the highest values at the 8th week of cultivation. Non-significant differences were detected among substrates for production of leaves ($p = 0.0920$); meanwhile production of stems showed statistical significances between zeolite ($148.0 \text{ g plants}^{-1}$) and the mixture of zeolite and soil ($190.4 \text{ g plant}^{-1}$, $p = 0.0422$). The highest plantibody concentration in leaves ranged $56.7-71.5 \mu\text{g mL}^{-1}$ showing significant differences between zeolite and soil ($p = 0.0219$). The expression pattern of the plantibody was similar in all substrates reaching maximum values of plantibody amount (mg m^{-2}) at 8th week. The expression level of plantibody ranged 0.19 ± 0.05 (soil)- $0.13 \pm 0.02\%$ (zeolite). The optimal moment for purifying plantibody was at the 7th week when zeolite was used as substrates. Results of purification experiments performed to corroborate that plantibody produced by plants cultivated in zeolite was functional allow confirming a value of plantibody recovery higher than 40%, high level of plantibody purity and non-statistical differences in the antibody affinity constant. As concise outline, results obtained from this study provide information to conclude that although zeolite experiment results were lower than those observed in the mixture of zeolite and soil and soil, zeolite can be used as substrate for cultivation of transgenic tobacco plants employed in plantibody large-scale production under assessed conditions.

Key words: Hepatitis B surface antigen, plantibody, transgenic plants, zeolite

INTRODUCTION

Tobacco plant cultivation is a common practice in conventional agriculture. However the use of this plant specie for large-scale production of molecules with a high biological value such as antibodies is a relatively novel activity (Hiatt *et al.*, 1989; Larrick and Thomas, 2001). Two important discoveries enable plant scientists to develop new procedures for crop improvements. The discovery of the natural gene transfer mechanism used by the *Agrobacterium tumefaciens* to introduce genetic material

into the plant genome (Van Larebeke *et al.*, 1974; Zambryski *et al.*, 1983) and the capacity to regenerate a whole plant from a single cell without changing cell genetic features (Birch, 1997). Both discoveries enable several laboratories generating transgenic plants with a stable expression of foreign genes (Bevan *et al.*, 1983; Lindbo, 2007).

Transgenic plants have several potential advantages for the production of recombinant proteins compared with microbial cells (Billman-Jacobe, 1996), mammalian cells (Hood *et al.*, 2002) and transgenic animals

(Houdebine, 2002). Among these advantages are: production of the raw material on an agricultural scale, reduced capitalization cost relative to fermentation methods and increased biological safety since plants do not serve as host for animal and human pathogens (Ponsteins *et al.*, 1996; Kusnadi *et al.*, 1998; Timko and Cahoon., 1999; Landgridge, 2000; Giddings, 2001).

However, cultivation of plants for producing drug substances could be affected by pests and diseases. Toxins released by microorganisms would contaminate raw materials affecting the quality of the purified protein and consequently the animal and human health. An alternative to mitigate or reduce these risks is using a well characterized, safe and controlled substrate such as zeolites. The problem is that in agronomic terms, this substrate has been used for cultivation of plants but never for large-scale cultivation of transgenic plants that would be employed in recombinant protein production. To address this problem, an *Agrobacterium*-mediated transformed *Nicotiana tabacum* plant to produce a functionally active engineered antibody was employed in this study (Ramirez *et al.*, 2003). This hepatitis B surface antigen (HBsAg)-binding antibody (plantibody PHB-01) recognizes the amino-acid sequence CKTCTT located at the HBsAg "a" determinant (Le Bouvier *et al.*, 1972; Heermann and Gerlich, 1991; Valdes *et al.*, 2003) and then it is used in the hepatitis B vaccine production. According to Ramirez *et al.* (2003) PHB-01 can be detected in seedlings and transgenic plants up to 0.16-0.20% of Leaf Total Soluble Protein (LTSP).

Thus, the aim of this paper was to investigate the biomass production and kinetic of expression of the PHB-01 in *Nicotiana tabacum* plants cultivated in zeolite, a mixture of zeolite and soil (w/w) and soil, to corroborate whether or not zeolite can be used as controlled substrate for transgenic tobacco plant cultivation under confined conditions and for estimating the optimal moment for biomass harvest, extraction and purification of the PHB-01. The subject matter would be interesting and contemporary for researchers that works on the development of transgenic plants employed in the production of recombinant proteins for veterinary and pharmaceutical uses.

MATERIALS AND METHODS

Transgenic plant source: Transgenic tobacco plants expressing the anti-HBsAg antibody (CB.Hep-1) generated by Ramirez *et al.* (2003) were used in this study. In this report, a tandem expression vector strategy to produce a full mouse monoclonal antibody (CB.Hep-1) in

this plant specie was employed. The clone 23 was chosen among different transgenic tobacco plant clones for further experiments and production of the PHB-01, due to its growth characteristics and PHB-01 production level.

Substrates: Substrates assessed were natural granulated zeolite (ϕ 1-3 mm), a mixture of this natural granulated zeolite and soil (w/w) and soil. Zeolites are aluminosilicate members of the family of microporous solids. It has a porous structure that can accommodate a wide variety of cations, such as Na^+ , K^+ , Ca^{2+} , Mg^{2+} and others. These positive ions are rather loosely held and can readily be exchanged for others. Zeolite can also act as water moderators, in which it will adsorb up to 55% of its weight in water and slowly release it under the plant demand. This property can prevent root rot and moderate drought cycles.

Chemical and mineralogical compositions of zeolite used in this study:

The chemical composition of zeolite (Tasajeras, Cuba) employed in this study was as follow (% in weight): SiO_2 (66.62), Al_2O_3 (12.17), Fe_2O_3 (2.08), CaO (3.19), MgO (0.77), Na_2O (1.53), K_2O (1.20), PPI (11.02), for a total of 98.58. The ratio of $\text{SiO}_2/\text{Al}_2\text{O}_3$ (mol) was 9.29. The total cationic exchange capacity expressed as CICT meq/100 g was 138.69 and for each element was Ca^{2+} 94.48, Mg^{2+} 4.13, Na^{2+} 32.49 and for K^+ 7.59. The mineralogical composition of zeolite was Clinoptilolite (49%), Mordenite (12%), Montmorillonite, $(\text{Na,Ca})_0.33(\text{Al,Mg})_2\text{Si}_4\text{O}_{10}(\text{OH})2\text{nH}_2\text{O}$, (very few); Calcite, CaCO_3 , (very few); Quartz, SiO_2 , (very few); Feldespart, Fe-aluminosilicates, (undetermined). The hardness was 1.08 ± 2.1 and results of chemical resistance (oxidability) was NaCl ($3.13\pm 1.9\%$), HCl ($3.39\pm 0.5\%$), NaOH ($2.50\pm 1.3\%$), H_2O ($2.38\pm 1.2\%$), Cl_2 ($1.58\pm 2.19\%$). The characteristics of main granular materials were (Mean \pm CV): particle density (2120.0 ± 1.45 kg cm^{-3}), apparent density (1021.0 ± 1.76 kg cm^{-3}), porosity (0.51 ± 0.4) and shape factor (0.64 ± 1.97) (Marquez *et al.*, 2000).

Generation of seedlings: *Nicotiana tabacum* L., seeds were cultivated in a mixture of organic substrate, rice, earthworm humus and 2% of natural granulated zeolite (ϕ 1-3 mm) using the floating tray method. Seedlings were fertilized with 0.5 g L^{-1} of the Ultrasol 18-6-18 NPK-fertilizer solution (SQM, Mexico) at days 7th and 21st, respectively and pruned at 25th day after seedtime. Subsequently, pruning was repeated every 48 h up to seedling transplant into the green-containment for producing vegetal biomass (leaves and stems) and PHB-01.

Transgenic plant cultivation and maceration of leaves:

Tobacco plants were grown in a green house under controlled conditions at the Center for Genetic Engineering and Biotechnology in each substrate. Plants were fertilized, dropping four times per day during 4 min, with the Polyfeet 12-43-12 NPK-fertilizer solution (Haifa-Chemical, Israel) and the Polyfeet 19-19-19 NPK-fertilizer solution (Haifa-Chemical, Israel). For biomass production calculation; the weight of fresh leaves and stems were measured using ten plants per each substrate. Samples of leaves, 15 plants (five plants in triplicate), were randomly selected for maceration. Briefly, 5 g of each group of leaves were macerated using 3 mL of 150 mM PBS/0.56 mM ascorbic acid pH 5.5.

Experimental design: A random block design was employed for comparing biomass, PHB-01 and LTSP among assessed substrates. In this design, each block was divided in the same number of treatments (three, zeolite, mixture of the zeolite and soil (w/w) and soil). Plant densities used was always 20 plants per m² in a whole cultivable area of 19.2 m² per each substrate. This number of experiments and size of treatment areas were selected for avoiding the variability in the fertilization between both block extremes (Fig. 1). The number of plants manually harvest for measuring the biomass production (leaves and stems) were 30 (10 in triplicate) every week per substrate, meanwhile for measuring the PHB-01 and LTSP concentration were 15 plants (5 in triplicate) every week per substrate.

Plantibody purification: The PHB-01 purification was performed as collateral experiment for corroborating the influence of the zeolite on plantibody production. The

Zeolite	Zeolite and soil (w/w)	Soil
Soil	Zeolite	Zeolite and soil (w/w)
Zeolite and soil (w/w)	Soil	Zeolite

Area: 19.2 m²
 Plant density: 15p m⁻²
 Sampling frequency:
 plant (10 in tripeate)/weel/substrate for measuring
 the biomass production 15 plants (10 in triplicate)/week/substrate
 for determining the PHB-01 and total protein concentration

Fig. 1: Random block experimental design employed for studying the biomass and PHB-01 production in the zeolite, mixture of the zeolite and soil (w/w) and soil

extraction buffer 150 mM PBS/0.56 M Ascorbic acid, in a ratio of 400 mL per every kg of leaves, was used to extract proteins. This material was processed using a blender 990-220 (Hamilton Beach Commercial, Washington, NC, USA) and separated by centrifugation at 1,051.38×g in HIMAG centrifuge SCR7B (Hitachi, Tokyo, Japan). Tobacco extract of plants cultivated in zeolite were applied to a Streamline 25 column containing recombinant protein A Streamline adsorbent (Amersham-Biosciences, Uppsala, Sweden). Adsorption and elution buffers used were 150 mM PBS; conductivity 12 mS cm⁻¹; pH 8 (300 cm h⁻¹) and 100 mM citric acid (100 cm h⁻¹) (Merck, Darmstadt, Germany); pH 3, respectively. Elution fractions were neutralized with 2 M Tris-HCl (Merck, Darmstadt, Germany). This purification by affinity chromatography was done in triplicate.

Plantibody purity measurement by SDS-PAGE: The purity of the purified PHB-01 was analyzed by gel electrophoresis on a 12.5% (w/v) SDS-PAGE (Laemmli, 1970) followed by Coomassie staining. In all cases, 20 µg of protein were applied.

Estimation of PHB-01 concentration by Enzyme-Linked Immunosorbent Assay (ELISA): A polystyrene (PE) microplate (Costar, Cambridge, USA) was coated with 10 µg well⁻¹ of HBsAg in 0.1 M NaHCO₃ buffer for 20 min at 50°C. After this step, samples were added to the plate in 150 mM phosphate buffered saline solution (PBS)/0.05% Tween 20 buffer and incubated for 1 h at 37°C. After several washings with 150 mM PBS/0.05% Tween 20 buffer, the plate was incubated for 1 h at 37°C with a horseradish peroxidase conjugate (Sigma Chemical Co., St. Louis, USA). Reaction was then revealed using 100 µL well⁻¹ of 0.05% O-phenylendiamine and 0.015% H₂O₂ in citrate buffer, pH 5.0 and stopped with 50 µL well⁻¹ of 1.25 M H₂SO₄. The absorbance was measured in a Multiskan ELISA reader (Labsystems, Helsinki, Finland) using a 492 nm filter (Leyva *et al.*, 2007).

Determination of total proteins: Protein concentration was determined following the method described by Lowry *et al.* (1951) using bovine serum albumin as standard material. The curve range was from 10 to 100 µg mL⁻¹ and the absorbance of samples was measured at 730 nm in a UV/Visible Ultrospec 2000 Spectrophotometer (Pharmacia Biotech, Cambridge, England).

Affinity constant determination: The affinity constant was determined by the method described by Beatty *et al.* (1987). Microtiter plates were coated with the

rec-HBsAg and incubated with PHB-01. Plates were sequentially incubated with a horseradish peroxidase-antibody conjugate and the reaction was revealed using OPD as substrate and 0.015% H₂O₂ in citrate buffer; pH 5.0. Reaction was stopped by adding 50 mL of 2 M H₂SO₄. The amount of antibodies adherent to the antigen on the plates was reflected by the enzyme product measured by optical density at 492 nm using an ELISA reader (Labsystem, Helsinki, Finland).

Statistical analysis: ANOVA tests were carried out using the Statgraphic program (version 5.0) and p-values less than 0.05 were considered to be of statistical significance. The Tukey's multiple range test was used for determining experimental variants with statistical differences.

RESULTS AND DISCUSSION

Cultivation of modified genetically crops have been created to increase productivity of cultivations by mean of the resistance to plagues, illnesses, herbicides, droughts and high salinity; to increase the quality of products improving their nutritional content or retarding the maturation of fruits, but also for the production of medications such as therapeutic antibodies (Stoger *et al.*, 2005).

Currently, there are three techniques that allow obtaining transgenic plants: protoplast transformation, particle bombardment and transformation by mean of the *Agrobacterium tumefaciens* (Morikawa *et al.*, 1994; De la Riva *et al.*, 1998). The technique used to express the PHB-01 was the transformation with the *Agrobacterium tumefaciens* (Ramirez *et al.*, 2003). This agrobacterium is a gram-negative rod-shaped bacteria closely related to nitrogen-fixing bacteria, which resides at root nodules in legumes (Van Larebeke *et al.*, 1974; Zambryski *et al.*, 1983; Birch, 1997). The useful use of the *Agrobacterium tumefaciens* as a tool in plant genetic engineering is due to it takes advantages of its host by injecting DNA derived from the Ti (tumor inducing) plasmid into the host, causing plant creating galls, which excrete opines that bacteria use as an energy source (Zambryski *et al.*, 1983). Therefore, results of this study would be of interest for researchers, who used this specific transformation method. In such sense, it is important to point out that the huge majority of transgenic plants transformed to produce antibodies in the world have been transformed by the *Agrobacterium tumefaciens* method (De Muyneck *et al.*, 2010).

In parallel, other studies have demonstrated a list of products released by microorganisms that would contaminate raw materials and final products and as

consequence the animal and human health. For instance, bacteria, yeasts and moulds are highly efficient and would be a serious problem for manufacturers when soil is used. There are several factors that enable these microorganisms to colonize a wide range of plants and products: some species can grow over a wide pH range, enabling them to survive in very acidic environments and can tolerate relative extremes temperatures (0-47°C). Many species are also xerotolerant (able to grow in environments with very low water activity), while other are osmophilic and halophilic, which are able to grow in environments with high osmotic pressure due to the presence of sugar or salt, respectively. Taking as consideration this information, authors investigate in this study the biomass production and expression of the PHB-01 in *Nicotiana tabacum* cultivated in zeolite for corroborating whether or not zeolite can be used as well characterized and controlled substrate for the transgenic tobacco plant cultivation under confined conditions as an alternative to mitigate risks of contamination with microorganisms and uncontrolled level of nutrients in soil (Campbell and Davies, 1997).

The reason why zeolite was choose as substrate for this study was because it is a mineral of the aluminum-silicates moisturized group, composed by aluminum, silica, hydrogen and oxygen. It is composed of pores and corner-sharing aluminosilicate tetrahedrons, joined into 3-dimensional frameworks. The structure of pores is characterized by cages approximately 12Å in diameter, which are interlinked through channels about 8Å in diameter, composed of rings of 12 linked tetrahedrons (Kaduk and Faber, 1995). These channels allow the easy movement of resident ions and molecules into and out of structure. The zeolite has large vacant spaces and the aluminium results in a negative charge, which is balanced by positively charged cations. In outline, investigations developed in several countries have proved the effectiveness of this substrate for the cultivation of short cycle-vegetables (Polat *et al.*, 2004; Giritch *et al.*, 2006) and as an alternative approach, which promising from the standpoint of ecological safety. However, cultivation of plants for producing recombinant proteins on large-scale using the zeolite under confined conditions have been very poorer studied.

As specific results of the study, the highest production of leaves was observed in the soil variant (228.8 g plant⁻¹). This value was obtained at the 8 th week of cultivation (Table 1, Fig. 2). While, the lowest value was identified in zeolite (168.4 g plant⁻¹), nevertheless, this result did not showed statistically differences among these three substrates (p = 0.0920). The production of

Table 1: Summary of the PHB-01 production in *N. tabacum* plants cultivated in the zeolite, mixture of zeolite and soil (w/w) and soil

Parameters	Soil	Zeolite+soil	Zeolite	p-values
Highest production of leaves (g plant ⁻¹)	228.3	211.3	168.4	0.092
Time to reach the highest production of leaves (weeks)	8	8	8	-
Highest production of stems (g plant ⁻¹)	180.8	190.4	148	0.0422
Time to reach the highest production of stems (weeks)	8	8	8	-
Highest PHB-01 concentration in leaves (µg mL ⁻¹)	54.8±8.8	70.88±9.3	56.98±3.7	0.0219
Time to reach the highest PHB-01 concentration in leaves (weeks)	6	7	7	-
Highest LTSP concentration in leaves (mg mL ⁻¹)	39.3±1.8	39.4±2.4	41.9±5.2	0.2853
Time to reach the highest LTSP concentration in leaves (weeks)	7	7	7	-
Highest PHB-01 yield in leaves (µg g ⁻¹)	32.8±8.8	42.5±9.3	34.2±3.7	0.3281
Time to reach the highest PHB-01 yield in leaves (weeks)	6	7	7	-
Highest amount of the PHB-01 in leaves (mg PHB-01 m ⁻²)	96.5	125.6	76.7	-
Time to reach the highest amount of the PHB-01 m ⁻² (weeks)	8	8	8	-

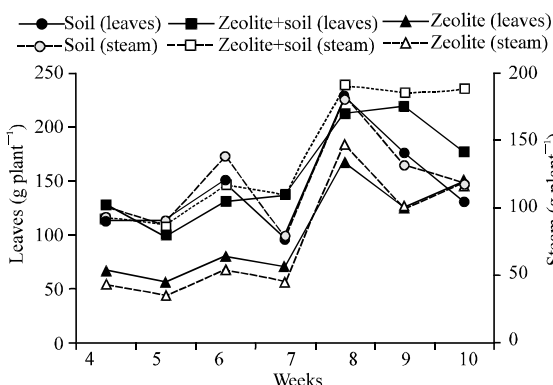


Fig. 2: Production of leaves and stems in tobacco plants cultivated in zeolite, mixture of zeolite and soil (w/w) and soil

leaves in plants cultivated in the soil substrate was slightly above, up to 8-35%, higher than in the mixture of zeolite and soil (w/w) and zeolite, which corroborated that zeolite is able to promote the growth of plants and production of leaves but with lower efficiency than in soil and in the mixture of both substrates (Fig. 2).

The analysis of the production of stems, expressed as gram of stem per plant evidenced that the highest value was observed when the mixture of zeolite and soil (w/w) was used (190.4 g plant⁻¹) (Table 1, Fig. 2). This value was also obtained at the 8th week of cultivation and significant differences were observed between soil and the mixture of both substrates and with zeolite (p = 0.0422). Thus, the use of zeolite has a remarkable influence on the stem production under these conditions of cultivation, confinement, fertilization type and fertilization schedule, used in this study.

The highest concentration of the PHB-01 in leaves was also observed in soil and the mixture of soil and zeolite (w/w). It was 1.25 fold higher than results observed in zeolite, which was statistically corroborated (p = 0.0219). However, the calculated difference was within the coefficient of variation of the ELISA used for estimating the PHB-01 concentration (Leyva *et al.*, 2007).

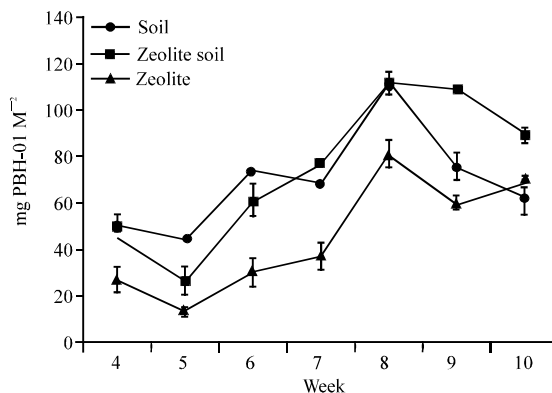


Fig. 3: Amount of PHB-01 in leaves of tobacco plants cultivated in zeolite, mixture of zeolite and soil (w/w) and soil

Considering these results, the best moment for harvesting leaves was at 8th week (Table 1).

The analysis of the kinetic of expression of the PHB-01 showed the same profile in all cases (Fig. 3), showing a PHB-01 expression level almost invariable during the whole experiments in each substrate (Soil (0.19±0.05%), Zeolite+soil (0.17±0.03%) and Zeolite (0.13±0.02%). In summary, both parameters (PHB-01 and LTSP concentration) varied in the same proportions over the time of the experiment. In general, the highest values of PHB-01 yield were observed at 8th week in all substrates (Fig. 3) and statistical differences were observed among the 8th week and the rest of the weeks (p<0.05). The analysis of LTSP production verified that this parameter was statistically similar in all cases (p = 0.2853). Values of LTSP ranged 39.6±0.9-41.9±0.8 mg mL⁻¹ of leaf extract (Table 1).

As a consequence, the yield of the PHB-01 was also compared among assessed substrates. The range of the highest yield was from 34.0±3.5 to 37.6±8.9 µg of PHB-01 g⁻¹ of leaves (Table 1, Fig. 3). The highest amount of the PHB-01 expressed as mg of plantibody per squared meter of substrate shown the lowest value in the zeolite variant (Table 1). In such sense, other experiments have

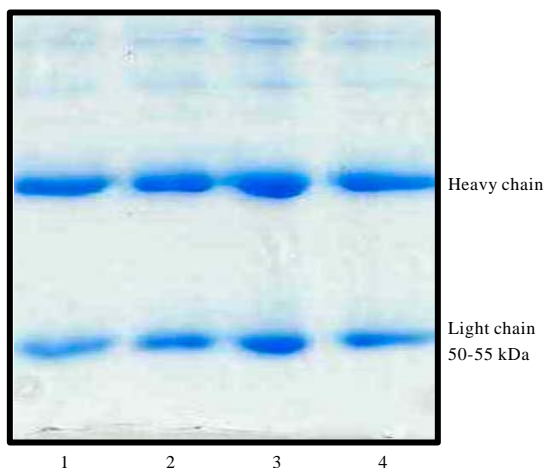


Fig. 4: Coomassie blue stained SDS-PAGE of PHB-01 purified from plants cultivated in zeolite under reducing conditions. Lane 1, PHB-01 purified from plants cultivated in zeolite (first group of plants). Lane 2, PHB-01 purified from plants cultivated in zeolite (second group of plants). Lane 3, PHB-01 purified from plants cultivated in zeolite (third group of plants). Lane 4, PHB-01 previously purified and used as reference material

to be carried out for determining reasons of the relatively lower productivity of plants in zeolite, more generally, for identifying and confirming optimal cultivation conditions. We hypothesize that other fertilizer solutions and fertilization protocols would help for increasing the production of biomass and PHB-01 in zeolite.

To corroborate if PHB-01 could be undoubtedly purified from leaf extracts of plants cultivated in zeolite under assessed conditions, PHB-01 purification was performed by affinity chromatography as main purification step. Results allowed confirming that immunoglobulins purified from plants cultivated in zeolite was not affected by cultivation of plants under conditions assessed in this study. A value of PHB-01 recovery higher than 40% ($40.5 \pm 1.24\%$) was obtained in purification experiments using three groups of plants cultivated in zeolite as well as a high level of purity ($97.5 \pm 1.5\%$) and no differences in the affinity constant ($3.56-9.21 \times 10^8 \text{M}^{-1}$) was also demonstrated (Fig. 4). The affinity constant of the plantibody was compared with the mouse counterpart of the plantibody produce by ascitic fluid ($p = 0.4835$).

CONCLUSIONS

As conclusion, although results observed in zeolite experiments were lower than those observed in the mixture

of zeolite and soil and soil, zeolite can be employed as a well characterized and controlled substrate for large-scale cultivation of transgenic tobacco plants for the production of plantibodies under confinement conditions.

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