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Assessment of a *N. tabacum* L., Variety using Natural Zeolite as Substrate and Confined Conditions for Consistent Biomass, Protein and Plantibody Production

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Abstract: This study describes an assessment of a *N. tabacum* L., variety for the analysis of the consistent production of biomass, Leaf Total Soluble Protein (LTSP) and plantibody. To perform this research, seedlings of genetic modified plants were transplanted and grown in zeolite in a green-containment of 1320 m^2 of cultivable area to be analyzed during a cropping year. Comparison of morpho-agronomic characters of this variety cultivated under these conditions revealed similarity in qualitative characters but differences in plant height and leaf weight with those reported for this variety cultivated soil and open field. A first-round result using a random block design demonstrated that 15 plants m^{-2} was the best assessed density to perform further experiments. The maximum biomass and LTSP production was observed during the spring, demonstrating statistical differences among seasons of the year ($p<0.05$). Biomass yield showed a strong correlation with temperature ($r = 0.918$) and humidity ($r = 0.891$), whereas correlation coefficient for LTSP production also shows strong correlation with temperature ($r = 0.832$) but a weak correlation with humidity ($r = 0.585$). Summarizing, the use of this variety under the assessed conditions would allow a production of 100.77 tons of biomass and 3.91 tons of LTSP per cultivable hectare each year. The plantibody expression corroborated 0.10±0.09% of expression level and a recombinant protein productive potential of 391.5 kg ha^{-1} in a whole cropping year. Thus, these results tested the hypothesis that this variety allows a consistent production of biomass and plantibody in the assessed conditions.

Key words: *Nicotiana tabacum* L., plantibody, transgenic plants, tobacco biomass production, zeolite

INTRODUCTION

Plant molecular farming would offers advantages over well established technologies for recombinant protein production (Joseph and Jini, 2011; Wei *et al*., 2006; Behrooz *et al*., 2008a, b; Srivastava *et al*., 2011). The use of transgenic plants as bioreactors seems to be more economic and has low risk of contamination by mammalian viruses, oncogenes and bacterial toxins (Herbers and Sonnewald, 1999; Crosby, 2003; Menkhaus *et al*., 2004; Abranches *et al*., 2005).

In that sense, there are several factors than should be considered in regard to the efficiency of this relatively new technology. For instance, some plants accumulate greater protein quantities than others. For instance, soybeans contain 40% of their weight as protein compared to 2% for potato tubers. Thus, if a given protein is expressed at an equal percentage of total protein in these two crops, more products will be produced by soybeans for same weight of each (Delaney, 2003). Regarding inside plant protein stability, there are several proteins that have been stable after a long-term storage. In general, relatively seed desiccated environments are preferred over higher moisture tissues due to a lower proteolytic activity (Fieldler and Conrad, 1995; Bai and Nikolov, 2001; Stoger *et al*., 2002). Technological issues such as easy genetic transformation would also contribute to crop selection and to advantages of this system (Sharma *et al*., 2001; Malabadi and Nataraja, 2007).

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However, there are several areas demanding attention as well. (1) Quality substrate components. Plant cultivation in natural soil yield higher biomass but a satisfactory assurance of batch quality uniformity is difficult to establish, open field cultivation may also elicit pharmacological effects, if transgenic product is ingested, for humans, disruption of soil microbes and soil erosion. (2) Environmental regulations in relation to genetically modified pollen spread (Dale *et al*., 2002; Snow, 2002). (3) Complexity of proteins, carbohydrates, phenols and alkaloids mixture that should be separated from transgenic products (Menkhaus *et al*., 2004).

In spite of demanding areas, tobacco plant has been widely employed as transgenic plant because it is easily transformed (Cramer *et al*., 1996; Cramer *et al*., 1999). Although, a comprehensive study about its potentiality for biomass and protein production using natural granulated zeolite as substrate under confined agriculture conditions and is lacking in the literature.

In this study, an assessment of a *N. tabacum* L., variety productive potential (Ramirez *et al*., 2003; Gomez *et al*., 2010) was assessed using natural granulated zeolite as substrate in a green-containment for demonstrating the consistency of biomass, leaf total soluble protein and recombinant protein production. This tobacco variety was selected for this research because it had shown resistance to *Peronospora tabacina*, *Phytophthora nicotianae*, *Pseudomonas syringae*, *Orobanche ramosa* L. and environmental necrosis; a green leaf biomass yield of 14.6 tons ha^{-1} in natural soil and capacity for cultivation in several regions and regimens.

MATERIALS AND METHODS

Duration of the study and transgenic plants source: The assessment of the consistent biomass, protein and plantibody production was performed during two cropping years. The amplified DNA of the antibody Heavy Chain (HC) and Light Chain (LC) were purified, digested and ligated into the plant expression vector pHES74 which contains the signal sequence of the sweet potato (*Ipomoea batatas*) sporamin storage protein. The HC and LC expression cassettes from the pHES74 were sequentially cloned into the binary vector pDE1001 and the resulting plasmid was used to transform *N. tabacum* by *Agrobacterium tumefaciens*-mediated gene transfer procedure (Ramirez *et al*., 2003; Gomez *et al*., 2010).

Seedling production: The *Nicotiana tabacum* L., variety 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 18-6-18 NPK-fertilizer solution (Ultrasol, Mexico) at days 7th and 21st, respectively and pruned at day 25th after seedtime. After this time (day 25th), the prune was repeated every 48 h up to seedling transplant (about 10 cm height) into the green-containment for pilot-scale biomass production.

Plant density study: A random block design (Snedecor and Cochran, 1956) was employed for determining the best plant density. In this design, each block was divided in the same number of treatments. Plant densities studied were 15, 20, 25 and 28 plants m^{-2}, respectively in a treatment area of 2 m^2; therefore, each plant density was studied 4 times. This number of experiments, treatment areas and experimental design were selected because of the fertilization variability between both block extremes could be high.

Green-containment culture conditions: Ten centimeter high seedlings were transplanted and grown in a green-containment of 1320 m^2 of cultivable area (Azrom, Israel) under controlled conditions and using natural granulated zeolite (ϕ 1-3 mm) as substrate. Seedlings were manually transplanted at a rate 15 plants m^{-2} and fertilized dropping 66.6 g L^{-1} of 18-18-18 NPK-fertilizer solution (Meristem, Spain) at a flow rate of 5 L min^{-1} during 4 min, 3 times a day. Then, leaves were manually harvested at the age of 6-7 weeks after transplanting to be selected, weighed and macerated.

Leaf processing for protein determination: A randomized harvest of 47 plants per each cultivable section of 168 m^2 was employed for leaf processing. This number of plant represented 1.8% of plants cultivated in this section (2520 plants). Subsequently, all central leaves were harvested and grouped to be cut in portions of 5 g, wetted and macerated by dropping extraction buffer (150 mM Phosphate-Buffered Saline (PBS)/0.56 M ascorbic acid; pH 8) at room temperature. The extraction buffer in a ratio of 0.4 mL g^{-1} of leaf was used to extract proteins and solid green particles were then removed by centrifugation at 1200x g for 1 min in a Universal 16 centrifuge (Hettich Zentrifugen, Tittingen, Germany).

Protein determination: Total protein concentration was determined by the method described by Lowry *et al*. (1951), using bovine serum albumin as standard material. The calibration curve ranged 100-500 μg mL^{-1}.

Plantibody determination: A polystyrene microplate (Costar, Cambridge, USA) was coated with 10 μg $well^{-1}$ of the hepatitis B surface antigen in 100 mM $NaHCO_3$; pH 9.6

buffer for 20 min at 50°C. After this step, samples were added to the plate in 150 mM PBS; pH 7.2/0.05% Tween 20 and incubated for 1 h at 37°C. Several washes with 150 mM PBS; pH 7.2/0.05% Tween 20 were done. The plate was incubated for 1 h at 37°C with a horseradish peroxidase conjugate (Sigma Chemical, St. Louis, USA). The reaction was revealed using 100 μL $well^{-1}$ of 0.05% O-phenylenediamine and 0.015% H_2O_2 in citrate buffer; pH 5 and stopped with 50 μL $well^{-1}$ of 125 mM H_2SO_4. Absorbance was measured in a Multiskan ELISA Reader (Labsystems, Helsinki, Finland) using a 492 nm filter (Leyva *et al*., 2007). Calibration curve ranged 3.12-50 ng mL^{-1}. The mAb standard used was the IgG2B-070305 supplied by the Cuban Center for Genetic Engineering and Biotechnology.

Statistical analysis: The plant density analysis was done by calculating the coefficient of variation of each treatment and the variance analysis by a multi factor-ANOVA among treatments at 95% confidence level. Biomass yield and LTSP (leaf total soluble protein) production were evaluated by the, non-parametric ANOVA, Kruskal-Wallis test (Kruskal and Wallis, 1952) and a simple factor-ANOVA test, respectively. The significance level (α) was 0.05 for both tests. The Kruskal-Wallis test was used for comparing results that do not followed a normal distribution according to Kolmogorov-Smirnov's test (Stephens, 1974) and do not show variance homogeneity according to the Cochran C test. The relation between temperature and humidity with biomass yield and LTSP production was analyzed by the correlation coefficient (r). The plantibody expression level was statistically compared using a simple factor-ANOVA test and Duncan's test (multiple range test). The significance level (α) employed was also 0.05. The computer program used for the statistical analyses was the STATGRAPHIC PLUS, 5.00, 1994-2000.

RESULTS AND DISCUSSION

The inconvenient of the production of recombinant proteins in microorganisms and mammalian cells might be overcome by mean of transgenic crop production because of the large-biomass production capacity, enormous accumulated experience cultivating plants and low risk of contamination with human pathogenic viruses (Kusnadi *et al*., 1997; Srivastava *et al*., 2011). In that sense, tobacco plant is used as model for the easy genetic transformation of plants with human genes (Tso, 2006). However, researchers could discard this alternative for large-scale production of proteins with pharmaceutical purpose due to low level of expression of the target protein and difficults to isolate it with high purity and recovery. Obviously, this perception will disappear shortly after robust purification methods can be developed which in combination with the high biomass yields could stimuli the use of these plants for large-scale protein production.

Another key point is the discussion on contention barriers and source of contaminations, the considerable public debate on the fact that pollen carried on the wind could pollinate wild varieties of the modified plant, producing hybrids with unknown characteristics (Smyth *et al*., 2002), the loss of biodiversity and soil degradation (Barton and Dracup, 2000) and potential contamination of plants with fluids and excrements of animals infected with pathogenic viruses would makes necessary the use of controlled substrates and green-containments to cultivate transgenic plants. In general, it has been concluded that process validation under GMP is more feasible under greenhouse conditions and thus greenhouse cultivation should therefore be recommended for the production of vaccines from plants. Besides, many agricultural procedures currently applied to greenhouse cultivation are very convenient for the establishment of standard operation procedures for biomass production in plant expression systems. Irrigation and fertilization are finely controlled by programmable machines that deliver water and nutrients through customized schedules to sustain plant needs. Not only year round cultivation is possible in greenhouses but, besides, the lower dependence from weather conditions would assist to both having a more consistent production of biomass and a more reproducible content of the target recombinant protein.

Nevertheless, the use of greenhouses for large scale production of plant-made pharmaceuticals have not been widely considered by industry because it would result in capital investments that otherwise are mostly avoided in open cultivation. It should be observed, however, that a cost-benefit analysis will probably reveal that, in return of modest investments that are currently estimated in up to 20 USD per square meter range for standard greenhouses, biomass production there would bring a lot of advantages over open field cultivation (Knablein, 2003; Pujol *et al*., 2007).

In regard to substrate, zeolites are materials featuring three-dimensional crystalline structures, uniform pore dimensions and internal cavities of regular size and shape. Several reports have described that natural zeolite have many useful properties, such as a high cation exchange capacity, absorption and emission of water, ammonium absorption and inhibiting of nitrate leaching (Lippmaa *et al*., 1981). This mineral has intensively been

Fig. 1: Plantation of the *N. tabacum* L., variety cultivated in natural soil

used for plant cultivation (Chen and Gabelman, 1990; Pushkina *et al*., 1996) nevertheless there is not report on large-scale application of natural granulated zeolite for tobacco cultivation in the literature.

Considering this information, this article discusses about the assessment of a *N. tabacum* L., variety for the consistent production of vegetal biomass and proteins using natural granulated zeolite under confined conditions.

Summary of the morpho-agronomic characterization of plants: In order to know the impact of the application of the natural granulated zeolite (ϕ 1-3 mm) and confined agriculture conditions on Morpho-Agronomic Characters (MAC), biomass and protein yield of a particular *Nicotiana tabacum* L., variety, a two year-round cropping study was performed under these conditions. The biomass yield in natural soil (16.4 tons (green leaf) ha^{-1}) and the capacity for cultivation under several regions and regimes were some of the main criteria considered for the selection of the *N. tabacum* L., variety used in this to study. Qualitative and quantitative MAC of the *N. tabacum* L., variety cultivated in natural granulated zeolite were compared with those reported for the cultivation of this variety using natural soil in open field. Pictures of both kinds of cultivations are illustrated in Fig. 1 and 2. In general, this variety showed same qualitative MAC in both conditions. Differences were only observed in the plant height and leaf weight (quantitative MAC). The reported plant height and leaf weight values for plants cultivated in natural soil and open field were 2.03 and 2.20 fold higher to that seen in natural granulated zeolite and confined conditions, respectively (Table 1). In our opinion, differences found in these two quantitative MAC (plant height and leaf weight) in regard to these parameters measured in the same plants cultivated in natural soil and open field were mainly consequences of the plant density and confinement conditions used and not of the substrate employed.

Table 1: Qualitative and quantitative MAC of the *N. tabacum* L., variety cultivated in natural soil and natural granulated zeolite

Qualitative and quantitative MAC	Natural soil and open field	Natural zeolite and green-containment
Plant habit	Ellipsoidal	Idem
Leaf surface	Middle undulated	Idem
Leaf shape	Long ovulate	Idem
Leaf color	green	Idem
Leaf angle (°)	58.5	42.11±12.89
Central leaf longitude (cm)	51.2	43.06±7.66
Central leaf width (cm)	27.5	26.69±5.19
Leaf weight (g $plant^{-1}$)	328.4	161.0±37.08
Central leaf longitude-width ratio (fold)	1.86	1.61
Stem diameter (cm)	1.8	1.70±0.17
Plant height (cm)	281.4	127.4±19.72
Plant diameter (cm)	59.3	52.9±9.34
Nnmber of useful leaves	17.8	12.07±1.67
Between nudes distance	7.9	10.40±1.93
Average yield of leaves (tons ha^{-1})	16.4	24.9
Average yield of stems (tons ha^{-1})	Non-determined	12.9

Plant density study: Cultivation of this *N. tabacum* L., variety using natural soil in open field is usually carried out at 5- 6 plants m^{-2} but this parameter must be optimized in correspondence with the subject of the research and cultivation method. One of the main subjects of the employment of tobacco transgenic plants for recombinant protein production is its capacity to generate a high biomass yield with a high content of proteins. Thus, the optimization of the plant density becomes a critical parameter to be assessed.

A preliminary experiment for estimating the best plant density was done for determining the minimum number of plants yielding same biomass production per cultivable area. It was performed for reducing the size of the seed bank and seedling generation stage. The multi factor-ANOVA tested whether there was significant difference among means of biomass yield between and within the studied groups of plant densities (15, 20, 25, 28 plants m^{-2}) and the contribution of each factor (random block design and density). Results demonstrated a biomass yield ranged 14.35 - 18.10 (leaf) and 9.56-10.88 (stem) kg per each plant density block. Since no p-values were no less than 0.05 (Leaf (block $p = 0.145$; density $p = 0.466$); Stem (block $p = 0.102$; density $p = 0.825$) there were no significant difference among means of biomass yield of four assessed groups of plant densities (Table 2). Plants cultivated at 15 plants m^{-2} showed more robust stems than plants cultivated at higher plant densities.

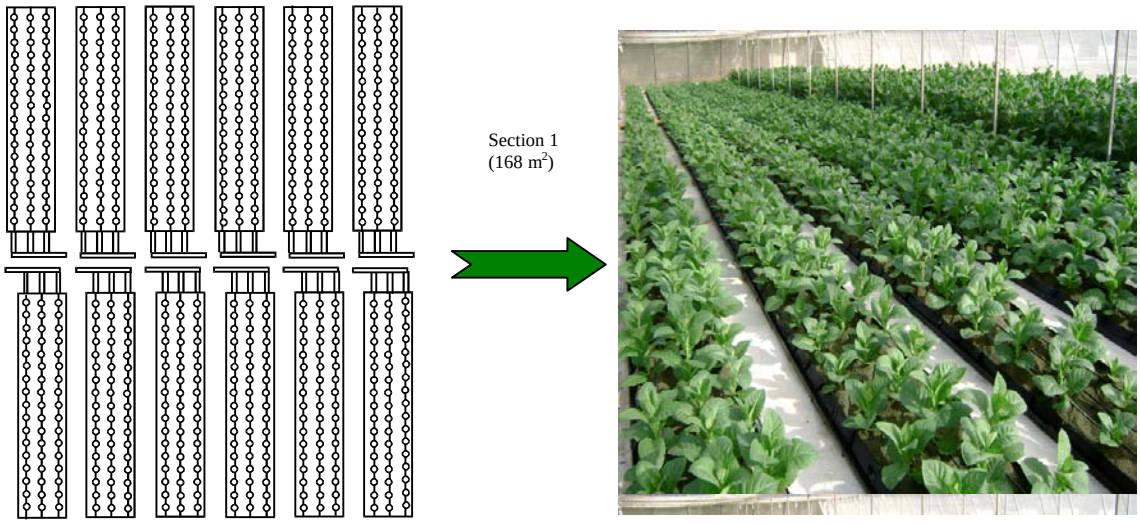

Fig. 2: Clean-green containment for the *N. tabacum* L., variety biomass and protein production using natural granulated zeolite as inert substrate

Table 2: Results of the *N. tabacum* L., variety plant density study in natural granulated zeolite using a random block design

Part of the plant	Block (4×2 m^2)	15 p m^{-2}	20 p m^{-2}	25 p m^{-2}	28 p m^{-2}	Total	Average
Leaf	Block I (kg)	3.80	3.70	3.45	3.35	14.30	3.58
	Block II (kg)	4.45	2.35	3.75	3.10	13.65	3.41
	Block III (kg)	4.60	5.05	5.65	3.30	18.16	4.65
	Block IV (kg)	3.25	4.55	5.25	4.61	17.65	4.41
	Total (kg)	16.10	15.65	18.10	14.35		
	Average/Block (kg)	4.03	3.91	4.53	3.59	-	-
	SD	0.62	1.18	1.09	0.68	-	-
	Coefficient of Variation	15.47	30.19	24.03	19.05	-	-
	Biomass per plant (g plant^{-1})	134.17	97.81	90.50	64.06	-	-
	Number of plants	120	160	200	224	-	-
Source	Sum of squares	Df	Mean square	F-ratio	p-value	Total	Average
A: Blocks	4.47927	3	1.49309	2.30	0.1458	-	-
B: Density	1.80527	3	0.60175	0.93	0.4663	-	-
Residual	5.83981	9					
Total (corrected)	12.1243	15					
Part of the plant	Block (4×2 m^2)	15 p m^{-2}	20 p m^{-2}	25 p m^{-2}	28 p m^{-2}	Total	Average
Stem	Block I (kg)	2.46	2.66	1.96	2.10	9.18	2.30
	Block II (kg)	2.80	1.56	2.50	1.30	9.16	2.04
	Block III (kg)	2.46	1.70	2.76	3.10	10.02	2.51
	Block IV (kg)	2.60	3.56	3.66	3.06	12.82	3.11
	Total (kg)	10.32	9.48	10.88	9.56		
	Average/Block (kg)	2.58	2.37	2.72	2.39	-	-
	SD	0.16	0.93	0.71	0.86	-	-
	Coefficient of Variation (%)	6.23	39.32	26.09	36.04	-	-
	Biomass per plant (g plant^{-1})	86.00	59.25	54.40	42.68	-	-
	Number of plants	120	160	200	224	-	-
Source	Sum of squares	Df	Mean square	F-ratio	p-value	Total	Average
A: Blocks	3.0846	3	1.0282	2.78	0.10270	-	-
B: Density	0.3316	3	0.11053	0.30	0.82590	-	-
Residual	3.3346	9					
Total (corrected)	6.7508	15					

Differences found in the leaf and stem weight per plant could be explained by a better access to nutrients, water and day light. Interestingly, the plant density of 15 plants m^{-2} allowed a biomass (leaf) yield (24.9 tons ha^{-1}) higher that described in natural soil and open field (14.6 tons ha^{-1}) at 5 plants m^{-2}.

Once the plant density of 15 plants m^{-2} was defined for following experiments; the progression of the plant height, leaf longitude and leaf width was examined. The progression of these parameters in the *N. tabacum* L., variety cultivated in natural granulated zeolite under confined conditions over the time is illustrated in Fig. 3.

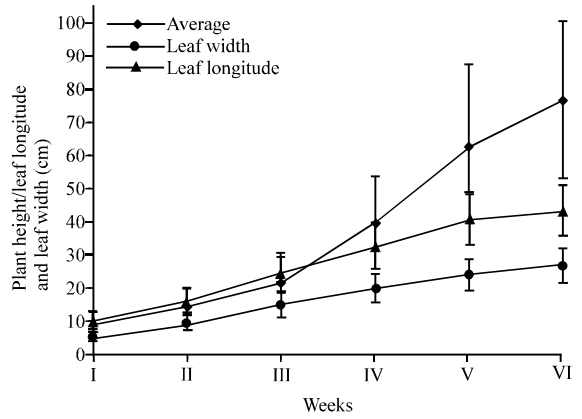

Fig. 3: Height of the plants; and leaf longitude and width progression of the *N. tabacum* L., variety over the time cultivated in natural granulated zeolite and confined conditions. These curves represent the average and the standard deviation of each parameter

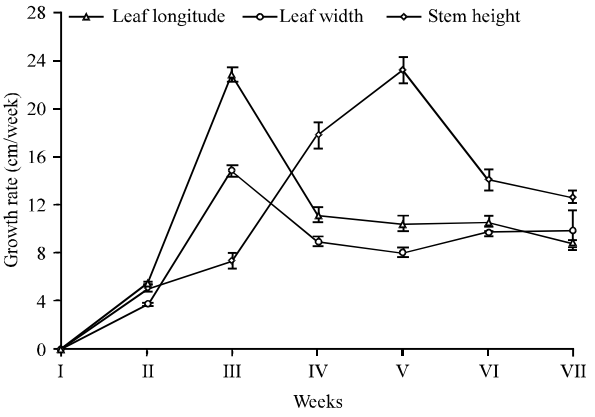

Fig. 4: Plant growth-rate, the number of plants measured was 893 distributed (a random) in 47 plants per section. These curves represent the average and the confidence level of the stem height, leaf longitude and width

The plant height progression clearly demonstrated a biphasic curve where no considerable changes were observed in the first two weeks, up to 6.1 cm week^{-1}, followed by a linear progression up to the 6th week. The average increment observed in this linear phase, from 3rd to 6th week, was up to 18.6 cm week^{-1} allowing an average maximum height of 76.6±24.15 cm (flower decapitated) and describing the progression equation y = 14.25x - 12.63.

The central leaf longitude and width showed a lower increment than the height increment of the plant. Mathematic equations that describe these parameters were y = 7.07x+2.93 and y = 4.42x+1.16, respectively. The progression of the leaf longitude was estimated as an increment of 7.17 cm week^{-1}, whereas just 4.44 cm week^{-1} was measured for the leaf width. In summary, the maximum longitude and width of the central leaf corresponded with 56.20% and 34.84% of the maximum height of the plant, respectively (Fig. 3).

The profile of the plant growth-rate was also studied in this work. Figure 4 illustrates the average of the plant height, leaf longitude and leaf width growth-rate profiles. The profile of these parameters showed a rapid growth-rate in the first three-five weeks, followed by a rapid decrease. The maximum height growth-rate was observed at 5th week, while the maximum leaf longitude and width growth-rate values were observed at the 3rd week. Finally, the growth-rate decreased up to 51.23% soon after the time needed to achieve the maximum growth-rate for each parameter.

Biomass and LTSP production by the *N. tabacum* variety: Tobacco plant growth and biomass yield are highly dependent on the climate. In general, sunlight, rainfall, relative air humidity and temperature are considered as the most important factors (Ferguson and Burke, 1991; Schrader *et al*., 2004). These climatic factors usually affect relevant parameters of plants such as nitrogen uptake and photosynthesis. Because tobacco plant is a C_3 plant species (Charles-Edwards and Ludwing, 1974), it is therefore, very sensitive to high temperatures. Photosynthesis of C_3 plants is maximized when the temperature is 15-25°C and the net carbon fixation rate is considerably affected by the photorespiration process. Thus, under high temperature conditions there is a marked reduction of the carbohydrates supplying carbon skeletons for other synthesis.

In this assessment, the net biomass yield ranged 140.5±23.4-191.6±35.4 g plant^{-1}, showing significant differences among seasons of the year (p = 0.00005). The maximum biomass yield was obtained during the spring season, 191.6±35.4 g plant^{-1} and this parameter indicated a moderate strong correlation with the inside green-containment temperature (r = 0.918) and humidity (r = 0.891). The equation of fitted linear model was biomass = -35.96+6.21x temperature (Table 3).

The average value of LTSP production ranged 26.6±12.5-92.0±58 g kg^{-1} of leaves at 6th week after transplant in a season dependent way, showing significant differences among seasons of the year as well (p = 0.00045). The highest value was also measured in the spring while no statistical differences were detected among summer, winter and autumn (p = 0.1140). The LTSP

Table 3: *N. tabacum* L., variety biomass and LTSP production. These plants were cultivated at 15 plants m^{-2} and harvested 6 weeks after the transplant. The statistical test was carried out at 95% confidence level and n = 18 per each season. Values of temperature and humidity were always measured inside the green-contaiument three times a day. R is the correlation coefficient

Seasons	Temperature Min-Max/Mean (°C)	Humidity Min-Max/Mean (%)	Average biomass yield (g $plant^{-1}$)	Average LTSP production (g kg^{-1} of biomass)
Winter	16-37/27.4	4-95/28.8	140.5±23.4	28.5±12.1
Spring	23-42/34.9	17-99/55.9	191.6±35.4	92.0±58.0
Summer	32-41/33.4	39-99/70.0	172.3±38.9	30.6±8.1
Autumn	20-38/32.2	17-99/63.0	145.7±17.3	26.6±12.5
Whole year	16-42/24.6	4-99/41.9	161.0±37.8	44.0±40.3
p-values	-	-	0.00005	0.00045
r (temperature)	-	-	0.84360	0.69270
r (humidity)	-	-	0.79536	0.34305

Table 4: Recombinant protein productive capacity by the *N. tabacum* L., variety

Seasons	Biomass yield (tons ha^{-1})	LTSP production (kg ha^{-1})	Considering a HTPEL of 1% (kg ha^{-1})	Considering a HTPEL of 0.1% (kg ha^{-1})	True plantibody expression level (%)	True plantibody production (kg ha^{-1})
Winter	21.07	771.49	0.77	7.71	0.13±0.09	1.00
Spring	28.74	1938.47	1.93	19.38	0.07±0.03	1.35
Summer	25.84	644.74	0.64	6.44	0.10±0.04	0.66
Autumn	25.12	560.46	0.56	5.60	0.15±0.13	0.84
Whole year	100.77	3915.16	3.91	39.15	0.10±0.09	3.91

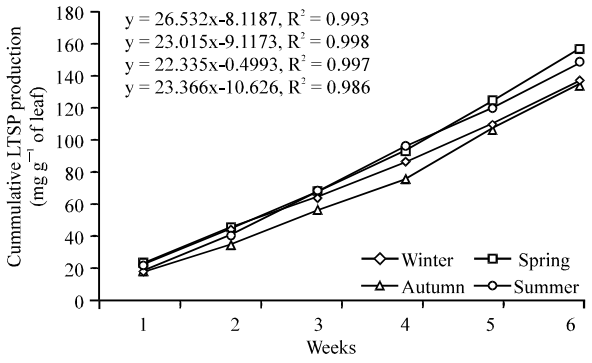

Fig. 5: Cumulative LTSP production in the *N. tabacum* L., variety over time cultivated in natural granulated zeolite and confined conditions. R^2 represents the determination coefficient

production showed a moderate strong correlation with the temperature (r = 0.832) as well but a weak correlation with humidity (r = 0.585), describing the equation; LTSP = 18.75+0.55 x humidity. The full assessment of biomass and LTSP yield during two cropping years evidenced an average per year of 161.0±37.8 g of leaves $plant^{-1}$ and 44.0±40.3 g of LTSP kg^{-1} of leaves (Table 3).

Considering that climate conditions (temperature, luminosity, etc.) can speedup or holdup the plant development, the cumulative LTSP production expressed as mg of LTSP g^{-1} of leaves per week was also compared among seasons of the year. Results revealed that the cumulative LTSP production trend did not show significant differences among seasons, according to results of a simple factor-ANOVA test (p = 0.96000). This parameter showed a linear increment over the time (determination coefficient = 0.986-0.998) achieving the maximum value at 6th week (Fig. 5). Thus, the particular environmental conditions (of each season) inside the green-containment did modify the cumulative LTSP production trend.

The average biomass yield also demonstrated the maximum productivity in the spring, followed by the summer and autumn. These seasons reported a biomass yield of 28.74, 25.84 and 25.12 tons ha^{-1}, respectively, whereas the minimum biomass productivity was observed in the winter with only 21.07 tons ha^{-1}. Regarding the LTSP production, spring reported again the maximum LTSP productivity value with 1.93 tons ha^{-1} which represented 2.51 fold the value for winter (the second highest LTSP producer season). The minimum average value of LTSP production (0.56 tons ha^{-1}) was detected in the autumn. Therefore, this tobacco variety would allow a consistent net biomass production of 100.77 tons ha^{-1} and 3.91 tons of LTSP ha^{-1} during a cropping year under this cultivation conditions (Table 4).

In summary the correlation analysis among air temperature and humidity of the green-containment with biomass and protein production demonstrated a strong correlation among biomass yield, temperature and humidity but in contrast with the optimal temperature reported for tobacco cultivation, the average temperature was higher than 27°C in all seasons of the year, reaching values up to 34.9°C during the spring, time in which the maximum biomass yield and protein production were achieved. The LTSP production correlated with the temperature but did not correlate with humidity. Thus, best conditions for cultivating this variety seem to be ≤34.9°C and 55.9% of air humidity. These results are in

contradiction with those that described that tobacco plants are quite sensitive to temperature, air and humidity, as well as the type of substrate (a temperature of 20-30°C is the best for plant growth, a humidity of 80-85% and soil without a high level of nitrogen are also indicated).

Plantibody production: Finally, the use of plants as bioreactors has an attractive potential for the production of transgenic proteins. The recent development of purification methods for large-scale purification of recombinant proteins (Menkhaus *et al*., 2004) demands transgenic plants with high biomass yield and capacity for cultivation during a whole cropping year under any regimen and conditions. This principle has been demonstrated by the success of a diverse repertoire of proteins. However, a number of issues remain to be addressed before plant bioreactors can be accepted in preference to the established platforms.

In general, the yield of recombinant proteins produced in plants depends on three main factors. (1) The intrinsic limitations of the host and expression system. (2) Limitations imposed by the level of transgene expression and (3) Limitations imposed by the stability of the recombinant protein. In that sense, protein stability is probably the most important factors limiting yields in molecular farming and this can be addressed at least in part by appropriate sub-cellular targeting. For example, recombinant antibodies targeted to the secretory system generally accumulate to much higher levels than those synthesized in the cytosol and further yield increases occur when they are retained in the endoplasmic reticulum rather than secreted to the apoplast.

Several techniques have been developed for the expression of proteins in stably transformed plants (where the gene is incorporated into the plant genome). However the level of expression is still quite low. Nowadays, more than 40 recombinant proteins have been expressed in plants. Their levels of expression are really low, between 0.0005-0.3% of LTSP. One of the most successfully examples is the expression of a transgenic protein as fusion protein, p24/IgACα2-C3 which allows an increase in the expression level up to 1.4% (Arntzen *et al*., 2005).

In this study, an assessment of the *N. tabacum* L., variety stably transformed by the *Agrobacterium tumefaciens* method (Zambryski *et al*., 1983; Ramirez *et al*., 2003) for expressing a plantibody directed against the hepatitis B surface antigen was performed to corroborate its recombinant protein productive potential. Before proceeding to the assessment of plantibody expression level, two expression level sceneries were theoretically examined (Table 4). These sceneries were considered because most of the recombinant proteins expressed in transgenic plants are expressed at a low level of the LTSP (0.1%) while 1% was considered as a protein expression level appropriated with the recovery of current purification technologies (Kusnadi *et al*., 1997). In that sense, an amount of 3.91 kg (0.1% expression level) of the recombinant protein produced by hectare of the cultivable area may satisfy the amount of plantibody, employed for the vaccine manufacturing required in a year. Therefore, the reproduction of this low expression level scenery would make attractive the use of this variety for plantibody production (Ramirez *et al*., 2003; Valdes *et al*., 2003; Mila *et al*., 2009; Gomez *et al*., 2010). For this estimation, 50% was considered as average recovery of the plantibody purification process. Obviously, this lowest expression level scenery will have a negative influence on size of the cultivation areas, human resources employed and purification cost.

As results, the average expression level determined for this plantibody was 0.10±0.09% of LTSP and consequently a production of 3.91 kg ha^{-1} per year. Again, the best season of the year was the spring, showing significant differences with the winter and autumn ($p = 0.022$). Differences among the winter, autumn and summer were also observed in this parameter (Table 4). Even when there is a large heterogeneity is found in the quantity and quality of the antibody produced the huge majority of reports also demonstrated a very level of expression of antibodies in most plant systems described so far, specially when CaMV 35S, cauliflower mosaic virus 35S, is used as promoter in *Nicotiana tabacum* (De Muynck *et al*., 2010). However, this result should be carefully considered because of the expression level of a recombinant protein usually is more dependent of the expression system employed and not of the host. Summarizing, the degree of characterization and the demonstrated consistence in the biomass and protein yield by the *N. tabacum* L., variety used in this work let us to propose this variety as a proper candidate for transgenic protein production. Therefore, improving the plantibody production yield in this tobacco plant variety is a crucial subject that will have a significant impact on the economic feasibility of this variety as bioreactors.

CONCLUSIONS

Comparison of MAC of the assessed *N. tabacum* L., variety cultivated under these conditions revealed similarity in qualitative MAC but differences in plant height and leaf weight with those reported for this variety cultivated soil and open field. The spring, followed by summer and autumn, was the best season for the production of biomass, LTSP and plantibody. The

particular environmental conditions of each season inside the green-containment did modify the cumulative LTSP production trend. This *N. tabacum* L., variety could consistently be used for the production of transgenic proteins for human use in natural granulated zeolite under confinement conditions during a year-round cropping with independence of climate conditions, because it allows a consistent net biomass production of 100.77 tons ha^{-1} and 3.91 tons of LTSP ha^{-1} per year under this cultivation conditions.

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